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Intracellular Cyclic AMP Concentration Responds Specifically to Growth Regulation by Serum*

(transformation/reversion/mouse fibroblasts/serum requirement/density-dependent growth inhibition)

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ABSTRACT **Intracellular concentrations of cyclic AMP increase when cells are deprived of serum. Studies with the mouse fibroblast line 3T3, an SV40-transformed subline of 3T3, and six different revertant lines derived from this clone show that a marked increase in cyclic AMP occurs only when the serum concentration is reduced below the minimum necessary for growth of a given line. Conversely, density-dependent inhibition of growth is not accompanied by an increase in cyclic AMP concentration in any line.**

The regulation of proliferation of fibroblast cultures is complex. Well regulated cell lines, such as the mouse line 3T3, are sensitive to inhibition by at least three different environmental signals: contact with other cells (1–3), deprivation of serum (4, 5), and absence of a solid substrate (6). SV40 virus transformed 3T3 clones may lose sensitivity to all three signals, and thus grow despite cell–cell contact (2), grow even when serum is reduced by 10-fold (7), and form spherical colonies when suspended in methyl cellulose gel (8, 9) (Table 1). This loss of growth control can be reversed. By applying negative selection pressures to transformants, we have obtained cell lines, called revertants, which exhibit growth control comparable to that of normal cells (7, 9–11). Some revertants have regained sensitivity to contact-regulations and anchorage-regulations without regaining a high serum requirement for growth. We term these "density-revertants" (10, 11) (Table 1). Other revertants have regained a high serum requirement for growth and, in addition, sensitivity to either contact alone, or to both contact and anchorage. We term these "serum-revertants" (7) (Table 1).

Many studies have suggested a possible inhibitory role for 3′:5′-cyclic AMP (cAMP) in the regulation of proliferation of fibroblast cultures (12–19). When cAMP is measured directly in cultures of normal and transformed cell lines, the concentration of cAMP is consistently found to be about half as high in growing cultures of transformed lines (13–17). Studies on synchronized cultures of 3T3 have shown that cAMP is twice as high in interphase 3T3 as in mitotic 3T3 cells (18, 19), suggesting that a fall in cAMP may be required during at least part of the cell cycle if cells are to proliferate. In this paper we use 3T3, SV3T3, density-revertants, and serum-revertants to examine which of the three signals regulating proliferation is mediated through intracellular changes in concentration of cAMP.

Abbreviation: TCA, trichloroacetic acid.

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MATERIALS AND METHODS

Culture Conditions. Cells were grown at 37° in a water-saturated atmosphere of 10% CO_2–90% air in Dulbecco's modification of Eagle's medium (Gibco H 21) supplemented with 10% calf serum (Colorado Serum Co.) and 50 μg/ml of Gentamycin (Schering). Stock cultures were carried sparse in 28.3 cm^2 Falcon tissue culture plates, with medium changes twice a week.

Cell Lines. All revertant cell lines were derived from SV101, a clone of SV40-transformed Swiss 3T3 cells (10). Density-revertants have a low saturation density in 10% calf serum. Density-revertants isolated with FrdU are designated FlSV and those isolated with BrdU and UV light are designated BuSV (7, 9, 10).

Serum-revertants are unable to grow in 1% calf serum, and grow to low saturation density in 10% calf serum (7). The serum-revertants isolated in 1% calf serum are called LsSV, while serum revertants isolated in 10% agamma-depleted calf serum are called AγSV.

Cyclic AMP Assay. Cells were seeded at densities of 3.5 to 7.0 × 10^3 cells per cm^2 in either 10% or 1% calf serum and the intracellular cAMP levels determined 2 days later. To begin the assay, the medium was carefully removed from plates containing a total of at least 10^6 cells. Without rinsing, the cells were fixed with 2 ml per plate of cold 5% trichloroacetic acid (TCA) and incubated at 4° for an hour. Then the TCA suspensions were transferred to a cold centrifuge tube, pooled with a 5% TCA wash of the plates, and pelleted at 10,000 rpm for 5 min at 4° in a Sorvall centrifuge. The supernatant was treated 5 times with 2 volumes of water-saturated acidified ether and lyophilized. The dry residue was solubilized in 0.2 ml of 0.2 M acetate buffer (pH 4), and 0.1-ml samples were assayed for cAMP content exactly as in Gilman (21).

Cell protein was measured by the method of Lowry (22), using bovine-serum albumin as a standard.

