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Cyclic Nucleotides and Growth Regulation in BHK Cells (41259)

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Abstract. The role of cyclic nucleotides in the hormonal regulation of proliferation was evaluated in BHK cells. Sparse quiescent cultures in low (0.5%) serum could be stimulated to enter DNA synthesis by addition of physiological concentrations of insulin or epidermal growth factor (EGF). The activity of the two hormones together was greater than either alone, but was less than additive. Their effects were maintained for at least 3 days and led to as high as a sixfold increase in cell number. Dexamethasone was inhibitory under most conditions, but stimulated in the presence of high levels of insulin. NSILA-S (nonsuppressable insulin-like activity) also stimulated DNA synthesis, whereas $PGF_{2\alpha}$ and several other hormones were ineffective. Serum, EGF, insulin, and NSILA-S all decreased cAMP levels. However, $PGF_{2\alpha}$, which was not mitogenic, also decreased cAMP levels. Also, the combination of EGF and insulin lowered cAMP levels to the same extent as 10% serum but was much less effective in stimulating growth. Cyclic GMP levels were exceedingly low in BHK cells (3.5 fmole/mg protein). In exponentially dividing cultures, insulin and EGF had no effect on cGMP levels. Transformed BHK cells had sixfold-elevated cGMP levels, whereas cAMP levels were unchanged. Both cyclic nucleotides increased in confluent cultures. These studies suggest that a fall in cAMP accompanies growth activation but is not sufficient to induce DNA synthesis. In addition, they provide further support against a mitogenic role for cGMP.

A number of different hormones have been implicated in the regulation of growth of cultured fibroblasts (1). For example, fibroblast growth factor (FGF) (2), epidermal growth factor (EGF) (3, 4), glucocorticoids (5), insulin (6), $PGF_{2\alpha}$ (7), and others have been cited as activators of serum-starved, quiescent fibroblasts. Numerous studies have further suggested cyclic nucleotide involvement in growth stimulation (for reviews see (8–10)). Falling levels of cAMP and rising levels of cGMP were generally found to accompany growth stimulation by serum, and the effects of these nucleotides on transport and macromolecular synthesis led to the postulate that they were negative and positive "pleiotypic mediators," respectively (11, 12). These views are consistent with studies in malignantly transformed fibroblasts in which cAMP levels decreased and cGMP increased as compared with their untransformed counterparts (13). More recently the involvement of cGMP in these phenomena has been seriously questioned (14, 15), in part because of the inability to reproduce some of the earlier findings. Studies with cAMP have also shown some inconsistencies with the postulated regulatory role (7). One approach to shed more light upon this controversy is to study the effect of hormonal stimulation of quiescent fibroblasts upon cyclic nucleotide levels.

The present studies explore the actions of insulin, EGF, and several other hormones in baby hamster kidney (BHK) fibroblasts. The effects of these agents on cyclic nucleotide levels are examined and compared with those of serum. It is shown that all of the agents which stimulate growth also lower cAMP levels. However, inconsistencies in the data suggest that the fall in cAMP may not be the only event required for the release from quiescence. Measurements of cGMP revealed exceedingly low levels in normal BHK cells. The levels were unaffected by the hormones but were elevated in polyoma-transformed cells.

Materials and Methods. *Cell Cultures.* Normal BHK cells and the polyoma-

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transformed cells derived from them were a gift from the laboratory of Dr. G. Di Mayorca, Abraham Lincoln School of Medicine, Chicago. Attached cultures were grown at 37° in a 5% CO_2 atmosphere in closed Falcon flasks in Dulbecco's modified Eagle's medium containing 10% tryptose broth and 10% calf serum (complete medium). Stock cultures, from which experimental cells were obtained, were passaged twice a week. They were cultured for 6 weeks, after which new stock cultures were initiated from the frozen state. Sparse quiescent (serum-starved) cultures were induced by plating 7×10^5 cells per 25 cm^2 flask in 5 ml of complete medium. After 1 day, the medium was replaced with 5 ml medium containing only 0.5% calf serum. The medium was replaced 48 hr later with 5 ml of the same fresh medium and 24 hr later the experiments were begun by addition of 10% serum or hormones.

