Regulation of Cyclic Adenosine 3′:5′-Monophosphate-binding Protein in N-18 Mouse Neuroblastoma Cells

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ABSTRACT

The regulation of cyclic adenosine 3′:5′-monophosphate (cAMP)-binding protein in N-18 neuroblastoma cells in tissue culture was studied by the covaleat incorporation of [γ-32P]cyclic adenosine 3′:5′-[32P]monophosphate, together with the techniques of sucrose dodecyl sulfate-alkylacylamide gel electrophoresis and autoradiography. Greater than 95% of the total cAMP binding activity of N-18 neuroblastoma cells was identified as being regulatory subunits of the type 1 (R) and type II (R2) species, with R1 being the predominant form of the two (R1/R2 = 3:1). The specific activity of R1, but not of R2, increased 3-fold when cells were grown in medium containing 1% rather than 10% fetal calf serum. Under the same conditions, the specific activity of acetylcholinesterase increased 3- to 5-fold. The increase in R1 was inversely related to the serum concentration in the medium and was specific for cells at the stationary phase of growth. An increase in intracellular cAMP, concurrent with the increase in R1, was also observed. Morphological examination of met-anaphase-phase neuroblastoma cells maintained in medium containing 1% fetal calf serum suggested the presence of a high proportion of highly-differentiated cells. It is proposed that the regulatory control of R1-cAMP-binding protein by serum may involve modulation of intracellular cAMP and that the expression of R1 may be used as a biochemical index of differentiation in mouse neuroblastoma cells.

INTRODUCTION

N-18 neuroblastoma cells grown in culture are predominantly round and tightly refractile (1, 2). Under appropriate experimental conditions, these cells can undergo differentiation, a process grossly defined by increases in neurite outgrowth and acetylcholinesterase activity. A vast variety of agents or treatments have been shown to induce differentiation in neuroblastoma cells (3, 9, 12, 14, 16-18, 23, 28-28, 33, 34). In general, these agents or treatments also cause a slowing of cell growth. The precise relationship between cell growth and differentiation is not clear. It has been suggested that (a) morphological differentiation may be a direct function of the inhibition of cell growth (25, 26), (b) inhibition of cell division is necessary, although by itself not sufficient, for cell differentiation (28, 29) (the inhibition of cell growth is not the cause of neurite extension but rather is itself a result of the induction of differentiated functions incompatible with rapid growth (12), or finally (c) it is possible to dissociate inhibition of cell growth from biochemical differentiation of neuroblastoma cells (14).

The N-18 neuroblastoma cells undergo morphological and biochemical changes characteristic of differentiated neurons when grown in medium supplemented with 1% rather than the normal 10% FCS (3, 34). These changes are accompanied by a decreased growth rate and an increase in neurite outgrowth. Treatment on a variety of mammalian cell systems have indicated an inverse relationship between cellular growth and intracellular cAMP concentration (25). It has been suggested that lower levels of cAMP in rapidly dividing cells may have a significant role in the increased growth rate (14). Previous studies on the regulation of cAMP-binding activity in mouse neuroblastoma cells have demonstrated the induction of cAMP-binding protein either by cAMP or by agents which increase intracellular cAMP (29, 30, 35).

The purpose of this paper is to characterize the possible relationships between serum concentration and the levels both of cAMP-binding protein(s) and of intracellular cAMP in N-18 neuroblastoma cells and (b) determine whether these relationships can be explained merely as functions of altered growth properties induced by changes in the serum concentration or whether they are in fact a characteristic property of the differentiated state in N-18 neuroblastoma cells.

MATERIALS AND METHODS

Materials. cAMP, IBMX, neostigmine, and other biochemicals were obtained from Sigma Chemical Co., St. Louis, Mo. Acid phosphatase (potato) was obtained from Calbiochem-Behring Corp.; La Jolla, Calif. [γ-32P]cAMP and [3H]meotethionine were obtained from New England Nuclear, Boston, Mass. B-NaB4H4][32P]cAMP was obtained from ICN Pharmaceuticals, Inc., Irvine, Calif.; purity of the compound was checked by thin-layer chromatography using Cellulose Fcoated thin-layer chromatographic sheets (EM-Laboratories, Inc., Elmsford, N. Y.) in a solvent system of N-butyrolactophenol acid H2O (5:2:3 v/v/v). [14C]acetylcholinesterase was obtained from American Pharmaceutical Chemical Corp., Arlington, Va. Cell extracts were obtained from Grand Island Biological Co., Grand Island, N. Y.

