

Secondary pulsed field gel electrophoresis: a new method for faster separation of larger DNA molecules

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ABSTRACT

A novel technique, which we call secondary pulsed field gel electrophoresis (SPFG) has been developed. In SPFG, short pulses are applied in the direction of net migration of the DNA in addition to the reorienting pulses used in conventional pulsed field electrophoresis (PFG). Experimental results show that SPFG extends and improves the electrophoretic resolution of DNA for molecules from 0.5 megabase pairs to over 10 megabase pairs in size. This improved resolution is obtained with dramatically shorter run times. Thus SPFG appears to circumvent a number of the key limitations in previous PFG protocols.

INTRODUCTION

Gel electrophoresis is the most widely used technique for analytical fractionation of macromolecules. Pulsed-field gel electrophoresis (PFG) has been developed to separate DNAs up to about 10 megabase pairs (Mb) in size (1–4). However, it is estimated that human chromosomal DNAs range in size from about 50 to 250 Mb, more than five times the size of the largest DNA molecules that have been resolved to date by PFG (4). In addition, conventional PFG separations involving molecules larger than 3 Mb typically require electrophoretic runs of one week or more. It is known that PFG parameters such as pulse time, electrical field strength, temperature, and agarose type and concentration are interdependent (5, 6). Changing these parameters to increase the speed of separations often results in reduced resolution. Therefore, methods that could allow more rapid fractionation of megabase DNA with improved resolution would be attractive and significant.

To accomplish this goal, we have developed a complex pulsed-field configuration in which a short secondary pulse is applied in addition to the primary reorienting pulses in conventional homogeneous or inhomogeneous PFG. The secondary pulse is parallel to the direction of net migration and is called the secondary pulsed field. We call the new technique secondary pulsed field electrophoresis (SPFG). For very large DNAs, SPFG provides improved DNA separations in shorter time periods.

METHODS and MATERIALS

DNA samples

Bacteriophage λ DNA concatemers were prepared as previously described (6). Chromosomal DNAs from *Saccharomyces cerevisiae* strain YNN295 and *Schizosaccharomyces pombe* wild type strain 972h⁻ were prepared as described (7). Sizes of *S. pombe* are 5.74 Mb, 4.7 Mb, and 3.5 Mb. Chromosomal DNAs of *Pichia scolyti* strain NRRL Y-5512 (8) and *Pichia mississippiensis* strain NRRL YB-1294 (9) were prepared using methods similar to those for *S. cerevisiae* (10). *Chlamydomonas* chromosomal DNAs were a generous gift of Dr. John L. Hall of Rockefeller University. *Neurospora crassa* chromosomal DNAs were prepared from strain 4716 which was obtained from the Fungal Genetics Stock Center, Department of Microbiology, the University of Kansas Medical Center. This strain does not contain cell walls, which means that genomic DNA can be prepared in a manner similar to that used for *S. cerevisiae*. An *N. crassa* colony was inoculated in 5 ml of Vogel-N supplemented with 2% Mannitol, 0.75% Yeast Extract, and 0.75% Nutrient Broth (Difco) at 30°C with shaking overnight. The overnight culture was used to inoculate 250 ml of Vogel-N medium, which was inoculated with shaking overnight at 30°C. Cells were pelleted by centrifugation at 4000 rpm for 10 min and resuspended in 50 mM Na EDTA (pH 8.0) containing 1% sorbitol. Then the cell suspension was diluted to 1×10^8 cells/ml, add 1 mg/ml zymolase 100T, and mixed with an equal volume of 1% of low gelling temperature agarose (InCert agarose, FMC). The mixture was pipetted into an insert mold. The filled mold was placed at 4°C for 15 min. Then, inserts were put into an equal volume of 7.5% 2-mercaptoethanol in 0.5 M EDTA, pH 9.0, for 24 h at 37°C, washed twice with equal volumes of 50 mM EDTA, kept in ESP (0.5 M EDTA, pH 9.0; 1% Lauroyl Sarcosine, Sigma L5125; and 1 mg/ml proteinase K) at 50°C for 48 h, and finally stored in ESP at 4°C.

Bacteriophage λ DNA provides regularly spaced DNA bands at known 48.5 kb size intervals up to about 1.2 Mb. The sizes of *S. cerevisiae*, *P. scolyti*, and *P. mississippiensis* chromosomes are given in reference (11); molecular weights of *S. pombe* and *N. crassa* DNAs are given in references (3) and (4), respectively.