Cell number. Attached cells were removed by incubation with 1 ml of 0.05% trypsin and 0.6 m*M* EDTA in a balanced salt solution at 37°. Following detachment, 4 ml of cold complete medium was added, and cell number was determined in a model F_N Coulter counter.

Thymidine incorporation. Eight to ten hours after addition of serum or hormones, [^{3}H]thymidine (10 μCi/flask, 67.5 μCi/μmole) was added to each flask. Fourteen to sixteen hours later (24 hr after activation) the cells were removed with trypsin-EDTA and the suspension was pipetted several times to disperse clumps. Aliquots containing 2 to 8×10^5 cells were removed and sedimented. The cells were suspended, swollen, and applied to microscope slides as described previously (16). The slides were air dried and then fixed for 10 min in cold 7% trichloroacetic acid, followed by 10-min washes each with 70% ethanol and 95% ethanol. After drying, the slides were dipped in Kodak NTB emulsion, exposed in the dark at 4° for 5 days, and developed. The percentage of labeled nuclei (labeling index) was determined microscopically in at least 200 cells, in each of the two droplets. All experiments were performed with duplicate Falcon flasks.

Cyclic nucleotide determinations. The culture medium was decanted, followed by the addition of 5 ml ice-cold 0.3 *N* perchloric acid, containing tritiated cyclic nucleotides to permit correction for losses during chromatography. The flasks were stored at −70°. Prior to chromatography, cells were lysed by thawing and freezing five times. To determine both cGMP and cAMP content in the same samples, the perchloric acid extracts were first clarified by centrifugation and the nucleotides were then purified by sequential chromatography over acidic alumina and neutral Dowex-50 columns (17). In experiments where only cyclic AMP was measured, the acidic alumina columns were omitted and Dowex-50 columns, equilibrated with 0.1 *N* HCl, were used. Column fractions containing the cyclic nucleotides were lyophilized and resuspended in 0.5 ml of the appropriate assay buffer. cGMP was measured by radioimmunoassay using a modification (18) of the acetylation method of Harper and Brooker (19). The cGMP antiserum was a gift from Dr. J. Bomboy, V. A. Hospital, Nashville, Tennessee. cAMP was measured by the competitive protein binding method of Gilman (20) using peak II protein kinase from rabbit skeletal muscle. Results are expressed as femtomoles per 10^6 cells for cGMP and picomoles per 10^6 cells for cAMP.

Materials. The following compounds were obtained from the sources indicated: insulin, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), dexamethasone, cAMP, cGMP (Sigma Chemical Co., St. Louis, Mo.); [^{3}H]thymidine (ICN, Irvine, Calif); Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, N.Y.); [^{3}H]cAMP, [^{3}H]cGMP (Schwarz/Mann, Orangeburg, N.Y.); 8-Br-cGMP (Boehringer Mannheim, Mannheim, West Germany); 3-isobutyl-1-methylxanthine (Aldrich Chemical Co., Milwaukee, Wisc.). Glucagon was a gift from Eli Lilly & Co., Indianapolis, Indiana. EGF was a gift from Dr. Stanley Cohen. Nonsuppressable insulin-like activity (NSILA-S) was a gift from Dr. E. R. Froesch.

Results. *Hormonal effects on release from quiescence.* The mitogenic effect of a number of hormones on quiescent BHK

cells was first characterized. Sparse quiescent cultures were prepared in low serum and then either 10% serum, insulin (10^{-7} *M*), or EGF (25 ng/ml) was added. The ability to induce DNA synthesis was analyzed autoradiographically following a 16- to 18-hr exposure to [^{3}H]thymidine beginning 6–8 hr after stimulation. The results of this series of studies showed considerable variability between experiments. Serum stimulated the incorporation in 60–90%, insulin in 15–45%, and EGF in 6–35% of the cells. The variation was apparently related to the age of the cultures (Fig. 1). The older the cultures, the greater was the effect of each of the agents. This was especially striking with EGF which had almost no effect in young cultures. With increasing age of the cultures it also became increasingly difficult to induce complete quiescence (saline control, Fig. 1). After 40 days in culture, 17% of the serum-starved cells entered S phase in the absence of hormone or serum addition.

