Pulse time and agarose concentration affect the electrophoretic mobility of cccDNA during electrophoresis in CHEF and in FIGE

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ABSTRACT

Circular DNAs have been shown to migrate in an unusual manner during field inversion gel electrophoresis (FIGE) and orthogonal field alternating gel electrophoresis (OFAGE). We studied the effect of varying pulse time and agarose concentration on the electrophoretic mobility of supercoiled (ccc) DNAs ranging from 2 kbp to 16 kbp during FIGE and contoured homogeneous electric fields (CHEF). Both supercoiled and linear molecules display a minimum mobility as a function of pulse time in a CHEF apparatus. Linear and cccDNAs of the same size are differently affected by pulse time. Pulse-time dependence was observed for cccDNAs in both systems. Pulse-time dependence in FIGE is very small at a 1.0% agarose concentration, but is pronounced in 0.8% or 1.2% gels.

INTRODUCTION

In continuous field (CF) agarose gel electrophoresis the electrophoretic mobility (μ) of a DNA molecule is influenced by its size (1–4), topology (5–8), applied field strength, ϵ , (4,6,8–10), ionic environment (1,4,6,7,11), agarose concentration, A (3,4,8), loading mass (12), and temperature (1,4,8,13). These parameters can be adjusted to give maximal resolution for DNAs up to 20 kbp. Pulsed-field gel electrophoresis (PFG) extends the separation range of agarose gel electrophoresis to larger DNAs (14). During PFG, the mobilities of different-sized fragments of DNA are altered in a size-dependent manner in response to the variations in the electric field. PFG technology has introduced new factors that affect the migration of DNA molecules. Two new factors introduced by PFG are pulse time and reorientation angle. It is not clearly understood how these new factors interact with factors previously studied in CF electrophoresis.

Few studies on the migration of circular DNAs during PFG have been reported (15–18). Mathew et al. (17) suggested that reorientation times might be a function of both size and topology of the DNA molecule undergoing PFG. Electrophoretic studies involving different topological forms of DNA could be meaningful to theories about the migration of DNA in agarose during PFG. Using field-inversion gel electrophoresis (FIGE) (19), Levene and Zimm (16) studied the migration of open circular (oc) DNAs at various field strengths. They showed that open circular (oc) DNAs from 2.9 kbp to 56 kbp, when subjected to a 1-s forward (P_f) and 0.25-s reverse pulse (P_r), display greater electrophoretic mobilities than when the pulsing is omitted. Shorter pulses do not alter the mobilities of ocDNAs. Thus, varying pulse times can cause alterations in the value of μ for one topological form of circular DNAs. Studies using orthogonal field alternating gel electrophoresis (OFAGE) (20) configurations suggest that changes in the mobility of circular topoisomers in relation to linear DNAs are caused by pulsing of the electric fields (15,17,18). In the double inhomogeneous (di) OFAGE configuration, the mobility of supercoiled (ccc) DNAs is

constant over a wide range of pulse times (15). The degree of supercoiling also was shown to have a dramatic effect on plasmid migration. Beverley (18) showed that a complex behavior is observed in OFAGE for cccDNAs of 30 kbp and 85 kbp. Directional and quantitative differences are observed for cccDNA mobilities when compared with larger yeast chromosomes, with which they co-migrate. There are no systematic reports about the electrophoretic mobility of circular DNAs undergoing PFG in electrophoresis using a contoured homogeneous electric field (CHEF) (21).

The present study investigates the influence of pulse time and agarose concentration on the electrophoretic mobility of cccDNAs undergoing FIGE and CHEF. We observed a pulse-time-dependent behavior in the migration of all sizes of cccDNAs studied. This pulse-time dependency was influenced by agarose concentration.

