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Site-selective 8-Cl-cAMP which causes growth inhibition and differentiation increases DNA (CRE)-binding activity in cancer cells*

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Control mechanisms of normal differentiation are disrupted in cancer cells but can be restored by treatment with site-selective cAMP analogs. The cellular events associated with such changes entail compartmental redistribution of the cAMP-dependent protein kinase type II regulatory subunit, RII_β. The results of this study indicate that the molecular mechanisms of action involve changes in specific DNA-binding activity of putative transcription factors. Gel retardation analyses revealed that nuclear extracts from cells of various human cancer cell lines [colon cancer (LS-174T), gastric cancer (TMK-1), and leukemia (K-562)] and rodent pheochromocytoma (PC12) show a concentration-dependent increase in binding activity to a synthetic DNA that contained the cAMP-responsive element 5′-TGACGTCA-3′ after treatment with 8-Cl-cAMP. Such an increase in cAMP-responsive element binding activity was not observed in the 8-Cl-cAMP-unresponsive MKN-1 gastric cancer cells. These findings indicate that the antitumor activity of site-selective cAMP analogs may reside in the induction of transcription factors that restore normal gene regulation in cancer cells.

Gel retardation; Transcription factor; cyclic AMP responsiveness

1. INTRODUCTION

In mammalian cells, gene expression is altered in response to stimulation by hormones. Stimuli whose action is mediated through adenylate cyclase and cAMP-dependent protein kinase influence the transcriptional efficiency of several genes through the binding of specific factors to distinct *cis*-acting DNA sequences located in the 5′-noncoding (promoter) region [1]. Recently, a region containing the cAMP-responsive element (CRE) has been shown to bind specific nuclear proteins from various tissues [2–4]. In recent reports [5–7] we demonstrated that human and rodent cancer cells show growth inhibition and changes in morphologic appearance and biochemical properties after treatment with site-selective cAMP analogs. These effects were not mimicked by previously studied analogs such as dibutyryl-cAMP. Of the two known cAMP-binding sites in protein kinase regulatory subunits, the C-8 halogen analogs preferentially bind to site 1 (site B) of type II protein kinase [8,9]. The apparent underlying cellular mechanism of 8-Cl-cAMP, the most potent growth

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Abbreviations: CRE, cAMP-responsive element; RII_β, type II cAMP-dependent protein kinase regulatory subunit

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inhibitor, is to promote translocation of type II cAMP-dependent protein kinase regulatory subunit (RII_β) (see [10] for nomenclature) from the cytoplasm to the nucleus [11].

Site-selective cAMP analogs also have induced differentiation in several leukemic cell lines [12]. The molecular events underlying these changes have not been elucidated but are suggested to involve specific factors that play a regulatory role in transcription [13]. In this study, we examined whether or not the regulatory role of site-selective cAMP analogs on the growth and differentiation of cancer cells would involve modulation of gene transcription. To assess such transcriptional modulation, we examined the change in the DNA (CRE)-binding activity of nuclear extracts prepared from cancer cells after treatment with 8-Cl-cAMP.

2. MATERIALS AND METHODS

2.1. *Materials*

The cell lines used in this study were cancer cells, such as LS-174T (human colon carcinoma) [5,11], K-562 (chronic myelogenous human leukemia) [12,13], PC12 (rodent pheochromocytoma), and TMK-1 and MKN-1 (8-Cl-cAMP-responsive and -unresponsive human gastric cancers, respectively) [14], that were demonstrated previously to be responsive to treatment with site-selective cAMP analogs. The cells were grown under standard conditions and treated for 1–3 days with 8-Cl-cAMP at indicated concentrations as previously described [5,12]. 8-Cl-cAMP was kindly provided by R.K. Robins (Nucleic Acid Research Institute, Costa Mesa, CA).

The CRE-containing oligonucleotide used in this study contained the TGAG sequence as the first 5′ to 3′ downstream bases and GTCA as the next sequence. A 3-fold repeat of this sequence with a complementary second strand and a 5′-dinucleotide overhang was synthesized (MIM no. 001, Synthecell Corporation, Gaithersburg, MD). The double-stranded trioctamer of CRE was labeled with ^{32}P at the 5′-end using T4 kinase [15]. Cold competition assays were performed using (i) a 200-bp oligonucleotide containing CRE sequences (SS-200) prepared from the promoter region of somatostatin-CAT fusion gene [Δ-71 CAT; kindly provided by M. Montminy (Salk Institute, La Jolla, CA)] and (ii) a 323-bp segment of the early promoter region of SV40 DNA (SV40-323) containing no CRE sequences [16].

