Dual Blockade of Cyclic AMP Response Element- (CRE) and AP-1-directed Transcription by CRE-transcription Factor Decoy Oligonucleotide

GENE-SPECIFIC INHIBITION OF TUMOR GROWTH*

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Alteration of gene transcription by inhibition of specific transcriptional regulatory proteins has important therapeutic potential. Synthetic double-stranded phosphorothioate oligonucleotides with high affinity for a target transcription factor can be introduced into cells as decoy cis-elements to bind the factors and alter gene expression. The CRE (cyclic AMP response element)-transcription factor complex is a pleiotropic activator that participates in the induction of a wide variety of cellular and viral genes. Because the CRE cis-element, TGACGTCA, is palindromic, a synthetic single-stranded oligonucleotide composed of the CRE sequence self-hybridsizes to form a duplex/hairpin. Herein we report that the CRE-palindromic oligonucleotide can penetrate into cells, compete with CRE enhancers for binding transcription factors, and specifically interfere with CRE- and AP-1-directed transcription in vivo. These oligonucleotides restrained tumor cell proliferation, without affecting the growth of noncancerous cells. This decoy oligonucleotide approach offers great promise as a tool for defining cellular regulatory processes and treating cancer and other diseases.

Eukaryotic transcription is regulated by the interplay of various protein factors at promoters (1, 2). It has been shown that prokaryotic repressors can function as negative regulators of eukaryotic promoters (3, 4). This observation suggests that displacement of activating proteins might provide a general strategy for gene-specific repression in eukaryotes. Several approaches have been undertaken to control eukaryotic gene expression through such displacement.

In one approach, trans-dominant mutants are generated that interfere with the function of transactivators. Mutants are generated that retain the ability to bind to cis-regulatory DNA sequences but that have dysfunctional transcriptional activation domains. These mutant transcription factors compete with their functional, wild-type counterparts for binding to the enhancer sequences and prevent the activation or repression of the target gene. Although this strategy has been successful, in vitro (5–8), the generation of such mutants is not always possible. The transcription factor must be well characterized such that the activation domain(s) is identified and can be mutated. Also, even with sufficient knowledge to generate such mutants, difficult gene therapy procedures would be required to express these proteins in vivo.

Promoter competition strategy has also been utilized whereby plasmids containing cis-acting elements in common with the targeted gene are introduced in high copy number into cells (9). At high copy number, a majority of the transcription factors can be competitively bound away from the native enhancer sequences with gene expression accordingly regulated. Because these plasmids must be stably maintained at high copy number in target cells, a requirement that is difficult to achieve in vivo, this approach has also been limiting.

Another alternative is to employ oligonucleotides to form triple helices with enhancer elements. Pyrimidine oligonucleotides were found to bind in a sequence-specific dependence to homopurine sites in duplex DNA by triple helix formation and had sufficient specificity and affinity to compete with site-specific DNA binding proteins for occupancy of overlapping target sites (10). However, such oligonucleotide-directed triple helix formation has not been shown in cells in vitro or in vivo.

A more successful oligonucleotide-based approach has been the use of synthetic double-stranded phosphorothioate oligonucleotide containing a cis-transcription element that can penetrate cells, can bind sequence-specific DNA-binding proteins, and can interfere with eukaryotic transcription in vivo (11, 12).

The CRE1 (cyclic AMP response element)-transcription factor complex is a pleiotropic activator that participates in the induction of a wide variety of cellular and viral genes (13). Because the CRE cis-element, TGACGTCA (13), is palindromic, a synthetic single-stranded oligonucleotide composed of the CRE sequence self-hybridsizes to form a duplex/hairpin. We sought to ascertain whether the CRE-palindromic oligonucleotide can penetrate cells, bind sequence-specific DNA-binding proteins, and interfere with the CRE-directed transcription in vivo.

Because there are many cAMP-regulated genes and because they are ubiquitous in all cell types, the use of CRE decoy could be harmful to cells and organisms. Surprisingly however, the CRE decoys were harmless for normal cells but were potent inhibitors for cancer cell growth in vitro and in vivo.

