The effect of DNA concentration on mobility in pulsed field gel electrophoresis

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ABSTRACT

The effect of DNA concentration on pulsed field gel electrophoretic mobility was studied for human genomic DNA prepared in agarose inserts at 8–800 $\mu\text{g/ml}$ and digested to completion with *Not* I. An eighth of each 100 μl insert was used to produce DNA loads of 0.1 to 10 μg per lane. The mobility of single copy restriction fragments, as detected by hybridization, was largely concentration independent when DNA concentrations were 80 $\mu\text{g/ml}$ or less. However, at DNA concentrations of 200 $\mu\text{g/ml}$ and greater, dramatic effects of DNA concentration are evident. In the worst case, at 800 $\mu\text{g/ml}$, the apparent size of a DNA fragment is almost 2.5 times its true size. At constant DNA concentrations, increasing the DNA mass loads by loading larger insert slices had no further effect on DNA electrophoretic mobility, although the bands were broader for bigger insert slices. Thus, for precise and accurate sizing in pulsed field gel electrophoresis the DNA concentration in agarose inserts should not be greater than 80 $\mu\text{g/ml}$ (10^7 diploid human cells/ml agarose insert).

INTRODUCTION

Pulsed field gel electrophoresis (PFG, 1) is a technique which can separate DNA molecules up to 10 megabases (Mb) in size. PFG is a powerful tool for establishing long range restriction maps of large regions of chromosomal DNA, and for helping to localize disease genes. Since its introduction, numerous experimental studies have focused on optimizing the various configurations and parameters (including pulse times, voltage gradient, reorientation angle, temperature, and agarose concentration) which are critical for high-resolution separations (2–14). It has also been noted (7,8) that mobility of DNA molecules in PFG is quite sensitive to DNA concentration. However, this effect has not been quantitated. Here, the effect

of DNA concentration on PFG mobility was characterized under conditions comparable to those used in typical mammalian physical mapping experiments.

MATERIALS AND METHODS

Preparation of DNA Inserts and Restriction Enzyme Digests

Human DNA was prepared in low gelling-temperature agarose inserts (100 μl) from an Epstein-Barr virus-transformed human lymphoblastoid cell line, Gus 570, (Dr. J. Gusella, Mass. General Hospital, Boston) using from 10^6 to 10^8 logarithmically growing cells/ml (15,16). Cell concentration was determined with a hemacytometer. DNA concentration was estimated as follows: 6×10^9 bp genomic DNA/diploid cell + 1.66×10^4 bp mitochondrial DNA $\times 5 \times 10^3$ copies = 6.08×10^9 bp total DNA/diploid cell. 6.08×10^9 bp total DNA/cell $\times 660$ gram/mole bp $\times 1$ mole/ 6.02×10^{23} bp = 6.67 picograms total DNA/diploid cell. For logarithmically growing cells, this value was increased by 20% to account for the increased DNA content of cells in S and G2 phases to yield an average of 8.0 picograms DNA/cell. This resultant value of 8.0 picograms DNA/cell was used for estimating DNA concentrations in agarose inserts. Restriction enzyme digests of human DNA were performed with 20 units *Not* I/ μg DNA for 4 hr at 37°C. One eighth slice of each insert (0.1 to 10 μg DNA) was loaded in each PFG lane. *S. cerevisiae* DNA inserts were prepared from the diploid strain YPH 274 at concentrations between 3.5×10^8 and 1.4×10^{10} cells/ml (approximately 5.2–210 $\mu\text{g/ml}$ DNA for stationary phase cells) as described (15).

PFG

Inhomogeneous PFG was performed on a Pulsaphor apparatus (Pharmacia-LKB) using the orthogonal electrode configuration as described elsewhere (1, 7). Homogeneous PFG was performed on a BioRad CHEF II DR apparatus. Gels were cast from 1.0% agarose (Seakem LE Agarose, FMC) and were run at 14°C in

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0.5×modified TBE buffer (45 mM Tris base/45 mM boric acid/0.2 mM EDTA, pH 8.2). Lambda DNA ladders (48.5 kb), or *S. cerevisiae* and *S. pombe* chromosomal DNA markers (approximately, 100, 50, and 10 µg/ml respectively, and all from FMC Bioproducts) were included as size standards. Pulse times, run times and the voltage employed for each run are indicated in the figure legends.

DNA Transfer and Hybridization

DNA in agarose gels was nicked with 310 nm light from a Photodyne UV transilluminator for 90 sec, then denatured in 0.5 M NaOH/0.5 M NaCl for 1 hr, and neutralized in 0.5 M Tris-HCl, pH 7.5/1 M NaCl for 1 hr. DNA was transferred to nylon membranes (Nytran, Schleicher & Schuell) by capillary action with 10×SSC.

