DNA Sequencing with [α-^{33}P]-Labeled ddNTP Terminators: A New Approach to DNA Sequencing with Thermo Sequenase™ DNA Polymerase

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ABSTRACT

A new approach to DNA sequencing is described. The method is based on the use of [α-^{33}P]-labeled dideoxyribonucleoside triphosphate terminators and Thermo Sequenase™ DNA polymerase in cycle sequencing. Thermo Sequenase DNA polymerase incorporates ddNTPs as efficiently as dNTPs, allowing the use of low concentrations of these nucleotides in DNA sequencing. Because only the properly terminated chains are labeled and visualized on autoradiography of the sequencing gels, the sequence results are free of background. The intensity of DNA bands generated are remarkably uniform, which makes reading of DNA sequences easy. By staggered loading of the sequencing gel (at 2–3-hour intervals), it is possible to sequence DNA at least 450 to 500 nucleotides. Exposure time for autoradiography with [α-^{33}P] labels is much shorter than with [^{35}S] and does not substantially compromise autoradiographic resolution. Data can be obtained after only 12 hours of exposure of an X-ray film. Moreover, cycle sequencing requires very small amounts of single- or double-stranded template. Consequently, it is even possible to generate sequence data from a single bacterial colony. The details of the protocol are presented in a stepwise manner, and some important parameters to be considered for sequencing with this method are discussed.

INTRODUCTION

The dideoxy chain termination method of Sanger et al. (20) has proven to be very effective in obtaining DNA sequence information from either a single-stranded or a denatured double-stranded template DNA (2,5). Improvements in DNA sequencing methodology are being introduced constantly. These improvements include the introduction of T7 DNA polymerase with its high processivity, which allows generation of sequence data that are relatively free of background (4,21). Similarly, *Taq* DNA polymerase has been used in DNA sequencing, especially when a limited amount of template is available (7). In this procedure, the primer is initially labeled with [^{32}P] using [γ-^{32}P]ATP. The labeled primer is then used in a thermal cycler for polymerase chain reaction (PCR) (9). The amount of template required is reduced 10–100-fold. Furthermore, the introduction of Δ*Taq*® DNA polymerase (Amersham, Arlington Heights, IL, USA) (1) has extended DNA sequencing to the standard two-step [α-^{35}S]dATP-based sequencing protocol (14), to cycle sequencing (6–8,10) or to direct cycle sequencing (J. Fan and R.S. Ranu, unpublished). Δ*Taq* DNA polymerase is a truncated version of *Taq* DNA polymerase in which the first 235 amino acids have been deleted (1). This deletion results in the loss of 5′ → 3′ exonuclease activity, but the enzyme still retains thermostability.

Recent studies of Tabor and Richardson (22) suggest that the substitution of phenylalanine with tyrosine at position 762 in *E. coli* polymerase I eliminates the discrimination between dNTPs and ddNTPs. Amersham has used analogous substitution in a thermostable DNA polymerase to create Thermo Sequenase™ DNA polymerase, which is thermostable at 95°C (15,19). The enzyme does not discriminate between dNTPs and ddNTPs and has neither the 3′ → 5′ nor the 5′ → 3′ exonuclease activities (19). Consequently, Thermo Sequenase DNA polymerase, like the T7 DNA polymerase, incorporates ddNTPs into DNA at rates comparable to that of dNTPs. The enzyme produces sequences that have uniform band intensities (15,19). These properties make this enzyme ideal for generating high-quality DNA sequences, comparable to those generated with Sequenase® T7 DNA polymerase (Amersham).

