REGULATION OF PHOSPHOENOLPYRUVATE
CARBOXYKINASE AND TYROSINE TRANSAMINASE
IN HEPATOMA CELL CULTURES

III. Comparative Studies in H35, HTC, MH1C1, and RLC Cells

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ABSTRACT

The ability of N', O'-dibutyryl cyclic AMP (DBcAMP) to regulate a number of metabolic events in four lines of cultured rat hepatomas has been examined. Although dexamethasone induces tyrosine transaminase in all four lines, DBcAMP induces this enzyme normally only in H35 cells. A slight increase in transaminase activity was seen with MH1C1 cells and HTC cells, but no effect was detectable in RLC cells. In contrast, phosphoenolpyruvate carboxykinase activity is increased by both agents in H35 and MH1C1 cells, but neither had any effect in HTC or RLC cells.

DBcAMP caused a rapid inhibition of the growth rate and DNA synthesis and an increase in protein content in both H35 and MH1C1 cells but not in HTC or RLC cells. The effect of DBcAMP on DNA synthesis in MH1C1 cells could be reversed by deoxycytidine as is also the case with H35 cells.

The resistance of HTC and RLC cells to DBcAMP was not due to reduced uptake or deacylation as judged by studies with [3H]DBcAMP. The cyclic nucleotide appears to enter the cells by passive diffusion as the intracellular concentration approaches that in the medium within 30-60 min. Possible explanations for the differential responses observed are discussed.

INTRODUCTION

In the past few years increasing emphasis has been placed on the use of cultured cells in studies of biochemical regulatory mechanisms in eukaryotic systems. A limited variety of cell lines derived from chemically induced rat hepatomas1 (1-4) have been placed in culture and have proven to be especially useful in studies of the mechanism of regulation of specific protein synthesis by horm-

1 Although the RLC cells were originally derived from normal rat liver, at the time of their use in our experiments, they appear to have become a spontaneous hepatoma. Injection of these cells into rats produces rapid tumor formation (5), and as a result, we have considered them to be hepatoma cells.
mones and cyclic AMP (cAMP). There does appear to be some justification in using cultured hepatoma cells as a model system to study the control of normal liver function. In the more "well-differentiated" hepatomas (1), especially the Reuber H35 (6, 7) and MH1C1 (3, 8-11), a number of normal hepatic processes appear to persist as well as certain features of the regulatory circuits controlling these processes.

One of the major purposes for using cultured cells in these studies has been to search for or to generate variants in which some aspect of the regulatory system under investigation is selectively altered or deleted. Such variants could provide an important means of dissecting out the component parts of various regulatory processes (e.g., induction of tyrosine transaminase (EC 4.2.1.13) by glucocorticoids, insulin, and cAMP, etc.), as has been so successfully exploited in prokaryotic systems. To date, this potential has only been modestly explored with hepatoma cells either through extensive cloning (12), by repassage of the tumor cells in animals (13), or by somatic cell hybridization (14-18). The results, however, do provide support for the expectation that potentially useful regulatory variants may be obtained by suitable manipulation of cultured hepatoma cells.

We have approached this problem by analyzing the effects of N6,02'-dibutyryl cyclic AMP (DBcAMP) on a number of metabolic processes in four hepatoma cell lines to determine whether any of these might already be variant in their responses. As will be described in this report, certain differential responses to DBcAMP have been observed providing support for the specific expectation that variants with selective alterations in the cAMP regulatory system may in fact be obtained from existing cultured hepatoma cells.

MATERIALS AND METHODS

Cell Growth

All cell lines were grown in monolayer culture as described previously for Reuber H35 cells (6, 7, 19, 20) except that the final concentration of calf serum was 10% instead of 5%. The number of cells was determined with the aid of a hemacytometer or, in some cases, by measuring DNA content after harvesting by agitation with 1 mM EDTA (20). For the latter measurements the modified diphenylamine method of Burton was used (21). Protein was estimated by the method of Lowry et al. (22).

Enzyme Assays: After reaching confluency, cells were placed in serum-free medium 16-20 h before additions. 5 h after the various additions were made, the cells were harvested by scraping with a rubber policeman and collected by centrifugation (6, 7, 19, 20). Lysis was accomplished by three cycles of freezing and thawing in 0.15 M KCl-1 mM EDTA. Tyrosine transaminase and phosphoenolpyruvate (PEP) carboxykinase (EC 4.1.1.32) activities were assayed in the supernatant fraction after centrifugation of the lysates at 20,000 g for 20 min as described previously (23).

Incorporation of[^3H]Deoxyadenosine: The incorporation of[^3H]deoxyadenosine into cold trichloracetic acid-precipitable material was measured using the filter paper disk method of Mans and Novelli (24). Better than 90% of the radioactivity in this fraction could be accounted for as DNA nucleotides (25). The distribution of radioactivity incorporated from[^3H]deoxyadenosine into the DNA of the different cell lines was found to be analogous to that found previously in H35 DNA (25).

