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26. The methylation status of the Hha I site located just upstream of the KI and KII sites [U. Storb and B. Arp, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 6642 (1983); K. J. Nelson, E. L. Mather, R. P. Perry, *Nucleic Acids Res.* **12**, 1911 (1984)] was studied with the same PCR assay as the one described in Fig. 3. Bone marrow fractions C′ and D, representing the cell populations engaged in the process of κ light chain rearrangement, were sorted from heterozygous animals (*21*) and the proportion of each allele assessed before and after Hha I (methylation-sensitive) digestion. No obvious difference in methylation between the wild-type and the mutant allele was observable in this assay (*18*).
27. These results differ from those obtained by Takeda *et al.* (*4*) with the intronic κ enhancer knock-out; in mice homozygous for that mutation, κ rearrangement is completely blocked. The total number of B cells in these mice was about half that in normal mice, and only λ-expressing B cells were detected in the periphery. Whether the deletion of the intronic κ enhancer is sufficient by itself to completely abolish accessibility and rearrangement remains an open question. The neomycin gene left in the κ locus at the site of the enhancer might by itself contribute to making the block of rearrangement observed in Takeda *et al.* complete (*4*). The sole insertion of the neomycin gene at the 3′ end of the enhancer also shows some inhibition (*4*). We note that in the knock-out experiments with the Igμ intronic enhancer, inhibition of rearrangement is much more profound when the neomycin gene is left in the locus (*5*). See also P. Artelt *et al.*, *Gene* **99**, 249 (1991), for a possible silencing effect of the neomycin gene.
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31. The 3′ primer has been previously described [M. S. Schlissel and D. Baltimore, *Cell* **58**, 1001 (1989)]. PCR amplification was performed in a volume of 50 μl in a Perkin-Elmer 9600 thermal cycler for 30 cycles. Each cycle consisted of 1 min at 94°C and 2 min at 72°C. The reaction buffer contained 10 mM tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate, 25 pmol of each primer, and 1 U of Taq polymerase (Perkin- Elmer).
32. We thank B. Van Ness for providing us the pSPIg8 plasmid; R. Kühn for the E14.1 ES clone; A. Egert for advice and technical help; C. Steinberg for helpful comments and critical reading of the manuscript; R. Torres, Y.-R. Zou, F. Huetz, and L. Quint for encouragements and discussions; J.-P. De Villartay for phosphorimager quantifications; S. Schaal for bone marrow analysis; and C. Garcia for cell sorting experiments. This work was partly supported by a short-term European Molecular Biology Organization fellowship.

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Electrophoretically Uniform Fluorescent Dyes for Automated DNA Sequencing

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A class of dyes, BODIPY fluorophores, has been identified for automated DNA sequencing that has improved spectral characteristics compared with conventional fluorescein and rhodamine dyes. Single and double BODIPY dye primers were characterized in commercially available DNA sequencers and showed uniform electrophoretic mobilities and high fluorescence intensities. The improved physical properties of BODIPY dye primers were demonstrated by direct base-calling from the unprocessed fluorescent signals and improved heterozygote analyses of mixed-base populations. The high sensitivity of BODIPY dye primers requires at least 33 percent less reagent consumed per reaction than conventional dye primers, which should affect the costs of large genome-sequencing efforts.

Families of fluorescent dyes form the basis of multicomponent DNA detection assays. In automated DNA sequencing, up to four dyes are attached to oligonucleotides (*1*) that are enzymatically extended by DNA polymerase to generate a set of nested fragments. DNA sequence data are obtained after electrophoretic separation of the DNA and excitation, detection, and processing of the raw fluorescent signal by a computer. The generation and interpretation of these mixed fluorescent signals are therefore the central elements of current DNA sequencing technology.

Dyes suitable for high-throughput genome-scale DNA sequencing require the combined properties of physical stability, minimally overlapping emission spectra, high fluorescence intensity, and uniform electrophoretic mobilities. Although physically stable, the currently available set of fluoroscein and rhodamine dyes do not meet the remaining criteria and require both chemical and software corrections to produce optimal data (*1*).

