Antisense Protein Kinase A RIα Inhibits 7,12-Dimethylbenz(*a*)anthracene-Induction of Mammary Cancer: Blockade at the Initial Phase of Carcinogenesis

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ABSTRACT

Purpose: **There are two types of cyclic AMP (cAMP)-dependent protein kinase (PKA), type I (PKA-I) and type II (PKA-II), which share a common catalytic (C) subunit but contain distinct regulatory (R) subunits, RI *versus* RII, respectively. Evidence suggests that increased expression of PKA-I and its regulatory subunit (RIα) correlates with tumorigenesis and tumor growth. We investigated the effect of sequence-specific inhibition of RIα gene expression at the initial phase of 7,12-dimethylbenz(αa)anthracene (DMBA)-induced mammary carcinogenesis.**

Experimental Design: **Antisense RIα oligodeoxynucleotide (ODN) targeted against PKA RIα was administered (0.1 mg/day/rat, i.p.) 1 day before DMBA intubation and during the first 9 days post-DMBA intubation to determine the anticarcinogenic effects.**

Results: **Antisense RIα, in a sequence-specific manner, inhibited the tumor production. At 90 days after DMBA intubation, untreated controls and RIα-antisense-treated rats exhibited an average mean number of tumors per rat of 4.2 and 1.8, respectively, and 90% of control and 45% of antisense-treated animals had tumors. The antisense also delayed the first tumor appearance. An increase in RIα and PKA-I levels in the mammary gland and liver preceded DMBA-induced tumor production, and antisense down-regulation of RIα restored normal levels of PKA-I and PKA-II in these tissues. Antisense RIα in the liver induced the phase II enzymes, glutathione *S*-transferase and quinone oxidoreductase, c-fos protein, and activator protein 1 (AP-1)- and cAMP response element (CRE)-directed transcription. In the mammary glands, antisense RIα promoted DNA repair processes. In contrast, the CRE transcription-factor decoy could not mimic these effects of antisense RIα.**

Conclusions: **The results demonstrate that RIα antisense produces dual anticarcinogenic effects: (*a*) increasing DMBA detoxification in the liver by increasing phase II enzyme activities, increasing CRE-binding-protein phosphorylation and enhancing CRE- and Ap-1-directed transcription; and (*b*) activating DNA repair processes in the mammary gland by down-regulating PKA-I.**

INTRODUCTION

Cancer is a potentially preventable disease. Although causative factors for the genesis of human malignancies have been recognized for many years, the identification of compounds that prevent cancer is much more recent (1–3). A surprising variety of chemicals can protect against neoplastic, mutagenic, and other toxic effects of many types of carcinogens (1, 4). A striking feature of the chemoprotectors is that they belong to unrelated chemical classes such as substituted phenols, azo dyes, coumarins, sulfur compounds (disulfiram, isothiocyanates, carbone disulfide), flavones, indoles, retinoids, tocopherols, selenium compounds, and nucleotides (5, 6).

Oligodeoxynucleotides (ODNs) have been described as naturally occurring biological inhibitors of gene expression in prokaryotes and eukaryotes (7, 8). Antisense ODNs are short synthetic nucleotide sequences formulated to be complementary to a specific gene or RNA message. Through binding of these ODNs to a target DNA or mRNA sequence, transcription or translation of the gene can be selectively blocked, and the disease process generated by that gene can be halted (9–12).

Another approach to altering gene transcription by ODNs is inhibiting transactivation of specific transcription factors by introducing double-stranded ODNs into cells as decoy *cis*-elements to bind the factors and alter gene expression *in vivo* (13, 14). Both approaches were used to affect cyclic AMP (cAMP) signaling in the cell (15, 16).

Varying the ratio of two isoforms of cAMP-dependent protein kinase, PKA-I and PKA-II, which are distinguished by different regulatory (R) subunits (RI *versus* RII), has been linked to cell growth and differentiation. Increased expression of RI/PKA-I correlates with active cell growth and cell transformation, whereas a decrease in RI/PKA-I and an increase in RII/PKA-II are related to growth inhibition and differentiation-maturation (17).

Recently, it was shown that modulating the expression of these PKA isozymes might offer a novel approach to regulating cell division and developing antitumor drugs (18–20). It was demonstrated that treating different tumor cell lines with antisense ODNs targeted against the RIα subunit of PKA caused inhibition of cell proliferation *in vitro* (21, 22) and tumor growth *in vivo* (15, 23, 24). A single administration of RIα antisense to nude mice bearing LS-174T human colon carcinomas resulted in an almost complete suppression of tumor growth for 7 days (15). It was also shown that the synthetic, single-stranded, cAMP response element (CRE) palindromic ODN, which self

Received 10/21/03; revised 2/9/04; accepted 4/5/04.
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hybridizes to form a hair-pin/duplex, can compete with CRE enhancers to bind transcription factors and, specifically, to interfere with CRE- and activator protein 1 (AP-1)-directed transcription *in vivo* (16). These ODNs restrained tumor cell proliferation without affecting the growth of noncancerous cells (16).

Studies from this laboratory have previously shown that a cAMP analog, *N*6,02′-dibutyryl cAMP, administered to rats 1 day before and during 90 days after 7,12-dimethylbenz(*a*)anthracene (DMBA) intubation resulted in a marked reduction in tumor production (6).

In the present study, we investigated the ability of antisense RIα and CRE-decoy ODNs to prevent and inhibit DMBA mammary carcinogenesis. To elucidate the mechanism of chemoprevention by these ODNs and to identify the stages(s) of carcinogenesis (initiation or promotion) affected by these compounds, we first investigated the effect of these ODNs on the early stages of DMBA carcinogenesis. The bioactivation of DMBA to electrophilic metabolites that interact with nucleophilic regions of target-cell DNA is considered an important prerequisite for the initiation of DMBA-induced tumorigenesis, and inactivation/detoxification of DMBA through the induction of electrophile-processing phase II enzymes is the dominant mechanism of action of naturally occurring carcinogenesis inhibitors (25–27). In this study, we investigated whether the ODNs described above could serve as anticarcinogens by altering the metabolic fate of a DMBA carcinogen through modulation of the activities of phase II drug-metabolizing enzymes, or whether they work at the level of DNA-repair processes (28–31).

