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Separations of open-circular DNA using pulsed-field electrophoresis

(DNA topology/agarose gels/electrophoretic mobility of DNA)

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ABSTRACT **The effect of high electric fields on the gel-electrophoretic mobility of open-circular DNA in agarose differs dramatically from that on linear molecules of the same molecular weight. At high fields, sufficiently large circular forms are prevented from migrating into the gel whereas linear molecules and smaller circular DNAs migrate normally. This effect is strongly field dependent, affecting circular molecules of decreasing size with increasing field strength. We have studied this effect with a series of plasmid DNAs ranging from 2.9 to 56 kilobase pairs using continuous and reversing-pulse electric fields. Application of reversing pulses abolishes the effect under certain conditions and supports the model for the gel electrophoresis of open-circular DNA where circular forms are trapped by engaging the free end of an agarose gel fiber.**

We show here that cycles of forward and reverse electric field pulses eliminate a troublesome anomaly in the electrophoretic mobility of open-circular DNA molecules in agarose gels. This anomaly prevents the separation of large open-circular DNA molecules by conventional electrophoretic methods.

Although it is well known that the properties of circular DNA in gel electrophoresis differ considerably from those of linear DNAs (1, 2), perhaps less well known is the fact that open-circular DNAs differ sharply in their electrophoretic behavior with respect to both supercoiled and linear molecules at high field strengths. Mickel *et al.* (1) observed that, at voltage gradients of 4–8 V/cm, open-circular molecules 20–30 kilobase pairs (kbp) in size no longer migrated as well-defined bands in agarose gels. Further, they observed that open circles significantly larger than this critical size had negligible mobilities, forming a band at the top of the gel. The critical size for the onset of this phenomenon decreases with increasing field strength, a fact that can place severe limitations on the voltage gradients that may be used to achieve separations of circular DNAs in size ranges of interest.

Gel-electrophoresis data obtained with pulsed and continuous electric fields are presented here for a series of open-circular DNA molecules ranging in size from 2.9 kbp to 56 kbp. The ability of reversing field pulses to abolish the effect is shown to be dependent on the width of the reversing pulse; experiments that are done with very short pulses yield mobilities the same as those observed with a continuously applied field. The data suggest that during electrophoresis, open-circular forms become ensnared by gel fibers, obstacles that may be negligible in low fields but trap open-circular DNA in high fields.

MATERIALS AND METHODS

Plasmid DNA Preparation. Plasmid DNA, generously provided by Marcin Filutowicz (Department of Biology, UCSD), was prepared from *Escherichia coli* strain HB101 by a modification of the alkaline lysis technique described by Maniatis *et al.* (3) and was twice purified by centrifugation through CsCl–ethidium bromide density gradients. Preparations containing at least 90% open-circular forms were obtained by partial digestion of the plasmids with pancreatic DNase I in the presence of ethidium bromide (4). Reaction mixtures contained DNA at 0.3 mg/ml in 20 mM Tris·HCl (pH 7.5), 2 mM $MgCl_2$, 5% (vol/vol) glycerol, ethidium bromide at 1.0 mg/ml, and pancreatic DNase I at 0.1 μg/ml (Calbiochem) and were incubated in the dark at 37°C for 1 hr. The DNA was extracted three times with phenol, once with chloroform, and three times with ether. Traces of organic solvents were removed by incubating the DNA in an uncapped tube for 1–2 hr at 37°C. Linear plasmid DNA was prepared by incubating DNA at 0.3 mg/ml with 1–10 units of the indicated restriction enzyme in the appropriate digestion buffer as suggested by the supplier at 37°C followed by the organic-extraction procedure described above.

