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A conditionally immortalized cell line from murine proximal tubule

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A conditionally immortalized cell line from murine proximal tubule. We have developed a conditionally immortalized murine cell line with proximal tubule characteristics (*ts*MPT) and a background suitable for genetic manipulations. *ts*MPT was derived from the F_1 progeny of crosses between: [1] a transgenic mouse harboring a γ-interferon (IFN-γ)-inducible, temperature sensitive SV40 large T antigen gene (*ts*A58) and [2] mice of the 129/SvEv strain, the background from which most embryonic stem (ES) cells are derived. Under permissive conditions (33°C and in the presence of IFN-γ), *ts*MPT cells grow rapidly as monolayers with a doubling time of 23 hours; the large T antigen can be detected by immunocytochemistry and by Western blotting. When transferred to non-permissive conditions (39°C, without IFN-γ), the cells undergo differentiation coinciding with the disappearance of the large T antigen. By electron microscopy, *ts*MPT cells are polarized and show microvilli at their apical surface. *ts*MPT cells express brush border enzymes γ-glutamyl transpeptidase and carbonic anhydrase IV. They possess Na^+-dependent transport systems for P_i, D-glucose and L-proline as well as an amiloride-insensitive Na^+-H^+ exchanger. Intracellular cAMP generation is stimulated by parathyroid hormone but not by arginine vasopressin. Angiotensinogen mRNA and protein are present in *ts*MPT with markedly higher levels at non-permissive conditions. *ts*MPT cells should be a useful model for investigation of the functional features of the proximal tubule epithelium in relation to cellular differentiation.

Recent observations have shown that molecular variants of the angiotensinogen gene constitute inherited predispositions to essential hypertension [1]. The evidence obtained so far is statistical in nature and therefore does not provide a functional clue. The underlying mechanism may involve circulating as well as tissue renin-angiotensin systems (RAS). The proximal tubule is the major site of sodium reabsorption by the kidney [2]. Sodium is reabsorbed primarily via the Na^+-H^+ exchanger but also cotransported with glucose, phosphate and amino acids. All the components of renin-angiotensin system, including angiotensin II receptors are present in the proximal tubule, suggesting that this local RAS may participate, in an autocrine/paracrine fashion, in sodium homeostasis and blood pressure regulation [3, 4].

Key words: angiotensinogen, cell culture, differentiation, transgenic mice, proximal tubule, SV 40 large T antigen.

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We have developed a murine cell line of proximal tubule origin (*ts*MPT cells), which is conditionally immortalized by the expression of an inducible transgene, allowing control of its proliferative potential to induce either division or cellular differentiation. The cells were derived from the kidneys of mice that were the F_1 progeny of crosses between female mice of the 129/SvEv background and a male mouse homozygous for a transgene consisting of a DNA segment encoding the temperature-sensitive large T antigen of SV40 (*ts*A58) under the control of the mouse major histocompatibility complex H-$2K^b$ class 1 promoter, which is inducible by γ-interferon (IFN-γ) [5, 6]. The 129/SvEv strain was chosen because it is the background from which most embryonic stem (ES) cells used for the development of transgenic animals are derived [7]. The cells are immortalized due to the expression of the SV40 large T antigen. They proliferate under permissive conditions (33°C, in the presence of IFN-γ) and differentiate under non-permissive conditions (39°C, without IFN-γ). *ts*MPT cells express angiotensinogen and angiotensin converting enzyme, primarily under non-permissive conditions, thus providing a model system to probe the genetics of angiotensinogen expression in the proximal tubule.

METHODS

Selection of proximal tubules

Kidneys were removed aseptically from several adult mice and the cortex dissected and cut into 1 mm^3 pieces. These were incubated for 15 minutes at 37°C in K1 medium containing 0.1% collagenase, 0.01% DNase and 0.1% soybean trypsin inhibitor. K1 medium consists of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM): Ham F12, supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, 5 μg/ml selenium, 36 ng/ml hydrocortisone, 10^{-8} M triiodothyronine, and 10 ng/ml epidermal growth factor [8]. The medium was replaced with fresh collagenase-containing medium and the incubation continued for an additional 15 minutes, followed by the addition of 0.05% trypsin/EDTA. After 10 minutes incubation at room temperature, the supernatant was centrifuged at 600 g for 10 minutes. The pellet was resuspended in 10 ml of ice-cold K1 medium and washed twice. The final cell pellet was resuspended in DMEM:Ham F12 with 10% fetal calf serum (FCS) at a density of 10^5 cells/ml and plated in collagen-coated T-25 flasks. For the first 24 hours, the cells were incubated at 37°C to allow cell attachment. Thereafter, the medium was changed to serum-free K1 medium supplemented with 10 U/ml

murine γ-interferon (IFN-γ) and the cells were transferred to a 33°C incubator with 5% CO_2, 95% air. Since passage three, the cells have been maintained in DMEM supplemented with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10 U/ml IFN-γ. The medium was replaced every two to three days and the cells passaged at confluency, using 0.05% trypsin/EDTA. To induce differentiation, 48 to 96 hours before experiments, the culture medium was changed to one without IFN-γ and the cells were transferred to 39°C and grown on culture dishes coated with type I collagen. For electron microscopy and for measurement of electrical resistance, cells were grown on collagen-coated filter inserts. For immunocytochemistry and for measurements of Na^+-H^+ exchange activity, cells were grown on collagen-coated coverslips. An established SV40 transformed murine cortical cell line (MCT) was used as control.

General experimental conditions

In most studies, the experiments were performed under non-permissive conditions, resulting in maximum differentiation, and compared to those performed in parallel, under permissive conditions, which allow rapid proliferation in an undifferentiated state. In general, cells were grown to a sub-confluent stage on a collagen type I-coated surface, at 33°C and in the presence of IFN-γ. Half of the plates were then transferred to 39°C and the culture medium was changed to one without IFN-γ. The other half was kept at 33°C. For some studies, the cells were evaluated under non-permissive conditions only. All studies were performed approximately 72 hours after transfer to non-permissive conditions, during a time period when the large T antigen is undetectable and the cells are completely viable.

Electron microscopy

Cells were grown on 0.45 μm pore size collagen-coated polycarbonate Nulcepore® filters. In these experiments, the media placed in both chambers was buffered with the addition of 10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), and was replaced daily to prevent the development of a pH gradient across the monolayer. Cells were fixed with 2.5% glutaraldehyde/1% paraformaldehyde in cacodylate buffer (pH 7.4). They were post-fixed with 1% osmium tetroxide in cacodylate buffer, dehydrated with a graded series of alcohol and embedded in epoxy resin. Cross-sections of the monolayers were cut with a diamond knife. The thin sections were counterstained with uranyl acetate and lead citrate. The specimens were observed and photographed with a JEOL 100S transmission electron microscope (JEOL, Tokyo, Japan).

Gel electrophoresis and Western blots

Cells grown in collagen-coated 60 or 100 mm culture dishes were washed twice with ice-cold phosphate buffered saline (PBS), scraped and collected by centrifugation. The pellet was homogenized in a small volume of a lysis buffer (50 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.2% NP-40, 0.5 mM PMSF), incubated on ice for 60 minutes, followed by two freeze-thaw cycles. Following centrifugation at 10,000 g for 10 minutes, the supernatant was transferred to new tubes and frozen at −70°C. The antigens were immnunoprecipitated prior to electrophoresis. The antibody was added to the cell lysate (5 μl per 100 μl cell lysate) and incubated for two hours at 4°C. Protein A Sepharose (20 μl diluted in the same volume of NETN buffer containing 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 25 mM Tris-HCl, pH 8.0) was added to the mixture and incubated overnight at 4°C. After washing four times in PBS, the pellet was resuspended in loading buffer and subjected to electrophoresis.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [9]. Following separation, protein bands were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P*) according to Towbin et al [10]. Non-specific sites were blocked by incubating the membranes for at least two hours at room temperature in a blocking buffer consisting of Tris buffered saline, pH 7.4, 0.2% Tween 20 (TBS-T), and 3% bovine serum albumin (BSA). Following two washes in TBS-T, a 1:500 to 1:1000 dilution of the primary antibody (in blocking buffer) was added to the membranes, followed by incubation at room temperature for two hours. After three washes in TBS-T, the second antibody was added (1:5,000 to 1:10,000 dilution of an HRP-conjugated anti-mouse or anti-rabbit IgG, and the membranes were incubated for two hours at room temperature. Following four washes in TBS-T, the protein bands were visualized using the ECL chemiluminescence detection method. Membranes were briefly exposed to radiographic film for a permanent record.

