A Novel Instrument for Separating Large DNA Molecules with Pulsed Homogeneous Electric Fields

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A new instrument has been developed for the electrophoretic separation of large DNA molecules that can independently regulate the voltage of each of 24 electrodes and allow the magnitude, orientation, homogeneity, and duration of the electric field to be precisely controlled. Each parameter can be varied at any time during the electrophoretic process. Thus distinct sets of conditions can be combined to optimize the separation of various fragment sizes in a single run. Independent control of electrode voltage allows all of the fields to be generated with electrodes arranged in a closed contour, independent of a particular geometry. This device increases both the resolution in any size range and the speed of separation, especially for DNA molecules larger than 3 megabases.

GEL ELECTROPHORESIS IS WIDELY used for the separation of biological macromolecules based on their size, charge, and conformation. In conventional agarose gel electrophoresis in which a single homogeneous electric field is used, the largest DNA fragments that can be resolved are about 50 kilobase pairs (kb). In 1984, Schwartz and Cantor (*1*) introduced the concept that large DNA fragments (50 to 2000 kb) could be separated in agarose gels by using two alternating electric fields. This technique allowed the separation of intact chromosomes from microorganisms (*2*) and opened the possibility of long-range mapping of mammalian genes (*3*). However, the nonhomogeneous electric fields used caused the velocity of DNA molecules to vary with position in the gel; thus it was difficult to compare samples run in different lanes and measure quantitative aspects of DNA migration. Several investigators realized that using homogeneous fields would eliminate these nonuniform mobilities (*4–6*). In particular, Chu *et al.* (*6*) applied the principles of electrostatics in using a hexagonal array to generate homogeneous electric fields in pulsed-field gel (PFG) electrophoresis. However, these apparatuses are limited in the orientation and number of different electric fields that they can generate during a single gel run. For example, the CHEF system (*6*) is limited to two alternating electric fields at a fixed reorientation angle. The vertical PFG electrophoresis system (Geneline, Beckman Instruments, Inc.) also does not permit active control of the reorientation angle and further, the set reorientation angle varies with position in the gel.

We have developed a programmable, autonomously controlled electrode gel electrophoresis (PACE) apparatus in which all of the electric field parameters can be precisely controlled by the independent regulation of the voltages on each electrode. This apparatus can generate an unlimited number of electric fields of differing voltage gradients, orientations, and durations sequentially in time. We show that (i) linearity of separation can be improved by the use of switch-time ramping; (ii) resolution can be increased through the incorporation of reverse-field states into pulsed-field regimens; and (iii) speed of separation can be increased by the manipulation of reorientation angles.

The PACE instrument consists of three major components; a computer to store and recall field parameters, a controller to generate the electrode voltages, and a gel box. The gel box contains 24 electrodes arranged in a closed hexagonal contour as described in (*6*). Our choice of a hexagonal contour for the electrode array was based on convenience, and in principle, any other closed contour can be used. Electric fields are generated by sending sets of data representing the desired voltage values from the computer to the controller. In the controller the digital information is converted to analog voltages that are amplified by the high-voltage amplifiers. The 24 high-voltage amplifier outputs actively impress the desired voltages on the electrodes, thus determining the electric field (*7, 8*). The desired voltage values are determined from Poisson's equation. We refer to the set of voltages that corresponds to a given electric field as a voltage state. The ability to activate voltage states in any sequence at any time has allowed us to execute all of the previous types of PFG electrophoresis. Separations of large-size DNAs in standard PFGs are achieved by alternating between voltage states; the duration of a voltage state is called the switch time.

