Kor J Physiol (1994) 28(2), 233-240

Transformation of Rabbit Proximal Tubule Cells by Strontium Phosphate Transfection with a Plasmid Containing SV40 Early Region Genes

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=ABSTRACT=

In this study, it was investigated whether immortalized proximal tubule cells transformed with pRSVT could survive through the numerous passages. Results were as follows:

1. The cells transfected with pRSVT formed rapidly growing, multilayered colonies within 2 weeks in a hormone defined medium. Domes were also observed in some of the cultures.

2. r-glutamyl transpeptidase activity was equivalent to that observed in primary renal proximal tubule cell cultures.

3. Transformed cells with pRSVT form tubules in matrigel following 20 passages.

4. Genomic DNA of transformants was digested with either the restriction enzyme Xba or BamH1. A band of approximately 7.5kb was detected with Xba. Three BamH1 bands were detected at approximately 15 kb, 6.5 kb, and 3 kb.

Key Words: Cell culture, Proximal tubule cell, Transformation

INTRODUCTION

Established animal cell lines are advantageous for biochemical studies in that large homogenous populations of cells can be cultured. The use of established kidney cell lines for renal function studies is well documented in previous studies conducted with the dog kidney epithelial cell line, MDCK(Taub et al, 1979). However, MDCK cells lack transport systems distinctive of the renal proximal tubule(such as the Na/glucose cotransport system) and have an arginine vasopressin sensitive adenylate cyclase typical of cells in the distal tubule(Waqar et al, 1985). The porcine kidney epithelial cell line, LLC-PK_1, which has also been extensively studied, does possess a Na/glucose cotransport system typical of proximal tubule cells(Devies et al, 1985). However, LLC-PK_1 like MDCK possess an arginine vasopressin sensitive adenylate cyclase typical of the distal tubule rather than a parathyroid hormone(PTH) sensitive adenylate cyclase typical of the proximal tubule(Taub, 1985). Also, fructose bisphosphatase activity is absent in LLC-PK_1(Matsuo et al, 1987). In addition this cell line lacks an intact gluconeogenic pathway(which is typical of the reanl proximal tubule).

Primary rabbit kidney proximal tubule cells are an important in vitro model system for defining the mechanism by which these regulatory factors control growth and function

(Taub & Sato, 1980). Primary rabbit kidney epithelial cell cultures are potentially powerful tools for studying the effect of transformation and the mechanisms which regulate renal growth and function. Unlike established kidney cell lines(in which the normal genotype maybe already be altered), primary cell lines closely resemble normal cells in vivo genotypically, as well as phenotypically. An understanding of growth regulation in normal renal proximal tubule cells is essential in the process of identifying the alterations which occur in these regulatory mechanisms as a consequence of transformation.

Primary rabbit kidney proximal tubule cells can readily be subcultured on a 1 : 2 or a 1 : 4 basis, so that confluent monolayers are obtained. These first passage cultures can then be subcultured in a similar manner. However third passage cultures cannot be obtained. The cells can be transformed with plasmids containing specific genes of interest including oncogenes, which may influence not only growth but also the expression of differentiated function. Genetic variants can also be isolated which are altered with regards to their ion transport systems and/or signal transduction pathways. Moreover, hormonally defined serum free culture conditions can be obtained, in which the effects of hormones can be examined in a precise, reproducible manner.

This study was designed to examine the possibility that immortalized proximal tubule cells that could survive numerous passage could be obtained by transformation with the plasmid pRSVT which contains an RSV promotor and the SV40 early region genes.

METERIALS AND METHODS

Primary cell cultures

Rabbit kidney proximal tubules were prepared from the kidneys of adult male New Zealand white rabbits as previously described (Chung et al, 1982, Han, 1993). The purified proximal tubules were treated for 2 minutes at room temperature with 0.05mg/ml collagenase (type IV, Worthington) and suspended in serum-free DME/F12(50 : 50 mixture) supplemented with 5μg/ml insulin, 5μg/ml transferrin and 5×10^{-8}M hydrocortisone. After distributing the material into 60 mm dishes, the cultures were maintained in a humidified 5% CO_2/95% air environment at 37°C. Newly confluent monolayers were utilized for transformation studies.

