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Expression of the helix-loop-helix protein, Id, during branching morphogenesis in the kidney

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Expression of the helix-loop-helix protein, Id, during branching morphogenesis in the kidney. Id, a member of the helix-loop-helix protein family, is an inhibitor of transcriptional activation by basic-helix-loop-helix proteins. In the developing mouse kidney, Id mRNA was observed as early as 12.5 days post-coitum (dpc) specifically in the condensed mesenchyme surrounding the ureteric buds by *in situ* hybridization. At 14.5 dpc, Id mRNA was localized to the collecting tubules and developing glomeruli while the surrounding mesenchyme lacked Id hybridization. From birth to day 10 postnatal, Id mRNA is localized to the collecting tubules, immature glomeruli and renal pelvis. In the adult kidney, Id mRNA was detectable by Northern blot analysis but no cell type-specific localization was noted by *in situ* hybridization. These results indicate a role for HLH-bHLH proteins in the differentiation of the epithelial structures of the kidney.

The basic helix-loop-helix (bHLH) proteins function as transcriptional activators for a variety of cellular processes [1, 2]. These proteins form homodimers or heterodimers via their HLH domain allowing the juxtaposed basic domains to bind to the E box consensus sequence found in many promoters [3]. For example, one of the best characterized bHLH proteins, MyoD, binds to the enhancers of numerous skeletal muscle specific genes and activates their expression [1].

Recently a related class of proteins has been identified which have HLH domains but lack the basic DNA binding domain [4, 5]. The HLH proteins can heterodimerize with bHLH proteins, but the resulting complexes are unable to bind DNA. Thus, HLH proteins are repressors of transcriptional activation by bHLH proteins [4, 6]. This relationship is best understood for the *Drosophilia* HLH protein *extramacrochaetae*. Extensive evidence has shown that this gene antagonizes the function of the bHLH protein *achaetae in vivo* and is able to repress its DNA binding activity *in vitro* [7, 8].

Unfortunately, the role of HLH proteins in mammalian development is not well understood. The first mammalian HLH protein identified, Id, has been shown to heterodimerize with the ubiquitously distributed bHLH proteins E12 and E47 [4]. In addition, overexpression of Id in tissue culture prevents myogenic and lymphoid differentiation [2, 4, 6] and the up-regulation of bHLH protein expression in lymphoid cells [2].

In order to better understand the venue of Id's function *in vivo*, we have studied the expression of this gene during embryonic development in the mouse. By Northern blot hybridization, Id was found at high levels in the embryonic kidney, liver, muscle and brain, with much lower levels seen in the adult [9]. *In situ* hybridization analysis of Id in the developing nervous system showed that Id is expressed at high levels in dividing undifferentiated neuronal precursors, while it was absent from dividing neurons which expressed differentiated traits. This indicated that Id was involved in maintaining the undifferentiated state of neurons [9] and not related to proliferation *per se*.

Since the nervous system is a highly specialized structure whose morphogenesis is distinctly different from that of many other tissues [10], it was of interest to study what role Id may play in other organs. Since Id was found at high levels in the embryonic kidney, a tissue whose development unlike the brain is mediated by epithelial/mesenchyme interactions and branching morphogenesis [11], we have further investigated the expression pattern of Id by *in situ* hybridization.

Methods

Mice

(CBA/J × C57Bl/6J) F1 female mice were placed with males of the same genotype and checked the following morning for the presence of a copulatory plug. The age of embryos resulting from these matings was calculated by designating 0.5 dpc (days post-coitum) as the day the copulatory plug was observed. The day of birth was designated as pn 0 (postnatal day 0). All animals were sacrificed either by cervical dislocation or decapitation.

Pretreatment of tissue for in situ *hybridization*

Kidneys or embryos were fixed overnight in a freshly made solution of 4% paraformaldehyde in phosphate buffered saline. The following day, they were dehydrated through graded ethanols, cleared with 1:1 toluene:ethanol and finally 100% toluene. The tissue was infiltrated with Paraplast plus with 4 × 30 minute changes, embedded and allowed to cool. After the paraffin has set, the blocks are placed in the cold room where they can be stored indefinitely. Sections, 6 μm in thickness, were prepared, floated

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out in boiled, distilled water, and mounted on 3-aminopropyl-triethoxysilane treated slides [12]. The mounted slides were baked at 39°C overnight and then stored desiccated at 4°C.

Preparation of single stranded Id mRNA probes

The full length cDNA clone used for transcription has been previously described [4]. The insert of 928 nucleotides was cloned into the SmaI and EcoRI site of Bluescript SK- (Stratagene, La Jolla, California, USA) and large scale plasmid recovery performed. To prepare the template to transcribe antisense mRNA, the plasmid was cut with BamHI, gel purified, and the DNA recovered from the agarose utilizing GeneClean (Bio101, La Jolla, California, USA). The sense strand template was prepared similarly except the Id containing plasmid was cut with EcoRI.

