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Separation of closed circular DNA from linear DNA by electrophoresis in two dimensions in agarose gels

Ariella Oppenheim*

Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20205, USA

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ABSTRACT

A method that provides an easy, rapid, and reproducible way for separating closed circular DNA species from linear DNA and nicked circles is described. The method is based on the difference in mobility of form I (supercoiled) DNA and form II (nicked circles), and the differential mobility of relaxed circular DNA in the presence and absence of ethidium bromide (EtdBr). It can be used for detection or for purification of plasmid, episomal, or viral DNA from the bulk of cellular DNA, or from other DNA mixtures.

INTRODUCTION

Autonomously replicating supercoiled DNA species are not easily detected in cell extracts such as Hirt supernatants of eukaryotic cells (1). Usually, nonintegrated episomes are demonstrated following digestion with several "no cut" and "single cut" restriction enzymes (2). DNA species with unknown molecular weight and unknown restriction pattern cannot be analyzed by the available techniques. Therefore, I have developed simple, rapid methods that facilitate detection and isolation of closed circular DNA present in a mixture of linear fragments. The methods can be also employed in a variety of studies on reactions of DNA by helping in detection of intermediates of replication, presence of intertwined circles, reaction products of topoisomerases and other reactions in which closed circular species are involved.

The method for detection of supercoiled DNA is based on the difference in mobility between form I (supercoiled) and form II (nicked circles). The DNA is run in the first dimension as form I. It is then nicked by exposing the gel to high intensity ultraviolet (UV) irradiation in the presence of ethidium bromide (EtdBr) and the DNA is run in the second dimension as form II. Relaxed, closed circles are detected due to their different mobilities in the absence and presence of EtdBr (3, 4).

To exploit this property the gel is run in one dimension in the absence of EtdBr and in the second dimension in its presence.

METHODS

DNA of bacteriophage λ, digested with the restriction endonuclease Hind III, and DNA of pBR322 (5) and of SV40 were purchased from BRL. Plasmid pML2 DNA (kindly supplied by Drs. Lusky and Botchan (6)), was prepared in E. coli HB101 by the standard methods. Closed circular, relaxed SV40 DNA was prepared by a partial digestion with λ int gene product (7).

Electrophoresis was performed using 0.8% agarose gels of variable sizes. The gels presented in Figs. 1 and 2 were 25 cm x 20 cm, 0.5 cm thick. Samples were applied at 2 start lines, at 2.5 cm and at 13.5 cm, using every third well. The gel was run in TE buffer (40 mM Tris, 20 mM Na acetate, 2 mM EDTA, pH 7.8). The gel was run for 4 hrs at 100 V (constant voltage) (Fig. 2) or for 18 hrs at 20 V (Fig. 1). It was then cut cross-wise at 11 cm from the start and immersed in TE buffer containing 1 μM EtdBr for 30 min. From this stage on, one-half of the gel was kept in the dark (Figs. 1, 2, middle). The other half was photographed (Figs. 1, 2, top), and irradiated by UV light (5.4×10^7 ergs/cm^2) to accomplish significant nicking of the supercoiled species. For UV source I used 2 UV germicidal bulbs mounted in parallel at 2.5 cm from the gel, without filters. Both gel-halves were then turned at 90° with respect to the original orientation and were run overnight (in 2 separate apparatuses) in TE buffer containing 1 μM EtdBr, at 20-25 V.

RESULTS

A mixture of linear DNA fragments of different sizes (λ DNA cut by Hind III) and SV40 DNA form I and II (nicked circles) were separated by electrophoresis in agarose gel (Fig. 1, top, lane 1). The gel was then incubated in 1 μM EtdBr for 30 min and run in the second dimension with 1 μM EtdBr in the running buffer. The linear fragments and SV40 form II DNA became arranged along a diagonal line, as their mobility was almost unchanged from the first to the second dimension (Fig. 1, middle, lane 1). The negatively supercoiled SV40 DNA became partially relaxed in the presence of EtdBr and it moved more slowly in the second dimension relative to the linear fragments. For comparison, SV40 DNA (forms I and II) was run alone in lane 2.