Combinations of EGF and insulin generally stimulated more cells to synthesize DNA than either alone, but their actions were considerably less than additive (Fig. 3, Table I). This suggests that the two hormones act upon the same class of cells and that some additional cells may require both hormones to surpass their threshold for stimulation.

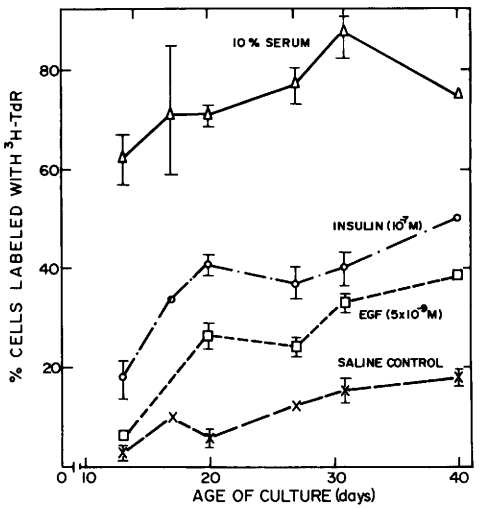

FIG. 1. Effect of age of cultures upon stimulation of DNA synthesis by serum, insulin, and EGF. Preparation of quiescent cultures, thymidine labeling, and autoradiography were as described under Materials and Methods. The age of the cultures refers to the time from the planting of stock cultures from the frozen state until the start of the quiescence protocol. Points are the means and standard deviations of four determinations.

The dose–response relationship for insulin and EGF and combinations of the two on DNA synthesis are seen in Fig. 2. Insulin exhibited measurable activity at 10^{-11} *M* and marked stimulation at 10^{-10} *M*. The curve was biphasic, perhaps indicating that insulin at high concentrations was acting by binding to noninsulin receptors. EGF was effective at levels as low as 10^{-10} *M*.

Dexamethasone was also tested for its ability to stimulate DNA synthesis. Glucocorticoids have been shown previously to be effective in some fibroblasts cell lines (5), especially in combination with FGF (2). In the present studies we found that dexamethasone (100 ng/ml) alone or in the presence of EGF, had no effect or was slightly inhibitory. However, it enhanced the effect of high concentrations of insulin (Fig. 2). With combinations of insulin and EGF, dexamethasone also stimulated at the higher hormone concentrations.

The action of several other hormones

TABLE I. EFFECT OF VARIOUS AGENTS ON THE LABELING INDEX IN BHK CELLS AFTER RELEASE FROM QUIESCENCE

Addition	Labeling index (percentage)
BSA	14.3 ± 1.0
Insulin (10–8 *M*)	32.5 ± 1.3
EGF (10 ng/ml)	31.9 ± 0.6
NSILA-S (10 ng/ml)	41.1 ± 0.2
$PGF_{2\alpha}$ (200 ng/ml)	15.1 ± 1.7
Insulin + EGF	41.1 ± 1.5
Insulin + $PGF_{2\alpha}$	30.0 ± 2.0
EGF + $PGF_{2\alpha}$	24.5 ± 2.3
EGF + NSILA-S	47.4 ± 3.0
10% calf serum	54.2 ± 1.0

Note. BHK cells were released from quiescence by various agents at 0 time. At 6 hr [^{3}H]thymidine was added for 18 hr. The labeling index was determined as described under Materials and Methods. Each value is the mean ± SEM of three determinations.