The most commonly used DNA sequencing method involves a two-step procedure introduced by Tabor and

Table 1. Sequencing Protocol

1. Prepare a master reaction mixture containing the following components: 2 μL of reaction buffer; 50–500 ng (or 25–250 fmol) of DNA template; 0.5–2.5 pmol of primer; and H_2O to adjust to a final volume of 20 μL. Thermo Sequenase preparation, 2 μL (4 U/μL).
2. Four Termination Mixtures: Prepare four 0.5-mL vials, each labeled A, C, G or T, and transfer 2 μL of the dGTP termination master mixture to each vial. Then add 0.5–1.0 μL (0.23–0.46 μCi) of each of the respective [α-^{33}P]-labeled ddNTPs to each tube.
3. Sequencing Reaction Mixtures: Transfer 4.5 μL of the master reaction mixture (from step 1) to each termination tube (A, C, G and T) prepared in step 2. Mix samples by brief centrifugation and overlay with 10–20 μL of mineral oil. Place tubes in a thermal cycler.
4. Thermocycling: Perform an initial denaturation at 94°C for 3 min; subject samples to 30–50 cycles at 94°C for 30 s, at 55°C for 30 s and 72°C for 60 s (described in the figure legend).
5. Add 4 μL of stop solution to each sequencing reaction. Cap samples and heat at 95°C for several minutes. Cool on ice before loading on a sequencing gel.

Richardson (21). In the first step, the primer or a small stretch of DNA is labeled. In the second step, DNA synthesis is carried out in the presence of the four mixtures of dNTPs and ddNTPs (21). The products are then resolved by high resolution polyacrylamide gel electrophoresis in the presence of urea (17). The procedure described in this paper uses a new approach; it takes advantage of the two main features of Thermo Sequenase. First, the four [α-^{33}P]-labeled dideoxyribonucleoside triphosphate (ddNTP) terminators are used to both terminate chain growth and label reaction products. As a result, a single step is needed for labeling and termination reactions. Second, the thermostability of the enzyme is exploited for use in cycle sequencing (7,10), allowing the use of very small amounts of double- or single-stranded template. Cycle sequencing (7,10) is a process in which repeated cycles of thermal denaturation and polymerization with a single primer amplify the amount of product made in the DNA sequencing reaction. Because labeled ddNTPs are used, only the properly terminated DNA chains are labeled and subsequently visualized by autoradiography of the sequencing gel. The present method is similar, in principle, to the fluorescent dye-terminator approach (13). Our results show that the sequence data generated by this method are remarkably free of background. We also show that the sequence data can be obtained directly from a plasmid-containing single bacterial colony without any purification of the template. The method will be introduced in the form of a sequencing kit by Amersham (Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit).

MATERIALS AND METHODS

Reagents Used in DNA Sequencing Protocol

The radiolabeled terminators consisted of 0.3 μM [α-^{33}P]ddGTP, 0.3 μM [α-^{33}P]ddATP, 0.3 μM [α-^{33}P]ddTTP and 0.3 μM [α-^{33}P]ddCTP (each with a specific activity of 1500 Ci/mmol or 0.45 μCi/μL) and were obtained from Amersham.

The dGTP termination master mixture contained 15 μM each of dATP, dCTP, dGTP and dTTP (Amersham).

Thermo Sequenase DNA polymerase was used at a concentration of of 4 U/mL with 0.0006 U/mL of *Thermoplasma acidophilum* inorganic pyrophosphatase in buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM DTT, 0.1 mM EDTA, 0.5% Tween® 20, 0.5% Nonidet® P-40 and 50% glycerol (Amersham).

The reaction buffer consisted of 260 mM Tris-HCl (pH 9.5) and 65 mM $MgCl_2$.

Primers

The primers used in the study are described in the legends to Figures 1 and 2.

The sequencing protocol is described in Table 1.

DNA Sequencing from PCR-Amplified Template

A 336-bp target DNA sequence was amplified from ptACC2 plasmid (16) using standard techniques (J. Fan and R.S. Ranu, unpublished). DNA was resolved by agarose gel electrophoresis and the DNA band was excised from the agarose. The DNA was purified by SpinBind® column method (FMC BioProducts, Rockland, ME, USA), and the DNA fragment was cloned into a TA cloning vector, pCR®II (Invitrogen, San Diego, CA, USA). This plasmid was called ptTA336ACC. Similarly pcPHSta9 was constructed from a PCR-amplified target sequence from pcPHS49 (3).

