Original Article

Expression pattern of the rat *Lim-1* homeobox gene suggests a dual role during kidney development

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ABSTRACT This study describes an *in situ* hybridization and immunohistochemical analysis of *Lim-1* homeobox gene expression during kidney development in the rat. *Lim-1* is expressed at all stages of mesonephric and metanephric kidney development. In the metanephros, *Lim-1* gene mRNA is first found at day 13 in the ureteric bud, but not in uninduced mesenchyme. Expression in the mesenchyme can be seen only after mesenchymal cells have condensed around the ureteric bud tips and primary vesicles have formed. Experiments with mesenchymal explants induced to differentiate *in vitro* by high levels of basic FGF in the absence of ureteric bud also indicate that *Lim-1* expression is correlated with tubulogenesis and this experimental model faithfully reproduces its expression *in vivo*. During mesenchymal differentiation Lim-1 protein and mRNA were found in comma- and S-shaped bodies, proximal and distal tubules, and collecting ducts. *Lim-1* mRNA and Lim-1 protein were seen transiently at early stages of glomerulus formation. In the fully differentiated kidney *Lim-1* gene products disappear from mesenchymal derivatives but persist in the collecting ducts which are derived from the ureteric bud. These data suggest a dual role for the *Lim-1* homeobox gene in the developing kidney, a transient developmental function in the mesenchyme and a maintenance function in the ureteric bud and its derivatives. Further we suggest that *Lim-1* is not directly involved in mesenchymal induction but may participate in its epithelial transformation at later stages as its expression in mesenchyme begins only after the formation of primary vesicle.

KEY WORDS: *Lim-1, kidney, epithelial-mesenchymal transformation, tubulogenesis*

Introduction

The developing rat kidney provides a useful model system for studying numerous problems in developmental biology (for review, see Bard *et al.*, 1994; Patterson and Dressler, 1994; Lechner and Dressler, 1997). A variety of genes has been implicated in kidney development and differentiation, and some of these have been shown by gene disruption techniques to be essential for metanephric kidney development. The latter group includes *WT-1* (Kreidberg *et al.*, 1993), *c-ret* (Schuchardt *et al.*, 1994), *wnt-4* (Stark *et al.*, 1994), *Lim-1* (Shawlot and Behringer, 1995), *BMP-7* (Dudley *et al.*, 1995; Luo *et al.*, 1995), *Pax-2* (Torres *et al.*, 1995), *BF-2* (Hatini *et al.*, 1996), *GDNF* (Durbec *et al.*, 1996; Moore *et al.*, 1996; Pichel *et al.*, 1996; Sanchez *et al.*, 1996) and integrin α8B1 (Muller *et al.*, 1997). Of these, knock-outs of *WT-1*, *c-ret* and its ligand *GDNF*, *Lim-1*, and *Pax-2* are characterized by the complete absence of metanephric kidney.

Rat Lim-1 protein (Furuyama *et al.*, 1994) (named Lhx-1 in the mouse) belongs to the family of LIM homeodomain proteins which are thought to be involved in cell fate specification in many developmental situations (for review see Sanchéz-Garcìa and Rabbits, 1994; Dawid *et al.*, 1995). The *Xlim-1* gene in *Xenopus* is expressed in the Spemann organizer during the gastrula stage, then transiently in the notochord, and in late embryos and adults primarily in the CNS and the kidney (Taira *et al.*, 1992,1994; Karavanov *et al.*, 1996). A similar pattern of expression occurs in the mouse (Barnes *et al.*, 1994; Fujii *et al.*, 1994; Karavanov *et al.*, 1996). Mice carrying a disruption of the *Lim-1* gene (Shawlot and Behringer, 1995) fail to develop the head and die early in embryo-

Abbreviations used in this paper: BMP, bone morphogenetic protein; CNS, central nervous system; EGF, epidermal growth factor; FGF, fibroblast growth factor; GDNF, glial cell-derived growth factor.

