# High-Resolution Separation and Accurate Size Determination in Pulsed-Field Gel Electrophoresis of DNA. 1. DNA Size Standards and the Effect of Agarose and Temperature†

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ABSTRACT: Pulsed-field gel electrophoresis (PGF) subjects DNA alternately to two electrical fields to resolve DNA ranging from 10 000 base pairs (10 kb) to 10 000 kb in size. The separations are quite sensitive to a variety of experimental variables. This makes it critical to have a wide range of reliable size standards. A technique is described for preparing mixtures of bacteriophage DNA oligomers that span a size range from monomer to more than 30-mer. The relationship between size and mobility of oligomers of different bacteriophage DNA monomers is generally self-consistent. Thus, these samples can serve as primary length standards for DNAs ranging from 10 kb to more than 1500 kb. They have been used to estimate the size of the chromosomal DNAs from various *Saccharomyces cerevisiae* strains and to test the effect of gel concentration and temperature on PFG. DNA resolution during PFG is slightly improved in agarose gels with small pore sizes, in contrast to continuous electrophoresis where the opposite is observed. PFG mobility is surprisingly sensitive to changes in the running temperature.

Pulsed-field gel electrophoresis (PFG)[1] separates DNA molecules in agarose matrices by subjecting them to electric fields that alternate in two directions (Schwartz et al., 1983; Schwartz & Cantor, 1984). The larger the molecule, the larger the fraction of each cycle needed before net translation can occur, and thus the smaller the net translational motion (Cantor et al., 1987; Southern et al., 1987). DNA molecules that have been resolved by PFG range in size from ordinary restriction fragments, less than 10 kilobase pairs (kb), to intact chromosomal DNAs up to more than 5 million base pairs (Mb) (Schwartz & Cantor, 1984; Van der Ploeg et al., 1985; Carle & Olson, 1985; Smith et al., 1986, 1987b; Vollrath & Davis, 1987; Orbach et al., 1988; Mathew et al., 1988b).

PFG separations are quite sensitive to a number of experimental variables. To evaluate this and to determine the size of unknown DNAs, it is essential to have a broad range of DNA size standards. Here we describe the preparation of a number of different tandemly annealed bacteriophage DNA oligomers that cover the molecular weight range from 10 kb to 1.5 Mb. We demonstrate that the PFG mobilities of different samples are generally consistent. Thus, these samples can serve as accurate size standards for PFG experiments. They also show that the resolution of PFG can be better than 5% in size, even for DNA molecules as large as 1 Mb under specific conditions. In this paper we also use such DNA oligomers to evaluate the effects of agarose concentration and temperature on PFG separations. The effects of electrical field and temporal parameters are described in accompanying papers (Mathew et al., 1988a; Cantor et al., 1988).

## Materials and Methods

*Samples.* Bacteriophage DNA oligomers were made from $\lambda cI_{857}$ (monomer = 48.5 kb; Sanger et al., 1982), λvir (42.5 kb; see Results), P2 (31.8 kb; Chattoraj & Inman, 1972), and P4 (11.6 kb; Younghusband et al., 1975). Bacteriophages P2 and P4 were gifts of Richard Calendar. Virions isolated from cesium chloride gradients were directly mixed with low gelling agarose (SeaPlaque, FMC) in SM buffer [100 mM NaCl, 8 mM $MgSO_4$, and 50 mM Tris-HCl (pH 7.5) containing 0.01% gelatin] to yield final concentrations of 0.5% agarose and bacteriophage ranging from 10 to 150 μg of DNA/mL. The liquid mixture was pipetted into 0.1-mL plastic molds (Schwartz et al., 1983) and allowed to solidify at 4 °C for at least 15 min. The agarose plugs were then pushed into modified ESP [0.1 M $Na_2EDTA$ (titrated to pH 9.5 with NaOH), 1% sodium lauroyl sarcosine, and 1 mg/mL proteinase K].

Bacteriophage lysis and DNA annealing reactions were combined by incubating the samples in modified ESP at 50 °C for varying periods of time with gentle shaking. A minimum of 8 h was required for the formation of satisfactory ladders of the lower oligomers, while 20 h sufficed for steps ranging up to the 25-mer. The ladders did not degrade on incubation for up to 80 h. Samples were usually prepared by incubation for 50 h. Sample concentrations of 10–30 μg/mL worked effectively for the production of λ ladders ranging from monomer to 15-mer, while 80–150 μg/mL produced ladders extending to the 30-mer. Samples can then be stored at 4 °C in the same buffer for several months.

Yeast strains D273 and DBY782 are described by Schwartz and Cantor (1984); YN295, a derivative of YP80, was obtained from Ronald Davis; X2180-1A was obtained from the Yeast Genetics Stock Center (University of California at Berkeley). Yeast chromosomal DNAs were prepared by embedding cells in agarose and digesting away all non-DNA components as described by Schwartz and Cantor (1984).

*Pulsed-Field Electrophoresis.* Electrophoresis was carried out in either 33 or 55 cm square horizontal, submarine ap-

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[1] Abbreviations: PFG, pulsed-field gel electrophoresis; kb, kilobase pairs; Mb, megabase pairs; ESP, 0.5 M $Na_2EDTA$, 1% sodium lauroyl sarcosyl, and 1 mg/mL proteinase K; TBE, 0.1 M Tris–borate and 0.2 mM EDTA, pH 8.4; μ, electrophoretic mobility; $P_E$, agarose pore size.

