Pulsed homogeneous orthogonal field gel electrophoresis (PHOGE)

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ABSTRACT

A versatile system (PHOGE) has been developed that allows resolution of molecules of DNA of megabase pair size by the use of homogeneous, orthogonal, pulsed fields. The resulting electrophoreograms have characteristics that differ from those produced by other systems for pulsed field electrophoresis. Molecules in a two-fold range of sizes can be separated with maximum resolution, or a much larger range of sizes may be separated with lower resolution but with a linear relationship of mobility to size from 50 kb, or below, to at least 1 Mb. Straight lanes and large useable gel areas, characteristic of PHOGE, are also valuable for mapping procedures or for any other circumstance in which large numbers of samples of DNA are to be directly compared. Existing models cannot explain the results obtained, because a stage of the molecular reorientation appears to result in a rate of migration greater than that occurring by reptation. We suggest a mechanism that might account for the resolution observed and also suggest that the resolution achieved by existing OFAGE-type systems may be the result of the superimposition of PHOGE and FIGE separatory mechanisms. No maximum size of molecules that may be resolved by the PHOGE system has yet been determined.

INTRODUCTION

Conventional electrophoresis resolves DNA molecules by a process of gel filtration (1). Above a certain size, however, all molecules, irrespective of length, migrate at similar rates and little resolution is achieved (2,3). It is thought that such molecules, the lengths of which are large relative to the gel pore size, adopt a conformation that is extended in the direction of the electric field and then migrate by a process termed reptation (4-6). The limit of efficient resolution by conventional electrophoresis is ca. 50 kb.

In 1983, Schwartz et al. (7) reported an electrophoretic system in which much larger molecules of DNA could be resolved by periodically altering the direction of the applied field. The observed resolution was proposed to be a consequence of the requirement for reorientation in the new direction of the field before migration by reptation could occur. The time taken for reorientation would be size-dependent, so that larger molecules would spend less time reptating than smaller ones and therefore would migrate a lesser
distance and be resolved (7,8). The now widely used derivative of this system (9) involves the alternating application of inhomogeneous fields using orthogonally oriented arrays of point electrodes and is termed "orthogonal-field-alternation gel electrophoresis" (OFAGE). It was reported (7,9) that in these original OFAGE systems, the application of homogeneous, orthogonal fields resulted in poor resolution. Pulsed field electrophoretic systems applying homogeneous fields oriented at 120° (the "CHEF" system, 10) or 110° (gel rotation system, 11) to each other have been developed and result in similar resolution, but produce straight lanes on electrophoretograms. We will refer to this group of systems, which use the same principles for resolution, as the OFAGE-type systems. A model has recently been proposed (11) to describe the mechanism by which resolution is achieved by these systems. According to this model, molecules retain their extended conformation when the field direction is changed and migrate in the new direction led by their formerly trailing ends. Resolution is thus achieved by a "ratcheting" mechanism, is dependent upon molecular length and requires an angle greater than 90° between the alternating fields. The inhomogeneity introduced into at least one of the applied fields is thought to have been necessary in the original OFAGE systems so that the molecules would experience field angles greater than 90°.

Another system for pulsed field electrophoresis that resolves large DNA molecules is "field inversion gel electrophoresis" (FIGE) (12). In this technique a homogeneous field is applied, the polarity of which is periodically reversed. Provided that the field is applied for a longer time or at a higher voltage gradient in one direction, size-dependent resolution is achieved. The electrophoretograms produced by FIGE show that the rate of migration is at a minimum for DNA molecules of a size that is dependent upon the pulse time; both smaller and larger species exhibit greater mobility. Bands may eventually be arrayed in an order monotonically increasing with size by the use of a pulse-time "ramp" (steady increase of applied pulse times throughout the duration of the electrophoresis). The resolution of large molecules of DNA by FIGE cannot be explained by the "ratcheting" mechanism proposed for the OFAGE-type systems. Instead, a mechanism involving a resonance phenomenon was proposed (12) and was recently modelled (13).

