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A Mutant of 3T3 Cells with Cyclic AMP Metabolism Sensitive to Temperature Change

(fibroblasts/adhesiveness/cell shape/prostaglandin E_1/dibutyryl cAMP)

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ABSTRACT **A mutant line of 3T3 (mouse fibroblast) cells with temperature-sensitive cyclic AMP (cAMP) metabolism was isolated by selecting for low substratum adhesiveness after a change in temperature. Although the mutant is identical in behavior to the parent cell at constant temperature, a rise or fall in temperature causes a fall in intracellular cAMP levels within seconds, followed by loss of adherence to the culture dish and retraction of cell processes. The mechanism of this fall in cAMP level is at least in part excretion into the medium. The decrease in adhesiveness and retraction of processes can be blocked by analogues of cAMP or agents that elevate intracellular cAMP. The properties of this mutant imply that cAMP is a direct regulator of cell shape and adhesiveness.**

3′:5′-Cyclic AMP (cAMP) levels in fibroblasts are an important factor in the control of cell shape (1–4), growth (3–6), motility (7), adhesiveness to substrata (8), and the cell cycle (9). Transformed cells have low cAMP levels (5). The rapid growth, low adhesiveness, altered morphology, and abnormal motility of these cells appear to be related to their low levels of cAMP. Some other properties of transformed cells do not appear to be under cAMP control (3, 10, 11). To distinguish the properties of transformed cells that are due to low cAMP levels from those due to other effects of transformation, we decided it would be helpful to have cells that have low cAMP levels due to host mutations. To isolate such cells we took advantage of the fact that cells with high levels of cAMP are characterized by firm adherence to the substratum, and cells with low cAMP levels are loosely adherent (8). This paper describes the properties of a mutant cell line whose cAMP levels, adherence to substratum, and shape change when the temperature at which it is grown is altered. We call the mutant 3T3 $cAMP^{tcs}$-1.

MATERIALS AND METHODS

Swiss 3T3 cells (mouse fibroblast) were generously supplied by Dr. Howard Green. These cells were maintained in Dulbecco–Vogt's modified Eagle's medium supplemented with penicillin and streptomycin (50 units/ml) (Flow Laboratories) and 10% calf serum (Colorado Serum Co.) at 37° in 95% air and 5% CO_2. Electron microscopy and agar culture methods (12) failed to show the presence of mycoplasma in the parent or mutant cells. SC-2964 (1-methyl, 3-isobutyl xanthine) was a gift of G. D. Searle and Co., and prostaglandin E_1 a gift of the Upjohn Co. ICR-170 and ICR-191 (13) (acridine dye mutagens) were gifts of Dr. H. J. Creech, Institute for Cancer Research, Philadelphia, Pa. cAMP levels were determined by the method of Steiner *et al.* (14) as modified by D'Armiento *et al.* (15). The cells were not washed before extraction with cold 5% trichloroacetic acid.

Abbreviations: PGE_1, prostaglandin E_1; Bt_2cAMP, dibutyryl cyclic adenosine monophosphate; BtcAMP, *N*-monobutyryl cyclic adenosine monophosphate; SC-2964, 1 methyl, 3-isobutyl xanthine.

RESULTS

Mutant Selection. 3T3 cells were planted in a 75-cm^2 plastic flask and treatment with mutagens was begun at a density of 1.0×10^4 cells per cm^2. We first added ICR-170 at 1 μg/ml for 24 hr and followed it with ICR-191 in fresh medium at 1 μg/ml for 24 hr. Cells were then maintained for 3 days at 39.5° with daily medium changes. On the fourth day the flask was placed at 23° for 15 min, and then shaken gently by hand 4 or 5 times. Medium containing loosely adherent cells was poured into a new flask and incubated at 39.5°. 60 Clones emerged in the new flask after 6–7 days. The new flask was then placed at 23° and each clone was observed under an inverted phase contrast microscope as the cells gradually cooled toward room temperature. After 5–10 min, we noted retraction of cell processes and rounding of the cell bodies in only one of the 60 clones. The cells in this clone were removed by forcefully spraying the medium present in the flask onto the cells through a 1-ml pipette held in a piggyback pipetter. After transfer to a new vessel at 37° the cells attached, flattened out, and grew normally as long as the temperature remained constant. These cells were cloned and stored frozen. The temperature-sensitive phenotype has remained stable for over 6 months of continuous culture.

