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Optimized conditions for pulsed field gel electrophoretic separations of DNA

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ABSTRACT

Quantitative measurement of DNA migration in gel electrophoresis requires precisely controlled homogeneous electric fields. A new electrophoresis system has allowed us to explore several parameters governing DNA migration during homogeneous field pulsed field gel (PFG) electrophoresis. Migration was measured at different switch times, temperatures, agarose concentrations, and voltage gradients. Conditions which increase DNA velocities permit separation over a wider size range, but reduce resolution. We have also varied the angle between the alternating electric fields. Reorientation angles between 105° and 165° give equivalent resolution, despite significant differences in DNA velocity. Separation of DNA fragments from 50 to greater than 7000 kilobases (Kb) can easily be optimized for speed and resolution based on conditions we describe.

INTRODUCTION

The observation that alternating the orientation of the electric field during electrophoresis enables large DNA molecules to be separated in agarose (1) has led to new applications of electrophoresis in molecular biology and genetics (2,3,4). Pulsed field gel (PFG) electrophoresis can separate DNAs ranging to at least 7 megabase pairs (Mb) (5,6). The theoretical basis for the PFG separations of large DNAs remains largely speculative, as neither the shape of the molecules in the gel, nor the mechanism by which they move is known. However, quantitative and reproducible separations can be achieved by maintaining constant voltage gradients and homogeneous electric fields throughout the run (7,8,9).

Several reports have described factors which influence DNA migration in PFG electrophoresis. The importance of the switching interval was originally noted by Schwartz and Cantor in

the initial report of the method (1). Temperature (10) and field inhomogeneity (1) have each been proposed as crucial aspects of DNA separation. Field strength has been shown to be critical in separating molecules greater than 2 Mb (6). There is a growing reliance on PFG electrophoresis and interest in the physical properties of DNA that produce the separation. However, the factors which influence homogeneous field PFG electrophoresis have not been fully characterized.

We have described an electronic gel controller that permits continuously variable independent regulation of all 24 electrodes in a closed-contour array gel box, the programmable autonomously-controlled electrodes (PACE) electrophoresis gel system (11). We have used this apparatus to characterize several parameters which influence the mobility of DNA in agarose gels. These data delineate optimal conditions for separating DNA in the range of 50 to greater than 7,000 Kb, and are relevant to models attempting to explain the migration of DNA in homogeneous field pulsed field gels.

MATERIALS AND METHODS

Preparation of DNAs for Electrophoresis.

Lambda concatamers were prepared using CsCl banded phage particles as described (13). Yeast chromosomes were prepared according to published procedures for Saccharomyces cerevisiae, strain D273-10B (1), and Schizosaccharomyces pombe, strain 972 h- (6).

Preparation and Photography of Gels.

Gels were cast 13 cm square in 0.5 x TBE (3) using FMC SeaKem LE agarose. Agarose plugs containing DNA samples were cast in the gel by supporting them against a comb while the liquid agarose was poured and allowed to solidify. After electrophoresis gels were stained with ethidium bromide (1 ug/ml) and photographed using a Wratten 23A filter and 1-2 minute exposure with Polaroid type 55 positive/negative film with illumination at 300 nm. For Southern hybridizations, gels were irradiated for one minute at 254 nm before alkaline transfer to nylon membrane and hybridization as described (6). Migration

distances were measured using the negative. All figures show the full length of each gel (13 cm).

Electrophoresis.

The electrophoresis chamber contains 24 electrodes in a closed-contour, with the dimensions of the chamber and the positions of the electrodes as described by Chu, et al. (7). The voltage of each electrode is independently and actively controlled by separate digital-analog converter (DAC) circuits. A control unit containing the 24 DAC circuits is regulated by an IBM-PC. The controlling unit which drives this gel box actively controls each electrode independently. Its circuitry and use in PFG electrophoresis have been described (11). Electrophoresis takes place in homogeneous fields, with uniform migration of any sized fragment across the entire width of the gel. Constant temperature was maintained by circulating the electrophoresis buffer, 4 liters total, in coiled plastic tubing through a cooling bath (3). Buffer temperature was read using two thermometers mounted in the electrophoresis chamber at the buffer inlet and outlet. Voltages were confirmed by measurement directly at each electrode with a voltmeter at the beginning and near the end of each run. Unless otherwise specified all gels were 1% agarose, run at 6 V/cm (13° C), with switching every 90 seconds between two electric fields whose orientation differs by 120°.

RESULTS

Effects of Switch Intervals on DNA Mobility.