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§ The abbreviations used are: CRE, cyclic AMP response element; DOTAP, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl sulfate; CREB, CRE-binding protein; EMSA, electrophoretic mobility shift assay; CAT, chloramphenicol acetyltransferase; FTIC, fluorescein isothiocyanate; TPA, 12-O-tetradecanoylphorbol-13-acetate; PKA, cAMP-dependent protein kinase; R, regulatory subunit of PKA; C, catalytic subunit of PKA; PEPCK, phosphoenolpyruvate carboxykinase; MDR, multidrug resistance; kb, kilobase(s).
EXPERIMENTAL PROCEDURES

Oligonucleotides—CRE-decoy and control oligonucleotides used in the present studies were phosphorothioate oligonucleotides. Their sequences are as follows: 24-mer CRE palindromic, 5'-TGAGCTCA TGACGTCA TGACGTCA-3'; 24-mer CRE mismatch control, 5'-TGCGTCA TGACGTCA TGACGTCA-3'; and 24-mer nonsense-sequence palindromic, 5'-CTAGCTAG CTAGCTAG CTAGCTAG-3'. The synthesis of the 24-mer phosphorothioate oligonucleotides was carried out using β-cyanoethylphosphoramidite chemistry on automated DNA synthesizer (Amersham Pharmacia Biotech Oligo Pilot II). Deprotection and purification of the oligonucleotides followed the experimental procedures previously described (14). Analysis of the purified oligonucleotide was carried out using capillary gel electrophoresis and polyacrylamide gel electrophoresis. The purity of the oligonucleotide based on capillary gel electrophoresis was 95% full-length and n = 1, n = 2 products.

Treatment of Cells in Culture with CRE Oligonucleotides—Cells (0.25–1 × 10^5 cells/well) were plated in 6-well plates containing the growth medium at 37 °C. To increase the delivery of oligonucleotide into the cell, cationic lipid (DOTAP) (Boehringer Mannheim) was used in the oligonucleotide treatment. The CRE-decoy and control oligonucleotides were added (1 day after seeding) to the cells at varying concentrations (50–200 nM) in the presence of DOTAP. At 5 h of incubation, the medium was removed, and fresh medium without oligonucleotide and DOTAP was added. Cells were harvested at indicated times, and cell numbers were counted in duplicate by a Coulter Counter.

Production of Stable Transfectants—MCF-7 cells (3 × 10^5 cells/60-mm dish) were transfected with 6 μg of KCREB plasmid (kindly provided by Richard H. Goodman) and a dominant negative mutant of CREB using DOTAP. Stably transfected cells were selected by growing cells in the presence of Geneticin (400 μg/ml) (G418, Life Technologies, Inc.). The G418-resistant colonies were isolated after 3 weeks of selection.

Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared by the method of Dignam et al. (15). EMSA assay was performed by a method of Fried and Crothers (16). Briefly, nuclear extracts (5 μg of protein) were preincubated with poly(dI-dC)-poly(dI-dC) (2 μg), dithiothreitol (0.3 mM), and reaction buffer (12 mM Tris, pH 7.9, 2 mM MgCl2, 60 mM KCl, 0.12 mM EDTA, and 12% glycerol) with or without CREB antisera (1–2 μl) for 30 min at 4 °C. 32P-labeled oligonucleotides (double-stranded oligonucleotides with one copy of CRE, 5'-AGAGAT-TGCGTGACGTAGAAGCTAG-3'; Oct1, 5'-TGCGTGACGTAGAAGCTAG-3'; and SP1, 5'-ATTCGATCggggcggggcccggg-3', Promega) were then added, and the reaction mixtures were incubated for 10 min at 37 °C. The reaction mixtures were then separated on a 4% nondenaturing polyacrylamide gel at 200 V for 2 h. The gel was dried and autoradiographed. AbCREB, CREB antibody (Santa Cruz Biotech.), was used for supershift.

