Stable Triple-Stranded DNA Formation and its Application to the SNP Detection

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Abstract

We have found that a short stretch (30mer or larger) of triple-stranded DNA structure formed at the terminus (or very near) of linear DNA molecules is unusually stable, withstanding heat treatment at as high as 95°C. The stable triple-stranded structure is formed only when deoxyoligonucleotides are complementary to the strand terminating with 5′-phosphate and not to the strand terminating with 3′-OH. Presence of a single mismatched base in the complementary deoxyoligonucleotides drastically reduces the stability. We show that these unique properties of the terminal triple-stranded structure can be applied to the detection of single nucleotide polymorphisms in genomes without DNA dissociation and/or hybridization.

Key words: RecA protein; stable triple-stranded DNA; deoxyoligonucleotide probe; SNPs detection

1. Introduction

Triple-stranded DNA has been generating increased interest not only because of its unique structural characteristics and possible biological significance but also because of its potential utility as a tool for DNA analysis.1–3 There have been several studies reporting the use of triple-stranded DNA as a tool to cleave specific sites in DNA molecules, to knock out specific genes and to correct mutations.4–15 Triple-stranded DNA structure particularly with short deoxyoligonucleotides has generally been considered unstable after removal of the protein.16 During the course of studies on RecA-mediated triple-stranded DNA formation with complementary deoxyoligonucleotides (40 to 120mer), we found that triple-stranded structures at the terminus (or near terminus) of double-stranded DNA is unusually stable against heat, provided that the sequence of the single strand deoxyoligonucleotide is complementary to the 5′ terminal sequence of the double-stranded DNA. This heat stability is drastically increased, to levels even higher than that of double-stranded DNA, when excess complementary deoxyoligonucleotides are present in the reaction mixture. Furthermore, we have found that the heat-stable terminal triple-stranded structures are formed with completely matched complementary deoxyoligonucleotides, but not with those carrying a single mismatched base regardless of its position.

In this manuscript, we describe the basic characteristics of terminal triple-stranded DNA, and show that our findings can be applied to single nucleotide polymorphism (SNP) detection without DNA dissociation and/or hybridization.

2. Materials and methods

2.1. Materials

M13mp18RF DNA and pBR322 DNA were purchased from Takara-Bio (Shiga, Japan). pBR322 DNA with a substituted base was prepared by PCR using primers (40mer), one of which contained a mismatched base, and Scal pBR322 DNA fragment as a template. The PCR products were purified by agarose gel (1%) electrophoresis and DNA was eluted from the corresponding band using a DNA extraction kit (QIAGEN, Hilden, Germany). Deoxyoligonucleotides (HPLC purified grade) were custom-synthesized by Sawady Technology (Tokyo, Japan). Deoxyoligonucleotides were labeled at 5′

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terminus with T4 polynucleotide kinase and \(^{32}\)P-ATP using a kit (MEGALABEL\textsuperscript{TM} Labeling kit, Takara-Bio). RecA protein was purchased from Epicenter Technologies (Madison, USA). ATP-\(\gamma\)S and proteinase K were obtained by Roche Diagnostics (Mannheim, Germany).

### 2.2. Standard reaction for the triple-stranded DNA formation

A reaction mixture (50 \(\mu\)l) containing a deoxyoligonucleotide complementary to target DNA (5 pmol), RecA protein (30 \(\mu\)g), ATP-\(\gamma\)S (4.8 mM), Mg-acetate (2 mM) and Tris–acetate (30 mM, pH 7.2) was first incubated for 5 min at 37°C. The mixture was then combined with a mixture (50 \(\mu\)l) containing target DNA (10 \(\mu\)g), Mg-acetate (20 mM) and Tris–acetate (30 mM, pH 7.2) and incubated for another 60 min. The reaction was stopped by adding 5 \(\mu\)l SDS (10\%, v/v), 5 \(\mu\)l EDTA (500 mM, pH 8.0) and 0.5 \(\mu\)l proteinase K (22 mg/ml), further incubated for 30 min at 37°C, and subjected to phenol/chloroform treatment. Excess deoxyoligonucleotides were removed through Sephacryl S-400 HR column (Amersham-Bioscience, Uppsala, Sweden) which had been equilibrated with TE buffer (10 mM Tris–HCl pH 7.5, 1 mM EDTA).