The switching interval has been shown to have a dramatic influence on the mobility of large DNA in PFG gels (1,6). We therefore explored this effect to optimize DNA separations in the size range of 50-1000 kb. Figure 1A shows photographs of lambda concatamers and chromosomes from the yeast strain D273-10B, separated in 1% agarose gels at different switch times. As previously noted (1,3,6) as the switch time is increased, DNA molecules of increasing size are separated from the bulk of slowly moving unresolved material. Velocity (cm/hr) is the most useful expression of migration for predicting the migration of

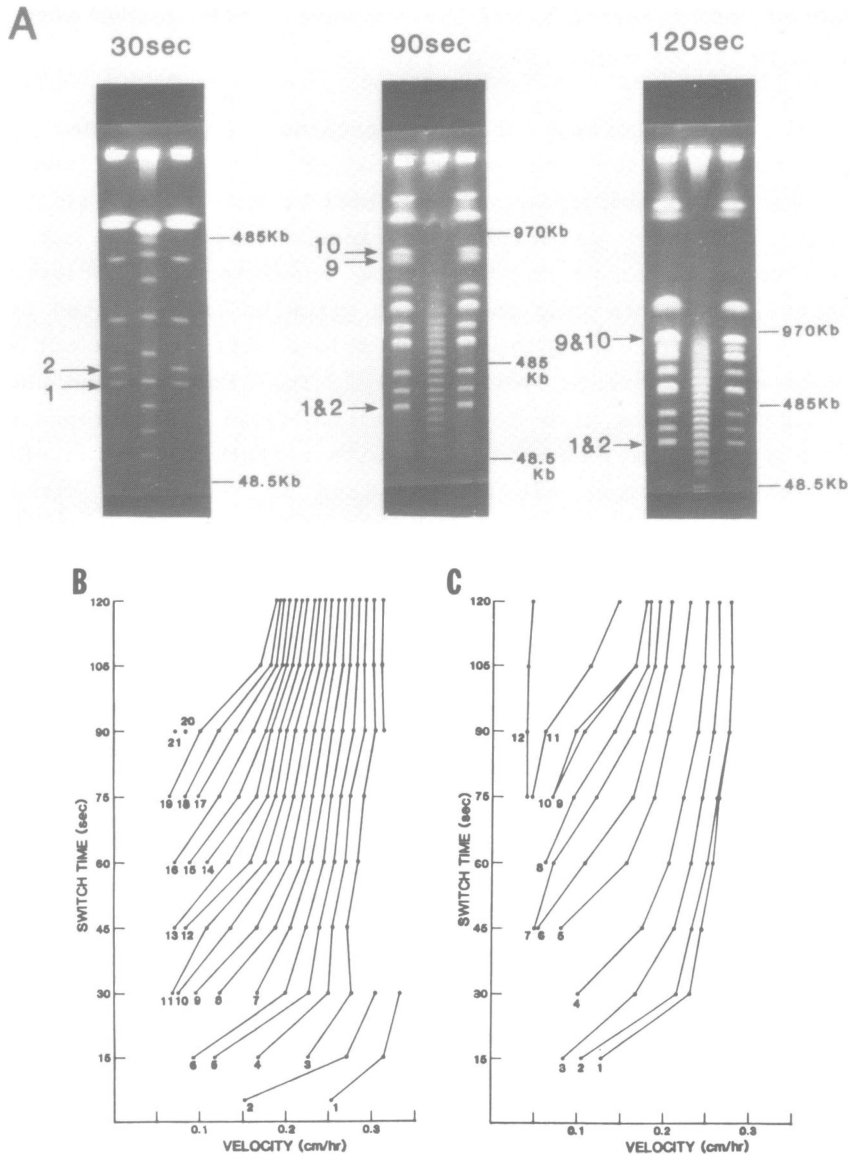


Figure 1 Effect of switch time on migration of yeast chromosomes and lambda DNA.
A. Ethidium bromide staining of lambda concatamers flanked by chromosomes of yeast strain D273-10B. 13 cm long, 1% agarose gels were run at 6 V/cm (13° C) Switching intervals (seconds) are indicated. The 30 second gel was run for 37.2 hr, the 90 second gel was run for 33.6 hr., and the 120 second gel was run for 37 hr. Arrows indicate pairs of yeast chromosomes separated only at

specific switch times and refer only to the relative order of bands and not chromosome assignments.
B. Velocity of lambda concatamers versus switch time. Numbers indicate the number of lambda molecules in the concatamer. All gels were 1% agarose and run at 6 V/cm at 13° C. Distances migrated by the individual members of the lambda ladder were measured off photographic negatives and velocities (cm/hr) calculated.
C. Velocity of yeast chromosome bands versus switch time. Gels and calculations as in 1B. Numbers indicate the order of the yeast chromosome bands as by arrows in figure 1A.

specific sized molecules (e.g., how long to run a gel intended to move the 200 kb molecules 10 cm). Identical gels were run at a variety of switch times and the velocity of each size class of lambda concatamer was plotted as a function of switch time (figure 1B). Figure 1B demonstrates that as the switch time increases, each molecular weight class asymptotically approaches a maximum velocity. Additionally, with increasing switching intervals the velocities of different sized molecules converge. This behavior has two practical consequences. First, as the switch time is increased beyond that necessary for separation, little additional change in velocity occurs. Thus, the distance run by the lambda monomers in the gels of figure 1A (run for closely matched lengths of time) is nearly the same with different switch times. Second, maximal separation between molecules in a given size range occurs closest to the switch time required to separate the DNAs from the unresolved region. For example, the greatest distance between molecules consisting of lambda 3-mers and 4-mers is achieved just after they have begun to resolve with a 15 second switching interval. Molecules of lambda 16-mers and 17-mers are best separated with a 75 second switch interval as they first fully resolve.