Cell Cultures. N-18 mouse neuroblastoma cells were grown as monolayer cultures in Dulbecco's modified Eagle medium (with 4500 mg glucose per liter, without sodium pyruvate) supplemented with 10% FCS. Cells were maintained at 37° in

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a water-jacketed CO₂ incubator (95% air:5% CO₂). During the initial 1 to 2 h of a buffer containing penicillin (50 units/ml) plus streptomycin (50 µg/ml). Control experiments demonstrated that the presence of these antibi-

totics in the culture medium had no effect on either the cell

morphology or the cAMP-binding activity. Periodic tests of the cultures for Mycoplasma and bacteria were negative. Upon

reaching stationarity phase of growth, cells were centrifuged, flushing the cells off their growing substratum with a stream of medium, diluting the cell suspension 1:10 to 1:30, plating in Petri dishes.

Differentialination. In this study, differentialination, defined as the morphological appearance of neurites >50 µm in length and the biochemical expression of increased acetylcholinesterase activity, was induced by growing cells in medium supplemented with 1% rather than the usual 10% FCS. Morphological studies demonstrated that >96% of the cells grown in medium containing 10% FCS existed in the round undifferentiated form, while >95% of the cells extended lengthy neurites when grown in medium containing 1% FCS. However, it should be noted that these 2 morphologically distinct cell forms can do coexist in any given culture; the ratio of these 2 forms varies as a function of cell growth and serum concentration in the medium. In view of this consideration, we decided to label populations of neuroblastoma cells according to the serum concentration in the growth media. Thus, NB₁₀ and NB₁ represent neuro-

blastoma cells grown in medium supplemented with 10% and 1% FCS, respectively. The term differentiated cells, when used, refers to NB₁₀ cells at stationary phase which have elevated levels of acetylcholinesterase activity and extensive neurite outgrowth.

![Fig. 1](image)

Fig. 1 is a photomicrograph of NB₁₀ (A, B, and NB₁ (C) cells at early stationary phase of growth. Two features should be noted. (a) The density of NB₁₀ cells was approximately 2.5 times that of NB₁ cells. The saturation densities of NB₁₀ and NB₁ cells were approximately 6 x 10⁵ and 2 x 10⁶/25 cm², respectively. (b) At high proportion of cells with neurites and the length of neurites (50 to 200 µm in length) were distinct features of the NB₁₀ cells. It is of interest that in SDS-polyacy-

lamide gel electrophoresis, no distinct difference in the Coom-

assie blue stained pattern of proteins from NB₁₀ and NB₁ cells was detected.

Cell Growth. Cell growth was measured by the number of viable cells attached to the growing surface. It should be noted that the replicates of cell multiplication and shedding of cells into the medium. Occasional cell growth was also studied by the incorporation of [3H]thymidine into TCA-

insoluble material. Cells grown in 60-mm Petri dishes were labeled for 30 min with 5 µCi [3H]thymidine per ml. The cell layer was then washed 5 times with cold PBS, and 0.7 ml of 10% TCA was added. The amount of radioactivity present in the TCA-precipitable material was determined by liquid scintilla-

tion spectrometry. For cells in the logphase phase of growth, the incorporation of [3H]thymidine gave a fairly good index of the rate of increase in cell number.

Cell Fractionation. Unless otherwise stated, cells at an early stationary phase of growth were used; all procedures were conducted at 4°C. Cells were resuspended twice with 5 ml of PBS and scraped off the Petri dishes with a rubber policeman. Cells were sedimented by centrifuging at 1000 x g for 2 min and washed once with PBS. The cell pellet obtained from ten 100-

mm Petri dishes was resuspended in 1 5 to 2.0 ml of a buffer containing 0.32 M sucrose, 1 mM EDTA, 50 µg phenylmethyl-

sulfonyl fluoride per ml, and 10 mM Tris-HCl (pH 7.4). Cells

were broken by sonicating the cell suspension for 1 min at 4°C with a Heat System-Ultrasonics sonicator equipped with a stepped microtip (50-watt power output; 50% duty cycle, pulsed). The 100,000 x g supernatant obtained from cell homogenates was then centrifuged at 4°C for 1 hr, the resulting pellets discarded, and the above procedure repeated for additional 0.25 hr. The entire supernatant was then centrifuged at 100,000 x g for 1 hr at 4°C. The major supernatant was removed and the pellets discarded. Protein concentration was determined by the method of Lowry et al. (22) using bovine serum albumin as standard.

