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Metanephric mesenchyme contains multipotent stem cells whose fate is restricted after induction

DORIS HERZLINGER[1,*], CHIZUKO KOSEKI[1,†], TAKASHI MIKAWA[2,†] and QAIS AL-AWQATI[1]

[1]*Departments of Medicine and Physiology, Columbia University College of Physicians and Surgeons, New York, NY 10032, USA*
[2]*Department of Cell Biology and Anatomy, Cornell Medical College, New York, NY 10021, USA*

*Present address: Department of Physiology and Biophysics, Cornell Medical College, New York, NY 10021, USA
†Permanent address: National Institute of Cardiovascular Research, Osaka, Japan

Summary

At least fourteen epithelial cell types of the mammalian nephron develop from the metanephric mesenchyme. To distinguish whether this single embryological primordium contains a heterogenous population of committed renal cell lines or a multipotent stem cell, the *lac-Z* gene was introduced into individual renal progenitors by retroviral mediated gene transfer. The differentiated fate of *lac-Z*-tagged daughters derived from single metanephric mesenchymal cells was characterized after cytodifferentiation. We found that the metanephric mesenchyme contains multipotent stem cells that can generate at least three distinct cell types; glomerular, proximal and distal epithelia. After induction the fate of this multipotent cell becomes restricted to populate a single nephron segment.

Key words: metanephros, lineage analysis, stem cells, renal development.

Introduction

Regulation of the volume and composition of the body fluids of multicellular organisms is mediated by the heterogenous transporting epithelia of the kidney. Each nephron is composed of seven discrete segments each of which has one or more unique epithelial cell types (Fig. 1A). Hence, the formation of the nephron requires the generation of these unique epithelial cell types. In addition, these diverse cells have to be correctly associated and aligned into a filtering glomerulus and a segmented renal tubule, and finally, this nephron will have to join the collecting tubule system which is derived from a different lineage, the ureteric bud (Saxen, 1987). To begin to analyze the regulatory mechanisms guiding the generation of renal cell diversity, we have traced the lineage of distinct cell types back to a common multipotent progenitor.

Metanephric kidney development begins when a caudal outgrowth of the Wolffian duct, the ureteric bud, induces undifferentiated mesodermal cells of the metanephric blastema to proliferate and coordinately express epithelial cell adhesion molecules, (Fig. 1B); Grobstein, 1953, 1957; Saxen et al., 1983; Klein et al., 1988; Aufderheide et al., 1987; Vestweber et al., 1985; Vainio et al., 1989). The mesodermal cells need only 24 hours of contact with inducing tissue to progress through advanced stages of nephrogenesis (Lehtomen et al., 1983; Ekblom, 1981). Following induction, the cells aggregate and condense around the outgrowth of the ureteric bud to form a "renal vesicle". This rapidly changes its shape to form an S-shaped body which is the first evidence of segmentation of the primitive nephron. When embryonic development occurs in vivo, the S-shaped body is invaded by primitive blood vessels at one pole which eventually becomes the glomerulus. Grobstein discovered that the metanephric mesenchyme can be induced in vitro to form renal vesicles, S-shaped bodies, segmented nephrons including joining with the ureteric bud (Grobstein, 1953, 1957). However, no vascularization of the glomerulus occurs. It was also noted that heterologous inducers such as embryonic spinal cord could also allow progression of the mesenchyme through these stages. This in vitro system has allowed detailed study of the cellular events of nephrogenesis (reviewed in Saxen, 1987).

Renal epithelial cell diversity may arise from the expansion of several predetermined renal cell lines present in the metanephric mesenchyme or may be generated from a multipotent renal progenitor present in this primordium. To discriminate between these possibilities, the differentiated fate of the progeny derived from individual metanephric mesenchymal cells was analyzed. We found that the metanephric mesenchyme contains multipotent stem cells capable of generating glomerular and tubular epithelial cells.

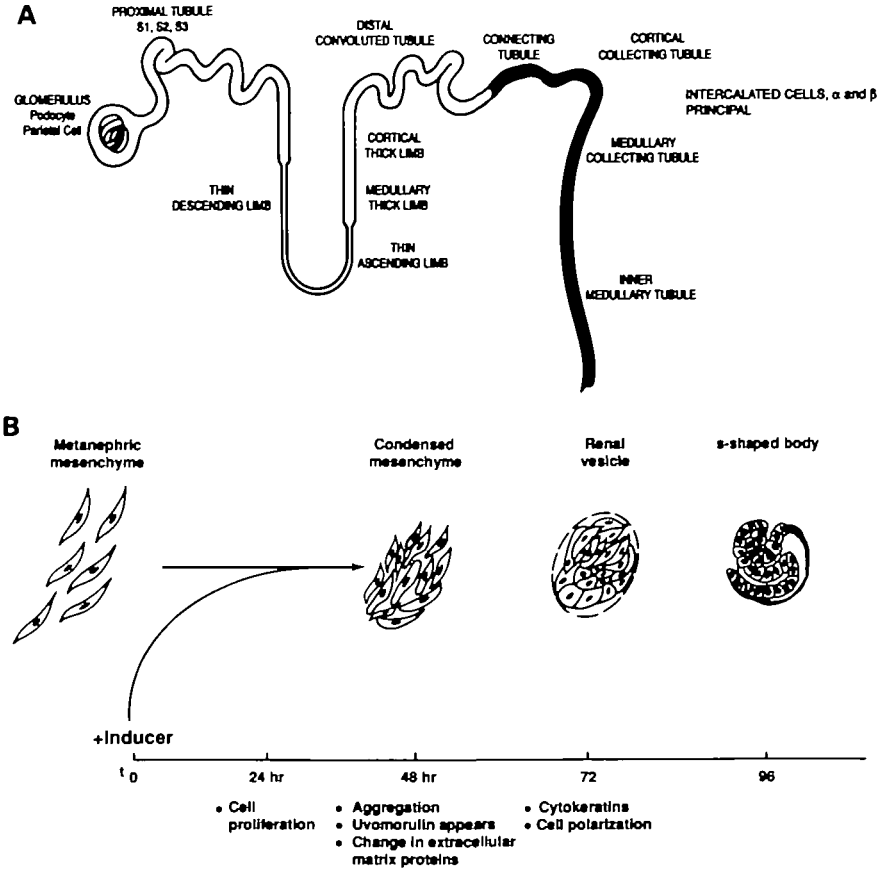


Fig. 1. (A) A schematic model of the mature nephron showing the known segments and their specific epithelial cell types. (B) A schematic model for early development of the nephron showing the stages before a mature nephron appears with the time course of appearance of specific markers.

