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Growth of Madin–Darby canine kidney epithelial cell (MDCK) line in hormone-supplemented, serum-free medium

(renal epithelium/prostaglandins/mammalian cell growth)

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ABSTRACT **Madin–Darby canine kidney (MDCK) cells can grow in synthetic medium supplemented with five factors—insulin, transferrin, prostaglandin E_1, hydrocortisone, and triiodothyronine—as a serum substitute. These five factors permit growth for 1 month in the absence of serum and a growth rate equivalent to that observed in serum-supplemented medium. Dibutyryl adenosine 3′,5′-cyclic monophosphate substitutes for prostaglandin E_1 in the medium. Potential applications of the serum-free medium are discussed. The medium permits a defined analysis of the mechanisms regulating hemicyst formation by hormones and permits the growth of primary kidney epithelial cell cultures in the absence of fibroblast overgrowth.**

The presence of serum in tissue culture media has interfered with studies concerning the regulation of animal cell growth and differentiated function *in vitro*. However, the synthetic media generally utilized for animal cell lines were originally designed with the assumption that a serum supplement would be added (1). Recently, Sato and coworkers (2–4) have demonstrated, by using a number of cell types, that serum can be replaced as a growth supplement by specific hormones and accessory factors, which differ according to cell type. Once an identification has been made, for any particular cell type, of the factors that permit growth in the absence of serum, novel studies concerning the mechanism of hormone action will be possible.

The kidney is a complex organ, consisting of cells whose growth (5, 6) and differentiated functions (6) are affected by hormones. Each of the numerous types of epithelia in kidney tubules has unique transport properties. Hormones play an important role in regulating solute transport by the tubule epithelia. The kidney is also a producer of hormones (notably prostaglandins and renin) and is thus often referred to as an endocrine organ in its own right (6). The release of these renal factors and other hormones into the kidney is aimed at regulating kidney function so as to maintain a state of homeostasis in the adult organism. Toward these ends, the kidney must make compensatory adjustments in response to its own injury; presumably, hormones are also involved in renal hypertrophy, including factors produced by the kidney itself (5).

Differentiated kidney epithelial cell lines provide a convenient system for the study of hormonal regulation of these kidney functions. The Madin–Darby canine kidney (MDCK) cell line, for example, bears close resemblance to transporting epithelia present in the kidney. Confluent MDCK monolayers form multicellular hemicysts (groups of cells slightly raised from the tissue culture dish surface) (7). Hemicyst formation has been attributed to the vectorial transport of salt and water from the mucosal surface of the cells (facing the culture medium) to the serosal surface of the cells (facing the disk surface) (8). Furthermore, MDCK cells have hormonal responses typical of kidney epithelia. Treatment with arginine vasopressin, prostaglandin E_1 (PGE_1), or glucagon stimulates adenosine 3′,5′-cyclic monophosphate (cAMP) production by MDCK cells (9). In order to study regulation by these and other hormones in tissue culture, a serum-free growth medium would be desirable in which all the hormone concentrations are known. This paper reports the long-term growth of MDCK cells in a serum-free culture medium supplemented with insulin, transferrin, PGE_1, hydrocortisone, and triiodothyronine (T_3). The utilization of the medium to support the growth of primary kidney epithelial cell cultures and to examine the regulation of hemicyst formation is demonstrated.

MATERIALS AND METHODS

Cells and Maintenance. MDCK cells (10), obtained from J. Holland, (University of California at San Diego, La Jolla, CA), were routinely incubated in a humidified 5% CO_2/95% air mixture at 37°C. The growth medium, a 50:50 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (SF-DME/F12), was supplemented with 10 mM Hepes, sodium bicarbonate at 1.1 mg/ml, penicillin at 92 international units/ml, streptomycin at 200 μg/ml, ampicillin at 25 μg/ml, and 10 nM $Na_2SeO_3 \cdot 5H_2O$. Triple-distilled water was used for medium preparation. Stock cultures were maintained in SF-DME/F12 supplemented with 5% horse serum and 10% fetal calf serum.

