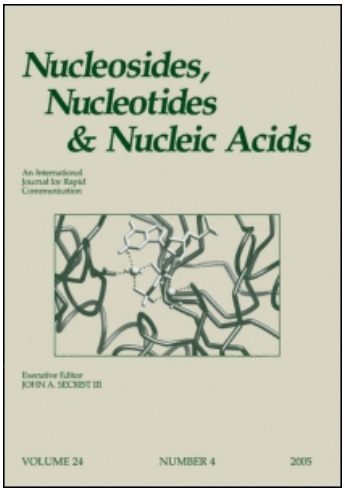

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A Nano-Circular Single-Stranded DNA as a Novel Tool for SNPs Detection

Tatsuo Ohmichi[ab]; Aki Takashima[c]; Naoki Sugimoto[ac]
[a] Frontier Institute for Biomolecular Engineering Research (FIBER), Konan University, Kobe, Japan [b] I.S.T Corporation, Shiga, Japan [c] Department of Chemistry, Faculty of Science and Engineering, Konan University, Kobe, Japan

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A NANO-CIRCULAR SINGLE-STRANDED DNA AS A NOVEL TOOL FOR SNPs DETECTION

Tatsuo Ohmichi ▫ *Frontier Institute for Biomolecular Engineering Research (FIBER), Konan University, Higashinada-ku, Kobe, Japan and I.S.T. Corporation, Shiga, Japan*

Aki Takashima ▫ *Department of Chemistry, Faculty of Science and Engineering, Konan University, Higashinada-ku, Kobe, Japan*

Naoki Sugimoto ▫ *Frontier Institute for Biomolecular Engineering Research (FIBER), Konan University, Higashinada-ku, Kobe, Japan and Department of Chemistry, Faculty of Science and Engineering, Konan University, Higashinada-ku, Kobe, Japan*

▫ *Analysis of single nucleotides polymorphisms (SNPs) is very important for the elucidation of gene-based physiological differences. For high-throughput applications, detection systems need to be highly selective, highly sensitive, and simple. In this study, we investigated whether synchronous transcription and translation from nano-circular single-stranded DNA (ssDNA) might be useful for the detection of SNPs. A nano-circular probe ssDNA was designed to contain codons for a $(His)_6$ peptide, and the sense mRNA transcribed from this ssDNA contains a Shine-Dalgarno sequence, a start AUG codon, 6 His codons (CAU), and a stop UAG codon. The entire circular ssDNA is 55 nt and contains T_{20} as a linker sequence and a binding site for the target SNP-containing DNAs. Our results that show SNPs can be detected by the cell-free synthesis of the $(His)_6$ peptide from this ssDNA. Because this method allows sequence distinction, signal amplification, and easy detection in one system, it should improve the efficacy of high-throughput gene analysis.*

Keywords SNPs, Nano-Circular DNA, Cell-Free Peptide Synthesis, MALDI-TOF Mass

INTRODUCTION

Single nucleotides polymorphisms (SNPs) are very important for elucidation of gene-based physiological differences, such as individual susceptibility to diseases and responses to drugs. To detect SNPs, hybridization on arrays and polymerase-mediated single-base primer extension (mini-sequencing) have been developed using chemical

Address correspondence to Naoki Sugimoto, Professor of Chemistry, Faculty of Science and Engineering, Konan University, 8-9-1 Okamoto, Higashinada–ku, Kobe, Japan; Fax: +81-78-435-2539; E-mail: sugimoto@konan-u.ac.jp

approaches.[1–3] However, when the mismatch between a target and probe DNA on the array is stable, the selectivity of hybridization is low. Also, it is not easy to distinguish the molecular weight of a single-base primer extension, which causes low sensitivity. Thus, improved selectivity, sensitivity, and simplicity are needed for the detection of SNPs. Here we describe a novel approach to detect SNPs by synchronous translation and transcription of an oligopeptide from a nano-circular single-stranded DNA (ssDNA) in a cell-free system. This nano-circular ssDNA contains codon sequences for a $(His)_6$ peptide as well as a binding site for target DNAs containing SNPs. This strategy allows for high selectivity, high sensitivity, and simple detection of SNPs.

