Contrasting Expression Patterns of Three Members of the *myc* Family of Protooncogenes in the Developing and Adult Mouse Kidney

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Abstract. The *myc* family of protooncogenes encode similar but distinct nuclear proteins. Since N-*myc*, c-*myc*, and L-*myc* have been found to be expressed in the newborn kidney, we studied their expression during murine kidney development. By organ culture studies and in situ hybridization of tissue sections, we found that each of the three members of the *myc* gene family shows a remarkably distinct expression pattern during kidney development. It is known that mesenchymal stem cells of the embryonic kidney convert into epithelium if properly induced. We demonstrate that N-*myc* expression increases during the first 24 h of in vitro culture as an early response to induction. Moreover, the upregulation was transient and expression levels were already low during the first stages of overt epithelial cell polarization. In contrast, neither c-*myc* nor L-*myc* were upregulated by induction of epithelial differentiation. c-*myc* was expressed in the uninduced mesenchyme but subsequently became restricted to the newly formed epithelium and was not expressed in the surrounding loose mesenchyme. At onset of terminal differentiation c-*myc* expression was turned off also from the epithelial tubules. We conclude that N-*myc* is a marker for induction and early epithelial differentiation states. That the undifferentiated mesenchyme, unlike stromal cells of later developmental stages, express c-*myc* demonstrates that the undifferentiated mesenchymal stem cells are distinct from the stromal cells. The most astonishing finding, however, was the high level of L-*myc* mRNA in the ureter, ureter-derived renal pelvis, papilla, and collecting ducts. In the ureter, expression increased, rather than decreased, with advancing maturation and was highest in adult tissue. Our results suggest that each of the three members of the *myc* gene family are involved in quite disparate differentiation processes, even within one tissue.

THE *myc* family of protooncogenes is a multigene family dispersed in the genome, and includes three well-defined members: c-, N-, and L-*myc* (2). These genes encode related nuclear proteins and display a similar overall structure and organization (6, 14, 30, 38, 44, 45). All three genes are conserved as distinct sequences not only during vertebrate evolution (e.g., 14, 34, 41, 58) but also across the broad phylogenetic gap between Trypanosomes and vertebrates (13). This strong evolutionary conservation indicates that the *myc* protooncogenes play fundamental roles in some basic physiological processes (15, 39). There is considerable evidence suggesting that *myc* genes and other nuclear protooncogenes are important in cell cycle control (e.g., 22, 32, 33, 37, 53). However, there are also indications that these and other protooncogenes during embryonic development could have additional functions (1, 10, 37, 43). This view is supported by the findings that the expression patterns of these protooncogenes do not always correlate with high rates of cell proliferation during normal development (16, 29, 42, 49, 52, 60). In the mouse embryo, the expression of the N-*myc* protooncogene is not a marker for cellular proliferation but rather for early stages of differentiation in some cell types during organogenesis of the mouse (42). Although c-*myc* expression was observed to be correlated with cellular proliferation during expansion and folding of partially differentiated epithelial cells in the mid-gestation mouse embryo (52), c-*myc* is not always a marker for proliferation processes. For example, in the gastrulating mouse embryo c-*myc* is selectively downregulated in the most highly proliferative tissue, the primitive ectoderm (16). Apart from limited data derived from Northern blot analysis of newborn and postnatal stages of mouse development (55, 60), no information is available for L-*myc* expression during embryonic development.

One of the tissues with a particularly high expression of c-*myc*, N-*myc*, and L-*myc* in newborn mice is the kidney, and it was reported that their expression was greatly reduced (c-*myc*, N-*myc*) or completely shut off (L-*myc*) in kidneys of adult mice (46, 60). Hence, the developing kidney provides a suitable model system to study the role of the *myc* family of protooncogenes in normal organogenesis. The embryonic mouse kidney, in particular, offers some valuable advantages for such an analysis. The development of the mammalian kidney is morphologically well documented (27, 31, 48) and much is known about the nature of the different cell lineages. Kidney organogenesis involves the interplay of at least four different cell populations: the ingrowing and branching epi-

thelial ureter, mesenchymal cells, which convert into either a new epithelium (23) or to interstitium (4), and endothelial cells, which form the blood vessel system (20). The conversion of the mouse metanephrogenic mesenchyme to epithelium and to interstitium can be induced in vitro in the absence of the ureter cells and without continuity to blood vessels.

Our previous study on in vivo development suggested that N-*myc* mRNA expression is restricted to mesenchymal cells that have been induced to convert into epithelium (42), but it was not clear to what extent N-*myc* expression was influenced by embryonic induction. Therefore, we have here used the in vitro culture system for differentiation of the metanephric mesenchyme to epithelium (23) to investigate this issue. Since all three *myc* genes are expressed in the newborn kidney (60), it was important to compare the expression of c-*myc*, L-*myc*, and N-*myc*. Initial Northern blot studies of early kidneys raised the possibility of a coexpression of c-*myc*, N-*myc*, and L-*myc* at the early developmental stages. However, a more detailed analysis by Northern hybridization and in situ hybridization of in vivo and in vitro development revealed that each of the three members of the *myc* protooncogene family showed a remarkably distinct expression pattern.

Materials and Methods

Tissues and Organ Cultures

Hybrid mouse embryos NMRI × 129 were used. The day of the vaginal plug was designated as day 0. Mice were killed by cervical dislocation and tissues for RNA isolation were frozen on dry ice immediately after dissection. Embryonic and adult ureters were isolated by microsurgically removing the parts that emerge from the hilus of the kidney. For transfilter culture, the kidney anlagen were isolated from 11-d-old embryos. After microsurgically removing the ureter bud, the metanephric mesenchyme was cocultured with spinal cord as inductor tissue (23, 50) in I-MEM medium (Gibco Laboratories, Eggenstein, FRG) supplemented with transferrin and 10% FCS as described by Klein et al. (35, 36). Uninduced mesenchymes were collected in medium supplemented with 10% FCS but were frozen on dry ice within 5 h. From all transfilter cocultures, spinal cord was removed before freezing the mesenchymes on dry ice.

Tissues for in situ hybridization were embedded in Tissue-Tek OCT (Miles Scientific/Bayer Diagnostics, München, FRG) and frozen in liquid nitrogen. The inducer tissue was removed from the transfilter cultures prior to embedding. Cryostat sections (5 μm) were collected on poly-L-lysine-coated (Sigma Chemical Co., Deisenhofen, FRG) slides, fixed in freshly prepared 4% paraformaldehyde (Serva Biochemicals, Heidelberg, FRG) in PBS for 15 min, and stored desiccated at −20°C.

Isolation of Total RNA

Total RNA from intact embryonic or adult kidneys and ureters was isolated according to Chirgwin et al. (9) as described previously (42). Briefly, tissues were homogenized in 4 M guanidinium thiocyanate either with a Dounce tissue homogenizer or with an Ultra-Turrax T25 homogenizer (IKA Labortechnik, Staufen, FRG), and RNA was then purified by ultracentrifugation (SW41 Ti rotor; Beckman Instruments Co., Stuttgart, 35,000 rpm, 20°C, 16 h). The smaller mesenchyme tissues collected from transfilter cultures were homogenized in Eppendorf tubes containing 4 M guanidinium thiocyanate by extensive vortexing. The mesenchyme homogenate was subsequently squeezed through a 22-gauge needle, and RNA was purified by ultracentrifugation (SW50.1 rotor; Beckman Instruments Co. 42,000 rpm, 20°C, 16 h) through a 1.3-ml CsCl cushion.

Hybridization Probes

Four different probes were used in the study. The 2.3-kb N-*myc* probe was the previously used (42) p277.3 probe of DePinho et al. (14) spanning the third exon and part of the second intron. The 540-bp pML2 probe for L-*myc*, inserted in both directions into pSP65, contains the 3′ untranslated region of exon 3 (38). The c-*myc* probe pMC3 was constructed by subcloning a 2.5-kb Xba I/Hind III fragment into both pGEM3-blue (Promega Biotec, Madison, WI) and Bluescript KS (Stratagene Corp., La Jolla, CA). The pMC3 fragment contains exon 2 and 3 including intron 2 of the murine genomic clone isolated from pCHTK2-myc (56). The three probes (p277.3, pML2, pMC3) were specific for the different members of the *myc* family and each of them detected a single transcript of the correct size when hybridized to RNA by Northern analysis. The fourth probe was the 1.95-kb Sal I/Eco RI fragment of pMC1 carrying the 5′ terminus of the murine 18S rRNA gene (24). Since steady-state levels for β-actin mRNA are not constant during all stages of organogenesis (17, 42) we used the 18S rRNA probe instead of the β-actin probe as a control in Northern hybridizations.

