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Separation of chromosomal DNA molecules from yeast by orthogonal-field-alternation gel electrophoresis

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ABSTRACT

A simple agarose-gel apparatus has been developed that allows the separation of DNA molecules in the size range from 50 kb to well over 750 kb, the largest size for which size standards were available. The apparatus is based on the recent discovery that large DNA molecules are readily fractionated on agarose gels if they are alternately subjected to two approximately orthogonal electric fields. The switching time, which was on the order of 20-50 sec in our experiments, can be adjusted to optimize fractionation in a given size range. The resolution of the technique is sufficient to allow the fractionation of a sample of self-ligated λ DNA into a ladder of approximately 15 bands, spaced at 50 kb intervals. We have applied the technique to the fractionation of yeast DNA into 11 distinct bands, several of which have been shown by DNA-DNA hybridization to hybridize uniquely to different chromosome-specific hybridization probes. In this paper, we describe the design of the apparatus, the electrophoretic protocol, and the sample-handling procedures that we have employed.

INTRODUCTION

Our knowledge of the structural and functional organization of chromosomal DNA has long been limited by the difficulty of fractionating mixtures of large DNA molecules or even assaying heterogeneous DNA samples for the presence of such molecules. Routine preparative and analytical techniques for manipulating DNA are only effective for molecules of approximately 100 kb or less, while the average amount of DNA per chromosome is on the order of 1000 kb in lower eukaryotes such as yeast and over 100,000 kb in higher plants and animals. Nonetheless, numerous efforts have been made to characterize chromosome-sized DNA molecules in organisms such as yeast and Drosophila, primarily by electron microscopy, velocity sedimentation, and measurements of visco-elasticity. Although these techniques have met with some success--for example, in providing evidence that the chromosomes of lower eukaryotes probably contain a single linear molecule of duplex DNA (1,2)--they have not led to any detailed knowledge of the composition of the highly heterogeneous samples of large DNA molecules that are produced by the gentle lysis of cells

or nuclei. The single chromosome-sized DNA molecule that has been well characterized was isolated from a mutant yeast strain that harbors a ring chromosome containing about half of the genetic material present on a wild-type copy of chromosome III. This molecule could be purified as a 190 kb supercoiled circle and shown to be free of protease- or RNase-sensitive junctions (3).

Gel electrophoresis has appeared to offer the best opportunity to study heterogeneous populations of large DNA molecules, but efforts to apply this technique to chromosome-sized molecules have until recently met with little success. Using conventional agarose slab gels, combinations of low matrix densities and low voltage gradients have allowed the separation of bacteriophage DNA's from λ (48.5 kb), T5 (125 kb), T4 (170kb), and G (~750 kb), but in the best experiments the mobility difference between T4 and G was only about 10% (4,5). In general, large DNA molecules migrate with size-independent mobilities in conventional gels, presumably because they present an effective cross-section to the gel matrix that is independent of their lengths. Against this background, it was recently reported by Schwartz et al. (6) that much improved resolution of large DNA molecules could be obtained on agarose slab gels if the molecules were alternately subjected to two approximately orthogonal electrical fields. The basic idea behind the method is that small molecules have a mobility advantage in such an apparatus because they can turn corners in the gel matrix more rapidly than large molecules.

We were encouraged by this report to experiment with a variety of gel apparati and experimental protocols that involved alternating applications of orthogonal fields, and we report here the development of an easily constructed apparatus that resolves the DNA molecules present in yeast chromosomes into a series of widely spaced bands. The sizes of the smaller of these molecules were estimated by comparing their mobilities with those of a variety of size standards, which cover the range up to 750 kb. We also obtained excellent resolution between still larger molecules, but because we lacked suitable size standards, we have not yet been able to estimate the sizes of the largest molecules that were separated. In addition to presenting a detailed description of our electrophoresis protocol, we demonstrate that DNA samples that are suitable for experiments of this type can be prepared by conventional techniques and require surprisingly little special handling.

MATERIALS AND METHODS

Apparatus

The Gel Box. A schematic of the gel apparatus is presented in Figure 1. The region showing the actual gel box, including the placement of the gel and the electrodes, is drawn to scale. The box itself has internal dimensions of 38.1x38.1x5.1 cm (15x15x2 inches). Each of the four electrodes lies on the bottom of the box, supported from above by cross bars that are not shown. The lengths of the negative electrodes are 16.5 cm, while the positive electrodes are 2.5 cm long. All the electrodes are 100% platinum wire that is 0.4 mm in diameter (26 gauge). The short vertical lengths of platinum wire leading to the main sections of the electrodes are insulated from the buffer with silicone sealant. The gel, which is 10.2x10.2 cm, is supported on a glass plate of the same dimensions that is 3.2 mm thick and lies directly on the bottom of the box. The precise positioning of the gel and the electrodes is critical. As shown in Figure 1, the gel is centered in the box with its sides parallel to the walls. The electrodes, however, are deployed at 45° angles relative to the gel.