The velocity of the bands representing chromosomes of the S. cerevisiae strain D273-10B have been plotted in figure 1C. This strain contains two different pairs of chromosomes with closely related sizes which serve to illustrate the role of switch times in separation. These chromosomes are indicated by arrows in figure 1A. The smallest two chromosomes, numbered bands 1 and 2, are clearly separated with 30 second switching, but as the velocities converge at longer switch times they run as an unresolved doublet. A second pair of bands, numbered bands 9 and

Table I
Size Estimation of Yeast Chromosomes

Band Number	Est. Size (Kb)	S.D.(± Kb)
1	238	1.3
2	263	2.7
3	335	2.3
4	427	2.3
5	581	5.3
6	681	10.0
7	774	6.4
8	832	5.5
9	913	6.8
10	936	2.3
11	1051	26.0

Comparisons of migration were made for yeast chromosomal bands and lambda concatamers run side-by-side at switch times of from 30 to 120 seconds. Estimations reflect measurements taken from three to six gels. Yeast chromosomes larger than band 11 could not be sized in these experiments. S.D. represents standard deviation.

10, is distinguishable with 90 second switching but cannot be resolved in runs of other switch times. Thus the region of greatest resolution can be positioned at different DNA sizes by selection of appropriate switch times.

Use of Different Switch Times in Sizing Yeast Chromosomes.

It has been common practice to estimate the size of an unknown fragment by comparing its migration to that of a known DNA size marker. Because molecules of different sizes will co-migrate at certain switch times, as seen in figure 1A, comparisons of size must be made at switch times which result in maximum separation in the specific size range of the unknown fragment. To estimate the sizes of the yeast chromosomes in the strain D273-10B, we superimposed figures 1B and 1C, and made comparisons at switch times appropriate for the different sizes. The sizes calculated for the yeast bands that fall within the size range covered by our lambda concatamers (48.5-1164 Kb) are presented in Table I. The high degree of reproducibility between runs in this gel system is indicated by the low standard deviations of these measurements.

Relationship Between DNA Length and Velocity.

Having calculated the sizes of the yeast chromosome bands, we have used them as additional size markers. Figure 2 shows the relationship between size and velocity for DNA in a 1% gel with

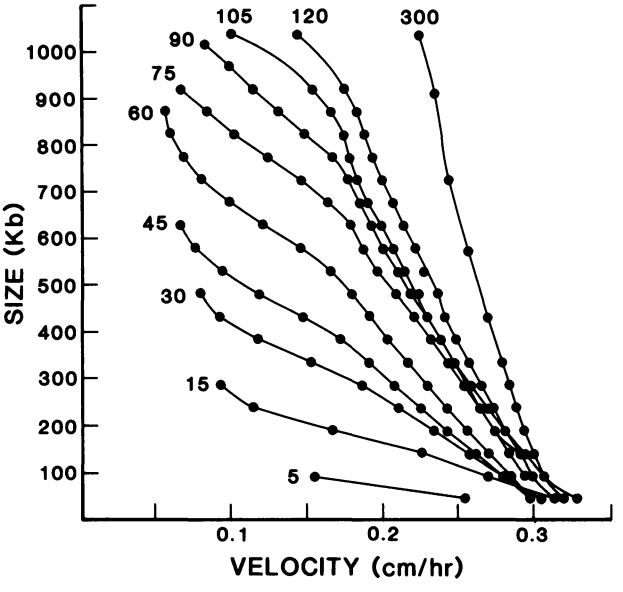


Figure 2 Velocity of DNAs from 50 to 1000 kb at different switch times. All data come from 1% gels run at 6 V/cm at 13° C. Numbers indicate switching intervals (seconds).

switch times of from 5 to 300 seconds. Any single switch time produces a plot with a linear region covering a specific range of DNA sizes [Figure 2 and ref. (6)]. Electrophoretic separation of a specific size range of molecules is achieved by manipulation of switch times according to figure 2. Thus, by varying only the switch time we have separated DNAs in the range of from 1 kb to over 2 Mb, [see below and ref. (11)].

Effects of Temperature on PFG Separations.

We have measured the effects of different temperatures on the migration of DNA in pulsed field gels of identical composition and switch time. We find a dependence of velocity on temperature, as shown in figure 3A. DNA over the range of 50-1000 Kb moved almost twice as fast when run at 34° C compared to 4° C. The velocities of lambda monomer under these conditions are listed in Table II. This behavior may reflect the two-fold decrease in the viscosity of water over this temperature range. While the high temperature gels ran faster, they generally yield