### 2.3. Analysis of SNPs in human genomic DNA

The region of 950 bp sequence of human genomic DNA (GenBank accession number AC004465) in which an SNP site of interest is positioned at 40 nt from the terminus in three human genomic DNA preparations was amplified by PCR. The genomic DNA preparations were kindly supplied by Drs Y. Ohnishi and Y. Nakamura, the University of Tokyo. The PCR products, prepared after 25 cycles of PCR using the genomic DNA (200 ng) and PCR primers (GenBank accession number AC004465, nt 2701–2720 and nt 3631–3650), were subjected to triple-stranded DNA formation with a set of labeled deoxyoligonucleotides. The reaction was performed by incubating a reaction mixture (20 \(\mu\)l) which contained amplified DNA (400 ng), a labeled probe (1 pmol), RecA protein (3 \(\mu\)g), ATP-\(\gamma\)S (4.8 mM), Mg-acetate (20 mM) and Tris–acetate (30 mM, pH 7.2) for 60 min at 37°C. After removal of RecA protein and excess deoxyoligonucleotides through a TE buffer equilibrated Sephacryl S-400 HR column (two cycles), samples (20 \(\mu\)l) were incubated at 85°C for 10 min in the presence of a mixture of four cold deoxyoligonucleotides (10 pmol, each). The samples were then subjected to heat treatment at 80°C (85°C for the experiments shown in Figs 5 and 6) for 10 min (25°C for 10 min for the controls). The deoxyoligonucleotides were then removed through Sephacryl S-400 HR column (one cycle), and the samples were subjected to electrophoresis on agarose gel (1%) and autoradiographed.

### 3. Results and Discussion

#### 3.1. The polarity of the third strand in terminal triple-strand formation

We performed an experiment in which M13mp18RF DNA (7249 bp) linearized by digestion with SnaBI was incubated with RecA protein and one of four \(^{32}\)P-labeled 60mer deoxyoligonucleotides (oligo-1, 2, 3 and 4, Fig. 1A). The labeled deoxyoligonucleotide sequences were complementary to the terminal sequences of the double-stranded DNA. After removal of RecA protein, the products were electrophoresed and autoradiographed. Triple-stranded DNA formed with a deoxyoligonucleotide (oligo-1) complementary to the 5’-phosphate terminal sequence (nt 1270–1329) of the double-stranded DNA or that formed with oligo-3 identical to the 3’-OH terminal sequence (nt 1210–1269) displayed signal at the position where linear M13mp18RF DNA was expected to migrate (Fig. 1B, lane 1 or lane 3, respectively). In contrast, no signal was detected when the double-stranded DNA was incubated with oligo-2 identical to the 5’-phosphate terminal sequence (nt 1270–1329) or oligo-4 complementary to the 3’-OH terminal sequence (nt 1210–1269) (Fig. 1B, lane 2 or lane 4, respectively). Similar results were obtained when the terminal sequence of linearized pBR322 DNA or pUC118 DNA was used as a target DNA for the formation of the triple-stranded molecules (data not shown). These results strongly suggest that a triple-stranded structure at the ends of a double-stranded DNA is formed with deoxyoligonucleotides complementary to the 5’-phosphate terminal sequences of both strands, but not with those complementary to the 3’-OH termini, and that the triple-stranded structures thus formed are stable even after the removal of RecA protein. We estimate that 80–90% of the target DNA is converted to triple-stranded structure under the conditions we employed (data not shown).

#### 3.2. Effect of the position of triple-stranded structure relative to the DNA terminus

The stable triple-stranded DNA structure seems to be limited to those formed at the very terminus of double-stranded DNAs. SnaBI digest of M13mp18RF DNA was subjected to triple-stranded DNA formation with labeled deoxyoligonucleotides complementary to different sequences at or near the DNA terminus (Fig. 2A). As seen in Fig. 2B, when the 3’ position of the complementary deoxyoligonucleotides (60mer) was moved from the terminus (oligo-1, lane 1) to 10 (oligo-5, lane 2), 20 (oligo-6, lane 3) and 30 nt (oligo-7, lane 4) to the inside of the DNA molecule, the stability of triple-stranded structures formed was dramatically decreased in proportion to the distance from the DNA terminus. Essentially no stable triple-stranded DNA was formed with a deoxyoligonucleotide complementary to DNA sequences
inwardly located 30 nt from the terminus (Fig. 2B, lane 4). The results indicate that stability of triple-stranded DNA is greatly affected by the position of the triple-strand, with those structures formed closer to the 5' terminus of DNA demonstrating greater stability. As one might expect, we have also found that the stability of triple-stranded DNA is dependent upon the size of the deoxyoligonucleotides employed. Linearized M13mp18RF DNA was subjected to triple-stranded DNA formation with labeled deoxyoligonucleotides (oligo-1 to 4) used for triple-stranded DNA formation. Nucleotide numbers are those registered in GenBank (Accession number X02513). Lane 1, DNA with a deoxyoligonucleotide (oligo-1) complementary to the 60 nt 5' terminal sequence (nt 1270-1329); lane 2, DNA with a deoxyoligonucleotide (oligo-2) complementary to the 3'-OH strand at the same terminus; lane 3, DNA with a deoxyoligonucleotide (oligo-3) complementary to the 60 nt 5' terminal sequence (nt 1210-1269); lane 4, DNA with a deoxyoligonucleotide (oligo-4) complementary to the 3'-OH strand at the same terminus. (C) Ethidium bromide patterns of DNA products. Positions of the size markers (HindIII digested λ DNA fragments) in kb are shown at the left side of each figure. For details, see Section 2.