Agarose gel electrophoresis

One percent agarose gels (Seakem LE, FMC) were cast in $1 \times$ TBE buffer (0.1 M Tris base, 0.1 M Boric acid, 0.2 mM

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Na₂EDTA·2H₂O). The electrophoresis tank, 40 cm×40 cm, was cooled by a 2219 MultiTemp II thermostatic circulator (Pharmacia-LKB). During electrophoresis, the temperature was maintained at 11 ± 1°C. After electrophoresis, the gel was stained with 0.5 μg/ml ethidium bromide in TBE for 10 min, followed by 30 min of destaining in TBE. Gels, together with a fluorescent ruler, were photographed through a filter using Polaroid film under UV illumination.

RESULTS

SPFG apparatus

Two types of SPFG apparatus were used for the experiments described here. One of these designs is outlined in Fig. 1. In this case, the electrophoresis apparatus was adapted from a commercial Pulsaphor Electrophoresis Unit (Pharmacia-LKB). The primary pulsed field was generated using either the conventional point electrode configuration for double inhomogeneous electric fields or the clamped homogeneous hexagonal electrode configuration (CHEF) for homogeneous electric fields. Agarose gels measured 20×20 cm. A special electrode frame for the secondary pulsed field was installed in the buffer chamber. This frame contained 6 platinum electrodes; 3 were mounted at the position labeled NE (the top of the gel), and 3 were mounted at the position labeled SW (at the bottom of the gel). Diodes isolated the electrodes from reverse currents. The secondary pulse was generated independently of the primary pulses by using a programmable time controller and an additional power supply. The secondary pulsed field was superimposed on the primary pulsed field. The pulse timing is shown in Fig. 2.

A second design was implemented using a multi-functional PFG instrument constructed in this laboratory. Fig. 3 shows a block diagram of this system. The application of primary and secondary pulse fields was implemented through microprocessor-controlled switching of voltages applied to a 24×24 cm square array of 24 electrodes. Voltage regulators supplied stable

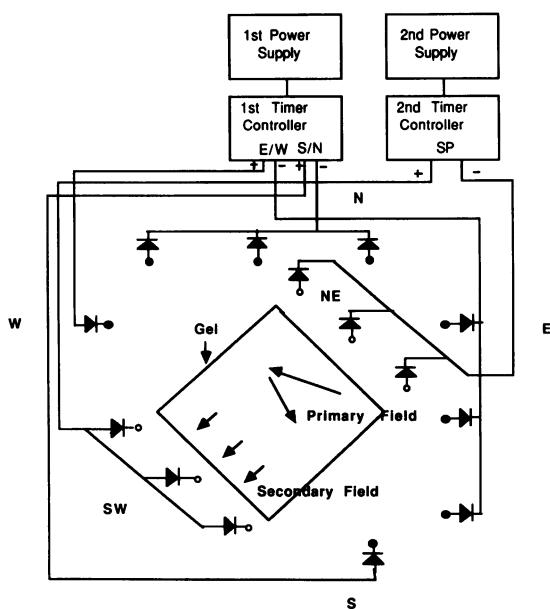


Figure 1: Schematic diagram of an SPFG experimental apparatus based on a modified Pulsaphor system. E/W, S/N, and NE/SW indicate the two conventional PFG fields and the secondary pulse fields, respectively.

electrode potentials. Desired fields, reorientation angles, and pulse times were generated by clamping the potential of each electrode, and thus these parameters could be flexibly modified to meet the requirements of specific experiments. (We will be happy to supply detailed plots for this apparatus for any reader wishing to duplicate it). We have previously demonstrated the usefulness of this kind of clamped electric field in a square array (12), and a similar hexagonal design was described subsequently (5). This apparatus was used to generate pulses like those shown in Fig. 4. The difference in the pulsing in Figs. 2 and 4 is that, in the latter, the primary pulsed field was switched off during the secondary pulse. Experiments in this apparatus used homogeneous primary pulsed fields with reorientation angles of 120 degrees.

