High-sensitivity two-color detection of double-stranded DNA with a confocal fluorescence gel scanner using ethidium homodimer and thiazole orange

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Received October 4, 1990; Accepted December 7, 1990

ABSTRACT

Ethidium homodimer (EthD; λ^Fmax 620 nm) at EthD:DNA ratios up to 1 dye:4–5 bp forms stable fluorescent complexes with double-stranded DNA (dsDNA) which can be detected with high sensitivity using a confocal fluorescence gel scanner (Glazer, A.N., Peck, K. & Mathies, R.A. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 3851–3855). However, on incubation with unlabeled DNA partial migration of EthD takes place from its complex with dsDNA to the unlabeled DNA. It is shown here that this migration is dependent on the fractional occupancy of intercalating sites in the original dsDNA-EthD complex and that there is no detectable transfer from dsDNA-EthD complexes formed at 50 bp: 1 dye.

The monointercalator thiazole orange (TO; λ^Fmax 530 nm) forms readily dissociable complexes with dsDNA with a large fluorescence enhancement on binding (Lee, L.G., Chen, C. & Liu, L.A. (1986) Cytometry 7, 508–517). However, a large molar excess of TO does not displace EthD from its complex with dsDNA. When TO and EthD are bound to the same dsDNA molecule, excitation of TO leads to efficient energy transfer from TO to EthD. This observation shows the practicability of 'sensitizing' EthD fluorescence with a second intercalating dye having a very high absorption coefficient and efficient energy transfer characteristics.

Electrophoresis on agarose gels, with TO in the buffer, of preformed linearized M13mp18 DNA-EthD complex together with unlabeled linearized pBR322 permits sensitive fluorescence detection in the same lane of pBR322 DNA-TO complex at 530 nm and of M13mp18 DNA-EthD complex at 620 nm. These observations lay the groundwork for the use of stable DNA-dye intercalation complexes carrying hundreds of chromophores in two-color applications such as the physical mapping of chromosomes.

INTRODUCTION

Interest in the clinical use of compounds that bind strongly to DNA as antitumor agents has led to extensive study of intercalators (1). Appropriately designed dimers of intercalating compounds such as acridines (2–4), various 7H pyridocarbazoles (5) and 3,8-diamino-6-phenylphenanthridinium (6–8) show high DNA binding affinities, greater by several orders of magnitude than those of the corresponding monomers. The length and character (ionic, aliphatic, rigid or flexible) of the chains linking the chromophores has a profound effect on the affinity and mode of binding of these derivatives to the DNA. Derivatives with linkers bearing positive charge(s) are bound much more tightly than those with neutral linkers and linker chain length of ≥ 10Å leads to bisintercalation. In general, the bisintercalating derivatives cover four DNA base pairs. Rigid linkers significantly reduce the rate of disintercalation.

Some dimeric (or oligomeric) intercalators bind essentially irreversibly to DNA. A trimer with seven positive charges, consisting of three substituted acridines linked by a positively charged poly(aminoalkyl) chain, binds to DNA with intercalation of all three chromophores with a K_{app} value in 0.1 M Na^+ at pH 5 of over 10^{14} M^{-1} (9). This value is comparable to the affinities for DNA of such strong repressors as *lac*. Nielsen et al. (10) showed with radiolabeled compounds that di-, tri-, tetra- and hexa-9-acridinylamines are so tightly associated with double-stranded DNA (dsDNA) that they are not removed during electrophoresis in polyacrylamide or agarose gels. Glazer et al. (11) exploited the stability of a tight complex between the highly fluorescent bisintercalator ethidium homodimer (EthD) and dsDNA to detect picogram amounts of DNA on agarose gels with a recently developed laser excited confocal fluorescence gel scanner (11–13).

Glazer et al. (11) pointed out that stable complexes of DNA with fluorescent dyes such as EthD could be used in a wide spectrum of applications as highly fluorescent probes carrying hundreds of dye molecules. In many applications, such as the physical mapping of chromosomes, multi-color labeling of dsDNA would be highly advantageous (14). A probe such as

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biotinylated dsDNA-EthD (or other strongly bound bis-intercalator) would serve as the fluorescent label for site-specific detection of a hybridization probe on a chromosome and a second dye with distinguishable fluorescence emission would be used for the general staining of chromosomes.

