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Inhibition of Transcription at a Coding Sequence by Alkylating Polyamide

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Abstract: Transcription from DNA sequence-specifically alkylated by a hairpin polyamide (ImPyPy-γ-ImPyLDu86, **1**) was investigated. High-resolution polyacrylamide gel electrophoresis demonstrated that conjugate **1** alkylated a 993-bp DNA fragment, in accordance with the Py−Im recognition rule, predominantly at the one match site on the GFP-encoding strand and at four sites (I′−IV′) on the noncoding strand. Alkylation of DNA inhibited the formation of full-length mRNA and caused the transcription of truncated mRNA. Polyacrylamide gel electrophoresis demonstrated that the length of the truncated mRNA was consistent with the alkylated site on the coding strand. Complete inhibition of full-length mRNA synthesis was observed in the presence of 50 nM **1**. In clear contrast, the hydrolyzed derivative of **1**, designated **2**, produced no truncated mRNA, nor did it significantly retard transcription: >80% transcription of full-length mRNA was observed at 50 nM. These results clearly indicate that inhibition of transcription can be achieved with alkylating Py−Im polyamide even in the coding regions of genes.

Introduction

Synthetic transcription factors and activators are of particular importance in molecular medicine because they can regulate the expression of specific genes.[1] Oligodeoxynucleotides are one such agent, and they recognize and bind to a broad range of sequences with high affinity and specificity by forming a triple helix.[2] Although oligonucleotides and their analogues interfere with gene expression,[3] the triple-helix approach can only be used with purine-rich tracts and is limited by poor cellular uptake. Several pyrrolo[2,1-*c*][1.4]benzodiazepine derivatives and cell-permeable ligands with oligosaccharide moieties that interfere with the binding of transcription factors cannot target a broad range of DNA sequences.[4]

On the basis of earlier work on the concept of minor-groove sequence information readout,[5] Dervan et al. designed and synthesized minor-groove-binding hairpin polyamides that contain *N*-methylpyrrole (Py)−*N*-methylimidazole (Im), which uniquely recognize each of the four Watson−Crick base pairs.[6] A pairing of Im opposite Py targets the G−C base pair, and Py−Im targets the C−G base pair.[7] Py−Py degenerately targets T−A and A−T base pairs.[7,8] The binding constant and sequence specificity of the Py−Im polyamide is comparable to that of a transcription factor. In fact, it has been demonstrated that the combination of Py−Im hairpin polyamides, which competitively bind to regulatory sequences, and transcription factors inhibits the expression of 5S ribosomal RNA and human immunodeficiency virus (HIV) genes.[9] Because gene expression is usually

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controlled by a combination of multiple common transcription factors, inhibiting gene expression by binding Py−Im polyamides to regulatory sequences places potential limitations on the design of polyamides. Because the target sequence must be unique to a specific gene, it should contain part of the recognition sequence of the transcription factor together with the unique flanking sequences. In contrast, targeting Py−Im polyamide to the coding region is fairly straightforward in terms of selecting a unique sequence. Moreover, many types of mutations responsible for cancer have been found in coding regions. However, the inhibition of transcription by simple Py−Im hairpin polyamides may be difficult because the polyamide is removed from duplex DNA by the transcribing RNA polymerase. Gottesfeld et al. recently demonstrated that hairpin polyamides inhibit the passage of the elongating polymerase through nucleosomal DNA.[10] However, finding an appropriate target sequence in the nucleosome structure, the alkylation of which will efficiently inhibit transcription, may be difficult because the chromatin positioning of specific genes is uncertain.

Recently, we demonstrated that hybrid molecules, derived from duocarmycin A and Py−Im hairpin polyamides, alkylated a predetermined seven-base-pair (bp) sequence on a 450-bp DNA fragment.[11] Furthermore, we have shown that Py−Im hairpin polyamide−cyclopropapyrroloindole (CPI) conjugates with a vinyl linker (ImPyPy-γ-ImPyLDu86, **1**) efficiently alkylate double-stranded DNA.[12] In this work, we investigated the transcription from DNA sequence-specifically alkylated by the hairpin polyamide **1**. Polyacrylamide gel electrophoresis (PAGE) of the transcription products demonstrated that **1** can efficiently inhibit transcription precisely at the alkylated site, thus producing truncated mRNA.

