Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 4347–4351, May 1995
Biophysics

Fluorescence energy transfer dye-labeled primers for DNA sequencing and analysis

(Förster energy transfer/oligodeoxynucleotide synthesis/primer labeling/fluorescent tags)

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Communicated by I. Tinoco, Jr., University of California, Berkeley, CA, January 6, 1995

ABSTRACT **Fluorescent dye-labeled DNA primers have been developed that exploit fluorescence energy transfer (ET) to optimize the absorption and emission properties of the label. These primers carry a fluorescein derivative at the 5′ end as a common donor and other fluorescein and rhodamine derivatives attached to a modified thymidine residue within the primer sequence as acceptors. Adjustment of the donor–acceptor spacing through the placement of the modified thymidine in the primer sequence allowed generation of four primers, all having strong absorption at a common excitation wavelength (488 nm) and fluorescence emission maxima of 525, 555, 580, and 605 nm. The ET efficiency of these primers ranges from 65% to 97%, and they exhibit similar electrophoretic mobilities by gel electrophoresis. With argon-ion laser excitation, the fluorescence of the ET primers and of the DNA sequencing fragments generated with ET primers is 2- to 6-fold greater than that of the corresponding primers or fragments labeled with single dyes. The higher fluorescence intensity of the ET primers allows DNA sequencing with one-fourth of the DNA template typically required when using T7 DNA polymerase. With single-stranded M13mp18 DNA as the template, a typical sequencing reaction with ET primers on a commercial sequencer provided DNA sequences with 99.8% accuracy in the first 500 bases. ET primers should be generally useful in the development of other multiplex DNA sequencing and analysis methods.**

The human genome project is driving the development of high-speed and high-throughput DNA sequencing and analysis methods (1–3). Currently, four-color DNA sequencing on slab gels with fluorescent primers or terminators is the most commonly used method (4, 5). Recently, we introduced the technique of capillary array electrophoresis coupled with laser-excited confocal-fluorescence scanning, which provides one approach for increasing the speed and throughput of DNA sequencing and analysis (6–8). However, here, as in other sequencing methodologies, the detection sensitivity is limited by the spectroscopic properties of the available dyes for labeling the sequencing fragments. A requirement of four-color sequencing is that the emission maxima of the four fluorophores be distinctive. This criterion is met by the dyes currently used for four-color sequencing (5, 9). The trade-off, however, is that these dyes do not have equivalent molar absorbances in the range of excitation wavelengths available with an instrument having a single, argon-ion laser. For example, primers labeled with the rhodamine dyes yield far weaker signals than those labeled with the fluorescein dyes. To compensate for this effect, one must use more template DNA in the reactions involving rhodamine dye-labeled primers. One way to solve this problem is to use multiple lasers (10, 11). A second approach is to improve the spectroscopic properties of the dyes used for labeling.

To increase the sensitivity of DNA detection in four-color sequencing, ideally one would like each of the four dyes (*i*) to exhibit strong absorption at a common laser wavelength, (*ii*) to have an emission maximum at a distinctly different wavelength, and (*iii*) to introduce the same relative electrophoretic mobility shift of the DNA sequencing fragments. These criteria are inconsistent with the spectroscopic properties of single fluorescent dye molecules having high-emission quantum yield. However, these objectives are met by energy transfer (ET) primers, whose design, synthesis, and application to four-color DNA sequencing are presented here.

Fluorescence ET is mediated by a dipole–dipole coupling between chromophores that results in resonance transfer of excitation energy from an excited donor molecule to an acceptor (12). Förster established that the ET efficiency is proportional to the inverse sixth power of the distance between the two chromophores. Thus, fluorescence resonance ET has been used extensively as a spectroscopic ruler for biological structures (13), and ET-coupled tandem phycobiliprotein conjugates have found wide application as unique fluorescent labels (14). Earlier, we showed that polycationic heterodimeric fluorophores that exploit ET and that have high affinities for double-stranded DNA offer advantages over monomeric fluorophores in multiplex fluorescence-labeling applications (15–17). We show here that the ET primers are markedly superior to single dye-labeled primers in DNA sequencing, and we anticipate that they will find broad use in multiplex PCR-based mapping and sizing protocols.

