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Site-selective cAMP analogs induce nuclear translocation of the R^{II} cAMP receptor protein in Ha-MuSV-transformed NIH/3T3 cells

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Site-selective cAMP analogs, depending on the position of their substituents on the adenine ring, selectively bind to either site 1 or site 2 of the known cAMP binding sites of protein kinase. Treatment of Harvey murine sarcoma virus-transformed NIH/3T3 cells with such site-selective analogs results in growth inhibition and phenotypic reversion, and the combination of a C-8 thio or halogen analog (site 1 selective) with an N^6 analog (site 2 selective) produces a synergistic effect. We report here that the growth inhibitory effect of the analogs correlates with the nuclear translocation of the R^{II} cAMP receptor protein, the regulatory subunit of protein kinase type II. The transformed NIH/3T3 cells contained no detectable level of R^{II} in the nucleus, whereas nontransformed NIH/3T3 cells exhibited a high level of nuclear R^{II}. Within 30 min after treatment of the transformed cells with the site-selective analogs, immunofluorescence against the R^{II} protein markedly increased in the cell nucleus. The nuclear translocation of the R^{II} cAMP receptor protein is an early event in the reverse transformation of the fibroblasts treated with site-selective cAMP analogs.

cAMP; Protein kinase; Reverse transformation

1. INTRODUCTION

cAMP in mammalian cells functions by binding to its receptor proteins, R^{I} and R^{II}, the regulatory subunits of cAMP-dependent protein kinases, type I and type II [1,2]. The type I and type II protein kinases differ only in their regulatory subunits while the catalytic subunits are identical [3–5]. The physiological role of protein phosphorylation by the catalytic subunit is well established [3,4], but the only documented function of the cAMP receptor protein is inhibition of the catalytic subunit activity [3]. There are, however, an increasing number of observations that suggest selective functional roles for the cAMP receptor proteins [6–10].

At the nuclear level, the function of the cAMP-dependent protein kinase catalytic subunit has been shown to involve chromosomal protein phosphorylation [11]. The role of cAMP receptor proteins in gene regulation has also been suggested [12–14].

We have recently reported that the site-selective cAMP analogs, which are selective for either one of the two different cAMP-binding sites on the cAMP receptor protein [15,16], in combination synergistically demonstrate growth inhibition and

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phenotypic reversion of the Harvey murine sarcoma virus (Ha-MuSV)-transformed NIH/3T3 clone 13-3B-4 cells and that the growth inhibitory effect of these analogs correlates with an increase of the R^{II} cAMP receptor protein and a decrease of the R^{I} receptor protein [17,18].

In the present work, we investigated, by the use of biochemical and immunocytochemical methods, the intracellular compartmentalization of the R^{II} cAMP receptor protein during treatment of the transformed 13-3B-4 cells with the site-selective cAMP analogs.

2. MATERIALS AND METHODS

2.1. *Materials*

cAMP and N^6,$O^{2'}$-dibutyryl cAMP were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). N^6-Monobutyryl cAMP and 8-methylthio-cAMP were obtained from Sigma (St. Louis, MO). All other cAMP analogs were synthesized at the Nucleic Acid Research Institute (Costa Mesa, CA). 8-N_3-[^{32}P]cAMP (60.0 Ci/mmol) was obtained from ICN (Irvine, CA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG was from Cappel Laboratories (Cochranville, PA). ^{14}C-Methylated molecular mass marker proteins were obtained from Bethesda Research Laboratories (Bethesda, MD).

2.2. *Cell culture*

NIH/3T3 clone 13-3B-4 cells were grown in the absence or presence of additives (cAMP analogs) in serum-free, chemically defined medium [18] (Dulbecco's modified Eagle's medium-HAM's F12 (nutrient mixture F12-HAM; 75:25) supplemented with bovine insulin (5 μg/ml), transferrin (5 μg/ml), histidine-HCl (42 μg/ml), glutamine (292 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and Hepes (20 mM, pH 7.3); the medium was changed every 48 h. The additives were provided every 48 h, starting at day zero of culture in the serum-free medium. The cells were grown in humidified incubators in an atmosphere of 10 CO_2.