Here we describe the properties of three clones (A, B, and D) of the mutant cell line isolated from this first selection. Subsequently many other isolates with the same phenotype have been obtained by the identical selection procedure with or without ICR-170 and ICR-191. These later isolates have not been characterized biochemically.

Effects of Temperature Change. Fig. 1 is photographs of the mutant cells 0.5 and 15 min after removal from the 39.5° incubator. Under this procedure the temperature initially declined by about 1°/min. At the time of removal from the incubator, cells with both flattened and spindly morphology were evident in the culture. Within the first 30 sec an occasional cell began to change in shape. In general, at least 40% of the mutant cells showed easily recognizable shape changes in 10–15 min. These changed cells ultimately became rounded and were easily detached from the plastic by gentle agitation. We observed contraction and detachment of both the spindly and the very flattened cells. The latter are presumed to have large areas of attachment to the substratum. During the rounding process, numerous spindly, string-like extensions

were seen with phase contrast (at ×200) between the retracting cell process or body, and the substratum or an adjacent cell. These string-like extensions are very similar in appearance to those seen when normal 3T3 cells retract due to trypsin treatment. When the mutant cells were confluent, the contraction process initially began with the formation of holes in the monolayer. These holes gradually increased in diameter; the retracting edges were formed by rounded cell bodies.

After a temperature shift the cells did not permanently remain in the detached or rounded form. After 1 hr of incubation most of the detached cells reattached to the surface of the flask and began to spread out. By 2 hr they resembled the cells before the temperature was lowered. We found there is no critical temperature at which the change in cell shape occurs. Rounding up and detachment was observed when cells were shifted from 39.5° to 37°, 39.5° to 33°, 37° to 33°, 37° to 23°, or 31° to 23°. Apparently the cells are sensitive to the transient event. Exposure to room air, light, or gentle motion at constant temperature failed to cause any rounding.

We also examined the effect of raising the temperature. The cells were initially grown at 39.5° and then placed at 23°. As expected, the cells rounded up, detached, and then reattached and spread out. The cells were left at 23° for 12 hr.

