Induction of Adenosine 3′,5′-Monophosphate Binding Proteins by N^6,$O^{2'}$-Dibutyryladenosine 3′,5′-Monophosphate in Mouse Neuroblastoma Cells. Analysis by Two-Dimensional Gel Electrophoresis†

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ABSTRACT: Adenosine 3′,5′-monophosphate (cAMP) binding proteins from mouse neuroblastoma cells were purified by cAMP-affinity Sepharose resin and were analyzed by two-dimensional polyacrylamide gel electrophoresis. The two cAMP binding proteins R1 and R2 were identified on the second-dimensional slab gels. The isoelectric points of these proteins are 5.4 (R1) and 5.2 (R2), and both proteins have a molecular weight of 48 000–50 000. These two cAMP binding proteins, identified in the cellular fractions by the photoaffinity labeling techniques, are located above actin (p*I* = 5.4 and 43 000 M_r) on the second-dimensional slab gels. Analysis of cellular fractions, cytoplasmic, particulate, and nuclear nonhistone proteins, from [^{35}S]methionine-labeled cells on two-dimensional gels, shows the presence of two cAMP binding proteins, R1 and R2. N^6,$O^{2'}$-Dibutyryladenosine 3′,5′-monophosphate (Bt_2cAMP) induced only one cAMP binding protein, R1, from three- to eightfold in these fractions. Bt_2cAMP stimulated the synthesis of cAMP binding protein R1 without affecting R2. cAMP binding protein R1 is 0.06 and 0.2% of the total cytoplasmic proteins in the control and Bt_2cAMP treated cells and R2 is 0.06% in both cells. Chromatin isolated from Bt_2cAMP treated cells also showed an increase in [^{3}H]cAMP binding. Bt_2cAMP also affected the levels of 42 nuclear nonhistone proteins: 23 proteins increased and 19 proteins decreased. The increase in the nuclear cAMP binding protein and the changes in other nonhistone proteins may affect the transcriptional activity of some specific genes, thus causing biochemical changes in neuroblastoma cells.

Bt_2cAMP[1] treatment causes morphological differentiation of mouse neuroblastoma cells by increasing neurite formation (Prashad et al., 1977; Furmanski et al., 1971) and causes biochemical changes by increasing enzyme activities (Waymire et al., 1972; Richelson, 1973) and by protein modifications (Prashad et al., 1977). In mouse neuroblastoma cells the levels of cAMP binding proteins were increased when treated with Bt_2cAMP (Prashad et al., 1977; Prashad & Rosenberg, 1978), prostaglandin E_1 (Prasad et al., 1975), and phosphodiesterase inhibitors (Prasad et al., 1976). cAMP binding proteins were increased in the cytoplasmic and particulate fractions of the cells treated with Bt_2cAMP, and the induced-cAMP binding proteins increased in the cytoplasm 15 h after Bt_2cAMP treatment (Prashad & Rosenberg, 1978).

In this present report the purified cAMP binding proteins and photoactivated cAMP binding proteins were analyzed by two-dimensional polyacrylamide gel electrophoresis to determine the number of cAMP binding proteins and their location among the cellular proteins. The cellular fractions, cytoplasmic, particulate, and nuclear nonhistone proteins, from untreated control and Bt_2cAMP treated [^{35}S]methionine-labeled cells were analyzed by two-dimensional polyacrylamide gel electrophoresis. The cAMP binding proteins were increased from three- to eightfold in the cellular fractions from the Bt_2cAMP treated cells, and the cAMP binding proteins were identified by the photoaffinity labeling method. The increase in the nuclear cAMP binding proteins was also shown by an increase in the binding of [^{3}H]cAMP to chromatin. Part of these results has been presented in abstract form (Prashad et al., 1978).

Materials and Methods

Materials

[^{35}S]Methionine (389 Ci/mmol), [^{3}H]cAMP (37.7 Ci/mmol), and [^{32}P]cAMP (17.7 Ci/mmol) were obtained from New England Nuclear, and activated Sepharose was obtained from Pharmacia. Phenylmethanesulfonyl fluoride was obtained from Sigma and (6-chloropurinyl)ribose 3′,5′-monophosphate was obtained from Boehringer Mannheim. Acrylamide and bis(acrylamide) were obtained from Eastman Kodak Chemicals.

Methods

[^{35}S]*Methionine Labeling.* The cholinergic S20 clone of mouse neuroblastoma cells (Amano et al., 1972) was grown in monolayer culture. Cells (3×10^6) were plated per 100-mm plate containing 10 mL of Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum and other supplements and conditions as described (Prashad & Rosenberg, 1978). For Bt_2cAMP treatment, 1 mM Bt_2cAMP was included in the media at the time the cells were plated. Two days after plating the cells were labeled with [^{35}S]methionine as follows. Cells were rinsed with warm DMEM (without methionine and serum), and then 10 mL of warm DMEM (without methionine) with 10% serum was added to plates. Bt_2cAMP was added to the Bt_2cAMP treated cells, and the cells were incubated without methionine at 37 °C for 1 h. Unlabeled methionine was added to a final concentration of 5 μM, and 125 μCi of [^{35}S]methionine (389 Ci/mmol, New England Nuclear) was added per plate. The cells were incubated at 37 °C for 20 h. The uptake of [^{35}S]methionine is generally 25–30% in both control and Bt_2cAMP treated cells,

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[1] Abbreviations used: cAMP, adenosine 3′,5′-monophosphate; Bt_2cAMP, N^6,$O^{2'}$-dibutyryladenosine 3′,5′-monophosphate; cAMP-affinity resin, N^6-(2-aminoethyl)-cAMP–Sepharose; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; IEF, isoelectric focusing.

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and 77–84% of this is incorporated into proteins as determined by trichloroacetic acid precipitation.

Cell Fractionation. [^{35}S]Methionine-labeled cells were rinsed with warm DMEM containing an excess of unlabeled methionine (200 μM) and then with cold 0.9% NaCl containing 1 mM phenylmethanesulfonyl fluoride (PMSF). The subsequent steps were carried out at 4 °C and all the buffers contained 1 mM PMSF. Cells from each plate were scraped in 2 mL of homogenization buffer, containing 0.05 M Tris-HCl, pH 7.4, 0.25 M sucrose, 3 mM $MgCl_2$, and 4 mM 2-mercaptoethanol.

Cells were homogenized in a glass Dounce homogenizer, and the homogenate was centrifuged at 2000 rpm for 5 min at 4 °C in a Sorvall centrifuge. The pellet was used to purify nuclei. The supernatant was centrifuged at 10000 rpm for 15 min; the supernatant is designated as the "cytoplasmic fraction" and the pellet is designated as the "particulate fraction". The cytoplasmic and particulate fractions were treated with RNase and DNase and prepared for two-dimensional gel electrophoresis (O'Farrell, 1975).

Purification of Nuclei and Isolation of Nonhistone Proteins. The crude nuclear pellet was washed twice with homogenization buffer containing 1 mM PMSF and 0.25% Triton X-100. The nuclei were resuspended in 3 mL of the same buffer, mixed with 14 mL of 2.4 M sucrose in 0.05 M Tris-HCl, pH 8.0, and 0.01 M $MgCl_2$, and centrifuged at 20000 rpm in SW 27.1 Beckman rotor for 90 min at 5 °C. The pellet contains purified nuclei.