For Northern blot analysis, each insert was purified from an agarose gel and labeled with 50 μCi [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham-Buchler, Braunschweig, FRG) using an oligolabeling kit (Pharmacia-LKB, Freiburg, FRG) according to the manufacturer's instruction. The reaction was stopped by adding 40 μl TNE (10 mM Tris-Cl, 10 mM NaCl, 2 mM EDTA, pH 8.0) followed by ethanol precipitation for 15 min on dry ice. Probes were labeled to an approximate specific activity of 2–5 × 10^9 cpm/μg. The *myc*-specific probes were used for Northern hybridization at a final concentration of 1 × 10^6 cpm/ml. The purified 18S rRNA-specific probe was labeled to an approximate specific activity of 3 × 10^8 cpm/μg using a nicktranslation kit (Bethesda Research Laboratories, Gaithersburg, MD) according to the supplier's instruction, and was used at a final concentration of 1 × 10^5 cpm/ml.

To prepare cRNA probes, the templates (p277.3, pML2, and pMC3) were linearized with the appropriate restriction enzymes at a site in the polylinker sequence downstream of the insert, with respect to the RNA polymerase promoter to be used. The transcripts produced represent the 3′ untranslated region of the three different *myc* genes in the region where they display the least degree of homology (38). Single-stranded sense and antisense RNA probes were synthesized in the presence of 100 μCi of [α-^{35}S]-UTP (1250 Ci/mmol; Amersham-Buchler) using either SP6 (Amersham-Buchler), T7 (Genofit, Heidelberg, FRG) or T3 (Stratagene Corp.) RNA polymerase according to the method of Melton et al. (40) as described previously (42). Specific activities varied between 8 × 10^8 and 2 × 10^9 dpm/μg RNA. Probes were reduced in size to an average length of 50–200 bp by limited alkaline hydrolysis (11) and were used for in situ hybridization at a final concentration of 1 × 10^6 dpm/μl.

Northern Hybridization

Northern hybridization of 10 μg of total RNA was performed as described previously (42) with the exception that herring sperm DNA was omitted from the hybridization solution. All filters were hybridized consecutively with probes specific for N-, L-, and c-*myc*, and 18S rRNA. Stringent washes were carried out in 0.1× SSC/0.1% SDS at 42°C for 15 min (*myc* probes) and in 0.1× SSC/0.1% SDS at 68°C three times for 30 min (18S rRNA), respectively. Before rehybridization filters were stripped of previous probes by washing in 0.005 M Tris-Cl, 0.002 M EDTA, 0.1× Denhardt's solution (0.002% BSA, 0.002% Ficoll, 0.002% polyvinylpyrollidone), pH 8.0 at 65°C for 2 h with one change of solution.

Autoradiographs in the linear range were scanned one dimensionally with an Ultroscan XL laser densitometer using the Gelscan XL program (Pharmacia-LKB). The signals were corrected for loading variations using densitometric measurements of the 18S rRNA controls and were expressed as a probe/control ratio.

In Situ Hybridization

In situ hybridizations were carried out according to Hogan et al. (26) as described by Mugrauer et al. (42), with the following modifications. The incubation step in 0.2 M HCl was omitted; Proteinase K (Sigma Chemical Co.; 200 μg/ml in 0.1 M Tris-Cl, 0.1 M EDTA, pH 8.0) was used instead of predigested pronase, and after hybridization (50°C, 16 h) and posthybridization treatment including RNase digestion final washes were performed at 50°C in 2× SSC for 30 min and in 0.1× SSC for 30 min. No signals above background level were observed in the routinely performed control hybridizations employing sense transcripts of comparable specific activity. Slides were exposed to Ilford K2 emulsion (Ilford Ltd., Basildon, Essex, UK) 9 d in the case of N-*myc* and c-*myc*, and between 9 and 33 d for L-*myc*. After development, the slides were stained with 0.02% toluidine blue and examined on a Leitz Diaplan microscope using brightfield and darkground illumination.

Detection of Cell Proliferation by Bromodeoxyuridine Immunohistochemistry

Pregnant and newborn mice were injected intraperitoneally with a single dose of 5-bromo-2'-deoxyuridine (BrdU; Serva Biochemicals) dissolved in 1× PBS, pH 7 (200 μg/g body weight). After 1 h, the animals were killed and embryonic, newborn, and adult kidneys were removed, embedded in Tissue-Tek O.C.T. (Miles Scientific), and frozen in liquid nitrogen. 5-μm cryostat sections were fixed in cold methanol for 10 min at −20°C and washed twice in PBS (5 min). Subsequently, the DNA was denatured by immersing the sections in 2 N HCl for 30 min at room temperature. This incubation step was necessary because the mouse-generated anti-BrdU antibody used is directed against single-stranded DNA containing BrdU. After neutralization in 0.1 M sodiumborate, pH 8.5 (2 min) and two washes in PBS (5 min each) sections were incubated with FITC-conjugated anti-BrdU antibody (Becton-Dickinson, Heidelberg, FRG) for 30 min at room temperature in a humidified chamber. The antibody was used either 1:10 diluted in PBS or undiluted. After a final wash in PBS (twice for 5 min) slides were mounted in ELVANOL embedding medium (35) for fluorescent microscopy. Sections from unlabeled kidneys served as negative controls whereas intestine removed from BrdU-treated animals was chosen as a positive control tissue.

Results

Northern Blot Analysis of myc Gene Family during In Vivo Kidney Development

Northern hybridization to RNA obtained from intact kidneys of embryonic, newborn, and adult mice showed that the probes used detected single transcripts of the expected size: 2.9 kb for N-*myc*, 2.4 kb for c-*myc*, and 3.7 kb for L-*myc* (Fig. 1). The hybridization signals for the three genes were strongest in the earliest developmental stages studied and declined thereafter. N-*myc* expression decreased rather early during kidney development, whereas c- and L-*myc* expression declined more slowly (Fig. 1). In adult kidneys, the signals were very weak, even after long exposure times, as also found by others (55, 60).

Since the expression of all three genes was strongest at early stages of kidney development, the expression was studied in an in vitro culture system which allows detailed investigation of the development of kidney tubules, glomeruli, and stroma. Undifferentiated metanephric mesenchyme, a homogenous stem cell population from the kidney anlagen of 11-d-old mouse embryos, can be experimentally induced to convert into epithelium in the absence of the ureter cell lineage. In such cultures, the mesenchyme is induced to differentiate by a heterologous inductor tissue, the embryonic spinal cord (23). After 24 h of coculture of mesenchyme and spinal cord the majority of the mesenchymal cells become induced and the differentiation process will proceed even after removal of the inductor. The expression of N-*myc*, c-*myc*, and L-*myc* mRNA was studied during this in vitro development.

Northern Blot Analysis of N-myc, L-myc, and c-myc mRNA during In Vitro Development of the Mesenchyme

Northern blot analysis of total RNA isolated from transfilter cultures at different stages of differentiation revealed that N-*myc*, c-*myc*, and L-*myc* were not coordinately expressed during in vitro kidney development. N-*myc* was transiently expressed during the conversion of the metanephric mesenchyme into kidney tubule epithelium. Expression was moderately up-regulated during induction (approximately three-

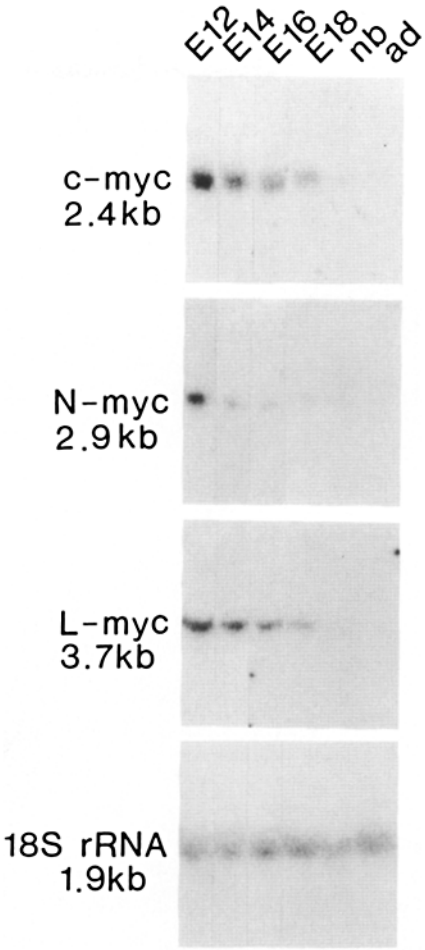

Figure 1. Northern blot analysis of c-, N-, and L-*myc* mRNA expression during murine kidney development. Total cellular RNA (10 μg per lane) from different embryonic stages (*E12*, *E14*, *E16*, *E18*), newborn (*nb*) and adult (*ad*) mice were hybridized to probes detecting the three different *myc* protooncogenes. Control hybridizations were performed with a probe specific for the 18S rRNA. Autoradiography times were 2 d for c-*myc*, 3 d for N-*myc*, 4 d for L-*myc*, and 3 h for 18S rRNA.