Experimental Section

General Methods. T7 RNA polymerase was purchased from Promega. The plasmid pQBI63 and Ex Taq DNA polymerase were from Takara. The Thermo Sequenase Primer Kit for DNA sequencing was from Amersham Biosciences. MinElute PCR Purification Kit was from Qiagen. RNase inhibitor was from Wako Chemicals. Inorganic pyrophosphatase and other biochemicals were from Sigma. All DNA oligomers were from JBioS. Electronspray ionization mass spectra (ESIMS) were recorded on a PE Sciex API 165 mass spectrometer. High-resolution PAGE of Texas Red-labeled DNA was performed on a Hitachi 5500-S DNA sequencer. Polyamides were purified by high-performance liquid chromatography (HPLC) with a Jasco PU-980 HPLC pump, a UV-975 HPLC UV/VIS detector, and a Chemcobond 5-ODS-H column (4.6 × 150 mm). Polymerase chain reaction (PCR) was performed on a Perkin-Elmer GeneAmp PCR System 2400. E-Gel 1.2% agarose gel was from Invitrogen.

Py−Im Polyamides. Py−Im polyamide−CPI conjugate **1** was synthesized by previously described methods,[12] and its purity and identity were established by analytical HPLC, ESIMS, and ^{1}H-nuclear magnetic resonance. The hydrolyzed Py−Im polyamide−CPI conjugate **2** was prepared by incubating **1** in sodium phosphate buffer (50 mM, pH 7.0) at 37 °C for 48 h and was purified by HPLC; the structure verified by ESIMS. HPLC was performed using 0.1% acetic acid and a 0−50% acetonitrile linear gradient (0−40 min) at a flow rate of 1.0 mL/min, with detection at 254 nm. Polyamides **1** and **2** were eluted at 33.0 and 30.3 min, respectively. **2**: ESIMS, *m*/*z* calculated for $C_{50}H_{56}N_{15}0_{11}$ [M^+ + H] 1042.1; observed 1041.4. Polyamides were purified by preparative HPLC, lyophilized, and stored at −20 °C.

Preparation of 5′-Texas Red-Labeled DNA Fragments. 5′-Texas Red-modified 993-bp DNA fragments containing the T7 promoter and the entire coding region of red-shift green fluorescent protein (GFP) were prepared by PCR using the pQBI63 plasmid and 5′-Texas Red-modified and unmodified 20-mer primers. For the 5′-end-labeled noncoding strand: 5′-Texas Red-labeled-CCCCAAGGGGTTATGCTAGT-3′ and 5′-GGTGATGTCGGCGATATAGG-3′ (GFPpro). For the 5′-end-labeled coding strand: 5′-Texas Red-labeled-GGTGATGTCGGCGATATAGG-3′ and 5′-CCCCAAGGGGTTATGCTAGT-3′. The DNA fragments used for the synthesis of RNA markers were prepared by PCR from pQBI63, the GFPpro primer, and one of the following primers: 5′-TTCTATTAACAAGGGTATCACCTTC-3′ (for the 458-mer), 5′-GAAGTCAATACCTTTTAACTCGATTC-3′ (for the 481-mer), and 5′-TTTGTGTCCCAGAATGTTGC-3′ (for the 511-mer). After incubation for 25 thermal cycles, the products were purified by filtration with a MinElute PCR Purification Kit, and their concentrations were determined by UV absorption at 260 nm. Their lengths and purity were confirmed by E-gel 1.2% agarose gel electrophoresis.

High-Resolution Gel Electrophoresis of 1-Treated 993-bp DNA Fragments. The 5′-Texas-Red-labeled DNA fragment (2 nM duplex concentration) was alkylated by various concentrations of **1** (5, 10, 30, 50 nM) in a 50 mM HEPES buffer (pH 7.0) containing 10% dimethylformamide in 50 μL at 4 °C for 12 h. The reaction was quenched by the addition of DNA oligomer (1 mM, 1 μL) and heating for 5 min at 90 °C. The DNA was dried by vacuum centrifugation. The pellet was dissolved in loading dye (formamide with fuchsin red, 4 μL), heated at 94 °C for 20 min, and then immediately cooled to 0 °C. A 2-μL aliquot was subjected to electrophoresis on a 5% denaturing polyacrylamide gel using a Hitachi 5500-S DNA sequencer. Fluorescent bands were analyzed using FRAGLYS2 (version 2.2, Hitachi) and NIH Image (version 1.61) on a Power Macintosh G3.