Gel Electrophoresis. Electrophoresis was carried out in a conventional horizontal submarine apparatus (CBS Scientific, Del Mar, CA) with electrophoresis buffer (7.7 mM H_3BO_4/2.3 mM $Na_2B_4O_7$/1.0 mM Na_2EDTA, pH 8.5) circulated at 600 ml/min by a Masterflex peristaltic pump (Cole Parmer, Chicago, IL) to a buffer reservoir in series with a glass condenser serving as a heat exchanger. The buffer temperature was monitored with a thermometer and maintained constant at 17.5 ± 0.2°C by circulating water from a temperature-controlled water bath through the heat exchanger. Reversing-pulse electric fields were generated by switching the contacts of the leads to the electrophoresis unit across the poles of a JMF2-1000 DPDT mercury-wetted relay (Potter and Brumfeld, Princeton, IN) connected alternately to the positive and negative terminals of the power supply. The relay was driven by a Wavetek (San Diego, CA) model 184 sweep generator in experiments where the reversing pulses were longer than 30 ms. Short pulses (<30 ms) were obtained using a Datapulse (Culver City, CA) model 101 pulse generator. In both cases, the pulse widths were set with an oscilloscope and high voltage probe connected in parallel with the electrophoresis unit. With the short pulses used in these experiments (2–250 ms), we have found that the voltage regulation circuitry present in conventional electrophoresis power supplies leads to instabilities and highly distorted pulses. The power supply used in these experiments is an old tube-based design giving reliable performance and rectangular pulses free from ringing over the entire range of pulse widths. We have since found that similar suitable designs are still available, such as the Kepco (Flushing, NY) HB series of power supplies. The rise and decay times of the field pulses will generally be functions of the buffer composition; under the conditions described here, the time constants for rise and decay of the field were approximately the same, <500 μs.

Agarose gels were prepared from SeaKem Laboratories (Rockland, ME) LE agarose (FMC, lot 61,975) by boiling a weighed suspension of the agarose powder in electrophoresis buffer and cooling the hot agarose to 65°C before readjusting the mass of the solution with distilled water. The gels were

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cast on 20-cm × 20-cm partially sandblasted glass plates in an oven maintained at 65°C, using 150 ml of the agarose solution. The solution was allowed to remain in the oven for 10–15 min before being removed to room temperature and allowed to cool with the comb in place overnight. Subsequently, the gels were either used immediately or stored at 5°C. DNA samples were diluted with electrophoresis buffer and contained DNA at 5 μg/ml, 4% (vol/vol) Ficoll, and 0.005% methylene blue. This mixture (5 μl) was loaded into a well of dimensions 6.75 mm × 0.75 mm × 3.5 mm. The DNA was initially electrophoresed into the gel with a steady field and without buffer circulation to minimize the possibility of band broadening due to repeated electrophoresing of the DNA into and out of the gel or to mixing of the sample with the buffer above the well. After a delay time, chosen so that the product of the electric-field strength and the time of electrophoresis was constant and equal to 9000 V·s/cm, the circulating pump and relay were started simultaneously. Under these conditions, the temperature reequilibrated within 15 min. Gels were stained with ethidium bromide at 0.5 μg/ml for 1 hr and destained in deionized water for 30 min before photographing.

RESULTS

Size Dependence of the Gel Mobility of Circular DNA. As illustrated in Fig. 1, the effect of increasing electric fields on the mobilities of open-circular plasmids in 0.5% agarose is dramatic. The gel in Fig. 1*A*, electrophoresed at an applied field of 1.0 V/cm, shows that all except the largest of the circular species have mobilities within a factor of four of those of the corresponding linear DNAs. At 7.5 V/cm, more typical of the voltage gradients normally used in agarose-gel electrophoresis of DNA, only the smallest circular molecule migrates normally, as shown in Fig. 1*B*. The three largest circular plasmids are present in extremely sharp bands at the top of the gel, just below the well, and the open-circular form of pMF10 is present in a smear ≈2 cm long. These results are in agreement with the data of Mickel *et al.* (1), which indicated that there is a critical size at which open-circular DNA migrates as a smear and above which the open-circular forms have negligible mobilities. Following a suggestion of these authors, we tentatively attribute this mobility reduction to the trapping of open-circular forms. Minor contaminants present in the lanes corresponding to open-circular plasmid DNAs in this and in a subsequent figure are primarily linear fragments present in comparable amounts in closed-circular (unnicked) preparations of the plasmids.