Two hundred and fifty microcuries of S^{35}UTP (NEN, Boston, Massachusetts, USA; 1320 Ci/mM) were evaporated to dryness in a Speed vac concentrator and transcription was initiated by adding one microgram of template DNA, 8 μl of nucleotide mix (2.5 mM each of ATP CTP, and GTP), 4 μl of 5X T3/T7 transcription buffer (BRL, Gaithersburg, Maryland, USA), 2 μl of 0.1 M dithiothreitol, 40 units of RNAsin (Promega, Madison, Wisconsin, USA), 25 units of T7 RNA polymerase (Promega) for antisense strand preparation or 25 units of T3 RNA polymerase (BRL), and diethylpyrocarbonate (DEPC) treated water to 20 μl. The reaction was placed at 37°C for one hour, then 20 units of RNAsin were added with 50 units of RNAse free DNAse (Boehringer Mannheim, Indianapolis, Indiana, USA) and incubated at 37°C for another 15 minutes. The mixture was extracted with phenol/chloroform and precipitated by adding 0.8 μl of 1 M dithiothreitol, 2 μl of 3 M sodium acetate, pH 5.2 and 50 μl of 100% ethanol. The sample was then reprecipitated twice as before [9]. The RNA was again pelleted in a microcentrifuge and redissolved in 50 μl of DEPC treated water. One μl of this solution was reserved to check transcript length on a denaturing acrylamide gel. Fifty microliters of 0.2 M sodium carbonate, pH 10.2, were added to the RNA and the sample incubated at 60°C for 55 minutes in order to hydrolyze the transcripts to an average fragment length of 150 base pairs. This reaction was precipitated by adding 3 μl of 3 M sodium acetate, pH 6.0, 5 μl of 10% acetic acid and 300 μl of ethanol and placed at −80°C for one hour. The RNA was pelleted, dried and dissolved in 100 μl of 10 mM Tris, 1 mM EDTA, pH 8.0 with 2 μl of 1 M dithiothreitol added.

In situ *hybridization*

Slides with attached sections were deparaffinized and hydrated to 0.85% saline. They were equilibrated in 1× PBS for five minutes and postfixed in 4% paraformaldehyde for 20 minutes. Following removal of the paraformaldehyde with 2× five minute PBS washes, the slides were treated for eight minutes with 20 μg/ml Proteinase K in 50 mM Tris-HCl, 5 mM EDTA, pH 7.5. The slides were again postfixed in 4% paraformaldehyde. In order to reduce background, the slides were acylated for 10 minutes with 625 μl acetic anhydride in 350 ml, 0.1 M triethanolamine, pH 8. The salts were removed from the sections with successive washes of PBS, 0.85% saline and DEPC treated water. The slides were dehydrated to 100% ethanol and allowed to air dry [9].

For prehybridization, the sections were ringed with Duco cement (Dupont), then 1 ml of prehybridization solution (4× SET, 0.02% Ficoll-400, 0.02% polyvinylpyrrolidone, 0.1% Pentex BSA (ICN, Costa Mesa, California, USA), 500 μg/ml sheared and denatured salmon sperm DNA, 600 μg/ml yeast total RNA and 50% deionized formamide) was carefully placed over the sections and allowed to sit undisturbed in a humid chamber for three hours. For hybridization, 35 ng of probe were mixed with 350 μl of deionized formamide and incubated at 80°C for one minute. After the samples were quenched on ice, 350 μl of 2× hybridization buffer (8× SET, 0.04% Ficoll-400, 0.04% polyvinylpyrrolidone, 0.2% Pentex BSA, 200 μg/ml sheared, denatured salmon sperm DNA, 200 μg/ml yeast total RNA, 20% dextran sulfate), 7 μl of 10% SDS, and 7 μl of 1 M dithiothreitol were added. Fifty microliters of this mixture was overlaid over each section, then the slides were coverslipped and placed on Whatman paper soaked with 4× SSC/50% formamide in a metal pan. The chamber was sealed with plastic wrap, then placed at 50°C for 16 to 18 hours [9].

To remove any unhybridized probe, the slides were soaked in formamide wash buffer (50% formamide, 1× SSC, 0.01 M dithiothreitol) until the cover slip floated off, then moved into formamide wash buffer at 50°C for 30 minutes. To remove the formamide, the slides were washed in 0.5× SSC for 30 minutes. The remaining unhybridized, single stranded probe was digested with 5 U/ml RNAse T1, 100 μg/ml RNAse A in 3.5 × SSC for 30 minutes at 37°C. After 2 × 10 minute washes with 3.5 × SSC, the remainder of unhybridized probe was removed with a two hour wash in 0.1× SSC. The sections were dehydrated through an increasing ethanol/0.3 M sodium acetate, pH 5.5 series and air dried [9]. The slides were exposed to B-max x-ray film (Amersham, Arlington Heights, Illinois, USA) for two days, then dipped in NTB-2 x-ray emulsion (Kodak, Rochester, New York, USA). The slides were exposed for a time based on the autoradiogram intensity, developed in D-19 developer (Kodak) and fixed in Kodak fixer. The excess fixer is washed from the emulsion with a 20 minute wash in running dH_2O, the slides were dehydrated to ethanol, lightly counterstained with hematoxylin and eosin, and mounted. The hybridization was visualized with a Wild Leitz Ortholux microscope fitted with a dark field condenser. All timepoints were repeated at least twice with tissue prepared from different animals.

