

Experimental gene therapy of human colon cancer

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Background. Gastrin regulates growth of human colon cancer cells by activation of the cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA). Gastrin and 8-Br-cAMP, a membrane-permeable cAMP analog, inhibit growth of HCT116 cells; both stimulate growth of LoVo cells. This dual effect on growth may be explained by relative amounts of the regulatory subunit ($R_{I\alpha}$ or $R_{II\beta}$) of PKA within the cancer cells. Antisense oligodeoxynucleotides (ASO) to either $R_{I\alpha}$ or $R_{II\beta}$ inhibit protein translation of the target mRNA by sequence-specific binding; subsequently, cellular PKA content and the cAMP-mediated growth may be altered. We determined whether ASO to either the $R_{I\alpha}$ or $R_{II\beta}$ subunit altered the cAMP-mediated growth of HCT116 and LoVo human colon cancer cells.

Methods. HCT116 cells were treated with $R_{II\beta}$ ASO (15 $\mu\text{mol/L}$, 4 days) and then treated with 8-Br-cAMP (25 $\mu\text{mol/L}$); tritiated thymidine incorporation was measured after 24 hours, and the cell number was determined on alternate days. Protein and mRNA levels of the $R_{II\beta}$ subunit were determined by Western and Northern blotting, respectively. Similar studies with an ASO against the $R_{I\alpha}$ subunit were performed on LoVo cells.

Results. $R_{II\beta}$ ASO reversed the cAMP-mediated inhibition of growth of HCT116 cells, and $R_{II\beta}$ ASO decreased the protein level of the $R_{II\beta}$ subunit. $R_{II\beta}$ ASO did not alter the basal growth of HCT116 cells. $R_{I\alpha}$ ASO reversed the cAMP-mediated stimulation of growth of LoVo cells.

Conclusions. The regulatory subunits of PKA are potential targets to alter growth of human colon cancer cells. Gene therapy directed to alter specific steps in signal transduction pathways may provide new therapeutic strategies. (SURGERY 1994;116:189-96.)

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THE EFFECT OF GROWTH FACTORS and hormones is mediated by specific binding to cell surface receptors and subsequent alteration in the intracellular levels of small-molecule second messengers, such as 3',5'-cyclic adenosine monophosphate (cAMP).¹⁻² Many peptide hormones, including gastrin, have been shown to use the cAMP signal transduction mechanism to regulate growth.³⁻⁷ cAMP activates the cAMP-dependent protein kinase (protein kinase A [PKA]), a heterodimer composed of two regulatory and two catalytic subunits.^{8,9} It has been shown that there are several isomers

of the regulatory subunit.¹⁰ The relative ratio of these regulatory subunits of PKA (growth-stimulatory [$R_{I\alpha}$] or growth-inhibitory [$R_{II\beta}$]) dictate whether cell growth will be stimulated or inhibited on activation of the cAMP pathway.¹¹⁻¹³ Halide-substituted cAMP analogs easily penetrate cell membranes and lead to PKA activation.¹⁴⁻¹⁶ We have found that gastrin and 8-Br-cAMP, which bind equally to both $R_{I\alpha}$ and $R_{II\beta}$, inhibit growth of HCT116 human colon cancer cells; 8-Br-cAMP stimulates growth of LoVo human colon cancer cells.⁴

Sequence-specific antisense oligodeoxynucleotides (ASO) are effective means of blocking translation of cellular protein from messenger RNA transcripts.^{17,18} ASO bind to the target mRNA and, through either mRNA digestion by RNase H or ribosome competition, inhibit protein translation and decrease the cellular content of the targeted protein.^{19,20} ASO directed against the $R_{I\alpha}$ decrease the protein level and lead to differentiation and inhibition of growth of a leukemia

