Bioorganic Chemistry **29,** 223–233 (2001)
doi:10.1006/bioo.2001.1213, available online at http://www.idealibrary.com on IDEAL®

Photo-Cross-Linked Oligonucleotide Duplex as a Decoy-DNA for Inhibition of Restriction Endonuclease Activity

Akira Murakami,[1] Yoko Yamamoto, Masataka Namba, Reiko Iwase, and Tetsuji Yamaoka

Department of Polymer Science and Engineering, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan

Received January 30, 2001; published online August 9, 2001

As a novel type of regulator molecule for DNA-recognizing proteins, a photo-cross-linked oligonucleotide duplex was designed and synthesized. The molecule regulated the activity of a restriction endonuclease by being recognized as a substrate. This type of regulating molecule is regarded as a decoy-DNA. 4,5′,8-[4-Aminoethylaminomethyl]-trioxalen (aeAMT) was conjugated with an oligodeoxyribonucleotide (ODN) at the 5′-end and the aeAMT was cross-linked with the thymine residue of the complementary oligonucleotide upon UVA irradiation. The terminally cross-linked oligonucleotides, singly clipped (SC) decoy-DNA, acquired thermal stability. An oligonucleoside phosphorothioate (OPT) was also introduced as one or both components, yielding three types of decoy-DNAs, SC-ODN-ODN (SC.DD), SC-OPT-ODN (SC.SD), and SC-OPT-OPT (SC.SS). The SC decoy-DNAs inhibited the function of the restriction endonuclease, *Aat*II, in a sequence-specific and concentration-dependent manner with an appreciable IC_{50} value (1.3 μM for SC.DD, 0.016 μM for SC.SD, 0.002 μM for SC.SS). The SC decoy-DNAs were found to be effective for regulating the DNA recognizing proteins.

Key Words: oligonucleotide; photo-cross-linking; decoy-DNA; restriction enzyme; enzyme inhibitor; oligonucleoside phosphorothioate.

INTRODUCTION

Nucleic acids, oligonucleotides and their derivatives have been intensively studied for the development of novel types of therapeutic reagents (*1–4*). Antisense oligonucleotides are the first substances for such purposes and the oligonucleoside phosphorothioate (OPT) became the first antisense drug (*5*). Antisense RNA, which appeared several years later, is regarded as another promising type of nucleic acid drug. Ribozymes, which hydrolyze RNA in a sequence-specific manner, could be powerful gene-regulating reagents using effective carrier materials (*6,7*). Aptamers, which interact with proteins in a sequence-specific manner, are also a hopeful candidate as a new type of nucleic acid drug (*8*). Recently, it has been reported that the cellular proliferation

[1] To whom correspondence should be addressed. Fax: +81-75-724-7814. E-mail: akiram@ipc.kit.ac.jp.

0045-2068/01 $35.00

was effectively regulated with a double-stranded oligonucleotide with a specified sequence. The trial focused on the sequence specific regulation of the transcription factors (TF) (*9–16*). TF recognizes the added duplex instead of recognizing the cellular DNA, which resulted in an ineffective transcription by TF. Such a double-stranded oligonucleotide functioned as a decoy molecule of TF, and such oligonucleotide is called a decoy-DNA. By now, other than the double-stranded oligonucleotides, various types of decoy-DNAs have been reported. They include (A) the terephthalamide linker (*17*), (B) the disulfide cross-linking (*18,19*), (C) the dumbbell type (*20–22*), (D) the ethylene glycol bridge (*23,24*), and (E) the stilbene bridge (*25*). With these materials, the decoy approach will be further studied to regulate cellular functions by regulating TF.

In designing a decoy-DNA, there are several requirements: stability of the serum/cell, simple and specific methods of preparation, and sequence specificity. This study is concerned with the development of new types of decoy-DNAs and consists of three aspects that fulfill these requirements. First, the preparation of stable decoy-DNAs was carried out by using a photoreactive reagent, the psoralen derivative, and subsequent photoirradiation. The psoralen derivative attached to the 5′-end of the oligonucleotide was cross-linked with a thymine residue at the 3′-terminus of another oligonucleotide to form a clamped double-stranded oligonucleotide (*26,27*). Second, the OPTs were used as one of the components of the decoy-DNAs. OPTs are regarded as the most effective antisense molecules (*3*). It is much more stable in serum than oligonucleotides (ODNs) and is easily incorporated by cells, though the detailed mechanism is still unknown. Also, it has been reported that OPT interacts with biological substances in a sequence-dependent or independent manner (*3,28*). Therefore, OPT could add its interesting characteristics to the chimera decoy-DNA (*10,29*). Third, in order to evaluate the clipped decoy-DNA, restriction enzymes were chosen as a DNA recognizing protein (*30,31*). From these aspects, several interesting results were disclosed.

