reports in their combination of characteristics. They therefore support the notion that the evolution of the group might have followed different morphological trends in different areas. After an origin in tropical areas, different lineages migrated to higher latitudes and fairly diverse dicotyledons attained an essentially worldwide distribution by 115 million years ago, that is, 10 million years after the first appearance of pollen in the fossil record. Third, the isolated, almost monospecific remains, in an association dominated by gymnosperms and pteridophytes, as well as the nature of the sediments (fluvial and volcanic, that is, from unstable environments) seem to indicate that the angiosperms were a marginal component of the flora during their early radiation. Primitive angiosperms probably were opportunists related to unstable environments (*7*) or successional elements in the first steps toward an assemblage dominated by gymnosperms and pteridophytes.

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Separation of Large DNA Molecules by Contour-Clamped Homogeneous Electric Fields

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Electric fields can be manipulated by a method in which multiple electrodes are arranged along a closed contour and clamped to predetermined electric potentials. This method may be applied to a broad range of problems in the separation of macromolecules by gel electrophoresis. DNA molecules as large as 2 megabases can be well separated with a contour-clamped homogeneous electric field alternating between two orientations 120° apart. The pattern of separation is independent of position in the gel, which is an advantage over previous methods. DNA less than 50 kilobases can be separated without distortion even at high voltage with a nonalternating contour-clamped homogeneous field. Decreased band broadening in DNA less than 200 bases can be achieved with a contour-clamped inhomogeneous field.

GEL ELECTROPHORESIS IS CAPABLE of separating macromolecules such as proteins and nucleic acids on the basis of size, charge, or conformation. Most applications use a single pair of electrodes to generate the electric field. Such a field is necessarily constrained to be uniform and oriented in a single direction. Conventional techniques are therefore limited in many respects. For example, they cannot resolve DNA fragments much larger than 50 kb (*1*).

The recent introduction of new electrode configurations that generate electric fields in alternating orientations has allowed the separation of large DNA molecules up to 2 megabases (Mb) in size (*2–4*). The technique has been used to separate chromosomes from yeast and other organisms. A problem with these methods is that the electric field is not uniform, so that DNA molecules migrate with mobilities and trajectories that depend on where in the gel the samples are loaded. Comparison of multiple samples across the gel is therefore difficult.

These limitations can be overcome by applying a contour-clamped homogeneous electric field (CHEF) that alternates between two orientations. The electric field is generated by a method in which multiple electrodes are arranged along a polygonal contour and clamped to predetermined electric potentials. The method applies the principles of electrostatics to gel electrophoresis. In particular, the electric field vector is confined to two dimensions and has two components, $E_x(x,y)$ and $E_y(x,y)$. To simplify the problem, the electric field may be expressed as the negative gradient of a single function, the scalar potential field $\phi(x,y)$ (*5*),

$$E_x(x,y) = -\partial\phi(x,y)/\partial x \quad (1a)$$

$$E_y(x,y) = -\partial\phi(x,y)/\partial y \quad (1b)$$

A homogeneous electric field is generated by two parallel, infinitely long electrodes. If one electrode is located along the x axis ($y = 0$) and the other is separated by a fixed distance ($y = a$), the potential field between the electrodes is

$$\phi(x,y) = \phi_0 y/a \quad (2)$$

where ϕ_0 is the voltage applied across the electrodes. Substitution of Eq. 2 into Eq. 1 shows that the corresponding electric field is homogeneous and oriented perpendicular to the electrodes,

$$E_x(x,y) = 0 \quad (3a)$$

$$E_y(x,y) = -\phi_0/a \quad (3b)$$

It is impractical to use infinitely long electrodes, but it is possible to produce a homogeneous electric field with a finite system. A solution is to use multiple elec-

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trodes arranged along a polygonal contour in which two faces of the polygon coincide with the positions of the infinite electrodes. The electrodes along $y = 0$ and $y = a$ are clamped to the potentials 0 and ϕ_0, respectively. The remaining electrodes located at intermediate positions are clamped to intermediate potentials as determined by Eq. 2. Thus, positions along the contour are clamped to potentials equal to those generated by two infinite electrodes. It follows that everywhere inside the contour the potential field will also be equal to that generated by two infinite electrodes (*6*). If an infinite number of electrodes are arranged along the contour, the electric field can be made exactly homogeneous. In fact, we have found that a relatively small number of electrodes can generate an excellent approximation of the desired field.