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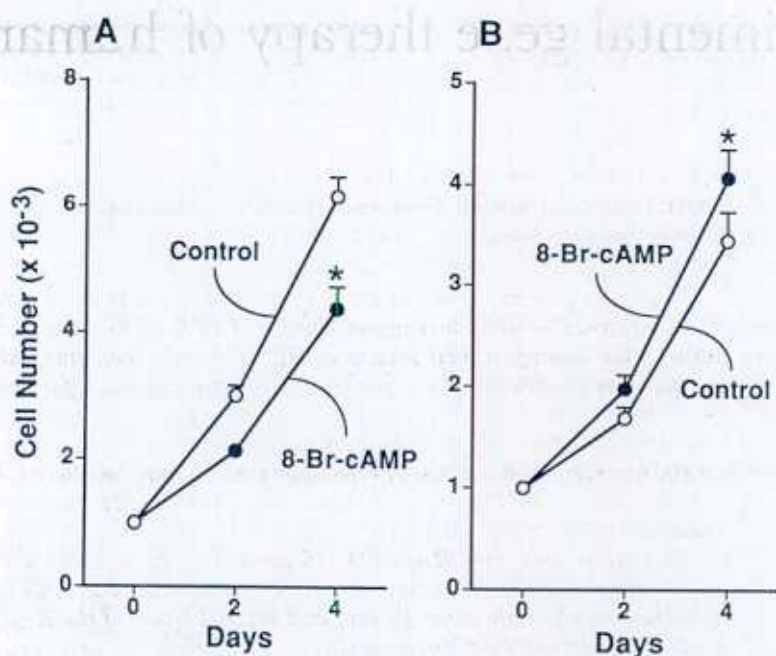


Fig. 1. Growth study of HCT116 (A) and LoVo cells (B) after treatment with 8-Br-cAMP (25 $\mu\text{mol/L}$). * $p < 0.05$ versus control.

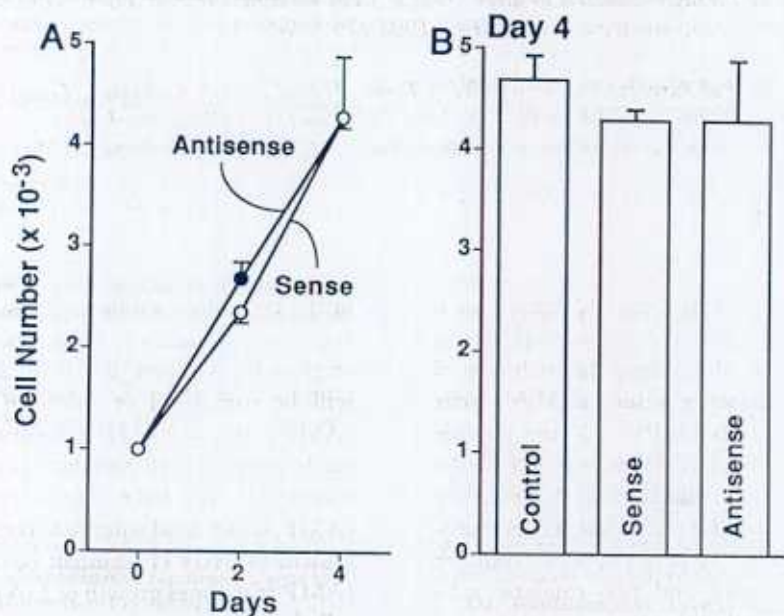


Fig. 2. Growth study of HCT116 cells (A) after treatment with R11 β sense or ASO (15 $\mu\text{mol/L}$) and total cell number after 4 days of treatment (B) with untreated (Control) cells for reference.

cell line.²¹ Furthermore, manipulations in the relative ratio of R1 α and R11 β lead to alterations in rate of growth and degree of differentiation in mouse cells.²²

Our hypothesis was that the opposite effect of 8-Br-

cAMP on the growth of either HCT116 or LoVo cells was a result of differences in the amounts of the regulatory subunits of PKA. Therefore we examined whether ASO against either the R1 α or R11 β subunit would