Buffer recirculation occurs through two sets of evenly spaced holes (6.3 mm diam.) in the bottom of the box. Beneath these sets of holes, there are manifolds that connect the holes to the external buffer-recirculation loop (see below). In the presence of the high rates of buffer flow that we employ, there is a tendency for the gel to separate from the glass plate. Our standard solution to this problem is to glue a narrow strip of Velcro along one edge of the glass plate with silicone glue; when the agarose gels, the embedded Velcro bonds this edge of the gel firmly to the plate. If the Velcro-bonded edge is placed upstream in the buffer flow, the entire seal between the gel and the glass plate is stabilized. Velcro is a consumer product that is available at fabric-supply stores.

Electrical circuitry. The switching is achieved by a simple external circuit that contains a double-pole/double-throw relay and an electrical timer. A wide variety of standard products can be used both for the relay and for the timer, neither of which is subject to particularly demanding performance requirements. For a relay, we have used a heavy-duty power relay, rated for use at 120/240 V AC and 30 amps with a coil that is activated by 120 V AC (Deltrol controls, Series 900 DPDT relay 20241-83). We have found that any power relay rated at this level works well under the electrophoretic conditions that we describe here (see below). It should be noted, however, that arcing between relay poles is more of a problem when DC power is being

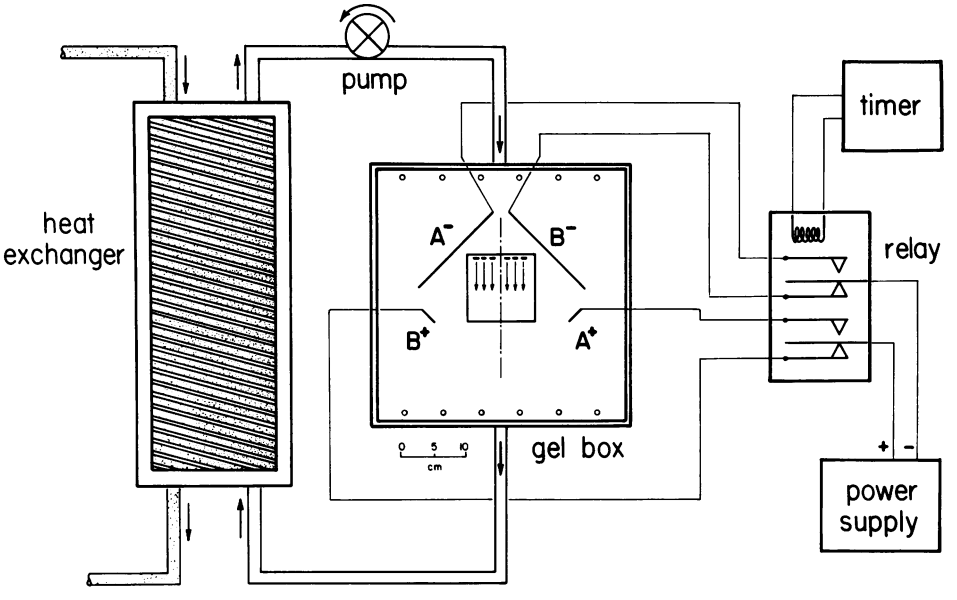

Figure 1. A Schematic Diagram of the Gel Apparatus. Only the region showing the gel box, the placement of the electrodes, and the gel itself is drawn to scale.

switched than in typical AC applications. If gels are run at significantly higher powers than those that we describe, relays of this type will arc visibly during switching. For a timer, any laboratory timing device that can be programmed to control a single circuit (repeating cycles of 120 V AC for 20-60 sec followed by 0 V for the same interval) can be employed to activate the relay's coil (for example, a Chrontrol Model CT-4, available from most scientific supply houses, meets these requirements).

The only special requirement for the power supply is that it must be able to deliver at least 150 mA, and preferably 200 mA, at 300 V. Since we control the temperature of the gel during the run, the conductance of the apparatus is constant, and it is irrelevant whether the power, the voltage, or the current is regulated. Our experience is predominantly with a DanKar Model DK203 unit that regulates only the voltage.

The circuit itself is represented schematically in Figure 1. The timed-AC outlet from the timer is wired to the coil of the relay. The positive lead from the power supply goes to the common pole on one side of the relay while the negative lead goes to the common pole on the other side. The electrode B^+ is wired to the relay pole that becomes connected to the

positive common pole when the relay is in its relaxed configuration, while the electrode B^- is wired to the analogous negative pole. Similarly, the electrodes A^+ and A^- are wired to poles that are connected to the positive and negative common poles when the relay's coil is activated.