The purified nuclear pellet was rinsed once with homogenization buffer and resuspended in 20 mM Tris-HCl, pH 7.5, plus 1 M NaCl. The nonhistone proteins (NHP) were isolated by the method of Langan (1967) as described by Kamiyama & Wang (1971). In our method, the NHP supernatants from two Bio-Rex 70 treatments were pooled, and proteins were precipitated with 2 volumes of cold ethanol at −20 °C for 24 h. The precipitated NHPs were collected by centrifugation at 12000 rpm for 30 min. The NPHs were treated with DNase and RNase and prepared for two-dimensional gel electrophoresis (O'Farrel, 1975).

Purification of cAMP Binding Proteins by cAMP-Affinity Chromatography. The N^6-(2-aminoethyl)-cAMP derivative was prepared as described (Dills et al., 1975) and activated Sepharose was obtained from Pharmacia. The derivative was coupled to activated Sepharose as described (Dills et al., 1975).

[^{35}S]Methionine-labeled cells were homogenized in 0.05 M Tris-HCl, pH 7.4, 0.25 M sucrose, 3 mM $MgCl_2$, 4 mM mercaptoethanol, and 1 mM PMSF. The homogenate was centrifuged at 2000 rpm for 5 min to remove nuclei, and then the supernatant was centrifuged at 10000 rpm for 15 min. The supernatant cytoplasmic fraction was passed through a DEAE-cellulose (Whatman DE-52) column (1.5 × 30 cm). The column was washed with 100 mL of buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 4 mM 2-mercaptoethanol, and the proteins were eluted with 500 mL of a linear gradient of 0–0.3 M KCl in the above buffer. Both control and Bt_2cAMP treated cell fractions eluted between 0.06 and 0.065 M KCl show a major cAMP binding protein peak. Fractions containing cAMP binding activity were pooled and passed through the cAMP-affinity column. The cAMP binding proteins were eluted with 30 mM cAMP in Mes buffer (Dills et al., 1975). cAMP binding proteins were extensively dialyzed against 5 mM Tris-HCl, pH 7.4, and 1 mM 2-mercaptoethanol at 30 °C and lyophilized. The lyophilized [^{35}S]methionine-labeled cAMP binding proteins were dissolved in lysis buffer (O'Farrell, 1975) and were analyzed by two-dimensional gel electrophoresis.

Photoaffinity Labeling. The photoaffinity labeling method (Antonoff & Ferguson, 1974) was used to stabilize the [^{32}P]cAMP–binding protein complex by a covalent bond, which is resistant to $NaDodSO_4$. In these experiments unlabeled cells were fractionated into cytoplasmic, particulate, and nuclear nonhistone proteins as described above. The photoactivation reaction mixture consists of 250–300 μg of protein in homogenization buffer with 5 mM EDTA and 1 mM [^{32}P]cAMP (17 Ci/mmol; New England Nuclear) in a final volume of 100 μL. This reaction mixture was incubated for 1 h in ice and then transferred into a small pH disposable cup with a magnetic stirrer. The reaction mixture with an ice jacket was placed on a stir plate and was irradiated at a distance of about 5 cm with a 25W Germicidal lamp (GE; predominantly 253.7-mm radiation) for 30 min. To the reaction mixture solid urea was added to 9 M, and then an equal volume of lysis buffer (O'Farrell, 1975) was added. Photoactivated samples were analyzed by two-dimensional gel electrophoresis. Irradiation of these samples for 15 min, 30 min, and 1 h gave similar results.

Preparation of Chromatin and [^{3}H]cAMP Binding Assay. Nuclei were purified from control and Bt_2cAMP treated cells as described above. Purified nuclei were used to prepare chromatin as described by Spelsberg & Hnilica (1971). The DNA content in chromatin was determined by Burton's method (1956). The binding of [^{3}H]cAMP to chromatin proteins was assayed (Gilman, 1970) in a total volume of 400 μL containing 50 mM sodium acetate, pH 4.0, 1 mM 2-mercaptoethanol, and 12.5 mM [^{3}H]cAMP (37.7 Ci/mmol; New England Nuclear). The reactions were started with the addition of chromatin proteins and incubated in ice for 1 h and then processed as described (Prashad & Rosenberg, 1978).

Two-Dimensional Gel Electrophoresis. Two-dimensional gel electrophoresis was used as described by O'Farrell (1975) with slight changes. The first-dimensional isoelectric focusing gels (IEF) were run at a constant 400 V for 15 h; this gave the most reproducible and superimposable results (Prashad et al., 1977). The top of the IEF gels was rinsed 3 times with H_2O to remove the unfocused proteins and then extruded into $NaDodSO_4$ buffer to equilibrate. These rinses remove the proteins which otherwise stick to the IEF gel during equilibration and cause streaks on the second-dimensional slab gels. The IEF gels were equilibrated in $NaDodSO_4$ buffer for 1 h. The second-dimensional 10% polyacrylamide–$NaDodSO_4$ slab gels were run at a constant 80 V until the dye ran off the gels (Prashad et al., 1977). The slab gels were fixed in 50% Cl_3AcOH for 1 h, stained in 0.1% Coomassie blue in methanol–H_2O–acetic acid (5:5:1) for 30 min, and destained in 7.5% acetic acid. The gels were dried and exposed to Kodak XR2 or X-omat-MA film.

Quantitation of cAMP Binding Proteins. The cAMP binding proteins R1 and R2 were quantitated to determine the amounts of R1 and R2 in the total cellular proteins, the ratio between R1 and R2, and the percent increase in R1 and R2 in Bt_2cAMP treated cells. The quantitation was done by two methods. (1) A total of 0.5×10^6 trichloroacetic acid precipitable cpm from control and Bt_2cAMP treated cells were used for two-dimensional gel electrophoresis. The second-dimensional slab gels were fixed in trichloroacetic acid and stained. The stained spots R1, R2, and actin were cut out from the same gels and were treated with 5% protosol, in scintillation liquid containing 5 g of PPO and 0.5 g of dimethyl-POPOP in 1 L of toluene, at 37 °C for 12 h, and the radioactivity was determined. Actin was included to determine the effect of

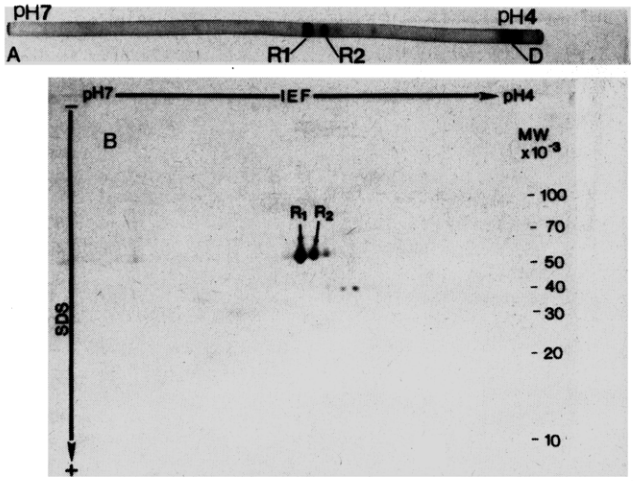

FIGURE 1: Analysis of purified cAMP binding proteins on two-dimensional polyacrylamide gels. A [^{35}S]methionine-labeled cytoplasmic fraction from Bt_2cAMP treated cells was passed through a DEAE-cellulose and cAMP–Sepharose affinity column as described under Materials and Methods. cAMP binding proteins were eluted from the cAMP-affinity column with 30 mM cAMP, and the fractions were dialyzed extensively against 5mM Tris-HCl, pH 7.4, and 0.4 mM 2-mercaptoethanol at room temperature to remove cAMP. The presence of excess cAMP effected the pH gradient of isoelectric focusing gels. The dialyzed [^{35}S]methionine-labeled cAMP binding proteins were lyophilized and dissolved in lysis buffer A (O'Farrell, 1975) and analyzed by two-dimensional gel electrophoresis. (A) Coomassie blue stained first-dimensional IEF gel of [^{35}S]methionine-labeled cAMP binding protein purified by cAMP-affinity column. IEF was stained as described (Prashad et al., 1977). (B) Autoradiogram of the second-dimensional $NaDodSO_4$ slab gel of [^{35}S]-methionine-labeled cAMP binding proteins (5×10^3 cpm) purified by cAMP affinity column. R1: basic cAMP binding protein with a p*I* of 5.4–5.5. R2: acidic cAMP binding protein with a p*I* of 5.26–5.4. D: isoelectric focusing dye.

