Fluorescence decay measurements for determining the relative content of ethidium bromide to DNA in situ in cell nuclei

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Summary. A fluorometric method for the determination of the amount of ethidium bromide (EB) bound to DNA in situ in cell nuclei is discussed. Even when the EB content was very small, the molar ratio of DNA-phosphorus (DNA-p) to dye (P/D ratio) could be estimated by measuring the lifetime of the transient fluorescence of the EB-DNA complex as a function of the P/D ratio. To examine the relationship between the fluorescence intensity, lifetime, and P/D ratio, polyacrylamide gel film containing 4.7 mM DNA-p was used as a model DNA tissue, and its fluorescence was measured using a nanosecond microfluorometer. The fluorescence intensity showed a maximum at P/D = 6. The fluorescence lifetime increased with the P/D ratio, and this was accompanied by a proportional increase in the quantum efficiency. Thus, the lifetime value was an effective parameter for the determination of the P/D ratio in situ in tissue. When this approach was applied to tissue sections of mouse liver treated with solutions of EB at concentrations of 10 and 50 μg/ml, the fluorescence lifetimes on cell nuclei were 18.9 and 17.4 ns with P/D ratios of 20 and 12, respectively, as based on the model-tissue experiments. When the P/D ratio was 20, the concentration of EB in the nucleus was approximately 1.5 mM, i.e., 60 times higher than that in the staining solution.

Materials and methods
DNA was extracted from calf thymus and purified according to the method of Zamenhof et al. (1954). The concentration of DNA-p was checked colorimetrically at 260 nm, taking its molar extinction coefficient to be 6200 M⁻¹ cm⁻¹. EB (Wako Chemicals, Osaka, Japan) was dissolved in 5 mM Tris-HCl buffer, pH 7.2, at a concentration of 0.1 mg/ml and used as a stock solution. This solution was diluted with Tris-HCl buffer to obtain the concentrations required for staining. Polyacrylamide gel film with a thickness of 0.1 mm was made according to the technique of Chikamori et al. (1980), who modified the method of van Duijn and van der Plieeg (1970). The film contained 1.65 mg/ml DNA (4.7 mM DNA-p). This film was immersed in the staining medium containing EB at a concentration of 0.5, 2, 5, 20, 50, or 200 μg/ml for 30 min and then in Tris-HCl buffer for 1 min, before being mounted on a glass slide. For in situ specimens, tissue sections of mouse liver were prepared. The extracted liver was frozen with liquid nitrogen, cut into 7 μm-thick sections in a cryotome, and fixed in cold acetone-methanol (1:1). After treatment with RNase, each section was immersed in the staining medium containing EB (10 or 30 μg/ml) for 30 min, rinsed with Tris-HCl buffer for 1 min, and mounted on a glass slide according to the routine procedure in our laboratory.

A microspectrophotometer (Zeiss UMSP-1, equipped with a detector for fluorescence measurement) was used to measure the concentrations of EB in the film. The complex was fluoresced by excitation with a probe light, and the fluorescence was detected using the same instrument. Fluorescence decay was measured with an epi-illumination fluorescence microscope equipped with a multichannel photoncounter and nitrogen-pulsed laser (Iwata et al. 1985). This system responds to temporal emission for a period of 100 ns with a time resolution of 2.5 ns. The spot diameter of the excitation beam was set at 100 μm on the film and at 10 μm on the nucleus. All of the measurements were carried out at room temperature.

Introduction
An intercalating fluorochrome, ethidium bromide (EB), is widely used for the histochemical determination of DNA in cells and tissue. The quantum efficiency of the fluorescence of the DNA-EB complex varies depending on the molar ratio of DNA-phosphorus (DNA-p) to dye (P/D ratio; LePecq and Paoletti 1967). Therefore, for a quantitative assay of DNA using EB fluorescence, it is necessary to estimate the P/D ratio in the specimens being studied. However, in practice, colorimetric determination of the EB concentration in tissue sections is difficult, because the extinction of EB is too small for it to be distinguished from the background absorption due to tissue components.