Four spectrally resolvable dipyrromethene boron difluoride (BODIPY) dyes have been identified (Fig. 1) that show uniform electrophoretic properties under a variety of polyacrylamide gel conditions. Initially, the substitution of a BODIPY-labeled universal sequencing primer (Fig. 2A) for the same primer labeled with the corresponding fluorescein or rhodamine dye (*2*) generated termination reactions products that migrated approximately ¾ to 1 base faster by gel electrophoresis (Fig. 3, A and B). Substitution of BODIPY dyes labeled with various linker-arm modifications (Fig. 2A) revealed the optimal configurations that mimicked the mobility pattern of software-corrected fluorescein or rhodamine dye primers (Fig. 3, A and C) (*3*). The combination of these four BODIPY dye primers yielded high-quality sequencing data when analyzed with mobility software correction; however, the spacing pattern of the BODIPY dye primer set was further improved in the beginning of the sequencing run when no software correction was applied at all (*4*). Further evaluation of other BODIPY-linker combinations yielded an optimal dye primer set (*5*). Thus, a set of four BODIPY-linker constructs was identified that generated excellent sequence data when analyzed without mobility correction and gave an indistinguishable spacing pattern compared with software-corrected conventional sequencing reactions.

Comparison of normalized, overlapping emission spectra revealed that the bandwidths of the BODIPY dye primer set are narrower than their respective conventional dye primer counterparts (*6*), resulting in lower noise cross-talk between the instrument's collection filters (Fig. 4). The signal strength of BODIPY dyes, measured by fluorescence spectroscopy and an ABI 373A

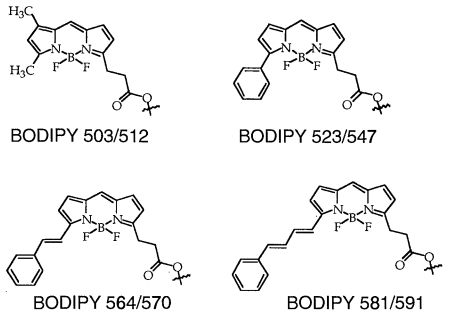

Fig. 1. Chemical structures of four BODIPY dyes for automated DNA sequencing. BODIPY dyes (4,4-difluoro-4-bora-3α,4α-diaza-*s*-indacene-3-propionic acid) are indicated followed by their approximate absorption/emission maxima. BODIPY 503/512 (5,7-dimethyl-BODIPY), BODIPY 523/547 (5-phenyl-BODIPY), BODIPY 564/570 (5-styryl-BODIPY), and BODIPY 581/591 [5-(4-phenyl-1,3-butadienyl)-BODIPY].

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instrument, gave results comparable to those for the four conventional dyes (*7*).

To improve the emission intensity, we constructed doubly labeled dye primers and evaluated them for fluorescence energy transfer (ET). To achieve efficient ET and maximal signal, we systematically substituted oligonucleotides with the acceptor dye at base increments away from either a 5-carboxyfluorescein (FAM) donor (0 to 3 bases apart) or a BODIPY 503/512 donor (1 to 10 bases apart) (*8*). We observed that ET efficiency decreased with increasing distance and decreased with decreasing spectral overlap between donor and acceptor dyes (*9*).

A 3-base separation between either the FAM donor (FET-3) or the BODIPY 503/512 donor (BET-3) (Fig. 2B), and acceptor dyes was observed to give the greatest signal enhancement for BODIPY 564/570 and BODIPY 581/591 dyes, consistent with FAM-TAMRA (F3T) and FAM-ROX (F3R) dye pairs (*10*). BET-3 dye primers, however, showed considerably greater ET efficiencies (*11*) and signal enhancements over FET-3 dye primers (*4*). This observation was unexpected because FAM showed a greater spectral overlap with BODIPY dyes than BODIPY 503/512. BET-3–BODIPY 564/570 and BET-3–BODIPY 581/591 primers had signal enhancements of 180% and 360% over the single BODIPY primers (*7*) and ET efficiencies greater than 98%.