Strontium phosphate transfection of rabbit kidney proximal tubule cells

The plasmid, pRSVT, is SV40 ori-construct containing the SV40 early region genes and the Rous Sarcoma Virus long terminal repeat(Fig. 1). Confluent monolayers of primary proximal tubule cells were transfected with the pRSVT plasmid, utilizing the strontium chloride transfection method(Sack, 1981). Control cultures were also subjected to the same transfection procedure without a plasmid.

Primary rabbit proximal tubule cells were established as previously described(Han, 1993). At 18 hours prior to transfection, the cells were

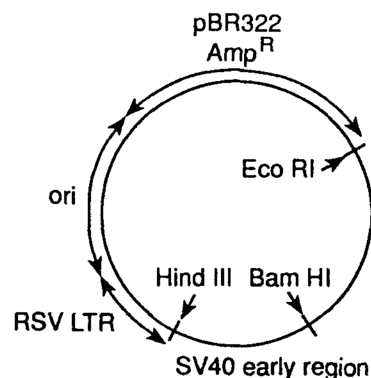


Fig. 1. Restriction map of plasmid pRSVT.(pRSVT, an SV40-ori-plasmid in which the Rous sarcoma virus long terminal repeat directs expression of the SV40 large-T-antigen coding region)

passaged. At 3 hours prior to transfection, the cells were refed with 4ml of medium which had been equilibrated in the incubator overnight. The 2M $SrCl_2$ solution was prepared in distilled water and filter sterilized. A 2x HEPES balanced saline(HBS; 8.18g of NaCl, 5.95g of HEPES, 0.2g of $Na_2HPO_4 \cdot 7H_2O$, in 500 ml H_2O) was adjusted to pH 7.7-7.9 with NaOH, filter sterilized and stored at 4°C. Glycerol was 15%(wt/vol) in HBS; the solution was filter sterilized and stored at 4°C.

To prepare precipitates, solutions were warmed to 37°C. 5 μg of DNA in 30 μl of solution was transfected by consecutively mixing 190 μl of sterile distilled water, 31 μl of 2M $SrCl_2$, and 30 μl of DNA in a sterile polypropylene plastic tube(Falcon 2059: Becton Dickson Labware, Oxnard, Calif. USA) and then shaken gently for 30 seconds at 37°C. This solution was added dropwise to the dish whilst swirling to prevent high local concentrations of phosphates. The dish was returned to the incubator.

After 2 hours, the cells were rinsed twice with serum free hormone defined medium to 37°C, incubated with 1.5 ml of 15% glycerol for 30 seconds at room temperature, rinsed 3 times with serum free hormone defined medium, refed, and returned to the incubator(Holland et al, 1960). The medium was changed the following morning with normal growth medium.

The cultures were subcultured three times on a 1 : 4 basis into culture dishes containing medium supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, 5×10^{-8}M hydrocortisone, 10 ng/ml EGF, 14 μg/ml bovine pituitary extract, 10units/ml heparin and 30 μg/ml bovine serum albumin(BSA) or 10% fetal calf serum(passage 1)

Isolation of transformants by subculturing

Within 4-8 weeks, colonies of tightly packed, multilayered cells measuring 1 cm or more in diameter were observed on dishes containing pRSVT-transfected cells, but not on the control dishes. Cloning cyclinders were placed around the colonies which were then individually dissociated by sequential treatment of trypsin EDTA and trypsin inhibitor. The cells were cultured and expanded under the previously described conditions. The ability of the transformed cells to form colonies on a background of senescing normal cells was the only selective procedure used.

Preparation of genomic DNA

Confluent cells were removed from the dishes by trypsinization and were collected from the culture dishes(Chung et al., 1982). These cells were centrifuged for 5 minutes at 500 xg and the supernatant was discarded. Cells were resuspended with 5 ml ice-cold PBS, centrifuged for 5 minutes at 500 xg and the supernatant was discarded. The cells were then resuspended in 1 volume of digestion buffer(100 mM NaCl, 10 mM TrisHCl, pH 8.0, 25 mM EDTA, pH 8.0, 0.5% SDS, 0.1 mg/ml protein kinase K). This sample was incubated with shaking at 500°C for 18 hours in tightly capped tubes and then throughly extracted with an equal volume of phenol/chloroform/isoamyl alcohol. After centrifuging for 10 minutes at 1700 xg, the aqueous layer was transferred to a new tube. 1/2 volume of 7.5 M ammonium acetate and 2 (original) volumes of absolute ethanol was added to the tube. The DNA was resuspended in TE buffer until dissolved and then stored at 4°C(Douglas, 1993)

