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A stable double-stranded DNA–ethidium homodimer complex: Application to picogram fluorescence detection of DNA in agarose gels

(electrophoresis/fluorescence imaging/fluorescent probes/laser gel scanning)

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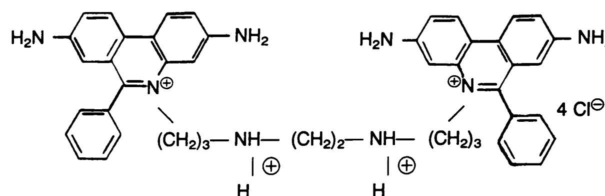
ABSTRACT **The complex between double-stranded DNA and ethidium homodimer (5,5′-diazadecamethylene)bis(3,8-diamino-6-phenylphenanthridinium) cation, formed at a ratio of 1 homodimer per 4 or 5 base pairs, is stable in agarose gels under the usual conditions for electrophoresis. This unusual stability allows formation of the complex before electrophoresis and then separation and detection in the absence of background stain. Competition experiments between the preformed DNA–ethidium homodimer complex and a 50-fold molar excess of unlabeled DNA show that approximately one-third of the dye is retained within the original complex independent of the duration of the competition. However, dye-extraction experiments show that these are not covalent complexes. After electrophoretic separation, detection of bands containing 25 pg of DNA was readily achieved in 1-mm thick agarose gels with laser excitation at 488 nm and a scanning confocal fluorescence imaging system. The band intensity was linear with the amount of DNA applied from 0.2 to 1.0 ng per lane and with the number of kilobase pairs (kbp) per band within a lane. Analysis of an aliquot of a polymerase-chain-reaction mixture permitted ready detection of 80 pg of a 1.6-kbp amplified fragment. The use of the ethidium homodimer complex together with laser excitation for DNA detection on gels is at least two orders of magnitude more sensitive than conventional fluorescence-based procedures. The homodimer–DNA complex exemplifies a class of fluorescent probes where the intercalation of dye chromophores in DNA forms a stable, highly fluorescent ensemble.**

The experiments described here were initiated by the intriguing observation that after agarose gel electrophoresis of mixtures of DNA restriction fragments and ethidium bromide some of the dye remained with the DNA. This observation held for mixtures at initial DNA concentrations ≥0.075 μg/μl and ethidium bromide concentrations ≥0.04 μg/μl. At a 10-fold lower concentration of these components the residual fluorescence was no longer visible. During electrophoresis, the ethidium cation and the DNA polyanion should move to opposite electrodes. The retention of dye on the DNA implies that the rate of dissociation of ethidium bromide from certain binding sites on the DNA must be slow compared with the electrophoresis time. This suggests that a fluorescent cationic intercalator with a much higher affinity for double-stranded DNA might remain bound to the DNA throughout the course of electrophoresis and allow very sensitive detection of DNA on gels because free dye would be removed quantitatively in the electrophoresis.

Ethidium homodimer (EthD) is an intercalator that meets these requirements (1).



Ethidium homodimer
(5,5′-diazadecamethylene)bis(3,8-diamino-6-phenylphenanthridinium) dichloride dihydrochloride

Gaugain *et al.* (2) showed that in 0.2 M Na^+ the homodimer bound to strong binding sites in double-stranded DNA with an affinity constant almost 1000 times higher than ethidium bromide (EthD, $K = 2 \times 10^8\ M^{-1}$; ethidium bromide, $K = 1.5 \times 10^5\ M^{-1}$) with a stoichiometry of one homodimer per 4 base pairs (bp). As previously determined for ethidium bromide, the fluorescence properties of DNA-bound EthD are not sensitive to the base composition of the DNA (3). From viscometric measurements, the lengthening of the DNA helix caused by the binding of EthD was of the same order of magnitude as that caused by the intercalation of ethidium bromide. It was concluded that EthD binds to DNA by intercalating only one of its two phenanthridinium chromophores (2). The ratio of the fluorescence quantum yield of the EthD–DNA complex to that of unbound EthD was determined to be 40 (2). The high affinity of EthD for DNA, the large enhancement of fluorescence emission in the complex, and the insensitivity of the binding to the base composition of the DNA were exploited to determine DNA concentrations in solution from 1 to 10 ng/ml (3).