In this report, we explore further some of the properties of dsDNA-EthD complexes and describe procedures for two color-detection of DNA on agarose gels. We show that complexes of DNA with EthD (λ_{max}F 620 nm) are stable in presence of excess of the monointercalator thiazole orange (TO, λ_{max}F 530 nm (15)) and exploit this finding to demonstrate high-sensitivity two-color dsDNA detection on agarose gels. The structures of EthD and TO are given in Fig. 1. The information gained in these studies is directly relevant to diverse applications of DNA-dye complexes as general probes.

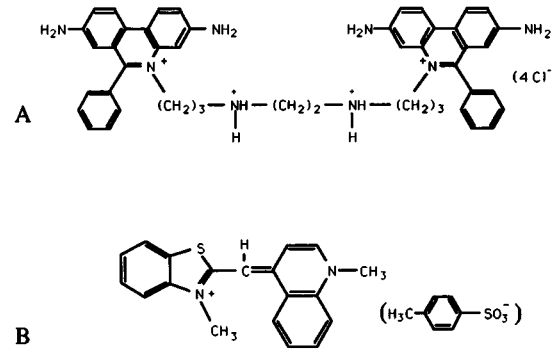


Figure 1. Structures of (A) ethidium homodimer and (B) thiazole orange.

MATERIALS AND METHODS

DNA samples

λDNA HindIII fragments, 1 kilobase pair (kbp) DNA ladder, and the double-stranded intact plasmids M13mp18 and pBR322, were obtained from Bethesda Research Laboratories and ultrapure calf thymus DNA from Sigma.

Reagents

EthD (lot 9A; ϵ = 8,900 M^{-1} cm^{-1} at 492 nm; ref. 16) was obtained from Molecular Probes. TO (ϵ = 30,000 $M^{-1}cm^{-1}$ at 476 nm; ref. 15) was a generous gift of Dr. Diether Recktenwald of Becton-Dickinson. EthD was prepared as a 1 mg/ml stock solution in 0.04 M Tris-acetate/1 mM EDTA, pH 8.2 (TAE buffer), and TO as a concentrated solution at 0.7 mg/ml in methanol. Prior to use, the TO solution was diluted to 0.1 mg/ml in TAE buffer. All dye solutions were stored in the dark at 4°C. Agarose gels were prepared from ultrapure agarose (BRL) and Ficoll (type 400) was obtained from Sigma.

Linearization of plasmid DNA

Both M13mp18 and pBR322 plasmids (3−5 μg) were cut at a single site with *Hin*dIII (6−10 units) using the Boehringer-Mannheim restiction kit. The linear dsDNA was then extracted with phenol and recovered by precipitation with sodium acetate/ethanol. Analysis by agarose gel electrophoresis showed nearly complete single cutting of the plasmids.

DNA-dye complex formation and agarose gel electrophoresis

Mixtures of DNA, EthD, and/or TO at various DNA:dye ratios were prepared in 4 mM Tris-acetate/0.1 mM EDTA, pH 8.2 under reduced illumination at 23°C. All diluted samples of DNA or dye solutions were also prepared in this buffer. Glazer et al. (11) demonstrated that DNA-EthD complex formation was essentially complete within 30 minutes. Fluorescence emission spectroscopy, as well as previous data from Lee *et al.* (15), indicate that complex formation with TO is complete within that time. All dsDNA-dye mixtures were incubated for at least 30 minutes prior to application to gels. Ficoll (15% wt/vol in H_2O) was added to each sample at a ratio of one part of Ficoll to 3 parts sample (by volume) just prior to loading. Vertical (0.9%, wt/vol) agarose gels, 1 mm thick, 7 cm long with 5 mm wide wells, were prepared in TAE buffer. Aliquots of 4 or 8 μl (as indicated) of sample solutions were applied to the wells and electrophoresis performed in TAE buffer in a Bio-Rad Mini-Protean II gel apparatus at 8.6 V/cm in the dark. Where detection of DNA with TO was desired, the dye was added to the electrophoresis buffer at a concentration of 0.05 μg TO/ml. The best results were obtained by first pre-running the gel with TO in the buffer for 1.3 hours and then running the gels for 1.3 hours after sample application without changing the buffer. Gels run with no dye added to the buffer were pre-run for 1−2 hours to decrease background fluorescence and then run for 1 hour after application of samples.