METHODS and MATERIALS
1. Design and Operation of the Apparatus
Our objective was to construct an electrophoretic system in which there would be a regular alternation between pulses that establish a uniform
conformation for all molecules to be resolved and pulses that resolve pre-oriented DNA molecules of different sizes. For the sake of simplicity, we used homogeneous electric fields oriented at right angles; the characteristics of resulting electrophoretograms proved very useful.

The design of the electrophoretic system is outlined in Fig. 1. The electrophoresis tank, 40 cm square, consists of an upper chamber (containing a 20 x 25 cm agarose gel) and a lower chamber (serving as a reservoir for buffer) directly connected by a single 3 x 1 cm slot. Buffer circulated between the chambers is temperature-controlled (8°C) by means of a heat-exchange system. The heat-exchange system is refrigerated by a water-ethylene glycol mixture that is maintained at 0 °C and circulated by a Lauda WB-20/R refrigerated waterbath. Ten platinum point electrodes are mounted on each side, connected together via diodes as shown. The use of diodes (see, for example, reference 8) eliminates current flow through the non-powered electrode arrays. Voltage is applied to the electrodes by one of three circuits, determined by computer controlled actuation of two relays. Each of the three circuits contains a rheostat to enable independent attenuation of the voltage between each opposing pair of electrode sets.

Each cycle of pulses is divided into four phases: 1, electrode array A is powered by circuit Al, resulting in orientation and migration in the
"transverse" direction. Either the applied voltage or the duration of the pulse, or both, is greater for phases 1 and 3 than for phases 2 and 4 (see below). 2, electrode array B is powered by circuit B resulting in reorientation and migration of DNA molecules (as marked on Fig. 1). We define the direction from B" to B" as the "longitudinal" direction, that in which net migration and size-dependent fractionation occurs. 3, electrode array A is again powered, but this time by circuit A2, which results in a field of identical magnitude, but polarity opposite to that applied by circuit A1. 4, electrode array B is again powered, exactly as in phase 2. At the termination of phase four, therefore, all molecules should be in the same orientation as that in which they began phase 1, but will have advanced longitudinally by the distance allowed by the resolving pulses of phases 2 and 4.

Unless otherwise stated, all electrophoresis was performed through 1% GTG agarose (FMC BioProducts, Rockland, ME), with 2 mm of buffer overlaying a gel of thickness 4 to 5 mm. The buffer was 0.5 x TBE electrophoresis buffer (TBE is 90 mM Tris base, 90 mM boric acid, 2 mM EDTA). DNA was visualized by staining of electrophoretograms with ethidium bromide upon completion of electrophoresis. Fields of 6 V cm\(^{-1}\) and a duration of transverse pulses double that of the longitudinal pulses were standardly used.

2. Preparation of DNA Samples

Yeast (Saccharomyces cerevisiae strain AB1380 received courtesy of D. Burke, Washington University, St. Louis, MO) chromosomes were prepared in blocks of low gelling temperature (LGT) agarose (from Bethesda Research Laboratories, Inc., Gaithersburg, MD) by a method based on that of Bellis, Pages and Roizes (14). Multimers of bacteriophage lambda DNA (monomeric size, 48.5 kb) were generated from lambda DNA, obtained from BRL, that had never been frozen. The solution of DNA was gently pipetted into a microfuge tube and heated to 70 °C for 5 min. It was then added to ice-cold TE to a final concentration of 0.1 or 0.2 mg ml\(^{-1}\). After storage on ice for 5 min the solution was warmed to room temperature. An equal volume of (50 °C) 1% LGT agarose made up in 0.5 x TBE was added and the solution was set on ice to form blocks. The blocks were placed in 5 volumes of 0.5 M EDTA, pH 8, and stored at 4 °C overnight. They were then equilibrated with, and stored in, 0.5 x TBE until they were used.

