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Characterization of the 'unusual' mobility of large circular DNAs in pulsed field-gradient electrophoresis

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ABSTRACT

Large circular amplified DNAs (30 and 85 kb) present in methotrexate-resistant Leishmania major appear to migrate anomalously in pulsed field-gradient electrophoresis (PFGE), exhibiting pulse time-dependent mobility and migrating along a different apparent path relative to the large linear chromosomal DNAs. Quantitative studies indicate that the relative pulse-time dependence is actually conferred by the mobility properties of the large linear DNAs. One contributing factor to the difference in migration path is variability in the intrinsic voltage-dependence of mobility of supercoiled and linear DNAs, in combination with the asymmetrical/inhomogeneous voltage gradients. Certain linear chromosomes exhibit a previously undescribed pulse-time dependence in the voltage-dependence of mobility. When enzymatically relaxed or physically nicked the large circular DNAs fail to leave the well using any pulse time, a property also observed in conventional electrophoresis. These findings are relevant to PFGE theory, and its application to the study of circular DNA amplification in Leishmania and other species.

INTRODUCTION

Circular DNAs are common elements of the genomes of prokaryotes and eukaryotes (1). Amplified genes present in drug-resistant organisms may also exist as extra-chromosomal circular DNAs, such as those present in drug-resistant Leishmania major and natural isolates of L. tarentolae (2-5). Two regions are amplified as circular DNAs in the methotrexate -resistant R1000 line of L. major and clonal derivatives thereof (2), the first example of a single cell bearing two separate amplifications. One of these, the R region, consists of a 30 kb circular species which contains the coding region for the bifunctional dihydrofolate reductase-thymidylate synthase of Leishmania (6,7). The other amplification, termed the H region, consists of a 85 kb circular DNA which consists of a large inverted repeat (2). Current data indicate that the H region encodes a drug resistance determinant, although the biochemical mechanism of resistance is

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unknown (5). Both of these extra-chromosomal circular DNAs were initially unstable in the absence of continued drug selection, and stabilized upon continued propagation in MTX (2). Gene amplification in Leishmania thus resembles this well-known phenomenon in cultured mammalian cells (2,5,8,9).

Recently, powerful new methods have become available for examining the chromosomal basis of gene amplification and stabilization in Leishmania. Very large DNA molecules can be separated electrophoretically by employing methods which periodically vary the directionality of the electric field. These "alternating field" methodologies include pulsed-field gradient electrophoresis (PFGE; 10), orthogonal field alternating electrophoresis (OFAGE; 11), field inversion gel electrophoresis (FIGE; 12), contour-clamped homogeneous electric field electrophoresis (CHEF; 13), and vertical PFGE (14). In the yeast Saccharomyces cerevisiae these large DNAs have been shown to correspond to the chromosomes previously defined by genetic methods (10,15). Similarly, alternating field electrophoresis of Leishmania DNAs reveals molecules whose sizes are similar to those observed in yeast, and by inference (in the absence of genetic mapping) these constitute the chromosomes (5,16-19).

It is known that circular DNAs migrate in an apparently "anomalous" fashion during PFGE (10) or OFAGE (4,11). For example, small circular DNAs (less than 16 kb) migrate in OFAGE in a pulse-time independent manner (20). I have utilized DNA from the MTX-resistant Leishmania to characterize the properties of large circular DNAs by PFGE, and quantitatively demonstrate that these large supercoiled circular DNAs also exhibit pulse-time independent mobility. However, in contrast to small circular DNAs, when the large circular DNAs are physically nicked or enzymatically relaxed they fail to enter the gel, a behavior very similar to that observed during conventional electrophoresis (26). This latter finding has important consequences to the use of PFGE methodology in the study of circular gene amplification.

METHODS

Cell Lines

The wild-type LT252 line of Leishmania major (21,22) and its MTX-resistant derivative R1000-3 line (2,23) were used in all experiments. After thawing, drug-resistant cells were propagated for no more than 2 weeks, due to the tendency of the amplified DNA of the R1000-3 line to rapidly stabilize (accompanied by the formation of multimeric forms of the originally monomeric circular amplified R DNA; 5).