The importance of using homogeneous

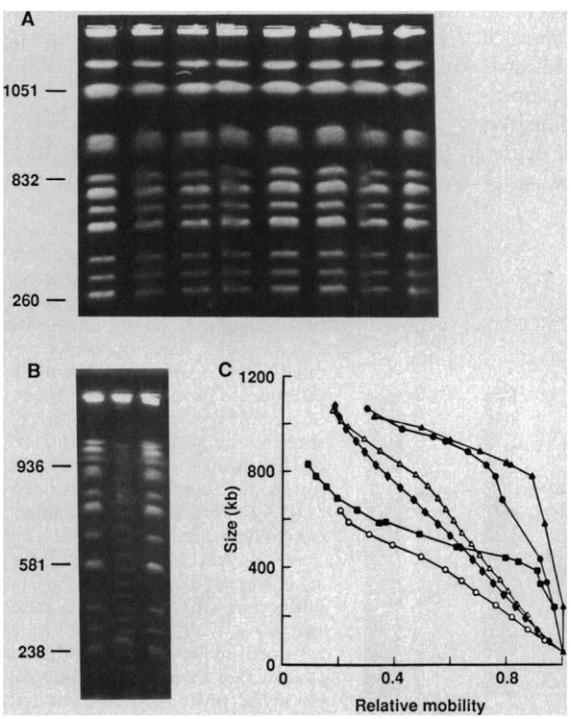

Fig. 1. (**A**) Separation of *S. cerevisiae* chromosomes (strain D273-10b) by pulsed-field gel electrophoresis (band markers in kilobases). Identical samples were loaded across the entire gel. The separation conditions were: 6 V/cm, 1% agarose, 120° reorientation, 90-s switch time, 30-hour run. (**B**) Separation of yeast chromosomes and lambda concatamers by PFG using ramped switch times. The conditions were identical to (A) except that the switch time was linearly ramped from 45 to 90 s during the 36-hour run. Phage lambda concatamers are in the center lane, flanked by *S. cerevisiae* chromosomes. (**C**) The mobility of DNA is plotted as a function of size for various PACE separation conditions. Migration distances for yeast chromosomes and lambda concatamers were measured and the mobility is expressed relative to the 48.5-kb lambda monomer. Separations using fixed switch times are shown for 45 (○) and 90 s (△). The mobility of the molecules separated using ramped switching shown in (B) is represented by (◆). The mobility of DNAs separated using several different multiple voltage state regimens (each of which included a reverse state) are depicted as follows: a run using three voltage states cycled counterclockwise (shown in Fig. 2B) (▲); and a three–voltage state run as in Fig. 2B, except that the switch times were 45 s for the two forward states and 22.5 s for the reverse state (■); a run that used three voltage states, cycled alternatively counterclockwise and clockwise (shown in Fig. 2C) (●).

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electric fields in electrophoresis has been previously demonstrated (*5, 6*). The ability of the PACE system to generate homogeneous electric fields for PFG electrophoresis by imposing proper voltage states is shown by the uniform migration of the *Saccharomyces cerevisiae* chromosomes across the entire gel (Fig. 1A). We have used this gel system to extensively characterize the resolution that can be achieved in standard homogeneous PFG electrophoresis. Among the parameters we have explored are switch-time interval, temperature, agarose concentration, reorientation angle, and voltage gradient (*8*). The resolution characteristic of this technique may be seen in Fig. 1A and is reflected in the slope of the open symbol curves of Fig. 1C. In Fig. 1C, the molecular weights are plotted with respect to the relative mobilities of DNA molecules; slopes closer to horizontal reflect increased resolution. There are two aspects of these standard PFG separations that we wanted to improve. First, there are deviations from a linear relation between molecular weight and mobility resulting in both compressed and expanded regions of separation (*9*). Second, we wanted to establish conditions that would yield large improvements in resolution. In field inversion gel (FIG) electrophoresis (*4*), linear mobility behavior can be achieved by progressively changing the durations of the states during the run (that is, switch-time ramping). We used switch-time ramping in PFG gels and achieved near-linear resolution over the entire range of separation from 50 to 1100 kb, as indicated by the regular spacing of the lambda concatamers (Fig. 1B) and the corresponding plot of the mobility (Fig. 1C). This high degree of linearity over an extensive size range allows improved molecular size estimations for unknown DNAs.

In an effort to obtain resolution beyond that available with standard PFG electrophoresis, we added an additional voltage state corresponding to the reverse-field configuration of FIG electrophoresis to the two equal duration forward states of standard PFG. Initially, these three alternating fields were cycled in a clockwise sequence. The resolution in a given molecular size range could be greatly increased, but there was also a size-dependent distortion (Fig. 2A). Measurement of the electric field indicated that the distortion was not caused by field inhomogeneity. When the fields were cycled counterclockwise, distortion in the opposite direction occurred (Fig. 2B), which suggested that the distortion resulted from the difference in switch times used for the forward and reverse states. Inequality in either switch time or voltage is required between the forward and reverse states to produce net forward movement. Superior resolution and straight lanes can be achieved simultaneously by alternating clockwise and counterclockwise cycling sequences. Figure 2C shows a twofold increase in resolution in the size range from 800 to 1000 kb obtained by cycling three states (Fig. 1C), compared with a standard two-state run. The size region for which there is increased resolution depends on the switch times used. Thus any size range can be selected by the choice of the forward and reverse switch times (*8, 9*). The mobility of DNAs with the region of expanded resolution in the 400- to 600-kb range is also shown in Fig. 1C.