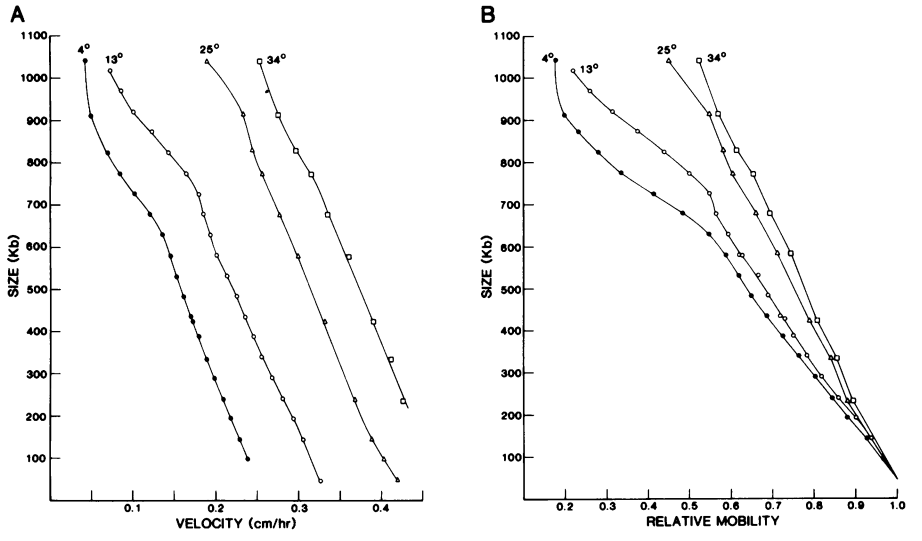


Figure 3 Temperature dependance of DNA migration.
A. Velocity of DNAs run in 1% gels at 6 V/cm with 90 second switching at different temperatures is plotted versus size. The 5° C gel was run for 37 hr, the 13 ° C gel was run for 30 hr, the 25° C gel for 16.8 hr and the 34° C gel for 12.5 hr.
B. Relative migration of DNAs at different temperatures. Migration of the DNAs represented in 1A were calculated relative to the distance migrated by the 48.5 kb lambda monomer.

poorer resolution than that obtained at lower temperatures. To compare the resolution (the distance between molecules of similar sizes) of gels run at different temperatures, we have analyzed plots of relative mobility, in addition to absolute velocity. Since DNA molecules move at different rates at the different temperatures, normalization permits direct comparison of resolution for the various conditions. Figure 3B displays the data of Figure 3A calculated as the distance travelled by each band normalized with respect to the lambda monomer. We have plotted migration rates relative to several lambda oligomers and find that the relationship described here, and in other figures, is unchanged (not shown). Figure 3B demonstrates that the increase in temperature from 4° C to 13° C diminishes the distance between molecules of similar sizes. Temperatures of 25° C and 34° C allow separation of an even greater size range, but with further reductions in resolution. Thus the increases in

Table II
Velocity of lambda with 90 second switching.

Voltage gradient (V/cm)	Temp. (°C)	Agarose conc. (percent)	Reorientation Angle (°)	Velocity (cm/hr)
3	13	1.0	120	0.10
6	5	1.0	120	0.25
6	13	1.0	120	0.31
6	25	1.0	120	0.42
6	34	1.0	120	0.48
9	13	1.0	120	0.59
6	13	0.6	120	0.46
6	13	1.4	120	0.21
6	13	1.8	120	0.16
6	25	1.4	120	0.32
6	25	1.6	120	0.27
6	13	1.0	105	0.41
6	13	1.0	135	0.24
6	13	1.0	150	0.17
6	13	1.0	165	0.09

temperature which increase velocity, permitting dramatic reductions in run time, clearly do so at the expense of resolution.

Effects of Agarose Concentrations on PFG Separations.

We have also measured the effect of differing agarose concentrations on DNA separation. Figure 4A shows photographs of gels of different agarose concentrations run at the same temperature and switching interval. As with temperature, agarose concentration affects the velocity of all sizes of DNA in the gel. For example, figure 4A shows the difference in distance covered by DNA in 1.4% and 1.8% gels which were run for identical lengths of time. Increased agarose concentrations do not uniformly affect the DNA markers, but retard the larger molecules to a greater extent. This is indicated by the shifts in the relative mobility of DNAs as plotted in figure 4B. Resolution is improved with increasing agarose concentrations, although the separation is restricted to a smaller range of DNA sizes. For example, with a 90 second switching interval, the region of optimal resolution of the 1% gel extends from approximately 700 Kb to 1000 Kb. In 1.8% agarose this region is found between 500 Kb and 750 Kb. Another aspect of separations at higher gel

