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Cyclic AMP Suppresses Expression of v-ras^H Oncogene Linked to the Mouse Mammary Tumor Virus Promoter

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SUMMARY: Clone 433.3 of NIH 3T3 cells is a stable carrier of the MMTV LTR:v-ras^H chimeric DNA. Only in the presence of dexamethasone (a synthetic glucocorticoid), 433.3 cells exhibit an induced level of p21 transforming protein and phenotypic transformation. $N^6,O^{2'}$-dibutyryl cAMP (DBcAMP) antagonized the effect of dexamethasone in a time - and concentration - dependent manner. DBcAMP (5×10^{-4}M) added 18 hr prior to the addition of dexamethasone (10^{-7}M) almost completely blocked the hormone effect: cells contained levels of p21 20% of that in the cells treated with dexamethasone alone, and formed flat, contact inhibited monolayers. On the basis of these results together with our previous data on mammary carcinomas in vivo, we postulate that cAMP may be an intracellular suppressor acting at a regulatory locus of both cellular and viral ras genes.

INTRODUCTION: In a recent report (1) we have shown that the growth of hormone-dependent mammary carcinomas, induced by 7,12-dimethylbenz(α) anthracene in Sprague-Dawley rats, is associated with hormone-dependent expression of the cellular ras^H (c-ras^H) oncogene. The p21 transforming protein of the ras gene product was a prominent in vitro translation product of mRNAs of the growing tumors, and a sharp reduction of the translated p21 protein preceded regression of these tumors following ovariectomy (1).

In previous work (2), we had observed that the tumor regression, following ovariectomy, was preceded by an elevation of cAMP in the neoplastic tissues. Moreover, injections of DBcAMP in the host increased the cAMP level and induced growth arrest or regression of the tumors, as did ovariectomy (3). The objective of this work was to evaluate whether an increase of intracellular cAMP levels could modulate the expression of the ras gene. To this end, we examined the influence of elevated levels of cAMP on the production of p21 induced by glucocorticoid hormone in the system developed by Hager and his co-workers (4,5).

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This model is a chimeric molecular construction consisting of the mouse mammary tumor virus long terminal repeat (MMTV LTR), which contains one or more DNA sequences known to bind the glucocorticoid-receptor complex (6), fused to the ras gene of Harvey sarcoma virus (v-ras^H) (7). In cells transfected with this molecular chimera, such as the NIH 3T3 cell clone 433·3, expression of v-ras^H is dependent upon a promoter sequence(s) within the MMTV LTR, which requires physiological concentrations of glucocorticoid hormones for efficient transcription (8). Najam et al. (in preparation) have adapted the 433.3 system to serum-free culture conditions where the glucocorticoid-induced synthesis of the v-ras^H transforming protein, p21 (9), as well as morphological transformation of the cells can easily be quantified.

RESULTS AND DISCUSSION: In the work reported here, 433.3 cells were grown on a poly-D-lysine-coated plastic substrate in defined medium containing transferrin, EGF, and insulin. Under these conditions, the transformed phenotype is observed in most of the cells within 3-4 days after the initial addition of physiological concentrations of dexamethasone (8).

As shown in Fig. 1A, flat, contact inhibited monolayers of 433.3 cells were observed in the absence of hormone. In the presence of dexamethasone at either 10^{-6}M (Fig. 1B) or 10^{-7}M (Fig. 1C) concentrations, the cells became round and refractile and eventually floated away from the substratum. We examined the effect of DBcAMP on the dexamethasone-mediated transformation in 433·3 cells. DBcAMP (5×10^{-4}M) added 18 hr prior to the dexamethasone (10^{-6}M) treatment blocked cell transformation (Fig. 1D). When DBcAMP (5×10^{-4}M) was added simultaneously with dexamethasone (10^{-6}M), approximately half of the cells became transformed. Moreover, the proportion of flat cells in the population increased according to the time during which the cells were exposed to DBcAMP prior to dexamethasone treatment: treatment with DBcAMP starting as short a time as 1 hr before dexamethasone treatment, for example, resulted in more flat cells than round cells. Thus, DBcAMP inhibited the dexamethasone-inducible transformation in a time-dependent manner.

