Nucleic Acids Research, 1993, Vol. 21, No. 24 5727–5735

Heterodimeric DNA-binding dyes designed for energy transfer: synthesis and spectroscopic properties

Scott C.Benson, Paramjit Singh and Alexander N.Glazer*
Department of Molecular and Cell Biology, 229 Stanley Hall, University of California, Berkeley, CA 94720, USA

Received August 12, 1993; Revised and Accepted November 3, 1993

ABSTRACT

Heterodimeric dyes are described which bind tightly to double-stranded (dsDNA) with large fluorescence enhancements. These dyes are designed to exploit energy transfer between donor and acceptor chromophores to tune the separation between excitation and emission wavelengths. The dyes described here absorb strongly at the 488 nm argon ion line, but emit at different wavelengths, and can be applied to multiplex detection of various targets. The chromophores in these dyes, a thiazole orange–thiazole blue heterodimer (TOTAB), two different thiazole orange–ethidium heterodimers (TOED1 and TOED2), and a fluorescein–ethidium heterodimer (FED), are in each case linked through polymethylene–amine linkers. The emission maxima of the DNA-bound dyes lie at 662 (TOTAB), 614 (TOED 2), and 610 nm (FED). The dyes showed a >100 fold enhancement of the acceptor chromophore fluorescence on binding to dsDNA and no sequence selectivity. In comparison with direct 488 nm excitation of the constituent monomeric dyes, in the heterodimers the fluorescence of the acceptor chromophores was greatly enhanced and the emission of the donor chromophores quenched by over 90%. The acceptor emission per DNA-bound dye molecule was constant from 100 DNA bp:dye to 20 bp:dye and decreased sharply at higher dye:DNA ratios.

INTRODUCTION

Two recent developments have prompted extensive study of non-covalent high affinity fluorescent DNA–dye complexes. The first is the demonstration that many such complexes are stable under a variety of conditions, particularly gel electrophoresis (see ref. 1 for a review). The second is the invention of a novel highly sensitive fluorescence scanner which employs 488 nm laser excitation and confocal detection (2,3). Applications of the stable DNA-intercalation complexes have included high sensitivity detection of products of the polymerase chain reaction (4), multiplex analysis of restriction fragments (5,6), and of complexes of double-stranded (dsDNA) with binding proteins (7).

For multiplex detection of DNA fragments in gel electrophoresis, for two-color analyses of DNA-protein complexes (7), for multi-color cytogenetic applications (8), for fluorescence activated cell sorting (9), and the like, it would be advantageous to have sets of dyes with a common, strong absorption maximum, but well-separated emission maxima. One approach to this objective is to exploit energy transfer and synthesize sets of heterodimeric molecules with donor chromophores which absorb at the same wavelength but with different acceptor chromophores having distinctive fluorescence emission maxima. For the success of this approach, the heterodimers must satisfy several requirements. In the DNA-bound form, the energy transfer from donor to acceptor needs to be very efficient, otherwise the emission from the donor will overlap emissions from other fluorophores which might be used concurrently. The donor should have a high absorption coefficient and the acceptor should have an adequate fluorescence quantum yield. The affinity of the dyes for dsDNA should be sufficient to permit electrophoretic separation of the dsDNA–dye complexes in the absence of free dye.

To test the energy transfer approach, we have synthesized four heterodimeric dyes and examined their spectroscopic properties in the free and dsDNA-bound form. The results presented here and in the accompanying paper (10) indicate that the approach is sound and that a wide range of structurally dissimilar chromophores can be used as donors or acceptors in dyes designed to bind tightly to DNA. These findings have strong positive implications for the successful design of other fluorescent DNA-binding dyes with large Stokes shifts to minimize background from scattering and autofluorescence and for the simultaneous detection of multiple targets.

MATERIALS AND METHODS

Materials

Lepidine, 3-methyl-benzothiazole-2-thione, 3,8-diamino-9-phenyl-phenanthridine, 1,3-diaminopropane, N,N′-tetramethyl-1,3-diaminopropane, N,N′-tetramethyl-1,2-diaminoethane, 1,3-diiodopropane, iodomethane, diethylenetriamine, fluorescein isothiocyanate isomer 1 (FITC), ethidium bromide, and anhydrous

* To whom correspondence should be addressed

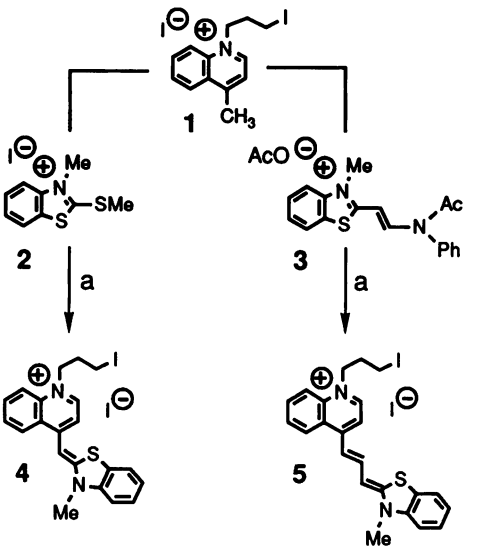


Figure 1. Synthesis of the asymmetric cyanine dye monomer intermediates. In step **a**, suspend **1** with equimolar **2** or **3** in absolute EtOH, warm to solubilize, add 1 equivalent triethylamine, stir at room temperature for 15 minutes, precipitate with ether, recrystallize from acetone/ether; yield 80% **4**, 60% **5**.

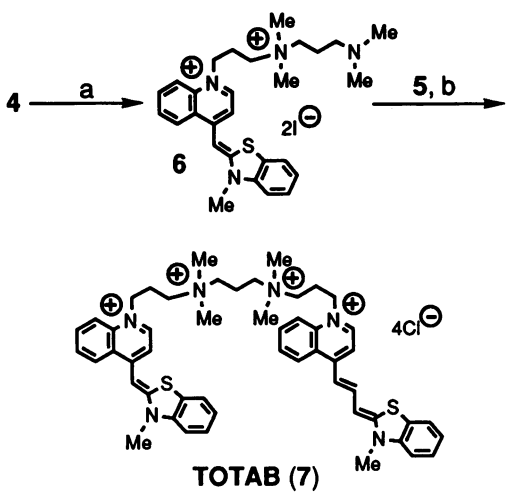
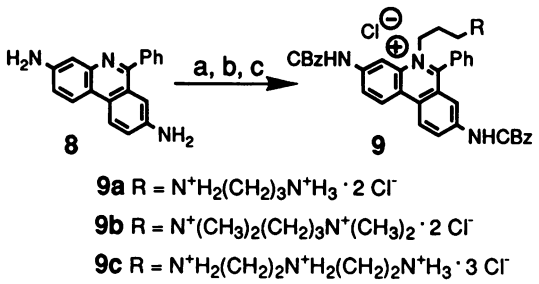


Figure 2. Synthesis of TOTAB heterodimer (**7**). (**a**) Suspend **4** in anhydrous MeOH, add 6 equivalents N,N′-tetramethyl-1,3-propanediamine, reflux 6 hours, precipitate with acetone/ether, recrystallize from MeOH:CH_2Cl_2 (1:10)/acetone; yield 80% **6**. (**b**) Suspend **6** in anhydrous MeOH, add 1 equivalent **5**, reflux 10 hours, precipitate with ether, triturate solid with MeOH:CH_2Cl_2 (1:10), flash column EtOAc:AcOH:H_2O saturated with NaCl (1:2:2 v/v); yield 60% **7**.

