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Establishment of renal proximal tubule cell lines by targeted oncogenesis in transgenic mice using the L-pyruvate kinase-SV40 (T) antigen hybrid gene

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SUMMARY

Targeted oncogenesis allowed us to obtain two cell lines which have been derived from the proximal tubule of kidney from transgenic mice harbouring the simian virus (SV40) large T and small t antigens placed under the control of the 5′ regulatory sequence from the rat L-type pyruvate kinase (L-PK) gene. The cell lines (PKSV-PCT and PKSV-PR cells) were derived from early (PCT) and late (Pars Recta, PR) microdissected proximal tubules grown in D-glucose-enriched medium. In such conditions of culture, both cell lines exhibited L-PK transcripts, a stable expression of SV40-encoded nuclear large T antigen, a prolonged life span but failed to induce tumors when injected sub-cutaneously into athymic (nu-nu) mice. Confluent cells, grown on plastic support or porous filters, were organized as monolayers of polarized cuboid cells with well developed apical microvilli and formed domes. Both cell lines exhibited morphological features of proximal tubule cells with villin located in the apical brush-border and subtantial amounts of hydrolase activity. By immunofluorescence studies using specific antibodies, aminopeptidase N appeared restricted to the apical microvillar domain, whereas the H2 histocompatibility antigen was distributed in the cytoplasm and lateral membranes. These results demonstrate that the proximal morphological phenotype has been fully preserved in these cultured cells derived from tissue-specific targeted oncogenesis in transgenic mice.

Key words: L-pyruvate kinase, SV40, proximal tubule, renal cultured cells, targeted oncogenesis

INTRODUCTION

Targeted oncogenesis in transgenic mice has been proposed as a powerful method of producing established lines of differentiated cells (Hanahan, 1988). The principal advantage is the avoidance of viral infection or transfection of primary or sub-cultured cells for the establishment of the cell lines. The expression of the oncogene is driven by a tissue-specific promoter, only active in differentiated cells or their precursors. Thus, a positive selective pressure maintaining a differentiated phenotype would be exerted on the immortalized cells. Moreover, depending on the tissue-specific promoter used, these models of transgenic mice are expected to provide new types of immortalized cells. Highly differentiated β-pancreatic cell lines derived from pancreatic tumors in which the production of insulin remains controlled by glucose (Efrat et al., 1988, 1991), neuronal cell lines (Mellon et al., 1990) and hepatoma cell lines (Antoine et al., 1992) have been established by this methodology.

We decided to derive epithelial tubule cells of the kidney using this approach. Cartier et al. (1992) have recently described LPK/Tag1 and LPK/c-*myc* transgenic mice carrying SV40 T antigen (Tag) or c-*myc* oncogenes, respectively, directed by the regulatory sequences of the L-type pyruvate kinase gene (L-PK, EC 2.7.1.40). This gene is physiologically expressed in the liver, kidney, small intestine (Imamura and Tanaka, 1982; Munnich et al., 1984; Noguchi et al., 1987) and endocrine pancreas (Cartier et al., 1992) and is subjected to transcriptional control: glucose and insulin excert positive effects, while cyclic AMP has a negative effect (Vaulont et al., 1986; Decaux et al., 1989). Previous studies have shown that a 3.2 kb of the rat L-PK gene 5′ regulatory sequence contains the elements necessary for tissue-specific expression and correct control of

transcription in liver, intestine and kidney (Tremp et al., 1989; Yamada et al., 1990; Cognet et al., 1991). As a result, L-PK/Tag1 and L-PK/c-myc transgenic mice develop diet-dependent malignancies of the endocrine pancreas and liver (Cartier et al., 1992), but kidney cancers are very rare. Thus, we used microdissected tubules from kidneys of these L-PK/Tag1 transgenic mice to derive the corresponding cell lines.

The present study describes the establishment of two immortalized epithelial cell lines, PKSV-PCT cells and PKSV-PR cells, derived from the convoluted portion (PCT) and the terminal straight part (Pars recta, PR), respectively, of superficial proximal tubules microdissected from the kidneys of L-PK/Tag1 transgenic mice. Both these cell lines exhibit nuclear large T antigen positivity when cultured in D-glucose-enriched medium and have retained their capacity to express the proximal tubule phenotype. By morphological and immunohistochemical studies, we showed that the cells grown on plastic support or on permeable filters formed confluent monolayers of polarized cells developing a brush-border which has retained the apical microvillar expression of villin and aminopeptidase N, both of which are reliable indicators of renal proximal tubule differentiation (Tauc et al., 1988; Maunoury et al., 1992).

MATERIALS AND METHODS

Transgenic mice

A vector containing a 2.7 kb *Bam*HI-*Bcl*I fragment of SV40 early region, including the coding sequences of the transforming large tumor (T) and small tumor (t) antigens (Tag), was placed under the control of a 3.2 kb fragment of the rat L-pyruvate kinase (L-PK) gene regulatory region in the 5′ flanking region (Cognet et al., 1987). The *Eco*RI-*Pvu*I fragment of the L-PK/Tag transgene was purified by restriction endonuclease digestion, agarose gel electrophoresis and binding to glass powder. Purified DNA fragments were microinjected into fertilized mouse eggs (Cartier et al., 1992). One line of transgenic mice (L-PK/Tag1 mouse) has been shown to express the transgene in a appropriate tissue-specific manner, i.e. in the liver, intestine, kidney and endocrine pancreas (Cartier et al., 1992).