Preparation of DNAs

Samples were prepared as described for trypanosomes (24), except that each agarose sample plug contained 10^7 promastigotes. Log or late log phase cells were used, although no differences in the chromosome pattern were observed among these or stationary phase cells (data not shown). Following protease digestion, the plugs were washed twice in 0.2 M Tris, 0.1 M EDTA, pH 8 (storage buffer), and kept at 4° until use. For enzymatic treatments the sample plugs were washed exhaustively in the storage buffer, and then washed twice at room temperature (2 hrs each) in enzyme buffer lacking magnesium, albumin and reducing agent, and then once with complete enzyme buffer prior to addition of enzyme. Enzymatic digestions were allowed to proceed overnight, and then the samples were washed twice in the storage buffer prior to electrophoresis. Topoisomerase I from calf thymus was purchased from BRL. Gamma-irradiation was performed using an ICN GR9 irradiator, containing a ^{60}Co source.

The 30 kb circular amplified R region contains a single Kpn I site (2), and was cloned into the KpnI site of a pUC vector. This recombinant is named pK300. Blot hybridization analysis was performed using probes specific for either the R or H region (2).

Pulsed field gradient electrophoresis

A PFGE gel box of the design originally described by Schwartz and Cantor (10) was used in these experiments, obtained from Biomedical Equipment, 445 E. 77th St., N.Y., N.Y. 10021. Unless otherwise indicated, the voltage on the north-south (non-uniform) axis was 320 V, and on the uniform east-west direction 135 volts. The pulse time is that interval at which the field directions were switched (1/2 cycle). For some experiments uniform voltage gradients were used. The temperature of the gel was maintained at 12-14° by recirculation of the buffer through a glass condenser. 1% agarose gels and 0.5X TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3) were used for all experiments. The samples were placed in wells cast along a angle of approximately 35° relative to the upper right corner of the gel, so that the net direction of migration was approximately perpendicular to the sample wells.

RESULTS

Identification of large supercoiled circular DNAs in PFGE

The chromosome patterns obtained following PFGE of the DNAs of the wild-type and R1000 lines of *L. major* are shown in Fig. 1A (40 second pulse time). The R1000 line exhibits additional intensely-staining DNAs relative to the wild-type, which appear to migrate somewhat out of the "track" evident for the remaining

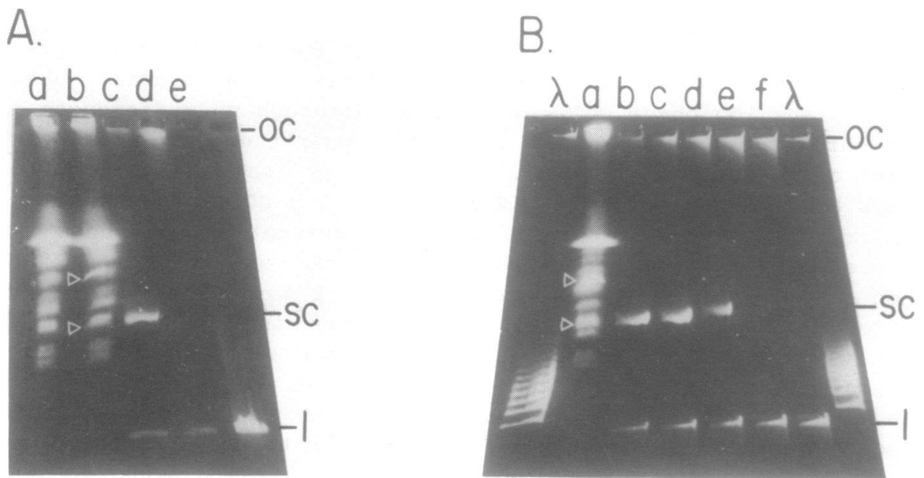

Figure 1. PFGE of _Leishmania_ DNAs. A. Mobility of chromosomal DNAs and circular topoisomers of a molecular recombinant containing the entire R region. The pulse time was 40 sec and electrophoresis was for 14 hr. Lane a, wild-type _L. major_; lane b, R1000-3; lane c, pK300; lane d, pK300 treated with topoisomerase I; lane e, pK300 linearized by digestion with Not I. The arrows in lane b indicate the position of the H (upper) and R (lower) circular amplified DNAs B. Effect of nicking on the mobility of pK300. L, lambda ladder; lane a, R1000-3 lanes b-f, pK300, gamma-irradiated with 0, 3000, 12000, 50000 and 200000 rads as described in the methods. The pulse time was 50 seconds and electrophoresis was for 15 hr. Topoisomers of the R-containing DNAs are indicated as: oc, open circle; sc, supercoiled circle; l, linear. The arrows in lane a indicate the position of the H (upper) and R (lower) circular amplified DNAs.

