Activation of the Simian Virus 40 (SV40) Genome Abrogates Sensitivity to AVP in a Rabbit Collecting Tubule Cell Line by Repressing Membrane Expression of AVP Receptors

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Abstract. To analyze the role of SV40 genome in the phenotypic alterations previously observed in SV40-transformed cell lines, we infected rabbit renal cortical cells with a temperature-sensitive SV40 mutant strain (tsA58) and compared the cell phenotypes at temperatures permissive (33°C) and restrictive (39.5°C) for SV40 genome expression. At both temperatures, the resulting cell line (RC.SVtsA58) expresses cytokeratin and uvomorulin, but epithelial differentiation is more elaborate at 39.5°C as shown by the formation of a well-organized cuboidal monolayer with numerous tight junctions and desmosomes. Functional characteristics are also markedly influenced by the culture temperature: cells grown at 33°C respond only to isoproterenol (ISO, 10^{-6} M) by a sevenfold increase in cAMP cell content above basal values; in contrast, when transferred to 39.5°C, they exhibit increased sensitivity to ISO (ISO/basal: 19.1) and a dramatic response to 10^{-7} M dDarginine vasopressin (dDAVP/basal: 18.2, apparent Ka: 5×10^{-9} M) which peaks 48 h after the temperature shift. The latter is associated with membrane expression of V2-type AVP receptors ($\sim$50 fmol/10^6 cells) which are undetectable when SV40 genome is activated (33°C). Clonal analysis, additivity studies, and desensitization experiments argue for the presence of a single cell type responsive to both AVP and ISO. The characteristics of the RC.SVtsA58 cell line at 39.5°C (effector-stimulated cAMP profile, lack of expression of brush-border hydrolases and Tamm-Horsfall protein) suggest that it originates from the cortical collecting tubule, and probably from principal cells.

To analyze at the cellular level the specialized functions that characterize the various segments of the renal tubule, several groups (Scott et al., 1986; Vandewalle et al., 1989; Arend et al., 1989) have recently transformed primary cultures of tubular cells by infection with a wild strain of simian virus 40 (SV40)[1] or with an adenovirus 12-SV40 hybrid, or by transfection with the early region of SV40 DNA. Using this approach, Scott et al. (1986) first immortalized rabbit thick ascending limb cells, and Arend et al. (1989) developed a rabbit collecting tubule cell line probably originating from intercalated cells. We (Vandewalle et al., 1989) have recently reported the establishment of three rabbit tubular cell lines maintaining properties of proximal cells for one of them (RC.SV1), and of more distal cells for the two others (RC.SV2 and RC.SV3). However, most of the con-

served functions including effector-stimulated cAMP production, enzymatic activities, and transport capacities were blunted after long-term culture of the infected cells. This might be due to deleterious effects of SV40 on the cell differentiation program (Cherington et al., 1986; Garcia et al., 1986; Amsterdam et al., 1988) and/or to a cell selection process occurring either at the time of viral infection or later on after several passages.

These results prompted us to study the influence of SV40 genome per se on the cell phenotype using a temperature-sensitive mutant of SV40 that can be rapidly activated or repressed (Chou, 1978, 1985; Isom et al., 1980; Banks-Schlegel and Howley, 1983; Handlogten and Kilberg, 1988). When infected cells are grown at a temperature permissive for the viral genome, they exhibit a transformed phenotype with rapid cell growth. When shifted to a restrictive temperature for the viral genome, they stop dividing and recover characteristics akin to those of parental cells. Since the temperature-dependent modulation of viral genome activity is rapid, it is possible to analyze at each passage the phenotypic alterations induced by the virus under conditions that practically exclude cell selection.

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1. *Abbreviations used in this paper*: AVP, arginine vasopressin; CT, calcitonin; dDAVP, dDarginine vasopressin; DPPIV, dipeptidylpeptidase IV; FK, forskolin; γGT, γ-glutamyltranspeptidase; ISO, isoproterenol; LAP, leucine aminopeptidase; MOI, multiplicity of infection; PTH, parathyroid hormone; SV40, simian virus 40.

Using the temperature-sensitive strain of SV40 tsA58 (Tegtmeyer, 1972) to infect rabbit cortical tubular cells, we have established a permanent epithelial cell line (RC.SVtsA58) displaying temperature-dependent profiles of effector-stimulated cAMP. Cells cultured at the permissive temperature (33°C) only responded to isoproterenol (ISO); when transferred to a restrictive temperature (39.5°C), they showed increased response to ISO and became highly sensitive to vasopressin (dDarginine vasopressin [dDAVP] and arginine vasopressin [AVP]) which induced a dose-dependent increase in cAMP content above basal values. These characteristics associated with the lack of expression of brush-border antigenic markers and Tamm-Horsfall protein, and with very low levels of brush-border hydrolases suggest that the cell line originates from the cortical collecting tubule and is related to principal cells. Appearance of the AVP response at 39.5°C, a phenomenon constantly observed over 50 passages, is not due to increased membrane fluidity but is explained by the membrane expression of AVP receptors that are not detectable at 33°C by binding studies. Thus, the model that we have developed provides the first demonstration that the activation of SV40 genome activation can directly inhibit membrane expression of receptors for a peptide hormone.

Materials and Methods

Establishment and Culture of the Cell Line

Suspensions of isolated renal cortical cells were first prepared as previously described (Poujeol and Vandewalle, 1985), under sterile conditions, by mechanical dissociation and sequential sieving of kidney cortices from male New Zealand white rabbits (500-700 g body weight). The isolated cells were then seeded at a concentration of 0.5×10^6 cells/cm^2 in 25-cm^2 culture flasks (Nunc, Roskilde, Denmark) and cultured at 37°C in 5% CO$_2$ containing atmosphere in an hormonally defined medium (DM:DME-HAM's F12 1:1 (vol/vol), transferrin, 5 µg/ml; sodium selenate, 30 nM; glutamine, 2 mM; dexamethasone, 5×10^{-8} M; insulin, 5 µg/ml; Hepes, 20 mM, pH 7.4). Medium was changed every other day until confluency (day 11). After trypsinization followed by neutralization with soybean trypsin inhibitor (Sigma Chemical Co., St Louis, MO), cells were seeded at the same density as above and grown at 37°C in MEM supplemented with 10% newborn calf serum. 2 d later, cells were washed twice with MEM and culture flasks were either mock-infected or infected (MOI = 100 pfu/cell) with the temperature-sensitive SV40 mutant tsA58 (Tegtmeyer, 1972). After 2 h adsorption at 37°C, the cells were washed and then grown at 33°C in 10% newborn calf serum-supplemented MEM. Foci of transformed cells were observed within 3 wk only in tsA58-infected flasks. Cell cultures were subsequently expanded and were adapted after the fifth passage to serum-free hormonally defined medium. Cell transformation was confirmed by stable nuclear expression of SV40 large T antigen detected by indirect immunofluorescence using specific rabbit polyclonal antibodies applied to acetone-permeabilized cells (Lafarge-Frayssinet et al., 1984). The cell line, referred to as RC.SVtsA58, was regularly cultured at 33°C and experiments were performed between the 15th and 50th passages.

The tsA58-transformed cell population was cloned at 33°C after the 20th passage. Cells were carefully trypsinized to obtain single cell suspension and seeded at a density of 10^3-10^4 cells in 100-mm Petri dishes (Falcon Labware, Lincoln Park, NJ). After 10 d of culture at 33°C, single colonies were isolated by trypsinization in a plastic ring sealed around the colonies by means of silicon grease (Lafarge-Frayssinet et al., 1984). Each cell colony was then expanded in defined medium. Six clones could be subcultured and reached confluency after 1 wk. cAMP studies were carried out on each clone between the 7th and 22nd passages after cloning.

For all biochemical studies, cells were seeded at a density of 2×10^4 cells/cm^2 in collagen-coated petri dishes or multiwell trays and initially grown at 33°C. Unless otherwise stated, studies were carried out comparatively on cells grown for 7 d at 33°C (referred to as "33°C condition") and on cells seeded at the same passage and grown in parallel at 33°C for 5 d but transferred to 39.5°C for 48 h (referred to as "39.5°C condition"). In

some experiments, cells were subsequently returned from 39.5°C to 33°C. Whatever the culture condition, medium was changed every other day.