Cell cultures

Tubule segments from various locations on the proximal tubule were isolated by microdissection (Vandewalle et al., 1981) and used to establish proximal cell lines. Three-month-old male transgenic mice fed a high-carbohydrate diet (75%) were killed under nembutal anesthesia, the abdominal cavity was opened under sterile conditions, and the kidneys removed. The renal capsule was removed and thin sections from the renal cortex were incubated for one hour at 37°C in medium (DMEM:Ham's F_{12} (1:1, v/v), 30 nM sodium selenate, 5 μg/ml transferin, 2 mM glutamine, 20 mM HEPES, pH 7.4) containing 20 mM D-glucose, 2% decomplemented fetal calf serum and 0.1% (w/v) collagenase (type 1, Sigma). The cortical slices were then rinsed in this medium, without collagenase, and tubule segments were microdissected out using fine sterile needles under a stereomicroscope at room temperature. The convoluted (PCT) and terminal (Pars Recta, PR) segments of superficial proximal tubules were isolated. Pools of two to five segments (0.1 to 0.5 mm) of PCT and PR tubules were rinsed twice in 20 ml of medium, transferred to 24-well trays precoated with rat tail collagen and 1 ml of fresh medium was added to each well. To ensure cell growth, the tubules were cultivated in a modified culture medium (CM: DMEM:Ham's F_{12} (1:1, v/v), 30 nM sodium selenate, 5 μg/ml transferin, 2 mM glutamine, 50 nM dexamethasone, 1 nM triiodothyronine, 10 nM epidermal growth factor, 2% fetal calf serum, 20 mM HEPES, pH 7.4) supplemented with 5 μg/ml insulin and 20 mM D-glucose at 37°C in 5% CO_2/95% air atmosphere. Cells began to grow after two to four days in culture; the medium was not changed until two weeks after seeding. After three weeks and two changes of medium, primary cultures reached confluency. The cells were then removed by incubating with 1 ml 0.05% trypsin/0.02% EDTA for 30 min at 37°C. The detached cells from each segment (PCT or PR) were pooled and seeded into three wells of 12-well trays. Cell growth accelerated after the first passage and medium was changed every four days. Two weeks later, confluent cells were trypsinized and seeded into 3 wells of 6-well trays. The resulting confluent cells were removed and seeded into 25 cm^2 culture flasks. At this stage, cells were routinely sub-cultured into 25 or 75 cm^2 culture flasks and the medium was changed every two days. Two renal cell lines, one derived from PCT and the other from PR microdissected tubules, were established and referred to as PKSV-PCT and PKSV-PR cells, respectively. A similar procedure was applied to microdissected segments from other parts of the renal tubule. None of them, including glomeruli, thick ascending limbs of Henle, distal convoluted or cortical collecting tubules, could be sub-cultured. As previously observed for isolated rabbit proximal tubule cells mock-infected with the wild-type SV40 strain LP (Vandewalle et al., 1989), proximal microdissected tubules from normal mice could not been sub-cultured for more than five passages.

All studies were performed between the 15th and 40th passages on sets of cells seeded on plastic Petri dishes, culture flasks or on semi-permeable transparent filters precoated with collagen (Transwell-COL, 0.4 μm pore size, 1.2 cm^2 diameter; Costar Europe Ltd., Badhoevedorp, The Netherlands, or Millicell-CM, 0.4 μm pore size, 0.6 cm^2 diameter; Millipore Continental Water systems, Bedford, MA).

Electrophysiological studies

Experiments were performed on confluent cells initially seeded on Millicell-CM filters. The transepithelial resistance (R) was measured (10 and 30 days after seeding) using the Millicell Electrical Resistance system (ERS, Millipore corporation, USA) and dual silver/silver chloride (Ag/AgCl) electrodes. The resistance area measured was deduced from the resistance of the same collagen filters devoid of cells and expressed as Ω/cm^2. Transepithelial voltage (V_t) was measured in parallel by applying square current pulses (± 20 mA, 2 s duration) through the Ag-AgCl electrodes.

Enzymatic studies

Enzyme activities were measured in renal cortical homogenates and the two established proximal renal cell lines. Homogenates from the kidney cortex of transgenic mice fed a high-carbohydrate diet were prepared as previously described (Vandewalle et al., 1989). Confluent cell monolayers grown on plastic Petri dishes were washed in phosphate-buffered saline (PBS) and resuspended in 20 mM Tris buffer, pH 8. All samples were stored at −80°C before use. γ-Glutamyl transpeptidase (γ-GT), aminopeptidase N (APN), dipeptidylpeptidase IV (DPP IV) and phosphoenolpyruvate carboxykinase (PEPCK) were measured as previously described (Vandewalle et al., 1989). Pyruvate kinase was determined by the procedure of Bücher and Pfleiderer (1955). Protein content was measured by the Bradford (1976) method with bovine serum albumin (BSA) as the standard.

Extraction of total tissue and cellular RNA

Mice were killed, and tissue samples immediately frozen in liquid

nitrogen. Total RNA was extracted from tissues by guanidine thiocyanate solubilization followed by centrifugation of the extract through a cushion of 5.7 M CsCl (Chirgwin et al., 1979). For cultured cells, RNA was extracted by the LiCl method described by Genton et al. (1987). The integrity of all RNA preparations stained with ethidium bromide was analyzed by electrophoresis in agarose/formaldehyde submarine minigels.

Reverse transcriptase/polymerase chain reaction (RT-PCR)

Total RNA was reverse transcribed into cDNA as described by Akli et al. (1991). Reverse transcription was performed in a total volume of 20 µl by incubating 1 µg RNA at 42°C for 30 min with 100 pmoles of random hexamer primers (pd (N6), Pharmacia), 20 units RNasin (Promega, Biotec), 400 µmoles deoxyribonucleotide triphosphate (dNTP), 200 units Moloney murine leukemia virus reverse transcriptase in PCR buffer (16.6 mM ammonium sulfate, 10 mM β-mercaptoethanol, 6.7 µM EDTA, 4.5 mM $MgCl_2$, 67 mM Tris-HCl, pH 8.8). After 5 min at 90°C, 70 µl of the same PCR buffer containing 10% (v/v) dimethyl sulfoxide, 60 pmoles of each specific primer, 250 µM dNTP and 1 µCi of [α-^{32}P]dCTP was added. After incubation at 80°C for 10 min, 10 µl *Taq* polymerase (0.2 unit/ml) was added. The PCR was taken through 30 cycles of denaturation at 92°C for 30 s, annealing at 55°C for 30 s, and primer extension at 72°C for 1 min. The specific primers were selected from exon 2 and 4 of the rat L-PK gene (Cognet et al., 1987). Samples (15 µl) of PCR products were run on a 6% polyacrylamide gel and screened by autoradiography.

