Reverse Transformation of Harvey Murine Sarcoma Virus-transformed NIH/3T3 Cells by Site-selective Cyclic AMP Analogs*

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Pierosandro Tagliaferri‡, Dionyssios Katsaros§, Timothy Clair‡, Leonard Necker§, Roland K. Robins§, and Yoon Sang Cho-Chung||

From the ‡Cellular Biochemistry Section, Laboratory of Tumor Immunology and Biology, and §Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892 and the ¶Nucleic Acid Research Institute, Costa Mesa, California 92626

Eighteen site-selective cAMP analogs modified at either the C-8 position or the C-6 position were tested for their growth regulatory effects on the Harvey murine sarcoma virus-transformed NIH/3T3 clone 13-3B-4 cells grown in a serum-free defined medium. All 18 analogs, when tested individually, exhibited an appreciable growth inhibitory effect at micromolar concentrations.

The most potent growth inhibitory analogs contained a thio moiety at the C-8 position. In general, C-6 analogs required 5-10-fold greater concentrations than C-8 analogs to produce the same degree of growth inhibition. The growth inhibition induced by these analogs was accompanied by a change in cell morphology; cells treated with the analogs exhibited the morphology characteristic of untransformed fibroblasts, while untreated cells retained a transformed phenotype.

The regulatory subunit of cAMP-dependent protein kinase, the cAMP receptor protein, has two different intrachain cAMP binding sites, and cAMP analogs modified at the C-8 position (C-8 analogs) are generally selective for Site 1, while analogs modified at the C-6 position (C-6 analogs) are generally selective for Site 2. Thus, C-8 and C-6 analogs were tested in combination to enhance the growth regulatory effect. Both growth inhibition and morphological change were enhanced synergistically by a combination of the C-6 and C-8 analogs. Two C-6 analogs or two C-8 analogs added together did not cause synergism.

For both growth inhibition and phenotypic change, C-8 thio analogs acted far more synergistically than C-8 amino analogs when cells were treated in combination with C-6 analogs, suggesting a response of the R II rather than the R I cAMP receptor protein. DEAE-cellulose chromatography revealed that the growth inhibition, in fact, correlates with an increase of the R II cAMP receptor protein and a decrease of the R I receptor protein. The growth inhibitory effect of the site-selective analogs was not due to the cytotoxic effect of adenosine metabolites as shown by the different behavior of 8-CI-cAMP compared with 8-CI-adenosine in 1) cell cycle effects and 2) release from growth inhibition.

It is concluded that the observed growth inhibition and phenotypic reversion of 13-3B-4 cells is most likely mediated through the cellular effector, the R II cAMP receptor protein.

Transformation of cells with RNA tumor viruses results in marked changes in cell morphology, growth-related properties, and numerous cellular components associated with transformation (1). It has been shown that one of the cell components that changes rapidly with transformation is the intracellular cAMP level and that reversal of transformation can be obtained by treatments with cAMP analogs (2). It was suggested that cAMP analogs work by increasing the cellular cAMP concentration. This is brought about by inhibition of phosphodiesterase through competition between the analog and endogenous cAMP. Thus, raising the cellular cAMP level is the analog effect generally believed to be involved in the reverse transformation process. There are, however, a number of studies (3-5) that indicate that a decrease or increase of cellular cAMP does not correlate with transformation or the reverse transformation process, suggesting that cellular effector(s) other than endogenous cAMP may be involved in the cAMP regulation of cell growth.

CAMP in mammalian cells functions by binding to its receptor protein, the regulatory subunit of cAMP-dependent protein kinase (6, 7). Two distinct isozymes, type I and type II protein kinases, having different regulatory subunits (R I, R II) but an identical catalytic subunit have been identified (8, 9). Differential expression of these isozymes has been shown to be linked to regulation of cell growth and differentiation (10-13).