Transient Transfection Assay of Somatostatin-Chloramphenicol Acetyltransferase Fusion Gene—Cells (5 × 10^5 cells/60-mm dish) were transfected with 3 μg of somatostatin-chloramphenicol acetyltransferase (CAT) fusion gene plasmid and 4 μg of CRE or control oligonucleotide using DOTAP. After 24 h, fresh medium was added, and the cells were harvested at 48–72 h, then assayed for CAT activity. When indicated, cells were also treated with forskolin (10 μM) for the final 24 h. Cell lysates were prepared as described by Gorman (17). Lysates (100 μg of protein) were incubated with 0.4 μl of [32P]dCTP according to a standard protocol for random prime labeling using Amersham Pharmacia Biotech multiprime DNA labeling kit. The specific radioactivity of labeled DNA equaled 3.7 × 10^6 cpm/μg DNA.

Induction of c-fos Gene Expression—Cells were treated with 150 nM CRE or control oligonucleotide for 2 days in the serum-containing medium (16). Cells were washed twice with phosphate-buffered saline. Cells were further incubated in the absence of oligonucleotides in the serum-free medium containing 100 ng/ml TPA (Sigma) at 37 °C. At various times, cells were harvested and fos mRNA was measured by Northern blotting.

RESULTS AND DISCUSSION

We examined whether a 24-mer single-stranded phosphorothioate oligodeoxyxynucleotide comprising a CRE palindromic (i.e. triplet copies of TGACGTCA, a CRE consensus sequence) can compete for binding the sequence-specific DNA binding proteins. We used 32P-labeled double-stranded oligonucleotide (unmodified) that contained the CRE element (Promega) and nuclear extracts from MCF-7 breast cancer cells in the electrophoretic mobility shift assay. As shown in Fig. 1A, protein binding to the 32P-labeled CRE probe was inhibited by the unlabeled CRE-palindromic oligonucleotide (lanes 2 and 3) but not by two-base mismatched control oligonucleotide, nonsense-sequence palindromic oligonucleotide containing no CRE, or Oct-1, AP-1, and Sp-1 sequences.

We next examined the ability of CRE-decoy oligonucleotide to penetrate cells and compete with the cellular CRE elements for binding transcription factors in vivo. The nuclear extracts from cells treated with the 24-mer CRE oligonucleotide (150 nM, for 2 days) demonstrated a marked decrease in formation of the CRE-protein complex in the mobility shift assay as compared with control (saline-treated) cells (Fig. 1B, lanes 4, 8, and 12). CREB (19) (CRE-binding protein) antibody caused supershift, indicating the presence of CREB protein within the labeled CREB-DNA complexes (Fig. 1B, lanes 2, 6, 9, and 10). The two-base mismatched control oligonucleotide treatment did not affect the CRE-protein complex formation (Fig. 1B, lanes 3, 7, and 11). A Western blot analysis for CREB protein in untreated and in CRE oligo-treated and control oligo-treated cells demonstrated no change in CREB protein level (Fig. 1C), indicating that the CRE-decoy treatment did not affect CREB
levels in the cell. These results were demonstrated in MCF-7 (breast carcinoma), MCF-10A (normal human mammary epithelial cell) and LNCaP (prostate carcinoma) cells. The above results demonstrate that the CRE-decoy oligonucleotide successfully competed with the cellular CRE enhancer for binding of sequence-specific DNA-binding proteins.
We next examined whether CRE oligonucleotides can modulate the transcriptional activity of sequence-specific DNA binding proteins in vivo. As shown in Fig. 1D, transfection of MCF-7 cells with somatostatin Δ71-CAT (20) plus CRE-palindromic oligonucleotide (lane CRE) resulted in a greater than 90% inhibition of the CRE-directed transcription compared with cells transfected with Δ71-CAT alone (lane C). Addition of the nonsense-sequence palindromic oligonucleotide that contains no CRE sequence (lane CREC) or the two-base mismatched control oligonucleotide (data not shown), which does not self-hybridize to form a duplex, had no inhibitory effect on the CAT activity. None of the oligonucleotides inhibited the simian virus 40 (SV40) enhancer (data not shown), which contains no recognizable CRE enhancer element.