To initiate primary cultures, the kidneys from 10-day-old mice (BALB/c) were minced into 1-mm-diameter pieces. Kidney cell suspensions were prepared from the mince by following the method of Leffert and Paul (11) with the modification that the cells were incubated in 0.3% EDTA/0.1% trypsin containing collagenase (Worthington) at 1 mg/ml. Prior to use, cells were centrifuged and resuspended in SF-DME/F12.

Cell Growth and Plating Efficiency. Exponentially growing MDCK cells were trypsinized by using 0.3% EDTA/0.1% trypsin. The cells were then treated with an equal volume of 0.1% soybean trypsin inhibitor, centrifuged, and resuspended in SF-DME/F12. After repeating this procedure, the cells were inoculated into tissue culture dishes containing medium. After an appropriate incubation period, the cells were trypsinized and counted with a Coulter Counter. The dosage response to added factors was assayed by measuring cell number after a 4-day incubation period. To determine the plating efficiency, MDCK cells were plated (500 cells per 60-mm dish), and 1 week later colonies were fixed with formalin, stained with 0.5% crystal

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Abbreviations: PG, prostaglandin; T_3, Triiodothyronine; SF-DME/F12, a serum-free 50:50 mixture of Dulbecco's modified Eagle's medium and F12 medium; cAMP, adenosine 3′,5′-cyclic monophosphate.

violet, and counted. Unless otherwise mentioned, all determinations were made in triplicate.

Materials. Hormones—including bovine insulin, human transferrin, triiodothyronine, and hydrocortisone—were obtained from Sigma. Prostaglandins (J. Pike, Upjohn), epidermal growth factor and fibroblast growth factor (Collaborative Research, Waltham, MA), purified human transferrin (original source Behring Diagnositics obtained from R. W. Holley, Salk Institute, San Diego, CA), purified bovine insulin (original source Eli Lilly; obtained from J. Lever, Salk Institute), and $Na_2SeO_3 \cdot 5H_2O$ (R. Ham, University of Colorado) were gifts. Dibutyryl cAMP and isobutyl methylxanthine were obtained from Sigma.

RESULTS

Growth of MDCK Cells in Hormone-Supplemented, Serum-Free Medium. MDCK cells grew at 1.5 doublings per day in SF-DME/F12 supplemented with 10% fetal calf serum (Fig. 1). When serum was deleted from the medium, the growth rate was reduced to 0.4 doublings per day. However, MDCK cells grew at 1.5 doublings per day in SF-DME/F12 when serum-free medium was supplemented with PGE_1, hydrocortisone, triiodothyronine, insulin, and transferrin (medium K-1). A final saturation density of 2.6×10^5 cells per cm^2 was obtained when cells were grown in either hormone- or serum-supplemented medium. The effect of the substitution of serum by the five components on clone formation at low density was also studied. In medium K-1 (SF-DME/F12 supplemented with the five components), the plating efficiency was 70% of that observed in serum-supplemented medium.

Although norepinephrine (10 μM), epidermal growth factor (5 ng/ml), fibroblast growth factor (100 ng/ml), as well as other factors (see below), were also growth stimulatory to MDCK cells when added to SF-DME/F12 supplemented with insulin and transferrin alone, none of these factors increased growth when added to medium K-1. This latter observation suggested that the five components in medium K-1 are sufficient to obtain the maximal growth rate of MDCK cells in SF-DME/F12.

The optimal concentrations of the hormones in medium K-1 were determined by assaying the effect of hormone concentration on MDCK cell growth as described in Fig. 2. PGE_2 was equally as effective as PGE_1 in increasing MDCK cell growth, whereas PGA_1 and $PGF_{2\alpha}$ were ineffective (Fig. 2*A*). The growth stimulatory effect of T_3 on MDCK cells depended upon the presence of PGE_1 (Fig. 2*A*), and was reduced in the presence of hydrocortisone. Thus, the optimal dosage of T_3 was determined in medium K-1 lacking hydrocortisone [i.e., SF-DME/F12 containing insulin, transferrin, and PGE_1 (Fig. 2*B*)]. Both purified insulin (5 μg/ml) and purified transferrin (5 μg/ml) caused growth stimulation equivalent to that observed in Fig. 2 *C* and *D*.