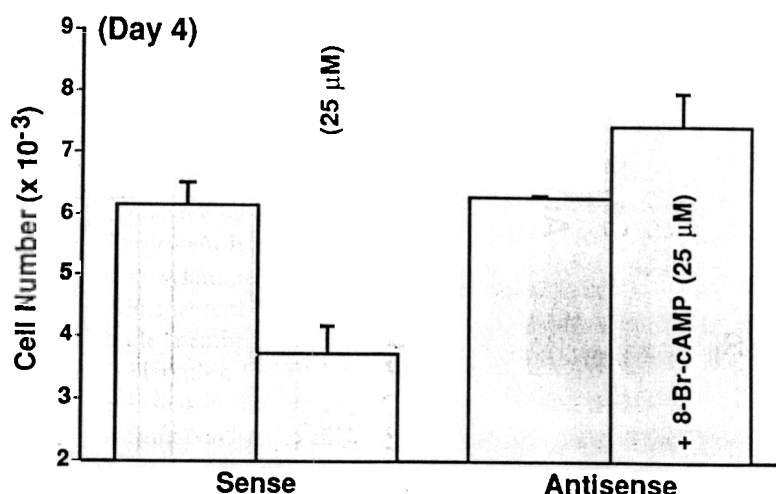


Fig. 3. Effect of 8-Br-cAMP (25 μmol/L) on growth of HCT116 cells pretreated with either R_{IIβ} sense or ASO (4-day pretreatment, 15 μmol/L). **p* < 0.05 versus without 8-Br-cAMP.

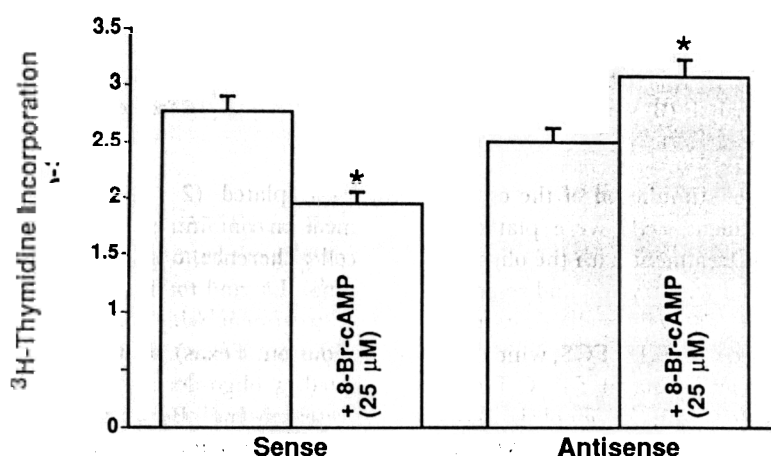


Fig. 4. Effect of 8-Br-cAMP (25 μmol/L) on tritiated thymidine incorporation in HCT116 cells pretreated with either R_{IIβ} sense or ASO (4-day pretreatment, 15 μmol/L). **p* < 0.05 versus without 8-Br-cAMP.

change the relative ratio of the regulatory subunits and subsequently alter the growth of HCT116 and LoVo human colon cancer cells.

MATERIAL AND METHODS

Cell culture. HCT116 and LoVo human colon cancer cells were obtained from the American Type Culture Collection (Rockville, Md.). HCT116 cells were cultured in McCoy's medium (Gibco Laboratories, Grand Island, N.Y.) supplemented with 5% fetal calf serum (FCS; Hyclone Laboratories, Inc., Logan, Utah). LoVo cells were cultured in Dulbecco's modified Eagle's medium (Gibco Laboratories) supplemented with 5% FCS. A humidified incubator at 37°C with an atmosphere of 95% air and 5% CO₂ was used. Passages 5 to 9 (HCT116) and passages 45 to 50 (LoVo) were used

for all studies to avoid differences in response after prolonged culture.