For epithelial characterization of the cell line, cells were cultivated on porous collagen-coated filters (CM and PCF, 0.4 µm, 12 mm; Millipore Continental Water Systems, Bedford, MA). They were plated at 10^4 cells/well and grown to confluency in the same conditions as on plastic. Transepithelial resistance (R), expressed as Ω .cm^{-2}, was measured using the Millicel Electrical Resistance System (ERS; Millipore Continental Water Systems) connected to dual silver/silver chloride electrodes. Unseeded collagen-coated filters incubated in the culture medium served as negative control to estimate the resistance of the filter. Transepithelial voltage (V$_T$) was measured in parallel by applying square current pulses ($\pm$ 20 µA, 2 s duration) through the electrodes.

In additional experiments, we also used, as control for the 33°C and 39.5°C culture conditions, an AVP-responsive distal cell line, RC.SV3, previously established in our laboratory (Vandewalle et al., 1989) after infection with the wild type SV40 strain LP.

Cell Growth Studies

Kinetics of cell growth were determined on cells seeded in individual 35-mm Petri dishes (1-2 $\times 10^5$ cells/dish). Every day from day 2, cells from quadruplicate dishes were separately harvested after addition of 1 ml Trypsin 0.05%-EDTA 0.02% and then counted in a hemacytometer. Cell viability was estimated by trypan blue exclusion.

The cell protein content was measured in parallel in the various culture conditions by the method of Bradford (1966) with BSA as reference. Results are expressed as µg protein per 10^5 cells.

DNA and protein synthesis were assessed, respectively, by ^{3}H (methyl) thymidine and ^{14}C (U) leucine incorporations into TCA-insoluble cell fractions. Cells cultured in 35-mm Petri dishes were pulsed at 37°C with ^{3}H methyl thymidine (1.25 µCi/dish, S.A. :25 Ci/mmol, CEN, Saclay, France) for 24 h, or with ^{14}C (U) leucine (0.2 µCi/dish, S.A. :320 mCi/mmol, CEN) for 30 min. All cultures were then rinsed three times with ice-cold PBS and incubated for 30 min at 4°C with 1 ml 5% TCA. Thereafter, TCA was discarded and the cells were further incubated for 30 min at 37°C with 1 ml 0.3 N NaOH. After neutralization with acetic acid, the cell lysates were counted in a scintillation counter. Results are expressed as cpm/10^6 cells.

Morphological and Immunomorphological Studies

Optical Microscopy. Morphology of the cultures was followed using an Olympus microscope equipped with phase-contrast device. Photographs were taken on cultures grown in plastic Petri dishes at both 33 and 39.5°C, fixed with 2% glutaraldehyde, and stained with Groat hematoxylin-0.5% phloxin.

Immunofluorescence. Identification of nuclear SV40 large T antigen in virus-infected cells was performed on cells grown on coverslips and fixed with acetone (5 min at 4°C). Cells were then washed in PBS and incubated for 30 min at room temperature with polyclonal rabbit antibodies specific for SV40 large T antigen, followed by FITC-labeled anti-rabbit Ig. Each incubation step was followed by three extensive washes in PBS. The preparation was examined with a Leitz microscope equipped with epifluorescence optics.

Expression of epithelial markers in cells grown on plastic or filters was searched for using mAbs specific for cytokeratin 18 (Amersham, Les Ulis, France) and uvomorulin (kindly provided by N. Peyrieras, Institut Pasteur, Paris). Cells cultivated on plastic were first permeabilized with methanol (10 min, 4°C), washed in PBS, and then incubated with the mAb followed by biotinylated species-specific anti-mouse Ig and FITC-labeled streptavidin (Amersham). Confluent cultures of cells grown on Millipore PCF filters were embedded in 10% gelatin in PBS, pH 7.4, and snap-frozen in liquid nitrogen. 2-4-µm transversal cryostat sections of the cell layer(s) were deposited on a glass slide, fixed in acetone, and processed as described above.

Expression of Tamm-Horsfall protein and apical hydrolases (leucine aminopeptidase [LAP], dipeptidylpeptidase IV [DPPIV], and neutral endopeptidase) was analyzed using mAbs previously produced in our laboratory (Brunisholz et al., 1986; Tauc et al., 1988) and incubated with methanol-fixed cells grown in plastic Petri dishes.

Transmission EM. Cells grown in collagen-coated plastic dishes were fixed in Karnovsky's fixative in 0.1 M sodium cacodylate buffer, pH 7.4, for 3 h at room temperature. They were postfixed in 1% osmium, stained en bloc with uranylacetate, dehydrated in a graded series of ethanols, and embedded in Epon 812. Ultrathin sections were performed on transversally

orientated confluent cultures, counterstained with uranyl acetate and lead citrate, and viewed in an EM 109 Zeiss electron microscope. Cells cultivated on PCF Millipore filters were processed as previously described (Vandewalle et al., 1989).

Biochemical Studies

Measurement of Enzymatic Activities. Enzymatic activities were measured in RC.SVtsA58 cells seeded in 60-mm Petri dishes and grown in the 33 and 39°C conditions described above. Before assay, cells were rinsed three times in ice-cold PBS, scraped off the dish, resuspended in 20 mM Tris buffer, pH 8, and stored at −70°C before use. γ-Glutamyltranspeptidase (γGT), LAP, DPPIV, and Na^+-K^+ ATPase activities were determined as previously described (Vandewalle et al., 1989). Protein content was measured according to Bradford (1966). Results are expressed as μmol per min per g protein.

Determination of cAMP Production. At the end of the culture period (day 7), sets of parental or cloned RC.SVtsA58 cells cultured in 12-well trays were transferred to 37°C to test their sensitivity to different hormones and compounds. Cells were first incubated for 20 min at 37°C in defined
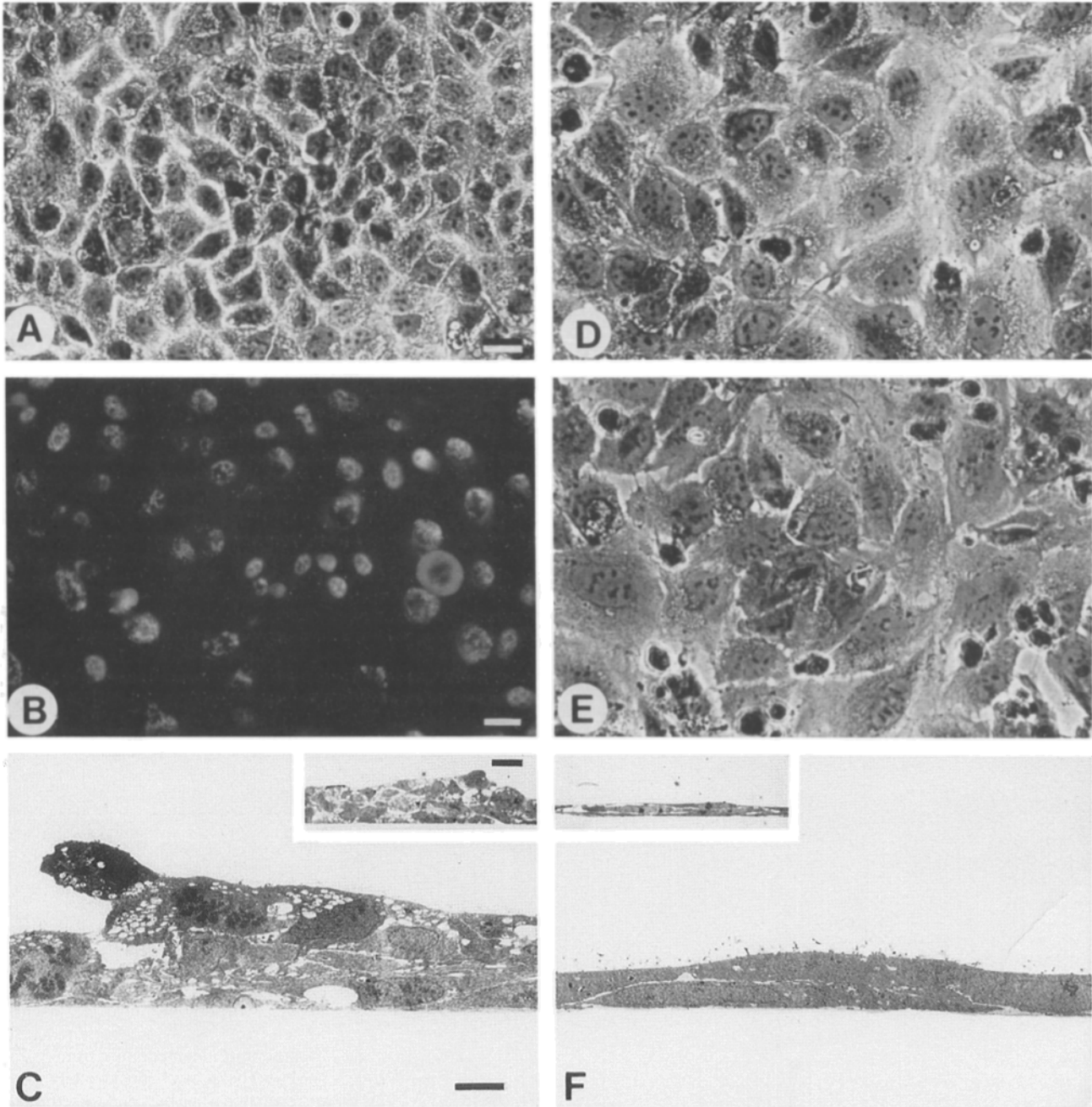