The relative effects of the individual components in medium K-1 on cell growth were compared by a hormone deletion study (Fig. 3). Omission of either PGE_1 or transferrin from medium K-1 had the most deleterious effects on cell growth (growth was inhibited by over 50%). However, the omission of hydrocortisone, T_3, or insulin resulted in less than 20% inhibition of growth; the growth inhibition resulting from the removal of T_3 was observed only after a 6-day time interval.

The requirement of each component in medium K-1 for long-term survival of MDCK cells in the absence of serum was examined in a similar manner. MDCK cells were inoculated into dishes containing medium K-1, or with medium K-1 lacking either insulin, transferrin, PGE_1, hydrocortisone, or T_3. After 5 weeks (after four subculturings and approximately 40 doublings), confluent monolayers of MDCK cells were observed with medium K-1 or with medium K-1 lacking either insulin, hydrocortisone, or T_3, whereas the MDCK cells did not survive with medium K-1 lacking either PGE_1 or transferrin. These hormone deletion studies suggest that PGE_1 and transferrin are the most critical of the five factors for the maintenance of MDCK cells in serum-free medium.

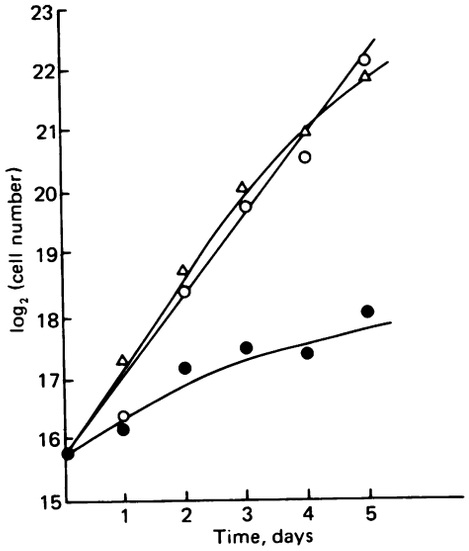

FIG. 1. Effect of medium supplementation on MDCK cell growth. MDCK cells were inoculated at 2.5×10^4 cells per 60-mm dish containing SF-DME/F12 supplemented with 10% fetal calf serum (△); PGE_1 at 25 ng/ml, 5 pM T_3, 50 nM hydrocortisone, insulin at 5 μg/ml, and transferrin at 5 μg/ml (○); or no additional factors (●).

Mechanism of Action of PGE_1. Because PGE_1 is one of the most critical components in medium K-1 for long-term survival and because it is a factor that specifically stimulates growth of these kidney epithelial cells, as opposed to other cell types, the mechanism by which PGE_1 enhances cell growth was of interest. The possibility that PGE_1 stimulates growth due to its effects on cAMP production in MDCK cells was studied. Thus, other agents known to affect cAMP production were also examined with respect to their effects on cell growth in SF-DME/F12 supplemented with insulin (5 μg/ml) and transferrin (5 μg/ml). Dibutyryl cAMP (0.5 mM) was not only growth stimulatory to MDCK cells, but it also substituted for PGE_1 in medium K-1, permitting optimal growth to occur. At a similar concentration, sodium butyrate had no effect on growth. Three other factors that affect cAMP metabolism in MDCK cells were also studied. Whereas isobutyl methylxanthine (0.5 mM) (a phosphodiesterase inhibitor) and glucagon (5 μg/ml) were also growth stimulatory, arginine vasopressin had no significant growth-enhancing effect.