Probes for hybridization were prepared by separating cloned

inserts from plasmid vectors by conventional agarose gel electrophoresis. After electroelution, these DNAs were ³²P-labeled by priming with random hexanucleotides (17). Prehybridization was for 2–4 hr at 65°C in 0.5 M sodium phosphate buffer (pH 7.2), 7% SDS, 1mM EDTA, and 100 µg/ml denatured salmon sperm DNA (18). Radiolabeled probe (10⁸ to 10⁹ cpm/µg) was added to the prehybridization solution, and hybridization was allowed to proceed for 14–18 hr at 65°C. Filters were washed to a final stringency of 0.5×SSC at 65°C, and exposed to X-ray film (Kodak XAR-5) at –70°C with two intensifying screens (Dupont Cronex Lightning-Plus) for 1–7 days.

DNA Probes

DNA probes pBS731B-C, pBS674E-D (both Dr. J. Wasmuth, University of California, Irvine), C4H (Dr. J. Gusella, Mass.

Figure 1. Southern blots of increasing human DNA concentrations fractionated by PFG. The concentration of DNA loaded is indicated above the lanes. For each lane, a one eighth slice of each insert (12.5 µl) was used, yielding DNA mass loads from 0.01 to 10 µg. Pooled single copy probes were used in the hybridizations to detect a range of fragment sizes (Nonidentical probe pools in each hybridization). In (A), an inhomogeneous electrode configuration was used for a 48 hr run at 10 volts/cm with a 120 sec pulse time, in (B), a homogeneous electrode configuration was used for a 48 hr run at 4.5 volts/cm with a 125 sec pulse time.

Table I. Apparent sizes (kb) of PFG fractionated restriction fragments with increasing DNA concentrations.

Gel Conditions	Fragment Size (kb)								
	Concentration of DNA loaded (µg/ml)								
	8	20	40	60	80	200	400	600	800
Inhomogeneous Gel 125 s pulse time	140	145	155	170	170	195	220	265	315
	380	390	400	410	410	445	475	510	560
		535	545	560	560	605	630	670	705
		730	740	750	750	765	785	805	820
Homogeneous Gel 125 s pulse time		70	75	75	75	95	115	145	175
		175	180	185	185	225	255	310	350
		410	415	420	420	465	510	560	625
				735	740	785	815	855	890
Homogeneous Gel 1000–3000 s pulse times		1100	1150	1250	1250	1450	1550	1650	1850
		1700	1850	1850	1850	2100	2200	2250	2500
Homogeneous Gel 3000–5000 s pulse times						1640	2000	2250	2500
		1640	1720	1750	1800	2370	2870	3120	3370

General Hospital, Boston), and D5 (Dr. P. Harper, University of Wales, Cardiff) were used in the hybridization for Figure 1A. DNA probes pBS678E-D, pBS854PB, pBS674E-D (all Dr. J. Wasmuth, University of California, Irvine), and D5 (Dr. P. Harper, University of Wales, Cardiff) were used in the hybridizations for Figures 1B, and 5. Probes pGSH8 (Dr. G. Stewart, Howard Hughes Medical Institute, Ann Arbor) and pPW518-1R (Dr. P. Watkins, Life Technologies, Gaithersburg) were used in the hybridizations for Figure 3.

RESULTS

Human DNA, in agarose inserts at concentrations between 8 and 800 $\mu\text{g/ml}$ was digested with *Not* I and fractionated by inhomogeneous or homogeneous PFG to resolve fragments from 50 kb to 1 Mb. Southern blots were hybridized with pooled single copy probes (Fig. 1). Radiolabeled lambda DNA was also

Figure 2. The effect of DNA concentration on electrophoretic mobility in homogeneous PFG. Restriction fragment sizes from Figure 1B are plotted as a function of the concentration of DNA loaded.

Figure 3. Southern blot of increasing human DNA concentrations fractionated by PFG with a 3000–5000 sec pulse ramp and hybridized with two single copy probes. The concentration of DNA loaded is indicated above the lanes. For each lane, a one eighth slice of each insert (12.5 μl) was used, yielding DNA mass loads from 0.01 to 10 μg . Electrophoresis was for 160 hr at 1.5 volts/cm. Two autoradiographic exposures are shown, a short exposure (left) and a long exposure (middle). Ethidium bromide-stained markers are shown on the right.

included in the hybridizations so that human DNA bands could be sized directly by comparison with the lambda DNA concatemers. Fragment sizes determined using the center of each band are summarized in Table I. Fragment sizes from Figure 1B are plotted in Figure 2 (fragment sizes from Figure 1A produce a qualitatively similar plot). The smallest band in Figure 1A was less than the lambda monomer standard; therefore, it could not be sized accurately and is excluded from Table I.