HBr/acetic acid were purchased from Aldrich and used without further purification. Ethidium homodimer was purchased from Molecular Probes. Anhydrous methanol, triethylamine, and pyridine were distilled from sodium and stored under nitrogen. Anhydrous nitrobenzene was freshly distilled from CaH_2.

All anhydrous reactions were run in oven-dried glassware under a nitrogen atmosphere. TAE buffer refers to 4 mM TAE pH 8.2, obtained by dilution of stock 2 M tris(hydroxymethyl)-methylamine–50 mM EDTA disodium adjusted to pH 8.2 by addition of acetic acid. Reactions were monitored by TLC (Merck A_{254}) under short and long wavelength UV light. Flash



9a R = $N^+H_2(CH_2)_3N^+H_3 \cdot 2\ Cl^-$

9b R = $N^+(CH_3)_2(CH_2)_3N^+(CH_3)_2 \cdot 2\ Cl^-$

9c R = $N^+H_2(CH_2)_2N^+H_2(CH_2)_2N^+H_3 \cdot 3\ Cl^-$

Figure 3. Synthesis of the propidium monomer intermediates. (**a**) Suspend **8** in anhydrous pyridine, add dropwise 2.2 equivalents carbobenzyloxychloride at 0°C, stir at room temperature for 12 hours, precipitate with ether/petroleum ether, suspend solid in CH_2Cl_2 and wash with 10% $NaHCO_3$, dry organic layer with $MgSO_4$, concentrate, flash column MeOH:CH_2Cl_2 (1:50). Recrystallize in CH_2Cl_2/ether. (**b**) Suspend tan solid in anhydrous nitrobenzene, add 5 equivalents 1,3-diiodopropane, heat at 160°C in sand bath for 4 hours, precipitate with ether, flash column MeOH:CH_2Cl_2 (1:10). (**c**) Suspend solid in anhydrous MeOH, add 10 equivalents 1,3-diaminopropane (for **9a**), N,N′-tetramethyl-1,3-diaminopropane (for **9b**), or diethylenetriamine (for **9c**), reflux 7 hours, precipitate with H_2O, suspend solid in EtOH and acidify with concentrated HCl, precipitate with ether, flash column EtOAc:AcOH:H_2O (6:3:2); typical yield 25% **9** from **8**.

chromatography was performed on 220–440 mesh silica gel 60 from Fluka. Intermediate products which gave single spots on TLC were identified by their ^{1}H-NMR spectra (see below) and by their UV/VIS absorption spectra measured with a Perkin Elmer Lambda 6 spectrophotometer.

Synthesis of cyanine dyes: thiazole orange (TO), thiazole orange homodimer (TOTO), and thiazole orange–thiazole blue heterodimer (TOTAB)

Reactive cyanine dye intermediates were synthesized as outlined in Fig. 1. Compound **1** was produced in high yield by alkylation of lepidine with 5 equivalents of 1,3-diiodopropane in refluxing dioxane, while compound **2** was formed quantitatively when 3 equivalents of iodomethane were reacted with 3-methyl-benzothiazole-2-thione in refluxing ethanol and precipitated with ether. Compound **2** was also used as an intermediate in the synthesis of **3** (11). Iodopropyl-lepidine **1** reacted in under 15 minutes with benzothiazole derivative **2** or **3** by the method of Brooker *et al.* (11,12) to produce the iodopropyl-thiazole orange derivative **4**, or the iodopropyl-thiazole blue derivative **5**, in good yield. Alkylation of lepidine with iodomethane and condensation with **2**, by the procedure described for the synthesis of **4**, produced thiazole orange (TO) in near-100% yield (13).

Compounds **4** and **5** were used to synthesize the cyanine heterodimer TOTAB. Compound **4** reacted with excess N,N′-tetramethyl-1,3-diaminopropane to form (N,N′-tetramethyl-1,3-propanediamino)propyl-thiazole orange derivative **6** in high yield (Fig. 2). Compound **6**, after purification by recrystallization, reacted with compound **5** to produce thiazole orange–thiazole blue heterodimer (TOTAB; **7**), in good yield. Dimerization of **4**, as described by Rye *et al.* (5), with 0.5 equivalents of N,N′-tetramethyl-1,3-diaminopropane in refluxing anhydrous methanol, led to nearly quantitative yield of thiazole orange homodimer (TOTO). Attempts to employ 1,3-diamino-propane as a linker to form cyanine dye dimers were unsuccessful. Tetramethyl-1,2-diaminoethane reacted with **4** in refluxing MeOH to produce quantitatively a (N,N′-tetramethyl-1,2-ethanediamino)propyl thiazole orange derivative with no dimer product. No monomeric starting materials were

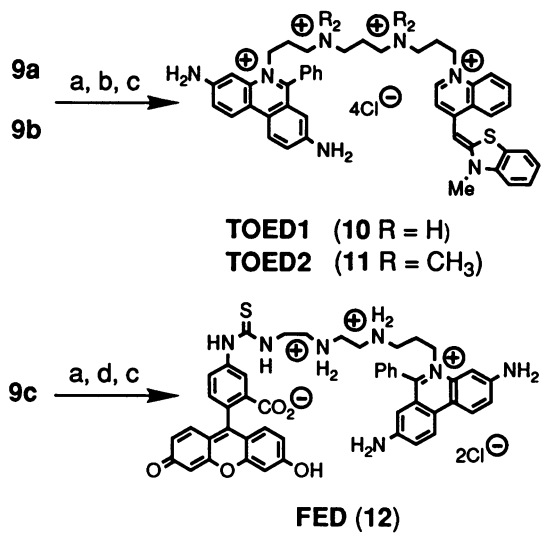


Figure 4. Synthesis of TOED1 (**10**), TOED2 (**11**), and FED (**12**) heterodimers (**a**) Suspend **9** in MeOH, add 3 equivalents 1N NaOH with stirring (4 equivalents of 1N NaOH for **9c**), precipitate/wash solid with H_2O, air-dry followed by 60°C drying in oven. (**b**) Suspend dry solid in anhydrous MeOH, add 0.7 equivalents **4** (1 equivalent **4** for **11**), reflux 10 hours, precipitate ether/petroleum ether; solid used without further purification. (**c**) Suspend solid in anhydrous HBr/AcOH, stir at room temperature for 1 hour, concentrate, suspend in CH_2Cl_2, precipitate with ether, flash column EtOAc:AcOH:H_2O (saturated with NaCl) (1:2:2 v/v). (**d**) Suspend solid in anhydrous MeOH, add 1.1 equivalents of FITC, stir at room temperature for 2 hours, acidify with concentrated HCl, precipitate with ether. Yield 40% **11**; 15–20% **10** and **12**.

detected in the purified TOTO or TOTAB (**7**) dimers by TLC in MeOH:CH_2Cl_2 1:1 (v/v) or EtOAc:AcOH:H_2O 1:2:2 (v/v).

In silica gel chromatography with EtOAc:AcOH:H_2O 1:2:2 (v/v) as the eluting solvent, saturation of the solvent with sodium chloride or addition of triethylamine (2% by volume) was necessary to remove the dimeric dyes from the column.

