Nucleic Acids Research, Vol. 20, No. 10 2471–2483

DNA sequencing with dye-labeled terminators and T7 DNA polymerase: effect of dyes and dNTPs on incorporation of dye-terminators and probability analysis of termination fragments

Linda G.Lee, Charles R.Connell, Sam L.Woo, Richard D.Cheng, Bernard F.McArdle[1], Carl W.Fuller[1], Nicolette D.Halloran[2] and Richard K.Wilson

Applied Biosystems, Inc., 850 Lincoln Centre Dr., Foster City, CA 94404, [1]United States Biochemical, 26111 Miles Road, Cleveland, OH 44128 and [2]Department of Genetics, Washington University School of Medicine, 4566 Scott Avenue, St Louis, MO 63110, USA

Received February 10, 1992; Revised and Accepted April 3, 1992

ABSTRACT

The incorporation of fluorescently labeled dideoxynucleotides by T7 DNA polymerase is optimized by the use of Mn^{2+}, fluorescein analogs and four 2′-deoxyribonucleoside 5′-*O*-(1-thiotriphosphates) (dNTPαS's). The one-tube extension protocol was tested on single-stranded templates, as well as PCR fragments which were made single-stranded by digestion with T7 gene 6 exonuclease. Dye primer sequencing using four dNTPαS's was shown to give uniform termination patterns which were comparable to four dNTPs. Efficiency of the polymerase also appeared to improve with the dNTPαS's. A mathematical model was developed to predict the pattern of termination based on enzyme activity and ratios of ddNTP/dNTPs. This method can be used to optimize sequencing reactions and to estimate enzyme discrimination constants of chain terminators.

INTRODUCTION

Sanger dideoxy DNA sequencing[1] has proved to be the most durable and efficient method of DNA sequencing and is the method of choice of most investigators in large scale sequencing programs.[2] 'Automated' or 'fluorescent' DNA sequencing refers to a variation of traditional Sanger sequencing in which fluorescent labels are covalently attached to the reaction products and data is collected during the polyacrylamide gel electrophoresis. Fluorescent sequencing can be divided into two categories: 'dye primer' sequencing, in which case the fluorescent dyes are attached to the 5′ end of the primer, and 'dye terminator' sequencing, in which case the fluorescent dyes are attached to the dideoxynucleoside triphosphates. Several companies (Applied Biosystems (ABI)[3,4], du Pont de Nemours & Co (Du Pont)[5], Pharmacia LKB Biotechnology Inc.[6], and Hitachi[7]) have produced automated DNA sequencers based on fluorescent dyes using dye primer and dye terminator sequencing.

In dye primer sequencing, four separate extension/termination reactions are carried out; each reaction contains four deoxynucleoside triphosphates (dNTPs) and one dideoxynucleoside triphosphate (ddNTP). In the ABI primer system, four separate dye primers are used, corresponding to each ddNTP. After the extension reaction, the four reactions are pooled and the nested set of oligonucleotide fragments are separated by electrophoresis. The dyes, and therefore the nucleotides, are distinguished from each other by their fluorescence emission spectra. In the Pharmacia system, a single dye primer is used in each of the four separate extension reactions. The reactions are not pooled but are run in four separate lanes in direct analogy to traditional, radioactive Sanger sequencing.

In dye terminator sequencing, the fluorescent label is incorporated into the DNA fragments in a single extension/termination reaction using an unlabeled primer. The products are separated, as with dye primer sequencing, by electrophoresis. Since the reaction is performed in one tube, both the Du Pont and the ABI terminator systems require four, spectrally resolvable fluorescent dyes.

There are advantages and disadvantages to both radioactive and fluorescent sequencing, and within fluorescent sequencing, between dye primer and dye terminator sequencing. Radioactive sequencing has the advantage of good signal-to-noise and a wide range in the amount of template needed for sequencing. The label can be incorporated many times in each oligonucleotide fragment during the extension by using radioactively-labeled dNTPs and the signal can be improved by longer film exposure times. Radioactive sequencing has the disadvantage of tedium, especially in the film analysis task, and the limitations in sequence ordering imposed by the requirement of combining data from four separate lanes on the polyacrylamide gel.

* To whom correspondence should be addressed

Fluorescent sequencing has the advantages of automated data collection and analysis and of high throughput. It has the disadvantage of high capital cost and, in all of the currently available chemistries, of incorporating a single fluorescent label in each olignucleotide fragment. The availability of *Thermus aquaticus* (taq) polymerase and cycle sequencing[8], however, has improved the range of the dye primer and dye terminator chemistries by linear amplification of the template and therefore, linear amplification of the signal.

Dye primers and dye terminator systems have their own set of advantages and disadvantages. One advantage of the dye primer system is that any DNA polymerase can be used as long as it accepts dideoxynucleoside triphosphates as substrates. The main disadvantage is the requirement for four separate extension reactions. In the DNA sequencing chemistry labor, dye-primers confer no time-saving advantage for fluorescent vs. manual sequencing.

Dye terminators offer the advantage of convenience. Synthesis of labeled primer is unnecessary, thereby allowing the use of any sequencing primer. Additionally, because only one extension reaction is needed for each sequence, the labor required to perform the extension reactions is reduced. Another advantage of this chemistry is that noise from 'false terminations', in which the oligonucleotide is terminated by a deoxynucleotide rather than a dideoxynucleotide, is eliminated, since such fragments are unlabeled and, therefore, invisible. A disadvantage of the chemistry is that each set of dye-labeled terminators must be tailored to a specific DNA polymerase. For any given enzyme, the pattern of termination from the sequence-dependent substrate specificities of the dideoxynucleotides tends to be less uniform for dye-labeled ddNTPs than for unlabeled ddNTPs. For a given amount of signal, uneven patterns cause increased noise, particularly in the underrepresented populations of oligonucleotide fragments (small peaks).

We previously have developed a set of dye-labeled terminators for taq DNA polymerase which utilized the same 3-amino-1-propyn-1-yl linker arm to attach the dye to the nucleotide as the original Du Pont system (Fig. 1). The Du Pont system utilizes a set of four dyes, each with a net −1 charge, and optimally uses T7 DNA polymerase with Mg^{2+}. The ABI taq terminators utilizes a set of four, zwitterionic rhodamine dyes[9]. Many variations on the dyes were made and tested with taq DNA polymerase, including varieties of fluorescein dyes, boron dipyridyl 'bodipy' dyes, and cyanine dyes. Rhodamine dyes were found to give the most acceptable results. Fluorescein dyes were poor substrates for taq polymerase. While the hydrophobicity of the rhodamine dyes appears to serve the dye-terminator well in the binding pocket of taq polymerase, the same characteristic causes problems in the gel electrophoresis. The rhodamine terminators cause 'compressions' in the data. That is, rhodamine terminators appear to stabilize hairpin structures in GC-rich regions, causing those fragments to migrate anomalously. The compression problem is entirely relieved by substituting 2′-deoxyinosine 5′-triphosphate (dITP) for deoxyguanosine triphosphate (dGTP). The use of dITP, however, results in a less even pattern of termination than results when dGTP is used.

This paper will focus on the development of a set of dye-labeled terminators for modified T7 DNA polymerase, a DNA polymerase developed by Tabor and Richardson[10] which is widely used in radioactive sequencing. The main advantage of T7 DNA polymerase over other DNA polymerases is its processivity and the striking evenness of termination patterns when manganese ions are used as a cofactor[11]. The addition of pyrophosphatase and Mg^{2+} makes the polymerase reaction essentially irreversible and solves the problem of disappearing peaks[12]. The main disadvantage is its lack of thermal stability. Therefore, for automated sequencing, the amplification of signal which is produced in taq cycle sequencing methods is unavailable. The purpose of developing a set of dye-terminators for T7 DNA polymerase was to capitalize on the characteristic processivity of the enzyme and to allow the use of any user-defined sequencing primer with the automated fluorescent sequencing instrument. The use of these dye terminators should provide another useful tool in the methods available for fluorescent sequencing.

MATERIALS AND METHODS

Instrumentation. Absorption spectra were obtained on an Hewlett-Packard Model 8451A UV-visible spectrophotometer. Fluorescence spectra were obtained on a Perkin-Elmer Model LS-5 fluorescence spectrophotometer. HPLC was performed using a Perkin-Elmer Series 4 Liquid Chromatograph. Columns (AX300, RP300) were obtained from Applied Biosystems, Inc. (ABI). PCR was perfomed on a Perkin-Elmer Cetus DNA thermal cycler or model 9600 DNA thermal cycler. DNA sequencing was performed on an ABI Model 373 DNA sequencer. Agarose gels were analyzed on an ABI Model 362 GENESCANNER.

DNA, oligonucleotides, nucleotides and enzymes. M13mp18 was obtained from Promega (Madison, WI). Oligonucleotides were prepared on an ABI Model 380B DNA synthesizer by Bill Guisti (ABI, Foster City, CA). The primers used were −21 primer (5′-TGTAAAACGACGGCCAGT), −40 primer (5′-GTTTT-CCCAGTCACGAC) and reverse primer (5′-CAGGAAACA-GCTATGACC). Nucleotides were obtained from Pharmacia LKB Biotechnology Inc., with the exception of (S_p)-dITPαS, which was a generous gift from Professor Fritz Eckstein (Max Planck Institut fur Experimentelle Medizin, Gottingen, W. Germany). The diasteriomeric mixture of dITPαS was synthesized by the method of Ludwig and Eckstein[13]. Sequenase® Version 2.0 T7 DNA polymerase, pyrophosphatase and T7 gene 6 exonuclease were obtained from United States Biochemical Corp. (Cleveland, OH). Sequenase® Dye-Primer sequencing kits were also obtained from USB. Pyrophosphatase was also obtained from Sigma Chemical Co. (St. Louis, MO). Amplitaq DNA polymerase was obtained from Cetus Corp. (Emeryville, CA).

