REGULATION OF PHOSPHOENOLPYRUVATE CARBOXYKINASE AND TYROSINE TRANSAMINASE IN HEPATOMA CELL CULTURES

III. Comparative Studies in H35, HTC, MH_1C_1, and RLC Cells

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ABSTRACT

The ability of N^6, $O^{2'}$-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 MH_1C_1 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 MH_1C_1 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 MH_1C_1 cells but not in HTC or RLC cells. The effect of DBcAMP on DNA synthesis in MH_1C_1 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 [^{3}H]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 cells derived from chemically induced rat hepatomas[1] (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 hor-

[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).[2] 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 MH_1C_1 (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 been only 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 $N^6,O^{2'}$-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,[3] 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 [^{3}H]DEOXYADENOSINE: The incorporation of [^{3}H]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 [^{3}H]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 [^{3}H]DBcAMP: [8-^{3}H]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 $N^6,O^{2'}$-dibutyryl-, N^6- and $O^{2'}$-monobutyryl-, and free cAMP was determined after chromatography of the remaining material on Whatman 1 MM paper with a solvent system containing 1 M ammonium acetate, pH 7.5, and ethanol (3:7.5 vol/vol) (26). Elution of the nucleotides was performed with water. Analysis of the composition of monobutyryl cAMP (MBcAMP) derivatives (i.e., N^6 and $O^{2'}$) was performed by two methods as described in the text.

COUNTING: Filters were counted in 5 ml toluene containing 4 g/liter 2,4-diphenyloxazole and 0.05 g/liter 1-4-bis[2-(5-phenyloxazolylphenyl)] benzene with an efficiency of 6%, while aqueous samples were counted in 15 ml Aquasol with an efficiency up to 30% depending on the quantity of the sample. Counting was performed in a Mark II liquid scintillation counter.

CHEMICALS AND RADIOISOTOPES: All tissue culture components were purchased from Grand Island Biological Co., Grand Island, New York or Flow Laboratories Ltd., Scotland. [^{3}H]Deoxyade-

[2] *Abbreviations used in this paper:* cAMP, cyclic 3′:5′-AMP; DBcAMP, $N^6,O^{2'}$-dibutyryl cyclic 3′:5′-AMP; MBcAMP, N^6- or $O^{2'}$-monobutyryl cyclic 3′:5′-AMP; PEP, phosphoenolpyruvate.

[3] A preliminary report of a portion of this work has been made (19).

nosine, [^{3}H]thymidine, and [8-^{3}H]DBcAMP were obtained from The Radiochemical Centre, Amersham, Buckinghamshire, England. Thymidine, deoxyadenosine, DBcAMP, and N^6-MBcAMP were supplied by Boehringer Mannheim Corp., New York. Aquasol 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 MH_1C_1 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 qualitatively 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 MH_1C_1 cells to the glucocorticoid (as has also been reported 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 MH_1C_1 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 MH_1C_1 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 MH_1C_1 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 MH_1C_1 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 carboxykinase 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)
MH_1C_1	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 MH_1C_1 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.

and at the same time generated a synergistic *increase* in tyrosine transaminase activity in MH_1C_1 cells. These results are analogous to those previously reported with H35 cells (6). DBcAMP added together with insulin did not elevate transaminase activity in MH_1C_1 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 MH_1C_1 cells has revealed the same nucleotide (GDP $\geqq$ 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 MH_1C_1 and HTC cells also did not change the results significantly from those shown in Table I.

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 MH_1C_1 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 MH_1C_1 cells (19, 20), also did not influence their rate of proliferation.

TABLE II

Effects of DBcAMP on Growth Rate and Protein Content in Various Hepatoma Cell Cultures

	Cell line	Control cells	DBcAMP-treated cells
		h	*h*
Doubling time	H35	30	50
	MH_1C_1	50	70
	HTC	36	36
	RLC	40	40
		mg/mg	*mg/mg*
Protein/DNA	H35	14.7	19.9
	MH_1C_1	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%.

Fig. 1 illustrates the fact that although the doubling time of MH_1C_1 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 MH_1C_1 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 MH_1C_1 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].)

EFFECT OF DBcAMP ON DNA SYNTHESIS: DBcAMP produces a marked inhibition of DNA synthesis (as measured by [^{3}H]deoxyadenosine