Compensatory renal hypertrophy

Unilateral nephrectomy or sham operations were performed on 12-week-old male mice under avertin anesthesia [13]. RNA was prepared from the remnant and control kidneys at 3, 6, 12, 18, 24 and 36 hours following surgery by the guanidium isothiocyanate/cesium chloride method as previously described [9]. Northern blots were performed with 10 μg of total RNA, and probed with the full length Id cDNA. All time points were analyzed in duplicate with RNA prepared from different animals.

Kidney regeneration following acute renal failure

Acute renal failure was induced by injecting 12-week-old male mice i.p. with 250 mg/kg body weight of folic acid in 150 mM $NaHCO_3$. Control animals were injected with comparable volumes of 150 mM $NaHCO_3$ [14]. RNA was prepared from the contralateral kidney at 1, 3, 6, 12, 18 and 24 hours following injection. Northern blots were performed with 10 μg of total RNA, and probed with the full length Id cDNA. All time points were analyzed in duplicate with RNA prepared from different animals.

Results

In order to understand the role of Id in the important developmental processes of epithelial/mesenchyme interactions and branching morphogenesis, *in situ* hybridization analysis of Id was performed on the developing mouse kidney. At 12.5 days postcoitum (dpc), Id expression was seen in the condensed mesenchyme surrounding the ureteric buds (Fig. 1 a, b), while the bifurcating ducts themselves exhibited little hybridization signal. In addition, the epithelial lining of the the bladder (Fig. 1 c, d) expressed Id mRNA at higher levels than the surrounding genital eminence. By 14.5 dpc, high levels of Id mRNA were seen in the developing collecting tubules derived from the ingrowth of the ureteric bud into the metanephric mesenchyme (Fig. 2 a, c), while the uninduced mesenchyme which differentiated into connective tissue exhibited little to no hybridization signal. S-shaped bodies expressed Id at appreciable levels in the flattened epithelial layer which gave rise to Bowman's capsule (Fig. 2d). More mature glomeruli lacked expression in Bowman's capsule; however, significant expression was observed over the region of the glomerular tuft (Fig. 2e). The condensed mesenchyme of the metanephric blastema still expressed Id at high levels (data not shown) when compared to the uncondensed mesenchyme which developed into connective tissue (Fig. 2 a, b, c).

By birth, in the mouse, most of the structures of the adult kidney were recognizable, although they were not structurally complete [15]. The renal pelvic mucosa and collecting tubules of the newborn kidney were strongly positive for Id mRNA; however, the morphologically-mature proximal convoluted tubules did not exhibit Id expression (Fig. 3 a, b, f). In the cortical regions of the kidney, a layer of cells situated several cell layers beneath the cortical surface expressed Id mRNA at higher levels than the cells at the cortical surface (Fig. 3d). Since these cells are so densely packed, it was difficult to ascertain whether epithelial, mesenchymal or both cell types were expressing Id. In general, silver grains were detected over the region of the glomerular tuft at birth; however there was heterogeneity observed in that some glomeruli lacked silver grains (Fig. 3e). The non-expressing glomeruli appear more morphologically mature which is consistent with the observation that little to no localized hybridization signal is observable in the kidney after 14 days postnatal (data not shown). In the ureter, the basal and intermediate cells of the transitional epithelia expressed Id at much higher levels than the surrounding smooth muscle layers (Fig. 3c).

By postnatal day (pn) 6, the Id hybridization signal was considerably weaker in the kidney. However, localized expression was still easily detectable in the pelvic mucosa, outer and inner medullary collecting tubules and deep and superficial cortical glomeruli (data not shown). By pn 10, only extremely weak signals are detectable, mostly in the collecting tubules (data not shown) and the cell type specific hybridization disappeared entirely by pn 14. It should be noted that although appreciable amounts of Id mRNA are detectable in the adult kidney by Northern blot hybridization, the levels are at least 10-fold lower than that observed in the 14 dpc kidney [9]. The lack of specific hybridization signal in the kidney after 14 pn is most likely attributable to low levels of basal expression throughout the kidney. By Northern blot hybridization, no increase in Id mRNA was found associated with either compensatory hypertrophy (Fig. 4a) or folic acid induced kidney damage (Fig. 4b) in adult kidneys from 30 minutes to 36 hours after treatment.

Discussion

Our previous study on Id mRNA expression in the brain indicated that Id plays a role in the maintenance of the undifferentiated state of developing neurons. However, by Northern blot hybridization, Id mRNA was found at appreciable levels in a number of other organ primordia [9]. Since the kidney is derived from a different cell lineage than the nervous system [11], it was of interest to further characterize the expression of Id during kidney development.