Figure 1. Morphological aspects of RC.SVtsA58 renal cells grown on plastic support. (*A, D,* and *E*) Phase-contrast microscopy of cells grown at 33°C for 7 d (*A*) or 5 d followed by 24 h (*D*) or 48 h (*E*) at 39.5°C. Note that the temperature shift induces a substantial increase in cell size. (*B*) Expression of large T antigen in cells grown at 33°C on coverslips, fixed in acetone, and stained with specific rabbit polyclonal antibodies. Note intense positivity of all nuclei. (*C* and *F*) Transmission EM of cells grown in 33°C (*C*) and 39.5° C (*F*) conditions. Transversal semi-thin sections of the cultures examined by light microscopy are shown in the insets. Note multilayer organization at 33°C and bilayer presentation with cell flattening at 39.5°C. Bars: (*A, B, D,* and *E*) 9 μm; (*C, F,* and *insets*) 5 μm.
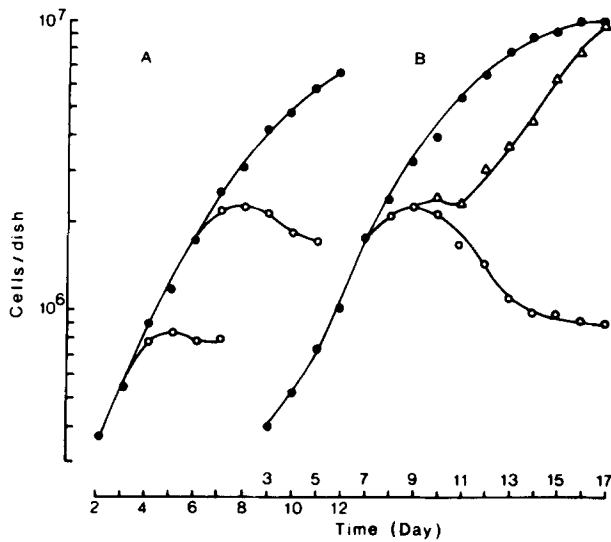
Figure 2. Cell growth kinetics. Representative growth curves of cells cultured at 33°C (permissive temperature) or 39.5°C (restrictive temperature). In *A*, cells initially grown at 33°C (*solid circles*) were transferred to 39.5°C (*open circles*) at day 3 or 6, or maintained at 33°C (*solid circles*). In *B*, cells initially grown at 33°C (*solid circles*) were transferred to 39.5°C (*open circles*) at day 7 and then returned to 33°C (*open triangles*) at day 9 or left at 39.5°C (*open circles*). Control cells were also kept at 33°C (*solid circles*). Note rapid cell growth at 33°C and, in contrast, complete arrest of cell growth at 39.5°C. Cell divisions resume when the cells are returned to 33°C (*B*). Each point is the mean of quadruplicate counts.

medium containing 0.1 mM 3-isobutyl 1-methyl xanthine (IBMX; Sigma Chemical Co.). Thereafter, they were incubated for 7 min at 37°C in 2 ml of the same defined medium supplemented or not with parathyroid hormone (PTH, 1-34 synthetic bovine parathormone; Calbiochem, Meudon, France), desmopressin (dDAVP; Ferring Pharmaceuticals, Malmoe, Sweden), arginine vasopressin (AVP; Sigma Chemical Co.), calcitonin (CT; Ciba Geigy, Rueil Malmaison, France), isoproterenol (ISO; Sigma Chemical Co.) or forskolin (FK; Sigma Chemical Co.). The amounts of hormones or compounds are given in text and figures. The reaction was stopped by rapid removal of the media, immediately followed by addition

of 1 ml ice-cold ethanol 95%-formic acid 5% solution. After 30 min incubation at 4°C, the supernatant was recovered, evaporated, and resuspended in 50 mM phosphate buffer, pH 6.2. Levels of intracellular cAMP were assessed in the deproteinated supernatants using a radioimmunoassay kit (no. 79830; Institut Pasteur, Lyon, France). All experiments were carried out in duplicate or triplicate. The number of cells per well was determined in parallel as previously described. Results are expressed as pmol cAMP formed per 10^6 cells.

Additivity studies were performed by incubating the cells with both dDAVP and ISO. In addition, in some experiments, the cells were preincubated for various periods of time before stimulation by the effectors with dDAVP (desensitization experiments), Bordetella pertussis toxin (Sigma Chemical Co.), or benzyl alcohol (Sigma Chemical Co.). All incubation conditions are indicated in the text and in the legends of tables and figures.

AVP Binding Studies

For these experiments, cells were seeded in collagen-coated 12-well trays and cultured under 33 and 39.5°C conditions. Binding studies were performed on day 7 at 37°C according to Roy and Ausiello (1981) with minor modifications. The cells were first incubated for 60 min at 37°C in DM supplemented with 2 mg/ml BSA (DM-BSA). After removal of the medium, 750 μl DM-BSA containing the appropriate concentration (1 or 2 × 10^{-8} M) of tritiated AVP (vasopressin, 8-L-arginine [phenylalanyl - 3,4,5-^{3}H(N)], S.A. :68.5 Ci/mmol, NEN, Paris, France) was added to each well. At the end of the incubation period (1-20 min), the medium was discarded and 1 ml of ice-cold isotonic saline was added. Cells were then scraped with a rubber policeman, poured on a glass filter (GF/B; Whatman Inc., Clifton, NJ), and washed with 15 ml ice-cold isotonic saline. The whole procedure did not exceed 40 s. Filters were then dried and counted in 8 ml scintillation liquid for 10 min. All measurements of total [^{3}H]AVP binding were performed in duplicate and were coupled with assessment of the nonspecific binding determined by incubating the cells in the presence of a 100-fold excess of unlabeled AVP or dDAVP (1 or 2 × 10^{-6} M).

Statistical Analysis

Results are the mean values ± SE of several experiments carried out in duplicate or triplicate. Statistical analysis was performed using Student's *t* test.

Results

Establishment of the RC.SVtsA58 Cell Line

When grown at 33°C, the cell population infected by the tsA58 SV40 strain displayed the properties of a transformed
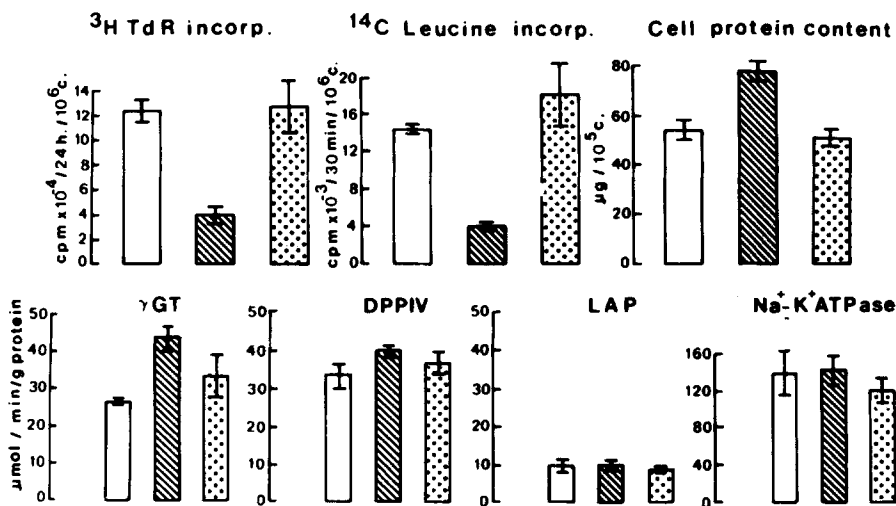