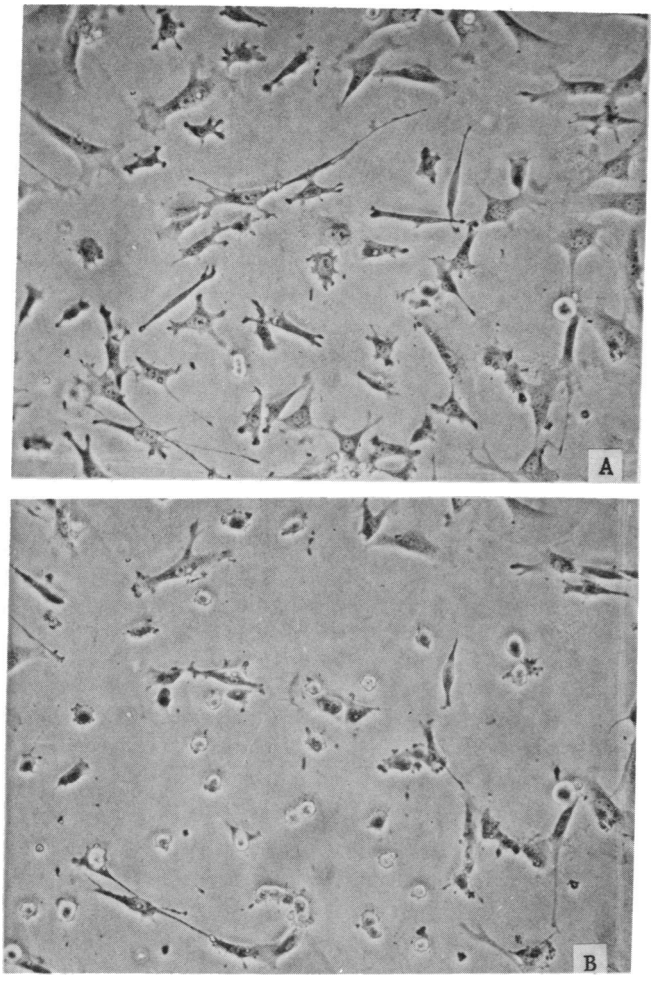

FIG. 1. 3T3cAMP^{ts}-1 (clone B) cells during temperature shift from 39.5°. (*A*) This ×50 phase contrast field shows the cells at the time they were removed from the 39.5° incubator without changing the medium. (*B*) This shows the same field after remaining at room temperature for 15 min. Note the large number of cells that have rounded and retracted their cell processes.

TABLE 1. *The effect of various agents on the shape change and growth rate of 3T3cAMP^{ts}-1 cells*

Additions	Min of pretreatment	% of cells rounding in 20 min	Generation time (hr)
None	—	50	20
0.5 ml serum-free medium	15	45	20
5.0 ml serum-free medium	15	42	(−)
5.0 ml 10% calf-serum medium	0	95	20
PGE_1 (50 μg/ml)	15	28	—
Cycloheximide (10 μg/ml)	15	45	(−)
cAMP (0.1 mM)	15	12	(−)
5′-AMP (0.1 mM)	15	10	(−)
5′-AMP (0.1 mM)	120	48	(−)
Bt_2cAMP (0.3 mM)	15	48	28
(0.5 mM)	15	32	30
(1.0 mM)	15	17	40
(0.3 mM)	1400	27	28
BtcAMP (0.1 mM)	15	43	22
(0.3 mM)	15	25	24
SC-2964 (0.1 mM)	15	39	22
(0.3 mM)	15	10	25
(1.0 mM)	15	3	32

Cells in 20-cm² plastic dishes (total medium volume of 5 ml) were exposed to the treatments listed for the indicated times. The rounding response is expressed as the percentage of the total number of cells per field (50–150) showing morphologic change after a 20-min exposure to room temperature from a growth temperature of 39.5°. The changed cells were counted in Polaroid phase contrast microscopic photographs taken at 0 time and 20 min after the cells were removed from the 39.5° incubator. Chronic growth rates were determined over a 2- to 3-day period in equally seeded 20-cm² plastic dishes, the cells being removed with 0.25% trypsin and counted on a Particle Data (model no. 112TA) cell counter. (—) Indicates a negative slope of the growth curve (cell death) at some time during the growth study. Solution concentrations were adjusted so that less than 0.5 ml of solution (in 39.5° serum-free medium) would be added to each dish to avoid overmanipulation and temperature change during preincubation.

Next morning the cells were still flattened. Then the flask was placed at 39.5°; within 15 min 50% of the cells had rounded up and detached.

Effects of Serum. In addition to responding to temperature change, these cells also responded to the addition of new serum. Changing the medium with new 10% calf serum alone causes this rounding phenomenon in many cells (Table 1). These experiments were done in a constant-temperature room to prevent any change in temperature. Medium change combined with a fall in temperature enhanced the number of cells responding. However, the presence of serum was not required to obtain a response to temperature shift; the magnitude of the response of cells preincubated in serum-free medium for 10–15 min appeared to be about the same as in the presence of serum. However, the contribution of serum is difficult to evaluate, due to the possibility that serum components remain bound to the cells after washing. Thus, a serum factor bound to the cells may be necessary for the temperature effect. We did not study the effect of prolonged incubation without serum because at least 20% of the parent and mutant cells die after exposure to medium with 0.5% serum for 24 hr.

