Rapid pulsed field separation of DNA molecules up to 250 kb

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ABSTRACT

Pulsed field gel electrophoresis (PFGE) is capable of resolving a wide size range of DNA molecules which would all co-migrate in conventional agarose gels. We describe pulsed field gel conditions which permit DNA fragments of up to 250 kilobases (kb) to be separated in only 3.5 h. The separations, which employ commercially available gel boxes, are achieved using conditions which deviate significantly from traditional pulsed field conditions. PFGE separations have been thought to require reorientation angles greater than 90° to be effective. However, reorientation angles of 90° and even less will resolve DNA fragments a few hundred kb and smaller ~5 × faster than with standard pulsed field conditions. The mobility of DNA fragments separated with 90° reorientation angles is switch time-dependent, as is seen for DNA run with the commonly used reorientation angle of 120°. With DNA fragments of several hundred kb and smaller, higher field strengths may be used, resulting in still greater increases in separation speed. The conditions described allow DNA from large insert bacterial clones, such as those using cosmid, Fosmid, P1, bacterial artificial chromosome (BAC), or P1-derived artificial chromosome (PAC) vectors, to be prepared, digested and analyzed on gels within a single working day.

INTRODUCTION

During conventional agarose gel electrophoresis, in which the electric field induces DNA to migrate in a constant direction, all DNA molecules larger than ~15–25 kb DNA migrate with nearly identical mobility. In contrast, pulsed field gel electrophoresis (PFGE) produces size-dependent mobility for large DNA molecules (up to 10 Mb) in agarose gels (1,2). PFGE produces this by causing the DNA to periodically alter its direction of migration, through regular changes in the orientation of the electric field with respect to the gel. With each change

in the electric field orientation, the DNA must realign its axis prior to migrating in the new direction (3,4). Thus, the path taken by DNA molecules during PFGE consists of a number of short steps, each taken in the direction of the switching electric fields. Electric field parameters, such as the direction, intensity, and duration of the alternating electric fields, are set independently for each of the different fields and are chosen so that the net migration of the DNA is down the gel. The difference between the direction of migration induced by each of the electric fields is referred to as the reorientation angle and corresponds to the angle that the DNA must turn as it changes its direction of migration each time the fields are switched.

The majority of pulsed field gel separations are performed in gel boxes which use a hexagonal array of 24 electrodes to produce homogeneous electric fields, the CHEF gel box (5). In early CHEF-type gel boxes the reorientation angle was fixed at 120° (5). However, for pulsed field gel boxes which alter the orientation of the field mechanically (6,7), or electronically by independent control of the electrodes (8), the reorientation angle can be varied by the user. It has been shown for large DNA molecules, such as yeast chromosomes, that as the reorientation angle is narrowed, for example from 120° to 106°, the migration rate of DNA down the gel is increased (9). This observation has allowed separation times for slow moving megabase (Mb) DNAs to be significantly shortened (8). However, this approach has not been systematically evaluated with regard to separation of smaller DNA molecules.

The development of bacterial cloning systems capable of maintaining fragments of a few hundred kilobases has stimulated interest in methods for the rapid analysis of such segments (10–12). These fragments, while at the small end of the size range for which PFGE is effective, still cannot be resolved in conventional, i.e. non-pulsed, gels. We have examined the electric field parameters which govern the migration of smaller DNA fragments in pulsed field gels to determine ways to increase the speed of separation. Fast separations can be obtained using conditions which deviate significantly from those in use for larger DNA molecules.