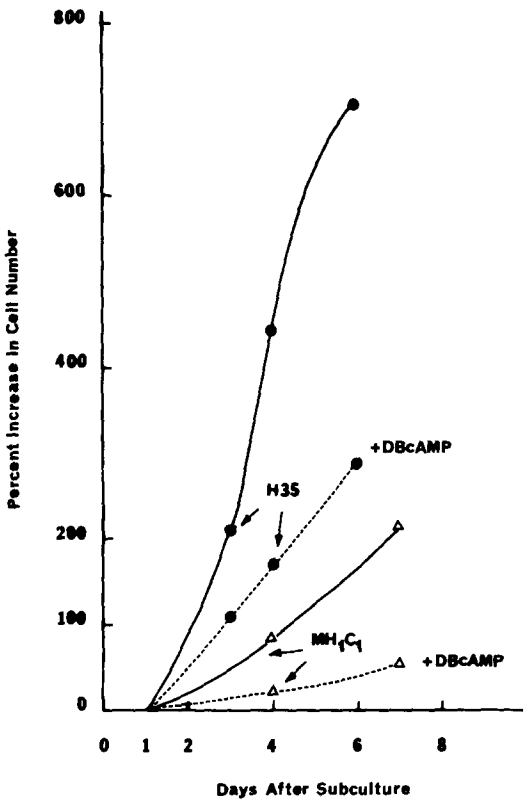

FIGURE 1 Time course of the effects of DBcAMP on the growth of MH_1C_1 cells. In the case of both H35 and MH_1C_1 cells, approximately 1.5×10^6 cells were placed in each flask at subculture (day 0). DBcAMP (0.5 mM) was first added 24 h after subculture (day 1) and again after medium changes on days 3 and 5 with H35 cells, and day 4 with MH_1C_1 cells. At the times indicated cells were frozen *in situ* and stored at −30°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; △——△, untreated MH_1C_1 cells; △---△, DBcAMP-treated MH_1C_1 cells.

incorporation) in both H35 (19, 25) and MH_1C_1 cells as seen in Table III. No significant effect of the cyclic nucleotide on DNA synthesis in either HTC or RLC cells was demonstrable as with the growth rate. Qualitatively similar results were obtained with [^{3}H]thymidine as the radioactive precursor (at high specific radioactivities [19, 25]).

REVERSAL OF DNA SYNTHESIS INHIBITION: We have found that deoxycytidine is capable of reversing the effects of DBcAMP on both growth rate and DNA synthesis in H35 cells (19). It was

TABLE III

Effect of DBcAMP on DNA Synthesis in Various Hepatoma Cell Cultures

Cell line	[^{3}H]Deoxyadenosine incorporation in: Control cells (cpm/h/10^4 cells)	DBcAMP-treated cells (cpm/h/10^4 cells)
H35	15.1	8.9
MH_1C_1	7.2	4.5
HTC	7.7	7.6
RLC	7.4	7.2

Cells were plated at a density of 2–4 × 10^5 cells per flask and tested during the logarithmic phase of growth (i.e., between 20–40 × 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 [^{3}H]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%.

of interest to determine whether this nucleoside was able to prevent the inhibitory effects of DBcAMP on DNA synthesis in MH_1C_1 cells as well. Addition of 1 mM deoxycytidine together with DBcAMP did, in fact, essentially completely prevent the inhibition of DNA synthesis in both H35 and MH_1C_1 cells (Table IV). No effect of DBcAMP was observed on DNA synthesis in HTC or RLC cells in the presence or absence of deoxycytidine.

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 deoxycytidine. Tyrosine transaminase is fully inducible by DBcAMP (0.5 mM) even in the presence of 1 mM deoxycytidine (19).

UPTAKE AND METABOLISM OF [^{3}H]DBcAMP: 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 [^{3}H]-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 [^{3}H]DBcAMP by all four cell lines reaches a rather constant level within 30 min after its addition. (*b*) No association of [^{3}H]DBcAMP (i.e.,

TABLE IV

Reversal by Deoxycytidine of Inhibition of DNA Synthesis by DBcAMP in Various Hepatoma Cell Cultures

Cell line	[^{3}H]Deoxyadenosine incorporation in: Control cells	DBcAMP-treated cells
H35	10.0	9.2
MH_1C_1	7.0	6.8
HTC	4.3	4.7
RLC	5.3	5.1

Cells were plated at a density of 2–4 × 10^5 cells per flask and tested during the logarithmic phase of growth (i.e., between 20 and 40 × 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) and deoxycytidine (1 mM) were added. 3 h later, 1.0 μCi of [^{3}H]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%.

TABLE V

Uptake and Metabolism of [^{3}H]DBcAMP by Various Hepatoma Cell Cultures

	Cell-associated radioactivity (dpm/mg protein)	Radioactivity associated per cell (dpm/cell × 10^6)
H35	2,500	1,000
MH_1C_1	3,400	1,620
HTC	1,670	980
RLC	2,480	890

	Percentage of total cell-associated radioactivity as DBcAMP	Cell-associated radioactivity as DBcAMP (dpm/cell × 10^6)
	%	
H35	59	590
MH_1C_1	39	630
HTC	60	590
RLC	56	500

H35, HTC, and RLC cells were grown for 4 days, while MH_1C_1 cells were grown for 6 days with regular changes of medium. After this period of time, 110,000 dpm/ml of [^{3}H[DBcAMP (0.5 mM) was added and 1 h later the cells were washed rapidly five times with ice-cold saline. Ethanol (90%) was then added and the cells were scraped, freeze-thawed several times, and centrifuged. The radioactivity of the supernatant was measured and analyzed as described in Materials and Methods. The data are the average of three experiments, each containing three flasks of the respective cell lines. The standard errors ranged from 5 to 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 [^{3}H]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 × 10^6. As can be seen, no significant differences in DBcAMP uptake were observed among the H35, HTC, and RLC cell lines, but MH_1C_1 cells exhibited a greater degree of cell-associated radioactivity.