BET-3–BODIPY 523/547, however, showed a gradual instability in denaturing, but not native gels compared with the single dye primer (*4*). A 6- to 10-base separation between the BODIPY donor and acceptor dyes corrected this phenomenon. Mixing experiments revealed that the mobility of BET termination reactions increased gradually with increased distance between donor and acceptor dyes (*4*). The combination of BET-6 and BET-3 dye primers showed the least mobility discrepancy, which was adjusted by shortening the BET-3 linker arm (Fig. 2B) to generate uniformly spaced termination reactions without software correction (*12*). BET-6–BODIPY 503/512 and BET-6–BODIPY 523/547 showed similar signal intensities compared with the single dye primers, and the latter BET-6 primer had an ET efficiency of 98%. The normalized, overlapping spectral profiles of BET-6/3 dye primers were indistinguishable from the single BODIPY dye primer spectra shown in Fig. 4, consistent with efficient ET (*4*). Overall, the strong signal enhancement of the weak-

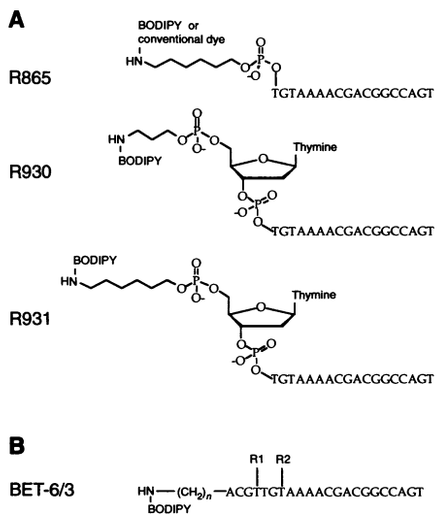

Fig. 2. Modifications of the universal (U) sequencing primer at the 5′ end. (**A**) Single dye-labeled primers. R865 (U-C_6), designated universal primer; C_6 linker, R930 (U^{+1}-C_3); and R931 (U^{+1}-C_6). (**B**) Double dye-labeled primers. Because different protecting groups block the linker-arm amines, BET primers were first labeled internally with BODIPY 503/512. After removal of the monomethoxytrityl group, BET primers were end-labeled with the BODIPY dye set. For BODIPY 503/512 and BODIPY 523/547 acceptor dyes, n = 6, R1 = CH_3, and R2 = $(CH_2)_6$NHBODIPY 503/512. For BODIPY 564/570 and BODIPY 581/591 acceptor dyes, n = 3, R1 = $(CH_2)_6$NHBODIPY 503/512, and R2 = CH_3.

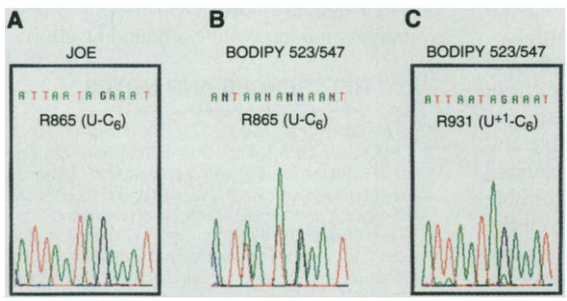

Fig. 3. Single BODIPY-labeled substitution experiment. DNA sequencing reactions were generated by Bst solid-phase sequencing (*15*) with the R865 primer labeled with FAM, TAMRA, and ROX plus (**A**) JOE, (**B**) BODIPY 523/547, or (**C**) R931 primer labeled with BODIPY 523/547. The 373A raw files were analyzed (*23*) with the DP6%{M13RP1} mobility-correction file.