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MATERIALS AND METHODS

All gels were 1% SeaKem LE agarose (FMC BioProducts, Rockland, ME), cast and run in $0.5\times$ TBE (with $1\times$ TBE being 89 mM Tris-borate, 25 mM EDTA) at 14°C unless otherwise noted. $0.5\times$ TAE, which was used for Figure 4B and C, is 20 mM Tris-acetate, 0.5 mM EDTA. Electrophoresis was performed in the CHEF Mapper and the DRIII gel boxes (Bio-Rad Laboratories, Richmond, CA). Note that the DRIII is not capable of generating reorientation angles of $<90^{\circ}$. Size markers employed were the 1 kb ladder and High Molecular Weight markers (Life Technologies, Gaithersburg, MD), a 5 kb ladder (Bio-Rad Laboratories) and Low-Range, Mid-Range I, and Mid-Range II markers and lambda ladders from New England Biolabs (Beverly, MA). Following electrophoresis, gels were stained with ethidium bromide ($1\text{ }\mu\text{g/ml}$) and photographed using Polaroid type 55 positive/negative film. Typical exposures for this slow film (ASA 50) were 60 s. Distance migrated from the well was measured from the negatives.

Fosmid and BAC mini-preps were carried out using an alkaline lysis mini-prep technique (Sheng *et al.*, manuscript in preparation) on 1.5 ml of overnight culture. DNA was resuspended in $20\text{ }\mu\text{l}$ of TE (10 mM Tris, 1 mM EDTA, pH 8.0) and restriction digestions were performed according to the protocol of the supplier of the enzymes (New England Biolabs). Reactions of $20\text{ }\mu\text{l}$ containing $5\text{ }\mu\text{l}$ of mini-prepped DNA and 3–5 units of enzyme were incubated for 1.5 h at 37°C .

RESULTS

Reorientation angles for separating small fragments

Figure 1 shows the mobility of DNA fragments when identical samples of from 1 to 50 kb were separated in pulsed field gels using reorientation angles ranging from 120° down to 75° , and otherwise identical conditions. For comparison, the mobility of these fragments in a conventional, i.e. non-switched, gel is also shown. Each of the pulsed field gels, including those using a 90° and 75° reorientation angle, is able to resolve a wider size range of DNA than is the conventional gel. Further, as is true for larger fragments, the mobility increases as the reorientation angle is reduced. For example, the 30 kb fragment migrates nearly twice as fast when separated using 75° , and 50% faster when separated using 90° , compared to the 120° reorientation angle gel. Clearly, separation of small DNA fragments can be accomplished faster through the use of smaller reorientation angles.

While none of these pulsed field gels produces the rapid mobility of the conventional gel, in which the DNA is always moving straight down the gel, the conventional gel is unable to resolve fragments larger than about 15 kb. It is also apparent from Figure 1 that as the reorientation angle is reduced, the size range of the fragments being resolved is progressively reduced as well. With the 1 s switch interval and other conditions employed here, the entire set of fragments in the 1–50 kb sample are well resolved in both the 120° and the 105° gels. However, the 90° gel fails to resolve fragments above 35 kb, while the 75° gel fails to separate fragments larger than 30 kb.

Dependence of mobility on switch interval

While we observed large increases in the speed of separation resulting from reduced reorientation angles, they are accompanied by a reduction in the size range of fragments which can be separated. Changes in reorientation angle have been noted to alter

the size range of separation for large DNA fragments as well (13). In pulsed field gels, the primary determinant of the size range which can be separated is the switch interval (1,7,9). If the size range of small ($<50\text{ kb}$) DNA separated with reorientation angles of around 90° is also governed by the selection of the switch interval, we may be able to take advantage of the speed afforded by reduced angles while still being able to govern the size range being separated. The relative mobility of DNA fragments ranging from 1 to 50 kb separated with a 90° orientation angle using several different switch intervals is shown in Figure 2. The mobilities of the fragments in these gels are modified by the changes in switch interval. Thus, as with larger DNAs, as the switch interval is lengthened, the size range of fragments which can be resolved is increased, though as indicated by the change in the slope of the mobility curve, the resolution of these fragments is altered. The 0.6 s gel separates fragments only up to 30 kb, while the 1.4 s gel separates all the fragments up to 50 kb. Note that with such short switch intervals, large differences in migration result from very small absolute changes in switch interval (Fig. 2).

