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Heterodimeric DNA-binding dyes designed for energy transfer: stability and applications of the DNA complexes

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ABSTRACT

Spectroscopic studies of the complexes of double-stranded (ds) DNA with the polymethylene–amine linked heterodimers thiazole orange–thiazole blue, thiazole orange–ethidium, and fluorescein–ethidium, in each case show efficient energy transfer from donor to acceptor chromophores (Benson,S.C., Singh,P. and Glazer,A.N. (1993) accompanying manuscript). A quantitative assay of the stability of such complexes during gel electrophoresis is presented. The off-rate of dye from complexes formed at an initial dsDNA bp:dye ratio ≥10:1 follows strict first-order kinetics. The $t_{0.5}$ values for the dissociation of a series of related dyes provide a quantitative criterion for the design of DNA-binding fluorophores. Complexes of dsDNA with the monomeric propidium and cyanine dyes, [1-(9-amino-4,7-diazanonyl)-3,8-diamino-6-phenyl-phenanthridinium bromide trihydrobromide] and (N,N'-tetramethyl-1,3-propanediamino)propyl thiazole orange [4-[3-methyl-2,3-dihydro-(benzo-1,3-thiazole)-2-methylidenyl]-1-(4,4,8-trimethyl-4,8-diazanonyl)-quinolinium diiodide], are much more stable than those with their widely used counterparts, ethidium and thiazole orange. Applications of the new dyes in post-staining of gels and in the multiplex detection of DNA restriction fragments are presented.

INTRODUCTION

In earlier studies exploiting a newly invented laser-excited confocal fluorescence gel scanner (1,2), we showed that dyes differing widely in structure, such as the phenanthridinium derivative, ethidium homodimer, and the asymmetric cyanine dyes, thiazole orange and oxazole yellow homodimers, bound with high affinity to double-stranded (ds) DNA to form fluorescent complexes stable to gel electrophoresis (3–6). Such high affinity complexes of dsDNA with dyes are valuable in the detection of DNA in solution (7), and in various applications dependent on gel electrophoresis. Current applications include multiplex detection of DNA restriction fragments (5,6,8), high sensitivity detection of the products of the polymerase chain reaction (3), sizing of individual dsDNA–dye fragments by flow cytometry (9,10), and study of protein–DNA interactions (11). The high stability of certain of the DNA–dye complexes opens up the possibility of other applications (e.g. in immunoassay, cell surface labeling, and fluorescence *in situ* hybridization) in which appropriately derivatized DNA–dye complexes may be used as multichromophore fluorescent tags (3).

Multiplex applications depend on the availability of dyes which preferably can be efficiently excited at a single wavelength but which emit at distinct well-separated wavelengths. In this and the preceding paper (12), we examine the possibility of generating appropriate dyes by exploiting energy transfer to tune the position of the fluorescence emission maximum. In each case, for dsDNA complexes with the heterodimeric dyes thiazole orange–thiazole blue heterodimer (TOTAB), two different thiazole orange–ethidium heterodimers (TOED1 and TOED2), and a fluorescein–ethidium heterodimer (FED), there is efficient energy transfer from the donor to the acceptor chromophore (12). This indicates that the criterion of emission wavelength tuning by energy transfer in dye–DNA complexes is adequately met by ligands with widely varying donor and acceptor chromophores. Complementary information on the stability of the DNA–dye complexes under conditions of electrophoresis is needed to guide the further synthesis of energy-transfer DNA dyes.

We describe here an electrophoretic assay which allows accurate measurement of the rate of dissociation of the DNA–dye complexes. We have applied this assay to compare the stability of complexes of various monomeric as well as homo- and heterodimeric dyes with dsDNA. The results of this assay provide information on the contribution of structural elements in the dye ligands which contribute to the binding to DNA, such as charge, spacing of charges, quaternization of linker amino groups, and possibility of intercalation. This information will guide the future design of fluorescent DNA ligands.

Finally, we describe the applications of certain of the new monomeric and dimeric dyes to the multiplex detection of dsDNA restriction fragments and to the detection of dsDNA on gels by post-staining.

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MATERIALS AND METHODS

Materials

The structures of the dyes used in this study are shown in Figure 1. The compounds are numbered in bold face and are identified in the text in this manner. The spectroscopic properties of the dyes in their dsDNA bound form are given in Table 1. TOTAB (**7**), TOED1 (**10**), TOED2 (**11**), FED (**12**), TOTO (**8**), thiazole orange (**4**), (N,N'-tetramethyl-1,3-propanediamino)-propyl thiazole orange (**6**), and (N,N'-tetramethyl-1,2-ethanediamino)propyl thiazole orange (**5**) were synthesized as described by Benson *et al.* (12). The monomeric phenanthridinium dyes, (1,3-propanediamino)propidium (**2**) and (diethylenetriamino)-propidium (**3**), were obtained by deprotection of compounds **9a** and **9c** described by Benson *et al.* (see fig. 3 in ref. 12) with HBr/acetic acid. Ethidium homodimer (**9**) was purchased from Molecular Probes and ethidium bromide (**1**) from Sigma. Tris−acetate−EDTA (TAE) buffers were prepared by appropriate dilution of a stock solution of 2 M tris(hydroxymethyl)-aminomethane−0.05 M Na_2 EDTA titrated to pH 8.2 with glacial acetic acid. DNA size standards were obtained from GIBCO BRL (Life Technologies, Inc., Gaithersburg, MD). M13mp18 plasmid was cut at a single site with *Hin*dIII using a Boehringer-Mannheim (Indianapolis, IN) restriction kit. The linear dsDNA was then extracted with phenol and recovered by precipitation with ammonium acetate/isopropanol. Analysis by agarose gel electrophoresis showed nearly complete single cutting of the plasmid. Stock double-stranded (ds) DNA solutions in 40 mM TAE buffer were stored at −20°C.