Identification of SV40-T antigen genes in transformants

Transformed cells were analyzed for the presence of the integrated SV40 early region DNA by means of Southern analysis(Southern, 1975). To summarize, genomic DNA(10 μg) was digested with restriction enzymes Bam H1 which cleaves a single site in the pRSVT plasmid and Xba which pRSVT is resistant to. The DNA digests were electrophoresed on 0.8% agarose gels. After electrophoresis, the DNA

was depurinated by soaking the gel in 0.25 M HCl for 15 minutes and then the DNA was denatured by placing the gel in a bath of solution containing 0.5 M NaOH and 1 M NaCl for 30 minutes. Finally, the gel was neutralized by bathing it in a solution containing 0.5 M TrisHCl, pH 7.4 and 3 M NaCl for 30 minutes at room temperature. Next procedures were followed as previously described (Han, 1993)

Enzyme assay

Brush border enzyme assays were conducted for the activities of r-glutamyl transpeptidase (Tate & Meister, 1974) and alkaline phosphatase(Linhardt & Walter, 1963) using L-glutamyl-p-nitroanilide and p-nitrophenylphosphate were utilized as substrates.

RESULTS

The cells transfected with pRSVT, formed rapidly growing, multilayered colonies within 2 weeks in a hormone defined medium(Fig. 2). Colonies of surviving cells were cloned utilizing cloning cylinders 6 weeks later. After 10 weeks, confluent monolayers of pRSVT transformants were obtained. Domes were also observed in some of the cultures(Fig. 3). These cells showed that their growth potential was well beyond that of normal progenitor cells. Three independent clones were tested for the presence of brush border enzymes activities. The activity of r-glutamyl transpeptidase was equivalent to that observed in primary renal proximal tubule cell cultures. r-glutamyl transpeptidase activity is a marker of the rabbit renal proximal tubule in vivo(Table 1). This study observed that following 20 passages, pRSVT transformants form tubules in matrigel(Fig. 4). This was also observed with primary rabbit kidney proximal tubule cells.

The following study examined the possibility that the immortalized rabbit kidney cells possess the SV40 early region genes. Genomic DNA, was isolated after clone 8 was subjected to 20 passages, and was digested with either the restriction enzyme Xba or Bam H1. After Southern blotting to a Zeta probe blotting membrane, the DNA was probed with the 1.2 Kb Hind III DNA fragment of the SV40 early region gene from pRSVT plasmid labelled by random priming(Church & Gilbert, 1984). A

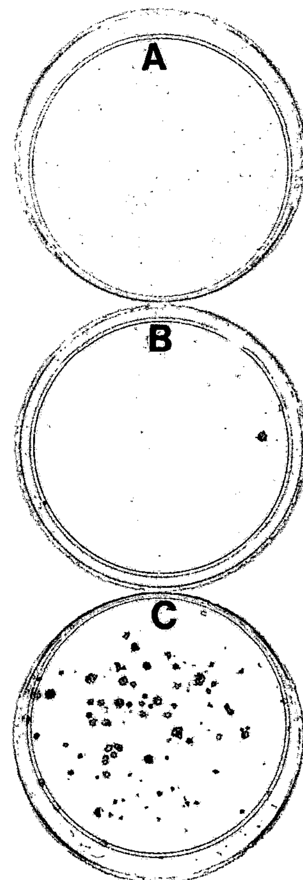
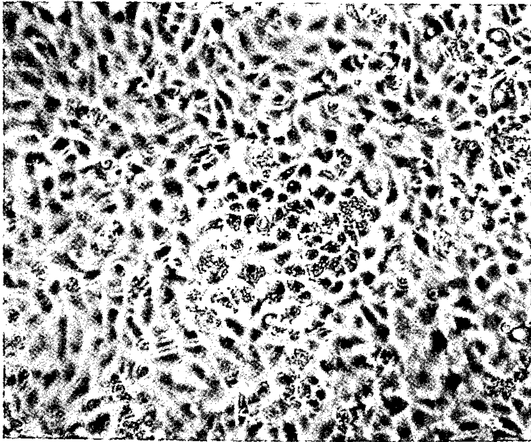