Because a mixture of type I and type II kinase isozymes is present in most mammalian cells (8, 9), selective modulation of these isozymes in intact cells may be a crucial function of cAMP. All past studies of the cAMP regulation of cell growth employed either a few earlier known cAMP analogs that require unphysiologically high concentrations (millimolar) or agents that raise cellular cAMP abnormally and continuously high levels (14-16). Under these experimental conditions, separate modulation of type I and type II kinase isozyme is not possible, since cAMP at high levels activates both isozymes maximally and equally without discrimination (6, 9, 17). Each regulatory subunit (R I, R II) of protein kinase isozyme contains two types of binding sites for cAMP, Site 1 and Site 2 (18, 19). These cAMP binding sites can be differentiated based on their cAMP dissociation rates (18, 19) and cAMP analog specificity (19). Site 1 is characterized by a slower dissociation rate and has a relative selectivity for cAMP analogs modified at the C-8 position on the adenine ring (C-8 analogs), while Site 2 has a faster dissociation rate and is more selective for analogs modified at the C-6 position.

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‡ To whom correspondence should be addressed: National Cancer Institute, National Institutes of Health, Bldg. 10, Rm. 6B38, Bethesda, MD 20892.

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(C-6 analogs). The Site 1- and Site 2-selective analog binding experiments established that binding of cyclic nucleotides at either site stimulates binding at the other site for both type I and type II protein kinase isozymes (20, 21). Furthermore, the site-selective analogs in appropriate combinations demonstrated synergism of binding and show specificity toward either type I or type II kinase (22, 23). This unique binding specificity of site-selective cAMP analogs is not mimicked by cAMP itself or by previously studied analogs.

Despite extensive studies in vitro, the studies in vivo of the effect of site-selective analogs in intact cells or tissues have been scarce (24-27). In the present studies, we, therefore, investigated the effect of site-selective cAMP analogs on the growth and morphology of the Harvey murine sarcoma virus DNA transfected, the NIH/3T3 clone 13-3B-4 cells. The experiments were carried out with the cells grown in a serum-free defined medium to avoid any possible interference of the analog effect that can be caused by the uncharacterized factors present in serum. By using site-selective analogs on the intact transformed cells, we can study the cellular cyclic nucleotide effector mechanisms involved in growth control because: 1) a new test is available to establish whether or not the cAMP receptor protein is the mediator of the response; 2) the analog binding to Site 1 and 2 is cooperative, and therefore, the analog sensitivity toward synergism for the binding to the cAMP receptor protein in intact cells can be measured; 3) if the analog combinations demonstrate synergism, lower total analog concentrations can be used to achieve the same cellular response obtained by using a single analog; and 4) we can selectively modulate one of two protein kinase isozymes present in intact cells to correlate the analog effect with a particular protein kinase isozyme.

**EXPERIMENTAL PROCEDURES**

**Materials—**cAMP, N6,2'-dibutyryl cAMP (Bt-cAMP), 8-Br-cAMP, and cGMP were from Boehringer Mannheim. N6-MonobutylcAMP (Bi-cAMP) and 8-Br-cGMP were obtained from Sigma. All other cAMP analogs were synthesized at the Nucleic Acid Research Institute, Costa Mesa, CA. Dulbecco’s modified Eagle’s medium, Ham’s F-12, glutamine, penicillin, and HEPES were purchased from Gibco Laboratories, and insulin, transferrin, histidine HCl, and poly-D-lysine were from Sigma.

**Cell Culture—**13-3B-4 cells, an NIH/3T3 clone that had been transfected with Harvey murine sarcoma virus DNA (kindly provided by Dr. D. R. Lowy, National Cancer Institute), were cultured in Dulbecco’s modified Eagle’s medium containing penicillin (100 units/ml) and streptomycin (100 μg/ml) and supplemented with 10% fetal bovine serum. Cells were then grown in 60-mm Petri dishes that had been coated with poly-D-lysine in the absence or presence of additives (cAMP analogs) in serum-free chemically defined medium. The serum free medium was composed of Dulbecco’s modified Eagle’s medium and Ham’s F-12 (nutrient mixture F-12 Ham) in a ratio of 75:25 and supplemented with bovine insulin (5 μg/ml), transferrin (5 μg/ml), histidine HCl (42 μg/ml), glutamine (292 μg/ml), penicillin (100 units/ml), streptomycin (100 μg/ml), and HEPES (20 mM, pH 7.3); the medium was changed every 48 h, and the additives were provided every 48 h. The cells were grown in humidified incubators in an atmosphere of 10% CO2.

For cell growth experiments, 2 x 10^4 cells/60-mm dish were seeded in serum-containing medium and, 24 h later (day zero), the medium was changed to serum-free medium, and the additives were added then and every 48 h thereafter. At the desired times, cell counts in duplicate were performed on a Coulter counter after harvesting cells with gentle trypsinization.