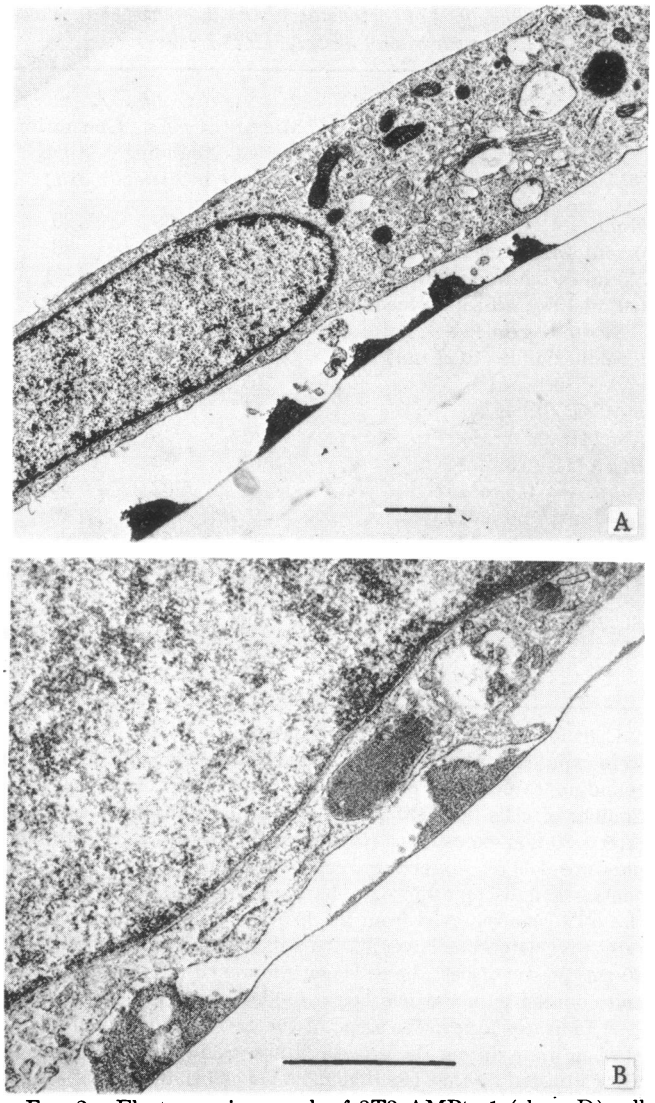

FIG. 2. Electron micrograph of 3T3cAMP^{tcs}-1 (clone D) cell sectioned perpendicular to the plastic "conditioned" growth surface. (*A*) Note the rounded mounds of dense granular material at the junction point with the plastic substratum. The mounds demonstrate a laminated structure and most of the cell attachments to the substratum appear to occur through these deposits rather than to the plastic itself. (Uranyl acetate–lead citrate stained, Epon-embedded, *bar* represents 1 μm). (*B*) A field similar to (*A*) but showing an intracytoplasmic vacuole containing material of the same general appearance and density as the deposits (*bar* represents 1 μm).

Refractory Period. Cellular contraction was not seen if the medium change or temperature shift followed within 1–2 hr of a previous event. But if the cells were retested in 3–4 days, they were once again fully responsive. During this refractory period the cells had very high levels of cAMP (see below).

Adhesiveness to Substratum. Previously we used trypsin treatment to quantitate the adhesiveness of cells to substratum. This method was too slow to use with the mutant cells. Removal by mechanical shaking lacked the sensitivity needed to measure the changes observed. We found that normal and mutant cells maintained at constant temperature were relatively resistant to removal by spraying the medium present in the dish back and forth onto the cells through a 5-ml pipette held in an automatic (piggyback) pipetter. After either temperature shift or addition of new serum the mutant cells were readily removed by spraying, indicating a marked loss of adhesiveness. The cells removed by spraying were completely viable. This decrease in adhesiveness occurred by 2–4 min, whether the cells were at low or high density.

Parent 3T3 Cells have never shown much alteration of shape or adhesiveness in response to temperature shift alone. On some occasions 1–5% of the parent cells showed some change in shape when the temperature was lowered. Occasional cellular rounding was also observed when medium containing fresh serum was added. We have also observed some cellular rounding when fresh medium was added to a line of polyoma-transformed mouse fibroblasts (PY-89).