Uptake and Metabolism of[^8-3H]DBcAMP: [8-3H]DBcAMP was added at 0.5 mM so that the medium contained 110,000 dpm/ml. 1 h later the medium was removed and the cells were washed five times rapidly with cold saline. Finally, ethanol was added and the cells were scraped, freeze-thawed twice, and centrifuged. Aliquots of the supernatant fraction were counted, and the radioactivity in N6,02'-dibutyryl-, N6- and O2'-monobutyryl-, and free cAMP was determined after chromatography of the remaining material on Whatman 1 MM paper using the filter paper disk method of Mans and Novelli (24). Better than 90% of the radioactivity in this fraction could be accounted for as DNA nucleotides (25). The distribution of radioactivity incorporated from[^3H]deoxyadenosine into the DNA of the different cell lines was found to be analogous to that found previously in H35 DNA (25).

Chemicals and Radioisotopes: All tissue culture components were purchased from Grand Island Biological Co., Grand Island, New York or Flow Laboratories Ltd., Scotland.[^3H]Deoxyade-
nosine, \[^{3}H\]thymidine, and \[^{8-3}H\]DBcAMP were obtained from The Radiochemical Centre, Amer-
sham, Buckinghamshire, England. Thymidine, deoxy-
adenosine, DBcAMP, and N\(^6\)-MBcAMP were sup-
plied by Boehringer Mannheim Corp., New York.
Aquadol was obtained from New England Nuclear
Corp., Boston, Mass. All other chemicals were of
analytical grade. 8-Thio cAMP analogs were kindly
provided by Dr. M. Stout of the ICN Nucleic Acid
Research Institute, Irvine, Calif. O\(^2\)-MBcAMP was
obtained from Sigma Chemical Co., St. Louis, Mo.
The purity of labeled and unlabeled DBcAMP
was 97%.

**RESULTS**

**Enzyme Activity Changes**

As illustrated in Table I, both H35 and MH1C1
cells contain tyrosine transaminase and PEP
 carboxykinase in amounts comparable to those
found in rat liver (27). Exposure of these cell
lines to dexamethasone for 5 h produces qual-
tatively similar changes in both enzymic activities
as is also the case in rat liver (27, 28). Thus, as
with H35 cells (6), the activity of the transaminase
rises two- to fourfold after exposure of MH1C1
cells to the glucocorticoid (as has also been re-
ported by Tashjian et al. with cortisol [10]). The
carboxykinase is also elevated two- to threefold
in both cell lines (as previously reported for H35
cells [6]). (Overnight exposure of H35 and MH1C1
cells to dexamethasone produces a somewhat
greater elevation of both enzyme activities.)

In striking contrast to rat liver (27, 29) and H35
cells (6), however, DBcAMP causes only a slight
increase in the activity of the transaminase in
MH1C1 cells. At the same time, the activity of
the carboxykinase exhibits the same increase
seen in other hepatic systems (6, 27, 29). Longer
exposure of MH1C1 cells to DBcAMP (up to 16
h), substitution of 8-methylthio-cAMP (30) or
concomitant addition of 1 mM theophylline did
not alter the observed responses.

Insulin causes a two- to threefold elevation of
tyrosine transaminase activity in MH1C1 cells but
does not influence carboxykinase activity (data
not shown). The simultaneous addition of insulin
led to a marked inhibition of the rise in PEP car-
boxykinase activity produced by dexamethasone.

| Table I | Enzyme Induction by DBcAMP and Dexamethasone in Various Hepatoma Cell Cultures |
|---------|--------------------------------------------------------------------------------|
| Cell line | Additions | Concentration | Tyrosine transaminase (u/mg protein) | PEP carboxykinase (u/mg protein) |
| H35     | None      | 36.3 (13)      | 51.4 (13)                         |
|         | DBcAMP 0.5 mM | 107.3 (12)   | 95.6 (12)                        |
|         | Dexamethasone 0.2 µM | 171.4 (8) | 94.9 (8)                        |
| MH1C1   | None      | 46.1 (16)*    | 36.6 (11)                         |
|         | DBcAMP 0.5 mM | 57.8 (18)*   | 72.7 (13)                        |
|         | Dexamethasone 0.2 µM | 113.8 (10) | 112.4 (10)                      |
| HTC     | None      | 19.3 (5)      | 4.5 (5)                           |
|         | DBcAMP 0.5 mM | 26.0 (6)   | 4.7 (6)                           |
|         | Dexamethasone 0.1 µM | 42.7 (6) | 4.9 (6)                           |
| RLC     | None      | 5.1 (5)       | 3.5 (5)                           |
|         | DBcAMP 0.5 mM | 6.2 (10)   | 3.7 (10)                          |
|         | Dexamethasone 0.1 µM | 13.7 (5) | 3.4 (5)                           |

Cells near confluency (6-8 days except 12-14 days with MH1C1 cells) were placed in
serum-free medium 16-20 h before addition of inducers. Cells were harvested 5 h after
additions for assays as described in Materials and Methods. Each value represents the
average of the number of observations shown in parentheses with standard errors
ranging from 5 to 15%.

* The disparity in the number of observations between the two enzymes is due to the
fact that the results of one experiment in which the carboxykinase was not assayed are
included.