Increased migration rate with higher voltage gradients

In addition to the reorientation angle, other factors are known to influence the rate of migration in pulsed field gels (9,13,14). Perhaps the most significant of these factors for separating small DNA fragments is the electric field strength, since small fragments can migrate under much higher voltage gradients than can be used for the PFGE resolution of large fragments (15,16). Figure 3 shows a comparison of the mobility of 1–50 kb DNA separated using a 90° reorientation angle and a 0.8 s switch

Figure 1. Pulsed field gel mobility of 1–50 kb DNA separated with different reorientation angles. DNA fragments consisting of 1 kb ladders and High Molecular Weight markers were run in each of the experiments using 1% LE agarose gels in $0.5\times$ TBE at 14°C using fields of 6 V/cm and a 1 s switch interval. Mobilities of fragments (cm/h) run in gels using reorientation angles of 120° (∇), 105° (Δ), 90° ($\circ$), and 75° ($\diamond$) are shown. In addition, fragment mobilities from a gel run with a static field of 6 V/cm (i.e. a non-pulsed field gel) are also shown (+). Run durations for the different gels were: 120° , 6 h; 105° , 90° and 75° , 5 h; non-switched gel 3.5 h.

interval with fields of either 6 (as was used for Figures 1 and 2) or 9 V/cm. All fragments show a dramatic increase in mobility at the higher field strength. For example, the 30 kb fragment migrates 2-fold faster at 9 compared to 6 V/cm.

With the increased field strength, the size range of resolved fragments is also altered; the 6 V/cm gel does not effectively separate fragments larger than 30 kb, while the 9 V/cm gel separates fragments up to 50 kb. This again mirrors the situation for pulsed field migration of larger DNA.

Figure 2. Influence of switch interval on PFG mobility for separations carried out using 90° reorientation angles. Relative mobilities are shown for DNA fragments run for 5 h in 1% LE agarose gels in 0.5×TBE at 14°C using fields of 6 V/cm and a 90° reorientation angle. Switch times used were, 0.6 s (Δ), 0.8 s ($\circ$), and 1.4 s (∇). Mobilities are shown relative to the 1 kb fragment.

Figure 3. Increased pulsed field gel mobility with increasing field strength for 1–50 kb fragments separated using 90° reorientation angles. Mobilities (cm/h) are shown for gels run at 6 V/cm (Δ) or 9 V/cm ($\circ$). Gels were 1% LE agarose in 0.5×TBE at 14°C using a 0.8 s switch interval. Run durations were: 6 V/cm, 5 h; 9 V/cm, 3.5 h. At 6 V/cm the 1 kb fragment migrated at 1.61 cm/h. At 9 V/cm the 1 kb fragment migrated 2.58 cm/h.

Rapid separation of fragments up to 25 kb

To demonstrate the usefulness of these principles in separating DNA of experimental interest, we carried out electrophoresis of DNA samples in several distinct size ranges. First, we digested a series of overlapping Fosmid clones with *Hind*III and ran the fragments in pulsed field gels using a 90° reorientation angle. Fosmid clones, like cosmids, are generated using lambda packaging extracts, and thus contain inserts averaging 40 kb (17). Figure 4A shows the separation obtained with a run time of 2.5 h. This degree of resolution is more than sufficient to verify that these clones overlap, by virtue of the presence of shared bands. In addition, note that *Hind*III digestion of each of the different samples yields large fragments which would not be resolved or sized without the use of pulsed fields. Accurate estimation of the total length of the cloned DNA requires determination of precise sizes for these large fragments.