RESULTS

cAMP Concentration in Growing Cultures. Previous studies on cAMP in growing cultures of 3T3 and SV3T3 (17) reported that 3T3 has about twice the cAMP concentration of SV3T3 (22 *versus* 10 pmol/mg of protein). We found exactly these same results (Table 2). In growing populations of most revert-

TABLE 1. *Sensitivity of mouse cell lines to different restrictions on growth*

Class	Line	Sensitivity to restriction* by Serum†	Density‡	Anchorage§
Normal	3T3	+	+	+
Fully transformed	SV3T3101	−	−	−
Serum-dependent revertant	AγSV4	+	+	−
Serum-dependent revertant	AγSV5	+	+	−
Serum-dependent revertant	LsSV1	+	+	+
Serum-dependent revertant	LsSV2	+	+	+
Density-dependent revertant	BuSV2	−	+	+
Density-dependent revertant	FlSV101	−	+	+

* (+), Restriction, leading to maintenance of cell number with little or no increase; (−), absence of restriction, leading to increase in cell number.

† To assay sensitivity to serum-dependent restriction, growth was measured after innoculation of cultures sparsely in 1% calf serum (7).

‡ To assay sensitivity to density-dependent restriction, growth was measured in cultures innoculated in 10% calf serum, and permitted to grow to confluence with periodic replacement of medium (9-11).

§ To assay sensitivity to anchorage-dependent restriction, cultures were innoculated as single cells suspended in methyl cellulose gel and the fraction of cells able to form visible colonies was determined after 21 days (9).

ant lines, cAMP concentration had returned to the high cAMP concentration characteristic of 3T3 (Table 2). However, the lines AγSV4 and AγSV5, which were selected for their inability to grow in 10% agamma-depleted calf serum (7), retained the low cAMP level of SV3T3101 (Table 2). Apparently, revertants which have acquired the ability to maintain a low saturation density need not always reacquire the ability to maintain a high cAMP level in growing cultures.

An involvement of cAMP in anchorage dependence is suggested by these data. Comparing Tables 1 and 2, lines able to grow in methocel have low cAMP levels while growing in 10% calf serum, and lines unable to do so have high cAMP levels while growing in 10% calf serum.

cAMP Concentrations in Serum-Restricted Cultures. Transformed SV101 cells and density-revertants can grow in 1% calf serum, but 3T3 and the serum revertants cannot (Table 1). All lines cultured in medium with 1% serum showed increased intracellular cAMP concentrations. However, lines unable to grow in 1% serum showed a greater increase in cAMP than lines which grow (Table 3).

In all five lines that did not grow, cAMP rose to more than 70 pmol/mg of protein (Table 3). In particular, cAMP rose to this exceptionally high level in the Aγ-serum revertants despite the fact that these cells have a low cAMP level while growing in 10% calf serum (Table 2). The transformed line and the two density-revertants, which all grew equally well in 1% calf serum, all showed a much smaller rise in cAMP.

TABLE 2. *cAMP concentrations in growing mouse cells*

Class	Line	cAMP concentration* (pmol/mg of protein) Average	Range
Normal	3T3	25	20-28
Fully transformed	SV3T3101	12	8-14
Serum-dependent revertant	AγSV4	13	10-15
Serum-dependent revertant	AγSV5	15	10-16
Serum-dependent revertant	LsSV1	20	17-22
Serum-dependent revertant	LsSV2	24	21-30
Density-dependent revertant	BuSV2	21	17-25
Density-dependent revertant	FlSV101	23	21-25

* All lines assayed while growing in sparse culture, at less than 2×10^4 cells per cm^2, in 10% calf serum.

Thus, when cultured in low serum and in the absence of contact, all eight lines showed a clear correlation between inability to grow, and a marked increase in cAMP concentration.

The specificity of this correlation was confirmed by the density revertants. cAMP rose no higher in these lines, which are serum-insensitive, than the level found in SV101 (Table 3). Taken together, these data indicate that at any serum level, a very high cAMP level is found only in cells that are unable to grow in that serum concentration.

cAMP Concentrations in Density-Restricted Cultures. To test whether density restriction also affected cAMP levels, cultures were innoculated in 10% calf serum and permitted to reach confluence. Medium was changed 3 times weekly even

TABLE 3. *cAMP concentrations of mouse cell lines cultured in medium with reduced serum*

Class	Line	cAMP concentration* (pmol/mg of protein) Average	Range
Normal	3T3	83	75-100
Fully transformed	SV3T3101	31	25-40
Serum-dependent revertant	AγSV4	67	60-75
Serum-dependent revertant	AγSV5	72	60-85
Serum-dependent revertant	LsSV1	68	52-70
Serum-dependent revertant	LsSV2	78	75-80
Density-dependent revertant	BuSV2	34	30-47
Density-dependent revertant	FlSV101	37	31-43

* All lines assayed 2 days after innoculation at sparse density in 1% calf serum. SV3T3101, BuSV2, and FlSV101 grow under these conditions of serum restriction, while 3T3 and the serum-dependent revertants do not. See Table 1.