chromosomes. These DNAs represent the amplified DNAs present in the R1000-3 line, as revealed by their intense hybridization with probes specific for the R (Fig. 2A,B) or H region amplifications (Fig. 2C,D). In addition to the intensely hybridizing material, both R and H region probes reveal weak hybridization to another species in both the wild-type and R1000 samples (marked by small arrows in Fig. 2). These represent the wild-type type chromosomes respectively encoding the and R and H regions (5; manuscript in preparation). The wild-type R and H chromosomes remaining within the R1000 line are similar in both size (Fig. 2A,B) and copy number to that observed in the wild-type line (Fig. 2 and reference 2), indicating that this line has undergone "conservative" gene amplification (5).

Several additional criteria were used to demonstrate the circular nature of the new DNA species detected in the R1000 line. First, these DNAs were resistant to exonucleases, such as

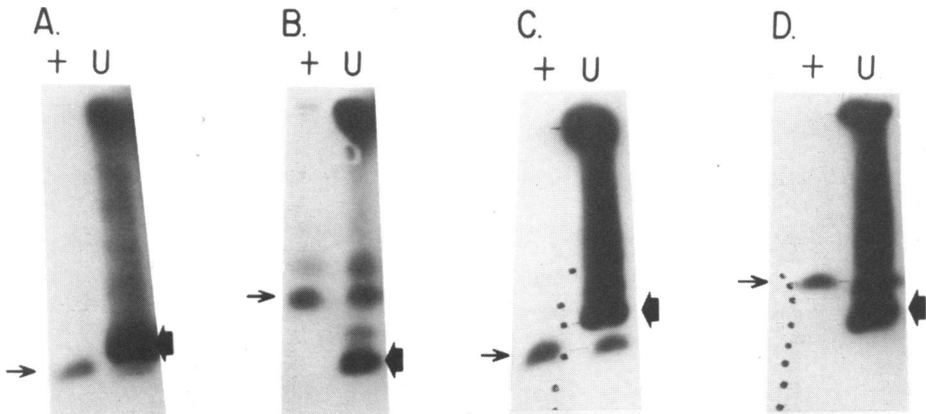

Figure 2. Mobility of circular amplified DNAs as a function of pulse time. Samples of wild-type L. major and the R1000-3 line were examined by PFGE, using pulse times as indicated below and 14 hrs of electrophoresis. The gels were blotted to Gene Screen Plus membranes and hybridized with radiolabeled probes specific for the amplified R (A,B) or H regions (C,D). A, 40 sec; B, 30 sec; C, 60 sec, D, 40 sec. +, wild-type LT252; U, the unstably MTX-resistant R1000-3 line. The position of the wild-type R or H chromosomes (approximately 500 and 900 kb, respectively) are indicated by small arrows, and the position of the amplified circular DNAs is indicated by large arrows. Residual hybridization to the sample well may reflect the formation of nicked circular DNAs during preparation or storage of the samples (see text). Additional hybridization evident represent either hybridization to the artificial "compression" band commonly observed in PFGE, or to small quantities of dimeric circular DNAs.

exonuclease III which completely digests the chromosomes (Fig. 3A), and exonucleases Bal 31, T4 polymerase (in the absence of deoxynucleotide triphosphates) and lambda exonuclease (data not shown). Secondly, the mobility of the circular DNAs exhibited sensitivity to low concentrations of ethidium bromide (0.05 ug/ml) which did not significantly affect the mobility of the chromosomal DNAs (Fig. 3B). Higher concentrations of ethidium bromide dramatically affect the mobility of both circular and linear DNAs. Thirdly, purified supercoiled circular amplified R and H DNAs, isolated from CsCl/ethidium bromide density gradients as described (2), migrate similarly to the novel species detected in PFGE (data not shown). Also, a 33 kb supercoiled circular recombinant plasmid bearing the entire 30 kb R region migrates in a manner similar to the 30 kb circular R DNA (Fig. 1A). Fourthly, these DNAs were sensitive to the action of

topoisomerase I (Fig. 3C). Hybridization experiments demonstrated that the topoisomerase I-treated amplified DNAs remained within the sample well (described below).