Growth studies. HCT116 cells (1000 cells/well, 96-well plate) were seeded in McCoy's medium containing 5% FCS. After 24 hours, medium was replaced with fresh medium containing 0.1% FCS, and cells were treated every 2 days with 15 μmol/L oligodeoxynucleotides (R_{IIβ} sense sequence, 5'-ATG-AGC-ATC-GAG-ATC-CCG-GCG-3'; R_{IIβ} antisense sequences, 5'-CGC-CGG-GAT-CTC-GAT-GCT-CAT-3'; R_{Iα} sense, 5'-ATG-GAG-TCT-GGC-AGT-ACC-GCC-3'; R_{Iα} antisense, 5'-GGC-GGT-ACT-GCC-AGA-CTC-CAT-3'; Midland Reagents, Midland, Texas). Cells were collected on later days with 0.25% trypsin (Gibco Laboratories) and were counted with a Coulter counter (Coulter Electronics, Hialeah, Fla.). For the

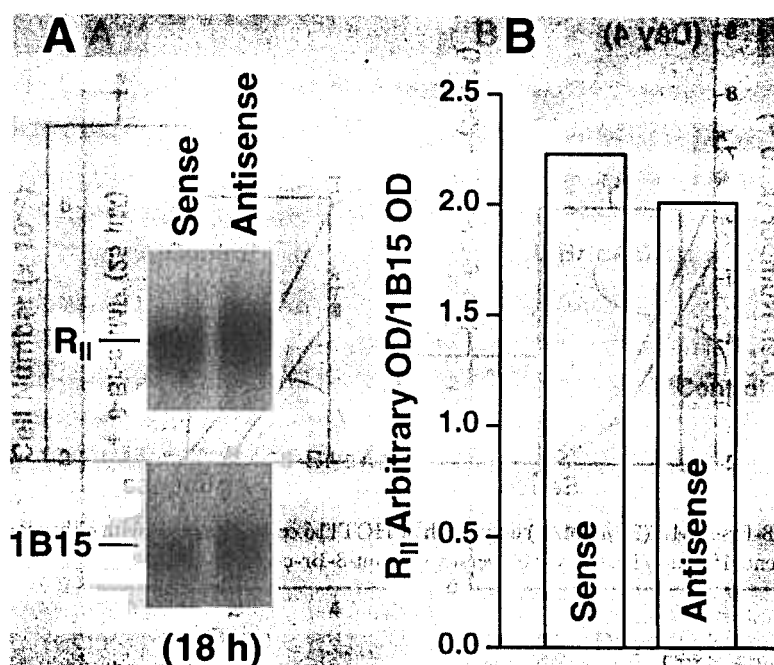


Fig. 5. Northern blot for $R_{11\beta}$ mRNA in HCT116 cells (A) treated with $R_{11\beta}$ sense or ASO (15 $\mu\text{mol/L}$, 18 hours) and densitometric analysis (B) with normalization to the constitutively expressed gene 1B15.

examination of the effect of stimulation of the cAMP pathway after ASO treatment, cells were plated as mentioned. After 4 days of treatment with the oligonucleotides, cells were collected with trypsin and reseeded into fresh 96-well plates (1000 cells/well) containing fresh medium supplemented with 0.1% FCS, which had been heat-inactivated by incubating at 54° C for 30 minutes. Cells were treated with 25 $\mu\text{mol/L}$ 8-Br-cAMP (Sigma Chemical Company, St. Louis, Mo.) or vehicle while still undergoing treatment every 48 hours with the respective oligodeoxynucleotide. Four days after the initiation of 8-Br-cAMP treatment, the cells were collected with trypsin and counted by Coulter counter.

Growth studies of LoVo cells were set up as mentioned with Dulbecco's modified Eagle's medium. Oligonucleotides to $R_{1\alpha}$ were used for growth studies and for the study involving pretreatment with the ASO and subsequent treatment with 8-Br-cAMP.