Synthesis of thiazole orange–ethidium (TOED1, TOED2) and ethidium–fluorescein (FED) heterodimers

These heterodimers were synthesized from 3,8-diamino-9-phenylphenanthridine (**8**) via intermediates **9a**, **9b**, and **9c** (Figs 3 and 4). In general, the synthesis of the reactive intermediates followed the method of LePecq and co-workers (14) who employed a dicarboethoxy or diacetyl protected form of compound **9a** to synthesize ethidium homodimer and an ethidium–acridine heterodimer. In the present synthesis, the phenanthridinium amino substituents were protected with carbobenzyloxy groups which were subsequently removed under mild acidic conditions. The phenanthridine ring nitrogen was alkylated with 1,3-diiodopropane rather than with 1,3-dibromopropane. The alkylation needed to be monitored carefully and stopped prior to completion to avoid excessive by-product formation. Alkylation with dibromopropane required higher temperatures and extended reaction times which resulted in excessive by-product formation and lower yields. After coupling **9a** or **9b** with compound **4**, the crude solid dicarbobenzyloxy protected intermediates were deprotected (anhydrous HBr/AcOH) to produce the thiazole orange–ethidium heterodimers TOED1 (**10**) and TOED2 (**11**). A poor yield of **10** from reaction of **9a** was due to production of substantial amounts of an undesired trimeric byproduct (double addition of **4** to **9a**) which was not observed in the reaction employing **9b**. Coupling compound **9c** with FITC produced a crude intermediate which was deprotected to give FED (**12**) with significant loss on purification by silica gel chromatography. No monomeric starting materials were detected in the purified dimers by TLC in MeOH:CH_2Cl_2 1:1 (v/v) or EtOAc:AcOH:H_2O 1:2:2 (v/v).

^{1}H NMR spectra and mass spectra

Proton nuclear magnetic resonance (^{1}H MR) spectra were recorded at the Department of Chemistry, University of California, on a 300 MHz NMR spectrometer (built by Rudi Nunlist) controlled by a Nicolet 1280 computer. The proton spectra were run in CD_3OD and the chemical shifts are reported in parts per million (ppm) relative to the residual proton resonance at 3.35 ppm.

Electrospray ionization mass spectrometry (ESMS) was run on a VG Bio-Q electrospray triple quadrapole mass spectrometer in positive ionization mode with a skimmer voltage of 20–70 electron volts (eV). For the ^{1}H NMR and ESMS samples, salt (NaCl) present in the dimers, added during final silica gel chromatographic purification, was easily removed by repeated suspension in absolute EtOH, centrifugation, removal of the supernatant, and addition of ether to precipitate the dye. For ESMS, samples of the dimers were further purified by ion exchange on Bio-Rad AG1-X8 anion exchange resin, chloride form, eluting with distilled H_2O to give the 4 Cl^- salt. ^{1}H NMR and ESMS data on compounds **5**, **6**, **7**, **9b**, and **11** are given below.

Iodopropyl thiazole blue (5). ^{1}HNMR δ 8.51 (d, 1 H, J = 8.1 Hz), 8.29 (t, 1 H, J = 12.6 Hz), 8.19 (d, 1 H, J = 7.2 Hz), 8.02 (d, 1 H, J = 8.1 Hz), 7.97 (dt, 1 H, J = 6.6, 0.9 Hz), 7.78 (d, 1 H, J = 7.8 Hz), 7.74–7.66 (m, 2 H), 7.55–7.54 (m, 2 H), 7.42–7.33 (m, 1 H), 7.11 (d, 1 H, J = 13.2 Hz), 6.54 (d, 1 H, J = 12.3 Hz), 4.64 (t, 2 H, J = 7.5 Hz), 3.83 (bs, 3 H), 3.32 (t, 2 H, J = 6.6 Hz), 2.45 (quintet, 2 H, J = 6.9 Hz).

(N, N'-tetramethyl-1,3-propanediamino)propyl thiazole orange (6). ^{1}HNMR δ 8.62 (d, 1 H, J = 8.7 Hz), 8.53 (d, 1 H, J = 7.2 Hz), 8.15 (d, 1 H, J = 8.7 Hz), 7.95 (dt, 1 H, J = 7.2, 0.9 Hz), 7.87 (d, 1 H, J = 7.8 Hz), 7.73 (t, 1 H, J = 7.8 Hz), 7.56–7.54 (m, 2 H), 7.41–7.35 (m, 1 H), 7.31 (d, 1 H, J = 7.5 Hz), 6.79 (s, 1 H), 4.64 (t, 2 H, J = 7.5 Hz), 3.95 (s, 3 H), 3.82-3.73 (m, 2 H), 3.52–3.43 (m, 2 H), 3.23 (s, 6 H), 2.56–2.45 (m, 2 H), 2.47 (t, 2 H, J = 6.9 Hz), 2.32 (s, 6 H), 2.11–1.97 (m, 2 H).

TOTAB (7). ^{1}H NMR δ 8.64 (bd, 2 H, J = 7.5 Hz), 8.42 (d, 1 H, J = 8.4 Hz), 8.33 (d, 1 H, J = 7.2 Hz), 8.21 (d, 1 H, J = 8.7 Hz), 8.19–8.06 (m, 2 H), 8.12 (t, 1 H, J = 12.9 Hz), 8.02 (dt, 1 H, J = 8.1, 0.9 Hz), 7.96 (dt, 1 H, J = 8.0, 0.9 Hz), 7.75 (bt, 1 H, J = 7.7 Hz), 7.71–7.59 (m, 5 H), 7.49–7.38 (m, 4 H), 7.30 (ddd, 1 H, J = 7.2, 6.6, 1.8 Hz), 6.99 (d, 1 H, J = 13.2 Hz), 6.89 (s, 1 H), 6.39 (d, 1 H, J = 12.6 Hz), 4.73 (bt, 2 H, J = 7.5 Hz), 4.65 (bt, 2 H, J = 7.5 Hz), 3.98 (s, 3 H), 3.85–3.74 (m, 4 H), 3.70 (s, 3 H), 3.57–3.49 (m, 4 H), 3.28 (bs, 6 H), 3.27 (bs, 6 H), 2.60–2.43 (m, 6 H); ESMS (skimmer voltage, 25 eV) *m/z* 446 ($[M\text{-}2(Cl^-)]^{2+}$, 70), 285 ($[M\text{-}3(Cl^-)]^{3+}$, 100), 205 ($[M\text{-}4(Cl^-)]^{4+}$, 20), 376 (12), 402 (5), 277 (40); (skimmer voltage, 60 eV) *m/z* 376 ($[C_{23}H_{26}N_3S]^+$, 100), 402 ($[C_{25}H_{28}N_3S]^+$, 50), 236 (35), 209 (45); molecular weight calculated for $C_{51}H_{60}N_6S_6Cl_4$, 962.