Organic reagents. Tricarballylic acid, methanesulfonic acid, *N*-hydroxysuccinimide, dicyclohexylcarbodiimide, 4-chloro-1,3-dihydroxybenzene and 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one were obtained from Aldrich Chemical Co. (Milwaukee, WI).

Fluorescent dyes. Bodipy dyes, bifluor, 5-carboxy-2′,7′-dichloro-fluorescein, 5-carboxy-2′,4′,5′,7′-tetrachlorofluorescein (ZOE), 5-(and 6-)carboxy rhodamine 110, 6-carboxytetramethyl rhodamine and 6-carboxy rhodamine X were provided by Molecular Probes (Eugene, OR). The synthesis of the dyes 5-(and 6-)carboxy-4,7-dichloro-2′,7′-dimethoxyfluorescein (BUB), 5-(and 6-)carboxy-4,7,4′,5′-tetrachloro-2′,7′-dimethoxy-fluorescein (LOU), 5-(and 6-)carboxy-4,7-dichlorofluorescein

(4,7-DCF), and 5-(and 6-)carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein (JOE) were originally described by Khanna and Ullman[14] and were synthesized at ABI with some modifications. The dyes 5-(and 6-)carboxy-4,7-dichloro-1',2',7',8'-dibenzofluorescein (NAN) and 5-(and 6-)carboxy-4,7,4',5'-tetrachloro-1',2',7',8'-dibenzofluorescein (DEB) and their 5-(and 6-)succinimidyl esters were synthesized at ABI by methods described elsewhere. For the dyes which have been characterized by NMR (FAM, JOE, all of the rhodamines), the 6-carboxy isomer was the faster-eluting isomer on reverse-phase HPLC columns. The uncharacterized dyes are designated as 'dye1' for the faster-eluted isomer or 'dye2' for the slower-eluting isomer. We believe the 6-carboxy isomer corresponds to 'dye1' and the 5-carboxy isomer to 'dye2'.

Synthesis of 9-(2',3'-dicarboxypropyl)-6-hydroxy-3H-xanthen-3-one (EVE). A mixture of 4-chlororesorcinol (1 g, 6.9 mmol), tricarballylic acid (1.2 g, 6.9 mmol) and methanesulfonic acid (10 mL) were heated to 80°C for 1 h. The red mixture was poured onto ice and the red-black precipitate was filtered and air-dried. The solid was dissolved in Hepes buffer (0.1 M, pH 7) and extracted with ethyl acetate. The organic phase was discarded, and the aqueous phase was acidified with conc HCl. The resulting slurry was extracted with ethyl acetate, dried ($MgSO_4$) and concentrated to a red solid (0.24 g).

Dye succinimidyl esters. The succinimidyl esters of 5-FAM, 6-JOE, 6-TMR and 6-ROX are commercially available from ABI. All of the remaining dyes were activated using dicyclohexylcarbodiimide and *N*-hydroxysuccinimide in ethyl acetate. A representative example is given below for the synthesis of NAN succinimidyl ester.

NAN succinimidyl ester. 5- (and 6-) carboxy-4,7-dichloro-1',2',7',8'-dibenzofluorescein (NAN, 2 mg, 4 mmol) was suspended in ethyl acetate. Dilute HCl was added and the dye (presumably the lactone form) became completely soluble in the organic phase. The layers were separated and the organic layer dried ($MgSO_4$) and filtered. To the dye solution was added *N*-hydroxysuccinimide (10 mg, 90 mmol) and dicyclohexylcarbodiimide (2 mg, 10 mmol). The reaction progress was monitored by thin-layer chromatography (silica gel, 600:60:16 dichloromethane: methanol:acetic acid). The succinimidyl esters had much higher R_f values (0.5, 0.6) than the free acids (0.1). In addition, the 5- and 6-carboxy isomers were separable by column chromatography under these solvent conditions.

Dye-labeled terminators. 2',3'-Dideoxynucleotide triphosphates with 3-amino-1-propyn-1-yl linker arms were kindly provided by John Van Camp at ABI and were prepared by methods described by Prober, *et. al.*[16] Rhodamine dye terminators were synthesized by John Van Camp by methods described elsewhere[9]. Fluorescein dye-terminators were prepared by methods essentially the same as described previously[16], with the modifications of a higher pH for the coupling reaction (aqueous solution, pH 9.5, instead of DMF/H_20 at pH 7) and the use of HPLC for the purification of the product dye-terminator. In general, the purification required two steps: an anion exchange column (ABI AX300: 40% acetonitrile (ACN)/ 60% triethylammonium bicarbonate (TEAB, 0.1 M), 20 min gradient to 40% acetonitrile/60% triethylammonium bicarbonate (1.2 M)) to separate the free dye, the unlabeled terminator and the labeled terminator. A subsequent, reverse-phase column (ABI RP300: 100% triethylammonium acetate (0.1 M), 20 min gradient to 75% TEAA (0.1 M)/25% ACN) was used to separate the 5- and 6-carboxy isomers of the dye-labeled terminators.

Sequencing reactions (fluorescein terminators). Annealing reaction: Five-fold concentrated sequencing buffer (5× SB) contained MOPS (200 mM, pH 7.5), NaCl (250 mM), $MgCl_2$ (50 mM), $MnCl_2$ (25 mM) and sodium isocitrate (75 mM). The annealing and extension reactions and subsequent ethanol precipitation were performed in one microcentrifuge tube (1.5 mL) for each template. The annealing mixture (6−12 μL) contained 2 μg of template DNA (0.8 pmol for M13mp18), universal or custom primer (1.6 pmol), and 5× SB (4 μL). The solution was incubated at 65°C for five minutes, then slowly cooled to room temperature over 20 min.

Extension reaction. To the annealed reaction mixture was added 4 μL of 2 mM dNTPαS (final concentration of 400 μM of each dNTPαS), and 4 μL of dye-labeled terminator solution [final concentrations are indicated for: ddA-LOU2 (1.2 μM), ddC-5ZOE (1.8 μM), ddG-5NAN (3.2 μM), ddT-6FAM (0.44 μM)]. T7 DNA polymerase (13 U) and inorganic pyrophosphatase (0.012 U) were added to the mixed solution. The final volume was adjusted to 20 μL. (In some cases, the concentrations of dNTPαS, terminators, and enzymes were reduced by factors of four, four and 32, respectively, without noticeable effect.) The extension reaction was incubated at 37°C for 10 min. To the reaction mixture was added a solution of NH_4OAc (9.5 M, 20 μL) and ethanol (95%, 90 μL). The DNA was pelleted by centrifugation (12,000×g) for 15 min and the contents decanted. The precipitated DNA was washed twice with 70% ethanol (300 μL), each wash consisting of brief mixing, a five-minute spin and removal of the wash solution. The precipitated DNA was dried in a vacuum centrifuge for 5−10 min.

Gel analysis using the ABI Model 373. The dried extension products were dissolved in a 5:1 solution of deionized formamide and 50 mM EDTA (4 μL). The microcentrifuge tube was heated to 95°C for two min to denature the DNA. The formamide solution was loaded onto a lane of the sequencing gel (8 M urea, 6% polyacrylamide) in a 373 DNA sequencer. The 373 had been modified to include a filter wheel with five bandpass filters, each of ten nm spectral bandwidth centered at 530, 545, 560, 580, and 610 nm. A modified instrumental software was written to spin the wheel so that one of two combinations could be chosen: 530, 560, 580, and 610, or 530, 545, 560, and 580. (The former combination is compatible with the commercially available fluorescent dye chemistry from ABI; the latter combination is optimized for the T7 DNA polymerase terminators.) Electrophoresis was conducted at constant power (35 W) for 14 h.

Sequencing reactions (rhodamine terminators). Reactions using the four rhodamine terminators, which were developed for taq DNA polymerase, were performed essentially as described above. The annealing mixture (4 μL) contained 1 μg DNA, primer (0.8 pmol), and buffer. The buffer consisted of Tris-HCl (final concentration in extension reaction of 20 mM, NaCl (50 mM), $MnCl_2$ (2 mM), and isocitrate (15 mM). The final concentration of dNTPαS was 200 μM each of dATPαS, dCTPαS, dITPαS, and dTTPαS. The concentrations of dye-labeled terminators

were: ddA-5-carboxyrhodamine 6G (0.1 μM), ddC-6-carboxy-rhodamine X (1.2 μM), ddG-5-carboxyrhodamine 110 (0.01 μM), and ddT-6-carboxytetramethylrhodamine (0.7 μM). The quantity of enzymes added were also slightly different: T7 DNA polymerase (2.5 U) and inorganic pyrophosphatase (1 U, from Sigma). The ethanol precipitation was performed with NaOAc added as the additional source of counter-ions.

Sequencing reactions (dye primers). Reactions using c^7dGTP were performed as described in the instructions in the Sequenase® Dye-primer Sequencing kit. Reactions using dNTPαS's were performed in the same manner with the substitution of twice the conc of dNTPαS's for dNTPs. Each primer was annealed to single-stranded M13mp18 DNA in a solution (4 μL for A and C, 11 μL for G and T) containing dye primer (0.4 pmol for A and C, 0.8 pmol for G and T), M13mp18 (0.25 μg for A and C, 0.75 μg for G and T) and 5× SB (1 μL for A and C, 3 μL for G and T). The mixture was heated to 65°C for 5 min and slowly cooled to RT over 20 min. Each extension-termination mixture (6 μL for A and C, 15 μL for G and T) contained dNTP solution [either dNTPs with c^7dGTP (170 μM) or dNTPαS's (340 μM)], and the appropriate ddNTP (0.55 μM). T7 DNA polymerase and pyrophosphatase (2 U/0.002 U for A and C, 4 U/0.004 U for G and T) were added and the reactions incubated for 10 min. Stop solution (15 μL of 20 mM EDTA, 1 M NaOAc, pH 8) was added to the T reaction and the contents of the A, C, and G tubes were added to the T tube. Ethanol precipitation was performed as described, above.