RESULTS and DISCUSSION

1. Characteristics of Electrophoretic Resolution

An electrophoretogram typical of those produced by the PHOGE system is shown in Fig. 2. In common with the various OFAGE-type systems (7-11) it
Fig. 2. Resolution of DNA by PHOGE. Alternating longitudinal (6 V cm$^{-1}$, 30 s duration) and transverse (6 V cm$^{-1}$, 60 s duration) pulses were applied to samples of yeast chromosomes (lane 2) and multimers of lambda DNA (lanes 1 and 3) for 64 h under the conditions described in Methods and Materials.

shows the following characteristic regions. A point of inflection divides two regions of differing resolution and, above a threshold size (that depends upon the pulse time: see below), there is a zone of compression that contains unresolved species. These regions, however, differ significantly in character from those produced by the other systems. The most striking differences are that the PHOGE inflection point is very clearly defined and that most of the resolution occurs in the region between the inflection point and the compression zone. The region containing species smaller than those at the inflection point shows relatively little resolution. For similar pulse times,
Fig. 3. Effect of variation of the ratio of the durations of transverse and longitudinal pulses. A, longitudinal field with linear pulse ramp of 10 to 30 s and transverse field with linear pulse ramp of 5 to 15 s; run 1080 cycles, i.e., 2160 longitudinal pulses. B, as A but using a transverse field ramp of 10 to 30 s. C, as A but using a transverse field ramp of 20 to 60 s. D, as A but using a constant transverse pulse duration of 60 s.

PHOGE resolves fragments of much greater size than do the other systems and its compression zone migrates a shorter distance into the gel.

Why does this system result in clear resolution of large species of DNA, whereas previous attempts using alternating homogeneous orthogonal fields of equal duration and magnitude failed to do so? As is illustrated by the following experiments, the difference in results is due to the longer duration of the transverse pulses as compared to the longitudinal pulses (at the same voltage gradient). A series of separations was performed that differed only in the ratio of the duration of the transverse pulses to that of the longitudinal pulses. The results are shown in Fig. 3. For this experiment a linear "ramp" of pulse times was employed (as has been used for FIGE, 12) in order to resolve a larger proportion of lambda multimers. The results show that when, as with existing OFAGE-type systems, equal pulse times were used (lane B), resolution was poor and bands were diffuse. These results are similar to those that were found for such OFAGE-type systems. A ratio of transverse to longitudinal pulse times of 0.5 (lane A) gave resolution similar
Fig. 4. Relationship between pulse time, sizes of molecules resolved and the distance of migration, per longitudinal pulse, of the inflection point in the electrophoretograms produced. Yeast chromosomes and multimers of lambda DNA were subjected to PHOGE at various durations, P (s), of pulses for between 25 and 44 h. The sizes of molecules that would be at the inflection point, $B_i$ (bp), and at the limit of resolution, $B_c$ (bp), were estimated by interpolation. The values, $S$, of the distance from the origin to the position of the (interpolated) inflection point per longitudinal pulse imposed, are also presented.

to that expected for conventional electrophoresis, but a ratio of 2 gave good resolution (lane C). When a constant transverse pulse time was used that was equal to twice that of the maximum longitudinal pulse (lane D) there was only a little improvement over the result obtained with a constant ratio of 2. These results suggest that it is the longer duration of transverse pulses, which presumably results in a greater proportion of molecules reaching a reptationally active conformation in the transverse direction, that accounts for the successful resolution of the PHOGE system. Furthermore, a two-fold excess of the duration of the transverse pulse almost maximizes the effect.

2. Relationship of Pulse Time to Sizes of Molecules Resolved

The range of sizes of effectively resolved molecules of DNA extends from the inflection point, corresponding to a size $B_i$ (base pairs), to the lower edge of the compression zone, corresponding to a size $B_c$ (base pairs). The sizes of molecules at $B_i$ and at $B_c$ were determined by electrophoresis of lambda DNA multimers and yeast chromosomes at various constant pulse times, $P$.

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(seconds): see Fig. 4. In all cases standard conditions were used and between 200 and 2000 cycles of pulses were applied. $B_I$ and $B_C$ were each proportional to $P$ throughout the range of pulse times investigated. A formal reorientation rate constant, $C_R = dB_I/dP = 14 \times 10^3$ bp s\(^{-1}\), can therefore be calculated for the conditions used, effective over the range of molecular sizes to at least 1 Mb. Similarly, one can calculate a rate constant, $C_M = dB_C/dP = 28 \times 10^3$ bp s\(^{-1}\), that defines the commencement of resolution from the compression zone. $C_M$ approximately equals $2C_R$ (and $B_C$ approximately equals $2B_I = 2C_R P$), again to at least 1 Mb. There relationships are helpful for choosing pulse times so as to obtain effective resolution over a desired range of sizes of DNA.