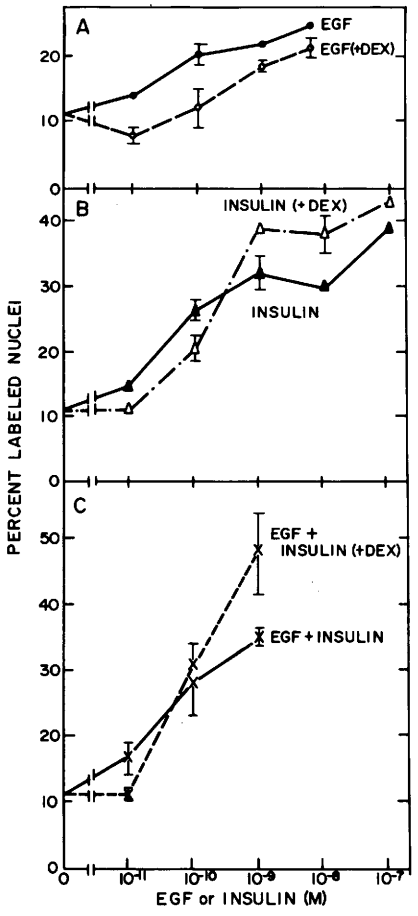

FIG. 2. Concentration dependence of cell activation by insulin and EGF in the presence and absence of dexamethasone. The ability to stimulate DNA synthesis of various concentrations of EGF (A), insulin (B), or the combinations of EGF and insulin (C) was tested. Dexamethasone (DEX), was 100 ng/ml. Cultures were obtained from the frozen state 28 days prior to the start of the experiment.

were also tested. Agents having little or no effect, alone or in combination with insulin, included $PGF_{2\alpha}$ (Table I) glucagon (10^{-7} *M*), isoproterenol (10^{-5} *M*), cGMP ($10^{-4}-10^{-7}$ *M*) and 8-Br-cGMP ($10^{-4}-10^{-7}$ *M*) (data not shown). Adenosine (5×10^{-5} *M*) stimulated slightly in some experiments. A partially purified preparation of NSILA-S was a potent stimulator, equal to that of insulin and EGF combined (Table I).

To determine whether insulin and EGF would also induce cell division and further rounds of DNA synthesis, cultures were treated daily with the agents for 3 days. Labeling index (Fig. 3) and cell number (Fig. 4) were monitored. The results demonstrated that these agents could support division and that the effects could be maintained for a 3-day period. Although none of the hormone combinations were as effective as 10% serum (10-fold increase), combinations of insulin, EGF, and dexamethasone induced a 6-fold increase in cell number in 3 days. Of interest was the finding that the combined action of insulin and EGF upon cell number was synergistic, though it was less than additive on DNA synthesis, suggesting that together these hormones may enhance the traverse of cells through G-2 and mitosis.

Hormonal effects upon cellular cyclic nucleotide levels. cAMP levels were first measured following a 10-min exposure to

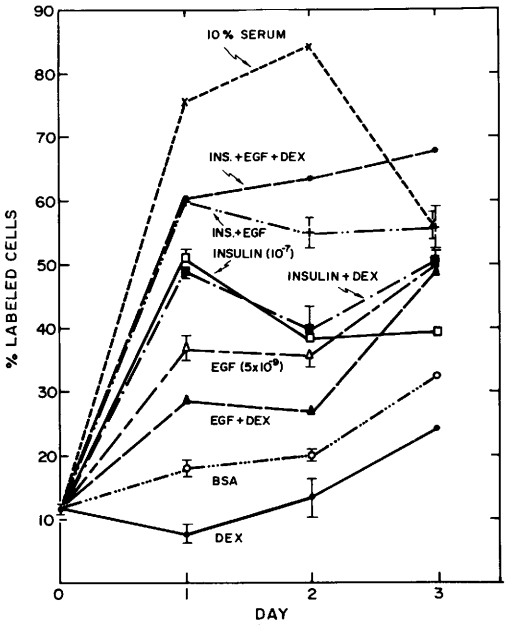

FIG. 3. Effects of insulin, EGF, and dexamethasone upon labeling index over a 3-day period. Cultures were obtained from the frozen state 40 days prior to the start of the experiment. Quiescent cultures were prepared as described under Materials and Methods. Zero time is taken as the time of hormone or serum addition. The medium was replaced at 27 and 53 hr with fresh medium containing 0.5% calf serum and the indicated hormones. [^{3}H]Thymidine was added 16 hr before harvest. Dexamethasone (DEX) concentration was 100 ng/ml. Points are the means ± SD of six determinations.