Relaxed closed circular DNA was obtained by a partial reaction of

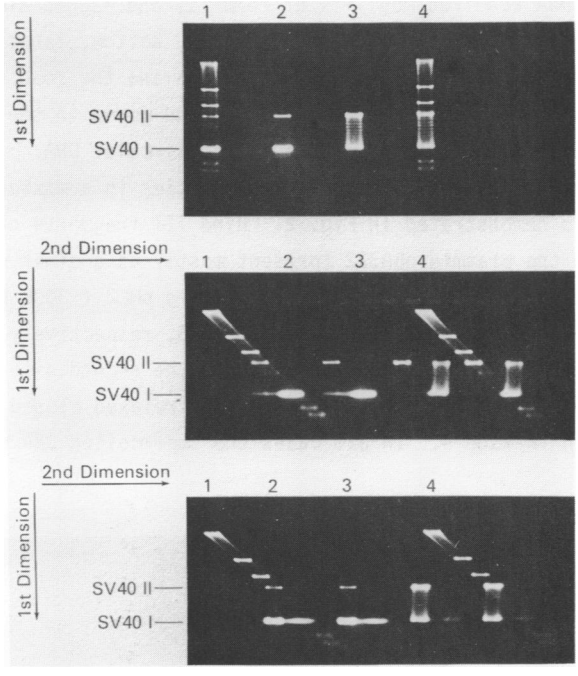

Figure 1: Separation of supercoiled and relaxed closed circles of SV40 DNA. Top: First dimension agarose gel was run without EtdBr and stained with 1 μM EtdBr. Lane 1, Hind III fragments of λ DNA and SV40 DNA; Lane 2, SV40 DNA; Lane 3, SV40 DNA, partially relaxed with λ *int* gene product; Lane 4, partially relaxed SV40 DNA and λ-Hind III fragments. Middle: Electrophoresis in the second dimension was in presence of 1 μM EtdBr, in the dark. Bottom: Second dimension electrophoresis was in presence of 1 μM EtdBr, after the gel was exposed to UV irradiation at 5.4×10^7 ergs/cm^2.

SV40 DNA with λ *int* gene product, with topoisomerase activity (7). The reaction mixture, which also contained intermediates of the topoisomerase reaction and nicked circular DNA, was then electrophoresed with (lane 4) and without (lane 3) λ Hind III fragments. In presence of EtdBr in the second dimension, the relaxed, closed DNA moved faster than, and separated from, the diagonal of the linear fragments and the SV40 nicked circles (Fig. 1, middle).

The separation of form I DNA from the diagonal of linear fragments was greatly enhanced by nicking the DNA before electrophoresis in the second dimension. Nicking was achieved by exposing the gel to high intensity UV light after it was incubated with EtdBr. A significant por-

tion of form I DNA acquired mobility of form II, and lagged significantly behind the diagonal of linear fragments (Fig. 1, bottom, lanes 1, 2). The majority of the relaxed, closed circular DNA and the topoisomerase intermediates also became nicked (lanes 3, 4) and moved in the second dimension with the same mobility as the nicked circular DNA.

Detection of different supercoiled DNA species in a mixture of linear fragments is demonstrated in Fig. 2. Hind III fragments of λ DNA were mixed with the plasmid pBR322 (present mostly as a dimer of 8724 bp), with SV40 DNA (5243 bp), or with the plasmid pML2 (~3000 bp), and electrophoresed as before (Fig. 2, lanes 1, 2, 3, respectively), without EtdBr in the first dimension and with EtdBr in the second dimension. For comparison, SV40 DNA (a mixture of supercoiled, relaxed closed and nicked circles) was run in lane 4. In all cases the supercoiled DNA was lagging

