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A Non-radioactive DNA Sequencing Method Using Biotinylated Dideoxynucleoside Triphosphates and ΔTth DNA Polymerase

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Abstract

We synthesized a set of four biotinylated dideoxynucleoside triphosphates (biotin-9-ddNTPs) and optimized the reaction conditions for non-radioactive cycle sequencing using modified Tth DNA polymerase (ΔTth) and a chemiluminescent detection system. The resulting sequencing ladders showed lower background compared to those with the conventional non-radioactive sequencing method which uses 5′-biotinylated primers, especially when PCR products were analysed. With our method, DNA sequences can be determined at any primer positions without preparing 5′-biotinylated primers for dideoxy chain-termination.

Key words: non-radioactive DNA sequencing; cycle DNA sequencing; thermostable DNA polymerase; biotinylated dideoxynucleoside triphosphate

1. Introduction

Dideoxy DNA sequencing has become a widely used method for DNA sequencing, and non-radioactive dideoxy sequencing methods using biotinylated primers and the chemiluminescent detection system has been developed.[1,2] However, one limitation of the previous non-radioactive method is that one has to prepare 5′-biotinylated primers for each sequencing reaction. It would seem to be more convenient if a DNA sequencing method using biotin-dideoxynucleoside triphosphates can be developed.

We have previously cloned a thermostable DNA polymerase gene from *Thermus thermophilus* HB8[3,4] and isolated a deletion mutant polymerase (ΔTth) lacking the 5′-3′exonuclease activity (to be published). This enzyme works well with the dideoxy DNA sequencing method, and has enough thermostability to perform cycle DNA sequencing. In this report, we synthesized a set of four biotin-9-ddNTPs and established the sequencing conditions which use four biotin-9-ddNTPs, ΔTth DNA polymerase and the chemiluminescent detection system.

2. Materials and Methods

2.1. Enzymes and chemicals

ΔTth DNA polymerase, M13mp18 single-stranded DNA (M13mp18 ssDNA), 2′-deoxy-, and 2′,3′-dideoxynucleoside 5′-triphosphates (dNTPs and ddNTPs) used were the products of Toyobo Co. (Osaka, Japan). Oligonucleotide primers for DNA sequencing and PCR were synthesized on a DNA synthesizer (Model 392, Applied Biosystems, USA). 7-Deaza-dGTP, dITP, and dUTP were obtained from Boehringer Mannheim (Germany). Other chemicals were of reagent grade obtained from commercial sources.

2.2. PCR amplification of DNA gyrase gene (gyrA)

A single colony of *Escherichia coli* HB101 harboring pGYRA was suspended in 10 μl of H_2O, heat denatured at 95°C for 5 min and centrifuged at 12,000 rpm for 3 min. The supernatant was added to a reaction mixture (100 μl) containing 20 pmol of each primer (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′, 5′-CGGATAACAATTTCACACAGGAAAC-3′), 200 μM each dNTPs, 2.5 units ΔTth DNA polymerase, 50 mM KCl, 10 mM Tris-HCl (pH 8.3) and 1.5 mM $MgCl_2$. After an initial denaturaion at 94°C for 5 min, the reaction mixture was incubated for 30 cycles of 30 sec at 94°C, 1 min at 60°C and 2 min at 72°C using the Thermal Cycler Model 480 (Perkin-Elmer, USA). Amplified products were purified and concentrated to 20 μl by a Centricon-100 (Amicon, USA) at 500 rpm for 30 min. Five microliters of concentrated DNA solution (approximately 250 fmol) was used for the sequencing reaction. Plasmid pGYRA containing the DNA gyrase gene of *Neisseria gonorrhoeae* was donated by Dr. Deguchi.

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2.3. *Sequencing reaction using biotin-9-ddNTPs*

Unless otherwise noted, 250 fmol of M13mp18 ssDNA or PCR product was added to the reaction mixture (17 μl) containing 5 pmol of primer (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′), 50 mM Tris-HCl (pH 8.8), 1.5–4 mM $MgCl_2$, 10 μM each set of four dNTPs (7-deaza-dGTP, dATP, dTTP and dCTP) and 4 units ΔTth DNA polymerase. Aliquots (4 μl) were added to 4 tubes each containing 2 μl of either 60 μM biotin-9-ddGTP, 90 μM biotin-9-ddATP, 900 μM biotin-9-ddUTP or 600 μM biotin-9-ddCTP. The mixture was incubated for 30 cycles of 30 sec at 95°C, 30 sec at 60°C and 2 min at 72°C using the Thermal Cycler Model 480 (Perkin-Elmer, USA). The reaction was terminated by 4 μl of stop solution (deionized formamide containing 0.1% bromophenol blue, 0.1% xylene cyanol and 20 mM disodium EDTA, pH 7.0).

