Estimation of Circular DNA Size Using \( \gamma \)-Irradiation and Pulsed-Field Gel Electrophoresis

Stephen M. Beverley
Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

Received September 7, 1988

A method is described for estimating the size of large circular DNAs found within complex chromosomal DNA preparations. DNAs are treated with low levels of \( \gamma \)-irradiation, sufficient to introduce a single double-stranded break per circle, and the resulting linear DNA is sized by pulsed-field electrophoresis and blot hybridization. The method is fast, reproducible, and very conveniently applied to the agarose-enclosed chromosomal DNA preparations commonly used in pulsed field electrophoresis.

Large circular DNAs are constituents of many genomes, including those of viruses, prokaryotes, and eukaryotes (1). Certain DNA amplifications in drug-resistant \textit{Leishmania} (2–5); T. E. Ellenberger and S. M. Beverley, in preparation) and cultured mammalian cells (6–9) also exist as circular DNAs. We have recently employed a new method for rapidly determining the size of large circular DNAs in complex chromosomal DNA preparations (5). In this approach, \( \gamma \)-irradiation is used to introduce limited breakage into chromosomal DNA prepared within agarose sample plugs, which converts supercoiled circular DNAs successively into open circular and then linear molecules. This approach is based on the classic method employing limited DNAse cleavage of circular DNAs. The size of the linearized DNA is then determined by separation with pulsed-field electrophoresis, followed by blot hybridization and comparison with appropriate linear DNA size markers.

In this report several parameters important in the application of this method are examined. The effects of \( \gamma \)-irradiation on DNA have been extensively studied (summarized in (10)). \( \gamma \)-Irradiation introduces a variety of lesions into DNA, including both single- and double-stranded breaks. The dose-dependence of the introduction of strand breaks into DNA is known to vary with different buffers (10), and thus it was necessary to establish this relationship in the buffers employed in this work. Moreover, the effect of enclosing the DNA within agarose sample plugs must also be determined, as this method is commonly employed in the preparation of large chromosomal DNA suitable for pulsed-field electrophoresis. These variables were examined and the results allow the estimation of the \( \gamma \)-irradiation dose appropriate for examining circular DNAs of differing sizes.

METHODS

\( \lambda \) DNA (cI857Sam7) was purchased from New England Biolabs. The 33-kb circular plasmid pK300 has been described (11). pK300 contains the entire 30-kb circular R region (2), which includes the bifunctional dihydrofolate reductase-thymidylate synthase gene (14,15) which is amplified in certain methotrexate-resistant lines of \textit{Leishmania major} (2). Agarose sample containing DNA of \textit{Escherichia coli} cells bearing pK300 were prepared as described (12). Chromosomes of the R1000-11 line were prepared in agarose plugs as described and stored at 4°C in 200 mM Tris, 100 mM EDTA, pH 8.0 (storage buffer; (11)).

\( \gamma \)-Irradiation

\( \gamma \)-Irradiation was performed using an ICN GR9 irradiator, which employs a \( ^{60} \)Co source. DNAs in solution or agarose sample plugs were placed in a standard 1.5-ml polypropylene microcentrifuge tube and then irradiated at ambient temperatures for the time necessary to obtain the desired radiation doses. Samples remained at room temperature for at least 30 min prior to electrophoresis.

Pulsed-Field Electrophoresis

Pulsed-field electrophoresis was performed using the method of Chu et al. (13), which is referred to as a con-
tour-clamped homogeneous electric field (CHEF) electrophoresis. Electrophoresis was performed in 0.5x TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3) at 4°C using a voltage gradient of 6 V/cm. The pulse times and lengths of electrophoresis were chosen to be appropriate for the DNA size range to be separated. Following electrophoresis sample plugs were sealed within the well, and the gel was stained with ethidium bromide prior to photography and processing for blot hybridization as described (2,11).

RESULTS

\(\gamma\)-Irradiation of Circular and Linear DNA Standards

Treatment of the 33 kb supercoiled circular plasmid pK300 in 200 mM Tris, 100 mM EDTA, pH 7.4, with increasing doses of \(\gamma\)-irradiation results in the successive generation of open circular followed by linearized DNA (Fig 1A). A dose of 5-7 krad converts about 50% of the supercoiled circle to open circular forms (Fig 1, lanes 4 and 5), while 25-50 krad results in complete conversion to open circular molecules (Fig. 1A, lanes 8 and 9). In contrast, generation of significant levels of the 33-kb linear form of pK300 begins to occur at 25-50 krad and occurs maximally at about 300 krad (Fig. 1A, lanes 13-15). At 200 krad and above, DNA less than 33 kb in size appears, indicative of the introduction of multiple double-strand breaks (Fig. 1A, lanes 12-15). A similar quantitative dependence of the relative introduction of single- and double-stranded breaks in DNA in different buffers has been observed in previous studies employing \(\gamma\)-irradiation (10).