Applicability of Serum-Free Medium to Study of Regulation of Hemicyst Formation and to Growth of Primary Cultures. MDCK cells maintained in medium K-1 not only were similar morphologically to cells in serum-supplemented medium, but also formed hemicysts as in serum-supplemented medium (Fig. 4). The effect of the five components in medium K-1 on hemicyst formation was examined (Table 1). No hemicysts were observed in monolayers grown to confluency in SF-DME/F12 containing only insulin and transferrin. However, when SF-DME/F12 was supplemented with PGE_1 in addition to insulin and transferrin, hemicyst formation was apparent. Hydrocortisone further increased the frequency of hemicysts, but only in SF-DME/F12 supplemented with PGE_1 in addition to insulin and transferrin (Table 1).

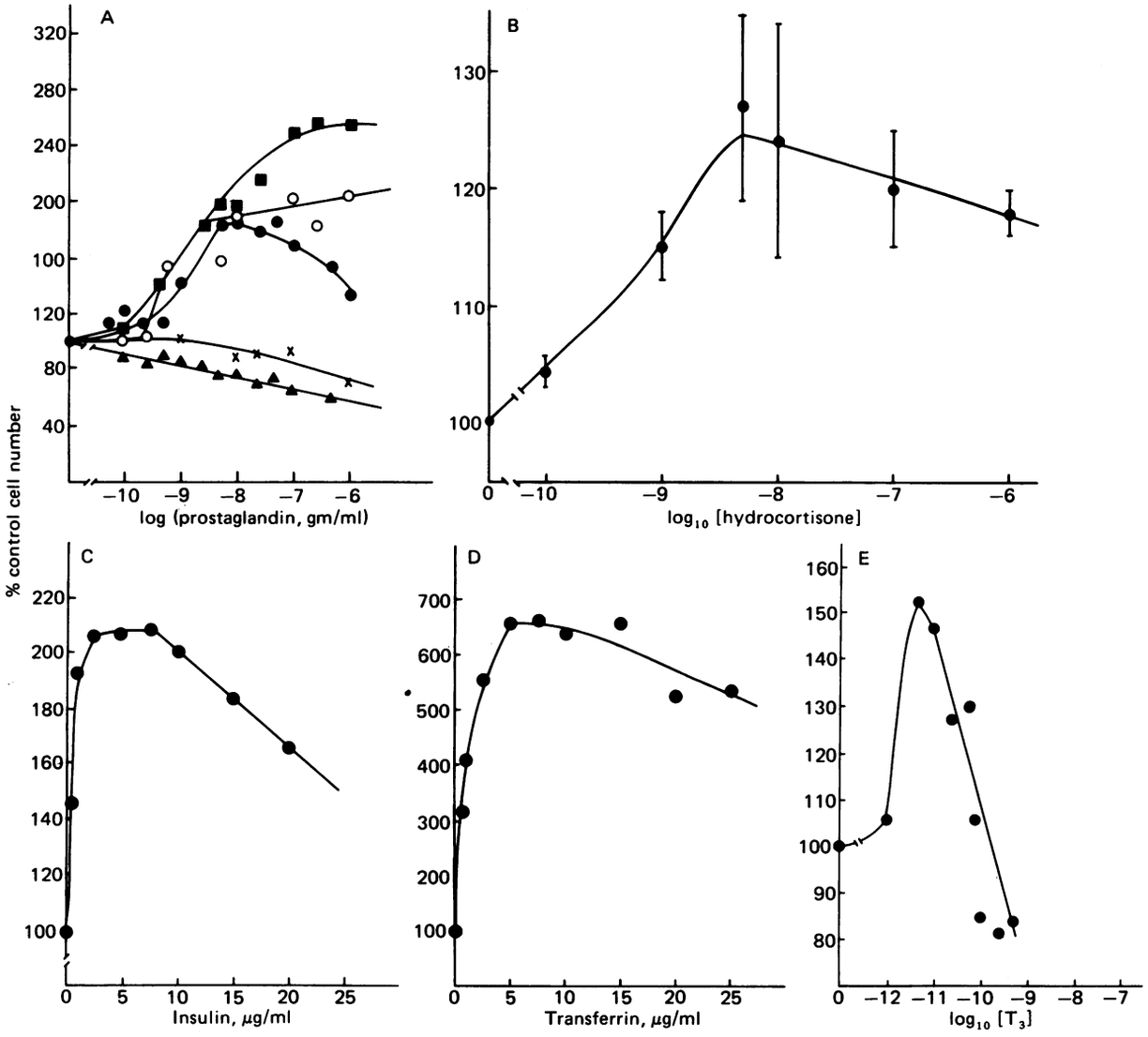

FIG. 2. Dependence of MDCK cell growth on hormone concentration. (*A*) The effects of PGE_1 (○), PGE_2 (●), PGA_1 (x), and $PGF_{2\alpha}$ (▲) on MDCK cell growth were compared in SF-DME/F12 supplemented with insulin (5 μg/ml) and transferrin (5 μg/ml). Dose–response curves were determined for (*A*) PGE_1 (■), (*B*) hydrocortisone, (*C*) insulin, (*D*) transferrin, and (*E*) T_3 in the presence of the other four components (insulin at 5 μg/ml, transferrin at 5 μg/ml, 5 pM T_3, 50 nM hydrocortisone, and PGE_1 at 25 ng/ml), with the exception that hydrocortisone was omitted for the T_3 dose–response. Determinations were made in triplicate. The cell number was normalized to the control cell number (the cell number in 35-mm dishes lacking the hormone being studied). In medium K-1 the final cell number was 4.4×10^5, whereas in medium K-1 lacking one component the control cell number was (*A*) 2.2×10^5 (−PGE_1), (*B*) 4.0×10^5 (−hydrocortisone), (*C*) 1.2×10^5 (−insulin), and (*D*) 5×10^4 (−transferrin), The control cell number in SF-DME/F12 supplemented with (*A*) insulin and transferrin and (*E*) insulin, transferrin, and PGE_1 was 1.8×10^5 and 3.0×10^5, respectively.