In Vitro Transcription. The alkylated 5′-Texas Red-labeled DNA fragment (2 nM, 200 μL) was prepared exactly as described above. The pellet was dissolved in 0.4 nM each of four deoxyribonucleoside triphosphates, 4 mM dithiothreitol, 2 mM spermidine, 2 mg of bovine serum albumin, 0.2 unit of inorganic pyrophosphatase, 20 units of RNase inhibitor, and 100 units of T7 RNA polymerase in 50 μL, and the transcription reaction was incubated at 37 °C for 1 h. The synthesized RNA markers were prepared by the same procedure. Transcription products were analyzed by 6% PAGE with 7 M urea, at 80 V, and were detected by ethidium bromide staining. The bands were photographed with a Kodak DC120 Zoom digital camera under UV light (302 nm) and analyzed by NIH Image (version 1.61) on a Power Macintosh G3. The yield of truncated mRNA was estimated by the intensity of the bands, which was corrected for the length of the mRNA: full-length mRNA, 873 nt; truncated mRNA, 457 nt.

Results and Discussion

Sequence-specific alkylation by **1** was investigated with a 5′-Texas Red-labeled 993-bp DNA fragment that encodes GFP under the control of the T7 promoter, using an automated DNA sequencer, as previously described (Figure 1).[11,13] High-resolution denaturing PAGE demonstrated that **1** alkylated predominantly at the A of the 5′-AGTC*A*-3′ (I) sequence on the GFP-coding coding strand (Figure 2a). On the noncoding strand, **1** alkylated predominantly at the A of 5′-TGTT*A*-3′ (I′), 5′-TGTC*A*-3′ (II′), and 5′-AGTC*A*-3′ (III′, IV′) (Figure 1b). The Im−L pair of **1** recognized the mismatched A−T base pair at the I′ site. The hairpin polyamide−CPI conjugate **1** effectively alkylated both strands according to the pairing rule for Py−Im

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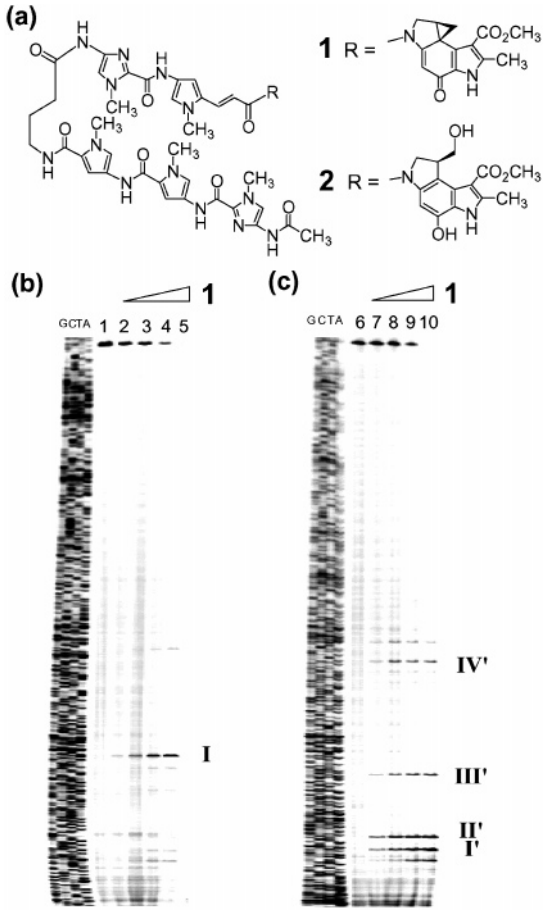

Figure 1. (a) Structures of **1** and **2.** (b, c) Thermally induced strand cleavage of 5′-Texas Red-labeled 993-bp DNA fragment alkylated by polyamide **1**. (b) DNA fragments derived from 5′-end-labeled coding strand (pQBI63) and (c) 5′-end-labeled noncoding strand (pQBI63) are shown. These two DNA fragments are complementary. Lanes 1−5 and 6−10: 0, 5, 10, 30, 50 nM **1**, respectively; G, C, T, A, lanes contain Sanger-sequencing products.