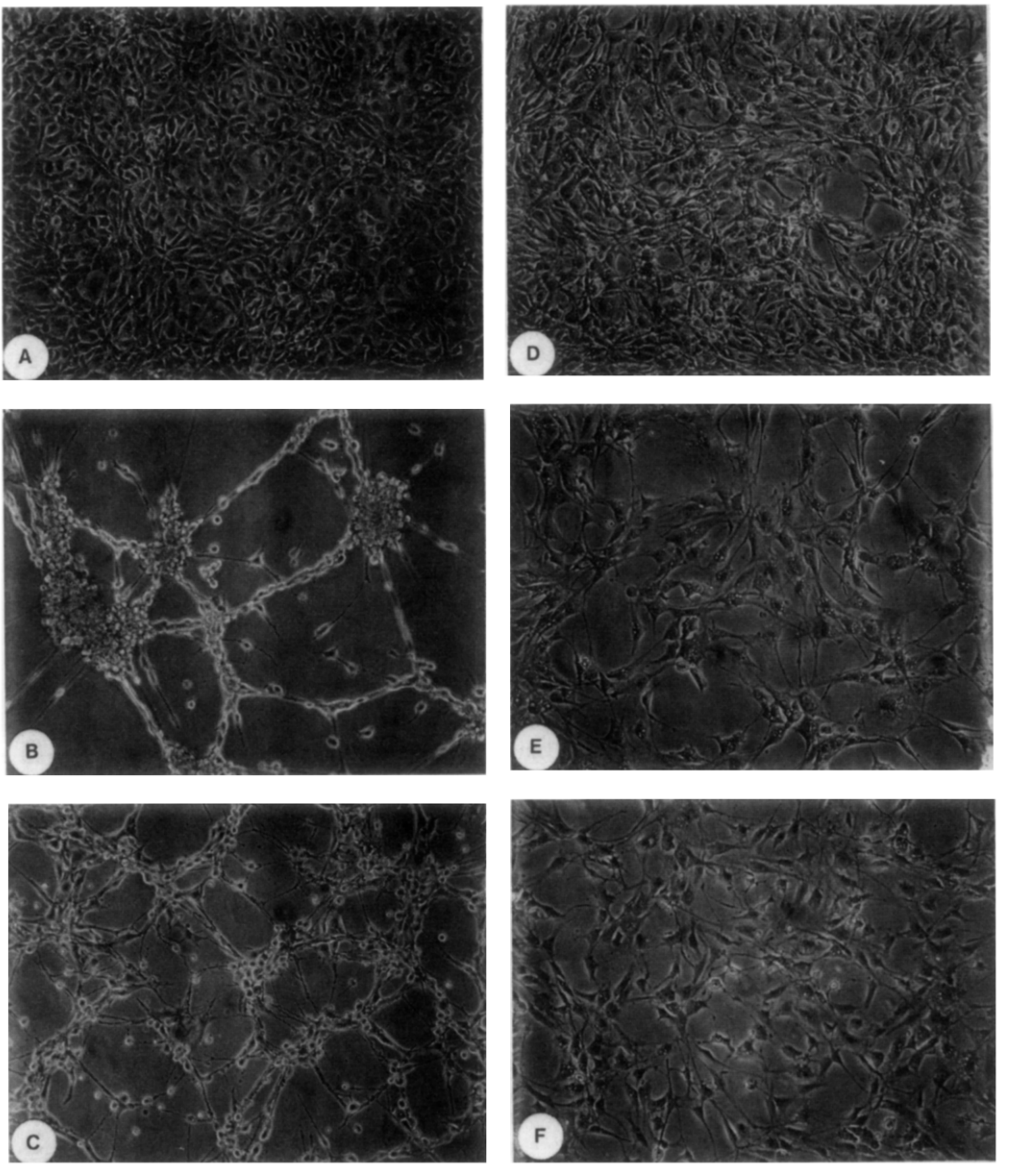

Fig. 1. Antagonistic effect between dexamethasone and DBcAMP on the phenotypic transformation of clone 433.3 of NIH 3T3 cells carrying MMTV-LTR:v-ras^H oncogene. The 433.3 cells were seeded onto poly-D-lysine coated 60 mm Falcon dishes (600,000 cells/dish). Cells were grown in the absence or presence of additives in the serum free medium DMEM (Dulbecco's modified Eagle medium)-HAM's F 12 (Nutrient Mixture F12-HAM) (75:25)] supplemented with bovine insulin (5 µg/ml), transferrin (5 µg/ml). EGF (epidermal growth factor) (10 ng/ml), Histidine-HCl (42 µg/ml), glutamine (292 µg/ml), Penicillin 100 U/ml), streptomycin (100 µg/ml), and fungizone (250 ng/ml); the medium was changed every 48 hours. The additives were provided every 48 hours, starting at day 1 after seeding (0 time). A, control, cells grown for 5 days with no additives; B and C, cells grown in the presence of dexamethasone 10^{-6}M and 10^{-7}M, respectively for 5 days; D and E, cells grown in the presence of DBcAMP 5 x 10^{-4}M, and 10^{-3}M, respectively, for 18 hrs, then grown for 5 days in the presence of dexamethasone 10^{-6}M + DBcAMP 5 x 10^{-4}M, and 10^{-3}M, respectively; F, cells grown in the presence of DBcAMP 5 x 10^{-4}M for 18 hrs, then grown for 5 days in the presence of dexamethasone 10^{-7}M + DBcAMP 5 x 10^{-4}M.

The inhibitory effect of DBcAMP on cell transformation was also dependent on its concentration relative to the concentration of dexamethasone. DBcAMP at 5×10^{-4}M concentration was more inhibitory against 10^{-7}M dexamethasone (Fig. 1F) than against 10^{-6}M dexamethasone (Fig. 1D), and 10^{-3}M DBcAMP (Fig. 1E) was more potent than 5×10^{-4}M DBcAMP (Fig. 1D) in the inhibition of 10^{-6}M dexamethasone effect. This inhibitory effect of DBcAMP was reversible: within a few days after removal of DBcAMP, cells appeared phenotypically transformed in the continued presence of dexamethasone.