Study of solute transport

Solute uptakes were determined as described previously [11]. Briefly, cells grown in collagen-coated 24 well plates were washed three times with 0.5 ml of a "washing solution" containing 137 mM choline chloride, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM Tris/HEPES, pH 7.4. Uptake measurements were started with the addition of uptake media to wells. The composition of uptake media was either the same as the washing solution, or choline chloride was replaced with NaCl and 0.1 mM of either [^{32}P]-K_2HPO_4, [^{14}C]-α methyl D-glucopyranoside (a non-metabolizable glucose analog), or L-[^{3}H]-proline were added. After five minutes incubation, the medium was removed by gentle suction, and uptake stopped by the addition of 0.5 ml of an ice-cold stop solution, containing 135 mM NaCl, 10 mM arsenate, and 5 mM Tris/HEPES, pH 7.4. After three washes, the stop solution was removed and the cells were solubilized in 0.5 N NaOH, preheated to 70°C. Aliquots of cell lysates were transferred to scintillation vials for counting.

Measurement of Na^+-H^+ exchange

The exchanger activity was determined by measurement of changes in intracellular pH (pH_i), in the presence of Na^+ in the extracellular buffer. The method is based on increased fluorescence intensity of the pH sensitive fluorescent probe SNARF-1 with increase in pH [12]. The cells were grown on collagen-coated coverslips and transferred to non-permissive conditions for 72 hours prior to the measurements. They were washed three times with a bicarbonate-free Na buffer (140 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM KCl, 5 mM glucose, 20 mM HEPES pH 7.4), then preloaded with 15 μM of the acetoxymethyl ester of SNARF-1 (SNARF-1/AM) in Na buffer for 15 minutes at 37°C. The coverslips were placed inside a perfusion chamber on the stage of an inverted microscope (Diaphot, Nikon), equipped with a temperature-controlled flow-through system. The same solution without the dye was continuously perfused through the chamber for 30 minutes to wash the extracellular dye. The flow rate of the

perfusates was kept between 1 to 1.5 ml/min. Cells were acid-loaded for one to three minutes with a Na buffer containing 10 mM NH_4Cl, followed by replacement of the buffer with a Na-free buffer (Na replaced with 140 mM N-methyl D-glucamine). Following the NH_4Cl pulse, there is a rise in intracellular pH (pH_i). When the NH_4Cl is washed out, a rapid drop in pH_i follows caused by the dissociation of NH_4 to NH_3 and H^+ and rapid diffusion of NH_3 out of the cell. Measurements were carried out at 37°C with a micro-spectrofluorometer as described previously [12, 13]. The unchopped excitation light was directed through a 515 ± 5 nm bandpass filter and thereafter reflected by a dichroic mirror centered at 555 nm into the 40 × objective of the microscope. The cells were illuminated from above and optical signals were acquired by a CCD camera and directed to a dichroic mirror centered on 610 nm. The mirror directed the fluorescence emission to photomultiplier tubes. The emitted fluorescent signals were filtered with bandpass filters centered at 640 ± 5 nm and 580 ± 5 nm, respectively. The ratio of 640:580 fluorescence emission was obtained on-line with an analog divider circuit and, along with the cell video image, was recorded on a video recorder. Autofluorescence was <1% of the fluorescence signal from SNARF-1-loaded cells. Background fluorescence at each wavelength was measured as the fluorescence from a clean, cell-free area on the coverslip, and was electronically subtracted from the signal from loaded cells. pH calibrations were performed at the end of each measurement by the use of nigericin/KCl calibration buffers with different pH of 6.8, 7.0, 7.2, 7.4, and 7.6, containing 10 μM nigericin. The composition of the KCl calibration buffers was the same as the Na buffer except that NaCl was replaced with 140 mM KCl and the final pH was titrated with 1 M KOH. Conversion from fluorescence ratio to pH was performed by constructing a calibration curve and analyzing the data by non-linear regression.

Immunocytochemistry

The cells were grown on collagen-coated coverslips and transferred to 39°C for 48 to 96 hours before study. They were washed three times with PBS, then fixed for two minutes with ice-cold acetone, followed by three washes with PBS. They were permeabilized for five minutes at room temperature with 0.5% Triton X-100 in PBS, washed with 0.5% bovine serum albumin in PBS, and blocked with the same solution for 20 minutes. Following incubation with primary antibodies (mouse monoclonal anti-large T antigen 1:40 dilution, rabbit polyclonal anti-mouse CA-IV 1:50 dilution) for one hour at room temperature, the cells were washed three times with PBS, followed by the addition of FITC-labeled secondary antibodies (anti-mouse or anti-rabbit IgG, both at 1:100 dilution). After one hour incubation in the dark at room temperature, the cells were washed with PBS and mounted on slides, using Prolong® antifade mounting solution and examined with either a Nikon microscope with epifluorescence capability or with a Nikon confocal microscope equipped with a Biorad laser light system. Acquired images were processed electronically and printed.

Measurement of adenosine 3′,5′-cyclic monophosphate (cAMP)

Cells grown in 12-well plates were placed at 39°C for 48 hours followed by transfer to serum-free DMEM for 15 hours prior to the experiments. They were preincubated for one hour with serum-free DMEM containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor, followed by the addition of parathyroid hormone (10^{-6} M) or arginine vasopressin (10^{-6} M). In some experiments, forskolin (10^{-5} M) was used as a positive control. After 10 minutes incubation at 37°C, the media were aspirated and the cells were washed 3 times with ice-cold PBS. Thereafter, 1 ml of a 100:1 (vol/vol) mixture of ethanol/1 N HCl was added to each well and cells were incubated overnight at 4°C. Following extraction, the ethanol was evaporated and the extract resuspended in 0.25 ml of ice-cold Tris-buffered saline, pH 7.4, containing 4 mM EDTA, and frozen at −20°C. cAMP was measured by radioimmunoassay.

Northern blot analysis

Total RNA was used for Northern blot analysis. Briefly, 10 μg RNA was denatured and electrophoresed on 1% agarose formaldehyde gels. Following overnight transfer to nylon membranes (Hybond N+), RNA was immobilized by UV cross-linking. The blots were pre-hybridized at least four hours at 42°C, in a solution containing 5 × SSPE (1 × SSPE: 3.6 M NaCl, 0.2 M Na_2HPO_4, 0.02 M EDTA), 50% formamide, 0.5% SDS, 5× Denhardt's solution (Ficoll, bovine serum albumin and polyvinylpyrrolidone, each at 1 mg/ml) and 50 μg/ml denatured salmon sperm DNA. Hybridization was performed at 42°C for 15 hours in a fresh solution, containing approximately 500,000 cpm/ml of an 850 bp cDNA probe, corresponding to exon-2 region of mouse angiotensinogen, as well as a 245 bp cDNA probe for mouse β-actin, both labeled by random priming. Thereafter, blots were washed twice for 15 minutes each at room temperature in 2 × SSC, 0.1% SDS, followed by one more 10 minutes wash at 65°C. The blots were autoradiographed at −70°C, using intensifying screens. To use additional probes, membranes were stripped by incubating in 0.1% SDS in water at 95°C for five minutes.