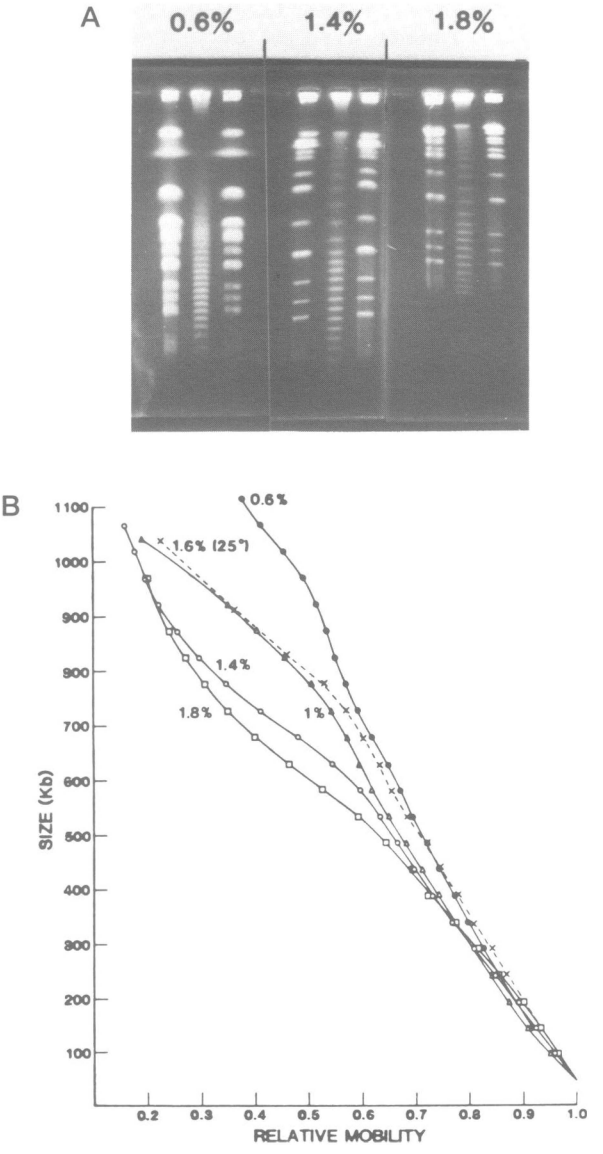


Figure 4 Effect of agarose concentration on DNA migration.
A. Ethidium bromide staining of gels of different agarose concentrations run at 6 V/cm (13° C) with a 90 sec switch interval. The 0.6% gel was run for 12.1 hr. The 1.4% and 1.8% gels were run simultaneously, side-by-side for 45.7 hr.
B. Relative migration of DNAs in varying agarose concentrations. Migration relative to the lambda monomer is plotted. The velocity of the lambda monomer was 0.487 cm/hr in 0.6% agarose, 0.30 cm/hr in 1%, 0.28 cm/hr in 1.4% and 0.156 in 1.8%.

concentrations is a decrease in the width of individual bands (i.e. band sharpening). This is illustrated by the higher molecular weight bands in the 1.8% gel in figure 4A which, though quite close together, are still distinguishable. This sharpening may result from stacking that occurs as the sample leaves the 0.5% agarose sample plug and meets the boundary of the higher concentration gels.

We have also measured the migration of DNA in 1.6% agarose run at elevated temperature (25° C). The increased velocity resulting from the elevated temperature compensates for the retardation of the high agarose concentration. The resulting velocities of DNA markers resemble those for DNA run in 1% gels at 13° C (Table II). The relative migration of the markers is indicated in figure 4B and closely matches that of the 1% gel. The DNA bands in such gels show the sharpness associated with higher agarose concentrations and thus represent improved separation conditions without requiring longer run times.

It has been reported that DNA molecules will exhibit non-monotonic migration behavior in high concentration agarose gels under high voltage gradients (12). We were therefore concerned about our assignment of sizes for the individual yeast bands in the higher concentration gels represented in figure 4. We separated a lane of yeast chromosomes under standard conditions in 1% agarose and subsequently re-ran this lane in a second dimension in 1.8% agarose. No reversal of migration occurred (not shown). We conclude that the migration reversal noted by Noolandi et al. (12), which we have confirmed for the agarose concentrations and voltage gradients they describe, does not affect the separations of molecules up to 1000 Kb plotted here.

Voltage Gradient Effects.

We have measured the migration of DNAs in 1% gels in electrical gradients of 3, 6, and 9 V/cm at switch times of 30 to 300 seconds. In each case the smallest DNA was run a similar distance to compare the ability of the gels to separate DNAs over the same size range. The velocity of all sizes of DNA is proportional to the voltage gradient. At each of the switch times tested, the smallest molecules moved 6 times faster at 9 V/cm, and 2 times faster at 6 V/cm than with a gradient of 3 V/cm

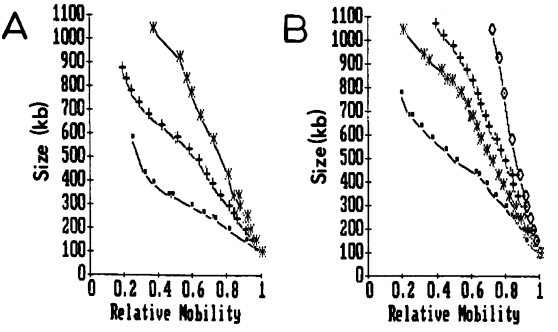


Figure 5 Separations of DNAs at different electric field strengths.
A. Relative migration of DNAs separated with 60 second switching at 3 (•), 6 (+), and 9 (✳) V/cm. 1% gels were run at 13° C and migration was calculated relative to the 97 kb lambda dimer. The length of the runs and the velocity of lambda dimers were: 3 V/cm: 60 hr. and 0.088 cm/hr; 6 V/cm: 37 hr and 0.28; 9 V/cm: 20 hr. and 0.58 cm/hr.
B. Migration of DNAs separated with 120 and 300 second switching at 3 and 6 V/cm. Gel conditions and relative migration is as in 5A. Run lengths and velocities of the lambda dimers were: 3 V/cm 120 second switch (•); 60 hr. and 0.093 cm/hr, 3 V/cm 300 second switch (+); 67.7 hr. and 0.081 cm/hr, 6 V/cm 120 second (✳); 67.9 hr. and 0.028 cm/hr, 6 V/cm 300 second (◇), 19.3 hr. and 0.31 V/cm.