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and at the same time generated a synergistic increase in tyrosine transaminase activity in MH1C1 cells. These results are analogous to those previously reported with H35 cells (6). DBcAMP added together with insulin did not elevate transaminase activity in MH1C1 cells significantly above that seen with insulin alone. The increase in PEP carboxykinase activity normally produced by DBcAMP was, however, partially suppressed by inclusion of the pancreatic hormone, as in H35 cells (6).

Analysis of the characteristics of PEP carboxykinase in MH1C1 cells has revealed the same nucleotide (GDP ≥ IDP > ADP + UDP), and divalent cation (Mn++ > Fe++ > Mg++) specificity as the enzyme from rat liver and H35 cells (6, 29).

The activity of tyrosine transaminase and especially PEP carboxykinase is much lower in HTC cells than in rat liver or H35 cells (Table I). As reported by others, however, dexamethasone does produce a marked increase in transaminase activity (2, 12). DBcAMP produced a marginal increase in transaminase activity as has also been observed by others (31), but PEP carboxykinase activity was not altered by any of the treatments employed.

The addition of theophylline at 1 mM alone or in combination with DBcAMP was without effect on either enzyme in HTC cells. Although more prolonged incubation with dexamethasone did produce a greater increase in transaminase activity, prolonged exposure to DBcAMP did not alter the response of this enzyme and neither changed the results obtained with the carboxykinase. Addition of the 8-methylthio analog of cAMP produced a slightly greater increase in transaminase activity than with DBcAMP (53% vs. 35%), but PEP carboxykinase still did not exhibit any change in activity.

In terms of both reduced basal activities and responses to inducers, RLC cells are analogous to HTC cells (Table I). The effects of dexamethasone on tyrosine transaminase in RLC cells are similar to those reported by others (4). Alterations of the protocol such as those discussed above with MH1C1 and HTC cells also did not change the results significantly from those shown in Table I.

The effects of DBcAMP on cell growth and protein/DNA ratio: We have previously reported that DBcAMP specifically reduces the rate of growth of H35 cells (20), and the results in Table II show that MH1C1 cells also exhibit an increase in generation time after exposure to DBcAMP. In contrast, neither HTC nor RLC cells respond to the cyclic nucleotide even with concentrations as high as 2 mM. Exposure of these cells to 8-substituted analogs of cAMP, which are potent inhibitors of growth in H35 and MH1C1 cells (19, 20), also did not influence their rate of proliferation.

Fig. 1 illustrates the fact that although the doubling time of MH1C1 cells is longer than that of H35 cells, the time course of the effects of DBcAMP on the growth rate of both cell lines is similar. The effect of DBcAMP on the growth of MH1C1 cells in this experiment was somewhat greater than that reported in Table II. A similar variation in the absolute degree of inhibition has been observed in H35 cells (20).

As is also the case in H35 cells (20), the content of protein per cell is increased in MH1C1 cells exposed to DBcAMP, but no effect could be detected in either HTC or RLC cells. The content of DNA per cell was not altered by growth of any of these cells in DBcAMP (20).

**Table II**

| Cell line | Control cells | DBcAMP-treated cells |
|-----------|---------------|----------------------|
| Doubling time | | |
| H35 | 30 | 50 |
| MH1C1 | 50 | 70 |
| HTC | 36 | 36 |
| RLC | 40 | 40 |
| Protein/DNA | | |
| H35 | 14.7 | 19.9 |
| MH1C1 | 14.1 | 20.0 |
| HTC | 12.5 | 12.6 |
| RLC | 13.6 | 14.0 |

DBcAMP (0.5 mM) was first added 24 h after subculture. The medium, with and without DBcAMP was changed on days 2 and 4. On day 5 the cells were harvested with 1 mM EDTA for determination of the cell number and the content of protein and DNA. The doubling times were estimated from growth curves of untreated cells constructed from data obtained in separate experiments (20). Each value is the average of five to six flasks with a standard error of less than 10%.

**EFFECTS OF DBcAMP ON DNA SYNTHESIS:** DBcAMP produces a marked inhibition of DNA synthesis (as measured by [3H]deoxyadenosine...
FIGURE 1 Time course of the effects of DBcAMP on the growth of MH1C1 cells. In the case of both H35 and MH1C1 cells, approximately $1.5 \times 10^6$ cells were placed in each flask at subculture (day 0). DBcAMP (0.5 mM) was first added 24 h after subculture (day 1) and again after medium changes on days 3 and 5 with H35 cells, and day 4 with MH1C1 cells. At the times indicated cells were frozen in situ and stored at $-30^\circ C$ until assay. Cell numbers were calculated from assays of DNA content (20). Each value is the average of three flasks with standard errors of less than 10% in each case. –-•, untreated H35 cells; •--•, DBcAMP-treated H35 cells; Δ--Δ, untreated MH1C1 cells; Δ--Δ, DBcAMP-treated MH1C1 cells.

The inability of HTC and RLC cells to respond to DBcAMP could result from several factors. The most obvious possibilities are that DBcAMP simply does not enter these cells or is not deacylated (32). In order to study both possibilities the uptake and metabolic fate of 0.5 mM [3H]-DBcAMP (labeled in the 8 position of the adenine ring) was measured.