Following induction, the progeny of the tagged cells were restricted in their fate, in that they populated only individual segments of the nephron.

Materials and methods

Preparation and growth of metanephric mesenchymal cells

Embryonic kidney rudiments were microdissected from gestation day 13 rat embryos (Sprague Dawley). The age of the embryos was counted from the day of the vaginal plug (day 0).

Primary metanephric mesenchymal cell cultures were prepared by removing the ureteric bud from isolated rudiments and incubating the remaining mesenchyme in 0.05% trypsin, 0.5 mM EDTA for 5 minutes at 0°C. Fetal calf serum (GIBCO) was added to a concentration of 10% to inactivate trypsin and cells were mechanically dissociated by gentle aspiration with a Pasteur pipette. The resultant single cell suspension was pelletted by low speed centrifugation (1500 revs/min) and plated at described densities on Costar tissue culture dishes (24 cluster plates or 30 mm dishes) in Dulbecco's modified Eagle medium (DMEM) including 10% fetal calf serum and grown at 37°C, 5% carbon dioxide.

Metanephric organ cultures were established by placing isolated gestation day 13 rudiments on collagen-coated transfilter systems (Transwell, pore size 3 μm, Costar Corp.) with DMEM including 20% fetal calf system in the bottom chamber. Cultures were grown at 37°C in the presence of 5% carbon dioxide.

For in vitro induction, transfilter organ cultures (Grobstein, 1953, 1957) were established with tissues isolated from gestation day 13 embryos. Each individual culture contained nephrogenic mesenchyme, microdissected free of ureteric bud, from two rat kidney rudiments and dorsal spinal cord, as an inducer. Mesenchyme and inducer were separated by 3.0 μm nucleopore filters and grown at 37°C, 5% CO_2 for 24 hour prior to infection with retrovirus. For long-term growth, in vitro-induced mesenchyme was transplanted under the left renal capsule of halothane-anesthetized neonatal rats (<48 hour after birth) (Koseki et al., 1991). Rat pups were reared for 2 weeks prior to killing.

Gene transfer

We used two replication-defective retroviral vectors to introduce *lac-Z* into the genome of renal progenitors. The BAG retroviral vector was generously provided by C. Cepko. This retrovirus contains the Moloney leukemia virus long terminal repeat (LTR) to drive *lac-Z* transcription and has been extensively characterized by Price et al. (1987). BAG retroviral stocks were prepared from medium harvested from confluent BAG ψ2 producer cells (provided by C. Cepko) as described by Price et al. (1987) and stored at −80°C until use. All virus was negative for helper virus activity. The CXL retrovirus was described previously (Mikawa et al., 1991) and it uses the spleen necrosis virus LTR for *lac-Z* transcription. CXL retroviral stocks were prepared from medium harvested from CXL producer cells, D17.2G#1-8, and stored at −80°C until use. All virus supernatants were not competent for replication.

Infective retroviral titre for both viruses was determined by incubating cultured NIH 3T3 fibroblasts and primary metanephric mesenchymal cells (2×10^4 cells per well of a 24 cluster Costar tissue culture dish) in serial dilutions of retroviral stock in the presence of 16 μg/ml Polybrene (Sigma) for 2 hour.

Virus was removed, cells were then fed with fresh medium and cultured for 48 hours before fixation and visualization of β-galactosidase activity. Renal progenitors contained in isolated gestation day 13 kidney rudiments and transfilter organ cultures were infected with BAG by immersion in retrovirus containing 16 μg/ml polybrene for 2 hour.

Histochemistry

Cultured cells and organ cultures were fixed with 2% paraformaldehyde/PBS for 2 hours and washed extensively in PBS. Neonatal rat kidneys containing transplanted mesenchymal cells were fixed by perfusion with 2% paraformaldehyde, 0.25% glutaraldehyde/PBS. Kidneys were immersed in fixative overnight, washed in PBS and 100 μm vibratome sections prepared. β-galactosidase activity was developed in all samples by immersion in a modified substrate (Pearson et al., 1963): 0.5 mg/ml X-gal (5-bromo-4-chloro-3-indolyl β-galactopyranoside), 3.5 mM $K_4Fe(CN)_6.3H_2O$, 3.5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$ for 16 hour at 4°C. Enzyme activity was stopped by washing and storing rudiments in 0.05 M carbonate-bicarbonate buffer, pH 10.3. Samples processed for plastic histological sectioning were dehydrated in graded alcohols, embedded in methacrylate (Historesin, L.K.B) and cut at 8 μm thickness.

Identification of nephron segments in thick sections

Nephron segments were identified based on the criteria of Tisher and Madsen (1986). Glomeruli were identified by their characteristic morphology. Proximal convoluted or straight tubules were identified as those segments having a tubule diameter of 50 μm. Occasionally, one was able to recognize brush borders in the cells near the surface of the section. Thick ascending limbs could not be distinguished from distal convoluted tubules, however, both had smaller tubule diameters than the proximal nephrons and averaged 27 μm. These segments were termed distal segments. It is well-known that branching occurs in the connecting tubule or early cortical collecting tubule. Hence, we were able to localize tagged cells to these segments when branching was observed. This branching was readily distinguishable from that in blood vessels because of the epithelial shape of the cells when observed at higher magnification especially in cross-sections. The detailed identification of these segments is described in Koseki et al. (1991).

Results

The recently developed technique of retroviral-mediated gene transfer (Price et al., 1987; Turner and Cepko, 1987; Jaenisch and Soriano, 1986; Sanes et al., 1986) was used to introduce the *lac-Z* gene into mesenchymal cells of the metanephric blastema. The first identifiable stage of rat metanephric kidney development occurs at gestation day 13 (E13) when the ureteric bud contacts the cells of the metanephric mesenchyme (Jokelainen, 1963). The efficiency of infecting cultured mesenchymal cells with BAG or CXL was very low compared to infection of cultured fibroblastic cell lines. The BAG virus supernate exhibited an infective titre of 4×10^4 colony-forming units per ml (cfu/ml) when assayed using NIH 3T3 cells; CXL had a titre of 1×10^5 cfu/ml using D-17 cells. However, the efficiency of infection using cultured metanephric mesenchyme was much lower; the BAG