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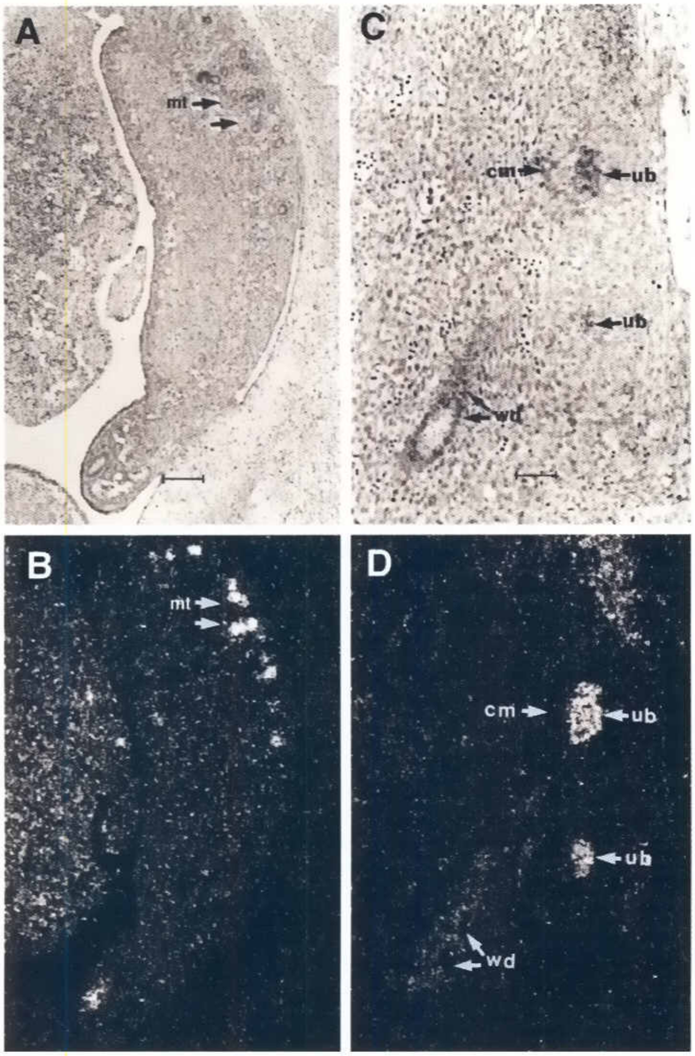

Fig. 1. ***Lim-1*** **gene expression in mesonephros and early metanephros.** *Sections were analyzed by in situ hybridization.* **(A and B)** *Expression in mesonephros 13 d.p.c.;* **(C and D)** *Expression in metanephros at 13.5 d.p.c. cm, condensing mesenchyme; mt, mesonephric tubules; ub, ureteric bud tips; wd, Wolffian duct. A, C- bright field; B, D-dark field. Bars, A and C, 100 µm; B and D, 50 µm.*

genesis, but the very few pups that survived through gestation to being stillborn also lack metanephric kidneys and gonads in addition to anterior head structures. These results indicated that the *Lim-1* gene is required for kidney development.

The aim of the current study is to analyze the essential role of *Lim-1* in kidney development in more detail. We show that *Lim-1* expression is transient in the mesenchyme and its derivatives but is continuous in the ureteric bud and its derivatives. These results imply distinct roles for the *Lim-1* gene in these two main interacting tissues which determine the process of kidney development. Since *Lim-1* transcripts first appear in the mesenchyme when primary vesicles have fully formed, we suggest that *Lim-1* function is not required in the responding tissue for the induction of the mesenchyme. In contrast, the expression of *Lim-1* during FGF-induced tubulogenesis *in vitro* suggests a role for *Lim-1* in this process.

Results

Lim-1 gene expression in kidney development in vivo

To investigate the role of *Lim-1* in kidney development we analyzed the expression of this gene by *in situ* hybridization and immunocytochemistry in the rat embryo. At 13.5 d.p.c., *Lim-1* RNA was detected in all mesonephric tubules (Fig. 1A,B), the mesonephric (Wolffian) duct, and the ureteric bud of the metanephric kidney (Fig. 1C,D). The process of induction has just begun since the first condensations of mesenchymal cells surrounding the two tips of the ureteric bud are apparent (Fig. 1C). At this stage, Lim-1 RNA is not detected in the mesenchymal cell population (Fig. 1D).