fold) reaching its maximum levels at 24–48 h of in vitro cultures and then declining to barely detectable levels (Fig. 2 *A*). In contrast, c-*myc* was already expressed in the uninduced mesenchyme and expression only gradually declined during in vitro culture. The c-*myc* pattern paralleled the proliferation profile of the transfilter cultures as judged by [^{3}H]thymidine incorporation (21, 51). In induced mesenchymes cultured for 120 h, c-*myc* expression dropped to ~30% of the preinduction level (Fig. 2 *B*). L-*myc* expression was readily detectable in all stages studied, although the hybridization signals were only about one third of those of N-*myc* and c-*myc*. The strongest hybridization signals were obtained in RNA isolated from uninduced mesenchymes and induced mesenchymes cultured for 120 h. Interestingly, L-myc expression was transiently downregulated during the induction process and returned to preinduction level at 48 h of in vitro development (Fig. 2 *A*). To ensure that the observed differences were not due to RNA isolation problems from the small tissues, different RNA isolations were prepared from three different pools of both uninduced and in-

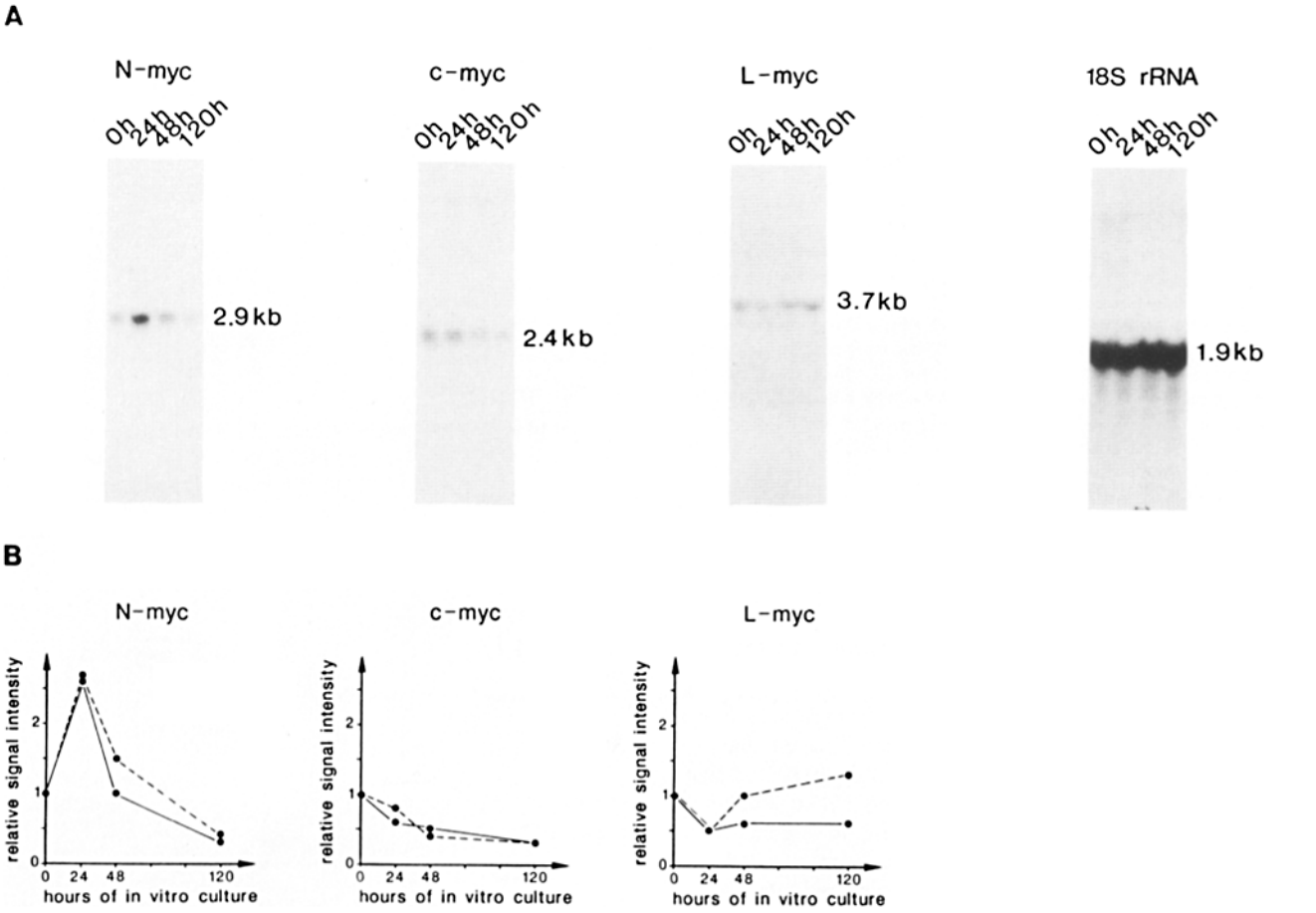

Figure 2. Uncoordinated expression of the *myc* family of protooncogenes during in vitro conversion of metanephric mesenchyme to epithelium. (*A*) Northern blot analysis of total RNA (10 μg per lane) isolated from uninduced metanephric mesenchyme (0 h) and induced mesenchymes cultured for 24, 48, and 120 h. The same filter was consecutively hybridized to radiolabeled DNA fragments specific for N-*myc*, c-*myc*, L-*myc*, and 18S rRNA. Exposure time was 25 h for N-*myc*, 42 h for c-*myc*, 74 h for L-*myc*, and 30 min for 18S rRNA. (*B*) Densitometric analysis of two different filters. Relative expression was established by dividing the area corresponding to the different protooncogene mRNAs by the area of the 18S rRNA transcript in the same 10-μg RNA sample.

duced mesenchyme cultured for 24 h, and from two pools of both induced mesenchymes cultured for 48 and 120 h. Samples from each preparation were analyzed on three separate filters and the above described expression pattern for the three genes was consistently found. The quantitative values of two consecutively analyzed filters are shown graphically in Fig. 2 *B*.

In Situ Hybridization of Transfilter Cultures

In vitro development of mesenchyme to epithelium and to stroma can be subdivided into several differentiation stages. After 24-h of induction, the morphological changes in the tissue are still minimal; a few mesenchymal cells begin to attach to each other and form condensed areas, whereas other mesenchymal cells comprise a loose cell population. At 36–48 h of in vitro development, the first definite signs of polarization of cells within the condensed areas occur (17, 36). After 72 h of culture, epithelial tubules can be clearly distinguished from the loose mesenchyme. At 120 h of in vitro development, the culture consists predominantly of fully developed kidney tubules and glomeruli displaying antigens characteristic of terminal differentiation (19).

After 24 h of induction, N-*myc*-positive clusters were detected in some areas of the mesenchyme (Fig. 3, *A* and *B*) and after 48 h of in vitro culture, N-*myc* expression was found exclusively in those areas where new condensates had appeared. N-*myc* was still present, although to a much lesser extent, in structures resembling S-shaped bodies. In areas where polarization of cells had already started, N-*myc* expression was selectively down-regulated (Fig. 3, *C* and *D*), and after 72 h of in vitro development few or no silver grains could be detected (Fig. 3, *E* and *F*). Sections from 120-h cultures were completely negative for N-*myc* (Fig. 3, *G* and *H*). Thus, N-*myc* expression during in vitro development is restricted to the very early epithelial differentiation stages. In contrast to N-*myc*, c-*myc* expression at 24 h of in vitro development was not confined to certain locations and silver grains, clearly above background level, were uniformly distributed over the whole section (Fig. 4, *A* and *B*). During the subsequent differentiation stages, however, c-*myc* expression became concentrated in the developing epithelial structures. It is clear from mesenchymes cultured for 42 and 72 h that no c-*myc* expression occurred in the mesenchyme surrounding the epithelial tubules (Fig. 4, *C–F*). In mesenchymes cultured for 120 h, c-*myc* expression was also turned