Dye terminator assay. Annealing reactions were performed as described in the sequencing reactions for the fluorescein terminators. Extensions reactions were performed with T7 DNA polymerase (13 U) and pyrophosphatase (0.012 U), dNTPαS (final concentration of 400 μM) and a single dye-labeled terminator: ddA-dye (6 μM), ddC-dye (4−8 μM), ddG-dye (8 μM), or ddT-dye (2−4 μM). Ethanol precipitations were performed using NH_4OAc as described above.

Processivity assay. A solution (144 μL) containing 5× SB (48 μL), M13mp18 (12 μg = 4.8 pmol), 5-carboxyfluorescein (FAM) labeled −21M13 primer (10 pmol) was annealed as described, above. The annealed primer-template was divided into two tubes. To one tube was added four dNTPαS's to a final concentration of 400 μM; to the other tube was added four dNTPs to a final concentration of 200 μM. The volume of each tube was adjusted to 60 μL. Each tube was incubated at 37°C for two minutes. Reactions were initiated by the addition of T7 DNA polymerase (13 U) and pyrophosphatase (0.012 U). Aliquots (6 μL) were removed at various time points (t=0, 0.5′, 1′, 2′, 3′, 4′, 8′ and 12′) and the extension reaction was stopped with a solution (12 μL) containing blue dextran (0.75%), NaOH (45 mM), glycerol (7.5%) and EDTA (12 mM). A portion (6 μL) of the reaction products at each time point was analyzed by denaturing (30 mM NaOH) agarose gel electrophoresis on the ABI model 362 GENESCANNER. The experiment was repeated using three-fold the amount of T7 DNA polymerase and pyrophosphatase (39 U and 0.036 U, respectively).

Generation of A-ladders with varying ratios of ddA/dNTPαS. A solution (44 μL) containing 5× SB (22 μL), M13mp18 (11 mg = 4.4 pmol) M13mp18 and FAM-labeled −21 primer (8.8 pmol) was annealed as described above. The solution was divided into aliquots of 4 μL into 1.5 mL microcentrifuge tubes. To each tube was added four dNTPαS's (final conc of 400 μM) and varying amounts of ddATP (final conc of 1 to 25 μM). The tubes were preheated at 37°C for two minutes. Each extension reaction was initiated by the addition of T7 DNA polymerase (4.3 U) and pyrophosphatase (0.004 U). After 10 min at 37°C, the reactions were stopped with EDTA (250 mM, 1 μL). An aliquot (3 μL) was removed from each extension reaction, mixed with formamide (3 μL) and loaded onto a sequencing gel on the ABI model 373 DNA sequencer.

PCR, T7 gene 6 exonuclease and sequencing. PCR Amplification. Ten-fold concentrated PCR buffer (10× PCR buffer) contained 500 mM KCl, 100 mM Tris-HCl (pH 8.3), 15 mM $MgCl_2$, 0.1% (w/v) gelatin. The dNTP mix contained 1.25 mM each of dATP, dCTP, dGTP and dTTP in Tris-HCl (10 mM, pH 8.0) and EDTA (0.1 mM). PCR was performed with bacteriophage lysate, DNA (0.01−1.0 mg), or bacterial colonies or bacteriophage plaques which were transferred to a small amount of water or TE buffer. Each template DNA which was to be sequenced with the fluorescein terminators required two PCR reactions to generate adequate template for sequencing. Each PCR reaction contained 10× PCR buffer (5 μL), dNTPs (8 μL), −40 primer (2.5 μL of 20 μM), reverse primer (2.5 μL of 20 μM), template DNA and taq DNA polymerase (1 U). The total volume was adjusted to 50 μL.

Thermal cycling was performed on either a Perkin-Elmer Cetus thermal cycler, or a Perkin-Elmer Cetus Model 9600 thermal cycler. If the former was used, each reaction was overlaid with light mineral oil (80 μL) and preheated (95°C) for 2 min. The subsequent thermal cycle (94°C for 30 s, 55°C for 30 s, 72°C for 60 s) was performed for 35 cycles. If the 9600 instrument was used, the oil was omitted and the thermal cycle (92°C for 10 s, 55°C for 60 s, 72°C for 60 s) was performed for 35 cycles.

Reaction with T7 Gene 6 exonuclease. Ten-fold concentrated T7 gene 6 exo buffer (10× exo buffer) contained Tris-HCl (500 mM,

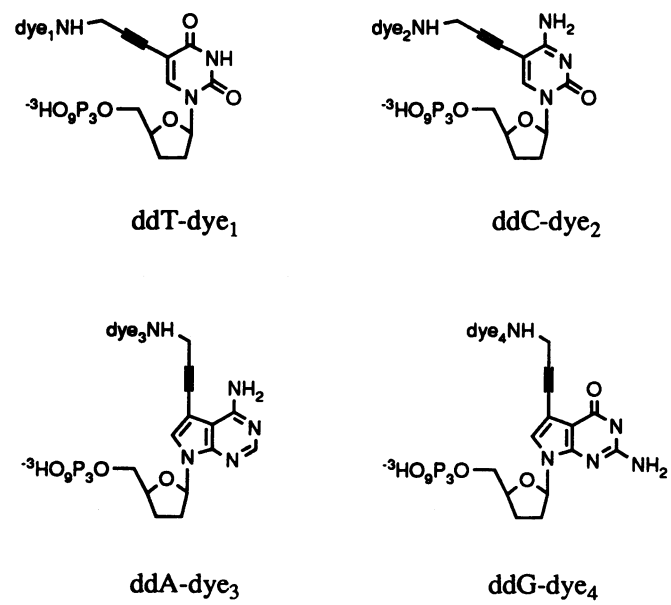


Fig. 1. Structures of the nucleotide portion of the dye-labeled terminators. The syntheses were originally designed and performed by Prober, et. al.[5]

pH 8.1), $MgCl_2$ (50 mM), KCl (200 mM), and 2-mercaptoethanol (50 mM). Each PCR reaction was transferred to a 1.5 mL microcentrifuge tube. To the tube was added T7 gene 6 exonuclease (120 U), 10 exo buffer (10 μL) and water, to a final volume of 100 μL. After incubation at 37°C for 30 min, a solution of phenol:chloroform (100 μL, 1:1) was added to each tube, the mixture was mixed well and centrifuged (13,000×g) for five min. The aqueous phases were transferred to a 1.5 mL microcentrifuge tube, and extracted once with water-saturated ether or chloroform.

Purification by PEG precipitation. PEG solution contained PEG-8000 (40% w/v) and $MgCl_2$ (10 mM). Two PCR samples were combined. To each combined sample was added NaOAc (16 μL, 3M, pH 4.8) and PEG solution (40 μL). The samples were mixed, incubated at RT for 10 min, and the DNA was pelleted by centrifugation (10,000×g) for 15 min at RT. All the supernatant was removed by aspiration. The DNA pellets were washed twice with 100% ethanol and dried briefly under vacuum. The DNA was dissolved in 7.5 μL of water. Each sequencing reaction required 6.5 μL of the DNA solution and utilized −21 primer.

RESULTS AND DISCUSSION

Dye Terminator Composition. Fig. 1 shows the structures of the nucleotide portion of the terminators. Initial experiments focused on using T7 DNA polymerase with the rhodamine-labeled set of dye terminators, which were developed for taq DNA polymerase, to examine the pattern of termination. Many variations in the composition of polymerase substrates and cofactors were tested, including the use of Mg^{2+} and Mn^{2+}, and the use of analogs of dNTPs. The best results with the rhodamine terminators, shown in Fig. 2, *panel a*, utilized Mn^{2+} and four

