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Separation of random fragments of DNA according to properties of their sequences

(denaturing gradient gel/electrophoresis/melting/bacteriophage λ DNA)

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ABSTRACT **The separation of DNA fragments by electrophoresis at high temperature in a denaturing gradient is independent of the length of the fragments. We have suggested that the basis of fragment separation is that each DNA molecule undergoes partial melting as it encounters a concentration of denaturants sufficient to melt its least stable sequence, while other sequences remain double stranded; in the partially melted configuration, DNA can continue migration only slowly. This model is consistent with the observation that fragments of λ phage DNA cleaved by different restriction endonucleases reach the same final depth in the gel if they contain the same least-stable sequence. A unique set of bands is produced from the electrophoresis of randomly fragmented DNA; this would be expected if there were a limited number of melting centers occupying discrete genetic loci. An intact DNA molecule penetrates about as deeply into the gel as the uppermost band after fragmentation; this would be expected only if the least-stable sequence controls the final depth of the whole molecule.**

We have shown (1) that electrophoretic migration of a restriction endonuclease fragment of bacterial or viral DNA into a denaturing gradient in polyacrylamide gels at 60°C is abruptly retarded when the fragment encounters a particular concentration of the denaturant. The denaturant concentration required for the retardation of each fragment of the genome is characteristic for that fragment and unrelated to its length. Because of the sharp reduction in mobility at some level for each fragment, the gel patterns hardly change once all fragments are retarded. Separation by this process, perpendicular to the direction in which the fragments have first been sorted by length, results in a two-dimensional display that is capable of resolving more than 350 fragments produced from the *Escherichia coli* genome by *Eco*RI. The combined effects of denaturant concentration and of temperature on mobility suggest that retardation is attributable to a structural change in the fragments from a double helix to the unstacked, or melted, configuration.

It does not appear that melting of the entire fragment is required for the abrupt drop in mobility for the following reasons. (*i*) The mobilities of completely separated, unstacked strands, inferred from published data and our measurements, are substantially larger than those we observe after retardation. (*ii*) The interval in denaturant concentrations between which any fragment undergoes its transition from high to low mobility is much smaller than the difference between the transition concentrations of any pair of fragments. Because *Eco*RI fragments are so large that they contain substantial sequence heterogeneity, there will be a large denaturant-concentration difference between the beginning and the completion of melting. (*iii*) The denaturant concentration at which whole λ DNA is retarded is nearly the same as that for its most easily retarded *Eco*RI fragment which suggests that whatever happens to that fragment is sufficient to retard uncleaved λ DNA. (*iv*) The appearance of more than one distinct mobility subtransition for some fragments suggests that the first retardation did not require a change in the entire molecule. These observations can be accounted for by assuming that the melting of a substantial section of any fragment or molecule is sufficient to effect a large reduction in mobility. To challenge this hypothesis, we have carried out a series of experiments comparing retardation after λ DNA is fragmented in different ways, both by digestion with restriction endonucleases and by shearing.

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MATERIALS AND METHODS

Restriction Enzyme Digestion. Wild-type phage λ DNA, prepared by repeated phenol extraction of CsCl-purified phage, was digested completely at a concentration of 30 μg/ml with *Eco*RI (New England BioLabs) or *Sma* I (Boehringer Mannheim) as specified by the suppliers. The reactions were stopped with EDTA (40 mM final concentration), and then equal volumes of the two digests were combined. Before loading onto gels, the digests were made 1.5 M in urea and 8% (vol/vol) in glycerol, and a trace of bromophenol blue was added.

Fragment Preparation. The *Eco*RI fragments of λ DNA were prepared by electroelution from a conventional preparative (40 mg/ml) polyacrylamide gel. The strips of gel containing each fragment were finely diced with a razor and loaded over glass wool into a glass tube to which a cellophane dialysis bag was tied at the bottom. Transfer was essentially complete in 20 hr with a field of 5 V/cm.

Sonication. To produce 1- to 3-kilobase fragments, 40 μg of DNA in 1 ml of Tris acetate buffer was sonicated continuously for 90 sec at output level 9 in a Branson cup horn. The size distribution was estimated by comparison in agarose gels with a *Hae* III digest of φX174 DNA.