The expression of the *Lim-1* gene in the mesenchyme can be seen for the first time in fully condensed groups of cells that form primary vesicles around the tips of the ureteric bud (Fig. 2A,B, 15.5 d.p.c., and 2C,D, 16.5 d.p.c.). The *Lim-1* gene is also strongly expressed in comma- and S-shaped bodies that develop from the mesenchymal cell condensates (Fig. 2C,D). In addition, *Lim-1* RNA continues to be present in the epithelial cells of the ureteric bud, but does not accumulate to as high a level in that tissue as in the mesenchymal derivatives. *Lim-1* expression could also be observed during early stages in the formation of the glomerulus (Fig. 2C,D).

The process of kidney development is dynamic and repetitive in that differentiation continues in the outer cortical layer as the metanephric kidney grows, while terminally differentiated structures are forming under this peripheral differentiating layer. In the uppermost cortex, the differentiation process repeats itself progressing through induction, condensation of mesenchymal cells, formation of primary vesicles, comma- and S-shaped bodies, and finally the generation of glomeruli and proximal and distal tubules. These terminally differentiated structures constitute the nephron and collecting ducts which form the functional units of the mature kidney (Saxén, 1987). Of these structures, only the collecting ducts are derivatives of the ureteric bud while all other structures are of mesenchymal origin.

We studied the expression of *Lim-1* RNA and protein during later kidney development at 19 d.p.c. and also at postnatal day 7 by *in situ* hybridization and immunohistochemistry. A clear difference was apparent in the expression of *Lim-1* between the cortical layer and the medulla in 19 d.p.c. kidneys. Both *Lim-1* RNA and the corresponding protein were seen in all the mesenchyme-derived epithelial structures as well as in the tubules in the cortical layer where differentiation occurs actively at this stage. In contrast, *Lim-1* expression in the medulla was seen only in the epithelial cells of the collecting ducts (Fig. 3A-F). A similar result was obtained by immunostaining of postnatal kidney (day-7), where pronounced *Lim-1* expression was seen in collecting ducts but much weaker staining in the thin, still differentiating cortical layer (Fig. 3G); in the rat, kidney differentiation proceeds until postnatal day 10 (Wachstein and Bradshaw, 1965). Furthermore, Lim-1 protein expression in the proximal and distal tubules showed a distinct gradient from cortex to medulla, with the highest number of positive cells close to the cortical layer and complete disappearance of *Lim-1* in the fully developed medulla, except for its continued presence in the collecting ducts (Fig. 3E,F).

Metanephric kidney development and differentiation is governed by the exchange of signals between the ureteric bud and mesenchymal cells. To summarize complex events briefly, the ureteric bud maintains and induces mesenchyme to form tubules