Measurement of angiotensinogen concentrations

After reaching a sub-confluent stage, cells were either maintained under permissive conditions or transferred to non-permissive conditions for 48 hours. Thereafter, culture media were changed to a serum-free DMEM with 20 mM HEPES buffer for 24 hours. The media were collected and the cells were washed twice with PBS, scraped and collected by centrifugation. The cell pellets were incubated for 60 minutes at 4°C in a lysis buffer containing 150 mM NaCl, 10 mM EDTA, 1 mM AEBSF, 1 mM 8-OH-quinoline, 0.5% NP-40 and 25 mM Tris-HCl, pH 7.0 and subjected to two freeze-thaw cycles. Following centrifugation, the supernatants were transferred to new tubes and their protein concentrations were adjusted to 1 mg/ml. The culture media were concentrated using Centricon 10 micro-concentrators. All samples were frozen at −80°C until assay. Angiotensinogen concentration was determined by radioimmunoassay of angiotensin I released after exhaustive digestion with purified murine submaxillary gland renin, in the presence of appropriate angiotensin-converting enzyme inhibitors. The results are expressed as ng Ang I per mg cell protein.

Other methods

Apical membranes were isolated from cells grown in several 225 cm^2 flasks, using a Mg^{2+} precipitation method as described by Brown et al [14]. γ-glutamyl transpeptidase was assayed colorimetrically by the method of Glossman and Neville [15].

Materials

H-2K^b*ts*A58 transgenic mice were obtained from Charles River Labs (Cambridge MA, USA). Mice with 129/SvEv background were obtained from Taconic (Germantown, NY, USA). Tissue culture supplies were purchased from GIBCO-BRL (Grand Island, NY, USA). Fetal bovine serum was purchased from Hyclone (Logan, Utah). Type I collagen was purchased from Collaborative Biomedical Products, Beckton Dickinson (Bedford, MA, USA). Nulcepore® filters were purchased from Costar (Cambridge, MA, USA). Protein A Sepharose was purchased from Pharmacia (Uppsala, Sweden). Polyvinylidene difluoride (PVDF) membranes (Immobilon-P®) were purchased from Millipore Corp. (Bedford, MA, USA). HRP-conjugated anti-mouse or anti-rabbit IgG, were purchased from Pierce (Rockford, IL, USA). The ECL chemiluminescence detection kit and cAMP radioimmunoassay kit (Biotrak®) were purchased from Amersham Corp. (Arlington Heights, IL, USA). Prime-It II random labeling kit (#300385) was purchased from Stratagene (La Jolla, CA, USA). Monoclonal antibody against large T antigen was purchased from Oncogene Science, Inc. (Uniondale, NY, USA). Monoclonal antibody against mouse ACE was purchased from QED Bioscience, Inc. (San Diego, CA, USA). Acetoxymethyl ester of SNARF-1 (SNARF-1/AM), Prolong antifade mounting solution, and Live/Dead cell viability assay were all purchased from Molecular Probes (Eugene, OR, USA). FITC-labeled anti-mouse or anti-rabbit IgG were purchased from Boehringer Mannheim (Indianapolis, IN, USA/Mannheim, Germany). Centricon 10 concentrators were purchased from Amicon Inc. (Beverly, MA, USA). Angiotensin I radioimmunoassay kit was purchased from Dupont NEN (Boston, MA, USA). MCT cells were a gift from Dr. Eric G. Neilson (Department of Medicine, University of Pennsylvania, Pittsburgh, PA, USA). Rabbit polyclonal anti-mouse CA-IV antibody was a gift from Dr. William S. Sly (St. Louis University, St. Louis, MO, USA).

Statistical analysis

The results are expressed as a representative experiment repeated at least twice or, when appropriate, as means ± SEM of several separate experiments. Data comparisons were made by Student's *t*-test. Values of $P \leq 0.05$ were considered to be significant.

RESULTS

Development of tsMPT cells

A male mouse, homozygous for a transgene carrying the temperature-sensitive large T-antigen of SV40 under the control of an IFN-γ inducible promoter (H-2K^b*ts*A58) mouse was crossed to females of the 129/SvEv background and several litters were obtained. The cells derived from such animals have been shown to proliferate as continuous cell lines at the permissive temperature of 33°C in the presence of IFN-γ; they differentiate at the non-permissive temperature of 39°C, and in the absence of IFN-γ [5, 6, 16]. The cells obtained from renal cortex were initially grown on collagen-coated dishes in K1 medium under permissive conditions. They reached confluency in approximately two weeks and appeared to be free of fibroblasts. Cells could be sub-cultured in the absence of serum, but their growth remained slow. After two passages in K1 medium, the cells have been grown in DMEM supplemented with 10% FCS. At passage three, cells were trypsinized and seeded at low density, resulting in the appearance of distinct colonies. Several clones were isolated and expanded. The clones were initially screened for the presence of alkaline phosphatase, an enzyme known to be localized to the brush border membrane. However, due to variable staining for alkaline phosphatase, this enzyme could not be used as a reliable means of identifying the clones of proximal tubule origin. We therefore relied on functional tests such as the presence of Na^+-dependent transporters and on immuno-staining characteristics to further identify the clones of interest. One clone (clone 831) was selected for further characterization and used to perform the studies reported here. This clone has now been maintained for over 50 passages, without any detectable change in its characteristics.

Growth characteristics of tsMPT cells

At the permissive temperature of 33°C and in the presence of IFN-γ, *ts*MPT cells proliferate adequately on plastic dishes or flasks as monolayers exhibiting contact inhibition. They have the characteristic cobblestone appearance of epithelial cells. When grown on collagen-coated dishes, they form "domes," suggesting the development of tight junctions and vectorial transport of water across cells (Fig. 1). We have observed "dome" formation primarily when cells are consistently grown and sub-cultured on collagen-coated flasks or dishes. Only occasional domes are seen in cells grown on plastic dishes without collagen. The domes appear at 33°C and persist when cells are transferred to 39°C. At 33°C, the doubling time of the cells is approximately 23 hours. When transferred to 39°C, in the absence of IFN-γ, the cells continue to divide for approximately 24 hours. Thereafter, they stop proliferating but remain differentiated, maintaining contact inhibition. Under such non-permissive conditions, cell viability remains >95% for at least 96 hours, as assessed by the Live/Dead cell viability assay.

Detection of SV40 large T antigen

To determine that *ts*MPT cells do in fact express the SV40 large T antigen, we performed Western blots on cell lysates, following immunoprecipitation of the antigen, using a specific monoclonal antibody. The protein was identified as a 94 kDa M_r band (Fig. 2). MCT cells, another SV40 transformed but not temperature-sensitive murine proximal tubule cell line also expressing the large T antigen, were used as control. When the *ts*MPT cells were kept in the absence of IFN-γ at 39°C for increasing time intervals, the intensity of the large T antigen band decreased rapidly and it could not be detected 48 hours or longer after transfer of cells to non-permissive conditions. The large T antigen was also detected by immunocytochemistry. At 33°C, the large T antigen was localized to the nucleus of the cells. Virtually all cells expressed the large T antigen (Fig. 3). At 39°C, the nuclear T antigen fluorescence was markedly reduced or undetectable and shifted to the cytoplasm where it took a patchy appearance, suggesting accelerated degradation of the antigen as described by Jat et al [5, 17].