The applicability of medium K-1 to growth of primary cultures of baby mouse kidney epithelial cells was also studied. The simultaneous growth of both fibroblasts and epithelial cells in primary cultures has been a long-standing problem in studies concerned with the functions of transporting epithelia. The growth of these two cell types in hormone-supplemented and serum-supplemented media was compared (Fig. 5). Microscopic observation indicated that 1 day after plating more than 99% of the attached kidney cells were epithelial in medium K-1 and in SF-DME/F12 supplemented with 10% fetal calf serum. However, after a 5-day period 13% of the population was fibroblastic in serum-supplemented medium, and less than 1% of the population was fibroblastic in medium K-1. After 11 days, the majority of the cells in primary cultures maintained in medium K-1 were epithelial, whereas fibroblast proliferation was apparently still suppressed (Fig. 5 *left*). In contrast, fibroblast overgrowth was observed in primary cultures of baby mouse kidney cells maintained in SF-DME/F12 supplemented with 10% fetal calf serum (Fig. 5 *right*).

The ability of medium K-1 to support the formation of baby mouse kidney clones at low densities was also examined (inoculum of 5×10^3 cells per 35-mm dish). Ten days after inoculation, only epithelial clones were observed in SF-DME/F12 without serum, with a plating efficiency of 1.2 ± 0.1% in medium K-1, compared to 0.03% ± 0.04% in SF-DME/F12 without hormones. In contrast, in SF-DME/F12 supplemented with 10% fetal calf serum, only fibroblast clones were observed at a plating efficiency of 0.42% ± 0.02%. These observations indicate that at low cell densities medium K-1 and SF-DME/F12 supplemented with 10% fetal calf serum are selective for kidney epithelial cells and fibroblasts, respectively.