Covariant Binding of 8-NH₂[3H]cAMP. Covariant binding of 8-

NH₂[3H]cAMP was performed as described previously (36).

The standard reaction mixture (final volume, 0.1 ml) contained 50

mm MES (pH, 6.2), 10 mM MgCl₂, 50 µM ATP, 1 mM to 1 µM 8-

NH₂[3H]cAMP (specific activity, 4 to 10 GB/mmol). To this solution, an amount of cytosol protein up to 200 µg. For studying the specificity of the incorporation of 8-NH₂[3H]cAMP, 10 µM cAMP and 1 mM BMA were added to the assay mixture. The presence of 1 mM IBMX by itself had no effect on the incorporation of 8-

NH₂[3H]cAMP. Samples were incubated for 60 min at 4°C in the dark and were then photolyzed for 10 min with a mineralize

UV-11 hard lamp at a distance of 8 cm. To each sample, 25 µl of a SDS stock solution containing 12% SDS, 0.5 to 10% Tris-HCl buffer (pH, 8.6, 10% mercaptoethanol, 5 mM EDTA, 25% glycerol, and a small amount of pyronin Y (a tracking dye for electrophoresis) was added. Samples were heated at 100°C for 5 min. The entire sample was then subjected to SDS-polyacryl-

amide gel electrophoresis (5 to 15%) gel electrophoresis (20). Gels were stained for protein with Coomassie blue, dried, and subjected to auto-

radiography. Apparent molecular weights of the radioactive bands were estimated by the method of Fairbanks et al. (11).

Standard protein (and their molecular weights) used were cytochrome c (12,500), chymotrypsigen (25,000), aldolase (40,000), ovalbumin (45,000), catalase (57,000), bovine serum albumin (65,000), and phosphorylase b (92,500). Autoradi-

ographs were scanned with a Schofield SD-3000 spectromet-

crodensitometer, and the peak heights of the optical tracing were used as a quantitative measure of the incorporation of [3H]cAMP. In most experiments, the results obtained by the densitom-

eter method were compared with results obtained by liquid scintillation counting of the dried gel slices. Similar results were obtained by the 2 methods.

Results

The presence of the ortho-8-NH₂[3H]cAMP-binding proteins in extracts of N-18 neuroblastoma cells. The apparent molecular weights of these proteins on SDS-poly-

acrylamide gel electrophoresis were 47,000, 52,000, and 54,000. In a previous study on the incorporation of 8-NH₂-

[3H]cAMP into proteins from several rat tissues, the M.W. 47,000 and M.W. 54,000 8-NH₂[3H]cAMP-binding proteins were identified as regulatory subunits of the type I and type II cAMP-dependent protein kinases, respectively (36). Studies on the cAMP-binding proteins of bovine cardiac muscle indi-

cated the occurrence of both a M.W. 54,000 and a M.W. 54,000.
50,000 B-N(3-51)PAP-binding protein as regulatory subunit of the type II kinase of bovine heart. Furthermore, the electro-phoretic mobility of the M.W. 54,000 component was affected by the state of phosphorylation of the protein (31).

In N-18 neuroblastoma cells, the quantitative incorporation of B-N(3-51)PAP into the M.W. 47,000, M.W. 52,000, and M.W. 54,000 proteins and the electro-horhic mobility of these proteins were unaffected by the presence of ATP (50 μm to 1 mm) or by preincubation of the samples with free phosphatase for 20 min at 30°C. DEAE-cellulose column chromatograph demonstrated the elution of the M.W. 47,000 B-N(3-51)PAP. CAM-binding protein with the type I CAM-dependent protein kinase peak, while the M.W. 52,000 and M.W. 54,000 proteins were eluted with the type II CAM-dependent protein kinase. Due to similarity in the apparent affinity of the M.W. 52,000 and M.W. 54,000 proteins for B-N(3-51)PAP, these clones, as well as a D5 clone, were transfected into the M.W. 47,000 and M.W. 52,000 and M.W. 54,000 B-N(3-51)PAP-binding proteins, respectively. No reference was made to the possible physiological functions of these proteins, e.g. associating with and inhibiting the catalytic activity of CAM-dependent protein kinase.