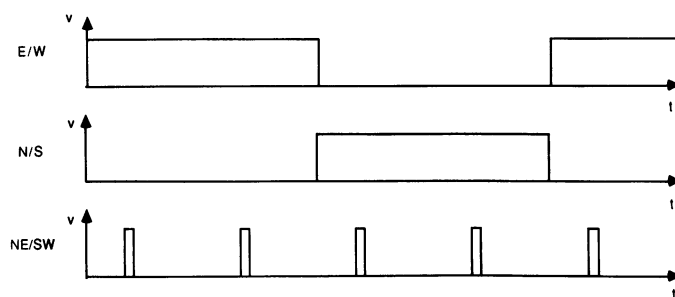


Figure 2: Pulse timing in the experimental apparatus of Fig. 1. A primary pulse is applied in the E/W or N/S directions indicated in Fig. 1 for a period of time (t). Short secondary pulses in NE/SW direction are superimposed at random on the primary pulse.

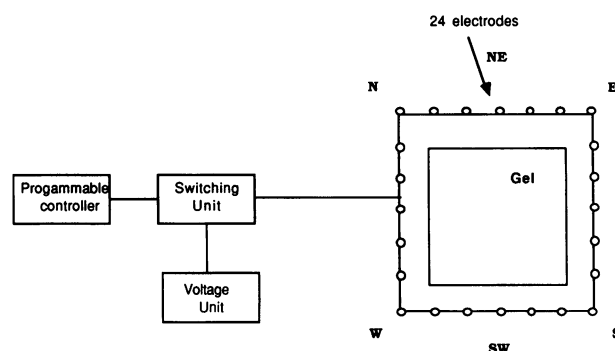


Figure 3: A block diagram of a multi-functional PFG system with individual programmable electrodes. See text for details.

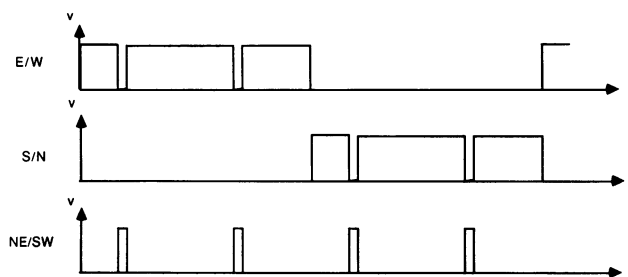


Figure 4: Pulse timing in the experimental apparatus of Fig. 3. A primary pulse is imposed in E/W or S/N directions for a period of time (t). The application of the secondary pulse in the NE/SW direction eliminates the primary pulse for the short duration of the secondary pulse.

Typical SPFG results

Results of typical secondary pulsed field electrophoresis experiments are shown in Figs. 5–7. In Fig. 5A, a pulse program was used that separated DNA molecules in the size range from 50 kb to 5.7 Mb. The primary field was increased progressively