MATERIALS AND METHODS

Chemicals. DMBA was obtained from Eastman Organic Chemicals (Rochester, NY). Polyclonal antibody for the PKA catalytic subunit and antibody for c-*fos* were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies for CRE-binding protein (CREB) and phospho-CREB were obtained from Upstate Cell Signaling Solutions (Charlottesville, VA).

Mixed-Backbone ODNs. ODNs used in the study are second-generation RNA/DNA mixed backbone (32) RIα antisense (HYBO165), 5′-[GCGU]GCCTCCTCAC[UGGC]-3′ targeted against the NH_2-terminal 8–13 codons of human PKA RIα (15); this ODN is a phosphorothioate ODN that contains segments of four 2′-*O*-methyl-oligoribonucleosides (RNA) at the 5′ and 3′ ends (bracketed); RNA/DNA mixed backbone antisense against rat RIα, 5′-[CCGC]TCTTCCTCAC[UGGC]-3′ (HYB0693); control ODNs, HYBO295, 5′-[GCAU]GCTTC-CACAC[AGGC]-3′, a four-base mismatched (underlined) ODN to HYBO165; and HYBO674, 5′-[NNNN]NNNNNNN[NNNN]-3′, a random-sequence ODN with bracketed segments representing 2′-*O*-methyl-RNA, and with n = A, T, C, or G. All interlinkages are phosphorothioate and the 24-mer CRE palindrome, 5′-TGACGTCATGACGTCATGACGTCA-3′, phosphorothioate ODN (16). These ODNs were kindly provided by Dr. Sudir Agrawal (Hybridon, Inc., Cambridge, MA).

Tumor-Induction Experiments. Virgin female Sprague-Dawley rats obtained from Taconic Farms (Germantown, NY) were randomly assigned to seven groups (35 rats/group) and were housed in a room artificially illuminated for 12 h each day and maintained at a temperature of 24 ± 1°C. All of the animals received Purina laboratory chow and tap water *ad libitum*. At 50 days of age, the rats received a single intragastric feeding of 20 mg of DMBA dissolved in 1 ml of sesame oil. Control animals received a single intragastric intubation of 1 ml of sesame oil only. Antisense RIα ODN, CRE-decoy ODN, or control ODNs were injected i.p. at concentrations of 0.1 mg/0.2 ml saline/rat at day 1 before DMBA intubation and during the first 9 days after DMBA intubation. Beginning 4 weeks after DMBA intubation, the rats were examined (palpated) twice weekly for mammary tumors and weighed monthly. Animals were killed by decapitation when moribund or 100 days after DMBA administration.

Preparation of Tissue Extracts. All of the procedures were performed at 0–4°C. Tissues were weighed, minced, and homogenized in a Teflon glass homogenizer with five volumes of buffer containing 20 mM Tris/HCl (pH 7.4), 100 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 5 mM $MgCl_2$, and proteinase inhibitor cocktail Set I (Calbiochem, La Jolla, CA). The homogenates were centrifuged in an Eppendorf microfuge for 10 min at 4°C, and the resulting supernatants were used as tissue extracts. Protein concentration was determined by the method of Lowry and Passonneau (33) using BSA as standard.

Immunoprecipitation of PKA Regulatory Subunits. The identification of PKA regulatory subunits (R) was carried out by photo-affinity labeling of protein samples with 8-*N*3-[^{32}P]cAMP followed by immunoprecipitation with the polyclonal antibodies (34) against RIα, RIIα, and RIIβ subunits of PKA. Then the solubilized immunoprecipitates were resolved by SDS/PAGE, transferred to nitrocellulose sheets, and exposed to X-ray film overnight for identification as described previously (15).

DEAE-Cellulose Chromatography. All of the procedures were performed at 0–4°C. The tissues were weighed, minced, and washed twice with ice-cold isotonic NaCl/P_i; were suspended in three volumes of 10 mM Tris/HCl (pH 7.1), containing 1 mM EDTA, 0.1 mM DTT, and protease inhibitor Cocktail Set 1 (Calbiochem) and kept on ice for 20 min; and were homogenized (70 strokes) with a Dounce homogenizer and centrifuged at 10,000 × *g* for 10 min. The resulting supernatants were used as the cell extracts for chromatography. The DEAE column (1 × 10) was equilibrated with 10 mM Tris/HCl (pH 7.1), containing 1 mM EDTA, 0.1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride. Tissue extracts (10 mg protein) were loaded onto the column and the column was washed with 30 ml of buffer. The column was developed with a 0–350-mM NaCl gradient in 10 mM Tris/HCl (pH 7.1), 1 mM EDTA, 0.1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride at a flow rate of 15 ml/h. Two-ml fractions were collected on ice and assayed for protein kinase activity.

Assay of Quinone Reductase Activity. The method of Prochaska and Santamaria (35) was used to assay quinone reductase activity. Briefly, before the samples were assayed, a cocktail containing 25 mM Tris-HCl (pH 7.4), 0.67 mg/ml BSA, 5 μM flavin adenine dinucleotide, 30 μM NADP+, 1 mM glucose-6-phosphate dehydrogenase, and 0.3 mg/ml MTT (3-[4,5-dimethylthiazo-2-yl]-2,5-diphenyltetrazolium bromide) was prepared; and, shortly before the assay began, 25 μl of 50 mM

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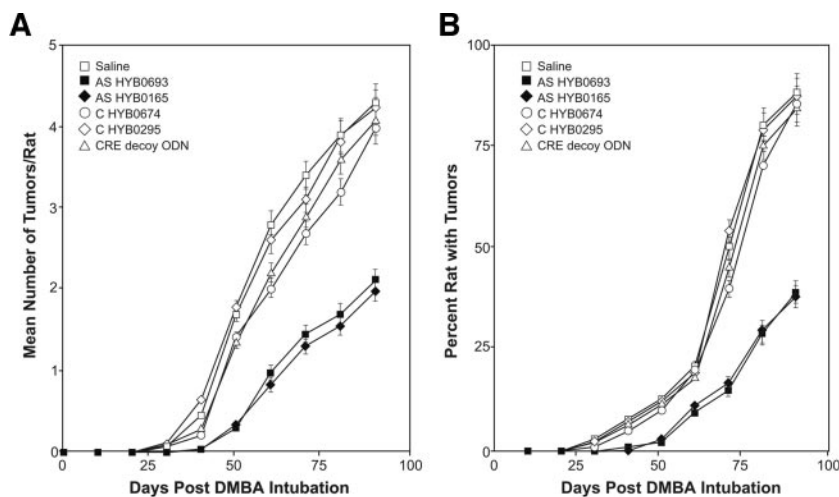