Absorption and fluorescence measurements on solutions

Absorption spectra were determined with a Perkin-Elmer Lambda 6 spectrophotometer. Fluorescence measurements were performed with a Perkin-Elmer model MPF 44B spectrofluorimeter connected to a Hitachi 057 plotter.

DNA detection in agarose gels by prestaining

Concentrated stock solutions of dyes (10^{-4} M in MeOH or DMSO) were stored at −20°C. Fresh diluted stock dye solutions (10^{-7} M) were prepared in 4 mM TAE, pH 8.2, for each experiment immediately before use. Because of the observed instability of the cyanine dyes in the TAE buffers (5), the diluted stock solutions were discarded after use.

The following exemplifies the procedure for the preparation of DNA−dye complexes in advance of electrophoresis. To obtain a final concentration of 0.2 ng λ DNA/*Hin*dIII ladder per μl, stock DNA (10 μg/ml; 2 μl) was added to an appropriately diluted dye solution in 4 mM TAE, pH 8.2, containing 50−100 mM NaCl (see below), to give a final volume of 75 μl of solution at a DNA bp:dye ratio of 10:1 (0.27 ng dsDNA/μl, corresponding to 4.19×10^{-7} M bp; 4.19×10^{-8} M dye). The mixture was incubated in the dark for 30 minutes and then 25 μl of 15% (w/v) aqueous Ficoll was added. Five μl aliquots of the final mixture (1 ng dsDNA) were loaded in each well.

Electrophoresis was performed in a Bio-Rad (Richmond, CA) Mini-Protean II cell on vertical, 1 mm thick, 0.9% agarose gels (Ultrapure™ agarose, Bethesda Research Laboratories). Gels were subjected to pre-electrophoresis for an hour prior to sample application. Samples were electrophoresed at 10 V/cm in 40 mM TAE, pH 8.2.

For the determination of dye off-rates, equal aliquots of a DNA−dye mixture were loaded into consecutive wells on a gel at appropriate time intervals to allow determination of the integrated fluorescence intensity of a given band as a function of the length of time of electrophoresis. Where possible, the integrated fluorescence intensities of two or three bands in each lane were summed. The slopes of plots of the summed values against time did not differ significantly from that of a plot of the integrated fluorescence intensity of the largest fragment band (23 kb).