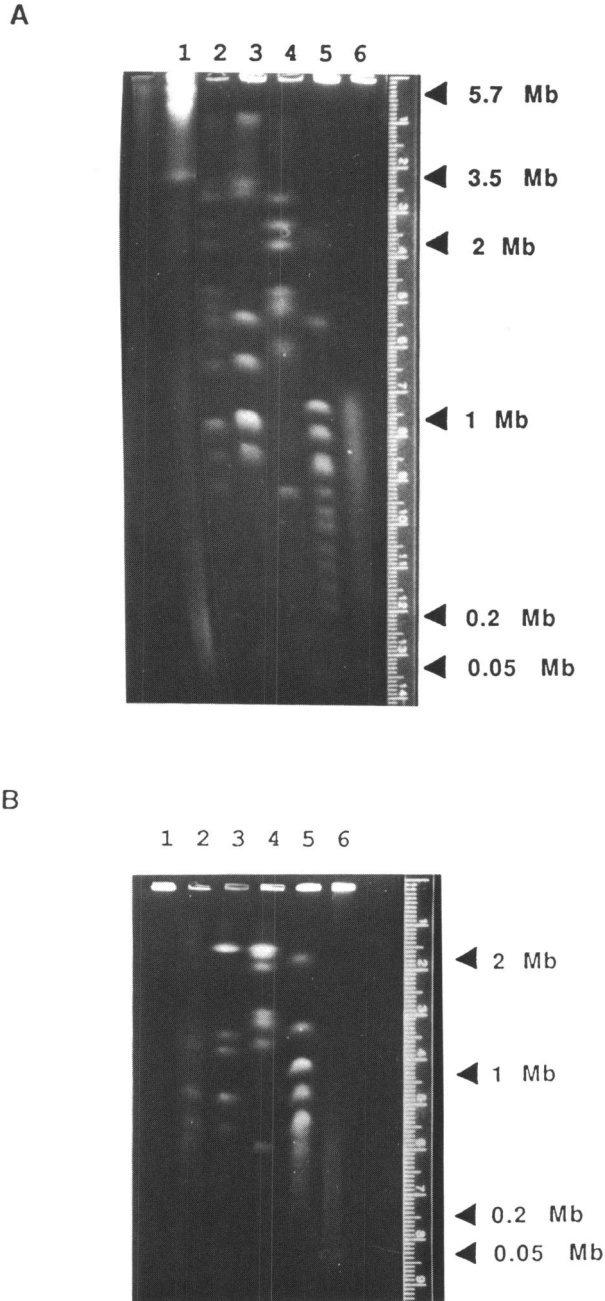


Figure 5: Separation of chromosomal DNA molecules in the size range between 50 kb and 5.8 Mb by SPFG. Samples are (lane 1) *S. pombe*, (lane 2) *Pichia* 1A which consists of a mixture of *P. scolyti* and *P. mississippiensis*, (lane 3) *P. scolyti*, (lane 4) *P. mississippiensis*, (lane 5) *S. cerevisiae*, and (lane 6) Lambda concatemers. Separation on a 1% agarose gel, in the multi-functional PFG system described in Fig. 3 using (A) a pulse program as follows (i) 4800 s, 2.2 V/cm primary pulses with 1:15 s (1 sec pulse every 15 sec), 6 V/cm secondary pulses for 12 h, (ii) 2400 s, 2.8 V/cm primary pulses with 1:15 s, 6 V/cm secondary pulses for 12 h, (iii) 240 s, 6 V/cm primary pulses with 1:15 s, 10 V/cm secondary pulses for 21 h, and (iv) 120 s, 6 V/cm primary pulses with 1:15 s, 10 V/cm secondary pulses for 10 h and (B) the same primary pulse program as shown in (A) but without secondary pulses.

from 2.2 V/cm to 6 V/cm and the primary pulse time decreased from 4800 s to 120 s. A secondary pulse was applied every 15 s initially at 6 V/cm and subsequently at 10 V/cm. Using this complicated scheme, excellent separation over a wide range of DNA sizes was obtained in 55 h. Fig. 5B shows results obtained with same primary pulses in absence of secondary pulses.

In Fig. 6A, a 600 s primary pulse time was used at a relatively high primary field strength (6.0 V/cm). In the absence of secondary pulsing such conditions normally fractionate only DNAs smaller than 2 Mb in size. The application of 12.5 V/cm field strength 1 s secondary pulses applied every 15 s has a

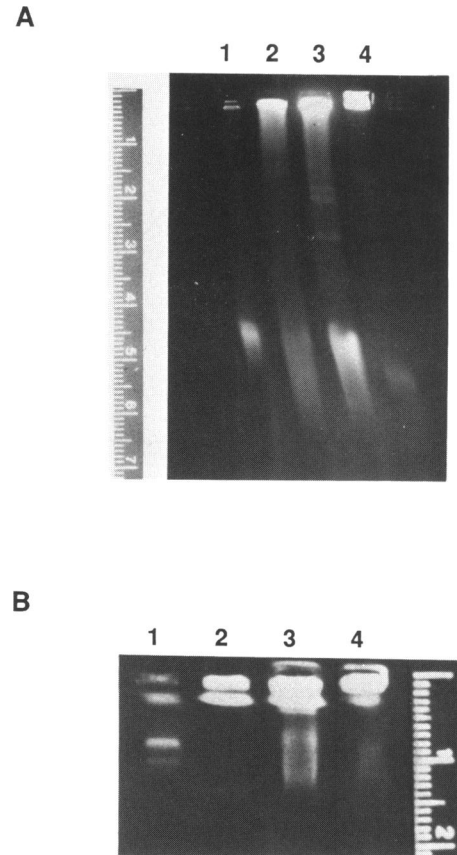


Figure 6: Separation of chromosomal DNAs by SPFG in 30 h. Samples are (1) *Pichia* 1A, (2) *S. pombe*, (3) *N. crassa*, and (4) *Chlamydomonas*. Separations used a 1% agarose gel in the modified Pulsaphor apparatus described in Fig. 1 with 600 s, 6 V/cm primary pulses for 30 h (A) without a secondary pulse and (B) with a secondary pulse of 1:15 s (1 sec pulse every 15 sec) at 10 V/cm.

