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Pulsed-Field Electrophoresis of Megabase-Sized DNA

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Success in constructing a physical map of the human genome will depend on two capabilities: rapid resolution of very large DNA and identification of migration anomalies. To address these issues, a systematic exploration of pulsed-field electrophoresis conditions for separating multimegabase-sized DNA was undertaken. Conditions were found for first liberating and then separating DNA up to 6 megabases at higher field strengths and more rapidly than previously reported. In addition, some conditions for transversely pulsed fields produced mobility inversion, in which increased size was accompanied by faster rather than slower migration. Importantly, anomalous migration could be identified by the presence of lateral band spreading, in which the DNA band remained sharply defined but spread laterally while moving down the gel. These results have implications for both practical applications and theoretical models of pulsed-field electrophoresis.

The haploid human genome contains 3,000 megabases (Mb) of DNA, with an average chromosome size of 130 Mb. The goal of constructing a physical map of the entire genome is now feasible with the development of pulsed-field electrophoresis (31). However, if this ambitious project is to be completed successfully, the technology must determine the sizes of large DNA molecules with both speed and reliability.

Speed has been a serious problem in resolving DNA larger than 3 Mb. Previous investigators have noted that unless the field strength is decreased as size increases, the DNA becomes trapped in the gel and fails to migrate. As a result, gels containing multimegabase-sized DNA have been run at low field strengths of less than 2 V/cm. Thus, run times of 3 to 6 days have been required to achieve adequate resolution (5, 24, 29, 36, 43).

Reliability in assessing DNA size has also been a problem. A number of systems are now capable of producing uniform migration across the gel, allowing lane-to-lane comparison with size markers (7, 9, 13, 20, 41). However, field inversion gel electrophoresis is plagued by the phenomenon of mobility inversion: at some point, increased size is accompanied by faster rather than slower migration (7). This can lead to wild errors in estimating the size of unknown DNA samples. Mobility inversion has also been suspected for transversely pulsed fields. Chromosomes IV and XII of *Saccharomyces cerevisiae* (1.6 and 2.2 Mb, respectively) have been reported to migrate in an inverted order (8, 28, 31). However, the presence of several hundred tandem repeats of rDNA on chromosome XII suggested that some sort of secondary structure could have led to anomalous migration.

To address the issues of speed and reliability, we focused our attention on the separation of the three chromosomes from *Schizosaccharomyces pombe*, which are 3.5, 4.7, and 5.7 Mb in size (18). A device based on the principles of contour-clamped homogeneous electric fields (CHEF) was built as a square array of electrodes capable of reorienting the electric field by arbitrary angles (12). The device permitted systematic exploration of electrophoresis parameters. Conditions were found that separated the chromosomes significantly more rapidly than previously reported. Furthermore, DNA could be liberated from the loading well under one set of conditions and then rapidly resolved under a second set.

The phenomenon of mobility inversion was also observed. Fortunately, inversion could be identified by the presence of lateral band spreading, in which the DNA band remained sharply defined but spread laterally while moving down the gel. Both speed and reliability should be enhanced by these results, facilitating the large-scale mapping of complex genomes.

MATERIALS AND METHODS

Preparation of DNA. *Escherichia coli* chromosomal DNA was prepared by methods described previously (35, 37). Yeast chromosomal DNA plugs were made from *S. cerevisiae* YNN 295 (kindly provided by Ron Davis) and *Candida albicans* ATCC 14053 by standard methods (43).

Attempts to prepare *S. pombe* DNA (strain ATCC 24811) with the same protocol resulted in unacceptable DNA degradation regardless of the growth stage of the yeast. Good-quality *S. pombe* plugs were finally obtained from a slightly modified protocol of Smith et al. (35). A 10-ml culture of *S. pombe* was grown at 30°C for 2 days to near-stationary phase in YPD medium (2% Bacto-peptone [Difco], 1% yeast extract, with 2% glucose added after autoclaving). The cells were cooled on ice for 10 min, spun at 1,000 × *g* for 10 min at 4°C, resuspended in 50 ml of ice-cold 50 mM EDTA (pH 8.0), spun down again, and resuspended in 25 ml of ice-cold Zymo buffer (40 mM sodium phosphate, 20 mM citric acid, 5 mM EDTA, 0.9 M sorbitol [pH 5.6]) to which was added 190 μl of 40-mg/ml Zymolyase 20T (ICN ImmunoBiologicals, Lisle, Ill.), yielding a final Zymolyase concentration of 0.3 mg/ml in the cell suspension. (Aliquots of Zymolyase 20T were dissolved at 40 mg/ml in water and stored at −20°C.) Keeping the sample cooled to 4°C up to this point appeared to be important in preventing DNA degradation. The mixture was then incubated at 37°C for 3 h.