Fig. 2. Transformation of normal rabbit kidney proximal tubule cells by SV40 early region genes. Photomicrographs of cultures of proximal tubule cells fixed and stained with crystal violet after 5 weeks of transfection with the plasmid pRSVT. No colonies were visible on the control plates(A). Tightly packed, multilayered colonies of variable size formed on the pRSVT-treated plates(B, C)



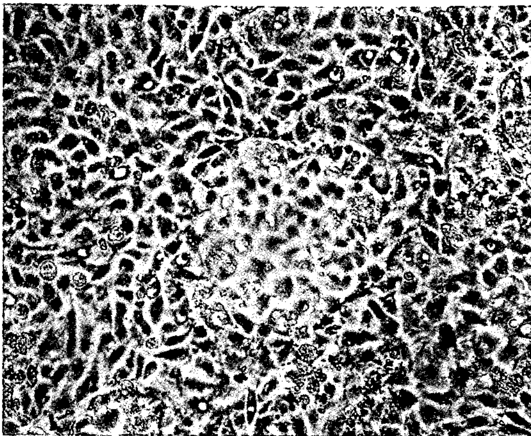


Fig. 3. Dome formation by pRSVT transformed cells

Table 1. Brush border enzyme activities of pRSVT transformed cells

	Alkaline phosphatase activity (nmoles p-nitrophenpol)	r-glutamyl transpeptidase activity (nmoles p-nitroanilide)
Primary proximal tubule cells	11.24±3.32	59.64±7.61
Transformant cells	25.34±5.27	56.54±9.48

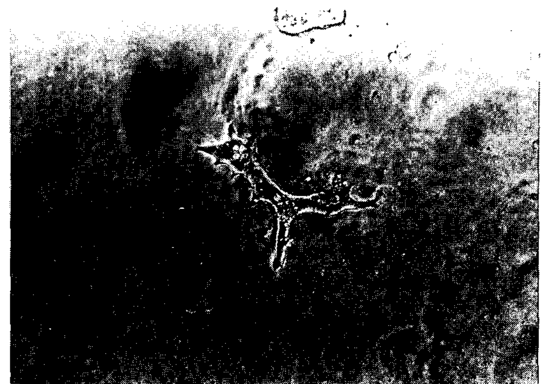


Fig. 4. Tubule formation by pRSVT transformants in matrigel

band of approximately 7.5 Kb was detected with Xba. Three Bam H1 bands were detected at approximately 15 kb, 6.5 kb and 3 kb, indicating that the transfected plasmid had integrated at three or more genomic sites(Fig. 5)

DISCUSSION

The main purpose of this study was to develop a culture model suitable for investigating the kidney proximal tubule functions. Since normal primary proximal tubule cells have a short life span, it was important to find a way of extending their replicative potential, without introducing genes, allowing for viral synthesis. Several groups have recently transformed primary cultures of renal tubular cells by infection with : a wild strain of simian virus(SV40), an adenovirus 12-SV40 hybrid, a temperature sensitive SV40 mutant strain, or the early region of SV40 DNA(Scott et al, 1986, Arend et al, 1989, Vandewalle et al, 1989).

DNA replication of SV40 DNA in infected monkey cells has served as a useful model system for mammalian chromosomal replication. Replication of the SV40 genome utilizes the

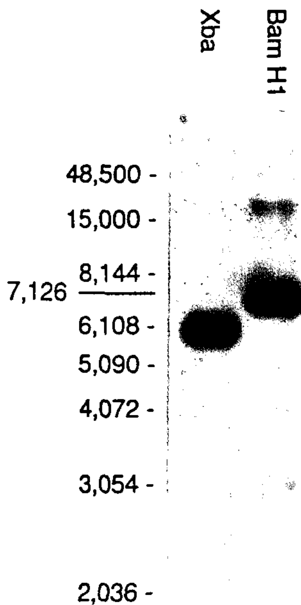


Fig. 5. Stable integration of the SV40 large-T-antigen(early region DNA) into the genome of primary rabbit kidney proximal tubule cells. Proximal tubule cells were transfected with 10μg of pRSVT. Cells were passaged 60 hours after transfection. One colony of morphologically transformed cells was trypsinized and expanded by repeated subculturing, usually at a subculturing ratio of 1 to 4. Cells from passage 20 of the T-antigen-transfected clone were used for Southern analysis. Lane 1 : genomic DNA digested with Xba. Lane 2 : genomic DNA digested with Bam H1.