3. Relationship of Size of Molecules at the Inflection Point to Their Distances of Migration

In addition to relating the size of the molecules present at the inflection point to the pulse time, it is possible to relate their size to the distance that they have migrated through the gel. For each of the electrophoretograms used to determine $B_I$ and $B_C$ for various values of $P$ we calculated the ratio, $S_I$, of the distance of migration of the molecules at the inflection point to the number of longitudinal pulses that had been imposed and plotted $S_I/B_I$ against $P$. The resulting graph (Fig. 4) is constant, with $S_I/B_I = 0.12$ nm bp\(^{-1}\) over the whole range of pulse times and, therefore, of sizes of molecules at the inflection point that we investigated. The contour length of DNA is approximately 0.3 nm bp\(^{-1}\) (15). Molecules at the inflection point, therefore, migrate a distance of $0.13/0.3 = 0.43$ times their contour length per reorientation, to at least 1 Mb.

4. Superimposition of PHOGE and FIGE

We assume that because PHOGE and FIGE mechanisms of resolution operate in directions of 90° (PHOGE) and 180° (FIGE) to the direction of a previous field pulse, these systems effect resolution of large DNA molecules by different mechanisms. It is possible, therefore, that the resolution achieved by PFGE systems that use intermediate angles, $\theta$ (90° < $\theta$ < 180°), between alternating fields may operate by a combination of the two mechanisms, giving resultant characteristics of resolution that are related to those of the individual components. We see evidence for such an interpretation in the biphasic nature of OFAGE electrophoretograms, i.e., two regions of resolution of different characteristics separated by a point of inflection (which is much less clearly defined than that of PHOGE). To test this idea we subjected samples of DNA sequentially to PHOGE and then to FIGE, each with a fixed duration of pulse. The results of one such investigation are shown in Fig. 5. Although it would
Fig. 5. Combination of PHOGE and FIGE. Samples of multimers of lambda DNA (lanes 1 and 3) and yeast chromosomes (lane 2) were subjected to electrophoresis as follows. A: PHOGE for 28 h, 52 min with 30-s longitudinal pulses in a field of 5.2 V cm⁻¹ and 30-s transverse pulses in a field of 6 V cm⁻¹. B: as A, followed by FIGE for 16 h, 40 min with 12-s forward pulses in a field of 6 V cm⁻¹ and 12-s reverse pulses in a field of 3 V cm⁻¹.

not be expected that the resolution resulting from such a sequence would be identical to the resolution from an OFAGE-type system, the resulting electrophoretogram does have the biphasic nature characteristic of OFAGE. On this electrophoretogram, molecules larger than those at the inflection point are resolved by PHOGE, whereas molecules smaller than those at the inflection point are resolved by FIGE. We propose that, on electrophoretograms produced by the various OFAGE-type systems (see ref. 11 for an example), the molecules between the inflection point and compression zone are separated primarily by the PHOGE resolution mechanism (this size range corresponding to the FIGE migration minimum), whereas the molecules smaller than those at the inflection point are resolved primarily by the FIGE resolution mechanism (this size range
corresponding to species which migrate rapidly and with poor resolution under PHOGE). The increased migration rate of large species of DNA under FIGE may contribute to the greater migration of the compression zone under OFAGE than under PHOGE. However, it remains possible that for $90^\circ < \theta < 180^\circ$, a third, distinct, mechanism (perhaps that proposed by Southern et al., 11) may operate.