MATERIALS AND METHODS

Chemicals were purchased from Applied Biosystems unless otherwise stated. Sequenase (Version 2.0) T7 DNA polymerase and other DNA sequencing reagents were manufactured by Amersham. Oligodeoxynucleotides were synthesized by the phosphoramidite method on an Applied Biosystems 392 DNA synthesizer.

Design and Synthesis of ET Primers. The structures of the four ET primers used here and a representative synthetic reaction are presented in Fig. 1. Oligodeoxynucleotides (18 bases long) with the sequence 5′-GTTTTCCCAGTCACGACG-3′ (the M13-40 universal forward sequencing primer) were synthesized with donor–acceptor fluorophore pairs separated by different distances. The 18-mer contains a modified base (T*) introduced by the use of 5′-dimethoxytrityl-5-[*N*-(trifluoroacetylaminohexyl)-3-acrylimido]-2′-deoxyuridine 3′-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite (Amino-

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Abbreviations: ET, energy transfer; FAM, 5-carboxyfluorescein; JOE, 2′,7′-dimethoxy-4′,5′-dichloro-6-carboxyfluorescein; TAMRA, *N*,*N*,*N*′,*N*′-tetramethyl-6-carboxyrhodamine; ROX, 6-carboxy-X-rhodamine; ddNTP, dideoxynucleoside triphosphate.

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QIAGEN Exhibit 1017
Page 1

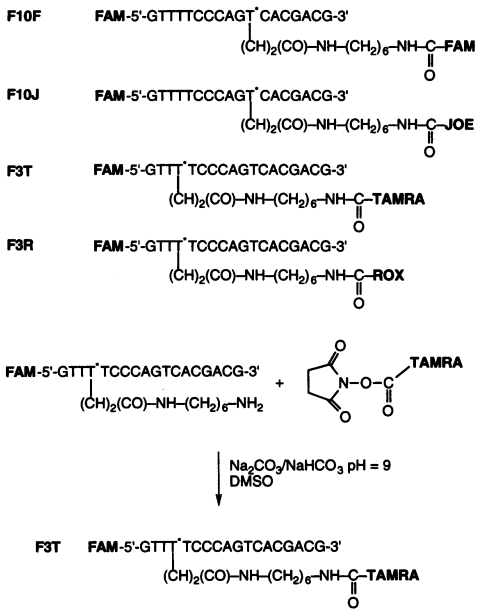

FIG. 1. Structures of the four ET primers (F10F, F10J, F3T, and F3R) and a synthetic scheme for the preparation of F3T.

Modifier C6 dT, Glen Research), which has a protected primary amine linker arm. The donor dye was attached to the 5′ end of the oligomer, and the acceptor dye was attached to the primary amine group on the modified base (T*). The ET primers are named using the abbreviation D-N-A, where D is the donor, A is the acceptor, and N is the number of bases between D and A. In all the primers prepared, 5-carboxyfluorescein (FAM or F in primer name) is selected as a common donor and 2′,7′-dimethoxy-4′,5′-dichloro-6-carboxyfluorescein (JOE or J), *N,N,N′,N′*-tetramethyl-6-carboxyrhodamine (TAMRA or T), and 6-carboxy-X-rhodamine (ROX or R) are selected as acceptors. We have synthesized 20 ET primers labeled with the same donor at the 5′ end and with different acceptors at different positions on the primer sequence. The spacing between the two chromophores is altered by varying the position of T* in the synthesis of each primer. We found that the electrophoretic mobility of the ET primers depends on the spacing between the donor and acceptor. Within a range of distances determined by the number of intervening bases that allow good ET, it is possible to adjust the electrophoretic mobility of the primers. Application of this principle leads to the selection of four ET primers (F10F, F10J, F3T, and F3R) with optimum fluorescence properties and similar electrophoretic mobilities for four-color DNA sequencing. The detailed evaluation of these four primers for DNA sequencing is described here. The complete synthesis, as well as the spectroscopic and electrophoretic properties of all 20 primers will be reported elsewhere.