Morphological studies

Indirect immunofluorescence

Nuclear large T antigen was identified in confluent cells grown on glass coverslips. The cells were washed in PBS, fixed with ice-cold methanol (30%)/acetone (70%) solution for 30 min at 4°C, rinsed with PBS, and incubated at room temperature for 60 min with a polyclonal rabbit antibody specific for large T antigen (kindly provided by Dr. Feunteun) The cells were washed with PBS, incubated with biotinylated anti-IgG, followed by incubation with streptavidin-fluorescein. Villin, aminopeptidase N (APN), and a class II histocompatibilty antigen (H2) were detected immunocytochemically as described by Reggio et al. (1983). Briefly, confluent cells cultured on glass coverslips or on Petri dishes were fixed with 3% paraformaldehyde and permeabilized or not with 2% Triton X-100. Cells were first incubated with 10 µg/ml of $BDID_2C_3$ monoclonal anti-villin antibody (Dudouet et al., 1987), polyclonal anti-APN antibody (3.01.01.75 antibody, kindly provided by Dr S. Maroux), or biotinylated anti-H2 monoclonal antibody (H141-30 antibody, anti-H2 D^b) (Lemke et al., 1979) in a humidified atmosphere. After washing, cells were incubated with fluorescein-conjugated anti-IgG antibodies (for $BDID_2C_3$), biotinylated species-specific anti-IgG (for anti-APN) and rhodamine-labelled streptavidin (for anti-APN and H141-30) (Biosys, France). All preparations were examined under a Zeiss photomicroscope equipped with epifluorescence optics. Specimens labelled with anti-APN and anti-H2 antibodies were also examined by confocal laser scanning microscopy (CLSM). Fluorescein- or rhodamine-labelled cells were visualized by excitation at 488 nm and emitted light above 515 nm was recorded. The cell monolayer was optically sectioned in horizontal planes (*x-y*) or vertical planes (*x-z*) every 0.8 µm. For these experiments nuclei were visualized by incubating cells with propidium iodine (2 µg/ml) during the last wash following the second incubation step. The images generated were photographed from the screen using a freeze-frame polaroid system.

Optical and ultrastructural studies

Confluent primary cultures and cell monolayers from the established cell lines grown on 24-well trays or 60 mm plastic Petri dishes were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7) for two hours at room temperature. Cells were rinsed in 0.1 M cacodylate buffer and viewed on the stage of an inverted microscope (Nikon, MDT). For ultrastructural studies, cells were grown on Costar filters coated with collagen. Confluent cells grown under different conditions were rinsed in PBS and fixed for two hours with 2.5 % glutaraldehyde in 0.1 M cacodylate buffer, as described above. The filters were then excised from the plastic holders, cut into small pieces, post-fixed in 1% osmium tetroxide, dehydrated in a graded series of ethanols and embedded in Epoxy resin. Ultrathin sections were cut from transversally orientated confluent monolayers, counterstained with uranyl acetate and lead citrate, and examined under a Philips electron microscope (EM 410). Cells for scanning electron microscopy were grown on semi-permeable filters and fixed and dehydrated in an ethanol series as previously described. The detached filters were placed in Freon 113 and dried after substitution with liquid CO_2 in a critical point dryer (Balzers, Hudson, NH). Dried filters were coated with gold and specimens were examined with a Jeol 35CS scanning electron microscope. The density of apical microvilli was estimated morphometrically on scanning electron micrographs. A space grid with interline spacing equivalent to 1 mm was applied on enlarged photographs (×12,000). The number of microvilli per μm^2 membrane surface was determined over three to five different areas from individual cell. Twenty (for PCT cells) to 21 (for PR cells) cells from three different passages were analyzed.

Protein extraction and immunoblot analysis

Villin immunodetection by Western blotting of cell extracts was performed using the procedure of Burnette et al. (1981) as modified by Coudrier et al. (1983). Immunoblots were performed with 50 µg of total cellular extract from confluent cells grown on plastic support.

RESULTS

Establishment of proximal cell lines

The expression of Tag transcripts in L-PK/Tag1 transgenic mice increased in the liver, intestine and kidney when the mice were fed a high-carbohydrate diet (Cartier et al., 1992). Microdissected tubules were therefore cultured in a D-glucose-enriched medium. Two cell lines (PKSV-PCT and PKSV-PR) derived from microdissected proximal convoluted (PCT) and Pars recta (PR) tubules were established and sub-cultured in a culture medium containing 20 mM D-glucose. The cells in primary culture were characterized by their homogeneous shape. Cells derived from proximal convoluted tubules have a cobblestone appearance (Fig. 1A), whereas cells derived from Pars recta tubules appeared elongated and aligned in a swirled pattern along their long axes (Fig. 1B). After the 7th passage, all the cells were nuclear large T antigen-positive when cultured in high D-glucose medium (Fig. 1C,D). In order to demonstrate that the endogenous L type PK gene was present within the cell lines, the L-PK transcripts were detected by PCR, as the L-PK signal from the two renal cell lines by Northern blot was very faint (not shown). As shown in Fig. 2, both cell lines expressed the L-PK transcripts as in the liver, and in

the whole kidney. No L-PK mRNA was detected in the brain, which was used as a negative control (Fig. 2).

PKSV-PCT and PKSV-PR cells exhibited rapid rates of cell doublings (20-30 hours), and they had a prolonged life-span, having been taken through more than 60 passages to date. These immortalized cell lines had no tumorigenic potential. No tumor was detected 9 weeks after a subcuteanous injection into congenitally nu/nu (nude) athymic mice (5×10^6 cells per mice, $n = 8$).

Both PKSV-PCT and PKSV-PR cells formed confluent monolayers on Petri dishes. Dome formation, considered as a feature of transporting epithelia in culture (Cereijido et al., 1981), occurred soon after the cell lines were established (before the 10th passage); it occurred more frequently in PKSV-PCT (Fig. 3A) than in PKSV-PR cells (Fig. 3B). The cells exhibited epitheloid shape, with an apical domain bearing many microvilli as shown by scanning electron microscopy (Fig. 3C and D). Morphometric analysis showed that the number of microvilli per membrane surface area was almost 3-fold higher in PKSV-PCT (29 ± 2 s.e.m. microvilli per μm^2, $n = 19$) than in PKSV-PR cells (10 ± 1 s.e.m. microvilli per μm^2, $n = 21$).