Figure 4. Effect of DNA concentration and pulse time on the PFG mobility of restriction fragments greater than 1 Mb. Restriction fragment sizes from two experiments are plotted as a function of the concentration of DNA loaded. Open symbols, DNAs fractionated with 1000–3000 sec pulse times; closed symbols, DNAs fractionated with 3000–5000 sec pulse times (both from Table I); circles, *Not* I fragments detected with probe pGSH8; triangles, *Not* I fragments detected with probe pPW518-1R.

Figure 5. Effect on PFG mobility of increasing DNA mass at constant DNA concentration. The fraction of each 100 μl insert loaded is indicated above the lanes. The concentration of DNA in inserts was 80 $\mu\text{g/ml}$, yielding DNA mass loads of 1 to 8 μg . Pooled single copy probes were used in the hybridizations to detect a range of fragment sizes.

At DNA concentrations between 8 and 80 $\mu\text{g/ml}$, there is a gradual increase in apparent DNA size with increasing concentration (Fig. 2 and Table I). This is slightly more pronounced in inhomogeneous than in homogeneous PFG. Over this concentration range the apparent size increase was less than 5% for all except the smallest fragments on each gel. At ten fold higher DNA concentrations (80–800 $\mu\text{g/ml}$) the apparent size of DNA fragments rose more sharply in both varieties of PFG, and the concentration-dependent increase was somewhat more pronounced in the homogeneous gel (Fig. 2 and Table I). Over the full range of DNA concentrations studied (8–800 $\mu\text{g/ml}$) the

Figure 6. Ethidium bromide-stained PFG containing different concentrations of *S. cerevisiae* (strain YPH 274) chromosomal DNA. Insert DNA concentrations ($\mu\text{g/ml}$) are indicated above the lanes. One eighth slice (12 μl) of each insert was loaded in each lane. Electrophoresis was for 48 hr at 4.5 volts/cm with a 125 sec pulse time using a homogeneous electrode configuration.

Figure 7. Effect of DNA concentration on PFG mobility of *S. cerevisiae* chromosomal DNAs. Bands 1, 5, 7, 12 (counting from the bottom) were sized from Figure 6.

apparent size increase of DNA fragments on the ranged from 21% for the largest fragment (735 kb) to 150% for the smallest (70 kb) (derived from homogeneous PFG values, Table I).

We next sought to determine the effect of DNA concentration on the electrophoretic mobility of fragments larger than 1 Mb. Homogeneous PFG was run with either a 1000–3000 sec pulse ramp (not shown) or a 3000–5000 sec pulse ramp (Fig. 3), and the fractionated DNA was hybridized with probes pGSH8 (locus D21S17) and pPW518–1R (locus D21S55) which recognize *Not* I fragments of approximately 1.2 and 1.8 Mb. Fragment sizes were extrapolated from *S. cerevisiae* and *S. pombe* chromosomal DNA markers and are summarized in Table I, and plotted in Figure 4. (No bands were detected with DNA concentrations of 8 $\mu\text{g/ml}$ on either gel, and fragments less than 1640 kb on the 3000–5000 sec PFG could not be sized because *S. cerevisiae* chromosomal DNAs smaller than 1640 kb were not resolved under these conditions.)

The rise in apparent molecular size with increasing DNA concentrations was greater for fragments run on the longer pulse

Figure 8. Relationship between the relative increase in DNA fragment size (apparent size divided by true size) and the true fragment size. Circles, data from Figure 1B; triangles, data from Figure 1A.

Figure 9. Optical density scans of Figure 1B. A) lanes containing 8, 80, and 800 $\mu\text{g/ml}$ human DNA. B) lambda DNA standard lane. Human DNA sizes were determined relative to the peaks of the lambda bands.

time PFG. For example, a 87% increase was seen in the 1.8 Mb fragment in the 3000–5000 sec PFG compared with a 35% increase for the same fragment in the 1000–3000 sec PFG. Over this same concentration range, the apparent size increase in the 1.2 Mb fragment was approximately 100% for the 3000–5000 sec PFG compared with 48% in the 1000–3000 sec PFG (assuming the same size for the 1.2 Mb band at 80 $\mu\text{g/ml}$ in both gels).

We next sought to determine if the retarded DNA electrophoretic mobility was due to DNA concentration or to the total mass of DNA loaded. A range of DNA mass was loaded by progressively increasing the size of the insert slices used. The DNA sample concentration in the inserts was constant at 80 $\mu\text{g/ml}$ while DNA mass loads ranged from 1 to 8 μg (Fig. 5). At constant DNA concentration, changes in DNA mass loads did not further alter the DNA electrophoretic mobility, although the bands were broader for the bigger insert slices. Similar results were found using inserts with a DNA concentration of 600 $\mu\text{g/ml}$ and DNA mass loads of 7.5 to 60 μg (data not shown).