The mechanisms of kidney development are well understood at the morphological level. In mammals, the precursor to the adult kidney, the metanephros, is composed of an undifferentiated mesenchyme which is induced to epithelize by the ingrowth of the ureteric bud. This epithelization produces the structures of the functional kidney including the glomeruli and accessory structures [11]. At all stages of development studied, Id mRNA was found at high levels in immature epithelial structures derived from epithelized mesenchyme and/or the ureteric bud; however, no hybridization was observed in stromal cells or their precursors, the uninduced mesenchyme [16].

By Northern blot hybridization, the 14 dpc kidney expressed Id at high levels which progressively decreased during development to the low levels seen in the adult [9]. By *in situ* hybridization, expression in the 14 dpc kidney was localized to the newly formed epithelial structures of the kidney, including the developing glomeruli and renal tubules. At birth, renal tubules are morphologically different from their adult counterparts, possessing simple mitochondria, an abundance of cytoplasmic granules and lipid inclusions [15]. Interestingly, renal tubules acquire their adult morphology coincident with the disappearance of Id expression at 14 dpn. This paradigm is also seen in glomeruli; immature glomeruli found in the peripheral layers of the newborn kidney exhibit high levels of Id mRNA and as these glomeruli mature, mRNA levels decline. However, an appreciable number of glomeruli lacked hybridization signal. This observation may indicate that the morphologically similar glomeruli are heterogeneous with respect to their differentiation state and/or molecular identity. It is interesting to note that such molecular heterogeneity has been observed in apparently morphologically identical kidney tubules [17]. The sharp decline of the Id hybridization signal observed for kidney RNA in Northern blots between birth and 4 weeks of age [9] is consistent with the disapearence of detectable Id transcripts after 14 dpn by *in situ* hybridization.

Similarly, Id mRNA was found in the basal and intermediate cells of the transitional epithelium of the ureter while it was absent from the superficial layer. The basal layer is the proliferative population of the transitional epithelium which produces cells of the intermediate layer. As the superficial layer is sloughed into the lumen of the ureter, it is replaced by the differentiation of the intermediate cells [18]. When the ureter is fully developed, the basal layer becomes relatively quiescent, only proliferating after chemical or mechanical damage to the mucosa. The observation that Id mRNA was absent from the most morphologically differentiated epithelia of both the kidney and ureter indicates that Id may play a role in the terminal differentiation of epithelial structures in the urogenital system.

