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Short communication

Quantitation of ethidium-stained closed circular DNA in agarose gels

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Abstract

The fluorescence of ethidium bromide (EB) bound to equimolar amounts of supercoiled form I and unstrained linear form III pBR322, SV40 and PM2 DNA in agarose gels has been measured by scanning a photographic negative of the gel with a microdensitometer. For SV40 and PM2 DNA, commonly used staining conditions cause both forms, i.e. linear and supercoiled, to fluoresce to the same extent. This obviates the need to use a correction factor for the fluorescence of form I DNA when measuring the amount of this form relative to the amounts of unstrained forms in agarose gels. In the case of PBR322 DNA, form I was found to fluoresce ~20% more than form III DNA. © 1997 Elsevier Science B.V.

1. Introduction

Closed circular DNA is an ideal substrate for studying drug- and protein-DNA interactions [1,2]. If an agent is able to break the sugar-phosphate backbone of DNA, supercoiled (form I) is converted into nicked circular (form II) and ultimately into linear (form III) DNA. Measurement of the relative amounts of the various forms as a function of time and/or concentration of cleavage agent can give valuable information about the rate and mechanism of the cleavage process.

Agarose gel electrophoresis is a convenient way to separate the various forms of

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closed circular DNA [3]. In order to visualize the DNA, the gel (after electrophoresis) is usually immersed in an ethidium bromide solution (EB) and photographed in the presence of UV light. The photographic negative can be scanned with a microdensitometer to give quantities that are proportional to the concentration of DNA [4,5].

Initially, EB binds by intercalation to the DNA. However, as intercalation sites become saturated, weaker binding, involving stacking on the outside of the helix, takes place [6]. It is known that the binding constant of intercalated EB towards forms II and III DNA is the same and independent of binding density [7]. However, the presence of supercoiling in form I causes the binding constant of intercalated EB towards this form to be dependent on the binding density. At low binding density, the binding constant of intercalated EB towards form I is greater than its binding constant towards forms II and III, while at high binding density, the opposite is true [7]. Thus, DNA forms present in a gel that has been exposed to a solution of EB may not have the same amount of bound ethidium, i.e., form I may have more or less bound EB than forms II and III. If this is the case, the observed fluorescence from form I cannot be used, without correction, to measure the amount of this form relative to the two unstrained forms.

In this report, we determine the relative amounts of EB bound to forms I and III of PBR322, SV40 and PM2 DNA in agarose gels that have been stained with an ethidium bromide solution.

2. Materials and methods

2.1. Materials

SV40 DNA, 5.3 kb (Gibco BRL), PBR322 DNA, 4.3 kb and PM2 DNA, 10.09 kb (Boehringer Mannheim) as well as the restriction enzymes, Hind III, Eco RV (Boehringer Mannheim) and Acc I (New England Biolabs) were purchased commercially and used without further purification. Low-melting molecular biology grade agarose and molecular weight DNA markers (0.125–21.2 kb) were purchased from Promega. The DNAs used contained 10–30% nicked circular form II DNA.

2.2. Preparation of the DNA

The closed circular DNAs (1.5 μ g in 60 μ l of the appropriate enzyme buffer) were linearized by cleavage at 37°C for 1 h with an excess of the restriction enzyme. Acc I was used for SV40, Hind III for pBR322, and Eco RV for PM2 DNA. As a control, a separate sample of linearized SV40 DNA was deproteinized to remove Acc I using the phenol extraction procedure described in Maniatis et al. [3]. Electrophoresis of deproteinized and undeproteinized samples showed that the presence of Acc I did not affect the electrophoretic mobility of linearized SV40 DNA in the gel. Comparison with molecular weight markers showed that cleavage of PM2 DNA with the restriction enzyme Eco RV produced linearized DNA with a molecular weight of 10.09 kb [8]. Samples of the uncleaved DNAs, at the same concentration as that of the cleaved DNA

(1.5 μg in 60 μl), were prepared in TAE buffer, 40 mM Tris-acetate, 1 mM EDTA, pH 8.0.