3.3. Stability of terminal triple-stranded DNA structures

To characterize the stability of terminal triple-stranded structures, triple-stranded DNA formed with M13mp18RF DNA and labeled 60mer deoxyoligonucleotide (oligo-3) was exposed to different temperature (for 10 min) after removal of RecA protein, and radioactivity associated with M13mp18RF DNA was measured. As seen in Fig. 3A, the deoxyoligonucleotide started to dissociate from the complex at 60°C (lane 2), and was completely dissociated at 75°C (lane 5). The estimated melting temperature was ~65°C. The melting temperature of a hybrid structure formed from the same deoxyoligonucleotide (oligo-3) and single-stranded M13mp18 DNA was ~75°C under the same conditions (data not shown). To our surprise, when unlabeled deoxyoligonucleotide (oligo-3) (10-fold excess over the labeled deoxyoligonucleotide) was added during heat treatment, the stability of the formed triple-stranded DNA seemed dramatically increased. At temperatures as high as 95°C, a substantial portion (~50%) of the deoxyoligonucleotide remained undissociated (Fig. 3B, lanes 1–9). No such stabilizing activity was observed when the same amount of unlabeled deoxyoligonucleotides (oligo-8) not complementary to the terminal DNA sequence was added (data not shown). The stabilizing effect of complementary deoxyoligonucleotides on terminal triple-stranded DNA is not fully understood at this time. The results are diagrammatically shown in Fig. 3C.
3.4. Detection of a single mismatched base

We found that the formation of terminal triple-stranded DNA can be used for the detection and identification of a single mismatched base in DNA molecules. The linearized pBR322 DNA (GenBank accession number J01749, 4361 bp) by digestion with ScaI (nt 3847–3846), in which the C–G pair at nucleotide position 3879 within the terminal 60 bp (nt 3847–3906, Fig. 4A, target DNA I) had been replaced to other 3 bp (target DNA II, III and IV), was subjected to triple-stranded DNA formation. Each target DNA was incubated with one of four labeled deoxyoligonucleotides complementary to the 60 nt terminal sequence but having the base substitution at the position corresponding to nt 3879 of the template DNA (Fig. 4A, oligo-8, 9, 10 and 11). In the standard condition, all target DNAs formed stable triple-stranded structures with any combination of deoxyoligonucleotides with or without the base substitution (Fig. 4B, left panels), indicating that the formation of stable triple-stranded molecules was possible even in the presence of a single mismatched base in the oligonucleotides at 25°C. However, when the triple-stranded molecules formed at 37°C were exposed to a higher temperature (80°C, 10 min) in the presence of excess amounts of corresponding unlabeled deoxyoligonucleotides, the triple-stranded structures formed with completely matched complementary oligonucleotides were observed (Fig. 4B, right panels), while incubation of the triple-stranded DNAs with deoxyoligonucleotides having a single mismatched base resulted in complete dissociation of the structures. Essentially, the same results were obtained with triple-stranded DNA with complementary deoxyoligonucleotides in which a single nucleotide (nt 3879) is deleted (Fig. 4A, oligo-12) or an extra nucleotide is inserted (Fig. 4A, oligo-13, an extra C is inserted at nt 3878–3879). The results are shown in Fig. 4D. Figure 4C and E are ethidium bromide patterns of DNA products. Lanes correspond to those shown in (B). Positions of the size markers (HindIII digested λ DNA fragments) in kb are shown at the left side of each figure. For details, see Section 2.