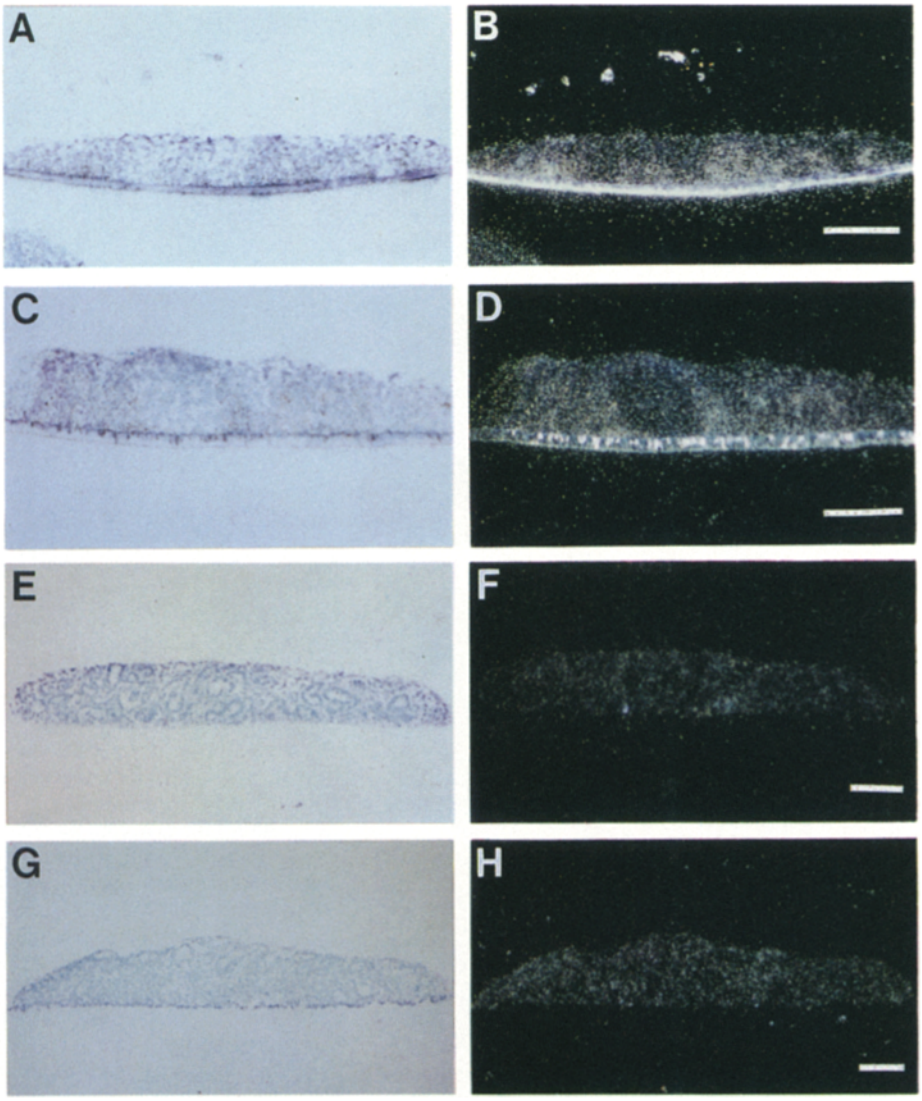

Figure 3. Localization of N-*myc* mRNA in sections of metanephric mesenchyme cultured in vitro. ^{35}S-labeled antisense cRNA probe was hybridized in situ to cryostat sections of mesenchyme cultured in vitro in the presence of inducer tissue for 24 h (*A* and *B*), 48 h (*C* and *D*), 72 h (*E* and *F*), and 120 h (*G* and *H*). The autoradiographs are shown in brightfield (*A*, *C*, *E*, and *G*) and darkground (*B*, *D*, *F*, and *H*) illumination. Autoradiography was for 9 d. N-*myc* is strongly expressed in condensed areas where conversion of mesenchyme to epithelium starts (*A* and *B*) and is downregulated during further epithelial differentiation. Cultures consisting predominantly of late and terminal differentiation stages (*E–H*) are devoid of silver grains above background. The bright line in *B* and *D* is the Nuclepore filter on which the kidney mesenchyme develops. In *F* and *H* the filter was lost during the procedure. Bars, 100 μm.

off in the epithelial cells, since only a few of the tubules remained weakly positive for c-*myc* (Fig. 4, *G* and *H*). Since in vitro differentiation of the kidney mesenchyme is asynchronous, the tubular structures that continue to weakly express c-*myc* most likely represent not yet fully differentiated kidney tubules.

Northern blot analysis suggested the presence of L-*myc* before induction, a subsequent slight decline, and a reappearance of the mRNA at the stage when epithelial cell polarization becomes evident. In situ hybridization revealed that L-*myc* expression occurred in only one of the three major segments of the nephron, in large, rounded multicellular bodies that resemble glomeruli rather than tubuli (Fig. 5, *A–D*). The glomeruli that develop in vitro lack endothelial cells and the podocytes form such large rounded glomerular bodies without endothelium (7, 19; Fig. 6). Thus, histological criteria suggest that the glomerular bodies were positive for L-*myc* (Fig. 5, *C* and *D*) explaining the clear signal in the 120-h cultures observed in Northern blots (Fig. 2). The hybridization signals for L-*myc* expression were, in general, much weaker than those for both c- and N-*myc*. To detect L-*myc* expression in the glomeruli of transfilter cultures the exposure time was 3.5 times longer than those for c- and N-*myc* (i.e., 33 as compared with 9 d).

Localization of c-myc and N-myc mRNA during In Vivo Kidney Organogenesis

Using in situ hybridization we next investigated whether the three *myc* genes showed the pattern predicted from the in vitro results also during in vivo development. The development in vivo differs from that in vitro in so far as two major additional cell lineages are present, the ureter lineage and the endothelium. The tips of the branching collecting ducts are responsible for induction in vivo, and thus a further notable difference is that continuous waves of induction occur in the upper cortex of the developing kidney, where the tips of the collecting ducts are located. As a consequence, the early and late differentiation stages for all cell lineages are located in different parts of the kidney. In each developing kidney, regardless of the embryonic age, the cortical area consists predominantly of undifferentiated mesenchyme and early differentiation stages. Such regions are readily distinguished from the more central parts of the kidney, which contain late