Establishment of the proximal tubular nature of tsMPT cells

Monolayer cells were stained for alkaline phosphatase, an enzyme localized to the brush border membrane (BBM) [8]. Alkaline phosphatase staining was found to be variable. In contrast, we have demonstrated a significant activity for γ-glutamyl transpeptidase, another BBM enzyme [15, 18, 19], in apical

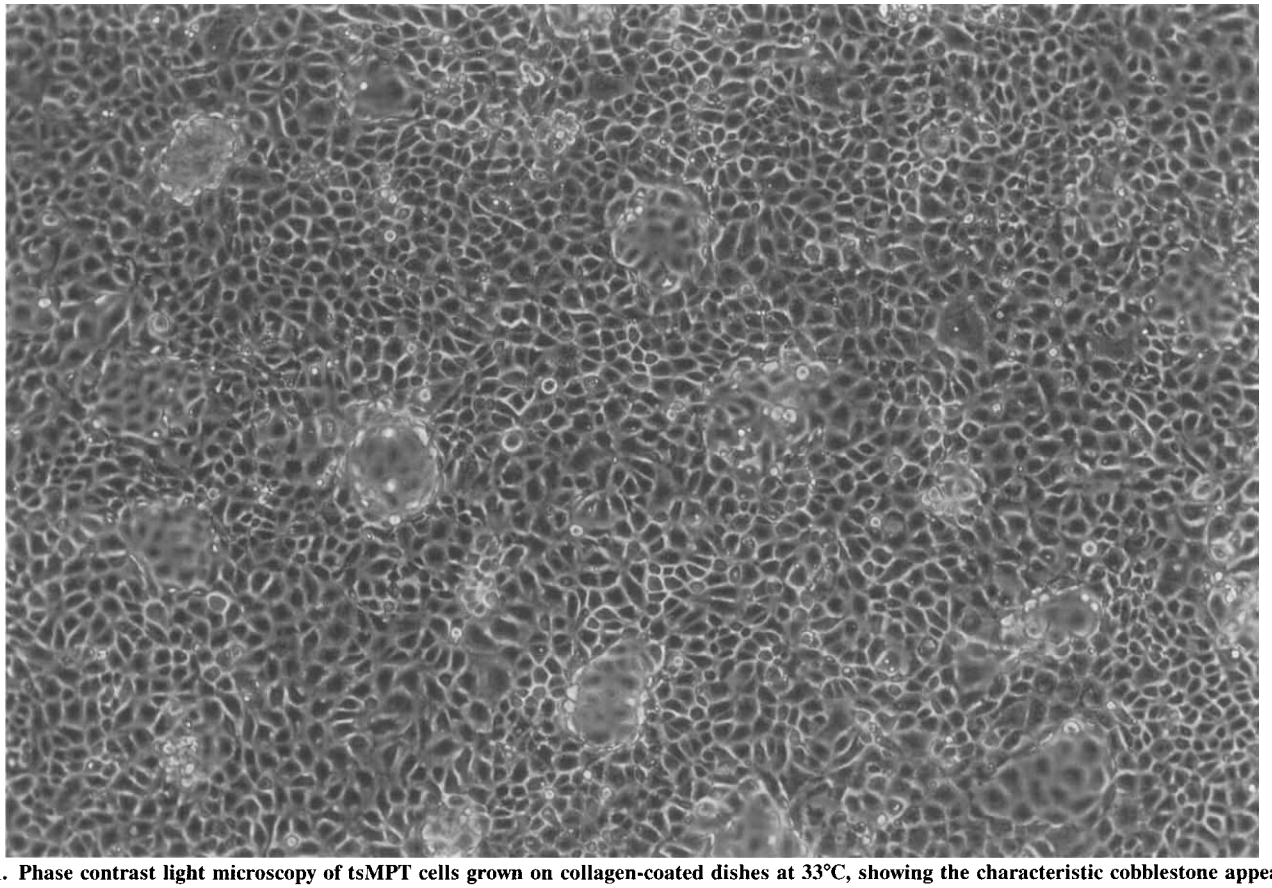

Fig. 1. Phase contrast light microscopy of tsMPT cells grown on collagen-coated dishes at 33°C, showing the characteristic cobblestone appearance and multiple "domes" (magnification ×100).

membranes isolated from *ts*MPT cells. The enzyme activity of the membrane fractions was higher at non-permissive compared to permissive conditions (34 ± 4 vs. 13.8 ± 0.2 nmol/g protein/min, respectively; $N = 2$). This difference was even more pronounced for cell homogenates (12.0 ± 1.6 vs. 2.2 ± 0.5 nmol/g protein/min, $N = 2$).

In addition to the cobblestone appearance and presence of "domes" described earlier, we measured the electrical resistance across *ts*MPT cells grown on collagen-coated permeable filter supports. In sub-confluent cells, the electrical resistance was not different from that measured across empty filters. It increased rapidly when cells became confluent, particularly after they were transferred to 39°C. The highest average electrical resistance, reached was 129 ± 18 Ohms/cm^2 ($N = 5$), a value similar to that obtained in other proximal tubule cell lines [20, 21]. Electrical resistance remained elevated for at least five days. These results indicate that the *ts*MPT cells form tight junctions, a feature of epithelial cells.

To examine the presence of ultrastructural features of proximal tubules, transmission electron microscopy was performed in *ts*MPT cells grown on collagen-coated filter supports. As shown in Figure 4, *ts*MPT cells, grow as monolayers both at 33°C and at 39°C. They are polarized with their basolateral membrane attached to the filter and the apical membrane facing the culture medium. They show tight junctions at their apical side. At 33°C, the cells appear flat with few organelles and sparse microvilli. At 39°C, cells are thicker with more mitochondria and other organelles. They possess more abundant and taller microvilli at their apical membrane. These ultrastructural features are consistent with those reported for other proximal tubule cells grown in culture [8, 22–24].

Determination of sodium-dependent solute transports

One of the major characteristics of the proximal tubule is the presence of specific Na^+-dependent transporters for phosphate, D-glucose and amino acids [19, 24]. We, therefore, determined the uptakes of P_i, α-methyl D-glucopyranoside (AMG, a non-metabolizable glucose analog) and that of L-proline, measured in the presence and absence of a Na^+ gradient, in cells grown on collagen coated plates. The uptake of all three solutes was Na^+-dependent, with markedly lower uptakes in the absence of Na^+ (Fig. 5). Both total uptake and the Na^+-dependent component of uptake were lower in cells grown on plastic without collagen (results not shown). Phosphate uptake was inhibited 64.9% by 5 mM phosphonoformic acid and 75% by 5 mM arsenate, both specific inhibitors of Na^+-P_i cotransport [19]. The uptake of AMG was similarly inhibited 38.6% by 0.5 mM phlorizin, a specific inhibitor of the apical Na^+/D-glucose cotransport [25]. This represents 100% inhibition of the Na^+-dependent component of transport.

Determination of Na^+-H^+ exchange

The presence of a Na^+-H^+ exchanger was determined with the pH-sensitive fluorescent dye SNARF-1 (Fig. 6). Cells were perfused with NH_4Cl, causing an increase in intracellular pH (pH_i).

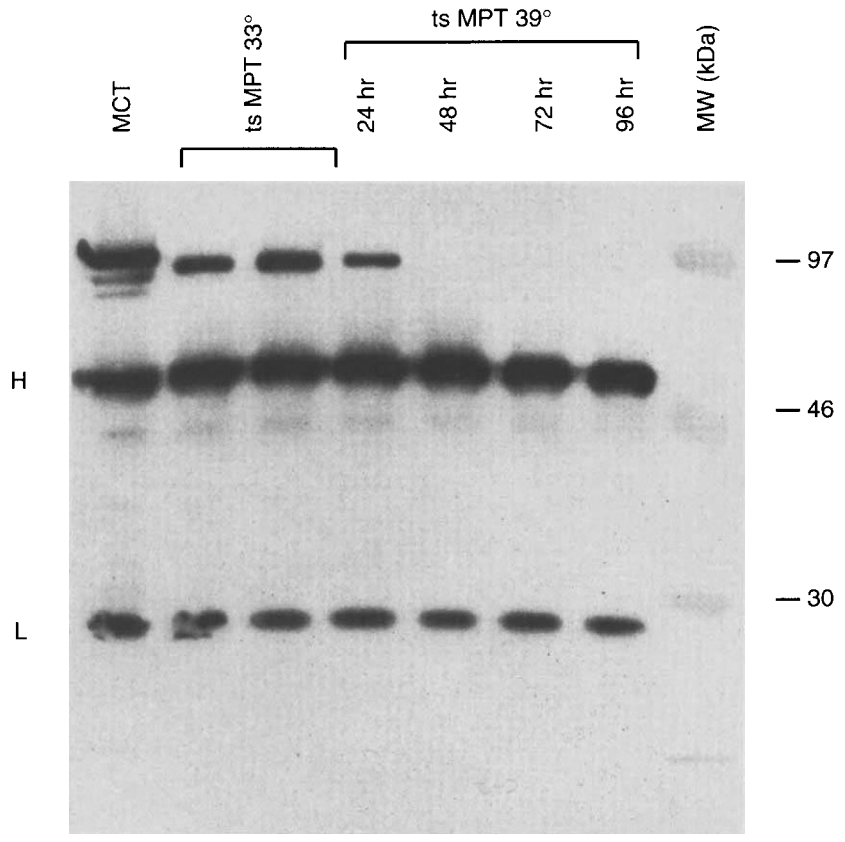

Fig. 2. Detection of SV40 large T antigen in tsMPT cells by Western blots. Cells were grown at 33°C or transferred to 39°C for different times before the experiment. Cell lysates were prepared and immunoprecipitated with the addition of a specific monoclonal antibody and protein A Sepharose (see **Methods**) and used for SDS-PAGE. Representative Western blot shows the large T antigen as a 94 kDa M_r protein band in cells grown at 33°C. The band becomes undetectable 48 hours after transfer of cells to 39°C, and remains undetectable for at least 96 hours. MCT (murine cortical tubule) cells also express the T antigen. Middle bands (H) correspond to IgG heavy chain and lower bands (L) to IgG light chain, respectively.