Buffer Recirculation and Cooling. The gels are run both at relatively high power (~60 Watts) and high current (~200 mA). Consequently, there is the potential both for substantial temperature increases and for buffer failure during a gel run (10-20 hr). We solve both problems at once by recirculating the buffer through a buffer loop that contains a simple heat exchanger. The buffer is pumped continuously at 250 ml/min using a high-capacity peristaltic pump (Cole Parmer Masterflex with a K-7553-00 drive and a K-7018-21 head using silicone tubing in the pump head that has an outside diameter of 11 mm and an inside diameter of 8 mm). The heat exchanger consists of two 15 m roles of polyethylene tubing (low density with an outside diameter of 6.4 mm and an inside diameter of 4.3 mm) that are wrapped side-by-side into 23 loops with diameters of approximately 20 cm; the loops are immersed in an insulated tank filled with water, which serves as a passive heat-exchange medium. One of the coils of polyethylene tubing is in series with the buffer-recirculation loop, while the coolant flows through the other coil. With this arrangement we maintain a gel temperature of approximately 14° C by keeping the coolant, which is water, at a temperature of 6° C and maintaining a flow rate of 400 ml/min. Obviously a wide variety of methods could be used to dispose of the heat produced in the electrophoresis tank. The only non-obvious feature of heat exchangers similar to the one described here concerns the choice of material for the tubing: if plastic tubing is to be employed, it should be thin-walled polyethylene tubing such as that described, since polyethylene has much higher heat conductivity than do most other common polymers.

Safety Considerations. This protocol involves potentially lethal combinations of voltage and current. We would like to call attention to the particular hazard posed by the recirculation of electrically hot buffer through a tubing loop that includes multiple plumbing connections. A prudent safety precaution would be to enclose the electrophoresis box, the pump, and all the tubing connections in an outer, water-tight box that would contain all the buffer in the event of a failure either of the tubing in the peristaltic-pump head or of any of the tubing connections. In addition, the tubing within the peristaltic-pump head should be a high-quality silicone tubing such as that described, and it should be changed regularly, to avoid tubing failures. Finally, the outer containment box should be well vented at the top to avoid accumulations of hydrogen gas.

Electrophoresis Protocol

Gel Preparation. The gels are 1.5% agarose, cast and run in 0.5xTBE (1xTBE= 90 mM Tris base, 90 mM boric acid, 2.5 mM Na_2H_2EDTA, unadjusted pH~8.2). We have employed standard grades of agarose from a variety of manufacturers without significant variations in the results. The gels are cast directly on the 10.2x10.2 cm glass plate to a thickness of 4 mm. We have used combs as thick as 1.6 mm without any observable broadening of the bands.

Sample Loading. The gels are loaded while submerged in the running buffer. The 1x dye mix in the loaded samples has the following composition: 5% (w/v) ficoll (Sigma, Type 400-DL), 0.02% bromphenol blue, 0.02% xylene cyanole FF, 40 mM EDTA [pH8]. The samples are gently pipetted into a plastic tube using a plastic pipet tip that has been cut off with a razor blade to produce an orifice that is ~1 mm in diameter. An appropriate quantity of the 5x dye mix is pipetted into the tube and the solution is gently stirred with the pipet tip until it is well mixed. The sample is then loaded by gently pipetting it into the submerged gel wells using a glass capillary that also has an orifice ~1 mm in diameter. As in the case of conventional agarose gels, the ratio between the sample volume and the well volume is not critical: we normally employ ratios between 0.3 and 0.8.

Electrophoresis Conditions. The apparatus and the electrophoresis protocol were designed around the limitations of power supplies of the type used for conventional electrophoresis experiments. Most such power supplies will only deliver maximum currents of 150 or 200 mA. We normally run the gels at a constant voltage of 300 V. Given this voltage, the composition of the running buffer, and the basic geometry of the gel box, the two variables that determine the current are the depth of buffer in the gel box and the temperature. Our standard protocol calls for a buffer depth of 1.5-1.7 cm and a temperature of 14° C, under which conditions the current is approximately 150 mA. If the gel is being run with a power supply whose maximum current is 150 mA, the buffer depth should be slightly reduced; this measure has little effect on the results as long as the gel is well submerged. Under the conditions described here, the voltage gradient in the center of the gel, measured along the axis of net migration is ~3 V/cm.

With respect to the running temperature, we can only say that we have consistently obtained the best results when the gels were run relatively cold. It is likely that comparable results are obtainable at higher temperatures, but a number of other variables (voltage, switching time, lenth of the run) would have to be re-optimized at the chosen temperature. We are convinced

that reproducible results can only be obtained if the gels are run at a constant temperature, and with the cooling system that we describe it is no more difficult to regulate the temperature at 14° than at ambient temperatures.

Gel Staining and Photography. Any standard technique can be employed to stain and photograph the gels. The method chosen, however, should have a demonstrated ability to detect 1 ng of uncleaved λ DNA when it is run on a single lane of a standard agarose gel. We achieve this sensitivity using a protocol that involves staining the gels with ethidium bromide for 10-30 min at a concentration of 0.1 μg/ml in 0.5xTBE, de-staining for 20 min in 0.5xTBE, and employing an ultraviolet transilluminator with a wavelength maximum at 300nm (Spectronics, Model TR-302). The combination of camera, film, and filters that we employ has been described previously (7). High-speed instant film (Polaroid Type 667) gives adequate results for day-to-day use with exposures of 30 sec or less, while the photographs for this paper were prepared using a wet-process film (Eastman Kodak No. 4155). The protocol for using the wet-process film was exactly as described except that in some cases, when maximal sensitivity was required, a lens with f=75 mm (rather than the usual 135 mm) was employed. This modification improves the detection of extremely faint bands by allowing the lens to be moved closer to the gel and, equally importantly, by concentrating the image onto a smaller area of the film.