3.5. Effect of the location of mismatched bases

A mismatched base can be detected at almost any location in the terminal DNA sequences within at least
120 bp. Triple-stranded DNA was formed at the terminus of \textit{Sna}BI-treated M13mp18 DNA with labeled 120 bp complementary deoxyoligonucleotides carrying matched (Fig. 5A, oligo-14) and mismatched bases (Fig. 5A, oligo-15 to 23). A mismatched base (G, guanine) was placed at different sites over the 120 bp sequence, including one located at the very terminus (oligo-23). Triple-stranded samples obtained at 37°C were then exposed to high temperature (85°C, 10 min) in the presence of unlabeled completely matched deoxyoligonucleotide. As seen in Fig. 5B, although all triple-stranded structures were stably maintained after incubation at a lower temperature (25°C, 10 min) (Fig. 5B, left panel), deoxyoligonucleotides with a mismatched base were eliminated almost completely from the triple-stranded DNA molecules by exposure to high temperature (85°C, 10 min) (Fig. 5B, right panel). Figure 5C shows the DNA products detected by ethidium bromide staining. Lanes correspond to those shown in Fig. 5B.

3.6. Analysis of SNPs in human genomic DNA

We applied our findings for the analysis of SNPs in human genomic DNA (GenBank accession number AC004465). The region of 950 bp sequences shown in Figure 6A in which an SNP site of interest is positioned at 40 nucleotides from the terminus was amplified by PCR (Fig. 6A). The amplified products from three human genomic DNAs with known allelic variations (C/T, C/C and T/T for human DNA I, II and III, respectively, see Fig. 6A) were then subjected to terminal triple-stranded DNA formation with a set of labeled deoxyoligonucleotides (oligo-24, 25, 26 and 27, see Fig. 6A) which cover the primer sequences and SNP sites, and are complementary to one of the terminal sequence of the DNA product amplified from the genomic DNA template. As seen in Fig. 6B and C, after brief heat treatment of the triple-stranded molecules formed at 85°C, very distinct patterns of the labeled products emerged, clearly distinguishing the three different polymorphisms.

4. Conclusions

In this article, we have shown that stable triple-stranded DNA can be formed at the termini of linear DNA molecules with oligonucleotides complementary to the DNA strand terminating with 5' phosphate. The formed structure is unusually stable, withstanding heat treatment at as high as 95°C when excess completely matched oligonucleotides are present. In this respect, it should be noted that Radding and his co-workers reported that the stability of the triple-stranded DNA formed over a long stretch of complementary bases (more than a thousand bases) is affected by the polarity of the third strand.\textsuperscript{17–19} At present, neither the specific molecular structure of terminal triple-stranded DNA nor the mechanism of its stability is clearly understood. Although we have faced difficulties in obtaining sufficient amounts of terminal triple-stranded DNA for structural analysis by means of CD spectrum or NMR or X-ray crystallography, this line of experiments is currently under way. If successful, the information obtained should provide vital clues to the interesting phenomena associated with terminal triple-stranded DNA structure.