Successful formation of spheroplasts was checked by a turbidity test: the addition of an equal volume of 1% sodium dodecyl sulfate (SDS) to a sample of the cell suspension should cause the turbidity of the suspension to clear. The spheroplasts were spun down at 1,000 × *g* for 10 min, at 4°C and resuspended in Zymo buffer to a final concentration of 10^9 spheroplasts per ml (OD_{600}, ≅30). The final volume of about 2 ml was then brought to a temperature of 37°C. An

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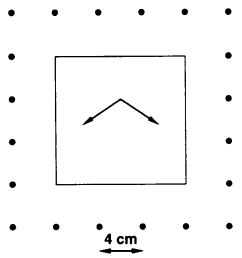

FIG. 1. Variable-angle CHEF device. The square array of 20 vertically placed electrodes was driven by an electronic circuit that allowed the electric field to be reoriented by an arbitrary angle. The migration of DNA was highly uniform across the width of the gel. A complete description of the device will appear elsewhere (12).

equal volume of 1% low-melting-temperature agarose dissolved in Zymo buffer and equilibrated at 50°C was added and mixed well. The suspension was distributed into plug molds (LKB 2015-404; Pharmacia LKB Biotechnology, Bromma, Sweden) and allowed to solidify either at 4°C for 30 min or at −20°C for 5 min. The solidified inserts were pushed out of the mold with a sterile glass rod into ESP (0.5 M EDTA [pH 9.5], 1% *N*-lauroylsarcosine, 1 mg of proteinase K [Bethesda Research Laboratories, Gaithersburg, Md.] per ml) and incubated for 16 to 24 h at 50°C with gentle shaking. Inserts were stored at 4°C in fresh ES (ESP without proteinase K). Inserts were dialyzed in TE (10 mM Tris, 1 mM EDTA, pH 8.0) for at least 1 day before loading.

Gamma irradiation of *E. coli* chromosomal DNA. In order to linearize the circular *E. coli* chromosome, a ^{137}Cs source was used to deliver doses of 500, 1,000, and 2,000 rads to *E. coli* DNA inserts (4, 22) suspended in 1 ml of TE (pH 8.0) in 1.5-ml microfuge tubes.

Pulsed-field electrophoresis. All gels were made from 0.4% agarose with a high tensile strength (chromosomal grade agarose; Bio-Rad Laboratories, Richmond, Calif.) and run at 12°C in either 0.5× or 1× TBE buffer (1× TBE is 90 mM Tris base, 88 mM boric acid, and 2 mM EDTA [pH 8.0]) for 20 h unless otherwise stated. The buffer was recirculated through a refrigeration unit (MGW Lauda RM-6; Brinkmann Instruments Co., Westbury, N.Y.) by a rotary aquarium pump (model 1021; Eheim, Berlin, West Germany) with silicone (0.25-inch [ca. 0.6 cm] diameter) tubing.

The pulsed-field electrophoresis was done either in a CHEF apparatus with a hexagonal array of 24 electrodes producing a field reorientation angle of 120° as previously described (9, 10) or in a variable-angle CHEF apparatus with a square array of 20 electrodes capable of arbitrary reorientation angles as described elsewhere (12). A schematic of the square electrode array is shown in Fig. 1.

The electric field strength was calculated from the potentials measured at the electrodes. For the vertical electrode arrays used here, which span the height of the buffer, this is close to the actual field strength in the gels.

Probes for *S. pombe* chromosomes. Probes for Southern blotting (40) were made by randomly primed polymerization from plasmid DNA by using ^{32}P-labeled dCTP (3,000 Ci/mmol; Amersham) (19). Three probes were made, one for each chromosome of *S. pombe*. The probe containing *mei2.8* is specific for chromosome I (3); *mei3.2* is specific for chromosome II (27); pNN4 contains rDNA sequences from *S. cerevisiae* (30) which cross-hybridize to rDNA on chromosome III of *S. pombe*. The plasmids were obtained from Douglas Vollrath, Whitehead Institute, Cambridge, Mass.