For morphological studies, 5 x 10^4 cells were plated onto the precoated 60-mm dishes in serum-containing medium and then shifted to serum-free medium as in the cell count experiment. Photographs were taken using a Leitz inverted microscope.

**Cell Cycle Analysis—**DNA histograms were generated on the FACS II (Becton Dickinson, Sunnyvale, CA) by using the DNA intercalating dye propidium iodide as described by Braylan et al. (28). The percentage of cells in each cycle phase was calculated by using a PDP-11/34 computer (Digital Equipment Corp., Maynard, MA) and software (Division of Computer Research and Technology, NIH) as previously described (29).

**DEAE-cellulose Chromatography of Protein Kinase—**The cAMP-dependent protein kinase holoenzymes and the regulatory subunits of 13-3B-4 cells were separated using DEAE-cellulose according to the method of Robinson-Steiner and Corbin (22). The cell pellets (2 x 4 x 10^6 cells), after two washes with phosphate-buffered saline, were hand homogenized with a Dounce homogenizer (60 strokes). The homogenates were centrifuged for 20 min at 10,000 g. The resulting supernatants (2-2.5 ml) were loaded on a 0.9 x 5.0-cm column pre-equilibrated with Buffer B. After washing, the column was eluted using a 60-ml total column gradient from 0 to 0.4 M NaCl in Buffer B with a 1.0-1.2 ml fraction volume.

**Protein Kinase Assay—**The activity of the cAMP-dependent protein kinase was determined by the method previously described (22). The reaction mixture contained a 50-μl sample and a 50-μl solution of 40 mM potassium phosphate (pH 6.8), 8 mg/ml calf thymus histone (type II-A, Sigma), 2 mM theophylline, 100 μM [γ-32P]ATP (-60 cpm/pmol), 20 mM magnesium acetate, and ±10 μM cAMP. The reaction mixture was incubated at 30 °C for 7 min in a shaking water bath. The reaction was stopped by the addition of 1.0 ml of ice-cold 20% trichloroacetic acid. After standing in an ice bath for 30 min, the samples were passed through Millipore filters (type HA, HAWP 02500). The filters were washed five times with 1 ml of 5% trichloroacetic acid and then counted with liquid scintillation in 7 ml of Flitron-X (National Diagnostics, Somerville, NJ).

**cAMP Binding Assay—**cAMP binding activity was measured by the method previously described (8) at cAMP exchange conditions (30, 31). The reaction mixture contained a 50-μl sample and a 50-μl solution of 100 mM potassium phosphate (pH 6.8), 2 mM EDTA, 10 mM theophylline, 2 μM NaCl, 2 μM [3H]cAMP (~20 cpm/mmol), and ±2 mM cAMP. After incubation at 23 °C for 1 h, the reaction was stopped by filtering through Millipore filters (type HA), and the filters were washed five times with 1.0 ml of 10% potassium phosphate (10 mM, pH 6.8) and then counted as for the protein kinase assay.

**Photoaffinity Labeling of CAMP Receptor Proteins—**The photoactivatable incorporation of 8-N3-[32P]cAMP was performed as previously described (32) with a minor modification. The reaction mixture (final volume, 50 μl) contained 10^-4 M 8-N3-[32P]cAMP ± 1000-fold excess unlabeled cAMP and samples (50-100 μg of protein) in Buffer Ten (0.1 M NaCl, 5 mM MgCl2, 1% Nonidet P-40, 0.5% sodium deoxycholate, 2 kallikrein-inactivating units/ml bovine sprotinin, and 100 μg/ml Tris-HCl, pH 7.3). The homogenates were boiled for 3 min, and centrifuged at 7700 g. The resulting bound material was analyzed by SDS-polyacrylamide gel electrophoresis.