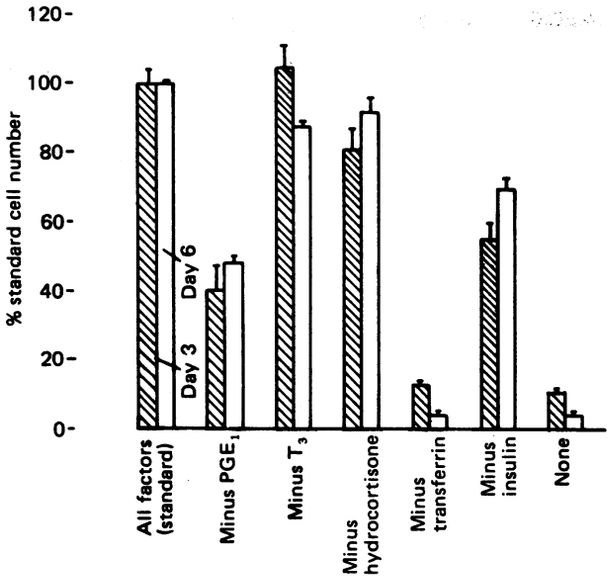

FIG. 3. Effect of deletion of a factor from hormone-supplemented medium on MDCK cell growth. MDCK cells were inoculated at 5×10^4 per 60-mm dish containing (*i*) SF-DME/F12 and five factors (PGE_1 at 25 ng/ml, 5 pM T_3, 50 nM hydrocortisone, transferrin at 5 μg/ml, and insulin at 5 μg/ml), (*ii*) SF-DME/F12 and four of the above factors, or (*iii*) SF-DME/F12 alone. Cell number was assayed in triplicate on days 3 and 6. In medium K-1 the cell number was 1.1×10^6 on day 3 and 3.7×10^6 on day 6.

DISCUSSION

A hormone-supplemented, serum-free medium has been designed that permits the growth of the kidney epithelial cell line MDCK and primary cultures of kidney epithelial cells in the absence of serum. In this medium (medium K-1) MDCK cells grew for over 1 month in the absence of serum; the growth rates of the cells were equivalent in medium K-1 and in serum-supplemented medium. The supplements in medium K-1, which include insulin, transferrin, PGE_1, hydrocortisone, and T_3, are essential for optimal growth of MDCK cells in the absence of serum. The hormone-deletion experiments suggest that PGE_1 and transferrin are more stringently required in medium K-1 than are the other components. Although the effect of T_3 on growth was minor, this component was added to the medium.

The hormones in medium K-1 are all known to affect kidney epithelial function *in vivo*, and conceivably these factors may also affect growth *in vivo*. The observation that PGE_1 was critical to survival of MDCK cells in the absence of serum is of particular interest because prostaglandins are produced in large quantities by the renal medulla (12). If the effect of prostaglandins on the *in vitro* growth of MDCK cells is similar to that *in vivo*, then renal growth may also be regulated by the kidney itself. For example, factors such as epidermal growth factor (13) that may regulate renal prostaglandin production may indirectly affect kidney growth *in vivo*.

However, renotrophic factors that have not yet been purified have also been implicated in regulation of kidney growth *in vivo*. For example, conditioned medium from cultured kidney epithelial cells presumably contains growth stimulatory and growth inhibitory factors for the same cells (14). The involvement of analogous renal growth stimulatory and inhibitory factors in renal hypertrophy has similarly been suggested (15). A serum-free medium for kidney epithelial cell growth *in vitro* may provide an excellent system for identifying novel growth factors.