Rapid separation of BAC clones

To assess our ability to separate larger fragments, we digested DNA from BAC clones containing human sequences. *Not*I excises the inserts from the vector in these clones, and therefore is used to establish the length of cloned fragments. Figure 4B shows a gel run in 3.5 h using a 90° reorientation angle and fields of 9 V/cm to analyze clones containing inserts ranging up to ~75 kb. The markers in this gel show clear separation from 4 kb to beyond 100 kb. This size range represents that encountered in analysis of phage P1 clones.

Finally, Figure 4C shows separation of even larger BAC clones performed in 3.5 h. Bands from both the BAC vector (7.3 kb) and the BAC inserts, which range up to 200 kb in this gel, are extremely clear and sharp. This gel is able to resolve clearly bands of the lambda ladder size marker up to 250 kb. Separation of such large fragments is carried out using a reorientation angle of 100°, instead of the 90° reorientation angle used for the smaller fragments in Figure 4A and B. In addition, this gel was cast from extra-low EEO agarose (SeaKem Gold), which is known to increase the migration rate of larger DNA during PFGE (18). These gel conditions will serve to distinguish BACs which differ in size by small amounts, or those which have very different *Not*I digestion patterns due to differences in the number or spacing of *Not*I sites within the cloned segment.

DISCUSSION

Role of the reorientation angle in pulsed field separations

Pulsed field gel electrophoresis involves many more variable parameters than does conventional agarose electrophoresis. Over time, the influence of each of these variables has come under investigation. The role of the angle between the alternating electric fields has received relatively little attention, in part because this angle is fixed at 120° in early versions of the widely used homogenous-field gel boxes. Significant increases in speed can be obtained though changes in the reorientation angle. Reorientation angle appears to influence DNA migration in much the same way that all other known variables do, namely changes in reorientation angle alter both the speed of migration, the size range separated, and the resolution obtained for any given separation (13). Early reports suggested that there was a finite limit to the changes which could be made in the reorientation angle. Specifically, it was shown that a 90° reorientation angle was ineffective for separating yeast chromosomes when all other

conditions were held the same (1). Further, a model was proposed to explain the inability of DNA to be resolved with a 90° reorientation angle (7). Subsequent work has shown these views to be simplifications. For example, a pulsed field system making exclusive use of 90° reorientation angles was able to resolve large DNA (19). Using a pulsing regimen comprised of repeating cycles of many different electric fields, we have achieved resolution of yeast chromosomes using angles smaller than 90° (B.Birren and E.Lai, unpublished observations). In fact, reorientation angle resembles other pulsed field parameters such as voltage, for which selection of an appropriate value depends on the size range of the DNA being separated. A much wider range of voltages are effective for separating smaller (<250 kb) DNA than can be used for large DNA. Similarly, a wider range of angles is apparently tolerated for separating small DNA molecules. Thus, the widely used reorientation angle of 120° represents just one point on a continuum of reorientation angles which are effective in pulsed field gel separations.

Other parameters increasing separation speed for these fragments

In addition to manipulating the reorientation angle we have employed additional changes in gel conditions to increase the migration rate for these small DNAs. In fact, the gel shown in Figure 4C differs in nearly every condition from those employed for separation of larger molecules. For example, we normally separate *Saccharomyces cerevisiae* chromosomes in gels of 1%

low electroendosmosis (low EEO) agarose run in 0.5×Tris–borate buffer. In contrast, for a gel such as that shown in Figure 4C we have used a lower agarose concentration, agarose with a reduced EEO, a different buffer and a lower buffer concentration. Each of these changes will incrementally increase the rate of DNA migration (13). By far the greatest increase in the speed of these separations has been obtained by increasing the electric field strength. While decreasing the reorientation angle from 120° to 90° increases the mobility of the 30 kb fragment by 50%, this rate is nearly doubled by the increase in field strength from 6 to 9 V/cm. The size-dependent effects of field strength which require low voltages for very large DNAs (15,16) permit very high field strengths to be used for smaller fragments (20).