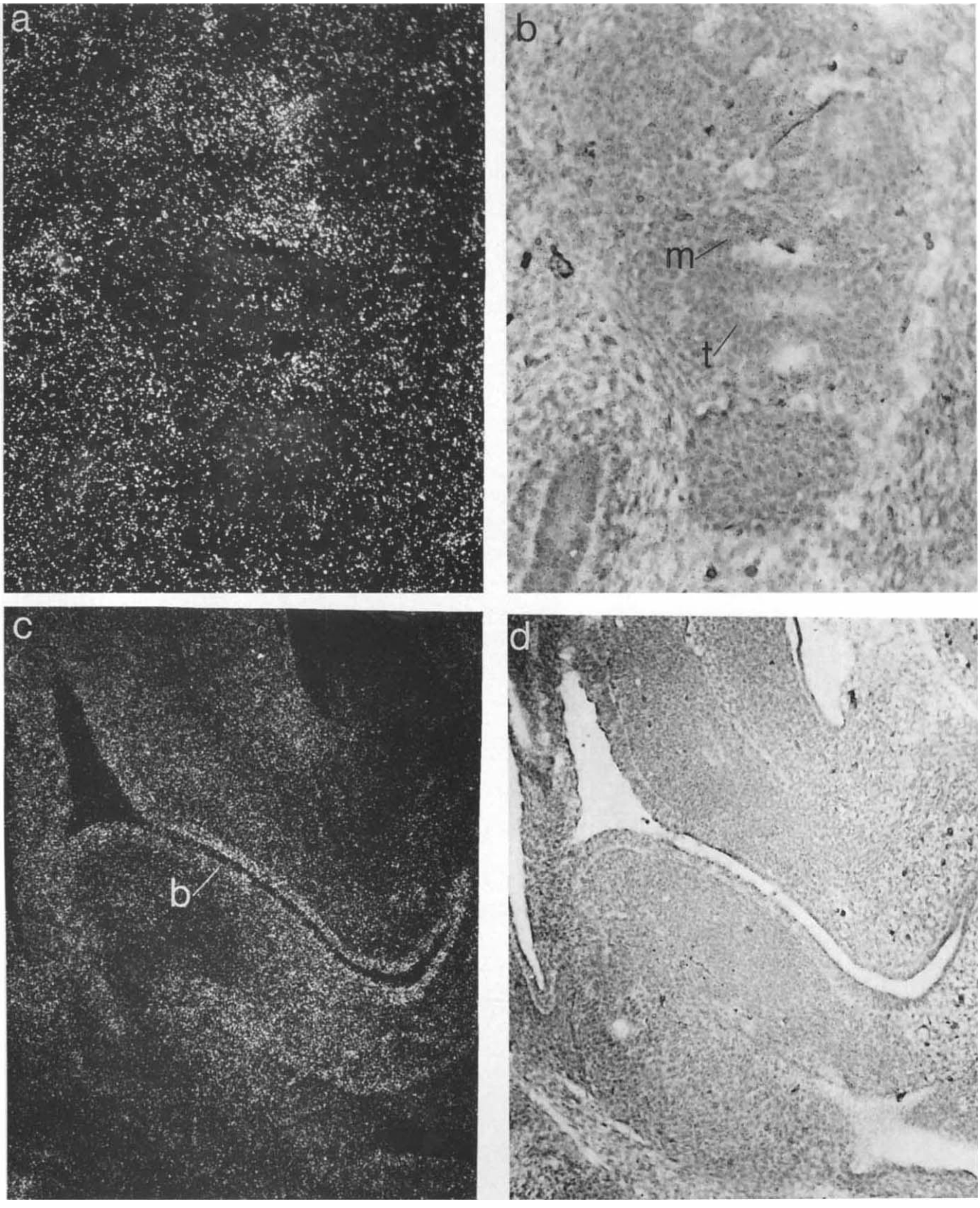


Fig. 1. In situ *hybridization analysis of Id mRNA to sagital sections through the excretory system of a 12.5 dpc mouse embryo.* **a.** Darkfield photomicrograph showing Id mRNA localized to mesenchymal condensations of the developing metanephros and not the bifurcating ducts (200×). **b.** Brightfield photomicrograph of a section adjacent to *a* stained with hematoxylin and eosin (200×). **c.** Darkfield photomicrograph showing Id transcripts localized to the lining of the developing bladder (100×). **d.** Brightfield photomicrograph of the same section as *c* lightly stained with hematoxylin and eosin (100×). Abbreviations are: M, metanephric blastema; B, lining of the bladder; t, bifurcating duct.