Fluorescence detection in agarose gels

Detection of DNA-dye complexes in agarose gels was accomplished with a laser-excited confocal fluorescence gel scanner which we have recently developed (11−13). Briefly, the gel is placed on a motor-driven scanning stage and the fluorescence is excited with a 1−5 mW 488 nm laser beam from a Spectra-Physics 2020 argon ion laser focused on the gel with a 16× microscope objective. The fluorescence is gathered by the same objective and passed through a confocal spatial filter to a photodetector by a dichroic beam splitter. For specific 'color' detection of the dyes in the gel, the fluorescence emission was passed through different spectral filters prior to reaching the detector. A single Schott RG610 long-pass color filter was used for detection of EthD and a GC495 long pass color filter coupled with a 530 nm bandpass interference filter was used for detection of TO. Imaging of gels containing both dyes was achieved by scanning each gel twice, exchanging the filter set between scans. The gel images were scanned with 134 μm square pixels and a typical scan time was 30 min.

Fluorescence measurements on solutions

Fluorescence measurements were performed with a Perkin-Elmer model MPF44B spectrofluorimeter connected to a Perkin-Elmer Hitachi 057 plotter.

RESULTS

Interaction of thiazole orange with dsDNA

By fluorimetric titration Lee *et al.* (15) showed that TO bound to dsDNA with a stoichiometry characteristic of monointercalators: one dye molecule per two base pairs. This finding was reproduced under the conditions used in this study. As shown in Fig. 2B, the titration of calf thymus DNA in 4 mM Tris-acetate/1 mM EDTA, pH 8.2 with TO showed a maximum enhancement of TO fluorescence emission at 1 dye: 2 bp. The fluorimetric titration is consistent with a K_{app} of the dye for the DNA > 10^6 M^{-1}. To examine the stability of the DNA-TO

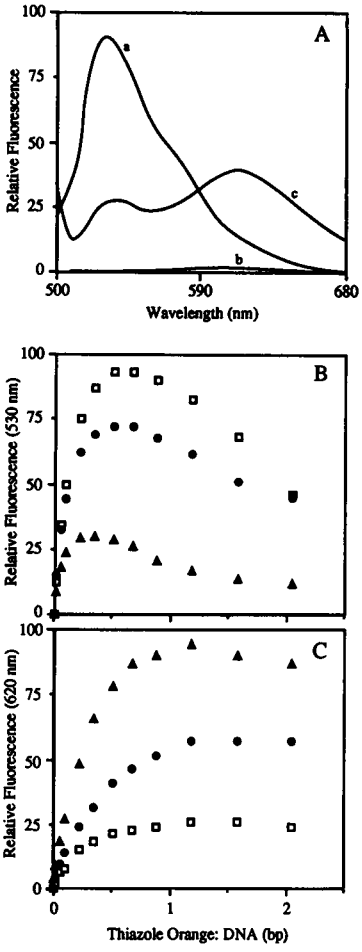


Figure 2. Fluorimetric titration of calf thymus DNA with TO in presence and absence of EthD. DNA (2.2 μg/ml, equivalent to 3.25 μM base pairs) in 4 mM Tris-acetate−0.1 mM EDTA, pH 8.2, was titrated with small volumes of 33 μM TO (from a stock solution in the same buffer). Excitation was at 488 nm. Panel A shows the fluorescence emission spectra of (a) DNA-TO complex at a DNA to dye ratio of 2 bp:1 TO, (b) DNA-EthD complex at a DNA to dye ratio of 4 bp:1 EthD and (c) DNA-EthD-TO complex at DNA to dye ratios of 4 bp:1 EthD and 2 bp:1 TO. Panels B and C plot the relative fluorescence emission at 530 and 620 nm as a function of the ratio of TO: bp DNA. Titrations were performed on calf thymus DNA solution in absence of EthD (□), in presence of EthD at a ratio of DNA bp:EthD of 20:1 (●) and of 4:1 (▲).

complex, mixtures containing TO (1.25 ng/μl) and λDNA/HindIII (5 ng/μl) were subjected to electrophoresis on agarose gels. No TO fluorescence was seen on imaging of the gels with laser excitation and confocal detection. Consequently, as demonstrated previously for the DNA-ethidium bromide complex (11), the DNA-TO complex dissociates on electrophoresis.

