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The Correlation of Plasma Membrane Microvilli and Intracellular Cyclic AMP Content in a Rat Epitheloid Kidney Cell Line

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Modulation of the intracellular concentration of cyclic AMP has been associated with a regulatory role in cell division, cell morphology, and physical properties of the plasma membrane. Untransformed rat kidney cells in culture exhibit epitheloid morphology, high intracellular cyclic AMP levels, and contact inhibition of growth. Untransformed rat kidney cells transformed with the Kirsten murine sarcoma virus exhibit a low cyclic AMP content, rapid growth rate, and a loss of contact inhibition. Scanning electron microscopy reveals a distinctive difference in the surface structure of the two cell types during G1 of the cell cycle. The surface of the transformed cell is covered with microvilli while its untransformed counterpart is devoid of microvilli. The presence of microvilli can be controlled as a function of temperature by two temperature-sensitive mutants of the Kirsten sarcoma virus (ts6t6 and ts371 cl 5). In the ts6t6 mutant, growth at 32°C results in a low cyclic AMP content and the presence of microvilli, while growth at 39°C results in a high cyclic AMP content and a decrease in microvilli. The opposite effect is seen with the ts371 cl 5 mutant. Correlation of cyclic AMP content with the presence of microvilli suggests that this surface phenomenon is a function of cyclic AMP concentration.

Key words: **temperature sensitive mutants, plasma membrane microvilli, cyclic AMP, scanning electron microscopy, cell synchrony, neoplastic, epitheloid cells, Kirsten murine, cell tissue culture**

INTRODUCTION

A comparison of the steady-state levels of cyclic AMP (cAMP)* in untransformed and transformed cells has resulted in the widely accepted concept that transformation lowers the intracellular concentration of cAMP. Numerous studies using cell culture systems have associated cAMP metabolism with a variety of in vitro transformation parameters such as lectin agglutinability, morphology, growth rate, cell density at confluence, and altered properties of the plasma membrane (1–7). If a correlation exists between these

Abbreviations: adenosine-3′, 5′-cyclic monophosphate, commonly referred to as cyclic AMP or cAMP; normal rat kidney, NRK; NRK cells transformed with wild-type Kirsten murine sarcoma virus, K-NRK; temperature sensitive, ts.

parameters and cAMP levels, a dose response relationship should exist between intracellular cAMP concentrations and observed biochemical, physiological, or anatomic responses associated with neoplastic transformation. Although normal and transformed cells may have similar relative cAMP levels during certain phases of the cell cycle (i.e., mitosis shows a decrease in cAMP), ar. absolute difference in cAMP concentration at a decision point in the cell cycle such as the G–S interface, could determine events to follow, as suggested by Friedman et al. (8).

In vitro methodologies theoretically offer ideal experimental conditions for the investigation of the relationship of cyclic nucleotides with transformation. However, such a straightforward approach is rarely realized due to variations in culture conditions, cloned variants, and assay conditions (9). In an effort to clearly define our culture conditions and assay methodologies as well as to circumvent the hazards associated with the exogenous supply of cAMP or derivatives to transformed cells in culture, we have used a murine epitheloid, contact inhibited cell (untransformed rat kidney NRK) in which transformation has been accomplished with temperature sensitive (ts) mutants of the Kirsten RNA tumor virus that can endogenously regulate cAMP levels as a function of temperature. In this study untransformed cells, wild-type viral transformed cells, and cells transformed by the ts mutants were assayed for growth rate, surface alterations such as microvilli, and cAMP concentration at a preconfluent level in the G1 phase of the cell cycle. Trivial effects of temperature (i.e., -Q 10 effects) on the ts mutants were ruled out since the two mutants employed demonstrate opposite responses to temperature shifts at subconfluence. In this manner we have been able 1) to demonstrate that the number of surface microvilli in the NRK cell line and its transformants are inversely correlated with the intracellular cAMP concentration, and 2) establish the inverse correlation of cAMP intracellular concentration to the rapidity of growth in a cell line of epitheloid morphology.

MATERIALS AND METHODS

Cell lines. Normal rat kidney (NRK); NRK transformed with wild-type Kirsten murine sarcoma virus (K-NRK); and two ts Kirsten MSV mutants, ts6t6 and ts371 cl 5, were the kind gift of Dr. E. R. Scolnick.