Quantitation of \(\gamma\)-Irradiation-Induced Breakage

\(\gamma\)-Irradiation of phage \(\lambda\) DNA was utilized to measure the dose-dependence of double-stranded breakage. Introduction of single-stranded breaks into this DNA does not alter the mobility of the phage DNA, whereas double-stranded breaks will generate fragments smaller than the intact phage, thereby decreasing the amount of intact DNA. Figure 1B shows that 100–150 krad irradiation reduces the level of intact \(\lambda\) DNA by about 50%. Assuming that the introduction of breakage is (i) random, (ii) follows a linear dose–response curve, and (iii) follows a poisson distribution, the rate of introduction of double-stranded breaks was determined to be approximately \(7 \times 10^{-5}/\text{kb}/\text{krad}\) under these conditions. A similar rate was determined from the pK300 data shown in Fig. 1A.

Abbreviation used: CHEF, contour-clamped homogeneous electric field.

\* Abbreviation used: CHEF, contour-clamped homogeneous electric field.
**Buffer Effects**

Irradiation of pK300 in 10 mM Tris, 1 mM EDTA, pH 7.4, results in the expected conversion to open circular and then linearized DNA forms (Fig. 1C). However, the conversion to open circles is 50% complete after only 0.6 –1 krad, and linearized DNA appears maximal at 40 krad. These dosages are about eightfold lower than required in the 200 mM Tris, 100 mM EDTA buffer (Fig. 1A). A similar effect was observed using λ DNA (not shown). The effect of buffers was examined on pK300 and λ DNA following irradiation, using the following buffers: 10 mM Tris, 1 mM EDTA; 200 mM Tris, 1 mM EDTA; 10 mM Tris, 1 mM EDTA, 500 mM NaCl; and 10 mM Tris, 1 mM EDTA, 4 mg/ml sorbitol, all at pH 7.4. The 500 mM NaCl-containing buffer showed a quantitative dependence of breakage very similar to that observed in 10 mM Tris, 1 mM EDTA (not shown), while the other three buffers exhibited sensitivities identical to that observed in 200 mM Tris, 100 mM EDTA (not shown).

**Irradiation in Agarose Sample Plugs**

The buffer effects described above, especially that observed with sorbitol-containing buffers, suggested that the sensitivity of DNAs to γ-irradiation within agarose plugs could differ from that observed in free solution. E. coli cells containing pK300 were cast in agarose plugs and processed to yield chromosomal DNA. This protocol yields intact DNAs enclosed by a cell-sized space within the agarose matrix (as opposed to simply casting free DNA in agarose), as employed in typical eukaryotic chromosomal preparations for pulsed-field electrophoresis. These sample plugs were washed extensively in either 10 mM Tris, 1 mM EDTA or 200 mM Tris, 100 mM EDTA, and irradiated with 5, 20, or 100 krad. The DNAs were electrophoresed, and the pK300 DNA visualized by blot hybridization (Fig. 2). The buffer dependence of irradiation sensitivity observed in solution is clearly evident in the agarose-enclosed DNAs; 100 krad in 10 mM Tris/1 mM EDTA yields mostly linear or degraded pK300 (Fig. 2A, lane 4), whereas 100 krad irradiation of samples in 200 mM Tris/100 mM EDTA results in mostly open circular and linear species (Fig. 2B, lane 4). Moreover, comparison of the results of Fig. 2 with those in Figs. 1A and 1C indicate that quantitatively similar levels of breakage occur in the two buffers, regardless of whether pK300 is free in solution or contained within E. coli-sized spaces within the agarose matrix. These data indicate that the irradiation-dependence of DNA breakage is comparable in free and agarose-enclosed DNAs.