The observation that the terminal triple-stranded structure which withstands at high temperatures is formed only with completely matched complementary
Figure 4. Detection of a single mismatched base. PBR322 DNA fragments (nt 3847–3846, 4361 bp) (200 ng), in which a single base within the terminal 60 bp (nt 3879) had been substituted by different bases (target DNA I, II, III and IV), were subjected to triple-stranded DNA formation with four labeled complementary deoxyoligonucleotides (nt 3847–3906, 60mer), each with a corresponding mismatched base (oligo-8, 9, 10 and 11). The DNA samples (20 µl) were incubated at 80°C in the presence of unlabeled completely matched deoxyoligonucleotide (10 pmol) (target DNA I with oligo-8, target DNA II with oligo-9, target DNA III with oligo-10 and target DNA IV with oligo-11). (A) Diagrammatic representation of target DNA (I, II, III and IV) in which a single mismatched base is introduced (top panel) and corresponding deoxyoligonucleotides used for triple-stranded DNA formation (bottom panel). Nucleotide numbers are those registered in GenBank (Accession number J01749). (B) Autoradiographic patterns of DNA samples with triple-stranded structure carrying a mismatched base. Left panel: after incubation at 25°C for 10 min. Right panel: after incubation at 80°C for 10 min. Deoxyoligonucleotides used for triple-stranded DNA formation are shown at the top of each lane. Positions of the size markers (HindIII digested λ DNA fragments) in kb are shown at the left side of each figure. (C) Ethidium bromide patterns of DNA products. Lanes correspond to those shown in (B). (D) Autoradiographic patterns of DNA samples with triple-stranded structure carrying a deleted or extra nucleotide. Target DNA I with triple-stranded structure formed with labeled oligo-8 (control), oligo-11 (mismatched), oligo-12 (deleted) or oligo-13 (inserted) was incubated in the presence of unlabeled oligo-8 (10 pmol) for 10 min at 80°C, subjected to electrophoresis on agarose gel (1%) and autoradiographed using X-ray film. Left panel: after incubation at 25°C. Right panel: after incubation at 80°C. (E) Ethidium bromide patterns of DNA products. Lanes correspond those shown in (D). For details, see Section 2.
Figure 5. Effect of the position of mismatched bases. M13mp18 SnaBI fragments (nt 1270–1329, 60 bp) (200 ng) were subjected to terminal triple-stranded DNA formation using a series of labeled complementary deoxyoligonucleotides (nt 1270–1389, 120 mer) with a single mismatched base (guanine) at different positions (oligo-14, 15, 16, 17, 18, 19, 20, 21, 22 and 23). After removal of RecA protein as well as excess deoxyoligonucleotides through a Sephacryl S-400 spin column, samples (20 µl) were incubated at 85°C in the presence of unlabeled completely matched deoxyoligonucleotide (oligo-14) (10 pmol), subjected to electrophoresis on agarose gel (1%) and autoradiographed using X-ray film. (A) Diagrammatic representation of M13mp18 SnaBI DNA fragment (top panel) and corresponding deoxyoligonucleotides (oligo-14 to 23) containing a mismatched base, as used for triple-stranded DNA formation (bottom panel). The exact positions of the mismatched bases are as follows. Oligo-14; no mismatched base, oligo-15; a mismatched base at nt 1280 (thymine is replaced by guanine), oligo-16; a mismatched base at nt 1290 (cytidine is replaced by guanine), oligo-17; a mismatched base at nt 1300 (cytidine is replaced by guanine), oligo-18; a mismatched base at nt 1310 (adenosine is replaced by guanine), oligo-19; a mismatched base at nt 1330 (adenosine is replaced by guanine), oligo-20; a mismatched base at nt 1350 (adenosine is replaced by guanine), oligo-21; a mismatched base at nt 1370 (cytidine is replaced by guanine), oligo-22; a mismatched base at nt 1378 (cytidine is replaced by guanine) and oligo-23; a mismatched base at nt 1389 (cytidine is replaced by guanine). Nucleotide numbers are those registered in GenBank (Accession number X02513). (B) Autoradiographic patterns of DNA samples after incubation at 25°C (left panel) and 85°C (right panel). (C) Ethidium bromide patterns of DNA products. Lanes correspond those shown in (B). For details, see Section 2.
Figure 6. Analysis of SNPs in human genomic DNA preparations. Sequences (950 bp) covering an SNP site in three human genomic DNA preparations were amplified by PCR. The PCR products were subjected to triple-stranded DNA formation with a set of labeled deoxyoligonucleotides (oligo-24, 25, 26 and 27) which cover the SNP site and are complementary to one of the terminal sequence of the genomic DNA. (A) Diagrammatic representation of DNA sequences with an SNP site (human DNA I, II and III) in which polymorphic bases with allelic variations are indicated (top panel) and deoxyoligonucleotides (60mer covering the SNP site, oligo-24, 25, 26 and 27) used for triple-stranded DNA formation (bottom panel). Nucleotide numbers are those registered in GenBank (Accession number AC004465). (B) Autoradiographic patterns of DNA samples after heat treatment at 25°C for 10 min. (C) Autoradiographic patterns of DNA samples after heat treatment at 85°C for 10 min. Throughout (B) and (C), lanes 1–4, human DNA I; lanes 5–8, human DNA II; and lanes 9–12, human DNA III. Positions of the size markers (HindIII digested λ DNA fragments) in kb are shown at the left side of each figure. For details, see Section 2.
and capacity to identify mismatched bases. Needless to say that as the signals obtained with completely matched oligonucleotides are very clear this procedure can be simplified and be higher throughput by combining with other techniques. Efficient and reliable methods of detecting single nucleotide substitutions in DNA molecules are now in great demand, especially for the analysis of SNPs in higher organisms such as humans. We believe that all the procedures described above based upon the unique nature of the terminal triple-stranded DNA, direct probing and detection of single base substitutions should make current DNA technologies to be more efficient and be utilized in a more versatile manner.

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