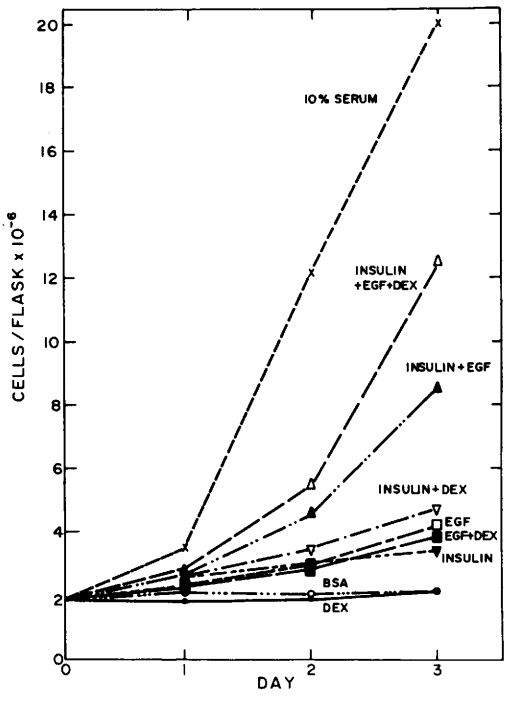

FIG. 4. Effects of insulin, EGF, and dexamethasone upon cell density over a 3-day period. Conditions are identical with those in Fig. 3. Cell numbers were determined as described under Materials and Methods. Values are the averages of three determinations.

insulin, EGF, or a combination of the two. In an attempt to distinguish between potential effects on adenylate cyclase and cAMP phosphodiesterase, these experiments were carried out in the presence or absence of 3-isobutyl-1-methylxanthine (IBMX), an inhibitor of cyclic nucleotide phosphodiesterases. The effects of IBMX on cAMP content were dose dependent (Table II). The level of cAMP was decreased about 25% with insulin and about 35% with EGF. The effects of insulin and EGF were additive. However, in the presence of IBMX, the effect of insulin, but not EGF, was abolished. This is consistent with the possibility that insulin acts through effects upon the phosphodiesterase rather than the adenylate cyclase.

The time course over a 30-min period of the effects of these hormones on cAMP levels as well as that of the effects of several other hormones is seen in Table III. Insulin (10^{-8} *M*) lowered cAMP levels

TABLE II. EFFECTS OF INSULIN AND EGF ON BHK cAMP LEVELS

Addition	pmole cAMP/10^6 cells	
	−IBMX	+IBMX
None	3.14 ± 0.27	8.50 ± 0.19
Insulin (10−7 M)	2.34 ± 0.18	7.78 ± 0.51
EGF (25 ng/ml)	2.03 ± 0.29	5.54 ± 0.25
EGF + Insulin	1.38 ± 0.25	5.18 ± 0.08
Carbachol (10−5 M)	2.20 ± 0.11	7.29 ± 0.36
IBMX (*μM*)		
50	6.10 ± 0.15	
100	8.50 ± 0.19	
500	15.20 ± 1.30	

Note. Quiescent BHK cells were treated with the indicated hormones in the presence or absence of 100 *μM* IBMX. After 10 min, the medium was decanted, 3 ml of ice-cold 0.3 *N* perchloric acid was added, and cAMP was purified and assayed. Each value represents the mean ± SEM from three flasks, each assayed in duplicate. Also shown are the effects of three concentrations of IBMX.

within 5 min and the effect persisted for the 30-min test interval. The action of EGF also persisted and EGF was more effective than insulin at both 15 and 30 min. Together the effect of the two hormones again appeared to be additive. The combined effect of the two hormones was equal to or greater than that of 10% serum.

NSILA-S also caused a significant decrease in cAMP at 30 min, under conditions where it was a potent stimulator of cell divi-

TABLE III. TIME COURSE OF THE CHANGES IN cAMP LEVELS AFTER RELEASE FROM QUIESCENCE

Addition	pmole cAMP/sample		
	5 min	15 min	30 min
None	7.4 ± 0.4	—	8.2 ± 1.1
Insulin (10−8 *M*)	5.9 ± 0.5	6.5 ± 1.2	6.1 ± 0.6
EGF (10 ng/ml)	6.2 ± 0.5	4.5 ± 0.3	5.5 ± 0.3
Insulin + EGF	5.1 ± 0.4	3.8 ± 0.4	4.7 ± 0.3
NSILA-S (10 μg/ml)	—	—	3.8 ± 0.4
$PGF_{2\alpha}$ (200 ng/ml)	6.5 ± 0.6	5.8 ± 0.2	6.4 ± 0.2
Insulin + $PGF_{2\alpha}$	7.7 ± 1.0	6.2 ± 0.2	5.3 ± 0.2
EGF + $PGF_{2\alpha}$	6.1 ± 0.3	4.8 ± 0.4	6.9 ± 0.8
EGF + NSILA-S	—	—	3.8 ± 0.4
10% calf serum	6.5 ± 0.6	4.9 ± 0.9	4.5 ± 0.1