The devices that apply these ideas experimentally are shown in Fig. 1. With a square or hexagonal contour, two opposing sides of the polygon are used to define the electric field orientation. Alternation in the orientation is achieved by electronic switching. A square array generates a reorientation in the electric field of 90°. A hexagonal array generates reorientation angles of either 120° or 60°, depending on the placement of the gel with respect to the hexagon and the assignment of polarity to the electrodes.

The intermediate electrodes may be clamped to the desired potentials by any method. The apparatus in Fig. 1 uses a series

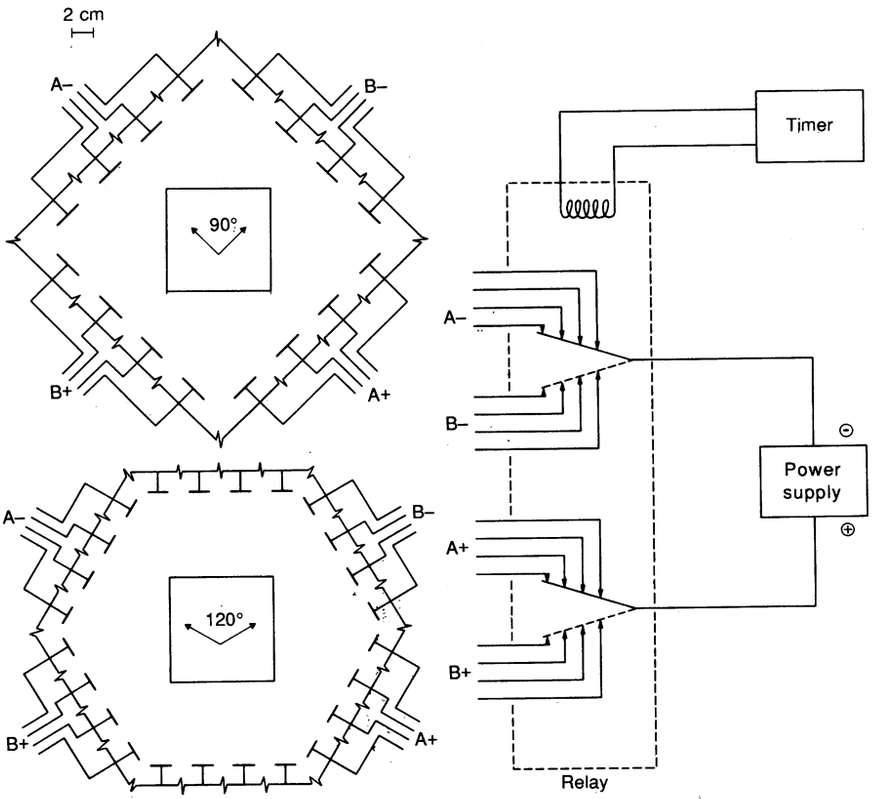


Fig. 1. Apparatus for alternating CHEF's. The gel and electrode configurations are drawn to scale. The electrodes (platinum wire, 0.030 inch in diameter) were suspended in 0.5× TBE buffer (45 m*M* tris, 45 m*M* boric acid, 1.25 m*M* EDTA, *p*H 8.3) and connected by a series of resistors (820 ohms, 2 W for the square array; 470 ohms, 2 W for the hexagonal array). Alternating orientations were generated by a timer (GraLab model 451 digital timer–intervalometer) that drove the switching relay. The hexagonal array is shown in the configuration for a reorientation angle of 120°. The buffer was circulated around the gel (*3*) and cooled to 9°C by being passed through a coil of silicone tubing placed in a water bath (Haake A81).