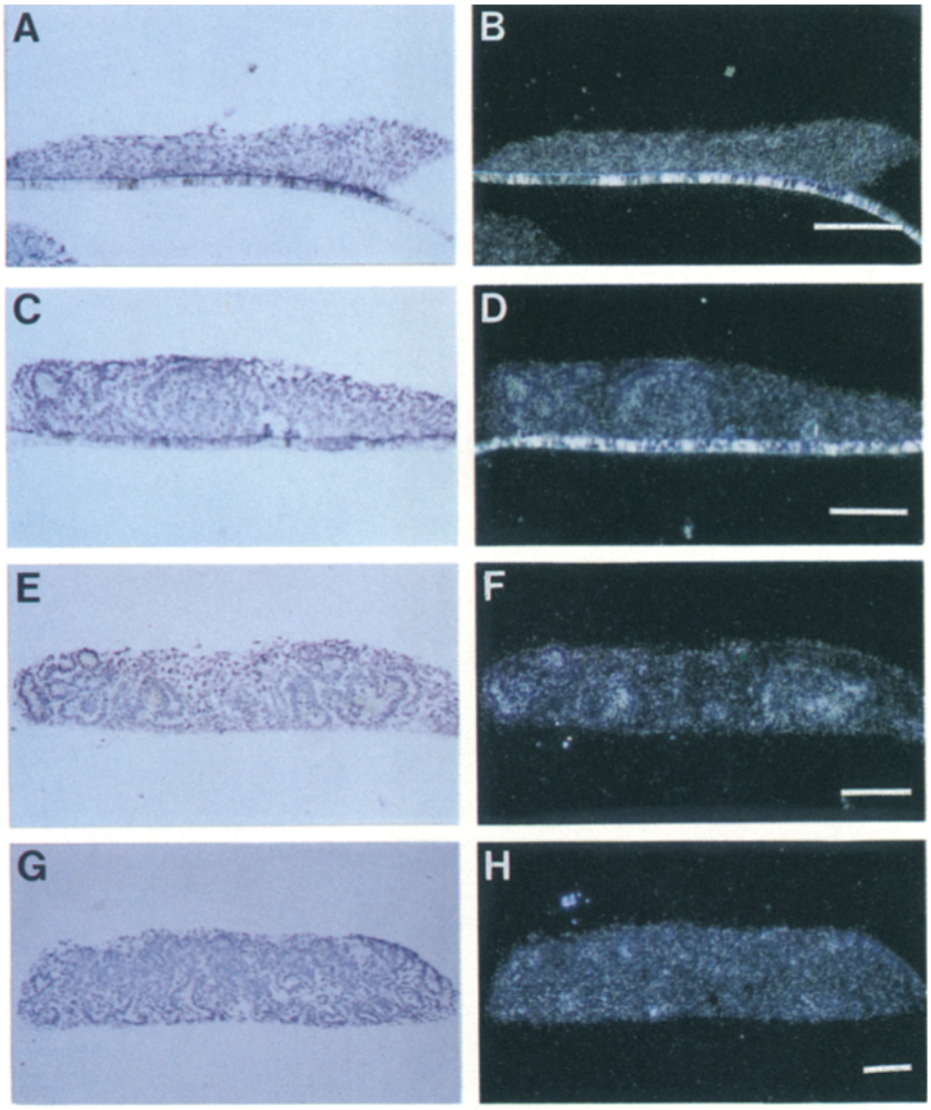

Figure 4. Distribution of c-*myc* mRNA during kidney differentiation in vitro. Cryostat sections of induced mesenchymes cultured for 24 h (*A* and *B*), 48 h (*C* and *D*), 72 h (*E* and *F*), and 120 h (*G* and *H*) were hybridized to the antisense c-*myc* probe, and exposed for 9 d. The autoradiographs were photographed under brightfield (*A*, *C*, *E*, and *G*) and darkground (*B*, *D*, *F*, and *H*) illumination. Note that the autoradiographic grains become concentrated in the developing epithelial structures (*C–F*) and that they are not detectable during terminal differentiation (*G* and *H*). The strong signal seen in *B* and *D* is the Nuclepore filter on which the kidney mesenchyme is cultured. In *F* and *H* the filter was lost during the procedure. Bars, 100 μm.

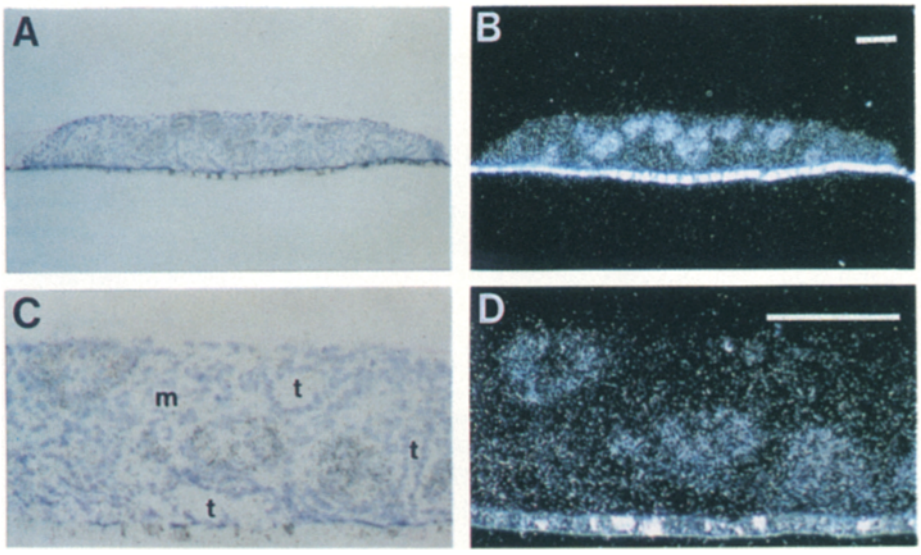

Figure 5. Localization of L-*myc* mRNA in transfilter cultures. Antisense strand L-*myc* probe was hybridized in situ to cryostat sections of induced mesenchymes cultured for 120 h, and exposed for 33 d. Autoradiographs are shown under brightfield (*A* and *C*) and darkground (*B* and *D*) illumination. In induced mesenchymes cultured for 120 h local clusters with high grain density are visible (*A* and *B*). Higher magnification of a section of induced mesenchyme kept in culture for 120 h reveals that L-*myc* is expressed exclusively in glomerular bodies consisting of podocytes only (*C* and *D*). Note that both well developed epithelial tubules (*t*) and the loose mesenchyme (*m*) surrounding tubules and glomerular bodies are devoid of silver grains. Bars, 100 μm.

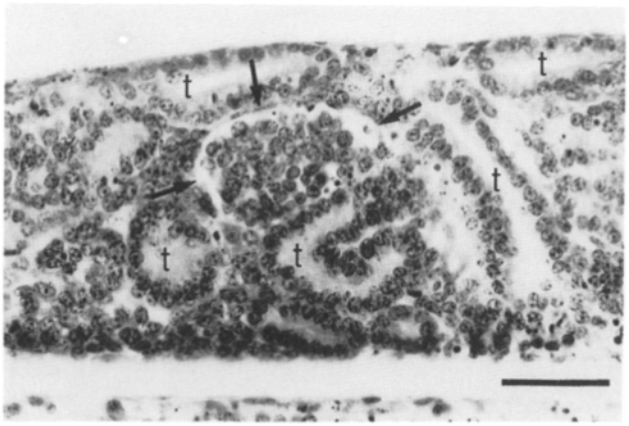

Figure 6. Histological section through an induced mesenchyme cultured for 120 h. Glomeruli that develop in vitro lack the endothelial cells and the podocytes therefore form large rounded structures. These avascular glomerular bodies are separated from the surrounding tissue by a characteristic narrow cleft (*arrows*). *t*, epithelial kidney tubules. Hematoxylin-eosin staining. Bar, 50 μm.

and terminal stages of epithelial differentiation surrounded by a fully differentiated stroma (4, 42).

Both c-*myc* and N-*myc* expression was found in cortical areas of developing kidneys, and no expression was seen in blood vessels or the ureter-derived structures. Although c-*myc* expression could be detected by Northern blotting in the ureter, no clear signals were seen for c-*myc* by in situ hybridization in the ureter cells. The reason for this apparent discrepancy is unclear. In contrast to N-*myc* (42; Fig. 7, *C* and *D*), c-*myc* expression was not completely restricted to the cortical area but rather extended towards the more central part of the kidney (Fig. 7, *A* and *B*). c-*myc* expression was detected in the kidney epithelium as well as in the uninduced mesenchyme. During the formation of the new epithelium from mesenchyme c-*myc* could be detected in both the early differentiation stages (condensates, comma- and S-shaped bodies; for description of stages of kidney tubulogenesis see reference 42) and late differentiation stages (tubule elongation). However, in fully differentiated tubules present in newborn kidneys, glomeruli of any developmental stage, and the interstitial cell compartment c-*myc* was not expressed (Fig. 7, *A* and *B*). In adult kidneys, the silver grains were uniformly distributed over the whole section and were only slightly above background (data not shown). Analogous to the in vitro situation, c-*myc* expression during in vivo kidney development closely followed the cell proliferation pattern. All c-*myc* positive cells were mitotically active as demonstrated by BrdU incorporation (Fig. 8). In contrast to c-*myc*, high-level of N-*myc* expression was confined to the very early differentiation stages of induced cells (condensates, comma- and S-shaped bodies) found exclusively in the kidney cortex (42; Fig. 7, *C* and *D*). Thus, matching the in vitro data, c-*myc* displays a broader expression pattern than N-*myc*. In the developing kidney c-*myc* is restricted to two cell populations, the uninduced mesenchyme and to the mesenchyme which converts to epithelium and its expression is shut off when kidney tubules terminally differentiate. Thus, c-*myc* expression seems to correlate with cellular proliferation during kidney development.

Distribution of L-myc mRNA during Kidney Development

The expression pattern of L-*myc* was notably different from those of c-*myc* and N-*myc*. In sections from 16-d-old embryonic kidneys, high density of silver grains, indicating the presence of L-*myc* mRNA, were concentrated in the collecting duct system, the papilla, the renal pelvis (Fig. 7, *E* and *F*), which are all derived from the ureter (48), and in the ureter itself (Fig. 9, *A* and *B*). Within the collecting duct system, high levels of L-*myc* expression could be detected only in the large papillary ducts that are situated in the medulla of the kidney and that fuse with the renal pelvis. In the straight collecting ducts of the kidney periphery, the hybridization signals were rather weak (Fig. 7, *E* and *F*). The papillary ducts represent a later developmental stage than the straight collecting ducts because they are generated during the first cycles of branching of the ingrowing ureter (see reference 48). Several of these papillary ducts eventually fuse to form the papillae which drains into the renal pelvis. Cells from these ureter-derived structures divided rather slowly as judged by BrdU immunohistochemistry. For example, the papillary ducts ceased their proliferative activity already at embryonic day 15 (Fig. 8). The papilla and the renal pelvis displayed the highest density of silver grains apart from the kidney ureter. In the collecting duct system the signals decreased from the medulla to the cortex and with advancing development. In the adult kidney all collecting ducts became negative. Only the papilla and the renal pelvis continued to express L-*myc* up to the adult stage. Neither N-*myc* nor c-*myc* were expressed in the renal pelvis, irrespective of the age of the kidney (Fig. 7, *A–D*).