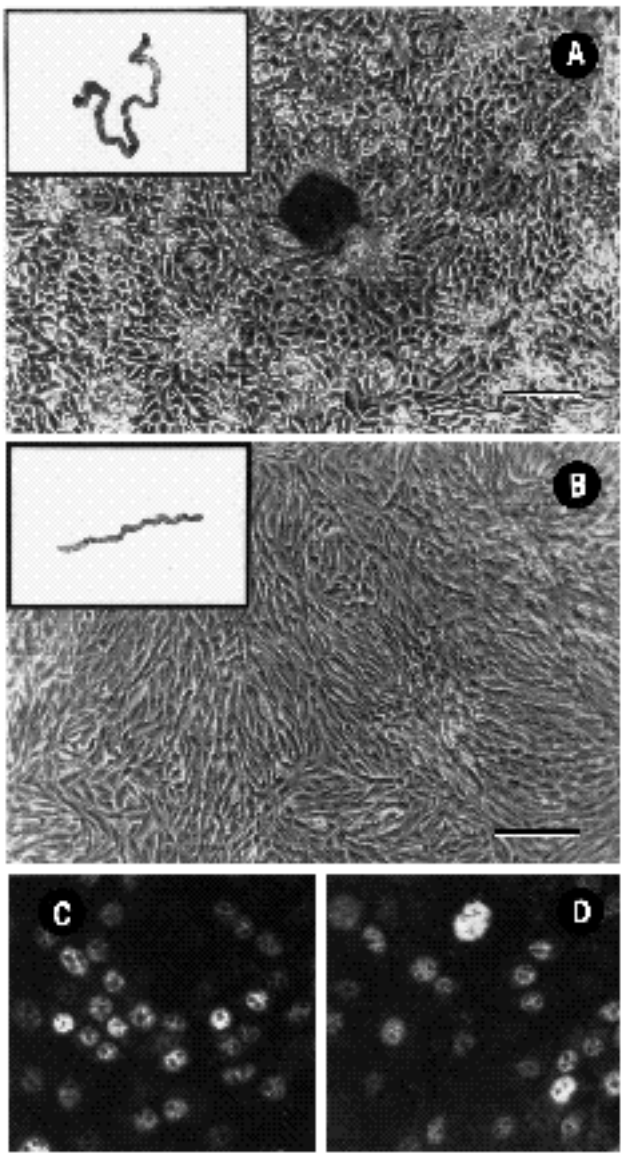

Fig. 1. Morphology of primary cultures of PK-SV cells and nuclear expression of SV40 large T antigen. (A-B) Phase-contrast microscope pictures of confluent primary cultures (day 30) from isolated microdissected proximal convoluted (PCT) and Pars recta (PR) tubules plated on plastic Petri dishes and grown in 20 mM D-glucose-enriched medium. Isolated microdissected PCT and PR tubules are shown in the insets. The confluent cells had a homogeneous epitheloid cell shape but the PKSV-PR cells (B) were more elongated than the PKSV-PCT cells (A).
(C-D) Confluent cells (7th passage) grown on glass coverslips and fixed with 60% methanol/30% acetone, were processed to detect large T antigen as described in Materials and methods. The nuclei of all PKSV-PCT (C) and PKSV-PR (D) cells contained large T antigen. Bars, 50 μm.

Morphological and enzymatic characteristics of proximal cell lines

The two renal cell lines have retained the principal features of in vivo proximal tubule cells. The proximal tubule origin of the cells was assessed by the expression of villin. This tissue-specific actin-binding protein (Louvard, 1989; Bretscher, 1991) is mainly expressed in epithelial cells developing brush-borders, particularly in renal proximal tubule cells where this protein is detected after the mesenchyme/epithelium conversion following the appearance of tubular structure (Maunoury et al., 1992). Immunofluorescence studies on permeabilized cells showed the presence of villin in microvilli of confluent cells from both cell lines (Fig. 4A and B). In accordance with the lower density of apical microvilli present in PKSV-PCT than PKSV-PR cells (Fig. 3), the cellular villin content per 50 μg of total protein extract, as determined by Western blot analysis, was sligtly lower in PKSV-PR (Fig. 4A, inset) than in PKSV-PR cells (Fig. 4B, inset). The apical microvillar location of villin was confirmed by CLSM analysis, which showed that villin is mainly restricted in the apical microvillar domains of the cells (Fig. 4C,D,E). Ultrastructural analysis of transverse sections of cells grown on porous filters indicated that both cell types were fully polarized. The cells, organized as monolayers (Fig. 5A,F), exhibited epitheloid shapes, with an apical domain bearing many microvilli (Fig.

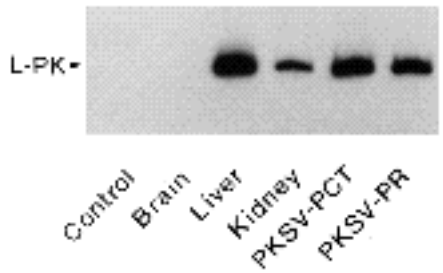

Fig. 2. L-type pyruvate kinase gene expression in PK-SV cells. Autoradiograms of PCR products obtained with RNA from brain, liver, kidney and the two renal cell lines (PKSV-PCT and PKSV-PR) grown in D-glucose-supplemented medium. As a negative control PCR amplification was performed in the absence of RNA.

5B,C,G,H) separated from lateral and basolateral domains by tight junctions (Fig. 5D,I). The PKSV-PCT cells (Fig. 5B,C) displayed more microvilli than the PKSV-PR cells (Fig. 5G,H). The electrophysiological studies (Table 1) indicated that PKSV-PCT and PKSV-PR cell monolayers developed a low transepithelial resistance and a small apical negative voltage. The transepithelial resistance was similar to that reported by Merot et al. (1988) for primary cultures of rabbit kidney proximal cells (ranging between 37.4 and 60.0 Ω/cm^2).