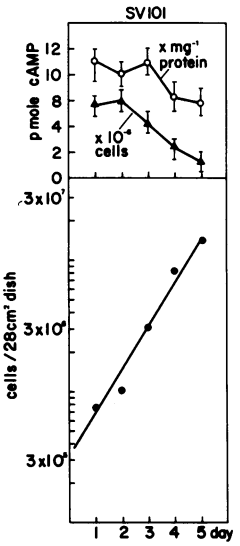

FIG. 1. Response of SV101 to density restriction. Cultures of SV101 were permitted to reach confluence in 10% calf serum. Medium was replaced every third day. Cells did not slow their growth at confluence. cAMP concentration fell slightly as cultures got very dense.

at confluence to prevent serum depletion from inducing an inadvertant rise in cAMP. Only SV101 was able to grow to high cell densities, since the serum revertants, as well as the density revertants and 3T3, were all subject to density regulation of proliferation (Table 1).

SV101 showed no increase of cAMP at confluence or beyond. Rather, levels declined somewhat in dense cultures of SV101 (Fig. 1). Previous reports have found a similar response for SV101 (13, 17). The drop in cAMP was not due to changes in cell volume at confluence, since the data remain qualitatively the same when expressed as cAMP/10^6 cells (Fig. 1).

In 3T3 cells, density inhibition of growth did not lead to an increase in cAMP at confluence (Fig. 2). Rather, 3T3 cultures showed a slight decline in cAMP, similar to the response of SV101. These results also cannot be due to changes in cell volume of either 3T3 or SV101, since expressing the data as pmol/10^6 cells did not change the result (Fig. 2). As the serum revertants LsSV2 and AγSV4 entered a state of density inhibition, they too showed a drop in cAMP concentration

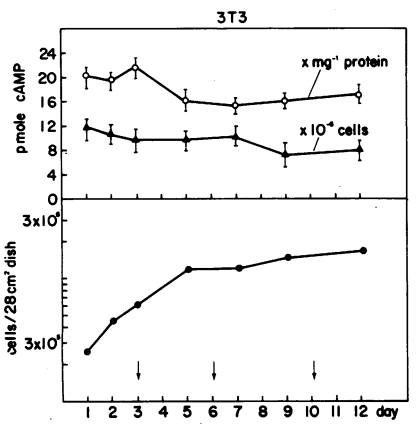

FIG. 2. Response of 3T3 density restriction. Despite regular changes of medium (*arrows*), proliferation of 3T3 ceases at confluence. cAMP does not increase in 3T3, even after a week at confluence.

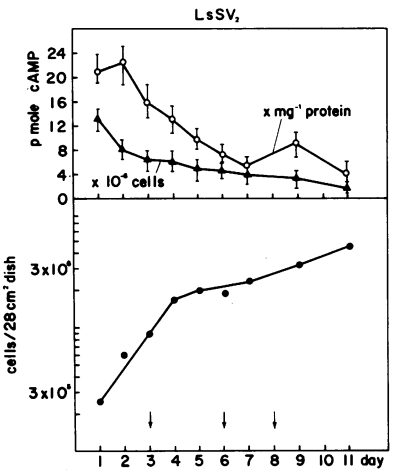

FIG. 3. Response of Ls serum revertant to density restriction. cAMP decreases as the revertant cells remain at a plateau due to density-inhibition of proliferation. Compare with Fig. 2.

(Figs. 3 and 4). Thus, cessation of proliferation due to sensitivity to density inhibition is not accompanied by an increase in cAMP, but rather by a continuing slight decrease. Recent results by Burstin *et al.* confirm these observations (20).

Kinetics of cAMP Fluctuations. To study the time course of changes in cAMP accompanying changes in serum concentration, sparse cultures of 3T3 and SV101 were shifted from 10% calf serum to either 1% calf serum or to serum-free medium and sparse cultures of 3T3 were shifted from 1% calf serum to 10% calf serum. Cell number, cell protein, and cAMP were measured at times after the shift.

Both lines were unable to grow in serum-free medium, and only SV3T3 was able to grow for more than 35 hr in 1% calf serum (Fig. 5). In serum-free medium, cAMP rose slowly in both lines, passing 70 pmol/mg of protein by 24 hr (Fig. 6). Apparently, when serum was dropped below the level sufficient for growth of SV101 cells, even these fully transformed cells attained the high level of cAMP characteristic of other cells under serum restriction.