Fig. 1 Effect of antisense (*AS*) regulatory subunit RIα, cyclic AMP response element-decoy oligonucleotide (*CRE decoy ODN*), and control oligonucleotides (*C HYB0674* and *C HYB0295*) on the 7,12-dimethylbenz(*a*)anthracene (DMBA)-induction of mammary tumors. DMBA was administered by intubation to six groups (each composed of 50 rats) of 50-day-old rats on day 0. The animals received ODNs (*AS HYB0693* and *AS HYB0165*) starting at day minus 1 (day −1) before DMBA intubation and, thereafter, during 9 days post-DMBA intubation, as described in "Materials and Methods." Induction of mammary tumors by DMBA was monitored as described in "Materials and Methods." *A,* average number of tumors per rat. *B,* percentage of rats with tumors.

menadione, dissolved in acetonitrile, was mixed with 25 ml of assay cocktail. Tissue extracts were added to 200 μl of complete reaction mixture, blue color developed, and the reaction was arrested after 5 min by the addition of 50 μl of solution containing 0.3 mM dicumarol in 0.5% DMSO and 5 mM potassium phosphate (pH 7.4). Absorbency was read at 610 nm.

Assay of Glutathione *S*-Transferase. The conjugation of allyl alcohol (2-propen-1-ol) with glutamine was measured by the disappearance of free sulfhydryl groups using 5,5′-dithiobis (2-nitro benzoate) (36). In addition to enzyme, the assay mixture included 0.1 M potassium phosphate (pH 6.5), 10 mM allyl alcohol, and 1 mM reduced glutathione.

Electrophoretic Mobility Shift Assay. Nuclear extracts were prepared by the method of Digman *et al.* (37). Electrophoretic mobility shift assay was performed by the method of Fried and Crothers (38). Briefly, nuclear extracts (5 μg of protein) were preincubated with 2 μg of poly(dI-dC)·poly(dI-dC) [poly(deoxyinosinic-deoxycytidylic acid)·poly(deoxyinosinic-deoxycytidylic acid], 0.3 mM DTT, and reaction buffer [12 mM Tris (pH 7.9), 2 mM $MgCl_2$, 60 mM KCl, 0.12 mM EDTA, and 12% glycerol] for 30 min at 4°C. ^{32}P-ODNs [double-stranded ODNs with one copy of CRE, 5′-AGAGATTGCCTGACGTCAGAGAGCTAG-3′; AP-1, 5′-CGCTTGATGAGTCAGCCGGAA-3′ (Promega Corp., Madison, WI)] were then added, and the reaction mixtures were incubated for 10 min at 37°C. The reaction mixtures were separated on a 4% nondenaturing polyacrylamide gel at 200 V for 2 h. The gels were dried and autoradiographed.

DNA Repair Assay. DNA repair assay was performed as described previously (39) using a 148-bp plasmid DNA probe derived from the bacterial CAT gene, which was end labeled and treated with cisplatin for 18–24 h, followed by incubation with nuclear extracts. DNA binding was performed in a 15–20-μl volume. Complexes for DNA-protein interaction were resolved on nondenaturing 6% polyacrylamide gel and then were dried and exposed to autoradiographic film. Competitor DNA consisting of supercoiled plasmid (pSV2NEO) was exposed to the DNA-damaging conditions as described and then purified by ethanol precipitation (31).

Western Blot Analysis. Proteins were separated on 12% SDS-polyacrylamide gel, and separated proteins were transferred onto nitrocellulose membrane using a semidry blotting system. Anti-CREB, phosphor-CREB, and c-fos antibodies, and horseradish peroxidase-conjugated antirabbit antibody (Amersham Pharmacia Biotech, Sunnyvale, CA) were used as primary and secondary antibodies, respectively. Immunodetection was performed using an enhanced chemiluminescence method (Amersham Pharmacia Biotech).

Statistics. Statistical significance was assessed when appropriate, with a paired Student's *t* test, with a significant difference taken as $P < 0.05$.

RESULTS

Inhibition of Mammary Tumor Production. The time course of tumor production in rats that received DMBA only and those that received DMBA plus antisense RIα or CRE-decoy ODN is shown in Fig. 1. At 90 days post-DMBA intubation, control rats had an average of 4.2 tumors per rat, whereas rats that received antisense RIα treatment had an average of 1.8 tumors per rat (Fig. 1*A*). In contrast, CRE-decoy ODN and control antisense ODN (HYB0674, a random-sequence RNA/DNA ODN; HYBO295, 4-bases mismatched ODN) produced no inhibitory effects on tumor formation (Fig. 1*A*). In rats that received DMBA only, the first tumors were found 30 days after DMBA intubation, and almost 80% of the rats had mammary tumors by 70 days post-DMBA intubation (Fig. 1*B*). By contrast, in the antisense-treated groups, tumor induction was markedly delayed (15 days; Fig. 1), and at day 90 (end of experiment), only one-third as many tumors appeared in antisense-treated rats as in control rats (DMBA only; Fig. 1*B*). The CRE-decoy and control antisense ODNs had no effect on tumor production (Fig. 1). ODN treatment produced no toxic effect as evaluated by hematocrit, body weight, and food consumption, and did not affect the animals' estrous cycles.