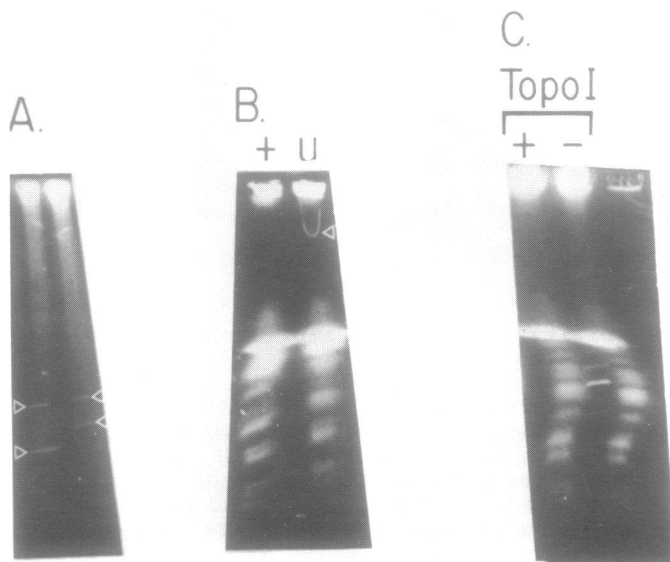

Figure 3. Effect of exonucleases, ethidium bromide and topoisomerase treatment on circular amplified DNAs in PFGE. The pulse time was 50 seconds in all experiments. A. Exonuclease III treatment. The left track shows a R1000-3 sample (as shown in Figure 1) was treated with exonuclease III (200 units/400 ul buffer plus sample plug) and analyzed by PFGE (20 hrs electrophoresis time). The right track shows a similarly treated sample of the stable R1000-11 line (2), which bears oligomers of the monomeric 30 kb R circle found in the R1000-3 line (unpublished data). The wild-type chromosomes evident in the samples evident in Fig. 1 have been digested, leaving the circular H (upper arrows in each track) or R amplifications (lower arrows). B. Effect of ethidium bromide on mobility. Samples of the wild-type (+) and R1000-3 (U) lines of L. major were washed in the buffer containing 0.05 ug/ml ethidium bromide, and electrophoresed for 15 hrs, with both the gel and buffer containing 0.05 ug/ml ethidium bromide. The unusually curved DNA evident near the origin (marked by arrow) in the R1000-3 line contains the circular amplified DNAs, as revealed by hybridization with specific R or H probes (not shown). C. Effect of topoisomerase I treatment. Sample plugs of the R1000-11 line were treated with topoisomerase I (+; units/400 ul buffer + plug) and analyzed by PFGE as described in part A, except that ethidium bromide was omitted. A control in which topoisomerase I was omitted is also shown (-). Note that the circular DNAs present in this sample exhibit a different migration path than the linear chromosomal DNAs.

Relative migration of large circular DNAs is dependent upon sample well position

The migration of the large amplified supercoiled circular DNAs relative to the large linear chromosomes in *Leishmania* varies dramatically with the sample well position. Fig. 4A shows the PFGE pattern obtained for samples of the R1000-3 line inserted into different gel slots, in which the the circular H amplification is readily visible (confirmed by hybridization analysis; data not shown). In the leftmost samples the circular H DNA migrates towards the center of the gel relative to the linear chromosomes, which migrate with a characteristic "bulge" (10,24). The mobility of the H circle is comparable to that of chromosomes approximately 1000 kb in size. In contrast, in the center of the gel the linear and circular DNAs appear to follow the same path, while at the right side the pattern is a very approximate mirror image of the leftmost samples, in which the linear chromosome migration is bowed to the right outer edge of the gel while the H circle migrates more closely to the center of the gel. Overall, it appears as though the circular DNAs migrate in an apparently straight line, whereas the large linear DNAs exhibit a complex and positionally-dependent pattern pattern. Additionally, the mobility of the circular H region relative to the chromosomal DNAs decreases across the gel, until in the rightmost sample the circular DNA migrates more slowly than the uppermost compression band. Blot hybridization experiments with probes specific for the R region revealed similar behavior for the 30 kb R circle (data not shown). Thus, under PFGE a complex behavior is observed, with both directional and quantitative differences in mobility of the 30 and 85 kb circular DNAs relative to the large linear chromosomal DNAs.