Other cAMP analogs, 8-Br-cAMP (10^{-5}M) and 8-N_3-cAMP (10^{-6}M), as well as cholera toxin (10 ng/ml), an agent that specifically increases intracellular levels of cAMP (10,11), all inhibited dexamethasone-induced cell transformation in 433.3 cells. Sodium butyrate (5×10^{-4}M), 5'-AMP (5×10^{-4}M), and 2',3'-cAMP (5×10^{-4}M) had no apparent effect.

Phenotypic alteration of 433.3 cells in response to glucocorticoid hormones has been correlated (4) with the increased synthesis of a 21,000 dalton transforming protein (p21), the product of the v-$\underline{ras}^{H}$ gene (9). We examined whether the inhibitory effect of DBcAMP on dexamethasone-induced transformation is associated with a decrease in p21 level. Levels of p21 in 433.3 cells were determined by Western blotting analysis. As shown in Fig. 2A, p21 bands [phosphorylated and dephosphorylated forms (9,15)] were detected from the extracts of 433.3 cells cultured for 18 hr in the presence of dexamethasone. The p21 band intensity was dexamethasone-concentration dependent. Dexamethasone concentrations at 10^{-5}M and 10^{-6}M, the p21 bands exhibited the highest intensities (Lanes 1 & 2) and the band intensity gradually decreased with decreasing concentration of dexamethasone (Lanes 3-5), and at 10^{-10}M dexamethasone, the band was negligible (Lane 6). The p21 bands were absent when control serum was used in place of the #Y13-259 antibody (data not shown). The effect of DBcAMP on the dexamethasone-induced p21 level is shown in Fig. 2B. Treatment of cells with DBcAMP (5×10^{-4}M) for 18 hr prior to dexamethasone (10^{-7}M) treatment resulted in a marked decrease in p21 level (Lane 2). Quantitation by densitometric tracings of autoradiograms showed that the level of p21 in DBcAMP treated