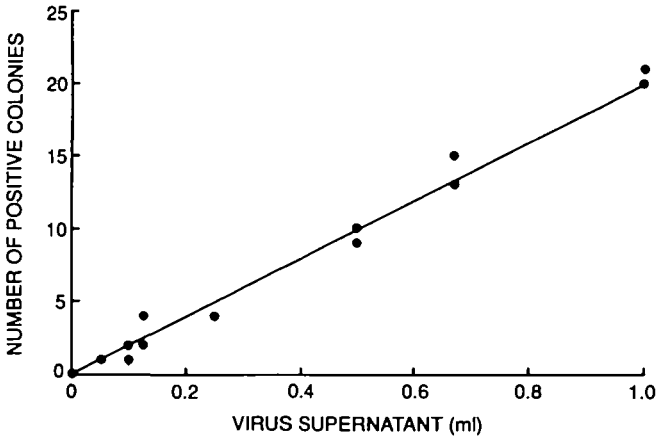


Fig. 2. Titration curve with BAG virus in monolayer cultures of metanephric mesenchyme. The supernatant from virus-producing cells was concentrated by ultracentrifugation approximately 3-fold. Primary mesenchymal cell cultures (2×10^4 cells/cm^2) were incubated in 1 ml of concentrated stock (3× solution) and dilutions of this stock, expressed in μl aliquots added to a final incubation volume of 1 ml. Four days later the wells were fixed and processed for the β-galactosidase reaction and the number of positive cells counted.

and CXL viral stocks that gave high titers when assayed on fibroblasts (4×10^4 cfu/ml and 1×10^5 cfu/ml respectively) produced only 8 and 4 cfu/ml in mesenchymal cells even though both cell types had similar doubling times of 24-36 hours.

A titration study was performed in which increasing volumes of concentrated BAG virus stock was added to the culture medium of isolated mesenchymal cells. The results, shown in Fig. 2, demonstrate that there was a linear relationship between virus concentration and number of cell colonies (virus titre). These results show that, although the virus infects mesenchymal cells inefficiently, there was no complex interaction between the two that would prevent its use as a marker for lineage analysis where clonal dilutions are needed. Additionally, once infected by retrovirus, cultured metanephric mesenchymal cells and their progeny expressed high levels of β-galactosidase activity demonstrating that tagging nephrogenic progenitors for lineage analysis by BAG infection was feasible. We found that β-galactosidase expression was stronger in cells infected by BAG than in those infected with CXL, hence most of the studies reported below were performed with the BAG virus.

To study the fate of a single uninduced mesenchymal cell and its progeny, microdissected E13 kidney rudiments were infected with limiting dilutions of the BAG retrovirus and grown as organ cultures. Such rudiments are composed of ureteric bud surrounded by $\sim2\times10^4$ metanephric mesenchymal cells that require contact with the inducing ureteric bud to undergo cytodifferentiation in vitro (Grobstein, 1957, 1956; Saxen et al., 1968). Isolated rudiments were incubated with producer cell supernatants containing 16 μg/ml of polybrene. The viral concentration used was determined to be near that

needed to infect 1 cell/rudiment. This concentration was obtained from the titration studies mentioned above. Note that the titration analyses were performed on mesenchymal cell cultures containing 2×10^4 cells, which is similar to the number of cells in each rudiment. The higher concentration used gave 8 infected cells in mesenchymal cell culture and the lower concentration resulted in less than 1 infected cell in the mesenchymal cell monolayer.

Freshly microdissected gestation day 13 rudiments were incubated with 10-fold dilutions of BAG virus. Rudiments were cultured for 3 to 7 days and scored for the presence and number of β-gal-positive cells (Table 1). No β-galactosidase-labelled cells were present in mock-infected control rudiments (Fig. 3A). Only a low percentage of rudiments incubated with BAG at 8 cfu/ml contained *lac-Z*-tagged cells when examined at either 3 or 7 days postinfection. Where present, positive rudiments had only a single cluster of labelled cells (Figs 3B and 4A); no rudiment contained more than one such tagged colony. The number of cells contained in such compact clusters of labelled cells increased with prolonged culture (Table 1). The Poisson distribution predicts that at clonal dilutions of retrovirus (1 cfu/rudiment) only 37% or less of samples incubated with virus will be infected. The low percentage of rudiments containing β-gal-labeled cells suggests that such a clonal dilution of retrovirus has been attained (Table 1). To document this further, we repeated these experiments by infecting mesenchymal rudiments with a lower titer of the virus (0.8 cfu/ml) and found that no positive colonies developed (Table 1) implying that we were below the concentration of virus needed to infect one cell per rudiment. This statistical analysis combined with the distinct spatial distribution and increased number of labeled cells present in colonies after long-term culture strongly supports the clonal derivation of labeled cells contained within individual rudiments.

The lower efficiency of infecting mesenchymal cells contained in rudiments is most likely due to steric hindrance imposed by the three-dimensional structure of the rudiment which could prevent virus penetration. Alternatively it could be due to reduced proliferation of the cells in the rudiment compared to those in monolayer culture. To ensure that the low efficiency of infectivity was not due to inactivation of the Moloney viral LTR, we assayed the infective titer of CXL retrovirus in freshly isolated E13 rudiments. CXL drives transcription of *lac-Z* using the spleen necrosis virus LTR. The virus supernate produced a titer of infection in mesenchymal monolayer culture of 4 infected colonies per well containing 2×10^4 mesenchymal cells, similar to the BAG virus. When used to infect E13 rudiments in organ culture, only 2 out of 300

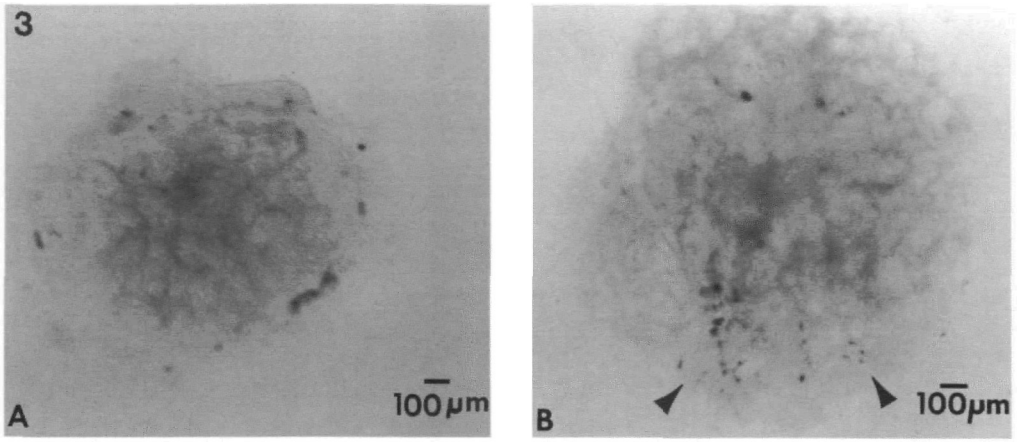
Fig. 3. Control (A) and BAG-infected metanephric organ culture (B,C) exhibiting a single *lac-Z*-tagged colony. Metanephric kidney rudiments were isolated, incubated with virus and cultured organotypically for 7 days prior to fixation and visualization of β-galactosidase activity as described in Table 1. (A) Whole mount of control organ culture mock infected by incubation in DMEM, 10% FCS (tissue culture medium used for ψ2 cell culture) containing 16 μg/ml polybrene. No *lac-Z*-tagged cells are observed. (×40). (B) Whole mount of organ culture incubated with BAG virus (8 cfu/ml) as described in Table 1. A single cluster of *lac-Z*-tagged cells is indicated (arrowheads). (×40). (C) Serial methacrylate sections (hematoxylin and eosin stained, 8 μm thick) of colony observed in Fig. 1B. *lac-Z*-tagged cells were observed only in area indicated by arrowheads (Fig. 1B). All *lac-Z*-tagged cells in this colony exhibit spindle-shaped morphology and are not integrated into renal tubules. (×200).