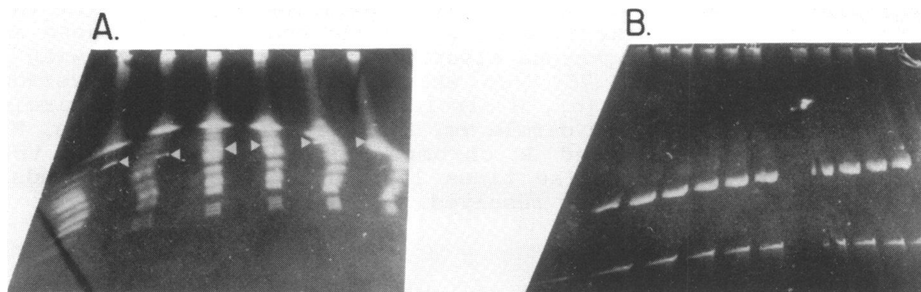

Figure 4. Effect of gel position on PFGE patterns of cellular and circular DNAs. PFGE was performed using a pulse time of 50 seconds for 15 hours. Identical samples were loaded in all lanes A. R1000-3 samples. Arrows indicate the position of the circular amplified H DNA (verified by hybridization). B. pK300.

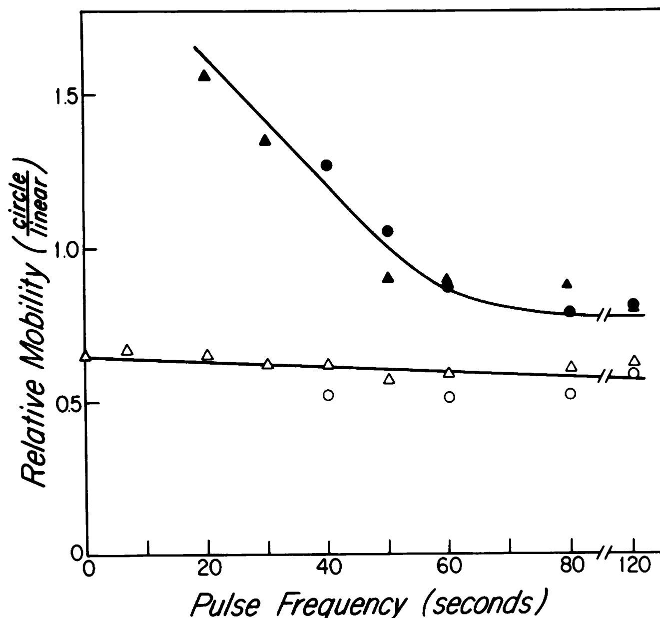

Figure 5. Quantitative dependence of relative mobility of circular R and H region DNAs upon pulse time. Samples of the R1000-3 line were electrophoresed for the same length of time (15 hr), temperature (12° C) and voltage gradients (see the methods). Additionally, the samples were loaded in the same gel position; this slot was the one which gave the most linear track of apparent migration (sample 3 from the left in Fig 3). The gels were blotted and hybridized successively with probes specific for either the R or H region amplifications. The mobility of the R and H circular, linear and chromosome DNAs were measured from the middle of the well to the middle of the hybridizing band, and the relative mobilities are shown in the figure. For the pulse time of 0 seconds the gel was placed in the PFGE box in a homogeneous electric field arrangement, with a voltage gradient of 6.75 V/cm which approximates the average obtained during PFGE. (o), H circle vs. H linear; (●), H circle vs. H chromosome; (△), R circle vs. R linear; (▲) R circle vs. R chromosome. The H and R chromosomes migrate within the compression region at pulse times less than 40 and 20 seconds, respectively, and were not resolved.