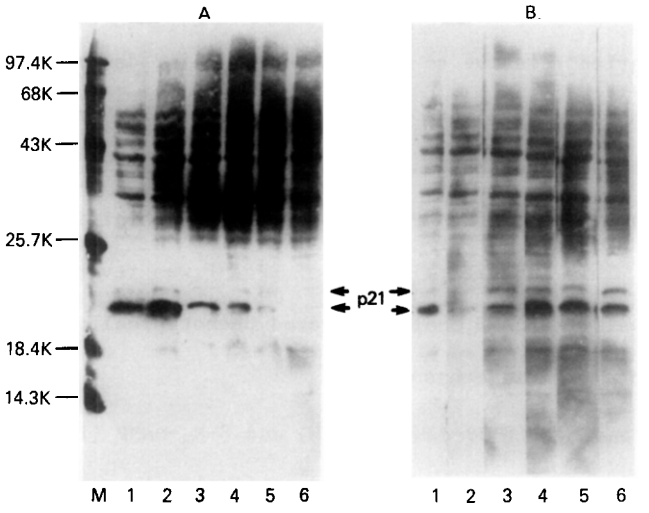

Fig. 2. Effect of dexamethasone and DBcAMP on p21 levels of clone 433.3 cells containing MMTV-LTR:v-ras^H oncogene. 433.3 cell pellets were suspended in ice-cold Buffer Ten (0.1M NaCl - 5mM $MgCl_2$-1% Nonidet P-40 - 0.5% Na deoxycholate - 0.1mM Dithiothreitol - 2 KIU/ml bovine aprotinin - 20mM Tris-HCl, pH 7.4) (5 x 10^6 cells/ml), vortexed and allowed to stand for 20 min at 4°C, passed through a 22-gauge needle 10 times, centrifuged at 750 x g for 20 min at 4°C, and the resulting supernatants were used as cell lysates. Cellular proteins were separated by 12% $NaDodSO_4$-PAGE (12) and transferred to nitrocellulose sheets (13) (0.2 μm, Schleicher & Schnell). Nitrocellulose sheets were washed and first incubated with 3% bovine serum albumin in NTE-NP40 (50mM Tris-HCl, pH 7.5 - 150mM NaCl - 2mM EDTA - 0.1% Nonidet P-40) for 3 hr at 37°C, and then sequentially incubated with media containing p21 monoclonal antiserum #Y13-259 (14) (or normal rat serum) for 16 hr at 4°C, rabbit anti-rat IgG (Cappel Lab.) for 2.5 hr in ice water bath, and 5 x 10^5 cpm/ml ^{125}I-protein A (Amersham Corp., specific activity ~ 30 mCi/mg) for 1 hr in ice water bath. The nitrocellulose sheets were air-dryed and exposed to Kodak XAR films for 12-36 hr at -20°C. A, cells grown for 18 hrs in the presence of dexamethasone at the concentrations 10^{-5}M (Lane 1), 10^{-6}M (Lane 2), 10^{-7}M (Lane 3), 10^{-8}M (Lane 4), 10^{-9}M (Lane 5), and 10^{-10}M (Lane 6). B, Lanes 1 and 4, cells grown for 18 hrs in the presence of dexamethasone 10^{-7}M and 10^{-6}M, respectively, + DBcAMP 5 x 10^{-4}M; Lanes 2 and 5, cells first grown for 18 hrs in the presence of DBcAMP 5 x 10^{-4}M and DBcAMP was removed, and grown for another 18 hrs in the presence of dexamethasone, 10^{-7}M and 10^{-6}M, respectively; Lanes 3 and 6, cells first grown for 18 hrs in the presence of dexamethasone 10^{-7}M and 10^{-6}M, respectively, and dexamethasone was removed, and grown for another 18 hrs in the presence of DBcAMP 5 x 10^{-4}M. M, [^{14}C] labelled protein molecular weight standards (Bethesda Research Laboratories, Inc.) 97.4K, phosphorylase B; 68K, Bovine serum albumin; 43K, ovalbumin; 25.7K α-chymotrypsinogen; 18.4K, β-Lactoglobulin; 14.3K, Lysozyme. Each Lane contained 100 μg of proteins for $NaDodSO_4$-PAGE.