protosol treatment on radioactivity. Actin spots from control and dibutyryl-cAMP treated cells show the same amounts of radioactivity. (2) The cAMP binding proteins R1 and R2 spots in the autoradiograms of cellular fractions from control and Bt_2cAMP treated cells were scanned by a Joyce Loebl microdensitometer. The area under the peak was determined by cutting out the peak and weighing it.

Results

Purification of cAMP Binding Proteins by cAMP-Affinity Column. We reported that Bt_2cAMP increased cAMP binding proteins in mouse neuroblastoma cells (Prashad et al., 1977) and cAMP binding proteins were increased in both soluble and particulate fractions (Prashad & Rosenberg, 1978). To determine the number of cAMP binding proteins and their electrophoretic position on two-dimensional gel electrophoresis, the cAMP binding proteins were purified from [^{35}S]-methionine-labeled cells by DEAE-cellulose and cAMP-affinity columns (Dills et al., 1975) and analyzed by two-dimensional gel electrophoresis (O'Farrell, 1975). cAMP binding protein fractions eluted from the DEAE-cellulose column between 0.06 and 0.65 M KCl show a major cAMP binding peak, and cAMP binding proteins from these fractions were purified by a cAMP-affinity column (Dills et al., 1975). Figure 1B shows that two proteins were eluted from the cAMP-affinity column. [^{3}H]cAMP binding to these proteins was further confirmed by the filtration method (Gilman, 1970), strongly indicating that the two proteins in Figure 1B are cAMP binding proteins. We designated these two cAMP binding proteins as cAMP receptor proteins R1 and R2 as shown in Figure 1B. cAMP binding protein R1 is more basic with a p*I* of 5.4–5.5, and R2 is more acidic with a p*I* of 5.26–5.40. These two cAMP binding proteins are separated by 0.24 p*I* units in a first-dimensional gel as shown in Figure 1A. Both cAMP binding proteins R1 and R2 have a molecular weight of about 48 000–50 000.

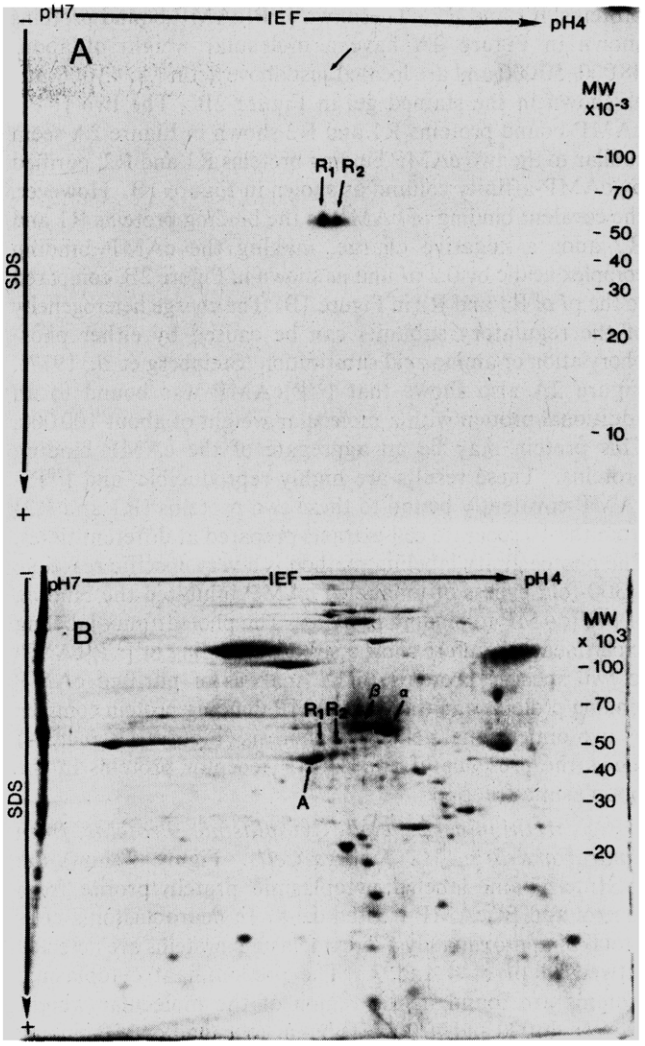

FIGURE 2: Identification of cAMP binding proteins in the cytoplasmic fraction by the photoaffinity labeling method. The photoactivation reaction mixture consists of 300 μg of unlabeled cytoplasmic proteins in the homogenization buffer with 5 mM EDTA and 1 mM [^{32}P]-cAMP. The reaction mixture was incubated for 1 h in ice and then irradiated for 30 min as described under Materials and Methods. The photoactivated mixture was made to 9 M urea, and then an equal volume of lysis buffer A was added and analyzed by two-dimensional gel electrophoresis. (A) Autoradiograms of the second-dimensional $NaDodSO_4$ slab gel of the photoactivated [^{32}P]cAMP–binding protein complex. (B) Stained gel of total cytoplasmic proteins after photoactivation. R1: basic cAMP binding protein with a p*I* of 5.4–5.5. R2: acidic cAMP binding protein with a p*I* of 5.26–5.4. A: actin (molecular weight 43 000). α: acidic subunit of tubulin. β: basic subunit of tubulin.

Identification of cAMP Binding Proteins in the Cytoplasmic Fraction by the Photoaffinity Labeling Method. cAMP binds to binding proteins by a noncovalent bond. In the photoaffinity labeling method the cAMP–binding protein complex is irradiated with ultraviolet light, and the photochemical reaction adds a covalent bond between cAMP and binding proteins. This complex is resistant to trichloroacetic acid and $NaDodSO_4$ (Antonoff & Ferguson, 1974). The photoaffinity labeling technique and two-dimensional gel electrophoresis were used to identify the cAMP binding proteins among the cytoplasmic proteins. The autoradiograph in Figure 2A shows that [^{32}P]cAMP was bound specifically to two cytoplasmic