However, under appropriate conditions, TO is an effective stain for the detection of DNA on gels. When a small amount of TO (0.05 μg/ml; 1.65×10^{-7}M) was added to the running buffer, λDNA/HindIII fragments containing as little as 20 pg DNA/band were readily detected. High sensitivity detection coupled with best band resolution required optimization of running conditions. Samples were premixed with TO at a DNA:dye weight ratio of 1:2, which approximates the stoichiometry of 1 dye: 2 bp and electrophoresis was carried out with 0.05 μg TO/ml in the buffer (see 'Materials and Methods'). Fig. 3 shows the scan of a gel loaded with amounts of DNA per lane from 2−0.25 ng. It is evident that staining with TO by this procedure allows detection

LANE 1
7.9×10^{-7}M bp λ DNA/HindIII
3.5×10^{-7}M thiazole orange
Incubate for 30 min
Load 4 µl (2 ng DNA)

LANE 2
3.95×10^{-7}M bp λ DNA/HindIII
1.75×10^{-7}M thiazole orange
Incubate 30 min
Load 4 µl (1 ng DNA)

LANE 3
1.98×10^{-7}M bp λ DNA/Hind III
8.75×10^{-8}M thiazole orange
Incubate 30 min
Load 4 µl (0.5 ng DNA)

LANE 4
9.9×10^{-8}M bp λDNA/HindIII
4.38×10^{-8}M thiazole orange
Incubate 30 min
Load 4 µl (0.25 ng DNA)

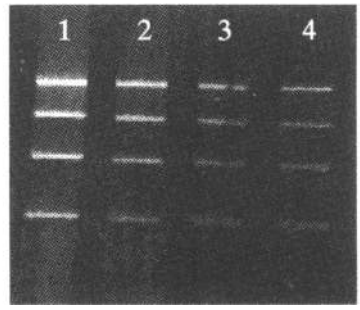


Figure 3. Electrophoresis of dsDNA-TO mixtures at varying DNA and dye concentrations but a constant initial DNA bp: TO molar ratio of 2:1. Electrophoresis was performed in TAE buffer containing 1.65×10^{-7} M TO.

of bands containing about 50-fold less DNA than the amount required for conventional detection with ethidium bromide.

Energy transfer from thiazole orange to ethidium homodimer

As illustrated by the fluorescence spectra in Fig. 2A, when both TO and EthD are bound to the same dsDNA molecule energy absorbed by TO is transferred to EthD. Fig. 2B, C shows the relative fluorescence emission at 530 nm and 620 nm as a function of TO:DNA ratio for DNA alone (□), for the DNA-EthD complex at 1 EthD:20 bp(●), and for the DNA-EthD complex at 1 EthD:4 bp(▲). In presence of EthD, the fluorescence emission at 530 nm (emission maximum for TO) is quenched, whereas that at 620 nm (emission maximum for EthD) shows a large increase. These data permit two conclusions. Excess TO does not displace EthD from dsDNA and energy is transferred efficiently from TO to EthD.

Transfer of bound ethidium homodimer between DNA molecules

In our previous study of the DNA-EthD complex, we demonstrated that over half of the complexed EthD could be transferred to a large excess of unlabeled DNA. The following experiments explore the influence on this phenomenon of variation in DNA and dye concentration at constant DNA:dye ratio and of variation in DNA:dye ratio.