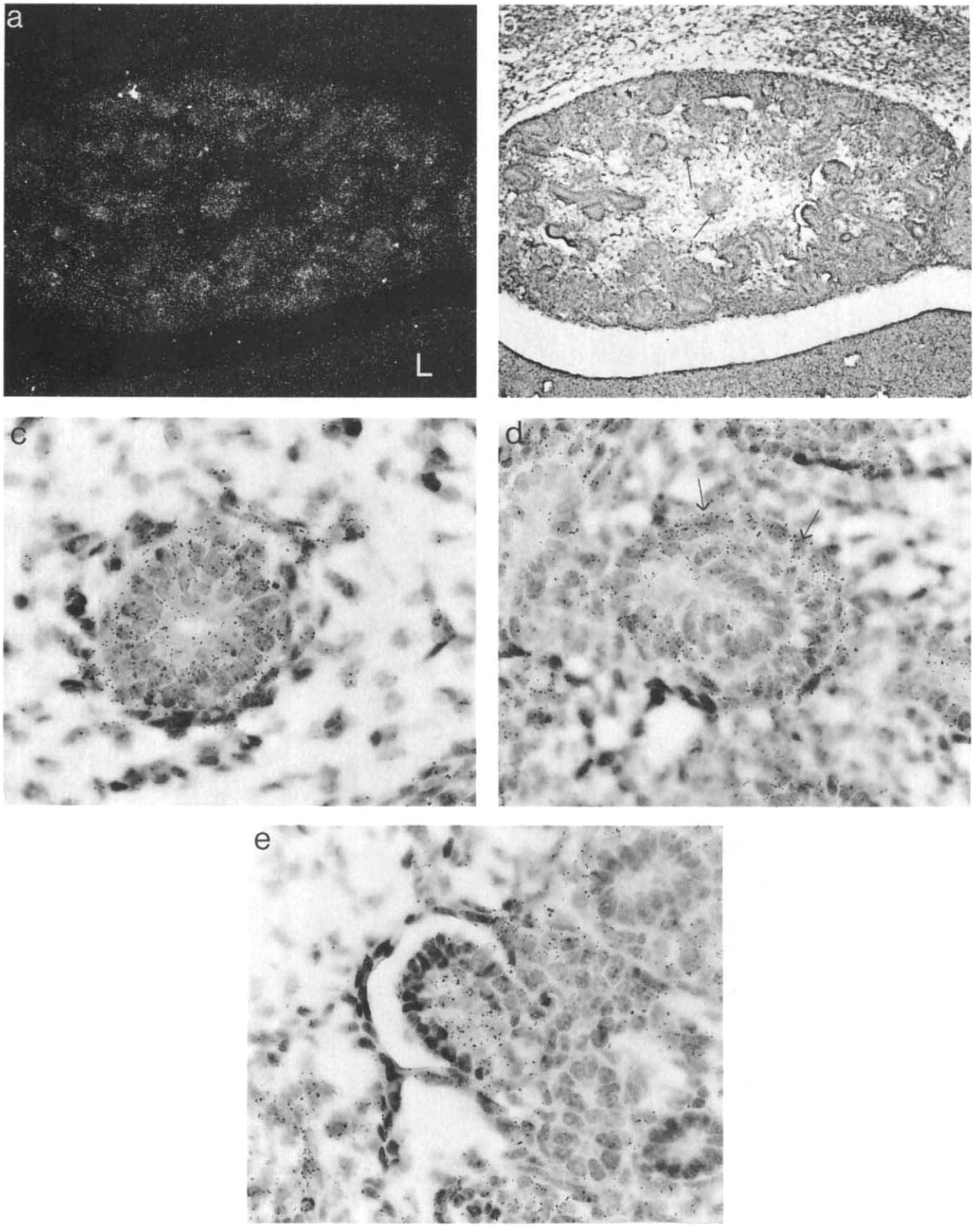
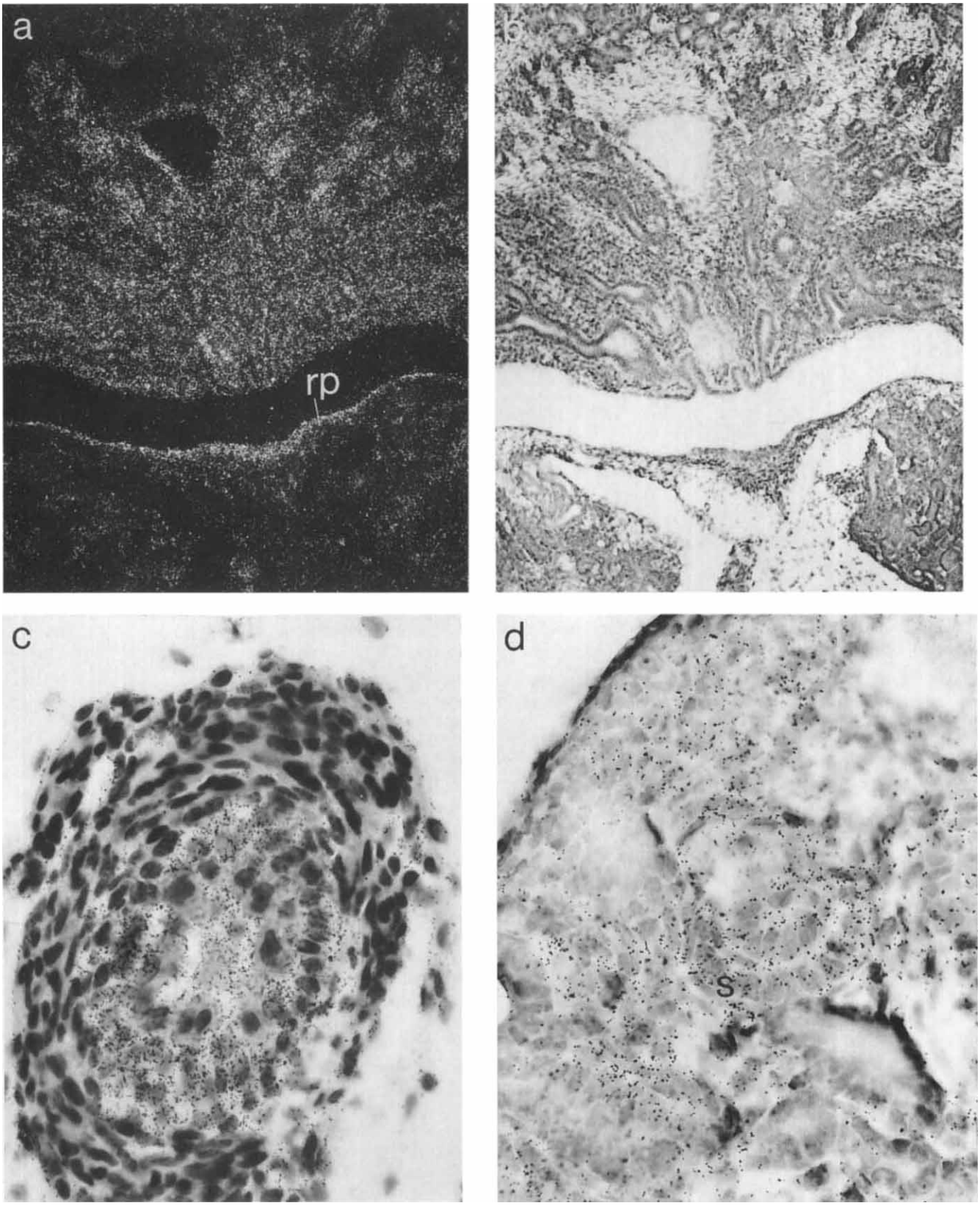


Fig. 2. In situ *hybridization analysis of Id mRNA in the 14.5 dpc mouse kidney.* **a.** Darkfield photomicrograph of a sagital section from a 14.5 dpc mouse kidney hybridized to the antisense strand of Id mRNA (100×). **b.** Brightfield photomicrograph of the same section shown in *a* stained with hematoxylin and eosin, arrows point to developing tubules (100×). **c.** Id localization over epithelial cells in a tubular arrangement surrounded by nonexpressing connective tissue (630×). **d.** Expression of Id mRNA in a S-shaped body of a 14.5 dpc kidney. Note the labeling over the precursor cells to Bowman's capsule (arrowheads) (630×). **e.** Expression of Id in a morphologically distinct glomerulus of a 14.5 dpc kidney. Note the labeling over the region of the glomerular tuft (630×). Abbreviation is L, liver.