Denaturing Gradient Gels. Denaturing gradient electrophoresis was carried out in 17 × 17 cm slab gels (2.5 mm thick) containing 40 mg of acrylamide and 1.04 mg of methylene bisacrylamide per ml of buffer (0.04 M Tris/0.02 M NaAcO/1 mM EDTA, pH 8 with acetic acid), and a linear gradient of a urea/formamide mixture that increased in the direction of DNA migration. Polymerization was initiated with ammonium persulfate (0.1 mg/ml) and 0.01% (vol/vol) *N,N,N′,N′*-tetramethylethylenediamine. The gradient was formed by mixing two similar acrylamide solutions, one of which contained 5.6 M urea and 32% (vol/vol) formamide. A linearly varying mixture was delivered by two digitally controlled syringe pumps which protected the outgassed solutions from contact with air except at the top meniscus of the slab. For two-dimensional gels, the DNA sample was first sorted by length on an agarose slab gel (10 mg/ml; 2.5 mm thick). The agarose strip was then sealed across the top of the gradient gel with hot agarose (10 mg/ml of buffer) and allowed to set 1 hr.

The gels were maintained at 60°C during electrophoresis by immersion in a well-stirred thermostated bath containing the anode. Both plates containing the slab were in contact with the flowing bath electrolyte; the solution was continuously circulated between the anode and cathode compartments. After electrophoresis for 2 hr, staining in a large volume of water containing ethidium bromide (1 μg/ml) removed most of the denaturant. The stained gels were photographed on 4 × 5 inch Kodak SO115 technical panchromatic film through a combined 650-nm short-pass interference filter and a Corning 3-67 long-pass glass filter with filtered 254-nm illumination. Agarose gels for two-dimensional separation were visualized with 365-nm illumination. Additional details are presented elsewhere (1, 2).

Virtually all of the fragments produced by sonication in this work reached their retardation depths in 10–12 hr, but large restriction fragments from λ DNA may require 18 hr.

Determination of DNA Distribution. The distribution of DNA along each channel in the gel shown in Fig. 2 was calculated from a two-dimensional densitometric scan of the negative. The scan, carried out by Perkin–Elmer using a model 1010 microdensitometer, provided an array of 680 × 777 data points, each representing a 100-μm square. Each point was converted to a relative DNA amount, assuming linearity between DNA and the intensity of fluorescence, by a relationship to film density similar to that used by Pulleyblank *et al.* (3). The DNA values were summed perpendicular to the axis of each channel and the distances in the channels were brought into equivalence by a slight length adjustment to match the λ DNA pattern at the left to the λ DNA channel at the right.

RESULTS

Restriction Endonuclease Fragments with Common Sequences. The hypothesis specifies that the point of retardation of any fragment in a denaturing gradient is determined by the properties of the most easily melted sequence in the fragment. Two fragments sharing the same most easily melted sequence would be expected to stop at the same gradient depth despite differences in segments flanking that sequence. The fragments prepared from λ DNA by digestion with two restriction endonucleases, *Eco*RI and *Sma* I, were compared. The known sites of cleavage by both enzymes are indicated in Fig. 1 *Upper*. Note that fragment *Sma* I A is entirely contained within *Eco*RI A. Thus, *Sma* I A can have no sequence more readily melted than does *Eco*RI A, and it is expected to move at least as deeply into the gel as *Eco*RI A before stopping. Of the *Eco*RI fragments, *Eco*RI E stopped nearest the top of the gel and must contain the most easily melted sequence in the λ genome. Because *Eco*RI E is entirely contained within *Sma* I B, the *Sma* fragment must also contain the same most easily melted sequence. Therefore, it, too, should migrate to the same depth near the top of the gel where denaturant concentration is least. Fragments *Sma* I D and *Eco*RI B span nearly the same region of the genome, and unless the mobility-controlling sequence is outside the region in which they overlap, they should attain the same final depth in the gel. Fragment *Sma* I C overlaps *Eco*RI F and most of *Eco*RI C. Because *Eco*RI F stops higher in the gel than does *Eco*RI C, the contribution of sequences in the *Eco*RI C region should not affect the final position of *Sma* I C, which therefore should migrate to the same final position as *Eco*RI F.