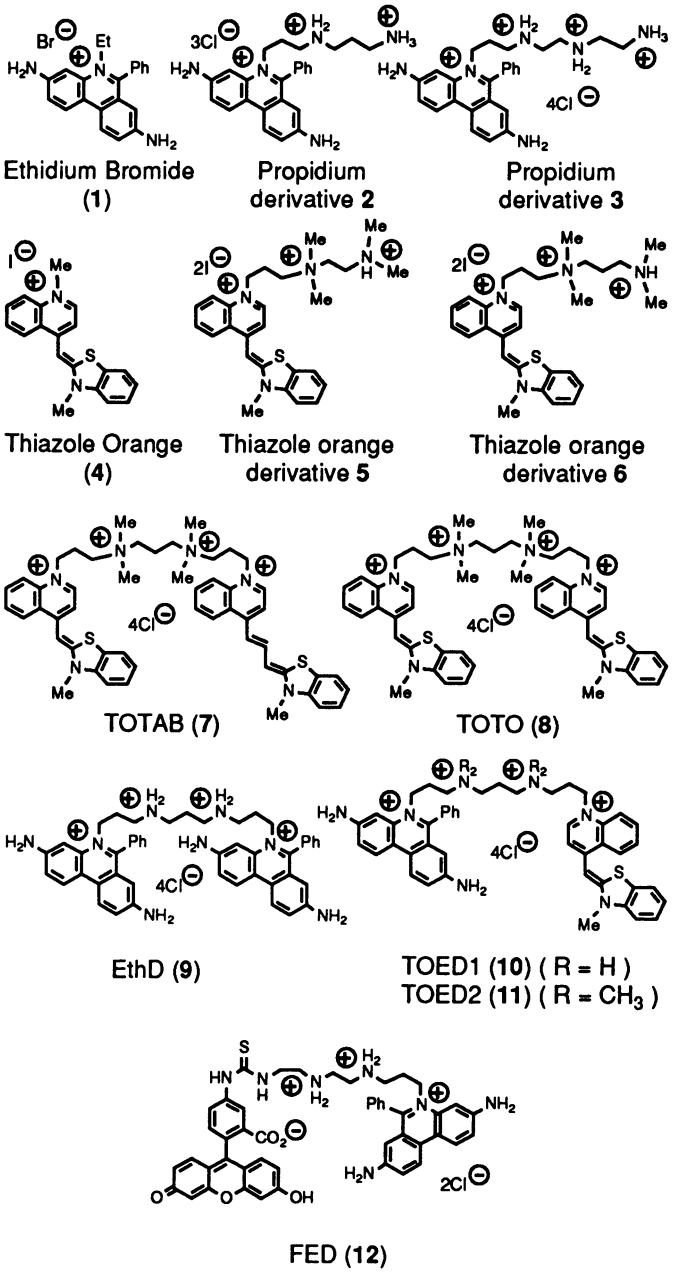


Figure 1. Structures of dyes forming fluorescent complexes with dsDNA. **1**, Ethidium bromide; **2**, (1,3-propanediamino)propidium; **3**, (diethylenetriamino)propidium; **4**, thiazole orange; **5**, (N,N'-tetramethyl-1,2-ethanediamino)propyl thiazole orange; **6**, (tetramethylpropanediamino)propyl thiazole orange; **7**, thiazole orange-thiazole blue heterodimer (TOTAB); **8**, thiazole orange homodimer (TOTO); **9**, ethidium homodimer (EthD); **10** and **11**, thiazole orange−ethidium heterodimers TOED1 and TOED2, respectively; **12**, fluorescein−ethidium heterodimer.

Table 1. Spectroscopic properties of dsDNA-bound dyes

Dye[a]	λ_{max}[b] (nm)	ϵ_M ($M^{-1}\,cm^{-1}\times10^{-3}$)	λ^F_{max}[c] (nm)	F_{free}/F_{bound}[d]
Ethidium bromide (1)	514	4.2	603	21[e]
Propidium 2	529	3.5	612	40
Propidium 3	530	3.5	610	30
Thiazole orange (4)	509[f]	52	527[f]	2100
Thiazole orange 5	515	62	532	3800
Thiazole orange 6	515	64	532	3800
TOTAB (7)	514, 646	60.8, 46.6	532, 662	140
TOTO (8)	513[f]	100	532[f]	3200[f]
EthD (9)	534[f]	5.3	616[f]	35[f]
TOED1 (10)	515	57.5	532, 610	380
TOED2 (11)	515	46.9	532, 614	220
FED (12)	492	48	518, 610	8

[a]Numbers refer to structures in Figure 1.
[b]Spectra recorded in 4 mM TAE pH 8.2 containing calf thymus DNA at 10 bp:dye unless otherwise specified.
[c]Emission spectra for 488 nm excitation recorded in 4 mM TAE pH 8.2 containing calf thymus DNA at 100 bp:dye.
[d]Fluorescence intensity recorded at the long wavelength emission maxima of the dyes.
[e]Ref. 3.
[f]Ref. 5.

Confocal laser-excited fluorescence gel scanner

Following electrophoresis, gels were scanned with a two-color confocal laser excited fluorescence imaging system previously described in detail (1,2,5,6). The data were transformed into MAC Image™ files to produce pseudoimages or transformed into Scan Analysis™ files to determine the integrated fluorescence intensity of the bands (5).

DNA detection in agarose gels by post-staining

Samples containing amounts varying from 5 to 30 ng of λ DNA/*Hin*dIII fragments were applied in duplicate sets to the same gel. After 45 minutes of electrophoresis, the gels were cut in half to separate the duplicate sets and stained in dye diluted in 40 mM TAE buffer. One half of a gel was soaked in thiazole orange (2×10^{-7} M) and the other half in (N,N'-tetramethyl-1,3-propanediamino)propyl thiazole orange (6) (2×10^{-8} M). One half of a second gel was soaked in ethidium (2×10^{-6} M) and the other half in (diethylenetriamino)propidium (3) (2×10^{-7} M). After soaking for 40 minutes, the gels were transferred for 20 minutes to a dye-free TAE solution and then scanned as described above.

Comment on the salt concentration of the sample buffer

With several of the dyes examined in this study, smearing of bands and some precipitation of DNA in the wells was noted when the complexes were formed in 4 mM TAE, pH 8.2. We had noted earlier for dsDNA-YOYO complexes, that such problems were greatly decreased by forming the complexes in 40 mM TAE (5). Therefore, we investigated band sharpness and signal intensity for identical samples of λ DNA/*Hin*dIII ladder prepared in 4 mM TAE containing varying concentrations of NaCl. Concentrations of NaCl below 50 mM had little effect on band sharpness. Both signal intensity and band sharpness were optimal for samples prepared in 80–100 mM NaCl in 4 mM TAE. The effect of salt is illustrated in Figure 2. The signal intensity decreased when the NaCl concentration in the 4 mM TAE exceeded 150 mM. Subsequent routine inclusion of 50–100 mM NaCl (as specified) in the 4 mM TAE sample buffer eliminated both sample precipitation and band smearing.