DNA transfer and hybridization. The DNA was transferred to a nylon membrane (0.45 μm; Nytran; Schleicher & Schuell, Keene, N.H.) by vacuum blotting (LKB 2016 VacuGene; Pharmacia LKB, Pleasant Hill, Calif.). A low transfer pressure of 15 to 20 cm of H_2O was used to prevent gel collapse. (Care must be taken so that the gel does not float away.) During the vacuum blotting procedure, the gel was first covered for 10 min with a layer of 0.25 M HCl to depurinate the DNA; then covered for 20 min in denaturing buffer (1.5 M NaCl, 0.5 M NaOH), followed by 20 min in neutralizing buffer (2 M NaCl, 1 M Tris [pH 5]); and finally covered for 2 h in 20× SSC (20× SSC is 3 M NaCl plus 0.3 M trisodium citrate). After blotting, the DNA was cross-linked to the membranes with 1.2 mJ of UV radiation (Stratalinker 1800; Stratagene, La Jolla, Calif.).

The nylon membrane was probed for the presence of specific sequences by standard methods (2). First, the membranes were incubated for 3 to 4 h in prehybridization buffer (25 mM potassium phosphate [pH 7.4], 5× SSC, 5× Denhardt's solution [1× Denhardt's solution is 0.02% each bovine serum albumin, Ficoll, and polyvinylpyrrolidone], 50 μg of salmon sperm DNA per ml, 50% formamide, 1% SDS). Probe DNA was boiled for 5 min added to 10 ml of hybridization buffer (prehybridization buffer plus 10% dextran sulfate) for a specific activity of 1×10^6 to 3×10^6 cpm/ml, and incubated overnight at 37°C.

Following hybridization, the nylon membranes were washed as follows: 5 min at 20°C in 2× SSC–0.1% SDS; 15 min at 20°C in 2× SSC–0.1% SDS; 15 min at 20°C in 0.5× SSC–0.1% SDS; and finally 30 min at 42°C in 0.1× SSC–0.1% SDS. X-ray film (Kodak XAR5) was exposed to the membranes for 24 to 72 h at −80°C with an intensifying screen.

RESULTS

High field strengths allow faster separation of large DNA. In order to find ways of separating very large DNA more rapidly, we directed our attention to the three chromosomes of *S. pombe*. After a systematic exploration of electrophoresis conditions, it was possible to achieve rapid resolution. Figure 2 shows data for three different sets of conditions appropriate for resolving DNA of 1 to 6 Mb. The four gels were run with different field strengths. Pulse times were adjusted to keep the size range of resolution roughly the same for each gel. Chromosomes from *E. coli*, *S. pombe*, *C. albicans*, and *S. cerevisiae* were run in parallel.

An increase in the field strength from 2 to 3.25 V/cm resulted in dramatically improved separation of the *S. pombe* chromosomes. A further increase in the field strength to 4.0 V/cm resulted in significant loss of the *S. pombe* bands, especially the two largest chromosomes. This problem was overcome by running the gel with a short pulse time leader: 500-s pulses for 2 h, followed by 660-s pulses for the remaining 18 h of the run. A plausible explanation for this phenomenon is that the large DNA was trapped in the loading well at high field strengths and long pulse times. A series of shorter pulses enabled the DNA to enter the gel more easily. Once in the gel, the DNA was able to continue its migration even with the longer pulse time. The same effect was also seen when a low-field-strength leader was used to allow the DNA to enter the gel (data not shown). It is likely that the debris remaining in the agarose plug after lysis of the cells in situ plays an important role in trapping the DNA in the loading well. For example, proteins remain-