The incorporation of B-N(3-51)PAP into R and R0 is both specific and quantitative. That the incorporation is specific is supported by the ability of 10-fold excess of CAM in the presence of 1 μM BIXM to displace the B-N(3-51)PAP. That the incorporation of B-N(3-51)PAP is quantitative was supported by the following experiments in Experiment 1, at a saturating concentration of the ligand (1 μM), the amount of specifically bound B-N(3-51)PAP and B-N(3-51)PAP, as determined by the niewoe (noncovalent) binding procedure of Guman (13), was the same as the total amount of radioactivity incorporated B-N(3-51)PAP. In Experiment 2, removal of endogenous sources of CAM by overnight diaphragm or by inactivation at 30°C for periods of up to 24 h had little effect on the amount of B-N(3-51)PAP incorporated. It should be noted that, in quantifying CAM-binding protein by the incorporation of B-N(3-51)PAP, the presence of endogenous sources of CAM does not present an insurmountable barrier due to its hydrolysis by phosphodiesterase (present in abundant amounts in extracts of N-18 neuroblastoma cells). That B-N(3-51)PAP was indeed relatively resistant to hydrolysis by phosphodiesterase was verified by the following observations: (a) Incubation of B-N(3-51)PAP with crude bovine heart phosphodiesterase for 10 min at 30°C had little or no effect on the chromatographic behavior of B-N(3-51)PAP on thin-layer chromatography (disc). (b) A second batch of B-N(3-51)PAP contained from ICN Pharmaceuticals contained traces of (3-51)P bound to both B-N(3-51)PAP and B-N(3-51)PAP, which could be separated from B-N(3-51)PAP on thin-layer chromatography. (c) The incorporation of B-N(3-51)PAP was not significantly affected by the presence of a phosphodiesterase inhibitor, BIXM, in the assay mixture. In Experiment 3, the rate of exchange of protein-bound ligand with free ligand in solution was investigated by the exchange of protein-bound [3-51]PAP with free B-N(3-51)PAP. A new 30% of the protein bound [3-51]PAP exchanged with the B-N(3-51)PAP in solution in a time course of less than 60 min at 4°C.

Reversal of Binding of B-N(3-51)PAP or [3-51]PAP. An alternative approach for the determination of CAM-binding property present in extracts of neuroblastoma cells was the noncovalent binding of B-N(3-51)PAP or [3-51]PAP (13). The experiments were performed with a final volume of 0.1 ml contained 50 mm MES (pH 6.2), 10 mm MgCl2, 10 pmol of B-N(3-51)PAP (specific activity, 2 to 3 X 108 dpm/mmol) or [3-51]PAP (specific activity, 30 to 50 Ci/mmol), and various quantities of cell extracts. Two μl Samples were incubated for 60 min at 4°C. To each sample, 3 ml of 50 mm MES (pH 6.2) was added. The entire sample was filtered through a Millipore filter (Type HAWP, 0.22 μm) followed by washing twice each with 5 ml of MES buffer (pH 6.2). The amount of [3-51]PAP bound to protein and hence retained by the Millipore filter was determined by liquid radioactivity spectrometry. All results were corrected for nonspecific binding, determined from the amount of radioactivity retained in the presence of 10 μM CAM and 1 μM BIXM.