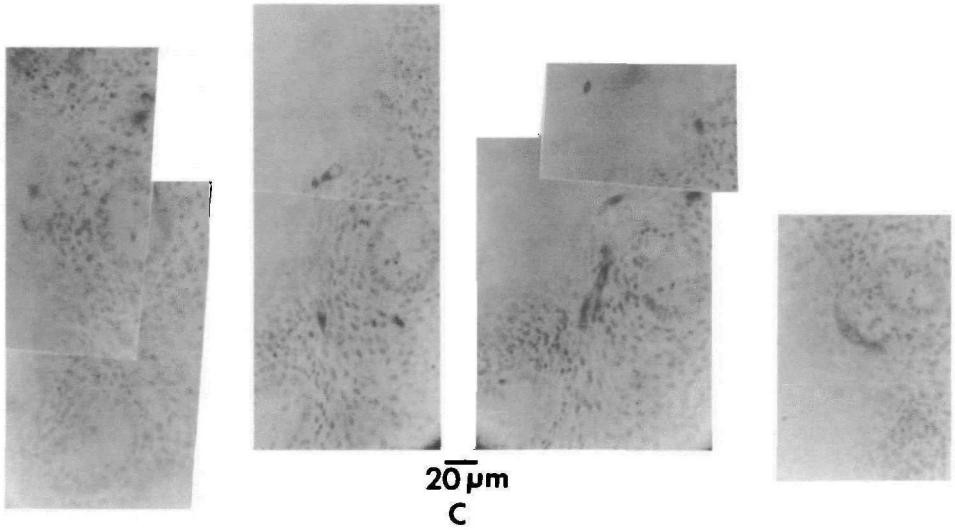
Fig. 4. BAG-infected metanephric organ culture exhibiting epithelial *lac-Z*-tagged colony. Metanephric kidney rudiment was isolated, incubated with BAG (8 cfu/ml) and cultured for 7 days prior to fixation and visualization of β-galactosidase activity as discribed in Table 1. (A) Whole mount of organ culture. *lac-Z*-tagged cells are present throughout an entire nephron; from its glomerulus (G) on through an extended length of the renal tubule (T). Adjacent to this nephron containing *lac-Z* cells along its entire length is a short tubule segment containing *lac-Z*-tagged cells (T_s). (×40). (B-M). Serial methacrylate sections (hematoxylin and eosin stained, 8 μm) of epithelial colony seen in whole mount above. (×250). (B-E) Ureteric bud branching into two primitive collecting tubule segments exhibiting tagged cells at transition to distal nephron. (F-M) The nephron exhibiting tagged cells from tubule through glomerulus can be followed. Tall columnar epithelia (H) are observed just distal to the compact, disorganized epithelia of the glomerulus (J,K). This nephron is no longer observed in panel M.

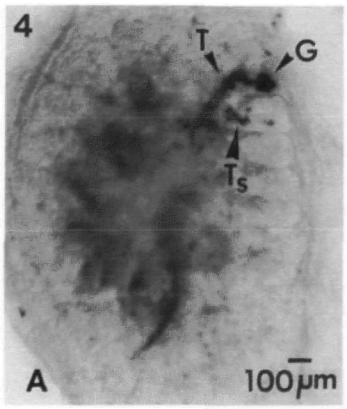
Table 1. *Efficiency of infecting renal progenitors with BAG in organ culture*

Viral dilution (cfu/ml)	No. of rudiments incubated with BAG	No. of rudiments with β-gal-positive cells (cell morphology)	No. of β-gal-positive cells/colony
(A) 3 day culture			
0.8	100	0	–
8.0	200	3	2 (avg)
(B) 7 day culture			
0.8	100	0	–
8.0	200	5 (spindleshaped)	<15
		3 (epithelial)	>50

Metanephric kidney rudiments (gestation day 13) were incubated with 1 ml of retrovirus at given titre. This titre is expressed as cfu/ml and was determined by infection with 10-fold dilutions of BAG retrovirus for 2 hours and grown as organ cultures for stated times prior to fixation and development of β-galactosidase activity.