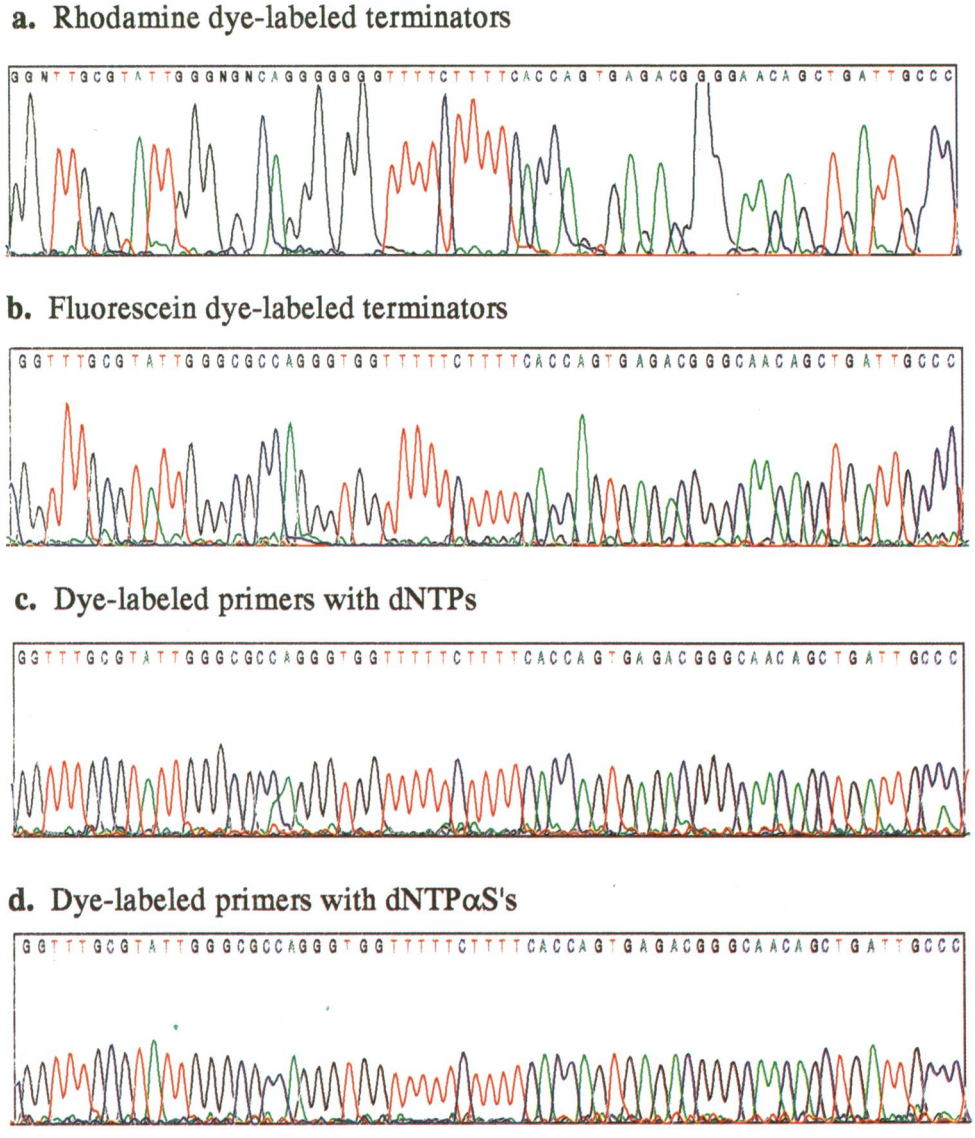


Fig. 2. Effect of different dyes and dNTPs on four-color sequencing with T7 DNA polymerase and Mn^{2+}. Single-stranded M13mp18 (1−2 μg) was annealed with −21 primer. The sequencing method utilized dye-labeled terminators (*panels a* and *b*) or dye-labeled primers (*panels c* and *d*). The dNTP solution contained dATPαS, dCTPαS, dITPαS and dTTPαS (*panel a*); four dNTPαS's (*panels b* and *d*); or four dNTPs (*panel c*).

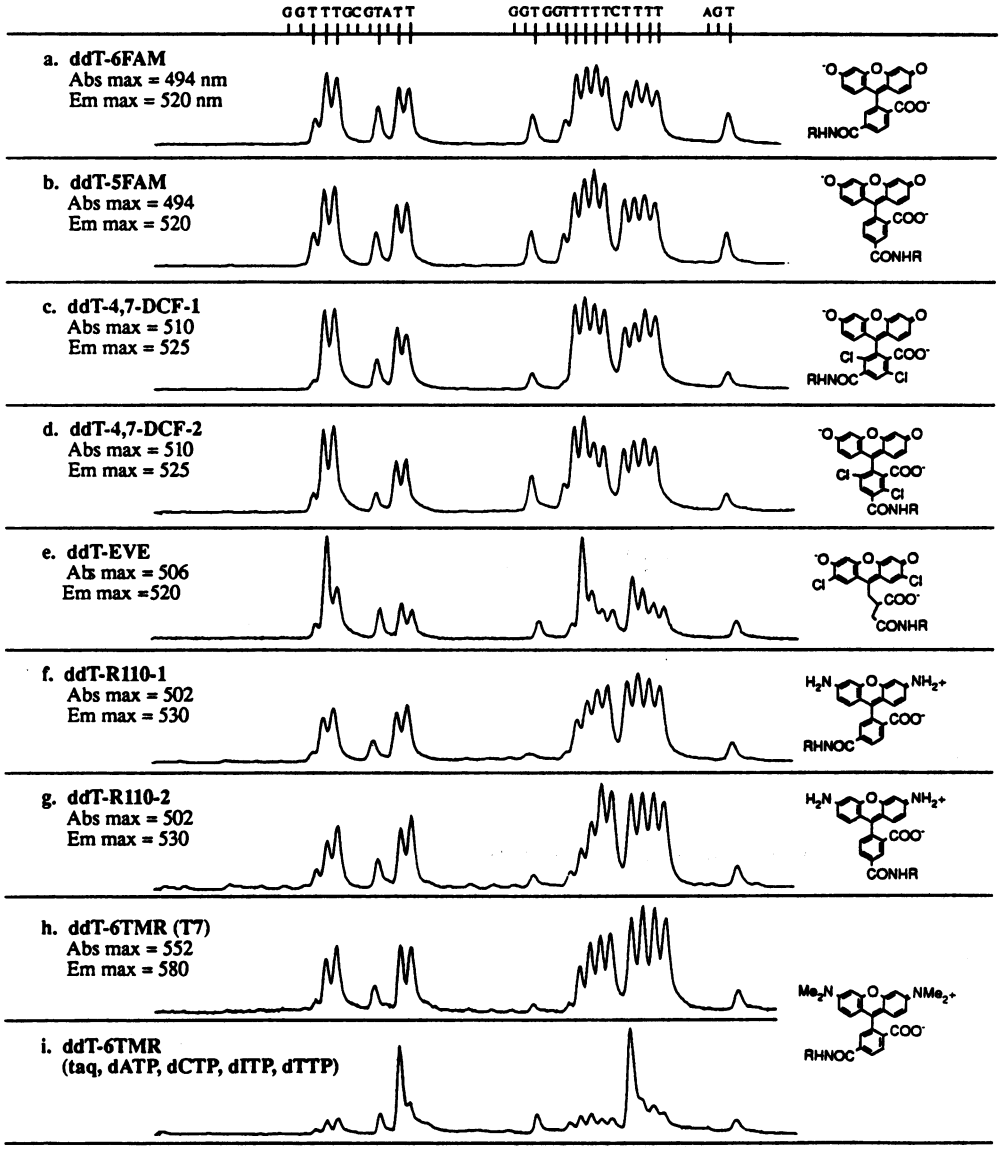


Fig. 3. Termination patterns of different dye-labeled terminators (ddT-dye). The structure of the nucleotide (R) is shown in Fig. 1. Each extension reaction contained −21 primer annealed to ss M13mp18, Mn^{2+}, Mg^{2+}, four dNTPαS's, T7 DNA polymerase and pyrophosphatase.

2′-deoxyribonucleoside 5′-*O*-(1-thiotriphosphates) (dNTPαS's): dATPαS, dTTPαS, dCTPαS, and dITPαS. The use of dITPαS alleviated the compression caused by the rhodamine terminators. For any of the dye terminators described in this paper, the use of four dNTPαS's generally gave more even patterns of termination with T7 DNA polymerase for dye-labeled ddCTP and dye-labeled ddTTP, and slightly less even patterns for dye-labeled ddGTP and dye-labeled ddATP. Use of analogs of dGTP, such as 7-deaza-2′-deoxyguanosine 5′-triphosphate (c^7dGTP) or 2′-deoxyinosine 5′-triphosphate (dITP), gave less even patterns of termination.

The termination pattern was quite acceptable for the rhodamine dyes (Fig. 2, *panel a)*, except for the sequences in which T or C follow G. In these sequences almost no signal is visible. Interestingly, the same sequences which gave the smallest peaks with T7 DNA polymerase also gave the smallest peaks when taq DNA polymerase was used with the rhodamine dye terminators.

The next step towards improving the pattern of termination was to change the type of dye. We investigated analogs of fluorescein dyes since many spectrally resolvable analogs were available. Negatively charged fluorescein dyes have several advantages over zwitterionic rhodamine dyes. Fluorescein dyes on terminators do not cause additional compressions in the gel electrophoresis and therefore do not require the use of dITP. The fluorescein terminators have a net −5 charge and migrate through the gel before the DNA sequence bands, while rhodamine terminators, with a net −3 charge, migrate during the sequence and must be removed thoroughly from the reaction mixture before loading onto the gel. Finally, fluorescein terminators produce a better pattern of termination with T7 DNA polymerase, as shown in Figs. 3−6.

Since dye-labeled ddTTP and ddCTP generally gave the least uniform pattern of the terminators, several dyes were tested on both nucleotides. In general, within a class of dyes, the smaller

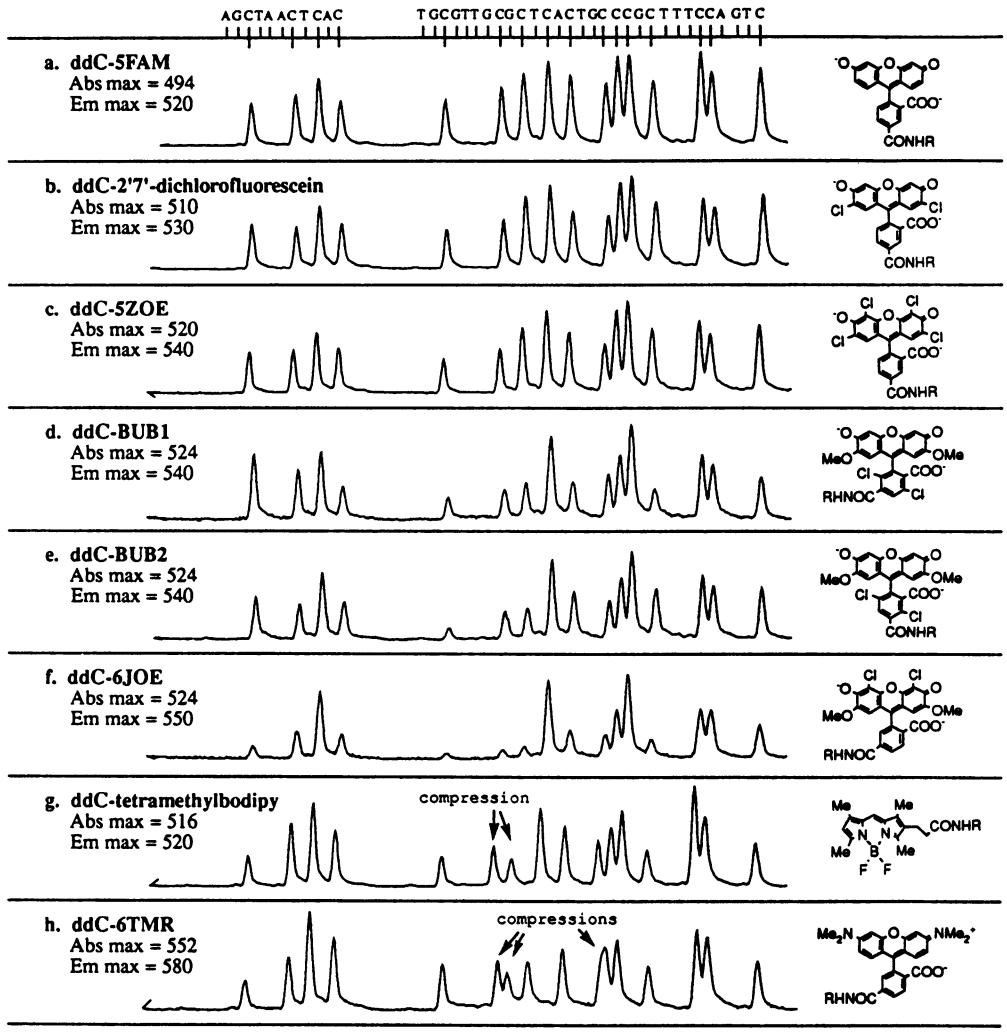


Fig. 4. Termination patterns of different dye-labeled terminators (ddC-dye). The structure of the nucleotide (R) is shown in Fig. 1. Each extension reaction contained −21 primer annealed to ss M13mp18, Mn^{2+}, Mg^{2+}, four dNTPαS's, T7 DNA polymerase and pyrophosphatase. The compressions for the zwitterionic dyes (*panels G* and *H*) are resolved when dITP or dITPαS is substituted for dGTPαS.