From the results of these studies the following points can be made: (a) The uptake of [3H]DBcAMP by all four cell lines reaches a rather constant level within 30 min after its addition. (b) No association of [3H]DBcAMP (i.e.,

TABLE III

| Cell line | Control cells (cpm/h/10^4 cells) | DBcAMP-treated cells (cpm/h/10^4 cells) |
|-----------|----------------------------------|----------------------------------------|
| H35       | 15.1                             | 8.9                                    |
| MH1C1     | 7.2                              | 4.5                                    |
| HTC       | 7.7                              | 7.6                                    |
| RLC       | 7.4                              | 7.2                                    |

Cells were plated at a density of $2-4 \times 10^5$ cells per flask and tested during the logarithmic phase of growth (i.e., between 20-40 $\times 10^5$ cells per flask) which was achieved 5 days after subculture. The medium was changed on days 3 and 5. After the last change of medium, DBcAMP (0.5 mM) was added. 3 h later, 1.0 µCi of [3H]deoxyadenosine (1 µM final concentration) was added to each flask and 1 h later the cells were harvested for assay (25). Each value is the average of four to five flasks with a standard error of less than 10%.

Reversal of DNA Synthesis Inhibition:

We have found that deoxyctydine is capable of reversing the effects of DBcAMP on both growth rate and DNA synthesis in H35 cells (19). It was of interest to determine whether this nucleoside was able to prevent the inhibitory effects of DBcAMP on DNA synthesis in MH1C1 cells as well. Addition of 1 mM deoxyctydine together with DBcAMP did, in fact, essentially completely prevent the inhibition of DNA synthesis in both H35 and MH1C1 cells (Table IV). No effect of DBcAMP was observed on DNA synthesis in HTC or RLC cells in the presence or absence of deoxyctydine.

The reversal of the effects of DBcAMP on DNA synthesis and growth does not result simply from inhibition of transport of the cyclic nucleotide by deoxyctydine. Tyrosine transaminase is fully inducible by DBcAMP (0.5 mM) even in the presence of 1 mM deoxyctydine (19).

Uptake and Metabolism of [3H]DBcAMP:
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van Rijn et al. Comparative Studies of Hepatoma Cells
Reversal by Deoxycytidine of Inhibition of DNA Synthesis by DBCAMP in Various Hepatoma Cell Cultures

| Cell line | [3H]Deoxyadenosine incorporation in: |  |  |
|-----------|-------------------------------------|---|---|
|           | Control cells | DBCAMP-treated cells |  |
| H35       | 10.0 | 9.2 |
| MH3C1     | 7.0  | 6.8 |
| HTC       | 4.3  | 4.7 |
| RLC       | 5.3  | 5.1 |

Cells were plated at a density of 2-4 X 10^6 cells per flask and tested during the logarithmic phase of growth (i.e., between 20 and 40 X 10^6 cells per flask) which was achieved 5 days after subculture. The medium was changed on days 3 and 5. After the last change of medium, DBCAMP (0.5 mM) and deoxycytidine (1 mM) were added. 3 h later, 1.0 μCi of [3H]deoxyadenosine (1 μM final concentration) was added to each flask. 1 h later the cells were harvested for assays (25). Each value represents the average of four to five flasks with a standard error of less than 10%.

...after washing) occurs with the culture flasks themselves or with cells that have been previously denatured by ethanol. (c) The washing procedure appears to be adequate since a very low and essentially constant amount of radioactivity is removed by the last two washings. Thus, the observed radioactivity most likely represents cell-associated DBCAMP and its metabolites. Table V compares this cell-associated radioactivity after incubation of [3H]DBCAMP with each cell line for 1 h. The amounts are expressed both as disintegrations per minute per milligram protein and as disintegrations per minute per cell X 10^6. As can be seen, no significant differences in DBCAMP uptake were observed among the H35, HTC, and RLC cell lines, but MH3C1 cells exhibited a greater degree of cell-associated radioactivity.

In all the cell lines a significant fraction of the DBCAMP was converted into MBcAMP (N6- and O2'-) as judged by paper chromatography. Radioactivity could be found not only in the two MBcAMP derivatives but also in cAMP and, to a lesser extent, AMP and adenosine. These metabolites were formed in roughly equivalent amounts in all the cell lines except for MH3C1 cells where about 50% more radioactivity was found in metabolites, but the proportions were similar. Thus, after an incubation period of 1 h, approximately 50% of the radioactivity in metabolites derived from DBCAMP was found in MBcAMP (N6- and O2'-), 10-20% in cAMP and the rest in AMP and adenosine.