proteins, R1 and R2. These two [^{32}P]cAMP-bound proteins shown in Figure 2A have a molecular weight of about 48 000–50 000 and are located just above actin (A; 43 000 M_r) as shown in the stained gel in Figure 2B. The two [^{32}P]-cAMP-bound proteins R1 and R2 shown in Figure 2A seem similar to the two cAMP binding proteins R1 and R2, purified by cAMP-affinity column as shown in Figure 1B. However, the covalent binding of cAMP to the binding proteins R1 and R2 adds a negative charge, making the cAMP–binding complex acidic by 0.2 p*I* unit as shown in Figure 2B, compared to the p*I* of R1 and R2 in Figure 1B. The charge heterogeneity of the regulatory subunits can be caused by either phosphorylation or amino acid substitution (Steinberg et al., 1977). Figure 2A also shows that [^{32}P]cAMP was bound to an additional protein with a molecular weight of about 100 000. This protein may be an aggregate of the cAMP binding proteins. These results are highly reproducible, and [^{32}P]-cAMP covalently bound to these two proteins (R1 and R2) from the 10 separate cell extracts prepared at different times. The photoaffinity labeling method is a very specific reaction: a 300-fold excess of unlabeled cAMP inhibited the binding of [^{32}P]cAMP to binding proteins. The photoaffinity labeling experiments therefore show a selective binding of [^{32}P]cAMP to two specific proteins. The analysis of purified cAMP binding proteins and the [^{32}P]cAMP–binding protein complex by two-dimensional gel electrophoresis (Figures 1B and 2A) shows the presence of two cAMP receptor proteins in the cytoplasmic fraction.

[^{35}S]Methionine-Labeled Cytoplasmic Proteins from Control and Bt_2cAMP Treated Cells. Figure 3 shows the [^{35}S]methionine-labeled cytoplasmic protein profile from control and Bt_2cAMP treated cells. In neuroblastoma cells a total of approximately 575 cytoplasmic proteins are detected between a p*I* of 4 and 7. The predominant cytoplasmic proteins are found in the region of the molecular weight between 40 000 and 70 000. Tubulin, actin, and cAMP binding proteins are found in this region. Figures 3A and 3B show that the cytoplasmic protein profiles of control and Bt_2cAMP treated cells are very similar and comparison of the protein shows an increase in only one protein, R1. Approximately 532 cytoplasmic proteins from HeLa cells have been reported (Peterson & McConkey, 1976).

The photoaffinity labeling results in Figure 2B show that cAMP binding proteins R1 and R2 are located just above the actin (A). Actin is used as an internal marker to locate cAMP binding proteins in the cellular fractions on the second-dimensional gels. The nonmuscle actin has a p*I* between 5.42 and 5.44 (Garrels & Gibson, 1978) with a molecular weight of 43 000. These properties of actin were also used as an internal marker to determine the p*I* and molecular weights of cAMP binding proteins R1 and R2 on the second-dimensional gels.

The [^{35}S]methionine-labeled cytoplasmic proteins from the control and Bt_2cAMP treated cells show cAMP binding proteins R1 and R2 located just above actin (A) in Figure 3. The R1 and R2 were quantitated by determining the radioactivity incorporated in these proteins as well as by scanning the autoradiographs with the Joyce Loebl microdensitometer. In Figure 3A the cAMP binding protein spots R1 (300 cpm) and R2 (300 cpm) are 0.06% of the total cytoplasmic proteins (0.5×10^6 cpm) applied on the first-dimensional gels. In control-cell cytoplasmic fractions the cAMP binding proteins R1 and R2 are found almost in equimolar ratio by radioactivity incorporation as well as by quantitation of the scans. In Figure 3B the cAMP binding protein spots R1 (1000 cpm) and R2

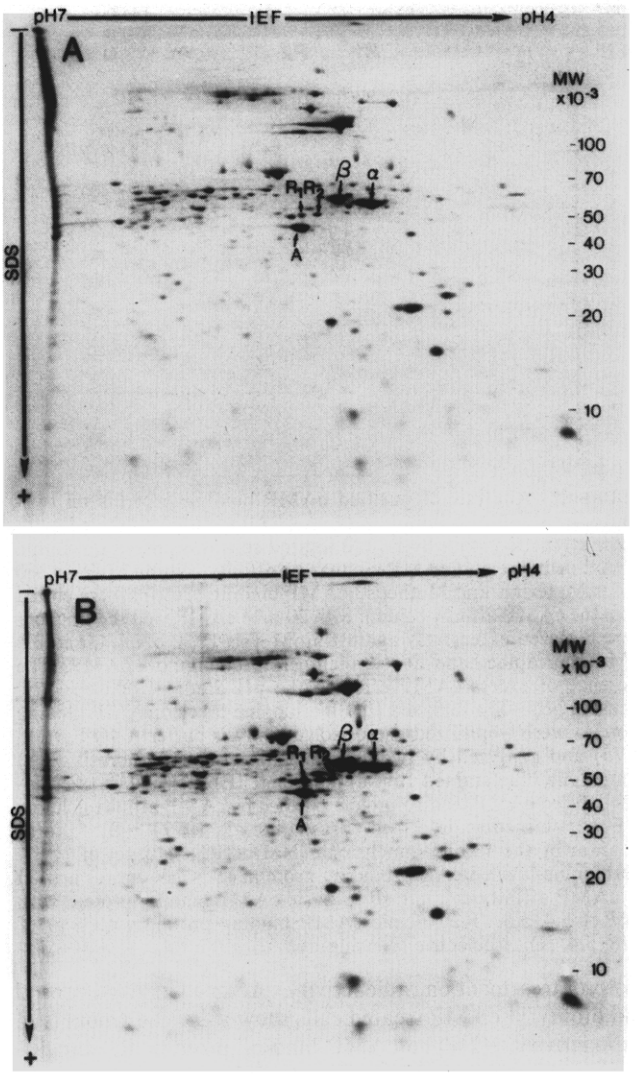

FIGURE 3: [^{35}S]Methionine-labeled cytoplasmic proteins from control and dibutyryl-cAMP treated cells. Cytoplasmic fraction was prepared from [^{35}S]methionine-labeled control and Bt_2cAMP treated cells. A total of 0.5×10^6 acid-precipitable cpm from control (A) and Bt_2cAMP treated cell (B) cytoplasm were analyzed by two-dimensional gel electrophoresis. Autoradiograms were exposed for 10 days. R1: basic cAMP binding protein with a p*I* of 5.4–5.5. R2: acidic cAMP binding protein with a p*I* of 5.26–5.4. A: actin (molecular weight 43 000). α: acidic subunit of tubulin. β: basic subunit of tubulin.

(350 cpm) are respectively 0.2 and 0.06% of the total cytoplasmic proteins (0.5×10^6 cpm) applied on the first-dimensional gels. The cytoplasm of Bt_2cAMP treated cells therefore has a ratio of R1 to R2 of 3:1, a threefold increase in the cAMP binding protein R1 without an effect on cAMP binding protein R2. These data confirm our previously reported results that Bt_2cAMP increased cAMP binding proteins by threefold (Prashad & Rosenberg, 1978). However, these results indicate that only one cAMP binding protein of the cytoplasmic fraction was increased by Bt_2cAMP treatment. These results are highly reproducible: the autoradiographs from 15 different cell extractions show an increase in cAMP binding protein R1. This increase in R1 was also observed when [^{35}S]methionine-labeled cells were analyzed from days 1, 3, 4, and 5. Increases in R1 were also observed when unlabeled Bt_2cAMP treated whole cell extract or cytoplasmic proteins were stained with Coomassie blue stain on second-dimensional $NaDodSO_4$ slab gels.