Figure 3. DNA and protein synthesis, protein content, and membrane enzymatic activities. Experiments were carried out at cell confluency of cultures grown in the various temperature conditions described: (*open bars*) 33°C; (*hatched bars*) 39.5°C; (*dotted bars*) return to 33°C. DNA and protein synthesis assessed, respectively, by measurements of [^{3}H] thymidine and [^{14}C] leucine incorporations into TCA-insoluble cell fractions, are markedly reduced after the shift in temperature from 33 to 39.5°C. These biochemical alterations are reversible when the cells are returned to 33°C after 48 h of culture at 39.5°C. Reciprocal variations of protein content are observed. Note that brush-border enzymatic activities remain extremely low in either condition and that Na$^+$-K$^+$-ATPase is not influenced by culture temperature. Values are the mean ± SE of five experiments performed in duplicate or triplicate, except for protein content figures which are the mean ± SE of nine determinations in each culture condition.
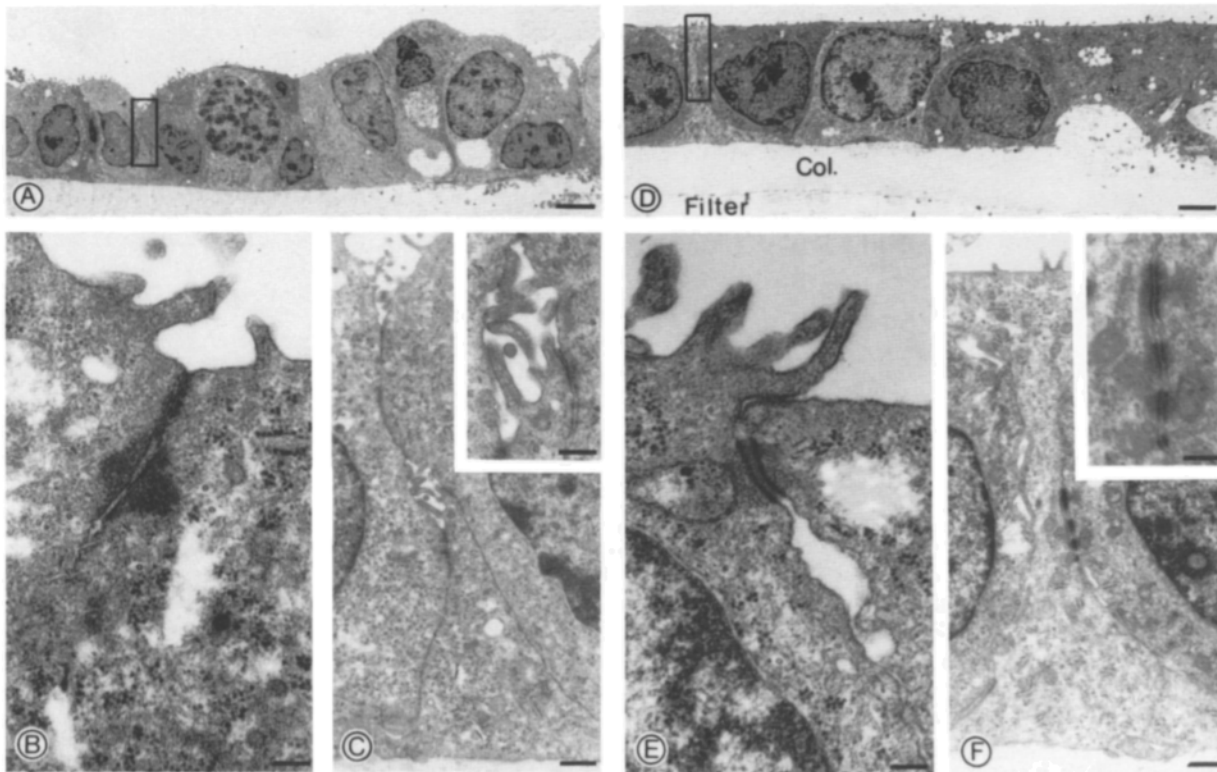
33°C   **39,5°C**

Figure 4. EM analysis of the epithelium formed by RC.SVtsA58 cells grown on porous filters. Cells were cultivated on PCF Millipore filters in 33°C (*A-C*) and 39.5°C (*D-F*) conditions. *C* and *F* are enlargements of the areas denoted by a rectangle in *A* and *D*, respectively. At 33°C, cells form an irregular mono- or bilayer and present a convex apical pole covered with numerous short cytoplasmic microprojections (*A*). *B* shows a tight junction and a diamond-shaped desmosome; these connecting structures were only detected in the upper cell layer near the cell apex. *C* shows an intercellular space with cytoplasmic projections limited by desmosomes (*C* and *inset*). At 39.5°C, cells form a regular monolayer composed of cuboidal cells with a flat apical pole and a limited number of apical microprojections (*D*; *Col.*, collagen). *E* is a representative illustration of tight junctions and desmosomes that were detected in the epithelium at 39.5°C. Compare the elaborate aspect of the junctions at 39.5°C with their atypical presentation at 33°C. Numerous desmosomes are visible in the lateral domain of adjacent cells (*F* and *inset*). Bars: (*A* and *D*) 3 μm; (*B* and *E*) 0.16 μm; (*C* and *F*) 0.27 μm; (*C* and *F, insets*) 0.16 μm.

cell line. First, it could be regularly subcultured in vitro up to the 50th passage at the time of the writing of this article, while mock-infected cells stopped dividing after the 4th passage. Second, SV40 specific large T antigen could be detected in 100% of the cell nuclei at various passages (Fig. 1 *B*). Finally, cells were able to form colonies when seeded in soft agar.

Cell Growth Kinetics

As shown in Fig. 2, cells cultured at the permissive temperature (33°C) grew rapidly with a doubling time of 53.5 ± 2.4 h (*n* = 9). Transfer of the culture to the restrictive temperature (39.5°C) after various times of culture at 33°C (Fig. 2, *A* and *B*) induced within 48 h an arrest of cell division
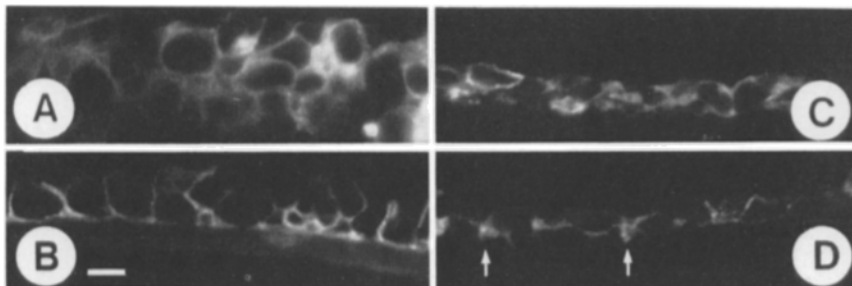

Figure 5. Expression of cytokeratin and uvomorulin in RC.SVtsA58 cells. Cytokeratin (*A* and *C*) and uvomorulin (*B* and *D*) were detected by immunofluorescence using specific mAbs, in cells grown on Millipore filters at 33°C (*A* and *B*) and 39.5°C (*C* and *D*). Note expression of cytokeratin in all cells of the multilayer at 33°C (*A*). Uvomorulin was concentrated in the basolateral domain at 33 and 39.5°C (*arrows*). This distribution is more readily identified at 33°C because the filter remained attached to the cell layer. Bar, 6 μm.