Southern Transfers. Any standard protocol can be used for transferring DNA from these gels to nitrocellulose as long as it includes--as most current protocols do--a step in which the DNA is partially degraded before it is denatured in the gel. To reduce the average molecular weight of the DNA, we soak the gels in 0.25 M HCl for 20 min at room temperature (8) before soaking them in 0.5 M NaOH/1.0 M NaCl and then proceeding by standard methods.

DNA Preparations

Yeast. Yeast DNA was prepared by a slight modification of a method that we have previously described (9). In broad outlines, the method involves preparing spheroplasts with zymolase, lysing the spheroplast suspension with N-lauroyl sarcosinate, and purifying the DNA with a preparative sucrose gradient. Only our minor modifications of the previously published protocol will be described here, along with a few comments on precautions that may be important for minimizing degradation of the larger chromosome-sized DNA molecules.

The current implementation of the procedure employs zymolase preparations

that have higher activity than those available at the time of our earlier publication. Using 0.1 mg of zymolase 60,000 (Miles Laboratories) for 1 g (wet weight) of cells, spheroplasting is generally complete in 30 min. The course of the spheroplasting reaction is easily followed under the light microscope by taking small aliquots of the cell suspension, mixing them with the lysis solution, incubating them for 5 min at 37° C, and comparing their appearance with that of a control that is diluted with 1.0 M sorbitol instead of with the lysis solution; when the cells are properly spheroplasted, lysis should produce a field that contains so little refractile material that it is difficult to find the focal plane. Once prepared, the spheroplasts must be mixed gently but thoroughly with the detergent solution: if mixing is insufficiently vigorous, large clots form that cannot be subsequently resuspended, while if the solution is agitated too vigorously, the largest DNA molecules are mechanically sheared. We use a mixing method in which the lysis buffer is placed in a 125 ml Erlenmeyer flask and swirled by hand while the spheroplast suspension is allowed to run down the side of the flask at a rate of 2-3 ml/min; an appropriate swirling action involves moving the flask back and forth along a semi-circular arc 10-15 cm in diameter at 10-20 cycles per minute.

For the sucrose gradient, we use the same solutions as previously described except that our current gradients are 15% sucrose at the top (rather than the 10% employed previously). The gradients are poured rapidly by hand, simply by pipetting the 15% solution on top of the 20% solution without taking any special care to avoid mixing at the boundary between the two solutions. The 50% pad is then underlaid onto the bottom of the tube, taking some care to produce a clear demarcation between the pad and the bottom of the 20% layer. The lysate is pipetted on top of the gradient through the large end of plastic 5 ml pipette, and the gradient is then run as described.

In order to minimize shear while collecting the gradients, we simply insert the large end of a 10 ml pipet to the bottom of the tube and gently withdraw the bottom 5-6 ml of the gradient, taking care not to disturb the hard pellet of polysaccharide that forms on the bottom of the tube. This fraction, which should be highly viscous, is then dialyzed and concentrated in the dialysis bag (to a final volume of ~20 ml per liter of cells), as described. The resulting sample should be sufficiently concentrated to load directly onto the gels; we normally load 10-20 µl of a sample prepared in this way onto a well that is 1 cm wide. We have stored yeast DNA samples for several months at 4° C without any detectable change in their properties.

The DNA cleaves well with most restriction enzymes if BSA is added to the digests at a concentration of 100 μg/ml.

All the gels shown in this paper employed DNA prepared from the ρ^o haploid yeast strain AB972 (ref. 10).

Bacteriophage. All bacteriophage DNA's were stored as phage lysates, which were generous gifts from D. Dykhuizen (T4 and T5) and W. Fangman (G). Aliquots of the phage lysates containing ~100 ng of phage DNA were lysed immediately before being loaded onto a gel, simply by including sufficient SDS in the ficoll-dye mix to give a final concentration of 1% in the gel sample. The inclusion of SDS causes anomalous behavior of the loading dyes early in the gel run, but has no detectable effect on the mobility of the DNA.

Lambda DNA Ladders. DNA from λcI857 (Bethesda Research Laboratories) was self-ligated at a concentration of 50 μg/ml using 0.2 Weiss units of T4 DNA ligase (Bethesda Research Laboratories) for a 15 μl reaction, carried out for 12 hr at 14° C in 66 mM TrisHCl [pH 7.6], 5 mM $MgCl_2$, 5 mM DTT, and 1 mM ATP. We loaded ~400 ng of DNA, prepared in this way, onto a gel well with a width of 1 cm.

Hybridization Probes. All three of the hybridization probes were prepared by nick-translation of DNA from entire lambda clones using α-^{32}P-dATP. The clones were from a lambda-yeast pool prepared by M.Y. Graham in the vector λMG14 using DNA from the yeast strain AB972. The identities of the probes were as follows: chromosome I, λPM4237 (cloned from the CDC19-CDC24 interval by H.Y. Steensma and D. Kaback; the original name of λPM4237 was λC1a); chromosome V, λPM910 (cloned by G. Brodeur using a URA3 probe); chromosome VI, λPM4235 (cloned by M. Graham using a SUP11 probe).