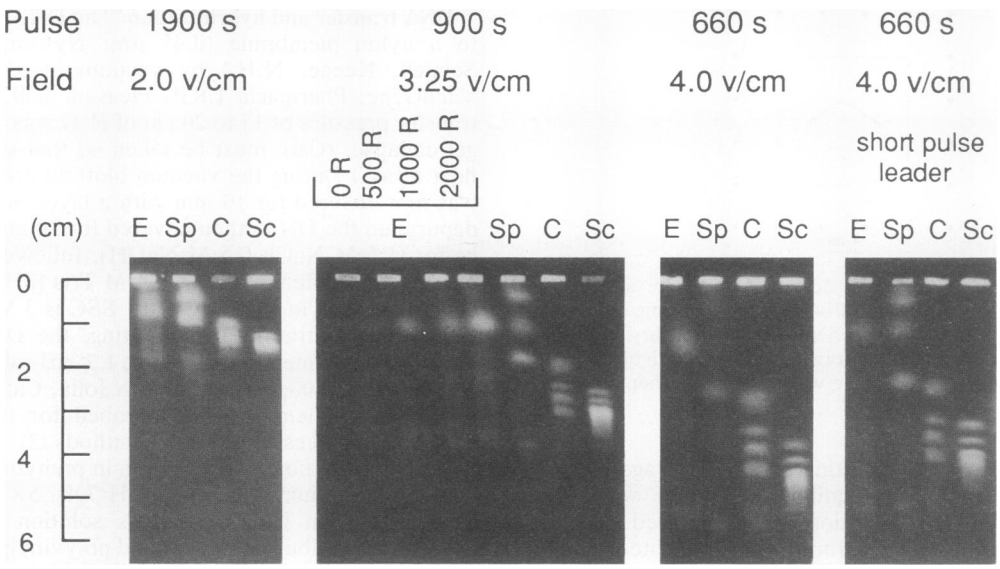

FIG. 2. Resolution of large DNA at different field strengths. Intact chromosomes from *E. coli* (E), *S. pombe* (Sp), *C. albicans* (C), and *S. cerevisiae* (Sc) were separated at three different field strengths. The pulse times were adjusted in the first three gels to maintain approximately the same separation pattern. The second gel shows linearization of the *E. coli* chromosome after exposure to gamma radiation at doses of 0, 500, 1,000, and 2,000 rads (R). In the third gel, run at 4.0 V/cm, the two largest *S. pombe* chromosomes remained trapped in the well. To allow entry into the gel, the fourth gel was run with a short pulse time leader (500-s pulses for 2 h, followed by 660-s pulses for 18 h). All gels were in 1× TBE buffer and run for 20 h with a reorientation angle of 120°.

ing bound to the DNA after protease treatment could retard mobility.

Bacteria can be a convenient source of size markers for very large DNA. *E. coli* contains a single circular chromosome, which has been physically mapped to be 4.7 Mb in size (23, 34). The circle may be linearized by gamma rays, which introduce random double-strand breaks in DNA. High doses produce multiple breaks, yielding a heterogeneous mixture of fragments. However, a dose of gamma radiation that introduces an average of one break per molecule yields a subpopulation of linear molecules of a single size, which was used in these experiments as a reference marker. The second gel in Fig. 2 shows how the circular *E. coli* chromosome, which is trapped in the loading well, is released from the well with increasing doses of gamma radiation by conversion to a linear molecule. The linearized *E. coli* chromosome comigrates with chromosome II of *S. pombe*, yielding an estimated size of 4.7 Mb for *S. pombe* chromosome II. This agrees with previous size estimates based on *Not*I digestion (18).

The resolving power, R, for a given set of electrophoresis conditions is best expressed as the separation between bands divided by the band width: R = band separation/band width. By this measure, the electrophoresis conditions described here were capable of resolving the two largest *S. pombe* chromosomes (4.7 and 5.7 Mb) by at least four bandwidths in 20 h.

The gels in Fig. 2 were run with different field strengths (E) and pulse times (T_P), keeping constant the product $E^{1.5} \times T_P$. Although the region of resolution was in the *S. pombe* size range for each case, some difference in the separation pattern was apparent. To determine the proper relationship between the variables, multiple gels were run, titrating the pulse time and the field strength (data not shown). The equation that preserved the resolution pattern most precisely was $W = E^{1.4} \times T_P$, where W is the window function. Its magnitude affects the size range or "window" of resolution: as W increases, separation is achieved for larger DNA and lost for smaller DNA. The same resolution may be maintained for different field strengths as long as the pulse time is also changed to keep W constant. The variation in the exponent was estimated to be 1.4 ± 0.1. Note that in addition to W, the size range of resolution is also a function of other parameters, including gelling material, buffer concentration, and temperature.

DNA molecules can migrate with an inverted size order in transversely pulsed fields. The effect of pulse time on the pattern of separation was examined for a constant field strength. Gels were run for a range of W values to produce different ranges of resolution. Figure 3 shows a series of gels run at different pulse times in a field of 3.1 V/cm. For a pulse time of 60 s, the chromosomes of *E. coli*, *S. pombe*, and *C. albicans* comigrated as a single band near the top of the gel. Only the six smallest chromosomes of *S. cerevisiae* were resolved. When the pulse time was increased to 180 s, the *E. coli* and *S. pombe* chromosomes remained unresolved, but there was some resolution of the *C. albicans* chromosomes and complete resolution of the *S. cerevisiae* chromosomes. Note that the *S. pombe* chromosomes migrated further down the gel than the slowest-moving *C. albicans* chromosomes.