2.2. *Preparation of nuclear extracts*

Nuclear extracts were prepared by previously published methods with a minor modification [11,17]. Briefly, a homogenate was prepared in 0.35 M sucrose solution containing 0.05 M Tris, pH 7.5, 5 mM EDTA, 2 mM mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamide. A Dounce homogenizer was used to disrupt cells while preserving nuclear and organellar integrity. The crude nuclear pellet (obtained by centrifuging at $5000 \times g$ for 10 min) was washed in phosphate-buffered saline, extracted with 0.4 M NaCl, and subjected to centrifugation at $25000 \times g$ for 60 min. The supernatant fraction from this centrifugation was used for gel mobility assays. When proteins were purified from this extract, ammonium sulfate was added to 45% saturation, the precipitate was collected in the cold by centrifugation at $16000 \times g$ for 20 min, and the solubilized pellet was dialyzed and used for assays. Protein concentrations were determined by the method of Lowry et al. [18] with bovine serum albumin as standard. The nuclear extracts were stored in aliquots at −80°C.

2.3. *Gel retardation assay*

The DNA-binding assay was performed by a method modified from that of Fried and Crothers [19]. Nuclear extracts at the indicated concentrations were incubated with 0.5–1.5 ng of labeled oligonucleotide for 30 min at 30°C. The poly dI·dC concentration (Sigma Chemical Co., St. Louis, MO) was titrated and found to be optimal for eliminating nonspecific mobility retardation in the concentration range of 1.5–5.0 μg per assay. Other DNA preparations were added when indicated, and the reaction was terminated by the addition of 2.5 μl of bromophenol blue and xylene cyanole (0.1% each in 50% glycerol). The samples were then applied to a 4% acrylamide gel (30:1 acryl/bis) and separated at 200 V for 1.5 h at room temperature. The gels were dried, and the gel mobility retardation was visualized by autoradiography using X-OMAT film (Eastman Kodak, Rochester, NY). Densitometry was carried out using a Hoefer Scientific Instruments GS 300 scanning densitometer and analyzed using the Macintosh version of the GS-370 data system.

3. RESULTS

LS-174T human colon cancer cells grown in culture in the presence of 8-Cl-cAMP (10 μM) demonstrated an increase in CRE DNA-binding activity in their nuclei (fig.1) and cytoplasm (data not shown). At least 25 μg of nuclear proteins or 30 μg of cytoplasmic proteins were required to demonstrate this activity. At protein concentrations below these limits, no gel retardation activity was shown in either control or treated cell extracts.

Formation of DNA-protein complexes with the ^{32}P-labeled CRE-containing oligonucleotide was also demonstrated in purified nuclear protein preparations from K-562 human leukemia and PC12 rat pheochromocytoma cell lines (fig.2A and B). The nuclear proteins from K-562 and PC12 cell lines treated with 8-Cl-cAMP showed a concentration-dependent increase in gel mobility retardation of the CRE trioctamer (lanes 2,4,6, and 8) when compared with the DNA binding in the untreated control nuclei (lanes 1,3,5, and 7).

The DNA-binding activity in 8-Cl-cAMP-

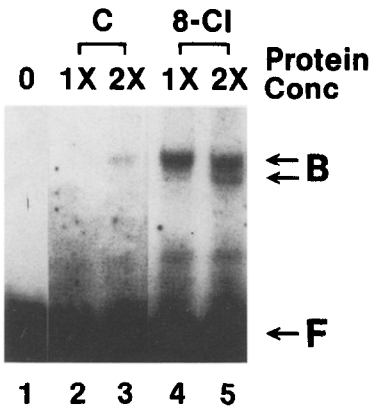

Fig.1. Binding activity of CRE-oligonucleotide with nuclear extracts from LS-174T colon cancer cells before and after treatment with 8-Cl-cAMP. Lanes: 1, DNA alone; 2 and 3, incubated with nuclear extracts from untreated control cells; 4 and 5, nuclear extracts from cells treated for 72 h with 10 μM 8-Cl-cAMP. Crude nuclear extracts were used. The letter B on the right of this and the subsequent two figures refers to bound DNA and the letter F to free DNA.

responsive (TMK-1) and -unresponsive (MKN-1) gastric cancer cell lines [14] is shown in fig.3. 8-Cl-cAMP treatment increased the DNA binding in IMK-1 nuclear extracts (fig.3A) but not in MKN-1 (fig.3B). In MKN-1, there was a decrease in DNA binding after 8-Cl-cAMP treatment.