Table I. Transepithelial Resistance and Voltage of RC.SVtsA58 Cells Grown on Porous Filters

	33°C		39.5°C	
Time from seeding	R	V_T	R	V_T
d	*Ω/cm^2*	*mV*	*Ω/cm^2*	*mV*
10	100 ± 6 (8)	−1.7 ± 0.3 (8)	116 ± 25 (9)	−0.6 ± 0.1 (9)
21	131 ± 17 (6)	−1.4 ± 0.4 (6)	94 ± 9 (9)	−0.8 ± 0.1 (9)

Transepithelial resistance (R) and voltage (V_T) were measured on confluent RC.SVtsA58 cells grown on Millipore filters. Cells were either cultivated at 33°C throughout the culture time or shifted to 39.5°C in the last 48 h of culture. Measurements were performed 10 and 21 d after seeding. Values are the mean ± SE of (n) determinations at three different passages.

followed by a progressive decrease in cell number due to cell death and detachment. After 48 h at 39.5°C, cell viability was not significantly lower than in parallel cultures maintained at 33°C (39.5°C: 86.1 ± 5%; 33°C: 90.3 ± 3.4%, n = 9); if the cultures were returned at this stage to 33°C, the inhibition of cell growth was fully reversible within 48 h (Fig. 2 B).

The arrest of cell growth at 39.5°C was associated with an overall reduction in DNA and protein synthesis as indicated by a 70% decrease in the incorporation of thymidine and leucine (Fig. 3), and with a substantial increase in individual cell size (Fig. 1, D and E) and protein content, which was 1.4-fold higher than at 33°C (33°C: 54.7 ± 4.1, 39.5°C: 78.0 ± 4.5 $\mu g/10^5$ cells, n = 9) (Fig. 3). Modifications of DNA and protein synthesis, cell size, and protein content were totally reversible when the cells were returned to 33°C (Fig. 3).

Epithelial Characterization of the Cell Line

Morphologically (Fig. 1), cells cultivated on plastic exhibited a cobblestone appearance at 33 and 39.5°C. By EM (Fig. 1, C and F), cells grown at 33°C tended to form multilayers; their counterparts grown at 39.5°C were markedly flattened and formed mainly mono- or bilayers. Epithelial differentiation was generally greater when cells were grown on porous supports, but remained more pronounced at 39.5 than at 33°C (Fig. 4). At 33°C, cells formed mainly bilayers (Fig. 4 A). The upper layer consisted of cuboidal cells sealed at their apex by tight junctions and desmosomes (Fig. 4 B). The latter were infrequent and characterized by a diamond-shaped atypical aspect (Fig. 4 B). The upper and lower cell layers were not connected by tight junctions or desmosomes but were separated by intercellular gaps. Intercellular spaces containing cytoplasmic projections were often observed in the epithelium. In the neighborhood of these formations, the lateral domains of adjacent cells presented desmosome-like structures (Fig. 4 C, inset). In contrast, cells shifted to 39.5°C in the last 48 h of culture formed a well-organized cuboidal monolayer (Fig. 4 D) with numerous typical tight junctions (Fig. 4 E) and desmosomes (Fig. 4, E, F, and F inset). In both culture conditions (33 and 39.5°C), the cells expressed cytokeratin 18 and uvomorulin. Cytokeratin 18 was detected in all cells cultivated on filters in the 33°C multilayer as well as in the 39.5°C monolayer (Fig. 5, A and C). The same observation was made for uvomorulin which was concentrated in the cell lateral domain (Fig. 5, B and D). Transepithelial electrical resistance measured after 10 d and 21 d of culture averaged 100 Ω/cm^2 at both culture temperatures (Table I).
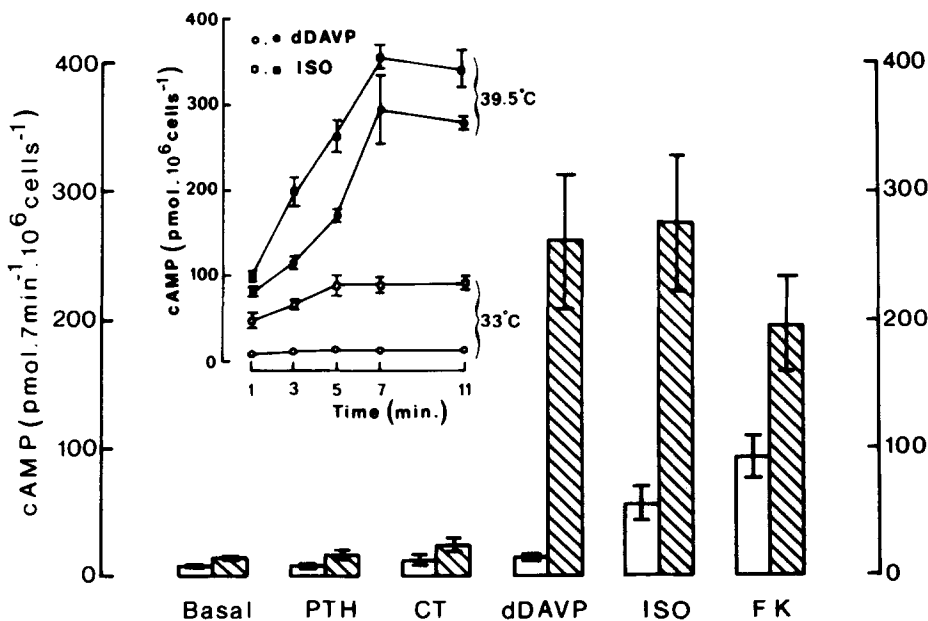

Figure 6. Profiles of effector-stimulated cAMP production. Experiments were performed on cells seeded at the same passage and grown in 33°C (*open bars*) and 39.5°C (*hatched bars*) conditions. Whatever the culture conditions, incubations in the absence (*Basal*) or the presence of the various effectors (*PTH*, 10^{-7} M; *CT*, 100 ng/ml; *dDAVP*, 10^{-7} M; *ISO*, 10^{-6} M; *FK*, 10^{-5} M) were carried out at 37°C for 7 min after preincubation of the cells with IBMX. Values are the mean ± SE of seven experiments performed in triplicate between the 24th and 45th passages. The upper insert graphs represent typical examples of time-course responses to dDAVP (10^{-7} M, *circles*) and ISO (10^{-6} M, *squares*) of cells seeded at the same passage and grown either in 33°C condition (*open symbols*) or in 39.5°C condition (*solid symbols*). Each point represents the mean value ± SE of triplicate measurements.
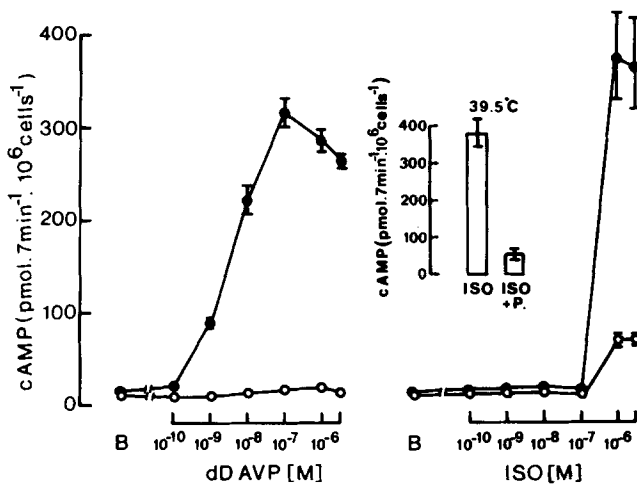
Figure 7. Dose-response curves depicting cAMP production upon stimulation by dDAVP and ISO. Experiments were carried out on cells seeded at the same passage and grown in 33°C (*open circles*) and 39.5°C (*solid circles*) conditions. Cells preincubated with IBMX at 37°C were stimulated at the same temperature (37°C) by increasing concentrations of dDAVP (*left panel*) and ISO (*right panel*). B stands for basal values. Each point is the mean value ± SE of three experiments. The bars of the insert in the ISO dose-response graph represent the mean value ± SE of four experiments in which cells grown at 39.5°C were stimulated with ISO in the absence or the presence of propranolol (*P.*, 10^{-5} M), a β-receptor antagonist. Note that propranolol inhibits almost completely the effects of ISO.
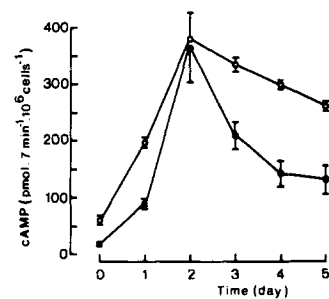

Figure 8. Time-course effects of culture at the restrictive temperature on RC.SVtsA58 cell responses to dDAVP and ISO. Cells initially grown at 33°C were transferred to 39.5°C (day 0) and studied for hormonal sensitivity every 24 h afterwards (days 0–5). cAMP was measured after stimulation at 37°C by dDAVP (10^{-7} M) (*solid circles*) or ISO (10^{-6} M) (*open circles*) in the presence of IBMX. Each point represents the mean value ± SE of triplicate measurements.