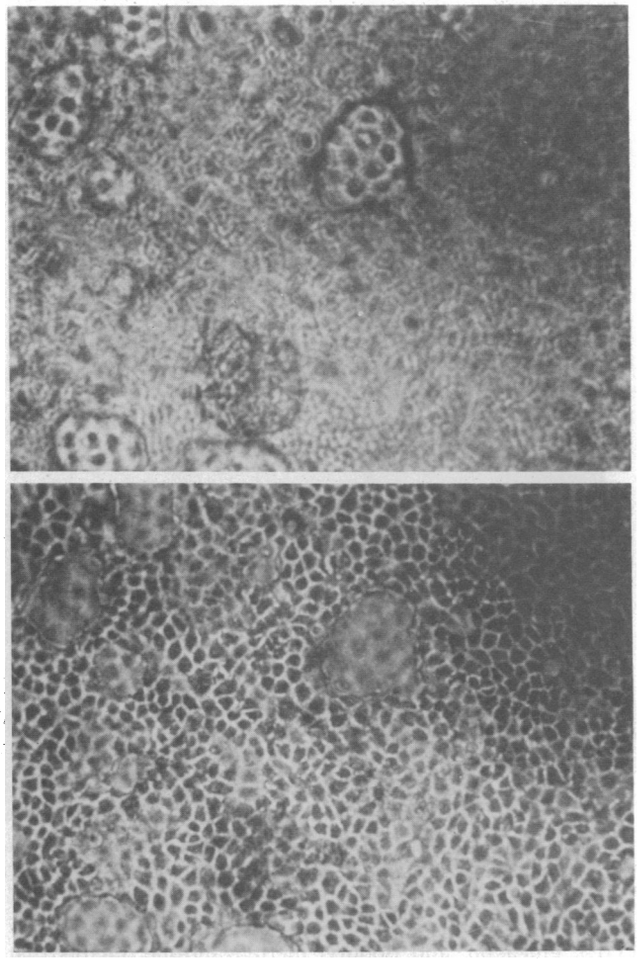

FIG. 4. Hemicyst formation by MDCK cells. MDCK cells were grown to confluency in SF-DME/F12 supplemented with 10% fetal calf serum. When hemicysts were observed in the culture dish, the cells in one microscope field were photographed in two different focal planes.

These studies have introduced the possibility that cAMP is involved in mediating the growth stimulatory effects of PGE_1 on MDCK cells. In medium K-1, dibutyryl cAMP effectively substituted for PGE_1 to permit optimal growth. In addition,

Table 1. Effect of hormones on hemicyst formation

Added components	Hemicysts per field	Percentage of monolayer as hemicyst
Insulin + transferrin (standard)	0	0
Standard + PGE_1	2.7 ± 2.1	8 ± 5
Standard + PGE_1 + hydrocortisone	10.4 ± 3.4	32 ± 10
Standard + hydrocortisone	0	0
Standard + PGE_1 + T_3	6.0 ± 3.8	18 ± 9
Standard + T_3	0	0
Serum	1.3 ± 1.3	12 ± 31

MDCK cells were grown to confluency in SF-DME/F12 supplemented with the components indicated above (insulin, 5 μg/ml; transferrin, 5 μg/ml; PGE_1, 25 ng/ml; hydrocortisone, 50 nM; T_3, 5 pM). The average number of hemicysts per field was estimated by counting 10 microscope fields at ×100 using a Nikon microscope. The hemicyst size was estimated from the diameter of 20 hemicysts, determined with a Nikon microscope grid, and compared to the total field size, also with the grid. The above estimations permitted a calculation of the percentage of monolayer as hemicyst.