2.3. Electrophoresis

Electrophoresis was carried out using a BRL model H5 horizontal gel electrophoresis apparatus in 1% agarose gels (~ 4 mm thick). The agarose solutions were prepared by heating a suspension of 0.55 g of low melting agarose in 55 ml of TAE buffer to boiling. Before the gel was poured, the weight of the boiled solution was readjusted to its weight prior to heating by the addition of water. After allowing the gel to set for 30 min at room temperature, identical amounts (60 μl) of cleaved and uncleaved DNA were mixed with 18 μl of loading buffer (30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol FF) and 13 μl samples were loaded alternately (uncleaved and cleaved) into the center 12 lanes of the gel. Electrophoresis was for 15 min at 150 V, at which point the voltage was reduced to 20 V. After 18 h, the electrophoresed gel was removed from the gel tray and immersed in 300 ml of aqueous ethidium bromide (0.5 $\mu\text{g}/\text{ml}$) for 30–40 min. Changing the length of the staining time in the range 30–50 min did not affect the quantitative results.

2.4. Photography and microdensitometry

The gel, without destaining, was placed on a UV transilluminator (Ultra Violet Products) and photographed with a Polaroid camera using type 55 positive/negative (ASA 100) film. In order to minimize fluorescence background from the gel, the camera lens was equipped with orange (Cokin A.030), red (Cokin A.003) and yellow (Polaroid 1 A) filters. Exposure times were, 5, 10 and 30 s (f/4.5), giving maximum absorbance values for the most intense bands of 0.51, 0.66 and 1.00 O.D., respectively, and backgrounds of 0.19, 0.20 and 0.23, respectively. After development for 20 s, the negative was fixed in a saturated sodium sulfite solution for 5 min, washed with water and air dried. The photographic negative was scanned with a Molecular Dynamics (Model 300A) microdensitometer and band intensities (area integrations) were determined using Image Quant software. A photograph of one of the gels used in the analysis is shown in Fig. 1.

2.5. Data and analysis

In alternate lanes, the integrated band intensities correspond to either form I and a small amount of form II (circular) or to form III (linear) DNA. The total amount of DNA is the same in all lanes, except for possible pipetting errors. However, the proportionality of intensity to the amount of DNA (C_i , $i = \text{I, II or III}$) may be different for form I than for the other forms. It was noticed that, for most of the gels, the total intensity in the first and/or last lanes was significantly less (sometimes by as much as half) than the intensity of the remaining lanes. This may be due to non-uniform illumination of the gel by the transilluminator or to optical effects caused by the camera used to take the photographs. We correct for these effects by fitting each form to a parabolic function of lane number

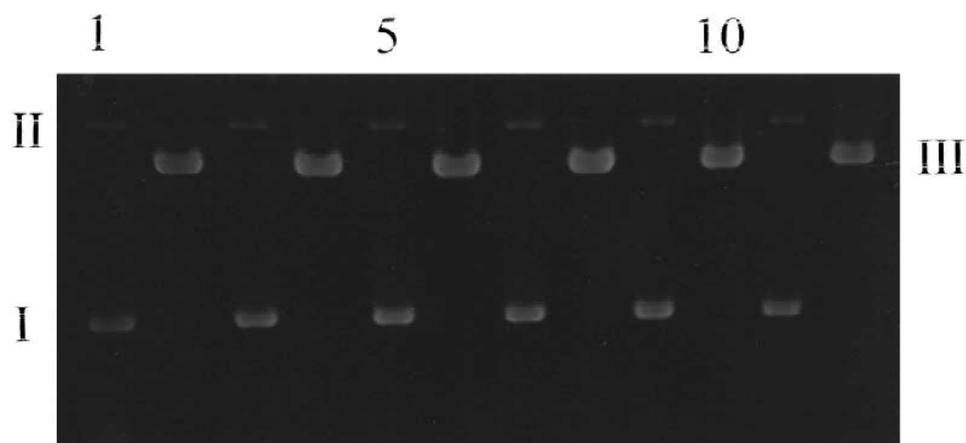


Fig. 1. Photograph of a typical agarose gel with pBR322 DNA. The odd lanes show uncut DNA (forms I and II) and the even lanes show DNA linearized with Hind III to produce form III DNA.

and using the parabola to calculate the intensities of forms that are missing in a given lane. Thus, spots for form I are found in lanes 1, 3, 5, 7, 9 and 11; the parabolic fit to these six intensities is used to generate spot intensities for form I in lanes 2, 4, 6, 8, 10 and 12. The same is done for form II. For form III, one has intensities for the even lanes and the parabolic fit is used to estimate intensities for form III in the odd-numbered lanes. An example of the fits is shown in Fig. 2.