At 300 s, three distinct *S. pombe* chromosomes were resolved, all migrating as fast as or faster than the slowest *C. albicans* chromosome. The *E. coli* chromosome comigrated with the middle *S. pombe* band. At 500 s, two of the *S. pombe* chromosomes comigrated with the *E. coli* chromosome, the third migrated slightly faster, and all *S. pombe* chromosomes now migrated more slowly than the *C. albicans* chromosomes. At 780 s, all three *S. pombe* chromosomes were well resolved, migrating more slowly than the *C. albicans* chromosomes, as expected from estimates of their sizes. At 900 s, the two largest *S. pombe* chromosomes did not appear in the gel, presumably remaining trapped in the

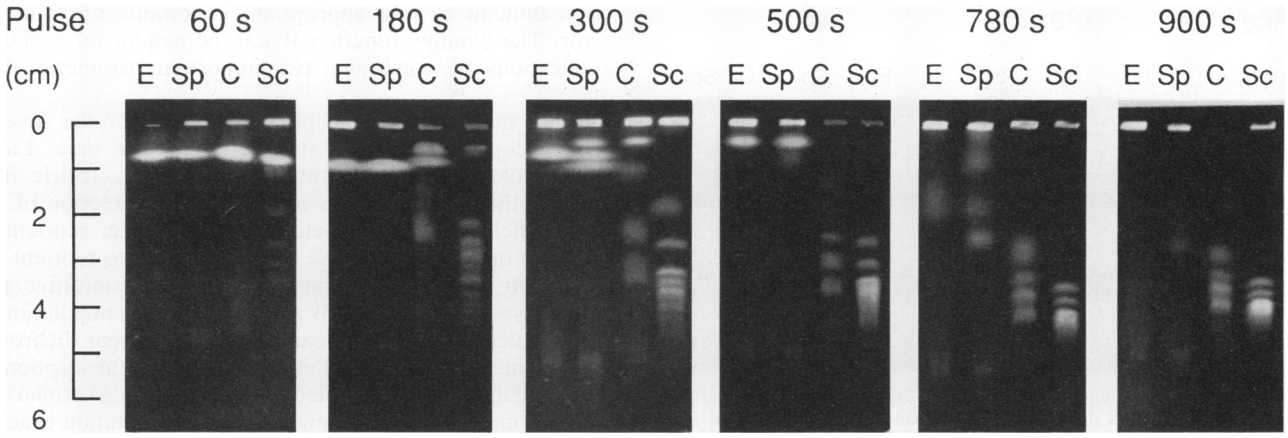

FIG. 3. Resolution of large DNA as a function of pulse time. The separation pattern changed dramatically as the pulse time was increased from 60 to 900 s. Note the lateral band spreading for the *S. pombe* (Sp), *E. coli* (E), and largest *C. albicans* (C) chromosomes in the 60-, 180-, and 300-s gels, and for the largest *S. cerevisiae* (Sc) chromosomes in the 60-s gel. All gels were in 0.5× TBE buffer and run for 20 h at a field strength of 3.1 V/cm and reorientation angle of 120°.

loading well. Entrapment of DNA in the wells can be caused by long pulse times as well as by high field strengths, as noted previously (Fig. 2).

This series of gels suggest that the migration of the DNA undergoes a size inversion under certain conditions. This was explicitly demonstrated by identifying individual *S. pombe* chromosomes with specific DNA probes. Figure 4 shows *S. pombe* chromosomes resolved with pulse times of 300 and 780 s, visualized by ethidium bromide and identified by a Southern blot probed with DNA sequences specific for each chromosome.

For a 780-s pulse time, the three *S. pombe* chromosomes migrated in the correct order for their expected sizes, with the largest (chromosome I) migrating most slowly and the smallest (chromosome III) migrating most rapidly. Note that there was significant smearing upward from each band, with