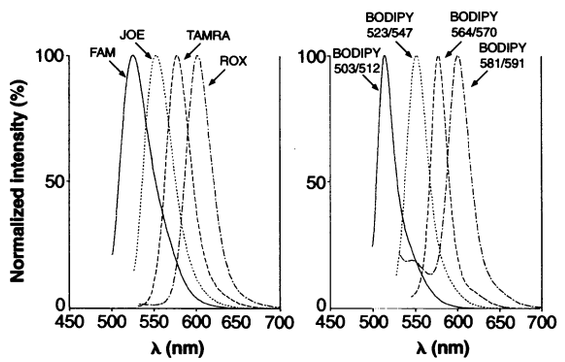

Fig. 4. Normalized emission spectra of four conventional dye primers and BODIPY dye primers.

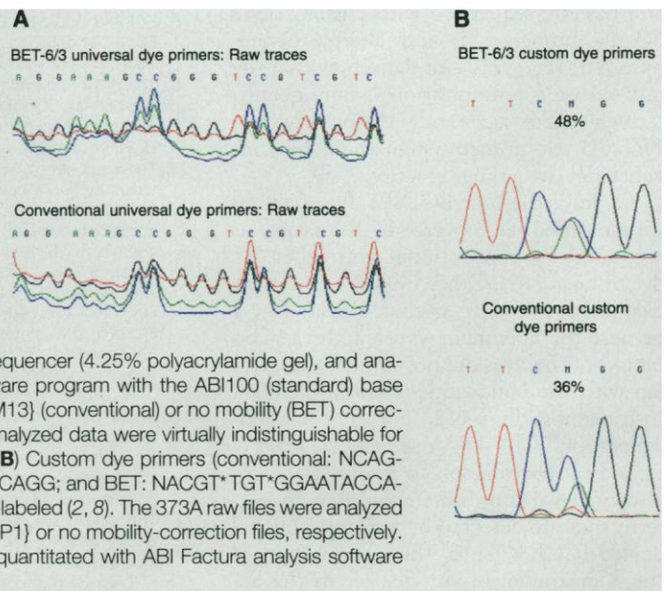

Fig. 5. BET-6/3 applications. (**A**) Comparison of raw traces near the primer peak for BET-6/3 and conventional dye primers. Thermo Sequenase (*16*) cycle sequencing reactions of M13 clones were generated from 0.4 pmol reactions for BET-3–BODIPY 564/570 and BET-3–BODIPY 581/591 compared with 0.8-pmol reactions for TAMRA and ROX, run on a 377A DNA sequencer (4.25% polyacrylamide gel), and analyzed by version 2.1.1 software program with the ABI100 (standard) base caller with either DP4%{-21M13} (conventional) or no mobility (BET) correction files, respectively. The analyzed data were virtually indistinguishable for both sequencing reads (*4*). (**B**) Custom dye primers (conventional: NCAGGAGGAATACCACATCCCGCAGG; and BET: NACGT*TGT*GGAATACCACATCCCGCAGG) were dye-labeled (*2*, *8*). The 373A raw files were analyzed (*23*) with either DP6%{M13RP1} or no mobility-correction files, respectively. Mixed-base positions were quantitated with ABI Factura analysis software (*24*).

er fluorescent dyes compared with the absence of significant enhancements of the normally stronger fluorescent dyes produced a set of four dye primers with roughly balanced signal intensities.

The sensitivity of the complete BET-6/3 primer set was examined by serial dilutions of DNA template with an ABI 377A DNA sequencer on a single gel, and sufficient signal was correctly analyzed even with a 16-fold reduction of concentration (*4*). This increased sensitivity of BET-6/3 dye primers now enables the direct loading of sequencing reactions onto gels without a laborious concentration step.

The unprocessed fluorescent signals generated from BET-6/3 sequencing reactions demonstrate the benefits of the uniform mobility, properly balanced signal outputs, and improved spectral purity. The raw data from BET-6/3 reactions generate a DNA sequencing pattern that is visually interpretable and agrees well with the corresponding analyzed data (Fig. 5A). In contrast, no discernible sequence pattern could be detected from the unprocessed signals of conventional primers. Comparative studies of conventional, single BODIPY, and BET-6/3 dye primers are under way to examine these benefits in relation to improved base-calling and readlength in large-scale sequencing (*4*).