We represent by I_I , I_{II} and I_{III} the intensities of forms I, II and III DNA, either

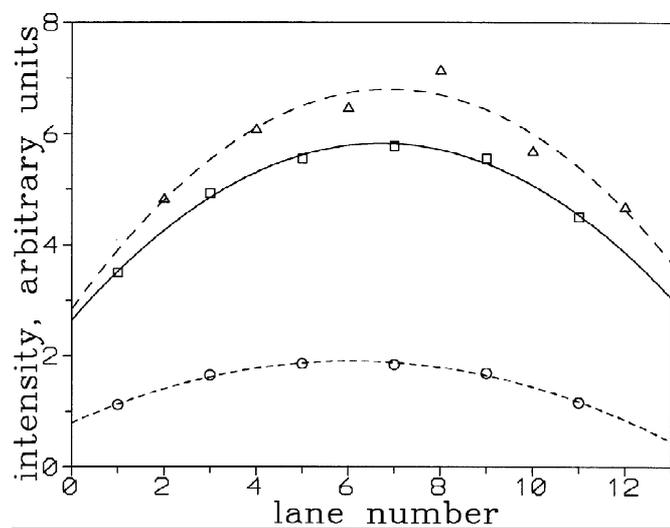


Fig. 2. Plot of spot intensity vs. lane number for the photograph in Figure 1. The lines are best parabolic fits to the intensities. The intensities of various forms are shown as squares (form I), circles (form II) and triangles (form III).

measured or interpolated, for a particular lane of the gel. Since $I_1/C_1 + I_{II}/C_{II} = I_{III}/C_{III}$ and $C_{III} = C_{II}$,

$$I_1 = \frac{C_1}{C_{II}} (I_{III} - I_{II})$$

which allows calculation of the ratio of staining coefficients. The twelve values obtained for the ratio $r = C_1/C_{II}$ are averaged and the expected error in the mean value, E , is calculated as,

$$E^2 = \frac{1}{6 \times 5} \sum_j (r_j - r_m)^2$$

because there are only six independent measurements of each intensity. Here, r_j is the value of C_1/C_{II} obtained from the intensities of lane j and r_m is the mean of the twelve values of r_j .

The results of 24 determinations of the ratio C_1/C_{II} , which has a value of 1.14 with a standard deviation of 0.03, are shown in Fig. 3. As shown in Fig. 3, the values of C_1 and C_{II} are significantly higher for pBR322 DNA than for the other DNAs. The 13 experiments with pBR322 give $C_1/C_{II} = 1.226$, with a standard deviation of 0.042. The 5 experiments with SV40 DNA give $C_1/C_{II} = 1.037$, with a standard deviation of 0.037, while the ratio for PM2 DNA, for 6 experiments, is 1.053, with a standard deviation of 0.016.

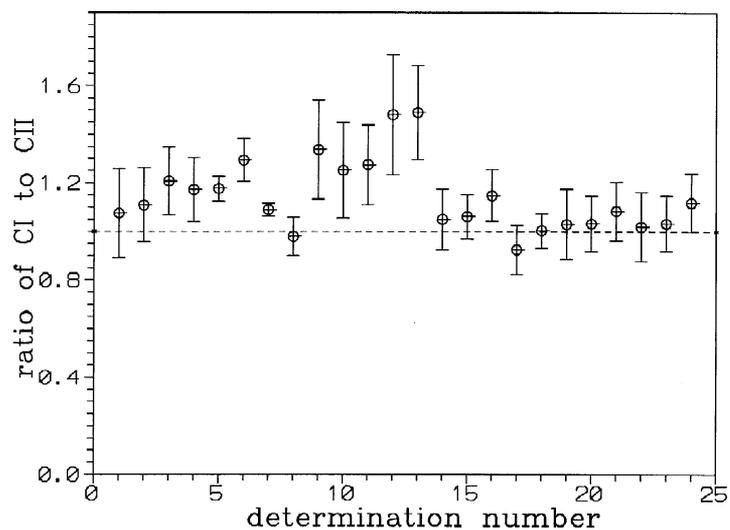


Fig. 3. The corrected intensity ratio of form I to forms II and III, C_1/C_{III} , with standard error plotted against determination number. The dashed line represents an intensity ratio of one. Determinations: 1–13, pBR322; 14–18, PM2 and 19–24, SV40 DNA.

3. Results and discussion

As is evident from Fig. 3, the ratio of staining efficiencies of supercoiled to unsupercoiled DNA is close to one for SV40 and PM2 DNA. For pBR322, it is about 1.2, which is significantly closer to unity than the ratio for DNA in solution at the concentration used [7]. The fact that the values for SV40 and PM2 are close to unity indicates that the presence of supercoiling does not significantly affect the staining of the DNA in the gel.