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paratus (the LKB Pulsaphor and a Pulsaphor prototype, respectively) using 20 × 20 cm gels centered in the apparatus with the sides of the gel set at a 45° angle to the sides of the square buffer chamber of the apparatus. In the Pulsaphor, diode-isolated cathodes were positioned at 11, 44, and 77% marks along adjacent sides and anodes at the 22% mark on the other two sides, relative to a reference point at the corner closest to the sample wells. The 55-cm prototype is identical with the Pulsaphor except for its size and the fact that four cathodes, set at 23, 46, 64, and 83%, were used. These symmetrical electrode positions are expected from calculations to produce electrical field gradients in both directions (Cantor et al., 1988). This experimental geometry has been called the double inhomogeneous configuration (Smith et al., 1986) or OFAGE (Carle & Olson, 1984). The angle between the applied fields increases from about 100° to about 140° in a typical experiment (Cantor et al., 1988). Nominal field strengths of 9–10 V $cm^{-1}$ were used, calculated as the applied voltage divided by the size of the buffer chamber (Mathew et al., 1988a). The buffer used was modified TBE [0.1 M Tris–borate and 0.2 mM EDTA (made from the disodium salt), pH 8.3]. Agarose concentrations ranged from 0.6 to 2.2% (w/v). Agaroses were all obtained from the FMC Corp. Seakem LE was used unless otherwise specified as low-gelling (SeaPlaque) or high-gelling strength (SKA 4864-30).

Mobility was measured from densitometric scans of gels. Gels were stained with ethidium bromide (1 μg/mL for 10 min) and photographed. The negatives were scanned on a Joyce Loebl Chromoscan 3. A clear plastic ruler was also photographed on the gel and scanned as an internal length standard. Estimates of migration distances were reproducible to within 2%. The distance traveled was taken as the component along the diagonal of the apparatus, parallel to the sides of the gel and perpendicular to the sample wells (Cantor et al., 1988). The mobility is calculated as the distance divided by the product of the running time and the nominal applied voltage, defined above.

## Results and Discussion

*Preparation of Oligomers of Different-Sized Bacteriophage DNAs.* The sizes of bacteriophage DNAs $\lambda cI_{857}$ (48.5 kb), P2 (31.8 kb), and P4 (11.6 kb) are known (Sanger et al., 1982; Younghusband et al., 1972; Chattoraj & Inman, 1975). The size of λvir was determined by restriction mapping using the enzymes *Bam*H1 and *Acc*I. This bacteriophage appears to have a spontaneous deletion of the b2 region that extends from about nucleotides 21 700 to 27 000 in the sequence of $\lambda cI_{857}$. We estimate the deletion to be between 5.5 and 6 kb, yielding a size of 42.5–43 kb. These bacteriophages have single-stranded cohesive ends of 12 (λ phages; Sanger et al., 1982) and 19 bases [P2 (Younghusband et al., 1972) and P4 (Chattoraj & Inman, 1975)]. The cohesive ends can be used to form oligomers of different, known, chain lengths held together by noncovalent interactions. Such concatemers are stable under typical gel electrophoretic conditions even over protracted time periods.

While bacteriophage concatemers can be made in solution, we find it more convenient to carry out all steps in situ in agarose since this greatly facilitates all subsequent sample handling. The other advantage of working in agarose is that it raises the apparent DNA concentration by molecular exclusion effects (Zimmerman & Harrison, 1985) and thus allows the use of lower DNA concentrations (Wang & Davidson, 1966). The essential step in the preparation is to lyse and anneal the bacteriophage in a modified ESP (Materials and Methods). Bacteriophages purified over a CsCl gradient are routinely used. This may be important for good ladder formation. We have also tried to use DNA ligase to link the oligomers covalently. However, this has not led to any improvement, and in some cases addition of the ligase apparently damaged the λ oligomers. It is possible that the level of nuclease contamination in some commercial DNA ligase preparations is too high to allow their use on such large DNA.