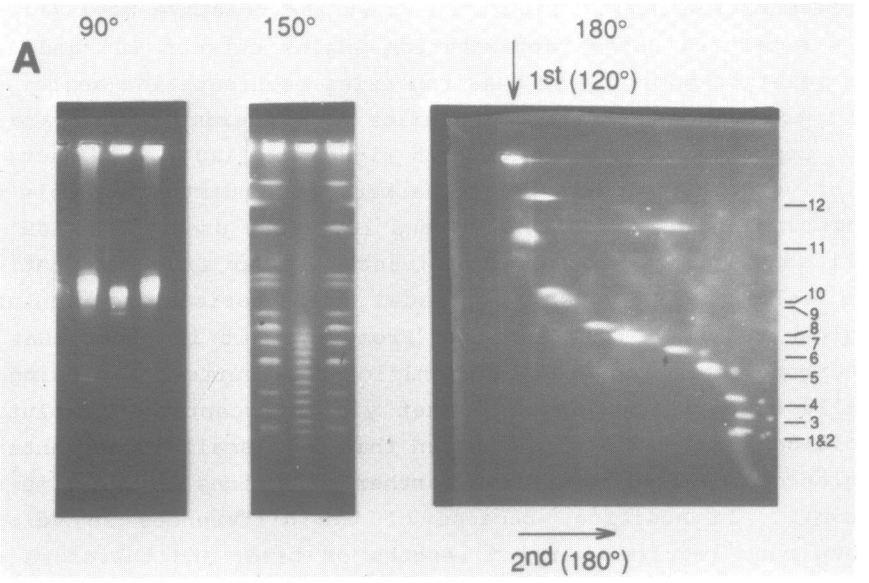
(figure 5 legend). Figure 5A shows the relative migration of DNA in field strengths of 3, 6, and 9 volts/cm when switched every 60 seconds. The best resolution (the greatest distance between consecutive lambda concatamers) is achieved at the lowest voltage gradient. However, at 3 V/cm linear separation extends only to about 450 kb with this switch time (Fig. 5A). Figure 5B displays separations at longer switch times. In every case examined, lower voltage gradients produce better separation over a narrower size range.

Effects of Altering the Reorientation Angle.

Chu, et al., (7), have demonstrated the necessity of using angles greater than 90° in PFG electrophoresis, but the behavior of DNA in fields switched between other reorientation angles has not been fully explored. We have measured the mobilities of DNAs separated using different reorientation angles between the alternate electric fields. Figure 6A shows several

representative gels. Figure 6B shows the relative mobilities of DNAs separated using reorientation angles between 90° and 180°. The relative mobility of DNAs run using reorientation angles from 105° to 165° is strikingly similar. For example, compare the 150° separation of figure 6A with figure 1A (120°). In fact, the relative mobility curve of the markers was indistinguishable when switching angles of 135°, 150° and 165° were used. The 105° and 120° separations did not significantly differ from this pattern. The velocities of DNAs separated with various reorientation angles are shown in Table II. From this, it is clear that PFG electrophoresis runs can be significantly shortened by using the smallest reorientation angle that yields acceptable resolution. Additional experiments have shown that even smaller reorientation angles can be used to achieve further reductions in run time (not shown). In addition, because of the differences in velocity these runs required varying lengths of time, and therefore each run involved very different total numbers of switches. The equivalence of separations achieved under these various conditions suggests that separation is not a simple function of the total number of switches (8).

DNA separated at the extremes of this range of angles exhibited markedly different behavior. As seen in figure 6A, 90° provides virtually no separation of DNAs from 50 to > 2000 Kb. To directly compare the migration of DNAs switching between 180° [i.e., field inversion (14)], and 120°, a lane of yeast chromosomes separated using 120° reorientation was re-run in a second dimension using field inversion. Separation by field inversion yields a dramatically different migration pattern, with regions at both the small and large end of the size range in which fragments of dissimilar sizes co-migrate. This non-monotonic relationship between size and migration has been noted for field inversion gels (14,15). The 2D gel of Figure 6A shows that a diffuse band running between the bands numbered 11 and 12 in the 120° pulsed field gel migrates much faster than both of these bands in the second dimension (field inversion). This molecule migrates as an approximately 1.5 Mb fragment in the 120° pulsed field gels, and as a 700 Kb fragment in the field inversion separation. Southern hybridization of such 2D gels



