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CYCLIC NUCLEOTIDES AND CELL GROWTH

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Growth induction in resting fibroblast cultures by serum or growth factors induces a fast, transient cGMP peak which may constitute the intracellular signal for growth. A similar cGMP peak occurs when 3T3 cells arrested at the restriction point or in G_0 by starvation for certain amino acids are induced for growth by readdition of the lacking nutrients. Both 3T3 and SV3T3 cells which are arrested randomly all around the cell cycle do not exhibit major changes in cyclic nucleotides after growth induction.

Determination of intracellular cAMP and cGMP levels in normal and transformed fibroblasts under different growth conditions shows that the transition between growing and resting state (G_0 arrest) is accompanied and probably induced by characteristic changes in cAMP to cGMP ratios. cGMP is decreased 2-5-fold in resting as compared to growing cultures, and increased 10-20-fold in activated cultures 20 min after serum induction. No major cGMP change was observed in growing, confluent, or serum-activated cultures of transformed cells.

Measurement of guanylcyclase under unphysiological conditions (2 mM Mn^{++}) in crude and purified membranes from 3T3 and SV3T3 cultures did not show increased enzyme activity in the transformed cells. Significant differences may only show up when synchronized cells pass through the restriction point in G_1 phase. As a hypothesis it is proposed that transformed cells have an activated guanylcyclase system or a relaxed cGMP-pleiotypic response mechanism at the restriction point of their cell cycle.

INTRODUCTION

Fibroblast cells in culture exist either in a growing state or in a state of relative quiescence. The transition between these two states is reversible and controlled in the presence of sufficient nutrients by growth-stimulating substances in serum. This transition is accomplished by a sequence of molecular events termed "pleiotypic response": stimulation of transport systems, polyribosome formation, protein synthesis, ribosomal and tRNA synthesis, and eventually the induction of DNA synthesis followed by cell division. These sequential changes are preceded by very fast alterations in cyclic nucleotide levels. A rapid decrease in cAMP was observed after serum induction of cell growth (1–3). From this and other evidence such as the well-known growth inhibition by exogenous addition of DB-cAMP to cell cultures, cAMP was implied in growth control. More recently a fast, transient cGMP peak was observed in lymphocytes and fibroblasts after growth induction by mitogenic substances (4–6). This suggested the possibility that cGMP might act as an intracellular signal for the induction of the pleiotypic and mitogenic program of the cell. In this paper we discuss some experiments and implications with regard to the cell cycle and the possible differences between normal and transformed cells.

METHODS

Cell Preparation

Growth and handling of cells were as described previously (5). Cells were grown at 37°C in a 10% CO_2 atmosphere in 10 ml of Dulbecco's modified Eagle's medium containing 10% calf serum in 9-cm tissue culture dishes unless otherwise stated. Conditions for arrest of cells by nutrient starvation were substantially as reported for 3T3-4A (8) and SV3T3 (9) cells.

Cell cycle analysis was performed with a cytofluorograph as described by Holley and Kiernan (8).

DNA synthesis. Cell cultures were labeled with 3 μCi/ml of [^{3}H] thymidine at either 3 μM for DNA synthesis or 1 μM for autoradiography and processed as previously described (5). Unless otherwise stated, labeling times were throughout the entire DNA synthetic period in synchronized cultures.

Cell numbers. Cell numbers were recorded in a Coulter counter.

Measurement of cyclic nucleotides. The medium from usually 10 cultures on 9-cm dishes was removed, cold 5% TCA added to the dishes, and cell extracts were purified by centrifugation, ether extraction, and chromatography over Dowex columns as previously described (7). ^{3}H-Cyclic nucleotides were added to standardize the recoveries. The cyclic nucleotides were estimated by the radioimmunoassay of Steiner et al. (10). To check the authenticity of the products assayed, half of each sample was digested with 3′-5′ cyclic nucleotide phophodiesterase, and any nondigested material that cross-reacted with the antiserum directed against cGMP and cAMP was deducted to yield the final values (approximately 0.1 pmol/10^6 cells for cAMP and 0.5 for cGMP). All points are the average of duplicate experiments which agreed to within 15%.