3
A
100 μm
B
100 μm


20 μm
C


4
T
G
Ts
A
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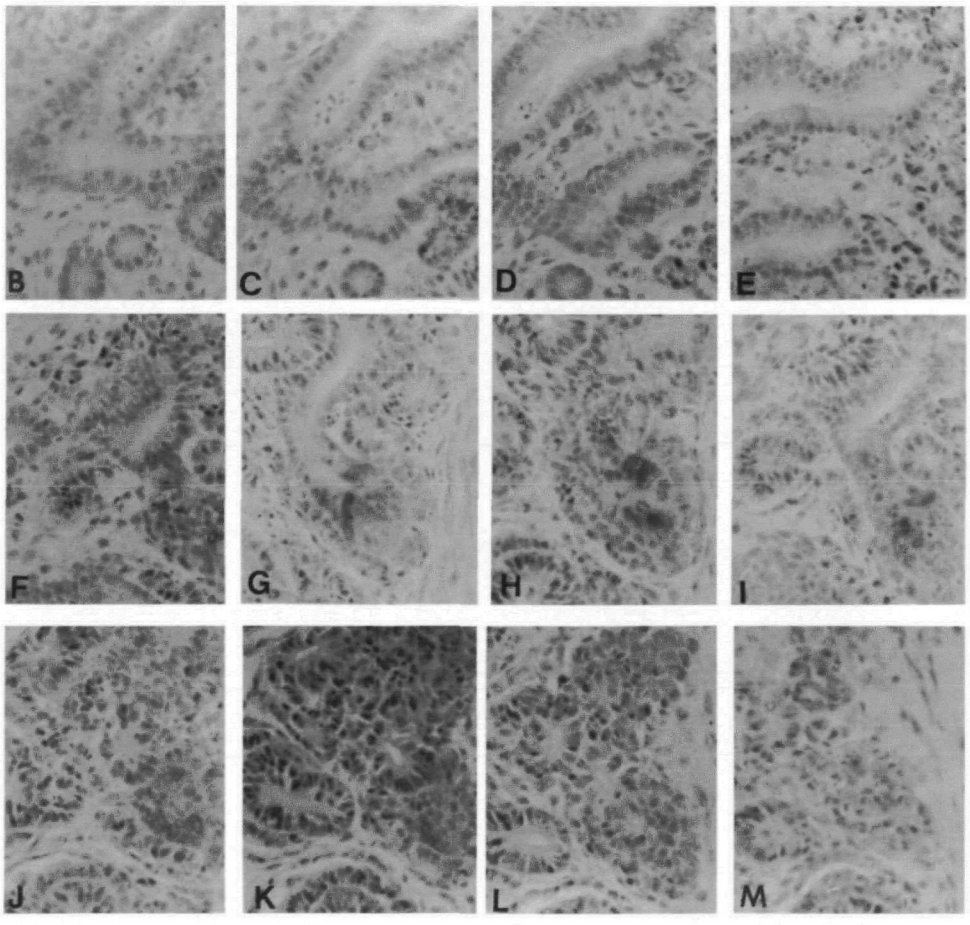
B
C
D
E
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H
I
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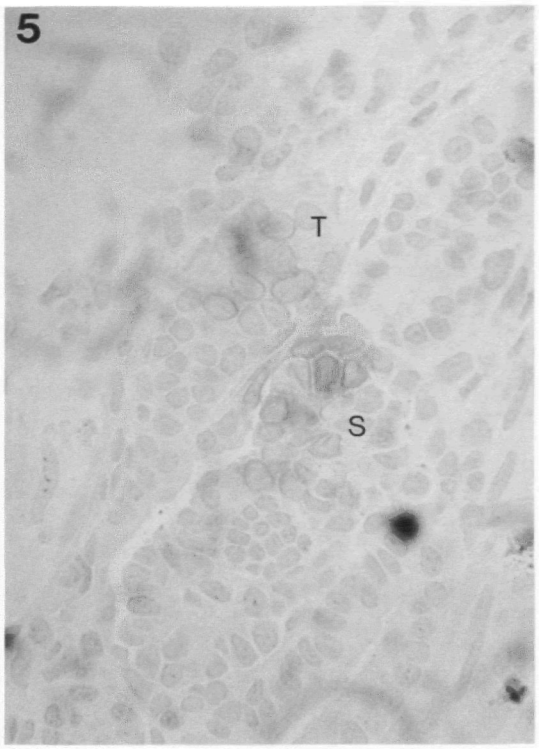
K
L
M
20 μm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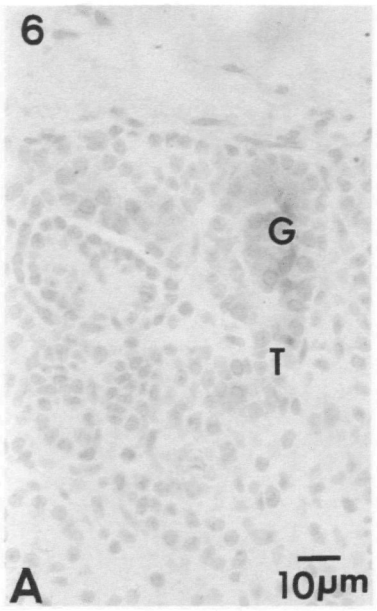
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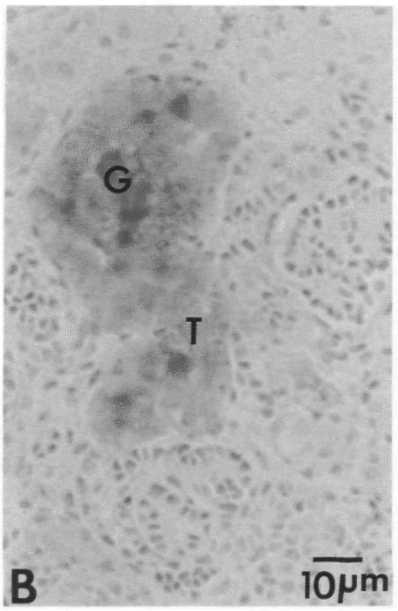
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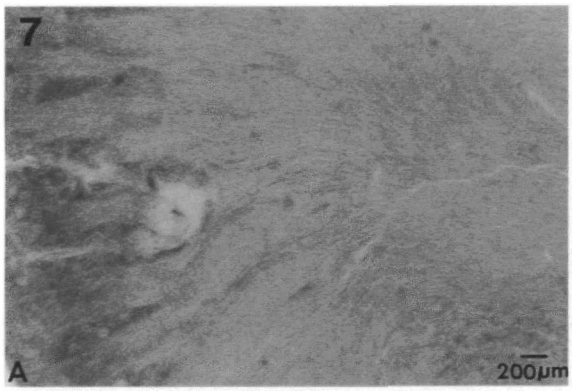
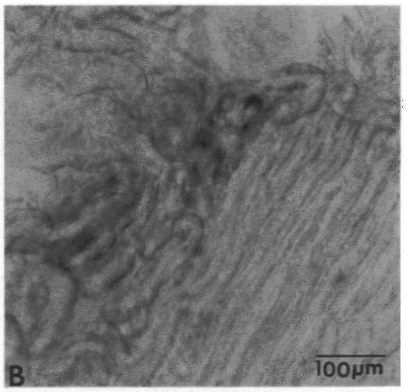