Tritiated thymidine incorporation. Cells were plated as mentioned for the study of ASO pretreatment. Twenty-one hours after the addition of 8-Br-cAMP, cells were pulsed with 1 $\mu\text{Ci/ml}$ of tritiated thymidine (Amersham Life Science, Arlington Heights, Ill.). After a 3-hour pulse, incorporated tritiated thymidine was precipitated with 10% trichloroacetic acid (Sigma Chemical Company). Cells were then solubilized with 1 N NaOH, and radioactivity was determined by liquid scintillation counting.

mRNA analysis by Northern blot. HCT116 cells

were plated ($2 \times 10^6/100$ mm plate) in McCoy's medium containing 5% FCS. After 24 hours to allow for cell adherence, oligodeoxynucleotides were added (15 $\mu\text{mol/L}$), and total RNA was collected after 18 hours of treatment with RNazol B (Biotecx Laboratories, Houston, Texas). Polyadenylated mRNA was then isolated by oligo-deoxythymidine cellulose (Collaborative Research Inc., Bedford, Mass.) column chromatography. Twenty micrograms was separated on a denaturing 1.25% agarose gel. After electrophoresis, RNA was transferred to a nitrocellulose membrane for subsequent Northern blotting. Probes were made by nick-translation of the complement DNAs for the $R_{11\beta}$ and cyclophilin genes with α - ^{32}P -deoxyadenosine triphosphate. After hybridization and later posthybridization washes for removal of the free probe, the membranes were exposed to x-ray film for autoradiography. The hybridization signals on the autoradiographs were analyzed quantitatively with a densitometer (Bioimage Visage 60; Bioimage, Ann Arbor, Mich.).

Protein analysis by Western blot. HCT116 cells ($2 \times 10^6/100$ mm plate) were cultured in McCoy's medium containing 5% FCS. After 24 hours to allow for adherence, cells were treated with oligodeoxynucleotides (15 $\mu\text{mol/L}$) for 48 hours. Cells were washed twice in phosphate-buffered saline solution (Gibco Laboratories) and collected into ice-cold phosphate-buffered saline solution. Cells were pelleted and resuspended in 0.5 ml of buffer "10" (Tris-HCl pH 7.4, 20 mmol/L; NaCl, 100 mmol/L; NP-40, 1%; sodium

deoxycholate, 0.5%; $MgCl_2$, 5 mmol/L; aprotinin, 0.4 mg/ml; soybean trypsin inhibitor, 0.5 mg/ml; leupeptin, 0.2 mmol/L; phenylmethyl sulfonyl fluoride, 0.2 mmol/L). Cells were lysed by passage through a 20-gauge needle and centrifuged (15000 rpm, 5 minutes, 4° C) to remove the membrane fraction. Protein concentration was determined with bovine serum albumin as a standard. Aliquots of the protein lysate (100 μ g) were loaded onto a 10% polyacrylamide gel and separated by electrophoresis. Proteins were then transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Clinical Div., Hercules, Calif.) by electrotransfer. Membranes were soaked in NTE-NP40 buffer (Tris-HCl pH 7.5, 12 gm; NaCl, 17.5 gm; Na-ethylenediaminetetraacetic acid, 1.7 gm; NP40, 2 ml/L) containing 3% bovine serum albumin for 18 hours at 4° C. A polyclonal rabbit antibody (1:1000 dilution) was added and incubated for 24 hours at 4° C. Membranes were washed with NTE-NP40 and subsequently incubated with ^{125}I -protein-A (1 mCi/ml; Amersham Life Science) for 1 hour at room temperature. Membranes were then washed twice with NTE-NP40, and radioactivity was detected by autoradiography.