Measurement of CAM activity. Measurements of intracellular CAM were done according to the method described by Brown et al. (7). The assay is based on the competition binding of [3-51]PAP and CAM to a preparation of CAM-binding protein (the type II CAM-dependent protein kinase eluted from DEAE-cellulose column) obtained from bovine heart. Briefly, 2 ml of ice-cold 6% TCA was added to neuroblastoma cells grown on 100-mm Petri dishes. A rubber policeman was used to scrape the cell debris off the Petri dish. The protein precipitate was "removed by centrifugation. The supernatant thus obtained was extracted 3 times each with 5 ml of water-saturated ether, ether-dried to dryness, and taken up in 100 μl of 50 mm MES (pH 6.2). The standard assay mixture (total volume, 0.1 ml) contained 50 mm MES (pH 6.2), 10 pmol [3-51]PAP (specific activity, 30 to 50 Ci/mmol), 400 μCi of the CAM unknown, and 30 to 40 μl of the binding protein (containing approximately 5 to 7 pmol of CAM binding capacity). Samples were incubated at 4°C for 60 min. The method used for separation of bound CAM from free CAM was identical to that described for determining [3-51]CAM binding activity in cell extracts. The amount of CAM present in the unknown was calculated from a standard curve generated by adding known concentrations of CAM to the assay mixture.

Acetycholinesterase Assay. Acetylcholinesterase activity was assayed by a radiometric method described previously (10, 21). All assays were carried out at 37°C with 5 μCi/1°C 14C-acetylthiocholine, unless otherwise stated. The standard reaction mixture contained 0.1 mm Thi-HCl, 1 mm sodium hydroxide, (pH 7.0, 5 μCi) 14C-acetylthiocholine, and various amounts of protein up to 200 μg/ml. At predetermined time intervals (usually 0.5 min apart), a 1-ml aliquot was removed from the assay flask, and the enzyme reaction stopped with 0.1 ml of 1.9 sodium phosphate buffer (pH 7.2). The amount of 14C-acetate present in the aliquot was determined by liquid radioactivity spectrometry. Results are expressed as nmol of 14C-acetate generated per min per mg protein for a substrate concentration of 10-4 M.
RESULTS
Cytochalasin CAMP-binding proteins of Nbhs and Nbhs cells were studied by the photoactivated incorporation of 8-N2-[3H]AMP and were characterized by: (a) the pattern of incorporation (Fig. 2); (b) the apparent affinity of this incorporation (Chart 1); and (c) a quantitative comparison of the amount of covalently incorporated 8-N2-[3H]AMP to the amount of reversibly bound 8-N2-[3H]AMP or [3H]AMP (Table 1).

Three protein bands, with apparent molecular weights of 47,000, 52,000, and 54,000, present in cytosols of Nbhs and Nbhs cells, were covalently incorporated 8-N2-[3H]AMP. The incorporation of 8-N2-[3H]AMP into cytosolic proteins of Nbhs cells differed from that of Nbhs cells in that there was a 2- to 4-fold increase in the amount of radioactivity incorporated into the M.W. 52,000 protein. Under the same experimental conditions, no significant difference in the amount of 8-N2-[3H]AMP incorporated into the M.W. 52,000 or M.W. 54,000 proteins of Nbhs and Nbhs cells was observed (Fig. 2).

The concentration-dependent photoactivated incorporation of 8-N2-[3H]AMP into R3 and R3 CAMP-binding proteins of Nbhs and Nbhs cells is shown in the form of semilog dose-response plots in Chart 1. No significant difference in the affinity of 8-N2-[3H]AMP for either the R3 (M.W. 47,000) or the R3 (M.W. 52,000 and M.W. 54,000) CAMP-binding protein of Nbhs and Nbhs cells was observed. The apparent dissociation constants (Ko) for the incorporation of 8-N2-[3H]AMP into R3 and R3 CAMP-binding proteins were 1 x 10^-7 M and 1 x 2 x 10^-7 M, respectively.

Chart 2. Effects of serum concentration on the amount of cytosol CAMP-binding protein, cell growth, and acetylcholinesterase activity in N18 neuroblastoma cells. Cytosols were assayed at concentrations of 10^-6 M [3H]AMP and grown in medium supplemented with various concentrations of FCS as indicated. Cells were harvested in an early stationary phase of growth (2.1 days after plating). Cytosolic activity was measured using a harmanate assay and expressed in terms of radioactivity per 10^-6 M [3H]AMP as noted in each bar. The incorporation of 8-N2-[3H]AMP into cytosolic proteins was done under normal growth conditions (10^-5 M [3H]AMP). The specific activity of acetylcholinesterase present in the 100,000 g supernatant cell homogenate was determined according to the method described. Results shown are representative of 3 independent experiments.