7
A
200µm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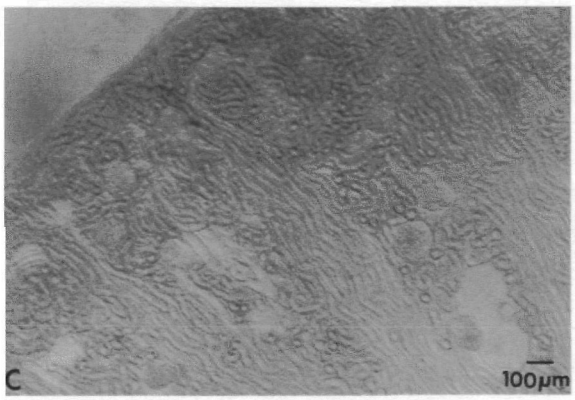
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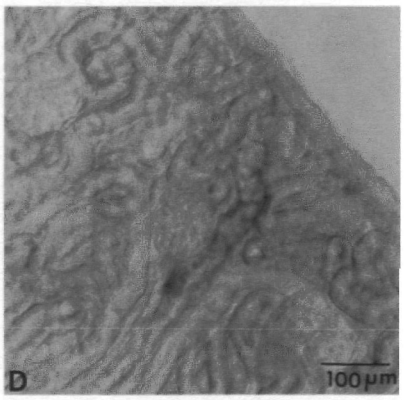
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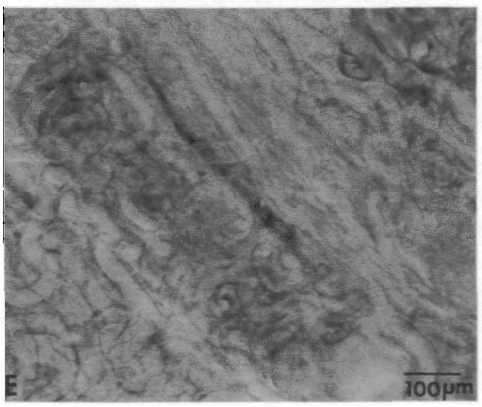
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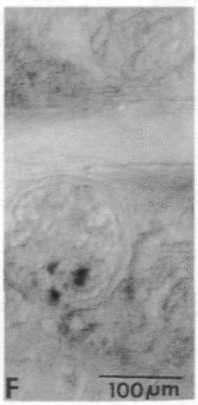
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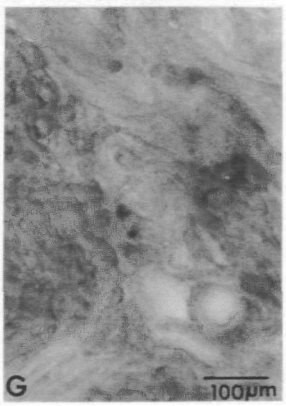
G
100µm

Fig. 5. Epithelial *lac-Z*-tagged colony in CXL-infected organ culture. Gestation day 13 kidney rudiment was incubated in CXL virus at a concentration of 4 cfu/ml as determined by titration on primary mesenchymal cell cultures. Rudiment was cultured for 7 days, fixed and processed as described in Materials and Methods. Labelled cells of the resultant single tagged colony are present in an early S-shaped body (labelled S) and adjacent mass of cells (labelled T). That these two cell masses are not part of one nephron is documented by serial sections (data not shown). β-galactosidase-positive cells line the glomerular crevice (arrow) and exhibit two distinct morphological characteristics; flattened epithelial cells destined to form the parietal epithelium of the glomerulus and larger cells organized as masses destined to form tubular epithelium.

Fig. 6. BAG-infected organ cultures exhibiting *lac-Z*-tagged colonies in developmentally advanced nephrons. Rudiments were isolated, incubated with virus and processed as described in Fig. 4. (A) Section of *lac-Z*-tagged colony located at the periphery of an organ culture. Two nephrons containing tagged cells were determined to be separate nephrons by serial sectioning. β-galactosidase-positive cells can be seen within a glomerulus (G) and extend into the renal tubule (T) emanating from that glomerulus. Tagged cells are also present in the tubular segment of an adjacent nephron. (B) β-galactosidase-labelled cells are present in this late S-shaped body showing an early glomerular structure (G). Tagged cells are seen to extend into the tubular segment (T). The disorganized, tightly packed labelled cells are characteristic of glomerular epithelia contained in avascular glomeruli of organ cultures. Labelled tubular cells are cuboidal or columnar in shape.

Fig. 7. Distribution of *lac-Z*-tagged induced mesenchymal cells cultured in neonatal kidneys (×120). Transfilter organ cultures were established with tissues isolated from gestation day 13 rat embryos. Mesenchymes were induced by co-culture with spinal cord for 24 hours and then infected with BAG virus (4 cfu/ml). Cultures were maintained in vitro for 48 hours and then grown for 2 weeks as explants on neonatal rat kidneys prior to fixation and development of β-galactosidase activity. Neonatal rat kidneys containing explants were sectioned and sections containing β-galactosidase-labeled cells identified by bright-field microscopy and examined further at high power (40×) with bright-field and Nomarski optics. (A,B) Low and higher power view of one colony showing the presence of a single colony which on higher power is seen to be a proximal tubule. (C,D) Low and high power view of a colony which on high power is seen to be that of a distal tubule. (E) A thin long labelled tubule which is identified as distal tubule. (F,G) Glomeruli containing labelled cells. These were taken from experiments when the infecting virus concentration was above clonal dilution (Koseki et al., 1991). Glomeruli were readily identifiable by their characteristic size and morphology; proximal tubule segments were identified by the presence of a well-defined brush border on high columnar epithelia present in tubules with large diameters of 50±2.5 μm; distal nephron segments were localized to medullary rays and identified as tubular segments with diameters of 27±0.7 μm exhibiting a low columnar epithelium lacking a well-defined brush border.

rudiments showed β-galactosidase-positive cells. Hence, it is unlikely that the structure of BAG is somehow limiting its infectivity.

Rudiments cultured for 7 days exhibited two distinct patterns of *lac-Z*-tagged cell colonies. Five rudiments contained β-gal-labeled cells that were spindle shaped with a maximum of 15 labeled cells in each rudiment (Fig. 3B). Histological sections of such rudiments demonstrated that the spindle-shaped cells exhibited fibroblastic morphology and were not integrated into nephrons (Fig. 3C). These fibroblastic clonal cell colonies may be derived from a renal stromal cell progenitor or may be the progeny of renal epithelial stem cells that have not terminally differentiated.

Three BAG-infected rudiments had single distinct colonies of labeled cells organized into nephrons (each colony had greater than 50 labelled cells) (Fig. 4A). Histological examination of such colonies demonstrated that *lac-Z*-labeled cells were contained in adjacent nephrons indicating that the progeny of a single mesenchymal cell can populate more than one nephron (Fig. 4A). Assuming that all β-gal-negative cells lack the *lac-Z* insert, the intermixing of *lac-Z*-tagged cells with unlabeled cells demonstrates that a nephron is not clonally derived from a single renal stem cell. Serial sections of the rudiment showed that labeled cells were present throughout single nephrons from the glomerulus to the most distal tubular segment - the junction with the ureteric bud (Fig. 4A-J). The localization of clonally derived *lac-Z*-tagged cells in a single nephron from its most proximal to distal segments indicates that the metanephric mesenchyme contains cells whose clonally derived progeny can populate the entire length of a developing nephron. Hence, this cell or a daughter cell has the capacity to differentiate into the diverse renal cell types that constitute different nephron segments.