2.4. *Sequencing reaction using 5′-biotinylated primers*

The reaction was carried out under conditions similar to those used for biotin-9-ddNTPs. 250 fmol of M13mp18 ssDNA or PCR product was added to the reaction mixture (17 μl) containing 5 pmol primer (5′-biotin-CGCCAGGGTTTTCCCAGTCACGAC-3′), 50 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 10 μM of each dNTP (7-deaza-dGTP, dATP, dTTP and dCTP) and 4 units ΔTth DNA polymerase. Aliquots (4 μl) were added to 4 tubes each containing 2 μl of 60 μM ddGTP, 90 μM ddATP, 900 μM ddTTP or 600 μM ddCTP. Then the mixture was incubated as in the above section.

2.5. *Other methods*

The sequencing reaction products (2 μl each) were electrophoresed at 30 W for 2 hr on 8% polyacrylamide gel, and blotted onto a dryed, positive-charged nylon membrane (Imaging High, Toyobo) according to the manufacturer's instructions. The chemiluminescent detection reaction was performed using a horizontal rolling apparatus (Rolling Mixer, Toyobo) at room temperature according to the manufacturer's instructions.

3. Results and Discussion

3.1. *Synthesis of biotin-9-ddNTPs*

Biotin-9-ddNTP was synthesized by a previously reported method using fluorescence-tagged ddNTP[5] with some modification. 5-Halogeno-2′,3′-dideoxypurine or -pyrimidine was coupled to *N*-trifluoroacetyl propargylamine under catalyst in dimethylformamide. The resulting nucleoside was converted to the 5′-triphosphate, and coupled with biotinyl-*N*-hydroxy succinimide and deprotected to generate the corresponding biotin-9-ddNTP. The structure of one of the biotin-9-ddNTPs (biotin-9-ddGTP) is shown in Fig. 1. ΔTth DNA polymerase could incorporate biotin-9-ddNTPs as well as ddNTPs, but there were some differences in the incorporation rate among the four biotin-9-ddNTPs, as in the following section. Recently, we learned that biotin-11-ddNTPs were listed in the latest catalogue of NEN Research (USA).

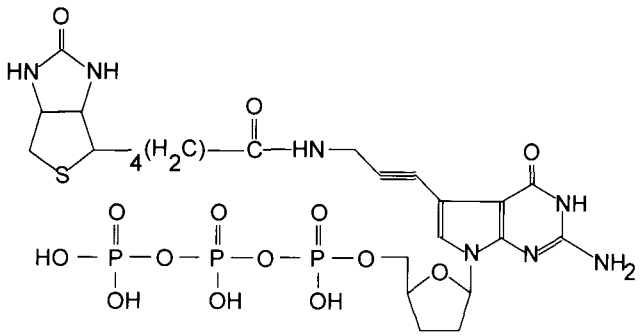

Figure 1. Chemical structure of biotin-9-ddGTP.

3.2. *Optimization of sequencing reaction conditions*

When the sequencing reaction was performed by using biotin-9-ddNTPs under conditions similar to those used for the 5′-biotinylated primers, the band intensities in lanes T and C were weaker compared with those of lanes A and G. As this is likely caused by differences in the activity of ΔTth DNA polymerase incorporating biotin-9-ddNTPs, we investigated the effect of the reaction conditions on activity. As shown in Fig. 2, the optimum $MgCl_2$ concentrations were different for the four biotin-9-ddNTPs, and the weak band intensities in lanes C and T could be improved by increasing the $MgCl_2$ concentration.

Another problem found in the biotin-9-ddNTP sequencing was that the band intensities became relatively weak in the stretch of T or G, but this was essentially solved by replacing 7-deaza-dGTP and dTTP with dITP and dUTP, respectively. Although the mechanism involved in the difference of band intensities is not known, it is apparent that the activity of ΔTth DNA polymerase incorporating biotin-9-ddNTPs is influenced by the surrounding DNA sequences. In Fig. 3A, the sequence patterns obtained when the $MgCl_2$ concentration for the C and T reactions was increased 3 and 4 mM, respectively, and that 7-deaza-dGTP and dTTP were replaced with dITP and dUTP are shown (right four lanes), in comparison with those obtained by the biotin-primer method (left four lanes).

To test whether the biotin-9-ddNTP method can be applicable to PCR products, a part of the *gyrA* gene was amplified and used as a template. The resulting sequence patterns are shown in Fig. 3B (right four lanes) in comparison with the patterns produced by the biotin-primer method (left four lanes). Essentially identical patterns were detected by both methods, but it was noted that