N4- and O4'-MBcAMP could not be reproducibly separated by chromatography on paper or ion-exchange thin layer plates despite the use of a variety of solvent systems. However, incubation of the MBcAMP fraction with 0.1 N NaOH after chromatography allowed a direct estimate to be made of the relative amounts of these two derivatives after rechromatography. N4'-MBcAMP is resistant to treatment with alkali but O4'-MBcAMP is quantitatively converted to cAMP which is readily separable from N4'-MBcAMP (33). Using this procedure, it was found that roughly equal amounts of N4'- and O4'-MBcAMP were formed in all four cell lines after incubation for 1 h with 0.5 mM DBCAMP.
Another method involving ultraviolet spectroscopy was employed to measure the relative amounts of N6- and O2'-MBcAMP, which did not require chemical treatment or rechromatography. For these experiments a larger quantity of cells was used and the MBcAMP fraction resulting from metabolism of DBcAMP was isolated as usual. The absorption maximum of the fraction after elution was determined between 250 and 280 nM, and the relative quantity of the two derivatives was calculated. N6-MBcAMP exhibits an absorption maximum at 272 nM and O2'-MBcAMP at 258 nM and, since the extinction coefficients of both compounds are similar, it is possible to estimate, approximately, the percentage of each present by determining the absorption maximum of the mixture (i.e., (272-λmax)/(272-258) × 100 = percent O2'-MBcAMP) (33). Estimates of the amounts of N6- and O2'-MBcAMP using this method were similar to those made with the alkali treatment procedure.

Estimates of the intracellular concentration of DBcAMP and its metabolites can be made if the cellular volume is known. Several experiments were conducted in an effort to measure cellular volumes, and the results varied to some extent as a function of the harvesting procedure, the age of the cells, and so forth. In any event, values between 4 and 6.5 × 10⁻¹² liters per cell for each of the lines were obtained in the different experiments.

Using these estimated cellular volumes, the intracellular concentration of DBcAMP and its metabolites can be calculated to be approximately 0.3 mM in all four cell lines. The calculated intracellular concentrations of the presumptively active compounds, N6-MBcAMP and cAMP (32), were roughly 0.05 mM and 0.03 mM, respectively, with the exception of MH1C1 cells where approximately 50% more DBcAMP was converted to these metabolites (see Table V).

**DISCUSSION**

The responses observed in the present experiments suggest that MH1C1 cells have retained the same regulatory mechanisms, with respect to those examined, as H35 cells, with one exception; the regulation of tyrosine transaminase by cAMP analogs. In contrast, the HTC and RLC cells both appear to have lost (or never possessed) key components in most of these regulatory processes. Although a weak elevation of transaminase activity can be achieved with DBcAMP in HTC cells, as Stellwagen has also reported (34), no other responses to cAMP analogs were observed in these cells and no responses at all were found with RLC cells.

The trivial possibility that these cells are resistant to DBcAMP because of reduced uptake of impaired conversion to N6-MBcAMP or cAMP would appear unlikely on the basis of the present results (see Table V). Furthermore, employment of 8-substituted analogs of cAMP which are active as enzyme inducers in H35 and MH1C1 cells (19, 20) did not produce any significant responses in HTC or RLC cells above and beyond those seen with DBcAMP. The 8-substituted analogs contain no butyric acid and are capable of activating protein kinase in vitro as well as inhibiting phosphodiesterase activity (30, 35). The defect in HTC and RLC cells would, therefore, appear to be in the intracellular response of these cells to cAMP (or N6-MBcAMP, etc.).

One obvious candidate for the key lesion is the cAMP-dependent protein kinase (36). There are no data, to our knowledge, with reference to this possibility in RLC cells but Granner has reported that both subunits of this enzyme (36) are present in HTC cells (37). Although there appears to be a diminished amount of the cAMP-binding subunit (and hence a reduced dependency on cAMP), it is possible to obtain stimulation of histone phosphorylation with HTC cell extracts in vitro (37). If the Km of the regulatory subunit for cAMP (or N6-MBcAMP) was altered, this could conceivably account for the requirement for higher concentrations of DBcAMP to

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4 We have tested N6-MBcAMP as an enzyme inducer and it is about as effective as DBcAMP in H35 cells, but has no effect in HTC cells. O2'-MBcAMP, in contrast, is inactive as an inducer. These results are consistent with N6-MBcAMP being an active intracellular inducer. Dr. Jon Miller at the ICN Nucleic Acid Research Institute has obtained identical results in studies of these analogs as inducers of the transaminase in adrenalectomized rats (personal communication). He has also found that among these three, only N6-MBcAMP is an effective activator of rat liver protein kinase and only DBcAMP and N6-MBcAMP are resistant to hydrolysis by phosphodiesterase. These results thus provide a logical explanation both for the lack of effect of O2'-MBcAMP and the activity of DBcAMP and N6-MBcAMP in H35 cells.
produce significant changes in tyrosine transaminase activity, but not for the lack of effect of cAMP analogs on PEP carboxykinase activity or DNA synthesis.

H35 cells possess a cAMP-dependent protein kinase as judged by in vitro assays and by the fact that histone phosphorylation can be stimulated in intact H35 cells by DBcAMP. MH1C1 cells also contain a protein kinase which is the same as in H35 cells both in terms of basal activity and cAMP stimulability. Examination of RLC cells for protein kinase activity will be conducted shortly. It should be stressed at this point that there is no direct evidence that the effects of cAMP on either enzyme induction or growth regulation are mediated by a cAMP-dependent protein kinase. However, there is at present no well-defined alternative mechanism by which cAMP has been shown to act in eukaryotic cells and, therefore, it seems only reasonable to suggest that protein kinase is involved. Indirect support for this suggestion is provided by the fact that there is a correlation between the ability of various analogs of cAMP to induce tyrosine transaminase and to activate rat liver protein kinase in vitro (7, 19, 20, 35). In addition, only cAMP analogs that are enzyme inducers are capable of regulating the growth of H35 and MH1C1 cells (19, 20).