Cell culture methods. All cell lines were maintained in Dulbecco's modified Eagle's medium (DEM) containing 10% Colorado calf serum (filtered, 30 mM tricine, pH 7.2), and penicillin-streptomycin (100 U/ml and 100 μg/ml, respectively). Cells were synchronized in early G1 by a combination of growth to confluence and starvation methods (8,10). Typically, cells were grown to confluence, trypsinized, and plated in DEM containing 10% calf serum at 1/2 the confluence level. The cells were grown to confluence (one generation) and then incubated in DEM containing 1% calf serum for 16 hr. Under these conditions NRK cells cease to grow and the growth of KNRK cells is severely retarded (i.e., 75% increase in the generation time). Cells were then released from G1 by trypsinization and replated at 1/3 confluence in DEM with 10% calf serum. By continuous one-generation growth periods to confluence with serum starvation, an average of 82% synchrony was obtained as determined by the increase in mg of DNA/flask with time.

Cell generation time. The minimum generation time (k) was calculated from the relationship where N_0 = the initial number of cells plated, N = the number of cells at

$$k = \frac{\ln (N/N_0)}{t},$$

time t. At various times after plating, three flasks were separately trypsinized and the cells were collected by low-speed centrifugation, resuspended in medium without serum, and counted with a hemacytometer. Protein was determined in each flask by a modification of the microburet method (11) and converted to cell equivalents. All values were averaged for each point represented. After time vs log of the cell number was graphed, the maximum slope was determined for each set of sequential points and the region of greatest slope change, usually the first five points, were analyzed by linear regression analysis to yield the calculated minimum generation time.

Cyclic AMP assay. cAMP was determined 4 hr after subculture or, where indicated, following confluence. Following media decantation and fixation with 5% trichloroacetic acid, the resultant precipitate was homogenized at 4°C using 4 strokes with a TRI-R homogenizer (Jamaica, New York) at #4 speed, then centrifuged at 20,000 g for 20 min. Elution of the supernatant off Dowex AG 50 X 4, 200–400 mesh, was with 0.1 N HCL. Recovery was from 60 to 85%, as judged by trace-labeled cAMP recovery. Two aliquots of each sample were assayed by the protein kinase binding procedure of Gilman (12) using ^{3}H-cAMP (22c/m mole; New England Nuclear) displacement. Each apparent value for an unknown was corrected for cAMP recovery by the addition of a known trace quantity of ^{3}H-cAMP at the trichloroacetic acid step. Radioactivity was analyzed with a Beckman LS-233 scintillation spectrometer. Nucleic acids were measured by comparing the $O.D._{260}$ of each flask with a standard curve using calf thymus DNA.

Scanning electron microscopy. All cells were synchronized and then plated and grown on coverslips as described. The coverslips were rinsed twice in Puck's saline G at 37°C for 20 min. Fixation was in Puck's saline G containing 3% glutaraldehyde and .05 M cacodylate buffer (pH 7.2) for 20 min at room temperature. Cells were then rinsed twice with Puck's saline G (37°C) and stored at 4°C in the same solution. Critical point drying was accomplished by graded ethanol dehydration with subsequent CO_2 exchange at 1,800 lb/in^2. Fixed and dried cells were first coated with carbon, then coated with paladium-gold (~ 100Å). Examination was performed on an ETEC scanning electron microscope at 20 kV. Evaluation of changes in surface morphology was accomplished by viewing 50 to 100 cells per preparation. The number of microvilli on the surface of each cell was scored from 0 to ++++ to denote, respectively, a lack of microvilli to a maximum number of microvilli. Highly rounded mitotic cells were not scored for microvilli.

RESULTS

The NRK cells are epitheloid in morphology, are devoid of surface microvilli, and exhibit contact inhibition of growth at confluence (Fig. 1a). Transformation by wild-type Kirsten murine sarcoma virus results in a rounded cell, extensively covered by microvilli (Fig. 1b), which continues to grow past confluence levels. Both NRK and K-NRK mitotic cells exhibit extensive microvilli, although K-NRK microvilli are more extensive (Fig. 2). As expected, intracellular cAMP concentrations during G1, were lower in K-NRK cells than in the NRK parent (Table I). The ts6t6 mutant is permissive for a shortened generation time at 32°C. At the nonpermissive temperature of 39°C the generation time is increased to a level actually higher than for NRK and there is a corresponding increase in cAMP concentration. General morphology and surface microvilli are also dependent on temperature. At the permissive temperature of 39°C (Fig. 3a), the ts6t6-infected cells