To prepare the ET primers, the donor FAM was introduced by using 6-FAM amidite in the last step of the oligonucleotide synthesis on a DNA synthesizer. After cleavage from the solid support and removal of the base-protecting groups, the primers were evaporated to dryness under vacuum (0.5 mm of Hg). To incorporate the acceptor dyes, 15–20 nmol of FAM-labeled, T*-containing oligonucleotides in 40 μl of 0.5 M $Na_2CO_3/NaHCO_3$, pH 9.0, buffer were incubated overnight at room temperature with an approximately 150-fold excess of the corresponding FAM, JOE, TAMRA, and ROX *N*-hydroxy succinimidyl esters in 12 μl of dimethyl sulfoxide. Unreacted dye was removed by size-exclusion chromatography on a Sephadex G-25 column (Pharmacia). The ET primers were then purified by standard electrophoresis methods (18) and desalted with an oligonucleotide purification cartridge. The single dye-labeled primers with the same sequence as that of the ET primers were prepared by the standard protocol using Aminolink 2 (18). The purity of the primers was shown to be >99% by polyacrylamide capillary gel electrophoresis. Primers were quantified by their absorbances at 260 nm and then stored in 10 mM Tris·HCl/1 mM EDTA, pH 8.0, at a final concentration of 0.4 pmol/μl.

The absorption spectra of the primers were measured on a Perkin–Elmer Lambda 6 UV/visible spectrophotometer, and the fluorescence-emission spectra were taken on a Perkin–Elmer model MPF 44B spectrofluorimeter. Samples were dissolved in 1× TBE (89 mM Tris/89 mM boric acid/2 mM EDTA, pH 8.0) containing 7 M urea. Quantum yields of the ET primers were determined by comparison with fluorescein (Φ_f = 0.93 in 0.1 N NaOH; refs. 19 and 20).

DNA Sequencing. DNA sequencing was performed by using M13mp18 template DNA and modified T7 DNA polymerase on an Applied Biosystems 373A DNA sequencer. Four reaction mixtures were run, one for each dye/dideoxynucleoside triphosphate (ddNTP) combination. The reaction mixtures containing ddCTP were run with the F10F primer, those containing ddATP were run with the F10J primer, those containing ddGTP were run with the F3T primer, and those containing ddTTP were run with the F3R primer. The working buffer was freshly prepared by mixing equal volumes of 400 mM Mops, pH 7.5/500 mM NaCl/100 mM $MgCl_2$ (10× Mops buffer) and 50 mM $MnCl_2$/150 mM sodium isocitrate (10× Mn buffer). One microliter of this buffer mixture was then combined with 1 μl of primer (0.4 pmol), the indicated amount of template DNA, and water to a total volume of 5 μl. The mixtures of primers and templates were annealed by heating at 65°C for 2 min and slowly (≈35 min) cooling to <30°C. Three microliters of the dNTP/ddNTP mixture (2.4 mM each of dGTP, dATP, dTTP, and dCTP with 8 μM specific ddNTP) was then added, and the reaction mixture was warmed to 37°C for 2 min. Then 2 μl of a freshly diluted mixture of T7 DNA polymerase (2 units/μl) and yeast pyrophosphatase (1.5 units/ml) was added, and incubation was continued at 37°C for 30 min. The four reactions were stopped and the mixtures for each sequence were then combined into one vial, and 4 μl of 3 M sodium acetate, together with 180 μl of 95% ethanol, was added to precipitate the DNA. After 15 min at −20°C, the precipitated DNA was collected by centrifugation (12,000 × *g*) for 15 min, and washed twice with 70% ethanol. The precipitated DNA was vacuum dried, resuspended in 5 μl of deionized formamide containing 8.3 mM EDTA, and heated at 95°C for 2 min. The denatured DNA was loaded onto a denaturing 8.3 M urea/6% polyacrylamide gel mounted in the Applied Biosystems 373A DNA sequencer equipped with argon-ion laser excitation. Electrophoresis was conducted at a constant power of 35 W for 12–14 h using Tris/taurine/EDTA buffer (21). The data were analyzed by using the Applied Biosystems 373A software (Version 1.2.0).