Relative migration of large circular DNAs is dependent upon pulse time

The pulse time-dependence of the mobility of the large circular amplified DNAs during PFGE is shown in Fig 2. At a pulse pulse time of 30 seconds the supercoiled circular R DNA migrates ahead of the 500 kb R chromosome, whereas at 40 seconds the

circular R region migrates slower than the R chromosome (Fig. 2A,B). A comparable behavior was seen for a 33 kb supercoiled circular recombinant molecule, pK300, bearing the entire R region (data not shown), and for the circular H DNA relative to the wild-type H chromosome (Fig. 2C,D).

The quantitative dependence of relative mobility upon pulse time was measured for both the R and H circular DNAs using PFGE, and with conventional electrophoresis under similar voltage gradients (Fig. 5). Due to the complex patterns evident in Fig. 4A for PFGE of circular and linear DNAs, measurements were made on samples electrophoresed starting from the same sample well (the middle well shown in Fig. 4), with the same voltage gradient, duration of electrophoresis, and temperature. At pulse times of 20-30 seconds the relative mobility is high, and progressively declines until a plateau of relative mobility appears at pulse times of 60 seconds or greater. This behavior is similar to that seen for small circular DNAs (<16 kb; 20).

The "anomalous" relative migration of circular DNA species is conferred by the anomalous migration of large linear DNAs

Several lines of evidence suggest that it is the linear DNAs which migrate unusually in PFGE, not the large circular DNAs. Fig. 5 also shows the relative mobility of the H and R circular DNAs relative to their 85 and 30 kb linear forms, which are present in the R1000-3 samples at low levels due to the presence of a small number of double strand breaks in these preparations (the linear nature of these 30 and 85 kb DNAs was confirmed by exonuclease III digestion). The mobility of the supercoiled circular DNAs relative to these smaller DNAs is independent of the pulse time, and is identical to the relative mobility obtained in conventional electrophoresis (equivalent to a pulse time of either zero or infinity). Moreover, the mobility of the supercoiled circular recombinant R plasmid relative to the linear form is nearly independent of position within the gel (Fig. 4B). The simplest interpretation of these data is that the large linear chromosomes exhibit dramatic positional and pulse time dependent migration, while the large supercoiled circular DNAs exhibit a relatively uniform mobility with respect to position and pulse time, a conclusion also recently found for small circular DNAs in OFAGE (20). Thus, the apparently "anomalous" migration of the supercoiled circular DNAs is actually an artifact of comparison with the large linear DNAs.

Differences in the apparent migration path can be caused by differences in the intrinsic voltage vs. mobility relationship for circular and linear DNAs

The basis for the complex relative migration patterns of DNAs during PFGE is currently not well understood. One explana-