In addition to the strong unexpected signals in ureter cells, weak positive signals were detected in the cortical area of 16-d-old embryonic (Fig. 7, *E* and *F*) and newborn kidneys where the early differentiation of tubules and glomeruli takes place. This pattern matches the in vitro data. The slightly elevated levels of expression probably represent developing podocytes, which during in vivo development form a thin, single-layered epithelium. In contrast, podocytes form large multilayered bodies in the in vitro cultures (7, 19), which explains why they are more readily visible as L-*myc* positive areas in the in vitro cultures (see Fig. 5).

Expression of myc Family of Protooncogenes in the Epithelium of Embryonic and Adult Ureter

The surprising expression pattern obtained for L-*myc* in intrarenal urothelium in 16-d-old embryonic and newborn kidneys prompted the study of the expression of each of the three *myc* genes in the ureter cell lineage in more detail. As can be clearly seen in sections from 16-d-old embryonic ureter, L-*myc* expression was restricted to the transitional epithelium of the ureter whereas the surrounding lamina propria and the smooth muscle cell layer are devoid of silver grains above background levels (Fig. 9, *A* and *B*). In contrast, N-*myc* was completely negative in each of the three layers of the ureter (Fig. 9, *C* and *D*). In vivo proliferation studies demonstrated that the mitotic activity of all three layers of the ureter was already rather low in 15-d-old embryonic kidneys (Fig. 8) and decreases further with ongoing development. In newborn and adult kidneys only a tiny fraction of the ureter cells still proliferated, in accordance with the fact

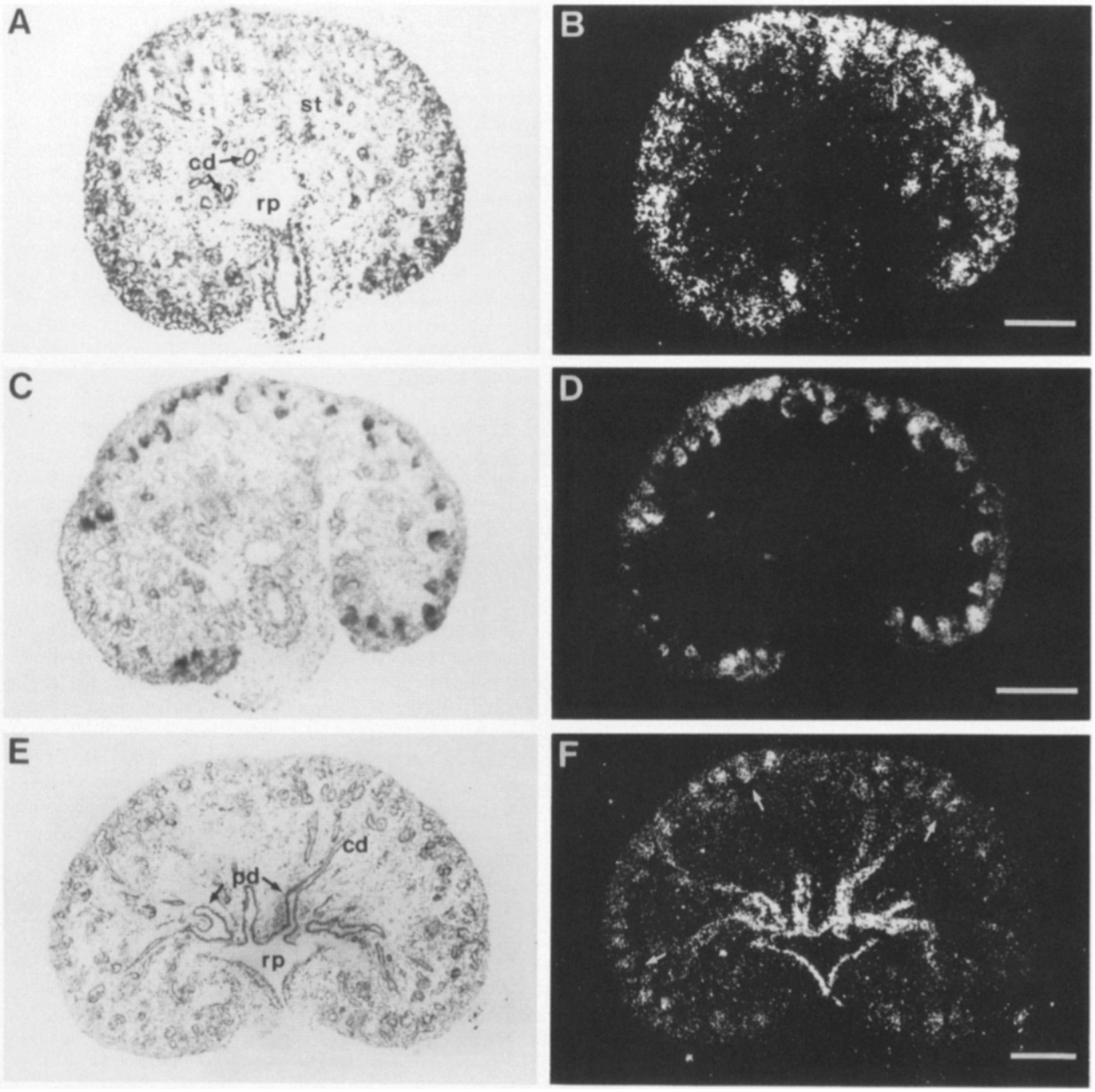

Figure 7. Expression of the *myc* family of proto-oncogenes in kidneys from 16-d-old mouse embryos, as revealed by in situ hybridization of cryostat sections. Brightfield (*A*, *C*, and *E*) and corresponding darkground (*B*, *D*, and *F*) micrographs are shown. (*A* and *B*) c-*myc* mRNA is broadly distributed in the kidney cortex due to expression in the uninduced mesenchyme present in the outermost cortical layer and in early and late differentiation stages of kidney tubule development present in the deeper layer of the cortex. Note that the stroma (*st*), the collecting ducts (*cd*), and the renal pelvis (*rp*) are c-*myc*-negative. (*C* and *D*) N-*myc* expression is restricted to local clusters in the kidney cortex representing early stages of kidney tubule differentiation as shown previously (42). (*E* and *F*) L-*myc* expression pattern differs from both those of c-*myc* and N-*myc*. Highest grain density is evident in the renal pelvis (*rp*) and in the papillary ducts (*pd*), whereas lower grain density is over straight collecting ducts (*cd*). Only weak hybridization signals can be seen in the kidney cortex (*arrows*). Bars, 200 μm.

that the transitional epithelium continuously renews itself but at a very slow rate. The in situ data were confirmed by Northern blot analysis of total RNA isolated from various developmental stages of ureter and the corresponding kidney (Fig. 10). The lanes representing different kidney stages displayed the expected expression patterns for all three *myc* protooncogenes (compare with Fig. 1) and served as internal controls showing that the correct hybridization conditions were used. In the extrarenal ureter, N-*myc* was not detectable in any stage, apart from a very faint signal at embryonic day 14. Although it was not possible to conclusively demonstrate c-*myc* expression by in situ hybridization, c-*myc* mRNA was present in the embryonic ureter but declined with advancing development (Fig. 10). The discrepancy between in situ hybridization and Northern blot analysis is most probably due to the fact that low-level expression of a gene is more easily