It was noted that the untreated MCF-7 cells exhibited relatively high levels of basal somatostatin-CAT activity, and forskolin treatment had only a small stimulatory effect (Fig. 1D). In contrast, LNCaP prostate cancer cells and noncancerous MCF-10A cells exhibited very low levels of basal CAT activity, and forskolin greatly stimulated CAT activity (5–10-fold) (data not shown). This forskolin-stimulated CAT activity was also almost completely abolished by the CRE oligonucleotide treatment (data not shown). The disparity in the basal CAT activity observed in different cells may reflect varying degrees of cAMP-dependent protein kinase activation and CREB-phosphorylation in the cell. Significantly, the CRE oligonucleotide was capable of inhibiting both the basal and cAMP-stimulated CAT activities.

A group of cAMP-responsive genes, such as somatostatin and phosphoenolpyruvate carboxykinase (PEPCK) contain the CRE which lies within the first 150 base pairs of the 5′-flanking region of the gene (13). Therefore, these elements could be regarded as basal enhancer, in addition to functioning as inducible enhancer (1). A role for the CRE as a basal transcription element was suggested in deletion analysis of the PEPCK promoter-regulatory region (22). When the CRE was deleted from the promoter, the basal level of gene transcription was reduced, and the responsiveness of the promoter to cAMP in hepatoma cells was abolished. CRE binding protein binds to the CRE in a cAMP-independent manner (13, 19). This binding, as it has been proposed (13), may stimulate basal transcription by interacting with proximal promoter element, such as the TATA box binding factor and/or RNA polymerase II. The cAMP-induced phosphorylation of CREB could lead to a higher order complex formation with the basic transcription factor. Thus, dual role for the CRE as both a basal and an inducible enhancer (1). A role for the CRE as a basal transcription initiation element was identified within the GC-rich region (24, 25). Such GC-rich regions have been associated with transcription initiation sites of many constitutively expressed housekeeping genes (26). Our observation that the CRE oligonucleotide inhibits the basal expression of RIIa and Ca genes (Fig. 1E) strongly indicates that the CRE oligonucleotide can indeed compete with the cis-CRE element in binding CREB. Because the CRE oligonucleotide can interfere with CREB binding to the cis-element, it is expected that the oligonucleotide could produce a more profound effect on the mRNA reduction under cAMP-induced conditions. Thus, the CRE oligonucleotide can interfere with both basal and cAMP-induced expression of the endogenous CRE-containing genes.

CREB is known to associate with (e.g. heterodimerize) a variety of other transcription factors (e.g. member of the Jun/Fos family) (27). The products of the proto-oncogenes jun and fos bind as a heterodimeric complex to a DNA sequence element TRE (AP-1) binding site (28), whereas CREB-1 homodimer and CREB-2/ATF heterodimer bind the CRE sequence (27). However, Jun/Jun homodimer binds to both CRE and TRE (24), and CREB-2 (ATF-2)/Jun heterodimer binds CRE (27, 29), and c-fos is cAMP-inducible (13). These data clearly demonstrate AP-1 and CRE cross-talk.

We therefore examined whether the CRE oligonucleotide
**Fig. 3.** Cellular uptake of FITC-conjugated CRE-decoy and control oligonucleotides. A and B, 6 h; C and D, 12 h; E and F, 24 h, respectively, after CRE oligonucleotide treatment of MCF-7 breast cancer cells; G and H, 6 h; I and J, 12 h; respectively, after CRE-oligonucleotide treatment of MCF-10A normal mammary epithelial cells; K and L, 12 h after control (nonsense-sequence palindrome) oligonucleotide treatment of MCF-7 cells. A, C, E, G, I, and K, fluorescent images; B, D, F, H, J, and L, phase contrast images corresponding to A, C, E, G, I, and K, respectively. Magnification, × 200.
treatment affects AP-1 binding. The nuclear extracts from cells treated with the CRE-palindromic oligonucleotide demonstrated a marked reduction in formation of the AP-1 DNA-protein complex in the mobility shift assay compared with control (saline-treated) cells (Fig. 1F, lane 4). Two-base mismatched control oligonucleotide treatment had no effect on the AP-1 DNA-protein complex formation (Fig. 1F, lane 3). By comparison, CRE-decoy oligonucleotide had no effect on Sp-1 or Oct-1 DNA-protein binding (Fig. 1F, lanes 8 and 12).