RESULTS

Basic Behavior of the Gels

The appearance of a typical gel, in which a single large well has been loaded with yeast DNA, is shown in Figure 2. This gel was run with a switching interval of 40 sec (A electrodes active for 40 sec, B electrodes active for 40 sec, ...), which optimizes the fractionation of the largest bands that we observed. Our overall analysis of the yeast pattern suggests that the 6 bands visible in Figure 2 (numbered 5-11) represent the upper half of a set of 11 well-resolved bands. As will be discussed below, the median band (no. 6) appears to have a size of ~700 kb. Because the average molecular weight of this yeast-DNA preparation is only ~300 kb, the smaller bands are

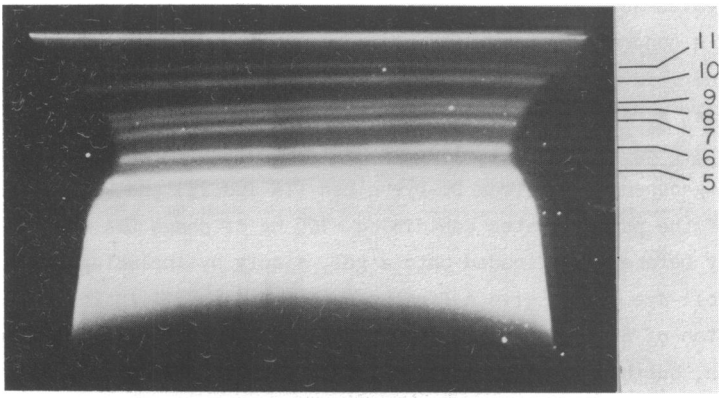

Figure 2. Fractionation of total yeast DNA with a switching interval of 40 sec. This gel was run for 8.6 hr, stained with ethidium bromide, and photographed as described in Materials and Methods. The numbering system for the yeast bands reflects our overall analysis of the yeast pattern. Bands are numbered in order of decreasing mobility; bands 1-4 are obscured in this photograph by the heterogeneous smear of partially degraded DNA.

obscured by the heterogeneous smear of partially degraded molecules.

The example in Figure 2 illustrates that the gels exhibit substantial edge effects that cause molecules that are loaded to either side of the gel's axis of symmetry to follow a complex trajectory. Nonetheless, the bands themselves are relatively straight over a large fraction of the gel, a property which greatly aids in comparing the patterns on different gels and in assaying for specific sequences by DNA-DNA hybridization. A possible explanation for this phenomenon is that the rate of net migration parallel to the gel's main axis may simply be determined by a molecule's size and the number of times that the field changes direction. In contrast, the detailed trajectory followed by a molecule may be insensitive to size, but highly responsive to local differences in the electric-field strengths generated by the two electrode pairs.

A second point that should be emphasized about gels such as the one shown in Figure 2 is that the duration of the gel run has non-trivial effects on the pattern of bands obtained. Because of the relatively complex field geometry, molecules do not migrate at a constant rate throughout an experiment. In particular, the angle between the field directions associated with the two electrode pairs is changing from ~90^{o} at the top of the gel to something substantially higher at the bottom. Consequently, the net mobility of all molecules decreases as they migrate down the gel since the net field along the

