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Comparison of Various Ultraviolet Sources for Fluorescent Detection of Ethidium Bromide–DNA Complexes in Polyacrylamide Gels

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Ultraviolet sources with output wavelengths of 254, 300, and 366 nm were compared for detection of ethidium bromide–DNA complexes in acrylamide gels. The 254- and 300-nm sources were both much more sensitive than the 366-nm source. The 254-nm source produced a great deal of photodamage, photonicking and photodimerization, and photobleaching, while the longer wavelength sources cause little damage or bleaching. The 300-nm source is clearly the most suitable source, providing high sensitivity and a relatively low amount of photodamage and photobleaching.

The examination of DNA on polyacrylamide or agarose gels using fluorescent staining with ethidium bromide (EB) is convenient and effective (1). Ultraviolet sources for exciting the EB–DNA complex are commercially available, with output at either 254 or 366 nm. Each of these sources has its shortcomings. The 254-nm source produces photodamage in the nucleic acid and causes photobleaching of the complex. The 366-nm source is relatively inefficient and gives a weak fluorescence of the complex.

The excitation maximum of an EB–DNA complex occurs in the range of 300 nm, and fluorescent lamps that emit in this range are commercially available. We have constructed an ultraviolet source using these 300-nm lamps in conjunction with a filter that passes the ultraviolet and blocks visible wavelengths. This system should be optimal for the fluorescent detection of EB–DNA complexes. In this paper, we compare the 300-nm source with commer[redacted]vailable 254- and 366-nm sources as a means of detecting E[redacted]A bands on an acrylamide gel.

[redacted]ETHODS

G[redacted]e. The 300-nm source was constructed [redacted] lamps (RPR-3000 Å).[1] These lamps

[redacted]pany, 954 Newfield Street, Middletown,

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have a spectral output centered on 3000 Å with a half-bandwidth of about 30 Å. The lamps with the appropriate ballasts (Universal No. 619) are housed in a 6 × 18 × 6-in. box covered by a 6 × 12-in. ultraviolet pass–visible blocking filter (Corning Glass No. 9228).[2]

Spectral measurements. The intensity of the 300-nm source and the two commercially available sources, 254-nm source (Model C51)[3] and 366-nm source (Model C50),[3] was measured by potassium ferrioxalate actinometry (2). The absorption of the filters was measured using a Cary Model 15 recording spectrophotometer. The fluorescence spectra were measured using a Perkin–Elmer Model MPF-3L recording spectrofluorometer.

DNA preparation. Covalently closed kinetoplast DNA networks were isolated from stationary-phase *Leishmania tarentolae* culture forms grown in Neo Y E medium as described previously (3). After the EB–CsCl equilibrium gradient, the dye was removed by isoamyl alcohol extraction, and the DNA was then dialyzed and ethanol precipitated. The DNA was resuspended in 10 mM Tris–HCl–0.1 mM EDTA (pH 7.9) at a concentration of 0.5 mg/ml and stored at 5°C with chloroform.

Restriction endonuclease digestion. Closed kinetoplast DNA networks were digested overnight at 37°C with excess Hae III restriction endonuclease (purchased from New England Biolabs) in 6 mM Tris–HCl (pH 7.4), 6 mM NaCl, 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol. The reaction was stopped by the addition of 0.1 vol of 2 M NaCl and 2 vol of cold ethanol. The DNA fragments were pelleted and redissolved in electrophoresis application buffer (25% glycerol, 5% sodium sarcosinate, 1 mM EDTA, 0.025% bromphenol blue, 0.075% xylene cyanole FF) at a concentration of approximately 200 μg/ml.

Acrylamide gel electrophoresis. An acrylamide gradient slab gel was prepared as follows: Two solutions of 10% acrylamide–0.3% bisacrylamide (*N,N'*-methylene-bis-acrylamide; purchased from BioRad Laboratories) and 3.5% acrylamide–0.12% bisacrylamide were prepared in 0.09 M Tris, 0.09 M boric acid, 2 mM EDTA (pH 8.3) with 0.06% ammonium persulfate and cooled. Addition of 15% glycerol to the 10% acrylamide solution was necessary to stabilize the density gradient. TEMED (*N,N,N',N'*-tetramethylethylenediamine; purchased from BioRad Laboratories) was added to initiate polymerization (7 μl of TEM[illegible] of acrylamide solution and a linear 24-ml acrylamide gradie[illegible]d in a 2-mm slab chamber by use of a standard sucrose [illegible] Redistilled water was then run slowly onto the up[illegible]rder to produce a smooth interface. After poly[illegible]ved and a 3.0% acrylamide stacking gel w[illegible]

[2] Corning Glass Works, Corning, N[illegible]
[3] Ultra-Violet Products, Inc., San[illegible]

The total gel dimensions were 11 × 14 × 2 mm. Samples (8 μl) were applied and electrophoresis was performed at 42 V for 16 hr at 25°C. Gels were stained by immersion in 1 μg of EB/ml in electrophoresis buffer for 30 min and were destained in electrophoresis buffer for 30 min.