Automated DNA sequencing has been routinely used for identifying heterozygosity (*13*). Analyses of mixed-base populations, however, can be problematic because of spacing errors or signal intensity differences from fluorescein and rhodamine dyes. To simulate heterozygous populations, two different molecular clones containing a 1121–base pair (bp) insert from the protease–reverse transcriptase region of human immunodeficiency virus–type 1 (HIV-1) were quantitated (*14*) and mixed (50:50), amplified by polymerase chain reaction (PCR), and directly sequenced with custom BET-6/3 dye primers (*15*) and Thermo Sequenase (*16*) (Fig. 5B). Our data show that the mixed base is both positionally and quantitatively more accurately defined by the BET-6/3 dye primer system than the conventional dye primer reactions.

Overall, the four BODIPY fluorophores we identified have overcome the problems presented by conventional and other ET dye primers (*10*). Other BODIPY dyes were examined, but were excluded from Fig. 1 because the maximum wavelength (λ maximum) of the dyes did not sufficiently overlap with the bandwidth of the ABI 373A instrument's filter (*12*, *17*). The second-generation 377A DNA sequencer, however, uses a spectrograph to resolve the fluorescent light into discrete wavelength patterns that are detected by a charge-coupled device (CCD). Thus, the optimization of the λ maximum of any dye set to the appropriate CCD pixels is now made possible by software manipulation.

Both single and double BODIPY-labeled primers should prove useful in other multicomponent genetic analyses, including sequencing by hybridization (*18*), in situ hybridization, multiplex PCR (*19*), sizing of DNA fragments, multiplex analyses of restriction fragment length polymorphisms and variable number of tandem repeats, and TAQMAN assays (*20*). Moreover, labeling strategies to directly synthesize BODIPY single and double dye primers (that is, phosphoramidite chemistry) should simplify and standardize the construction of fluorescent primers and probes. The identification of the BODIPY dye set should affect routine genetic analyses and large-scale sequencing efforts.