We then examined the effect of CRE-decoy oligonucleotide treatment on the expression of c-fos gene that is cAMP responsive (13). As shown in Fig. 1G, the CRE decoy brought about a marked decrease in the TPA-inducible mRNA level of c-fos. The control mismatched control oligonucleotide had no effect on the c-fos expression (data not shown). A cAMP-unresponsive gene, such as PKC-α, was not affected by the CRE oligonucleotide treatment (Fig. 1G). These results show that the CRE-decoy oligonucleotide treatment resulted in inhibition of transcription factor binding at two different cis-elements, the CRE and the AP-1.

The above results showed that the CRE-decoy oligonucleotides effectively competed with the cellular cis-element for binding the transcription factors and interfered with the function of transactivators in intact cells. To correlate such effects of decoy oligonucleotides with their cellular uptake, we incubated 32P-labeled 24-mer CRE-palindromic oligonucleotide or mismatched or nonsense sequence palindromic control oligonucleotide with MCF7 and MCF-10A cells. Cell-associated radioactivity was quantified. Within 5 h, about 10% of the total input oligonucleotide accumulated in the cell and the incorporation continued to rise thereafter, reaching 20–25% maximum levels at 24 h of oligonucleotide incubation (Fig. 2A). The amount and the rate of the incorporation of the oligonucleotides were similar between MCF-7 (Fig. 2A) and MCF-10A (data not shown) cells and between the CRE-decoy oligonucleotide and control oligonucleotide (Fig. 2A). Cell-associated DNA was isolated and analyzed by nondenaturing polyacrylamide gel electrophoresis. Up to 48 h of examination, the 24-mer CRE oligonucleotide accumulated in MCF-7 cells at a size consistent with the duplex/hairpin forms (Fig. 2B). Consistent with these data, the 24-mer CRE oligonucleotide exhibited a high melting temperature (Fig. 2C).

The intracellular distribution of the fluorescence signal at different times after the treatment of cells with FITC-labeled
CRE-decoy and control oligonucleotides is illustrated in Fig. 3. In MCF-7 breast cancer cells, within 6 h of treatment with CRE-palindromic oligonucleotide, a strong fluorescence labeling was observed in both cytoplasm and nucleus (Fig. 3, A and B). In addition, a large amount of labeling was also observed in the extracellular space. 12 h after the treatment, the nuclear fluorescence had become much more intense, and the extracellular fluorescence had largely disappeared (Fig. 3, C and D), and by 24 h, the intensity of fluorescence was reduced in both nucleus and cytoplasm (Fig. 3, E and F). This pattern of fluorescence was also observed in noncancerous MCF-10A cells except that the fluorescence had a more punctated appearance (Fig. 3, G, H, I, and J). The control oligonucleotide exhibited the same pattern of fluorescence as did the CRE-decoy oligonucleotide (Fig. 3, K and L).

Because there are many cAMP-regulated genes, and they are ubiquitously expressed, we examined whether the CRE oligonucleotide treatment could interfere with cell growth. Surprisingly, the CRE-decoy oligonucleotide produced selective growth inhibition of cancer cells without adversely affecting the normal cell growth (Fig. 4A). The 24-mer CRE-palindromic oligonucleotide produced potent growth inhibition in a variety of cancer cells including MCF-7 (breast cancer), A549 (lung carcinoma), LNCaP (prostate cancer), LS174T and SW480 (colon carcinomas) (data not shown), KB (epidermoid carcinoma) (data not shown), and multidrug-resistant (MDR) cancer lines of MCF7TH (MDR-breast cancer) and HCT-15 (MDR-colon carcinoma) (data not shown). In contrast, the CRE oligonucleotide had little (<20%) or no effect on the growth of noncancerous cells, MCF-10A (human mammary epithelial cell), L132 (human lung epithelial cell), Hs68 (human newborn foreskin fibroblast) (data not shown), and NIH/3T3 fibroblasts (data not shown). The growth inhibition of cancer cells was achieved at nanomolar concentrations of CRE oligonucleotide (IC50, 100–200 nM) without obvious cytotoxicity and accompanied by changes in cell morphology and appearance of apoptotic nuclei (programmed cell death) (data not shown). The growth inhibition was CRE-sequence-specific as the two-base mismatched control oligonucleotide (Fig. 4A) or the nonsense-sequence palindromic oligonucleotide that contains no CRE sequence (data not shown) had little (<30%) or no growth inhibitory effect.