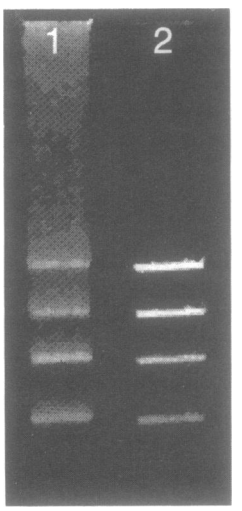


Figure 2. Effect of salt on sharpness and intensity of bands of λ DNA/*Hin*dIII restriction fragments complexed with TOTAB at an initial ratio of 10 bp:dye. For sample preparation protocol, see Materials and Methods. Lane 1, 5 μl (2ng) DNA–TOTAB complex in 4 mM TAE, pH 8.2, containing 5% Ficoll; lane 2, 5 μl (1ng) DNA–TOTAB complex in 4 mM TAE–100 mM NaCl, pH 8.2, containing 5% Ficoll.

RESULTS

Measurement of the rates of dissociation of the dsDNA–dye complexes

Quantitation of the off-rate of dye from a dsDNA–dye complex under the conditions of gel electrophoresis is of central importance in allowing the direct comparison of the relative stability of various dsDNA–dye complexes during electrophoresis, in providing a quantitative assessment of structure–binding affinity relationships for a series of closely related dyes, and in guiding the choice of dyes for post-staining of gels.

For the majority of the dyes investigated in this study, we have found that the decrease in fluorescence intensity of prestained dsDNA fragments on an agarose gel *versus* the length of time of electrophoresis was strictly first order (Figure 3). With two exceptions (TOED2 and TOTAB), for complexes formed at an

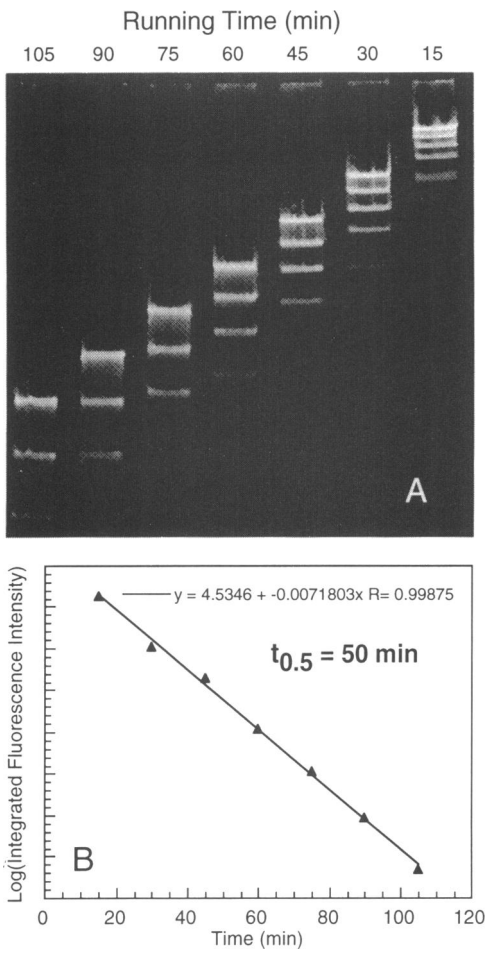


Figure 3. Dependence of fluorescence intensity of bands of λ DNA/*Hin*dIII−TOED complexes on time of electrophoresis. A mixture was prepared containing 0.6 ng/μl λ DNA/*Hin*dIII (1.26×10^{-6} M bp) and TOED1 (2.52×10^{-7} M; 5 bp:dye) in 4 mM TAE−80 mM NaCl, pH 7.7. At 15 minute intervals, in the course of electrophoresis, 5 μl (3 ng DNA) aliquots were loaded into consecutive wells on a 0.9% agarose gel. Panel **A** shows an image of the gel (see Materials and Methods). To obtain the plot in panel **B**, the fluorescence intensity of the bands was integrated, and summed within each lane, and the logarithm of the intensity plotted against time. The $t_{0.5}$ value determined from these data is given in panel B.

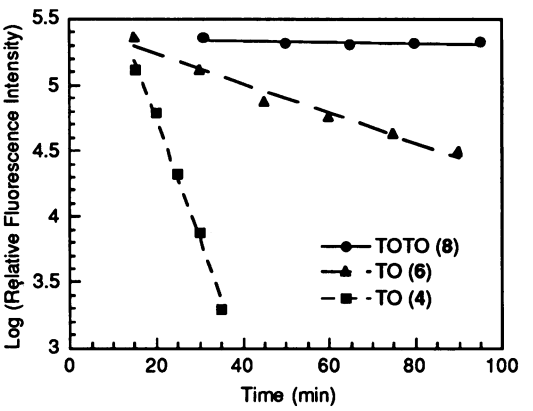


Figure 4. Determination of dye off-rates for dsDNA complexes with thiazole orange (**4**), (N,N′-tetramethyl-1,3-propanediamino)propyl thiazole orange (**6**), and TOTO (**8**). For experimental details, see Materials and Methods and Figure 3.