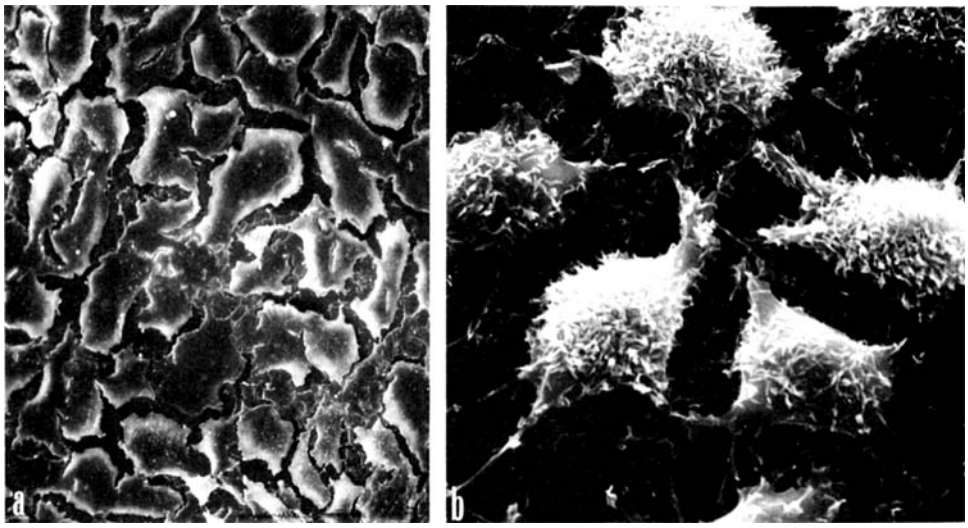

Fig. 1. Surface features of NRK vs K-NRK cells. (a) NRK cells (× 320) are flattened with irregular contours and virtually devoid of microvilli; (b) K-NRK cells (× 960) are rounded with extensive microvilli.

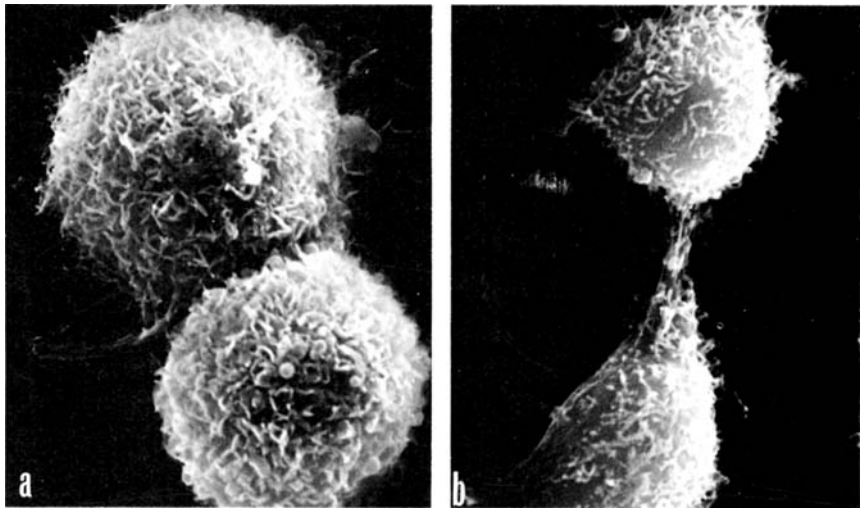

Fig. 2. Surface features of mitotic NRK and K-NRK cells. (a) K-NRK (× 1,600); (b) NRK (× 1,600).

closely resemble the parent NRK cells in epitheloid morphology and relative lack of surface microvilli. At 32°C, the permissive temperature that allows a faster growth rate and loss of contact inhibition of growth at confluence, there is a tendency for the cells to lose their flattened characteristics and to exhibit an absolute increase in the number of surface microvilli (Fig. 3b). The ts371 cl 5 mutant demonstrated a more complex growth pattern. In this ts mutant, the shorter generation time and lower cAMP concentrations were observed at a 39°C growth temperature in contrast to the t6st6 mutant (Table I).