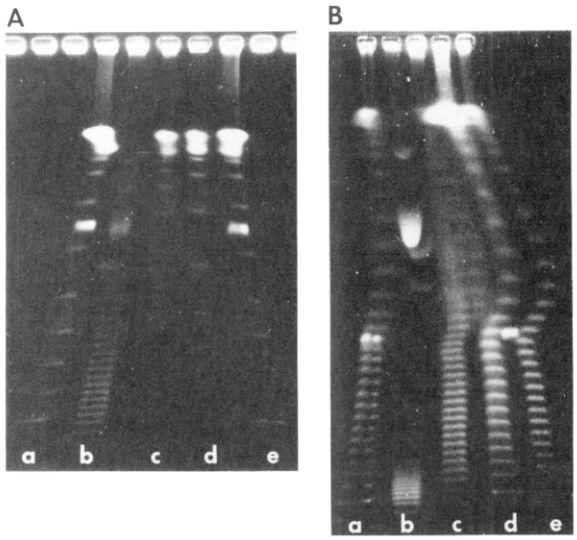


FIGURE 1: Size dependence of the mobility of DNA during PFG. Separation of tandemly annealed bacteriophage DNA ladders in 1% agarose was performed at 9.1 V $cm^{-1}$ for 72 h in 55-cm apparatus with a pulse time of (A) 25 and (B) 110 s: (Lanes a and e) λvir DNA (40.5 kb); (lane b) P4 DNA (11.6 kb); (lane c) P2 DNA (31.8 kb); (lane d) $\lambda cI_{857}$ DNA (48.5 kb). The smears about a fifth of the way down the gel correspond to the compression zone. The smear in the upper portion of the P4 ladder (lane b in the 110-s experiment) is probably due to the presence of knotted DNA (Wolfson et al., 1985). The bright band seen in lanes a and e is an unknown contaminant of this particular λvir preparation. Considerable sinuosity is seen in the upper half of the two right lanes of the 110-s experiment.

Examples of PFG analyses of bacteriophage concatemers are shown in Figure 1. To avoid overloading the gel and reducing resolution, it is necessary to load sections or even slivers of the agarose blocks. We recommend that not more than 2 μg of bacteriophage DNA be loaded in one lane. Low temperatures and moderate ionic strengths promote the association of sticky ends in a mixture of bacteriophage DNA molecules. This should result in a population of DNA oligomers, some linear and others circular. The expected distribution of oligomers is concentration dependent, with monomer circles favored in dilute solution and linear polymers favored in highly concentrated solution (Wang & Davidson, 1966; Dugaiczyk et al., 1975). In theory, at sufficiently high DNA concentrations, the sample should be skewed toward extremely long linear aggregates, ideally approaching infinite length. The actual, much shorter, distribution observed presumably reflects chain termination due to damaged single-stranded ends. We routinely use λ DNA concentrations of around 100 μg/mL to make ladders extending up to 1500 kb. For many PFG applications, a step size of around 50 kb is convenient, and so we use $\lambda cI_{857}$ as our standard in most experiments.

*Relationship between Mobility and DNA Size.* The electrophoretic mobility ($\mu$) of DNA is expressed as the distance moved per unit field in unit time. The PFG mobility of a DNA species was computed (as described under Materials and Methods) as the net distance migrated, parallel to the sides of the gel, divided by the nominal electrical field strength and

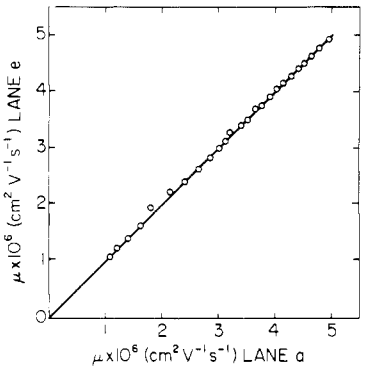


FIGURE 2: Reproducibility of DNA mobilities in different gel lanes. Mobilities of corresponding bands of the λvir samples in lanes a and e of Figure 1B (measured parallel to the edge of the gel) are plotted against one another. The line drawn has a slope of 1, expected if mobilities were identical in the two lanes. Points are data for measured mobilities of corresponding bands. Deviations from linearity are largest in the regions of extreme curvature but are never very large.

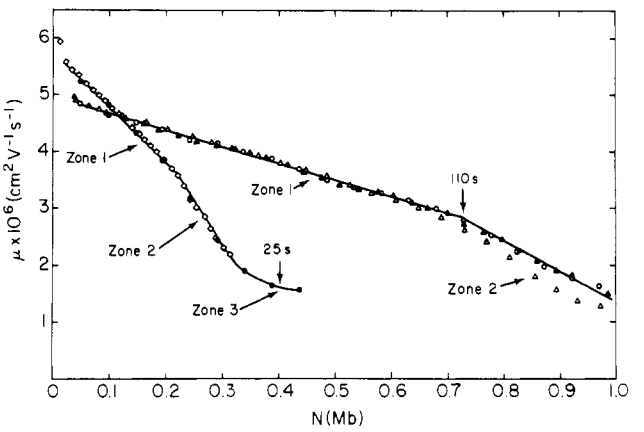


FIGURE 3: Mobilities of different DNA species during PFG as a function of their size: (○) λcI$_{857}$ DNA at 110 s; (△) λvir DNA at 110 s; (▲) P2 DNA at 110 s; (●) λcI$_{857}$ DNA at 25 s; (◇) P4 DNA at 25 s. The various zones of separation are indicated. Data are from the experiment shown in Figure 1.

the running time. Varying the running time by a factor of 2 did not alter the estimated mobility significantly, indicating that the apparent velocity of DNA is effectively constant through the course of an experiment.

In PFG only a nominal mobility can be estimated. In the limit of long pulse times, DNA will follow a zigzag path. Hence, the measured distance is less than that actually migrated. In the limit of short pulse times, the molecules presumably migrate directly along the diagonal in response to an average effective field, which is less than the field applied in one direction. At intermediate pulse times, no net translational motion may occur for a significant proportion of the pulse, and so the elapsed time of the experiment is an overestimate of the migration time. These differences must be considered when mobilities estimated in conventional and PFG electrophoresis are compared.