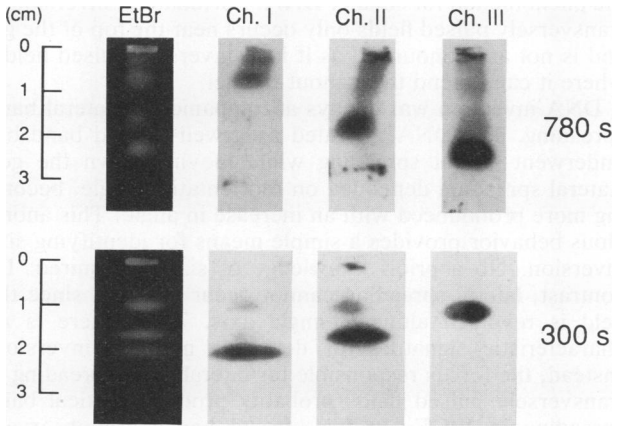

FIG. 4. Mobility inversion and band spreading of large DNA. Intact chromosomes from *S. pombe* were resolved at pulse times of 780 and 300 s, producing migration in the correct and inverted order, respectively. The left-hand panels show the gels stained with ethidium bromide (EtBr), and the remaining panels show Southern blots probed with DNA sequences specific for each chromosome (Ch.). Both gels were run in 0.5× TBE buffer at a field strength of 3.3 V/cm with a reorientation angle of 120° for 20 and 40 h, respectively.

the effect most pronounced for the largest chromosome. The smearing could be reduced by using a higher buffer concentration. These gels were run at the conventional buffer strength of 0.5× TBE, but gels run at 1× TBE showed significantly less band smearing. For example, the *S. pombe* chromosomes in Fig. 1 run in 1× TBE show less smearing than those in Fig. 4, which was run in 0.5× TBE.

For a pulse time of 300 s, the Southern blots demonstrated complete inversion in the order of migration. It is interesting that the probe for chromosome I also hybridized weakly to the bands for chromosomes II and III. Similarly, the probe for chromosome II hybridized to the band for chromosome III. It is possible that hybridization of chromosome I and II probes to the band for chromosome III is a "compression" phenomenon associated with the accumulation of partially degraded DNA in a broad range of sizes near the mobility minimum occupied by chromosome III. However, the probe from chromosome I also hybridized weakly to the band for chromosome II. Perhaps the larger chromosomes were entrained to comigrate with the smaller chromosomes, for example, by a mechanism dependent on intermolecular entanglement. The phenomenon occurred when the chromosome mobilities were inverted but was not evident when they were not inverted.

Conversely, the smearing that was seen at 780 s was not present at 300 s. One cause of smearing may be temporary entrapment of DNA molecules in the gel matrix. Shorter pulse times decreased the likelihood for entrapment in the gel, just as they did for entrapment in the loading well, perhaps by not allowing the DNA to reach a fully extended conformation more vulnerable to entrapment.

DNA inversion is accompanied by band spreading. DNA molecules undergoing size inversion were always observed to undergo an associated phenomenon of lateral band spreading. The DNA migrated as a sharp, sometimes even hypersharp, band but underwent lateral spreading perpendicular to the direction of migration down the gel. The greatest band spreading was always seen for DNA with the greatest anomalous mobility. This effect is easily seen in Fig. 4. It was also present in Fig. 3 for the *S. pombe* and *E. coli* chromosomes in the 180- and 300-s gels.

Furthermore, the band spreading was accentuated at larger reorientation angles. In Fig. 5, the reorientation angle

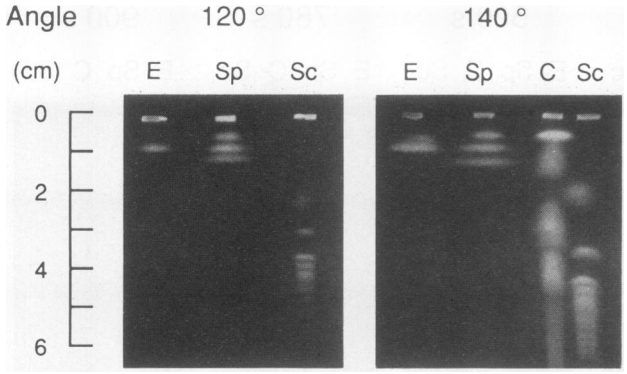

FIG. 5. Dependence of band spreading on reorientation angle. Electrophoresis was done at reorientation angles of 120° and 140° under conditions that resolved the *S. pombe* (Sp) chromosomes in an inverted order. To demonstrate the extent of band spreading, gels were run for 20 and 40 h, respectively. Other conditions were 0.5× TBE buffer, field strength of 3.3 V/cm, and pulse time of 300 s. E, *E. coli*; Sc, *S. cerevisiae*; C, *C. albicans*.

was increased from 120° to 140°, resulting in an increased lateral spreading of the *E. coli* and *S. pombe* chromosomes. By contrast, the noninverted *S. cerevisiae* chromosomes showed no band spreading. Note that the *C. albicans* chromosomes near the top of the gel showed band spreading, presumably due to size inversion, while the remaining chromosomes migrated down the gel normally.