When EB forms a complex with DNA, the fluorescence lifetime of EB increases markedly (Tao et al. 1970; Wahl et al. 1970). As the degree of this increase is a function of the P/D ratio, it can be used to estimate the P/D ratio of DNA in situ in cell nuclei. To examine the relationship between the fluorescence lifetime and the P/D ratio, polyacrylamide gel film containing a known amount of DNA was used as a model tissue, and the EB content in nuclei was determined on the basis of experiments using this model tissue.

Results and discussion
Free EB in the Tris-HCl buffer had two absorption maxima, i.e., at 300 and 480 nm, and its fluorescence emission peak was at 590 nm. When EB bound to DNA, the absorption peaks shifted to longer wavelengths, retaining an isos-
Fig. 1. Relationship between the fluorescence intensity and P/D ratio in polyacrylamide film treated with various concentrations of EB. The fluorescence emission due to excitation with the probe light at 510 nm was detected at 590 nm. F, total fluorescence intensity; RF, ratio of F to the EB concentration (in arbitrary unit).

bestic point at 510 nm, while the fluorescence peak did not shift appreciably. To avoid unexpected absorption of the light by the glass slide itself, the EB content in the film was determined at 510 nm with a molar extinction coefficient of 3800 M$^{-1}$ cm$^{-1}$, and the fluorescence emission was detected at 590 nm with a spectral band width of 20 nm. The relationship between the P/D ratio and the fluorescence intensity can be seen in Fig. 1, which shows both the total intensity (F) and the relative intensity (RF; the ratio of total intensity to dye concentration), the latter of which corresponds to the quantum efficiency of the complex. The F curve showed a maximum at a P/D ratio of about 6. This value indicates the number of binding sites of EB to DNA-p for intercalation in the model tissue, and it is slightly larger than that obtained in the DNA solution (Le-Péq and Paoletti 1967), but is consistent with the value obtained in the cellular DNA (Eisenhut and Choné 1979). On increasing the P/D ratio, the formation of the dye's array progressed into the DNA double strand, resulting in a decrease in energy transfer between EB molecules and an enhancement of the RF. This formation was also reflected in the decay profile of fluorescence emission, which showed an increased fluorescence lifetime.

For measurements of the fluorescence lifetime, the specimen was excited with a repetitive light pulse (wavelength, 338 nm; duration, 1.5 ns at half-height; repetition, 20 pulses/s) produced in the nitrogen laser. Fluorescence lifetime is the time that required for the intensity to drop to 1/e of its initial value. Typical profiles of the fluorescence decay at various P/D ratios are shown in Fig. 2. The fluorescence decay curve appeared to consist of a single component, and so the analytical lifetime was calculated by fitting a single exponential function to the data using a least-square program. Figure 3 shows the relationship between the lifetime and the P/D ratio. At a P/D ratio of about 50, the lifetime on the film was 21 ns. The lifetimes of the DNA-EB in gel film were shorter than those of the complex in water solution, e.g., 22.5 ns at P/D=50, and 20 ns at P/D=3 in Tris-HCl buffer (DNA-p, 50 μM). In a condensed system like the model tissue, the probability of energy transfer between the complex is greater than in a water solution, and the energy transfer quenches the fluorescence, resulting in a decrease in the lifetime. Since the fluorescence intensity of the free dye in the film was too weak to be distinguished from the background fluorescence, the observed lifetime at P/D=0 was erroneously longer than that obtained in the solution (1.5 ns). Thus, the lifetime was an effective parameter of the binding of dye to DNA, and it was possible to apply the fluorometric results obtained in the gel film for the estimation of the P/D ratio on nuclear DNA.

Liver-tissue sections of mice were treated with EB, and their fluorescences were analyzed. Genest et al. (1981) have shown that at P/D ratios of over 30, the fluorescence decay of the EB-chromatin complex in buffer medium consists of two exponential functions, i.e., a major decay portion...