RESULTS

Spectroscopic Properties of ET Primers. The absorption and emission spectra of the ET primers are presented in Fig. 2. The ratio of the absorbance at 260 nm to that at 496 nm of F10F is 1.3, while this ratio for the single FAM-labeled 18-mer is 2.2. This is consistent with the incorporation of two FAM molecules in F10F. Each ET primer exhibits the characteristic absorption of FAM at 496 nm, as well as strong absorption at 525 nm due to JOE in F10J, 555 nm due to TAMRA in F3T, and at 585 nm due to ROX in F3R. The fluorescence spectra of the ET primers are dominated by the acceptor emissions. While the emission maximum of F10F is at 525 nm, the emission of F10J with 488-nm excitation is Stokes-shifted to

QIAGEN Exhibit 1017
Page 2

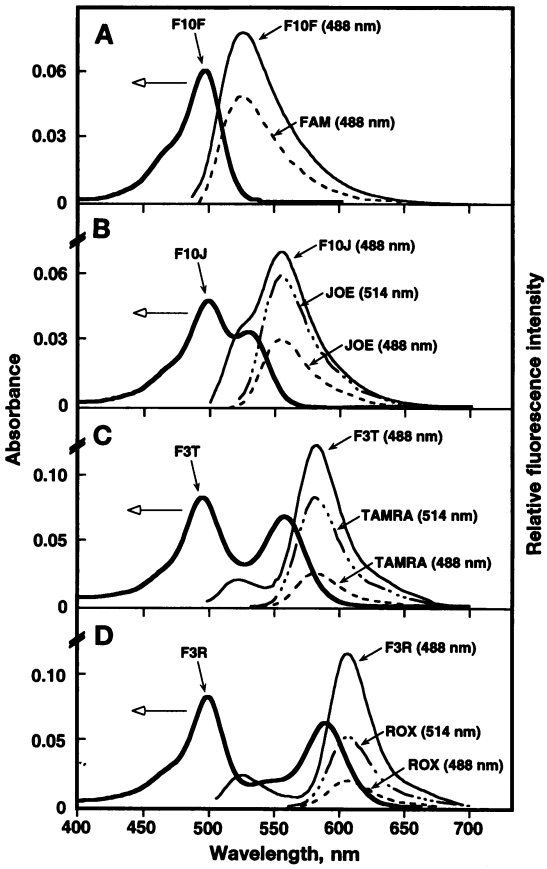

FIG. 2. Comparison of the fluorescence emission intensity of the four ET primers with the corresponding single dye-labeled primers at the indicated excitation wavelength (1× TBE/7 M urea). The thick lines indicate the absorption spectra of the indicated ET primers. (*A*) F10F vs. FAM, (*B*) F10J vs. JOE, (*C*) F3T vs. TAMRA, and (*D*) F3R vs. ROX. The emission spectra for each primer pair were determined in solutions having the same 260-nm absorbances, and the excitation powers at 488 nm and 514 nm were the same.