DNA Sequencing from a Single Bacterial Colony

A bacterial colony (ca. 1–1.5 mm diameter) from an overnight culture of JM 109 transformed with plasmid pcPHSta 9 was suspended in 5 μL of distilled water and heated at 95°C for 5 min. After cooling in an ice bath and briefly centrifuging at 10 000× *g* in a microcentrifuge, a 4-μL aliquot was removed and transferred to the master reaction mixture as the source of DNA template. The rest of the sequencing protocol is identical to that described in Table 1.

Other Methods

DNA sequencing polyacrylamide gels (6%, unless indicated otherwise) were prepared in Tris-borate buffer according to Sambrook et al. (17) or prepared using glycerol-tolerant gel buffer (TTE buffer; 100 mM Tris-HCl, pH 8.0, 30 mM taurine, 1 mM EDTA) (12). Autoradiography was performed using Kodak BioMax X-ray film (Scientific Imaging Systems, New Haven, CT, USA). The DNA primers used in the present investigation are described in the figure legends and were synthesized at the Colorado State University Macromolecular Resources Facility.

RESULTS AND DISCUSSION

The results in Figure 1 show sequence ladders generated with [^{33}P]-labeled terminators. The reaction mixtures were subjected to electrophoresis in a 6% denaturing polyacrylamide gel for 2 h (Panel A), 4.5 h (Panel B) and 7.5 h (Panel C). From this data, several points can be emphasized. DNA bands close to the primer are strong, allowing an unambiguous reading of sequence information (Panel A). Regardless of