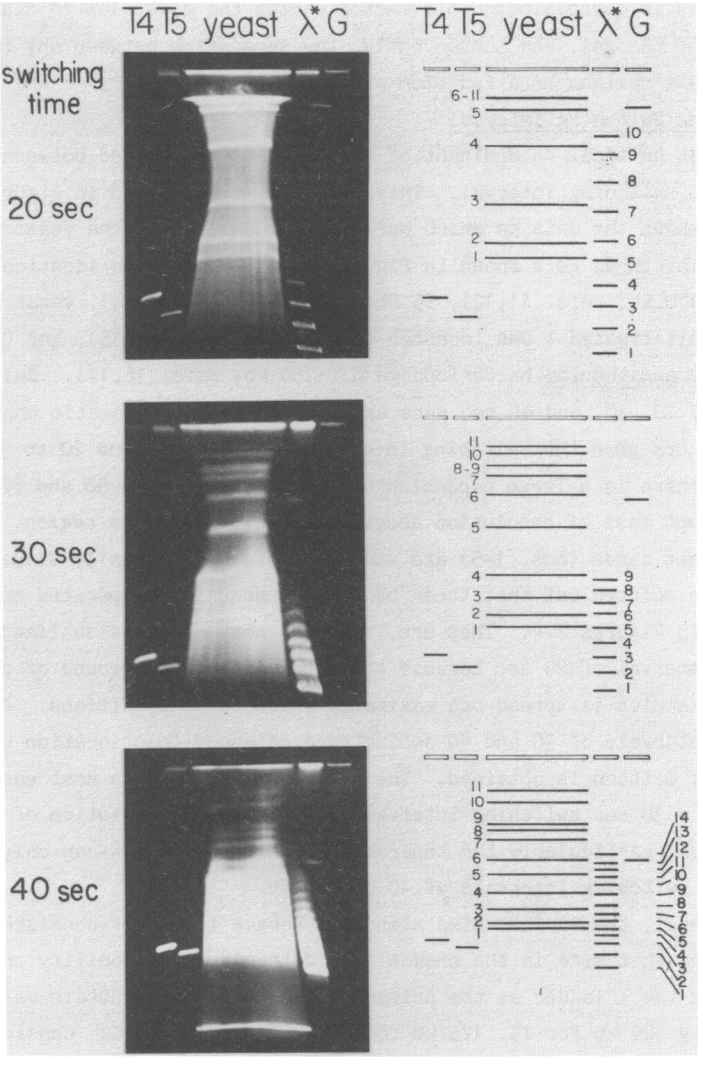

Figure 3. The effect of variable switching intervals on the fractionation of yeast and bacteriophage DNA's. The symbol λ* labels lanes in which samples of self-ligated λ DNA were fractionated. In the schematic representations of the gels, the yeast bands are numbered consistently, in order of decreasing mobility; the labeling of the λ ladders shows our interpretation of the number of monomers present in a given band. The schematic representations of all three gels are based on the analysis of at least two photographic exposures of the gel; heavily stained regions are more easily analyzed using short exposures, while faint bands are most easily detected in long exposures. The three gels were run for the following times: 20 sec switching interval, 18 hr; 30 sec, 15 hr; 40 sec, 12 hr. The strong band at the bottom of the third gel is due to one of the double-stranded RNA's of the yeast killer system.

main gel axis is decreasing. This factor causes the molecules to stack up at the bottom of the gel, and consequently, the separation between any two bands goes through a maximum as a function of time.

Effect of the Switching Interval

The most critical determinant of the separation obtained between two bands is the switching interval. This effect is illustrated in Figure 3, which also shows the data on which our overall analysis of the yeast pattern is based. The three gels shown in Figure 3 were loaded with identical samples of T4 DNA (170 kb; refs. 11,12), T5 DNA (125 kb; refs. 13,14), yeast DNA, a ladder of self-ligated λ DNA (monomer length 48.5 kb; ref. 15), and DNA from the Bacillus megatherium bacteriophage G (~750 kb; refs. 16,17). Switching intervals of 20, 30, and 40 sec were employed. The most dramatic change in behavior occurs when the switching interval is increased from 20 to 30 sec. At 20 sec, there is a large expansion of the region between 50 and 600 kb, with an abrupt loss of resolution above this range. In this region, the five smallest yeast bands (nos. 1-5) are widely separated and easily visualized. It should be pointed out that these bands are adequately separated on all the gels shown in Figures 2-4. They are, however, most easily visualized with a switching interval of 20 sec because the heterogenous background of partially degraded molecules is spread out maximally under these conditions. At switching intervals of 30 and 40 sec, a more balanced fractionation of the entire yeast pattern is obtained. The pattern as a whole is most easily observed at a 30 sec switching interval, although full resolution of the largest bands--particularly the separation of bands 8 and 9--can only be achieved at switching intervals of 40 or 50 sec.

In general, the various size standards behave in a self-consistent way, demonstrating that size is the predominant determinant of mobility on these gels. Using the λ ladder as the primary size standard, we obtain values of approximately 120 kb for T5, 175 kb for T4, and 670 kb for G. Considering the difficulty of reliably correcting for the curvature in our gels, this degree of internal consitency in the mobilities of these molecules is quite good. Furthermore, since T4 and G contain heavily glycosylated DNA, while T5 contains a series of single-stranded breaks at specific sites, the agreement between our results and those obtained by independent methods (primarily measurements of contour lengths in the electron microscope) suggests that this fractionation technique is not highly sensitive to minor variations in the physical and chemical structures of large DNA molecules. If the yeast DNA molecules obey the same size-mobility relationship that appears to hold for the λ ladder and the other bacteriophage DNA's, we would estimate the