5. Other Characteristics of the PHOGE System

The PHOGE system produces electrophoretograms with characteristics that differ from those produced by other PFGE systems. As can be seen in Fig. 2, the use of a constant pulse duration allows the high resolution of a narrow (two-fold) range of sizes of molecules of DNA. The introduction of ramped pulse times, as has been used for FIGE (11), allows (lower) resolution of a wide range of molecular sizes. An example of the use of a large range of pulse times is shown in Fig. 6. A ramp from 5 to 90 s allows resolution over a range of 50 kb to approximately 2 Mb (estimated sizes of largest yeast chromosomes from contour lengths of synaptonemal complexes, 16). A gradient of decreasing field intensity in the longitudinal direction may be introduced by inclination of the electrophoresis tank and by use of a wedge-shaped gel, with the result that bands are sharpened and the distance of migration of smaller fragments relative to larger ones (which retain their greater resolution) is reduced. In this case, the inflection point also becomes less clearly defined, presumably due to its shift to progressively smaller molecular size as the result of the reduced field intensity further along the gel (data not shown). PHOGE shows several characteristics conducive to the accurate determination of the sizes of DNA fragments. In addition to a linear relationship between mobility and molecular size (from 50 kb to at least 1 Mb) when ramped pulses are applied, gel lanes are both straight and parallel, as is characteristic of other homogeneous PFGE systems. A large useable gel area is available as a consequence of the limited migration of the compression zone and the direct comparability of lanes across nearly the entire width of the apparatus (data not shown). Care must be taken, however, to ensure that the apparatus is level and that the gel is of uniform thickness. Otherwise distortions, most notably net transverse migration of larger DNA species and differences between the migration rates of DNA in different lanes, can occur.

6. Suggested Mechanism of Migration and Resolution

Molecules in the compression zone have migrated little; presumably they have also reoriented little during longitudinal pulses. One can therefore assign a size-dependent interval of time for (essentially) non-migrational
Fig. 6. PHOGE resolution over a wide range of molecular sizes. Samples of multimers of lambda DNA (lanes 1 and 3) and yeast chromosomes (lane 2) were subjected to a linear pulse ramp of 5 to 90 s (10 to 180 s transverse) for 64 h in fields of 6 V cm$^{-1}$.

reorientation processes. For a molecule of size $B_C$, this interval of time ($T$) is the corresponding $P$, $T(B_C) = B_C/2C_R$. Molecules at the inflection point must also spend a corresponding but smaller period of time during each pulse undergoing (essentially) non-migrational reorientation processes, namely, $T(B_I) = B_I/2C_R = P/2$, i.e., half of the duration of the pulse. Nonetheless, the distance from the origin to $B_I$ is much more than half of the distance from the
Fig. 7. Proposed mechanism of migration and resolution by PHOGE.

- : Polarity of field (- - - +)
- : Reference point
a : Orientation of molecule at the end of a transverse field pulse
b to g : Orientation of molecule during the time-course of a longitudinal field pulse (f to g corresponds to reptation)
b' to g' : Positions of molecules relative to reference point upon completion of the next transverse field pulse (reorientation by same mechanism)

origin to the position of lambda monomers, which reorient rapidly and therefore (for P>4s) migrate for nearly the entirety of each pulse. Therefore, DNA molecules must migrate during reorientation, and must move more rapidly during the second, migrational phase of reorientation than during steady-state migration! Proposed mechanisms for PFGE cannot explain this result. We outline below, and in Fig. 7, a mechanism that might broadly account for the characteristics of migration and resolution observed under PHOGE.

At the start of each resolving pulse, molecules are considered to be fully oriented and reptating (see, however, 17) under the effect of the transverse field (Fig. 5: a). The longitudinal pulse commences and the molecules respond by forming numerous "V" sub-structures (b). If, as in the compression zone, this is as far as the reorientation process gets before the end of the pulse, little longitudinal migration results (b'). If the pulse persists, one "V" begins to dominate (c); eventually the whole molecule forms a single "V" (d). If the pulse ends during this phase of reorientation, greater net migration
is observed (c', d'). We assume that because the field has no transverse component, the "centre of mass" of the molecule cannot move transversely to the applied field during reorientation. The unified "V" must, therefore, form at the centre of the molecule. Continuation of the pulse results in resolution of this "V" to a linear conformation (d to e to f). We imagine that the reorientation of molecules at the inflection point corresponds to stage (f), i.e., just completed. The predicted migration per pulse of molecules at stage (f) is therefore 0.5 times the contour length of the molecules, close to the value observed. Further application of the field results in migration by reptation (g). We suggest that the contorted molecule of DNA straightens, from a dominant "V" to a linear conformation, relatively rapidly, as a kind of whiplash. The observed high rate of reorientation-dependent migration and high resolution near the inflection point would result.

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