Changes in PKA Regulatory Subunit Levels. We examined whether the RIα antisense could specifically affect the pattern of PKA subunits in mammary gland and liver. Although liver is not a target organ for DMBA-induced carcinogenesis, proximate and ultimate DMBA metabolites are formed in liver

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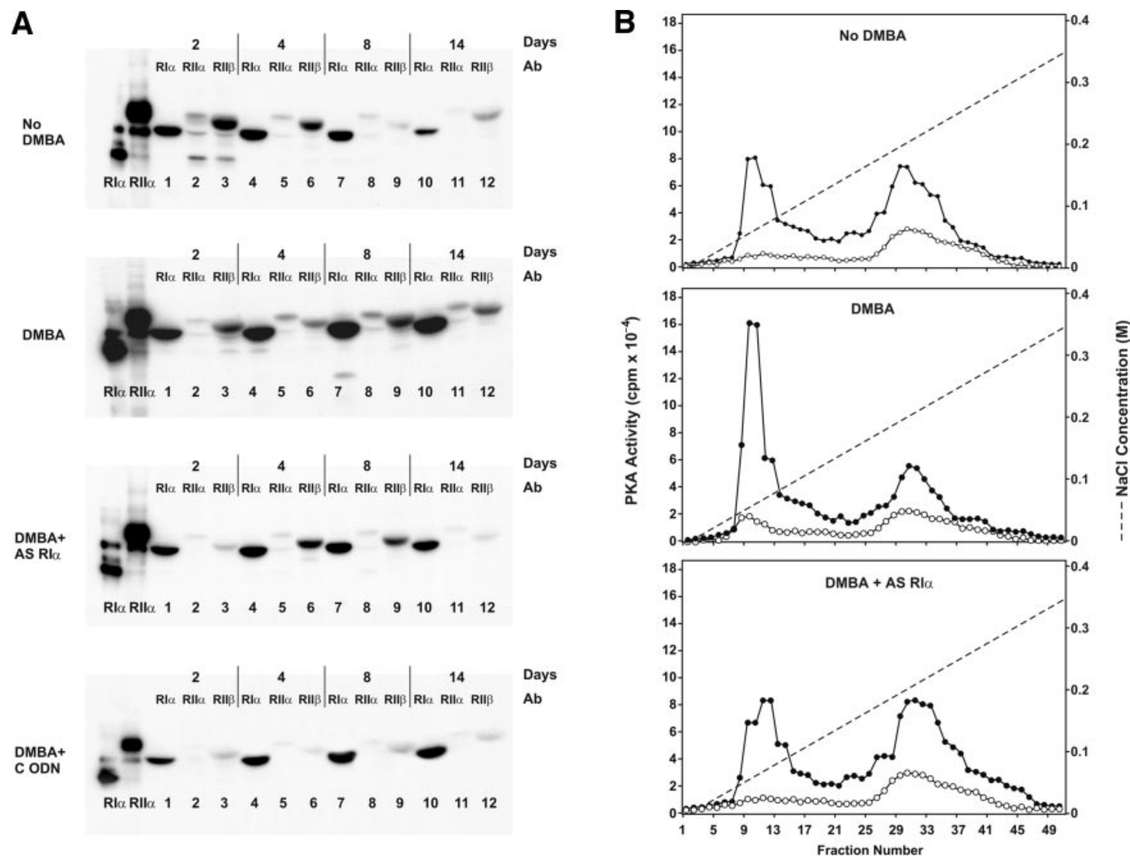

Fig. 2 Determination of protein kinase A (PKA) regulatory (R) subunits in the mammary gland (*A*) and PKA type I (PKA-I) and PKA-II determination in the liver (*B*), treated with no 7,12-dimethylbenz(*a*)anthracene (*No DMBA*; control); DMBA alone (*DMBA*); DMBA plus antisense RIα (*DMBA+AS RIα*; HYB0165), and DMBA plus control oligonucleotide (*DMBA+C ODN*; HYB0295). Animals were sacrificed at days 2, 4, 8, and 14 after gavage of DMBA and control (*No DMBA*) rats at respective time points, and extracts were prepared from mammary glands and liver. *A,* the PKA subunit level measurement by photo-affinity labeling, followed by immunoprecipitation and SDS-PAGE, as described in "Materials and Methods." *Lane RIα,* standard RIα from rabbit skeletal muscle, M_r 48,000 RI (sigma); *Lane RIIα*, standard RIIα from bovine heart, the Mr 56,000 RII (sigma). *Ab,* antibody. Data represent one of two separate experiments that gave similar results. *B,* PKA-I and PKA-II determination in the liver treated with *No DMBA, DMBA,* and *DMBA + AS RIα* (HYB0165) at day 4 post-DMBA-intubation, by DEAE-column chromatography (see "Materials and Methods"). ●, presence of cyclic AMP (*+cAMP*); ○, absence of cyclic AMP (*−cAMP*). Data represent one of two independent experiments that gave similar results.

cells that can be transported to mammary glands, resulting in DMBA-DNA adducts (40).

At days 2, 4, 8, and 14 after DMBA treatment, animals were sacrificed, and mammary glands and liver tissues were analyzed for the amounts of regulatory (R) and catalytic (C) subunits of PKA. The tissue extracts were photoaffinity labels with 8-N_3-[^{32}P]cAMP and were immunoprecipitated with antibodies (34) against RIα, RIIα, and RIIβ, and the immunoprecipitated proteins were resolved by SDS-PAGE (see "Materials and Methods"). In normal mammary glands of rats at age 52 days, RIα and RIIβ subunits were expressed as major regulatory subunits of PKA (Fig. 2*A, no DMBA, day 2*). However, the amounts and proportion of the two subunits changed markedly over the next 2 weeks, the age at which development and maturation processes occur rapidly in rats (Fig. 2*A, no DMBA, days 2–14*). DMBA treatment elicited a marked increase in RIα and a decrease in RIIβ (Fig. 2*A, DMBA*). Antisense RIα (HYB0165) treatment reversed these DMBA-induced changes in RIα and RIIβ levels back to the levels of DMBA-untreated normal mammary glands (Fig. 2*A, DMBA + antisense RIα*). These antisense effects were sequence-specific because the control ODNs could not mimic the antisense effect (Fig. 2*A, DMBA + control ODN*).

Using scanning densitometry and "NIH Image 1.63f" software, we quantified the bands of Fig. 2*A* and we determined the ratio of band intensity between RIIβ and RIα. As shown in Table 1, the ratio of RIIβ:RIα band intensity sharply decreased as early as 2 days post-DMBA treatment and, thereafter, progressively decreased during the 14 days post-DMBA intubation. Antisense RIα treatment elicited a time-dependent reversal of the DMBA-induced changes in RIIβ:RIα ratios maintaining the RIIβ:RIα ratio close to that of DMBA-untreated mammary glands (Table 1). By contrast, the control ODN had no effect on the DMBA-induced changes in the RIIβ:RIα ratios (Table 1), supporting the sequence-specific effects of antisense RIα. These results were reproduced by the Western blotting analysis of the

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Table 1 Antisense regulatory (R) subunit RIα reverses DMBA[a]-induced changes in the RIIβ:RIα ratio in mammary glands

The band intensities of RIα and RIIβ in Fig. *2A* were quantified (using scanning densitometry and "NIH Image 1.63f" software), and the data were presented as a ratio of RIIβ:RIα. The data represents one of two independent experiments that gave similar results.