In Chart 1, the incorporation of 8-N2-[3H]AMP into a series of CAMP-binding proteins of Nbhs and Nbhs cells was shown under control conditions. The reversible binding of 8-N2-[3H]AMP to a series of proteins of Nbhs and Nbhs cells was shown under control conditions. The reversible binding of 8-N2-[3H]AMP to a series of proteins of Nbhs and Nbhs cells was shown under control conditions. The reversible binding of 8-N2-[3H]AMP to a series of proteins of Nbhs and Nbhs cells was shown under control conditions.
general cellular biochemical processes related to growth of normal fibroblasts can be mimicked by treatment of these cells with CAMP or agents which increase intracellular CAMP in the presence of serum. Based on these and other similar observations, it has been suggested that lower levels of CAMP rapidly dividing cells may have a significant effect on the increased growth rate.

In our present study, cell growth was manipulated by controlling the serum concentration in the medium. To investigate the possibility of whether the effect of serum deprivation in the induction of Rb might involve a change in the ratio of Rb, we studied the relationship between the number of CAMP-death cells and the time course of their function in the growth curve (Chart 3). To measure the rate of cells was performed according to methods described. Results shown are representative of 2 independent experiments.

Kriel. The incorporation of 3H-N 

Further growth studies with the CAMP-binding protein demonstrated (3-6-fold (260%) increase in the Rb-CAMP-binding protein of stationary-phase Nb1 cells as compared to the rapidly dividing counterpart in logarithmic phase of growth (Chart 3). A qualitatively similar but quantitatively different "breakdown was made in Nb1 cells; the magnitude of increase in Rb of Nb1 cells, during their transition from logarithmic to stationary phase, was 1.5-4-fold (42%) increase. Under identical experimental conditions, no significant fluctuation in the amount of Rb-CAMP-binding protein was observed for the Nb4ov or Nb4 cells.

The mechanism by which serum regulates Rb-CAMP-binding protein appears to be related to intracellular CAMP concentrations. As illustrated in Chart 3, increases in CAMP levels were observed both during the transition from logarithmic to stationary phase and when cells were grown in medium containing 1% rather than 10% FCS.

The experiments presented above demonstrate that the Rb-CAMP binding activity of N-18 neuroblastoma cells can be regulated by incubation conditions in the growth medium. One possible interpretation of this result is that serum (or other factors) have a direct inhibitory effect on the expression of Rb. Alternatively, it is possible that the increased expression of Rb at low serum concentrations is key to differentiation and/or decreased cellular growth. In order to manipulate cell growth and differentiation without introducing variables such as serum concentration or time of growth in serum-containing media, N-18 neuroblastoma cells were plated at 6 different densities. At the end of a 3-day growth period, the density seeded cells...
quelled the stationary phase of growth, whereas the sparsely
seeded cells continued in logarithmic growth. The amount of R
CAMP-binding activity was then studied and compared. Results
are shown in Charts 4 and 5 and in Fig. 3.
Chart 4 illustrates the growth rate of NB-B, (A) and NB-R (B) cells at
different seeding densities. In addition to the aforementioned
effects of serum on cell growth and saturation density,
the rate of increase in cell count is also dependent on the
seeding density. Thus, densely seeded neuroblastoma cells,
compared to the sparsely seeded population, showed reduced
growth rates. Morphological observations of NB-B cells showed
a high proportion of cells with long neurites at the stationary
phase of growth.
A detailed examination of the incorporation of 8-6H2(32P)3-
CAMP into cytosolic proteins of NB-B and NB-R cells seeded at
various cell densities revealed the following. (4) For NB-R cells,
there were basically 2 patterns of incorporation reflected by
cells at the logarithmic and the stationary phase of growth.
Stationary-phase NB-R cells, regardless of seeding cell con-
centration, had significantly higher amounts of R-CAMP binding
activity than cells at the logarithmic phase of growth (Fig. 3;
Chart 5A). Under identical experimental conditions, little or no
change in the R-CAMP-binding protein was observed (2)
(unlike the tight coupling) of the expression of R-CAMP-binding
protein to the stationary phase of growth in NB-B cells, the
specific activity of R-CAMP-binding protein of NB-R cells did
not show dramatic changes as a function either of seeding cell
concentration or of logarithmic versus stationary phase of
growth (Fig. 3; Chart 5A). These results suggested that the
expression of R was specific for stationary-phase NB-R cells.
The fact that significant increases in R-B were not observed in
early logarithmic-phase NB-R or stationary-phase NB-R cells

Chart 4. Growth rates of 18 neuroblastoma cells at different cell and serum
concentrations. NB-B (A) and NB-R (B) cells were seeded at 6 different concen-
trations. Cells were started daily with a hemocytometer. Results are expressed
in number of cells per dish (approximately surface, 25 cm). Bars, saturation densities of NB-B and NB-R cells.