**RESULTS**

**Effect of cAMP Analog Concentrations on Growth Inhibition—**A variety of cAMP analogs, modified at the C-6 position or the C-8 position of the adenine moiety, at various concentrations, were tested for their growth inhibitory effect on 13-3B-4 cells. For morphological studies, Fig. 1 shows the dose-response characteristics of representative analogs. Generally, the analogs fit into two major categories based on their dose-response characteristics. The effects of analogs containing a thio derivative (8-thio-phosphoryl-cAMP, 8-thiomethyl-cAMP, 8-thioprpyl-cAMP), 8-Cl-cAMP, and 8-Br-cAMP varied directly linear with the concentrations (Fig. 1, A and B). Anologs that were modified at the C-6 position (Fig. 1, C and D) and 8-amino...
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cAMP (Fig. 1A) displayed hyperbolic dose-response curves. In general, the C-8 analogs exhibited 5-10-fold greater inhibitory effect compared with the C-6 analogs at given concentrations. Table 1 shows values for the growth inhibitory effect of 21 cyclic nucleotide analogs. The analogs are listed in order from the most to the least potent for growth inhibition for a given modification on the adenine ring. Analogs modified with a thio moiety at the C-8 position, such as 8-thio-p-chlorophenyl-, 8-thiomethyl-, and 8-thioisopropyl-cAMP, were the most potent inhibitors, exhibiting over 60% inhibition at 50 μM concentration. 8-Cl, 8-Br, and 8-aminomethyl-cAMP were the next most potent inhibitors, and all the N6 derivatives tested demonstrated poor growth inhibition at 50 μM concentration. When analogs were compared for their effects at higher concentration (100 μM), a similar order of efficacy was obtained within C-6 analogs or within C-8 analogs; however, the effect of some C-6 analogs, N6-carbamoyl-phenyl- and N6-butyryl-cAMP, became as potent as theo derivatives of C-8 analogs.

Effect of Analog Combination on Growth Inhibition—Previous studies have demonstrated that binding of a cAMP analog selective for either intrachain site on the regulatory subunit of protein kinase stimulated binding of a cAMP analog selective for the other site (20, 21). It was further demonstrated that two such site-selective analogs could also be used in combination to synergistically activate protein kinase (22, 23). Fig. 2 shows an experiment in which the Site 2-selective analog N6-butyryl-cAMP and the Site 1-selective analog 8-thiomethyl-cAMP were added alone, and in combination, to 13-3B-4 cells in culture. In Fig. 2A, the cell number was counted after the cells were treated for 3 days with 2.5 μM 8-thiomethyl-cAMP. The insets show the synergism quotients (23, 25). The synergism quotient was defined as the net growth inhibitory effect of an analog

Table 1

Effect of cyclic nucleotides on growth inhibition

The percentage growth inhibition values shown at 50 and 100 μM nucleotide concentrations were determined from the dose-response curve experiments like those shown in Fig. 1 and represent an average value obtained for each analog from two or more separate experiments.

| Cyclic nucleotide analog | Substituent | Growth inhibition at 50 μM | Growth inhibition at 100 μM |
|--------------------------|------------|---------------------------|---------------------------|
| cAMP                     |            | 0                         | 0                         |
| C-6 analog               |            |                           |                           |
| N6-Carbamoylphenyl       | N6-CO2-C3H7| 38                        | 60                        |
| N6-Butyryl               | N6-CO2-C3H7| 30                        | 54                        |
| N6-Carboxylethoxy        | N6-CO2-C3H7| 30                        | 54                        |
| N6,0-Dibutyryl           | N6-CO2-(CO2-C3H7)2| 23| 40                        |
| N6-Carbamoylmethyl       | N6-CO2-C3H7| 18                        | 30                        |
| N6-Carbamoylpropyl       | N6-CO2-C3H7| 16                        | 28                        |
| N6,N6-Diethyl            | N6-(C2H5)2| 15                        | 20                        |
| N6-Methoxy               | N6-O2-C3H7| 7                         | 20                        |
| C-8 analog               |            |                           |                           |
| 8-Thio-p-chlorophenyl    | 8-S-Cl     | 83                        | 95                        |
| 8-Thiomethyl             | 8-S-CH3    | 75                        | 85                        |
| 8-Thio-isopropyl         | 8-S-CH3    | 64                        | 80                        |
| 8-Chloro                 | 8-Cl       | 60                        | 70                        |
| 8-Br                     | 8-Br       | 54                        | 65                        |
| 8-Aminomethyl            | 8-NH2      | 50                        | 60                        |
| 8-Amino                  | 8-NH2      | 44                        | 60                        |
| 8-Amino-β-hydroxyethyl   | 8-NH-(CH2)2-OH| 35| 50                        |
| 8-Thio-pentyl            | 8-S-CH3    | 25                        | 45                        |
| 8-Methoxy                | 8-O2-C3H7  | 20                        | 40                        |
| cGMP analog              | 8-Bromo    | 0                         | 0                         |
| cGMP                     | 8-Br       | 0                         | 0                         |
The $N^\alpha$ analogs exhibited synergism with another C-8 thio derivative, 8-thioisopropyl-cAMP (synergism quotient = 1.5), and with 8-Cl-cAMP and 8-Br-cAMP (synergism quotient = 1.5–1.7). Only a limited degree of synergism was expressed, however, when $N^\alpha$ analogs were combined with 8-amino derivatives (average synergism = 1.2). Thus, the C-6 analogs acted far more synergistically when in combination with 8-thio-cAMP analogs than with 8-amino derivatives.