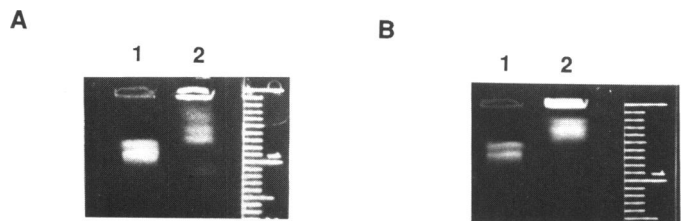


Figure 7: Separation of *S. pombe* chromosomal DNAs in 30 h. Samples are (1) *Pichia* 1A and (2) *S. pombe*. Separations used a 1% agarose gel in the modified Pulsaphor apparatus described in Fig. 1 with 4800 s, 3.3 V/cm primary pulses for 30 h (A) with 1:90 s (1 sec pulse every 90 sec), 12.5 V/cm secondary pulses and (B) without secondary pulses.

dramatic effect in the separation speed and the fractionation size range (compare to Fig. 6B where no secondary pulses were used). In 30 h, *N. crassa* chromosomal DNAs ranging from 4 Mb to what is estimated to be over 10 Mb in size were separated very well. *Chlamydomonas* chromosomal DNAs also were separated. However there is very poor resolution of the *S. pombe* chromosomal DNAs under those conditions. A series of experiments varying the strength of the primary and secondary fields showed that, unlike the comparably sized *N. crassa* and

Chlamydomonas chromosomes, separations of *S. pombe* DNAs are unusually sensitive to field strength in this range of pulse times.

Fig. 7 shows separations at lower primary field strength (3.3 V/cm). Here, separation of *S. pombe* chromosomes in 30 h was achieved using both SPFG and PFG, but improved resolution is seen with the former. Under these conditions, although *S. pombe* DNAs are well fractionated, *N. crassa* DNAs are not.

Effect of secondary pulsed field parameters

The sampling of SPFG experiments just presented indicates that secondary pulses have a dramatic effect on electrophoretic separations. An understanding of the full range of the characteristics of SPFG would require the analysis of a large number of variables including the angle between the primary fields, the direction of the secondary fields, the primary pulse time and field strength; the secondary field strength, pulse width, and pulse spacing; phase of the secondary pulses relative to the primary pulses, as well as typical experimental parameters such as temperature, gel concentration and ionic strength which affect ordinary PFG separations (6, 10, 13). If most of these variables were effectively decoupled from one another, this task would be relatively straightforward, albeit very tedious. However the results already presented indicate that the variables are interactive. For example, when added to primary pulses, secondary pulses allow separation of DNAs in a size range not possible with the primary pulses alone.

Initial explorations of SPFG have held gel concentration, gel temperature, and buffer ionic strength constant, and have ignored the relative phase of the primary and secondary pulses. Looking the pulse scheme shown in Fig. 2, the secondary field is applied in the presence of the primary field, while in the scheme of Fig. 4 the primary pulse field is turned off when the secondary field is on. This results in different angles between the primary and secondary fields in the two cases. No significant differences were seen with the two configurations. This case has not explored the effect of secondary pulse directions systematically. However a

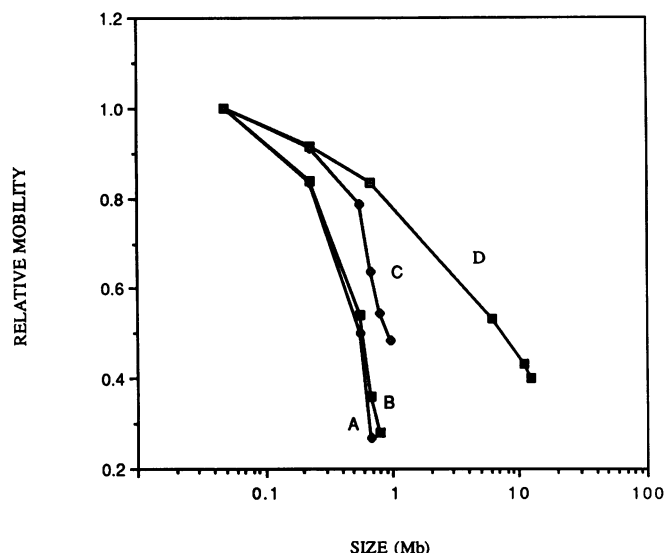


Figure 8: Relative migration of DNAs at different secondary pulse ratios. Separations used 1% agarose gels run for 12 h in the apparatus described in Fig. 1 with 100 s, 8.2 V/cm primary pulses, (A) without a secondary pulse, (B) with 1:60 s, 19 V/cm secondary pulses, (C) with 1:15 s, 19 V/cm secondary pulses, and (D) with 3:15 s, 19 V/cm secondary pulses.