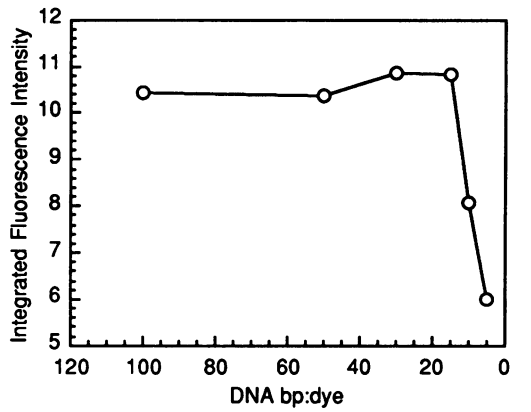


Figure 5. Dependence of the fluorescence of DNA−TOTAB complexes on the DNA bp:dye ratio. λ DNA/*Hin*dIII complexes with TOTAB, ranging from 2 to 100 bp:dye, were prepared in 4 mM TAE−100 mM NaCl by varying the DNA concentration at a constant dye concentration (for experimental details, see Materials and Methods) and subjected to electrophoresis on the same gel. The integrated fluorescence intensity of the 23 kb fragment band in each lane is plotted against the DNA bp:dye ratio.

initial ratio of DNA bp:dye of 5:1, plots of the logarithm of the integrated fluorescence intensity against time were linear (e.g. Figure 4). Since such plots represent the rate of loss of dye, the $t_{0.5}$ values provide an accurate measure of the relative stability of the dsDNA−dye complexes.

The dissociation of TOED2 and TOTAB complexes, formed at an initial ratio of DNA bp:dye of 5:1, showed at least two first order components. The $t_{0.5}$ values determined for these two dyes are not based on first order plots for dissociation. These values correspond to the time required from the initiation of electrophoresis for the loss of half of the dye from the complex. The off-rates for the various DNA−dye complexes are compiled in Table 2.

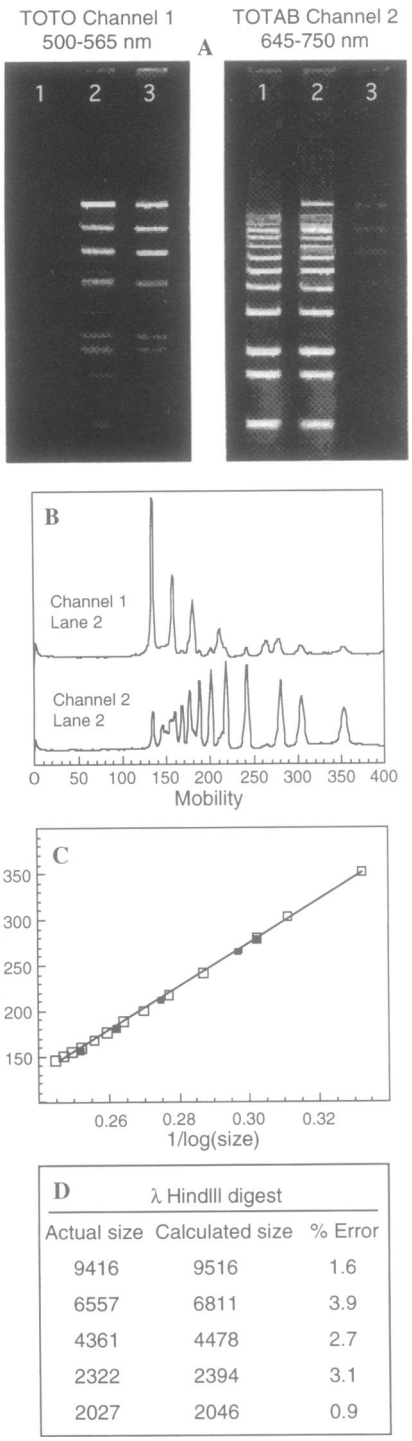
Some properties of DNA−TOTAB complexes

TOTAB shows the largest Stokes shift of any of the DNA-binding fluorescent dyes described to date. Consequently, it is an appropriate candidate for pairing in multiplex applications utilizing 488 nm excitation with dyes such as TOTO, which have a relatively small Stokes shift. With such applications in mind, the behavior of DNA−TOTAB complexes in gel electrophoresis was given particular attention.

Band fluorescence intensity at the end of 1 hour of electrophoresis as a function of initial DNA bp:dye ratio was studied for TOTAB complexes with λ DNA/*Hin*dIII fragments. The band intensity was constant per mole of dye for ratios from 100 bp:dye to 20 bp:dye and fell off at lower bp:dye ratios (Figure 5).

The dissociation of dye from the DNA−dye complexes during gel electrophoresis determines the sensitivity with which a given complex can be detected at the end of a particular time of electrophoresis. For example, with the detection system employed here, at the end of 1 hour, the limit for TOTAB was approximately 20 pg DNA per band as compared to >40 pg per band for TOED1 and 4 pg per band for TOTO (5).