In another experiment we determined if the effect of DNA concentration on the electrophoretic mobility of yeast chromosomal DNA was similar to that seen with human. Yeast chromosomal DNAs were prepared in agarose inserts at concentrations between 10.5 and 210 $\mu\text{g/ml}$ (between 3.5×10^8 and 1.4×10^{10} cells/ml) and separated by a homogeneous PFG with a 125 sec pulse time (Fig. 6). (We were not able to form inserts at higher concentrations of yeast.) The effect of DNA concentration on the electrophoretic mobility of yeast chromosomal DNA is plotted in Figure 7. At low DNA concentrations (10.5–105 $\mu\text{g/ml}$) there was very little increase in apparent molecular weight (ranging from 2.3–4.3%), consistent with the hybridization results of human DNA over a similar concentration range (from Table I). At the highest yeast DNA concentration used (210 $\mu\text{g/ml}$) the increase in apparent molecular weight ranged from 7.5% to 17.5%. By comparison, the apparent increase in molecular weight for total human DNA at 200 $\mu\text{g/ml}$ was somewhat higher, ranging from 6.8% to 36% in homogeneous PFG at the same pulse times (see Table I).

DISCUSSION

We have shown that the electrophoretic mobility of DNA in PFG is highly sensitive to the DNA concentration loaded. The effect of concentration on the apparent size of the DNA is so large that it can potentially lead to misidentification of DNA fragments. In the worst case, with a DNA sample concentration of 800 $\mu\text{g/ml}$ the apparent molecular size of a 70 kb restriction fragment was 2.5 times its true size. The relative size distortion at high DNA concentrations was greatest for the smallest DNAs on each pulsed field gel. For the same sized bands, the distortion worsened with longer pulse times. On any single pulsed field gel, the distortion decreased monotonically with DNA size. However, note that the distortion a 1.2 Mb band at 1000–3000 sec pulse times, a 42% increase, was greater than the distortion of the 730–735 kb bands at 125 sec pulse times, a 12% or 21% increase.

To quantitate the relationship between relative distortion and size, a semi-logarithmic plot of the relative increase in fragment size (apparent size at 800 $\mu\text{g/ml}$ divided by true size at 80 $\mu\text{g/ml}$ versus true size at 80 $\mu\text{g/ml}$) was constructed for the 125 sec PFG data (Fig. 8). The data was best fit with the regression: $y = 4.55 - 1.19 \log(x)$, with a correlation coefficient of 0.985.

To determine if this effect was due to progressively lower local DNA concentrations with increasing size, we scanned a Polaroid negative of the ethidium bromide-stained homogeneous (125 sec) PFG shown in Figure 1B. Scans of those PFG lanes containing 8, 80, and 800 $\mu\text{g/ml}$ DNA are shown in Figure 9. Local DNA concentration as measured by optical density rose continuously from 50 kb to 700 kb in the 80 $\mu\text{g/ml}$ lane and from 50 kb to 850 kb in the 800 $\mu\text{g/ml}$ lane. Thus, the enhanced PFG concentration dependence of smaller fragments is not due simply to higher concentrations of these fragments. A much more complex relationship must exist between DNA size and local DNA concentration. Simple models of DNA entanglement would predict that concentration effects would be most severe for the largest DNAs, in contradiction to what we observe. One picture that seems to rationalize our results is a cumulative effect on DNA mobility as a fragment migrates through a gel at high DNA concentrations. These cumulative effects might be less for yeast chromosomal DNA where there are only 16 different fragment sizes than for *Not* I-digested human DNA where there are thousands. The nature of this cumulative effect will need to be explored in future experiments.

The results presented here offer a clear warning that high DNA concentrations will not yield reliable sizing of mammalian DNA restriction fragments. Concentration effects are likely to be a major contribution to discrepancies between macro-restriction maps generated for the same physical region by different laboratories. We have shown that accurate sizing of PFG fragments is possible when using DNA concentrations that are not greater than 80 $\mu\text{g/ml}$. For normal human diploid logarithmically growing cells this concentration is achieved with 10^7 cells/ml. More caution is needed with aneuploid cells which can have a significantly higher DNA content. Presumably, the most reliable estimates of fragment size are obtained by using size standards that are comparable to the sample concentrations. Because the effects of DNA concentration on PFG mobility is so crucial, long range physical mapping results should be compared only when the DNA concentration is taken into account. All manuscripts which report long range physical mapping results should clearly indicate the concentration and mass of DNA used in PFG.

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