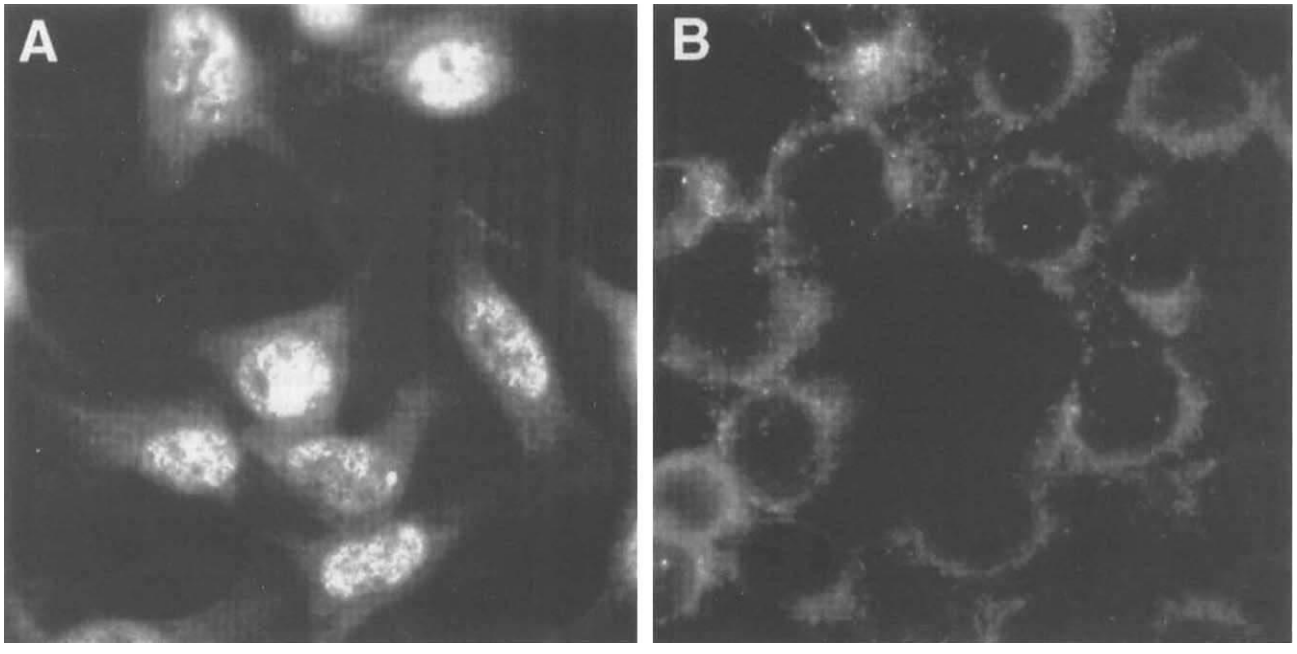

Fig. 3. Detection of SV40 large T antigen in tsMPT cells by immunocytochemistry. Cells were grown on collagen-coated coverslips, then either kept at 33°C (*A*) or transferred to 39°C for an additional 72 hours (*B*). Large T antigen is seen in the cell nucleus at 33°C but disappears from the nuclei at 39°C (magnification ×1,000).

Thereafter, NH_4Cl was removed from the perfusate and replaced with a Na containing buffer without NH_4Cl, resulting in a rapid drop of pH_i below the baseline. This was followed by a rapid recovery of pH_i secondary to H^+ efflux, suggesting the presence of a Na^+-H^+ exchanger in *ts*MPT cells. In experiments where the prefusate was a Na-free buffer, the pH_i recovery following the NH_4Cl pulse was much slower than that observed when a Na-containing perfusate was used (results not shown). Various