Effects of PGE$_1$, Bt$_2$cAMP, and Other Agents. The temperature-sensitive mutant cells responded to many agents that act through cAMP. In Table 1 it can be seen that preincubating cells with some of these agents (PGE$_1$, SC-2964, BtcAMP, Bt$_2$cAMP, or cAMP) markedly reduced the morphologic response to temperature shift. This was not the case with comparable concentrations of 5′-GMP, 5′-UMP, and cGMP, which presumably did not acutely raise intracellular cAMP levels. 5′-AMP blocked the contractile response after a 15-min, but not after a 2-hr, preincubation. If the incubation was continued for 24–48 hr, 5′-AMP killed both 3T3 and the mutant cells (Table 1). Therefore, we feel the inhibitory action of 5′-AMP is due to a nonspecific response of the cells, although 5′-AMP may block the rapid release of cAMP in the mutant. Cycloheximide treatment did not block the contractile response, suggesting that protein synthesis is not involved in this event.

Granular Deposits. After incubation of the mutant cells in the same dish for 3–5 days, granular deposits firmly adherent to the plastic became apparent under phase contrast microscopy. Similar incubation with 3T3 cells failed to show such changes. These deposits were rounded, and remained attached to the substratum even after forceful pipetting. In sparse cultures they appeared heaviest close to the cells. Mutant cells growing on this "conditioned" surface are less adherent, more spindly, and they detach and contract more rapidly than cells grown on an unconditioned surface. When 3T3 cells were planted on a surface conditioned by mutant cells, they too were somewhat more spindly, but they did not contract in response to temperature shift. Trypsin treatment would not detach the deposits, but mutant cells planted on the trypsin-treated surface were somewhat more adherent and less spindly in shape. Therefore, the deposits probably contain

TABLE 2. *Growth rates and saturation densities at various temperatures for 3T3cAMP^{tcs}-1 cells*

Temperature (°C)	Clone	Generation time (hr)	Saturation density (cells per cm^2) $\times 10^{-4}$
33	A	28	4.45
	B	28	4.85
37	A	18	4.00
	B	18	4.45
39.5	A	19	3.70
	B	20	2.80
	3T3	22	4.00

Cells were grown and counted as indicated in Table 1. *A* and *B* are clones of 3T3cAMP^{tcs}-1. The medium was changed every other day (10% calf serum media).

some protein. An electron micrograph of the deposits reveals that they are dense and finely granular (Fig. 2A). They appear as mounds on the plastic and measure 0.5–1.0 μm in diameter when sectioned perpendicular to the surface. Cytoplasmic vacuoles containing similar-appearing material occur inside the mutant cells (Fig. 2B).

Growth of Mutant Cells. As long as the temperature remained constant the mutant cells remained flattened and firmly attached. We measured the growth of two clones of cells at 33°, 37°, and 39.5°. The cells grew logarithmically at all temperatures, and, as expected, grew more slowly at 33° (Table 2). At each temperature they remained contact inhibited. They also failed to grow in agar. Thus, in growth characteristics they are indistinguishable from the parent 3T3 cells.

Cyclic AMP Levels in Mutant and 3T3 Cells. cAMP levels were measured in confluent cultures of normal and mutant 3T3 cells (Table 3). When maintained at 39.5°, both cell lines had similar levels of cAMP. However, when measured only 2 min after removal from the 39.5° incubator, the levels of cAMP in the mutant cells had significantly fallen (Table 4). By 10 min they reached their lowest value. The levels in the parent were unchanged.

Treatment of these mutant cells with serum also decreased cAMP levels, and the combination of serum addition and temperature shift further decreased these levels. PGE_1 raised cAMP in both the parent and mutant cells.

In an experiment shown in Table 4 the cells were removed from the 39.5° incubator for 5 min (at which time the temperature had fallen to about 34°) and then kept at 33°. The cAMP levels were measured for 3 hr. By 2 min the levels had fallen. They reached a minimum after 5 min and by 50 min had returned to normal. At this time the cells were firmly attached and could not be dislodged by shaking, but they were not yet flattened out. The cAMP levels did not remain at these normal levels but rose to about 200 pmol/mg of nucleic acid. After 3 hr the levels were once again normal and the cells appeared morphologically normal. A similar but earlier overshoot was seen when the cells were shifted down from 39.5° for only 5 min and then returned to 39.5°.