the dye, the better. In an attempt to find four spectrally resolvable dyes, however, variations on the dye structure were necessary to shift the emission maxima of the dyes to longer wavelengths. Figs. 3 and 4 show that T7 DNA polymerase only gave acceptable patterns with ddT-dye and ddC-dye when small dyes were used. The longer wavelength, and therefore, bigger, dyes, such as 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein (JOE) and 5-carboxy-4,7,4',5'-tetrachloro-2',7'-dimethoxyfluorescein (LOU), gave small peaks for Ts and Cs following Gs. The use of small dyes did not guarantee uniform patterns, however. Both bodipy and EVE dyes, which are smaller than the fluorescein analogs, gave relatively uneven termination patterns.

The best set of spectrally resolvable dyes for ddTTP-dye and ddCTP-dye were obtained with ddT-6FAM and ddC-5-carboxy-2',4',5',7'-tetrachlorofluorescein (ddC-5ZOE). With these in hand, we found that almost all the fluorescein dyes tested on ddATP and ddGTP produced acceptable patterns (see Figs. 5 and 6). Even the very large bifluor dye, which is a rhodamine dye attached to a fluorescein dye, produced a fairly even termination pattern on ddG (although it causes band compressions, as well). The two longest wavelength (and structurally most complex) dyes were reserved for the purine terminators. Fig. 2, *panel b* shows an example of single-stranded M13mp18 DNA sequenced using the four fluorescein dye terminators and a set of four bandpass filters which were chosen to spectrally resolve the new dye set.

Use of dNTP Combinations to Resolve Gel Compressions. Although the initial purpose of using dNTPαS's was to improve the pattern of termination, we soon observed that some sequence compression problems which normally required the use of c^7dGTP in radioisotopic or dye primer sequencing were completely resolved using the four dNTPαS's with the fluorescein dye terminators. By sequencing a hairpin region consisting of a 11 base pair stem and a six base loop with the 16 possible combinations of dNTPαS's and dNTPs, we found that dCTPαS was essential in resolving the compression. Furthermore, we observed that the combination of dCTPαS and dGTPαS was the most effective in resolving the compression, regardless of the composition of the remaining deoxynucleotides (Fig. 7). The use of dITPαS with three dNTPs also resolved the compression. For our purposes, the optimum effect of uniform pattern and absence of compression required the use of all four dNTPαS's.

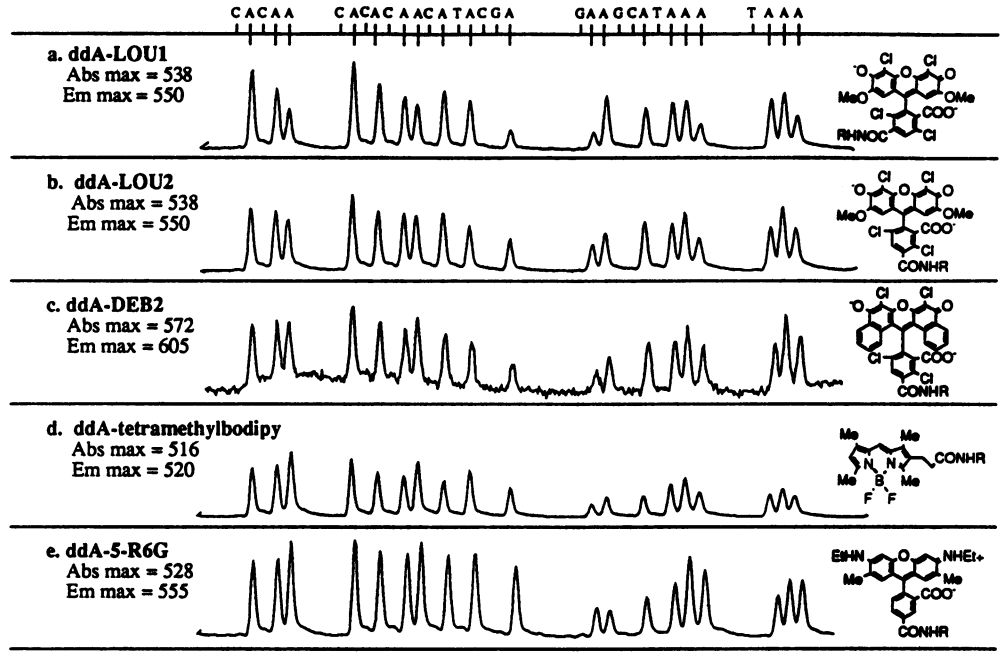


Fig. 5. Termination patterns of different dye-labeled terminators (ddA-dye). The structure of the nucleotide (R) is shown in Fig. 1. Each extension reaction contained −21 primer annealed to ss M13mp18, Mn^{2+}, Mg^{2+}, four dNTPαS's, T7 DNA polymerase and pyrophosphatase.

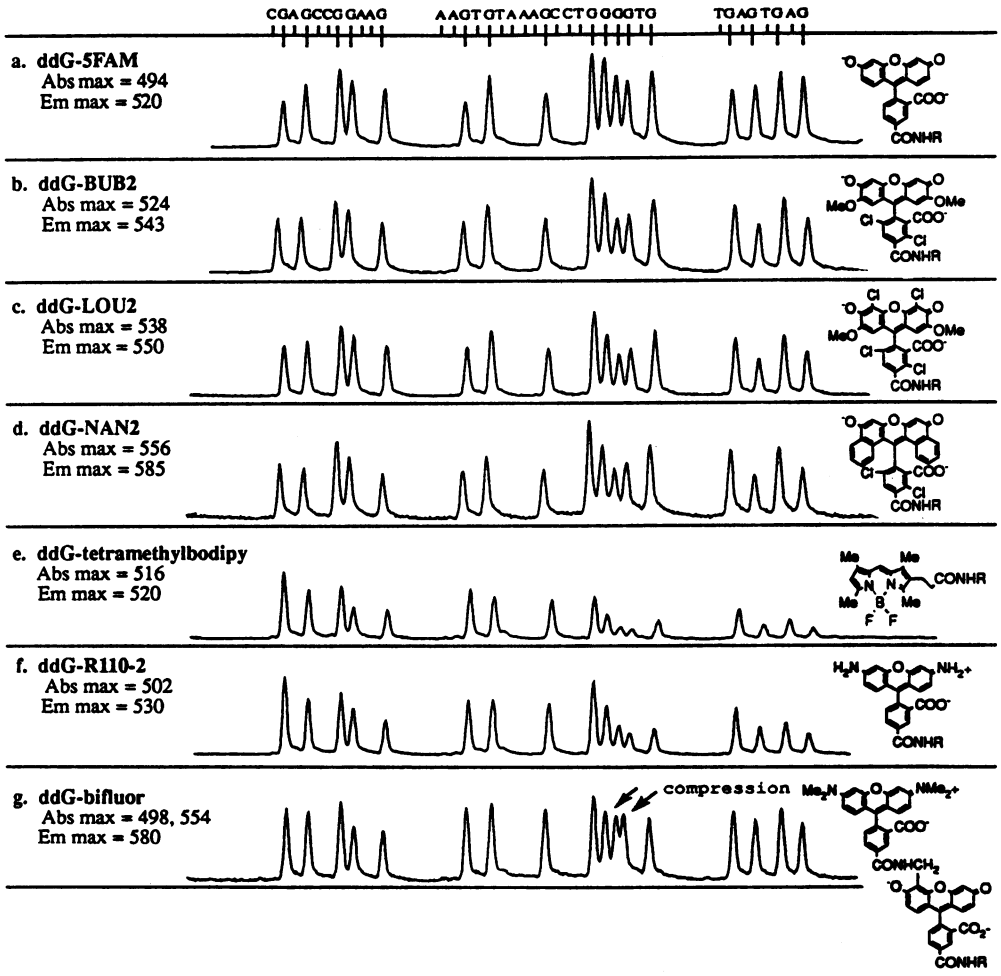
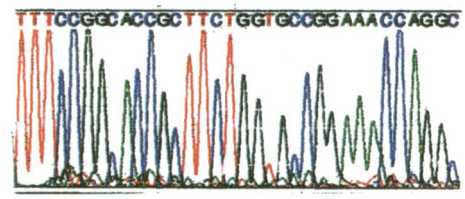
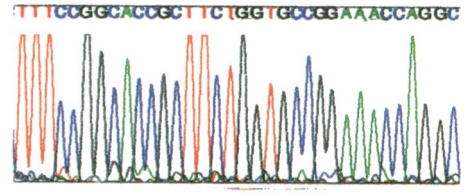


Fig. 6. Termination pattern of different dye-labeled terminators (ddG-dye). The structure of the nucleotide (R) is shown in Fig. 1. Each extension reaction contained −21 primer annealed to ss M13mp18, Mn^{2+}, Mg^{2+}, four dNTPαS's, T7 DNA polymerase and pyrophosphatase.