When serum was shifted to 1% no change in cAMP was seen for about 24 hr. The two lines then behaved differently. In SV101, cAMP did not rise beyond 30 pmol/mg of protein

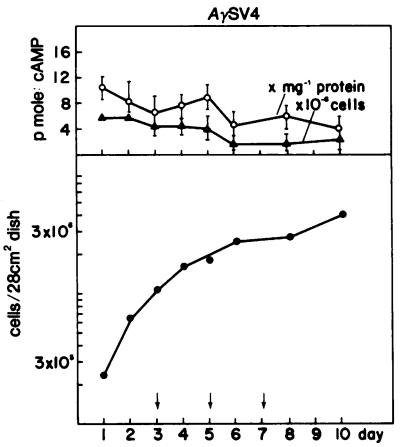

FIG. 4. Response of Aγ serum-revertant to density restriction. cAMP concentration in sparse AγSV4 is as low as in SV101 (see Fig. 1). cAMP falls further as AγSV4 cells reach confluence.

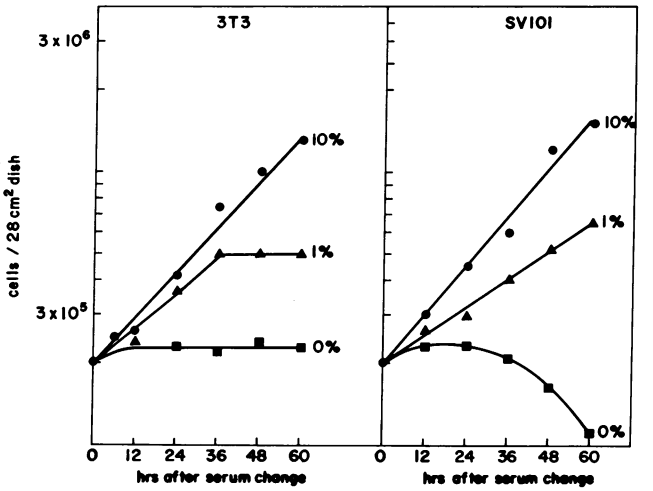

FIG. 5. Growth of 3T3 and SV101 after shift-down of serum. Sparse cultures of 3T3 and SV101 were innoculated in 10% calf serum; 2 days later, at 0 hr, medium was replaced with fresh medium containing 10% calf serum, 1% calf serum, or no calf serum. Neither line grew in the absence of serum. SV101 grew well in the presence of 1% calf serum.

even after 60 hr (Fig. 6*b*). In 3T3, cAMP rose beyond 70 pmol/mg of protein by 36 hr, as cell growth ceased (Fig. 6*a*).

When serum was shifted up from 1% calf serum to 10% calf serum, sparse 3T3 cultures responded with a very rapid drop in cAMP (Fig. 7).

DISCUSSION

We conclude from these data that growth control in cultured fibroblasts is mediated through both density-specific and serum-specific regulations, and that sensitivity to serum-restriction, but not sensitivity to density-restriction, is reflected in a great rise in cAMP.

Unless cells are kept from contacting each other during assays of serum deprivation, it is not possible to distinguish whether changes seen in cAMP are due to serum or contact effects. Conversely, serum must be constantly replaced at confluence in assays of density restriction.

Large increases in cAMP have been observed in cultured cells when serum is made limiting either by starvation or by lowering serum concentration (15, 23–25). Some (13, 14), but not other (17), previous reports also show a rise in cAMP in 3T3 as cells approach confluence. In view of the demonstrated high serum requirement of 3T3 as compared to transformants, the possibility must be considered that inadvertant serum-starvation occurred in confluent 3T3 cultures in which cAMP was found to rise, since the only density-dependent change in cAMP we observed was a slight but consistent decrease at confluence.

We have confirmed (Fig. 7) the observation that administration of fresh serum to serum-restricted cultures causes a drop in cAMP (14, 17, 18, 26). The rapidity of this drop compared with the slow rise in cAMP upon serum starvation is remarkable. This drop in cAMP is also inducible by proteases (18, 19), insulin (27), and Somatomedin (28). Insulin and Somatomedin have been shown to act by inhibiting adenylate cyclase in the cell membrane (27, 28), raising the hypothesis that serum stimulates growth through inhibition of membrane-bound adenylate cyclase.