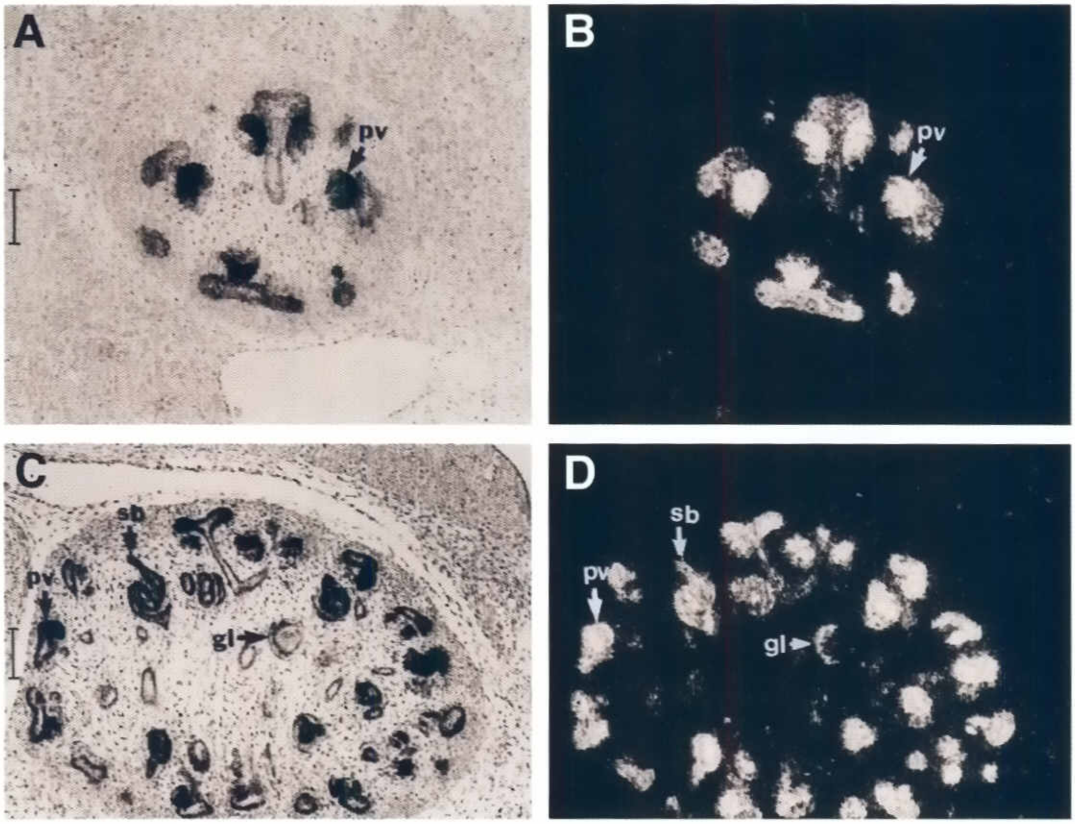

Fig. 2. Expression of the *Lim-1* gene in induced metanephric mesenchyme. *In situ hybridizations are shown of,* **(A and B)** *15.5 d.p.c. kidney;* **(C and D)** *16.5 d.p.c. kidney. A and C, bright field; B and D, dark field. pv, primary vesicle; sb, S-shape body; gl, glomerulus. Bars, 100 μm.*

while the latter send signals to the ureteric bud to induce branching. The data shown above suggest that *Lim-1* gene expression in kidney mesenchyme is not an early or direct consequence of primary ureteric bud signaling but is a late event in tubulogenesis.

Lim-1 expression in mesenchymal explants in vitro

To further investigate the relationship between mesenchymal differentiation and *Lim-1* expression, we examined these events in mesenchymal explants induced *in vitro* by cultivation in the presence of high levels of basic FGF. Under these conditions, mesenchyme maintains its condensed state and, after 12 days in culture, begins forming primitive tubules to a limited extent in the absence of ureteric bud cells (Perantoni *et al.*, 1995).

As shown in Figure 4, expression of *Lim-1* RNA (Fig. 4A,B) and protein (Fig. 4E) was found in the primitive epithelia of forming tubules on day 12 of culture in the presence of basic FGF, whereas uninduced mesenchyme at day 7 (Fig. 4C,D) of culture or earlier (data not shown) did not stain with anti-Lim-1 antibody. These observations indicate that *Lim-1* expression is correlated with tubulogenesis but cannot be detected in the mesenchyme at earlier stages of development. Furthermore, the presence of the ureteric bud is not required for the induction of *Lim-1* gene expression in the mesenchyme. Conditions that allow tubulogenesis favor the expression of *Lim-1* whether or not ureteric bud cells are present.

Discussion

Kidney development is distinguished by the sequential formation of three structures of putatively equivalent function, the pro-, meso- and metanephros. While these organs differ morphologically, their basic organization exhibits important similarities. The relationship between the successive kidneys has become clearer in the recent past when it was discovered that many of the same regulatory molecules are involved in the development of each kidney. The *Lim-1* gene is a good example of this repeated use. Studies in *Xenopus* (Taira *et al.*, 1994) and in zebrafish (Toyama and Dawid, 1997) have shown that *Lim-1* is strongly expressed in the pronephros and pronephric duct. In particular, the analysis in *Xenopus* showed that *Xlim-1* marks the lateral mesoderm well before the earliest pronephric anlage appears (Taira *et al.*, 1994). The *Xlim-1* gene is also expressed in the mesonephros of the adult frog (Taira *et al.*, 1992) and in the meso- and metanephros of the mouse (Fujii *et al.*, 1994). However, these studies did not address the question of which cell types or structures within the kidney express *Lim-1*.