Fig. 1 *Lower* shows that the positions of all the *Sma* I fragments were as expected. To identify the fragments in the denaturing gradient gel with respect to the genetic map, *Eco*RI and *Sma* I digests of λ DNA were combined and sorted by length on an agarose gel. The identities of the fragments in

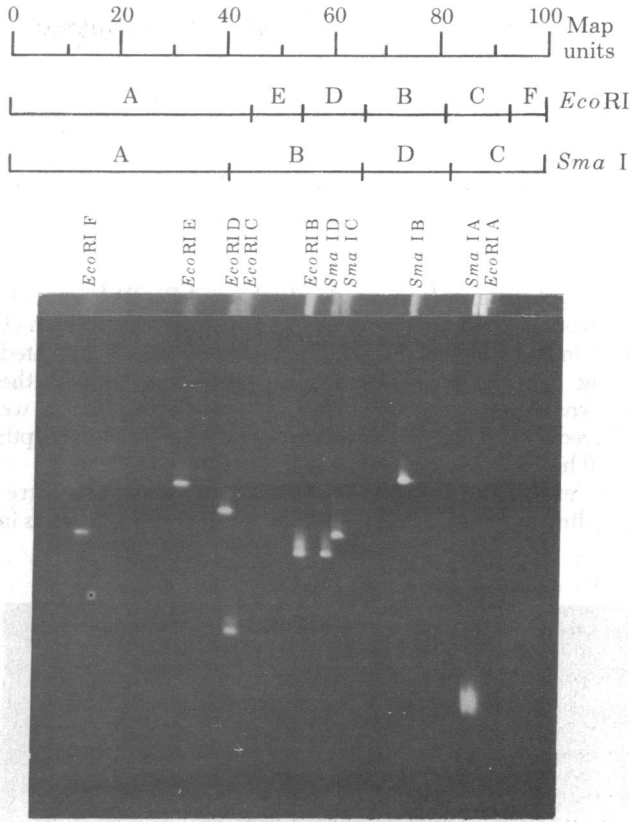

FIG. 1. (*Upper*) *Eco*RI and *Sma* I restriction fragment maps of λ DNA. Fragments are lettered from the left end of the λ genome. The full length of the λ genome, 100 map units, is 49,502 base pairs. Lengths of restriction fragments in base pairs are: *Eco*RI A, 21,938; *Eco*RI B, 7536; *Eco*RI C, 5902; *Eco*RI D, 5681; *Eco*RI E, 4845; *Eco*RI F, 3600; *Sma* I A, 20,077; *Sma* I B, 12,258; *Sma* I C, 8785; *Sma* I D, 8821. (*Lower*) Two-dimensional gel of mixed *Eco*RI and *Sma* I digests of λ DNA. One microgram of λ DNA digested with *Eco*RI was mixed with 1 μg of an *Sma* I digest of λ DNA and allowed to migrate during 20-hr electrophoresis at 2 V/cm in one channel of an agarose (10 mg/ml) slab gel. The ethidium-stained agarose strip was sealed across the top of an acrylamide (40 mg/ml) slab gel containing a linear gradient of denaturant increasing from zero at the top to 5.6 M urea/32% (vol/vol) formamide at the bottom. Electrophoresis was run at 60°C for 20 hr at 9 V/cm; the gel was stained with ethidium bromide. The direction of electrophoresis was from top to bottom. A photograph of the stained agarose strip with the starting positions of the restriction fragments after their initial separation by length is included as a reference above the two-dimensional gel.

decreasing order of molecular weight from right to left are shown above the photograph of the ethidium-stained agarose gel. The agarose gel was then placed across the top of a denaturing gradient gel and the fragments were electrophoresed downward. The following pairs of fragments moved to the same depth, as expected: *Eco*RI A and *Sma* I A, *Eco*RI E and *Sma* I B, *Eco*RI B and *Sma* I D, and *Eco*RI F and *Sma* I C. The model thus allowed us to understand the retardation properties of one set of restriction endonuclease fragments in terms of the behavior of an overlapping set of fragments.