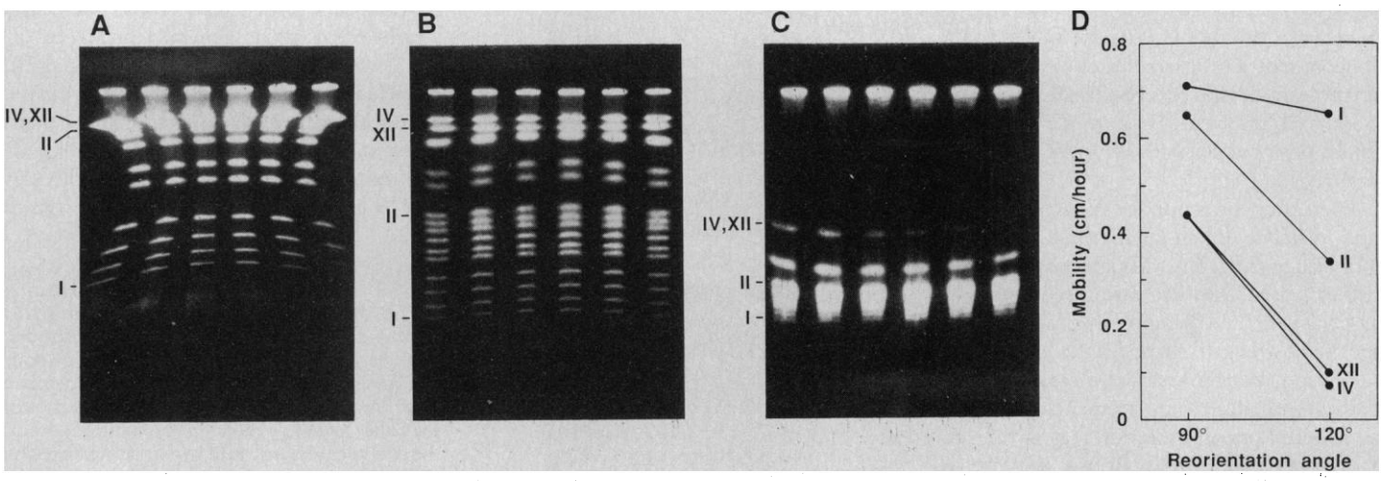


Fig. 2. Electrophoresis of yeast chromosomal DNA subjected to electrophoresis with (A) the OFAGE apparatus (*3*) for 48 hours, (B) the CHEF hexagonal-array apparatus for 24 hours, and (C) the CHEF square-array apparatus for 15.5 hours. In each case, the field direction was reoriented every 80 seconds, and the field strength was 6 V/cm. In the OFAGE apparatus, field strength was nominally defined as the voltage divided by the perpendicular distance between driving electrodes. Chromosomal DNA was prepared from *Saccharomyces cerevisiae*, strain YNN295, as described (*2*), except that the agarose inserts were washed with 50 m*M* EDTA (*p*H 8) after treatment with detergent and proteinase K. The gels were formed from 35 ml of molten 1% agarose (Baker standard low electroendosmosis, A426-5) in 0.5× TBE, poured onto a glass plate (10.5 by 10.5 by 0.2 cm), and confined to the plate by surface tension. Agarose inserts containing DNA were melted at 65°C for 5 minutes and loaded into the wells. After electrophoresis, the gels were stained in 0.5 μg of ethidium bromide per milliliter. Bands were identified by Southern blot through the use of specific sequence probes: chromosome II, LYS2 (*12*); chromosome IV, CEN4 (*13*); chromosome XII, GAL2. Chromosome I has consistently been the band with the highest mobility in other electrode configurations (*2, 4*). Current estimates for the physical sizes of chromosomes I, II, IV, and XII are 0.20, 0.95, 1.6, and 2.2 Mb, respectively (*14*). (D) Mobility of the chromosomes as a function of reorientation angle of the electric field. We calculated the average mobility by taking the net migration distance of the bands, multiplying by $\sqrt{2}$ for the square array and 2 for the hexagonal array to compute the true migration distance in the alternating field, and dividing by the electrophoresis time.