Note. BHK cells were made quiescent as described under Materials and Methods. Additions were made for varying time intervals, the medium was poured off, 3 ml of ice-cold 0.3 *N* perchloric acid was added, and cAMP was assayed. Each value represents the mean ± SEM from three flasks. cAMP in flasks without addition was 7.00 ± 0.2 pmole.

sion, equivalent to the combination of insulin and EGF (compare Tables I and III). By contrast, $PGF_{2\alpha}$ had no effect on cell division, but did lower cAMP levels to about the same extent as insulin (Tables I and III).

Initially we did not detect cGMP in BHK cells, even with amounts of cells calculated to contain levels $^1/_{10}$ those previously reported in fibroblasts. However, with appreciably larger numbers of cells (46×10^6) we found cGMP levels of about 1.0 fmole/10^6 cells (3.5 fmole/mg protein) (Table IV). By comparison, values in 3T3 cells have been reported to be at least two orders of magnitude higher (7, 13, 14). Control experiments with internal cGMP standards, as well as measurements with varying amounts of tissue, indicated that the large amounts of cell extract did not adversely affect the assay. The intracellular cGMP was further shown to be destroyed by cyclic nucleotide phosphodiesterase treatment. Since we found cGMP levels in a number of other tissues comparable to that reported by others, we concluded that the BHK cell line has extremely low cGMP levels.

Because of the technical difficulties in measuring cGMP, we could not determine the hormonal effects following release from quiescence on sparse cultures as described above for cAMP. The effects of hormones were tested instead in logarithmically growing cells (Table IV). BHK cells were grown to a concentration of about 15×10^6 cells/75-cm^2 flask. Hormones were added, and at short intervals thereafter perchloric acid was added. Perchloric acid extracts from three flasks were pooled, chromatographed, and assayed for cGMP. Insulin (10^{-8} *M*), EGF (10 ng/ml), and combinations of the two were without effect on cGMP levels after either 5 or 15 min of incubation (Table IV). NaN_3 (1 m*M*), a well known stimulator of guanylate cyclase (21), was also tested. This agent increased cGMP more than twofold in 5 min. The effect was diminished, but still evident at 15 min.

There has been a great deal of interest in the possible role of cyclic nucleotides in cell transformation. We have therefore compared the levels of both cAMP and cGMP in normal and polyoma-transformed BHK cells. The two cell lines had been derived from the same cell stock approximately 10 cell generations prior to the experiment. The comparison revealed essentially no difference in cAMP levels in the two cell lines (Table V). More interesting was the finding that cGMP levels were markedly elevated in the transformed cell line (Table V). Also shown in Table V are the effects of confluency. Consistent with the report by Miller *et al.* (14), with 3T3 cells, the levels of both cAMP and cGMP increased at confluency although the changes in cAMP were

TABLE IV. REGULATION OF cGMP IN BHK CELLS

Additions	Cyclic GMP (fmole/10^6 cells)			
	Normal[a]		Polyoma-infected[b]	
	5 min	15 min	5 min	15 min
BSA	1.45 ± 0.44[c]	1.14 ± 0.13	4.98 ± 0.57	4.01 ± 0.80
Insulin (10^{-8} *M*)	1.08 ± 0.09	1.05 ± 0.11	4.20 ± 0.12	4.77 ± 0.17
EGF (10 ng/ml)	1.22 ± 0.18	1.03 ± 0.12	4.64 ± 0.70	3.21 ± 0.20
EGF + insulin	1.52 ± 0.38	1.26 ± 0.15	—	2.93 ± 0.27
NaN_3 (1 m*M*)	3.77 ± 0.57	2.80 ± 0.10	—	5.32 ± 0.41

[a] Normal BHK cells were grown in 75-cm^2 flasks to a density of 15.3×10^6 cells. Additions were added for 5 or 15 min and then 0.3 *N* perchloric acid was added. The cells were scraped and the perchloric acid–cell suspensions from three flasks were pooled prior to chromatography and then were assayed in duplicate. This experiment was done three times.