Rapidly migrating compression zone. The gels in Fig. 1 and 2 contain a faint band of DNA near the bottom of the gel, at a position independent of the sample. The same band is seen in lanes for *E. coli*, *S. pombe*, *C. albicans*, and *S. cerevisiae* despite significant differences in chromosome number and size among those organisms. This band represents DNA degraded to random fragments that are large by conventional electrophoresis standards but too small to be resolved by the pulsed-field conditions used to resolve DNA from 1 to 6 Mb. The heterogeneous mixture thus appears as a distinct band but actually represents a fast-migrating compression zone. It is important to be aware of this phenomenon. For example, high levels of degradation of the *S. pombe* chromosomes may mimic a fourth band on pulsed-field gels. This can lead to misinterpretation, particularly if the conditions have been inadvertently chosen to cause the disappearance of the largest *S. pombe* chromosome.

DISCUSSION

Window function. A systematic study of key electrophoresis parameters allowed multimegabase-sized DNA to be resolved rapidly. *S. pombe* chromosomes were separated by at least four bandwidths in 20 h. Previous investigators have resolved *S. pombe* chromosomes with low field strengths of 1 to 2 V/cm, requiring inconveniently long run times of 3 to 6 days (5, 24, 36, 43). Our separation of *S. pombe* chromosomes at field strengths as high as 4 V/cm depended on a careful selection of pulse time, since small changes in that parameter strongly affected resolution. Since migration velocity varies roughly as the square of the field strength (1), a twofold increase in field strength leads to a fourfold increase in speed of resolution.

We found that the size range of resolution depends on the window function W, which is expressed in terms of the field strength (E) and the pulse time (T_P) as $W = E^{1.4} \times T_P$. It is often difficult to find appropriate conditions by trial and error. The window function W can be helpful for practical applications by combining two important parameters into one.

There may be a simple physical model for the inverse relationship between field strength and pulse time. Large DNA molecules are separated by a pulsed electric field because they are forced to reorient in the direction of the electric field. A stronger field exerts a stronger reorienting force, so that a shorter pulse time is required to reorient the DNA. Do direct measurements confirm this intuitive picture? Assessment of the bulk reorientation of a population of DNA molecules in the gel can be made by linear dichroism measurements, which exploit the differential absorption of polarized light to detect molecular orientation. Akerman and Jonsson measured the dependence of reorientation time on field strength for T2 DNA, which is 166 kb long (1). They found that the reorientation time scaled as $E^{-1.4 \pm 0.1}$, independent of the reorientation angle. This result is in excellent agreement with the window function exponent, which was determined independently by gel electrophoresis. Thus, the physical reorientation of the DNA molecules is a key determinant of resolution in a pulsed electric field.

Anomalous migration. In the course of these studies, we also discovered that the CHEF system is capable of producing mobility inversion. The phenomenon of DNA inversion is well described for field inversion gel electrophoresis (FIGE) (7). However, it is not peculiar to inversely pulsed fields but is also present in transversely pulsed fields. Previous reports of inversion between chromosomes XII and IV of *S. cerevisiae* were clouded by the possibility that secondary structure in chromosome XII led to the migration anomaly (8, 28, 31). In particular, chromosome XII has several hundred tandem repeats of the rRNA gene, rDNA. Our results indicate that inversion is not due to secondary structure. In *S. pombe*, the ribosomal DNA repeats are restricted to chromosome III, but all three chromosomes could be inverted with respect to each other. Thus, inversion has now been observed for DNA larger than 1 Mb, and it may occur for all sizes, although we have not yet detected the phenomenon for smaller DNA. Fortunately, inversion in transversely pulsed fields only occurs near the top of the gel and is not as pronounced as it is in inversely pulsed fields, where it can extend throughout the gel.

DNA inversion was always accompanied by lateral band spreading. The DNA migrated as a well-defined band but underwent lateral spreading while moving down the gel. Lateral spreading depended on reorientation angle, becoming more pronounced with an increase in angle. This anomalous behavior provides a simple means for identifying size inversion. No a priori knowledge of size is required. By contrast, lateral spreading cannot occur in FIGE since the field is reversed along a single axis. Thus, there is no characteristic signature for detecting mobility inversion. Instead, the forces responsible for lateral band spreading in transversely pulsed fields probably produce vertical band spreading in FIGE. In fact, broad bands are observed, particularly for sizes greater than a few hundred kilobases (28), a problem that seriously compromises resolving power.