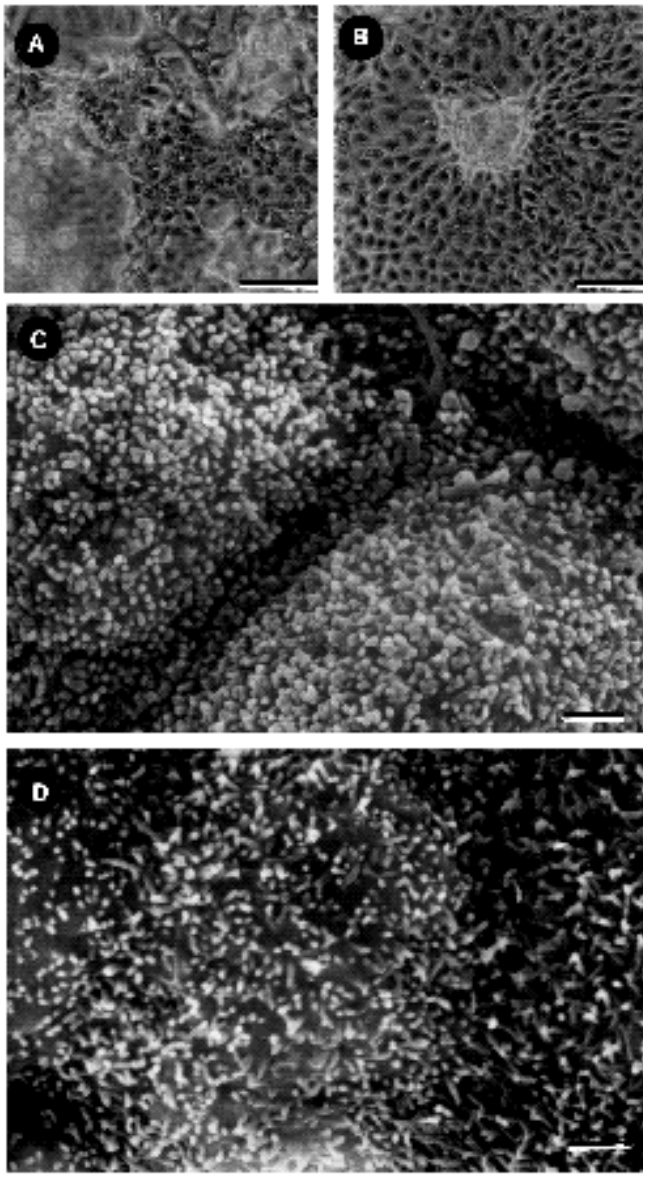

Fig. 3. Morphological aspects of proximal cell lines. PKSV-PCT (A, C) and PKSV-PR (B, D) cells were plated at 5×10^4 cells/ml on plastic Petri dishes and grown in medium supplemented with 10 mM D-glucose and 5 μg/ml insulin. The cells of both lines were cuboid and form characteristic domes (A, B). The abundance of dome formations was more important in PKSV-PCT (A) than PKSV-PR cells (B). The density of apical microvilli, illustrated by scanning electron microscopy, was greater in PKSV-PCT (C) than in PKSV-PR (D). Bars, A, B, 50 μm; C, D, 1 μm.

Table 2 summarizes the profiles of enzyme activity of the two renal proximal tubule cell lines. The total pyruvate kinase activity, which reflects the activity of the L and M_2 isoforms present in the kidney (Imamura and Tanaka, 1982), was 3-fold higher in PKSV-PCT cells and 5-fold higher in PKSV-PR cells than that of cortex homogenate (Table 2). The activity of PEPCK, a rate-limiting enzyme of the gluconeogenic pathway restricted to the proximal tubule (Vandewalle et al., 1981), although lower than in renal cortex homogenates, was present in cultured cells (Table 1). These results indicated that, in the conditions of culture required for proliferation (D-glucose-enriched medium), the cells had maintained a gluconeogenic capacity and an active glycolytic pathway.

Both PKSV-PCT and PKSV-PR cells also maintained cell-specific hydrolase activity (Table 2). γ-Glutamyl transferase, aminopeptidase N and dipeptidyl peptidase IV activities, normally concentrated in apical microvilli from enterocytes and renal-proximal tubule cells (Kenny and Maroux, 1982; Tauc et al., 1988), were lower than in cortex homogenates, but almost identical to that measured in primary cultures of proximal cells (Ronco et al., 1990).

The apical localization of aminopeptidase N was probed by indirect immunofluorecence with a specific anti-aminopeptidase N antibody. The results are illustrated in Fig. 6. In both cell lines the fluorescence appeared to be concentrated in the brush-border of the cells (Fig. 6A,B). CLSM analysis of PKSV-PCT and PKSV-PR cell monolayers, optically sectioned in vertical planes, showed that

Table 1. Transepithelial resistance and voltage of cell monolayers grown on filters

Days after seeding	PKSV-PCT cells	
	R (Ω/cm2)	V_t(mV)
10	37 ± 3 (10)	−0.4 ± 0.1 (10)
30	52 ± 5 (9)	−0.8 ± 0.2 (9)
	PKSV-PR cells	
	R (Ω/cm2)	V_t(mV)
10	28 ± 4 (9)	−0.2 ± 0.1 (9)
30	27 ± 4 (7)	−0.2 ± 0.1 (7)

Transepithelial resistance (*R*) and voltage (V_t) were measured on confluent PKSV-PCT and PKSV-PR cells grown on Millipore filters in medium supplemented with 20 mM D-glucose and 5 μg/ml insulin. Values are the means ± s.e.m. of (*n*) determinations at five different passages.

Table 2. Enzymatic activities in renal cortex homogenates and PKSV cell lines

	Homogenates	PKSV-PCT	PKSV-PR
Pyruvate kinase (6)	18.6 ± 1.1	54.6 ± 3.0	89.7 ± 4.6
Phosphoenolpyruvate carboxykinase (4)	38.7 ± 1.5	5.7 ± 1.6	4.3 ± 0.7
γ-Glutamyl transferase (4)	881.4 ± 69.5	219.8 ± 19.4	196.4 ± 26.6
Aminopeptidase N (5)	38.8 ± 3.5	20.5 ± 2.5	38.0 ± 9.8
Dipeptidyl peptidase IV (5)	52.4 ± 4.7	14.2 ± 1.9	12.5 ± 1.0

Enzymatic activities, expressed as μmol. min^{-1}.g $protein^{-1}$, are the means ± s.e.m. from (*n*) measurements performed on cortex homogenates (Homogenates) and confluent PKSV-PCT and PKSV-PR cells grown in medium containing 20 mM D-glucose and 5 μg/ml insulin.