Treatments	RIIβ:RIα ratio			
	2[b]	4	8	14
No DMBA	1.018	0.846	0.571	0.833
DMBA	0.423	0.253	0.264	0.126
DMBA + Antisense RIα[c]	0.571	0.718	0.792	0.662
DMBA + Control ODN[d]	0.367	0.268	0.298	0.139

[a] DMBA, 7,12-dimethylbenz(*a*)anthracene; ODN, oligonucleotide.
[b] Days post-DMBA intubation.
[c] Antisense RIα = HYB0165.
[d] Control ODN = HYB0295.

regulatory subunits, and the PKA catalytic subunit was unchanged in all of the experiments.

Liver tissue is relatively rich in RIα. Treatment with DMBA leads to additional increases in RIα levels in liver tissue. RIα levels markedly decreased in the livers of animals treated with DMBA plus antisense RIα. Changing RIα levels were reflected in column chromatography data of the liver PKA-I and PKA-II levels and in the decrease in PKA-II activity. Fig. 2*B* showed a marked increase in PKA-I at day 4 post-DMBA intubation and the reversal of this DMBA effect by antisense RIα treatment.

Activity of Glutathione *S*-Transferase and NAD(P)H: Quinone Oxidoreductase. As described above, our results indicate that antisense RIα inhibits DMBA carcinogenesis. A large body of evidence suggests that the induction of phase II enzymes is the dominant mechanism for the most synthetic and naturally occurring chemopreventives, (25–29, 41), and it is clear that monitoring phase II enzyme induction is a convenient way to screen for anticarcinogenic activity (29, 42, 43). We, therefore, examined whether the antisense RIα-induced changes in the levels of PKA regulatory subunits were causatively related to the induction of electrophile-processing phase II enzymes (intragastric glutathione *S*-transferase and quinone oxidoreductase).

The activity of these two enzymes was assayed in mammary gland and liver. All changes in enzyme levels are presented in the liver, however, because (*a*) liver enzymes provide a much more sensitive indication of a distant neoplasm in rats (44); (*b*) changes in rat liver enzymes are reported to be much more reproducible and reliable than changes in mammary enzymes during DMBA mammary carcinogenesis (45); and (*c*) metabolic activation and detoxification of DMBA *in vivo* are known to occur primarily in the liver.

Fig. 3, *A* and *B,* shows the time course of glutathione *S*-transferase and quinone oxidoreductase activity, respectively, in the livers of normal, DMBA-treated and DMBA plus RIα antisense (HYB0165)-, CRE-decoy-, or control ODN-treated rats. Immediately after DMBA intubation, levels of these enzymes became elevated, as expected from a host defense mechanism, but quickly decreased. But the RIα antisense treatment sustained this increase and maintained it at elevated levels for longer periods of time. The CRE-decoy and control ODN could not mimic these effects of antisense RIα (Fig. 3, *A* and *B*).

DMBA Degradation and Clearance. Urine analysis revealed a marked increase in low-molecular-weight aromatic compounds (possible products of DMBA degradation) after antisense RIα (HYB0165) treatment of rats that received DMBA (Fig. 3*C*). The presence of these compounds in urine was determined by the protein assay method of Lowry and Passonneau (33), which is based on the evaluation of aromatic base content in protein molecules. The complete disappearance of detectable aromatic compounds from urine after dialysis (data not shown) indicates that these substances have low molecular weight and are products of DMBA degradation. To evaluate the kidney function of rats under the experimental conditions, we

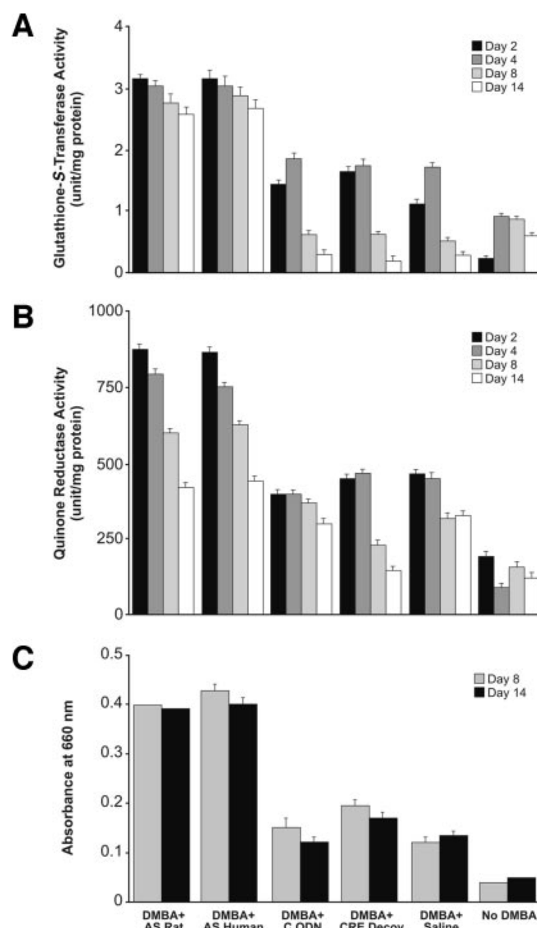

Fig. 3 Effect of 7,12-dimethylbenz(*a*)anthracene (DMBA) and antisense regulatory (R) subunits RIα on the phase II enzyme activities and DMBA clearance. *A,* time course of glutathione *S*-transferase. *B,* time course of quinone reductase. Enzyme assays were performed as described in "Materials and Methods." Specific activity of quinone reductase, nanomoles of reduced MTT (3-[4,5-dimethylthiazo-2-yl]-2,5-diphenyltetrazolium bromide) per mg of protein in 1 min. Specific activity of glutathione *S*-transferase, micromoles of free SH-groups that disappeared in reaction per mg of protein in 1 min. *C,* DMBA clearance. Levels of aromatic compounds in urine of rats after DMBA and antisense RIα treatment were assayed by the method of Lowry and Passonneau (33) at days 8 and 14 after DMBA gavage.