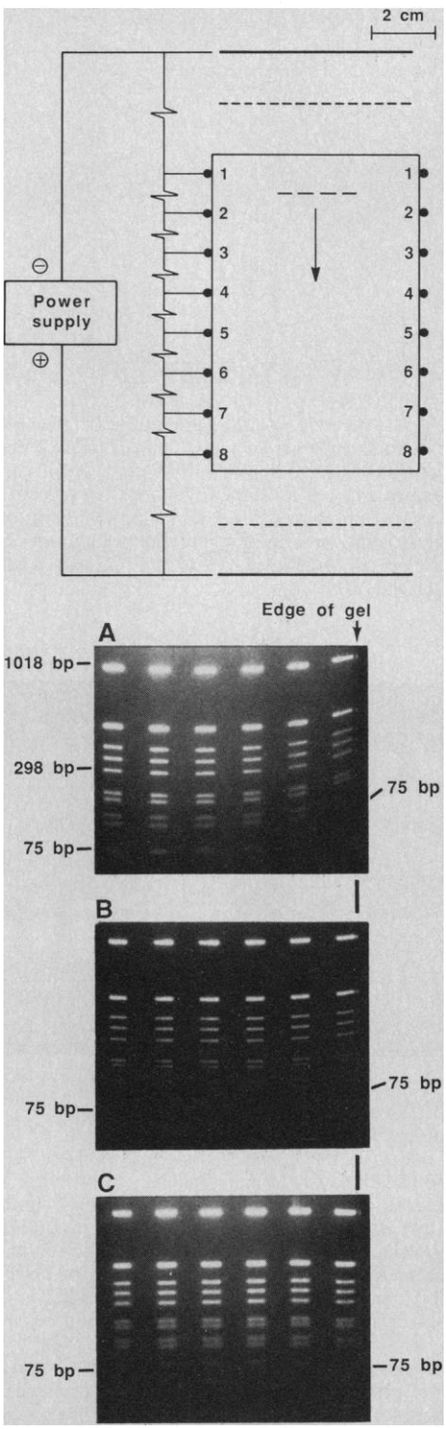
of equal resistors between the electrodes. When the resistances are small enough so that the electrical current passing through the electrophoresis buffer is much less than the current through the external resistors, the potentials at the electrodes assume the proper values. In practice, we found that even when the currents through the buffer and resistors were roughly equal, excellent results were obtained.

Homogeneous electric fields in alternating orientations were used to separate yeast chromosomal DNA (Fig. 2). Results from the orthogonal-field-alternation gel electrophoresis (OFAGE) apparatus (*3*) are shown for comparison. The polygonal electrode arrays could separate yeast chromosomal DNA. The pattern of separation was independent of position across the gel, and comparisons between multiple samples could be made with confidence. The uniformity across the gel in the square array was somewhat less than that seen in the hexagonal array, perhaps because the potential was clamped at fewer intermediate electrodes.

The pattern of chromosome separation changed dramatically with the reorientation angle of the electric field. In the OFAGE apparatus, different parts of the gel are subjected to different reorientation angles, mostly between 120° and 150°. In the hexagonal array the angle is 120°, and in the square array it is 90°. When the reorientation angle of the field was 120°, it was possible to obtain excellent resolution of DNA over the entire range of sizes extending up to 2 Mb. At 90°, there was a large increase in the mobility of the DNA and a concomitant loss in resolution. At 60°, the chromosomes moved even faster and none were well resolved. Carle *et al.* have also found poor resolution with small reorientation angles (*7*).

Even after correction for the actual migration distance in an alternating field, the mobility of the DNA through the gel matrix still increased with decreasing reorientation angle (Fig. 2D). Changing the pulse time or the field strength changed the pattern of resolution, but the marked increase in mobilities with decreasing reorientation angle remained. Loss of resolution may have been caused, at least in part, by the increase in mobility. The net separation between DNA molecules of different sizes is the accumulation of small separations produced with each reorientation of the field. At smaller angles, the molecules traveled faster down the gel, and the running time of the gel was necessarily shorter. Consequently, the molecules were subjected to fewer reorientations of the field and resolution decreased.

Schwartz and Cantor have concluded that a gradient in electric field strength is critical for achieving high resolution (*2*). The pulsed field gradient apparatus that they described has an array of electrodes arranged in a square. When the electric fields were generated between arrays of electrodes on opposite sides of the square, they found poor resolution of high molecular weight DNA. On the other hand, when the electric fields were generated between a single-point electrode and an array of electrodes, a strong gradient in field strength was produced across the gel and resolution improved dramatically.