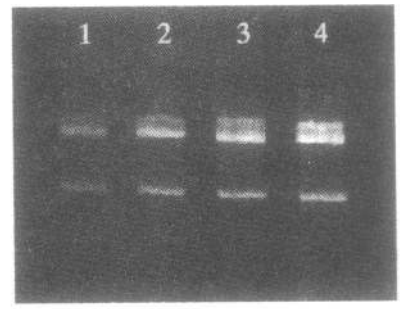
Fig. 4 illustrates the transfer of EthD from a M13mp18 DNA-EthD complex at a DNA bp:dye ratio of 11 to an equal concentration of pBR322 DNA. Over a 6-fold range of DNA concentrations, a constant fraction of dye (32%) was transferred to pBR322. The effect on the transfer of varying the ratio of DNA:dye is shown in Fig. 5. A complex of M13mp18 DNA-EthD at a DNA bp:EthD ratio of 50:1 transfers less than 10% of the bound dye to an equal concentration of pBR322 (Fig. 5, compare lanes 3 and 4). These experiments show that the extent of transfer of EthD between dsDNA molecules is strongly dependent on the fractional saturation of the binding sites on the DNA by the dye.

LANE 1.
3.94 x 10^{-7}M bp M13
3.54 x 10^{-8}M EthD
Incubate 30 min
3.94 x 10^{-7}M bp pBR322
Incubate 30 min
Load 4 µl (1 ng M13, 1 ng pBR322)

LANE 2.
7.88 x 10^{-7}M M13
7.88 x 10^{-8}M EthD
Incubate 30 min
7.88 x 10^{-7}M bp pBR322
Incubate 30 min
Load 4 µl (2 ng M13, 2 ng pBR322)

LANE 3.
1.58 x 10^{-6}M bp M13
1.42 x 10^{-7}M EthD
Incubate 30 min
1.58 x 10^{-6}M bp pBR322
Incubate 30 min
Load 4 µl (4 ng M13, 4 ng pBR322)

LANE 4.
2.36 x 10^{-6}M M13
2.12 x 10^{-7}M EthD
Incubate 30 min
2.36 x 10^{-6}M bp pBR322
Incubate 30 min
Load 4 µl (6 ng M13, 6 ng pBR322)



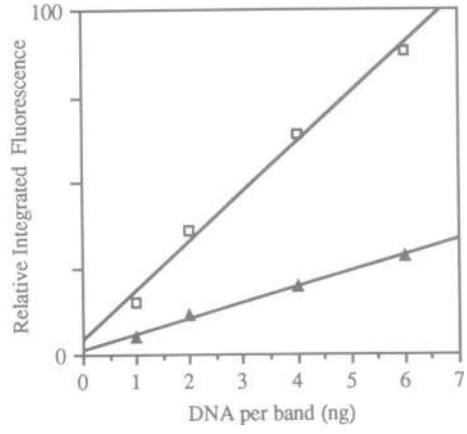


Figure 4. Extent of transfer of EthD from a preformed linearized M13mp18 dsDNA-EthD complex at a ratio of 11 DNA bp per dye to an equal weight of uncomplexed linearized pBR322 dsDNA at different absolute DNA and dye concentrations. The graph shows for each mixture the relative integrated fluorescence in each plasmid band. Electrophoresis was performed in TAE buffer containing no dye. The indicated order of components designates the sequence of mixing.

LANE 1.
2.02 x 10^{-7}M bp M13
3.94 x 10^{-8}M EthD
Incubate 30 min
Load 4 µl (0.5 ng M13)
M13 bp:EthD = 5:1

LANE 2.
2.02 x 10^{-7}M bp M13
3.94 x 10^{-8}M EthD
Incubate 30 min
1.82 x 10^{-6}M bp pBR322
Load 4µl (0.5 ng M13, 4.6 ng pBR322)
M13 bp:EthD:pBR322 bp = 5:1:46

LANE 3.
3 parts mixture A diluted with 5 parts buffer to give
1.01 x 10^{-6}M bp M13 DNA
1.97 x 10^{-8}M EthD
Load 8 µl (5.1 ng M13)
M13 bp:EthD = 50:1

LANE 4.
3 parts mixture A diluted with 5 parts pBR322 solution to give
1.01 x 10^{-6}M bp M13 DNA
1.97 x 10^{-8}M EthD
9.14 x 10^{-7}M bp pBR322
Incubate 30 min
Load 8 µl (5.1 ng M13, 4.6 ng pBR322)
Final M13 bp:EthD:pBR322 bp = 50:1:46