MATERIALS AND METHODS

Material Preparations

All DNA oligonucleotides were synthesized on solid supports using standard β–cyanoethyl phosphoramidite methods on an Applied Biosystems (ABI) model 391 DNA/RNA synthesizer. The 5′-phosphorylated 55-nt linear DNA was synthesized using a commercially available phosphoramidite reagent. The synthesized DNA oligonucleotides containing 5′-end dimethoxytrityl groups were removed from the solid support, and base blocking groups were removed by treatment with 25% ammonia at 55°C for 8 h. After drying in vacuum, the DNA oligonucleotides were passed through a Poly-Pak cartridge (Glen Research Co., Ltd.) with 2% trifluoroacetic acid (TFA) to remove the 5′-end dimethoxytrityl groups. After deblocking operations, the DNA oligonucleotides were desalted with a C-18 Sep-Pak cartridge column (Waters). The DNA oligonucleotides were purified by reverse-phase high-performance liquid chromatography (HPLC) on a TSKgel Oligo DNA RP column(Tosoh) with a linear-gradient of 0 to 50% methanol/H_2O containing triethylammonium acetate (pH 7.0). The final purity of the DNA oligonucleotides was confirmed to be > 99% by HPLC. The purified DNA oligonucleotides were desalted again with a C-18 Sep-Pak cartridge before use.

Single-strand concentrations of the DNA oligonucleotide were determined by measuring the absorbance at 260 or 280 nm at high temperature. Single-strand extinction coefficients were calculated from mononucleotide and dinucleotide data using a nearest-neighbor approximation.[4]

The purified DNAs were used in the ligation-cyclization reactions. A sequential one-step enzymatic ligation method using T4 DNA ligase and the corresponding 24-nt DNA splints was used to construct all of the circles. The ligation conditions were as follows: 1 μM template DNA for cyclization, 2 μM splint DNA, 0.1 U/μL ligase (US Biochemicals) in 50 mM Tris-HCl (pH 7.5) containing 10 mM $MgCl_2$, 10 mM dithiothreitol, and 100 μM ATP. The reaction mixture was incubated at 25°C for 1 h. The circular products were isolated by preparative denaturing

polyacrylamide gel electrophoresis (PAGE). The characterization of the circles was carried out as described previously[5] by nicking with S1 nuclease because the products nicked with S1 nuclease migrate at the same positions as the linear full-length precursor.

Transcription and Translation Reactions

Conditions for the rolling circle transcription reaction were as follows: 1 μM circular DNA, 2 U *E. coli* RNA polymerase holoenzyme or 25 U T7 RNA polymerase, 0.5 mM rNTP (ATP, CTP, GTP, and UTP) in 25 mM Tris-HCl (pH 8.1) containing 20 mM NaCl, 15 mM $MgCl_2$, 0.4 mM spermine-HCl, 100 μg/mL acetylated bovine serum albumin, 10 mM dithiothreitol, and 12.5 U/mL RNase inhibitor in a total reaction volume of 15 μL. The reaction mixture was incubated at 37°C for 2 h, and the reaction was stopped by the addition of 1 volume of stop solution (30 mM EDTA, 8 M urea). The products were analyzed by 8% PAGE.

The *E. coli* T7 S30 extract system (Promega) was used for the transcription and translation of the peptide. After template ligation with T4 DNA ligase, the crude DNAs were used for transcription and translation without any purification. The transcription and translation was carried out for 2 h at 37°C in a 25-μL reaction containing 3 μM crude template DNA, 100 μM amino acid mixture, and 7.5 U *E. coli* S30 extract. After terminating the reaction by freezing, the products were purified using a HiTrap™ Chelating HP column (Amersham Pharmacia Biotech). After washing column with phosphate-buffered saline (137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, and 1.47 mM KH_2PO_4 [pH7.4]) and 5 mM imidazole, the products were eluted with 200 mM imidazole. The eluted solution was separated into 1-mL fractions.