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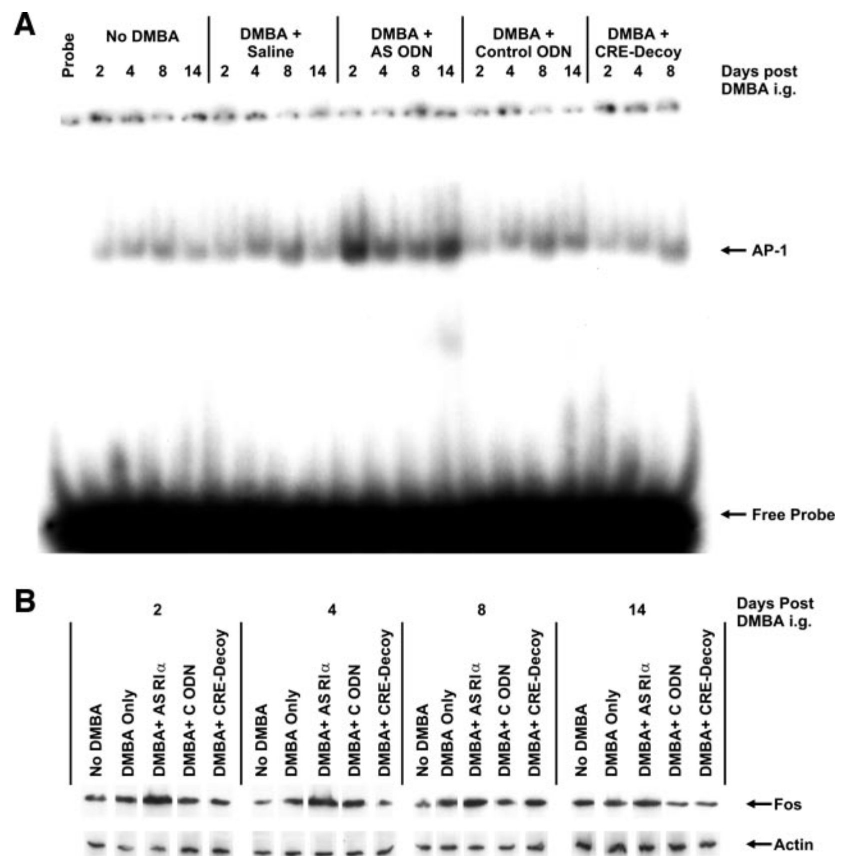

Fig. 4 Activation of activator protein 1 (AP-1)-directed transcription. *A,* induction of AP-1-binding activity by 7,12-dimethylbenz(*a*)anthracene (DMBA) and antisense regulatory (R) subunits RIα (HYB0165) in liver. Nuclear extracts were prepared and analyzed by electromobility-shift assay as described in "Materials and Methods" using 10 μg of nuclear protein samples and ^{32}P-labeled oligonucleotide (*ODN;* double-stranded AP-1). *B,* Western blot analysis of *c-fos* levels (*Fos*) in liver. At indicated times, rats were sacrificed and liver tissue was prepared as described in "Materials and Methods." *Actin* determination (polyclonal antibody; Santa Cruz Biotechnology) is shown for loading control. Data represent one of three independent experiments that gave similar results. *AS,* antisense; *CRE-Decoy,* cyclic AMP response element-decoy oligonucleotide (*CRE decoy ODN*); *i.g.,* intragastric; *C ODN,* control ODN.

assayed creatinine content in urine and found that it did not change in the course of the experiment and was maintained at normal levels. These results indicate that antisense RIα facilitated the DMBA clearance in the urine.

Induction of AP-1-Binding Activity and c-fos Level in Liver. A regulatory element, electrophile responsive element (EpRE), was found to be responsible for the induction of mouse glutathione *S*-transferase Ya and quinone oxidoreductase gene expression by a variety of chemical agents such as planar aromatic hydrocarbons, diphenols, phorbolester, phenobarbital, and electrophilic compounds (46). The electrophile responsive element (EpRE) is composed of two adjacent AP-1-like binding sites and was recently found to be activated by Fos/Jun heterodimeric complex (46).

To explore the effect of antisense RIα in phase II enzyme regulation, we examined the activation of AP-1 transcription in liver after DMBA and antisense RIα treatment (Fig. 4*A*). Nuclear extracts prepared from control and treated rat livers were analyzed by mobility-shift assay for AP-1 DNA binding activity, using ^{32}P-labeled consensus-sequence AP-1 ODN as a probe. Fig. 4*A* shows that, compared to nuclear extracts from untreated animals, DMBA exposure resulted in a slight increase of AP-1 binding activity, probably because of the host defense mechanism. AP-1 binding (Fig. 4*A*) activity of a normal liver is quite high compared to other organs because of the specific detoxification function of a differentiated liver. Antisense RIα (HYB0165) treatment produced sustained activation of Ap-1 binding. The results indicate that the nuclear extracts of liver contained Ap-1-binding transcription factors that form an uncharacterized DNA-protein complex that may be involved in the activation of phase II enzyme on treatment with DMBA and antisense RIα. In contrast, the CRE-decoy and control ODNs could not produce such effects. The specificity of induced AP-1 binding activity was shown by competition experiments; the complete competition of AP-1 binding activity was shown by a 25-fold molar excess of unlabeled AP-1 ODN.

Because c-fos, a transcription factor for Ap-1, is cAMP inducible, (47) and antisense RIα depletion of RIα induces the nuclear translocation of the catalytic subunit of PKA (48), resulting in increased CRE-directed transcription (47), we examined the effect of DMBA, antisense RIα, and CRE-decoy ODN on induction of this protein in liver. Western blotting showed that, in normal liver, the c-fos level was already quite high, but antisense RIα treatment of DMBA-treated rats led to an additional increase of *c-fos* levels (Fig. 4*B*). Quantification of bonds in Fig. 4*B* by scanning densitometry and "NIH Image 1.63f" software showed the increase in fos protein of 4.5-, 5.0-, 3.3-, and 3.4-fold over that of untreated controls during 2, 4, 8, and 14 days, respectively, after DMBA + antisense RIα treatment. During this period, DMBA intubation alone elicited only 1.5–1.8-fold increase in fos induction. By contrast, CRE-decoy and control ODNs could not mimic this antisense RIα effect on fos induction.