Each of the bacteriophage DNA samples consists of a set of molecules increasing in size by a constant increment. The spacing between adjacent bands of each DNA concatemer is fairly uniform, at least over the lower half of the gel (Figure 1), giving the appearance of a ladder.

The PFG configuration used [see Materials and Methods and Figure 1b of Cantor et al. (1988)] results in lanes that are symmetric about the diagonal of the buffer chamber. For samples in the central lanes of the gel, the distance migrated parallel to the edges of the gel by any DNA species is uniform across the gel irrespective of spreading or any lane sinuosity that may occur. Sinuosity is a deviation from a straight lane such as the curvature in the top of lane e in Figure 1B. Lane a in the same figure contains the same sample (λvir) as lane e but shows no sinuosity. Lane curvature, like that seen in lane e, is common in PFG. Its actual cause is unknown, but it appears to be exaggerated by the presence of air bubbles under the gel plates, which presumably lead to temperature variations due to the decreased local efficiency of heat exchange. Despite differences in the total distance migrated due to sinuosity, migration parallel to the sides of the gel in the direction of the resultant of the two alternate electric fields (Cantor et al., 1988) is almost identical for the two λvir lanes of Figure 1B as demonstrated in Figure 2. This uniform mobility makes it possible to compare, quantitatively, results from different central lanes on the same gel and, of course, from independent gel experiments.

The outside lanes of the gel tend to curve toward the sides of the gel, and lateral spreading of bands is often observed toward the bottom of the gels [for examples, see Smith et al. (1987a)]. These distortions are uniform and invariably directed outward, unlike the sinuosity in lane e of Figure 1B that loops away from and back to the diagonal. Uniform distortions usually increase with distance from the center of the gel and also with run distance and field strength. Thus, for quantitative analysis of mobility, only the central lanes were used, and runs were terminated at least 5 cm from the bottom edge of the gel to minimize distortions.

At fields of 5 V cm$^{-1}$ or lower, samples run in relatively straight lanes with little lateral spreading and few distortions. However, DNA molecules take much longer to move the same distance than at 10 V cm$^{-1}$ (Mathew et al., 1988a). The conflicting demands of time and aesthetics require a compromise. For separations in the range 100–1500 kb we usually employ 9.1–10 V cm$^{-1}$ fields for a 40-h running time.

The mobilities of ladders made from four different DNA monomers were compared at 9.1 V cm$^{-1}$ with pulse times of 25 (Figure 1A) and 110 s (Figure 1B) to determine whether DNA sequence influences mobility. Any anomalous electrophoretic behavior of DNA monomers might be amplified in the long oligomers. Plots of mobility versus molecular size for the two experiments are presented in Figure 3. Three regimes or zones are apparent. The first two zones exhibit a linear decrease in mobility with increasing DNA size. The slope in zone 2 is about twice that in zone 1. Still larger DNA molecules do not resolve at this pulse time and bunch together in the compression region, zone 3.

The mobilities of three DNAs, P2, P4, and λcI$_{857}$, are mutually consistent with their known sizes at 25-s pulse times, and most of the size range fractionated at 110-s pulse times. However, there is some significant divergence in the behavior of different samples in the high molecular weight range at 110-s pulse times. This is unlikely to reflect a small error in the estimation of the monomer sizes, since that would appear progressively, and not, as we observe, suddenly, at the inflection point between the two zones of separation. The slopes of the plots above the inflection point are similar for all the bacteriophage samples, indicating that the fractionation mechanism is sample independent in this zone as well.

The PFG migration of P4 is consistent with the migration of DNA restriction fragments and ligated plasmid ladders of known size (data not shown). Thus, the mobilities of oligomers of P2, P4, and λcI$_{857}$ can be used as size standards for PFG. In contrast to the self-consistent behavior of these three DNA samples, the fourth DNA, λvir, behaved anomalously. To be consistent with the other samples, the size of the λvir would