RESULTS

As we have found previously,⁴ 8-Br-cAMP inhibited the growth of HCT116 cells and stimulated the growth of LoVo cells (Fig. 1). After 4 days of treatment, 8-Br-cAMP decreased the total cell number of HCT116 cells by 29% compared with control and increased the total cell number of LoVo by 24% compared with control. The basal rate of growth of HCT116 cells under low-serum conditions was not altered by the addition of $R_{II\beta}$ ASO (Fig. 2). However, if HCT116 cells had been pretreated with the $R_{II\beta}$ ASO for 4 days, the growth response after treatment with 8-Br-cAMP was reversed (Fig. 3). The growth of HCT116 cells that had been pretreated with the $R_{II\beta}$ ASO was stimulated by 8-Br-cAMP with a resultant increase in cell number by 19% after 4 days. Similarly, the effect of 8-Br-cAMP on the rate of DNA synthesis of $R_{II\beta}$ ASO-pretreated HCT116 cells as measured by tritiated thymidine incorporation was also reversed (Fig. 4).

Examination of the mRNA level encoding the $R_{II\beta}$ subunit in HCT116 cells was examined by Northern blotting. After $R_{II\beta}$ ASO treatment for 18 hours, no alteration was detected in the level of mRNA for $R_{II\beta}$ (Fig. 5, A). Densitometric analysis of the autoradiograph, with the constitutive gene cyclophilin as a control, confirmed the observation that the antisense treatment did not alter the mRNA level (Fig. 5, B). However, determination of the protein level of $R_{II\beta}$ by Western blotting showed that $R_{II\beta}$ ASO treatment significantly lowered the PKA regulatory subunit content of HCT116 cells

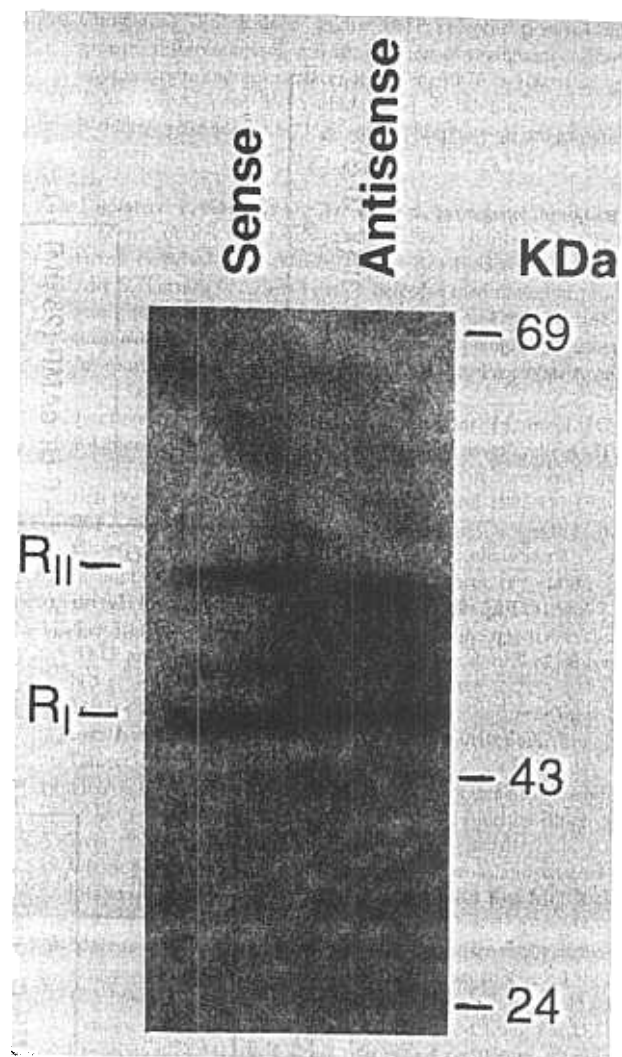


Fig. 6. Western blot of HCT116 cells for $R_{I\alpha}$ and $R_{II\beta}$ proteins after treatment with $R_{II\beta}$ sense or ASO (15 μ mol/L, 48 hours).