MATERIALS AND METHODS

Agarose gel electrophoresis

Agarose (Seakem LE, FMC) gels (11 cm×14 cm×3 mm) were prepared as described (22). A modified tris-borate buffer, TBEM (17), a tris-acetate buffer, TAE (22), or a tris-borate buffer, TBE (22), was used at either full (1×) or half (0.5×) strength. The agarose solution was stirred gently and cooled to 55°C before being poured. The gels were allowed to polymerize in the casting tray overnight at room temperature before being loaded. A Saran Wrap cover was used to prevent excessive dehydration of the gels. Programmable pulse controllers or power supplies were used to control the duration of the runs. Constant voltage was maintained for all experiments. Current and temperature were monitored throughout the runs. For CF electrophoresis, an International Biotechnologies Incorporated (IBI, New Haven, CT) model MRH horizontal gel box was used. The interconnecting port was kept open to maintain uniform pH during electrophoresis. For CF gels the initial temperature was 22°C, and the equilibrium temperature was 27°C ($\pm 3^\circ\text{C}$). Temperature was maintained by recirculating the running buffer by using a peristaltic pump set at a flow rate of 25 ml/min. Actual voltage gradients (ϵ) were measured directly with a voltmeter. The voltmeter reading was divided by inter-electrode distance.

FIGE gels were run in either an MRH box or an SE600 (Hoefer Scientific Instruments, San Francisco, CA) vertical slab box. Either a PC750 (Hoefer Scientific Instruments, San Francisco, CA) or a Minipulse (IBI, New Haven, CT) pulse controller was used to control pulsing time. A constant forward pulse:reverse pulse ($P_f:P_r$) ratio of 3:1 was maintained for each experiment. Temperature and voltage gradients were measured in the same manner as in CF gels. Ramped FIGE gels (14 cm×16 cm×3 mm) were run in an SE600. All ramps increased linearly with time from the initial P_f value to the final P_f value. The temperature was maintained at 13°C.

Electrophoresis in CHEF was done by using a Pulsaphor Plus system (Pharmacia/LKB, Piscataway, NJ) and a hexagonal electrode kit. The voltage gradient was estimated by dividing the voltage read from the power supply by the distance between electrodes. A correction factor of 0.8 was used, to produce a more accurate estimation of the value of ϵ within the buffer. The running temperature was maintained at 13°C.

Figure 1. Plots of the electrophoretic mobility of supercoiled and 1-kbp ladders as a function of size for electrophoresis in CHEF at different pulse times. A) supercoiled ladder; B) 1-kbp ladder. The insert on the upper right-hand corner contains the figure legends for both ladders, showing the curve that corresponds to each pulse time studied. Agarose concentration was 1%, and the runs lasted 16 h. Nominal voltage gradient was 6.4 V cm^{-1} , and the running buffer was 0.5×TBE.

Circular and linear DNA ladders

The supercoiled ladder was purchased from BRL (Gaithersburg, MD) (size (kbp) = 16.14, 14.17, 12.14, 10.10, 8.07, 7.05, 6.03, 5.01, 3.99, 2.97, 2.07). The ladder was separated into aliquots upon arrival and used at a concentration of 200 ng/10 μ l. To avoid accumulation of ocDNA in the ladder, the aliquots were frozen immediately and stored at -20°C until use. Aliquots were checked routinely on conventional gels for the presence of oc bands. Batches containing large amounts of ocDNAs were not used.

The following linear size standards were used for size estimation: 1) a lambda/*Hind*III digest (size (in kbp) = 23.130, 9.416, 6.557, 4.361, 2.322, 2.027, 0.564, 0.125); 2) a high molecular weight ladder (BRL, Gaithersburg, MD, size (in kbp) = 48.502, 38.416, 33.498, 29.942, 24.776, 22.621, 19.399, 17.057, 15.004, 12.220, 10.086, 8.612, 8.271); 3) lambda monomer (size (in kbp) = 48.50); and 4) a 1-kbp ladder (BRL, Gaithersburg, MD., size (in kbp) = 12.216, 11.198, 10.180, 9.162, 8.144, 7.126, 6.108, 5.090, 4.072, 3.054, 2.036, 1.636, 1.018, 0.517, 0.506, 0.396, 0.344, 0.298, 0.220, 0.201, 0.154, 0.134, 0.075). All linear standards were aliquoted and stored at -20°C upon arrival. Linear standards were heated at 65°C for 10 min, then chilled on ice for 2 min before being loaded.