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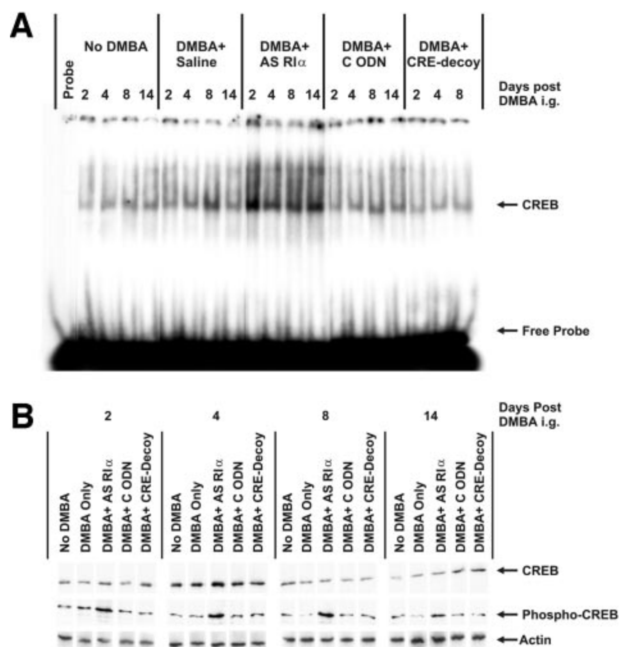

Fig. 5 Activation of cyclic AMP response element (CRE)-directed transcription. *A,* induction of CRE-binding activity by antisense regulatory (R) subunits RIα (HYBO165) in 7,12-dimethylbenz(*a*)anthracene (*DMBA*)-treated rats. Ten μg of nuclear extract were probed with ^{32}P-labeled oligonucleotide [double-stranded CRE with one copy of CRE (Promega)] and were analyzed by electromobility-shift assay. *B,* Western blot analysis of CRE-binding protein (*CREB*) and phospho-CREB levels in the liver. *Actin* determination is shown for loading control. Data represent one of three separate experiments that gave similar results. *AS,* antisense; *C ODN,* control ODN; *CRE-Decoy,* cyclic AMP response element-decoy oligonucleotide (*CRE decoy ODN*); *i.g.,* intragastric.

Induction of CREB Binding in Liver. As mentioned above, because c-fos, an Ap-1 binding protein, is a cAMP-inducible protein (47), we examined the effect of DMBA and antisense RIα on CRE-directed transcription. We examined CREB binding in liver after DMBA treatment and injections of antisense RIα, CRE-decoy ODN, and control ODNs. Nuclear extracts prepared from rat liver were analyzed by mobility-shift assay for CRE-DNA-CREB binding activity using ^{32}P-labeled consensus-sequence CRE ODN as a probe. Fig. 5*A* shows that antisense RIα (HYB0165) treatment produced a marked increase in CREB binding activity. The control ODNs and CRE-decoy ODN could not mimic the antisense RIα effect. The specificity of CREB binding was confirmed with the aid of excess unlabeled ODN. Western blot analysis of CREB protein in the liver of untreated, DMBA-treated, and antisense-treated animals demonstrated no change in CREB protein levels (Fig. 5*B*) but demonstrated an increase in phosphorylated CREB levels in the livers of animals treated with antisense RIα (Fig. 5*B*). Quantification of bands in Fig. 5*B* using scanning densitometry and "NIH Image 1.63f" software showed the increase in phospho-CREB protein of 3.4-, 3.6-, 4.0-, and 2.5-fold over that of untreated controls during 2, 4, 8, and 14 days, respectively, after DMBA + antisense RIα treatment. Treatment with DMBA alone, control ODN, and CRE-decoy ODN had no effect on phospho-CREB induction.

DNA Repair in Mammary Gland. It is known that DMBA-induced carcinogenesis starts with an interaction between DMBA electrophilic metabolites and nucleophilic regions of target-cell DNA. It has been shown that purified C subunit of PKA inhibits DNA repair, whereas H-89, a PKA inhibitor, or functional inactivation of PKA enhances DNA repair (31). We investigated whether antisense RIα, which down-regulates PKA I (Fig. 2), could activate DNA repair processes and whether that was part of its chemopreventive effect.

Antisense RIα treatment increased the recognition and binding of nuclear factors from mammary gland extracts to the cisplatin-damaged DNA (Fig. 6, *top right panel*). These effects of antisense RIα was not mimicked by the control ODN or CRE-decoy ODN. In contrast, the DNA repair system in liver did not respond to antisense RIα treatment. It is already activated in normal livers of DMBA-untreated animals (Fig. 6, *bottom right panel*).

DISCUSSION

For a tumor-bearing action of a carcinogen, the initial metabolic transformation in the body is of crucial importance. Metabolism of such a compound can proceed by different pathways, resulting in carcinogen activation or detox-

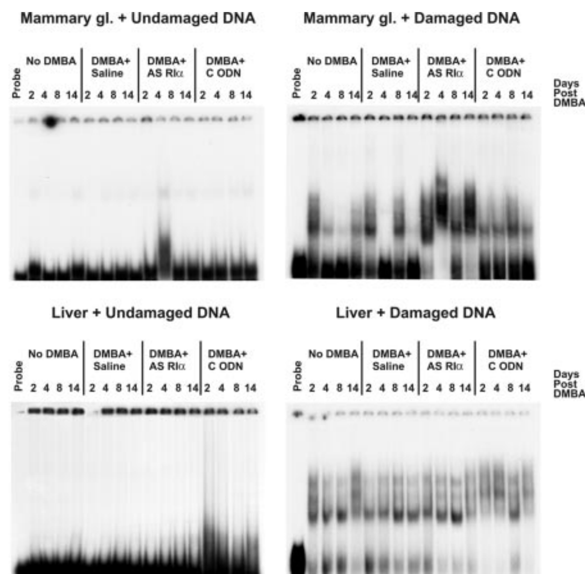

Fig. 6 DNA repair in mammary gland (*Mammary gl.*) and liver. The DNA repair assay was performed using a 148-bp plasmid fragment DNA probe derived from a bacterial *CAT* gene that was end-labeled and treated with 1.5 μM cisplatin for 18 to 24 h, followed by incubation with nuclear extract. DNA-protein complexes were resolved in 6% polyacrylamide gel as described in "Materials and Methods." *Upper left panel,* DNA repair assay in mammary gland with undamaged DNA. *Upper right panel,* DNA repair assay in mammary gland with DNA probe damaged with cisplatin. *Lower left panel,* DNA repair assay in liver with undamaged DNA. *Lower right panel,* DNA repair in liver with cisplatin-damaged DNA. Data represent one of two separate experiments that gave similar results. *AS RIα*, antisense regulatory subunits RIα; *C ODN,* control ODN.