555 nm, that of F3T is shifted to 580 nm, and that of F3R is shifted to 605 nm. In the case of F3R, the Stokes shift is over 100 nm. Fig. 2 also presents emission spectra of the single dye-labeled primers measured at the same 260-nm absorbances as the corresponding ET primers. Substantial enhancement of the ET primer emission intensity is observed compared with the corresponding single dye-labeled primers, indicating that efficient ET is occurring. After correcting for the absorption contributed by the fluorophores at 260 nm, the fluorescence intensity improvements derived from Fig. 2 are the following: F10F = 1.8 × FAM; F10J = 2.5 × JOE or, when JOE is excited at 514 nm, 1.4 × JOE; F3T = 5.3 × TAMRA or, when TAMRA is excited at 514 nm, 1.7 × TAMRA; F3R = 6.2 × ROX or, when ROX is excited at 514 nm, 2.3 × ROX. To evaluate the emission spectral purity of the four ET primers, their normalized emission spectra are presented in Fig. 3. It can be seen that the residual emission of FAM in F10J, F3T, and F3R is very small. On the basis of a comparison of the residual FAM emission in the ET primers with that of a FAM-labeled primer with the same sequence and length, the ET efficiency was calculated to be 65% for F10J, 96% for F3R, and 97% for F3T (22). The fluorescence quantum yields based on the integrated emissions from both donor and acceptor for the four primers relative to fluorescein were 0.82 for F10F, 0.63 for F10J, 0.15 for F3T, and 0.13 for F3R.

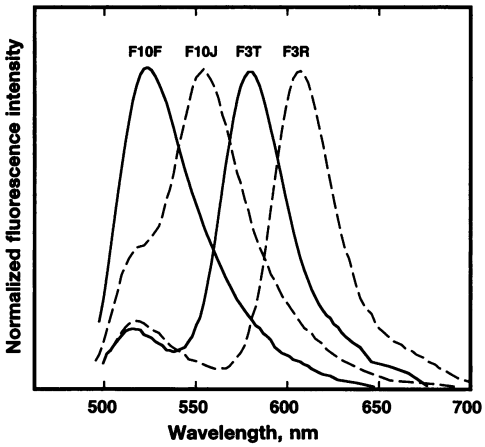

FIG. 3. Normalized fluorescence emission spectra of the four ET primers dissolved in 1× TBE/7 M urea.

DNA Sequencing with ET Primers. Single-base extension (ddTTP/dNTPs) experiments were performed to examine the relative mobility shift and detectability of DNA fragments generated with the ET primers. Fig. 4 presents raw fluorescence intensity traces from experiments on an ABI 373A DNA sequencer. The graphs in Fig. 4*A* were obtained by using M13-40 primers labeled with single dye molecules. The differences in mobility of the DNA fragments can be clearly seen. The TAMRA- and ROX-labeled fragments migrate about one nucleotide slower than the FAM- and JOE-tagged DNA fragments and have dramatically weaker intensities. The corresponding runs with the ET primers are presented in Fig. 4*B*. The mobilities of these DNA fragments are more closely matched (less than a quarter of a nucleotide difference).

To quantify the instrument sensitivity with the ET primers, reactions were carried out with a constant amount of primer (0.4 pmol) and various amounts of M13mp18 template DNA (0.05–1 pmol). A single gel was run for this entire DNA concentration range to permit a direct comparison between a single dye-labeled primer and its corresponding ET primer. Band intensities were measured by using the raw data from the optical filter that gave the strongest signal. Measurements were made by printing raw-data graphs with fixed vertical scales and by measuring peak height. This method essentially gave the same overall result as peak-area measurements. Graphs of several band intensities against quantity of template were made. The slopes of these graphs gave an indication of the instrument sensitivity per pmol of DNA. This method indicates that the sensitivity of the F10F primer is 160% that of the FAM primer. Similarly, the sensitivities of the F10J, F3T, and F3R primers are 360%, 400%, and 470% that of the JOE, TAMRA, and ROX primers, respectively. Additional estimates of the relative sensitivities can be made by measuring raw peak heights in sequencing experiments and by using the signal intensity values reported by the ABI software. These estimates all suggest that the sensitivity of the F10F and F10J primers is between 150% and 300% that of the FAM and JOE primers and that the sensitivity of the F3T and F3R primers is between about 350% and 500% that of the TAMRA and ROX primers. In experiments which included an excess of template DNA over primer, only a small fraction of either ET or single dye-labeled primer remained unextended. Thus, no significant difference was seen in the efficiency with which the ET primers were extended by DNA polymerase compared with single dye-labeled primers. The fluorescence correction matrix values obtained for the ET primers on the sequencer were also remarkably similar to those of the single dye-labeled primers,