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2. DNA synthesis reagents were purchased from Applied Biosystems (ABI), with the exception of 5′-amino-modifier C3, C6, and C12, and amino modifier C6 dT phosphoramidites, which were purchased from Glen Research (Sterling, VA). Oligonucleotides R865, R930, and R931 were synthesized trityl-on by either an ABI (Foster City, CA) model 380B or model 394 DNA synthesizer. FAM-*N*-hydroxyl succinimide (NHS), JOE-NHS, TAMRA-NHS, and ROX-NHS were purchased from ABI. BODIPY 523/547 propionic acid (PA) and all BODIPY–succinimidyl ester (SE) dyes were purchased from Molecular Probes (Eugene, OR). BODIPY-SE dyes were resuspended in anhydrous dimethyl sulfoxide or *N*,*N*-dimethylformamide (50 mg/ml), and BODIPY 523/547–PA was converted to BODIPY 523/547–SE according to the manufacturer's protocol. R865, R930, and R931 (0.4 μmol) were purified with Nensorb 20 columns according to the manufacturer's protocol (Du Pont, Boston, MA). R865 was resuspended in 160 μl of 0.25 M $NaHCO_3$–Na_2CO_3 buffer (pH 9.0) and divided into four portions. To each tube, 3 μl of either FAM-NHS, JOE-NHS, TAMRA-NHS, or ROX-NHS were added. For BODIPY additions, see (*21*).
3. BODIPY 503/512–R930, BODIPY 523/547–R931, BODIPY 564/570–R930, and BODIPY 581/591–R930 termination reactions mimicked the spacing pattern of R865 primer labeled with conventional dyes.
4. M. L. Metzker, unpublished results.
5. BODIPY 503/512, BODIPY 523/547, and BODIPY 564/570 contained a six-carbon linker, and BODIPY 581/591 contained a three-carbon linker.
6. The half-bandwidth of BODIPY 503/512 was 59% that of FAM, BODIPY 523/547 was 75% that of JOE, BODIPY 564/570 was 71% that of TAMRA, and BODIPY 581/591 was 89% that of ROX.
7. The signal strength of BODIPY dye primers was evaluated by fluorescence spectroscopy (range 71 to 100%) with a Hitachi Model F-4010 fluorescence spectrophotometer in 1× tris-borate EDTA buffer containing 7 M urea and by the 373A DNA sequencer (range 54 to 133%). Spectroscopy measurements were done in duplicate and determined by comparing the fluorescence intensity at λ maximum of BODIPY dye primers to conventional dye primers. FAM and BODIPY 503/512 were excited at 488 nm, and all remaining dyes were excited at 514 nm. The 373A measurements were determined by M13 cycle sequencing reactions of four different molecular clones. The relative intensity values were determined by normalizing the BODIPY dye signal to the remaining dye signals and comparing it to its normalized conventional dye signal.
8. The donor dye for FET primers was added with 6-FAM amidite. The leader sequences for FET-0 to FET-3 primers were 5′-FAM-T*GT, 5′-FAM-TT*GT, 5′-FAM-GTT*GT, and 5′-FAM-CGTT*GT followed by the primer sequence AAAACGACGGCCAGT. Primers were synthesized (0.2 μmol) with C6dT (T*) and were ethanol-precipitated. The leader sequences for BET-1 to BET-10 primers were 5′-NTT*GT, 5′-NTGT*, 5′-NTTGT*, 5′-NGTTGT*, 5′-NCGTTGT*, 5′-NACGTTGT*, 5′-NTTGTTTGT*, 5′-NTTGTGTTGT*, 5′-NTTGTCGTTGT*, and 5′- NTTGTACGTTGT* followed by the primer sequence AAAACGACGGCCAGT. Primers were synthesized (0.2 μmol) with either C3 or C6 amino link (N) and C6dT (T*) and resuspended in 400 μl of 0.01 N NaOH. To each tube, 20 μl of BODIPY 503/512-SE were added, incubated at 25°C for 10 min, ethanol-precipitated, incubated in 200 μl of 80% acetic acid for 20 min, and ethanol-precipitated (*21*).
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12. Although BET-3–BODIPY 576/589 showed better mobility in the first 50 bases of the chromatogram than BET-3–BODIPY 581/591, its λ maximum did not overlap with the instrument's filter.
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17. Other dyes examined were BODIPY 530/550 (5,7-diphenyl-BODIPY), BODIPY 558/568 [5-(2-thienyl)-BODIPY], BODIPY 576/589 [5-(2-pyrrolyl)-BODIPY], and BODIPY 589/616 {6-[({4-[5-(2-thienyl)-BODIPY-3- yl]phenoxy}acetyl)amino]hexanoic acid}.
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21. Primers were each resuspended in 160 μl of 0.25 M $NaHCO_3$–Na_2CO_3 (pH 9.0) buffer and divided into four portions. To three tubes, 5 μl of either BODIPY 503/512–SE, BODIPY 564/570–SE, or BODIPY 581/591–SE were added. To the fourth tube, 35 μl of 0.25 M $NaHCO_3$-Na_2CO_3 (pH 9.0) buffer and 30 μl of BODIPY 523/547–SE were added. Dye-labeling reactions were incubated at 25°C for 4 to 16 hours. After ethanol precipitation, dye-labeled primers were purified by reversed-phase high performance liquid chromatography (RP-HPLC), evaporated to ~20 μl, resuspended in deionized water, and diluted to 0.4 pmol/μl. The RP-HPLC hardware system was as described elsewhere (*22*) with the exception of a Beckman (Fullerton, CA) model 127 gradient solvent module and an ABI aquapore RP-300 column (4.6 mm by 250 mm). Gradient conditions for single and FET dye primers were as follows: 20% B, 5 min; 20% B to 40% B, 30 min; 40% B to 100% B, 18 min; and 100% B, 5 min. BET primers: 30% B, 5 min; 30% B to 100% B, 40 min; and 100% B, 5 min at a flow rate of 1.0 ml/min.
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