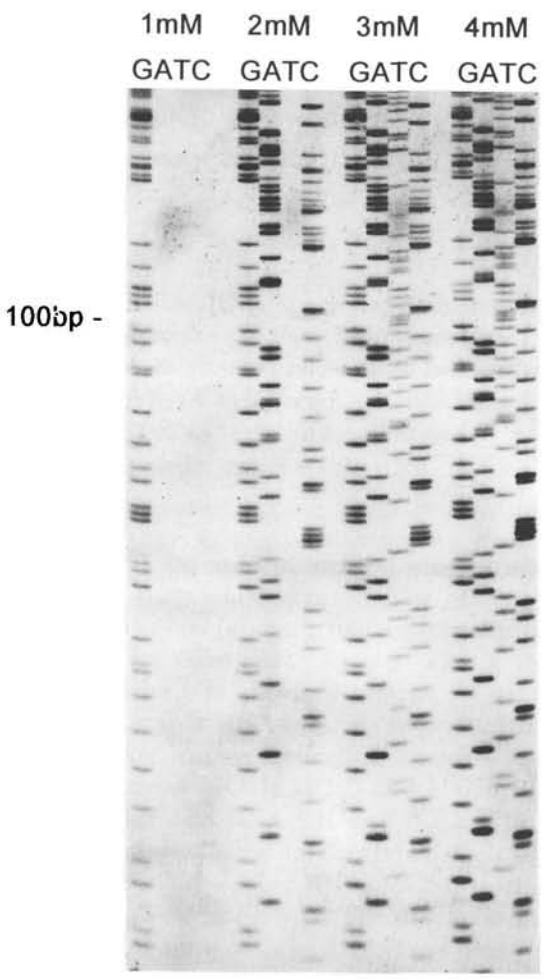

Figure 2. Effect of $MgCl_2$ concentrations on sequencing ladders by the biotin-terminator method. Sequencing was carried out as in Materials and Methods except for the $MgCl_2$ concentrations.

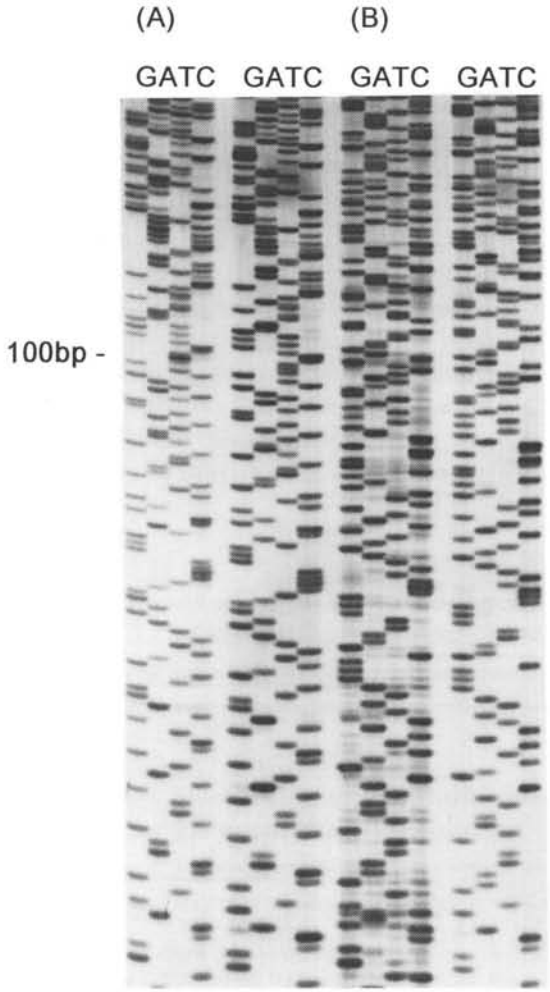

Figure 3. Comparison of sequencing ladders between the biotin-primer method and biotin-terminator methods. M13mp18 ssDNA(A) and PCR products of the *gyrA* gene(B) were used as templates.

the patterns produced by the biotin-terminator method gave lower background levels.

In summary, the biotin-terminator sequencing method that we established by synthesis of four biotin-9-ddNTPs offers two advantages over the conventional non-radioactive sequencing method which uses 5′-biotinylated primers: (I) DNA sequences can be determined from any primer site without preparing 5′-biotinylated primers, and (II) the resulting sequence ladders have lower background levels, so that PCR products can be used as templates.

References

1. Beck, S., O'Keeffe, T., Coull, J. M. et al. 1989, Chemiluminescent detection of DNA: application for DNA sequencing and hybridization, *Nucleic Acid Res.*, **17**, 5115–5123.
2. Tizard, R., Cate, R. L., Ramachandran, K. L. et al. 1990, Imaging of DNA sequence with chemiluminescence, *Proc. Natl. Acad. Sci. USA*, **87**, 4514–4518.
3. Oshima, T. and Imahori, K. 1974, Description of *Thermus thermophilus* comb. nov., a nonsporulating thermophilic bacterium from a Japanese thermal spa, *Int. J. Syst. Bacteriol.*, **24**, 102–112.
4. Asakura, K., Komatsubara, H., Soga, S. et al. 1993, Cloning, nucleotide sequence, and expression in *Escherichia coli* of DNA polymerase gene (*polA*) from *Thermus thermophilus* HB8 *Ferment. Bioeng.*, **76**, 265–269.
5. Prober, J. M., Trainor, G. L., Dam, R. J. et al. 1987, A system for rapid DNA sequencing with fluorescent chain-terminating dideoxynucleotides, *Science*, **238**, 336–341.