Densitometric analysis indicated that there is some variability in DNA-binding activity depending on the cell type relative to the protein concentration at which the binding saturation takes place. The quantification data of DNA binding with respect to protein concentrations are shown in fig.4. Saturation of DNA-binding activity in the nuclear proteins of LS-174T colon cancer cells was not observed, even at protein concentrations as high as 8 μg protein per assay, and the binding increased throughout the range of concentrations tested; the binding was 2-fold greater in the cells treated with 8-Cl-cAMP than in the untreated control cells. Both treated and untreated PC12 cells reached their saturation in DNA binding at 3–4 μg nuclear proteins per assy, and the binding activity was 3–5-fold greater in the treated cells than in the untreated control cells (fig.4B). The nuclear proteins from TMK-1 cells treated with 8-Cl-cAMP also showed progressively increasing binding activity at concentrations in the range of 3–16 μg protein per assay (2–3-fold increase over the control values) (fig.4C). In 8-Cl-cAMP-unresponsive MKN-1 cells, this increase in DNA binding was not observed in the treated cell nuclei as compared with untreated control cells, even up to 16 μg protein per assay (fig.4D).

Fig.5 shows competition of DNA-binding activity in LS-174T cancer cells using unlabeled DNA

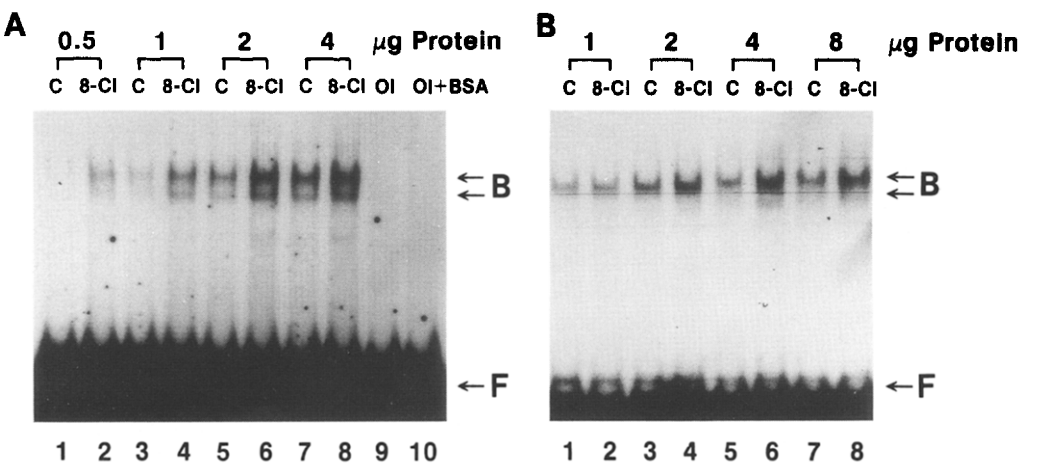

Fig.2. Comparison of 8-Cl-cAMP treatment on CRE DNA-binding activities in purified nuclear protein preparations from human K-562 leukemia and rodent Pc12 pheochromocytoma cell lines. A, K-562 cells; B, PC12 cells. Nuclear proteins were purified as described in section 2. Lanes: 2,4,6, and 8, nuclear proteins from 8-Cl-cAMP-treated cells; 1,3,5, and 7, nuclear proteins from untreated cells; 9, DNA alone; 10, DNA plus 10 μg bovine serum albumin. K-562 cells were treated overnight with 1 μM 8-Cl-cAMP and PC12 cells were treated for 72 h with 10 μM 8-Cl-cAMP.