Synergism of growth inhibition was only seen when a Site 1-selective analog was added with a Site 2-selective analog but not when two Site 1-selective or two Site 2-selective analogs were combined (Table II). When two C-6 analogs or two C-8 analogs were combined such that alone they exhibit synergistic effects on growth inhibition. When 2.5 $\mu$M 8-thiomethyl-cAMP were added in combination with N$^\alpha$-butyryl-cAMP (25 $\mu$M) when added alone, the open portion of bars represents percentage growth inhibition values for increasing concentrations of N$^\alpha$-butyryl-cAMP (A) and 8-thiomethyl-cAMP (B) when added alone. The height of the bars on the left of each pair represents the sum of the individual analog effects or the expected percentage growth inhibition if analogs were added together. The total height of the solid bar indicates the observed percentage growth inhibition when analogs were added in combination at the indicated concentrations. The differences between the heights of the paired bars reflect the magnitude of synergism of growth inhibition. The data represent means and standard errors of duplicate determination in three experiments. The insets show the synergism quotient at each concentration of analog combination. The synergism quotient was defined as previously described (23, 25). 8-S-CH$_3$, 8-thiomethyl-cAMP, N$^\alpha$-But, N$^\alpha$-butyryl-cAMP.

**TABLE II**

| Analog combination | Synergism quotient |
|--------------------|-------------------|
| N$^\alpha$-Carbamoylphenyl (10 $\mu$M) + N$^\alpha$-butyryl (10 $\mu$M) | 0.79 |
| N$^\alpha$-Carbamoylpentyl (50 $\mu$M) + N$^\alpha$-butyryl (10 $\mu$M) | 0.86 |
| N$^\alpha$-Carbonylethoxy (50 $\mu$M) + N$^\alpha$-carbamoylphenyl (10 $\mu$M) | 0.86 |
| 8-Thio-p-chlorophenyl (5 $\mu$M) + 8-thiomethyl (10 $\mu$M) | 0.76 |
| 8-Thiomethyl (5 $\mu$M) + 8-chloro (5 $\mu$M) | 1.01 |
| 8-Chloro (5 $\mu$M) + 8-bromo (10 $\mu$M) | 0.95 |

**Fig. 3.** Effect of cAMP analog on the morphology of Harvey murine sarcoma virus-transformed NIH/3T3 clone 13-3B-4 cells. A, untransformed NIH/3T3 cells grown in serum-free medium for 4 days; B and C, 13-3B-4 cells grown in serum-free medium for 4 days in the absence and presence of 500 $\mu$M BMAMP, respectively. × 40.
growth inhibition of 10–30% each, their synergism quotients were less than or equal to 1 (Table II).

Effect of cAMP Analogs on Cell Morphology—The growth inhibitory effect of cAMP analogs in 13-3B-4 cells correlated with a change in the cell morphology. As shown in Fig. 3, cells treated with Bt$_2$cAMP (500 μM) (panel C) exhibited a morphology characteristic of untransformed fibroblasts (panel A), while the untreated cells retained a transformed phenotype (panel B). Treatment of cells with N$^6$-butyryl-cAMP (100 μM), N$^6$-carbamoylphenyl-cAMP (100 μM), 8-thio-p-
The synergistic growth inhibitory effect of the C-6 and C-8 analog combination was also reflected in the change in cell morphology. As shown in Fig. 4, treatment of cells with either 100 μM 8-Br-cAMP (Fig. 4B) or 10 μM 8-Br-CAMP (Fig. 4D) for 4 days did not induce a change in the cell morphology; cells were round and refractile and eventually floated away from the substrate as did the untreated transformed cells (Fig. 4A). When the cells were treated in a combination of 8-Br-cAMP (100 μM) and 8-Br-CAMP (10 μM), however, the phenotypic transformation was inhibited (Fig. 4E); cells were flat and exhibited contact-inhibited monolayers just as cells treated with a high concentration of 8-Br-cAMP (500 μM) alone (Fig. 4C). The same synergism was demonstrated between the other C-6 analog, N6-butyryl-cAMP (50 μM), and the C-8 analog, 8-thiomethyl-cAMP (5 μM) (Fig. 4F), as well as with other combinations of C-6 and C-8 analogs that demonstrated the synergism of growth inhibition.