Transepithelial voltage remained equally low after the temperature shift (Table I).

Enzymatic Profile and Antigenic Markers

Enzymatic activities typical of the brush-border of the proximal tubule including γGT, LAP, and DPPIV remained very low at both temperatures (Fig. 3). Na^+-K^+ATPase was unaffected by the temperature shift. Tubular antigenic markers of the proximal tubule (LAP, DPPIV, neutral endopeptidase) and of the thick ascending limb of Henle's loop (Tamm-Horsfall protein) were not expressed on RC.SVtsA58 cells. These results suggested that this cell line derived from more distal tubular segments.

Functional Characterization of the RC.SVtsA58 Cell Line

Sensitivity to effector agents was assessed by measuring cAMP production at 37°C in cells previously grown either at 33 or at 39.5°C. As shown in Fig. 6, although FK induced a similar increase in cAMP content above basal values (33°C, FK/basal: 11.4; 39.5°C, FK/basal: 13.7), the profile and the magnitude of the effector-stimulated cAMP response markedly differed: cells maintained at the permissive temperature only responded to ISO (ISO/basal: 7.0, $P < 0.0025$); after 48 h of culture at the restrictive temperature, the cAMP response to ISO was greatly enhanced (ISO/basal: 19.1, $P < 0.0005$) and the cells became highly sensitive to dDAVP (dDAVP/basal: 18.2, $P < 0.0005$). Parathormone (PTH) and calcitonin (CT) were ineffective at both temperatures. The kinetics of cAMP production induced by ISO and dDAVP are

depicted in the insert of Fig. 6. Prolonged incubation with dDAVP (up to 11 min) of cells grown at 33°C did not increase cAMP production; under the same conditions, the plateau phase of ISO-stimulated cAMP response was reached in 5 min. When cells were cultured at 39.5°C, the peak production of cAMP induced by ISO or dDAVP was reached at 7 min. The effects of dDAVP and ISO on cAMP production in 33 and 39.5°C conditions were clearly dose dependent (Fig. 7). Half-maximal increase in cAMP content of 39.5°C grown cells occurred at 5×10^{-9} M dDAVP (apparent dissociation constant value, Ka), and maximal stimulation was achieved at 10^{-7} M. Concentrations of dDAVP up to 5×10^{-6} M failed to enhance cAMP production by cells cultured at 33°C. The concentration giving maximal stimulation and the apparent Ka of ISO were 10^{-6} M and 5×10^{-7} M, respectively, whatever the culture conditions, but the level of the plateau phase was approximately sixfold higher at 39.5°C. The response to ISO (10^{-6} M) of cells cultured at 39.5°C was inhibited by 79% in the presence of 10^{-5} M propranolol, thus, confirming that the effects of ISO were mediated by β adrenergic receptors. The cAMP production induced in response to dDAVP and ISO was also studied from 1 to 5 d after transfer of cultures to 39.5°C (Fig. 8). Peak responses were observed after 48 h and gradually decreased while remaining higher than at 33°C. This was related to cell detachment and cell mortality which reached 50% after 5 d of culture at 39.5°C.

Three types of experiments were finally carried out to show that a single cell type supported the AVP and ISO responses. First, the six stable clones derived from the whole transformed cell population after the 20th passage were all sensitive to both dDAVP and ISO (Table II). The amount of cAMP produced was of the same order of magnitude as that measured in the starting cell population. Second, the effects of dDAVP and ISO were not, or only partially, additive (Morel et al., 1982), as shown by experiments comparing cAMP production induced by both compounds (C) with the theoretical sum (A + B) of the amounts of cAMP produced under separate stimulation by each effector. The C/(A + B) ratio was 0.89 for a single clone, A4, that was recloned together with A1 which, with a ratio of 0.51, was used for comparison purposes. All five subclones derived from clone A4 were equally sensitive to dDAVP and ISO, as were their four counterparts derived from clone A1. Although this analysis does not completely rule out the presence in clone A4 of a minor subpopulation of cells only responsive to ISO which could not be subcloned, it does suggest that dDAVP and ISO

Table II. Additivity Studies: Effects of dDAVP and ISO on the cAMP Content in RC.SVtsA58 Cells

	cAMP (pmol/7min/10⁶ cells)			
	dDAVP (A)	ISO (B)	dDAVP + ISO (C)	C/(A + B)
RC.SV40tsA58 Clones	283.0 ± 23.1	379.8 ± 41.7	458.6 ± 31.4	0.69
A1	357.0 ± 68.1	247.9 ± 53.0	306.0 ± 85.2	0.51
A3	329.3 ± 12.1	286.8 ± 20.8	402.9 ± 32.8	0.60
A4	143.9 ± 23.7	172.6 ± 69.3	281.7 ± 88.9	0.89
A5	298.2 ± 13.4	218.3 ± 34.8	315.9 ± 26.4	0.61
A6	469.8 ± 86.9	486.0 ± 176.8	666.4 ± 216.9	0.70
A7	229.0 ± 64.5	167.2 ± 48.9	232.5 ± 59.2	0.59

Parental cells (*RC.S40VtsA58*) and the different clones cultured at 33°C were transferred to 39.5°C for 48 h before experiments. The cAMP content was measured in the presence of IBMX and dDAVP (10^{-7}M) and/or ISO (10^{-6}M). C/(A + B) represents the ratio of ISO + dDAVP-stimulated cAMP values to the theoretical sum of values measured under separate stimulation by each agent. Values are the mean + SE of three experiments performed in duplicate.

Table III. Homologous and Heterologous Desensitization Studies

	dDAVP	Inhibition	ISO	Inhibition
Untreated cells	363.6 ± 21.5	—	309.6 ± 20.3	—
dDAVP-pretreated cells	174.8 ± 15.2	52%	209.0 ± 14.1	32%

Cells grown in 39.5°C condition were preincubated with 10^{-7} M dDAVP for 1 h at 37°C or left untreated. Then, they were stimulated with either 10^{-7} M dDAVP or 10^{-6} M ISO for 7 min in the presence of IBMX. Values are the mean ± SE of four determinations.

share the same cell targets. Partial additivity of their effects could be explained by different levels of coupling with adenylate cyclase of dDAVP and ISO receptors. Finally, cells grown at 39.5°C for 48 h were first preincubated with dDAVP for 1 h at 37°C before a second exposure for 7 min to 10^{-7} M dDAVP or to 10^{-6} M ISO. This procedure reduced the response to dDAVP and ISO by 52 and 32%, respectively (Table III). The magnitude of homologous desensitization approximated that observed in vivo or in renal cell cultures (Rajerison et al., 1977; Roy et al., 1981; Wilson et al., 1988).

Analysis of the Mechanisms Modulating Temperature-dependent Response to dDAVP

To understand the appearance of a major response to dDAVP at the restrictive temperature, we investigated (a) the functioning of the transduction machinery at 33°C; (b) the density of AVP membrane receptors at both temperatures; and (c) the role of temperature per se and ensuing variations of membrane fluidity.

Because RC.SVtsA58 cells grown at 33°C were highly responsive to ISO, it seemed unlikely that the lack of dDAVP

Table IV. Effects of PGE2 on the cAMP Content in RC.SVtsA58 Cells

	cAMP (pmol/7 min/10⁶ cells)		
	Basal	PGE2	PGE2/basal
33°C	10.2 ± 1.2	138.0 ± 15.0	13.5
39.5°C	16.4 ± 2.0	112.4 ± 18.2	6.9

cAMP content was measured in RC.SVtsA58 cells cultured at 33°C or shifted to 39.5°C in the last 48 h of culture, in the basal conditions (*Basal*) or after stimulation by 10^{-5} PGE₂ (PGE₂, 37°C, 7 min). Values are the mean ± SE of four determinations.

response at 33°C was caused by deficient transduction. To confirm this interpretation, we first compared cell sensitivity to prostaglandin E₂, another compound known to activate adenylate cyclase in cultured cells derived from the rabbit collecting tubule (Grenier et al., 1982; Spielman et al., 1986). 10^{-5} M PGE₂ induced a major stimulation of cAMP production that was almost twice as high in cells grown at 33°C than in their counterparts grown at 39.5°C (Table IV). In addition, preincubation of the 33°C grown cells with pertussis toxin (0.5 μg/ml, 2 h), which catalyzes ADP-ribosylation of the α-subunit and thus uncouples the Gi from the receptors (Katada and Ui, 1982), failed to enhance dDAVP response (Fig. 9), which was not affected either by prolongation of preincubation with 0.25 μg/ml pertussis toxin up to 18 h. These results strongly suggested that transduction of the dDAVP signal was not blocked by overexpression of inhibitory Gi proteins, and led us to consider that the lack of dDAVP response at 33°C was due to the absence of AVP membrane receptors.