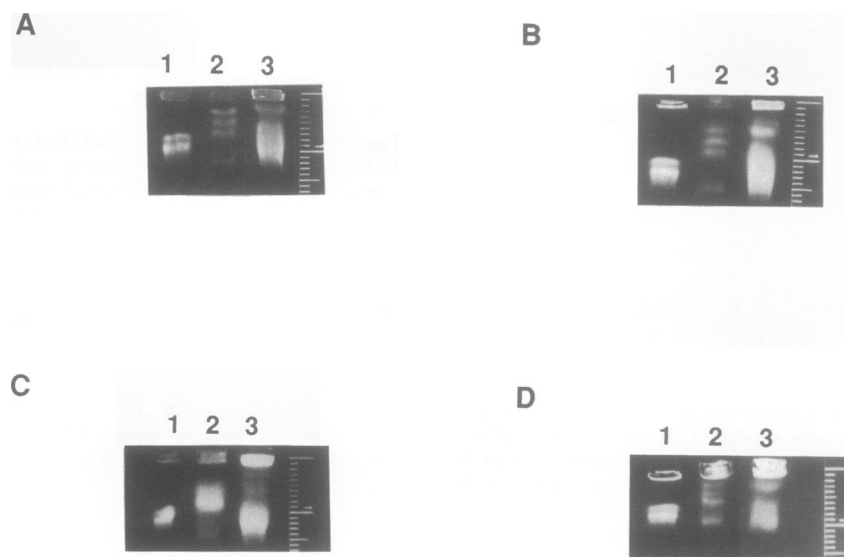


Figure 9: Effect of secondary field strength on SPFG separation. Samples are (1) *Pichia* 1A, (2) *S. pombe*, and (3) *N. crassa*. Separation used a 1% agarose gel in the apparatus described in Fig. 1 with 4800 s, 3 V/cm primary pulses for 48 h with (A) 0.1:1.6 s, 5 V/cm secondary pulses, (B) 0.1:1.6 s, 7 V/cm secondary pulses, and (C) 0.1:1.6 s, 8 V/cm secondary pulses, and (D) without secondary pulses. The smallest band in the *S. pombe* samples is presentable mitochondrial DNA.

recently published report (14) shows substantial effects of short secondary pulses oriented in directions quite different from the ones we use here.

In an initial set of experiments, the effect of secondary pulse width and period on the migration of DNA in pulsed field gel electrophoresis with identical primary pulse parameters was measured. Fig. 8 summarizes the mobility of various DNAs using a 100 sec, 8.2 V/cm primary pulse without secondary pulses or with a secondary pulse ratios (the ratio of secondary pulse length to primary pulse length) of 1 s : 60 s, 1 s : 15 s, and 3 s : 15 s using a secondary field strength of 19 V/cm. For DNA molecules smaller than 500 kb, the mobilities relative to the smallest lambda DNA band were similar, whether or not 1 s : 60 s secondary pulses were used. However, the use of 1 s : 15 s secondary pulses dramatically increased the relative mobilities of DNA molecules between 500 kb and 1 Mb. Secondary pulses (3 s : 15 s ratio) increased the relative mobilities of DNAs smaller than 1 Mb to make them less dependent on DNA size. However the same secondary pulses dramatically increased the mobilities of DNAs larger than 3 Mb and also allowed fractionation because these mobilities are size dependent.

The effect of secondary pulse amplitude on DNA migration in pulsed field gels was also characterized. Increasing secondary field strengths has effects similar to those observed in experiments with extended secondary pulse ratios (i.e. where secondary pulse width is comparable to the secondary pulse period). Fig. 9 shows the results of a set of experiments using *S. pombe* and *N. crassa* DNAs in which the secondary pulse amplitude was varied, while primary and other secondary pulse parameters were kept constant. It can be seen that application of 3 V/cm and 7 V/cm secondary pulses resulted in a progressive increase in the absolute mobilities of three visible bands of *S. pombe* and two visible bands of *N. crassa*. However, when the secondary pulse amplitude increased to 8 V/cm, the *S. pombe* bands coalesced. Surprisingly, the resolution of the *N. crassa* samples improved further, and four DNAs were now resolved. The identity of one chromosomal

DNA band (in size of 12.5 Mb (4)) was confirmed by hybridization experiments using the *N. crassa his 3* gene (data not shown).