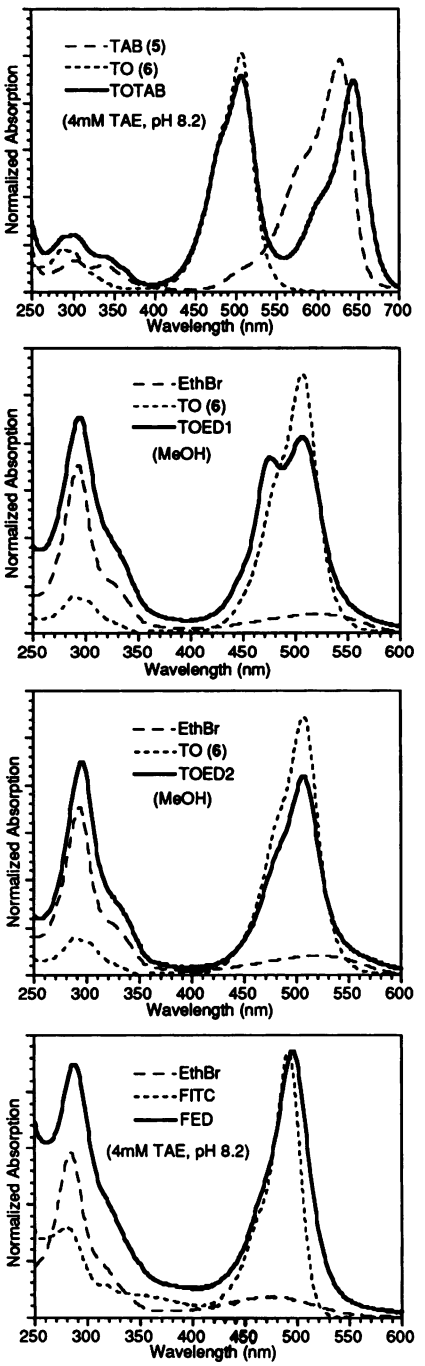


Figure 5. Absorption spectra of TOTAB (**7**),TOED1 (**10**), TOED2 (**11**), and FED (**12**), compared to monomers at identical concentrations in the same solvent. All spectra in MeOH and TAE (4 mM TAE buffer pH 8.2) were at room temperature with dyes at $\sim 4\times10^{-5}$ M.

*N,N'-dicarbobenzyloxy-(N,N'-etramethyl-1,3-propanediamino)-propidium (**9b**).* ^{1}H NMR δ 9.03 (d, 1 H, J = 9.3 Hz), 8.97 (d, 1 H, J = 9.3 Hz), 8.95 (bs, 1H), 8.31 (dd, 1 H, J = 9.3, 2.1 Hz), 8.08 (dd, 1 H, J = 9.3. 1.5 Hz), 7.93–7.85 (m, 5 H), 7.52–7.33 (m, 11 H), 5.33 (s, 2 H), 5.15 (s, 2 H), 4.87 (bt, 2 H, J = 8 Hz), 3.67–3.62 (m, 2 H), 3.58–3.52 (m, 2 H), 3.25 (t, 2 H, J = 7.8 Hz), 3.21 (s, 6 H), 2.95 (s, 6 H), 2.73–2.57 (m, 2 H), 2.40–2.25 (m, 2 H).

Table 1. Absorption data for the heterodimers[a]

Dye	Solvent	λ_{max} (nm)	ε (M^{-1} cm^{-1})
TOTAB	MeOH	302	22 000
		505	77 600
		633	101 000
	TAE	302	15 000
		506	57 100
		644	55 800
	TAE/DNA	514	60 800
		646	46 600
TOED1	MeOH	295	62 000
		476	50 500
		507	56 400
	TAE	288	53 600
		472	54 100
		507	sh
	TAE/DNA	485	22 000
		515	57 500
TOED2	MeOH	296	62 000
		507	60 100
	TAE	290	54 000
		510	43 200
	TAE/DNA	489	sh
		515	46 900
FED	MeOH	294	72 000
		486	13 200
	TAE	288	68 800
		495	72 300
	TAE/DNA	492	48 600

[a]All spectra in MeOH and TAE (4 mM TAE buffer pH 8.2) were at room temperature with dyes at $\sim 4\times10^{-5}$ M. Spectra of dyes bound to dsDNA (3×10^{-6} M dye) were with calf thymus DNA (3×10^{-5} M bp) at 10 bp:dye, in 4 mM TAE buffer pH 8.2, recorded at room temperature after incubationof the dyes with the DNA for 15 minutes in the dark.

*TOED2 (**11**).* ^{1}H NMR δ 8.65 (bd, 1 H, J = 8.1 Hz), 8.57 (d, 1 H, J = 9.3 Hz), 8.55 (d, 1 H, J = 7.2 Hz), 8.51 (d, 1 H, J = 9.3 Hz), 8.17 (d, 1 H, J = 8.7 Hz), 7.99 (t, 1 H, J = 7.5 Hz), 7.87–7.71 (m, 8 H), 7.65–7.52 (m, 5 H), 7.40–7.33 (m, 2 H), 6.88 (s, 1 H), 6.42 (d, 1 H, J = 2.4 Hz), 4.71 (bt, 2 H, J = 6.8 Hz), 4.64 (bt, 2 H, J = 7.5 Hz), 4.00 (s, 3 H), 3.81–3.74 (m, 2 H), 3.65–3.56 (m, 2 H), 3.55–3.43 (m, 4 H), 3.26 (s, 6 H), 3.19 (s, 6 H), 2.58–2.37 (m, 6 H). ESMS (skimmer voltage, 40 eV) *m/z* 430 ($[M-2(Cl^-)]^{2+}$, 90), 275 ($[M-3(Cl^-)]^{3+}$, 30), 376 (25), 371 (35), 236 (100); (skimmer voltage, 65 eV) *m/z* 376 ($[C_{23}H_{26}N_3S]^+$, 100), 371 ($[C_{24}H_{27}N_4]^+$, 60), 430 (12), 300 (70), 158 (90); molecular weight calculated for $C_{50}H_{59}N_7SCl_4$, 931.

Absorption spectra of the heterodimers

The absorption spectra of TOTAB (**7**), TOED1 (**10**), TOED2 (**11**), and FED (**12**) closely approximated those obtained by addition of the two monomer spectra taken at equal concentrations (see Fig. 5).

Direct determination of the molar extinction coefficients of the dimers based on dry weight was complicated by the presence of salts in the final purified products. Consequently, the correspondence between the absorption spectra of the monomer chromophores and of the heterodimers was used to calculate extinction coefficients for the latter at wavelengths where the agreement was best (see below). These values (Table 1) must be considered provisional, pending more accurate direct determinations.

For TOTAB, the extinction coefficient of **6** (ϵ_{507} = 76,000 M^{-1} cm^{-1}, MeOH) plus the extinction coefficient of thiazole blue (**5**; ϵ_{505} = 1,600 M^{-1} cm^{-1}, MeOH) was assigned the TOTAB dimer (ϵ_{505} = 77,600 M^{-1} cm^{-1}, MeOH). For TOED and TOED2, the extinction coefficient of **6** plus the extinction coefficient of ethidium bromide at 295 nm were added to give an extinction coefficient ϵ_{295} = 62,000 M^{-1} cm^{-1} (MeOH). The extinction coefficients of ethidium bromide and FITC in MeOH at 294 nm were added to estimate the extinction coefficient of FED (ϵ_{294} = 72,000 M^{-1} cm^{-1}, MeOH).

DNA solution

Calf thymus DNA (Sigma) was dissolved in TAE buffer, and sheared by repeated passage through a small gauge needle. The DNA was extracted with phenol, precipitated with ammonium acetate/isopropanol, suspended in 4 mM Tris−acetate−0.1 mM EDTA (TAE), pH 8.2, and stored frozen. The DNA concentration was calculated assuming 50 μg/ml per absorbance unit at 260 nm. The molar base-pair dsDNA concentration was calculated based on 635 gm/mole bp. Poly(dA)·poly(dT) and poly(dG)·poly(dC) were purchased from Pharmacia.