It is possible that the low activity of the carboxykinase in HTC and RLC cells is due to the fact that only the mitochondrial isozyme is present. The isozyme in the cytosol is subject to hormonal or dietary regulation in rat liver, but that in the mitochondrion is resistant to such regulation (38-40). Unfortunately, it is essentially impossible to distinguish between the cytosolic and mitochondrial isozymes by differences in nucleotide or metal ion specificities or on kinetic grounds (40, 41). The activity of the carboxykinase in these cells is also so low that, although theoretically possible (40), immunological differentiation would be technically difficult.

There are at least two other possible explanations for the variable responses to DBcAMP seen in the HTC, RLC, and MH1C1 cells. The first is that there are different protein kinases for each physiological response subject to regulation by cAMP and that one or more of these has been altered or lost during oncogenesis or continuous growth in culture. Thus, if three different protein kinases mediated the effects of DBcAMP on the transaminase, carboxykinase, and DNA synthesis, then the loss of or marked reduction in the first of these with the MH1C1 cells, a marked alteration in the first and loss of the second and third of these in HTC cells, and the loss of all three in the RLC cells could also explain the observed responses. (The “loss” of cAMP-dependent protein kinase would presumably relate to loss of or major reduction in the content of either the regulatory cAMP-binding or catalytic subunit [37].) Although the results in HTC cells are consistent with this possibility to some extent (34, 37), this does not seem very likely to be a major factor for several reasons. Although two, and possibly three, isozymes of protein kinase have been reported in rat liver, there is no significant difference in either the affinity for cAMP or in the protein substrate specificity among the isozymes (42, 43). Furthermore, there are clearly more than two to three discrete responses affected by cAMP in liver (6, 44-48). Finally, the same protein kinase appears to catalyze the phosphorylation of phosphorylase b kinase and glycogen synthetase I in skeletal muscle (36).

A more likely possibility would appear to be the deletion of or alteration in key protein substrates for the cAMP-dependent kinase. This would eliminate the arguments discussed above and could completely account for the differential responses observed. The fact that tyrosine transaminase is present and still responds to glucocorticoids in HTC and RLC cells also suggests that the defect in the regulation of the synthesis of this enzyme is relatively subtle. The fact that certain liver ribosomal proteins are subject to cAMP-dependent phosphorylation (49-50) and the evidence which strongly indicates that cAMP acts at the translational level in regulating the synthesis of tyrosine transaminase and PEP carboxykinase (39, 51-53) argue for a functional relationship between these events. It is not possible as yet, however, to do more than speculate about this relationship, as has been done with histone phosphorylation and enzyme induction (48). It is clear that if the differential responses observed in these studies result at least in part from protein substrate alterations, that attempts to determine whether ribosomal protein phosphorylation is in fact associated with enzyme induction in a cause-effect relationship would be greatly facilitated.

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8 W. D. Wicks. Unpublished observations.
6 T. A. Langan. Personal communication.
The fact that DBcAMP has very little effect on transaminase activity in MH1C1 cells in spite of the fact that the carboxykinase responds normally and both enzymes are elevated by dexamethasone provides additional convincing evidence that cAMP does not mediate the effects of glucocorticoids on enzyme induction (6, 27, 28, 39).

The pattern of response of PEP carboxykinase to dexamethasone in H35, MH1C1, HTC, and RLC cells is analogous to that reported for phenylalanine hydroxylase by Haggerty et al. (54). These results underscore the basic similarity of H35 and MH1C1 cells in terms of the regulatory processes studied to date. It will be of interest to see if phenylalanine hydroxylase can be induced in MH1C1 cells by DBcAMP as is the case in the H35 cells (54).

The mechanism by which DBcAMP inhibits the growth rate of MH1C1 cells appears to be analogous to that in H35 cells. The kinetics of inhibition of DNA synthesis and growth rate are similar in both cell lines and deoxycytidine reverses the effects of the cAMP analog in each (19, 20, 25).

Although detailed studies localizing the site of action of DBcAMP in the cell cycle of MH1C1 cells have not yet been performed, the present results are consistent with a prolongation of the DNA replicative phase of the cell cycle of these cells as in the case with H35 cells (19, 25). This effect would appear to account for both the inhibition of growth and increase in protein content in MH1C1 and H35 cells. Since cells take a longer time to traverse the cell cycle in the presence of DBcAMP, a proportionately greater amount of protein is synthesized per cell (20). The degree of increase in protein content in both cell lines correlates roughly with the increase in generation time.

The fact that the intracellular concentration of DBcAMP 30-60 min after its addition is only slightly less than that in the medium suggests that they may well provide starting material for the isolation of a large number of variants which could be of great assistance in the elucidation of the mechanism by which cAMP regulates specific protein synthesis and growth rate.

The excellent technical assistance of Janet McKibbin and Carolyn Bearg is gratefully acknowledged.