Calculation of electrophoretic mobility

After electrophoresis, gels were stained in ethidium bromide (100 ng/ml) overnight. Gels were destained in a 0.1% aqueous solution of H_2O_2 for 30 min. The migration (cm) was measured for circular and linear bands from photographic negatives. The electrophoretic mobility, μ ($\text{cm}^2 \text{V}^{-1} \text{s}^{-1}$), was calculated for each supercoiled and linear DNA by dividing its migration (cm) by the product of ϵ (V cm^{-1}) and the duration of the run (s). Each μ represents an average of measurements from at least two gels. Graphs of $\mu = f(\text{size})$ were prepared for the supercoiled ladder and the linear standards of each gel. These graphs were used to determine the crossover point (in kbp), which is the size at which linear and cccDNAs of the same size co-migrate.

RESULTS*Electrophoresis of cccDNAs in CHEF*

In all FIGE and CHEF gels, we found that the migration of the linear DNA ladders fit by using the SIZER program available on BIONET (23). SIZER considered all ladders a very good fit, with a standard error always below 0.280. SIZER strongly recommended against deleting any bands. SIZER-produced estimates of the cccDNA size by using the Southern (24), Spline (25), or Duggleby (26) methods, invariably judged the larger cccDNAs off-scale because there were no linear standards that displayed migration distances within 10% of the larger cccDNAs.

In Figure 1A, $\mu_{\text{ccc}} = f(\text{size})$ plots are shown for the various pulse times studied with the cccDNAs. The lowest values for μ_{ccc} were produced by a pulse time of 10 s. The largest values of μ_{ccc} in electrophoresis using CHEF were produced by the 25-s pulse time.

Figure 2. Plots of electrophoretic mobility of supercoiled DNAs as a function of pulse time and of crossover point as a function pulse time for electrophoresis in CHEF. A) μ_{ccc} plotted as a function of pulse time for select cccDNAs ranging from 5 kbp to 16 kbp (the smaller supercoiled DNAs were omitted for clarity, although their behavior is similar); B) crossover point plotted as a function of pulse time. The insert below A) is the legend for the sizes of the cccDNAs that correspond to each curve. Gels were 1% agarose and were run for 16 h at a nominal voltage gradient of 6.4 V cm^{-1} . Electrophoresis was done in $0.5\times\text{TBE}$.

The 3-s, 5-s, and 15-s pulse times produced approximately equal values of μ for all cccDNAs studied. Figure 1B is a plot of $\mu = f(\text{size})$ for a 1-kbp linear DNA ladder. Both 1-s and 10-s pulse times slowed the migration velocity of linear DNAs, although the 1-s pulse time slowed the larger (greater than 5 kbp) DNAs more than the smaller DNAs. The descending order of μ for the linear DNAs can be expressed as $25\text{ s} > 15\text{ s} > 5\text{ s} > 3\text{ s} > 10\text{ s} \approx 1\text{ s}$. For the 23.13-kbp λ /HindIII fragment, the sequence of decreasing values of μ was: $15\text{ s} \approx 25\text{ s} > 5\text{ s} \approx 3\text{ s} > 10\text{ s} > 1\text{ s}$. This sequence is similar to that observed for the 1-kbp ladder. The running conditions for this gel produced band compression of the high molecular weight fragments of the linear ladder. Therefore, the high molecular weight fragments of the linear ladder were not plotted.

The response to pulse time of cccDNAs studied was similar for all sizes (Fig. 2A). The crossover point was plotted as a function of pulse time in Figure 2B. All crossover point values shown in Figure 2B were smaller than the value obtained from a gel subjected to continuous electric fields (not shown). In general, increasing the pulse time lowered the value of the crossover point. The response of the crossover point to increasing pulse time was similar to the response to increasing ϵ or A (over 0.5%) observed by Serwer and Allen (8).

FIGE of cccDNAs

We conducted studies in CF electrophoresis (not shown) for comparison with FIGE and CHEF data. As reported in other studies (7,17), the crossover point was higher in TAE than in TBE running buffers. We found that the use of $0.5\times$ TBE or TBEM did not affect the crossover point in CF electrophoresis. The crossover point in TBEM at $A = 0.8\%$ was similar to the crossover point in TAE at $A = 1.0\%$.