We demonstrate here that EthD associated with double-stranded DNA is not removed on electrophoresis of the DNA–dye complex. The DNA–EthD complexes can be readily detected after electrophoresis in agarose gels by using laser excitation at 488 nm and a scanning confocal fluorescence imaging system with a sensitivity per band in the picogram range. With additional refinements, the detection limits should be similar to those attainable with radioactive labeling. We show further that in competition with a large excess of unlabeled DNA, a significant fraction of bound EthD is retained within the original DNA–EthD complex. These observations suggest a broad range of future applications for DNA labeled with this and similar dye molecules.

MATERIALS AND METHODS

DNA Samples. Standard mixtures, λ DNA *Hin*dIII fragments and 1-kilobase-pair (kbp) DNA ladder, were obtained

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Abbreviation: EthD, ethidium homodimer.

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from Bethesda Research Laboratories. A preparation of pUC18 purified on a CsCl gradient and a polymerase-chain-reaction mixture were provided by Jeffrey C. Gingrich (Human Genome Center, Lawrence Berkeley Laboratory, Berkeley, CA). The polymerase-chain-reaction mixture was obtained by using a primer, *Alu3* (CCTGTAATCCCAG-CACTTTG), with a yeast artificial chromosome containing a ≈350-kbp insert of human DNA derived from chromosome 21 and amplified for 30 cycles.

Reagents. EthD (ε = 8900 $M^{-1}{\cdot}cm^{-1}$ at 492 nm; lot 9A), obtained from Molecular Probes, was stored in the dark as a stock solution at 1 mg/ml in 0.04 M Tris acetate buffer, pH 8.4, at 4°C. Under these storage conditions, <4% decomposition of the dye was seen over a month, as determined by absorption spectroscopy. Ultrapure agarose was obtained from Bethesda Research Laboratories, and Ficoll (type 400) was from Sigma. The Geneclean kit was obtained from Bio 101 (La Jolla, CA). All other reagents were of the highest commercially available grade.

Complex Formation and Agarose Gel Electrophoresis. Mixtures of DNA (1.5–0.05 ng/μl) and EthD (0.3–0.015 ng/μl), at varying DNA/dye ratios, were prepared in 4 mM Tris acetate/0.1 mM EDTA, pH 8.2, under subdued illumination and kept in the dark. Fluorescence emission spectroscopy and gel scanning indicated that complex formation was complete within 30 min at room temperature. The mixtures were routinely incubated for 60 min before application to the agarose gel, unless otherwise indicated. Immediately before electrophoresis, one part of 15% (wt/vol) Ficoll in water was added to 3 parts of sample, by volume. Aliquots (4 μl) of sample were then applied to 5-mm-wide wells in 1-mm-thick, 7-cm-long, vertical 0.9% (wt/vol) agarose gels prepared in 0.04 M Tris acetate/1 mM EDTA, pH 8.4. Electrophoresis was performed in the same buffer in a Bio-Rad Mini-Protean II electrophoresis cell at 5 V/cm in the dark. Tracking dyes were not used; the commonly used dyes, xylene cyanole FF and bromophenol blue, are fluorescent and interfere in the detection. Gels were subjected to preelectrophoresis for 2–3 hr before sample application to decrease background fluorescence.