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CHART 5. Studies on the incorporation of 8-6H2(32P)3-CAMP, intracellular cAMP
concentration, and net growth as a function of seeding cell density. The various
symbols used identify the corresponding cell populations depicted in Chart 4.
Cells were harvested 3 days after plating; all assays were carried out according
the methods described. Note the difference in scales used for A and B.
activity in neuroblastoma cells is not known. Based on a num-
ber of studies (19, 32), as well as the results presented in this 
study, the possibility of cAMP serving as a second messenger
seems plausible. Measurable intracellular cAMP concent-
trations, expressed in pmol per mg protein, indicate elevated
levels of cAMP in NB14 cells compared to the NB104 cells.
Furthermore, if both the NB14 and the NB104 cells, there
appears to be a relationship between cells at stationary state
of growth and increased intracellular cAMP (Chart S).

DISCUSSION

One of the major contributions from cell culture studies was
the demonstration that proteins involved in cAMP metabolism
could be adaptive. Thus, in S-49 lymphoma cells, intracellular
phosphodiesterase activity fluctuates as a function of cAMP
concentration inside the cell; this effect of cAMP is mediated
through the activation of a cAMP-dependent protein kinase (4-
6, 15). The causal relationship of the cAMP-induced increase
in phosphodiesterase activity to the cAMP-mediated cytotoxic
effects in S-49 lymphoma cells is not clear. In mouse neuro-
blastoma cells, the addition of 8-Br-cAMP to the growth medium
causes morphological differentiation (28); it also produces in-
crease in cAMP phosphodiesterase and cAMP-binding pro-
tein (29). The precise relationship between these cAMP-in-
duced changes has not yet been resolved. Studies aimed at
delineating the role of cAMP-binding protein in the differentia-
tion of neuroblastoma cells have been hindered by the dual
action of 8-Br-cAMP in both synchronizing differentiation and inhibiting
growth. The use of 8-Br-cAMP in the mix concentration
range, its effects on the intracellular nucleotide pool, and
the growth inhibitory effects of butyrate certainly serve to
c complicate the issue even further.
In this report, we studied the regulation of cAMP binding
activity by serum concentration in the growth medium. The
results showed that the R, cAMP binding activity is negatively
regulated by serum and that the regulatory mechanism may
involve modulation of intracellular cAMP. That the induction
of Rl by serum deprivation is specific for cells at the stationary
phase of growth and was not observed in cells at the logarithmic
phase of growth suggests the possibility of a direct expres-
sion by serum or serum growth factor(s) on the expression of R.
Attempts were also made to dissociate the effects of slowing
cell growth from the effects of differentiation, a phenomenon
incompatible with rapid growth, on the expression of R cAMP-
binding protein. Using NB104 cells, a comparison was made
between the rapidly growing cells in logmodic phase of
growth and the predominantly quiescent cells in stationary
phase of growth; although some increase (40%) in R i was
noted in the stationary phase cells, its magnitude was not
nearly comparable to that observed in the NB104 cells. Fur-
thermore, the addition of 2% dimethylsulfoxide to the culture
medium inhibited cell growth by >30%, without producing an
increase in Rl cAMP binding activity. The increased expression of R, cAMP-binding protein in stationary phase cells is
consistent with the appearance of a significant population of highly differentiated and nondiving cells, suggests an intimate relationship between these 2 param-
ers. Nevertheless, in view of the varying degrees to which a
differentiated morphology (length of neurite, number of neurites
per cell) is expressed in cells in tissue culture, the direct relationship between neurite extension and the expression
of Rl, cAMP-binding protein remains to be documented at a single-
cell level.

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Fig. 1. Photomicrograph of stationary-phase NB+, (A) and NB−, (B) cells.