Product Characterization

The product peptide was characterized using matrix-assisted laser-desorption-ionization time-of-flight (MALDI-TOF) mass spectroscopy. A matrix solution of α-cyano-4-hydroxycinnamic was prepared at a concentration of 10 mg/mL in 497:3:500 (v/v) water/98% trifluoroacetic acid (TFA)/acetonitrile. For the MALDI analysis, the sample was dialyzed against water using a membrane filter, dried under vacuum, and diluted with 100 μL water. The dissolved sample was mixed at a 4:1 (v/v) ratio with the matrix solution. The MALDI/TOF-MAS analysis was performed on a Voyager-DE RP MALDI/TOF instrument (PerSeptive Biosystems) equipped with a nitrogen laser, a single-stage reflector, and a timed selector. The spectra were obtained in the positive-ion reflector mode with delayed extraction using standard conditions. The acceleration voltage was 20 kV, and the delay time (time between the laser pulse and the acceleration voltage pulse) was 100 ns.

RESULTS AND DISCUSSION

Designing Detection System

We constructed the system as shown in Figure 1 to satisfy the three key factors for distinguishing SNPs: 1) high selectivity, 2) high sensitivity, and 3) simple detection. For high selectivity, we used ligation of circularizable oligonucleotide ends after hybridization with a target DNA. In this system, a full match at the ligation site, such as the G–C base pair depicted in Figure 1, results in successful circularization of the probe DNA. On the other hand, when there is a single base pair mismatch at either side of the ligation junction, such as the T-C base pair shown in Figure 1, the probe DNA is not circularized with DNA ligase.

For high sensitivity, we amplified the signal by using rolling-circle transcription, which produces long RNAs with repeats of the sequence encoded by the circular DNA. Even small circular ssDNAs without a promoter sequence can be efficiently transcribed by bacterial and phage RNA polymerases.[5–9] The main requirement for successful rolling-circle transcription is the formation of a nano-circular ssDNA. Thus, as shown in Figure 1, the production of a long repeating RNA would result in the amplification of circularized ssDNA after recognizing the G–C match.

For simplicity of detection, we designed synchronous oligopeptide synthesis from the long repeating RNAs using a cell-free translation system. We suspected that a nano-circular ssDNA might be designed to encode sequences for an oligopeptide, and if it were transcribed and translated successfully, it would generate an oligopeptide. Such an oligopeptide can be easily detected by MALDI-TOF mass spectrometry, which is simpler and more sensitive than gel electrophoresis and recently has been shown to be useful in a high-throughput system.[10] Moreover, this synchronous translation in a cell-free system is expected to produce a large amount of oligopeptide because of the multiple translations from

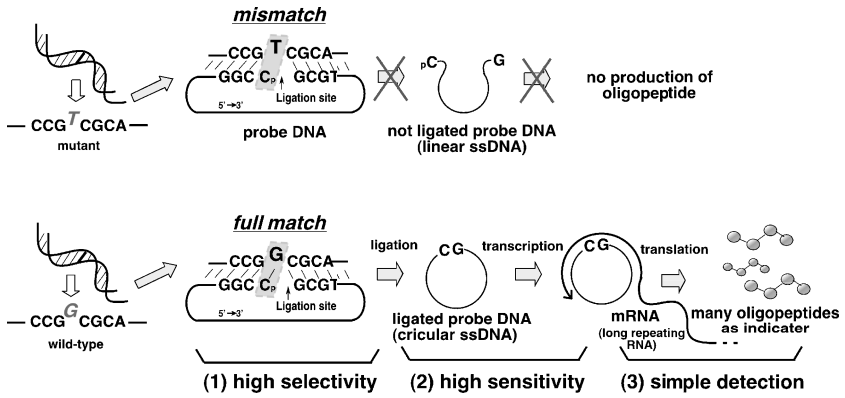

FIGURE 1 Schematic illustration of indicator oligopeptide synthesis for detection of SNPs using our synchronous system.

the long RNAs with repeated sequences. Thus, as illustrated in Figure 1, when there is mismatch in the hybridization step, there would be no oligopeptide detected. On the other hand, when the hybridization does not contain a mismatch, synchronous oligopeptide synthesis from the nano-circular ssDNA would be observed.

Effect of Hybridization Length on Selectivity

To determine whether it is possible to detect the SNPs using rolling cell-free peptide synthesis, we first used 14-, 16-, and 18-mer target DNAs (Figure 2a) to examine the effect of the length of hybridization on the selectivity of ligation. A 55-nt ssDNA probe was designed to encode an mRNA containing the Shine-Dalgarno sequence (rAGGAAACAGCU), which is a binding sequence for rRNA within the *E. coli* ribosome; a start AUG codon; codons for the indicator His_6 oligopeptide; a stop UAG codon; and T_{20} as a linker sequence. This probe does not, however, encode known RNA polymerase promoter sequences. The 14-, 16-, and 18-mer target DNAs were designed to form 7-, 8-, and 9-mer duplexes with or without one mismatch at the junction site.