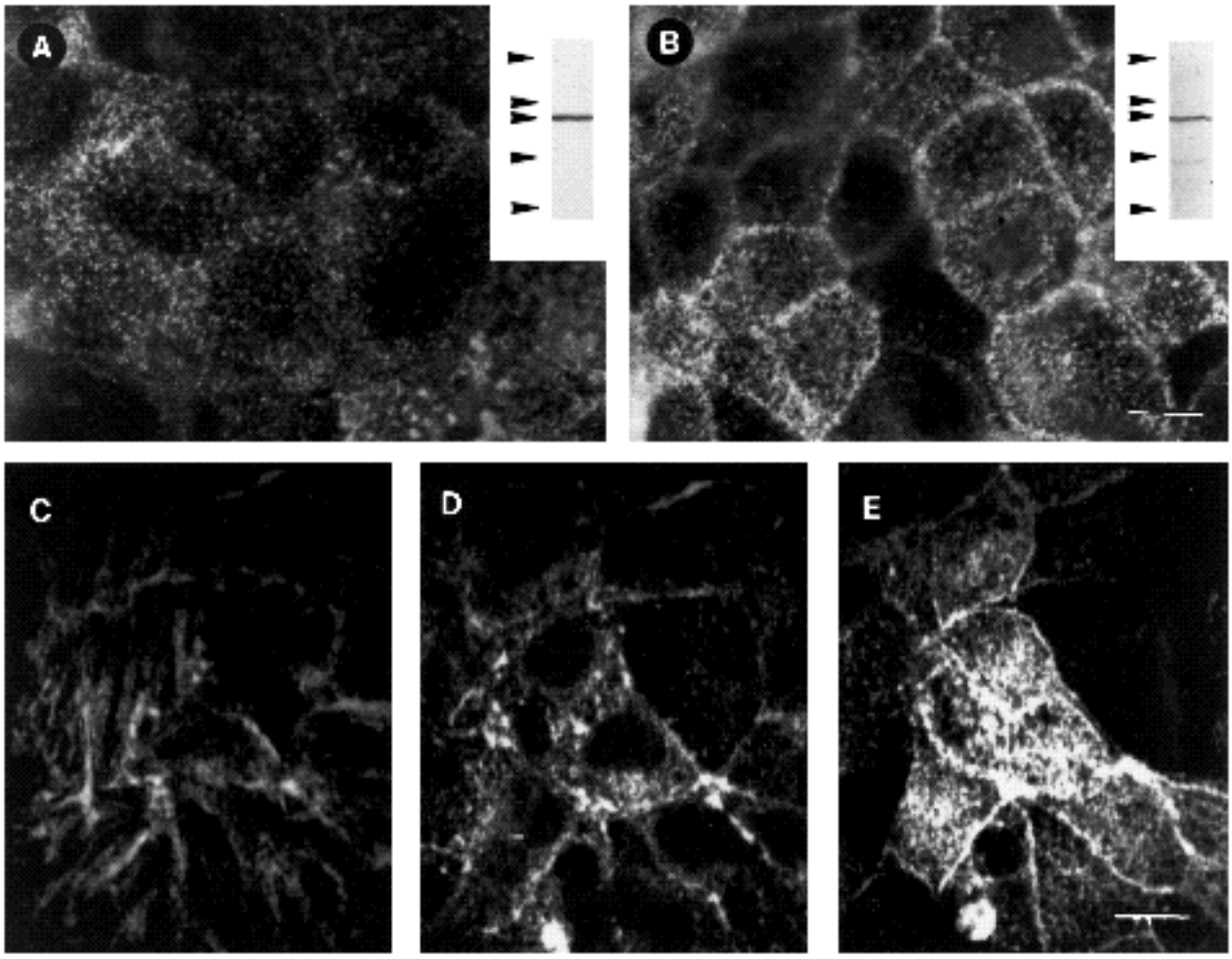

Fig. 4. Immunolocalization of villin in confluent PKSV cells. A monoclonal anti-villin antibody ($BDID_2C_3$) was used to localize villin in PKSV-PCT (A) and PKSV-PR cells (B). The microvilli were positive in both cell lines. Bar, 10 μm. (C to E) Representative illustrations by CLSM of the brush-border localization of villin within PKSV-PCT cells. Serials optical planes (*x*-*y*) parallel to the glass coverslips were generated by CLSM from the first plane (C) to the apex (E). (D and E) Three-dimensional reconstructions of the two (D) or three (E) upper planes. Villin, almost absent at the basolateral side (C), was detectable at the cell-cell contact area (D), and mostly located in the apical microvilli (E). Identical results were obtained with PKSV-PR cells (not shown). The amount of villin, assayed by Western blot analysis, were slightly higher in PKSV-PCT (A, inset) than in PKSV-PR (B, inset). Molecular size markers are indicated by arrowheads: myosin (180 kDa), β galactosidase (116 kDa), phosphorylase b (92 kDa), bovine serum albumin (68 kDa), actin (43 kDa). Bars, 10 μm.

aminopeptidase N was restricted to the apical domain of the cell plasma membrane (Fig. 6C,D). By contrast with the apical distribution of neutral aminopeptidase and villin (Fig. 4), CLSM analysis of PKSV cells also showed a lateral membrane localization and the presence of an intracytoplasmic pool of the H2 antigen (Fig. 6E,F), as was observed with classs I HLA in differentiated HT29 colonic carcinoma cells (Godefroy et al., 1988). Thus, these results demonstrate that the cells, maintained in a state of high rate proliferation by D-glucose-enriched medium, were fully polarized.

DISCUSSION

The two renal cell lines that we have established retain the principal morphological features and functions of proximal tubule cells. Both cell lines have well developed apical microvilli with substantial amounts of villin. This actin-binding protein is one of the major core proteins in microvilli of the brush-borders of intestinal (Mooseker, 1985) and renal proximal epithelial cells (Coudrier et al., 1988). Villin has been shown to be an early marker of intestinal and proximal tubule cell lineages (Robine et al., 1985; Dudouet et al., 1987; Maunoury et al., 1988, 1992). Thus, the finding that villin remained in the microvilli of rapidly growing PKSV proximal tubule cells also suggests that differentiation is not appreciably affected by cell growth.