(Fig. 6). The $R_{II\beta}$ subunit is a 52 kd protein, and intracellular content was significantly decreased after 48 hours of treatment with the $R_{II\beta}$ ASO when compared with the sense-treated cells. The $R_{I\alpha}$ subunit is 48 kd in size and is simultaneously detected by the antibody and shown for reference. The total intracellular content of this subunit was not altered by $R_{II\beta}$ ASO treatment.

When LoVo cells were pretreated with the $R_{I\alpha}$ ASO for 4 days, the effect of 8-Br-cAMP on growth was reversed (Fig. 7). Total number of cells pretreated with the $R_{I\alpha}$ ASO and then exposed to 8-Br-cAMP was decreased by 15% compared with control; the growth of sense-treated cells was stimulated, leading to a 22% increase in cell number. The rate of DNA synthesis paralleled this reversal of the effect on growth mediated by pretreatment with the $R_{I\alpha}$ ASO as seen by a 46% decrease in tritiated thymidine incorporation after 8-Br-

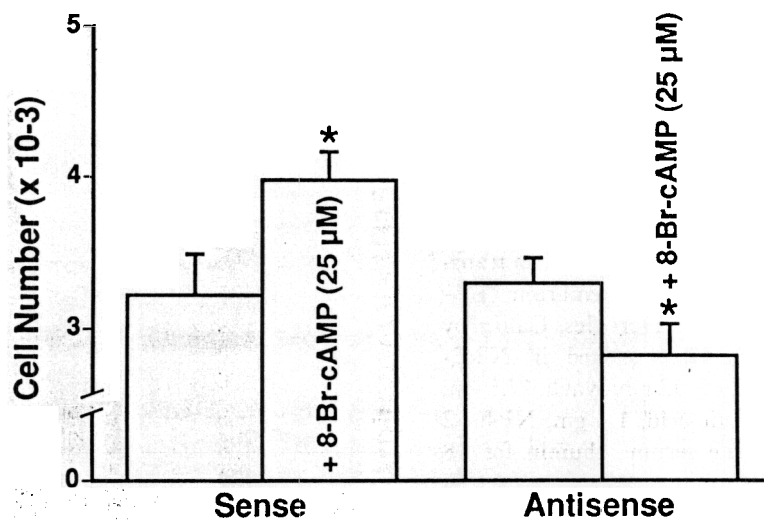


Fig. 7. Effect of 8-Br-cAMP (25 μmol/L) on growth of LoVo cells pretreated with either $R_{1\alpha}$ sense or ASO (4-day pretreatment, 15 μmol/L). * $p < 0.05$ versus without 8-Br-cAMP.

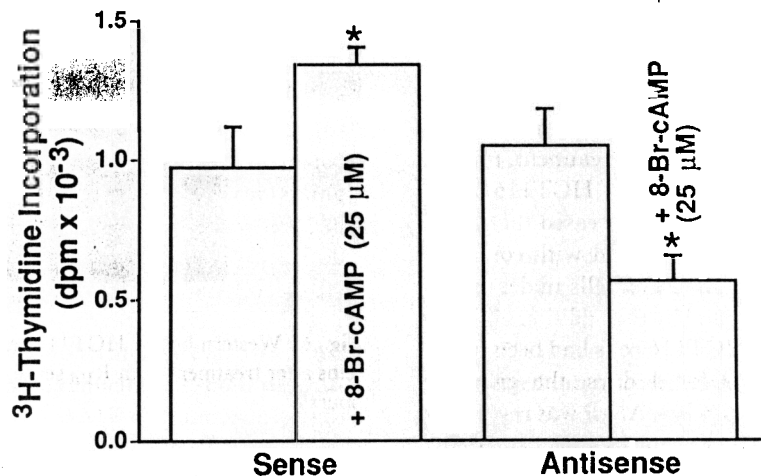


Fig. 8. Effect of 8-Br-cAMP (25 μmol/L) on tritiated thymidine incorporation in LoVo cells pretreated with either $R_{1\alpha}$ sense or antisense oligonucleotides (4-day pretreatment, 15 μmol/L). * $p < 0.05$ versus without 8-Br-cAMP.

cAMP treatment (Fig. 8). Treatment with the $R_{1\alpha}$ ASO did not alter the rate of growth of LoVo cells after 4 days of treatment (data not shown).