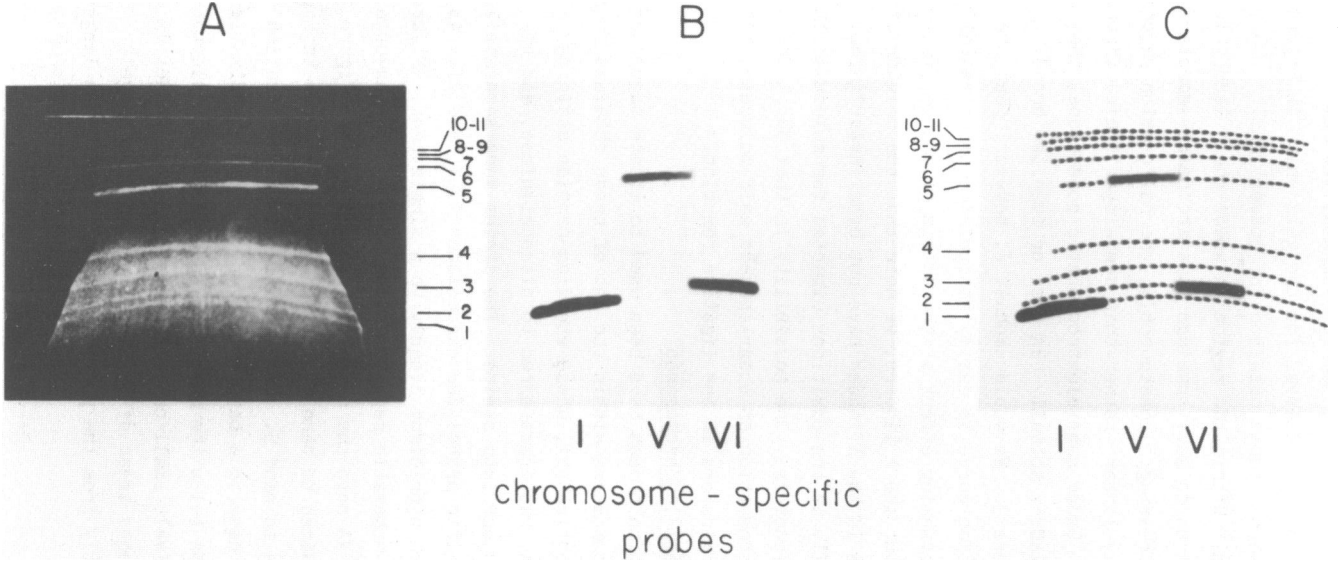

Figure 4. Analysis of the yeast banding pattern using DNA-DNA hybridization probes. A. The ethidium-bromide staining pattern of the gel on which the yeast DNA was fractionated; it was run for 15.5 hr with a switching interval of 30 sec. B. An autoradiogram showing hybridization to strips of a nitrocellulose sheet onto which the DNA from the gel shown in Figure 4A had been transferred; hybridization probes containing sequences specific to chromosomes I, V, and VI were employed. C. Superposition of a schematic representation of the ethidium-bromide staining pattern shown in Figure 4A onto the actual autoradiogram shown in Figure 4B.

following sizes for the six smallest bands: band 1, 260 kb; band 2, 290 kb; band 3, 370 kb; band 4, 460 kb; band 5, 580 kb; band 6, 700 kb.

The size-mobility relationships observed for the gels shown in Figure 3 do not even qualitatively resemble those obtained for conventional nucleic-acid and protein gels. Within the region in which the λ ladder is well resolved, the successive bands have spacings that are nearly constant rather than exponentially decreasing; consequently, the calibration curves for these gels are essentially linear (rather than semi-logarithmic) through the major region of the gel. Under some electrophoretic conditions, the inter-band spacing in the λ ladder actually has one, or even two broad maxima, as is evident in Figure 3. We would emphasize, however, that the detailed form of the calibration curve is easily manipulated experimentally, particularly because of the stacking effect at the bottom of the gel (see above). As a final point about the λ ladders, we note that the origins of the faint, off-register bands in the λ ladder have not been investigated. These bands fall almost exactly at the positions expected for molecules that contain 1.5, 2.5, 3.5, ... monomers. One possibility is that our λ DNA prepartion contains a small proportion of shear-induced half-molecules (17) that could serve as chain terminators in the ligation reaction.

Association of Bands with Specific Chromosomes

Three DNA-DNA hybridization probes have been used to locate sequences corresponding to specific chromosomes within the set of bands obtained from yeast DNA. The DNA from an entire gel, whose ethidium-bromide staining pattern is shown in Figure 4A, was transferred to nitrocellulose, and the nitrocellulose sheet was then cut into strips. The strips were separately hybridized to chromosome-specific probes for chromosomes I, V, and VI. As shown in Figure 4B, each probe hybridized cleanly to a single band. Figure 4C shows the superposition of a schematic representation of the ethidium-bromide staining pattern from Figure 4A onto the actual autoradiogram from Figure 4B: the chromosome I probe hybridizes to band 1, the chromosome VI probe to band 2, and the chromosome V probe to band 5. The fact that the hybridization occurs quite cleanly in each case to a band, with very little hybridization to the heterogeneous smear of DNA below the band, indicates that the molecules corresponding to these relatively small chromosomes are largely intact in our preparations. The heterogeneous smear of DNA in the 100-500 kb range apparently arises predominantly from the partial degradation of much larger molecules.

DISCUSSION

We have shown that total yeast DNA, even when prepared and handled by conventional methods, can be fractionated on an agarose gel into 11 well-separated bands by alternately applying two electric fields that are approximately orthogonal. The size of the molecule responsible for the smallest band in this pattern, which hybridizes specifically to a sequence from chromosome I, is estimated to be 260 kb. The middle band in the pattern corresponds to a molecule with an estimated size of 700 kb, while no estimates could be made for the upper bands since suitable size standards were not available. Because these bands have mobilities that are in the range expected for the chromosome-sized DNA molecules of yeast and because several single-copy clones of known yeast genes hybridize specifically to individual bands, we believe that the bands arise from the intact DNA molecules present in the various yeast chromosomes.