TABLE 3. *Cyclic AMP levels in 3T3 and 3T3cAMP^{tcs}-1 cells*

Treatment	cAMP 10 min after treatment (pmol/mg of NA) (± SEM)	
	3T3	3T3cAMP^{tcs}-1 (clone B)
None	42 ± 2.1	41 ± 2.0
Temperature shift (39.5° to 23°)	43 ± 1.7	20 ± 0.5
10% calf-serum media	—	26
10% calf-serum media with temperature shift	—	19
PGE_1*	80	122
PGE_1† with temperature shift	86	160

Values are expressed in picomoles of cAMP per mg of nucleic acid (*NA*). In the PGE_1 experiment cited, PGE_1 (30 μg/ml) was added in less than 0.5 ml of serum-free medium solution at 39.5°.

* PGE_1 was added 15 min before cAMP was extracted.

† PGE_1 was added 15 min before temperature shift (39.5°–23°) and 25 min before extraction.

TABLE 4. *Cyclic AMP levels in 3T3cAMP^{tcs}-1 (clone B) cells during equilibration after temperature shift*

Min after temperature shift	cAMP (pmol/mg of NA)	
	Cells equilibrated to: 33°	39.5°
0	42	42
2	29	27
5	23	24
20	25	73
50	48	68
95	213	106
185	46	50

Cyclic AMP levels were measured at the indicated times after equally seeded dishes of cells grown at 39.5° were shifted to room temperature for 5 min and then incubated at either 39.5° or 33°.

In addition to measuring the cAMP content of the cells, we also measured the cAMP content of the medium. In the experiments in Table 5 fresh medium was added to the cells 4 hr before the experiment was done. Both the normal confluent 3T3 cells and the mutant cells released cAMP into the medium during the 4-hr preincubation period. As can be seen from the table, 3T3 cells had a higher basal rate of loss of cAMP than the mutant cells. After the temperature shift the cAMP content of the medium of the mutant cells markedly increased, whereas the medium of the normal 3T3 cells did not contain any more cAMP. Thus, the fall in cAMP levels in the mutant cells was at least in part due to release into the medium.

In the experiments reported, cAMP was measured in unwashed cells by pouring off the medium and adding 5% trichloroacetic acid. With this procedure we found that 0.25 ml of medium remained in the dish. Since some cells release cAMP into the medium (see ref. 15 and above), the cell values should be corrected for the cAMP present in the remaining medium to obtain the true level of cAMP in the cells. An example of such a correction is shown in Table 5. This correction would not be necessary if the cells could be washed before extraction, but washing lowers the cAMP levels in some cells (15).

The release of cAMP by the mutant cells was not due to a nonspecific leakiness of the cells, for when the acid-soluble

TABLE 5. *Cyclic AMP levels in cells and media after temperature shift*

Cell	Total pmol cAMP in cells		Total pmol cAMP in media		Change in media cAMP (pmol)
	39.5°	Shifted to 23° (10 min)	39.5°	Shifted to 23° (10 min)	
3T3cAMP^{tcs}-1	38(35)	24(16)	116	326	+210
3T3	40(28)	41(32)	292	306	+14

Each figure represents the amount of cAMP in two 50-cm² dishes of cells containing 800 μg of nucleic acid; 8 ml of media (10% calf serum) had been added 4 hr previously (average of two experiments). The cells and media were extracted either at the constant growth temperature of 39.5° or after shifting to room temperature (23°) for 10 min. Numbers in parentheses are the cAMP levels in cells corrected for medium contribution (see *text*).

pool was labeled with 3.7×10^5 cpm of [^{3}H]adenine, about 1% of the label was found in the medium of shifted and unshifted cells.

DNA Synthesis. In confluent 3T3 cells the addition of serum or trypsin produces a rapid fall in cAMP levels followed by a synchronous wave of DNA synthesis 12–28 hr later (9, 16). We asked if the fall in cAMP in response to temperature shift would result in a similar stimulation of DNA synthesis. Confluent 3T3 and mutant cells were left without medium change for 2–3 days and then exposed to a temperature shift or to fresh medium containing 10% calf serum. A 1-hr pulse label of [^{3}H]thymidine was used to measure DNA synthesis at 24, 26, and 28 hr. Both 3T3 and mutant cells showed a 250-fold increase in DNA synthesis with the addition of new medium. Shifting from 37° to 33° caused no increase in DNA synthesis with 3T3 cells, and only a 5-fold increase with mutant cells. The failure of temperature shift to stimulate DNA synthesis markedly may be due to the brief fall in cAMP levels followed by the overshoot within 1 hr. Previously we found that Bt_2cAMP blocked the increase in DNA synthesis after trypsinization of confluent 3T3 cells (9).