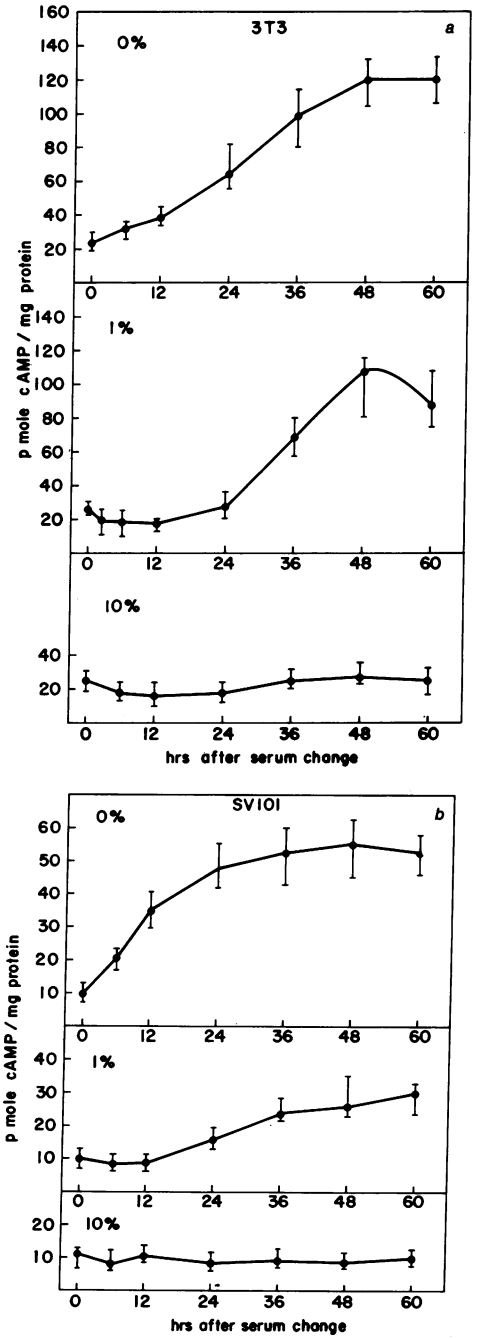

FIG. 6. Changes in cAMP concentration during serum restriction. cAMP was measured in sister cultures of those used in Fig. 5. (*a*) In growing 3T3 cultures, cAMP concentration in 10% calf serum is 20 pmol/mg of protein. One day after shifting to 1% calf serum, cAMP begins to rise and by 36 hr cAMP has passed 70 pmol/mg of protein. cAMP begins to rise immediately after serum is completely removed from 3T3 cultures. (*b*) In growing SV101 cultures, the cAMP concentration is 10 pmol/mg of protein. One day after shifting to 1% calf serum cAMP rises to 30 pmol/mg of protein, but gets no higher with time. cAMP begins to rise immediately after serum is completely removed, and eventually reaches about 60 pmol/mg of protein.

Density-Restriction Is Not Identical to Serum-Restriction. Normal 3T3 cells do not grow well in 1% calf serum and grow to low saturation density in 10% calf serum, while SV40 transformed 3T3 cells grow in 1% calf serum and grow dense in 10%

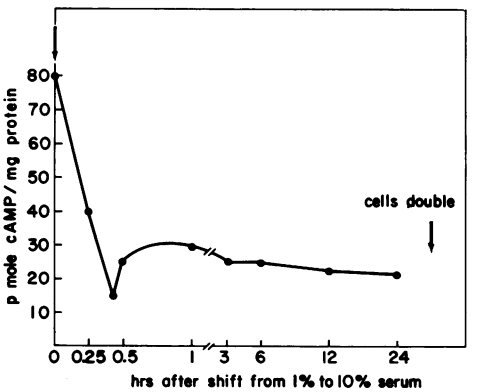

FIG. 7. Response of 3T3 to serum shift-up. 3T3 cells were plated sparsely in 1% calf serum. A day later (at 0 hr) medium was changed to 10% calf serum. The cells double 30 hr after shift-up. Within 15 min after the shift-up, the concentration of cAMP falls by 50%, and by 30 min the final lower concentration is reached.

calf serum. Density-revertant lines demonstrate that these serum-restrictions and density-restrictions are not identical, since they grow as well as SV3T3 cells in 1% calf but grow to a low saturation density in 10% calf serum.

The response of cAMP to these restrictions supports the hypothesis that they are not identical. The density-revertants have an SV101-like cAMP level in 1% calf serum, but a cAMP level similar to 3T3 cells in 10% calf serum. Serum revertants, which have a serum-response similar to that of 3T3, also show a rise in cAMP at low serum to a level comparable to that of 3T3. Taken together these data suggest that the mouse fibroblasts are subject to at least two distinct, separable modes of regulation of proliferation.

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