Although low, both cell lines also exhibited phosphoenolpyruvate kinase activity, a neoglucogenic enzyme present in early and late proximal segments of the renal tubule and absent in the more distal segments (Vandewalle et al., 1981). As another feature of proximal tubule cells, both cell

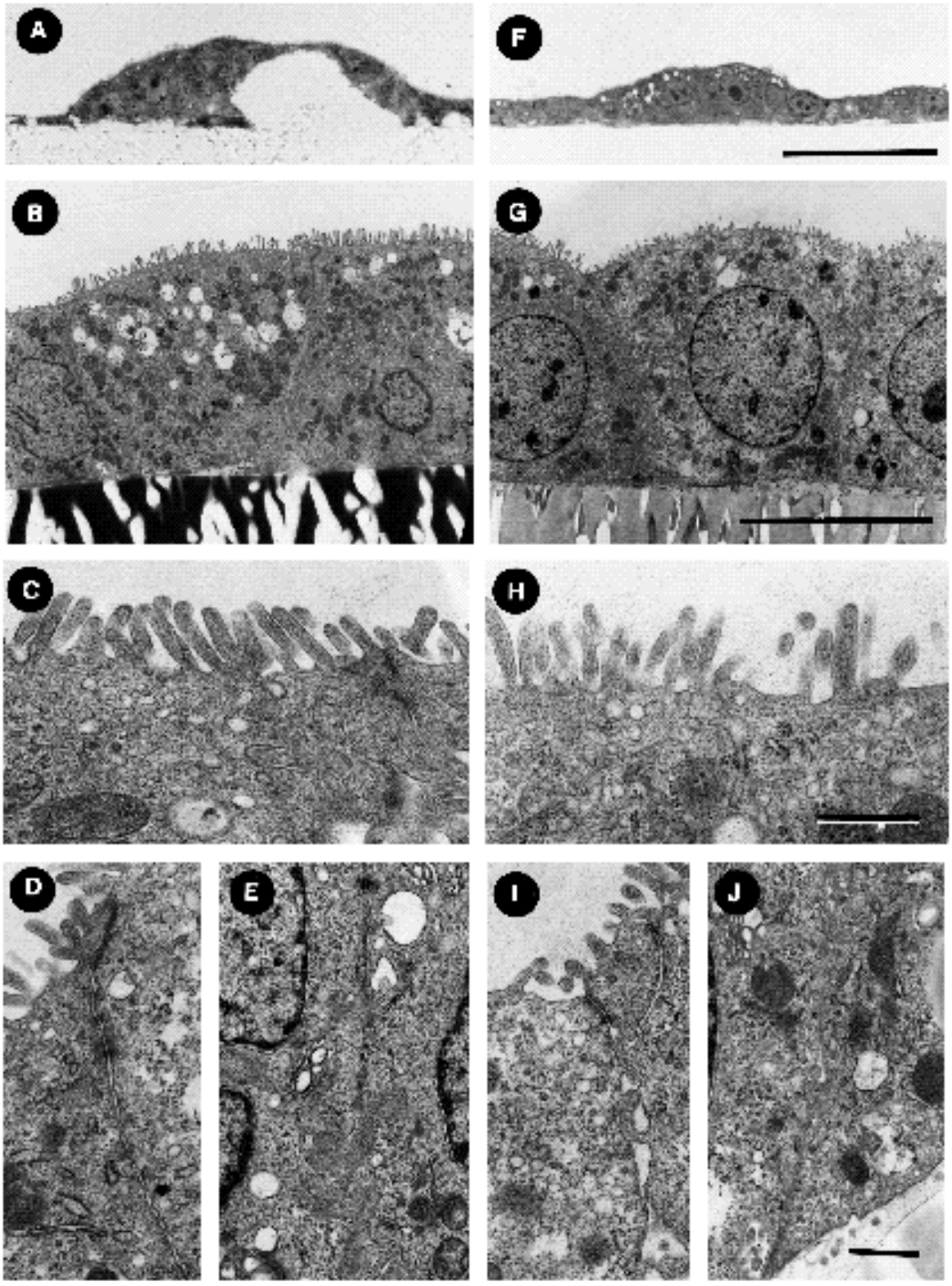

Fig. 5. Light and electron microscopic appearance of PKSV cells grown on plastic supports and porous filters. PKSV-PCT (A to E) and PKSV-PR cells (F to J) were grown to confluency on collagen-coated Petri dishes (A, F) or transparent Costar filters (B, C, D, E, G, H, I, J) in culture medium containing 20 mM D-glucose and 5 µg/ml insulin. Light microscopy of transverse semi-thin sections (A, F) showed that both cell lines formed confluent monolayers, with domes in the PKSV-PCT cells (A). These cells contained more apical microvilli than did PKSV-PR cells. The two cell lines, grown on porous filters, formed regular monolayers of cuboid cells with apical membranes bearing numerous microvilli (B, G). The microvilli are shown in C and H. The cells are separated by closely apposed lateral membranes (E, J) with tight junctions (D, I) and desmosomes (D, E, I). Bars, A and F, 50 µm; B and G, 10 µm; D, E, G, H, I, J, 1 µm.