cells (Fig. 2B, Lane 2) was 20% of that in control (Fig. 2A, Lane 3). This inhibitory effect of DBcAMP on p21 production was much reduced when DBcAMP was added simultaneously with dexamethasone (Fig. 2B, Lane 1) or at 18 hr post dexamethasone addition (Fig. 2B, Lane 3). The inhibitory effect of DBcAMP on dexamethasone-induced p21 production was also reduced when dexamethasone concentration was increased (Fig. 2B, Lanes 4-6). Thus, DBcAMP inhibited dexametha-

sone-induced p21 production in a time- and concentration-dependent manner. The decrease in p21 levels of 433.3 cells in response to DBcAMP treatment was further documented by immunoprecipitation of p21 from the ^{35}S-methionine-labelled cell lysates with monoclonal antibody #Y13-259 (14), which showed essentially the same results as the Western blotting analysis (data not shown).

This study presents the first evidence that cAMP and a steroid hormone may antagonize each other in the regulation of a viral oncogene expression. The inhibitory effect of cAMP on glucocorticoid-dependent expression of the viral *ras* gene in 433.3 cells seems to parallel our *in vivo* results (1) with hormone-dependent mammary carcinomas in rats, where an increased production of the c-*ras*^{H} mRNA appears to be dependent on the continuous presence of hormone. Hormone withdrawal following ovariectomy results in decrease of the *ras* mRNA concentration and tumor regression. Treatment of tumor bearing animals with DBcAMP also results in inhibition of c-*ras*^{H} expression followed by tumor regression (1). The suppressive effect of cAMP on *ras* gene expression was not, however, limited to the *ras* gene whose expression is under the control of mouse mammary tumor virus promoter. We showed in a recent report (16) that cAMP analogs also inhibit the expression of the *ras* gene of Harvey sarcoma virus having its own promoter. When clone 13-3B-4 of NIH 3T3 cells containing multiple copies of the v-*ras*^{H} oncogene (kindly provided by D.R. Lowy) was grown in the serum-free media, the cells exhibited a high level of p21 and the phenotypic transformation; addition of DBcAMP (10^{-3}M) and other cAMP analogs blocked the p21 production and the cell transformation.

That cAMP inhibits the expression of the viral *ras*^{H} gene of cultured fibroblasts having its own promoter or the MMTV promoter, and of the cellular *ras*^{H} gene of mammary carcinomas *in vivo* (1), suggests the action of cAMP at a regulatory locus that may be present in both cellular and viral *ras* genes. Cyclic AMP in this action may involve its receptor proteins (binding proteins) (17): the role of cAMP + receptor complex at the nuclear level has been suggested to be essential in the cAMP-induced regression of mammary carcinomas (18). Understanding the precise role of cAMP on the *ras* gene expression would

provide an insight into the intracellular mechanism that controls cellular proto-oncogene expression and neoplastic transformation.

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