The effect of pulse time and agarose concentration on μ vs. kbp plots for ccc and linear DNAs of similar size is presented in Figure 3. For cccDNAs in 0.8% gels (Fig. 3A), μ decreased in the following order: $0\text{ s} > 72\text{ s} \approx 120\text{ s} \approx 0.12\text{ s} > 1.2\text{ s} \approx 12\text{ s} > 36\text{ s}$. At $A = 1.0\%$ (Fig. 3B), the decreasing order became: $0\text{ s} > 72\text{ s} \approx 120\text{ s} > 1.2\text{ s} > 0.12\text{ s} \approx 12\text{ s} \approx 36\text{ s}$. For 1.2% gels (Fig. 3C), the behavior of μ_{ccc} with respect to pulse time became more complex. At a 0.12-s pulse time, the larger (size $> 7\text{ kbp}$) cccDNAs migrated even more quickly than in CF electrophoresis.

In Figure 3 D–F, $\mu = f(\text{size})$ is plotted for the linear DNA ladder. As the value of A was increased, the effect of pulse time on the mobilities of linear DNAs became more pronounced. There was a complex effect of A on μ_{linear} . At $A = 0.8\%$ (Fig. 3D) the lowest μ for all sizes was produced by a 36-s pulse time, whereas a 0.12-s pulse time produced the lowest μ 's at $A = 1.0\%$ (Fig. 3E). At $A = 1.2\%$ (Fig. 3F), a 0.12-s pulse time produced the lowest μ 's for DNAs greater than 5 kbp, whereas the smaller DNAs migrated slowest at a 12-s pulse time.

The plot of crossover point as a function of pulse time for FIGE gels of different A values is shown in Figure 4. At all values of A studied, there was an increase in the crossover point for the 0.12-s pulse time. The crossover point for pulse times of 1.2 s and greater

Figure 3. Plots of electrophoretic mobility as a function of size for supercoiled and 1-kbp ladders in FIGE at different agarose concentrations. A–C) supercoiled DNA ladder; D–F) 1-kbp ladder. Inserts on the upper right-hand corner of each plot show the legend containing the pulse time which corresponds to each curve. Gels were run at a voltage gradient of 5.71 V cm^{-1} for 7 (0.8% A), 8 (1.0% A), or 10 (1.2% A) h in $0.5\times$ TBE.

were similar at $A = 1.0\%$. At $A = 0.8\%$ and 1.2% , the crossover point was more dependent upon the pulse time, and displayed a complex behavior. This complex behavior was even more pronounced at values of A lower than 0.8% (not shown).

DISCUSSION

For the classes of DNAs studied, we have shown that the electrophoretic mobility of cccDNAs during PFG in FIGE and CHEF systems is influenced by pulse time. In addition, we have observed an interaction between pulse time and agarose concentration in FIGE. Depending on the agarose concentration, the influence of pulse time on μ can be great or small. The μ 's of small linear DNAs were also affected by these parameters, albeit in a different way. The mobilities of both supercoiled and linear molecules display a sharp minimum as a function of pulse time, in a CHEF apparatus (FIG 2A and 2B).