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ification. The fate of a carcinogen depends on the availability of the particular enzyme system in the cell. A good example of such a dual fate for a carcinogen is DMBA, one of the most carcinogenic of the polycyclic aromatic hydrocarbons. DMBA can be transformed into a carcinogenic compound by the following enzyme pathway: (*a*) cytochrome P450-dependent mono-oxygenases form epoxides in the bay region of DMBA (38–41); (*b*) epoxide hydrolase then transforms the epoxides into less reactive diols; and (*c*) another cytochrome P450 activates these diols by epoxidation, converting them to the carcinogenic diol epoxides, which subsequently adduct to adenine and guanine residues in DNA (49, 50). In another pathway containing protective (phase II) enzymes such as glutathione transferases, however, diols can be conjugated and, thus, removed. NAD(P)H:quinone oxidoreductase is another phase II enzyme that catalyzes the two-electron reduction and detoxification of quinones and their precursors (51). By catalyzing two-electron reductions, this enzyme converts quinones directly into hydroquinones and bypasses the one-electron-reducing pathways catalyzed by enzymes such as cytochrome P450 reductase, which generate semiquinone intermediates that lead to reactive oxygen species. These reactive oxygen species can contribute to DMBA bioactivation, cytotoxicity, and mutagenicity (52).

The results presented here show that antisense RIα can serve as a chemopreventive agent via its activation of phase II enzymes glutathione *S*-transferase and quinone oxidoreductase. Antisense RIα activates these enzymes in mammary gland and liver. However, the liver is a major organ responsible for carcinogen activation; therefore, the analysis of representative hepatic phase II xenophobic metabolizing enzymes presented here may provide adequate mechanistic information regarding the effect of RIα antisense on DMBA metabolism. Together with increased inactivation of DMBA predominantly in liver, antisense RIα produced a marked increase of DMBA clearance and excretion from the body, as demonstrated in the urine analysis.

Exposure of mammalian cells to a variety of chemical agents triggers the induction of xenobiotic metabolizing enzymes, glutathione transferases, glucoronyl transferases, and NAD(P)H:quinone reductase, which function as intracellular detoxification systems of mutagens, carcinogens, and other toxic compounds (3, 4). The structural diversity of the chemicals that induce these enzymes is remarkable and raises questions about the regulatory mechanism involved. A recent study of a mouse glutamine *S*-transferase Ya gene showed that chemical agents such as planar aromatic hydrocarbons, diphenols, phorbol esters, and electrophilic compounds all operate through a single *cis*-regulatory element to induce the expression of this gene (46). Induction of this gene and that of quinone oxidoreductase was found to be mediated by a new type of DNA regulatory element defined as electrophile responsive element (EpRE) and composed of two adjacent AP-1-like binding sites that bind and are transactivated by Fos/Jun (AP-1) complex (46). The finding that AP-1 activity is induced by chemical agents with diverse and unrelated structures raises the question of the molecular processes involved in this induction. The possibility exists that the various chemicals may produce, by diverse pathways, a common transduction signal responsible for AP-1 induction. This would mean that the downstream mechanism for all of the chemicals is the same (AP-1 activation), but upstream events can differ for compounds with different structures. The results obtained in this study show that antisense RIα produces anticarcinogenic action by activating AP-1-dependent transcription.

Previously the studies from this laboratory have shown that the antisense depletion of RIα promotes nuclear translocation of the catalytic (Cα) subunit of PKA (48). Thus, the AP-1 activation observed in antisense RIα-treated cells may be attributed to an increase in CRE transcription through increased phosphorylation of CREB by Cα, followed by an increase in the cAMP-inducible protein c-fos.

Important proof of involvement of the cAMP system in chemoprevention is the fact that the CRE-decoy ODN, which brings blockade of both CRE- and AP-1-directed transcription (16), did not exhibit anticarcinogenic effects. Even in some experiments, administration of this ODN in the early stages of DMBA treatment produced more tumors in animals than did treatment with DMBA alone. The CRE decoy restrained tumor-cell proliferation without affecting the growth of noncancerous cells. It appears that the CRE decoy can efficiently inhibit tumor growth in established tumors by blocking CRE-directed transcription, which is highly activated and causatively related to the growth of established tumors (16). Because of this ability, the CRE decoy cannot serve as a chemopreventive agent at the initial phases of DMBA-carcinogenesis, when activation of the AP-1 and CREB-binding system is of crucial importance.

Alterations in the isozyme pattern of cAMP-dependent protein kinases have been described previously, correlating the dominance of type I over type II kinase with active cell growth, cell transformation, or early stages of differentiation (17–20). These reports concern chemical carcinogenesis in colonic epithelium, viral transformation, testicular maturation and differentiation, isoproterenol stimulation of cardiac hypertrophy, and progression through the cell cycle of Chinese hamster ovarian cells.

The dominance of RIα observed in mammary gland after DMBA treatment was abolished by antisense RIα treatment starting at day 1 before DMBA intubation. Thus, suppression of RIα and an increase in RIIβ could be involved in the mechanism of early-phase inhibition of carcinogenesis. Antisense RIα, via decreasing RIα, restores the normal balance between regulatory subunits of PKA in mammary gland and induces the DNA repair system in mammary gland.

It has been shown that functional inactivation of PKA results in increased DNA repair and the acquisition of resistance to DNA-damaging anticancer drugs, and the purified C subunit of PKA inhibits DNA repair, whereas H-89, a PKA inhibitor, enhances repair synthesis (31), implicating a role for the cAMP signal transduction pathway in DNA repair.

Antisense RIα exerted dual inhibitory function in DMBA carcinogenesis: (*a*) it increased detoxification of DMBA in the liver via activation of phase II enzymes through increasing CREB phosphorylation and CRE- and Ap-1-directed transcription; and (*b*) it enhanced DNA repair in the mammary gland by down-regulating PKA-I.

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ACKNOWLEDGMENTS

We thank Dr. Sudir Agrawal (Hybridon, Inc., Cambridge, MA) for providing us with ODNs and Cheryl Pellerin of Palladian Partners, Inc., who provided editorial support under contract number N02-RC-37003 with the National Cancer Institute.

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