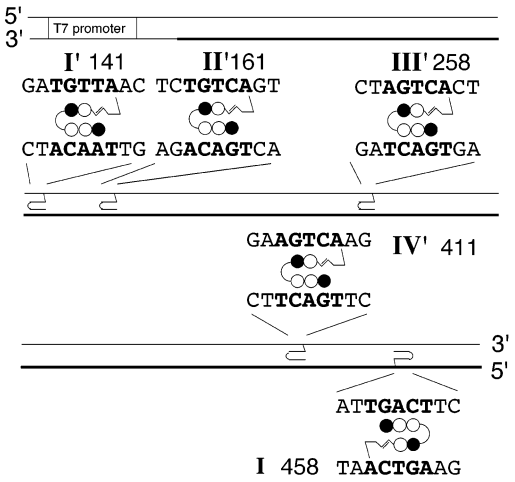

Figure 2. Sites of alkylation by **1** in 5′-Texas Red-labeled 993-bp DNA fragment. GFP-coding region containing DNA sequence and schematic presentation of binding polyamide **1**, together with the locations of the target sequences (in boldface). The filled and open circles represent Im and Py rings, respectively, and the curved line represents γ-aminobutyric acid. The numbers indicate the bases from the T7 promoter in this sequence. The bottom strand is the coding strand of GFP. The coding sequence of GFP is shown by a boldface line.

and Im−L.[12] Densitometric analysis of the bands of thermally cleaved DNA indicates that the efficiency of alkylation by **1**, which is the amount of DNA cleavage divided by that of the agent, was 10% at 30 nM. The ring-opened polyamide **2**, which was derived from **1** by hydrolysis and was purified by HPLC, lacks the ability to alkylate A or G in DNA. Polyamide **2** was characterized by ESIMS and HPLC. A concentration of 50 nM of **1** was sufficient to alkylate all the 5′-Texas Red-labeled coding strand. The sites of DNA alkylation are summarized in Figure 2.

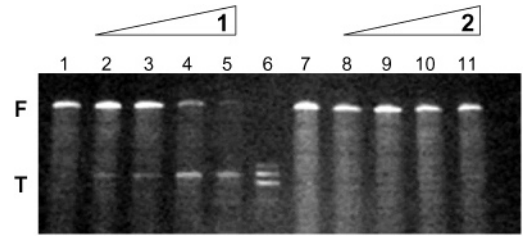

Figure 3. Effects on transcription of alkylation by polyamide. PAGE (6%) analysis of in vitro transcription of the GFP-coding region shows full-length and truncated mRNAs. Lanes 1−5: 0, 5, 10, 30, 50 nM **1**. Lane 6: authentic RNA markers 493, 463, 440 nt. Lanes 7−11: 0, 5, 10, 30, 50 nM **2**. F, full-length GFP mRNA; T, truncated GFP mRNA.

To examine the effects of **1** on transcription, the transcripts generated by T7 RNA polymerase in the presence of **1** were examined. After 1-h incubation, the products were analyzed by 6% PAGE. As shown in Figure 3, truncated mRNA was produced in addition to full-length mRNA in a concentration-dependent manner (lanes 2−5). The size of this truncated RNA was estimated to be 453−463 nt according to molecular markers of 493, 463, and 440 nt (lane 6). The length of the truncated mRNA was consistent with the alkylated site **I** (458 nt) on the template DNA (Figure 2). Furthermore, inhibition of full-length mRNA synthesis was apparent in the presence of 50 nM **1** (Figure 3, lane 5). Densitometric analysis indicated that the formation of full-length mRNA was 99% inhibited, thus allowing a truncated mRNA yield of 78%. These results clearly demonstrate that the inhibition of transcription by **1** occurred at the alkylated site within the coding region of GFP. Importantly, hydrolyzed **2** did not produce any truncated mRNA, nor did it significantly retard transcription: >80% formation of full-length mRNA was observed at 50 nM (Figure 3, lane 11). These results are consistent with the fact that polyamide lacking alkylating ability failed to inhibit the transcription of histone-free DNA by T7 RNA polymerase.[10] Low concentrations of alkylating polyamide **1** bound to the coding region are sufficient to inhibit transcription. In clear contrast, no inhibition of transcription was observed on the noncoding strand even though it had four alkylated sites. These results clearly indicate that alkylating polyamide directly inhibits transcription by T7 RNA polymerase and that this is not simply because alkylation prevents the melting of duplex DNA.

Effective DNA alkylation may be a promising technique with which to design new types of gene-regulating agents. We are currently investigating the regulation of GFP gene expression in living cells. Preliminary data on antitumor activity using a panel of 39 human cancer cell lines demonstrate that **1** has remarkably strong and unique biological activity against several cell lines. This paves the way for the development of "tailor-made antitumor agents" using sequence-specific alkylating Py−Im polyamides.

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