QIAGEN Exhibit 1017
Page 3

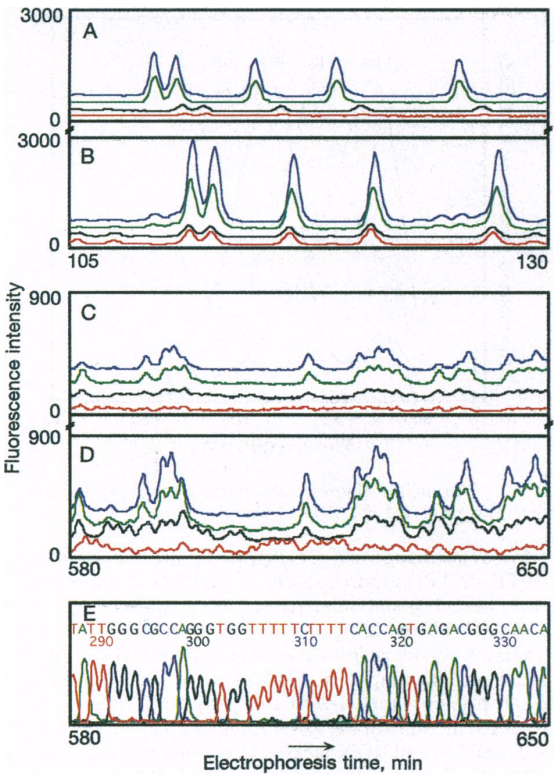

FIG. 4. Comparison of signal strengths and mobility shifts of the single dye-labeled primers and ET primers. A total of eight sequencing reactions with ddTTP/dNTPs were carried out by using 1 μg of M13mp18 DNA template and 0.4 pmol of primer and then loaded in eight adjacent lanes of the ABI 373A DNA sequencing gel. (*A*) Raw traces obtained when single dye-labeled primers were used. Colors correspond to the dye as follows: blue, FAM; green, JOE; black, TAMRA; red, ROX. The region shown corresponds to the DNA sequence located approximately 15–35 nt from the 3′ end of the primer. (*B*) Raw traces on identical scales obtained when using ET primers. Colors correspond to the dye as follows: blue, F10F; green, F10J; black, F3T; red, F3R. (*C* and *D*) Data from four-color sequencing reactions carried out with single-dye primers (*C*) or ET primers (*D*) on identical scales. For reference, the ET primer data in *D* are also shown in analyzed format in *E*. The reaction mixtures used for panel *C* included 0.4 pmol each of the FAM and JOE primers and 0.8 pmol each of the TAMRA and ROX primers, and the reaction mixtures for panel *D* and *E* included 0.4 pmol of each ET primer and a total of 6 μg of M13mp18 template DNA.

further confirming that the fluorescence emission of the ET primers is dominated by acceptor emission.

Typical raw fluorescence intensity traces for four-dye, single lane sequences are presented in Fig. 4 *C* and *D*. Shown here is a portion from the middle of the run spanning about 45 nt. On this intensity scale, the peaks from the red filter are barely discernible when the single ROX-labeled primer is used (Fig. 4*C*). In contrast, with the ET primers, all of the sequence-dependent intensity fluctuations are readily seen in the raw data (Fig. 4*D*). While four-color sequences analyzed with this instrument typically require 3-fold more template and 2-fold more primer in the reaction mixtures containing TAMRA- and ROX-labeled primers, the four reactions used for Fig. 4*D* contained equal amounts of ET primer and template. This change in reaction balance was made possible by the increased relative fluorescence intensities of the F3T and F3R primers.