The nature of the cell types expressing the *Lim-1* gene and the timing of its expression within the differentiation of the kidney is most effectively studied during development of the mammalian metanephros. The fully differentiated mammalian kidney is a complex structure composed of identical functional units, the nephrons. Each such unit comprises approximately 12 different cell types (Gilbert, 1994) which appear to constitute the final result of interactions between only four stem cell types, epithelial cells of the ureteric bud, loose mesenchymal cells, primary neuroblasts and stromal cells (Saxén, 1987; Sainio *et al.*, 1994). These four cell types interact in the main event in the differentiation of the metanephric kidney, the mesenchyme-to-epithelium transition. A number of regulatory molecules are involved in these events, as

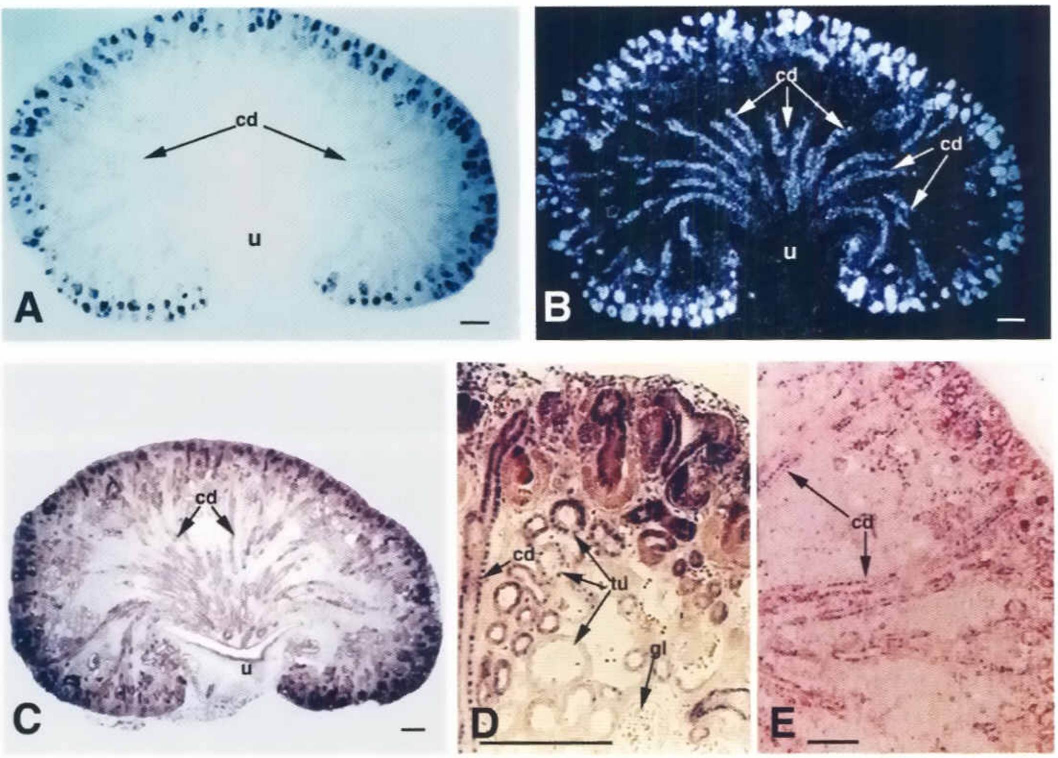

Fig. 3. *Lim-1* gene expression at advanced stages of kidney development. (A and B) *In situ hybrydization of kidney sections from 19 d.p.c. kidney;* **(C and D)** *immunostaining of Lim-1 protein in kidneys at 19 d.p.c. and at postnatal day 7* **(E)**. *tu, tubules; cd, collecting ducts; gl, glomerulus; u, urether. Bars, 200 μm.*

reviewed by Bard *et al.* (1994) and Lechner and Dressler (1997); the list of this factors is continually updated in the Kidney Development Database on the World Wide Web by A.J. Davies and A.W. Brändli. While more than 50 regulatory genes have been implicated in kidney development, only some of them have been shown by targeted mutation in the mouse to be indispensable for kidney formation. Some indispensable genes encode transcription factors, such as, *WT-1* (Kreidberg *et al.*, 1993), *Pax-2* (Torres *et al.*, 1995), and *Lim-1* (Shawlot and Behringer, 1995).