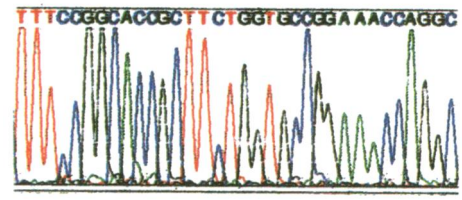
a. c^7dGTP, dATP, dCTP, dTTP: correct sequence, but uneven pattern



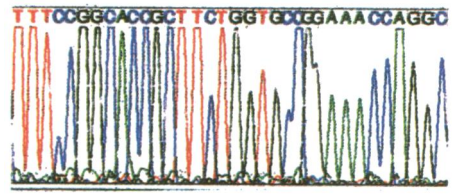
b. dATPαS, dCTPαS, dGTPαS, dTTPαS: correct sequence, good pattern



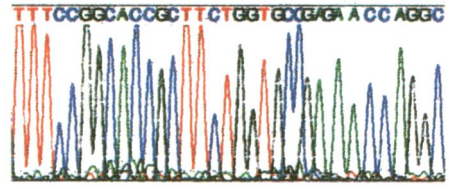
c. dCTPαS, dGTPαS, dATP, dTTP: correct sequence



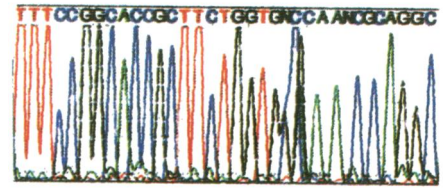
d. dCTPαS, dATP, dGTP, dTTP: correct sequence, but compressed



e. dGTPαS, dATP, dCTP, dTTP: incorrect sequence



f. dATP, dCTP, dGTP, dTTP: incorrect sequence



g. (S_p)-dITPαS, dATP, dCTP, dTTP: correct sequence

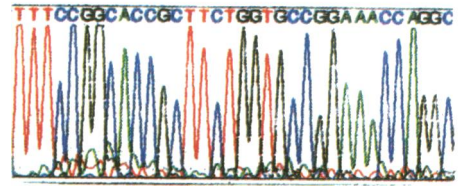


Fig. 7. Effects of different dNTP compositions on the resolution of a hairpin sequence. The sequence consisted of an 11 base pair stem and six base loop (5′-TTTCCGGCACCGCTTCTGGTGCCGGAAA-3′). Each extension reaction contained M13mp18 annealed to a 17-bp primer at base 6571, four fluorescein-labeled terminators, Mn^{2+}, Mg^{2+}, T7 DNA polymerase and pyrophosphatase.

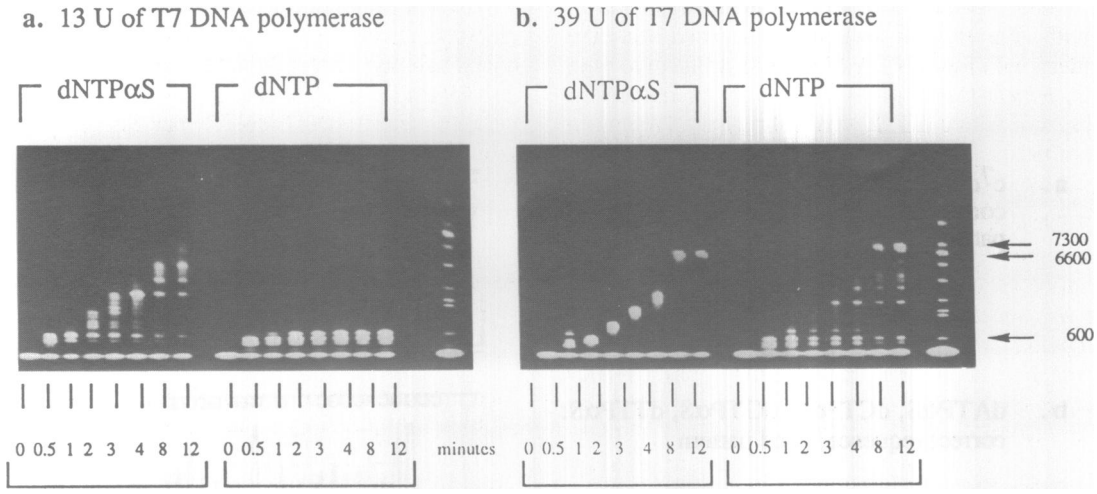


Fig. 8. Assay comparing the activity of T7 DNA polymerase with four dNTPαS's or with four dNTPs. The template was M13mp18 (12 μg) annealed with FAM-labeled −21 primer. The amount of DNA polymerase was 13 U (*panel a*) or 39 U (*panel b*). Aliquots were removed at 0.5, 1, 2, 3, 4, 8, and 12 min and added to stop solution. The extension products were examined on a denaturing (30 mM NaOH) agarose gel on the ABI model 362 GENESCANNER. The molecular weights of three bands in the size standard are indicated.

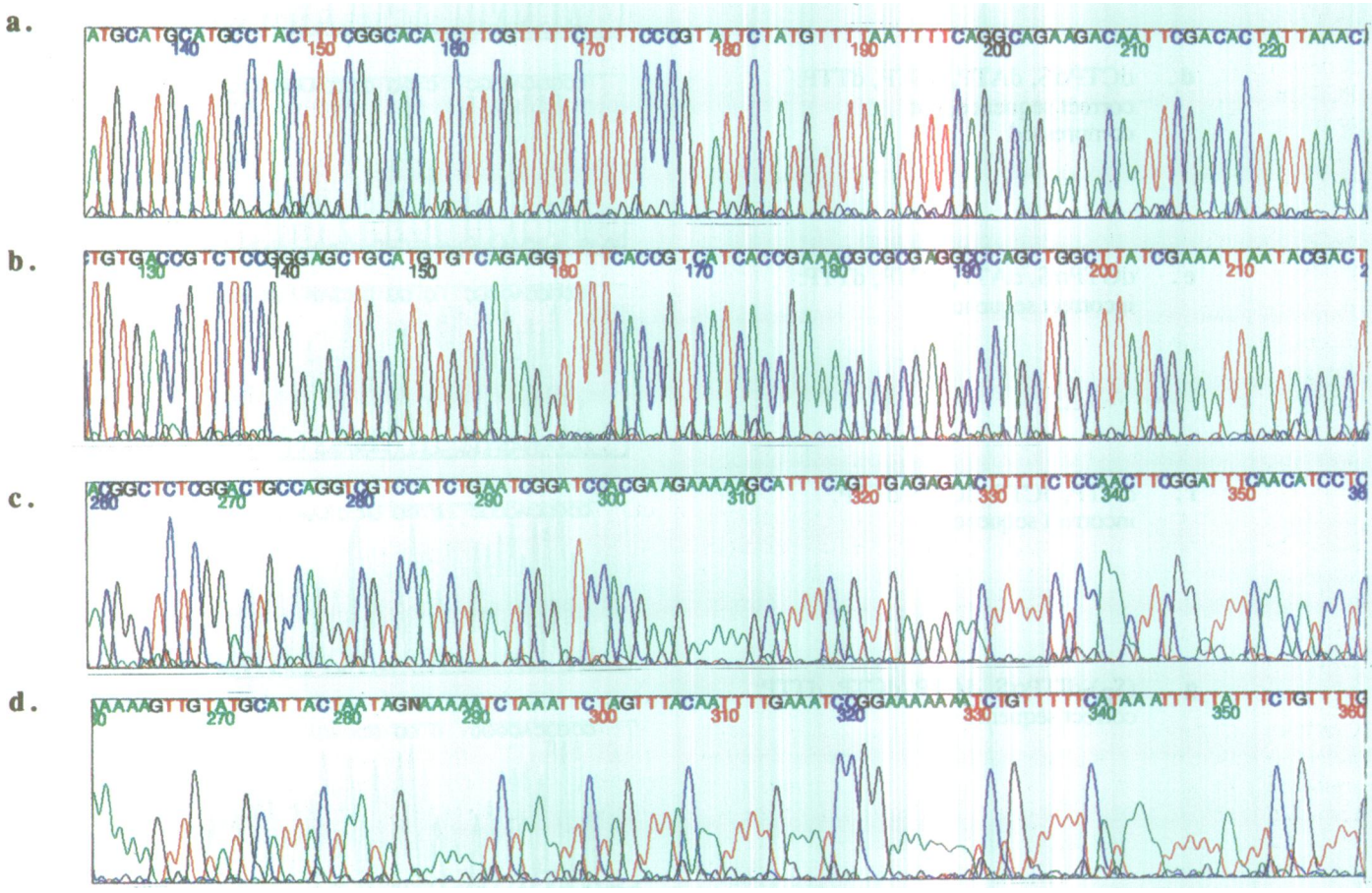


Fig. 9. Representative sequencing data from two PCR products (*panels a* and *b*) and from two single-stranded phagemids (*panels c* and *d*). The PCR samples were 500 and 4,500 base-pairs in length, respectively. Each PCR sample was digested with T7 gene 6 exonuclease, precipitated with PEG and sequenced with fluorescein-labeled terminators. Data is shown from approximately base 130 to 320 from the primer; useful data was obtained to base number 350. The phagemid samples were purified by standard methods[21]. Data is shown from base 260 to 360.

Dye-labeled Primer Sequencing with dNTPαS's. Initial experiments with dye primer sequencing showed that the termination pattern with four dNTPαS's was as uniform as with dNTPs (Fig. 2, *panels c and d*). A few minor compressions which were observed with c^{7}dGTP were entirely resolved when dNTPαS's were used.