Fluorescence emission spectra

These uncorrected spectra were measured with a Perkin Elmer MFP 44B spectrofluorimeter in ratio mode at room temperature with 488 nm excitation.

RESULTS

^{1}H NMR and mass spectrometry studies

From the ^{1}H NMR spectra, the resonance structures shown for the thiazole blue and thiazole orange chromophores (Figs 1 and 2) were determined to be the predominant form of the dyes on the basis of the significantly larger downfield shift of the triplet resonance corresponding to the methylene group (for example, δ 4.64 for **5** and **6**) attached to the quinolinium nitrogen in comparison to the singlet of the methyl group resonance attached to the benzothiazole nitrogen (δ 3.83 and 3.95, respectively).

Reaction of the monomers to give quaternized amino linkers in the dimers was indicated in the ^{1}H NMR spectra by the downfield shift of the methyl group resonances attached to the terminal tertiary nitrogen of the diamine side chain of the thiazole orange **6** and propidium **9b** intermediates upon forming the TOTAB and TOED2 heterodimers (from δ 2.32 and 2.95 to δ 3.28 and 3.19, respectively), and the resultant broad 6 proton resonance in the dimer spectra for the central methylene protons of the 3 propylene segments of the linker (δ 2.60−2.43 in **7** and δ 2.58−2.37 in **11**) due to psuedo-symmetry of the linker.

The linked structure of the TOTAB and TOED2 heterodimers was firmly established by electrospray mass spectrometry. The mass spectrum of TOED2 contained 2^+ and 3^+ molecular ions corresponding to loss of 2 and 3 chloride ions respectively, while the spectrum of TOTAB contained 2^+, 3^+, and 4^+ ions resulting from loss of chloride ions. In addition, higher skimmer voltages resulted in predominance of fragment ions corresponding to fragmentation of the dimers to give singly charged intact chromophores with attached dimethylaminopropyl groups (TOTAB, *m/z* 376 and 402; TOED2, *m/z* 376 and 371).

Inadequate amounts of pure TOED1 (**10**) and FED (**12**) were available for ^{1}H NMR spectroscopy. The ESMS spectra obtained for these two compounds were not interpretable. The structure of these chromatographically pure dimers is assigned on the basis of the synthetic route and the demonstration that the absorption spectra of these dimers in each case approach closely quantitatively the sum of the distinctive absorption spectra of their constituent monomers. The dimeric structure for TOED1 and FED is further supported by the fluorescence emission spectra which show energy transfer from the donor to the acceptor chromophore.

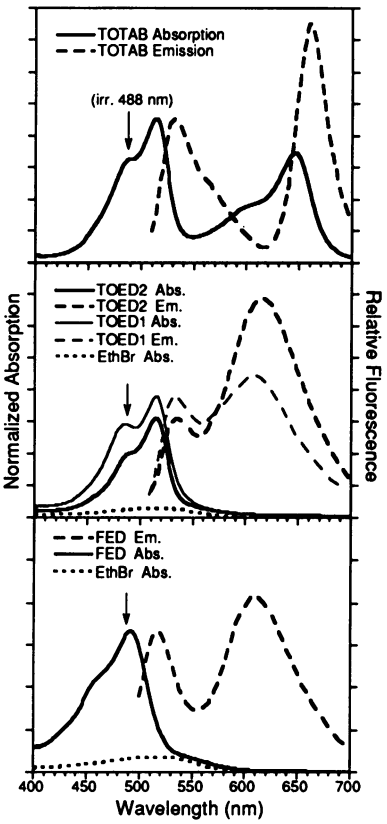


Figure 6. Visible absorption and fluorescence emission spectra of TOTAB (**7**),TOED1 (**10**), TOED2 (**11**), and FED (**12**) bound to dsDNA. The solid curves represent the absorption spectra of the dyes (3×10^{-6} M) bound to calf thymus DNA (3×10^{-5} M bp) at 10 bp:dye in 4 mM TAE buffer pH 8.2. The dashed curves represent the fluorescence emission spectra of the dyes (3×10^{-7} M) on excitation at 488 nm in 4 mM TAE pH 8.2 when bound to calf thymus DNA (3×10^{-5} M) at 100 bp:dye at room temperature after incubation of the dyes with the DNA for 15 minutes in the dark. *Arrow* indicates the excitation wavelength (488 nm).

Spectroscopic properties of free heterodimers and the dsDNA−heterodimer complexes

Absorption and fluorescence emission spectra of the heterodimers free and bound to dsDNA are shown in Fig. 6. The emission maxima and the fluorescence enhancements on binding to dsDNA are given in Table 2. TOTAB, TOED1, and TOED2 all showed over 100-fold fluorescence enhancements at their long wavelength emission maxima on binding to dsDNA. These enhancements compare favorably to the 21−40-fold fluorescence increase seen on binding of ethidium (15) or ethidium homodimer (16) to dsDNA.

The emission spectra of the dsDNA-bound heterodimers each show two fluorescence emission maxima, originating from the two different chromophores (Fig. 6). For example, the emission maxima of TOTAB at 532 and 662 nm originate from the thiazole

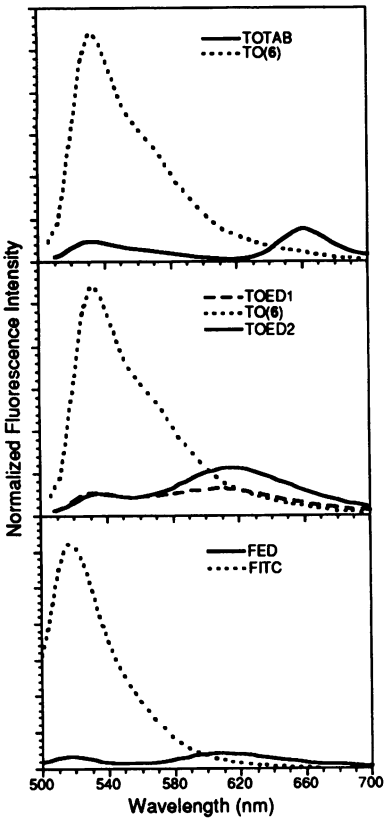


Figure 7. Comparison of the fluorescence emission spectra of calf thymus DNA complexes of donor chromophores (in monomers) with those of heterodimeric dyes containing the monomer. All spectra are normalized to the absorption of the donor chromophore. For each set of spectra, the DNA bp:dye ratio was 100 in 4 mM TAE, pH 8.2. (a) TOTAB (7) (2.5×10^{-7} M) *vs* equimolar thiazole orange monomer (6) in presence of 2.5×10^{-5} M bp DNA. (b) TOED1 (10) and TOED2 (11), both at 2.5×10^{-7} M, *vs* equimolar thiazole orange monomer (6). (c) FED (12, 1×10^{-7} M) *vs* equimolar FITC in presence of 1×10^{-5} M bp DNA.