Previous electrophoretic techniques were limited in the size range of molecules that could be separated in a single run and the speed with which a given separation could be achieved. With the PACE apparatus the resolution of large DNA molecules is independent of reorientation angle when the angle between the alternating fields is restricted to the range of 105° to 165° (*8*). However, the mobility of the molecules is inversely proportional to the reorientation angle. Thus the mobility at a reorientation angle of 105° is roughly 30% greater than that at 120° (*8*). In particular, smaller reorientation angles can be used for the separation of larger molecules, such as *Schizosaccharomyces pombe* chromosomes. A 3.2-fold increase in velocity can be achieved with a 96° reorientation angle at 2 V/cm. Under these conditions, *S. pombe* chromosomes were separated in 24 hours (Fig. 3A), compared with conditions previously reported (*9*) that required typical run times of 130 hours. The molecular size range over which separation can be achieved can be greatly increased when multiple switch-time regimens are used sequentially within a gel run. For example, DNA molecules from 240 to 7000 kb can be resolved in 4 days (Fig. 3B). This was achieved initially with a 1-hour switch time, followed by a 150-s switch time at an increased voltage gradient.

This instrument should have broad application in chromosome mapping and is also easily adaptable to the separation of proteins and other macromolecules. Its inherent versatility allows the development of new electrophoretic strategies for the isolation of large DNA molecules.

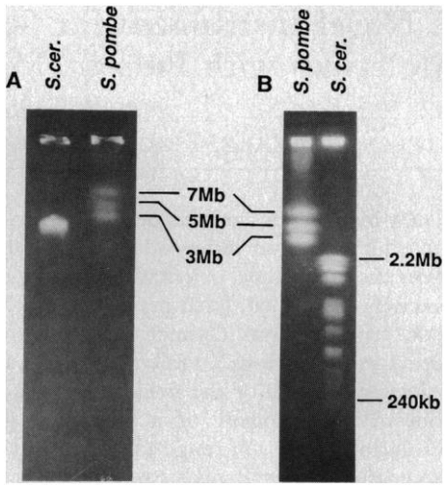

Fig. 3. Separation of DNAs ranging from 240 to 7000 kb. (**A**) *Saccharomyces cerevisiae* and *S. pombe* (strain 972 h-) chromosomes were separated in 1.0% agarose gel with a reorientation angle of 96°. Conditions used were 2 V/cm, 30-min switch time, and a run duration of 24 hours. (**B**) The gel (0.7% agarose) was run for 45 hours at 2 V/cm with a 45-min switch time, followed by 40 hours at 3 V/cm with a 150-s switch time.

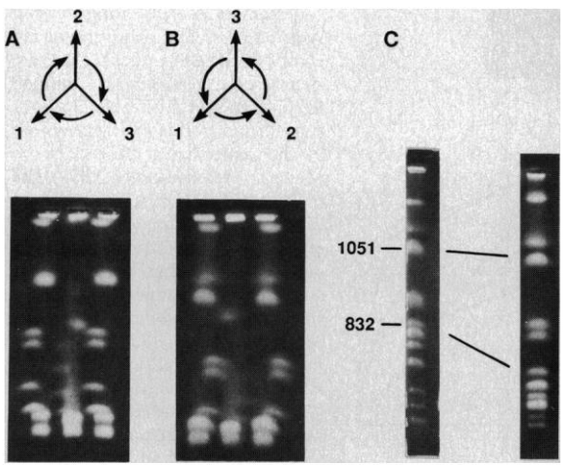

Fig. 2. (**A** and **B**) A size-dependent asymmetry of DNA migration (band markers in kilobases). Lambda concatamers, center, and yeast chromosomes, flanking, were separated in 1% agarose at 6 V/cm for 60 hours. The angular orientation of a state was measured with respect to the sample wells, that is, the line from the center of the gel toward the sample wells is defined as 0°. Combinations of three different voltage states were used. The duration and orientation of the states were as follows: state (i) 90 s, 240°; state (ii) 45 s, 0°; and state (iii) 90 s, 120°. The states were activated sequentially in either a clockwise order (A) or in a counterclockwise order (B), as indicated above the gels [that is, 1 to 2 to 3, as in (A)]. (**C**) Enhanced separation in the size range 800 to 1000 kb using multiple voltage states. The gel on the left was separated as in Fig. 1A. The gel on the right was run with a voltage gradient of 6 V/cm with a combination of three different voltage states. The duration and orientation of these states were: state (i) 90 s, 240°; state (ii) 45 s, 0°; and state (iii) 90 s, 120°. The voltage states were cycled in the following repeated sequence: i, ii, iii, i, iii, ii, i, iii.