[b] Polyoma-infected cells were grown in 75-cm^2 flasks to a density of 25.3×10^6 cells. The experiment was carried out as for the normal cells, but cell extracts were not pooled.

[c] Mean ± SEM of three experiments.

TABLE V. CYCLIC NUCLEOTIDE CONCENTRATIONS IN NORMAL AND POLYOMA BHK FIBROBLASTS[a]

Growth phase	Cells per flask (10^6)	Flasks pooled	Total cell number (10^6)	Total cAMP (pmole)	cAMP per 10^6 cells (pmole)	Total cGMP (fmole)	cGMP per 10^6 cells (fmole)
			Normal BHK				
Logarithmic	27.5	4	110	70.6 ± 8.3[b]	0.64	71 ± 11	0.65
2-Day confluent	37.8	2	75.6	56.6 ± 9.0	0.75	156 ± 13	2.06
4-Day confluent	36.6	2	73.2	71.7 ± 3.7	0.98	118 ± 9	1.61
			Polyoma BHK				
Logarithmic	25.4	1	25.4	17.6 ± 1.9	0.69	99 ± 13	3.89
2-Day confluent	34.35	1	34.4	47.0 ± 2.0	1.37	338 ± 22	9.83
4-Day confluent	40.9	1	40.9	25.4 ± 2.3	0.62	331 ± 25	8.09

[a] cGMP and cAMP were measured in normal BHK cells and in polyoma-transformed BHK cells during logarithmic growth phase or after 2 and 4 days of confluency. Cells were grown in 75-cm^2 flasks.
[b] Mean ± SEM from three experiments; in each, samples were assayed in duplicate.

modest. In transformed cells cGMP levels also rose at confluency whereas cAMP levels were elevated after 2 days of confluency and decreased after 4 days.

Discussion. Several observations in the present studies are of interest with regard to hormonal effects on cell proliferation. First, although the dose–response curve is biphasic, significant effects of insulin were obtained at relatively low, nearly physiological concentrations. This result is contrary to the often-stated view that effects of insulin in fibroblasts are only observed at high nonphysiological concentrations (22). Second, the sensitivity of the cells to insulin and EGF varied greatly with the time in culture. This is shown both by the increased sensitivity to all of the agents in high passage cells, and to the observation that there is a reversal in relative effects of EGF and insulin several days after release from quiescence. These results suggest that the effective hormonal combination capable of maximally stimulating cell growth is a dynamic property of the cell. Finally, the results with dexamethasone indicate that the hormone facilitates the effect of high concentrations of insulin, but that it has no effect at low insulin or upon EGF.

In the present studies, there was a general correlation between the ability of the hormones to release cells from the quiescent state and their ability to lower cAMP levels. The effect of insulin upon cAMP levels in BHK cells confirms the previous report by de Asua *et al.* (6). In addition, we found that EGF and NSILA also lower cAMP levels and stimulate growth in these cells. While these results support a role for decreased cAMP as a possible regulatory event in the release from quiescence, the data also suggest that the fall in cAMP by itself is not sufficient to stimulate growth. That is, $PGF_{2\alpha}$ caused a decrease in cAMP that was comparable to that of insulin but did not stimulate growth. Furthermore, insulin and EGF together lowered cAMP to the same extent as 10% serum but were less effective in stimulating growth. A similar discrepancy has previously been reported in 3T3 cells which were stimulated with $PGF_{2\alpha}$ and insulin (7). Also in an L6 myoblast system, a decrease in cAMP was insufficient to trigger growth (23).

The studies with cGMP produced the surprising finding that the levels in BHK cells were two to three orders of magnitude lower than had previously been reported for fibroblasts (7, 13, 14). Because of this, measurements on sparse quiescent cultures were not feasible. However, the studies carried out with dense cultures support the views of Miller *et al.* (14) and Nesbitt *et al.* (15) that cGMP is not a growth regulator in fibroblasts. Thus, the levels were unaffected by insulin and EGF and levels increased in normal confluent cultures. On the other hand, the significance of the

marked elevation in the cGMP levels of transformed cells requires further investigation.

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