Peptide patterns data, produced by partial proteolytic digest (Cleveland et al., 1977), indicate that Bt_2cAMP induced

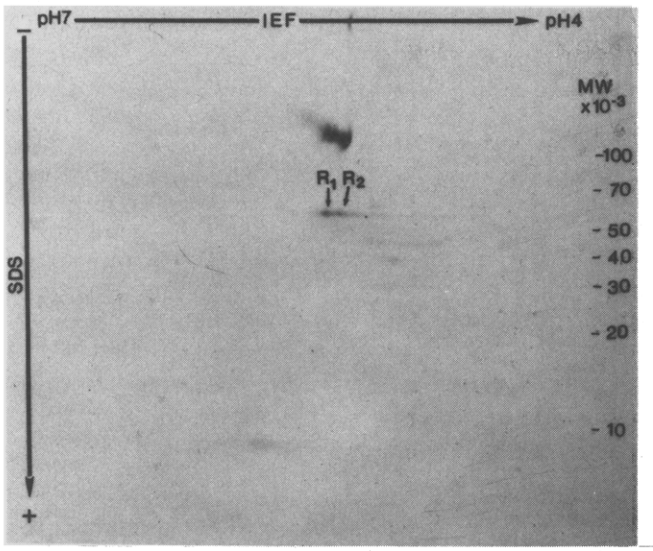

FIGURE 4: Identification of cAMP binding proteins in the particulate fraction by the photoaffinity labeling method. The photoactivation reaction mixture consists of 300 μg of unlabeled particulate fraction proteins in the homogenization buffer with 5 mM EDTA and 1 mM [^{32}P]cAMP. The reaction mixture was incubated for 1 h in ice and then irradiated for 30 min as described under Materials and Methods. The photoactivated mixture was made to 9 M urea, and then an equal volume of lysis buffer A was added and analyzed by two-dimensional gel electrophoresis. The autoradiograph of the second-dimensional $NaDodSO_4$ slab gel shows the photoactivated [^{32}P]cAMP–binding protein complex. R1: basic cAMP binding protein with p*I* of 5.4–5.5. R2: acidic cAMP binding protein with a p*I* of 5.26–5.4.

protein R1 is identical with the R1 of the control cells (unpublished experiments). Therefore, the increase in R1 in the Bt_2cAMP treated cells is due to the increase in the number of copies of R1.

cAMP Binding Proteins in the Particulate Fraction. As previously reported, Bt_2cAMP also increased cAMP binding proteins in the particulate fraction (Prashad & Rosenberg, 1978). In this report cAMP binding proteins among the particulate fraction proteins were identified on second-dimensional slab gels by the photoaffinity labeling method. The photoaffinity labeling experiment is described under Materials and Methods. The autoradiograph in Figure 4 shows that [^{32}P]cAMP also bound to two proteins (R1 and R2) in the particulate fraction. These two fractions have the same p*I* and molecular weight as the cytoplasmic binding proteins R1 and R2 as shown in Figure 3.

Effect of Bt_2cAMP on the Particulate Fraction Proteins. Figure 5 shows the [^{35}S]methionine-labeled particulate fraction proteins from the control and Bt_2cAMP treated cells. The general distribution of these proteins on two-dimensional gels is similar to cytoplasmic proteins; however, the intensity of [^{35}S]methionine incorporation is much lighter compared to that of cytoplasmic proteins (Figure 3). The protein "p" is found in large amounts and the β subunit of tubulin is less in the particulate fraction compared to the cytoplasmic proteins. Figure 5A shows two cAMP binding proteins R1 and R2 in the particulate fraction from control cells. The quantitative analysis of the scans of these autoradiographs shows that the R2 is present in larger amounts than the R1 protein and R1 and R2 show a ratio of 40:60. Figure 5B shows that Bt_2cAMP increased the R1 spot, and the quantitation analysis shows a fourfold increase in R1. However, the R2 protein is not affected by Bt_2cAMP treatment. These results are also highly reproducible, and the autoradiographs from five separate cell extractions as well as the extracts from days 1, 3, 4, and 5 show an increase in cAMP binding protein R1. Bt_2cAMP therefore

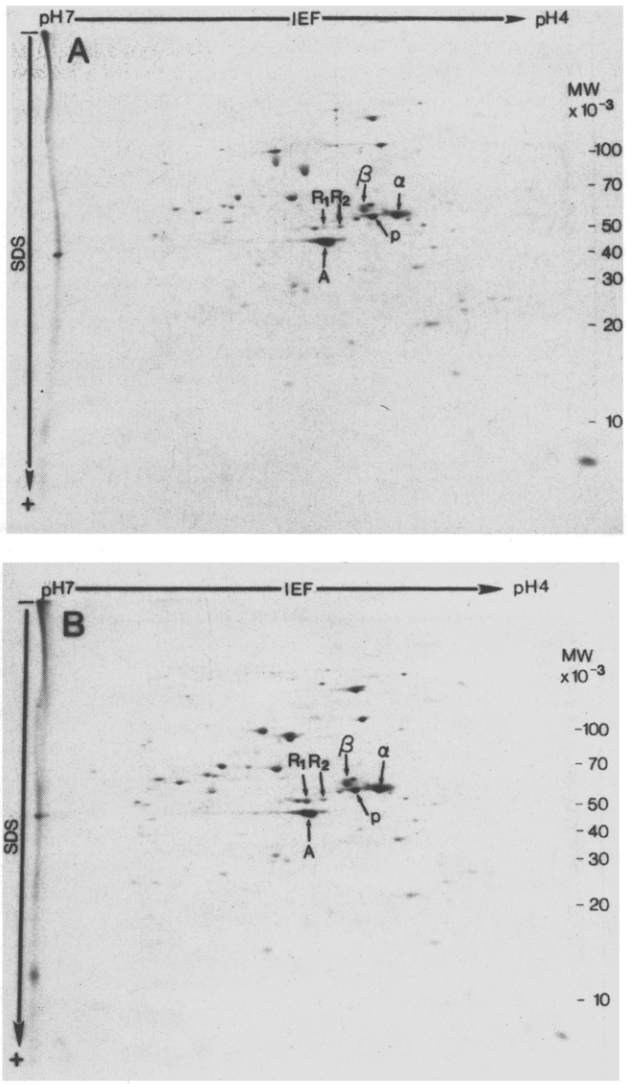

FIGURE 5: [^{35}S]Methionine-labeled particulate fraction proteins from control and dibutyryl-treated cells. Particulate fraction was prepared from [^{35}S]methionine-labeled control and Bt_2cAMP treated cells. A total of 0.5×10^6 acid-precipitable cpm from control (A) and Bt_2cAMP treated cell (B) proteins were analyzed by two-dimensional gel electrophoresis. Autoradiograms were exposed for 10 days. R1: basic cAMP binding protein. R2: acidic cAMP binding protein. A: actin (molecular weight 43 000). α: acidic subunit of tubulin. β: basic subunit of tubulin. P: an enriched protein of the particulate fraction.

increased only one protein, R1, in both cytoplasmic and particulate fractions, indicating a very specific effect.

Nuclear Nonhistone Proteins. Nuclear nonhistone proteins were isolated from [^{35}S]methionine-labeled control and Bt_2cAMP treated cells and were analyzed by two-dimensional gel electrophoresis.