Competition for EthD Between Preformed λ DNA *Hin*dIII Complexes and Excess pUC18 DNA. All solutions were in 4 mM Tris acetate/0.1 mM EDTA, pH 8.2, at 23°C. (Mixture A) A mixture of λ DNA *Hin*dIII fragments and EthD (100 ng of DNA plus 12.5 ng of EthD in 150 μl of buffer) was incubated for 60 min. pUC18 DNA (5 μg in 5 μl of buffer) was then added, and the mixture was allowed to stand for a further 60 min. Two control mixtures, B and C, were prepared as follows. Mixture B: a mixture of λ DNA *Hin*dIII fragments and EthD (100 ng of DNA plus 12.5 ng of EthD in 150 μl of buffer) was incubated for 60 min. Mixture C: a mixture of pUC18 DNA and EthD (5 μg of DNA plus 12.5 ng of EthD in 150 μl of buffer) was incubated for 60 min. At the end of the times indicated above, 50 μl of Ficoll (15% wt/vol in H_2O) was added to each of the three mixtures, and 4 μl of each sample was applied to an agarose gel. Additional experiments done in the manner described above included (*i*) a control mixture in which the λ DNA *Hin*dIII fragments (100 ng) and the pUC18 DNA (5 μg) were mixed before the addition of EthD, and (*ii*) mixtures of preformed λ DNA *Hin*dIII–EthD complexes with pUC18 DNA were prepared, as described for mixture A above, but incubated for periods up to 6 hr.

Removal of Bound EthD by the Geneclean Procedure. The procedure described below is based on the brochure provided by the manufacturer with the Geneclean kit. The recovery of λ DNA *Hin*dIII fragments was ≈50%. λ DNA *Hin*dIII fragments (0.4 ml; 4.76 ng of DNA/μl) were mixed with EthD (0.4 ml; 1 ng/μl), both in 4 mM Tris acetate/0.1 mM EDTA, pH 8.2, and kept in the dark for 60 min. To 0.4 ml of the above mixture were added 1.0 ml of 6 M NaI and 5 μl of Glassmilk suspension in sterile water. The DNA was allowed to bind for 15 min with periodic agitation. The Glassmilk was then pelleted by a brief spin in a microcentrifuge, and the NaI-containing supernatant was discarded. The pellet was resuspended by mixing in 900 μl of ≈50% (vol/vol) ethanol in Tris/EDTA buffer and then centrifuged again; this washing procedure was performed three times. The pellet was then resuspended in 40 μl of 4 mM Tris acetate/0.1 mM EDTA, pH 8.2. To one 8-μl aliquot of the eluate was added 142 μl of 4 mM Tris acetate/0.1 mM EDTA, pH 8.2, and to a second 8-μl aliquot was added 142 μl of the same buffer containing 10 ng of EthD. These mixtures were kept for an hour in the dark. Fifty μl of Ficoll (15% wt/vol in H_2O) were added to each sample, and a 4-μl aliquot of each sample was applied to an agarose gel. A suitably diluted aliquot of the portion of the original λ DNA *Hin*dIII–EthD mixture, not exposed to the Geneclean procedure, was also applied to the same gel as an additional control.

Fluorescence Detection and Quantitation of DNA–EthD Complexes on Agarose Gels. Fluorescence detection was performed with the confocal laser scanning fluorescence imaging system shown in Fig. 1. The fluorescence was excited with 46 mW of 488 nm light from a Spectra-Physics 2020 argon ion laser. A long pass dichroic beam splitter (Zeiss FT580) was used to reflect the laser beam down through a 100×, numerical aperture 1.3, oil immersion objective (Rolyn Optics) and onto the sample. The fluorescence emission was collected by the objective and passed through the beam splitter to the photodetector. The fluorescence emission passed through a spatial filter (200-μm pinhole, Melles Griot) to effect confocal detection and a long-pass color filter (Schott RG610) before reaching a photomultiplier tube (RCA 31034A). A computer-controlled dc servo motor-driven XY translation stage (Design Components) with a 6 in × 6 in (1 in = 2.54 cm) travel and 2.5-μm resolution was used to translate the gel past the laser beam at 3 cm/sec. A microcomputer (IBM PS/2 70-A21) with a Metra-Byte analog-to-digital board and a 8514/A graphic adapter was used to control the XY translation stage and to acquire and display images. The fluorescence images are pseudo-color encoded to represent different intensity levels and contrast stretched with a histogram equalization method (4) to enhance the images. To quantitate the image data, the image columns that enclose the DNA bands were extracted and integrated.