Further work will be required to rationalize the discrepancy between our band count of 11 and the genetically-defined chromosome count of 17 (refs. 19-21) for Saccharomyces, but we anticipate that the major source of the discrepancy will prove to be cases of co-migrating molecules. We cannot however, rule out the possibility that either our method of sample preparation or our electrophoretic protocol has a relatively abrupt size cut-off, and that we are not observing the DNA molecules corresponding to either the smallest or, more likely, the largest chromosomes. We are also unable to exclude the possibility that the yeast-DNA molecules that we observe are associated with proteins or other macromolecules that affect their electrophoretic mobilities. Although the DNA preparations have been heated to 65° C in the presence of 1% detergent and sedimented through a sucrose gradient containing 0.8 M NaCl, it cannot be assumed that their conformations are unaffected by residual associated factors. Although this possibility would not detract from the usefulness of the technique, it would undermine the basis of our size estimations. The excellent resolution that we obtain between λ concatemers of different molecular weight indicates that the fractionation technique itself is highly effective at separating large molecules of naked DNA.

With respect to the apparatus that we have described, the most critical aspect of the design--and the one for which we can provide the least compelling rationale--is the precise geometry of the gel in relation to the set of electrodes. As was previously reported by Schwartz et al. (6), we also have obtained superior results when only one member of each pair of electrodes is comparable in length to the gel. Considerable caution is required,

however, in extrapolating from a particular deployment of the electrodes to an assumed field geometry within the gel. A number of factors make it quite complicated to produce a uniform electric field in each direction, and the question of whether or not the fractionation technique actually depends on heterogeneity in at least one of the applied fields, as has been suggested (6), could only be resolved by more sophisticated experiments than we have done. As an illustration of the complexity of the problem, it may be pointed out that the simplest electrode geometry--two sets of full-length electrodes--fails to produce a uniform field in either direction despite the symmetry of the arrangement. There are several contributions to this effect, the largest of which arises from electrochemical activity at both ends of the electrodes that are turned off. Because the resistance of the platinum wire is essentially zero compared to that of the running buffer, the wires that form the inactive electrodes provide highly efficient paths for carrying current over most of the physical distance that separates the two active electrodes. Consequently, a substantial amount of the overall current is carried by a seven-step pathway that involves hydrogen production at the active negative electrode, ionic migration to the nearest end of an inactive electrode, oxygen production at that site, electron conduction down the inactive electrode, hydrogen production, ion migration to the active positive electrode, and finally oxygen generation. Obviously, a variety of discontinuous electrode configurations could be designed in order to minimize this effect. Although we have experimented with some such designs, we have not yet found any system that gives superior results to those produced by the simple electrode geometry that we describe here.

A surprising finding in this work is the ease with which DNA samples can be prepared and handled that contain useful representations of very large, intact molecules. It is obvious from our gel photographs that, as would be expected, there has been substantial loss of the largest molecules since much of the DNA is present in a heterogeneous smear in the 100-500 kb range and the largest bands have progressively less intensity rather than the progressively greater intensity that would be predicted if all the molecules were present in equimolar amounts. Nonetheless, it is also clear that there is a sufficient representation of intact molecules in the largest bands to make samples of this kind suitable for many types of experiment. The ease of analyzing the overall pattern is facilitated by the fact that the background of partially degraded molecules is centered rather far down the gel in a region where the bands are intense and easily visualized. A partial explanation for the

relatively low background in the upper region of the gel may lie in the longstanding observation that when a linear DNA molecule is broken by mechanical shear, it tends to break near the middle, producing two fragments that are much smaller than the parental molecule (18).

In discussing our experience with this method, we have deliberately emphasized experimental rather than theoretical issues. This choice stems from our opinion that, although it is not difficult to develop a heuristic model for the physical basis of this fractionation technique, the method currently rests on wholly empirical foundations. For that reason, we can make no claim that our current implementation is remotely optimal. Nonetheless, the method already works well enough to allow many previously unapproachable problems to be attacked. Furthermore, given the large number of relevant, interacting variables--voltage, temperature, field geometry, switching regime, matrix composition, and sample handling procedure--we expect that knowledge of the optimal way to carry out separations of large DNA molecules will only expand rapidly when variations on this technique come into widespread use. To facilitate the cross-referencing of future literature in this area we suggest orthogonal-field-alternation gel electrophoresis as a generic name for techniques that feature alternate applications of two approximately orthogonal fields. This name provides a self-explanatory description of the apparatus, is not biased towards any particular theory concerning the mechanism of fractionation, and lends itself to the manageable acronym OFAGE.

We have used the chromosome-sized DNA molecules of yeast as the test system for developing our OFAGE protocol because it provides a convenient source of ready-made molecules in a size range where the technique already performs well. Nonetheless, it would appear that there are no insuperable obstacles to using this method to expand greatly the sizes of the DNA molecules in higher organisms that can be directly analyzed. Particularly when coupled to rapidly improving techniques for fragmenting immense DNA molecules into pieces that are in the size range of yeast chromosomes (22), the OFAGE technique offers great potential for bridging the gap between the DNA molecules of plasmids and phages--which are the focus of current recombinant DNA technology--and the much larger molecules that store the vast majority of genetic information present within the cell.

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