That diverse cell types are derived from a single renal progenitor was shown in an analysis of three additional *lac-Z*-tagged colonies. In Fig. 5, an S-shaped body with its characteristic glomerular crevice is shown to contain β-gal-positive cells. The tagged cells are seen to be located among the flat epithelial cells destined to form the glomerulus as well as in the adjacent cell mass that will form the tubules. In addition, tagged cells are seen in an adjacent structure likely to be a section of another S-shaped body or renal vesicle. Two other colonies examined exhibited tagged cells in nephrons that were more developmentally advanced (Fig. 6A,B). Each figure contains a glomerulus whose morphology is characteristic of the avascular glomerulus that forms in organ culture (Saxen, 1987). The population of small disorganized β-galactosidase-positive cells seen within these glomeruli exhibit the characteristic morphology and location of renal podocytes. Renal tubules containing β-galactosidase-labelled columnar epithelial cells can be seen to emanate from the glomeruli containing tagged cells. These results further document the conclusion that renal stem cells present in E13 metanephric mesenchyme are able to generate daughters with morphological characteristics of glomerular and tubular epithelial cells. Thus the unrestricted position of clonally related cells along the entire developing nephron and the unique differentiated phenotype of

Table 2. *Localization of β-galactosidase-positive colonies in kidney*

Virus titer cfu/ml	Glm	PT	DT	Branched	Others	No. of colonies/ rudiments
4	0	4	6	0	1	11/24
8	4	24	28	16	18	90/64

Abbreviations: Glm, glomerulus; PT, proximal tubule; DT, distal tubule; Branched, branched tubule. The high titer experiments were previously reported in Koseki et al. (1991).

such progenies demonstrates that the metanephric mesenchyme of E13 rat embryos contains multipotent renal stem cells.

Lineage analysis experiments were performed later in development to examine the fate of multipotent renal progenitors after induction. Gestation day 13 nephrogenic mesenchyme, microdissected free of ureteric bud, was given a 24 hour inductive pulse by co-culture with E13 dorsal spinal cord (Grobstein, 1953, 1956). It is well known that embryonic spinal cord can act as a strong inducer of metanephric mesenchyme in vitro (Saxen, 1987). Such heterologously induced transfilter organ cultures were then infected with BAG and the progeny of *lac-Z*-tagged cells identified after 5-7 days of in vitro culture. In contrast to infection of uninduced progenitors (Table 1), only a small number (<6) of tagged epithelial cells were observed in each colony derived from an induced progenitor. The small size of the colonies suggests that the cells were tagged after the rapid phase of proliferation known to occur after induction (Saxen et al., 1983). However, the small yield of these experiments limited the amount of information that can be obtained. One possible reason for the low yield was the low abundance of factor(s) in the in vitro organ culture system. Since murine nephrogenesis continues in new born animals especially in the renal cortex, we devised a transplantation system where the metanephric mesenchyme could be cultured in the renal cortex of newborn animals (Koseki et al., 1991). Transfilter cultures were first induced for 24 hours in vitro, infected with serial dilutions of BAG retrovirus, and then transplanted under the capsule of neonatal rat kidneys for long-term culture. The efficiency of generating *lac-Z*-tagged cell colonies was greater with transfilter cultures maintained as in vivo transplants than with intact kidney rudiments. Using the limiting dilution found for in vitro cultures (8 cfu/ml), the majority of transplants now contained tagged cell colonies. This increased efficiency most likely reflects the greater viability of cells during in vivo culture. Clonal dilutions were obtained by reduction of the BAG virus titer to 4 cfu/ml; 45% (11/24) of the individual transplanted mesenchymes showed tagged colonies, similar to but slightly higher than that predicted for a clonal dilution (37%). However, there was never more than one colony per mesenchyme derived from one rudiment. Two representative sections from a kidney are shown in Fig. 7. At low magnification (Fig. 7A,C) it can be seen that a single colony was present. At higher magnification of the same two colonies, one is found to be a distal tubule, (Fig. 7D) and the other a proximal tubule (Fig. 7B). An additional distal segment is shown in Fig. 7E (at different magnification). The identification of the tubule segments was based on the diameter of these tubules as discussed in the Materials and Methods and in more detail in Koseki et al. (1991).

Such clonal colonies of induced cells were always contained in single nephrons and, most importantly, were always localized to single discrete nephron segments (Fig. 7). Of the eleven positive colonies, four had tagged cells in the proximal tubule, six in the distal tubule and one was in a tubule that could not be identified. There were no glomerular tagged cells (Table 2). These results demonstrate that following induction, the fate of renal cell progenitors becomes restricted to a single nephron segment. Using a higher concentration of virus, 8 cfu/ml, to ensure the production of a large number of colonies, most of the rudiments had positive colonies and some had more than one. There were 90 colonies in 64 rudiments. When these colonies were examined, we found (Table 2), 4% were localized to the glomerulus (Fig. 7F,G), 31% to the proximal tubule, 55% to the distal tubule and 18% were seen in segments that could not be definitively identified.

Discussion

Clonal derivation of lac-Z*-tagged cells*

Introduction of genes by infection with replication defective retrovirus provides a heritable tag that can be used to examine cell lineage. To demonstrate that tagged cells are derived from a single progenitor, it is necessary to show that only one cell was infected with the retrovirus. Formally, this is achieved by infecting the target organ with increasing dilution of virus until a concentration is reached which provides a single "hit". To satisfy this criterion we used a clonal dilution of retrovirus where less than one cell/experimental sample was infected. The efficiency of infecting metanephric mesenchyme by the BAG retrovirus was much lower than that seen for 3T3 fibroblasts, a cell line that was used in other studies of this virus and hence can serve to normalize the titre that we used to the published literature. Titration of virus on isolated mesenchymal cells in monolayer cultures showed a linear relation between viral concentration and the number of positive colonies, hence it was possible to experimentally

determine the concentration of virus needed to infect one or less than one cell in a cultured mesenchymal population containing 2×10^4 cells. However, when an intact rudiment containing the same number of cells was infected with such a limiting concentration of virus, the probability of obtaining infected colonies was two orders of magnitude lower. The percentage of E13 rudiments exhibiting positive colonies, using a titre that gave 8 cfu/ml in mesenchymal cells in culture, was well below the value predicted by the Poisson distribution for clonal virus concentration. Hence, it is very unlikely that more than one cell per rudiment was infected. Importantly, we never saw rudiments with more than one colony and each *lac-Z*-tagged colony was composed of cells that were spatially compact. Since each rudiment is composed of 2×10^4 cells and is about 1 mm in diameter, it is also unlikely that the colonies observed arose from the infection of more than one cell that were adjacent to each other. Finally, rudiments examined at 3 days had a similar number of colonies to those examined 7 days after infection. Only the number of tagged cells per colony increased. This provides additional evidence that the colonies observed were derived from a single infected progenitor.