LANE 5.
1.82 x 10^{-6}M bp pBR322
1.84 x 10^{-8}M EthD
Incubate 30 min
Load 4 µl (4.6 ng pBR322)
pBR322 bp:EthD = 100:1

MIXTURE A
2.69 x 10^{-7}M bp M13
5.26 x 10^{-8}M EthD
Incubate 30 min **Initial M13 bp:EtD = 5:1**
2.42 x 10^{-6}M bp M13
Incubate 30 min
Final M13 bp:EthD = 50:1

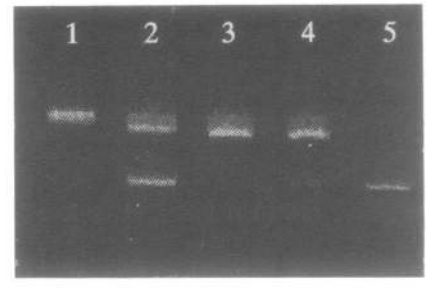


Figure 5. Dependence of extent of transfer of EthD from a preformed dsDNA-EthD complex to uncomplexed dsDNA on DNA bp:EthD ratio in the preformed complex. Electrophoresis was performed in TAE buffer containing no dye. The indicated order of components designates the sequence of mixing.

MIXTURE I.
5.29 x 10^{-7}M bp M13
1.04 x 10^{-7}M EthD
Incubate 30 min
M13 bp:EthD = 5:1

MIXTURE II.
5.29 x 10^{-7}M bp pBR322
2.51 x 10^{-7}M TO
Incubate 30 min
pBR322 bp:TO = 2:1

MIXTURE III.
2.02 x 10^{-7}M bp M13
3.94 x 10^{-8}M EthD
Incubate 30 min
M13 bp:EthD = 5:1
2.02 x 10^{-7}M bp pBR322
Incubate 30 min
M13 bp:EthD:pBR322 bp = 5:1:5

MIXTURE IV.
3 parts of mixtures I
and II were each diluted with
1 part of buffer and equal
volumes then mixed to give
1.98 x 10^{-7}M bp M13
3.90 x 10^{-8}M EthD
1.98 x 10^{-7}M bp pBR322
9.41 x 10^{-8}M TO
M13 bp:EthD:pBR322:TO = 5:1:5:2.1
Additional TO was added to
a final concentration of 2.58 x 10^{-7}M TO
Incubate 30 min
M13 bp:EthD:pBR322:TO = 5:1:5:6.5

LANE 1.
3 parts of mixture I
diluted with 1 part of
buffer to give
3.97 x 10^{-7}M bp M13
7.8 x 10^{-8}M EthD
Load 4 µl (1 ng M13)

LANE 2.
3 parts of mixture II
diluted with 1 part of
buffer to give
3.97 x 10^{-7}M bp pBR322
1.88 x 10^{-7}M TO
Load 4 µl (1 ng pBR322)

LANE 3.
Mixture III
Load 8 µl (1 ng M13)
(1 ng pBR322)

LANE 4.
MIXTURE IV
Load 8 µl (1 ng M13)
(1 ng pBR322)