DISCUSSION

The temperature-sensitive mutant reported in this paper has a number of unique properties. First, the cAMP^{tcs}-1 mutant displays the same response, a decrease in adhesiveness, withdrawal of cell processes, and rounding up, to either a rise or a fall in temperature. The entire response is transient and lasts but a few hours, the initial morphologic portion involving less than 15 min. The same response is obtained when the cells were shifted from 39.5° to 37°, 37° to 33°, or 33° to 23°. There is no critical temperature to which the cell responds. The result of the temperature shift is a fall in cAMP levels, a decrease in adhesiveness, and withdrawal of cell processes. This pleiotropic response to what is probably a single mutation is analogous to the loss of multiple properties in cAMP mutants of *Escherichia coli* (17).

The sequence of events when cells growing at 39.5° are placed at 23° is as follows: (*a*) within 30 sec cAMP levels are falling precipitously and by 2 min the levels have decreased to minimal values. Much of the fall in cAMP can be accounted for by nucleotide found in the medium; (*b*) by 2–4 min the adhesiveness of the cells is decreased so that they can be washed off with a pipette. At this stage a few of the cells begin retracting their processes; (*c*) from 5–15 min the cellular processes are withdrawn and the previously flat cells assume a rounded morphology. These morphologic changes are best observed in nonconfluent cultures where individual cells are readily observed. In confluent cultures the cells often come off in a sheet after the cAMP levels have fallen. Later the cells in the sheet can be seen to contract.

What is the evidence that cAMP controls cell shape and adhesiveness? Agents that raise cAMP levels increase the adhesiveness and alter the shape of transformed cells (1–4, 8); such cells originally have low cAMP levels (5). Treatment of cells containing elevated cAMP with serum or trypsin causes a fall in cAMP (16), a decrease in adhesiveness, and retraction of cell processes. A similar response is observed when the cAMP^{tcs}-1 mutant is shifted to a different temperature. The decrease in adhesiveness and change in shape is blocked by Bt_2cAMP and PGE_1.

It is not known whether the biochemical aspects of the contraction process can occur without a prior loss of adhesiveness. We propose that continued rigid structural forces such as cell-to-cell adhesion in confluent cultures mechanically inhibits morphologic contraction. But even in the presence of strong cell-to-cell attachments, loss of cell contact to the plastic dish is often followed by contraction of cells and rolling up of the cell sheet beginning at one edge.

A fall in cAMP levels can be due to a decrease in adenylate cyclase activity, a rise in phosphodiesterase activity, or release of cAMP from the cell. We have measured adenylate cyclase and phosphodiesterase activities but have not detected a change in either enzyme. We do find an increase in the cAMP content of the medium of magnitude sufficient to account for the fall in the cellular level. Previously we found changes in adenylate cyclase in chick cells transformed by a temperature-sensitive mutant of Rous sarcoma virus (18).

What is the nature of the mutation that results in the release of cyclic AMP? Frequently temperature-sensitive mutants have a protein whose activity is decreased by the change in temperature. Mutants defective in lipid metabolism (19) have also been isolated in which the composition of the membrane lipid can be altered by feeding the cells lipids of various types (20, 21). In these types of protein and lipid mutants the decrease in cellular activity occurs near some critical temperature and the characteristic response is unidirectional. The release of cAMP into the medium might suggest that 3T3 cAMP^{tcs}-1 cells are mutant in some membrane function. Alternatively, the change in temperature may perturb the cell so that the size of different pools of intermediates varies and is then readjusted. Perhaps because cyclic AMP is such a key regulatory molecule, its level is sensitive to a variety of metabolic disturbances.

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