Finally the effect of secondary pulse width on the migration of large DNA samples was examined. Figure 10 shows SPFG separations as a function of secondary pulse width at constant secondary pulse ratio. Long secondary pulses (10 s) had almost no apparent affect on mobility, while, as expected, short 1 s pulses increased the absolute mobilities of all DNAs examined.

DISCUSSION

The application of short electric field pulses in the direction of net migration can substantially improve the resolution of large DNA molecules in PFG. In SPFG, mobilities of large DNAs are sensitive functions of the width, period, and field strength of the applied secondary pulse. Appropriately chosen secondary pulse parameters sharply increased the resolution of DNA molecules larger than 3 Mb. The absolute mobilities of all DNAs also increase in SPFG, so that excellent separations can be achieved in shorter times than those required using conventional PFG.

Studies of SPFG as a function of field strength suggest that higher field strength helps to reduce running time by increasing the absolute electrophoretic mobility and increasing the resolution. At high field strengths (primary pulses 6 V/cm, secondary pulses 12.5 V/cm) *N. crassa* chromosomal DNAs have been separated with sharp resolution. *Chlamydomonas* chromosomal DNAs have also been separated at high field strengths. Surprisingly, *S. pombe* chromosomal DNAs, estimated to be similar in size to *N. crassa* chromosomal DNAs, are not well separated under conditions similar to those successfully employed with *N. crassa*. This may be due to the presence of special regions or structures in *S. pombe* chromosomes that are not found in *N. crassa*.

The results presented here are very encouraging for experimentalists, since they promise vistas of fast separations of large DNAs and suggest that the SPFG technique has the potential to separate DNAs over 10 Mb in size. However, they are also discouraging in that they reinforce current perceptions of how poor our fundamental understanding of DNA electrophoresis really is. Presumably the major effect of the secondary pulses is to release DNAs trapped in gel fibers. How the secondary pulse affects the dynamics of DNA in the gel is not known at the present time. Further macroscopic and microscopic experiments (15) as well molecular dynamics simulations (16) should help reveal the effect of secondary pulses on the shape and orientation of DNAs in agarose. However we have no quantitative understanding of how to use such information to optimize the many parameters in SPFG. There is no particular reason to think that the currently employed ranges of field angles, pulse times, pulse shapes, and field strength are anywhere near optimal. Perhaps the future of PFG will see increasing use of such kinds of complex field programs to achieve separations that are inconceivable by today's methods. It is tempting to try to draw an analogy between the present state of PFG and its possible future with the early days of nmr as it contrasts with today's ever so much more powerful multi-dimensional methods.

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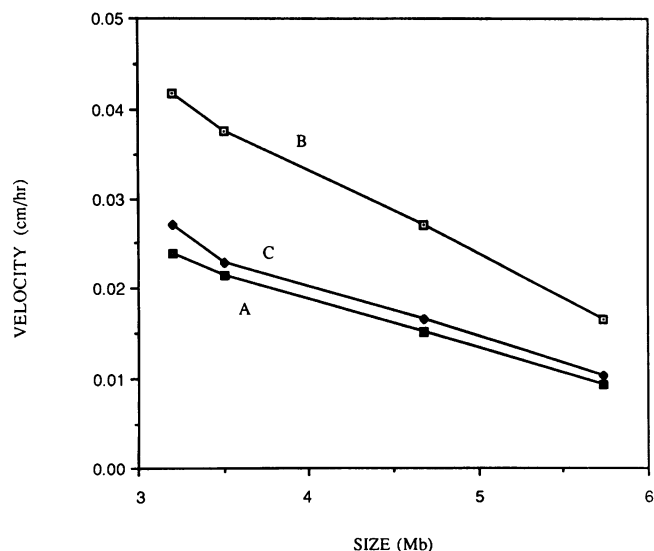


Figure 10: Velocity of *S. pombe* chromosomal DNAs as function of secondary pulse width. Separations used a 1% agarose gel, run for 48 h in a modified Pulsaphor apparatus with a 4800 s, 3.6 V/cm primary pulses (A) without secondary pulse, (B) with 1:100 s, 10 V/cm secondary pulses, and (C) with 10:1000 s, 10 V/cm secondary pulses.

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