The pulse-time-independent mobility of cccDNAs during FIGE in 1.0% TBEM gels observed here (Fig. 3B) is similar to that observed by Mathew et al. (17), Beverly (18), and Hightower et al. (15) in OFAGE. However, when A was varied, we show that the mobility of cccDNAs became increasingly pulse-time dependent, especially at higher values of A (Fig. 3C). Some pulsing regimes actually accelerate the larger supercoiled molecules (for example, a forward pulse time of 36 s, in Fig. 3C). This effect may be similar to that observed by Levene and Zimm (16) for ocDNAs. Mathew et al. (27) found a sharp transition in μ at approximately 1.1% agarose for linear DNAs in OFAGE. It is well established in CF electrophoresis that μ_{ccc} is greatly affected by A (5,8). Interestingly, studies showing pulse-time independence of μ_{ccc} were done at $A = 1.0\%$, which is within the transition region. Migration of ocDNAs in FIGE have also been shown to be pulse-time dependent (16). Whether this field dependence of μ_{ccc} can be attributed solely to topology and A or whether the reorientation angle of FIGE contributes to pulse-time dependence is unknown. Our CHEF results (Fig. 2A) also suggest pulse-time dependence of μ_{ccc} , although a limited number of pulse times were investigated. The point at which ccc and linear DNAs of the same size co-migrate (crossover point) in FIGE was pulse-time independent at $A = 1.0\%$ (Fig. 4). The crossover point was constant at approximately 8 kbp for P_f times between 1.2 s and 120 s. The exact value of the crossover point observed was higher than the 5 kbp value reported by Mathew et al. (17). However, the pulse-time independence is consistent with observations in OFAGE at $A = 1.0\%$ (15,18). Another observation consistent with OFAGE is that a very short P_f (0.12 s) caused an increase in the crossover point. Mickel et al. (7) found an inverse relationship between crossover point and A in continuous field electrophoresis. Our data showed a pulse-time-dependent behavior of the crossover point at $A = 1.2\%$ and $A = 0.8\%$, which suggests that there may be a pulse time/agarose interaction influencing the crossover point (Fig. 4). We also observed a pulse-time-dependent behavior of the crossover point in CHEF at $A = 1.0\%$ (Fig. 3B). This is in contrast to OFAGE (17) for the same value of A . Also in contrast to OFAGE, we found that the lowest values of μ_{ccc} in CHEF were produced by a 10-s pulse time (Figs. 1A and B). A 10-s pulse time produced the largest

Figure 4. Plot of crossover point as a function of pulse time in FIGE. The legend appears on the lower-right hand corner of the figure. For clarity, the lower left-hand corner of the plot shows an exploded view of the behavior of the crossover point at pulse times 0 s, 0.12 s, and 1.2 s. A constant 3:1 ratio was maintained for the forward and reverse pulse times, respectively. Gels were run at a voltage gradient of 5.71 V cm^{-1} for 8 h in $0.5 \times \text{TBE}$.

values of μ_{ccc} in OFAGE (17). A resonance-like effect may explain the behavior of μ_{ccc} in OFAGE (17). Further studies are needed to understand the behavior of μ_{ccc} in the various PFG systems studied.

Hightower et al. (15) have shown that cccDNAs undergoing OFAGE display a different size-mobility relationship when compared with linear DNAs. It has been shown that the μ 's of cccDNAs between 4 kbp and 16 kbp are similar to the μ 's of linear DNAs between 260 kbp and 2600 kbp. Our data from ramped FIGE (not shown) show that cccDNAs between 10 kbp and 16 kbp display μ 's in the same range as linear DNA μ 's of 300 kbp to 400 kbp. Together, these data support a different size-mobility relationship of cccDNAs during PFG as compared with linear DNAs. Clearly, PFG produces effects on cccDNAs that cannot be explained solely by their sizes. Serwer (28) proposed a gel hysteresis hypothesis to explain why circular DNAs migrate more slowly during PFG than linear DNAs of the same size. According to this hypothesis, if the reorientation angles are greater than 90° (but not greater than 180°), hair-like projections from the agarose gel fibers cause threading of all DNAs. Threading of linear DNAs is believed to be reversible. Reversible threading may be difficult for circular DNAs, however, and this may reduce the μ 's of circular DNAs.

Within the range of conditions studied, a complex interaction is observed between pulse time and agarose concentration. Although generalizations should be viewed as tentative until more extensive studies can be completed, it seems that lower values of A can be used to slightly increase μ_{ccc} and decrease running times. A pulse-time-independent behavior of μ_{ccc} can be achieved by using A within the transition region (approximately 1.0 % agarose).

Studies using a wide range of pulse times and agarose concentrations, analysis of Ferguson plots (29), and possibly logit transformations (30) would be useful to design mathematical equations for cccDNA migration during PFG. Ferguson plots allow the analysis of free electrophoretic mobility (μ_0) and coefficients of retardation (K_R) at various pulse times for different topological isomers (29). The study of these variables has produced improvements in the understanding of CF electrophoresis. A recent study using logit and probit analyses has yielded a mathematical equation for the electrophoretic mobility of linear DNA in CF agarose electrophoresis (30). It would be interesting to apply such mathematical methods to PFG gels and cccDNAs. These experiments could provide useful insight into the mechanisms of reorientation during PFG and practical parameters to obtain maximum resolution and gel performance.

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