Implications for theoretical models. The migration anomalies of inversion and band spreading have practical implications for long-range genomic mapping studies. They must be recognized in order to avoid errors in DNA sizing. In addition, they pose a challenge for understanding pulsed-field electrophoresis. Theoretical models must be capable of

predicting these phenomena if they are to be considered successful.

In the biased-reptation model, DNA is modeled as a segmental chain confined to a "tube" within the gel, moving along its axis through the tube in response to an electric field (14, 25, 26). It is striking that the reptation model successfully predicts both mobility inversion and lateral band spreading (33, 42). The critical determinants of DNA migration in the model are the pulse time, T_P, and the reorientation time of the DNA molecule, T_R, which increases linearly with DNA size. Four distinct migration domains, A, B, C, and D, are predicted by the model. (A) For very short pulse times, $T_P/T_R \ll 1$, DNA molecules comigrate on a low-mobility plateau near the top of the gel (Fig. 3, T_P = 60 s); (B) for moderately short pulse times, $0.1 < T_P/T_R < 1$, DNA molecules migrate in an inverted order and undergo concomitant lateral band spreading (Fig. 3, T_P = 300 s for *S. pombe* chromosomes); (C) for pulse times appropriate for the size range, $10 > T_P/T_R > 1$, DNA is well resolved in the proper order (Fig. 3, T_P = 780 s); (D) for very long pulse times, $T_P/T_R \gg 1$, DNA molecules comigrate on a high-mobility plateau corresponding to the continuous-field limit. The data presented here confirm the existence of these domains in support of the reptation model.

In the chain model of Deutsch and Madden (17), DNA is not constrained to move inside a tube but is modeled as a freely hinged chain that may adopt any configuration (16, 17). The actual behavior of individual DNA molecules observed in the microscope during gel electrophoresis (21, 32, 38) is mimicked closely by simulations in the chain model but not in simple versions of the reptation model. Moreover, the chain model correctly predicts successful resolution of large DNA in a pulsed field, consistent with domains C and D described above (15). However, the chain model has not yet been tested for the presence of mobility inversion and band spreading, domains A and B.

How do the data presented here support the competing theories? A simple model derived from minimal assumptions has general features common to both the chain and reptation models (11). Large DNA is pictured as a deformable "bag" which moves with a limiting mobility in a continuous electric field, adopts an orientation aligned with the field direction, and gradually reorients in response to a change in the field direction. Clearly, these are properties common to both the reptation and chain models. The bag model predicts all four migration domains including the phenomena of mobility inversion and band spreading reported here. This suggests that the chain model will be successful in predicting mobility inversion and band spreading. Conversely, the stringent constraints on DNA motion in the reptation model are not required for its success in predicting those phenomena.

DNA entrapment. What is the major barrier to separating even larger DNA? It appears to be DNA entrapment of one form or other. The phenomenon becomes significant once the DNA size becomes much greater than the characteristic dimensions of the gel matrix (28). Our experiments illustrated several forms of entrapment. Entrapment in the loading well occurred when the field strength was too great. This barrier could be surmounted by a prerun with either a shorter pulse time or smaller field strength.

A second form of entrapment occurred after entry into the gel. Further increases in field strength led first to band smearing, with DNA trailing back from each band, and then to a complete failure to migrate. This phenomenon was particularly prominent for DNA molecules larger than 3 Mb and could be decreased by increasing the ionic strength of the buffer from the conventional ionic strength of 0.5× TBE to 1× TBE. A plausible explanation is that smearing was produced by successive entrapment and release of DNA from the agarose matrix. Higher field strengths may aggravate smearing by making it more difficult for DNA to escape from a trapped configuration. Conversely, higher ionic strengths may reduce smearing by negating electrostatic forces and allowing more efficient elution of DNA from the agarose matrix. This "chromatographic" effect has been noted previously for large DNA (6) and even small DNA (39), but the phenomenon becomes critically important for multimegabase-sized DNA. In this report, we have used simple methods to avoid DNA entrapment: e.g., a prerun with either a short pulse time or low field strength, higher-ionic-strength buffer, and low temperature. New methods to reduce entrapment, such as the development of new gelling materials, are likely to be the best way to push the technology to even larger DNA sizes.

In conclusion, this report demonstrates that appropriate electrophoresis conditions can separate megabase-sized DNA rapidly but that inappropriate conditions may produce mobility inversion and lateral band spreading. Recognition of these migration anomalies will be important for accurate long-range genomic mapping and for evaluating theories of pulsed-field electrophoresis.

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