2.3. *Preparation of cell extracts*

All procedures were performed at 0–4°C. 13-3B-4 cell pellets, after two washes with phosphate-buffered saline, were suspended in buffer Ten (0.1 M NaCl, 5 mM $MgCl_2$, 1% Nonidet P-40, 0.5% Na deoxycholate, 2 KIU/ml bovine aprotonin, 20 mM Tris-HCl, pH 7.4) (2×10^7 cells/ml), vortexed, passed through a 22-gauge needle 10 times, allowed to stand for 20 min and centrifuged at $750 \times g$ for 20 min, and the resulting supernatants were used as cell lysates.

2.4. *Preparation of cell cytosol and nuclear fraction*

All procedures were performed at 0–4°C. The cell pellets (2×10^7 cells), after two washes with phosphate-buffered saline, were suspended in 0.5 ml buffer A (0.25 M sucrose, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM KCl, 20 mM Tris-HCl, pH 7.5) and homogenized with a dounce homogenizer (60 strokes). The cytosols and nuclear fractions were prepared as described [19]. The purified nuclear pellets were suspended in 100 μl buffer A and used as nuclear suspension.

2.5. *Photoaffinity labeling of cAMP receptor proteins*

The photoactivated incorporation of 8-N_3-[^{32}P]cAMP was performed as described [20]. The samples containing 50–75 μg of protein were subjected to 0.05% SDS-12% polyacrylamide gel electrophoresis [21]. The protein concentrations were determined by the method of Lowry et al. [22] with bovine serum albumin as standard.

2.6. *Western blotting of R^{II} cAMP receptor protein*

Cellular proteins present in the lysates (see above) were separated and transferred to nitrocellulose sheets [23] and first incubated with 3% bovine serum albumin [24] and sequentially incubated with medium containing the anti-R^{II} antibody [25,26] overnight at 4°C and 5×10^5 cpm/ml ^{125}I-labeled protein A for 1 h in an ice water bath. The nitrocellulose sheets were air dried and exposed to Kodak XAR film for 12 to 36 h at −20°C.

2.7. *Production and characterization of anti-R^{II} antibody*

The production and characterization of rabbit antibody against bovine heart R^{II} has been described in detail [25,26]. The antibody was affinity

purified using a glutaraldehyde cross-linked immunoabsorbent as described [27]. The monospecificity of antibody was confirmed as described in [28].

2.8. *Immunofluorescence experiments*

Cells were seeded at 2×10^4/1.5 ml onto 2-chamber tissue culture slides precoated with poly-D-lysine. After cells became attached, the medium was changed to the serum-free medium (see section 2.2) with or without additives. At desired culture times, the tissue culture chamber slides were fixed, the cells were permeabilized, and indirect immunofluorescence experiments were carried out as described [28,29] with the primary anti-R^{II} antibody and FITC-conjugated goat anti-rabbit second antibody. The slides were viewed on a Zeiss epifluorescence microscope. All photographs were taken by using identical exposure time with a $\times 40$ oil immersion objective with Kodak Tri-X film.

The specificity of staining of R^{II} antibody was assessed by the following: (i) preimmune IgG was used at the same concentration as that of immune anti-R^{II} IgG; (ii) fixed cells were incubated with the FITC-conjugated goat anti-rabbit IgG without preincubation with the primary antibody; and (iii) the antibody-blocking experiments were carried out by preabsorbing the anti-R^{II} antibody with R^{II} before staining.

3. RESULTS

3.1. *Photoaffinity labeling of cAMP receptor proteins*

The R^I and R^{II} cAMP receptor proteins in the cytoplasmic and nuclear fractions of the control cells (untreated) and the cells treated with site-selective cAMP analogs were identified by photoaffinity labeling with 8-N_3-[^{32}P]cAMP and electrophoresis and quantified by densitometric tracings of the autoradiograms.

As shown in fig.1A, the untreated 13-3B-4 cells contained a major cAMP receptor protein with an M_r of 48000 in both cytosol (lane 1) and nuclear fraction (lane 3). A low intensity band of 52–56 kDa receptor protein was also detected in the cytosol (lane 1), but the band was almost undetectable in the nuclear fraction (lane 3). The 48 kDa protein appears to be the R^I [1], and the 52–56 kDa proteins appear to be the R^{II} [30,31] cAMP receptor. These R^I and R^{II} bands represented the specific cAMP receptor proteins (the high affinity binding protein [1,2]), since these bands were absent when 1000-fold excess of unlabeled cAMP was present in the binding reaction (lanes 1+C, 2+C, 3+C, and 4+C). At 3 h after treatment of 13-3B-4 cells with N^6-butyryl-cAMP (25 μM) + 8-methylthio-cAMP (5 μM), the R^I cAMP receptor protein level in the cytosol decreased to 60% of that in the untreated cell cytosol; the R^I receptor level in the nuclear fraction, however, did not change (fig.1B). During this same period, the R^{II} receptor protein in the nucleus increased 10-fold over that of untreated cells, whereas the cytoplasmic R^{II} increased 3-fold (fig.1B). This resulted in an increase of the R^{II}/R^I ratio 5- and 10-fold in the cytosol and nuclear fraction, respectively, after the analog treatment.