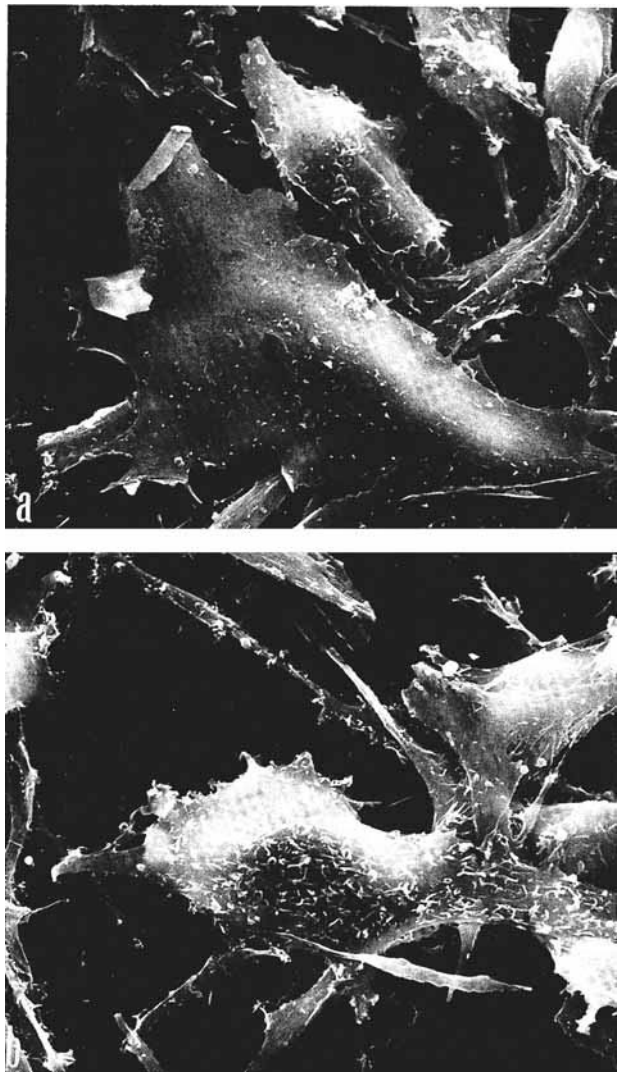

Fig. 3. Surface features of NRK cells transformed with the ts6t6 mutant as a function of temperature (× 1,200). (a) 39°C; (b) 32°C.

Apparently temperature sensitivity at the "nonpermissive" temperature is not complete, since the generation time is shorter and the cAMP concentration lower than NRK cells at 32°C. Morphology at 39°C more closely resembles wild-type K-NRK cells than the ts6t6 mutant line at its permissive temperature (32°C) with rounded cells and abundant microvilli (Fig. 4a). At 32°C the ts371 c1 5 cells exhibit the flattened appearance of NRK cells with sparse microvilli (Fig. 4b), although the cAMP content fails to attain the NRK levels. Temperature shift (39° to 32°C) from permissive to nonpermissive conditions (Fig. 4c) demonstrates the reversibility of the process. Within 14 hr cell morphology was similar to the NRK parent line and microvilli were virtually absent.

TABLE I. Correlation of Generation Time, Intracellular cAMP Concentration, and Surface Microvilli

Cell type	Growth temp. (°C)	Generation time (hr)	cAMP (pmoles/mg nucleic acid)	Surface microvilli
NRK	32°	26.4	284	0
	39	21.1	301	0
K-NRK	32	26.0	161	+++
	39	15.9	69	++++
Ts6t6	32	19.7	212	++
	39	25.4	320	0
Ts371 cl 5	32	20.3	214	+
	39	17.4	91	+++

DISCUSSION

A recent study using an indirect technique of microvillus visualization – dark field microscopy – previously has linked cAMP concentration and microvilli (13). In our study a comparison of cell generation times, number of surface microvilli as judged by the direct visualization technique, scanning electron microscopy, and quantitative cAMP levels in GI suggest that there is a correlation of these observed parameters in NRK and K-NRK cells. The higher the cAMP concentration, the fewer the number of microvilli, and the longer the generation time. Furthermore, the ts mutants exhibit overall intermediate levels of cAMP with ts6t6 having relatively high cAMP levels at 39°C and 32°C compared to ts 371 cl 5 at 39°C and 32°C. In addition the generation times at 39°C and 32°C for ts6t6 are longer than those of ts 371 cl 5 at 39°C and 32°C. Thus the ts mutants strengthen the correlation between cAMP, generation times, and surface microvilli. Moreover, since the permissive temperature for each ts mutant was reversed relative to the other, the effect of temperature on general metabolic processes cannot be argued as the originator of the observed effects, but is apparently controlling a specific mutant viral product that governs cAMP levels (14).

It can be argued that these cyclic AMP levels reflect only cells in G1, while generation times are determined over a number of cell divisions. However, it is thought that normal and transformed cells differ primarily in the length of G1 (8). Therefore, to relate cAMP and cell cycle length we compared cells in G1. Moreover, late G1 phase normal cells exhibit no surface microvilli (15), while transformed cells generally exhibit numerous microvilli throughout the cell cycle (16). Thus surface microvilli in G1 reflect cAMP concentration, which is itself a reflection of transformation. The ts 371 cl 5 mutant, however, continues to grow past confluency, despite an increase in cAMP concentration and a decrease in surface microvilli. Thus the relationship between changes in generation time, surface microvilli, and in vitro criteria of neoplasia with cAMP levels is not precise and the question remains of whether absolute levels of cAMP or a relative range of cAMP levels may be responsible for the observed surface morphology, growth rates, and contact inhibition.