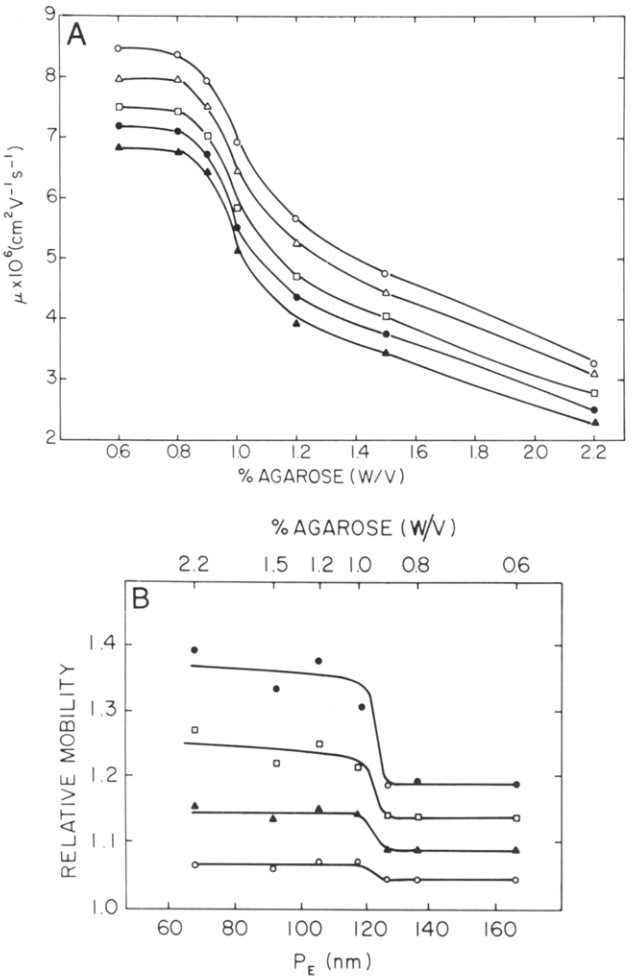


FIGURE 4: PFG mobility of $\lambda cI_{857}$ DNA as a function of agarose concentration. All gels were run at 10 V $cm^{-1}$ and 100-s pulse time in a 33-cm apparatus. (A) Mobility as a function of size: (○) 48.5 kb; (△) 145.5 kb; (□) 291 kb; (●) 388 kb; (▲) 485 kb. (B) Relative separations of different-sized DNAs as a function of maximum pore size, $P_E$, and agarose concentration (relative mobility of a 97-kb molecule versus that of larger molecules as indicated): (○) 145.5 kb; (▲) 291 kb; (□) 388 kb; (●) 485 kb.

have to be 40.5 kb, whereas the true size was determined by several restriction digests to be 42.5 kb. We have no explanation for this discrepancy, but it indicates that in PFG, as in ordinary electrophoresis (Hagermann, 1985; Koo & Crothers, 1987), complexities may arise from DNA sequence or tertiary structure. It is interesting that λvir is generated from $\lambda cI_{857}$ by deletion. The b2 region deleted in λvir is AT rich and contains DNA known to show anomalous mobility in normal electrophoresis (Ross & Landy, 1982). DNA at the λ bacteriophage replication origin also migrates anomalously in conventional electrophoresis, presumably due to DNA curvature (Zahn & Blattner, 1985). Perhaps the curvature in the b2 region somehow compensates for the replication origin curvature, and deletion of the b2 region eliminates this compensation.

*Effect of Agarose Concentration and Gel Type.* Figure 4A illustrates the PFG mobility of several size DNAs as a function of agarose concentration from 0.6% to 2.2% (w/v). The maximum pore size of the gel decreases progressively with increasing agarose concentration (Serwer & Hayes, 1986), and the mobility of the various DNA oligomers decreases concomitantly. In gel concentrations above 0.8% the decrease is particularly steep between 0.9% and 1.2% agarose. However, the mobility changes seen here are somewhat smaller than in conventional agarose gel electrophoresis. For instance, mobility

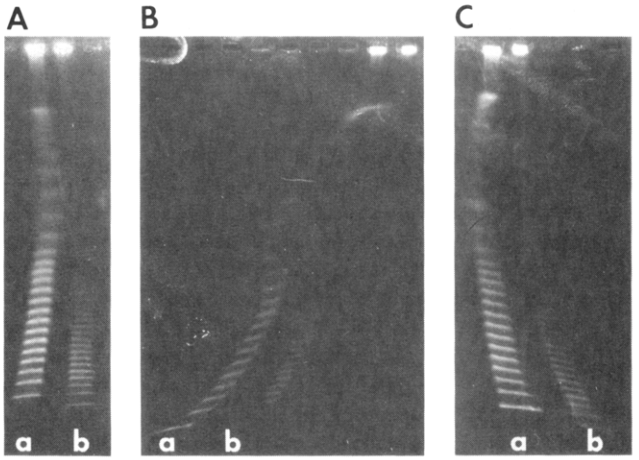


FIGURE 5: DNA mobility in gels cast with different types of agarose. All gels were run at 10 V $cm^{-1}$ and 100-s pulse time in a 33-cm apparatus for 40 h. (A) Low-gelling-temperature agarose; (B) LE agarose; (C) high-gel-strength agarose. Lanes contain (a) $\lambda cI_{857}$ DNA (48.5 kb) and (b) P2 DNA (31.8 kb).

of a 388-kb DNA in PFG decreased by a factor of 2.8 when the agarose concentration increased from 0.6% to 2.2% agarose. A corresponding change in pore size in conventional gel electrophoresis decreased the mobility of 3.2-kb DNA by a factor of 3.3 (Hervet & Bean, 1987).

An interesting effect is observed when the mobilities of fragments 200–500 kb in size, relative to a 100-kb reference size, are plotted against the agarose pore size (Figure 4B). The pore sizes used are actually estimates of the maximum pore radii in the matrix (Serwer & Hayes, 1986). Unfortunately, there is no accepted theory for obtaining the average pore sizes from the measured maxima. Over much of the range examined, the change in the relative mobility with pore size is quite small. However, a sharp transition in relative mobility is seen around 1.1% agarose, indicating that resolution is somewhat better for 1.2% agarose gels than for 0.9% gels. Further increases in agarose concentration merely decrease the mobility and hence increase the run time for comparable migration without improving the resolution.