Membrane Preparation and Determination of Guanylcyclase Activity

Membranes were prepared and guanylcyclase activity was determined as previously described (5). The radioimmunoassay was used to measure the formation of cGMP after a 10-min incubation at 37°C. In spite of relatively high backgrounds the method is reliable and gives reproducible results. All assays were done at 0.2 mM GTP and 2 mM Mn^{++} at pH 7.6 in the presence of phosphodiesterase inhibitors.

RESULTS

Fibroblasts can be arrested in the G_0 phase of the cell cycle not only by density inhibition or serum depletion, but also by starvation for certain nutrients. Some amino acids, such as methionine or leucine, tend to arrest 3T3 cells in a more random way all around the cell cycle, while others such as histidine and glutamine, bring about a more specific arrest in the G_1 phase as shown by analysis with a cytofluorograph (8). As judged from the time required to enter S phase after addition of the lacking amino acids, they seem to be arrested in the G_0 state, or – to use another terminology – at the restriction point (11).

We have followed the internal changes of cAMP and cGMP after adding back the lacking nutrients (Table I). A transient 10-fold cGMP increase with kinetics similar to those after serum induction was observed when the G_0-arrested cells were moving back into the cycle. Randomly arrested 3T3 or SV3T3 cells exhibit no major change in cGMP or cAMP after growth induction.

TABLE I. Kinetic Changes in Cyclic Nucleotide Concentrations After Addition of Depleted Nutrients

	3T3 starved + his + glu		SV3T3 starved + leu	
Time (min)	cGMP	cAMP	cGMP	cAMP
0	0.5	4.3	0.9	2.5
10	5.2	2.4	0.8	1.9
20	4.6	2.3	1.2	1.9
60	2.0	5.0	0.9	2.2

3T3: After 4 days of incubation in medium containing 10% of the normal concentration of histidine and glutamine (6.5×10^5 cells/dish), the regular concentrations of histidine and glutamine were added back and the cultures were isolated for cyclic nucleotide determinations at the stated times.
SV3T3: Simian virus 40-transformed 3T3-4A cells were grown to a density of 6.1×10^6 per dish, cell monolayers were washed twice, and medium containing 0.5% of the normal leucine concentration and 5% dialyzed serum were added back and incubated a further 2 days (5.5×10^6 per dish). Then regular concentrations of leucine were restored and cultures were isolated as above. Cell density 30 hr later was 9.9×10^6 per dish.
Cyclic nucleotide determination was performed by digesting half of each sample with 3′-5′ cyclic nucleotide phosphodiesterase. The values obtained were deducted to yield the final results. Duplicates agreed to within 15% and results are expressed in picomoles of cyclic nucleotide per 10^6 cells.

SV40-transformed fibroblasts cannot be arrested by nutrient starvation or density inhibition or serum depletion in a stable G_0 state as their untransformed counterparts. This lack of a quiescent G_0 state is also reflected in their internal cyclic nucleotide levels. Table II shows that no major changes are observed in transformed cells in growing subconfluent or confluent cultures or "serum-activated" cultures.

In contrast, normal fibroblasts show very pronounced changes, especially in their internal cGMP levels. Cyclic GMP is decreased 2-5-fold in resting as compared to growing cultures, and increased 10-20-fold in activated cultures 20 min after serum addition. These changes are even more pronounced for their cAMP to cGMP ratios, which reflect in a very sensitive way their different growth states (Table II).