One interpretation is that a gradient in the field is necessary for optimal resolution. However, the configuration of electrodes that produced a gradient in field strength also increased the effective reorientation angle to greater than 90°. Thus, a second interpretation of the data is that the observed improvement in resolution may not be due to the introduction of a gradient in field strength, but rather to an increase in the average reorientation angle. In fact, our data show that a gradient in field strength is not necessary for high resolution, whereas the reorientation angle is critical.

Field inversion gel electrophoresis is a modification of the field alternation method that has been applied to the separation of yeast chromosomal DNA (*7*). The electric field is periodically inverted so that a single pair of electrodes can be used to generate homogeneous electric fields. The reorientation angle is necessarily confined to 180°. The system resolves large DNA with a pattern of separation independent of position across the gel. However, for some pulse times, the fact that the migration of the DNA is not a monotonic function of size leads to the unexpected comigration of molecules that differ greatly in size. The problem can be avoided only by choosing an appropriate range of pulse times during electrophoresis.

By comparison, the CHEF system alternating in orientation with a single pulse time can produce excellent resolution of large DNA. Furthermore, the monotonic relation between migration and molecular weight seems to be generally preserved (*2, 3*). The physical variables of field strength, reorientation angle, and pulse time can be varied independently in a controlled manner. Understanding how these variables affect the migration of large DNA molecules may suggest ways to separate even larger

Fig. 3. Electrophoresis in a nonalternating CHEF. A standard horizontal gel apparatus (*15*) was modified by adding 16 vertical electrodes. The electrode array defined a closed contour surrounding the gel. The positive and negative electrodes were submerged in wells at the ends of the buffer tank. In a buffer tank of uniform depth, the equivalent electric field would be generated by electrodes substantially closer to the ends of the gel. This equivalent "electrical position" is indicated by the broken line. Shown are the results of electrophoresis (A) under conventional conditions, (B) with buffer recirculation, and (C) with buffer recirculation in a CHEF. For the CHEF, the resistances (960, 470, 470, 470, 470, 470, 470, 470, and 960 ohms) reflect the relative electrical distances between the contour electrodes. DNA samples (Bethesda Research Laboratories 5615 SA/SB) were subjected to electrophoresis in 0.7% agarose in 0.5× TBE containing 0.5 μg of ethidium bromide per milliliter at 7.25 V/cm for 110 minutes.