At 24 h after treatment with the analog combination, R^I further decreased while the R^{II} remained elevated, resulting in an increase of 6- and 15-fold in the ratio of R^{II}/R^I in the cytoplasm and nucleus, respectively, over those in the untreated control cells. Other combinations of N^6 (25 μM) with a C-8 thio or halogen derivative all brought about a similar increase in the R^{II}/R^I ratio to that shown in fig.1. Each of these analogs singly at the given concentrations was without effect but was effective at higher concentrations (N^6 analogs at 100 μM; C-8 analogs at 50 μM). Thus, the same synergism of C-6 and C-8 analog combination was demonstrated on the increase of the R^{II}/R^I ratio, especially in the cell nucleus, as was shown on the growth inhibitory effect [17].

3.2. *Western blotting of R^{II} cAMP receptor protein*

The effect of cAMP analog treatment of 13-3B-4 cells on the amount of the R^{II} cAMP receptor protein was determined by Western blotting analysis of R^{II}. The antiserum used was raised in rabbit against bovine heart R^{II} [28]. The affinity-purified anti-R^{II} antibody [27] exhibited monospecificity for antigen R^{II} from human mammary cancer cells grown in culture [28] as well as in nude mice [32]. As shown in fig.2, the anti-R^{II} antibody that specifically cross reacted with the R^{II} antigen (cf. lanes R^{II} and R^{II}+CS (control serum)) but not with the R^I antigen (lane R^I) detected the