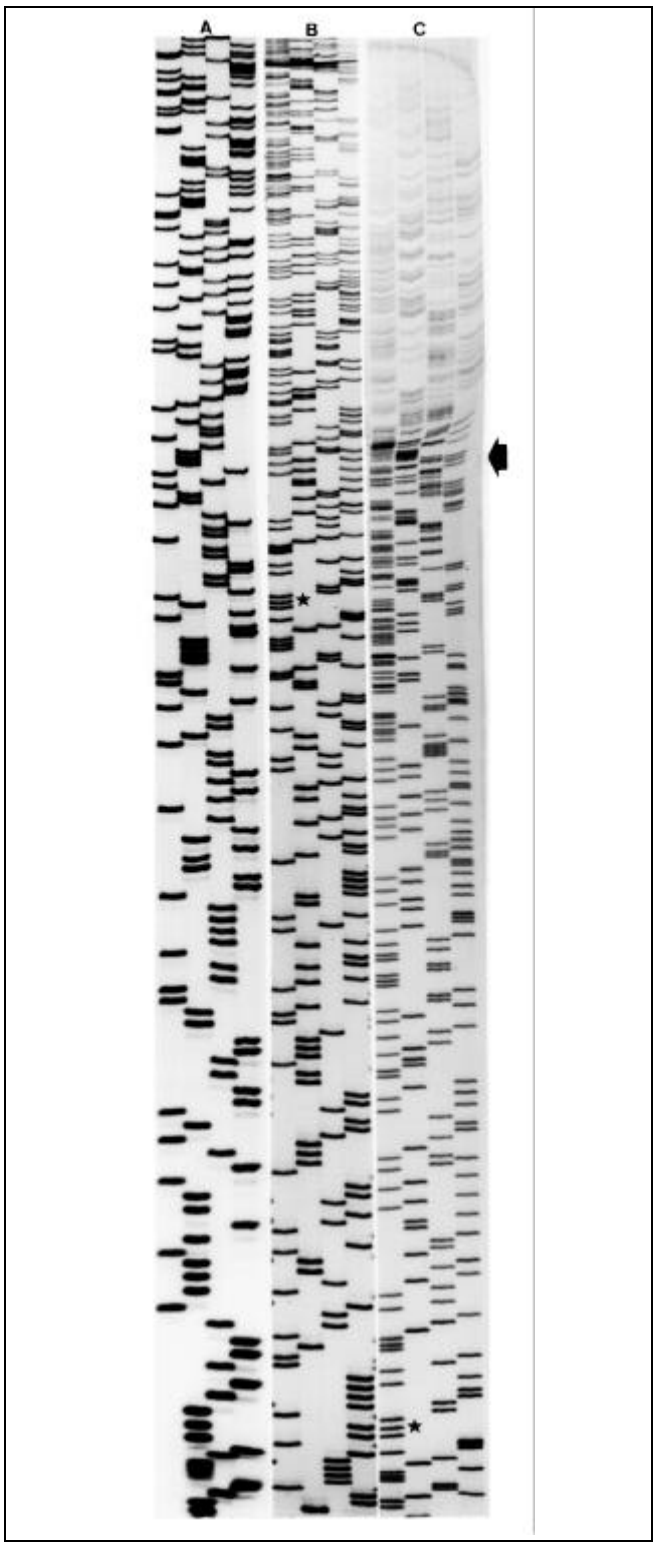

Figure 1. Cycle sequencing with [^{33}P]-labeled terminators. The reaction mixture, containing 100 ng of template DNA [plasmid pcPHS49 (3)] and 1 pmol of primer (5′-TCGTGCAACCGCGACCTC-3′), were prepared as described in Materials and Methods. After an initial denaturation cycle at 94°C for 3 min, the samples were subjected to 50 cycles at 94°C for 30 s (for denaturation), at 55°C for 30 s (for annealing) and at 72°C for 60 s (for extension). Four microliters of denaturing solution were added to each reaction tube. The samples were heated at 95°C for 3 min and cooled on ice. Aliquots (4 µL) were applied to the gel in each lane. Electrophoresis was carried out at 2000 V for 2 h (Panel A), 4.5 h (Panel B) or 7.5 h (Panel C) at 55°C. In each case, the gel loading order, from left to right, was GATC. Star in Panel B and Panel C indicates position of sequence alignment.