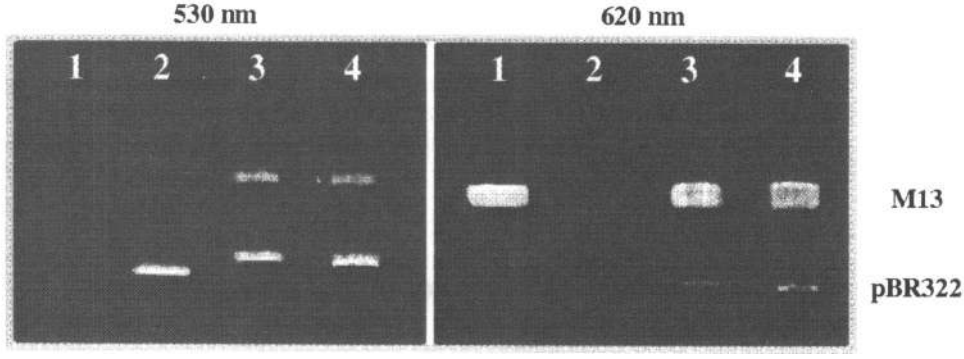


Figure 6. Two-color detection of DNA. Simultaneous detection of preformed linearized M13mp18 dsDNA-EthD complex and linearized pBR322 on an agarose gel after electrophoresis in buffer containing 1.65×10^{-7} M TO. Fluorescence of intercalated TO is detected at 530 nm and that of intercalated EthD at 620 nm. Lane 1, preformed M13mp18 DNA-EthD complex; lane 2, preformed pBR322 DNA-TO complex; lane 3, preformed M13mp18 DNA-EthD complex incubated with unlabeled pBR322 DNA ; lane 4, preformed M13mp18 DNA-EthD complex incubated with preformed pBR322 DNA-TO complex. The indicated order of components designates the sequence of mixing.

Two-color detection of DNA

The scans shown in Fig. 6 document convincingly that the DNA-EthD complex can be used effectively as a probe in situations requiring two-color detection of DNA. The detection sensitivity threshholds for the scans were set such that the weak signals from DNA-EthD at 530 nm and from DNA-TO at ≥620 nm were not detectable (see lanes 1 and 2). Mixtures of M13mp18 DNA-EthD (5 bp: 1 dye) complex were incubated with an equal weight of pBR322 both in the presence and absence of TO. Analysis of these mixtures by electrophoresis in the presence of TO (lanes 3 and 4) shows some M13mp18 DNA fluorescence at 530 nm due to intercalation of TO. However, the 530 nm signal is very weak relative to the ≥620 nm emission from the intercalated EthD. The pBR322 bands show strong fluorescence at 530 nm due to intercalation of TO. There is only a trace of fluorescence at ≥620 nm, indicating only a small amount of transfer of EthD from the M13mp18 DNA-EthD complex to the pBR322.

DISCUSSION

The experiments described above provide interesting insights into the dynamic behavior of complexes of dsDNA with the bisintercalator EthD. NMR studies of tightly bound

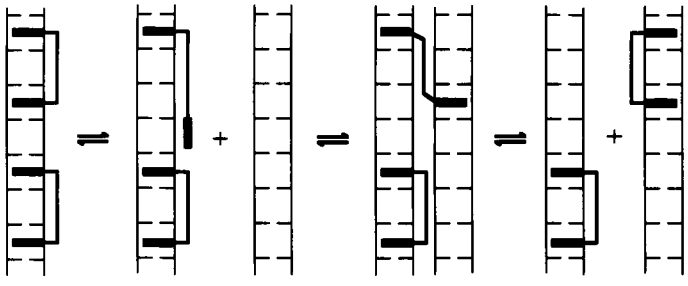


Figure 7. Proposed mechanism of transfer of EthD from a dsDNA-EthD complex with adjacent binding site occupancy to uncomplexed dsDNA.

bisintercalators show that the fraction of partially intercalated molecules in which one of the ring components is disintercalated is very small (16). Our studies on dsDNA-EthD complexes (ref. 11 and this study) show that no loss of bound dye takes place during electrophoresis. Consequently, the off-rate of EthD is much slower than the timescale (hours) of electrophoresis. Paradoxically, transfer of bound EthD from 'labeled' to 'unlabeled' DNA occurs within minutes of mixing. These observations can be reconciled if it is postulated that the transfer

of dye from one dsDNA molecule to another proceeds through intermediate complexes involving half-disintercalated dye molecules as illustrated in Fig. 7. The rate of such transfer reactions would be dependent on the fraction of bound bisintercalator in the half-disintercalated state. The known anticooperativity of ethidium intercalation into dsDNA (17) suggests that when two EthD molecules occupy adjacent positions, the disintercalation of the dye ring system flanked by two occupied intercalation sites (Fig. 7) would be most favored thermodynamically. Strong support for the mechanism diagrammed in Fig. 7 is provided by the observation that at low site occupancy, i.e., at low EthD:DNA bp ratios (1:50) there is little transfer of EthD between dsDNA molecules.