host replication machinery in conjunction with a single virus-encoded protein, the large tumor antigen(T antigen). The development of a cell extract that is capable of replication has led to the identification of the components of the eukaryotic replication machinery and the characterization of their biochemical functions (Stillman, 1989, Diffley, 1992). SV40 DNA replication has been considered to be highly species specific, as that T-antigen-mediated replication can be recreated in vitro with extracts from human and monkey cells but not with extracts from mouse cell. This apparent primate specificity of replication is mediated by species-specific functional interactions of DNA polymerase α-primase with SV40 T antigen (Murakami et al, 1986, Collins et al, 1993)

Calcium phosphate transfection has been widely used to introduce cloned and genomic DNAs and RNAs into cultured cell lines and intact animals(Gorman et al, 1983). This technique results in transient expression and stable integration of cloned or genomic DNAs at high copy numbers by using a simple procedure and also results in the efficient expression of stable integrants. However, primary culture of normal human brochial epithelial cells are lysed by calcium phosphate precipitates, and calcium ions at lower concentrations, in the presence of serum, induce normal human bronchial epithelial cells(Hennings et al, 1980). In addition, calcium inhibits the growth of primary human prostate cells. The biological regulatory activity of Ca^{2+} ions may also limit the use of calcium phosphate precipitates in other cell types. In order to avoid these problems associated with the use of calcium phosphate, the newly developed strontium phosphate procedure was used in this study.

All types of normal human cells appear to have finite life spans in culture(Hayflick & Moorhead, 1961). Human cells infected with the SV40 virus have extended life spans but, in most cases, undergo a crisis during which the rate of proliferation markedly decreases or ceases entirely(Sack, 1981). More recent reports indicate that this crisis does not always occur and its incidence and severity may vary with cell type. For example, human keratinocytes often become immortalized without the intervention of such a crisis(Sack, 1981). In this study there is also little or no indication of the culture crisis commonly seen in SV40-transformed cells.

Simian virus 40 is the best studied member of the papovavirus family small DNA tumor virus. This virus has small genomes(5-6 kilobases) that encode a total of 5 or 6 proteins. This is capable of lytic infection of permissive cells and can

permanently transform nonpermissive cells subsequent to integration of viral DNA into the host genome. Cellular transformation is dependant on the expression of the so called "early gene" of this virus. The proteins incoded by the early genes have been termed tumor antigens because of their immunoreactivity in tumor bearing animals. SV40 produces large (large T) and small (small t) tumor antigens by differential splicing of a single early region precusor RNA. The function of SV40 large T include : replication of viral DNA, transformation of established cell lines, immortalization and transformation of primary cells (Prives, 1990).

Current evidence indicates that transformation by this virus involves the information of complexes between the tumor antigens and normal cellular proteins. SV40 large T binds to the cellular growth suppressors p53 and the retinoblastoma(RB) protein(Marshall, 1991). There is evidence that the activity of the RB protein is controlled by phosphorylation. SV40 large T forms complexes exclusively with the hypophosphorylated form of RB(DeCaprio et al., 1989) and it has been hypothesized that the hypophosphorylated protein is active in growth suppression. In SV40-transformed cells, RB undergoes cell cycle-dependent dephosphorylation, release from, and rebinding to large T, suggesting that binding to large T may be regulated by phosphorylation and dephosphorylation(Ludlow et al, 1989). The biochemical functions of the p53 growth suppressor in the control of cell growth are not known. Binding of p53 has been shown to contain a transcriptional activation domain (Marshall, 1991). p53 is phosphorylated at multiple sites in vivo(Meek & Eckhart, 1988) and the major site(serine 312 in mouse p53) is phosphorylated in vitro by the cell cycle control kinase p34(Bischoff et al, 1990). p53 is phosphorylated to a greater extent in rat cells transformed by SV40 than in untransformed cells(Scheidtmann & Haber, 1990).

Although the immortalized primary proximal tubule cell lines described herein further evaluations will be required to investigate their physiological functions, these cells will be useful for studying the functions of the renal proximal tubule cells.

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