Closer inspection of the photographs in Fig. 1 reveals that the mobilities of the corresponding linear molecules are also field dependent. This field effect on the mobility of linear DNAs is well documented (5–9) and can be explained in terms of reptation models for gel electrophoresis by supposing that the DNA chain becomes oriented by the electric field due to biases imposed on the leading segment of the chain (10, 11). At large voltage gradients the high-field trapping phenomenon dominates other possible effects on the mobility of open-circular DNA, however.

The trapping effect is not due to an artifact of gel structure at the surface of the well, a conclusion based on the results of two-dimensional gel electrophoresis experiments (data not shown). A mixture of open-circular forms of pRK35, pMF10, and pMF34 were separated at low field strength (2.0 V/cm) in the first dimension and then electrophoresed under identical conditions at high field strength (10.0 V/cm) in the second dimension. Open-circular pMF10 (8.9 kbp) and pRK35 (20.9 kbp), which were well resolved in the first dimension, failed to migrate in the second. The open-circular form of pMF34 (2.9 kbp) migrated nearly the same distance in the two directions.

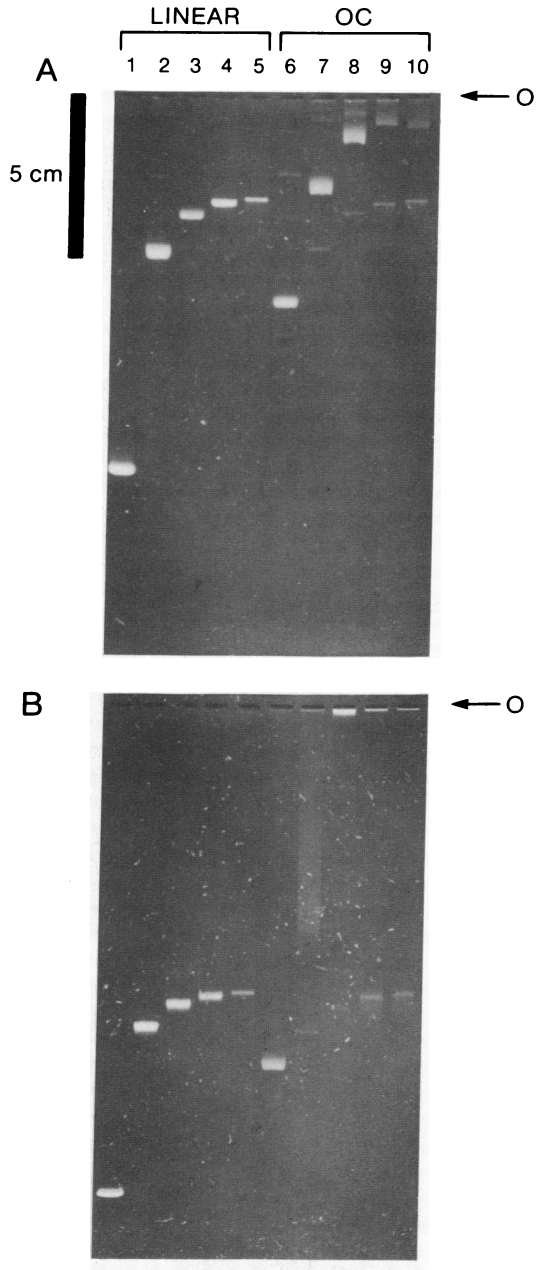

FIG. 1. Field-strength effects on the mobilities of linear and open-circular DNA. (*A*) Conventional gel electrophoresis of plasmid DNAs linearized at a unique restriction site (lanes 1–5) and open-circular plasmids (lanes 6–10) in 0.5% agarose at 1.0 V/cm for 25 hr. Plasmid DNAs are from left to right: pMF34 digested with *Pst* I (2.9 kbp), pMF10 digested with *Bam*HI (8.9 kbp), pRK35 digested with *Hpa* I (20.9 kbp), pR6K digested with *Bam*HI (38 kbp), pRK2 digested with *Bam*HI (56 kbp), open-circular (oc) pMF34, pMF10 (oc), pRK35 (oc), pR6K (oc), and pRK2 (oc). Faint, higher mobility bands corresponding to linear plasmid DNA are visible in lanes 8–10; these products were also present in small amounts in intact (unnicked) preparations of these plasmids. (*B*) Conventional gel electrophoresis of plasmid DNAs in 0.5% agarose at 7.5 V/cm for 3.33 hr. The three largest open-circular DNAs are trapped at the top of the gel and the open-circular form of pMF10 is present in the broad smear in lane 7.

The Effect of Reversing Pulses. Fig. 2 documents the ability of reversing field pulses to abolish the trapping of open-circular DNA at high fields [a technique used by Carle *et al.* (12) to achieve resolution of large linear DNAs]. The gel shown in Fig. 2*A* was electrophoresed at 10.0 V/cm with

forward pulses of duration, T_F, equal to 1.0 s and reversing pulses of duration, T_R, equal to 0.25 s. Under these conditions, all but one of the open-circular DNAs migrate as discrete bands, the exception being pMF10. The smearing of the pMF10 band is due to the initial application of a continuous field before actuating the function generator used to drive the relay coil. Under these conditions, open-circular pMF10 migrates some distance into the gel as a smear before the reversing pulses are applied; this smear then migrates downfield at a finite rate. The delay was chosen to minimize the possibility of band broadening due to repeated pulsing of the DNA into and out of the gel and could be reduced. The five open-circular plasmids are well resolved with this choice of cycle times, illustrating the value of this technique in improving the resolution of open-circular DNAs in agarose-gel electrophoresis.