The largest change in cGMP occurs 20 min after growth induction and seems to reflect more the transition of the internal cellular milieu from the state of quiescence to a state of growth, rather than the particular kind of mitogenic stimulus used. In Fig. 1 we compare the amount of DNA synthesis observed with the cGMP concentration measured at the height of the peak 20 min after growth induction, when levels are about 20-fold increased. These data were extracted from different series of experiments where we used either serum or different concentrations of fibroblast growth factors or amino acids as growth stimulus. There is a rather good linear correlation between the cGMP amount at 20 min and the amount of DNA synthesis induced in these cells. Only experiments in which large cAMP changes occurred (insulin or prostaglandin E_1 together with serum) did not fit this curve. In those cases it might be more appropriate to plot the cAMP to cGMP ratio vs. DNA synthesis.

If cGMP is involved in the control of cellular growth, it is important to measure not only internal cGMP levels, but also the enzymes probably involved in the generation of the transient cGMP peak: guanylcyclase and phosphodiesterase. This is of particular interest with respect to possible differences between normal and transformed cells.

TABLE II. Cyclic Nucleotides in Normal and Polyoma- or SV40-Transformed Fibroblasts Under Different Growth Conditions

	Normal				Transformed			
	labeled nuclei (%)	cA	cG	cA/cG	(%) l.n.	cA	cG	cA/cG
Growing	70–90	8	1–2	4–8	80–90	8	2	4
Confluent	0.5–2.0	16	0.4–0.8	>20	80–90	8	2	4
Activated (20 min serum)	70–90	4	4–10	< 1	80–90	6	2	3

Twenty culture dishes for cyclic nucleotide determinations wre isolated after 2 or 3 days on reaching a quarter to half of the final cell density (growing), after 8–9 days (confluent) (3–4 days after the cessation of DNA synthesis in nontransformed cells or at confluency for the transformed cells), or after 20 min following addition of fresh medium and 20% serum to confluent cultures (activated). The cyclic nucleotide amounts are expressed in picomoles per milligram cell protein. DNA synthesis is expressed by the percentage of cells with radioactively labeled nuclei after exposure to [^{3}H] thymidine for 24 hr before isolation of growing cultures or from 8 to 32 hr after the time of medium change for resting and activated cultures.
The results shown in this Table are the average taken from experiments with several different cell lines (3T3 BALB/c, 3T3-4A, BSC-1, BHK, SV3T3, PyBHK).

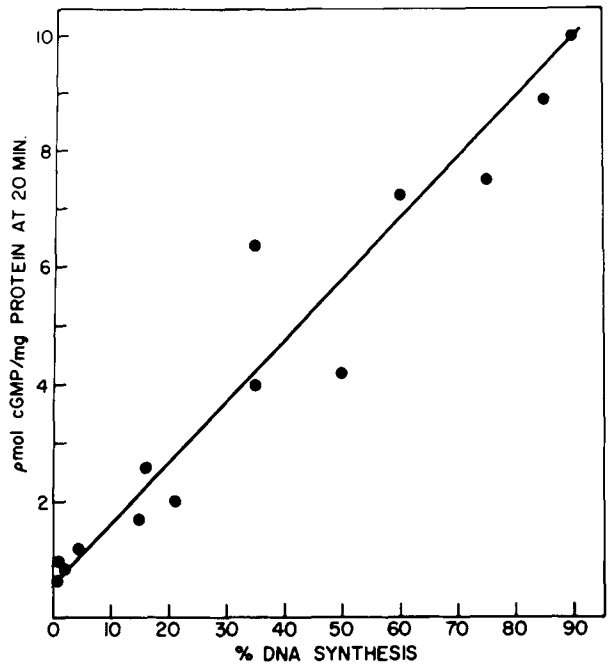

Fig. 1. Correlation between cGMP concentration measured 20 min after growth induction by serum, different concentrations of FGF, or amino acids. DNA synthesis as measured both by [^{3}H] thymidine incorporation and by autoradiography (percent labeled nuclei) is expressed as percentage of the maximal stimulation by 20% serum.