The retardation of the mobility of DNA restriction fragments complexed with TOTAB was virtually identical to that observed



D

λ HindIII digest

Actual size	Calculated size	% Error
9416	9516	1.6
6557	6811	3.9
4361	4478	2.7
2322	2394	3.1
2027	2046	0.9

Figure 6. Multiplex detection of DNA restriction fragments complexed initially either with TOTAB or TOTO at 20 bp:dye after 60 minutes of agarose gel electrophoresis. Lane 1, 4 ng 1 kb DNA ladder complexed with TOTAB; lane 2, 1 ng λ DNA/*Hin*dIII complexed with TOTO mixed with 4 ng 1 kb ladder mixed with TOTAB; lane 3, 1 ng λ DNA/*Hin*dIII complexed with TOTO. The simultaneously acquired fluorescence images of the gel from the 'green' and 'red' channels are shown in panel **A**. Panel **B** illustrates the fluorescence intensity plots derived from lane 2. For other details, see Results. Panel **C** is a plot of the mobilities of DNA–dye complexes, determined from the data in panel B, against 1/log(fragment size). The mobilities of the 1 kb ladder complexes with TOTAB were determined from the peak positions in the 'red' channel. These values (filled squares) were plotted against 1/log(fragment size) and a line fitted to the data by the method of least squares. The data points for the mobilities of the λ DNA/*Hin*dIII–TOTO complexes (empty squares) were used to calculate fragment sizes. A comparison of the calculated to the actual fragment sizes is given in Panel **D**.

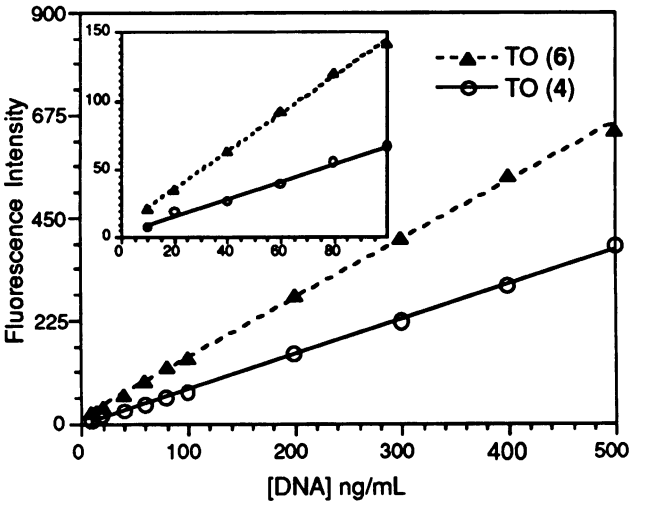


Figure 7. Quantitation of calf thymus DNA in solution with thiazole orange (**4**) (open circles) or with (N,N'-tetramethyl-1,3-propanediamino)propyl thiazole orange (**6**) (filled triangles). Dye (7.9×10^{-7} M) was added to DNA solutions in 4 mM TAE buffer at pH 8.2 at room temperature. Excitation was at 488 nm and, for each dye, fluorescence was measured at the emission maximum of its DNA complex. *Inset* shows the region of the titration curve from 10 to 100 ng DNA/ml in an expanded form.

in earlier studies for TOTO complexes (5). Relative to a dsDNA–dye complex formed initially at 100 bp:dye, mobility at 50 bp:dye was 1, at 20 bp:dye 0.99, at 10 p:dye 0.97, and at 5 bp:dye 0.92.

Multiplex detection of DNA fragments with TOTAB and TOTO

Since the retardation of DNA fragments on binding TOTAB was the same as that seen for TOTO, we anticipated that these two dyes could be used for multiplex detection and sizing of prestained restriction fragments.

For a model experiment, a 1 kb ladder was complexed with TOTAB and a λ DNA/*Hin*dIII ladder was complexed with TOTO. In each case, the bp:dye ratio was 20:1 to form complexes characterized by a single off-rate and to avoid subsequent transfer of dye between the ladders (3,5). Since the fluorescence intensity per bound dye for the DNA–TOTAB is much weaker than that for the DNA–TOTO complex (see fig. 7 in ref. 12), the mixed sample applied to the gel was adjusted to contain 4 ng of TOTAB-complexed DNA fragments and 1 ng of TOTO-complexed fragments. For DNA–TOTAB band detection, a 645 nm long pass filter installed in front of the red channel detector eliminated the emission from the DNA–TOTO fragments. An excellent separation of the emissions from the two dyes was achieved (see images and traces in Figure 6). A plot of the mobility data for the TOTAB complexes with the 1 kb ladder provided a standard curve which allowed the determination of the sizes of the λ DNA/*Hin*dIII fragments with high precision (Figure 6). Multiplex detection by the above procedure was also satisfactorily performed for sets of restriction fragments pre-labeled with TOTO and TOED2, respectively (data not shown).

Post-staining of gels

At present, the most frequent application of dyes which form fluorescent complexes with DNA is in post-staining of gels. Ethidium bromide is the dye generally used for this purpose. The choice of ethidium bromide is largely based on the efficient excitation of the DNA–ethidium complex with radiation from

a UV-transilluminator and detection of the visible fluorescence emission at about 600 nm. This is an insensitive procedure with a detection limit of about 1 ng DNA per band. With the advent of gel scanners with excitation and fluorescence detection capabilities in the visible region of the spectrum, routine detection of DNA with picogram per band sensitivity is attainable with suitable dyes.