RESULTS

Experiments were performed to establish the range of DNA and dye concentrations over which both qualitative and quantitative data on DNA components in agarose gels could be obtained. Formation of a red precipitate was noted at high

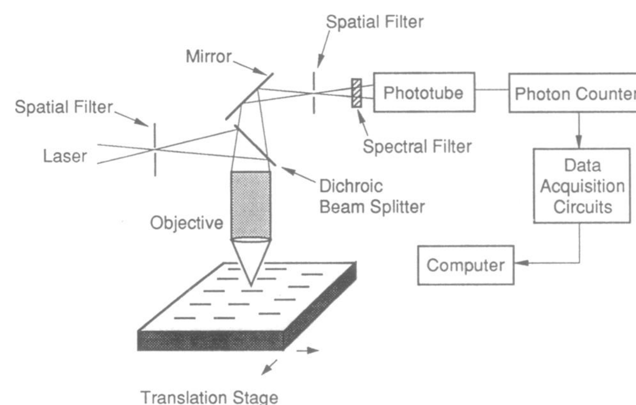

FIG. 1. Apparatus for laser-excited confocal fluorescence imaging of electrophoresis gels.

concentrations of DNA (150 ng/μl) and EthD (100 ng/μl). Down to DNA concentrations of 4 ng/μl and dye concentrations of 1 ng/μl (or higher), some aggregate was still detectable after electrophoresis as intensely fluorescent material in the portion of the gel immediately proximal to the well. Such material was not seen at lower DNA concentrations. *Consequently, further experiments were always done at DNA concentrations of 1.5 ng/μl or lower.* The fluorescence intensity of the DNA–EthD bands was at a maximum when the molar ratio of homodimer to DNA base pairs in the sample reached 1:4 or 1:5. Addition of more dye did not influence the band intensity significantly. Likewise, addition of DNA in excess of the 1:4 molar ratio of dye/base pairs had little effect on the fluorescence intensity. From these observations, a standard ratio of dye to DNA of 1:4 by weight (approximately 1 dye molecule per 5 bp) was adopted.

As illustrated in Fig. 2*A*, the electrophoretic patterns given by nanogram amounts of DNA–EthD mixtures with dye/DNA weight ratios of 1:4 were equivalent to those given by microgram amounts of DNA visualized in parallel experiments by conventional staining with ethidium bromide. For the 1-kbp-ladder DNA complexed with EthD, the amount of DNA per band is ≈60 pg. The binding of EthD leads to a small decrease in the mobility of the DNA fragments. For example, taking the mobility of a 6.56-kb fragment at an EthD/base pairs molar ratio of 1:40 as 1.0, the mobilities of this fragment at higher EthD/base pairs ratios of 1:20 and 1:4 are 0.99 and 0.90, respectively.

The dependence of the fluorescence intensity of λ DNA *Hin*dIII restriction fragment bands on the amount of DNA applied to the gel and on the size of the fragments is shown in Fig. 3. These data show that by comparison with appro-