In the early embryo, Id was not found in embryonic tissues prior to gastrulation, while after gastrulation it was found in all germ layers. Only later does its expression restrict to certain cell types [19]. Since Id expression was found in embryonic mesoderm [19], turned off in the undifferentiated mesenchyme, then turned on again in mesenchyme induced to epithelialize, it appears that Id

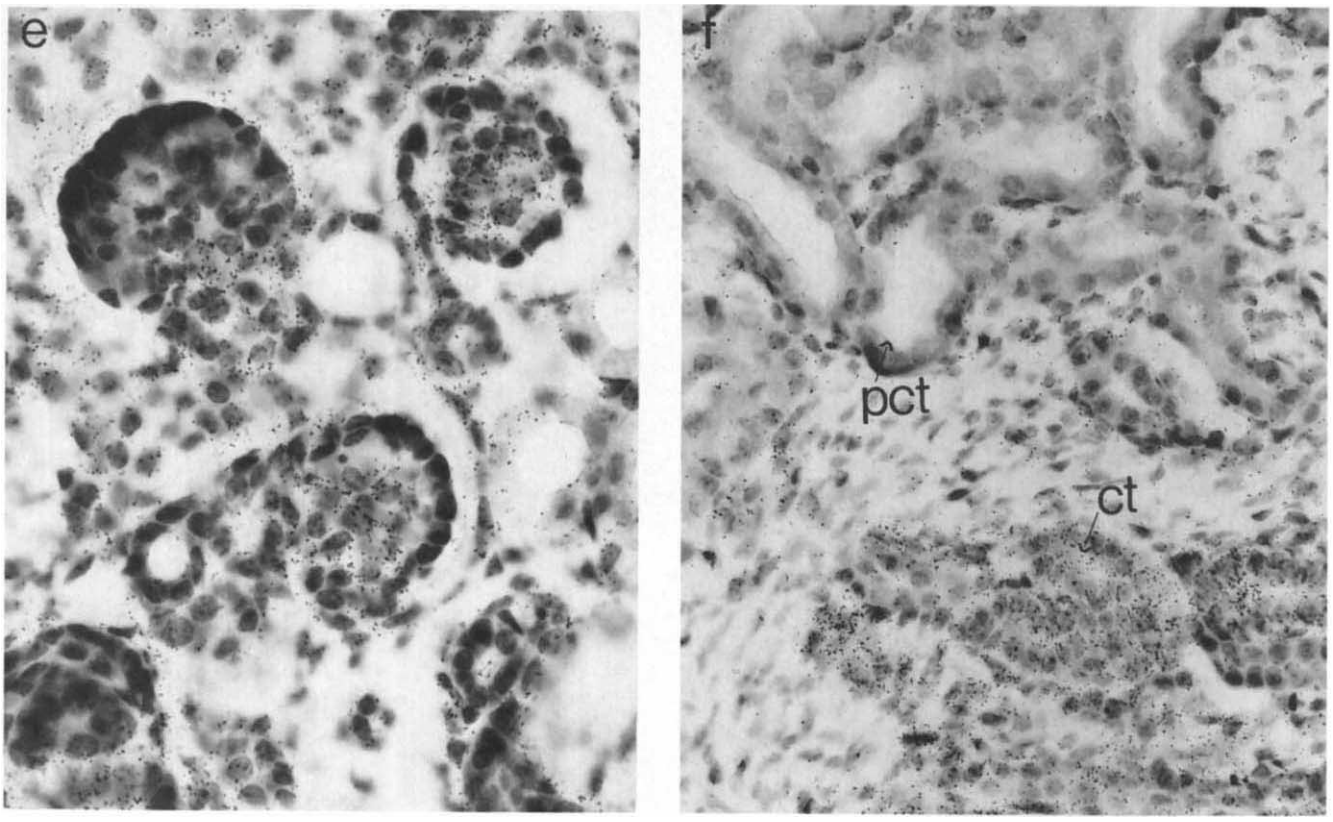


Fig. 3. In situ *hybridization analysis of Id mRNA in the newborn mouse kidney.* **a.** Darkfield photomicrograph of a frontal section from a newborn mouse kidney hybridized to the antisense strand of Id mRNA. The lining of the renal pelvis and collecting tubules are strongly positive for Id hybridization (100×). **b.** Brightfield photomicrograph of the section shown in *a* lightly stained with hematoxylin and eosin (100×). **c.** Brightfield photomicrograph of the ureter from a newborn mouse hybridized to the antisense strand of Id then stained with hematoxylin and eosin. Note the labeling over the transitional epithelial layer of the ureter (630×). **d.** Brightfield photomicrograph of the kidney cortex and capsule of a newborn mouse. Note the intense labeling over the condensed mesenchyme and S-shaped body. The labeling is much stronger in the deeper layers of the cortex while few silver grains are observed in the layers of mesenchyme nearest to the capsule (630×). **e.** Brightfield photomicrograph of a cluster of glomeruli found in the cortex of a newborn mouse kidney. The morphologically less mature glomeruli express appreciable amounts of Id mRNA in the intraglomerular mesangial region (630×). **f.** Bright field photomicrograph of the corticomedullary region of a newborn mouse kidney showing intense Id labeling over the collecting tubules while the proximal convoluted tubules exhibited little or no hybridization signal (400×). Abbreviations are: ct, collecting tubules; rp, renal pelvis; pct, proximal convoluted tubules.

has distinct functions at different times during development in a particular cell lineage.