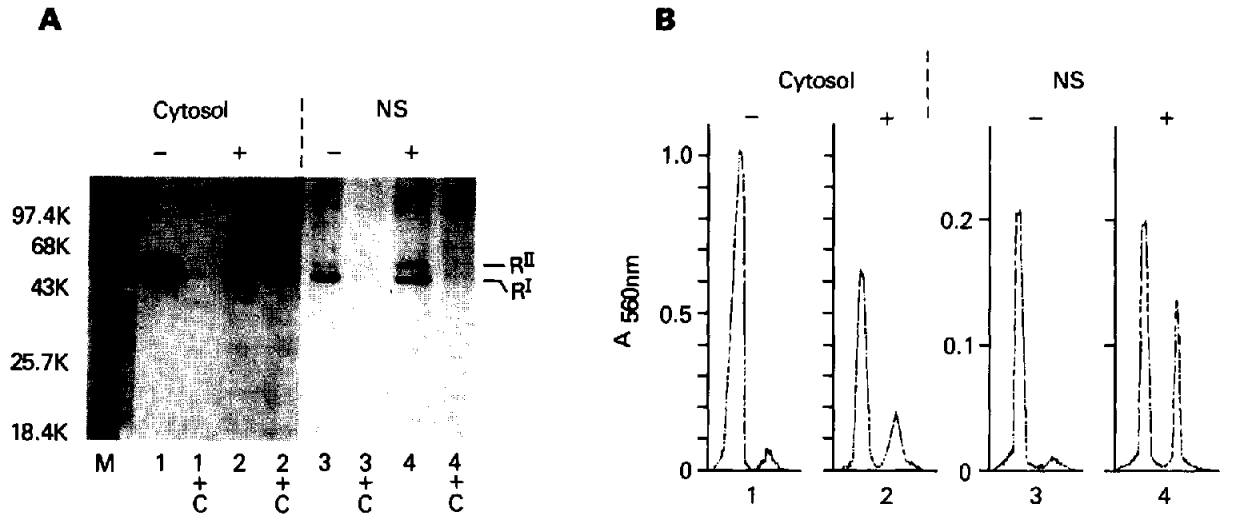

Fig.1. Photoaffinity labeling of cAMP receptor proteins in cytosols and nuclear suspensions (NS) from untreated and cAMP analog-treated 13-3B-4 cells. (A) Autoradiography of photoactivated incorporation of 8-N_3-[^{32}P]cAMP followed by electrophoresis; (B) densitometric tracings of autoradiograms (A) at A_{560nm}. The R^I level in the cytosol of untreated cells was set equal to 1.0 Å; the scale was expanded to differentiate the lower intensity bands in NS. Lanes 1 and 3, untreated control cells; lanes 2 and 4, cells treated for 3 h with 25 μM N^6-butyryl-cAMP + 5 μM 8-methylthio-cAMP; + C, the reaction performed in the presence of 1000-fold excess unlabeled cAMP. Positions of R^I and R^{II} were identified by coelectrophoresis of the purified preparations (Sigma) of R^I and R^{II} from rabbit skeletal muscle and bovine heart, respectively. M, ^{14}C-labeled molecular mass standard proteins.

52–56 kDa R^{II} receptor proteins from 13-3B-4 cells. The anti-R^{II} antibody also crossreacted with a lower kDa (30–39) R^{II} species that may be proteolytic fragments of R^{II} [5]. Both 52–56 kDa and 30–39 kDa R^{II} species were absent when the anti-R^{II} antibody was replaced by control serum (lane 2+CS), indicating that the proteins that crossreacted with the anti-bovine R^{II} antibody all represent the R^{II} subunits. The antibody detected markedly elevated levels of the 52–56 kDa R^{II} from the cells treated for 3 days with 50 μM 8-methylthio-cAMP (lane 2) or 100 μM

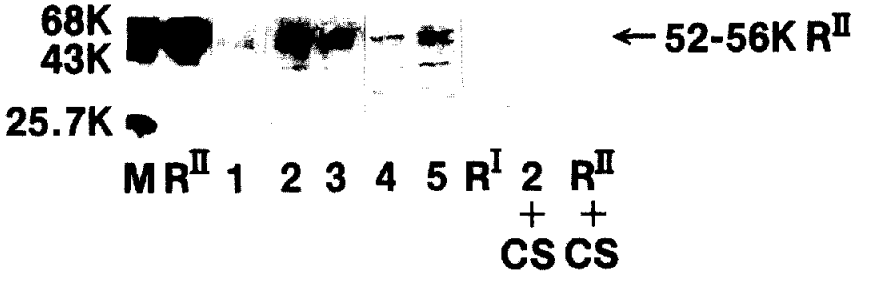

Fig.2. Western blotting of R^{II} cAMP receptor protein from untreated and cAMP analog-treated 13-3B-4 cells. Lanes: 1, untreated control cells; 2–5, cells treated for 3 days with 50 μM 8-methylthio-cAMP, 5 μM 8-methylthio-cAMP + 25 μM N^6-butyryl-cAMP, 5 μM 8-methylthio-cAMP, and 25 μM N^6-butyryl-cAMP, respectively. R^I and R^{II}, purified preparations (Sigma) of R^I and R^{II} from rabbit skeletal muscle and bovine heart, respectively; 2+CS and R^{II}+CS, cell extracts of lane 2 and R^{II}, respectively, were immunoblotted with control serum; M, ^{14}C-labeled kDa marker proteins. Each lane contained 75 μg of proteins (R^I and R^{II} were 5 μg proteins) for electrophoresis.