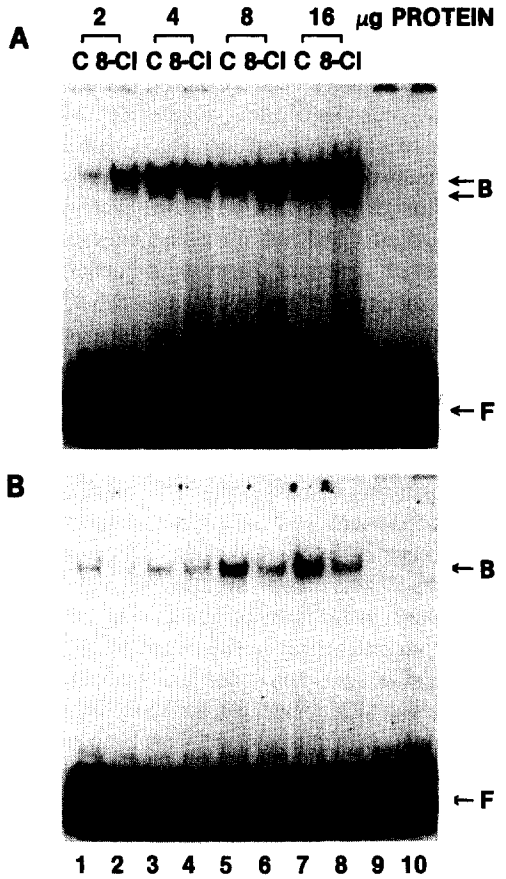

Fig.3. DNA gel mobility retardation of 8-Cl-cAMP-responsive and -unresponsive gastric cell lines. A, TMK-1 (responsive) cells; B, MKN-1 (unresponsive) cells. Purified nuclear proteins were used. Lanes: 1,3,5, and 7, nuclear proteins from untreated cells; 2,4,6, and 8, nuclear proteins from cells treated with 10 μM 8-Cl-cAMP for 72 h; 9 and 10, as in fig.2.

with or without CRE sequences. There was little or no reduction in the DNA-binding activity by SV40 promoter DNA, whereas somatostatin-CRE DNA caused a marked reduction (~60%) in the DNA-binding activity in the 8-Cl-cAMP-treated cells. DNA binding of control nuclear proteins was decreased onbly 15–25% by somatostatin-CRE (data not shown). Interestingly, the extent of competition is considerably greater in the treated cells than in the controls, indicating that binding affinity with DNA may play a role or that more than one factor may be present. The Coomassie blue-stained protein patterns after SDS-PAGE of the treated and untreated cell nuclear extracts were identical (data not shown).

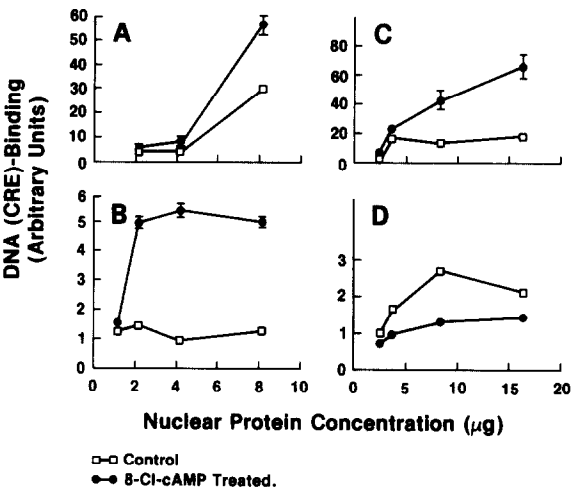

Fig.4. Protein concentration dependence of DNA complex formation of purified nuclear proteins from cells with and without treatment with 8-Cl-cAMP (10 μM for 72 h). A, LS-174T; B, PC12; C, TMK-1; D, MKN-1. The values are integrated areas using a densitometric analysis of the DNA gel mobility retardation and plotting the values (in arbitrary units, ordinate) against protein concentrations (abscissa). The values represent average ± SD of five determinations. (□——□) Untreated control; (●——●) 8-Cl-cAMP-treated.