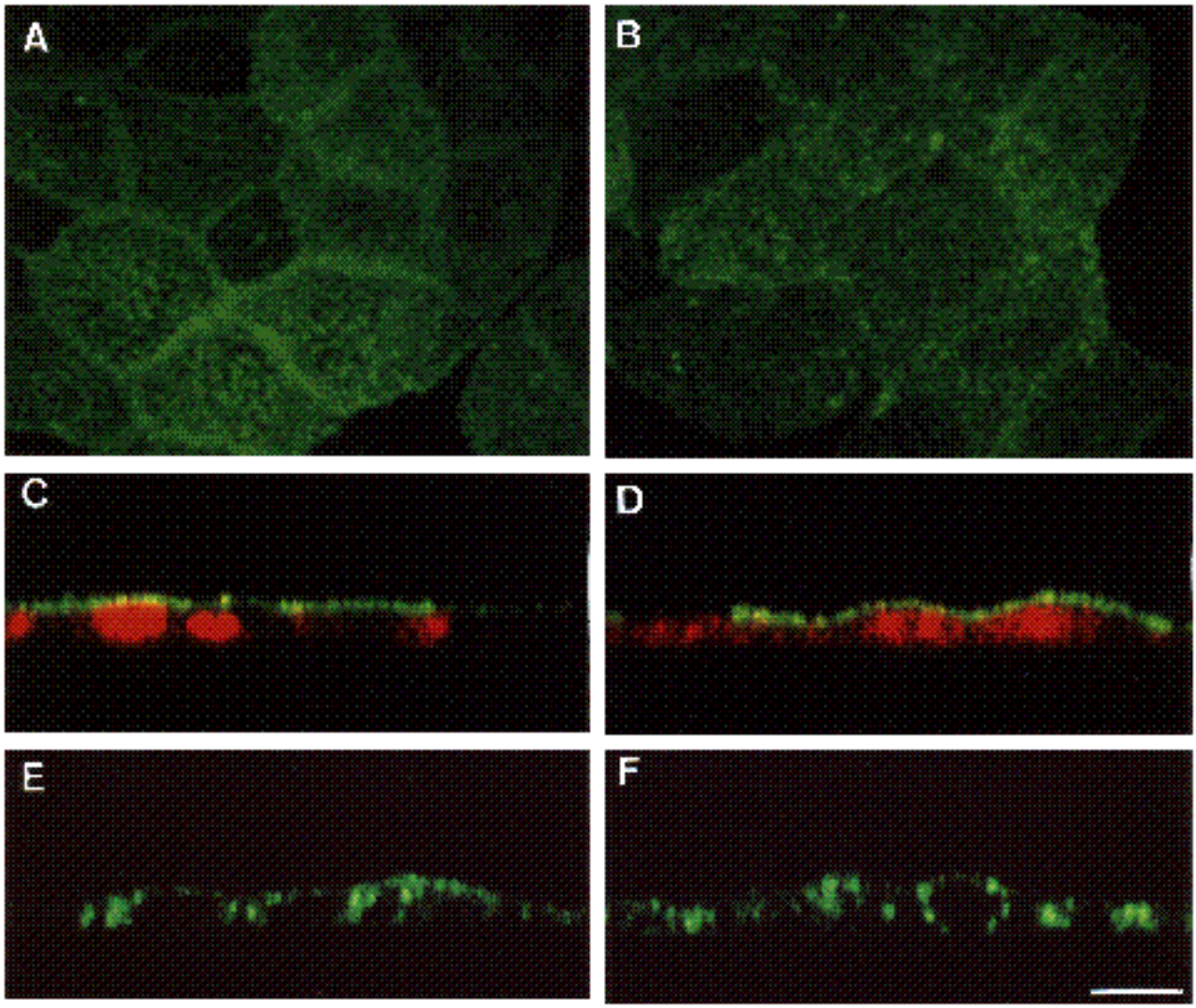

Fig. 6. Cellular distribution and localization of APN and H2 antigen in PKSV cells. PKSV-PCT (A, B) and PKSV-PR (C, D) cells seeded on plastic dishes were grown in D-glucose-enriched medium. Indirect immunofluorescence with the anti-APN showed apical labelling in both PKSV-PCT and PKSV-PR cells. On vertical (*x*-*z*) optical sections obtained by CLSM, the fluorescence remain stricly localized at the apical side of PKSV-PCT (B) and PKSV-PR cells (D). The position of the nucleus was visualized by labelling with propidium iodine. Identical results were obtained with PKSV-PR cells (not shown). (E and F) Illustrate the cytoplasmic and lateral membrane localization of the class II histocompatibilty antigen revealed by the anti-H2 D^b antibody. PKSV-PCT (E) and PKSV-PR (F) cells were analyzed on vertical (*x*-*z*) optical sections obtained by CLSM. Bar, 10 μm.

lines exhibited membrane-anchored hydrolase activities. Among them, aminopeptidase N was found exclusively localized in the apical domains of the cells delimited by tight junctions, as previously reported in proximal-like LLC-PK_1 cells (Louvard, 1980). Thus, the presence of a polarized expression of villin in microvilli and subtantial amounts of hydrolase activity at the aminopeptidase N location in the apical membrane domain, which are reliable enzymatic indicators of proximal and intestinal cell terminal differentiation (Kenny and Maroux, 1982; Ronco et al., 1990), strongly suggest that PKSV-PCT and PKSV-PR cells remain differentiated.

Kidneys contain small amounts of L type pyruvate kinase (Imamura and Tanaka, 1982; Munnich et al., 1984; Noguchi et al., 1987) and a recent immunocytochemical study by Domingo et al. (1992) showed that the L-type pyruvate kinase (PK) was localized exclusively in proximal convoluted (PCT) and straight proximal (PR) tubules from rat kidney. However, unlike the liver, the kidney principally produces the M_2 isoform of PK (Imamura and Tanaka, 1982). Electrophoresis of partially purified extracts indicates that the predominant isoforms in both PKSV-PCT and PKSV-PR cells are, indeed, the M_2 isoform and an L/M_2 hybrid (data not shown). Thus, the high PK activity measured in cultured PKSV proximal cells corresponds primarily to the M_2 isoform. The L-PK gene is probably regulated by D-glucose in the proximal convoluted and Pars recta tubules, as both PCT and PR tubule cells can be regularly sub-cultured in a D-glucose-enriched medium and all cells are nuclear Tag-positive. This was confirmed by PCR

amplification experiments showing that both proximal cell lines contain the L-PK gene.

The two immortalized proximal tubule cell lines are not tumorigenic and grow as single monolayers, unlike fetal hepatocytes derived from the same L-PK/Tag1 transgenic mice (unpublished data). This suggests that Tag is weakly oncogenic in renal tubule cells, either because it is not strongly expressed or because these cells are intrinsically resistent to transformation by Tag. Either of these characteristics could explain why immortalized proximal tubule cells are produced without any major change in their differentiation due to SV40 Tag-induced transformation.

In conclusion, immortalized, differentiated cell lines have been established from early and late portions of the proximal renal tubule cells from transgenic mice. The cells have maintained specific markers of polarity and also the main functions of proximal tubule phenotype which are described in the companion article (Lacave et al., 1993). Such proximal tubule cell lines are, therefore, promising models for further physiological, pharmacological and regulation studies of this important class of kidney cells.

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