The results we have obtained are readily explained on the assumption that at the low salt concentration (4–40 mM TAE) used in these experiments the proportion of bound bisintercalator in the half-disintercalated state increases with increase in the fractional saturation of binding sites in the DNA. In discussing the anticooperativity of the binding of ethidium to DNA, Friedman and Manning (18) wrote: "In absence of site bound ligands, the system is subject to stresses arising from the screened Coulombic interactions among polyion groups and from the high local concentration of the territorially bound counterions relative to the bulk solution. The smaller the average charge spacing on the polyion chain, the greater both stresses will be. Ligand binding increases the average spacing, both by neutralization, if the ligand is charged, and by chain lengthening, if the ligand intercalates. This increase in the average spacing reduces the electrostatic stress. It also reduces the concentration stress by causing the release of territorially bound counterions into the bulk solution. Thus, polyelectrolyte effects help drive the site-binding reaction. However, each newly bound ligand reduces both these stresses by less than the one before it, because the particular Coulombic interactions and the concentration gradients it reduces are smaller than before. This decrease in the reduction of stress as binding proceeds results in anticooperative binding." In addition to the polyelectrolyte effects, changes in vibrational entropy may well contribute significantly to the anticooperativity. A general empirical rule regarding DNA intercalative binding is that every second intercalation site in the DNA double helix remains unoccupied (19). From molecular mechanical simulations on double intercalation of 9-aminoacridine into a base-paired heptanucleotide, Rao and Kollman (20) concluded that vibrational entropy consideration could play an important role in excluding structures where intercalators occupy locations between adjacent base pairs. It is likely that such vibrational entropy considerations come into play, although to a quantitatively lesser degree, in situations where the occupancy of binding sites in a DNA double helix is high. From both empirical observations and thermodynamic considerations, it is evident that the problem of transfer of tight binding bisintercalators among DNA molecules can be minimized by maintaining a high ratio of DNA base pairs to bisintercalator molecules in the initial complex. It is also likely that bisintercalators can be found that will be bound so tightly that transfer will not be observed.

Displacement of EthD by a very large excess of TO does not take place and the fluorescence of TO bound to a dsDNA-EthD complex is largely quenched by energy transfer. Energy transfer from intercalated TO to intercalated EthD is predictable from the absorption and emission spectra of the bound dyes (Fig. 2A). The emission of TO has a large overlap with the absorption band of EthD. The finding that efficient energy transfer does take place is important for two reasons. If TO and EthD are used together, for example in a chromosome staining experiment such as that mentioned in the 'Introduction', it is reassuring to know that binding of some thiazole orange to a dsDNA probe preloaded with EthD would not displace the EthD, but would in fact facilitate probe fluorescence detection at the EthD emission maximum. Second, since such energy transfer is seen with other pairs of DNA-bound dyes (21), one could envisage 'sensitizing' EthD (or other intercalated fluorophores) with another intercalating dye having a very high absorption coefficient and other spectral characteristics appropriate for energy transfer.

Since EthD is not displaced from dsDNA by an excess of TO, these dyes can be used together in applications demanding two-color labeling of DNA, such as the physical mapping of chromosomes. For example, the smallest biotinylated probes used for mapping on human metaphase chromosomes by fluorescence *in situ* hybridization are about 1 kilobase in length or, if they consist of shorter fragments of DNA, add up to about 1 kilobase (e.g., 22). It may be possible to use much smaller biotinylated probes and to detect the hybridization signal with avidin complexed to biotinylated linearized DNA of a vector such as M13mp18 of 7250 bp carrying some 1800 molecules of EthD or other suitable stably bound fluorescent dye. An application of this type requires that the fluorescent dye remain bound to the probe dsDNA through the staining and washing steps and that staining with a second spectroscopically distinguishable dye can be performed to allow clear visualization and identification of chromosomes. The studies presented here show that these demanding requirements can indeed be met.

ACKNOWLEDGMENTS

H.S.R. is a recipient of a predoctoral fellowship from the Department of Health and Human Services Training Grant 5 T 32 GM 07232. This research was supported in part by a grant from the Lucille P. Markey Charitable Trust, by the National Science Foundation Grants DMB 88-16727 and BBS 87-20382, and by the Director, Office of Energy Research, Office of Health and Environmental Research, Physical and Technological Research Division of the U.S. Department of Energy under contract DE-FG-03-88ER60706.

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