Individual identical gel slots were cut out and used for calibration of the different uv sources.

Photography. The gels were transilluminated (ultraviolet source below and camera above) and photographed through a barrier filter. The barrier filter consisted of a thin glass sheet, a Wratten 23 A filter, and a "green" interference filter (Schott FAL).[4] The camera was a Polaroid Model MP-3 with a 105-mm Prontor lens (*f* stop 4.5). Kodak Tri-X (4 × 5-in.) sheet film was used and developed with Kodak D-76 developer. The bands in the photographs were traced with a Joyce–Loebl densitometer.

Photodamage measurements. Photonicking of DNA produced by the different ultraviolet sources was measured by irradiation of form I SV40 DNA in a quartz cuvette in the presence of 1 μg/ml of EB and buffer. After exposure to a given dose of ultraviolet irradiation, the nicking of the DNA was determined by velocity sedimentation of the DNA in a 5–20% alkaline sucrose gradient. The gradients were centrifuged for 90 min at 45,000 rpm in a Beckman SW50L rotor. The form I and form II DNA were well resolved. Similar results were obtained for the conversion of form I to form II DNA on neutral sucrose gradients, indicating that the majority of the photonicking was directly induced by the ultraviolet irradiation. The conversion of form I to form II DNA is a single nick process obeying a Poisson distribution (4). The D_{37} (the ultraviolet dose that converts all but 37% of the form I DNA to form II DNA) was readily calculated from these measurements.

The production of photodimers produced by various doses of ultraviolet irradiation was measured directly. ^{3}H-Labeled *E. coli* DNA was irradiated in a quartz cuvette in the presence of 1 μg/ml of EB and buffer. After exposure to a given dose of ultraviolet irradiation, the DNA was precipitated with cold 5% trichloroacetic acid. The DNA was hydrolyzed with formic acid and the photodimer content was determined (5). The net amount of photodimers is the result of the rate of photodimer formation and the rate of photodi[illegible]truction; both of these processes are wavelength dependen[illegible]est is in the rate of photodimer formation, but, as a pr[illegible]stantial amounts of photodimer must be created f[illegible]s. The initial rate of photodimer formation (*[illegible]* of thymine dimerized per uv dose, can be[illegible]ement of photodimer content as a

RESULTS

The optimal wavelength for exciting fluorescence from an EB–DNA complex is in the 300-nm range. Figure 1A shows that excitation at 300 nm produces substantially more fluorescence at 590 nm than excitation at 254 nm (down threefold) or excitation at 366 nm (down sevenfold). A slight correction for the decreased spectral output of the xenon lamp used in the spectrofluorometer may shift the excitation maximum to somewhat shorter wavelengths, but the maximum should remain close to 300 nm. The fluorescence of EB without DNA present is less than 1/10 the fluorescence of an EB–DNA complex. The fluorescence emission is centered on 590 nm for all wavelengths of ultraviolet excitation. The fluorescence emission for excitation at 300 nm is shown in Fig. 1B.

The optimum detection of EB-stained DNA bands on acrylamide gels should be accomplished by excitation at 300 nm. The fluorescence

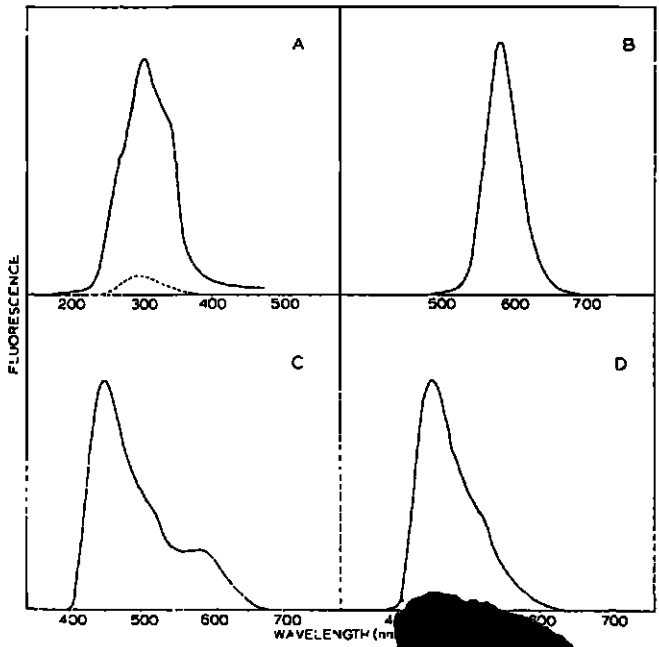

FIG. 1. The excitation and fluorescence emission s[illegible]mplexes in solution and in polyacrylamide gels. (A) The fluorc[illegible] at 590 nm. The solid line is the excitation spectrum for a[illegible]e is the excitation spectrum for EB without DNA pr[illegible]th is 300 nm. (B) The fluorescence emission [illegible]e fluorescence emission spectrum for an [illegible] The fluorescence emission spectrum f[illegible] The fluorescence is in arbitrary uni[illegible]