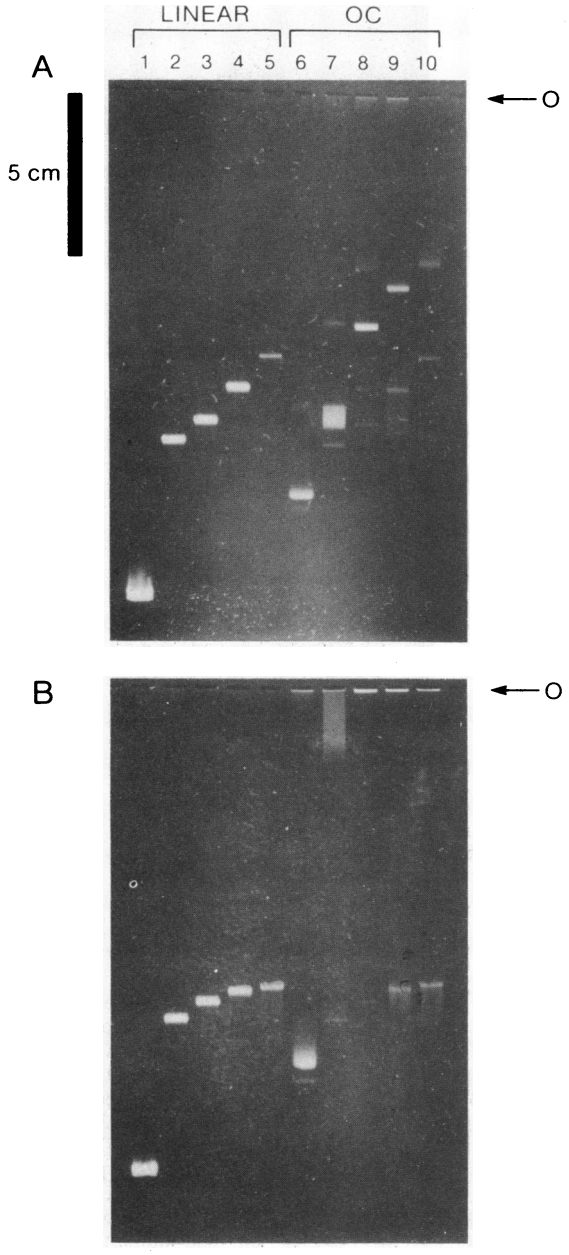

FIG. 2. Resolution of open-circular DNA using reversing pulses. (*A*) Electrophoresis of plasmid DNAs in 0.5% agarose at 10.0 V/cm for 4.17 hr with forward pulses of duration T_F equal to 1.0 s and reverse pulses of T_R equal to 0.25 s. Lane numbers correspond to the same plasmid DNAs as in Fig. 1. All of the open-circular species migrate as sharp single bands with the exception of pMF10, which runs as a smear under the continuous-field initial conditions that existed prior to turning on the pulse generator. (*B*) Plasmid DNAs electrophoresed under the same conditions as in *A*, except that T_R equals 2 ms and that the time of electrophoresis was 2.5 hr. This electrophoresis pattern is essentially the same as that observed with a continuous constant-polarity field.

It might be expected that the applicability of the technique would depend on the values of T_F and T_R; evidence that this is the case is shown in Fig. 2*B* where T_F is again equal to 1.0 s but T_R is now equal to 2.0 ms. With this choice of pulse widths, the electrophoresis pattern is essentially indistinguishable from that with a continuously applied electric field, indicating that the increased mobility of open-circular DNAs can only be obtained with a certain range of values for T_R.

DISCUSSION

The technique described here is an outcome of our efforts to understand the gel-electrophoresis behavior of DNA in detail. Our results show that trapping of open-circular DNA in agarose gels can occur at large voltage gradients, a phenomenon that may have important implications for interpreting the gel mobilities of other topological forms of DNA and also of protein–DNA complexes containing loop-like structures.

Indications that open-circular DNAs can be trapped in agarose and similar gels have been present in the literature for some time. Fuke and Thomas (13) showed that linear DNA can be separated from circular DNA by casting a mixture of the two forms in agar gels. By measuring the amounts of the different forms of DNA bound after extensively washing the agar gel with buffer, they found that up to 10 times more circular DNA than linear DNA was retained by the gel. Although the results of those experiments strongly suggest that circular molecules, unlike linear DNA, can become topologically linked with the gel network, true topological linkage with the gel fibers seems implausible in the case of gel electrophoresis. The data of Mickel *et al.