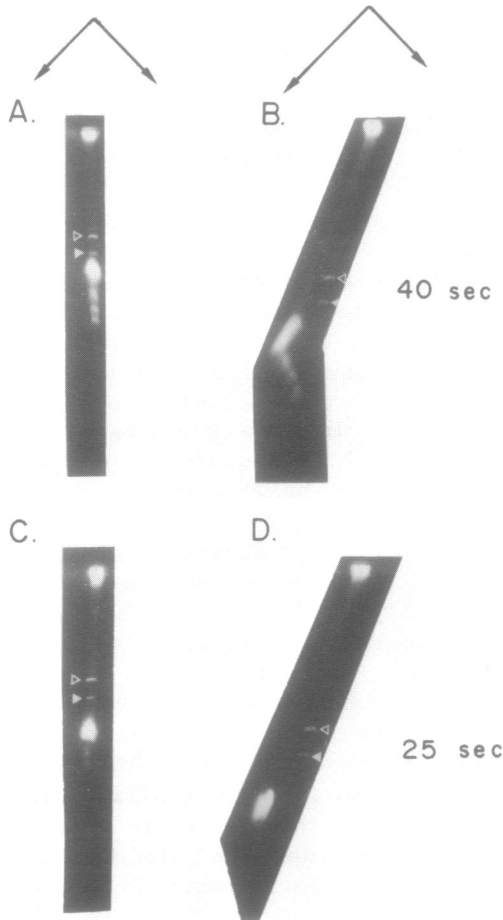

Figure 6. Directional Effects of Symmetric and Asymmetric Homogeneous Electric Fields on the Mobility of Circular and Linear *Leishmania* DNAs. The PFGE box was rewired so as to provide homogeneous electric fields, alternating at a 90° angle. Symmetric fields were 7.5 V/cm and asymmetric fields were 6.25 and 8.75 V/cm in the directions indicated by the small and large arrows above the figures. Samples were electrophoresed for 15 hr at 9° C using the indicated pulse times. The ethidium bromide-stained gels are shown. A. 40 sec pulse time, symmetric fields; B. 40 sec pulse time, asymmetric fields; C. 25 sec pulse time, symmetric fields; D 25 sec pulse time, asymmetric fields. The open arrows indicate the position of the circular H amplified DNA, the filled arrows the position of the circular R amplified DNA (verified by subsequent hybridization).

tion is that the voltage gradients attained during electrophoresis become distorted, though this view cannot easily account for the fact that linear and circular molecules applied in the same sample plug follow different apparent migration paths (Fig. 4A). Several studies have shown that electrophoretic mobility (proportional to the distance migrated divided by the product of time and applied voltage gradient) increases with voltage (32,33). Interestingly, this relationship varies among molecules of the same size, but of different conformations (26-28). Because PFGE employs different voltage gradients in each direction, it seemed likely that differences in the voltage-dependence of migration of circular vs. linear molecules could be one factor contributing to differences in the apparent migration path.

Due to complexities introduced by inhomogeneous electric fields, the effect of voltage gradient was examined using homogeneous alternating electric fields, oriented at a constant 90° angle. When the voltage gradients in both directions were identical (7.5 V/cm), circular and linear DNAs migrated along the same straight path (Fig. 6A,C). In contrast, when the voltage gradients were different (6.25 and 8.75 V/cm), the large circular DNAs migrated in a direction different than the majority of the linear molecules, migrating preferentially in the direction of the lower voltage gradient. Interestingly, Johnson and Grossman (27) have shown that in 1% agarose gels as the voltage gradient increases the mobility of linear DNAs increases more than that of supercoiled circular DNAs of identical lengths, up to voltage gradients of 6.6 V/cm. These data predict that the circular DNAs should appear to migrate towards the direction of the lower voltage gradient, as observed in our experiments (Fig. 6).

The direction of migration of the supercoiled circular DNAs relative to the majority of linear DNAs was independent of pulse time (Fig. 6B. vs. 6D, 25 and 40 secs; data for 60 sec not shown). Interestingly, at certain pulse times some of the smaller linear chromosomal DNAs also exhibit migration out of the apparent "track" followed by the majority of the linear DNAs (Fig. 6B,D), suggesting that the voltage-dependence of mobility for certain linear DNAs may be a function of the pulse-time.

Large open circular DNAs fail to migrate in PFGE or conventional electrophoresis

In PFGE topoisomerase-I treated pK300 fails to enter the gel (Fig. 1A, lane d). Because this DNA was loaded into the sample slot in free solution, this effect is not due to "trapping" of circular DNAs cast in agarose (25). Neither does it represent

catenation of supercoiled circular DNAs into a larger, interlocked network, as topoisomerase I does not catalyze this reaction (31). Similarly, the large amplified circular DNAS are lost after topoisomerase I (Fig. 3C) or topoisomerase II treatment, and correspondingly, hybridization analysis reveals material exclusively in the sample slot (data not shown). Again, this result cannot be due to agarose "trapping", as the cellular DNAs are not directly cast in agarose but instead are present in a cell-sized cavity formed within the agarose. The introduction of increasing amounts of single-stranded breaks by gamma-irradiation of pK300 causes the progressive loss of the supercoiled circular form, accompanied by increasing material at the well (presumably nicked circles) and eventually of the linear species (Fig. 1B). Similar results were obtained with the large circular DNAs in the R1000 line. Thus, the failure of large relaxed circular DNAs to migrate in PFGE appears to be solely dependent upon the lack of supercoiling.

Using conventional electrophoresis with buffers and voltage gradients similar to those in PFGE (6-7 V/cm), the 33 kb relaxed circular form of pK300 also failed to enter the gel. Similar results for large circular DNAs in conventional electrophoresis have been presented (26, 29). In contrast, smaller relaxed circular DNAs do enter agarose gels, either in conventional electrophoresis (26), OFAGE (20) or PFGE (data not shown). The precise molecular weight cutoff for entry into the gel is highly dependent upon ionic strength and voltage gradient (26,29,30). Nonetheless, it appears that as for supercoiled circular DNAs, the mobility properties of open circular DNAs are similar under PFGE and conventional electrophoresis.