Figure 2b shows the denaturing PAGE of the ligation products using 2-μM target DNA and 1 μM DNA probe. We found that the ligation yield of full-matched base pairings (G–C) at the junction site of an 18-mer target DNA was 56.8%. However, the 18-mer DNA with a C–T mismatch also showed the ligated band. When the hybridization length with a C–T mismatch at the junction site was

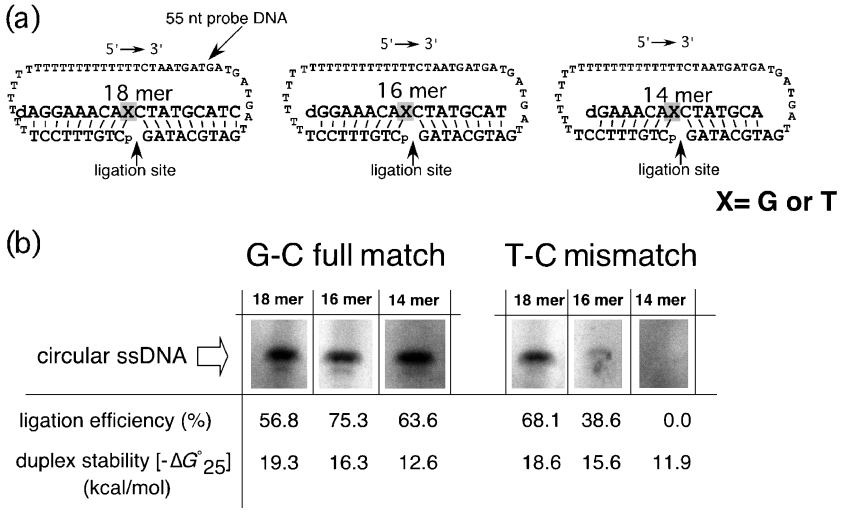

FIGURE 2 (a) Schematic illustration of the probe and linear target DNAs used for detection single mismatch. The X in the 18-, 16-, and 14-mer target DNAs shows the nucleotide designed to detect the single mismatch. In the case of a full match, X = G. On the other hand, when X = T, it is a mismatch. (b) Denaturing 10% PAGE showing the ligation efficiency of template DNAs using 18-, 16-, and 14-mer target DNAs. G and T correspond to a full match and a mismatch, respectively.

14-mer, the ligated band disappeared. These data indicate that long hybridization between the target and probe DNAs is not useful for SNP detection.

To investigate the relationship of the thermodynamic stability of hybridization and the ligation efficiency, we predicted the thermodynamic stability between the target and probe DNA using the nearest neighbor model.[11–16] As shown in Figure 2b, the value of ΔG°_{25} for the 18-nt DNA with a C–T mismatch is −11.9 kcal/mol, and it is the most unstable of all of the target DNAs analyzed. Thus, the results suggest that the ligation efficiency is controlled by the stability of hybridization between the target and probe DNA.

Transcription Reactions from Nano-Circular ssDNA

Next, we investigated whether the 55-nt oligopeptide-encoding circular DNA could be transcribed with T7 RNA polymerase. Reaction for 1 h at 37°C with T7 RNA polymerase generated long single-stranded RNAs (Figure 3). The observed band migrated as a smear between 1500-and 4000-nt single-stranded RNA markers. The product RNA corresponded to almost 70 end-to-end repeats of the RNA encoded by the circular ssDNA. In contrast, the linear DNA did not result in the production of RNA. These results indicate that the nano-circular ssDNA produces a long mRNA with repeating coding sequences that have the potential to synthesize a large amount of peptide.