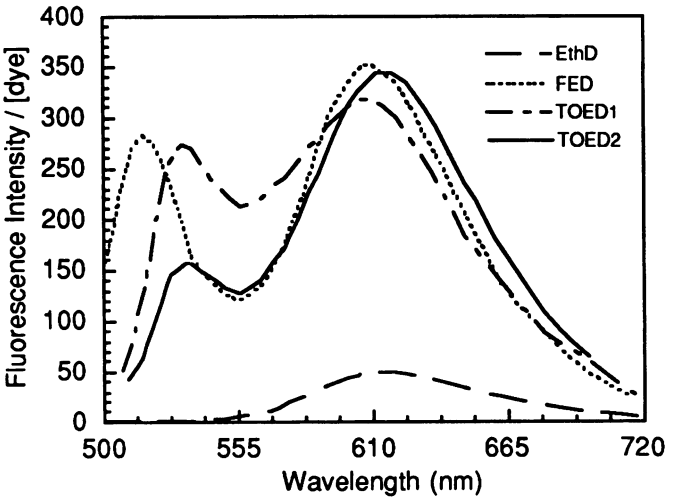


Figure 8. Comparison of the fluorescence emission spectra of equimolar solutions of TOED1 (10), TOED2 (11), FED (12), and ethidium dimer (EthD) in presence of calf thymus DNA at 100 DNA bp:dye, with 488 nm excitation. All other conditions as in Figure 7.

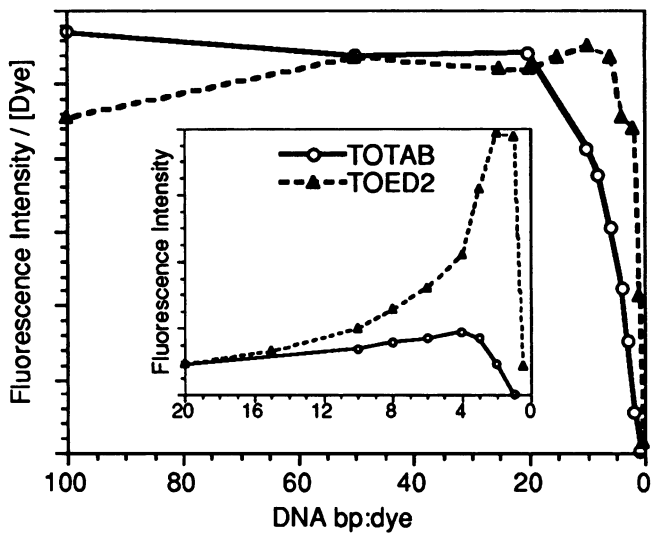


Figure 9. Titration of calf thymus DNA with TOTAB (7) and TOED2 (11) normalized per mole of total dye and monitored at the thiazole blue emission maximum (662 nm) for TOTAB and the ethidium emission maximum (610–614 nm) for TOED2. Titrations were performed by adding concentrated aliquots of stock dye solutions to DNA (5×10^{-6} M bp) in 0.5 ml of 4 mM TAE and incubating the mixture for 15 minutes prior to recording each spectrum. *Inset* shows the total relative fluorescence emission as a function of DNA bp:dye ratio.

orange and thiazole blue moieties, respectively. As illustrated in Fig. 7, in all four DNA-bound heterodimers, the short wavelength emission is quenched by over 90% relative to the emission of the appropriate monomeric chromophore derivative, i.e. the energy transfer within the heterodimers is >90%.

For the DNA-bound dyes, a comparison of absorption and emission spectra (with 488 nm excitation) clearly demonstrates efficient energy transfer (Fig. 6). Direct comparisons based on relative fluorescence emission from each of the two chromophores can be made because the fluorescence quantum yields of the DNA-bound monomeric thiazole orange and ethidium chromophores are similar (13,15). For example, at 488 nm for dsDNA-bound TOTAB, the thiazole blue chromophore accounts for only 3% of the total absorption and 97% is due to the thiazole orange moiety. However, when dsDNA–TOTAB is excited at 488 nm, the ratio of thiazole blue to thiazole orange emission is 1.66. The 488 nm absorption of dsDNA–TOED1 is dominated by the thiazole orange chromophore (at 488 nm, $\epsilon_{M\ (donor)}$: $\epsilon_{M\ (acceptor)}$ = 12:1), but 488 nm excitation yields 1.17-fold higher emission from the ethidium than from the thiazole orange chromophore. For DNA-bound TOED2 (488 nm, $\epsilon_{M\ (donor)}$: $\epsilon_{M\ (acceptor)}$ = 8:1), 488 nm irradiation resulted in 2.2-fold more ethidium than thiazole orange emission. For FED, although fluorescein absorption dominates 488 nm absorption of DNA-bound FED (488 nm, $\epsilon_{M\ (donor)}$:$\epsilon_{M\ (acceptor)}$ = 11:1), 488 nm irradiation resulted in 1.25 fold higher ethidium than fluorescein emission.

Another measure of the effect of the increased absorption cross-section in heterodimers at 488 nm on the fluorescence emission is evident from the following comparison. At DNA bp:dye ratio of 100:1, on 488 nm excitation the ~ 610 nm fluorescence emission from the ethidium chromophore in TOED1, TOED2, and FED is 10–12 times stronger *per ethidium* than the corresponding emission in ethidium homodimer (Fig. 8).

Table 2. Fluorescence properties of free and dsDNA-bound dyes

Dye	λ^F_{max}(free)[a] (nm)	λ^F_{max}(bound)[b] (nm)	F_{bound}/F_{free}[c]
TOTAB	660	532, 662	380
TOED1	630	532, 610	220
TOED2	635	532, 614	140
FED	518	518, 610	8

[a]Dye (2×10^{-6} M) in 4 mM TAE, pH 8.2.
[b]Dye (2×10^{-7} M) bound to DNA (2×10^{-5} M) at 100 bp:dye in 4 mM TAE pH 8.2.
[c]Fluorescence intensity measured at the λ^F_{max} of the acceptor chromophore.

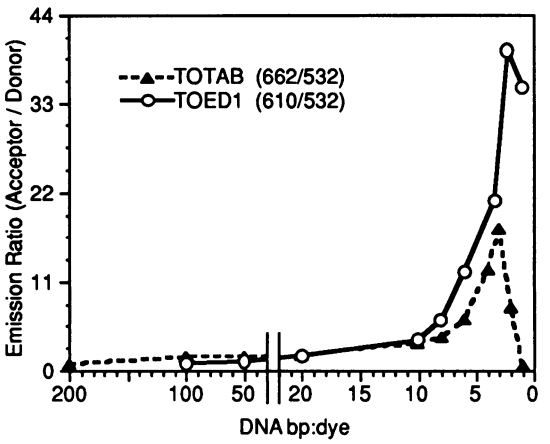


Figure 10. Titration of calf thymus DNA (5×10^{-6} M bp) by TOTAB (7) and TOED (10) by the procedure described in Figure 9, plotted as the ratio of acceptor to donor chromophore fluorescence intensities at the emission maxima in relation to bp:dye, normalized per mole of dye added. For TOTAB the thiazole blue acceptor emission at 662 nm is compared to the thiazole orange donor emission at 532 nm. For TOED1, the ethidium acceptor emission at 610 nm is compared to the thiazole orange donor emission at 532 nm.

Titration of dsDNA with with TOTAB, TOED1, and TOED2

The results of titration of calf thymus DNA with TOTAB and TOED2, as monitored by the fluorescence at the acceptor emission maxima, are shown in Fig. 9. The results obtained with TOED1 and TOED2 were very similar. Consequently, only the results with TOTAB and TOED2 are discussed. For both these heterodimers, the fluorescence emission per mole of added dye was essentially linear from 100 DNA bp:dye to 20 bp:dye, but diminished sharply at lower DNA bp:dye ratios. As shown in the inset in Fig. 9, for TOTAB the absolute fluorescence intensity increased to a maximum at 4–5 bp:dye and for TOED2 at 2 bp:dye and declined at lower bp:dye ratios. For both dsDNA–TOTAB and dsDNA–TOED2 complexes, emissions of both donor and acceptor were almost completely quenched when the DNA bp:dye ratio reached 0.5 bp or lower.