Id mRNA was found in kidney structures which are partially differentiated by both functional and morphological criteria. Unfortunately, even though a wide variety of differentiation specific genes have been localized to the adult kidney, the expression pattern of relatively few of these genes has been characterized during development. It should be noted that some of the genes expressed at high levels in the adult kidney such as αB crystallin are up-regulated as Id expression is turned off [20]. Interestingly, the promoter of αB crystallin contains an E box which has been shown to function as an enhancer [21]. Since E boxes are the *cis* elements that bHLH proteins bind [1], it is plausible that Id influences the expression of αB crystallin in kidney cells.

Id mRNA levels were not affected by either compensatory hypertrophy or damage induced regeneration. During compensatory hypertrophy, the increase in renal mass is predominatly due to the production of more cytoplasm by pre-existing cells [22]. Initially, it was surprizing that Id was not up-regulated during folic-acid induced kidney regeneration. In contrast to compensatory hypertrophy, the damaged tubule cells begin to divide and express a variety of proliferation specific proteins [14]. It is possible that these dividing cells do not dedifferentiate significantly during this process. Indeed, vimentin, a marker for undifferentiated renal tubules, is not found in tubules recovering from folic acid induced damage [23].

Id was localized to a variety of structures in the developing kidney including both the mitotically active developing glomerulus and the relatively quiescent renal pelvis. Id appears to play a role shortly after the epithelial-mesenchyme transition since its expression first arises shortly after the induction of epithelial structures. Even though the cellular localization of bHLH transcription factors has not been demonstrated in the kidney, the localization of Id suggests a role for the HLH-bHLH protein family in the process of terminal differentiation of the kidney. Interestingly, mRNA for a HLH protein Heir-1 and a bHLH protein, REB have also been found at high levels in the adult rat kidney by Northern analysis further suggesting a role for this important family of regulatory molecules in the kidney [24, 25].

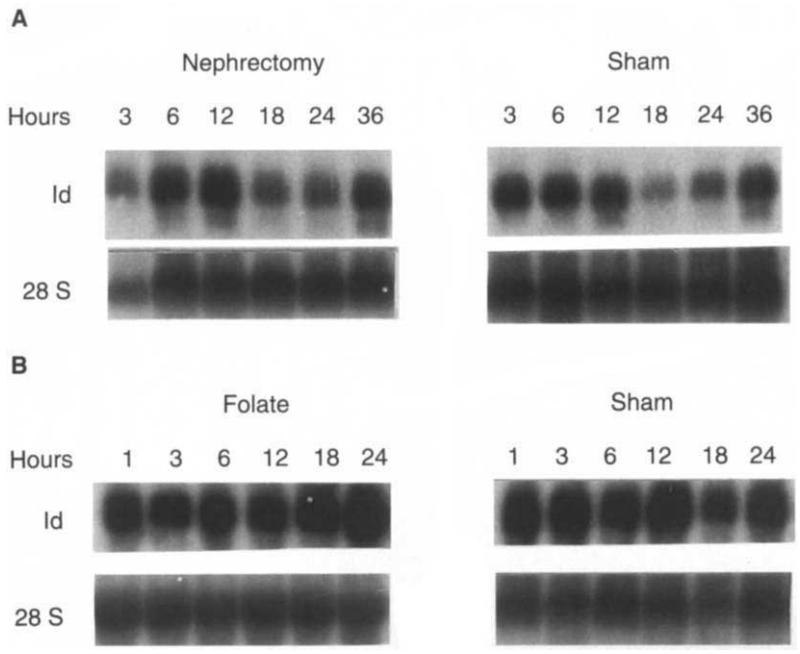


Fig. 4. A. *Northern blot hybridization analysis of RNA obtained from animals experiencing renal hypertrophy following unilateral nephrectomy or sham operation.* **B.** Northern blot analysis of RNA obtained from the kidney of animals either injected with folic acid or the sodium carbonate vehicle.

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