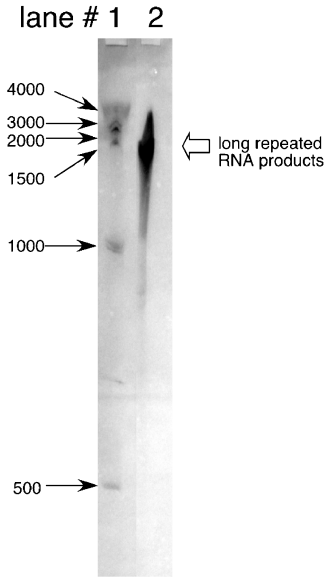

FIGURE 3 Denaturing 8% PAGE showing the product RNA transcribed from the DNA template by T7 RNA polymerase. Lane 1 shows the RNA size markers, and lane 2 shows the multiple RNA transcribed from the circular ssDNA template by T7 RNA polymerase.

Detection of SNPs by $(His)_6$ Peptide Synthesis

Finally, we examined the abilities of the circular and linear ssDNAs to produce the $(His)_6$ peptide as detected by MALDI-TOF mass spectrometry. Following ligation between the 14-mer target DNA and the 55-nt probe DNA (unpurified except for desalting by ethanol precipitation) using T4 DNA ligase, the reaction mixture was transcribed and translated using *E. coli* T7 S30 extract. Next, after isolating the products using a His-trap chelating column, the final products were identified by MALDI-TOF mass spectrometry. A peak was not observed in the reaction containing a combination of target DNA with a C–T mismatch and the probe DNA, indicating that the linear ssDNA cannot produce the $(His)_6$ oligopeptide (Figure 4a). In contrast, the combination of target DNA with the G–C full match and the probe DNA showed a single peak (Figure 4b) corresponding to the size of chemically synthesized $(His)_6$ peptide. Thus, this system can clearly distinguish a single mismatch using oligopeptide synthesis from the nano-circular ssDNA in a cell-free system. Because the nano-circular ssDNA can produce and amplify not only the $(His)_6$ peptide described in this report but also any other oligopeptide, this method has the potential to selectively, sensitively, and simply

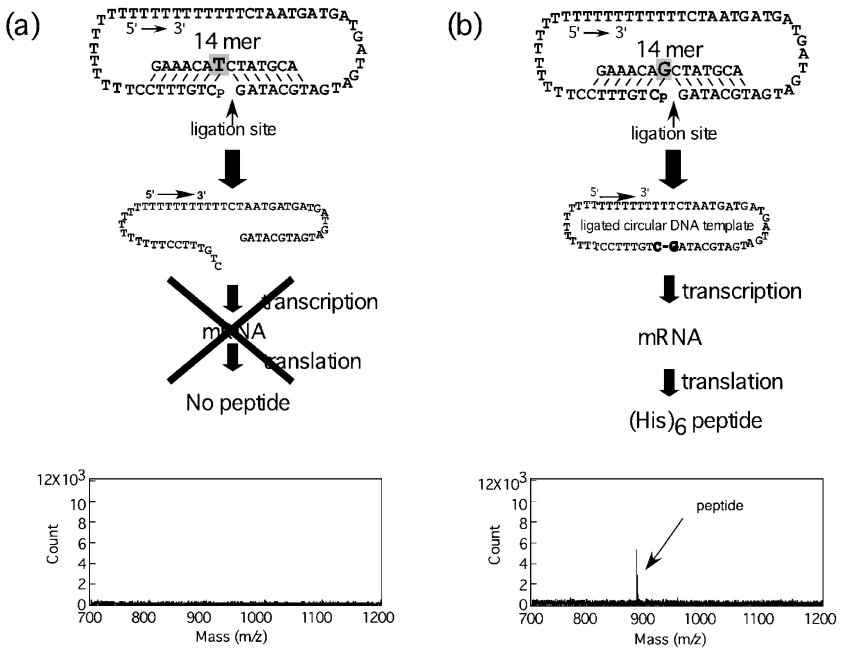

FIGURE 4 MALDI-TOF mass spectra of the products from a full match and mismatch at a ligation site between the probe and the target DNA. The illustration also shows the sequence of events for each condition. (a) In the case of a CT mismatch at the 5′-end of the probe DNA, there is no signal on the MALDI-TOF mass spectru. (b) In the case of the full-match (GC base pair) at the 5′-end of the probe DNA, MALDI-TOF mass spectrum showed one peak corresponding to the $(His)_6$ peptide.

detect any SNPs. These attributes should allow the system to be applied to high-throughput genetic screening.

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