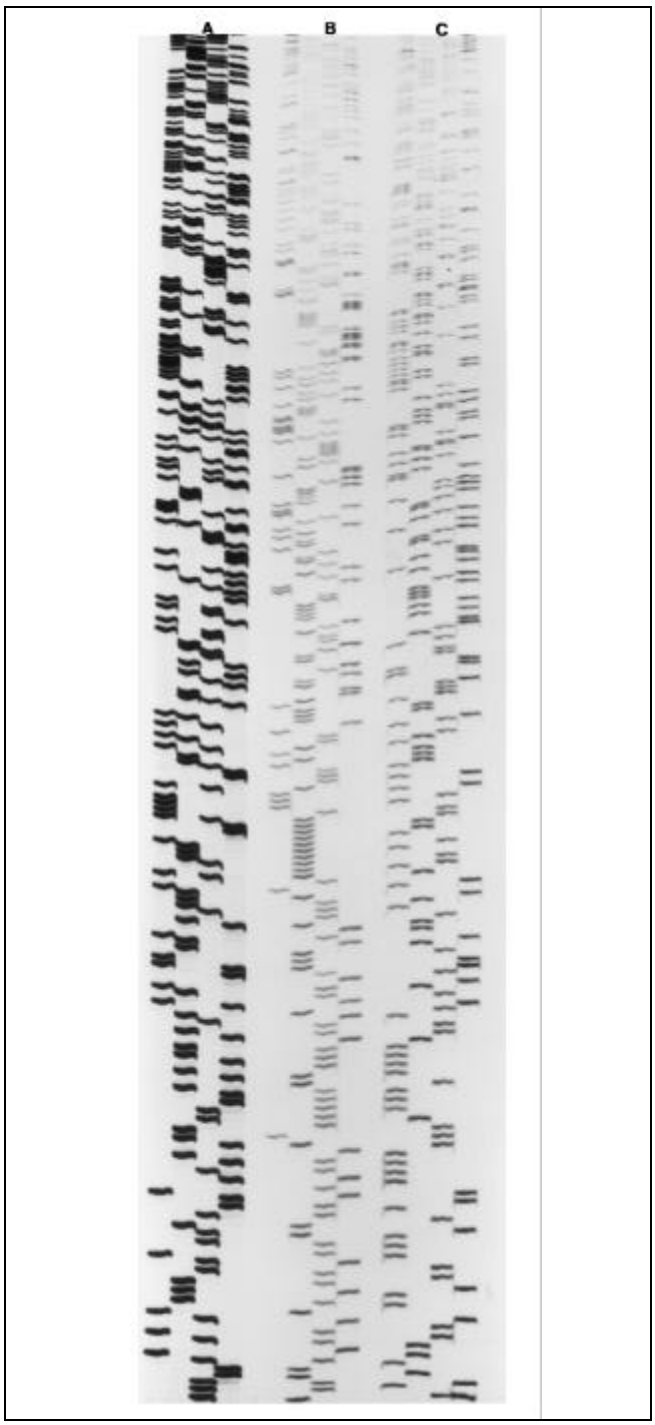

Figure 2. Cycle sequencing with a single-stranded or a double-stranded template (generated by PCR) and DNA template from a single bacterial colony. Reaction mixture for Panel A (M13mp18) and Panel B (ptTA336ACC) contained 100 ng of template DNA and 1 pmol of M13 (-20) primer (5′-GTAAAACGACGGCCAGTG-3′). Thermocycling conditions were similar to that described in Figure 1. The reaction mixture in Panel C contained DNA template from a bacterial colony; other components of the sequencing reaction were identical to those in Panels A and B. After an initial denaturation step at 94°C for 3 min, the samples were subjected to 42 cycles at 94°C for 30 s (for denaturation), 55°C for 30 s (for annealing) and 72°C for 60 s (for extension). Four microliters of denaturing solution were added to each reaction tube. The samples were heated at 95°C for 3 min and cooled on ice. Aliquots (4 µL) were applied to the gel in each lane and subjected to electrophoresis in a 6% sequencing gel at 2000 V for 3.5 h at 55°C. Gel loading order in each panel, from left to right, was GATC. Template in Panel A was M13mp18; in Panel B, ptTA336 ACC; and Panel C, pcPHSta 9 isolated from a single colony.

the length of time of electrophoresis, the sequencing results are relatively free of background ghost bands and other artifacts. The intensities of the bands generated are quite uniform, which makes reading of sequences easy. There is no evidence of compression in the results presented in Figure 1 (Panels A–C). The data clearly show that the intensities of the bands representing low molecular weight DNA are strongest, and the intensities decrease as the chain lengths increase. This is because many more low molecular weight chains are terminated than higher molecular weight chains. Despite this difference, the sequence data of the higher molecular weight DNA is still clearly defined and can be read without difficulty even in tracks after 7.5 h of electrophoresis (Panel C). By using staggered loading (at an interval of 2–3 h) of the gel (11), it is reasonably possible to obtain sequence data to at least 450–500 nucleotides.

A distortion in the sequence bands beyond about 500 nucleotides can be seen in Figure 1C (indicated by an arrow on the right). This distortion is the result of the interaction of glycerol (present in the polymerase solution) with borate in the gel buffer (12). When samples are run on a gel buffered with glycerol-tolerant gel buffer (TTE buffer), these distortions are completely avoided (results not shown). Such a gel can tolerate any amount of glycerol in the samples and can provide additional sequence data.

Results in Figure 2 compare sequence data from a single-stranded template (M13) and a double-stranded DNA template. The double-stranded template in this instance is a plasmid cloning vector that contains a 336-bp, PCR-amplified sequence. In both cases, sequence data is generated using the same M13 (-20) primer. Once again band uniformity and background-free sequence data are observed. The intensity of the DNA sequence of the single-stranded DNA template in Panel A is darker than that observed in Panel B with the double-stranded DNA template. This is not surprising, since priming and extension on a single-stranded template is somewhat more efficient than on plasmid templates under these conditions. The method has also been used to sequence PCR products directly (results not shown).