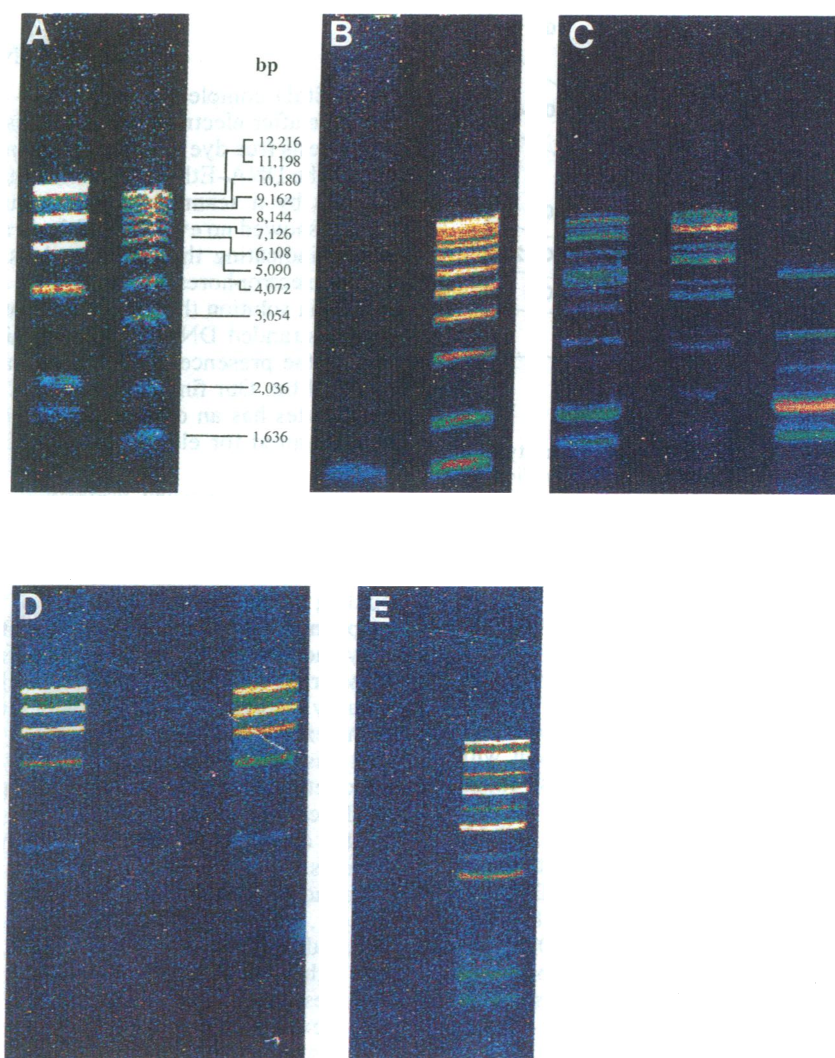

FIG. 2. Fluorescence detection of DNA–EthD complexes on agarose gels by laser excitation at 488 nm and a confocal fluorescence imaging system. Low-intensity levels are represented by deep-blue cold-tone colors, and high-intensity levels are represented by bright-red warm-tone colors. (*A*) Left lane, λ DNA *Hin*dIII fragments (load: 2 ng of DNA plus 0.5 ng of EthD); right lane, 1-kbp-ladder DNA (load: 1 ng of DNA plus 0.25 ng of EthD). (*B*) Left lane, polymerase-chain-reaction amplification mixture (load: total DNA not determined plus 0.25 ng of EthD); right lane, 1-kbp-ladder DNA (load: 2 ng of DNA plus 0.5 ng of EthD). (*C*) Competition for EthD between preformed λ DNA *Hin*dIII–EthD complexes and a 50-fold molar excess of pUC18 DNA. Left lane, preformed λ DNA *Hin*dIII–EthD complexes after incubation for 60 min with pUC18 DNA; middle lane, λ DNA *Hin*dIII–EthD complexes; right lane, pUC18 DNA after 60-min incubation with EthD. Each sample contained the same amount of EthD. (*D*) Effect of the Geneclean procedure on λ DNA *Hin*dIII–EthD complexes formed at an initial weight ratio of DNA/EthD of 4.75:1. Left lane, λ DNA *Hin*dIII fragments eluted from Glassmilk and incubated with fresh EthD at a weight ratio of DNA/EthD of 10:1 for 60 min (load: 2 ng of DNA plus 0.2 ng of EthD); middle lane, λ DNA *Hin*dIII fragments eluted from Glassmilk to which no fresh EthD was added (load: 2 ng of DNA); right lane, control λ DNA *Hin*dIII–EthD mixture at a weight ratio of DNA/dye of 4.75:1 that had not been subjected to the Geneclean procedure (load: 2.38 ng DNA plus 0.5 ng of EthD). (*E*) λ DNA *Hin*dIII fragments. Left lane, load: 4.8 ng of DNA plus 0.25 ng of ethidium bromide; right lane, load: 4.8 ng of DNA plus 0.25 ng of EthD.