**Effect of 8-Cl-cAMP and 8-Cl-adenosine on Growth and Cell Cycle Progression**—We examined whether the growth inhibitory effect of the site-selective cAMP analog reflects a cytotoxic effect due to an adenosine metabolite. Fig. 5 shows time courses of 8-Cl-cAMP and 8-Cl-adenosine in their growth inhibition and release from the inhibition. While the untreated control cells showed a logarithmic increase in cell number, cells treated with either 8-Cl-CAMP or 8-Cl-adenosine exhibited a marked reduction in cell growth and eventually stopped replicating within 3–4 days. Upon release of treatment, the cells treated with 8-Cl-cAMP for up to 9 days resumed growth within a few days, and the rate of cell growth became similar to that of untreated control cells, whereas the 8-Cl-adenosine–treated cells remained growth–inhibited up to 2 weeks after the release from the treatment. Thus, the growth inhibition produced by 8-Cl-cAMP and 8-Cl-adenosine was mediated through two different mechanisms, the former by a decrease in the rate of replication without affecting cell viability and the latter by cell killing.

We examined whether the reduced cell proliferation observed in 13-3B-4 cells after treatment with the analogs is due to a specific block in one phase of the cell cycle. As shown in Table III, the fractions of cells in G1, S, and G2/M phases were not appreciably different between the control cells (untreated) and the cells treated with N6-butyryl-cAMP (50 μM) + 8-thio-methyl-cAMP (5 μM), N6,02'-BT2-cAMP (1 mM), or 8-Cl-cAMP. Thus, the inhibition of cell growth induced by the cAMP analogs was not associated with a specific block in one phase of the cell cycle. However, 8-Cl-adenosine treatment induced an appreciable increase of the cell population in G1 phase with a marked reduction in S phase (Table III). These data, combined with those from the release experiments (Fig. 5), confirm that the growth inhibition produced by 8-Cl-cAMP treatment was not due to its adenosine metabolite. In fact, by high performance liquid chromatography analyses, 8-Cl-adenosine was not detected in either cell extracts or medium from the cells treated with 8-Cl-cAMP for 48–72 h (data not shown).

**Effect of cAMP Analogs on the Levels of R' and R" cAMP Receptor Proteins**—The synergistic effect demonstrated on the growth inhibition and phenotypic reversion of 13-3B-4 cells by the C-6 and C-8-thio derivatives of cAMP analogs in combination indicated a response of type II protein kinase rather than type I kinase present in the cells.

The relative proportions of free R' and R" and holoprotein kinases, type I and type II, were determined using DEAE-cellulose chromatography. Chromatography of the cytosols from treated and untreated 13-3B-4 cells is shown in Fig. 6. Catalytic subunit was eluted before the start of the NaCl gradient (results not shown). The untreated cells (Fig. 6a) showed two major peaks (peaks 1 and 2) of cAMP-dependent protein kinase activity that were coincident with peaks of cAMP binding activity. Peak 1 eluted at 0.07 M NaCl, and peak 2 eluted at 0.22 M NaCl, and the kinase and binding activities of peak 1 were ~3-fold that of peak 2. In addition, there were two minor cAMP binding peaks (peaks 3 and 4) with no cAMP-dependent protein kinase activity, eluted at 0.13 and 0.30 M NaCl, respectively. Radioautography after photoaffinity labeling the fractions of the eluents with 8-azido-[32P]cAMP (32) and performing NaDodSO4-polyacrylamide gel electrophoresis (33) showed that peaks 1 and 3 contained R', whereas peaks 2 and 4 contained R" (Fig. 6, c and d). These results suggest that peaks 1 and 2 are similar to types I and II holoprotein kinases, and peaks 3 and 4 are