We have measured guanylcyclase activity in 3T3 cells previously and found that the enzyme activity was 10-20-fold increased by Triton treatment of plasmamembrane fractions and that this Triton-stimulated activity comigrated with adenylcyclase and 5′-AMPase of the plasma membrane on sucrose gradients (12). We also found evidence for a direct stimulation of membrane-bound guanylcyclase by fibroblast growth factor. This latter result has to be regarded as a soft fact, since we had problems recently to reproduce it consistently with our present FGF and membrane preparations for reasons that are not yet clarified. We are currently trying to find which variables are involved.

In Table III the guanylcyclase activity of 3T3 and SV3T3 membrane preparations is compared under standard conditions at 0.2 mM GTP and 2 mM Mn^{++}. Under these conditions the activity in SV3T3 cells was not increased, but rather decreased by about 30–50% as compared to 3T3 cells.

DISCUSSION

When untransformed fibroblasts cease growing due to a lack of serum growth factors, the entire cell population becomes arrested at a specific point (G_0 or restriction point[11]) in the cell cycle. Artificial deprivation of certain nutrients, however, can arrest cells predominatly in G_0 or randomly around the entire cell cycle (8, 9).

Our experiments show that the early, transient changes in internal cyclic nucleotide concentrations upon growth activation of the resting cultures occur only in the G_0-arrested cultures (Table I). In contrast, the randomly arrested cells exhibit no significant change in cAMP or cGMP after reactivation, since only a small fraction of the cells passing through early G_1 phase could produce these early changes.

We have shown previously that the transient 10-20-fold cGMP increase is observed only when cells move out of G_0 by activation with serum or fibroblast growth factor FGF or when synchronized cells move through early G_1 phase in the cell cycle (5, 7, 13). No major change was observed during the rest of the cycle. The experiments with amino acid starvation and reactivation demonstrate that cells that are arrested randomly or at least not in a G_0 state will not exhibit these changes in cyclic nucleotides which seem to reflect the cellular state (presumably of the plasmamembrane) during the cycle rather than to depend on the specific type of mitogenic stimulus used.

This conclusion is supported by the fact that in a variety of fibroblast cells the quiescent G_0 state is characterized by a very high cAMP to cGMP ratio (Table II) (14). This ratio drops to below 1.0 in a 20 min activated state, mainly due to the approximate

TABLE III. Guanylcyclase Activity

	pmol cGMP/min/mg protein		
	Cytoplasmic supernatant	Microsomal pellet	Plasmamembrane pellet
3T3	20	17	35
SV-3T3	23	9	23

One or two hundred 9 cm dishes with confluent 3T3 or SV3T3 cells were harvested and microsomal pellets or purified plasmembrane fractions prepared as previously described (7). Guanylcyclase activity was measured at 2 mM Mn^{++} and 0.2 mM GTP, pH 7.6, by determining the cGMP synthesized in 10 min at 37°C with a radioimmune assay.

20-fold rise in cGMP, whenever cells are stimulated by serum, growth factors, or nutrients to move out of G_0 and back into the cycle for proliferation. Many transformed cells have lost the ability to rest in a G_0 state of quiescence and therefore show low cAMP to cGMP ratios under all growth conditions. Figure 2 summarizes these conclusions in a simplified way.

We have proposed that cGMP is the intracellular mediator of the positive pleiotypic and mitogenic program of the cell (5, 13). Although both in lymphocytes and in fibroblasts the prominent transient cGMP peak is one of the earliest responses in these cells to growth activation, this remains a hypothesis until the underlying biochemistry has been worked out. However, Fig. 1 shows that there is at least some reasonably good correlation between the height of the cGMP peak at 20 min after growth activation and the amount of DNA synthesis observed in these cultures. The autoradiography data show that a 20–30% cGMP peak will only stimulate about 20–30% of the cells for DNA synthesis. Since we measure the average cGMP content of the total population, we can assume that in this percentage of cells a full cGMP peak (100%) was produced. It seems reasonable that only those cells will be pushed out of the G_0 state where the cGMP signal reaches a certain threshold. The reason for this heterogeneity within a given cell population is totally unclear. It could be due to differences in the number or activity of receptors and guanylcyclase molecules in the cell membrane or to variations in cell size and therefore internal cGMP concentration.