This work was supported by The Netherlands Organization for the Advancement of Pure Research (Z.W.O.), by United States Public Health Service Grant no. AM 16753-01, by a grant from the American Diabetes Association, and by funds from the General Research Support Grant of the University of Colorado Medical Center.

Received for publication 7 May 1973, and in revised form 28 September 1973.

REFERENCES

1. PITOY, H. C., C. FERANO, P. A. MOSE, and V. R. POTTER. 1964. Hepatomas in tissue culture compared with adapting liver in vivo. Natl. Cancer Inst. Monogr. 13:229.

2. THOMPSON, E. B., G. M. TOMKINS, and J. F. CURRAN. 1966. Induction of tyrosine-α-ketoglutarate transaminase by steroid hormones in a newly established tissue culture line. Proc. Natl. Acad. Sci. U. S. A. 56:296.

3. RICHARDSON, U. I., A. H. TASHJIAN, and L. LEVINE. 1969. Establishment of a clonal strain of hepatoma cells which secrete albumin. J. Cell Biol. 40:236.

4. GERSCHENSON, L. E., M. ANDERSON, J. MOLSON, and T. OKIGAKI. 1970. Tyrosine transaminase induction by dexamethasone in a new rat liver cell line. Science (Wash. D.C.). 170:859.
5. OSHIRO, Y., L. E. GERSCHENSON, and J. A. DiPAOLI. 1972. Carcinomas from rat liver cells transformed spontaneously in culture. Cancer Res. 32:2877.

6. BARNETT, C. A., and W. D. WICKS. 1971. Regulation of phosphoenolpyruvate carboxykinase and tyrosine transaminase in hepatoma cell cultures. I. Effects of glucocorticoids, N\textsuperscript{6},O\textsuperscript{2′}-dibutyryl adenosine 3′,5′-monophosphate and insulin in Reuber H35 cells. J. Biol. Chem. 246:7201.

7. WICKS, W. D., C. A. BARNETT, and J. B. MCKIRBIN. 1973. Interaction between hormones and cyclic AMP in regulating specific hepatic enzyme synthesis. Fed. Proc. In press.

8. RUGSTAD, H. E., S. H. ROBINSON, C. YANNONI, and A. H. TASHJIAN. 1970. Metabolism of bilirubin by a clonal strain of rat hepatoma cells. J. Cell Biol. 47:703.

9. BANCROFT, F. C., L. LEVINE, and A. H. TASHJIAN. 1969. Serum albumin production by hepatoma cells in culture: direct evidence for stimulation by hydrocortisone. Biochim. Biophys. Res. Commun. 37:1028.

10. TASHJIAN, A. H., F. C. BANCROFT, U. I. RICHARDSON, M. B. GOLDLUST, F. A. ROMMEL, and P. OFNER. 1970. Multiple differentiated functions in an unusual clonal strain of hepatoma cells. In Vitro. 6:322.

11. ROMMEL, F. A., M. B. GOLDLUST, F. C. BANCROFT, M. MAYER, and A. H. TASHJIAN. 1970. Synthesis of the ninth component of complement by a clonal strain of rat hepatoma cells. J. Immunol. 105:396.

12. LEVISOHN, S. R., and E. B. THOMPSON. 1972. Tyrosine aminotransferase induction regulation variant in tissue culture. Nat. New Biol. 235:102.

13. DELUCA, C., E. J. MASSARO, and M. M. COHEN. 1972. Biochemical and cytogenetic characterization of rat hepatoma cell lines in vitro. Cancer Res. 32:2435.

14. SCHNEIDER, J. A., and M. C. WEISS. 1971. Expression of differentiated functions in hepatoma cell hybrids. I. Tyrosine aminotransferase in hepatoma-fibroblast hybrids. Proc. Natl. Acad. Sci. U. S. A. 68:127.

15. THOMPSON, E. B., and T. A. GELEHRTER. 1971. Expression of tyrosine aminotransferase activity in somatic cell heterokaryons. Evidence for negative control of enzyme expression. Proc. Natl. Acad. Sci. U. S. A. 68:2589.

16. WEISS, M. C., and M. CHAPLAIN. 1971. Expression of differentiated functions in hepatoma cell hybrids. Reappearance of tyrosine aminotransferase inducibility after the loss of chromosomes. Proc. Natl. Acad. Sci. U. S. A. 68:3026.
AMP in rat liver. Biochim. Biophys. Acta. 264:177.

30. MUNYAMA, K., R. J. BAURR, D. A. SHUMAN, R. K. ROBINS, and L. N. SIMON. 1971. Chemical synthesis and biological activity of 8-substituted adenosine 3':5'-cyclic monophosphate derivatives. Biochemistry. 16:2390.

31. GRANNER, D. K., L. R. CHASE, G. D. AURBACH, and G. M. TOMKINS. 1968. Tyrosine aminotransferase. Enzyme induction independent of adenosine 3':5'-cyclic monophosphate. Science (Wash. D.C.). 162:1018.

32. POSTERNAK, T., E. W. SUTHERLAND, and W. F. HENION. 1962. Derivatives of cyclic 3':5'-adenosine monophosphate. Biochim. Biophys. Acta. 65:558.