DISCUSSION

ASO are effective means of specifically altering protein levels by inhibition of translation of select mRNA; specific cellular functions may thus be altered. Our findings show that ASO targeted against the inhibitory isoform of PKA ($R_{11\beta}$) not only abolishes the inhibition of growth of HCT116 human colon cancer cells medi-

ated by 8-Br-cAMP but also reverses the effect of this agent on growth and leads to stimulation of growth. The analysis of the mRNA encoding the $R_{11\beta}$ protein indicated that the effect of the ASO was not likely by digestion of the mRNA but was more likely to have been caused by ribosomal competition. The analysis of the protein level by Western blotting shows that the $R_{11\beta}$ protein level is significantly decreased after treatment with the ASO. Conversely, the growth of LoVo cells is normally stimulated by activation of the cAMP pathway; but using ASO targeted against the $R_{1\alpha}$ subunit,

the growth response to 8-Br-cAMP is reversed, leading to inhibition of growth after treatment with 8-Br-cAMP.

Colon cancers are a heterogeneous group of neoplasms, and the development of a single therapeutic modality is difficult. The growth of various colon cancer cells may be either stimulated or inhibited after activation of the cAMP pathway. We must understand the central mediators of signal transduction that control the growth of cancer cell lines so that we may specifically target these mediators by new methods of treatment, such as gene therapy. ASO targeted against the stimulatory regulatory subunit of PKA ($R_{1\alpha}$) inhibit growth of cells that are stimulated to grow by activation of the cAMP pathway. Therefore altering the intracellular PKA content by gene therapy techniques may lead to a broadly applicable means of treatment of colon cancer.^{23, 24}

Several early trials of gene therapy have recently been initiated as a means to alter the basic cellular nature of both normal and neoplastic cells.²⁵ With new techniques of gene therapy available to introduce foreign genes into a specific population of cells, it is possible to alter the basic characteristics of cells, such as growth.²³ A clear understanding of the mechanisms involved in growth are required before targeting a specific cellular protein. Our findings show that alteration of signal transduction pathways, such as the regulatory subunits of PKA, lead to significant changes in cell growth and may contribute to a new therapeutic strategy for colon cancer.

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DISCUSSION

Dr. Raymond J. Joehl (Chicago, Ill.). Do you have an estimate of how many cells from both cell lines were actually "infected" with your sense and antisense oligonucleotides to show the efficacy of your treatment? You have shown inhibition of growth and a few other measures of degree of infection.

Second, have you looked at this particular enzyme and therapy for both sense and antisense infection in an in vivo model?

Dr. Bold. In answer to your first question, we have not studied what percentage of cells take up the antisense or small DNA fragments, although this has been examined in a variety of cells. I cannot give a specific number for these cells because they have not really been studied, but most other cells do take up small segments of DNA readily, and almost 100% incorporation of oligonucleotides is possible.

In answer to your second question, we have not done any in vivo studies, although we are in the process of generating cell lines that have been manipulated through vectors to alter the regulatory subunits. We are in the process of examining that.

Dr. Eric D. Whitman (St. Louis, Mo.). We have done some

work with antisense, and there are many issues concerning the breakdown of the oligonucleotides, especially in vivo. Many synthetic analogs, specifically, thioate esters, have been made. Although they last longer, the problem is that they may not inhibit or stimulate in the nucleus or next to the nucleus as well as you would like. Have you looked at any synthetic analogs in antisense?

Dr. Bold. No, we have not. You have identified some of the problems with antisense experiments. This type of experiment is useful in determining a potential target in initial in vitro studies before the use of other methods of gene therapy to alter that target for further in vivo studies. But we have not examined any other modifications, such as phosphothioate esters, to look at the effect of those on the antisense-mediated growth regulation.