The software must also compensate for the different electrophoretic mobility shifts introduced by the different dyes. Considering the small mobility differences between DNA fragments generated with ET primers, we first tried applying no mobility corrections. The result was accurate for the first 80–100 nt, after which miscalls were obtained about once every 20 nt. Application of small mobility shift corrections resulted in much more accurate sequence, an example of which is shown in Fig. 5. The software correctly assigned all of the first 450 bases where the first miscall occurred (four A residues called where three A residues is correct). No other miscalls occurred within the first 510 nucleotides, giving a sequence accuracy of over 99.8% for 500 bases. This sequence was obtained by using a total of 0.6 μg (0.24 pmol) of M13 template DNA. This is approximately one-fourth the amount of template DNA required to give similar sequence accuracy with single dye-labeled primers. Sequences obtained when using more DNA (up to 6 μg total) were equally accurate, and a sequence obtained by using only 0.3 μg of template DNA had similar accuracy up to about 400 nt.

DISCUSSION

Fluorescence ET is a well known and useful spectroscopic phenomenon that has been exploited extensively for a variety of biological analyses (13, 23–27). The fluorescence ET concept is potentially valuable for the development of multiplex genetic analysis methods. Such multiple fluorescent labeling experiments have conflicting requirements which are inconsistent with the use of single fluorophores. However, we have shown here that fluorescence ET can be used to design and synthesize a class of fluorescent labels that have nearly ideal spectroscopic properties. For this approach to be successful, the donor and acceptor dyes must be kept sufficiently far apart to avoid self-quenching, while being close enough for efficient ET. To control the separation between the donor and acceptor, we have used the oligonucleotide primer as a "scaffold" to place the dyes at defined positions having specific separation distances. The ET primers (F10F, F10J, F3T, and F3R) all have strong absorption at 488 nm, while exhibiting distinct emission spectra with maxima at 525, 555, 580, and 605 nm, respectively (see Fig. 3). The chosen spacings provide from 65% to 97% ET. The weak emission at ≈525 nm in the spectra of the ET primers (F10J, F3T, and F3R) testifies to the ET efficiency. The quantum yields of the F10F, F10J, F3T, and F3R primers are 0.82, 0.63, 0.15, and 0.13 respectively, while the quantum yields of the single dye-labeled primers are 0.82 for FAM, 0.58 for JOE, 0.26 for TAMRA, and 0.30 for ROX. Thus, the F10F and F10J primers do not exhibit any quenching, while the quantum yields of F3T and F3R primers drop by only 50%. Although the ET primers having two chromophores that are close together exhibit some self-quenching, their fluorescence emissions are still much stronger than those of the single dye-labeled primers because of their enhanced absorbances at the excitation wavelength. In addition to characterizing the spectroscopic properties of the ET primers, we have demonstrated their practical utility for DNA sequencing.

Four-color DNA sequencing is a demanding application because one desires intense signals, minimal cross-talk between the fluorescence detection channels, and small correctable mobility shifts. Compared with single dye-labeled primers, the ET primers exhibited similar cross-talk between the four-color channels and smaller mobility shifts. Most important, the use of ET primers dramatically increases the observed signal strengths. Depending on the detailed excitation conditions, the signal strengths of the ET primers were from 1.8 to 6 times greater than those of the single dye-labeled primers. Thus, ET primers should be superior for DNA sequencing. Sequencing runs performed on the Applied Biosystems 373A DNA sequencer confirmed this expectation. The signal strengths of the sequenced fragments were from 1.5 to 5 times higher than those of the single dye-labeled fragments. With the ET primers, the amount of DNA template required for