MATERIALS AND METHODS

Reagents

Trioxalen was purchased from Wako Pure Chemicals Inc. (Osaka, Japan). Reagents for the oligonucleotide synthesis were purchased from Glen Research Inc. Reagents for the gel electrophoresis were purchased from Nacalai Tesque Co. (Kyoto, Japan) and the restriction enzymes (*Aat*II and *Eco*RI) were purchased from Toyobo Inc. (Osaka, Japan). The other reagents were purchased from Wako Pure Chemicals Inc. and used without further purification. The reversed phase HPLC was operated on a reversed phase column (Capcell Pak 4.5mm i.d. × 150 mm, Shiseido Co., Tokyo, Japan) using acetonitrile gradient in 0.1 M TEAA as follows: 0 to 50% (20 min), 50 to 50% (5 min), 50 to 0% (3 min). Polyacrylamide gel electrophoresis (PAGE, 18%) was operated in 0.1 M TBE buffer containing 7 M urea. Agarose gel electrophoresis (AGE, 1.5%) was operated in 0.1 M TBE buffer. DNA fragments were visualized by staining with ethidium bromide. UV melting profiles of the decoy-DNAs were obtained by a Hitachi UV spectrophotometer (U2000A, Hitachi Co. Tokyo, Japan) equipped with a programmed thermo-controller with the rising rate of 1.0°C/min. The samples were prepared in 0.1 M phosphate buffer (pH 7.0) and the concentration was

fixed at 2 μM. The circular dichroism (CD) spectra of the 2 μM decoy-DNAs were obtained using a circular dichroism spectrophotometer (CD-703, JASCO Co., Tokyo, Japan) in 0.1 M phosphate buffer (pH 7.0) at 37°C.

Preparation of Photo-Cross-Linked Oligonucleotide Duplex

Oligodeoxyribonucleotides and OPTs were synthesized using an automatic DNA synthesizer (ABI 391, PE Biosystems Inc.). To the 5′-hydroxy group of ODN (or OPT) on controlled pore glass support, 4,5′,8-(4′-aminoethylaminomethyl)psoralen was introduced according to the carbonyldiimidazole (CDI) protocol (*32*). These psoralen-introduced oligonucleotides (Ps-ODN, Ps-OPT) were purified by reversed-phase HPLC. Photo-cross-linking of the Ps-ODN (or Ps-OPT) with its complementary ODN (or OPT) was carried out in 25 mM phosphate buffer (pH 7.0) at 4°C on a transilluminator (365 nm, 1.1 mW/cm^2). After irradiation, the solution was subjected to PAGE and the corresponding bands were excised under UV light illumination. The band was crushed and extracted using 1 M TEAA (1 mL $\times$ 3). The solution was passed through a Sep Pak cartridge followed by the elution with 30% acetonitrile in 0.1 M TEAA (pH 7.0). An aliquot of the photo-product dissolved in 0.1 M phosphate buffer (pH 7.0) was irradiated by a black light (254 nm, 0.9 mW/cm^2) at 4°C for 30 min and the solution was then analyzed by PAGE followed by a UV shadowing protocol.

Restriction Enzyme Reaction

The substrate, linearized pUC19, was prepared by treating the plasmid pUC19 (191 μg) with *Eco*RI (10 U) in 0.1 M NaCl/50 mM Tris–HCl (pH 7.0)/10 mM $MgCl_2$ at 37°C for 1 h. The DNA was subjected to *Aat*II treatment in the presence of various concentrations of decoy-DNAs. The reaction mixtures were incubated at 37°C for 1 h and incubated at 65°C for 15 min followed by AGE. The AGE patterns were evaluated by an image scanner and by the freeware, NIH Image ver.1.62 (http://www.yk.rim.or.jp/aisoai/molbio-j.html).