Since the efficiency of infecting mesenchymal cells in intact kidney rudiments was low, we used a second retrovirus to ensure that this low efficiency of infection was not due to an inhibition of Moloney LTR driven β-gal transcription. The CXL virus, which uses the spleen necrosis virus LTR to drive β-galactosidase transcription, had an infective titre that was similar to BAG in cultured mesenchymal cells and, importantly, in E13 rudiments. Hence, the low number of colonies is a characteristic of the rudiments rather than the LTR of the infecting virus.

This poor efficiency of infection, observed with two different retroviruses, most likely reflects difficulties with access of the virus particle to the cells contained in intact metanephric rudiments. In fact, we obtained a better efficiency of infecting mesenchyme in transfilter culture conditions (Table 2) than in intact rudiments. A likely explanation is that cells contained in mesenchyme for transfilter cultures are exposed by the dissection required to remove the ureteric bud.

Identification of multipotent progenitors

Retroviral tagging of cells contained in E13 metanephric mesenchyme demonstrate that this primordium contains multipotent stem cells; single tagged cells gave rise to daughters that exhibited glomerular and tubular phenotypes. Cells tagged at this stage of development have not yet been induced by the ureteric bud. An analysis of the number of cells present in *lac-Z*-tagged colonies over time supports this contention. We found that the number of cells present in tagged colonies observed 3 days after infection could not generate the number of tagged cells observed 7 days post-infection if cells exhibited a constant rate of proliferation (Table 1). This observation is compatible with previous studies demonstrating that mesenchymal cells undergo a burst of proliferation following induction. Thus the dramatic increase in cell number in epithelial colonies observed 3 versus 7 days post-infection suggests that induction occurred during this time. Interestingly, the number of cells seen in fibroblastic colonies observed 7 days after infection *is* compatible with what would be predicted from a constant rate of proliferation. These data suggest that such fibroblastic cell colonies may be derived from un-induced renal stem cells.

Fate of cells tagged after induction

Lineage analysis of renal progenitors present later in development was performed by tagging metanephric mesenchymal cells after induction in vitro. After extended culture as explants on neonatal rat kidney cortices the progeny derived from induced cells was studied. In contrast to the earliest stem cells, induced mesenchymal cells generated daughters that were localized to a single, discrete nephron segment. Thus, the progeny of induced mesenchymal cells exhibit a restricted differentiated phenotype suggesting that after induction, a mesenchymal cell may no longer be multipotent. Alternatively, it is possible that an induced cell is still multipotent, but may be influenced by positional effects present in the primitive nephron unit. To distinguish between these two possibilities it would be necessary to transplant an induced mesenchymal cell from one pole of the renal vesicle (or S-shaped body) to another, a formidable task. Cells transplanted from near the ureteric bud to the opposite pole would then have a "choice" to differentiate into glomerular epithelial cells even though they would have formed the distal tubule were they left in their position. If such transplants acquire the fate associated with their position, then the restriction that we observed would have been due to purely local factors rather than to a predetermined genetic program.

Renal development

Retroviral tagging of renal progenitors has allowed us to generate and answer questions regarding the early cellular events in nephrogenesis. Tagged cells were a minority of the cells of any nephron regardless of the stage of infection. This demonstrates that several progenitor cells are required to form a nephron. With the demonstration that several cells are needed to form a nephron, one can posit two hypotheses. In one the nephrogenic cells need to be all stem cells. The ureteric bud would induce them to become glomerular or tubular progenitors which eventually terminally differentiate. In this model, all cell types of the nephron would be "born", perhaps simultaneously, during induction by the ureteric bud. In a second hypothesis, one stem cell is needed which following induction can secrete factors that can convert uncommitted mesenchymal cells to form the different segments. In this model, there would be a "birth order" of renal cell types and this birth order is integral to the development of the nephron.

When mesenchyme was first induced in vitro and then infected with retrovirus, only a small percentage of tagged colonies were located in the glomeruli and the

percentage of β-galactosidase-positive segments seemed to be increased in proximal to distal order (Table 2). Since retroviral integration can only occur in actively dividing cells (Simon et al., 1982), the low percentage of glomerular β-galactosidase-containing colonies might reflect a decreased rate of proliferation of glomerular cells or a smaller number of these cells at the time of BAG infection. When terminal differentiation begins in many developing systems, progenitor cells exhibit reduced proliferation (Holtzer et al., 1975). Hence, the low percentage of glomerular cells could reflect the onset of differentiation at the time of infection. This analysis suggests that the glomerular progenitors are first to progress through terminal differentiation. Ekblom et al. (1980, 1981) found that glomerular terminal differentiation antigens appeatred at least 24 hours before the onset of expression of more distal antigens supporting the existence of birth order. It will be important to develop direct methods to test this notion.

The observation that tagged cells derived from early, uninduced mesenchymal cells can populate two adjacent nephrons implies that the original infected cell and/or its immediate descendents were able to migrate, at least for a short distance to join an adjacent nephron-forming aggregate, as seen in Fig. 5. When cells were infected after induction, however, tagged cells were seen in only one segment of single nephrons. Hence, following induction, cell motility must have been restricted. A likely mechanism to account for this observation is the rapid appearance of cell adhesion molecules and extracellular matrix components after induction (Saxen et al., 1983; Vestweber et al., 1985; Aufderheide et al., 1987; Klein et al., 1988; Vainio et al., 1989). Collagen and laminin on the one hand and uvomorulin on the other could prevent any cell "entrapped" in one renal vesicle from migrating to a neighboring renal vesicle. This restricted mobility may place a renal progenitor cell in an environment that would limit its developmental options. The interactions of the cells that form the renal vesicle must be quite complex. It is well known that glomeruli appear only in that pole of the renal vesicle that is furthest away from the ureteric bud (Saxen, 1987). How such positional information contained in the early renal vesicle is translated into nephron segmentation remains to be determined.

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