In continuous electrophoresis, resolution improves with increasing pore size (McDonell et al., 1977; Fangman, 1978; Serwer, 1981). In PFG on the other hand, the opposite is true. The agarose transition region is centered around a 125-nm pore size and is independent of DNA size up to 500 kb. This suggests that the structural features of the DNA seen by the agarose pores are independent of DNA size, consistent with the assumption in most models of PFG that DNA molecules are highly extended (Mathew et al., 1988a). The transition region may represent a shift from one fully extended DNA configuration to another. Presumably, in a loose gel, DNA molecules can still experience some local curvature within the gel pores, while in tighter gels this becomes less likely. The persistence length of DNA under our conditions is ~50–70 nm (Harrington, 1978; Cantor & Schimmel, 1981). This is probably within a factor of 2 of the average pore radius.

Gels cast with low-melting agarose and high-gel-strength agarose can also be used for PFG. The resolution obtained is similar to that seen with low electroendosmosis (LE) agarose (Figure 5). Gels cast with these modified forms of agarose differ in pore size from standard LE agarose (Serwer, 1983). The fact that resolution is not significantly altered by changing the gel type is consistent with the relative insensitivity of PFG separation to pore size.

*Effect of Temperature.* Figure 6 is a plot of mobility versus DNA size for gels run at 10, 15, and 20 °C. It reveals that

Table I: Assignment and Sizes of Yeast Chromosomal DNAs

| band | chromosome[a] | molecular size[b] (kb) | | | | molecular recombination | |
|---|---|---|---|---|---|---|---|
| | | D273 | D782 | YN295 | X2180-1A | size[c] (kb) | length[c] (cM) |
| 1 | I | 275 | 275 | 225 | 250 | 198 | 98 |
| 2 | VI | 275 | 290 | 295 | 280 | 225 | 137 |
| 3 | III | 360 | 360 | 375 | 360 | 311 | 137 |
| 4 | IX | 450 | 450 | 450 | 440 | 495 | 198[d] |
| 5 | VIII | 610 | 600 | 555 | 600 | 635 | 183[d] |
| 6 | V | 610 | 600 | 610 | 600 | 709 | 233 |
| 7 | XI | 705 | 685 | 680 | 670 | 763 | 242[d] |
| 8 | X | 793 | 760 | 745 | 740 | 835 | 200[d] |
| 9 | XIV | 793 | 790 | 785 | 780 | 864 | 283 |
| 10 | II | 850 | 790 | 815 | 810 | 948 | 244 |
| 11 | XIII | 930 | 930 | 915 | 930 | 991 | 229 |
| 12 | XVI | 945 | 950 | 945 | 940 | 1047 | 176[d] |
| 13 | XV | 1110 | 1110 | 1100 | ND[e] | 1285 | 361 |
| 14 | VII | 1110 | 1110 | 1120 | ND[e] | 1146 | 391 |
| 15 | IV | 1840[f] | 1800[f] | 1640[f] | ND[e] | 1571 | 485 |
| 16 | XII | 2215[f] | 2200[f] | 1900[f] | ND[e] | 2194 | 594[d] |

[a] Assignments based on those of Carle and Olson (1985) for strain AB972 and Schwartz and Cantor (1984) for strains D273-10B/A1 and DBY782. [b] Sizes based on comparison with $\lambda cI_{857}$ concatemers. [c] From Mortimer and Schild (1985), assuming a haploid genome size of 14 000 kb. The estimates are from a map of *S. cerevisiae* made with all available data. Some uncertainties arise due to the number of different strains used. [d] Lower limit of recombination length. [e] ND = not determined. [f] Sized on the basis of ethidium-stain intensity. Note that this probably yields a lower limit on the size.

PFG mobility is very sensitive to changes in temperature; hence, careful thermostating of the gel during electrophoresis is critical. In conventional electrophoresis, the temperature dependence of the mobility of DNA is largely due to variations in the solvent viscosity (Serwer & Allen, 1984; Hervet & Bean, 1987). The mobility increase seen in PFG on increasing the temperature from 15 to 20 °C (21%) is almost twice as much as the decrease in viscosity (11%) over the same range.

To examine temperature and viscosity effects independently, PFG mobility at 20 °C in the presence of 4.85% sucrose was compared to that at 15 and 20 °C without sucrose. The solvent viscosity in the first two cases is the same. The mobility measured in the sucrose-containing buffer is significantly higher than that in buffer alone at 15 °C, while it is significantly less than the mobility in buffer alone at 20 °C. These results indicate that the effect of bulk viscosity on PFG is not large enough to account for the temperature dependence of PFG mobility fully. Additional thermal effects must be involved. It is possible that the viscosity sensed by DNA while reorienting is a local microviscosity distinct from the bulk viscosity. Alternatively, the temperature dependence may reflect the activation energy of forming DNA configurations required for reorientation. For example, melted regions are required if the DNA forms hairpin structures.