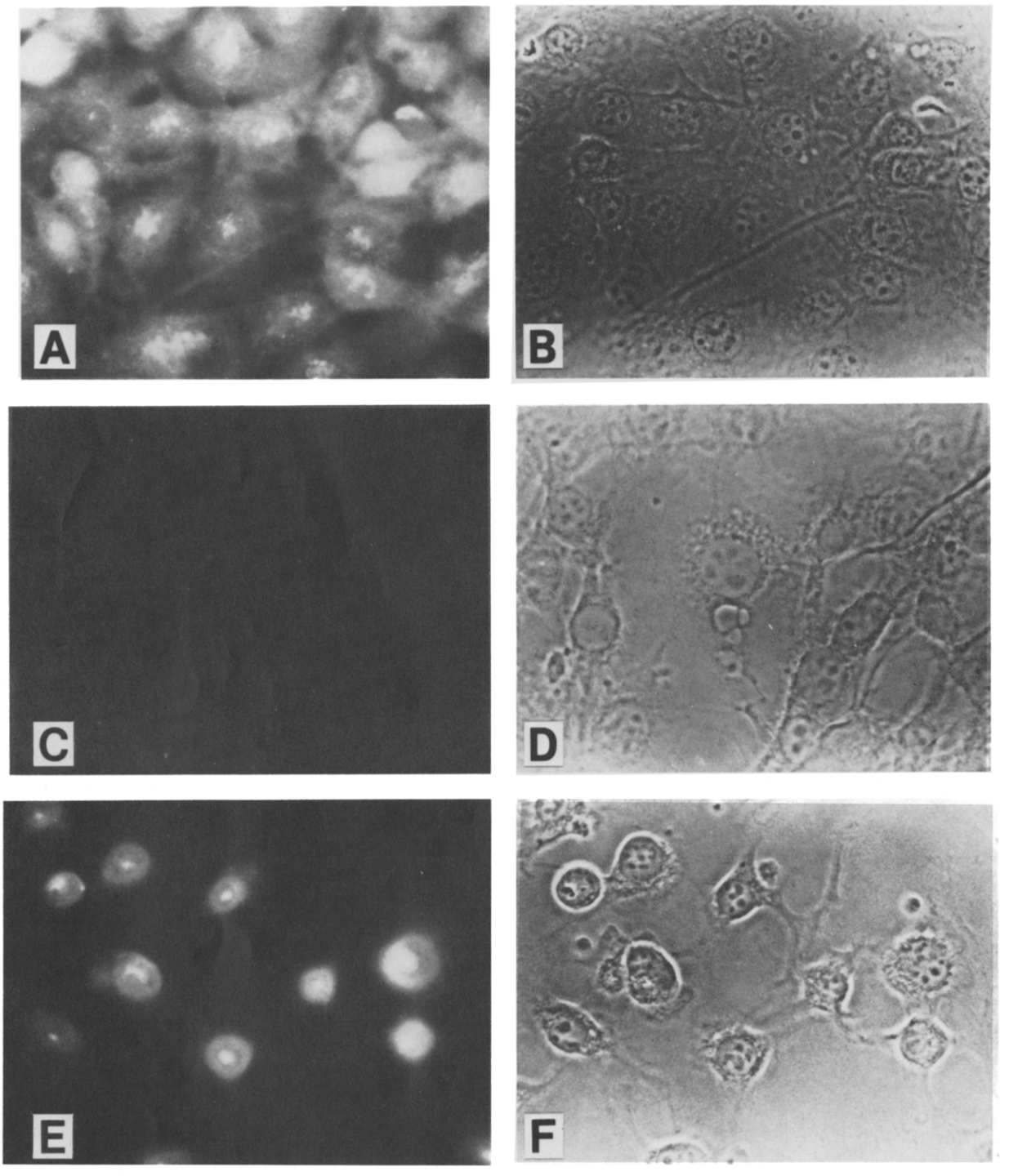

Fig.3. Immunocytochemical localization of R^{II} cAMP receptor protein. (A,B) Untransformed NIH/3T3 cells grown in serum-free medium for 24 h; (C,D) Ha-MuSV-transformed NIH/3T3 clone 13-3B-4 cells grown in serum-free medium for 24 h; (E,F) 13-3B-4 cells treated for 30 min with 25 μM N^6-butyryl-cAMP + 5 μM 8-methylthio-cAMP; (B,D,F) phase-contrast pictures of A, C and E, respectively, × 720.

N^6-butyryl-cAMP (not shown) compared with that in untreated cells (lane 1). However, the amounts of lower kDa R^{II} did not change after treatments with these analogs. Thus, specific increase of the 52–56 kDa R^{II} proteins that crossreact with the anti-bovine R^{II} antibody correlated with the growth inhibitory effect of the analogs on 13-3B-4 cells [17]. Furthermore, a synergistic increase in the band intensity of the R^{II} was observed when the cells were treated with a combination of N^6 and C-8 analogs, which exerted synergism in the growth inhibition. Thus, treatment with 5 μM 8-methylthio-cAMP added to 25 μM N^6-butyryl-cAMP (lane 3) or 25 μM N^6-carbamoylphenyl-cAMP (not shown) resulted in an intense R^{II} band, whereas each of these analogs alone, as shown with 5 μM 8-methylthio-cAMP (lane 4) and 25 μM N^6-butyryl-cAMP (lane 5), brought about a low intensity band of R^{II}. Quantification by densitometric tracings of the autoradiograms revealed that the analog treatments brought about a 5- to 6-fold increase in the amount of 52–56 kDa R^{II} compared with that in the untreated control cells. The increase in the R^{II} detected by the immunoblotting with the anti-bovine R^{II} antibody, therefore, correlated with the analog's growth inhibitory effect and confirmed the results previously obtained with DEAE-cellulose chromatography [17].