spectrum of an EB–DNA complex in a polyacrylamide gel excited by 300-nm irradiation is shown in Fig. 1C. In addition to the emission at 590 nm, which appears in this spectrum as a shoulder, there is a major fluorescence emission peak centered at 450 nm. This fluorescence emission peak at 450 nm is due to the fluorescence of the polyacrylamide gel itself. Figure 1D shows the fluorescence spectrum of EB-stained polyacrylamide without DNA present. Clearly, it is important to filter out the fluorescence emission due to the polyacrylamide gel. 9863

The uv pass–visible block filter (Corning Glass ~~9228~~), used as an excitation filter, transmitts 300 nm of uv irradiation with about 70% efficiency. It is important to note that this filter, although it efficiently blocks most visible wavelengths, does transmit long wavelengths in the red. The 300-nm lamps (RPR 3000 Å) used in our source have a spectral output component in the red, which must be eliminated by the barrier filter on the camera.

The three-element barrier filter used on the camera has a glass sheet to absorb short-wavelength uv and protect the cutoff filter that follows. The second element is a sharp-cutoff filter (Wratten 23-A) that absorbs wavelengths shorter than 580 nm. This element effectively reduces the fluorescence due to the polyacrylamide gel. The third element is a broadband "green" interference filter (Schott FAL). The objective of this filter is to block the transmission of red emission from the 300-nm lamps. The filter we use has a band pass centered at 565 nm and a half-bandwidth of 70 nm with transmission of 70%. It should be noted that interference filters of this type may have different band passes and center wavelengths. It is important that combination of the interference filter and sharp cutoff provide a suitable band pass for photography.

The outputs of the uv sources were measured by potassium ferrioxalate actinometry. In comparing these different sources where the wavelengths vary and thus the individual photons emitted vary in energy, it is most appropriate to use the photon flux (einsteins per square centimeter per minute) as the basis of comparison. The outputs of the various sources are shown in Table 1. The output of the 300-nm source is the lowest of

TABLE I

INTENSITIES AND PHOTODAMAGE PARAMETERS FOR THE UV SOURCES

Source wavelength (nm)	Output (nE/min cm^2)	Photonicking D_{37} (nE)	Photodimerization k (%/μE)
254	390	390	8.40
300	290	5,700	0.10
366	660	28,000	<0.001

the three (0.75 as great as the 254-nm source and 0.44 as great as the 366-nm source). It is very difficult to adjust the output of the sources, so our comparisons are made on the unequal sources.

The main objective of our comparison was the detection of EB-stained DNA bands in a polyacrylamide gel. As a test system, a sample of network kinetoplast DNA from *Leishmania tarentolae* that had been completely digested with Hae III restriction endonuclease was used. This digest provides a series of discrete bands which can be separated easily on a 3.5–10% acrylamide gradient gel. The heterogeneous banding pattern is due to a sequence microheterogeneity among the kinetoplast DNA minicircles which has been described elsewhere (7–9). Identical samples were electrophoresed in adjacent channels on a single slab gel and stained with EB. The individual channels were cut into separate strips and photographed with different sources and under various conditions. Figure 2 shows that both the 254- and 300-nm sources produce highly fluorescent EB–DNA bands (Figs. 2A and B), while the 366-nm source produces much less fluorescence (Fig. 2C). It should be borne in mind that the output of the 254-nm source is about one-third greater than that of the 300-nm source, while the output of the 366-nm source is greater than twice the output of the 300-nm source. The most apparent difference between the 254-nm source and the 300-nm source is in the photobleaching of the EB–DNA bands. With the 254-nm source there is a noticeable decrease in the intensity of the EB–DNA bands even during the period of time required to obtain suitable photographs. Figure 2D shows the bands as they