RESULTS

The structure of a cross-linked double-stranded oligonucleotide is shown in Fig. 1. As a photo-cross-linker, 4,5′,8-[4-aminoethylaminomethyl]-trioxalen (aeAMT) was

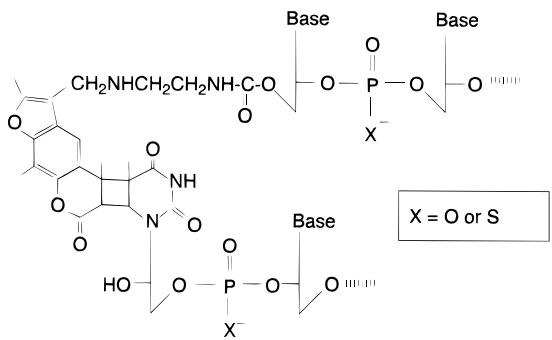

FIG. 1. Structure of singly clipped double-stranded DNA.

conjugated with oligonucleotides at the 5′-end. The method of the conjugation of aeAMT to the oligonucleotides adopted a CDI activation protocol, and the average introduction yield was 70%. Three equivalents of nonlabeled complementary ODN (or OPT) was added to the aeAMT-conjugated ODN (or OPT) and the photo-cross-linking reaction by UVA irradiation (365 nm) proceeded in a time-dependent manner. After 30 min, ca. 75% of the Ps-ODN (or Ps-OPT) was cross-linked with the complementary ODN (or OPT). The photoproducts were successfully purified by PAGE. When the photoproducts were irradiated by UV-254 nm, the appearance of two bands that correspond to the original components was observed (Fig. 2). In this study, three types of terminally cross-linked double-stranded DNA, namely singly clipped DNAs, were designed and synthesized as shown in Table 1. They were the singly clipped ODN/ODN duplex (SC.DD), the singly clipped OPT/ODN duplex (SC.SD), and the singly clipped OPT/OPT (SC.SS). In order to evaluate the sequence dependency, both mismatched and scrambled sequences were also adopted. As control double-stranded DNAs, the nonclipped ODN/ODN duplex (NC.DD) and nonclipped OPT/OPT (NC.SS), were also prepared by making an equimolar mixture of the corresponding oligomers.

Figure 3 shows the melting profiles of a typical singly clipped decoy-DNA, SC.DD. Whereas the nonclipped duplex showed a fairly steep melting curve with a distinct melting point, a singly clipped decoy-DNA showed a gradually increased melting profile without a distinct melting point. The structures were further studied by circular dichroism spectroscopy (CD) as shown in Fig. 4. The CD spectra of SC.DD at 37°C showed the positive Cotton effect around 280 nm and a small negative Cotton effect around 243 nm, which is characteristic of the B-form DNA and was similar to NC.DD. However, in that case, the magnitude of the positive Cotton effects around 280 nm decreased to ca. 50%. As for the SC.SD, the CD spectrum was further changed when compared to that of NC.DD. The wavelength that gives the maximum Cotton effect was red-shifted by 5 nm and the magnitude decreased to ca. 70% of NC.DD. The CD spectrum of SC.SS suggested a largely deformed B-form (*33*).

The ability of the singly clipped double-stranded DNA to regulate a DNA-binding enzyme was studied by evaluating the cleavage efficacy of the linearized pUC19 (2686 bp) by *Aat*II. The enzyme cleaves the DNA at a single site (5′-TGACGTCA) to yield two fragments, 465 and 2221 bp. Figure 5 showed the typical results of the AGE pattern. In the presence of the decoy-DNAs, the cleavage by *Aat*II was clearly suppressed depending on the types, sequence, and concentration of the decoy-DNAs. The types of inhibitory effects were examined by Lineweaver–Burke plots (Fig. 6) and the efficacy was evaluated by the IC_{50} values (Table 2).

DISCUSSION

In this study, new types of decoy-DNAs were prepared and their ability as a decoy-DNA for a DNA recognizing protein, restriction endonuclease, was examined. As such enzymes recognize the double-stranded DNA, the decoy-DNA must be double-stranded throughout the inhibitory reactions. In order to maintain the decoy-DNA as double-stranded, it is a rational idea to covalently cross-link two strands at a certain site which is apart from the protein recognition one. This idea was adopted in this study and a psoralen derivative that can covalently cross-link with pyrimidine bases