Figure 6 shows that approximately 300 NHP are detected which range in molecular weight from 10 000 to over 100 000. The predominant NHP are found in two molecular weight regions. One set of predominant NHP is located between 10 000 and 40 000 molecular weight with a p*I* range between 4.0 and 5.5, and the other is located between 80 000 and over 100 000 molecular weight with a p*I* range between 5.0 and 5.80. The NHP are the acidic proteins; therefore, the isoelectric point of the predominant proteins between 4.0 and 5.8 is expected. In contrast, the predominant cytoplasmic proteins are located between 40 000 and 70 000 molecular weight with a p*I* between 5 and 7.0. Actin is the only predominant NHP located between 40 000 and 70 000 molecular weight. A total

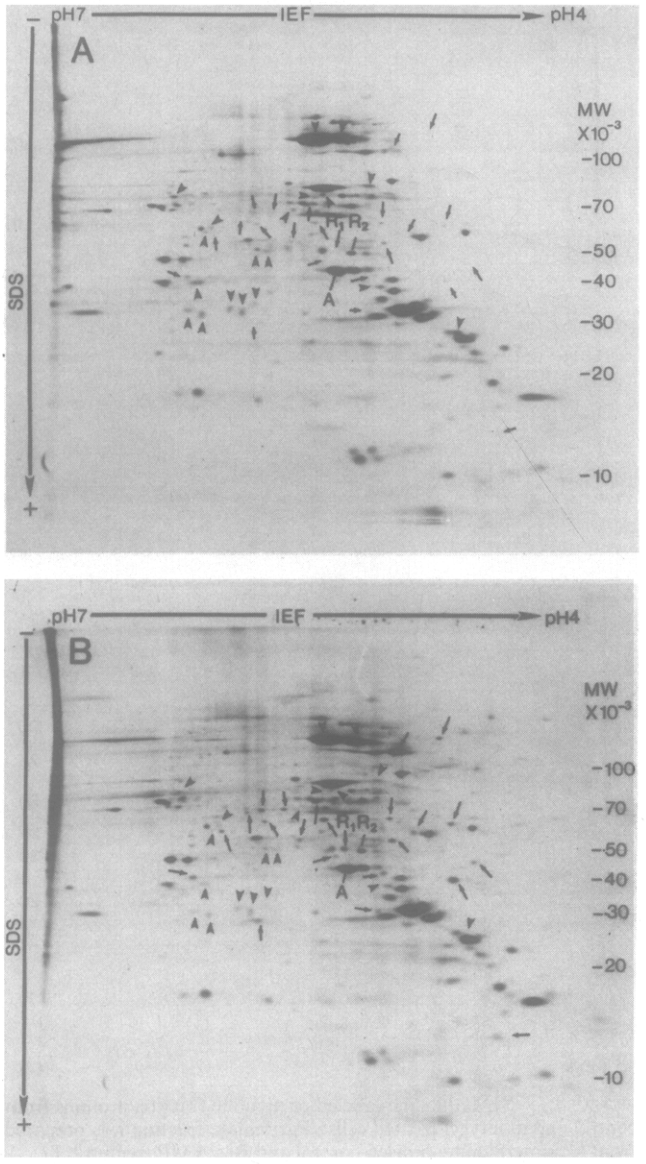

FIGURE 6: [^{35}S]Methionine-labeled nuclear nonhistone proteins from control and dibutyryl-cAMP treated cells. [^{35}S]Methionine nuclear nonhistone proteins were isolated from purified nuclei as described under Materials and Methods. A total of 0.5×10^6 acid-precipitable cpm from control (A) and Bt_2cAMP treated cell (B) nuclear nonhistone proteins were analyzed by two-dimensional gel electrophoresis. Autoradiograms were exposed for 10 days. R1: basic cAMP binding protein with a p*I* of 5.4–5.5. R2: acidic cAMP binding protein with a p*I* of 5.26–5.4. A: actin (molecular weight 43 000). Arrows indicate the protein spots that increased in Bt_2cAMP treated cells and arrowheads indicate the protein spots that decreased in Bt_2cAMP treated cells.

of 500 NHP were reported from HeLa cells (Peterson & McConkey, 1976).

Effect of Dibutyryl-cAMP on Nonhistone Proteins. Bt_2cAMP caused quantitative changes in the nuclear nonhistone proteins. Bt_2cAMP treated cells show a decrease in 19 proteins as shown by the arrowheads in Figure 6. Bt_2cAMP also caused quantitative increases of 23 proteins as shown by the arrows in Figure 6. Bt_2cAMP therefore specifically affected 43 proteins out of 300 NHP. The increases and decreases in the NHP may be due to changes in their synthetic rates and/or degradation rates in the presence of cAMP. Bt_2cAMP affected the NHP more than the cytoplasmic and particulate proteins. One of the most predominant effects of Bt_2cAMP in the nucleus is the increase in a protein, R1, with a molecular weight of 48 000–50 000, with a p*I* of 5.4 as shown

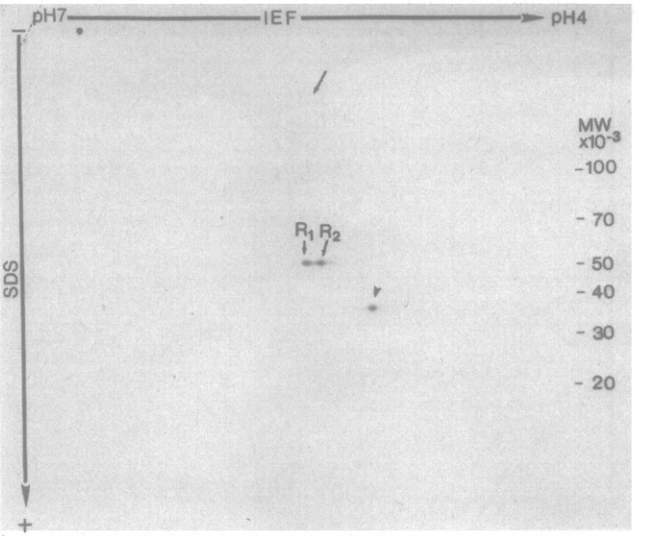

FIGURE 7: Identification of cAMP binding proteins in the nuclear nonhistone proteins by the photoaffinity labeling method. The photoactivation reaction mixture consists of 300 μg of unlabeled total nonhistone proteins in the homogenization buffer with 5 mM EDTA and 1 mM [^{32}P]cAMP. The reaction mixture was incubated for 1 h in ice and then irradiated for 30 min as described under Materials and Methods. The photoactivated mixture was made to 9 M urea, and then an equal volume of lysis buffer A was added and analyzed by two-dimensional gel electrophoresis. The autoradiograph of the second-dimensional $NaDodSO_4$ slab gel shows the photoactivated [^{32}P]cAMP–binding protein complex. R1: basic cAMP binding protein with a p*I* of 5.4–5.5. R2: acidic cAMP binding protein with a p*I* of 5.26–5.4. The arrow shows a protein of high molecular weight (110 000). The arrowhead shows a protein of low molecular weight (35 000).

in Figure 6B. The induced nuclear protein R1 resembles the cAMP binding protein R1 of the cytoplasm (Figure 3). The quantitation of the scans of the autoradiographs (Figure 6) shows that R1 protein increased by eightfold in the Bt_2cAMP treated cells. This increase in the R1 protein may be due to the translocation of induced cAMP binding protein R1 from the cytoplasm.