The cycle sequencing method (6–8,10) used in the experiments presented in Figures 1 and 2 is based on the premise that repeated cycles of thermal denaturation, primer annealing and polymerization produce greater amounts of product in a DNA sequencing reaction. Unlike PCR, the amplification uses a single primer. The amount of product DNA synthesized would increase linearly with the number of cycles. Consequently, proportionally less DNA template is necessary to generate sequence data. This being the case, we reasoned that it may be possible to sequence plasmid DNA directly from a bacterial colony. Results in Figure 2C show the data obtained from a single colony without any special pretreatment or DNA purification other than releasing it from the cells by heating at 95°C. Again the M13 (-20) primer was used in this experiment as in Panel B. The quality of results, uniformity and intensity of the DNA bands in Panel C are comparable with results presented in Panel B (Figure 2). These results

clearly suggest that, with an appropriate primer, it should be possible to sequence DNA from individual colonies of transformants.

Besides the clarity of data generated with the [^{33}P]-labeled terminators, other factors need to be pointed out. The energy level of [^{33}P]-labeled nucleotides is 0.249 MeV compared with 0.167 MeV for [^{35}S]-labeled nucleotides. This results in a significant increase in the signal, greatly reducing exposure time during autoradiography. This increased sensitivity is obtained without substantially compromising the high resolution generally associated with [^{35}S] label in DNA sequencing gels. An overnight (12 h) exposure to an X-ray film is adequate. A half-life of 25.4 days for [^{33}P], compared with 87.4 days for [^{35}S] label, does not pose any special problem, since it is possible to extend the X-ray film exposure time to 24 or 36 h and still obtain high-quality DNA sequence data (data in Figures 1 and 2 are from 36-h exposures). More importantly, the amount of radioactive label required for each sequence totals less than 1 μCi. This is 80% less than the amount usually recommended for sequencing with deoxynucleotides labeled with any isotope. Moreover, [^{33}P] is easier to monitor than [^{35}S] and is disposed of through decay.

In the case of Sequenase T7 DNA polymerase, when a sequence close to the primer is desired, it is desirable to perform the sequencing reaction in the presence of Mn^{++} (4,21). With the [^{33}P]-labeled terminator system described in this paper, there is no need to set up special conditions to derive the sequence close to the primer. In fact, the first base after the 3′-end of the primer is always visible on suitable gels (Figure 1A). It is, however, necessary to include *Thermoplasma acidophilum* inorganic pyrophosphatase in the sequencing reaction, otherwise pyrophosphate may react with and remove the [^{33}P]-labeled 3′-dideoxyribonucleotide of some products. As a result, some of the bands may become progressively weaker with increasing reaction times.

All polymerases can stop at regions of secondary structure or high GC content. These sequencing artifacts show up in an autoradiograph as bands across all four lanes (14). Our data clearly show that such artifacts are avoided by using the labeling method described here since such "stopped" chains will not be labeled. Most sequencing protocols use standard cloning vectors with M13 primers, so sequencing conditions for these primers are well-defined, especially the annealing temperature. For other sequencing projects, adequate design of primer(s) with 50% GC content and an annealing temperature close to 50°–55°C will assure good results. Therefore, specific cycling parameters used will depend on primer sequence and the amount of template DNA.

We have shown that it is clearly possible to obtain sequence information from a single bacterial colony (Figure 2, Panel C). We point out that results from single colonies may vary from plasmid vector to plasmid vector especially when copy numbers are low. A well-designed primer is still an important consideration. We speculate that it may be possible to generate sequence data from a plaque, although we have not yet tried it.

Finally, the procedure described in this paper represents a new approach to DNA sequencing. The protocol is simple and can be carried out in a standard biochemically oriented laboratory, without the need for any new specialized equipment. It offers a significant reduction in the amount of radioactivity required for sequencing and offers easy-to-read results with uniform band intensities without the common sequence artifacts.

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