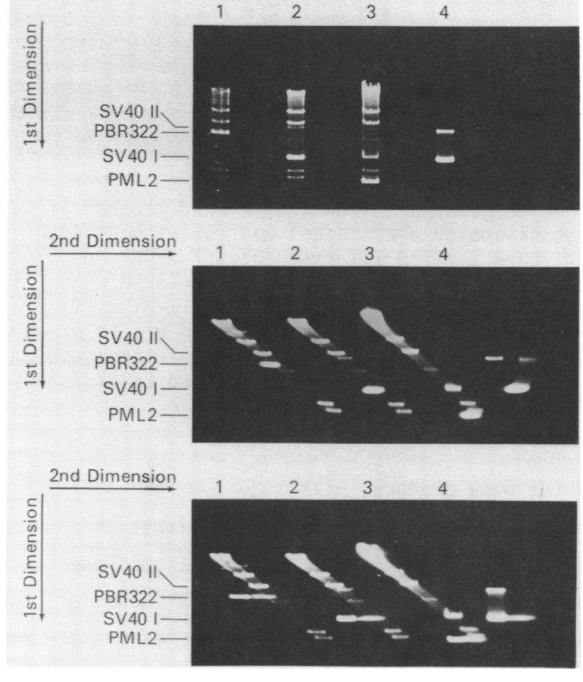

Figure 2: Separation of supercoiled DNA of different molecular weights. The gels were run as described for Fig. 1. Lane 1, λ-Hind III fragments and pBR322 (dimer) DNA; Lane 2, λ-Hind III fragments and SV40 DNA; Lane 3, λ-Hind III fragments and pML2 DNA; Lane 4, partially relaxed SV40 DNA. Top: First dimension electrophoresis without EtdBr. Middle: Second dimension with 1 μM EtdBr. Bottom: Second dimension with 1 μM EtdBr, after UV irradiation.

behind the diagonal formed by the linear fragments in the second dimension, due to the presence of EtdBr (Fig. 2, middle). As before, UV irradiation before the second run greatly enhanced the separation of the species that were originally supercoiled. Following the UV nicking, a small amount of a higher polymer of pBR322 (presumably a tetramer) could also be detected (Fig. 2, bottom, lane 1).

To optimize conditions for the UV nicking, SV40 DNA was run in 4 replicated groups. In each group DNA was run at 3 different levels: 125, 250, and 500 ng/lane. Electrophoresis was performed in the absence of EtdBr until the supercoiled DNA moved approximately 3 cm from the origin. The gel was then incubated with EtdBr for 30 min. It was cut lengthwise into 4 pieces, each containing 1 group of 3 lanes with the 3 levels of DNA. One piece was kept in the dark and the others were exposed to UV irradiation (lamp output at 180,000 ergs/sec/cm^2) for 30 sec, 90 sec, and 300 sec, respectively. The pieces were recombined and electrophoresis was continued in the same direction until form I separated from the UV-nicked form II. The gel was photographed, and the negatives were scanned with a Joyce-Loebl densitometer. Analyses of the scans are depicted in Fig. 3. Under the conditions that I have used, some nicking occurred at 90 sec. By 5 min the majority of DNA molecules were nicked at least once. The dose response was independent of DNA level at 125-500 ng/lane, probably because DNA concentration per gel area was the same.

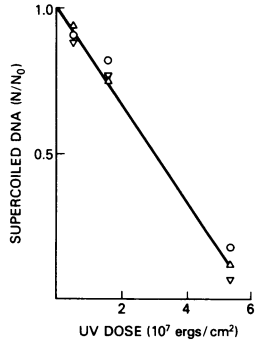

Figure 3: UV nicking of supercoiled DNA in an agarose gel: Dose response. The experiment was conducted as described in the text. SV40 DNA was applied to the gel at ⊙——⊙ 125 ng/lane; △——△ 250 ng/lane; ▽——▽ 500 ng/lane.

DISCUSSION

Intercalation of ethidium by DNA reduces the average rotation angle in the double helix (8, 9). The mobility of linear DNA fragments in agarose gels is almost unchanged in the presence of the dye (data not shown). Linear fragments of different size (at least up to 23.7 kbp, the largest λ Hind III fragment) form a straight diagonal line when electrophoresed in two dimensions, first without and then with EtdBr. Nicked DNA circles apparently have sufficient freedom of rotation around the single strand at the nick, and their mobility relative to the linear fragments is also not altered. However, because of the topological constraints, the closed circular molecules gain positive superhelical turns in EtdBr. Therefore, in presence of a low concentration of EtdBr, the negatively supercoiled form I DNA becomes partially relaxed and its mobility decreases (3, 4). The relaxed DNA becomes positively supercoiled and its mobility increases (4).