* (1) clearly indicate that trapping occurs under conditions of conventional gel electrophoresis in high fields. The observation of these authors that the critical size for the onset of trapping of open circles decreased with increasing field strength led them to propose a model where open-circular forms become hung on the ends of agarose gel fibers. Mickel *et al.* (1) argued that their "hoop-and-stick" hypothesis provided a plausible basis for the size dependence, because larger molecules with their greater charge would not have sufficient thermal energy to completely free themselves from the agarose fiber, becoming effectively trapped in the gel. In the case of electrophoresis with strong electric fields, molecules ensnared in this way may become trapped because the probability of drifting upfield out of the trap depends exponentially on the voltage gradient through a Boltzmann factor. With this view, it is not surprising that occasional reversals of the electric field should diminish these high-field effects. One other prediction of this model that is consistent with our data is that the trapping of circular DNAs depends strongly on the width of the reversing pulse. This is because a finite time is required for molecules to reverse the path that led to the trap. In fact, the trapping effect can also be overcome by periodically turning off the field; this was pointed out to us by P. Serwer (14). A final point is that the effect of reversing-field pulses on circular DNAs is contrary to the effect on the mobility of linear DNAs observed by Carle *et al.* (12). Reversing-field pulses increase the high-field mobility of open-circular DNA whereas the sequences of forward and reversing pulses that enhance the resolution of large linear DNAs have the effect of reducing the electrophoretic mobility of linear molecules in a certain size range.

We do not presently know the details of the trapping mechanism at the molecular level. The fact that there is an intermediate range of field strengths where the relaxed-circular DNA band smears suggests that the traps in the gel are either fairly dilute or that they are quite heterogeneous in nature. It is certainly possible that features of the gel structure other than free ends, such as kinks in agarose fibers, may also be involved in trapping the DNA.

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