A comparison of donor to acceptor fluorescence intensity is shown in Fig. 10. As sites on the DNA become saturated with dye, the emissions of both donor and acceptor chromophore *per mole* of added dye become increasingly quenched; this is particularly pronounced for the donor emissions. Whereas the ratio of donor:acceptor emission was nearly constant from 100 DNA bp:dye to 20 bp:dye, donor emission is more severely quenched than acceptor emission at lower DNA bp:dye ratios.

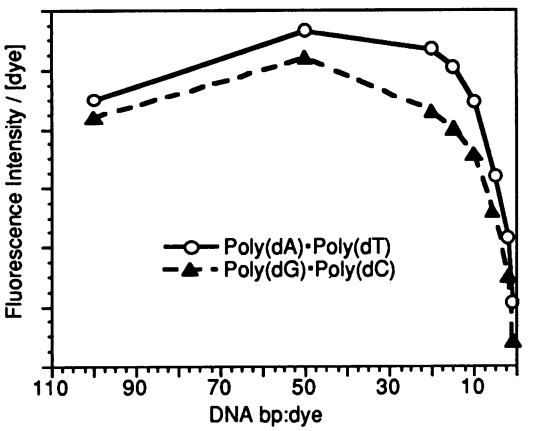


Figure 11. Titration of poly(dA)·poly(dT) and poly(dG)·poly(dC) with TOED1 (10), normalized per mole of dye and monitored at the ethidium fluorescence emission maximum. Titrations were performed by adding concentrated aliquots of stock dye solutions to the DNA (1×10^{-5} M bp) in 0.5 ml of 4 mM TAE containing 100 mM NaCl and incubating the mixture for 15 minutes prior to recording each spectrum.

Binding of TOED to poly(dA)·poly(dT) and poly(dG)·poly(dC)

Titration of poly(dA)·poly(dT) and poly(dG)·poly(dC) with TOED1 gives no evidence of base-pair specificity. The titration curves, as monitored by the fluorescence of the ethidium emission at 610 nm, are shown in Fig. 11. The titration curves are very similar with a slightly higher emission observed for the dye bound to poly(dA)·poly(dT) as compared to that bound to poly(dG)·poly(dC). The emission maxima of the two chromophores in the bound heterodimer were affected by the base composition of the DNA. At DNA bp:dye ratio of 100:1, the emission of the thiazole orange chromophore in the complex with poly(dA)·poly(dT) is at 536 nm, and in the complex with poly(dG)·poly(dC) it is at 548 nm. The corresponding values for the ethidium emission are 616 and 604 nm.

DISCUSSION

In previous studies, we have shown that ethidium homodimer [EthD; 5,5′-(diazadecamethylene)bis(3,8-diamino-6-phenylphenanthridinium tetrachloride)] (4) and the asymmetric cyanine homodimers, TOTO (1,1′-(4,4,7,7-tetramethyl-4,7-diazaundecamethylene)-bis-4-[3-methyl-2,3-dihydro-(benzo-1,3-thiazole)-2-methylidene]-quinolinium tetraiodide) and YOYO (an analogue of TOTO with a benzo-1,3-oxazole in place of the benzo-1,3-thiazole) (5,6), form complexes with dsDNA stable to electrophoresis. These complexes allow high sensitivity detection and quantitation of dsDNA in restriction fragment mapping, detection of polymerase chain reaction products, and band shift experiments (4–6). Moreover, we have demonstrated that sensitive multiplex detection, sizing, and quantitation of sets of dsDNA fragments labeled with different fluorescent dyes can be successfully performed. For example, a dsDNA ladder prelabeled with ethidium homodimer can be used to size a mixture of restriction fragments pre-labeled with TOTO by mixing the samples and co-electrophoresis on agarose or acrylamide gels (5). Laser excitation at 488 nm and simultaneous confocal fluorescence detection at 610–750 nm (dsDNA–EthD emission)

and 500–565 nm (dsDNA–TOTO emission) permitted rapid detection of dsDNA fragments with sensitivities comparable to those attainable with end-labeling with ^{32}P (5).

To extend the range of such applications, we have synthesized several heterodimeric dyes with the chromophores chosen to serve as a donor–acceptor pair to generate sets of dyes which could be efficiently excited at a single wavelength but with well-separated emission maxima dictated by the choice of the acceptor chromophore. In this exploratory study, we have largely confined ourselves to the well-studied ethidium and cyanine chromophores, and to previously investigated modes of linking these chromophores together modes known not to interfere with the binding of the chromophores to dsDNA. In the most radical departure from previously investigated types of chromophores incorporated into DNA-binding dyes, we examined a heterodimer which incorporates fluorescein as a donor and ethidium as an acceptor.

The choice of chromophores paired in each heterodimer is based on the following considerations. The spectroscopic properties of the chromophores are such that the emission spectrum of the dye chosen as donor overlaps extensively the absorption spectrum of the acceptor. The donor chromophores all have high absorption coefficients at the 488 nm argon ion laser line. The donor chromophores either have high fluorescence quantum yields when bound to dsDNA (ethidium, thiazole orange), or both when free and when tethered to dsDNA (fluorescein). This ensures that, at least for the donor, other non-radiative decay processes do not compete efficiently with energy transfer to the acceptor chromophore.

Polymethylene–amine linkers were used in all four heterodimers. The nucleophilic amino groups in the linker precursors provide convenient reactive sites for coupling to chromophores. Similar linkers have been employed previously to synthesize various homodimeric and heterodimeric compounds that bind tightly to dsDNA (see ref. 17 for a review). The multiple positive charges on the linkers in aqueous solution contribute to the binding affinity of such heterodimers to dsDNA (18). The linkers were chosen to give an extended length of about 11 Å. This length was selected in the event that both chromophores in one or another of the heterodimers would adopt a bis-intercalation mode of binding to dsDNA. An 11 Å separation of chromophores allows bisintercalation without violation of the 'nearest neighbor exclusion principle' (e.g. 19).

In the heterodimers synthesized for this study, we included three types of chromophores: the well-known phenanthridinium dye, ethidium, two unsymmetrical cyanine dyes (thiazole orange, thiazole blue), and fluorescein. Ethidium monomer binds to dsDNA strongly by intercalation with affinity constant of about 10^6 and a fluorescence enhancement of about 21-fold (15). Thiazole orange monomer binds to dsDNA with a similar affinity and a fluorescence enhancement of over 3000 (13). Nothing is known of the molecular details of the interaction of thiazole orange with dsDNA. The interaction of thiazole blue with dsDNA has not previously been explored in detail. Fluorescein is negatively charged and shows little affinity for dsDNA. It was hoped that the combination of a chromophore that binds strongly to dsDNA (ethidium, thiazole orange) and a polycationic linker attached at an appropriate site would result in high affinity binding to dsDNA even if the contribution of the second chromophore to binding were modest. As shown by the spectroscopic and titration measurements presented here, and by the extensive gel electrophoresis experiments described in the accompanying paper (10), TOTAB and TOED2 bind to dsDNA with an affinity comparable to that of ethidium homodimer (K_{diss} 2×10^{-8} in 0.2 M Na^+; 16), TOED1 with a somewhat lower affinity, and FED relatively weakly. Comparison of TOED1 (Fig. 4, **10**) and TOED2 (Fig. 4, **11**) shows that alkylation of the amino groups in the linker markedly enhances the stability of the dye–dsDNA complex (10). Titration of polyA:T and polyG:C with TOED1, monitored by measuring fluorescence emission intensity, did not reveal significant base-pair preference.