Processivity of T7 DNA Polymerase with dNTPαS's. A qualitative idea of how T7 DNA polymerase behaves when all four dNTPs are substituted with dNTPαS's was obtained in a method analogous to that of Tabor and Richardson[10]. A fluorescent dye-labeled primer was annealed to single stranded M13mp18 DNA and chain elongation was initiated by the addition of T7 DNA polymerase, pyrophosphatase and either four dNTPαS's or four dNTPs. Aliquots of the reaction mixture were removed at various time points, the enzyme activity stopped with EDTA and the products analyzed by denaturing agarose gel electrophoresis on the ABI Model 362 GENESCANNER (Fig. 8). The extension reaction with dNTPαS's showed a very different profile than when dNTPs were incorporated. At the lower concentration of T7 DNA polymerase, the enzyme seemed to 'pause' at about base 600 when dNTPs are used. With dNTPαS's, the enzyme synthesized longer extension products, even at the lower enzyme concentration. At higher enzyme concentrations, reactions with either of the dNTP solutions proceeded more efficiently, although the dNTPαS extensions left fewer pauses. These results can be interpreted as higher enzyme efficiency with Mn^{2+} when dNTPαS's are used.

Sequencing of PCR Products with Gene 6 Exonuclease Digestion. PCR products are effectively sequenced by cycle sequencing[17], or by digestion with T7 gene 6 exonuclease[18] to provide single-stranded template. We show here two examples of PCR products sequenced by the latter method (Fig. 9). The PCR products were treated with T7 gene 6 exonuclease (120 U) for 30 min followed by a phenol/chloroform protein precipitation. The reaction products were purified by PEG precipitation before sequencing.

Probability Analysis of Termination Products. The amount of dideoxynucleotide and deoxynucleotides in Sanger sequencing are generally adjusted by trial and error so that a reasonable amount of signal from the beginning to end (balance) is obtained. However, Tabor and Richardson's work with T7 DNA polymerase and Mn^{2+}, which showed almost perfectly uniform peak sizes, suggested that peak sizes and balance could be described mathematically[12]. By making an initial assumption about the probability of termination, we have derived equations which relate the probability of termination to the size of the nth peak. Four-color, one-pot dye-terminator reactions can also be described, and the balance for each color can be adjusted independently.

Analysis of a Repeating Sequence: Total Signal and Balance. Consider a repeating sequence, ACGTACGT.... This sequence is representative of a typical random sequence. Assume that the probability of termination at a ($p_a \leq 1$), the probability of termination at C is p_c, etc. We can write Equation 1, where R_N is an enzyme-dependent discrimination constant that describes the extent of discrimination of the enzyme between each ddNTP and dNTP pair. For T7 DNA polymerase, which does not discriminate against dideoxynucleotides when Mn^{2+} is used, the value of R is unity for each ddNTP/dNTP pair ($R_A = R_C = R_G = R_T = 1$), and is independent of sequence. The value of R is also unity for each ddNTP/(S_p)-dNTPαS pair. Taq polymerase, by contrast, discriminates much more against dideoxynucleotides, and the value of R_A for the unlabeled nucleotide pair, ddATP/dATP, is 0.0003 (based on the ratio of ddATP/dATP used in sequencing[19]). For taq, the values of R vary for each ddNTP/dNTP pair and is dependent on local sequence.

$$p_n = R_N \frac{[\text{ddNTP}]}{[\text{dNTP}]} \quad (1)$$

Assume that the total amount of primed template is D_o. The amount of terminated primer in the first band is:

$$d_1 = p_a D_o$$

That is, the molar amount of DNA in the first peak is the product of the probability of termination and the initial amount of primed template. The molar amount of 3′ hydroxyl on the extended primer which is available to continue polymerization is reduced by the amount consumed in the first peak. Therefore, the molar amount of the next peaks are:

$d_{1a} = p_a Do$ (d_{1a} designates the first A peak)
$d_{1c} = p_c D_o (1-p_a)$ (d_{1c} designates the first C peak)
$d_{1g} = p_g D_o (1-p_a)(1-p_c)$
$d_{1t} = p_t D_o (1-p_a)(1-p_c)(1-p_g)$
$d_{2a} = p_a D_o (1-p_a)(1-p_c)(1-p_g)(1-p_t)$

We can define the probability of not terminating at the ACGT four-base sequence as (1−p). Therefore, we can write:

$$1-p = (1-p_a)(1-p_c)(1-p_g)(1-p_t) \quad (2)$$

Using Equation 2, we can continue the series to arrive at an equation which describes the size of the nth A peak in either the dye-primer or dye-terminator system:

$$\begin{aligned} d_{2a} &= p_a D_o (1-p) \\ d_{3a} &= p_a D_o (1-p)^2 \\ d_{na} &= p_a D_o (1-p)^{n-1} \end{aligned} \quad (3)$$

For example, take the case of an A reaction using traditional primer-labeled sequencing in our repeating four-base sequence. Since there is no ddCTP, ddGTP or ddTTP in the reaction, we can set p_c, p_g, and p_t to zero. Substituting these values into Equation 2, Equation 3 can be written as the simpler form of Equation 4. In order to maximize the size of the nth peak relative to the nth peak for different termination probabilities, the first derivative of Equation 4 is set to zero, as shown in Equation 5.

$$d_{na} = p_a D_o (1-p_a)^{n-1} \quad (4)$$

$$0 = \frac{(d_{na})}{d(p_a)} = -p_a D_0 (n-1)(1-p_a)^{n-2} + D_0 (1-p_a)^{n-1}; \; p_a = \frac{1}{n} \quad (5)$$

To maximize the size of the 500th peak, or 125th A peak in our repeating sequence, the probability of termination (p_a) is set to 1/125. For T7 DNA polymerase the enzyme discrimination constant, R, is unity for each ddNTP/dNTP or ddNTP/(S_p)-dNTP pair, so the ratio of each ddNTP/dNTP should be 1/125, or 0.008.

For the dye terminator system, the enzyme constants vary by a factor of approximately three or four, depending on the local sequence. In general, the discrimination constants are smaller for the dye-labeled nucleotides following a series of Gs. The values for each constant, R, were estimated by testing a range of concentrations of each terminator with a fixed concentration

Table 1. Ratios of ddNTP-dye/dNTPαS in single- and four-color reactions; values of enzyme discrimination constants (R_B) for dye-terminators

Dye Terminator	Single-color reactions: ratio of ddNTP-dye/(R_p,S_p)-dNTPαS (μM/μM)	R_B	Four-color reactions: p_n	ratio of ddNTP-dye/(R_p,S_p)-dNTPαS (μM/μM)
ddA-LOU2	6/400	0.3	0.0016	1/400
ddC-5ZOE	9/400	0.2	0.0016	2/400
ddG-NAN2	8/400	0.2	0.0032	3/400
ddT-6FAM	2/400	0.8	0.0016	0.4/400

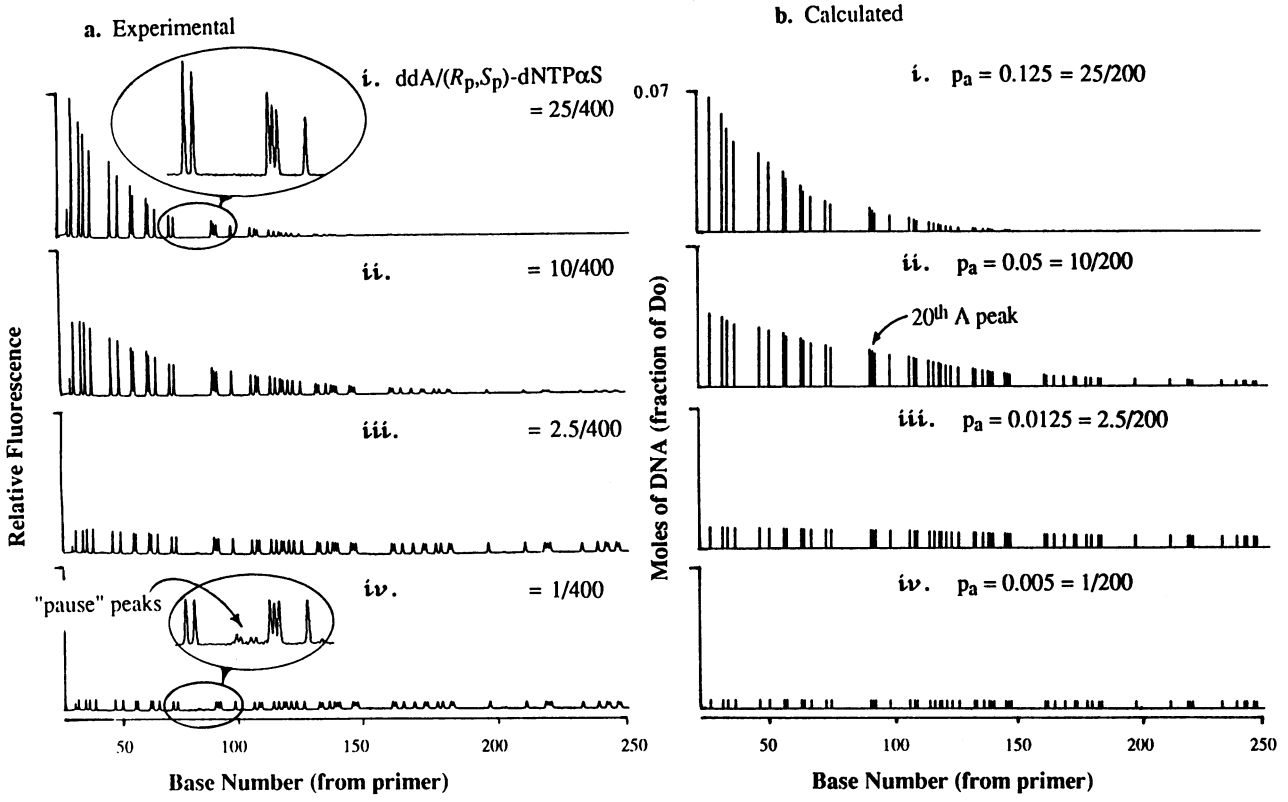


Fig. 10. Comparison of experimental *(panel a)* and calculated *(panel b)* termination patterns with variable ratios of ddATP/dNTPαS. The experimental extension reactions contained M13mp18, FAM-labeled −21 primer, Mn^{2+}, Mg^{2+}, T7 DNA polymerase, pyrophosphatase and ratios of ddATP/(R_p,S_p)-dNTPαS varying from 25 μM/400 μM to 1 μM/400mM. (The ratios of ddATP to the *active* isomers of the dNTPαS's were from 25 μM/200 μM to 1 μM/200 μM.) The fluorescence patterns were collected through the 580 nm filter; all the peaks are shown on the same scale. The first peak in each panel is the 5th A peak following the primer. The calculated termination patterns were obtained by entering the M13mp18 sequence, varying the termination probabilities of A-termination from 0.125 to 0.005 and setting the probabilities of terminating in C, G and T to zero. The linear regression analysis of each fluorescent peak vs. its corresponding calculated molar quantity gave correlation coefficients for *panels i.–iv.* of 0.995, 0.989, 0.969, and 0.969.

of dNTPαS in a single color extension reaction (i.e., for the ddA-dye terminator, $p_c = p_g = p_t = 0$, $p = p_a$ in Equation 2). The ratio of ddNTP/dNTPαS which produced the biggest peak at base 500 was chosen as 'optimal'. The 'optimal' ratio of dd/dNTPαS and a value of p_n of 0.008 were used in Equation 1 to arrive at values for R given in Table I.