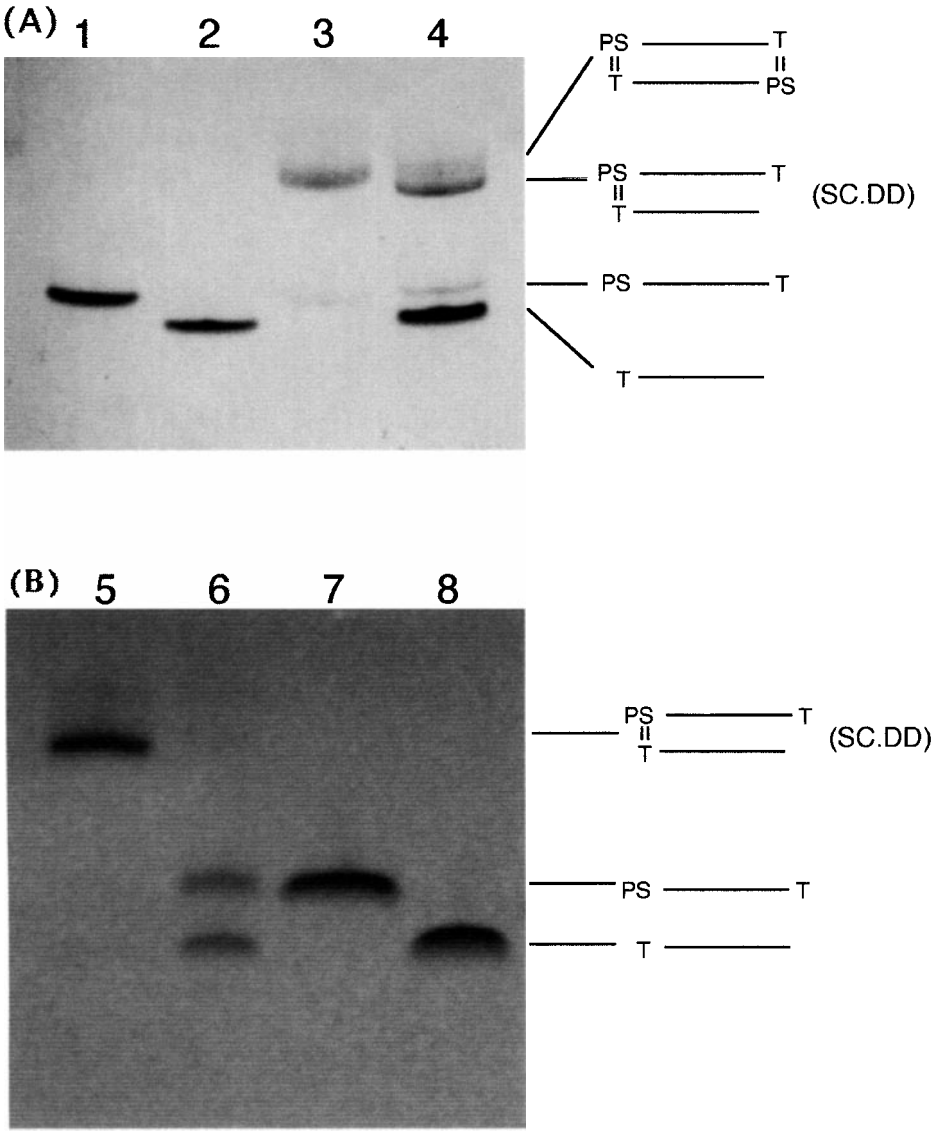

FIG. 2. Photoreactions of psoralen-conjugated oligodeoxyribonucleotide with the complementary strand. (A) Photo-cross-linking reaction, (B) photo-cleavage reaction. Sequence: Ps-ODN 5′-CCATGACG-TCATGGT, c-ODN 5′-CCATGACGTCATGGT. (A) Lane 1, Ps-ODN; lane 2, ODN; lane 3, Ps-ODN (irradiated); lane 4, Ps-ODN + c-ODN (irradiated). Irradiation: 365 nm, 1.1 mW/cm^2, 30 min, 25 mM phosphate buffer (pH 7.0), 18% denatured polyacrylamide gel electrophoresis, 400 V, 3 h. (B) Lane 5, SC.DD; lane 6, SC.DD (irradiated); lane 7, Ps-ODN; lane 8, c-ODN. Irradiation: 254 nm, 9.5 mW/cm^2, 30 min, 25 mM phosphate buffer (pH 7.0), 18% denatured polyacrylamide gel electrophoresis, 400 V, 3 h.