DISCUSSION

Large supercoiled circular DNAs appear to exhibit unusual electrophoretic mobilities in PFGE, relative to that of the large linear chromosomes. These include a positional and pulse-time dependent relative mobility, and altered directionality of migration. However, I have shown by several criteria that the mobility of the large (30 and 85 kb) supercoiled circular molecules in PFGE appears to be largely unaffected by pulse time and gel position, and is comparable to that observed under conventional electorphoresis. This suggests that the "unusual" mobility is an artifact of comparison with the large linear DNAs, which clearly exhibit unusual mobility patterns under a variety of alternating field methodologies (10-13). Similar conclusions have been obtained for smaller circular DNAs in OFAGE (20).

The complex apparent migration paths of chromosomal DNAs in

PFGE are well known, though recent improvements such as CHEF (13) which employ symmetrical and nearly homogeneous voltage gradients yield much straighter migration paths. Nonetheless, attempts to understand the unusual paths may provide some insight into the biophysical basis for PFGE separation. One factor which may significantly affect the mobility of DNAs of varying length and topology in PFGE is the interaction of the non-uniform voltage gradients commonly used with molecules exhibiting differences in the voltage-dependence of mobility. It is well known that the relationship between mobility and voltage gradient varies among linear DNAs of different sizes and among circular topoisomers (26-29). The data shown in Fig. 6 suggests that voltage-dependent effects also occur in alternating field electrophoresis, in a manner consistent with the results obtained from conventional electrophoresis of circular DNAs (27). Interestingly, these results also indicate a voltage- and pulse time-dependence of the mobility of certain Leishmania linear DNAs. It seems possible that the interactions between the intrinsic voltage- and pulse-time dependence of the electrophoretic mobility of DNAs, in conjunction with variations in the the applied voltage gradients, may contribute to the complex patterns obtained in many PFGE apparati. For example, consideration of voltage gradients accurately predicts the relative directionality of circular vs. linear DNAs observed in the CHEF apparatus that we have employed, although the quantitative magnitude of the difference in directionality is much less due to the relative uniformity of the alternating electric fields (unpublished data).

The different properties of circular and linear DNAs during PFGE can be used to identify the circular DNAs in complex cellular DNA preparations (4,5 and this work). Diagnostic properties include migration outside the apparent "track" of the linear DNAs in response to variations in voltage gradient, pulse time-dependent mobility, exonuclease resistance, and ethidium bromide and topoisomerase sensitivity. Once identified, appropriate manipulations can be used to separate circular DNAs from the chromosomal DNA, either by using short or long pulse times, or by using asymmetric voltage gradients (Fig. 6).

The failure of large relaxed circular DNAs to enter gels in conventional electrophoresis has previously been described (26,29,30). One explanation for this phenomenon is the threading or "hoop and stick" model for the trapping of open circular DNAs (26). Interestingly, large open circular molecules can enter the gel in FIGE (using a certain range of pulse times; 29), an observation which is consistent with the hoop and stick model in which the reversed field "unthreads" the open circular molecule

from the agarose matrix. Presumably, the angle between the alternating fields in PFGE is insufficient to overcome threading of open circular DNAs. This is also true for CHEF electrophoresis, in which the angle is 120° (unpublished data).

The observation that large relaxed or nicked circular DNAs fail to enter the gel, either under PFGE or conventional electrophoresis, has important implications to the experimental analysis of gene amplification by these methods. This is because amplified DNAs from certain lines of *Leishmania* and cultured mammalian cells fail to enter the gel (unpublished data). Possible sources for this behavior include 1) integration into large linear chromosomal DNAs or 2) formation of new large linear DNAs, of sizes beyond the current limits of resolution; 3) formation of large catenated networks of circular DNAs; and 4) as shown in this work, the formation of large nicked or open circular molecules. Thus, it is necessary to discriminate among these possibilities when hybridization to the sample well is observed. Studies addressing this phenomenon in the case of *Leishmania* circular amplification are in progress.

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