We have employed fields of only 9 V/cm in this work, having used commercially available gel boxes, yet undoubtedly further increases could be obtained with yet higher field strengths. Fields as high as 50 V/cm have been successfully used for pulsed field separations of small DNA fragments (20). In this regard, we are clearly limited by the currently available hardware. The PACE gel box (8), as well as the Bio-Rad DRIII and CHEF Mapper, are only able to generate fields up to 9 V/cm. The Bio-Rad DRII, which does not offer variable reorientation angles, is also not capable of providing field strengths greater than 6 V/cm, and therefore is not appropriate for the separations we describe. We anticipate that subsequent generations of pulsed field hardware will make higher field strengths available. It should also be noted

Figure 4. Separations of experimental samples using low reorientation angles. (A) 2.5 h separation of fragments up to 25 kb. Fosmid DNAs from mini-preps were cleaved with *Hind*III and loaded onto a 1% LE agarose gel in 0.5×TAE. Markers included a 1 kb ladder on the left, lane 1, and a 5 kb ladder on the right. The gel was run at 14°C for 2.5 h using fields of 9 V/cm, a switch interval of 0.4 s, and a reorientation angle of 90°. The 1 kb marker fragment has migrated 8.1 cm from the well. (B) 3.5 h separation of molecules up to 100 kb. Mini-prepped BAC DNA was cleaved with *Not*I to excise the inserted segment of human DNA. Digested DNA was run at 14°C, for 3.5 h in a 0.8% gel of SeaKem LE in 0.5×TAE, with a 90° reorientation angle using fields of 9 V/cm and a switch interval of 2 s. The far left lane contains lambda ladders, and the Mid-Range I marker of New England BioLabs is immediately adjacent. The far right lane contains the Low-Range marker, with the Mid-Range II immediately adjacent. Lanes 1–4 contain DNA from different BAC clones. The BAC vector band of 7.3 kb is visible in all lanes, and has migrated 7.8 cm from the wells. (C) 3.5 h separation of molecules up to 250 kb. Mini-prepped BAC DNA was cleaved with *Not*I to excise the inserted segment of human DNA. Digested DNA was run for 3.5 h in a 0.8% gel of FMC Gold Agarose in 0.5×TAE, with 100° reorientation angles using fields of 9 V/cm and a switch interval that was linearly ramped from an initial value of 3 s to a final value of 5 s. Lambda ladders were run in the left lane as size markers. Lanes 1–4 contain BACs of ~120, 100, 190 and 150 kb. The BAC vector band of 7.3 kb is visible in all lanes. A ruler indicates distance (cm).

that the rotating gel, or rotating electrode, gel boxes, that do offer variable angles, are not capable of switching at the high rates needed for these separations.

As with the pulsed field separation of larger DNA fragments, all changes which alter the rate of migration also alter the size range and the resolution of the separation. This is clear from Figures 1 and 3, in which the changes in reorientation angle and field strength change the size limit of the fragments that can be successfully separated in the gels, as well as the slope of the curves, which reflects resolution. To compensate for these unintended effects resulting from our optimization for speed, we have altered the switch interval used for the separations. An early review by Carle and Olson described the brinkmanship of selecting pulsed field gel conditions (21). The gel conditions described here place the gels even closer to the 'brink' of disappointment than is commonly encountered during traditional pulsed field separation of larger molecules. Thus, a very narrow window of conditions is effective for separating a desired size range. This is especially true for the switch interval, since a seemingly small change in the switch interval leads to a failure to separate the fragments as required (see Figure 2). In addition, since all the other parameters related to pulsed field migration, such as temperature, buffer type and concentration, agarose type and concentration, etc., will also affect resolution, seemingly minor variation or inattention to these variables may likewise prevent optimal separation of the desired size range. Finally, it should be pointed out that the resolution seen with these gels is not as high as that obtained with traditional slower pulsed field gel conditions. However, as demonstrated, the resulting bands are sharp, the resolution is more than adequate for many applications and the increased speed often make these the conditions of choice.

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