The observations presented in the present study suggest a temporal hierarchy in the activity of two of these genes, *Pax-2* and *Lim-1*, in methanephric mesenchyme. In metanephric kidney development, *Pax-2* gene expression is first detected immediately after induction in the early mesenchymal condensates surrounding the branching ureteric bud tips (Dressler *et al.*, 1990). We find the earliest expression of the *Lim-1* gene in the mesenchymal derivatives at a later stage, after primary vesicles have fully formed from these condensates (Figs. 1C,D and 2A,B). These observations suggest that *Lim-1* is downstream of *Pax-2* in the regulatory events in mesenchymal differentiation in the development of the metanephros. Additional support for this conclusion comes from data on *Pax-2* expression in *Lim-1* knockout mice since such embryos at 9.5 d.p.c. were positive for *Pax-2* antibody staining in the mesonephric region (Shawlot and Behringer, personal communication).

Our analysis further shows that *Lim-1* expression is transient in the mesenchymal derivatives of the metanephros (Fig. 3A,B,F,G) whereas it is persistently expressed in the collecting ducts, the derivatives of ureteric bud. In the mesenchymal derivatives, Lim-1 expression was found in differentiating epithelial structures, but both the corresponding RNA and protein disappeared from the epithelial cells of the proximal and distal tubules and the glomeruli as these structures become fully differentiated (Fig. 3F,G). This transient expression in mesenchymal derivatives as opposed to persistent expression in collecting ducts implies two different roles for *Lim-1* in kidney differentiation. We suggest that *Lim-1* participates in maintenance processes in the ureteric bud and collecting ducts. In the mesenchyme, the beginning of its expression only in fully formed primary vesicle (Figs. 1C,D and 2A,B) exclude its participation in early inductive processes in mesenchyme and suggests for it a transient role during mesenchyme differentiation. Here it may be involved in regulation of later events of mesenchymal-epithelial transformation including epithelial differentiation. While *Lim-1* is expressed continuously in the collecting ducts, it is repressed in another ureteric bud derivative, the ureter (Fig. 3B,D).

In this respect *Lim-1* differs from *Pax-2* which is required in both early and late processes of mesenchymal-epithelial transformation (Rothenpieler and Dressler, 1993). Observations made with the aid of mesenchyme explants that differentiate *in vitro* under the influence of high levels of basic FGF (Perantoni *et al.*, 1995) support this conclusion by showing that *Lim-1* expression is characteristic of the differentiation of tubule epithelium under these conditions, as it is *in vivo* (Fig. 4).

In conclusion, the data presented in this paper implicate the *Lim-1* gene in a dual role in two distinct epithelial components of the developing metanephric kidney, a possible maintenance role in the ureteric bud derivatives, and a transient developmental role in mesenchymal structures that is limited to periods of active differentiation.