*Yeast Chromosome Sizes.* The use of bacteriophage DNA concatemers as size standards requires their validation against DNAs sized independently. Sizes of DNAs from T4 (170 kb; Kim & Davidson, 1974; O'Farrell et al., 1980) and *Bacillus megatherium* bacteriophage G (750 kb; Donelli et al., 1975; Krasin, 1979) have been estimated on the basis of their contour lengths in electron micrographs. We have used $\lambda cI_{857}$ ladders to obtain sizes of 170 kb for T4 and 630 kb for bacteriophage G. Carle et al. (1984) have reported estimates of 170 kb for T4 and 670 kb for bacteriophage G also using λ ladders. We can, therefore, use these concatemers with reasonable confidence as size standards up to 700 kb.

We have further verified the self-consistency of ladders by using them to estimate the sizes of fragments resulting from total restriction nuclease digests of the *Escherichia coli* K12 genome (Smith et al., 1987a). Fragments generated by different enzymes, when sized by using λ size markers, add to yield the same genome size of 4.7 Mb. This is almost identical with the size reported by Kohara et al. (1987) on the basis

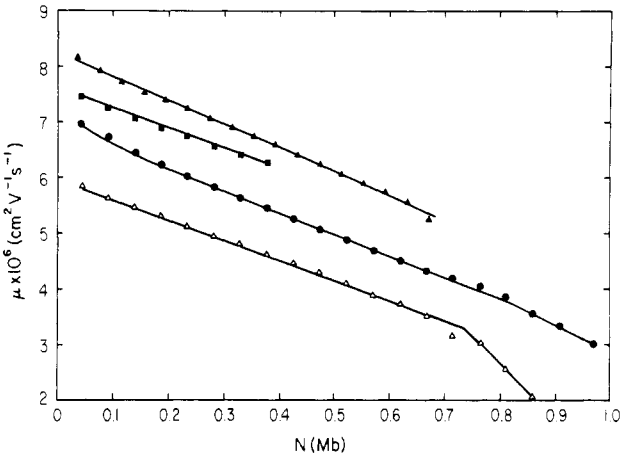


FIGURE 6: Effect of temperature and viscosity on $\lambda cI_{857}$ DNA mobility. All gels were run in 1% agarose at 10 V $cm^{-1}$ and a pulse time of 100 s in a 33-cm apparatus. Temperatures were (△) 10 °C, run for 40 h; (●) 15 °C, run for 40 h; (▲) 20 °C, run for 30 h; and (■) 20 °C in buffer containing 4.85% sucrose, run for 43 h. The viscosity of a 4.85% sucrose solution at 20 °C is the same as that of water alone at 15 °C (1.139 cP).

of mapping the *E. coli* genome with a library of overlapping clones. Furthermore, fragments from different strains, related by simple inversions, also yield a constant genome size despite variation in the sizes of the individual fragments produced on digestion (Smith et al., 1987a). Since the largest fragments were sized at 1 Mb, the ladders are presumably accurate to at least this size.

The bacteriophage DNAs can be used to determine the size of naturally occurring DNAs, which can then serve as secondary size standards. We have used $\lambda cI_{857}$ ladders to size all but the two largest chromosomes of four common *Saccharomyces cerevisiae* strains as shown in Figure 7. The results are presented in Table I. The molecular weights of the two largest chromosomes, estimated by the intensity of ethidium staining of the gels following electrophoresis, are likely to be lower limits, since 100% recovery is assumed. Our size estimates for the six smallest chromosomes are similar to those made by Carle and Olson (1984). While separations of the larger chromosomes have since been reported (Carle & Olson, 1986), no estimates of their sizes have been made. The largest chromosome in *S. cerevisiae* invariably migrates

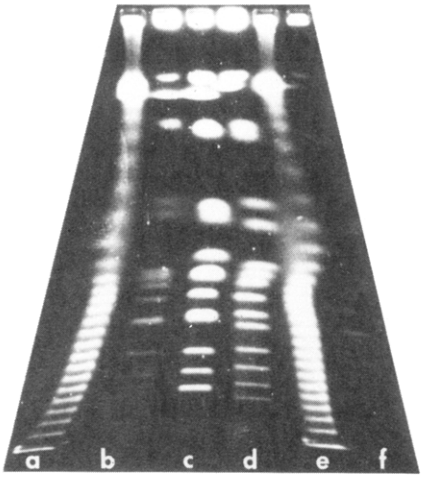


FIGURE 7: Sizing of yeast chromosomal DNAs. The gel was run in 1% agarose at 9.1 V $cm^{-1}$ and a pulse time of 130 s in a 33-cm apparatus for 52 h. Lanes contain (a) $\lambda cI_{857}$ DNA, (b) yeast strain D782, (c) yeast strain D273, (d) yeast strain YP80, (e) $\lambda cI_{857}$ DNA, and (f) yeast strain CF22. A negative of the photograph was scanned to construct a calibration curve of mobility vs $\lambda cI_{857}$ size, and this allowed the determination of the sizes of the first 14 bands in the yeast chromosomal lanes. A separate calibration plot was constructed by using the ethidium-staining intensities of bands 10–14, and this was used to estimate the lower limit of the sizes of the top two bands.

much faster than the smallest *Schizosaccharomyces pombe* chromosome (Smith et al., 1987b). The latter has been sized at 3.8 Mb,[2] which puts an upper limit on the size of *S. cerevisiae* chromosome XII. On the basis of gels that resolve the three *S. pombe* chromosomes very well, we estimate an upper limit of 2.5 Mb for chromosome XII, which is consistent with the results shown in Table I.