3.3. *Immunofluorescent localization of R^{II} cAMP receptor proteins by monospecific anti-R^{II} antibody*

The ability of anti-bovine R^{II} antibody to crossreact with the R^{II} antigen of 13-3B-4 cells made possible the immunocytochemical localization of R^{II} cAMP receptor proteins. As shown in fig.3A and B, untransformed NIH/3T3 cells grown for 24 h in the serum-free medium exhibited an extensive staining against the R^{II} antibody. The antibody produced prominent staining of the nucleus. In addition, a granular staining of a perinuclear area was often visible as was shown in the studies [33] of immunofluorescent localization of the R^{II} cAMP receptor proteins in Madine-Darby bovine kidney cells and rat fibroblasts. In contrast to untransformed NIH/3T3 cells, the transformed 13-3B-4 cells exhibited drastically reduced fluorescent labeling (fig.3C,D). Thus, transformation resulted in apparent loss of the cAMP receptor protein that crossreacts with the R^{II} antibody. Strikingly, however, treatment of 13-3B-4 cells with N^6-butyryl-cAMP (25 μM) + 8-methylthio-cAMP (5 μM) rapidly recovered the immunofluorescent staining in the cells. As early as 30 min after treatment with the analog combination, the cells exhibited an intensive fluorescent labeling, especially at the nucleus (fig.3E,F); at 5 h after the treatment, the immunofluorescent staining in the cells reached its maximal intensity and at 24 h, the fluorescence became less intense (not shown).

Treatment of cells with either 25 μM N^6-butyryl-cAMP or 5 μM 8-methylthio-cAMP alone for 5 h caused little or no immunofluorescent staining, whereas these analogs alone at higher concentrations (N^6-butyryl-cAMP (100 μM) and 8-methylthio-cAMP (50 μM)) elicited an intense staining (not shown). The same synergism was demonstrated with other combinations of N^6 and C-8 analogs, which showed synergism in the growth inhibition and phenotypic reversion [17].

4. DISCUSSION

We have shown here that the nuclear accumulation of the R^{II} cAMP receptor protein is an early event in the growth inhibition and phenotypic reversion of the Ha-MuSV-transformed 13-3B-4 cells treated with the site-selective cAMP analogs.

The transformed 13-3B-4 cells contained a markedly reduced level of the R^{II} cAMP receptor protein compared with their nontransformed counterpart, NIH/3T3 cells. Within 30 min after treatment of 13-3B-4 cells with the site-selective cAMP analogs, the R^{II} immunofluorescence labeling sharply increased, especially in the cell nucleus. The combination of a site 1 (C-8 thio or halogen analog) and a site 2 (N^6 analog) selective analog, which caused a synergism in growth inhibition [17], demonstrated a synergistic enhancement of accumulation of R^{II} in the nucleus. The nuclear binding of R^{II} was clearly demonstrated by a more intense immunofluorescence staining at the nucleus than in the cytoplasm. The Western blotting experiments demonstrated that the anti-R^{II} antibody specifically detected the 52–56 kDa R^{II} of 13-3B-4 cells. Thus, the increase in the immunofluorescent staining observed in the analog-treated cells was due to the increase of the

52–56 kDa R^{II} antigen in the cells. The photoaffinity labeling of cAMP receptor proteins with 8-N_3-[^{32}P]cAMP detected the R^{II} receptor only in the cytoplasm but not in the nucleus of the transformed 13-3B-4 cells. The early increase of the R^{II} in the nucleus of the analog-treated 13-3B-4 cells may therefore represent translocation of the R^{II} from the cytoplasm to the nucleus.

The site-selective cAMP analogs, which are known to demonstrate site-selectivity [15] and specificity toward the R^{II} receptor protein in vitro [16,34], induced the nuclear translocation of R^{II} in intact cells in vivo. Compartmentalization in the cell of the cAMP-dependent protein kinase and of its regulatory subunit has been implicated to play a role in the regression of hormone-dependent mammary cancer [32,35] and of disorders arising due to viral transformation of fibroblasts [36]. Understanding the ultimate mechanism of cAMP action in the modulation of gene activity would provide insights into the regulatory mechanism for cell proliferation and transformation.

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