To further link cAMP and growth parameters of the ts371 cl 5 mutant, we measured cAMP levels in superconfluent cultures at 39°C and 32°C. The cAMP levels were: 39° = 185 pmoles/mg N.A., 32° = 105 pmoles/mg N.A. The cell densities at each temperature reflect

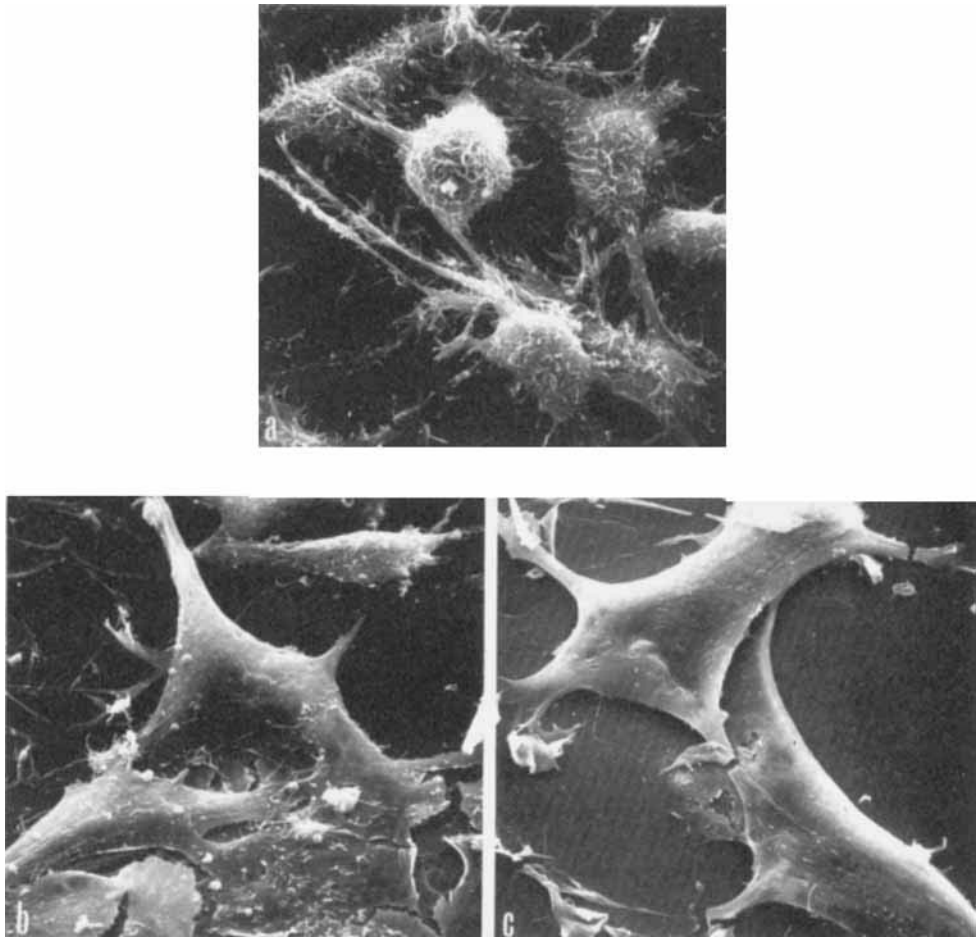

Fig. 4. Surface features of NRK cells transformed with the ts371 cl 5 mutant as a function of temperature (× 960); (a) 39°; (b) 32°; (c) temperature shift from 39° to 32°C at 14 hr.

the cAMP levels – that is, a higher density with lower cAMP levels. However, the densities at 32° and 39°C are nearly equal to those exhibited by K-NRK at 39°C and 32°C, which indicates that overall absolute cAMP levels regulate final cell density and perhaps microvilli. We are examining surface microvilli on the superconfluent cells to determine whether the correlation exists at high cell densities as well.

Similar reversion behavior of cAMP levels with various cell densities has been reported by Burstin et al., for ts clones of SV3T3 (17). Both ts SV3T3 clones used showed intermediate cAMP levels compared to normal and wild-type transformed cells, as in the NRK system we have used. Both systems need further quantitation of transformation parameters and cAMP levels rather than qualitative comparisons. By quantitatively establishing where cAMP is correlated with specific morphologic and biochemical lesions of neoplastic transformation, the role of cAMP may be elucidated in this biological phenomenon.

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