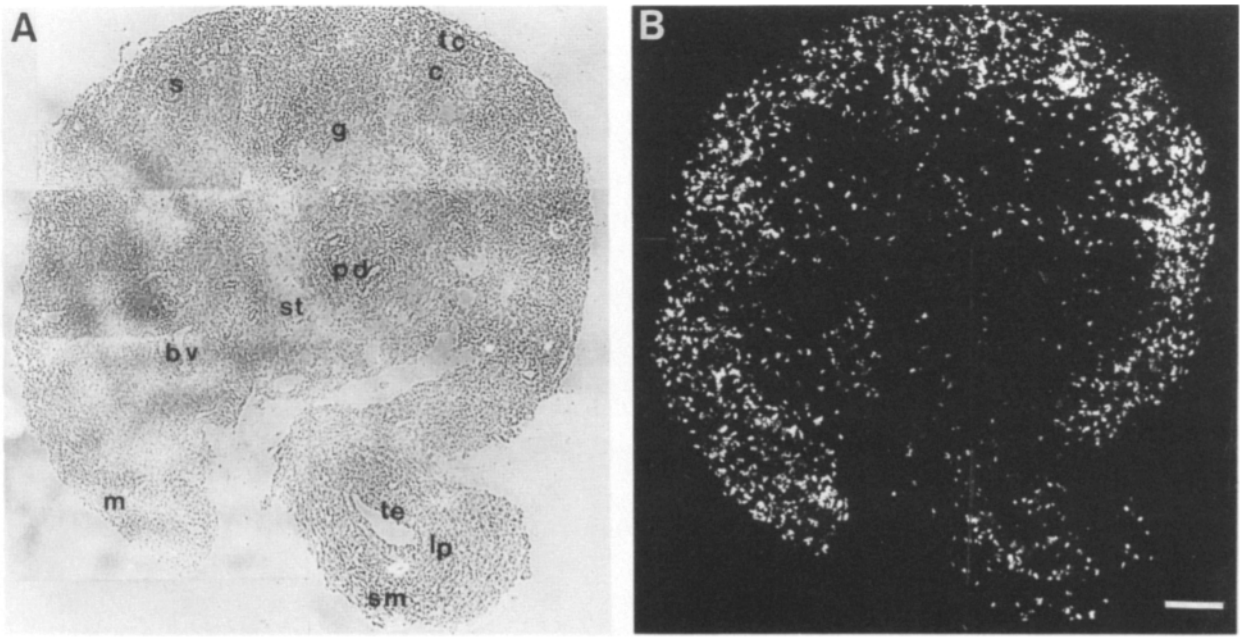

Figure 8. Immunofluorescent localization of proliferating cells during in vivo kidney development. A cryostat section of a 15-d-old embryonic kidney is shown in phase-contrast (*A*) and as fluorescent photograph (*B*). Highest rate of proliferation is found in the nephrogenic zone of the kidney cortex, including uninduced mesenchyme (*m*), the early stages of epithelial differentiation (*c*, condensate; *S*, S-shaped body) as well as the tips of the collecting ducts (*tc*). Low level of mitotic activity is seen in all three layers of the ureter (*te*, transitional epithelium; *lp*, lamina propria; *sm*, smooth muscle cell layer), some blood vessels (*bv*) and late stages of epithelial differentiation which are located more centrally underneath the nephrogenic zone. Note that neither stroma (*st*) nor glomeruli (*g*) nor papillary ducts (*pd*) show any notable proliferative activity. Bar, 100 μm.

detected by Northern blot analysis. In each stage of ureter development studied the strongest hybridization signals were obtained with the L-*myc* probe. Unexpectedly, the level of L-*myc* expression increased rather than decreased during advancing development and maximum intensity was seen in the ureter from the adult stage (Fig. 10).

Discussion

The spatial and temporal expression pattern of three members of the *myc* family of protooncogenes, c-*myc*, N-*myc*, and L-*myc*, was analyzed by in situ hybridization in the developing mouse kidney. We here report that each of the three *myc* protooncogenes shows a remarkably distinct stage- and cell type–specific expression pattern, although some overlap in areas of expression occurred during the early stages of kidney tubule differentiation. We found that N-*myc* was upregulated during induction of kidney tubule development and only transiently expressed during kidney tubulogenesis, whereas c-*myc* was already expressed in the uninduced mesenchyme and continued to be expressed also at later differentiation stages. The most remarkable finding, however, was the high level of L-*myc* expression in the urothelium of the adult kidney, a tissue with a very low mitotic rate and fully differentiated. These results indicate that each of the three *myc* genes might be involved in quite disparate differentiation pathways, even within one tissue. Furthermore, the remarkable L-*myc* expression in the urothelium raises the interesting possibility that some protooncogenes may participate in physiological processes unrelated to cellular proliferation or differentiation.

The data on the expression of the *myc* protooncogenes during embryonic kidney development are important for the understanding of the molecular basis of the early stages of kidney development. In the developing kidney, a major driving force for differentiation is the interaction between epithelial ureter cells and mesenchymal stem cells. As a result of this interaction, a large fraction of the metanephric mesenchyme will convert into an epithelium (5, 19, 23). A smaller portion of the mesenchyme differentiates to fibroblast-type cells comprising the stromal compartment of the kidney (4). Since the de novo formation of an epithelium from a homogenous mesenchymal cell population can also be induced in vitro, the embryonic kidney mesenchyme has been much used to study the generation of epithelial cell polarity (reviewed in 5, 18). Markers for the early molecular response to induction of the metanephrogenic mesenchyme into epithelium have long been sought and for the N-*myc* clearly seems to represent one such marker. Interestingly, N-*myc* expression is only transiently upregulated as an early response to induction of the metanephric mesenchyme. Based on our previous in vivo study for N-*myc* the result could already be anticipated (42), but since a completely different expression pattern for N-*myc* was reported for human embryonic kidneys (25) it became important to clarify the issue. In contrast to our results, the report on human kidneys showed expression of N-*myc* in well-differentiated tubuli and glomeruli, and some expression was also seen in differentiated stroma. By the use of in vitro cultures it was now possible to define how induction influences N-*myc* expression, and the data are in accordance with our previous study of in vivo development (42). The current study shows that N-*myc* is expressed at a

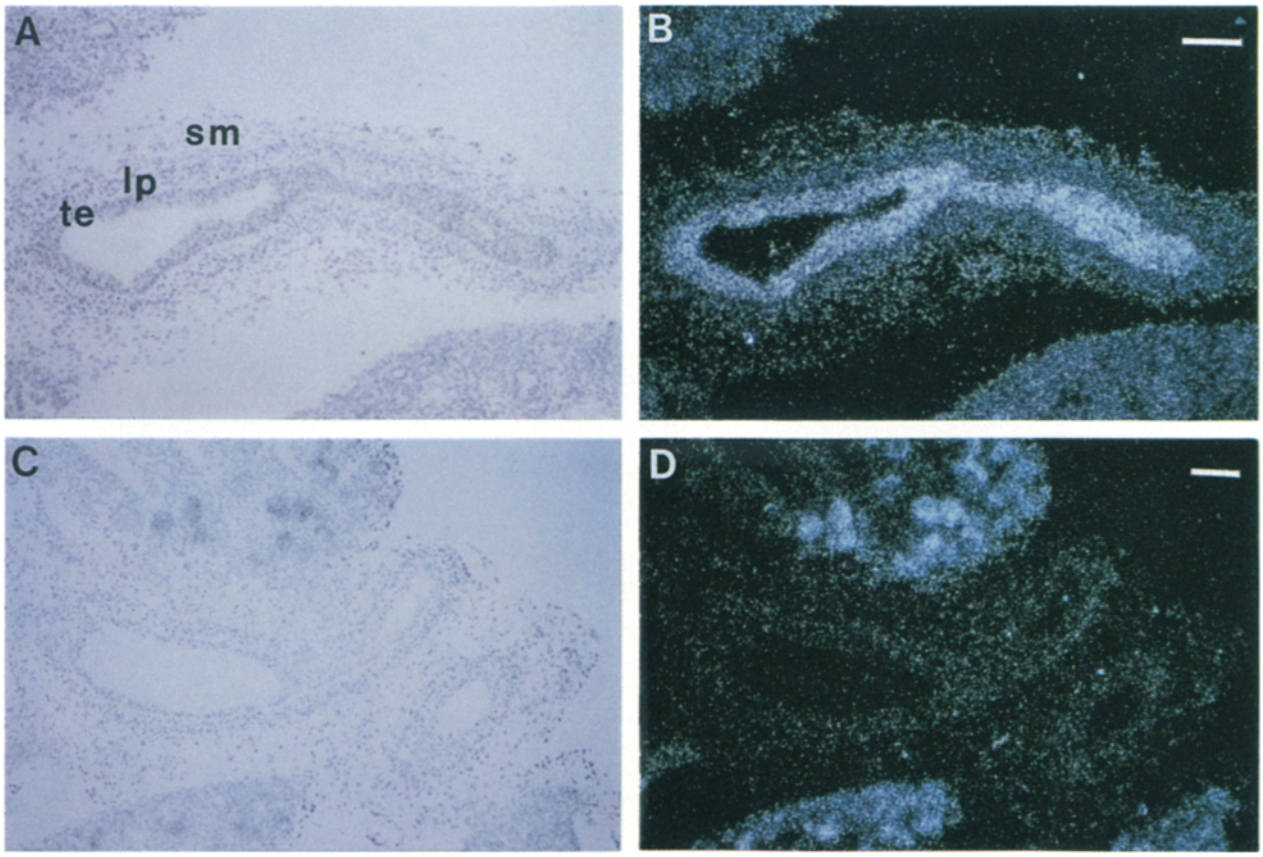

Figure 9. Expression of L-*myc* protooncogene mRNAs in the embryonic ureter. Autoradiographic exposure was 10 d for L-*myc* and 9 d for N-*myc*. Brightfield (*A* and *C*) and darkground (*B* and *D*) micrographs of cryostat sections of 16-d-old embryonic ureter hybridized in situ with antisense strand cRNA probes. (*A* and *B*) L-*myc* transcripts are clearly localized in the transitional epithelium (*te*) of the ureter. Both the lamina propria (*lp*) and the smooth muscle cell layer (*sm*) are negative for L-*myc*. (*C* and *D*) N-*myc* is not expressed in either of the three layers of the ureter. Note hybridization signals in the adjacent cortical parts of the kidney. Bars, 200 μm.