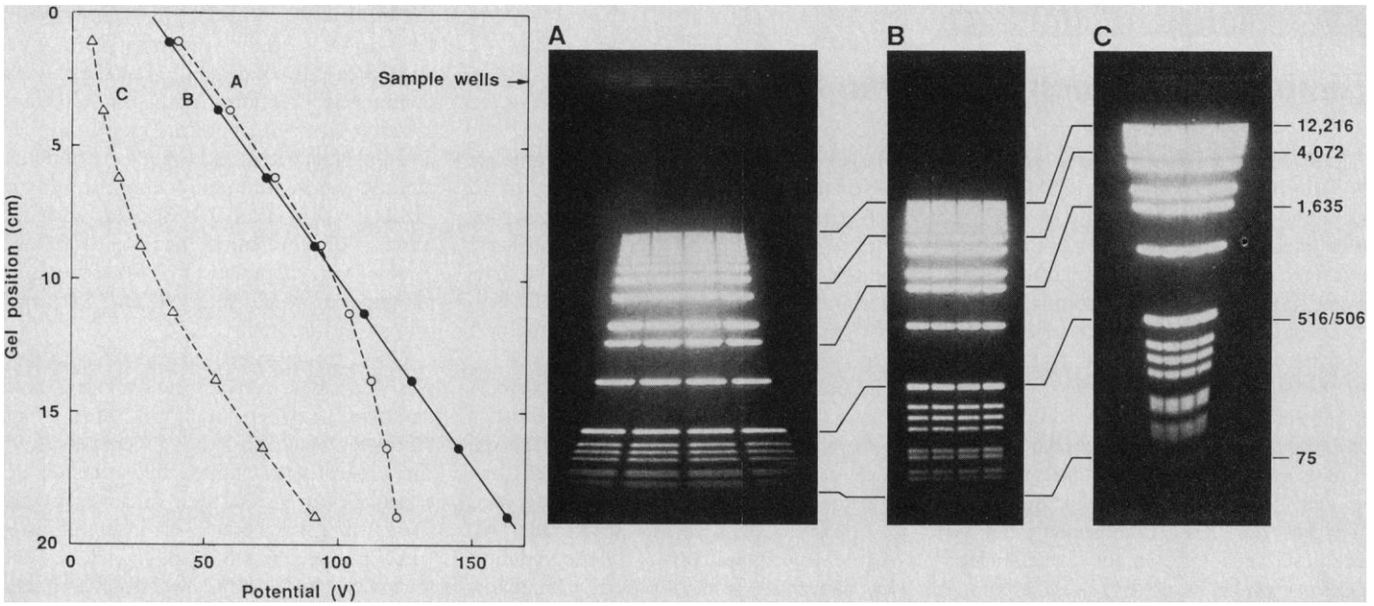


Fig. 4. Electrophoresis in nonuniform electric fields. The graph at left shows the electric potential as a function of position, measured at the contour electrodes. In the apparatus shown in Fig. 3, the electric field was manipulated to give (A) negative, (B) zero, or (C) positive gradients in field strength. The resistances from the negative to positive pole were: (A) 820, 390, 330, 270, 180, 120, 82, 51, and 100 ohms; (B) 960, 470, 470, 470, 470, 470, 470, 470, and 960 ohms; and (C) 100, 51, 82, 120, 180, 270, 330, 390, and 820 ohms. DNA samples were subjected to electrophoresis as in Fig. 3 with 200 V across the driving electrodes for 180, 120, and 200 minutes, respectively.

DNA. Current theories for the gel electrophoresis of DNA predict improved size resolution if the electric field is reoriented with appropriate pulse times (*8*). These theories do not limit the size of molecules that can be resolved.

In the conventional electrophoresis of DNA less than 50 kb, increasing the voltage permits adequate resolution in a shorter time. However, one disadvantage is that the migration pattern across the gel becomes distorted, with the effect most pronounced at lower molecular weights. This problem can be eliminated by using a nonalternating CHEF. Figure 3 shows the modification of a standard horizontal gel apparatus. When the gel was run conventionally at high voltage, the migration pattern was significantly distorted. Recirculation of the buffer dissipated much of the uneven heating across the gel and improved the migration pattern. The combination of a CHEF and heat dissipation removed the residual distortion.

Contour-clamping can be used for electrophoresis in nonuniform fields. A negative field strength gradient decreasing from 7.76 to 1.0 V/cm produced a number of effects on the migration of the DNA (Fig. 4). (i) The mobility of the DNA decreased as it moved through the gel, and electrophoresis could be prolonged to permit increased resolution of the larger DNA. (ii) The negative gradient counteracted band-broadening, with the benefit most pronounced for species of low molecular weight, because the leading edge of the band was subjected to a weaker electromotive force than the trailing edge. (iii) The migration of the DNA assumed a curvilinear trajectory with a progressive increase in the width of the lanes down the gel (*9*). Thus, a weak gradient adjusted to counteract band-broadening can improve resolution; a strong gradient adjusted to keep small DNA molecules on the gel can provide more time to separate larger molecules on the same gel. The zero and positive field gradient gels emphasize these effects by comparison.

Secondary structure in DNA may be identified by using contour-clamped fields. Biological systems can generate DNA molecules in forms such as supercoiled circles or branched DNA. Such molecules display anomalous mobility shifts with respect to linear DNA in response to voltage changes (*10, 11*). To separate DNA with secondary structure from a mixture of linear DNA, a square electrode array can be used to generate a CHEF with different field strengths in the two orientations. DNA with secondary structure will appear as spots displaced from the arc of linear molecules. Because the field orientation is switched electronically, there is no need to manipulate the gel.

We have described a general method in which multiple electrodes arranged on a closed contour and clamped to the appropriate potentials are used to manipulate the electric field. By use of this method the electrophoretic separation of DNA can be controlled in ways not previously accessible. The method may have application to a broad range of problems in the separation of macromolecules.

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