In all the cell lines a significant fraction of the DBcAMP was converted into MBcAMP (N^6- and $O^{2'}$-) 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 MH_1C_1 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 (N^6- and $O^{2'}$-), 10–20% in cAMP and the rest in AMP and adenosine.

N^6- and $O^{2'}$-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. N^6-MBcAMP is resistant to treatment with alkali but $O^{2'}$-MBcAMP is quantitatively converted to cAMP which is readily separable from N^6-MBcAMP (33). Using this procedure, it was found that roughly equal amounts of N^6- and $O^{2'}$-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 N^6- and $O^{2'}$-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. N^6-MBcAMP exhibits an absorption maximum at 272 nM and $O^{2'}$-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., $(272\text{-}\lambda_{max})/(272\text{-}258) \times 100$ = percent $O^{2'}$-MBcAMP) (33). Estimates of the amounts of N^6- and $O^{2'}$-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 can be calculated to be approximately 0.3 mM in all four cell lines. The calculated intracellular concentrations of the presumptively active compounds, N^6-MBcAMP and cAMP (32), were roughly 0.05 mM and 0.03 mM, respectively, with the exception of MH_1C_1 cells where approximately 50% more DBcAMP was converted to these metabolites (see Table V).

DISCUSSION

The responses observed in the present experiments suggest that MH_1C_1 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 N^6-MBcAMP[4] 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 MH_1C_1 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 N^6-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 N^6-MBcAMP) was altered, this could conceivably account for the requirement for higher concentrations of DBcAMP to

[4] We have tested N^6-MBcAMP as an enzyme inducer and it is about as effective as DBcAMP in H35 cells, but has no effect in HTC cells. $O^{2'}$-MBcAMP, in contrast, is inactive as an inducer. These results are consistent with N^6-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 N^6-MBcAMP is an effective activator of rat liver protein kinase and only DBcAMP and N^6-MBcAMP are resistant to hydrolysis by phosphodiesterase. These results thus provide a logical explanation both for the lack of effect of $O^{2'}$-MBcAMP and the activity of DBcAMP and N^6-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[5] and by the fact that f_1 histone phosphorylation can be stimulated in intact H35 cells by DBcAMP.[6] MH_1C_1 cells also contain a protein kinase which is the same as in H35 cells both in terms of basal activity and cAMP stimulability.[5] 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 MH_1C_1 cells (19, 20).[5]

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 MH_1C_1 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 MH_1C_1 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 f_1 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.

[5] W. D. Wicks. Unpublished observations.

[6] T. A. Langan. Personal communication.

The fact that DBcAMP has very little effect on transaminase activity in MH_1C_1 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, MH_1C_1, 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 MH_1C_1 cells in terms of the regulatory processes studied to date. It will be of interest to see if phenylalanine hydroxylase can be induced in MH_1C_1 cells by DBcAMP as is the case in H35 cells (54).

The mechanism by which DBcAMP inhibits the growth rate of MH_1C_1 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 MH_1C_1 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 MH_1C_1 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 the various cells are probably freely permeable to the cyclic nucleotide. The concentration of N^6-MBcAMP can be estimated to be about 0.05 mM in all but the MH_1C_1 cells where it is about 0.08 mM. The cAMP concentration is roughly 0.03 mM in all but the MH_1C_1 cells where it is proportionately higher. The concentrations of N^6-MBcAMP and cAMP are not far above those of free cAMP achieved in rat liver after exposure to glucagon (45, 47). Although these are only crude estimates of the intracellular concentration of DBcAMP, N^6-MBcAMP, and cAMP, they suggest that the intracellular responsiveness of H35 and MH_1C_1 cells is similar to that of normal rat liver. Indeed, minimal effects of DBcAMP on both enzyme induction and growth rate can be obtained with exogenous concentrations as low as 10–20 μM (6, 19), which is well within the range of hormonally induced fluctuations in hepatic cAMP concentrations (45, 47) and, in view of the present results, the intracellular concentration of active metabolites of DBcAMP (i.e., presumably N^6-MBcAMP and cAMP) would be expected to be even less than that of DBcAMP in the medium.

Although the present hepatoma cell lines constitute only a modest set of variants, comparative analysis of their responses to DBcAMP 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.

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