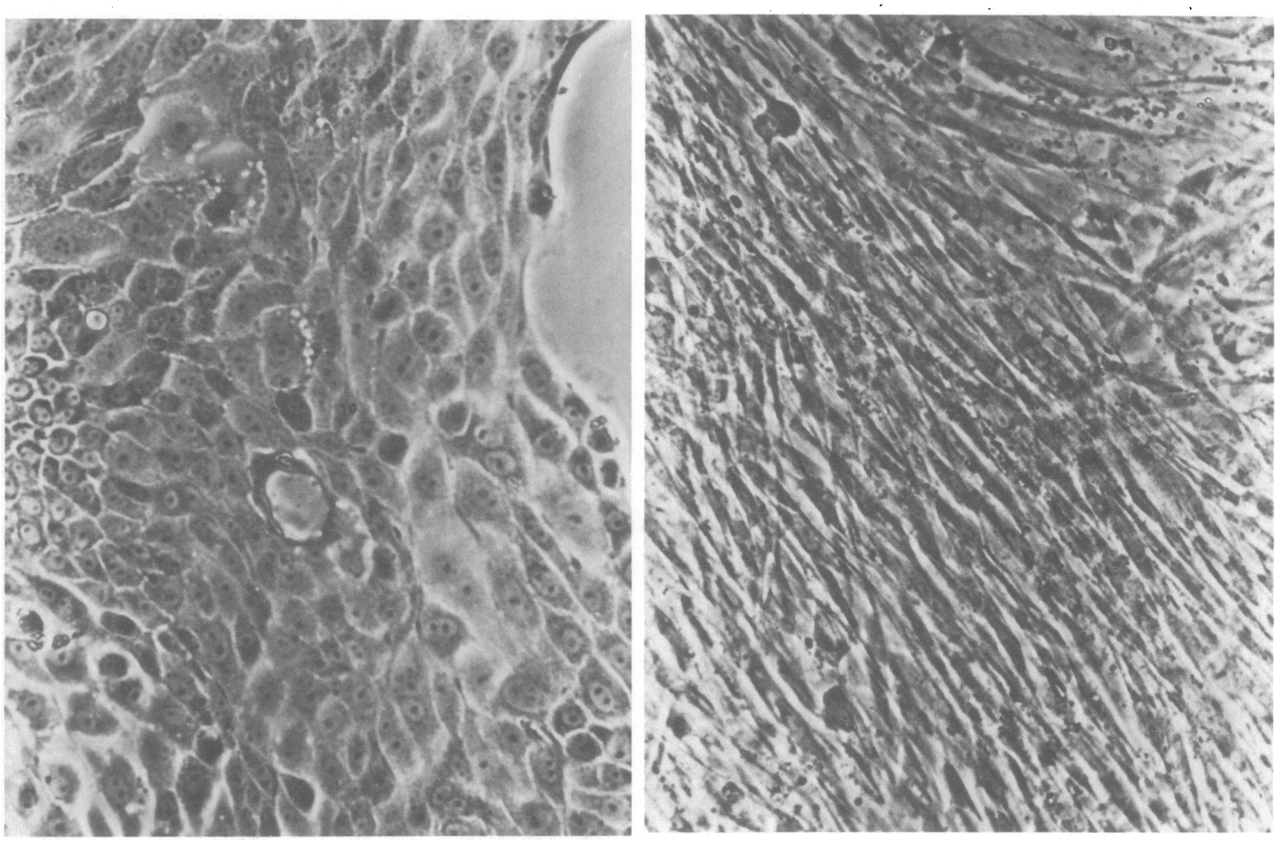

FIG. 5. Primary baby mouse kidney cultures. Baby mouse kidney cells were distributed at 10^4 cells per 35-mm dish containing either (*Left*) medium K-1 or (*Right*) SF-DME/F12 supplemented with 10% fetal calf serum. Eleven days later, representative microscope fields of the cultures were photographed.

PGE_2, glucagon, and isobutyl methylxanthine, factors that increase cAMP production by MDCK cells (9), also enhanced growth. Arginine vasopressin did not have a significant growth stimulatory effect on MDCK cells, although it increases adenylate cyclase activity (9). Thus, when assuming that the effect of PGE_1 on cell growth is mediated by cAMP, the existence of distinct cellular compartments of cAMP in MDCK cells might be considered. The association of increased intracellular cAMP levels with growth of these kidney epithelial cells is contrasted to that of fibroblasts, whose growth has generally been associated with decreased intracellular cAMP (16).

Possible applications of the hormone-supplemented, serum-free medium for MDCK cells have been presented. First, the hormone-supplemented medium permits the maintenance of primary kidney epithelial cell cultures without fibroblast overgrowth. Secondly, the medium will permit an analysis of the mechanisms by which hormones regulate transepithelial solute transport by the MDCK cell line. Previous studies indicated that the formation of hemicysts by MDCK cells is correlated with the vectorial transport of salt and water (8). In this study we demonstrate that the formation of hemicysts by MDCK cells in SF-DME/F12 depends upon the hormone components in the medium.

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