Certain of the dyes examined in the present study are particularly well-suited for high sensitivity detection of DNA. The fluorescence enhancements of ethidium analogues bearing polycationic chains upon binding to dsDNA were essentially the same as that for ethidium. The same holds for the polycationic thiazole orange analogues *versus* thiazole orange (see Table 1). The loss of all of these dyes from preformed complexes with dsDNA during electrophoresis follows strict first order kinetics (for example, see Figure 4). The relative stabilities of the complexes are conveniently assessed by comparing the $t_{0.5}$ values (Table 2). It is evident from this comparison that the ethidium and thiazole dyes bearing polycationic substituents bind much more strongly to dsDNA than do ethidium and thiazole orange, respectively. As anticipated from these results, dsDNA was detected with higher sensitivity with these dyes than with ethidium or thiazole orange, both in solution and on post-stained gels.

A comparison of the titration of dsDNA in solution with excess thiazole orange (**4**) or (N,N'-tetramethyl-1,3-propanediamino)propyl thiazole orange (**6**), both at about 10^{-6} M dye, is shown in Figure 7. With both dyes, a linear dependence of fluorescence emission on dsDNA concentration was obtained from 10–500 ng dsDNA/ml. Compound **6** gave a three-fold higher fluorescence signal than did thiazole orange (**4**). Moreover, the determination of dsDNA concentration could be readily extended to lower DNA concentrations with this dye.

Post-staining of gels with **6** at a concentration 10-fold lower than with thiazole orange (2×10^{-8} M *vs* 2×10^{-7} M) gave a 14-fold more sensitive detection of dsDNA fragments. Post-staining with (diethylenetriamino)propidium (**3**) at a concentration 10-fold lower than with ethidium (2×10^{-7} M *vs* 2×10^{-6} M) gave a 1.7-fold higher sensitivity of dsDNA detection on gels.

DISCUSSION

Fluorescence-based detection of nucleic acids depends on the availability of dyes which bind to these polymers with high affinity and large fluorescence enhancement. The list of such dyes is very short. In the Introduction, we listed the many applications, already described, of high affinity fluorescent complexes of dsDNA with certain homodimeric dyes (EthD, TOTO, YOYO; see Figure 1 for structures). New dyes are needed to increase the versatility of multiplex applications, for more sensitive detection of nucleic acids in capillary electrophoresis (e.g. 14,15), or in post-staining of gels. Addressing this need, we have examined the synthesis, spectroscopic properties, and binding to dsDNA of several dyes designed for energy transfer. In addition, we have examined new monomeric dyes in a search for dyes for the detection of nucleic acids superior to ethidium bromide (13) and thiazole orange (4,16). In this study, we chose dyes excited efficiently at 488 nm.

The spectroscopic studies on the complexes of dsDNA with TOTAB, TOED, TOED2, and FED in solution showed highly efficient energy transfer from the donor to the acceptor chromophore in each of these dyes (12). However, these studies provide no information on the stability of pre-formed complexes of these dyes with nucleic acids under conditions of electrophoresis. We describe here an assay which provides this information and, in consequence, provides guidance on the design of new DNA ligands. In this assay, samples of a preformed DNA–dye complex are applied to a gel at intervals in the course of electrophoresis. The off-rate of dye from the complex is determined from the change in the integrated fluorescence intensity of the DNA–dye bands as a function of length of time of electrophoresis.

Table 2. Off-rate ($t_{0.5}$) values for the dissociation of the dsDNA–dye complexes under conditions of gel electrophoresis

Dye[a]	$t_{0.5}$[b] (min)
Ethidium bromide (**1**)	3.6
Thiazole orange (**4**)	4.3
FED (**12**)	21
Thiazole orange **6**	22
Thiazole orange **5**	26
Propidium **2**	31
TOED1 (**10**)	50
Propidium **3**	75
TOED2 (**11**)	81[c]
TOTAB (**7**)	137[c]
EthD (**9**)	140
TOTO (**8**)	664

[a]Numbers refer to structures in Figure 1.
[b]Determined as described in the text. DNA-dye complex formed initially at 5 bp:dye unless otherwise noted. All assays employed λ DNA/*Hin*dIII fragments at a load of 2-6 ng except for the assayof ethidium bromide (**1**) and thiazole orange (**2**) which employed linearized M13 DNA at a load of 50 ng.
[c]The value given is for dye complexed to λ DNA/*Hin*dIII initially at 20 bp:dye. For complexes formed at 5 bp:dye, the loss of dye doesnot follow pseudo-first order kinetics. A faster component with a $t_{0.5}$ of ~55 min. is observed.