33. FALBRIARD, J. G., T. POSTERNAK, and E. W. SUTHERLAND. 1967. Preparation of derivatives of adenosine 3',5'-phosphate. Biochim. Biophys. Acta. 148:99.

34. STELLWAGEN, R. H. 1972. Induction of tyrosine aminotransferase in HTC cells by N6-O2'-dibutyryl adenosine 3':5'-monophosphate. Biochem. Biophys. Res. Commun. 47:1144.

35. BAUER, R. J., K. R. SWIATEK, R. K. ROBINS, and L. N. SIMON. 1971. Adenosine 3':5'-cyclic monophosphate derivatives. II. Biological activity of some 8-substituted analogs. Biochem. Biophys. Res. Commun. 45:526.

36. KREBS, E. G. 1972. Protein kinases. Curr. Top. Cell. Regul. 5:299.

37. GRANNER, D. K. 1972. Protein kinase. Altered regulation in a hepatoma cell line deficient in adenosine 3':5'-cyclic monophosphate:binding protein. Biochem. Biophys. Res. Commun. 46:1516.

38. NORDLIE, R. C., F. E. VARICCHIO, and D. D. HOLTEN. 1965. Effects of altered hormonal states and fasting on rat liver mitochondrial phosphoenolpyruvate carboxykinase levels. Biochim. Biophys. Acta. 97:214.

39. WICKS, W. D. 1971. Differential effects of glucocorticoids and adenosine 3':5'-monophosphate on hepatic enzyme synthesis. J. Biol. Chem. 246:217.

40. BALLARD, F. J., and R. W. HANSON. 1969. Purification of phosphoenolpyruvate carboxykinase from the cytosol of rat liver and the immunological demonstration of differences between this enzyme and the mitochondrial phosphoenolpyruvate carboxykinase. J. Biol. Chem. 244:5625.

41. HOLTEN, D. D., and R. C. NORDLIE. 1965. Comparative studies of catalytic properties of guinea pig liver intra- and extramitochondrial phosphoenolpyruvate carboxykinases. Biochemistry. 4:723.

42. CHEN, L.-J., and D. A. WALSH. 1971. Multiple forms of hepatic adenosine 3':5'-monophosphate-dependent protein kinase. Biochemistry. 10:3614.

43. YAMAMURA, H., A. KUMON, K. NISHIYAMA, M. TAKEDA, and Y. NISHIZUKA. 1971. Characterization of two adenosine 3':5'-monophosphate-dependent protein kinases from rat liver. Biochem. Biophys. Res. Commun. 45:1560.

44. SNELL, K. 1971. The regulation of rat liver l-alanine-glyoxylate aminotransferase by glucagon in vivo. Biochem. J. 123:657.

45. JOST, J. P., A. HAE, S. D. HUGHES, and L. RYAN. 1970. Role of cyclic adenosine 3':5'-monophosphate in the induction of hepatic enzymes. I. Kinetics of the induction of rat liver serine dehydratase by cyclic adenosine 3':5'-monophosphate. J. Biol. Chem. 245:2551.

46. REYNOLDS, R. D., D. F. SCOTT, V. R. POTTER, and H. P. MORRIS. 1971. Induction of tyrosine aminotransferase and amino acid transport in Morris hepatomas and in adult and neonatal rat liver. Cancer Res. 31:1580.

47. EXTON, J. H., S. B. LEWIS, R. J. HO, G. A. ROBISON, and C. R. PARK. 1971. The role of cyclic AMP in the interaction of glucagon and insulin in the control of liver metabolism. Ann. N. Y. Acad. Sci. 185:85.

48. LANGAN, T. A. 1971. Cyclic AMP and histone phosphorylation. Ann. N. Y. Acad. Sci. 185:166.

49. EIL, C., and I. G. WOOL. 1971. Phosphorylation of rat liver ribosomal subunits: Partial purification of two cyclic AMP activated protein kinases. Biochem. Biophys. Res. Commun. 43:1001.

50. LOER, J. E., and C. BLAT. 1970. Phosphorylation of some rat liver ribosomal proteins and its activation by cyclic AMP. FEBS Lett. 10:105.

51. WICKS, W. D., and J. B. MCKINNON. 1972. Evidence for translational regulation of specific enzyme synthesis by N6-O2'-dibutyryl cyclic AMP in hepatoma cell cultures. Biochem. Biophys. Res. Commun. 48:205.

52. BUTCHER, F. R., J. E. BECKER, and V. R. POTTER. 1971. Induction of tyrosine aminotransferase by dibutyryl cyclic AMP employing hepatoma cells in tissue culture. Exp. Cell Res. 66:231.

53. CHUAH, C. C., and I. T. OLIVER. 1971. Role of adenosine cyclic monophosphate in the synthesis of tyrosine aminotransferase in neonatal rat liver. Release of enzyme from membrane-bound polysomes in vitro. Biochemistry. 10:2990.

54. HAGGERTY, D. F., P. L. YOUNG, G. POPJAK, and W. H. CARNES. 1973. Phenylalanine hydroxylase in cultured hepatocytes. I. Hormonal control of enzyme levels. J. Biol. Chem. 248:223.