Finally, we should like to propose a new molecular hypothesis to explain the growth properties of transformed cells. This hypothesis is outlined in Figs. 1, 3, 4. We propose that transformed cells have an activated guanylcyclase system or a relaxed cGMP pleiotypic response mechanism either permanently or only at the restriction point of their cell cycle early in the G_1 phase.

This would explain at least three prominent properties of transformed cells such as SV3T3: (1) their low serum requirement, since they would not need growth factors to activate guanylcyclase for the delivery of the cGMP signal at the restriction point; (2) their insensitivity to density inhibition or serum depletion: delivery of the cGMP signal or

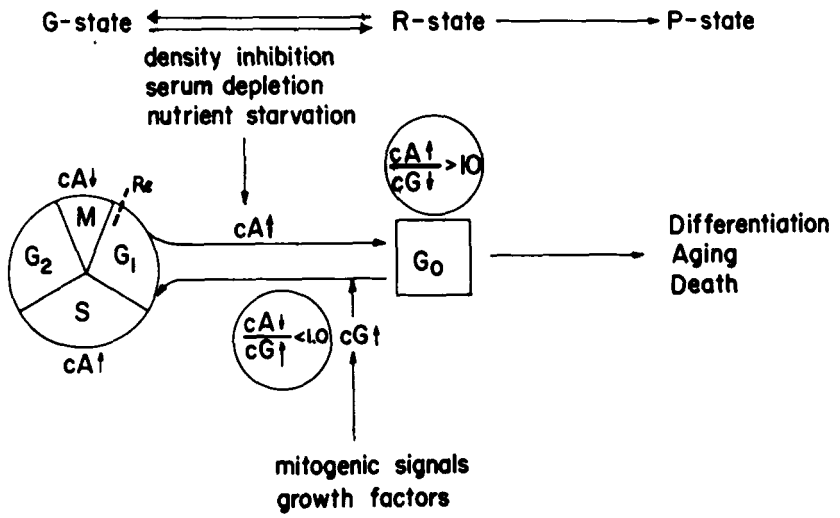

Fig. 2. Scheme of the cell cycle and the major cAMP-cGMP changes. G, growing state; R, resting state (arrest in G_0 or in an alternative concept at the restriction point Re); P, final programmed state.

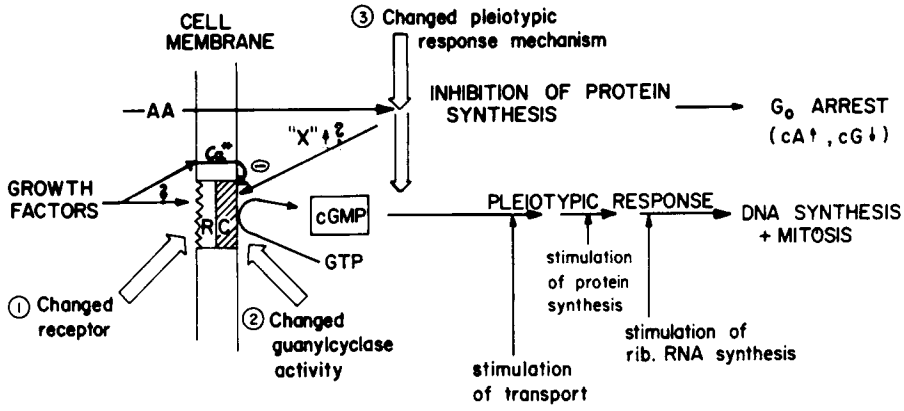

Fig. 3. Possible molecular changes in transformed cells.