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**Fig. 5. Comparison of the effect of 8-Cl-cAMP and 8-Cl-adenosine on the growth inhibition and release of inhibition of 13-3B-4 cells.** O, untreated control cell; △, V, cells treated with 8-Cl-cAMP (50 μM) for 5 and 9 days, respectively; △, V, cells treated with 8-Cl-adenosine (30 μM) for 5 and 9 days, respectively. 1 × 10⁵ cells/60-mm dish were seeded, and 24 h later (day 0), the medium was removed, and fresh medium and the additives were added then and every 48 h thereafter. The arrow indicates removal of additives. The triplicate cell count for each experimental point never varied by more than 10%.

**Table III**

| Treatment            | Cell population |
|----------------------|-----------------|
|                      | G1   | S    | G2/M |
| Control              | 60   | 18   | 22   | 100  |
| N6-butyryl-cAMP (50 μM) + 8-S-Br-cAMP (5 μM) | 58   | 19   | 23   | 37   |
| Control              | 60   | 17   | 23   | 100  |
| N6,02'-BT2-cAMP (1 mM) | 52   | 23   | 25   | 43   |
| Control              | 60   | 53*  | 19   | 22   | 24   | 45   | 50   |
| 8-Cl (50 μM)         | 63   | 54   | 15   | 22   | 24   | 22   | 45   | 50   |
| 8-Cl-adenosine (50 μM) | 73   | 69   | 7    | 12   | 20   | 19   | 35   | 40   |

* The paired numbers were derived from two separate experiments.
similar to free R' and R'' subunits found in mammalian tissue cytosols (8, 35).

When the cells were treated for 3 days with N^6-butylr-cAMP (25 μM) + 8-thiomethyl-cAMP (5 μM), the chromatographic pattern was considerably altered (Fig. 6b). Both cAMP-dependent protein kinase activity and cAMP binding activity of peak 1 decreased to 50% of those in the untreated cells, while the cAMP-stimulated kinase and cAMP binding activities of peak 2 increased to 2- and 3-fold, respectively, over that of untreated cells. In addition, peak 4 cAMP binding activity increased to 3-fold over that of untreated control cells, while peak 3 remained without appreciable change. Thus, decrease of type I holoenzyme (peak 1) was accompanied by an increase of both type II holoenzyme (peak 4) and R'' subunit (peak 4). A similar change in the elution profile was observed when the cells were treated with other combinations of N^6 + C-8 analog that showed synergism in growth inhibition, whereas each of these analogs alone exhibiting little or no growth inhibition caused no apparent change in the elution profile (data not shown). Thus, the same synergism of N^6 + C-8 analog combination was observed in protein kinase activity as that observed for growth inhibition and phenotypic change. When intact untreated cells were washed just before collection with the analog combination-containing medium, the elution profile was the same as that of cytosol from untreated cells. Thus, the change in peaks 1, 2, and 4 observed in the analog-treated cells was not a consequence of residual analog from the medium interacting with cytosol during cell homogenization.

The increase in peaks 2 and 4 observed after the analog treatment suggests that the analog caused both dissociation and increase of type II protein kinase. Furthermore, the presence in the treated cells of a considerable amount of type II holoenzyme (peak 2) suggests that peak 2, at least in part, may contain a partially dissociated form of holoenzyme, such as R_2C (36), which may not be resolved from R_2C (8) by DEAE-cellulose chromatography.

**DISCUSSION**

The results presented here are the first unequivocal demonstration that site-selective cAMP analogs are capable of exerting a major regulatory effect on the growth of transformed fibroblastic cells.

We demonstrated here that the site-selective cAMP analogs regulated cell growth at micromolar concentrations. Previous studies of cAMP effect on cell growth, using the earlier studied analogs, reported the effective analog concentrations in an unphysiologic millimolar range (14-16). Thus, the new site-selective analogs are active in their in vivo effect at much lower concentrations.