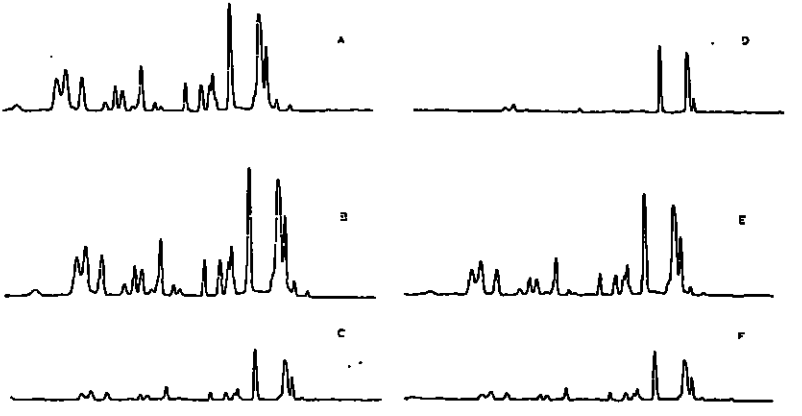

FIG. 2. The pattern of fluorescent EB–DNA bands on polyacrylamide gels excited with various uv sources for different periods (A) Excitation with a 254-nm source photographed immediately; (B) excitation with a 300-nm source photographed immediately; (C) excitation with a 366-nm source photographed immediately; (D) excitation with a 254-nm source for 20 min prior to photography; (E) excitation with a 300-nm source for 20 min prior to photography; (F) excitation with a 366-nm source for 20 min prior to photography. All photographs were traced with a Joyce–Loebl densitometer.

appear after a 20-min exposure to the 254-nm source. Virtually all of the smaller bands are bleached out. In comparison, little photobleaching occurs with the 300-nm source, even after 20 min (Fig. 2E). Similarly, there is virtually no photobleaching with the 366-nm source.

Damage of the DNA in the bands due to irradiation is also a major consideration. Single-strand breaks (nicks) are a dominant type of photodamage induced by uv irradiation of EB–DNA complexes. The conversion of a closed circular, form I DNA (SV40, 5100 base pairs) to an open circular, form II molecule requires only a single nick. This target DNA provides a sensitive detector for photonicking. Measurements of photonicking were performed in buffer with EB present. The uv dose required to nick all but 37% of the form I molecules using the various sources is shown in Table 1. The extent of photonicking with 254-nm source is 14 times higher than that with the 300-nm source for the same dose. The 366-nm source produces very little photonicking, 71 times less than the 254 source.

The production of pyrimidine dimers is another prevalent type of uv damage. The production of pyrimidine dimers in a solution of DNA with EB was measured directly. Table 1 shows that the rate of photodimerization is about 700 times higher with the 254-nm source than with the 300-nm source. No photodimerization was detected with the 366-nm source.

DISCUSSION

This comparison of the three different uv sources clearly indicates that detection of EB–DNA bands with either the 254- or 300-nm source is very sensitive and clearly superior to that with the 366-nm source. The 300-nm source is slightly more sensitive, as indicated by the similar band patterns (Figs. 2A and B) produced with only 75% the intensity of the 254-nm source. On the other hand, the sensitivity of the 300-nm source might be expected to be three times as great as that of the 254-nm source on the basis of the excitation spectrum (Fig. 1). This spectrum may require some correction due to the decrease in output of the xenon lamp in the spectrofluorometer at short wavelengths.

A major difference in the sources is observed in terms of photodamage. The 254-nm source is vastly more damaging, both in terms of photonicking and photodimerization, than the longer wavelength sources. Photodamage is a major consideration, particularly if the DNA samples are to be recovered and used for further analysis. An investigator may find any photodamage of the DNA unacceptable and, therefore, fluorescence visualization impractical. Although photodamage occurs with the 300-nm source, the rate of this damage is relatively slow and most photographic procedures can be performed without significant damage. On the other hand, the rate of damage by the 254-nm-source irradiation

precludes practically all fluorometric detection without substantial damage.

Photobleaching, which is rapid and presents a problem when samples are irradiated with a 254-nm source, is very slight with the longer-wavelength sources. The process of photobleaching is interesting because the bands can be restrained to fluorescence intensities near their original values (G. Fareed, personal communication; unpublished data). It is apparent that all bands are not photobleached at the same rate (Figs. 2A and D). Apparently, the photobleaching is not simply the result of photodamage because the bands can be restained. Most probably, excitation of the DNA, due to the uv irradiation, results in the dissociation of the EB from the DNA. This would explain the difference in photobleaching between the 254-nm source and the longer-wavelength sources. The 254-nm irradiation is much more strongly absorbed by the DNA than the longer-wavelength irradiation. This excitation of the DNA may result in the dissociation of the EB. Of course, the fluorescence need not require the excitation of the DNA, and probably is due mainly to absorption by the EB.

In comparing these three uv sources, it is apparent that the 300-nm source has clear advantages in terms of sensitivity and reduced photodamage. The 254- and 366-nm sources each represent several compromises in terms of either photodamage or sensitivity. From the excitation spectrum, a 300-nm source would be expected to be optimal. The commercial availability of 300-nm lamps at nominal expense make a 300-nm uv source a most attractive means of detecting EB-stained DNA bands on polyacrylamide gels.

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