In determining the ratios of the eight reagents (four dNTPs, four dye-labeled ddNTPs) in a one-pot extension reaction, the brightness of the dyes must also be considered. Although all the dyes have good quantum yields ($\Phi > 0.5$), the longer wavelength dyes are not excited near their absorbance maxima by the argon ion laser and are dimmer than the shorter wavelength dyes. Best results in four-color sequencing are obtained when the values for the termination probabilities (p_a, p_c, p_g, p_t) are set higher for those terminators with dim dyes, and lower for the brighter dyes.

Using Equation 3, we can see that the size of the n^{th} peak depends on the probability of not terminating at n bases (1-p). When p_a, p_c, p_g and p_t in Equation 2 are small, the higher order terms can be ignored, and:

$$p \cong p_a + p_c + p_g + p_t \quad (6)$$

That is, we can vary the values of p_a, p_c, p_g and p_t independently without changing the size of the n^{th} peak as long as their sum is a small constant, p.

With the T7 DNA polymerase terminators, the dyes on A, C and T are approximately equivalent in brightness, but the dye

on G is about half as bright. Therefore, we can arrange the ratios in Equation 6 so that the probability of termination in G is twice that of any other base, and so the sum of the termination probabilities is 0.008 ($p_a + p_c + p_g + p_t = p = 0.008$). Table I gives the ratios for single and four color reactions required to maximize the 500th nucleotide peak and the estimated enzyme discrimination constants, R.

The pattern of termination for a specific sequence can be calculated from the sequence, the ratio of ddNTP/dNTP, and the termination probabilities. We have written a software program which requests sequence (e.g., AGCCAA), termination probabilities (p_a, p_c, p_g, p_t) and amount of template (D_o). The program calculates the quantities of DNA in each peak (d_n) based on Equation 3. The data can be displayed in a spreadsheet and plotted in a bar graph format to generate the familiar sequencing pattern. Fig. 10, *panel b* shows the results of a calculation to generate the A termination pattern with M13mp18 for different values of p_a. The M13 sequence was entered, the probabilities of termination in C, G and T were set to zero (p_c, p_g, $p_t = 0$), and the probability of termination in A was varied from 0.125 to 0.005. Fig. 10, *panel a* shows the results of a sequencing experiment using single-stranded M13mp18 DNA, dye-labeled primer, four dNTPαS's, T7 DNA polymerase, and varying concentrations of ddATP. T7 DNA polymerase and dye primers were used since the enzyme discrimination constants are independent of sequence and best fit the model. The ratios of ddATP/dNTPαS were varied to give the same termination probabilities as in Fig. 10, *panel β*, calculated using Equation 1, where $R_A = 1$, and where the concentration of dNTPαS is divided by two, since only half of the dNTPαS's are the active (S_p)-diasteriomers[19].

The experimental and calculated patterns correlate well. The correlation coefficients, given in Fig. 10, are the results of a comparison between the calculated molar quantities and the actual fluorescence of each peak (measured in peak height). The 'pause' peaks, or nonspecific terminations, become increasingly apparent at lower values of p_a (Fig. 10, *panel a*: *i* vs. *iv*). The probability of pausing can also be assigned a value (p_p), and the size of the pause peak is the product of p_p and the amount of available primed template. So, if the pause occurs at position n, the size of the pause peak would be $p_p D_o (1-p_a)^{n-1}$. For a given extension incubation time, the pause peaks should increase for decreasing values of p_a.

Each termination probability (p_a) maximizes one peak relative to the corresponding peak for different termination probabilities. For example, in Fig. 10, *panel b.ii.*, the value of p_a is 0.05 or 1/20. Using Equation 5, we can see that the 20th A peak is maximized relative to the 20th A peak in *panels b.i.* or *b.iii.*

CONCLUSIONS

Dideoxy terminators labeled with analogs of fluorescein provide acceptable quality DNA sequencing data on single-stranded DNA with the ABI model 373 DNA sequencer. The main advantages of this method are the simplicity in performing the extension reactions and the ability to use any unlabeled primer. The main disadvantage is the need for fairly high quantities of template DNA (0.8 pmol) as compared to the amount needed for cycle sequencing to achieve adequate signal-to-noise for the automated base-calling software. The termination patterns for this set of terminators is more uniform than either the Du Pont terminators or the ABI taq terminators. The uniformity translates to better signal-to-noise in the sequencing data for adequate signal. The lack of thermal stability of T7 DNA polymerase, however, makes the simple cycling protocols which are so convenient for sequencing double-stranded templates out of reach. This problem may be solved in part by using PCR amplification and T7 gene 6 exonuclease as a means of preparing template DNA. In addition, we are currently working on variations of the alkaline denaturation method[20] as a method to sequence double stranded plasmid DNA using fluorescein terminators.

An additional advantage of this chemistry is that the use of four dNTPαS's instead of c^7dGTP with T7 DNA polymerase appears to be a promising method to resolve gel compressions. That dCTPαS appears to be the main component in destabilizing hairpin structures suggests investigation of analogs of dCTP may provide additional nucleotides which are useful for DNA sequencing. In addition, the use of four dNTPαS's with Mn^{2+} appears to improve the efficiency of the enzyme, suggesting that the usefulness of the lesser-used nucleotides (e.g., dITP, c^7dITP) may be improved with thiotriphosphate versions.

Modeling the typical DNA sequencing pattern with probability analysis addresses such sequencing issues as maximizing signal, optimizing protocols, and understanding the effects of chemical structures in the extension reactions. Measurement of the termination pattern to arrive at the enzyme discrimination constants may be a simple way to evaluate chain terminators such as AZT or ddI without separately measuring K_m or V_{max} values.

REFERENCES

1. Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc. Nat. Acad. Sci. USA* **74**, 5463–5467.
2. Hunkapiller, T., Kaiser, R.J., Koop, B.F., Hood, L. (1991) *Science* **254**, 59–67.
3. Smith, L.M., Sanders, J.Z., Kaiser, R.J., Hughes, P., Dodd, C., Connell, C.R., Heiner, C., Kent, S.B.H., Hood, L.E. (1986) *Nature* **321**, 674–679.
4. Connell, C., et. al. (1987) *BioTechniques* **5**, 342–348.
5. Prober, J.M., Trainor, G.L., Dam, R.J., Hobbs, F.W., Robertson, C.W., Zagursky, R.J., Cocuzza, A.J., Jensen, M.A., and Baumeister, K. (1987) *Science*, **238**, 336–341.
6. Ansorge, W., Sproat, B., Stegemann, J., Schwager, C., Zenke, M. (1987) *Nucleic Acids Res.* **15**, 4593–4602.
7. Kambara, H., Nishikawa, T., Katayama, Y., and Yamaguchi, T. (1988) *Bio/Technology* **6**, 816–821.
8. *(a)* Carothers, A.M., Urlaub, G., Mucha, J., Grunberger, D. and Chasin, L.A. (1989) *BioTechniques* **7**, 494–499. *(b)* Murray, V. (1989) *Nucleic Acids Res.* **17**, 8889.
9. Patent Corporation Treaty PCT/US90/05565 April 1991.
10. Tabor, S. and Richardson, C.C. (1989) *J. Biol. Chem.* **264**, 6447–6458
11. Tabor, S. and Richardson, C.C. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 4076–4080.
12. Tabor, S. and Richardson, C.C. (1990) *J. Biol. Chem.* **265**, 8322–8328.
13. Ludwig, J., and Eckstein, F. (1989) *J. Org. Chem.* **54**, 631–635.
14. Khanna, P., Ullman, E.F. (1982) US Patent 4,318,846.
15. Patent Cooperation Treaty PCT/US90/06608 May 1991
16. Prober, J.M., Dam, R.J., Robertson, C.W., Jr., Hobbs, F.W., Jr. and Trainor, G.L. Eur. Pat. Appl. (1988), O 252,683.
17. Tracy, T.E. and Mulcahy, L.S. (1991) *BioTechniques* **11**, 68–75.
18. (a) Straus, N.A. and Zagursky, R.J. (1991) *BioTechniques* **10**, 376–384. (b) Ruan, C.C., and Fuller, C.W. (1991) *Comments*, **18**, 1–8, United States Biochemical Corp., Cleveland, OH.
19. Innis, M.A., Myambo, K.B., Gelfand, D.H. and Brow, M.D. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 9436–9440.
20. Eckstein, F. (1985) *Annu. Rev. Biochem.* **54**, 367–402.
21. Chen, E.Y. and Seeburg, P.H. (1985) *DNA*, **4**, 165–170.
22. Vieir, J. and Messing,J. (1987) *Methods in Enzymology*, **153**, 3–11.