The efficacy of the analogs tested in the present studies can be related to three basic analog properties: high lipophilicity, low K_m for protein kinase activation, and stability to low K_m cAMP phosphodiesterase hydrolysis. Analogs with hydrophobic substituents such as an alkyl or aryl group have been shown to be highly lipophilic, whereas analogs with more hydrophilic substituents such as 8-amino derivatives show the lowest lipophilicity (25). Our data showed no clear correlation between the hydrophilic or hydrophobic substituents of analogs and the efficacy for growth inhibition (Table I). Likewise, the growth inhibitory effect of the analogs did not appear to be directly related to the resistance of the analogs toward the phosphodiesterase. The low K_m phosphodiesterase I_	ext{m} values for N^6 derivatives are generally much greater than those of C-8 derivatives (25, 37). However, C-8 analogs, which are much less resistant than N^6 analogs toward phosphodiesterase, showed the greater growth inhibitory effect. The synergistic effect demonstrated by C-6 and C-8 analog combinations further argues against the role of phosphodiesterase in the analog effect. These analogs in combination exerted growth inhibition at concentrations at least one-tenth below the reported (25) I_	ext{m} for the low K_m phosphodiesterase; at these low concentrations, the analogs would not be metabo-
ized to produce toxic products.

The absence of a role for adenosine metabolites in the analog effect was also experimentally demonstrated by direct comparison of the effect of 8-Cl-adenosine to that of 8-Cl-cAMP. The different behavior between 8-Cl-adenosine and 8-Cl-cAMP was demonstrated in 1) cell cycle effects and 2) release from growth inhibition. It seems, therefore, highly unlikely that the phosphodiesterase significantly contributes to the analog-mediated growth inhibition.

The analog effect appears to be more related to low $K_r$ for protein kinase activation. C-8 analogs, having lower or similar $K_r$ values (23, 25) as cAMP, were 5–10 times more potent in growth inhibition than N° analogs which possess 10 times or greater $K_r$ values (23, 25) than cAMP. The growth inhibitory effect of the analogs was related to selective activation of type II protein kinase, that is the inhibition brought about an increase of R° and type II protein kinase with a decrease of R' and type I protein kinase. Thus, the analog efficacy correlated with its ability to selectively activate type II protein kinase over type I kinase. In fact, -8-thio analogs that show preferential activation of type II over type I kinase (23) exhibited greater potency of growth inhibition than 8-amino analogs that preferentially activate type I protein kinase (23).

The synergistic effect of the C-6 and C-8 analog combination demonstrated on growth inhibition further supports that an analog's efficacy is dependent on its ability to selectively activate type II protein kinase. It has been shown that bovine heart type II and rat heart type I protein kinase exhibit a different C-8 analog specificity for stimulation of binding and synergism of activation when combined with a C-6 analog (22). Specifically, when used in combination with C-6 analogs, those analogs with a sulfur atom attached at C-8 act more synergistically for type II protein kinase, and the analogs with a nitrogen atom attached at C-8 exhibit greater synergism for type I protein kinase (22). Our results showing that the synergism of growth inhibition by N° analogs when combined with 8-thio analogs far exceeds that by N6 analogs in combination with 8-amino derivatives, therefore, suggests a response of type II rather than type I protein kinase present in 13-B4 cells.

The synergistic effect of the N° and C-8 analog combination demonstrated in 13-B4 cells is similar to that previously shown by Beebe et al. (25) in isolated rat adipocytes that primarily contain type II protein kinase isozyme. Beebe et al. (25) demonstrated that, both lipolysis and protein kinase activation, C-8 thio analogs act more synergistically than C-8 amino analogs when incubated with adipocytes in combination with C-6 analogs, a characteristic of type II protein kinase. The efficacy of individual analogs in adipocytes versus 13-B4 cells, however, showed some inconsistencies. In 13-B4 cells, N°-butyryl-cAMP was a relatively potent growth inhibitor and N°-carbamoylpropyl-cAMP was a weak inhibitor, whereas, in adipocytes, N°-carbamoylpropyl-cAMP was the most potent activator of all C-6 and C-8 analogs tested, and N°-butyryl-cAMP was quite ineffective. It appears that, in both systems, the analogs exert their effects through binding to type II protein kinase. However, the efficacy of the analogs shown in lipolysis may not be directly comparable to the analog efficacy in the complex phenomenon of growth inhibition.

From these studies, we suggest that a mere decrease or increase in cellular cAMP does not determine cell transformation or reverse transformation, respectively, but that the cellular cAMP effector(s), CAMP-dependent protein kinase, plays an important role in these processes. Site-selective cAMP analogs thus provide us with an important biological tool for understanding the mechanism of cell transformation.

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