To determine whether this increased nuclear R1 is a cAMP binding protein, the NHP were isolated from the unlabeled Bt_2cAMP treated cells and were photoactivated with [^{32}P]-cAMP as described in the photoaffinity labeling experiments. Figure 7 shows that [^{32}P]cAMP bound to two proteins, R1 and R2, and these two proteins superimpose with the two proteins located just above the actin in the stained gel with a molecular weight of 48 000–50 000. These data show that R1 and R2 are the cAMP binding proteins associated with the nuclear acidic proteins. Figure 7 also shows that [^{32}P]-cAMP was also bound to a protein of molecular weight higher than 100 000, shown with an arrow, which may be an aggregate of cAMP binding proteins. [^{32}P]cAMP was also bound to a protein of a molecular weight of 35 000–40 000, shown in Figure 7 with arrowhead. This low molecular weight cAMP binding protein was not seen in the cytoplasmic fraction.

These results are also highly reproducible: the photoaffinity labeling experiments gave similar results from three different extractions. The effect of Bt_2cAMP on NHP also gave similar results from six different extractions, and these effects were also noticed from the days 3, 4, and 5 cell preparations.

[^{3}H]cAMP Binding to Chromatin. Figure 6 shows that the cAMP binding protein R1 increased among the NHP from the Bt_2cAMP treated cells. The increase of the cAMP binding protein in the nucleus was further investigated by determining the binding of [^{3}H]cAMP to chromatin. Chromatin was

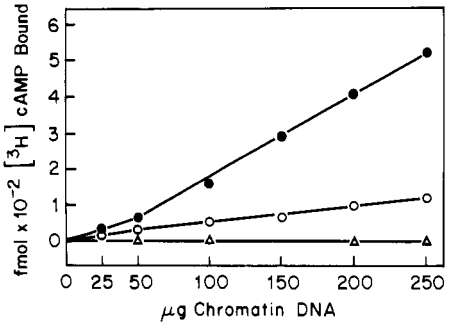

FIGURE 8: [^{3}H]cAMP binding to chromatin from control and Bt_2cAMP treated cells. Chromatin was prepared from purified nuclei of control and Bt_2cAMP treated cells, and the binding of [^{3}H]cAMP to chromatin proteins was assayed as described under Materials and Methods. Chromatin from control cells (○); chromatin from Bt_2cAMP treated cells (●); a 300-fold excess of unlabeled cAMP was included before the addition of [^{3}H]cAMP to the chromatin from Bt_2cAMP treated cells (△).

isolated from unlabeled control and Bt_2cAMP treated cells and was washed with 0.35 M NaCl to remove cytoplasmic contaminating proteins (Spelsberg & Hnilica, 1971). Figure 8 shows a three- to fourfold increase in the [^{3}H]cAMP binding to chromatin from Bt_2cAMP treated cells compared to chromatin from control cells. The [^{3}H]cAMP binding increased with increasing concentrations of chromatin. Unlabeled cAMP in 300-fold excess inhibited the binding of [^{3}H]cAMP by 99% to chromatin from Bt_2cAMP treated cells as shown in Figure 8. These data indicate the specific binding of [^{3}H]cAMP to chromatin-associated cAMP binding proteins. Five different chromatin preparations showed similar results with little variation in cAMP binding.

Bt_2cAMP treatment did not increase cAMP-dependent protein kinase activity in the chromatin compared to control cells chromatin (unpublished experiments). This increase in cAMP binding protein seems to be independent of protein kinase activity. The data of [^{35}S]methionine-labeled NHP (Figure 6), the photoaffinity labeling experiment (Figure 7), and [^{3}H]cAMP binding (Figure 8) strongly indicate an increase in cAMP binding proteins in the chromatin from Bt_2cAMP treated cells.

Discussion

The studies in this paper show that Bt_2cAMP increased only one of the two cAMP binding proteins present in the cytoplasmic, particulate, and nuclear nonhistone protein fractions. Bt_2cAMP shows a very specific effect: out of 575 proteins (p*I* = 4–7) in the cytoplasmic fraction only one protein, cAMP binding protein R1, increased by threefold. The induced cytoplasmic cAMP binding proteins may be translocated to the membranes (particulate fraction) and the nucleus, thus increasing and localizing the cAMP binding proteins in these fractions.

The Bt_2cAMP induced cAMP binding proteins show the biochemical properties of the regulatory subunit of cAMP-dependent protein kinase, in that adenine nucleosides and nucleotides do not inhibit the binding of [^{3}H]cAMP binding to these proteins (Prashad & Rosenberg, 1978) and that these induced binding proteins also inhibit the activity of the catalytic subunit (unpublished results). Control and Bt_2cAMP treated cells show the same amounts of the total cAMP-dependent protein kinase catalytic activity (Prashad et al., unpublished experiments), suggesting that Bt_2cAMP stimulated the expression of the gene for the regulatory subunit cAMP binding protein without affecting the expression of the gene for the catalytic subunit.

In vivo phosphorylation of cytoplasmic proteins did not show the phosphorylation of either R1 or R2 (data not shown). Autophosphorylation of the regulatory subunit of type II protein kinase has been reported, whereas autophosphorylation of the regulatory subunit of type I protein kinase has not been observed (Walter et al., 1977; Hofmann et al., 1975; Erlichman et al., 1974; Maeno et al., 1974). Steinberg et al. (1977) reported for the first time the phosphorylation of the regulatory subunit of type I protein kinase from S49 lymphoma cells. If R1 and R2 are unmodified proteins, then these two proteins belong to the regulatory subunit of type I protein kinase.

Peptide pattern data, produced by partial proteolytic digest (Cleveland, 1977), support the proposal that the differences in charge heterogeneity among R1 and R2 are due to amino acid composition (unpublished experiments). The regulatory subunit of type I protein kinase consists of two proteins, R1 and R2, with the same molecular weight but with different p*I*. Our results show that Bt_2cAMP increased only one cAMP binding protein, R1, suggesting that cAMP binding proteins R1 and R2 are the product of two different genes.

We previously reported that Bt_2cAMP induced cAMP binding proteins required RNA and protein synthesis (Prashad & Rosenberg, 1978). However, the exact mechanism by which cAMP increases cAMP binding proteins remains unknown. The increase in the binding proteins could be due to an increase in the rate of its synthesis or a decrease in the rate of its degradation. It has been reported in other systems also that Bt_2cAMP induced tyrosine aminotransferase activity in rat liver is accompanied by a corresponding increase in the level of translatable mRNA coding for this enzyme (Ernest & Feigelson, 1978). Hepatic mRNA coding for phosphoenolpyruvate carboxykinase also rapidly increased during induction of enzyme activity by Bt_2cAMP in vivo (Iynedjian & Hanson, 1977). We reported that Bt_2cAMP increased cAMP levels in mouse neuroblastoma cells (Prashad & Rosenberg, 1978), activated cytoplasmic cAMP-dependent protein kinase in vivo, and caused additional phosphorylation of cytoplasmic proteins compared to untreated control cells (Prashad et al., unpublished experiments). The increase in intracellular cAMP levels may also activate nuclear cAMP-dependent protein kinases, phosphorylating nuclear nonhistone proteins which may enhance the transcription of the gene for cAMP binding protein R1. In eucaryotes cAMP has been implicated in the regulation of the phosphorylation of some nuclear nonhistone proteins (Kleinsmith, 1975). The state of phosphorylation of these proteins may play a role in the regulation of transcription (Allfrey et al., 1975; Kleinsmith et al., 1975). The other mechanism may be that cAMP interacts with chromatin through cAMP binding proteins, leading to a selective stimulation of cAMP binding protein gene transcription analogous to β-Gal induction by cAMP in *Escherichia coli* (Pastan & Perlman, 1970).