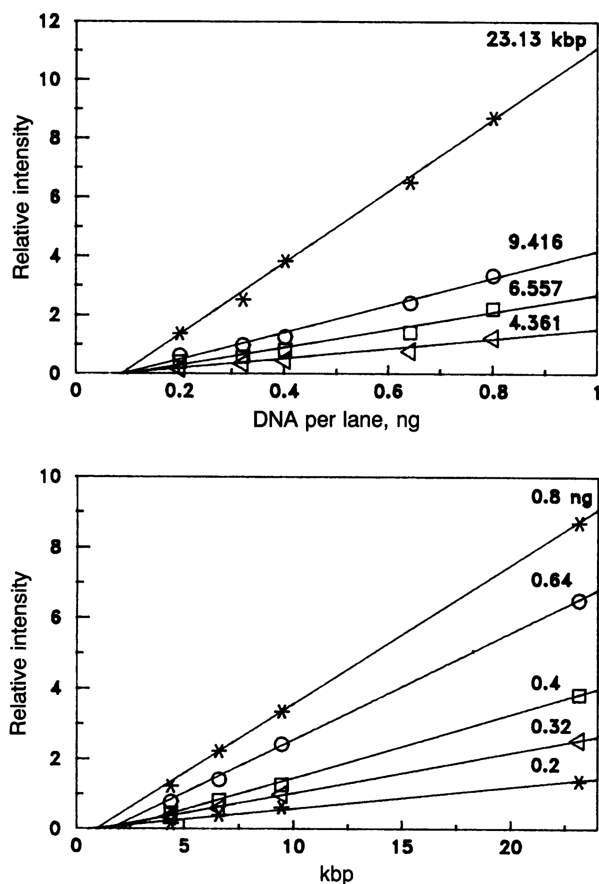

FIG. 3. (*Upper*) Dependence of the fluorescence intensity of λ DNA *Hin*dIII–EthD complexes on the amount of sample applied per lane. (*Lower*) Dependence of the fluorescence intensity of these complexes on the number of kbp. All samples contained a constant-weight ratio of DNA to EthD of 4:1. Lines were determined by a least-squares fit. Errors in the intensities are on the order of ±0.5.

priate standards, the sizes and amounts of DNA fragments in mixtures of unknown composition can be determined with a total amount of material ranging from 100 pg to 1 ng, depending on the complexity of the mixture and the size range of the fragments. For example, Fig. 2*B* shows the detection of about 80 pg (quantitated by comparison with standards) of a 1.6-kbp fragment in a polymerase-chain-reaction amplification mixture.

Because no indications of dissociation of the DNA–EthD complexes were seen in the above experiments, it was of interest to see whether in the presence of unlabeled DNA the dye would remain in the original complex or would redistribute. In Fig. 2*C* a 50-fold molar excess of pUC18 DNA was added to the preformed λ DNA *Hin*dIII–EthD complex, and the mixture was allowed to stand for varying periods of time before analysis by agarose gel electrophoresis. One-third of the dye remained with the λ DNA *Hin*dIII fragments after an hour of incubation with the competing DNA (Fig. 2*C*). No further loss of dye to the competing DNA was seen even after 6 hr of incubation. If the competing DNA and the λ DNA *Hin*dIII fragments were mixed at the 50:1 molar ratio before adding EthD, the dye was virtually quantitatively bound by the pUC18 DNA.

As noted above, a large fraction of the bound dye is not available for transfer from the original complex. A challenge posed by this observation was to find conditions for the removal of the tightly bound dye without damage to the DNA. The quantitative removal of bound dye was achieved by using NaI Glassmilk adsorbent. Based on the amount of Glassmilk-treated DNA loaded on the gel (Fig. 2*D*), the upper limit for residual EthD would be ≈5%. The results presented in Fig. 2*D* show that the DNA eluted from the adsorbent was indistinguishable from the starting material in its band pattern on agarose gel electrophoresis and in its capacity to bind newly added EthD.