In comparing our results with the classic measurements of Bauer and Vinograd [7], the differences in experimental conditions must be taken into account. These authors studied ethidium binding in solution, and found that the ratio of the amount bound to supercoiled DNA to the amount bound to unsupercoiled DNA varied markedly as a function of the relative concentrations of ethidium and DNA. The explanation for this variation relates to the fact that ethidium initially binds by intercalation which, for low binding densities, is favored by supercoiling. In our experiments, a gel containing a small amount of DNA (total of 3 μg in 12 lanes) was immersed in 300 ml of a relatively concentrated EB solution (0.5 $\mu\text{g}/\text{ml}$). It may be that under these conditions, the bulk of EB is externally bound [9]. The amount of externally bound EB at saturation should depend on the number of available external binding sites, or on the available surface area of DNA. For the open circular or linear forms, this area is proportional to the length of the DNA, but the length-dependence for supercoiled DNA may be different. A staining ratio near unity implies that many more external binding sites are available for forms II and III than for form I in the gel. The ratio may increase with DNA length, making external binding more important in forms II and III relative to form I. This may be the reason that the two longer DNAs, SV40 and PM2, have staining ratios that are closer to unity while the shortest DNA, pBR322, has a staining ratio that is larger than unity.

Capturing the image of the DNA that has been stained with EB can be done with an electronic camera having a charge coupled device (CCD) [10], or, as is usually the case, a Polaroid camera using a silver halide-based film. This study shows that with either method of image capture, it is unnecessary to apply a correction factor for determining the amount of form I relative to the two unstrained forms for DNA, unless the DNA is as small as pBR322 DNA.

Previously, we measured the DNA cleavage kinetics of a group of enediyne antitumor agents [11] and a series of cationic porphyrin complexes [12] using data taken from agarose gels. The value of $C_{\text{I}}/C_{\text{II}}$ used in those studies was 0.8. While this study shows that the earlier employed value is not correct, the main conclusions drawn from those investigations are not seriously affected by the small change in the value of the parameter.

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References

- [1] Gale, E.F., Cundliffe, E., Reynolds, P.E., Richmond, M.H. and Waring, M.J., The molecular basis for antibiotic action, John Wiley and Sons, New York, 1981.
- [2] Lilley, D.M.J., DNA: Sequence structure and supercoiling, *Biochem. Soc. Trans.*, 12 (1984) 127–140.
- [3] Maniatis, T., Fritsch, E.F. and Sambrook, J., *Molecular cloning: a laboratory manual*, 2nd ed., Cold Spring Harbor Laboratory Press, New York, 1989.
- [4] Prunell, A., Strauss, F. and Leblanc, B., Photographic quantitation of DNA in gel electrophoresis, *Anal. Biochem.*, 78 (1977) 57–65.
- [5] Ribeiro, E.A., Larcom and Miller, D.P., Quantitative fluorescence of DNA-intercalated ethidium bromide on agarose gels, *Anal. Biochem.*, 181 (1989) 197–208.
- [6] LePecq, J.-B. and Paoletti, C., A fluorescent complex between ethidium bromide and nucleic acids, *J. Mol. Biol.*, 27 (1967) 87–106.
- [7] Bauer, W. and Vinograd, J., The interaction of closed circular DNA with intercalative dyes, *J. Mol. Biol.*, 33 (1968) 141–171.
- [8] Streeck, R.E., Zachau, H.G., A long-range and two short-range periodicities are superimposed in the 1.076-g/cm³ satellite DNA from calf thymus, *Eur. J. Biochem.*, 89 (1978) 267–279.
- [9] McMurray, C.T., Small, E.W. and van Holde, K.E., Binding of ethidium to the nucleosome core particle. 2. Internal and external binding modes, *Biochemistry*, 30 (1991) 5644–5652.
- [10] Löbrich, M., Ikpeme, S., Haub, P., Weber, K.J. and Kiefer, J., DNA double-strand break induction in yeast by x-rays and α -particles measured by pulsed-field gel electrophoresis, *Int. J. Radiat. Biol.*, 64 (1993) 539–546.
- [11] Kishikawa, H., Jiang, Y.-P., Goodisman, J. and Dabrowiak, J.C., Coupled kinetic analysis of cleavage of DNA by esperamicin and calicheamicin, *J. Am. Chem. Soc.*, 113 (1991) 5434–5440.
- [12] Sehlstedt, U., Kim, S.K., Carter, P., Goodisman, J., Vollano, J.F., Nordén, B. and Dabrowiak, J.C., Interaction of cationic porphyrins with DNA, *Biochemistry*, 33 (1994) 417–426.