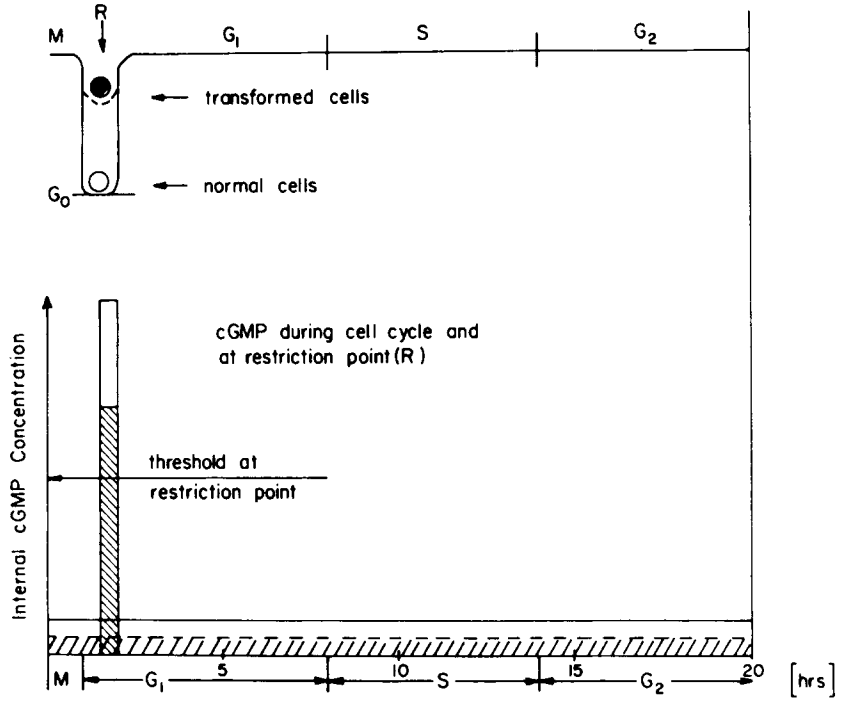

Fig. 4. Cyclic GMP during cell cycle and at restriction point (R)

activation of the positive pleiotypic program prevents arrest in a G_0 state or at the restriction point; and (3) their inability to become arrested in a stable G_0 state of quiescence by any other means such as amino acid starvation, in contrast to untransformed fibroblasts.

It is still an open question if growth factors like FGF activate membrane-bound guanylcyclase in a direct way via an external receptor similar to the adenyl cyclase system. Certainly other possibilities such as the indirect activation by a stimulated Ca^{++} influx should be considered as well.

In experiments with temperature-sensitive 3T3 mutants we could show that FGF did not activate the cells at low temperature where they exhibit transformed growth properties, but did activate the normal phenotype at the high temperature. If this is due to a lack of binding to surface receptors or to a failure to activate the guanylcyclase is not yet known, but it certainly points to a temperature-sensitive surface change for which FGF is a useful probe (15). The experiments with amino acid starvation demonstrate a difference between normal and transformed cells which reminds us of the "stringent-relaxed" regulation in Escherichia coli. Transformed cells behave in some way like relaxed mutants. This could be due to a change in the regulatory properties of the guanylcyclase system or to some other change in their pleiotypic response mechanism.

As shown in Table III, we did not detect an increased basal guanylcyclase activity in SV3T3 cells as compared to 3T3 cells nor a significant difference in Triton-released activity (not shown) under our assay conditions. But we consider such experiments as very preliminary and irrelevant for the hypothesis stated before for two reasons: (1) we have to be aware that the guanylcyclase assay has been done under conditions for optimal in vitro activity (2 mM Mn^{++}) which may be quite different from the actual in vivo conditions. Differences between normal and transformed cells with respect to the guanylcyclase system may show up if this enzyme system is more carefully studied under a variety of conditions; and (2) the SV3T3 cells used were unsynchronized cultures. If the guanylcyclase would be activated only at the restriction point early in the G_1 phase as a consequence of some membrane change during the cell cycle, this change in activity would not be detected in these assays where we measure an average over the entire cell cycle – for the same reason as discussed above for the internal cGMP levels in the case of unsynchronized growing or randomly arrested cultures. Clearly, studies with synchronized transformed and untransformed cells are needed in order to prove or disprove this hypothesis.

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