To test this hypothesis, we carried out binding studies using tritiated AVP. We first verified that AVP induced a dose-dependent increase in cAMP content in 39.5°C cultured cells similar to that caused by dDAVP (identical apparent Ka = 5×10^{-9} M) and was inactive on 33°C grown cells (Fig. 10 A). Specific binding of [³H] AVP was not observed when cultures were maintained at 33°C (Fig. 10B). EDTA-suspended cells prepared from these cultures also failed to bind [³H]AVP (data not shown), so that the lack of binding could not be attributed to unaccessibility of AVP receptors on cells often organized as a multilayer epithelium. By contrast, time-dependent specific binding was observed on cells grown for 48 h at 39.5°C. As shown in Fig. 10 B, binding reached a maximum (53.8 ± 4.4 fmol/10⁶ cells) after 3 min incubation and from then on decreased slightly to reach a plateau phase stable for at least 20 min. Membrane-bound [³H] AVP could be displaced in a time-dependent
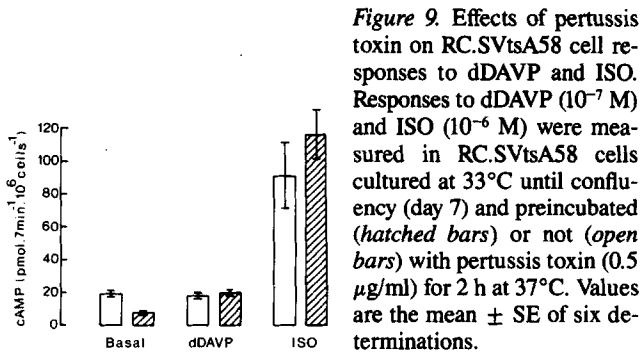
Figure 9. Effects of pertussis toxin on RC.SVtsA58 cell responses to dDAVP and ISO. Responses to dDAVP (10^{-7} M) and ISO (10^{-6} M) were measured in RC.SVtsA58 cells cultured at 33°C until confluency (day 7) and preincubated (*hatched bars*) or not (*open bars*) with pertussis toxin (0.5 μg/ml) for 2 h at 37°C. Values are the mean ± SE of six determinations.
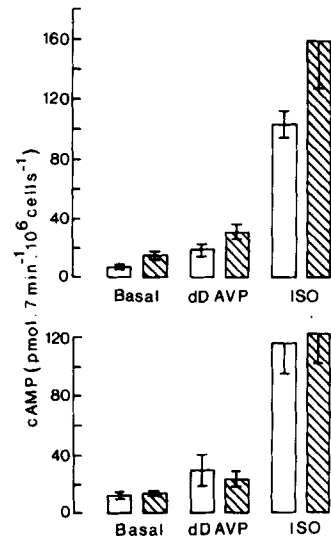

Figure 11. Specific effects of increases in temperature and membrane fluidity on cell responses to dDAVP and ISO. (*upper graph*) The AVP-responsive RC.SV3 cells (transformed by a nontemperature-sensitive wild strain of SV40) were grown in 33 (*open bars*) and 39.5°C (*hatched bars*) conditions according to the protocol described for the RC.SVtsA58 cell line. cAMP was measured at 37°C in the absence (*Basal*) or the presence of dDAVP (10^{-7} M) or ISO (10^{-6} M) after preincubation of the cells with IBMX. Values are the mean ± SE of three experiments performed in triplicate. Note that the temperature shift fails to enhance effector-stimulated cAMP production compared to basal values. (*lower graph*) cAMP production induced by dDAVP (10^{-7} M) and ISO (10^{-6} M) was measured in RC.SVtsA58 cells cultured at 33°C until confluency (day 7) and preincubated in the presence (*hatched bars*) or in the absence (*open bars*) of benzyl alcohol (40 mM, 20 min, 37°C). Values are the mean ± SE of three experiments performed in triplicate. Note that benzyl alcohol fails to restore cAMP production stimulated by dDAVP.

fashion by 1 μM unlabeled AVP or dDAVP: after 20 min incubation with 10 nM [^{3}H] AVP, binding was reduced by 55% within 3 min and by 80% after 20 min by incubation with unlabeled ligands (Fig. 10 C). The fact that the two curves were strictly superimposable strongly suggested that the cells expressed only V2 type receptors.

Finally, since temperature and membrane fluidity have been shown to influence hormonal responses (Hall and Grantham, 1980; Hebert and Andreoli, 1980), we investigated the potential role of an increase in temperature per se (i.e., independently of SV40 inactivation) in the response to AVP of tsA58 cells shifted to 39.5°C. We first studied under 33 and 39.5°C conditions, a previously reported cell line, RC.SV3 (Vandewalle et al., 1989), in which dDAVP induces a 3.3-fold increase in cAMP content when the cells are cultured under routine conditions (37°C). As shown in Fig. 11 (*upper graph*), the basal, dDAVP- and ISO-stimulated cAMP values were increased at 39.5°C in the same proportion (39.5°C/

33°C: 1.5–2.2) as basal values in RC.SVtsA58 cells (Fig. 6), so that the stimulated over basal cAMP ratios were not augmented by the temperature shift (33°C: dDAVP/basal, 2.8; ISO/basal, 15.3; 39.5°C: dDAVP/basal, 2.1; ISO/basal, 10.7).
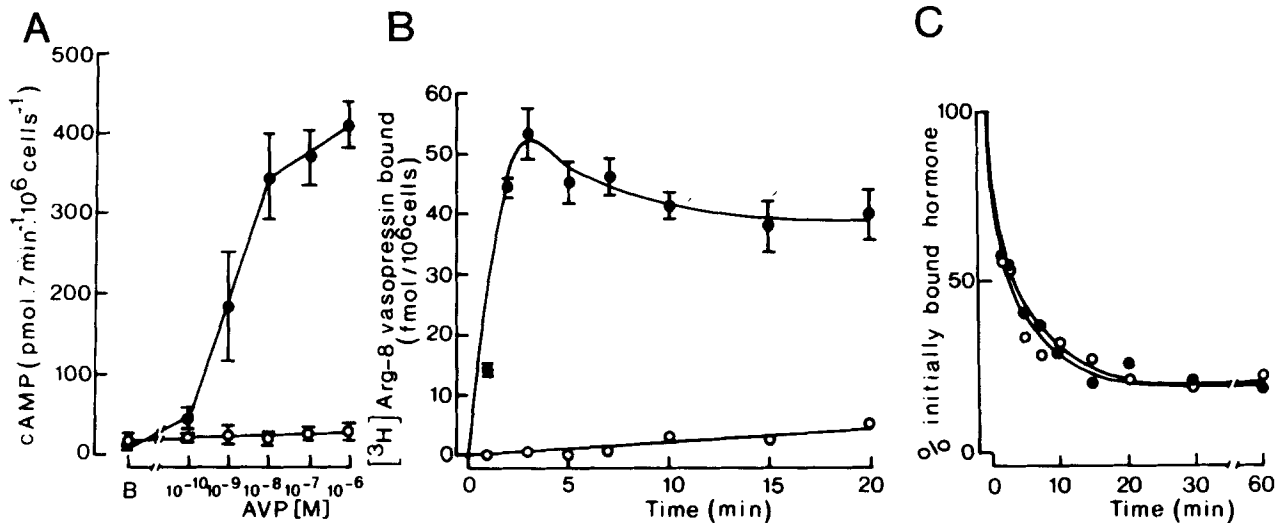

Figure 10. Dose-response curve of cAMP production in AVP-stimulated RC.SVtsA58 cells and AVP binding studies. (*A*) The AVP-stimulated cAMP dose-response curve was constructed as in Fig. 5 by incubating the cells previously cultured in 33°C (*open circles*) and 39.5°C (*solid circles*) conditions with increasing concentrations of AVP. Each point is the mean value ± SE of triplicate measurements. (*B*) The amount of [^{3}H]AVP specifically bound by cells cultured in 33 (*open circles*) or 39.5°C (*solid circles*) conditions was studied after various incubation times in the presence of 20 nM [^{3}H]AVP. Nonspecific binding was measured in the presence of a 100-fold excess of unlabeled AVP. Each point represents the mean specific binding value ± SE of three separate experiments performed in duplicate. At equilibrium, nonspecific binding represented 38% of the total binding. (*C*) Reversibility of hormonal binding. Cells cultured in 39.5°C condition were incubated in the presence of 10 nM [^{3}H]AVP for 20 min. Unbound free hormone was discarded and incubation medium containing 1 μm AVP (*solid circles*) or 1 μM dDAVP (*open circles*) was added to each well. At different time periods from 2 to 60 min, the supernatant was eliminated and replaced by 1 ml ice-cold isotonic saline. Cell-bound radioactivity was measured and expressed as percent of the total initial binding.