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7. There are 24 identical sets of digital-to-analog converter (DAC)–amplifier combinations, one for each electrode. The electronics can switch between fields up to 1 kHz. There was no delay of voltage change among the electrodes when electric fields were switched. The voltage range for each electrode is +150 to −150 V and up to 75 mA of current can be supplied to each electrode. The voltage can be controlled in steps of 55 mV. The output voltages of the electrodes were measured at the beginning and near the end of each gel run with a high-impedance digital voltmeter. The components used for the control circuitry are as follows: DACs are all National Semiconductor DAC0830LCN, low-voltage operational amplifiers are all Precision Monolithics, Inc., OP-07, and high-voltage amplifiers are either Burr-Brown 3583JM or Apex Microtechnology PA83. All gels shown were cast with FMC SeaKem LE agarose and are 13 cm square and less than 1 cm thick. The conditions for sample preparation as well as casting and running the gels are as in (*8*). Temperature control during electrophoresis was accomplished by recirculating the electrophoresis buffer through a heat exchanger, which was immersed in a temperature-controlled water bath. All gels were run at 14°C as measured in the electrophoresis buffer.
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Two Mechanisms for the Extinction of Gene Expression in Hybrid Cells

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When two different mammalian cell types are fused to generate a stable hybrid cell line, genes that are active in only one of the parents are frequently shut off, a phenomenon called extinction. In this study two distinct, complementary mechanisms for such extinction of growth hormone gene expression were identified. In hybrids formed by fusing fibroblasts to pituitary cells, pituitary-specific proteins that bind to the growth hormone promoter were absent. In addition, a negative regulatory element located near the rat growth hormone promoter was specifically activated.

THE GROWTH HORMONE GENE IS EXpressed only in specialized pituitary cells called somatotrophs (*1*) and has provided an excellent system for studies of cell-type specificity of gene expression (*2–9*). Since neither fibroblasts nor hybrids between fibroblasts and pituitary cells express detectable levels of growth hormone mRNA (*10*), growth hormone also provides an excellent model for study of extinction of gene expression in cell hybrids.

Detailed characterization of the rat growth hormone (rGH) gene has identified both trans-acting factors and cis-acting DNA sequences thought to be responsible for cell type–specific expression. Extracts from rat pituitary cells contain proteins that bind specifically to sites in the rGH promoter between approximately −95 and −60, and between −140 and −115, relative to the start of the mRNA (*2–6*). A protein originally called GC1 (*2*) appears to bind to both sites. There is direct evidence from in vitro transcription experiments that this protein, which is found only in pituitary cells, is required for activation of the rGH promoter (*6*). An additional protein, found in both pituitary and nonpituitary cell types, binds only to the latter, distal site and will be referred to here as GC2 (*3*). Analogous results have been obtained from analysis of proteins binding to the human growth hormone (hGH) promoter (*7*), which is similar in sequence to the rGH promoter in this area.

A cis-acting, cell type–specific negative regulatory element, termed a silencer, has also been mapped just upstream of the rGH promoter (*8, 9*). The silencer acts to repress rGH promoter activity in nonpituitary cells, but has no effect on expression in pituitary cells. It seems that a combination of positive and negative regulatory elements generates the correct, cell type–specific expression of the rGH gene.

To investigate the mechanism of extinction of rGH expression, we used hybrid cell lines formed by fusing a hypoxanthine-guanine phosphoribosyl transferase–deficient ($HGPRT^-$) derivative of rat pituitary GH_4 cells to a thymidine kinase–deficient (TK^-) derivative of mouse fibroblast L cells (LTK^-). Several independent stable hybrids were subcloned and screened for the presence of the rat and mouse growth hormone (GH) genes by DNA blotting with a full-length rGH cDNA probe (*11*). Both genes were present in all of the subclones, demonstrating that they are true hybrids (Fig. 1A). The hybrids and parental cell lines were also analyzed for the presence of rGH mRNA. As expected (*10*), there was a high level of rGH mRNA in the GH_4 cells, but no detectable rGH mRNA in either the LTK^- cells or the hybrids (Fig. 1B).

We used the deoxyribonuclease I (DNase I) protection assay to test nuclear extracts of both parents and the hybrid lines PT1CL5 and PT1CL6 for rGH promoter binding proteins (Fig. 2). As expected, extracts from GH_4 cells generated footprints at both the proximal GC1 binding site and the distal binding site for GC1 or GC2 (labeled GC2 in Fig. 2). In addition, a third footprint was seen between −240 and −220. We examined a number of cell types and found this protein, which we call GC3, only in pituitary cells (*12*), although there is evidence that some nonpituitary cells may contain a protein that interacts with this area (*4, 5*).

Extracts from LTK^- cells and the hybrid PT1CL5 showed a footprint at the distal GC2 binding site, but not the proximal GC1 binding site or the GC3 binding site (Fig. 2). Essentially identical results were obtained with the independent hybrid line PT1CL6. The presence of GC2 in the LTK^- cells was somewhat unexpected (*3, 5*), but is in agreement with results obtained with the hGH promoter (*7*). To determine whether the LTK^- extracts contained an activity that prevented binding of the pituitary proteins, the two parental extracts were mixed in various proportions and used for footprinting. As would be expected if GC1 and GC3 were simply absent in the LTK^- cells, both the proximal GC1 footprint and the GC3 footprint were observed at the expected GH_4 extract concentrations. We conclude that the expression or activity of

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