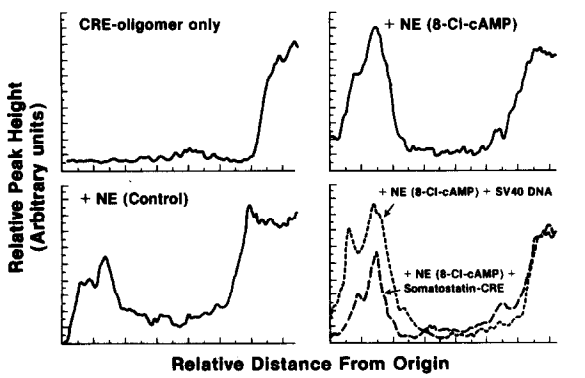

Fig.5. Competition of gel retardation with CRE-containing DNA (SS-200) and DNA without CRE (SV40-323). Purified nuclear proteins (4 μg) from control and 8-Cl-cAMP-treated (10 μM for 72 h) LS-174T colon cancer cells were incubated with ^{32}P-labeled CRE-oligonucleotide in the presence or absence of unlabeled SS-200 or SV40-323 (see section 2). Densitometric tracings of the DNA gel mobility retardation are shown by relative peak heights in arbitrary units (ordinate) against relative distance from origin (abscissa).

4. DISCUSSION

Evidence presented in this work showed that a cAMP-dependent factor(s) capable of specific binding to a synthetic CRE oligonucleotide increases in cancer cells after treatment with a site-selective cAMP analog, 8-Cl-cAMP. The results are directly related to the responsiveness of cancer cells to treatment with the cAMP analog. The increase in DNA gel mobility shift was found in cancer cells, such as LS-174T colon carcinoma, TMK-1 gastric cancer, and K-562 leukemia, that have been shown [5,11–14] to be biologically responsive [growth inhibition, biochemical and morphological changes, induction of differentiation (leukemia), etc.] to 8-Cl-cAMP treatment. A gastric cancer cell line (MKN-1) which is biologically unresponsive to 8-Cl-cAMP, as indicated by continued growth during treatment [14], did not show an increase in such nuclear factor activity (fig.3).

Responsiveness to normal control mechansism may be modified or lost in cancer cells [20–22]. The link between transcriptional control and cAMP-directed pathways is the CRE [1]. Our demonstration of an increase in DNA (CRE)-binding activity in only those cancer cells that are biologically responsive to the cAMP analog treatment suggests that the antineoplastic effect of site-selective cAMP analogs, which has been shown to be due to biological modulation rather than cell killing [5–7,11–14], may reside in restoration of CRE-related transcriptional control in malignancy.

The cellular events underlying the growth inhibitory effect of site-selective cAMP analogs involve differential regulation of type I versus type II cAMP-dependent protein kinase isozymes [5–7]. 8-Cl-cAMP, in the inhibition of the growth of human colon cancer cells in culture, brought about an enhancement of the RII_β nuclear translocation and transcriptional activation of RII_β receptor gene [11]. Nuclear translocation of RII cAMP receptor protein, which has also been observed previously during cell development [23], tumor regression [24,25], and reverse transformation [26–28], therefore suggests the possible role of this protein in gene regulation. It has been postulated [29] that the RII cAMP receptor proteins that bind cAMP [30] and DNA [31,32] and have amino acid homology with the *Escherichia coli* catabolite gene activator protein [33] could directly stimulate gene expression.

It has been reported [34] that a 43-kDa phosphoprotein binds specifically to the region of the somatostatin gene known to contain CRE. This protein is suggested to be a substrate of both protein kinase C and cAMP-dependent protein kinase, and its binding to DNA is not cAMP dependent. An increase of a transcriptional factor(s) upon cAMP stimulation has been shown in normal liver and hepatoma cell cultures using the cAMP-regulated gene encoding phospho*enol*pyruvate carboxykinase [35]. The relationship, if any, of these nuclear proteins to the protein(s) we detected is not known.

Ultimate proof that the factor(s) we have detected are causally involved in the cAMP-mediated signal, however, will require the use of purified factor(s) and a suitable transcription system. Nevertheless, our findings may provide insight into the understanding of the fundamental mechanisms of gene control in cell proliferation and differentiation, and also importantly, they may lead to useful treatment protocols to identify responsive tumors whose growth can be controlled by the use of site-selective cAMP analogs, either alone or in combination with conventional chemotherapeutic agents.

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