In addition to the transcriptional mechanism favoring selective gene expression, the posttranscriptional modification of the primary transcript, the transport of the mRNA into the cytoplasm and translation might be controlled by cAMP. The increase in cAMP binding protein R1 can also be caused by their stability in the presence of cAMP as a cAMP–binding protein complex. One other possibility is that directly or indirectly the cAMP binding proteins may be regulating the expression of its own gene as "autogenous regulation" (Goldberger, 1974).

Chromosomal nonhistone proteins from Bt_2cAMP treated cells show an increase in 23 nonhistone proteins and a decrease in 19 proteins. The effect of Bt_2cAMP on these proteins may

be due to a change in their synthetic rates and/or their stability in the presence of cAMP. At present, we cannot distinguish whether phosphorylation of nonhistone proteins and/or increase in the nuclear cAMP binding proteins caused the induction as well as the repression of the 42 nonhistone proteins.

Several lines of evidence suggest that the proteins associated with the chromatin play a regulatory role in eucaryotic cells (Bonner et al., 1968; Stein et al., 1974). The nonhistone proteins have been implicated in the positive control of transcription of chromatin (Gilmour & Paul, 1969; Spelsberg et al., 1971; Stein et al., 1974; Kostraba & Wang, 1972; Teng et al., 1971). Our results show that cAMP binding proteins were increased in the chromatin from Bt_2cAMP treated cells and the cAMP binding proteins are among the nuclear acidic proteins with a p*I* of 5.26–5.40. The association of cAMP binding proteins with the chromatin nonhistone proteins may constitute an important aspect of its mechanism of action. Two peaks of cAMP binding activity have been detected in a rat liver nuclear acidic protein preparation (Rikans & Ruddon, 1973). Further, approximately 70% of the cAMP binding activity in the nucleus has been ascribed to a nuclear acidic protein with physical and biochemical characteristics of the regulatory subunit of cAMP-dependent protein kinase. Several peaks of specific cAMP binding activity whose material when eluted bind to the DNA column were also found, which did not correspond to peaks of protein kinase activity (Johnson et al., 1975). Our results show an increase in the nuclear cAMP binding protein R1, from Bt_2cAMP treated cells, suggesting the nuclear translocation of induced cytoplasmic cAMP binding proteins. The nuclear translocation of the induced cytoplasmic cAMP binding site and catalytic subunit has been reported in other systems (Allfrey et al., 1975; Jungmann et al., 1975; Costa et al., 1976; Castagna et al., 1975).

Transcriptionally active regions of chromatin are susceptible to digestion by DNase I (Weintraub & Groudine, 1976; Garel & Axel, 1976; Levy et al., 1977). The DNase I digestion of neuroblastoma chromatin shows that cAMP binding proteins are associated with the transcriptionally active regions of chromatin (unpublished experiments), indicating that cAMP binding proteins may be involved in the positive control of transcription. The biological significance of nuclear cAMP binding proteins may be analogous to procaryotes, in which the cAMP–receptor protein complex binds to DNA and stimulates the transcription of genes (Anderson et al., 1974).

It appears likely, on the basis of several recent studies, that certain effects of cAMP in the differentiation of eucaryotic cells are due to the ability of cAMP to influence transcription. In neuroblastoma cells, the increase in the nuclear cAMP binding proteins and the quantitative changes in the nonhistone proteins may be involved in the increase in the enzymes tyrosine hydroxylase (Richelson, 1973), choline acetyltransferase (Waymire et al., 1972), acetylcholinesterase (Simantov & Sachs, 1975), and poly(A)-containing RNA (Bondy et al., 1974).

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Sequence Complexity of Polyadenylated Ribonucleic Acid from Soybean Suspension Culture Cells†

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ABSTRACT: The sequence complexity of total poly(A) RNA from a higher plant system, soybean cultured cells, was determined. Labeled cDNA synthesized from the poly(A) RNA hybridized exclusively with the unique sequence component of total soybean DNA. Analysis of the hybridization reaction between cDNA and the poly(A) RNA template revealed three abundance classes in the poly(A) RNA. These classes represent 18, 44, and 38% of the poly(A) RNA and contain information for approximately 60, 1900, and 30 000 different 1400-nucleotide RNA molecules. From these results, the total sequence complexity of poly(A) RNA was estimated to be 4.5×10^7 nucleotides. Saturation hybridization of labeled unique DNA with RNA showed that the total cell RNA represents 12.4% of the unique DNA sequence complexity, or 6.4×10^7 nucleotides, while poly(A) RNA represents 8.7% of the unique DNA sequence complexity, or 3.3×10^7 nucleotides. Thus, it is estimated that 50–70% of total RNA sequence complexity is contained in poly(A) RNA in these cells.

Many eucaryotic genomes are very complex, containing 10–100 times more information than is expressed during the lifetime of the organism. Cellular control mechanisms must therefore exist to select genetic information to be expressed at various times. To characterize such differential gene expression, it is important to measure the total amount of unique DNA sequence complexity that is represented in RNA at different developmental stages, in various differentiated tissues, or in response to various environmental stimuli.

Several studies have estimated RNA sequence complexity using either saturation hybridization of labeled unique DNA with RNA (Davidson & Hough, 1971) or analysis of the hybridization kinetics of poly(A)[1] RNA to a labeled cDNA probe copied from the RNA (Bishop et al., 1974). A general pattern for RNA sequence complexity in higher eucaryotes has emerged from these reports. The complexity of cytoplasmic or polysomal mRNA represents approximately 1–3% of the unique genome complexity (Galau et al., 1974; Bishop et al., 1974; Ryffel & McCarthy, 1975; Hastie & Bishop, 1976; Bantle & Hahn, 1976; Kleiman et al., 1977). Approximately 10–30% of the unique genome sequence complexity is represented in total cellular RNA or nuclear RNA (Grouse et al., 1972; Liarakos et al., 1973; Hough et al., 1975; Bantle & Hahn, 1976; Kleiman et al., 1977). As all of these studies were done in animal systems, and in view of the differences in development and differentiation between plants and animals, we have investigated RNA sequence complexity in a higher plant system (Silflow & Key, 1977). In another report using a higher plant system, Goldberg et al. (1978) reported that the nuclear RNA of whole tobacco leaves contains approximately 19% of unique DNA sequence complexity and is 3.6 times more complex than polysomal RNA.

Previous studies on soybean genome organization have provided values for genome size and unique DNA sequence

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[1] Abbreviations used: poly(A) RNA, RNA which contains poly(adenylic acid); AMV, avian myeloblastosis virus; cDNA, complementary DNA; PAS, sodium *p*-aminosalicylic acid; TNS, sodium triisopropylnaphthalenesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Me_2SO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetate; Cl_3AcOH, trichloroacetic acid; PB, phosphate buffer; HAP, hydroxylapatite; A_{260} unit, quantity of material contained in 1 mL of a solution which has an absorbance of 1 at 260 nm, when measured in a 1-cm path length cell; C_0t or R_0t, DNA or RNA concentration in moles of nucleotides per liter times the time in seconds; $C_0t_{1/2}$ or $R_0t_{1/2}$, C_0t or R_0t value at which 50% of the reaction is complete; T_m, temperature at which 50% of the duplex structure has melted; nt, nucleotide; ntp, nucleotide pairs.

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