We have examined the possibility that tight DNA–dye complexes are also formed in mixtures of ethidium bromide and DNA in the concentration range examined in this study. Mixtures of λ DNA *Hin*dIII fragments with dyes were prepared at an ethidium bromide or EthD concentration of 0.063 ng/μl and at a DNA concentration of 1.2 ng/μl. Aliquots containing 4.8 ng of DNA were subjected to agarose gel electrophoresis. The highest molecular weight restriction fragment represents ≈3.3 ng of this DNA. As shown in Fig. 2*E*, *no detectable ethidium bromide was retained by any of the DNA fragments, whereas EthD remained quantitatively bound by the DNA.*

DISCUSSION

DNA–EthD complexes were easily detected by their fluorescence after electrophoresis on agarose gels. During electrophoresis free dye would migrate in a direction opposite to that of the DNA–EthD complex. High-sensitivity imaging of the gels by a scanning confocal fluorescence microscopy system showed no evidence of dye trailing back from intense bands, indicating that no slow loss of dye was occurring during electrophoresis. Gaugain *et al.* (2) concluded from studies in solution that EthD binds at strong binding sites in double-stranded DNA with an affinity constant of 2×10^8 M^{-1} in the presence of 0.2 M Na^+ and that the bound dye covers 4 bp. Our findings show that the dye bound at such strong sites has an off-rate that is very slow relative to the time required for electrophoretic separation of DNA fragments.

Competition between preformed EthD–DNA complexes and excess unlabeled DNA showed only partial transfer of bound dye to the competing DNA. There are two possible interpretations of these results. (*i*) There may be two tight-binding modes for EthD. In one of these modes both phenylphenanthridinium moieties are intercalated; in the other, only one is intercalated. Dye bound in the latter mode can be transferred to competing DNA. Such transfer may not necessarily involve the dissociation of the EthD from the original complex and binding to a new DNA fragment. It is possible that this bifunctional intercalator dye forms a bridged complex between two DNA molecules and that such a complex mediates the transfer of the dye. This may be the mechanism for the crosslinking observed at high DNA and dye concentrations. (*ii*) A second interpretation may be that all of the dye is bound by intercalation of a single phenylphenanthridinium moiety, but that the strength of the association of the residual bound dye increases as dye is removed from adjacent sites on the EthD–DNA complex. Resolution of these questions requires further studies. All of the dye in the EthD–DNA complexes was extractable under the conditions of the Geneclean procedure, showing the absence of covalent bonds between the dye and DNA.

The use of the EthD–DNA complex together with the confocal fluorescence imaging system described here represents another approach to the detection and quantitation of double-stranded DNA fragments. In conventional electrophoresis of DNA on agarose gels, ethidium bromide is either added to the running buffer or the gel is stained after electrophoresis. Either procedure results in high background interference in fluorescence detection, coupled with modest sensitivity because of the low affinity of ethidium bromide for DNA. An additional problem is the need to dispose of substantial quantities of a mutagenic dye. These problems are all resolved by our EthD staining procedure. When photo-

graphic film is used for fluorescence detection, the sensitivity is low and the nonlinear response of the film complicates quantitation (5). Our laser-excited fluorescence detection method is advantageous because it offers improved sensitivity especially when optimized excitation conditions are used (6). At the extreme end, single-molecule fluorescence detection is possible (7, 8). High-quality display and computer analysis is also readily achieved. The combination of micrometer spatial resolution and low detection limits suggests that the sensitivity can be easily improved by using thinner gels and smaller sample spots to approach the detection limits of autoradiography with no sacrifice in electrophoretic resolution. The procedures used here can be readily modified for the detection and quantitation of restriction fragments or other DNAs in high-performance capillary electrophoresis. The EthD–DNA complex may also replace radiolabeled DNA in gel retardation experiments designed to detect high-affinity DNA-binding proteins (9).

These observations on the DNA–EthD complex suggest the possibility of a general class of fluorescent probes. In the DNA–EthD complex, the DNA functions as a very specific rigid scaffold for holding many dye fluorophores at a specific distance and orientation such that radiationless processes competing with fluorescence emission are greatly reduced. Thus, stable complexes of EthD with DNA can be used in a wide spectrum of applications as highly fluorescent probes carrying hundreds of dye molecules. For example, complexes of EthD with biotinylated DNA could be used in the numerous procedures that exploit the biotin–avidin or streptavidin technology. DNA–EthD complexes tailed with single-stranded DNA sequences could be used as specific hybridization probes for the detection of DNA sequences complementary to that of the tails. It is likely that a wide variety of dyes may be assembled into DNA matrices to make fluorescent probes with a wide variety of useful properties.

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