low level in the uninduced mesenchyme, but increases three to fivefold within the first day of induction of differentiation in vitro. Furthermore, mesenchyme induced for 24 h in vitro also contains cells which are still uninduced, and the increase in N-*myc* mRNA is therefore probably much higher for each individual induced area. The results obtained with in situ hybridization strongly support this view. Hence it is possible to distinguish between induced and not yet induced cells already at this stage by analyzing N-*myc* expression. Markers for epithelial cell polarization, uvomorulin (59) and the A chain of laminin and its receptor (17, 36, 57), appear some 12–24 h later in vitro, at the stage when onset of epithelial cell polarity can be detected also by morphological means. At this stage, N-*myc* mRNA level already declines somewhat, and the strong hybridization signals found by Hirvonen et al. (25) in well-differentiated tubuli and glomeruli in human embryonic kidneys are thus difficult to understand.

Of the analyzed mRNAs, N-*myc* was the only one whose expression increased in response to induction. c-*myc* was strongly expressed already before induction in the mesenchymal stem cells, and also stayed expressed longer than N-*myc*. c-*myc* was, however, more rapidly downregulated in those cells becoming stroma than in those cells that convert to epithelium. These results establish a molecularly defined difference between the undifferentiated mesenchymal stem cells and the stromal cells that surround developing epithelial cells. The finding that c-*myc* is expressed by all mesenchymal stem cells but not by the stromal cells support the view that conversion of mesenchyme to stroma, a process that is accompanied by chances in the expression of adhesion proteins (4, 35), is a true differentiation process although it can not be completely ruled out that c-*myc* expression merely reflects the mitotic activity of the mesenchymal cells. Since N-*myc* rather than c-*myc* is transiently upregulated during early tubulogenesis it might be of interest to study N-*myc* expression during experimental kidney regeneration, a process somewhat similar to tubule development in the embryo. It has been shown that c-*myc* expression is moderately altered during kidney regeneration (3, 12, 47).

In conclusion, the current data on N-*myc* and c-*myc* expression contribute to our knowledge about the development of both the epithelial and stromal cell lineage. Since the uninduced mesenchyme serves as a permanent pool of stem cells for the differentiating new kidney tubules it is not surprising that this cell population is characterized by a high mitotic activity. In addition, the formation of new kidney tubules is also accompanied by proliferation processes in the differentiating epithelial tubule cells. However, the stromal cells do not proliferate any longer in contrast to the induced mesenchymal cells that become epithelium. c-*myc* expression, which is found in the uninduced mesenchyme and in early and late differentiation stages of tubulogenesis but neither in the stromal cell compartment nor in the terminally differen-

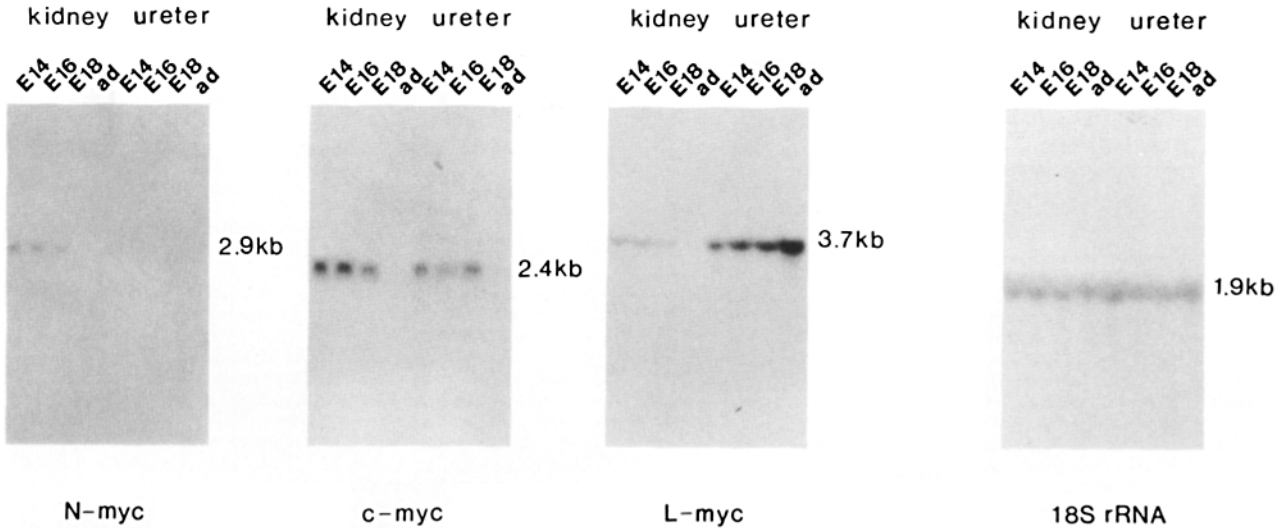

Figure 10. Northern blot analysis of *myc* family gene expression in the developing and adult ureter. Total RNA (10 μg per lane) isolated from different embryonic (*E14, E16, E18*) and adult (*ad*) ureter stages and the corresponding kidney stages were consecutively hybridized with radiolabeled DNA probes specific for N-, c-, L-*myc*, and 18S rRNA. Before rehybridization filters were stripped off the previous signals. Note that L-*myc* expression reaches the highest level in the adult stage. Exposure time was 4 d for N-*myc*, 2 d for c-*myc* and L-*myc*, and 3 h for 18S rRNA.

tiated and mitotically silent kidney tubules, may thus be related to the proliferative status of the developing cells. Therefore, in the embryonic kidney, c-*myc* expression could be involved in growth control as also suggested by Schmid et al. (52). Yet, the data for N-*myc* and especially for L-*myc* reveal that the expression of the other *myc* genes does not correlate with cell proliferation. It is true that, in the developing kidney, N-*myc* expression also coincides with proliferation but it is the onset of differentiation, rather than a burst of mitotic activity, which distinguishes the induced mesenchyme from the uninduced mesenchymal stem cells.

The most astonishing finding for L-*myc*, the third member of the *myc* family studied, was its strong expression in a large part of the structures derived from the ureter. Initially, L-*myc* expression in the developing ureter cells was noticed in sections of 16-d-old embryonic kidneys by in situ hybridization. This expression was not picked up by Northern blot experiments using whole embryonic kidneys by Zimmerman et al. (60) or by us, most likely because the ureter-derived structures make up only a small part of the kidney, and the descending ureter has been routinely removed for these types of studies. Therefore, this strong but locally restricted L-*myc* expression remained unnoticed. Surprisingly, we found that L-*myc* expression in the ureter cells continuously increased with advancing development and was highest in adult kidneys. Since the mitotic activity of the ureter cells behaves exactly inversely there is no simple relationship between L-*myc* expression and cell proliferation. The expression was initially in all parts of the collecting duct system but with progress of development, it became confined to the ureter, the urothelium of the renal pelvis and the papilla.

The expression of L-*myc* mRNA in adult kidney ureter and pelvis epithelium is interesting since there are only a few reports that protooncogenes are expressed in adult cell types. The kidney ureter epithelium is renewed continuously but at a very slow rate, and the proliferation rate is extremely low. Therefore, it seems very likely that the high expression of L-*myc* in the ureter cells is neither related to cell proliferation nor to embryonic development. In line with this finding, it was recently shown that some mitotic cells lack L-*myc* proteins (28). The expression of L-*myc* in normal adult cells is nevertheless a provocative finding since L-*myc* has been shown to transform cells (8, 15). Apparently, overexpression in some cells can be harmful if other genes are simultaneously expressed, whereas constitutive expression in another cell type with a different set of genes turned on is not. These results raise several issues about the possible role of L-*myc* in normal physiological processes. Expression of L-*myc* is not necessarily a sign of malignancy or proliferative activity. It will now be interesting to learn how many normal cell types in adult organs express L-*myc* or other genes of the *myc* protooncogene family.

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