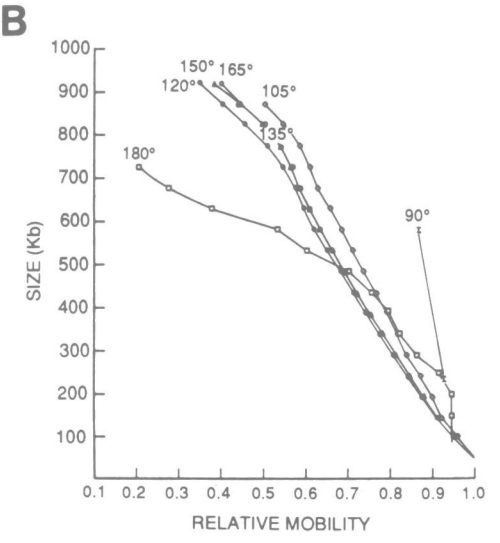


Figure 6 Pulsed field gel separation of DNA with differing reorientation angles.
A. Ethidium bromide stained gels containing DNA separated with field angles varying by 90°, 150°, and 180°. 1% gels were run at 6 V/cm at 13° C with 90 second switching for the 90° gel (run for 12.1 hr) and for the 135° gel (run for 44.7 hr). The 180° separation was accomplished by first running yeast chromosomes in a standard 1% gel PFG run with 90 second switching. After slicing off the appropriate lane, it was recast in 1% agarose and run for

16 hr using equivalent field strengths (6 V/cm) with pulses of 67 sec in the forward direction and 22.5 sec in reverse. The diffuse band running between bands 11 and 12 has been shown to be chromosome XII by Southern hybridization.
B. Relative migration of DNA separated at different reorientation angles. 1% gels were run at 6 V/cm (13° C) and switched every 90 seconds. Migration is calculated relative to the lambda monomer. Run lengths were: 90°, 12.1 hr; 105°, 19.5 hr; 120°, 33.6 hr; 135°, 44.7 hr; 150°, 68.22 hr; 165°, 94.2 hr; 180°, 16hr.

reveals that this band is chromosome XII of S. cerevisiae. Olson and co-workers have shown that while this chromosome can migrate faster than the 1.5 Mb chromosome in pulsed field gels (17), its actual size varies between 2 and 3 mb, depending on the strain (Carle, Link, and Olson, personal communication). Our results, in addition to those of reference (17) demonstrate that in pulsed field electrophoresis there can also be a non-monotonic relationship between size and mobility as has been found in FIGE.

Table II summarizes the rates of migration of lambda monomers under many of the conditions tested. The relative length of gel runs using the different conditions reported may thus be calculated.

Separation of S. pombe chromosomes by PFG.

We have also characterized the migration of S. pombe chromosomes as a function of the variables described above. The electrophoretic properties of these DNAs are largely predictable from the migration of the smaller S. cerevisiae chromosomes. For example, the migration of the S. pombe chromosomes follows the relationship between switching interval and voltage gradient described for the smaller DNAs we have studied. Figure 7 shows gels containing S. pombe chromosomes separated with different switch times and voltage gradients. S. pombe chromosomes were identified by Southern blot hybridizations with chromosome specific probes. Surprisingly, we find that small changes in switch time produce dramatic differences in the relative migration of the S. pombe chromosomes. In gels run at 2 V/cm, the two slowest S. pombe chromosomes are not completely resolved with a switch time of 25 minutes. Increasing the switching time to 26.5 minutes results in separation of the two slower chromosomes, though they still run far behind the fastest. With 30 minute switching, the three S. pombe chromosomes are more

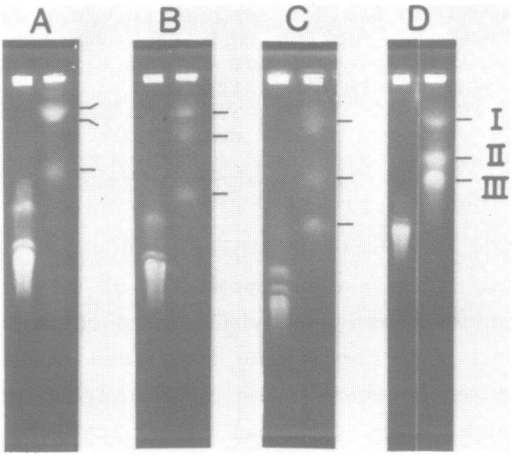


Figure 7 Migration of S. pombe chromosomes in PFG.
Samples containing chromosome preparations of S. cerevisiae, left, and S. pombe, right, were separated in 0.7% agarose at 13°C with a reorientation angle of 106°, unless otherwise stated. The positions of the S. pombe chromosomes were determined by Southern hybridization with chromosome specific probes, and are indicated with Roman numerals.
A. Separation was carried out at a voltage gradient of 2 V/cm, a switching frequency of 25 min., and a run length of 85 hr.
B. Separation was carried out at a voltage gradient of 2 V/cm, a switching frequency of 26.5 min., and a run length of 85 hr.
C. Separation was carried out at a voltage gradient of 2 V/cm, a switching frequency of 30 min., and a run length of 95 hr.
D. Separation was carried out at a voltage gradient of 1.5 V/cm, a switching frequency of 60 min., and a run length of 114 hr, with a orientation angle of 120°.

equally spaced in the gel. With the longer switch times and lower voltages described in reference (6), the two leading chromosomes run close together and well ahead of the third. Thus, the spacing of the three S. pombe chromosomes, relative to each other, shows striking variation with different switch times. The migration of S. pombe DNA varies with gel temperature and agarose concentration much as we have shown above for smaller DNAs (not shown).