In TOTAB and in FED, comparison of the spectroscopic properties of the bound *versus* the free dyes indicates that in each case the two chromophores of the heterodimer interact very differently with dsDNA. The absorption maximum of the thiazole orange moiety of TOTAB shifts from 506 (free heterodimer) to 514 nm on binding to dsDNA, whereas that of the thiazole blue chromophore shows a much smaller shift, 644 nm (free) to 646 nm (bound) (see Table 1). Similarly, the fluorescence emission maximum of thiazole blue shifts little on binding, 660 nm in the free heterodimer and 662 nm in the bound state. The much smaller fluorescence enhancement of thiazole blue (relative to that shown by thiazole orange) seen on binding to dsDNA also supports the view that the thiazole blue chromophore has fewer contacts with DNA than the thiazole orange chromophore.

Similar conclusions emerge from analysis of the spectroscopic properties of the FED–dsDNA complex. There was little change in positions of the absorption or fluorescence maxima of the fluorescein chromophore on binding to dsDNA. It appears that the negatively-charged fluorescein chromophore interacts weakly, if at all with the DNA.

In summary, this exploratory survey of heterodimers designed for tight binding to dsDNA with efficient energy transfer is very encouraging. In the dsDNA-bound form, each of the four heterodimers shows efficient energy transfer as shown by a greater than 90% quenching of donor emission and a large enhancement of acceptor emission. Efficient energy transfer is observed irrespective of whether only one or both chromophores interact tightly with dsDNA. As shown in the accompanying paper (10), two of the heterodimers, TOTAB and TOED2 interact sufficiently tightly with dsDNA to dissociate very slowly during electrophoresis and consequently these dyes can be exploited in multiplex applications.

This report describes the first examples of a valuable class of molecules specifically designed to exploit energy transfer for the high sensitivity multiplex detection of nucleic acids. There is substantial literature on energy transfer between donor and acceptor chromophores covalently attached at distinct sites on dsDNA, but a relative paucity of publications on energy transfer between non-covalently bound chromophores. Le Pecq and coworkers (16) coupled an ethidium through its ring nitrogen through a $-(CH_2)_3-NH-(CH_2)_3-NH-(CH_2)_3-$ bridge to the 9-amino group of a quinacrine chromophore to form a heterodimer with high affinity for dsDNA and demonstrated energy transfer from the quinacrine to the ethidium in the DNA-bound dye. Energy transfer between two different dye molecules bound to the same dsDNA fragment has been observed in a number of instances. Efficient Förster fluorescence energy transfer was seen when dimethyldiazaperopyrenium (donor) and ethidium (acceptor) were bound to poly d(A–T) (20). Similar observations were reported on energy transfer between dsDNA-bound thiazole orange (donor) and ethidium homodimer (acceptor) (21) and Hoechst 33342 (donor) and acridine orange (acceptor) (22). The diversity of potential donor and acceptor

chromophores in molecules that bind non-covalently to dsDNA has barely been tapped.

ACKNOWLEDGEMENTS

We thank Nell Shimasaki for performing a number of the spectrofluorimetric studies of DNA–dye interactions. We are indebted to Aberra Fura and Julie A.Leary for the mass spectrometry determinations. We are grateful to Richard A.Mathies and Hays S.Rye for advice and helpful discussions throughout the course of this work. This research was supported in part by a grant from the Director, Office of Energy Research, Office of Health and Environmental Research of the U.S. Department of Energy under contract DE-FG-91ER61125.

REFERENCES

1. Glazer, A.N. and Rye, H.S. (1992) *Nature*, **359**, 859–861
2. Quesada, M.A., Rye, H.S., Gingrich, J.C., Glazer, A.N. and Mathies, R.A. (1991) *Biotechniques*, **17**, 616–625
3. Mathies, R.A., Scherer, J.R., Quesada, M.A., Rye, H.S. and Glazer, A.N. (1994) *Rev. Sci. Instrum.*, submitted.
4. Glazer, A.N., Peck, K. and Mathies, R.A. (1990) *Proc. Natl. Acad. Sci. U.S.A.* , **87**, 3851–3855
5. Rye, H.S., Yue, S., Wemmer, D.E., Quesada, M.A., Haugland, R.A., Mathies, R.A. and Glazer, A.N. (1992) *Nucleic Acids Res.* , **20**, 2803–2812
6. Rye, H.S., Yue, S., Wemmer, D.E., Quesada, M.A., Haugland, R.A., Mathies, R.A. and Glazer, A.N. (1992) *Methods in Enzymology*, **217**, 414–431
7. Rye, H.S., Drees, B.L., Nelson, H.M.N. and Glazer, A.N. (1993) *J. Biol. Chem.*, in press
8. Ledbetter, D.H. (1992) *Human Mol. Gen.*, **1**, 297–299
9. Shapiro, H.M. (1988) Practical Flow Cytometry. Second Edition. Alan R. Liss, Inc., New York
10. Benson, S.C., Mathies, R.A. and Glazer, A.N. (1993) *Nucleic Acids Res.*, **21**, 5720–5726.
11. Brooker, L.G.S., Keyes, G.H. and Williams, W.W. (1942) *J. Am. Chem. Soc.*, **64**, 199–210
12. Brooker, L.G.S., White, F.L., Keyes, G.H., Smyth, G.P. and Oesper, P.F. (1941) *J. Am. Chem. Soc.*, **63**, 3192–3203
13. Lee, L.G., Chen, C. and Liu, L.A. (1986) *Cytometry* **7**, 508–517
14. Gaugain, B., Barbet, J., Oberlin, R., Roques, B.P. and Le Pecq, J.B. (1978) *Biochemistry*, **17**, 5071–5078
15. LePecq, J.B. and Paoletti, C. (1967) *J. Biol. Chem.* **27**, 87–106
16. Gaugain, B., Barbet, J., Capelle, N., Roques, B.P. and Le Pecq, J.B. (1978) *Biochemistry*, **17**, 5078–5088
17. Wakelin, L.P.G. (1986) *Medicinal Res. Revs.*, **6**, 275–340
18. Becker, M. McC. and Dervan, P.B. (1979) *J. Am. Chem. Soc.*, **101**, 3664–3666
19. Assa-Munt, N., Denny, W.A., Leupin, W. and Kearns, D.R. (1985) *Biochemistry* **24**, 1441–1449
20. Mergny, J.-L., Slama-Schwok, A., Montenay-Garestier, T., Rougée, M. and Héléne, C. (1991) *Photochem. Photobiol.*, **53**, 555–558
21. Rye, H.S., Quesada, M., Peck, K., Mathies, R.A. and Glazer, A.N. (1991) *Nucleic Acids Res.*, **19**, 327–333
22. Ludwig, M., Hensel, N.F. and Hartzman, R.J. (1992) *Biophys. J.*, **61**, 845–857