For the dyes shown in Figure 1, dissociation of dye from complexes formed at dsDNA bp:dye ratios ≥20:1 followed strict first order kinetics. The off-rates are tabulated in Figure 1. These data provide insight into relationship between structure and binding affinity. For example, in going from the thiazole orange to the (N,N'-tetramethyl-1,3-propanediamino)propyl thiazole orange (**6**) complex with dsDNA, the $t_{0.5}$ increases from 4.3 to 22 minutes. This is consistent with the anticipated contributions to the binding affinity from counter ion release (17) and the additional electrostatic interactions of the polycationic substituent in **6** with the negative potential in the grooves of the DNA duplex (18). Addition of the thiazole blue moiety to **6** to form TOTAB substantially increases the $t_{0.5}$ to 137 minutes. The thiazole blue must participate in additional favorable contacts with the dsDNA. However, the TOTO–DNA complex, with a $t_{0.5}$ of 664 minutes, is much more stable. This indicates that the second thiazole orange chromophore contributes more to the stabilization of the complex than does thiazole blue. Spectroscopic studies lend support to this interpretation. For TOTAB in TAE buffer, the absorption maxima for the thiazole orange and thiazole blue chromophores lie at 506 and 644 nm, respectively. In the dsDNA–TOTAB complex these maxima are at 514 and 646 nm, respectively; i.e. the absorption maximum of the thiazole blue portion of the dye is shifted much less than that of the thiazole orange portion on binding to dsDNA (fig. 5 in ref. 12).

Similar observations hold for the phenanthridinium dyes. In going from ethidium, to (1,3-propanediamino)propidium (**2**), to (diethylenetriamino)propidium (**3**), the $t_{0.5}$ increases from 3.6, to 31, to 75 minutes (Table 2). A $t_{0.5}$ of 140 minutes is seen for ethidium homodimer (**9**), indicating a significant contribution from the binding of the second ethidium moiety to the dsDNA. In contrast, the formation of the fluorescein thiourea derivative

of **3** leads to a change in $t_{0.5}$ from 75 minutes for **3** to 21 minutes for FED, indicating the effect of the loss of one positive charge and the addition of a negatively charged fluorescein, a chromophore which has no affinity for dsDNA.

Alkylation of the amino groups in the linker has a large effect on the stability of the dsDNA complex with thiazole orange-ethidium heterodimers (Figure 1, compounds **10** and **11**). The dsDNA-TOED1 complex has a $t_{0.5}$ of 50 minutes and compared to a $t_{0.5}$ of 81 minutes for the dsDNA−TOED2 complex (Table 2).

Prior to this report, thiazole orange, with a fluorescence enhancement of over 3,000-fold on binding to dsDNA and a high extinction coefficient (16), allowed the most sensitive detection of dsDNA on post-staining of gels (4) or in capillary gel electrophoresis (14). The off-rate assay showed that **6** binds to dsDNA substantially more strongly than thiazole orange. The two dyes have similar spectroscopic properties and fluorescence enhancements on binding to dsDNA (12). Indeed, **6** is superior to thiazole orange for fluorometric detection of dsDNA in solution (Figure 7) and improves the sensitivity of dsDNA detection by post-staining gels > 10-fold over that attainable with thiazole orange.

The $t_{0.5}$ of 137 minutes for the dsDNA−TOTAB is similar to that for the dsDNA−EthD complex (140 minutes). The latter complex has been used successfully in multiplex detection of DNA restriction fragments on gels as well as other applications (3,6). The effect of the binding of TOTAB on the mobility of dsDNA restriction fragments is equivalent to that seen resulting from the binding of TOTO (5,6). As a consequence of energy transfer, the dsDNA−TOTAB complex has a huge Stokes shift the largest reported for any DNA−dye complex. For the dsDNA-bound dye, excitation at the absorption maximum of the thiazole orange chromophore (514 nm) leads predominantly to emission from the thiazole blue moiety at 662 nm (fig. 6 in ref. 12). This dye is therefore well-suited to multiplex applications in conjunction with TOTO. The dsDNA−TOTO complex fluoresces maximally at 532 nm (5). A model multiplex experiment, sizing of dsDNA restriction fragments pre-labeled with TOTO with pre-labeled dsDNA−TOTAB standards (Figure 6), led to accurate size determination for the 'unknown' fragments.

We have recently used TOTAB in multiplex experiments to determine the stoichiometry of complexes formed by dsDNA with heat shock transcription factor (HSF) (11). In this study, an engineered fragment of HSF, containing the trimerization and DNA-binding domains and a single carboxyl-terminal cysteine residue, was specifically labeled on the cysteine residue with fluorescein-5-maleimide. For gel shift experiments, the DNA−fluoresceinated protein complex was labeled with TOTAB prior to electrophoresis. At the conclusion of electrophoresis, the gel was scanned and the emissions from the fluorescein (530 nm) and TOTAB collected in separate channels allowing quantitation of the relative amounts of protein and DNA in each band. The results demonstrated that the formation of higher order dsDNA−HSF complexes occurs by successive addition of trimers of HSF (11). The large Stokes shift of the dsDNA−TOTAB complex ensured minimum crosstalk between the fluorescein and TOTAB emissions.

As indicated in the above discussion, the studies presented here have led to the discovery of a monomeric dye superior to previously described monomeric fluorophores for the high sensitivity detection of dsDNA and to dimeric dyes valuable in diverse multiplex applications. The assay for quantitation of the rates of dissociation of dsDNA−dye complexes will undoubtedly prove very valuable in guiding future synthetic work on high affinity fluorescent nucleic acid ligands.

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