These properties of DNA and DNA-ethidium complexes were exploited in a two-dimensional electrophoretic system to detect closed circular DNA in a mixture of DNA molecules of different sizes. Under such conditions, the supercoiled circles lagged behind the diagonal of linear fragments whereas the relaxed circles ran ahead (Figs. 1, 2, middle.)

The number of positive superhelical turns introduced into the closed molecules depends on the amount of dye intercalated. Thus, if the concentration of EtdBr is increased, form I DNA first loses its supercoils and relaxes and then starts becoming supercoiled again, in the positive direction. Mobility of supercoiled DNA in agarose gels depends on the number of superhelical turns and is independent of the direction of these turns (4). Therefore, good separation between form I DNA and the linear fragments in the second dimension can only be achieved in optimal EtdBr concentration and under conditions of improved resolution (low voltage, reduced temperature). Optimal EtdBr concentration for maximum relaxation depends on the amount of supercoiled DNA in the band, which may be unknown. I obtained maximum relaxation at 0.5-1.0 μM EtdBr, using 100-500 ng supercoiled DNA per lane.

To increase resolution and simplify the method, a step of DNA nicking and conversion of a significant portion of form I DNA to form II was introduced between the 2 runs. This step greatly increased the detection level of supercoiled DNA as that DNA moved into the clear area behind the diagonal line. This is demonstrated in Fig. 2, where a slow migrating

species of pBR322 was resolved after the UV treatment (bottom, lane 1). The degree of nicking was not critical over a wide range, as long as detectable amounts of form I DNA were converted to form II. Incomplete nicking caused the originally supercoiled DNA to resolve into 2 (detectable) bands in the second dimension.

For identification and separation of form I DNA, the nicking step is therefore of great advantage. However, analyzing relaxed, closed circles is better accomplished when this step is omitted (and the gels protected from undue exposure to light in the presence of EtdBr). As relaxed, closed DNA becomes positively supercoiled in EtdBr, its separation from the diagonal of linears is not dependent upon a narrow optimum of EtdBr concentration. Under those conditions, closed, relaxed circles can be identified and separated from nicked circles present in the original DNA preparation (Fig. 2, middle).

Separation of DNA in two-dimensional agarose gels has been used by several authors to investigate specific properties of DNA. It was used in analysis of restriction patterns (10), in analysis of intertwined catenated dimers (11), in demonstrating double and single strand breaks (12) and in separating positively supercoiled circles from negatively supercoiled ones (4). The present method, of two-dimensional agarose gels in conjunction with UV nicking, was developed primarily for separating supercoiled DNA from linear fragments. However, theoretical considerations and preliminary studies suggest that intertwined circles could also be easily analyzed by this method. In addition, for mixtures of different multimeric DNA circles (such as during SV40 replication), the present method provides a quick assay for the different species. This is important since usually form I dimer has a similar mobility to form II monomer, and similarly higher multimeric species of different forms tend to run together.

The use of UV nicking of DNA on the gel simplifies the detection of supercoiled species. A similar approach has been independently developed for separating knotted DNA circles from simple circles (H. Nash, personal communication).

The method is very sensitive for detection of autonomously replicating supercoiled DNA in extracts of eukaryotic cells such as Hirt supernatants (1). Following appropriate labeling, when Hirt supernatants are run on gels they leave a smear of radioactive background, presumably due to the presence of randomly sheared cellular DNA. By the present

method the supercoiled DNA is moving out of that radioactive background. Therefore, supercoiled DNA is detectable even if it contains less radioactivity than the background in the smear. The level of detection can be further increased by using this two-dimensional gel system in conjunction with blotting and hybridization by the Southern method (13).

In conclusion, this method will probably be useful in a variety of studies in which rapid detection or isolation of closed circular double-stranded DNA (plasmids, non-integrated extrachromosomal episomes, or viruses) are desirable. It could also be employed in studies on reactions of DNA, such as intermediates of DNA replication, studies on topoisomerases and other reactions, enzymatic or others, in which closed circular DNA species are involved.

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*On leave from The Hebrew University--Hadassah Medical School, Jerusalem, Israel.

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