CRE oligonucleotide treatment also inhibited in vivo tumor growth. Treatment of nude mice bearing HCT-15 human MDR colon carcinoma with 24-mer CRE oligonucleotide (0.1 mg/mouse, intraperitoneal, daily, 5× week for 4 weeks) resulted in greater than 85% inhibition of tumor growth as compared with the saline-treated control tumors without causing systemic toxicity (Fig. 4B). Two-base mismatched control oligonucleotide had no growth inhibitory effect (Fig. 4B).

The growth inhibition may have been because of actions other than blockade of CRE gene transcription, as nonspecific binding of oligonucleotide or its degradation products to biological targets has been shown (30). As discussed below, however, our data show that the binding of decoy oligonucleotide at the transcription factor DNA-binding domain is clearly related to the inhibition of cell growth. First, in undifferentiated F9 teratocarcinoma cells, a cell line that is unresponsive to cAMP, the CRE decoy produced no growth inhibition (Fig. 4C). This suggests that the decoy may act as growth inhibitor, at least in part, through binding to CREB because the CRE is nonfunctional in F9 cells although CREB is present (31). Second, KCREB, a CREB mutant that contains a mutation of a single amino acid in the DNA-binding domain, is known not to bind to native CRE sequences (32). Cancer cells overexpressing KCREB exhibited decreased cell growth as compared with parental cells and showed little or no response to the decoy oligonucleotide treatment (Fig. 4D).

We demonstrated here that the synthetic single-stranded CRE oligonucleotide of palindrome structure functioned as effective and stable transcription factor decoys to alter gene expression in vivo. Importantly, the CRE-decoy oligonucleotides achieved gene-specific regulation in vivo, leading to selective inhibition of cancer cell growth without adversely affecting the growth of normal cells.

The specificity of the growth inhibitory effect of the CRE-decoy oligonucleotides on cancer cells is supported by several lines of evidence. (i) The CRE-decoy oligonucleotide produced growth inhibition of cancer cells but not normal cells, in vitro and in vivo, whereas mismatched control oligonucleotides or nonsense-sequence palindromic oligonucleotide that self-hybridizes but contains no CRE, did not inhibit growth. (ii) Administration of CRE-decoy oligonucleotides, but not mismatched oligonucleotides, markedly inhibited CRE DNA-protein complex formation, CRE-directed transcription activity, and endogenous cAMP-responsive gene expression in both cancer cells and normal cells. (iii) Cellular uptake of decoy oligonucleotides and control oligonucleotides was similar for cancer cells and normal cells. (iv) The specific growth inhibitory effect toward cancer cells correlated with induction of phenotypic change and apoptosis.

Importantly, the CRE-decoy oligonucleotides not only blocked the CRE-PAK pathway via repression of the PKA genes but also brought about the blockade of AP-1-PKC pathways by inhibiting c-fos gene expression that is CRE-responsive. This dual blockade of two important signal transduction pathways could be causally related, at least in part, to the CRE oligonucleotide-inhibition of cancer cell growth.

CREB, a critical regulator of immediate early gene transcription, has been shown to be activated by growth factors (33) and play an important role in the acquisition of the metastatic phenotype of human melanoma cells (21). Although the exact mechanism of action remains to be elucidated, our results suggest that the CRE-decoy oligonucleotides may provide a powerful means of combating cancers by regulating the expression of cAMP-sensitive genes.

Acknowledgments—We thank Matthew C. Ellis for critical reading of the manuscript, Richard H. Goodman for providing KCREB plasmid, and C. A. Stein for providing LNCaP cells and protein kinase C-a cDNA.

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