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Page 4

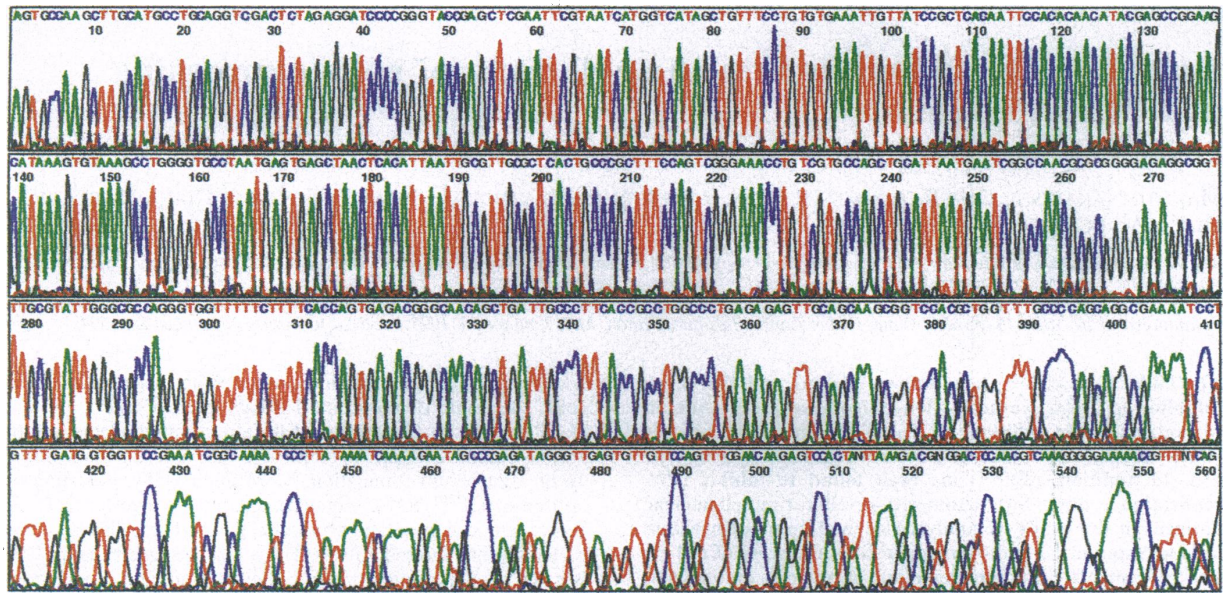

FIG. 5. DNA sequencing profile of single-stranded M13 DNA when using the four ET primers and Sequenase 2.0. Primer concentration: 0.4 pmol; DNA template: 0.6 μg (0.15 μg for each extension reaction). Comparison of this sequence with the known sequence of M13mp18 indicates a single error within the first 510 nt.

high-quality sequencing when using Sequenase is reduced to only 0.6 μg, one fourth of the amount recommended (>2 μg) when using the single dye-labeled primers. Thus, it should be possible to perform direct sequencing on small amounts of DNA template that would ordinarily require cycle sequencing. In addition, with ET primers the signal strength is still quite strong at more than 560 nucleotides beyond the primer. Thus, improvement of the resolving ability of the gel or using longer gels to achieve longer read-outs would now be more advantageous. The increased signal strength available with ET primers should also facilitate DNA sequencing and analysis using capillary array electrophoresis (6, 7).

In summary, the unique spectroscopic properties of ET primers make them valuable in all areas where multiple fluorescent tags are to be distinguished spectroscopically. In addition to DNA sequencing, applications to DNA fragment sizing, forensic identification, short tandem repeat (STR) typing, DNA sequencing by hybridization (28), and *in situ* hybridization are but a few examples of the potential applications of ET primers.

Note Added in Proof. We have recently demonstrated that ET primers can also be used to perform PCR amplification of short tandem repeat alleles followed by multiple-color sizing (29).

We thank the members of the Berkeley High-Sensitivity DNA Analysis Project and Dr. John Chase from Amersham Life Science Corporation for many valuable interactions. This research was supported by the Director, Office of Energy Research, Office of Health and Environmental Research of the U.S. Department of Energy under contract DE-FG-91ER61125. J.J. was supported by a Human Genome Distinguished Postdoctoral Fellowship sponsored by the U.S. Department of Energy, Office of Health and Environmental Research, and administered by the Oak Ridge Institute for Science and Education. Financial support from the Amersham Life Science Corporation is also gratefully acknowledged.

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Page 5