DNA molecules larger than 3 Mb respond to changes in temperature, agarose concentration, and reorientation angle in the same manner as do molecules smaller than 3 Mb. In contrast, the separation of the larger S. pombe chromosomes is extremely sensitive to the voltage gradient (5,6). We find that the

sensitivity to voltage gradient is proportional to DNA size (data not shown). For example, with one hour switching all three S. pombe chromosomes are completely separated at 2 V/cm. However, as the gradient is increased from 2 to 2.5 V/cm the two slowest moving S. pombe chromosomes cease to separate, though the fastest band continues to move as expected. At 4 V/cm none of the S. pombe bands are resolved, though S. cerevisiae chromosomes continue to be well separated.

DISCUSSION

Chu, et al. (7), have established the importance of electric field homogeneity in achieving PFG separations with uniform migration across the entire gel. We have characterized factors which influence the migration of DNA in homogeneous fields during PFG electrophoresis. Switching time is the single most important factor determining the migration of DNA in PFG electrophoresis. Plots of migration versus size yield an "S" shaped curve which contain a region which approximates linearity. This linear region of separation can be directed to size ranges whose center is 3 Kb (data not shown) to at least 4 Mb by altering the switch time. Little or no separation is achieved between molecules in the non-linear regions at the bottom and top of the curve. In practical terms, this linear region of the separation curve can occupy a relatively small portion of the gel (Figure 7). Thus, it is essential that molecules fall well within this linear region when using PFG electrophoresis to establish the size of unknown DNA fragments. This is especially important given that molecules of vastly different sizes can co-migrate, figure 6 and ref. (17).

A region of heightened resolution is found just before the non-resolved large material (see figure 1 and ref. 6) in gels of constant switch intervals. By choosing the switch time to make use of the region of increased resolution, pulsed field gels are capable of yielding extremely accurate estimations of unknown fragment sizes. However, gels of separate switch times are required to establish the size of unknown fragments in different size regions. Biphasic separations, such as shown in figures 1 and 2, reflect a presently uncharacterized relationship between

the size of the DNA molecules found at the inflection point of the curve and the switch time. Migration distances which directly reflect fragment size can be extended throughout the length of pulsed-field gels by progressively changing, i.e. ramping, the switch times (11).

Appropriate electrophoresis conditions must depend on the size of the molecules being separated. Use of the shortest switch time which permits separation of the desired size range, from figure 2, will provide maximum resolution. Increasing the rate of migration by raising the temperature or lowering the agarose concentration is generally not useful due to the concomitant decrease in resolution. Fragments less than 1 mb can be separated more quickly by increasing the voltage gradient to 9 V/cm. However, we find that the ability to discriminate between S. cerevisiae bands 9 and 10 is lost at the higher voltage. Decreased run times with minimal loss of resolution can be achieved by altering the reorientation angle. Table II indicates that a 30% increase in the rate of migration occurs when the reorientation angle is decreased from the standard 120° to 105°. Figure 6B indicates that there is little difference in the resolution between these conditions.

Figure 5 indicates that changes in voltage gradient alter migration in ways reminiscent of switch time changes. It has been suggested that the relative migration of DNA in PFG separations reflects the product of the switch time and voltage gradient (5,6), expressed as the "effective switch time". We have found examples where similar separation, judged by plots of relative migration versus size, results from electrophoresis conditions which maintain a constant product of the switch time and voltage gradient. For example, gels run at 3 V/cm with 60 second switching closely resemble those run at 6 V/cm with 30 second switching. However, matched relative mobility can also result from gel runs in which the product of the switch time and voltage gradient varies (while other parameters are constant). For example, the separation achieved in gels of 6 V/cm with 90 second switching (figure 2) is virtually indistinguishable from gels of 3 V/cm and 300 second switching (figure 5B). We have shown that changes in other parameters alter the migration of DNA

much as do changes in switch time alone. Raising the gel temperature from 4° C to 13° C shifts the relative mobility as if the switching time were lengthened from 70 to 90 seconds. Thus, a true "effective switch time" is a complex function which must include factors in addition to switch time and voltage. A mathematical expression of DNA migration with meaningful predictive value will emerge only with extensive controlled studies.

Separation of large DNAs (greater than 3 Mb) requires reduced voltage gradients. Large DNAs can migrate in agarose gels at higher voltage gradients if they first enter the gel at low voltage (11). However, we have not been able to achieve separation across the complete size range of S. pombe chromosomes in a homogeneous field above 2 V/cm. While the literature contains reports of S. pombe chromosomes separated at higher voltages (5,16), these were carried out using a gel apparatus in which the voltage gradient is not uniform, and with the positions of the movable electrodes not reported. Thus, no valid comparison can be made between these different voltage data.

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