We then examined the effects on tsA58 cells grown at 33°C of benzyl alcohol, a local anesthetic that increases membrane fluidity. Although the change brought about by the concentration selected (40 mM) is equivalent to that induced by an upwards temperature shift of 6–7°C (Carrière and Le Grimellec, 1986; Friedlander et al., 1987), we could not detect any change in the effect of AVP on cAMP production (Fig. 11, *lower graph*). These results thus suggest that the appearance of AVP response after the temperature shift in RC.SVtsA58 cells is essentially, if not exclusively, attributable to SV40 genome inactivation through induction of membrane expression of AVP receptors.

Discussion

We report the establishment and the characterization of a rabbit tubular cell line immortalized by infection with a temperature-sensitive strain of SV40. This cell line was produced to analyze the role of SV40 genome in the phenotypic alterations that we had previously observed after transformation by the wild strain of SV40 (Vandewalle et al., 1989). Indeed, the use of a temperature-sensitive strain makes it possible to compare repeatedly at each passage, on the same cell population, the phenotypes expressed at the permissive ("transformed state") and restrictive ("normal state") temperatures, under experimental conditions that exclude cell selection and progressive dedifferentiation.

RC.SVtsA58 exhibits growth kinetics akin to those observed in placental (Chou, 1978) and hepatocyte (Chou and Schlegel-Haueter, 1981) cell lines transformed by another temperature-sensitive strain of SV40. Shift from the permissive to the restrictive temperature induces arrest of cell growth, associated with reduced DNA and protein synthesis, which is totally reversible when the cells are returned to 33°C. At both temperatures, the cells show epithelial features including cytoplasmic expression of cytokeratin 18 and prevailing lateral localization of uvomorulin. However, the temperature shift induces dramatic changes in epithelial organization, whatever the support used for culture. Cells grown on porous filters form mainly bilayers at 33°C, and poorly defined tight junctions are detectable only in the upper layer. In contrast, cells shifted to 39.5°C compose within 48 h a well-organized cuboidal monolayer with numerous tight junctions and desmosomes. Nevertheless, transepithelial resistance remained relatively low, probably as a consequence of increased membrane fluidity due to the 6.5°C increase in temperature.

Functional Characterization and Origin of the Cell Line

The biochemical, immunomorphological, and functional studies showing (a) minimal level of brush-border hydrolases; (b) lack of Tamm-Horsfall protein, a marker of the thick ascending limb of Henle's loop; (c) lack of sensitivity to PTH and CT, which act on the proximal (PTH), the ascending limb of Henle's loop (CT and PTH) and the distal tubule (CT and PTH) (Morel, 1981); and (d) high sensitivity to dDAVP and ISO, all indicate that tsA58 SV40 infection has transformed a cell population which, at the restrictive temperature, exhibits properties of the cortical collecting tubule. Cloning, additivity, and desensitization studies further indicate

that the RC.SVtsA58 cell line is monoclonal at least with regard to dDAVP and ISO responses.

Two points deserve particular discussion. First, the cell line was derived from a primary culture of tubular cortical cells expressing essentially proximal cell functions (Vandewalle et al., 1989). This unexpected result may be explained by (a) the presence of a 10–15% contamination by distal cells in the initial isolated cell preparation; and (b) preferential transformation and/or selection of these distal contaminants in serum-free hormonally defined medium, as suggested by previous observations made in our laboratory on cells transformed by a wild type SV40 strain (Vandewalle et al., 1989). Second, the above experiments indicating that a single cell type accounts for the sensitivity to dDAVP and ISO cannot be immediately reconciled with the interpretations proposed at present on the properties of the various cell types which make up the distal tubule (Kaissling and Kriz, 1979; Morel, 1981; Madsen and Tisher, 1986). Indeed, principal cells are known to be responsive to vasopressin (review in Morel and Doucet, 1986), as indicated by the induction of water channels by vasopressin, but it is only assumed, based on the correlation between the frequency of intercalated cells and the responsiveness to isoproterenol along the distal nephron, that only A (α) and B (β) subtypes (Schwartz and Al-Awquati, 1985; Schuster et al., 1986) of intercalated cells are sensitive to isoproterenol. One may therefore hypothesize that RC.SVtsA58 is a principal cell line as indicated by (a) the magnitude of the AVP response; (b) its inhibition after preincubation with the hormone (desensitization); and (c) the similarity of dDAVP apparent Ka values calculated for RC.SVtsA58 cells, microdissected collecting tubules (Torikai and Kurokawa, 1983; Chabardes et al., 1988) and primary cultures of collecting tubule cells (Garcia-Perez and Smith, 1984). The sensitivity to isoproterenol of the RC.SVtsA58 cell line may be interpreted first as an aberrant response indicative of altered cell differentiation. Similar observations have been made in other transformed cell lines: for instance, Arend et al., (1989) have recently derived from immunodissected collecting tubule cells a cell line (28A) exhibiting properties of intercalated cells in which of all the hormones tested calcitonin produced the greatest increase in cAMP production. It is nevertheless surprising that the response to isoproterenol of RC.SVtsA58 cells increased at 39.5°C, under conditions which inactivate SV40 and augment cell differentiation. This may suggest alternatively that infection with the tsA58 SV40 strain has selectively transformed a population of principal cells actually sensitive to both dDAVP and ISO. In that case, response to ISO would extend the list of characteristics common to principal and intercalated cells, including shared expression of antigenic markers (Garcia-Perez and Smith, 1983; Spielman et al., 1986; Poujeol et al., 1987), which favor a close origin of the two cell populations.

Control of AVP Response by SV40 Genome

Binding experiments clearly show that 33°C grown cells fail to respond to AVP because of the lack of membrane expression of AVP receptors; they further indicate that the dramatic AVP response appearing at 39.5°C is induced by expression of V2-type AVP receptors. In addition, the temperature-dependent increase in membrane fluidity has no or little effect

on AVP-induced cAMP production at 39.5°C. These results demonstrate that SV40 genome activation (or inactivation) can repress (or induce) the expression of AVP membrane receptors.

It is noteworthy that SV40 activation has different effects on cell response according to the effector used: whereas it turns off AVP response, it almost doubles PGE_2 stimulation and reduces only by 60% the effects of ISO on cAMP production (probably by decreasing the number of ISO receptors without changing receptor affinity as suggested by identical apparent Ka at 33 and 39.5°C). These discrepant observations suggest, although they do not prove, that the absence of AVP receptors at 33°C is not merely the harmful consequence of cell proliferation induced by SV40 activation, but more probably reflects a specific effect of SV40 genome on the expression of AVP membrane receptors. This effect may take place at various stages of receptor synthesis and intracellular traffic from gene transcription, known to be altered by SV40 integration (Schutzbank et al., 1982; Morris et al., 1985), to translocation of the receptor from Golgi vesicles to the cell membrane.

In conclusion, using the temperature-sensitive SV40 strain tsA58, we have established a collecting tubule cell line which, at the restrictive temperature, becomes highly sensitive to AVP due to the membrane expression of AVP receptors. This feature of principal cells has remained remarkably stable over 50 passages of the whole cell population as well as in the various clones derived from it. The RC.SVtsA58 cell line, therefore, appears as a promising tool to study the molecular biology of the AVP receptor, modulation of the transduction signals, and molecular mechanisms of AVP effects on water permeability. Because of the rapid shift from the transformed (33°C) to the "normal" (39.5°C) phenotype, this kind of cell line may also contribute to unravel the cell alterations associated with the transformation process.

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