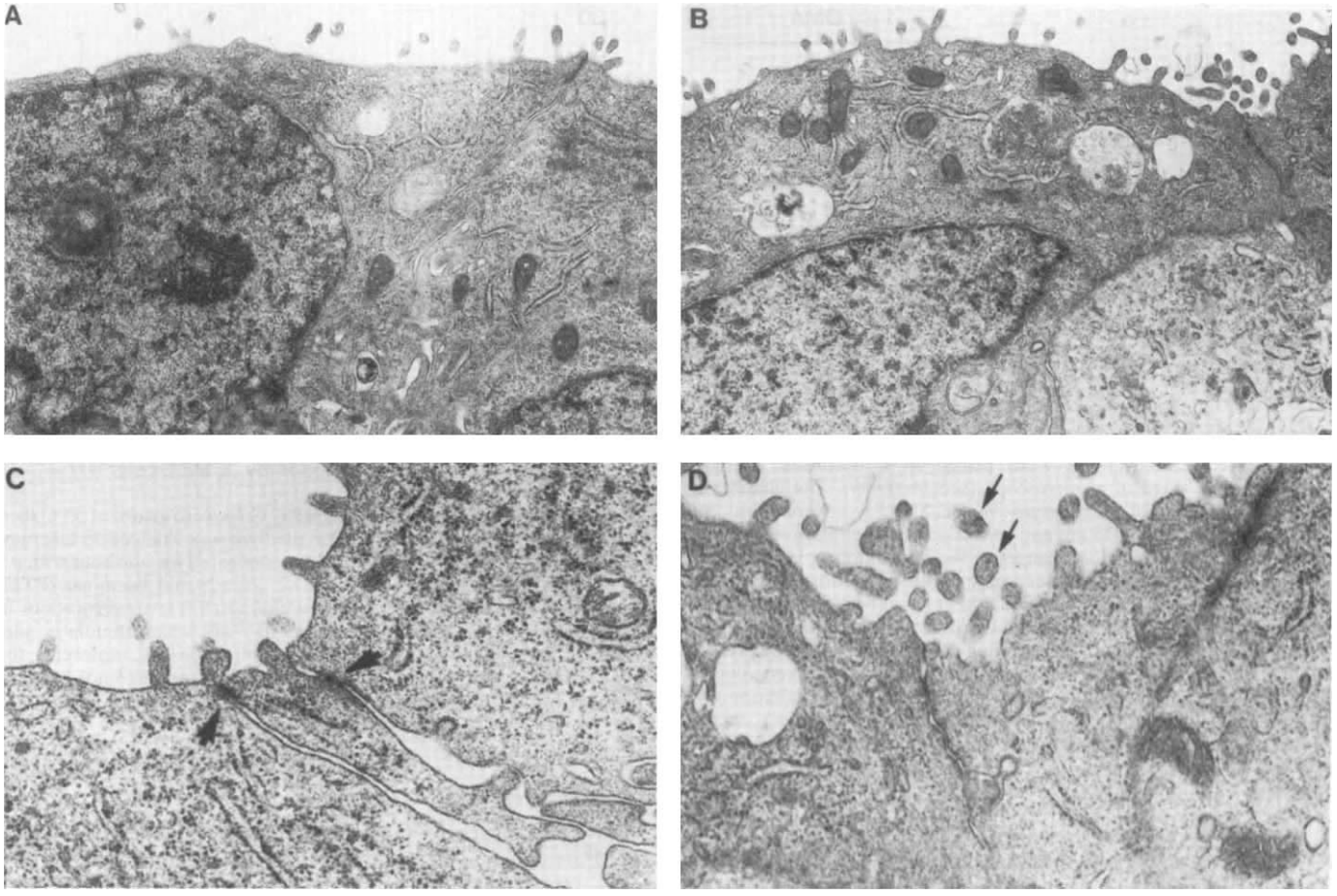

Fig. 4. Transmission electron microscopy of tsMPT cells. Cells were grown on permeable filter supports coated with collagen type IV and kept either at 33°C (A) or transferred to 39°C before processing (B,C and D). (*A*) Cross-section of *ts*MPT cells grown under permissive conditions, showing a polarized structure, few microvilli, and a tight junction. (*B*) Cells under non-permissive conditions show more abundant microvilli and an increased number of mitochondria at their apical pole. (*C*) *ts*MPT cell at 39°C, seen at high magnification, showing the presence of junctional complexes (large arrowheads). (*D*) *ts*MPT cells at 39°C, seen at high magnification, showing well developed microvilli (small arrows) (magnification: 19,200× for A and B; 52,000× for C and D).

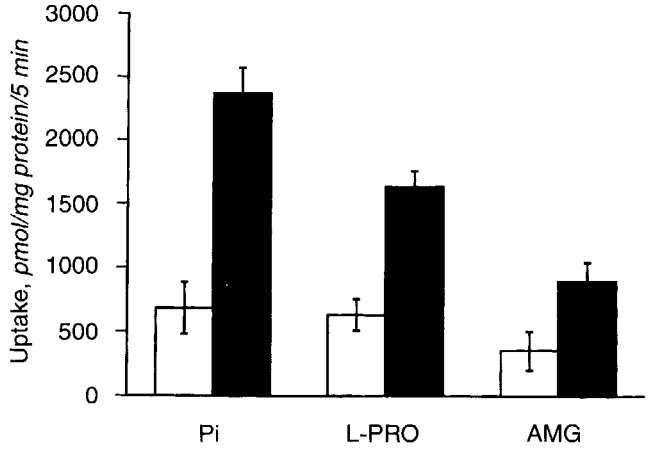

Fig. 5. Determination of sodium-dependent uptakes of solutes in tsMPT cells. Cells were grown at 33°C on collagen-coated multiwell plates, then transferred to 39°C for 48 hours before uptake measurements. Uptake was determined after five minutes incubation at 37°C, using media containing 137 mM Na (■) or choline (□) and 0.1 mM of each tested solute. Results are mean ± SEM of three separate experiments, each performed in quadruplicate. AMG is α-methyl D-glucopyranoside.

Na^+-H^+ exchanger isotypes show different sensitivities to amiloride or its more potent analogs such as ethylisopropyl amiloride (EIPA) or dimethylamiloride (DMA). The concentration at which these compounds inhibit the Na^+-H^+ exchanger can, therefore, be used to differentiate between the amiloride sensitive (NHE-1) and insensitive (NHE-3) exchanger isotypes [26]. In a second experiment, following the return of pH_i to baseline, the cells were again pulsed with NH_4Cl and both the NH_4Cl solution and the subsequent perfusate contained 1 μM DMA. The addition of 1 μM DMA did not result in a change in the rate of Na^+-H^+ exchange, as assessed by the slope of the pH_i recovery curve. The concentration of DMA used here is two orders of magnitude higher than its IC_{50} for inhibition of the ubiquitous amiloride-sensitive Na^+-H^+ exchanger (NHE-1 isoform), which is present on the basolateral membrane of the proximal tubule. The results indicate the existence of an amiloride insensitive form of Na^+-H^+ exchanger (NHE-3 isoform) in *ts*MPT cells.

Determination of cyclic AMP generation by parathyroid hormone

In the proximal tubule, cyclic 3′,5′-adenosine monophosphate (cAMP) is the major second messenger for parathyroid hormone

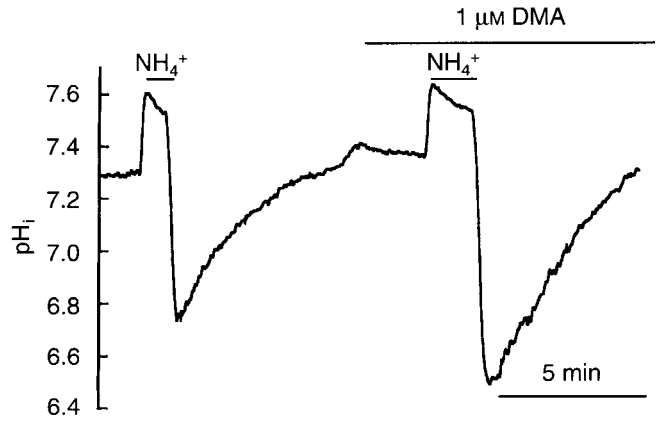

Fig. 6. Measurement of Na^+-H^+ exchange in tsMPT cells. Cells grown on collagen-coated coverslips were placed in a perfusion chamber mounted on the stage of a microscope (see **Methods**). The changes in fluorescence intensity of the pH-sensitive dye, SNARF-1, were monitored after single excitation at 515 nm and collection of the emitted light at 640 and 580 nm, and expressed as changes in the intracellular pH (pH_i) over time. pH_i was derived by conversion of 640/580 fluorescence ratios, following calibration with KCl-nigericin buffers of known pH. Perfusion of the cells with 10 mM NH_4Cl for one minute, causes an increase in pH_i. Changing the perfusate to a Na-containing medium without NH_4Cl results in intracellular acidification, followed by a rapid pH_i recovery due to acceleration of H^+ efflux by extracellular Na^+. Subsequent addition of 1 μM dimethylamiloride (DMA) to the perfusates has no effect on pH_i recovery, indicating the presence of an amiloride-insensitive Na^+-H^+ exchanger.

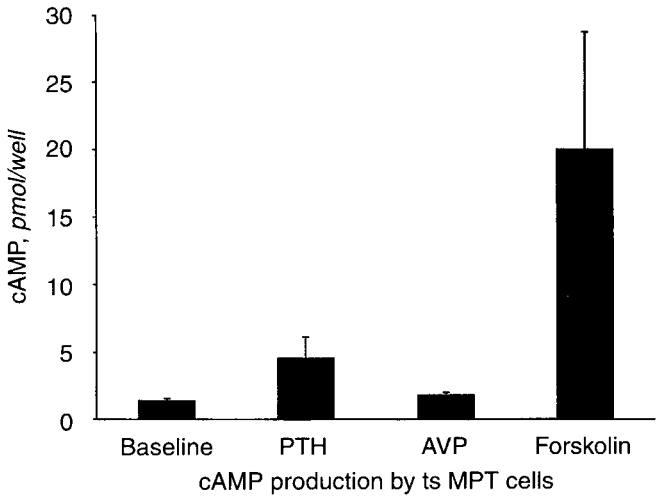

Fig. 7. Cyclic AMP production by tsMPT. Cells were grown at 33°C then transferred to 39°C for 48 hours. The medium was changed to a serum-free medium for the last 15 hours and pre-incubated for one hour with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX). Parathyroid hormone (PTH) and arginine vasopressin (AVP) were added at 10^{-6} M concentrations in a medium containing IBMX. Forskolin (10^{-5} M), which stimulates adenylate cyclase, was used as positive control. Each bar represents the mean ± SD of measurements from 6 to 9 wells. The difference between PTH and control-treated cells is significant ($P < 0.05$, group t-test).

(PTH). By contrast, in the distal tubule and the collecting duct, cAMP is the second messenger for arginine vasopressin (AVP). We therefore used the differential stimulation of cAMP production by these hormones to further confirm the proximal tubular origin of *ts*MPT cells. The cells were exposed to bovine PTH (1-34) or to AVP for 10 minutes and intracellular cAMP produced was measured by radioimmunoassay. There was a significant increase in cAMP production in cells exposed to 10^{-6} M PTH but no change after addition of 10^{-6} M AVP (Fig. 7).