The utility of accurate size standards in an analytic technique based on size is obvious. The actual molecular weights resolved in a given experiment can be tuned by a suitable choice of pulse times and electric field strength (Mathew et al., 1988a). The bacteriophage DNA concatemers can serve as primary size standards in the range of 10–1500 kb with an accuracy of better than 5% throughout this size range. The fact that the size–mobility relation is monotonic in the double inhomogenous PFG configuration allows the comparison of bands on different gels provided a size standard is run on each gel. Note that other pulsed-field configurations such as field inversion (Carle et al., 1986) do not always show a monotonic relationship between mobility and size (Ellis et al., 1987).

In addition to providing size standards, bacteriophage DNA ladders allow some of the experimental parameters important in high-resolution PFG separations to be examined. The nature of the gel matrix is not terribly important. The mobility of DNA in PFG decreases with decreasing pore size, but to a smaller extent than in continuous electrophoresis. Resolution is almost independent of pore size, except for a sharp change centered around 125 nm. On the other hand, mobility is extremely sensitive to temperature, indicating that some step in the PFG process has a significant activation energy. Bacteriophage ladders have also been used to study the influence of pulse time and electric field strength and shape on PFG (Mathew et al., 1988a; Cantor et al., 1988).

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**Registry No.** Agarose, 9012-36-6.

[2] J.-B. Fan, C. L. Smith, Y. Chikashige, O. Niwa, M. Yangida, and C. R. Cantor, unpublished observations.

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# High-Resolution Separation and Accurate Size Determination in Pulsed-Field Gel Electrophoresis of DNA. 2. Effect of Pulse Time and Electric Field Strength and Implications for Models of the Separation Process†

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ABSTRACT: Bacteriophage DNAs annealed into linear oligomeric concatemers were used to examine the quantitative pulsed-field gel electrophoretic behavior of different-sized DNAs as a function of electrical field strength and pulse time. Three zones of resolution are observed for increasingly larger DNAs. In the first two zones, the electrophoretic mobility decreases linearly with increasing DNA size. The separation in zone 2 is roughly twice that in zone 1. The largest DNA molecules do not resolve at all and migrate in a compression zone. Mobility in zone 1 increases linearly with the electric field strength and decreases with the inverse of the pulse time. The behavior of DNA in zone 2 is qualitatively similar. However, the effect of field strength and pulse time on the separations in each zone is quite different. The results for zone 1 are generally consistent with the predictions of several existing physical models of pulsed-field gel electrophoresis, but no model accounts for all of the observed behavior in the three zones.

Conventional agarose gel electrophoresis of DNA, like gel filtration, is a sieving technique. The driving forces, gravitational in gel filtration and electrical in electrophoresis, are proportional to the size of the macromolecule. Frictional forces are also proportional to size, and so the velocity of particles in both techniques is size independent (Olivera et al., 1984). Separation of molecules smaller than the maximum pore size of the matrix occurs because the smaller the molecule, the larger the fraction of pores it can enter. The size limit for sieving of DNA is a few hundred base pairs (bp), where DNA lengths approach the size of the pores. Larger molecules move through the matrix in a stretched out form by a reptation process with a velocity that is inversely proportional to length (Lumpkin & Zimm, 1982).

DNA molecules with sizes above about 20 000 base pairs (20 kb)[1] do not resolve well in conventional agarose gel electrophoresis at finite field strengths because of electrical orientation effects (Lumpkin et al., 1985). However, the introduction of pulsed fields dramatically extends the range of agarose gel electrophoretic separations (Schwartz et al., 1983). In this technique, DNA is subjected alternately to two electrical fields at different angles for a time called the pulse time, $\tau$. The molecules must presumably change direction prior to net translational motion each time the field is switched. Larger molecules take longer to change direction and have less time to move during each pulse, so they migrate slower than smaller molecules. We can operationally define a reorientation time as that portion of the pulse that does not result in net translational motion. The exact meaning of this time will depend on the particular physical model used to describe the reorientation process.

Pulsed-field gel electrophoresis (PFG) should resolve molecules that can complete reorientation within the pulse time. Larger molecules, which are unable to reorient rapidly enough, will presumably achieve an average orientation midway between the two applied fields (Schwartz & Cantor, 1984). Molecules so small that their reorientation time is short

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[1] Abbreviations: kb, kilobase pairs; Mb, megabase pairs; $\tau$, pulse time; $E$, electric field strength; TBE, 0.1 M Tris, 0.1 M borate, and 0.2 mM EDTA, pH 8.4; $\mu$, electrophoretic mobility; $N$, molecular size; $N_i$, size of DNA species at the inflection point between zones 1 and 2; $N_c$, size of the smallest DNA species in the compression zone; $\mu_i$, mobility of the DNA species at the inflection point between zones 1 and 2; $\mu_c$, mobility of the compression zone; $m$, number of pulses; $h$, length of the DNA molecule; $\theta$, angle between the midline of the gel and the applied field; $r$, distance moved in PFG; $r_0$, distance moved in continuous electrophoresis; $\mu_0$, mobility of DNA in continuous electrophoresis.

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