**Example: Sizing of Circular DNAs in Methotrexate-Resistant Leishmania**

As an example of the use of this technique, I show the analysis of the extrachromosomal circular H region present within the R1000-11 line of L. major (2). This highly methotrexate-resistant line also bears a stable amplification of the R region, encoding the bifunctional dihydrofolate reductase-thymidylate synthase (2,14,15). The biochemical mechanism of resistance encoded by H region is unknown, although current data indicate that it bears a functional drug resistance element which does not mediate decreased methotrexate uptake ((16); Ellenberger and Beverley, in preparation). Similar results have been presented for the amplified H region DNA present in certain laboratory stocks of Leishmania tarentolae (5).

Chromosomes of the R1000-11 line were irradiated with increasing levels of γ-radiation and the DNAs were separated by pulsed-field electrophoresis. Hybridization of an H region-specific probe to a blot of this gel is shown in Fig. 3. Without irradiation, strong hybridization is observed in the sample well, and weaker hybridization to a DNA labeled “sc” (Fig. 3, lane 1). The “sc” DNA exhibits mobility properties characteristic of supercoiled circular DNAs, such as pulse-time dependent mobility (relative to linear DNA size standards) and altered migration direction, and is resistant to exonuclease III treatment ((11,17); data not shown). The material remaining within the well probably corresponds to nicked circular H DNAs, as large open circular DNAs (>30 kb) do not enter the gel under the high voltage gradients utilized (6 V/cm; (11)). Following irradiation, progressively reduced levels of the supercoiled DNA are found, and a new DNA species appears with an apparent molecular
weight of about 85 kb (the nicked circular species remains in the well, as discussed above). The new 85-kb DNA is linear, as revealed by mobility characteristics (lack of altered migration path or pulse-time dependence) and sensitivity to exonuclease (not shown). These data show that the amplified circular H region is 85 kb in length in the R1000-11 line, which is also its length within the unstable R1000-3 parent of this line (2). A similar estimate for the size of the intact H circular DNA has been obtained by electron microscopy (18). Interestingly, the hybridization to the linear 85-kb DNA following 50-300 krad is more intense than that observed to the supercoiled circular species, indicating that at least some of this linear DNA must have arisen from the material retained in the sample well in the absence of irradiation. This provides evidence that some portion of the material hybridizing to the well corresponds to the 85-kb circular DNA. Further discussion concerning the structure of the amplified R and H region DNAs within the R1000-11 line will appear elsewhere.

The experiment shown in Fig. 3 provides additional controls for the γ-irradiation method. First, the relative dose-dependence for the appearance of the linearized forms of the amplified H region DNA of Leishmania is consistent with the values determined earlier with control DNAs in free solution. Secondly, at high irradiation doses the hybridization signal is completely removed from the sample well, indicating that a complete chase of the well DNA can be obtained. The dose required for this chase is also consistent with calculations based upon the size of the H circular DNA and the dose-dependence of strand breakage following irradiation of samples stored in 200 mM Tris, 100 mM EDTA buffer.

DISCUSSION

With limited γ-irradiation it is possible to introduce a controlled number of double-stranded breaks within DNA, allowing the generation of linearized forms from circular DNAs whose size may be readily estimated by electrophoretic techniques. The γ-irradiation approach is especially convenient for introducing breakage into circular DNAs found within the complex chromosomal DNA preparations commonly employed in pulsed-field electrophoresis, as one simply irradiates the DNA directly in the agarose sample plug with the desired dose. In comparison, enzymatic cleavage methods require diffusion of the enzyme into the sample plug, a process which is difficult to control sufficiently to obtain limited and reproducible cleavage (Beverley, unpublished observations). A similar approach employing irradiation from a radioactive Cs source has recently been presented (9).

If the approximate size of the circular DNA to be measured is known, one can use the values presented earlier to estimate the appropriate radiation dose necessary for linearization. With circular DNAs of unknown length, it is necessary to employ radiation doses over a wide range. Otherwise, dosages appropriate for smaller circular DNAs will introduce multiple breaks in larger DNAs, and their presence will be overlooked.

ACKNOWLEDGMENTS

I thank members of my laboratory for advice, discussions, and radiolabeled probes, D. E. Dobson, T. E. Ellenberger, and G. M. Kapler for comments on this manuscript, D. D. Rogers for technical assistance, and the Radiobiology Department of the Harvard School of Public Health for use of their γ-irradiator. This work was supported by NIH Grant AI 21903. SMB is a Burroughs-Wellcome scholar in Molecular Parasitology.

REFERENCES