Detection of carbonic anhydrase IV

Carbonic anhydrase isozyme IV (CA-IV) is localized to the lung and to the brush border membrane of proximal tubules in the kidney [27]. We therefore used this enzyme as a marker to further characterize *ts*MPT cells. By immunocytochemistry, using confocal microscopy, CA-IV was detected in a linear distribution on the cell membrane, both under permissive (33°C, with IFN-γ) and non-permissive conditions (39°C, without IFN-γ). Compared with cells kept at 33°C, the fluorescence intensity was higher at the membrane location in cells transferred to 39°C for 96 hours (Fig. 8).

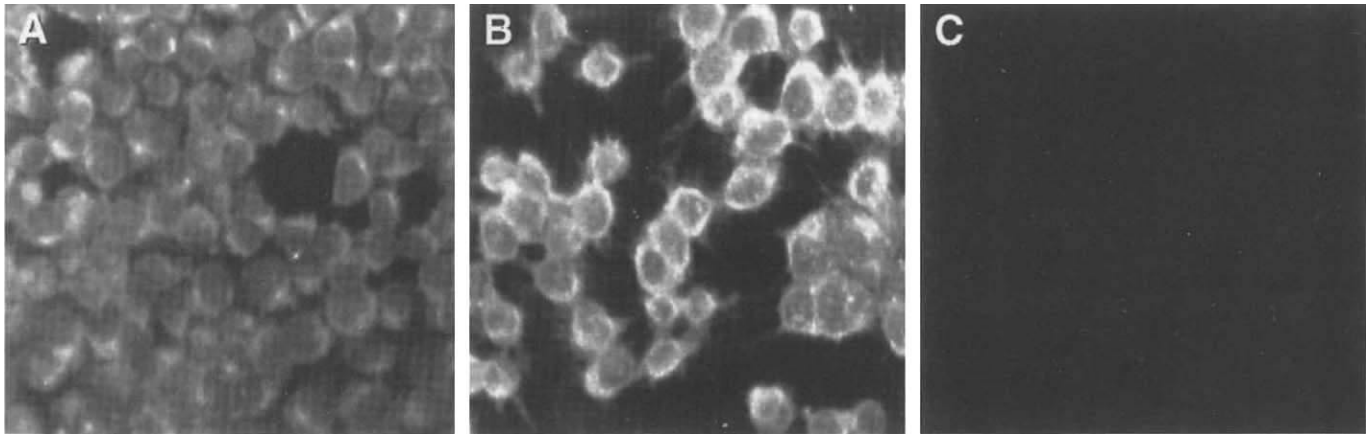

Fig. 8. Detection of carbonic anhydrase IV in tsMPT cells. Cells were grown on collagen-coated coverslips, then either kept at 33°C or transferred to 39°C for an additional 72 hours and CA-IV was detected with a specific rabbit polyclonal antibody. (*A*) Cells kept at 33°C, showing faint staining with CA-IV. (*B*) Cells at 39°C, showing strong linear fluorescence for CA-IV, corresponding to the cell membrane. *C*. Pre-immune serum. (Confocal microscopy. Magnification ×600.

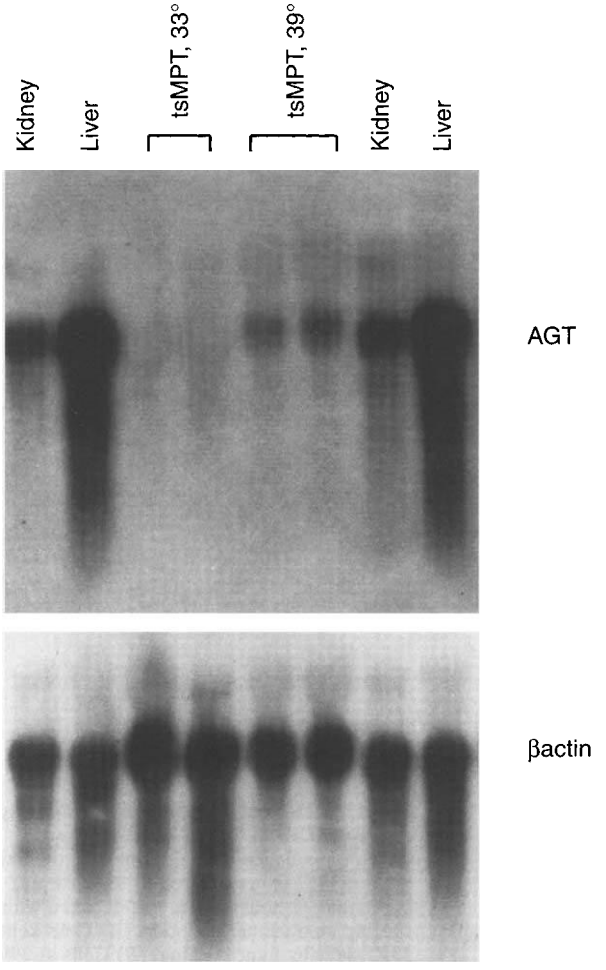

Fig. 9. Detection of angiotensinogen mRNA by Northern blots. Ten micrograms of total RNA were electrophoresed on a 1% agarose-formaldehyde gel, followed by transfer to a nylon membrane. The membrane was hybridized with a random-labeled 850 bp cDNA from the exon-2 region of mouse angiotensinogen, then stripped and re-hybridized with a 245 bp cDNA probe to mouse β-actin. A representative autoradiogram is shown. The mRNA for angiotensinogen is seen as a 1.6 kb band in *ts*MPT cells placed at 39°C but only a faint band is seen in cells kept at 33°C. Angiotensinogen mRNA is also abundant in liver and in kidney. There is no difference in the β-actin mRNA expression, suggesting equal RNA amounts loaded at each lane.

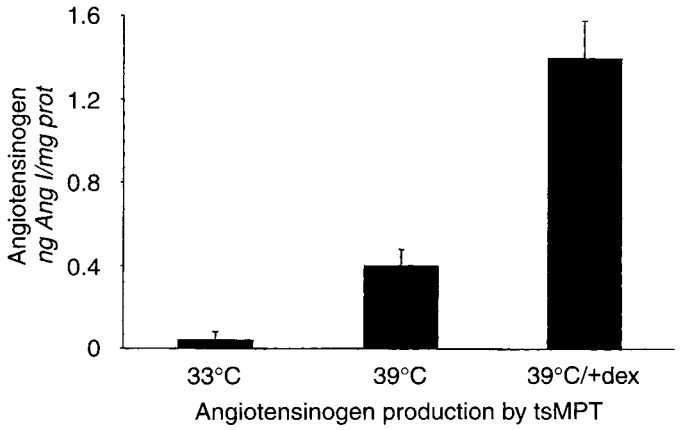

Fig. 10. Angiotensinogen production by tsMPT cells. Cells were transferred to non-permissive conditions for 48 hours, followed by an additional 24 hours incubation in serum-free media. Some cells were incubated with 10^{-6} M dexamethasone for 15 hours prior to assay. Angiotensinogen concentrations were determined in cell lysates by indirect radioimmunoassay. Results are mean ± SD of three plates for each condition.

Detection of angiotensinogen mRNA and protein

In addition to the liver, many tissues have been shown to synthesize angiotensinogen. In the kidney, angiotensinogen is localized mainly to the proximal tubules [4, 28]. Because of our interest in the angiotensinogen gene and its regulation, and to evaluate if *ts*MPT cells are suitable for studies of the regulation of the tissue renin angiotensinogen system, we investigated the presence of mRNA for angiotensinogen in *ts*MPT cells. Angiotensinogen mRNA was detected on Northern blots, using total RNA from cells. In cells kept under permissive conditions (33°C, with IFN-γ), the angiotensinogen mRNA abundance was very low. In contrast, in cells transferred to non-permissive conditions (39°C, without IFN-γ), angiotensinogen mRNA abundance was markedly increased. There was no difference in β-actin mRNA, which suggests that the differences observed are not the result of different amounts of RNA loaded at each lane (Fig. 9).