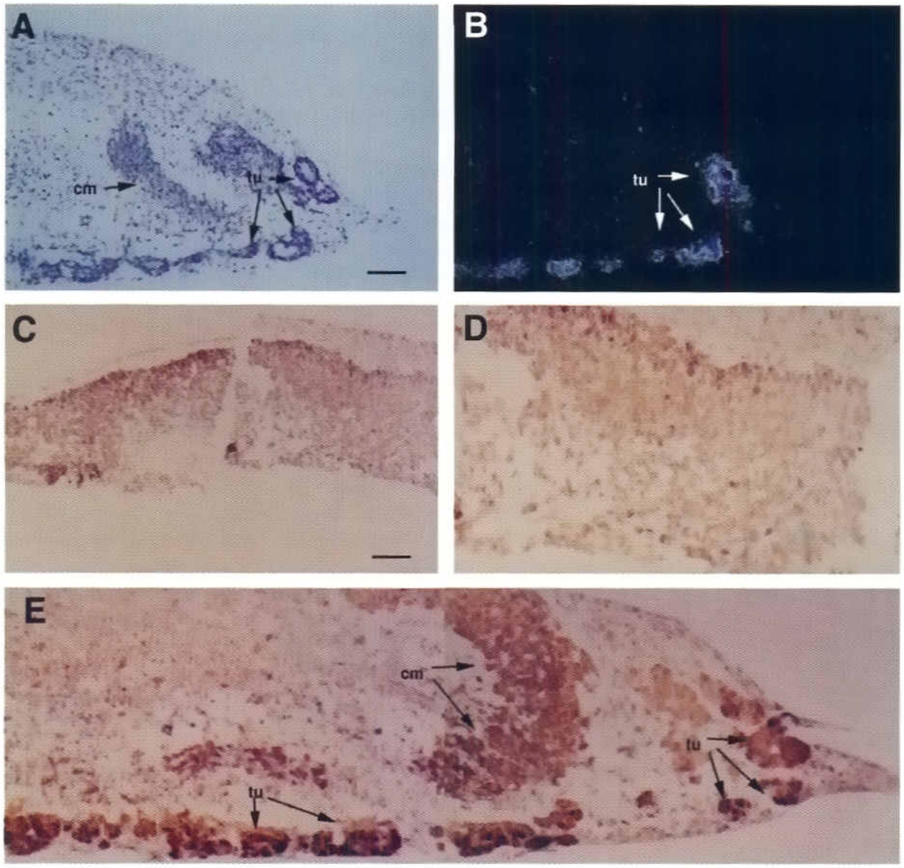

Fig. 4. *Lim-1* gene expression in mesenchymal explants. *Uninduced mesenchymes were cultured in the presence of basic FGF, as described (Perantoni et al., 1995).* **(A and B)** *In situ hybridization;* **(C, D and E)** *immunostaining. A, B, E, 12 day culture, showing tubulogenesis; C, D, seven days in culture in the presence of FGF showing mesenchymes before the beginning of tubulogenesis. tu, tubules; cm, condensing mesenchyme. Bars, A, B, C, 100 µm; D, E, 50 µm.*

Materials and Methods

Antibodies

A polyclonal rabbit antibody produced against the fusion of GST to the C-terminal part of Xlim-1 protein has been described and shown to cross-react with Lim-1 protein in other vertebrates (Karavanov *et al.*, 1996).

Immunohistochemistry

Rat embryonic and adult kidneys were fixed by two methods either in MEMFA (100 mM MOPS, 1 mM $MgSO_4$ 2 mM EGTA, 3.8% formaldehyde) for 1 h followed by two 15 min methanol washes and rehydration in PBS for 2x15 min (Karavanov *et al.*, 1996), or in 4% PFA as described earlier (Perantoni *et al.*, 1995). Paraffin embedding and sectioning were done as described (Karavanov *et al*, 1996). PFA-fixed sections after deparaffinization were boiled in 6 M urea in a microwave oven at 90% power for 5 min (Cattoretti *et al.*, 1992). This procedure greatly enhances signal-to-background ratios for PFA-fixed tissues. Sections were blocked in 2% Boehringer-Mannheim blocking reagent in 0.1M maleic acid, 0.15M NaCl, Ph 7.4, for 1 h at room temperature. Anti-Xlim-1 IgG was diluted 1/200 in Boehringer-Mannheim blocking reagent and applied to sections overnight at 4°C. Following 3x10 min washes in PBS, sections were treated with secondary antibody conjugated to alkaline phosphatase (Boehringer-Mannheim; 1/100) for 1 h at room temperature, and washed three times for 10 min in PBS. Staining was developed using NBT and BCIP (Boehringer-Mannheim) using the manufacturer's protocol. After dehydration, sections were mounted in Permount (Fisher).

In situ hybridization

Full length rat *Lim-1* cDNA probe for hybridization *in situ* was a generous gift of Dr. Hemin Chin. The protocol of *in situ* hybridization was described earlier (Perantoni *et al.*, 1995).

Rat kidney mesenchyme culture

Microsurgically separated uninduced 13 d.p.c. kidney mesenchymes were cultured *in vitro* in the presence of basic FGF (100 ng/ml) as described earlier (Perantoni *et al.*, 1995).

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