Angiotensinogen protein was also detected by indirect radioimmunoassay in cell lysates and in media. Its concentration increased as a function of time in serum-free medium (not shown) and remained markedly lower in cells kept at 33°C in the presence of INF-γ, compared to cells transferred to 39°C for 72 hours in the absence of IFN-γ (Fig. 10). Addition of dexamethasone (10^{-6} M) to culture media for 15 hours resulted in a significant increase in angiotensinogen concentrations in cell lysate (from 0.4 ± 0.04 to 1.4 ± 0.08 ng Ang I/mg protein, $P < 0.05$, $N = 3$).

Detection of angiotensin I converting enzyme (ACE)

ACE has been shown to be localized to the brush borders of proximal tubule and small intestine [29]. Using a specific monoclonal antibody, ACE was detected in *ts*MPT cells by immunocytochemistry combined with confocal microscopy (Fig. 11). The enzyme was present on the cell membranes; only faint staining was seen in cells kept under permissive conditions. ACE immunostaining was much stronger in cells placed under non-permissive conditions.

DISCUSSION

Genetic variations of the angiotensinogen gene may predispose to the development of essential hypertension [1]. Angiotensinogen is the major substrate for renin leading to the formation of angiotensin II. The latter stimulates sodium reabsorption in the proximal tubule, the principal site of renal sodium reabsorption. It is believed that increased sodium retention by the kidney may play a role in the genesis of essential hypertension.

Recent observations have confirmed the existence of a local RAS in the proximal tubule [4, 28] resulting in markedly elevated concentrations of angiotensin I and II in this tubular segment [30, 31]. This local RAS may act in an autocrine/paracrine fashion to regulate tubular sodium reabsorption. A continuous cell line such as *ts*MPT with controllable proliferative potential and a select

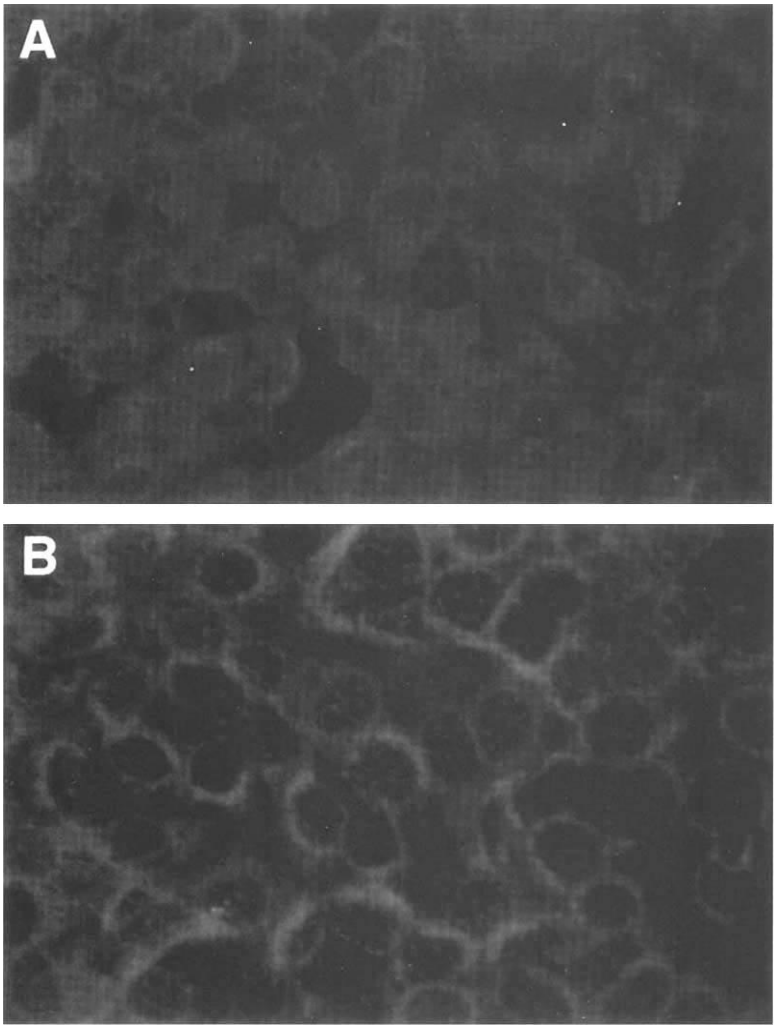

Fig. 11. Detection of angiotensin converting enzyme (ACE) in tsMPT cells. Cells were grown on collagen-coated coverslips, then either kept at 33°C or transferred to 39°C for an additional 72 hours. ACE was detected with a specific monoclonal antibody. (*A*) Cells kept at 33°C showing faint staining. (*B*) Cells at 39°C, showing strong membrane staining for ACE (confocal microscopy; magnification ×600).

genetic background should prove amenable to genetic manipulations. With the development of similar cell lines from other tissues of the same animals, it will become possible to perform and compare the impact of various genetic manipulations of the angiotensinogen or other genes, in parallel experiments, at the cellular level and in whole animals.

The *ts*MPT cells have the added advantage that the large T antigen expression is under the control of a promoter that can be induced by IFN-γ. Removal of IFN-γ results in complete suppression of the T antigen expression and differentiation within 48 hours (Fig. 2 and 3). In another murine proximal tubule cell line, the MCT [32], the transformation by SV40 is not conditional, and this may prevent the expression of a fully differentiated phenotype. Another cell line harboring an SV40 large T antigen under the control of a regulatory sequence of the rat L-pyruvate kinase gene, requires glucose-rich media for growth. Growth in glucose-free medium is necessary for the disappearance of the T antigen and differentiation [21]. Recently, Tang et al [23] reported a rat proximal tubule cell line developed by incorporation of a temperature-sensitive SV40 large T antigen gene into primary cultures. Although these various cell lines express components of the RAS, down-regulation of the large T antigen and differentiation are either not fully achieved or only partially achieved by a shift in temperature [10, 21, 32]. The *ts*MPT cells express many characteristics of proximal tubular cells: (*1*) They form polarized monolayers with distinct apical and basolateral domains. (*2*) They show microvilli at their apical surface. (*3*) They form tight junctions as seen by electron microscopy and as evidenced by the maintenance of an electrical resistance across the cell monolayer. (*4*) They contain γ-glutamyl transferase and carbonic anhydrase IV on their apical membrane. These enzymes are known to be localized to the brush border of proximal tubules [15, 27]. (*5*) They possess sodium-dependent transport systems for phosphate, D-glucose and amino acids as well as an amiloride-insensitive Na^+-H^+ exchanger. These transport systems are very similar to those described for established proximal tubule cell lines [11, 24]. (*6*) Cyclic AMP production in *ts*MPT cells is responsive to parathyroid hormone and not to arginine vasopressin. However, we were unable to demonstrate a significant inhibition of the Na^+-P_i cotransport with parathyroid hormone, suggesting that the Na^+-P_i cotransporter in these cells is most likely of the ubiquitous or type I variety [33]. Nevertheless, by virtue of their special background and the ability to divide rapidly under permissive conditions,

*ts*MPT cells remain an excellent model system for genetic manipulations. [7] The presence of angiotensinogen mRNA in *ts*MPT and the production of angiotensinogen protein, as well as the presence of ACE, are other features that support the proximal tubular nature of these cells. They also provide a potential target for genetic manipulation of the local RAS to study the physiological consequences of such alterations.

In summary, we have developed a murine proximal tubular cell line from the progeny of transgenic mice of the 129/SvEv background, harboring the temperature sensitive, IFN-γ inducible SV40 large T antigen. These cells display many characteristic features of proximal tubular cells, including the presence of a RAS. Angiotensinogen expression by *ts*MPT is markedly enhanced under non-permissive conditions. *ts*MPT should prove useful for studies of the role of angiotensinogen in the proximal tubule by gene targeting techniques, taking advantage of its special 129/SvEv background and rapid replication.

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