Random Fragments. Equilibrium melting of λ DNA as a function of increasing temperature appears to proceed in a large number of more or less discrete steps corresponding to the cooperative melting in the λ molecule of 34 domains with lengths of 620–3230 base pairs (4). Random shearing of λ DNA to an average length of roughly 2000 base pairs will produce a distribution of fragment sizes with widely varying end points. However, if the depth of retardation in the denaturing gradient is determined by the most easily melted sequence in each

fragment, all fragments carrying the lowest melting-temperature domain will accumulate at the same depth; all that carry the next higher melting-temperature domain (but not the lowest) will accumulate somewhat more deeply, etc. Thus, the distribution of DNA in the gel will form a series of bands determined by the progression of domains. Similarly, random shearing of individual restriction endonuclease fragments would be expected to give a gel distribution corresponding to a subset of the bands given by the random fragments of whole λ DNA.

The gel patterns from randomly sheared *Eco*RI fragments and from λ DNA are shown in Fig. 2, and the distribution of DNA in each channel, inferred from the negative, is presented in Fig. 3. Since there were no clear differences between the patterns of gels subjected to 10- and 14-hr electrophoresis, we inferred that all fragments had reached their retardation depth by 10 hr.

By examining both the original negatives and the corresponding plots of DNA distribution, we discerned 25 bands in

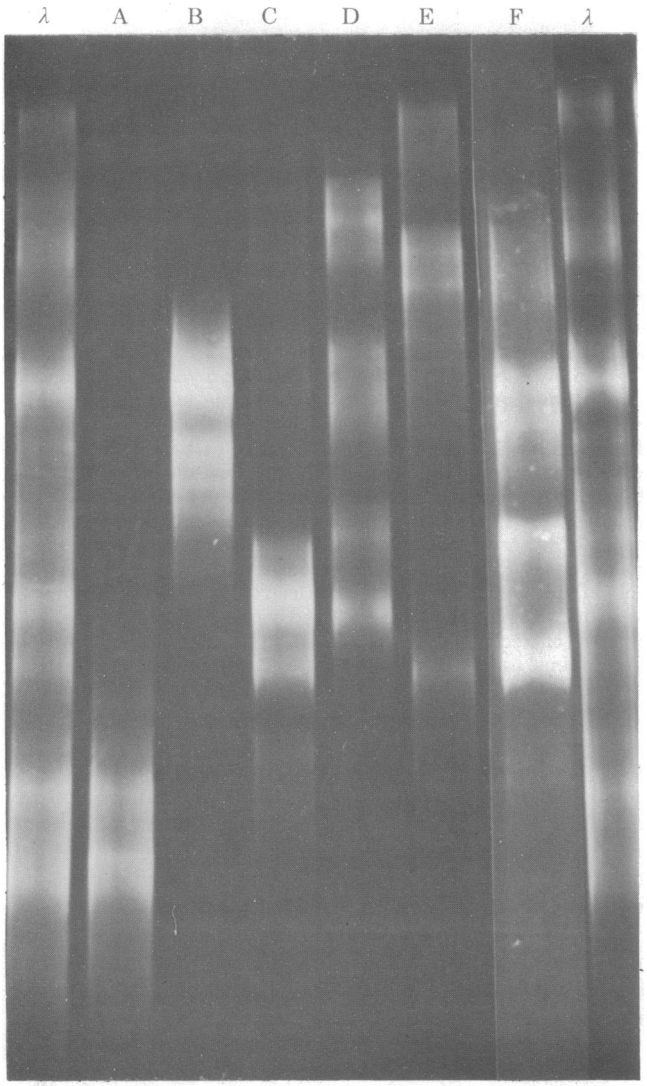

FIG. 2. Banding patterns of sonicates of λ DNA and *Eco*RI fragments in denaturing gradient gel electrophoresis. Four micrograms of λ DNA and 1–1.4 μg of *Eco*RI fragments A–F, sonicated to 1–3 kilobases, were electrophoresed at 60°C for 12 hr at 9 V/cm in an acrylamide gel (40 mg/ml) containing a gradient of denaturant that linearly increased from 1.4 M urea/8% (vol/vol) formamide at the top to 5.6 M urea/32% (vol/vol) formamide at the bottom. The gel was stained with ethidium bromide.

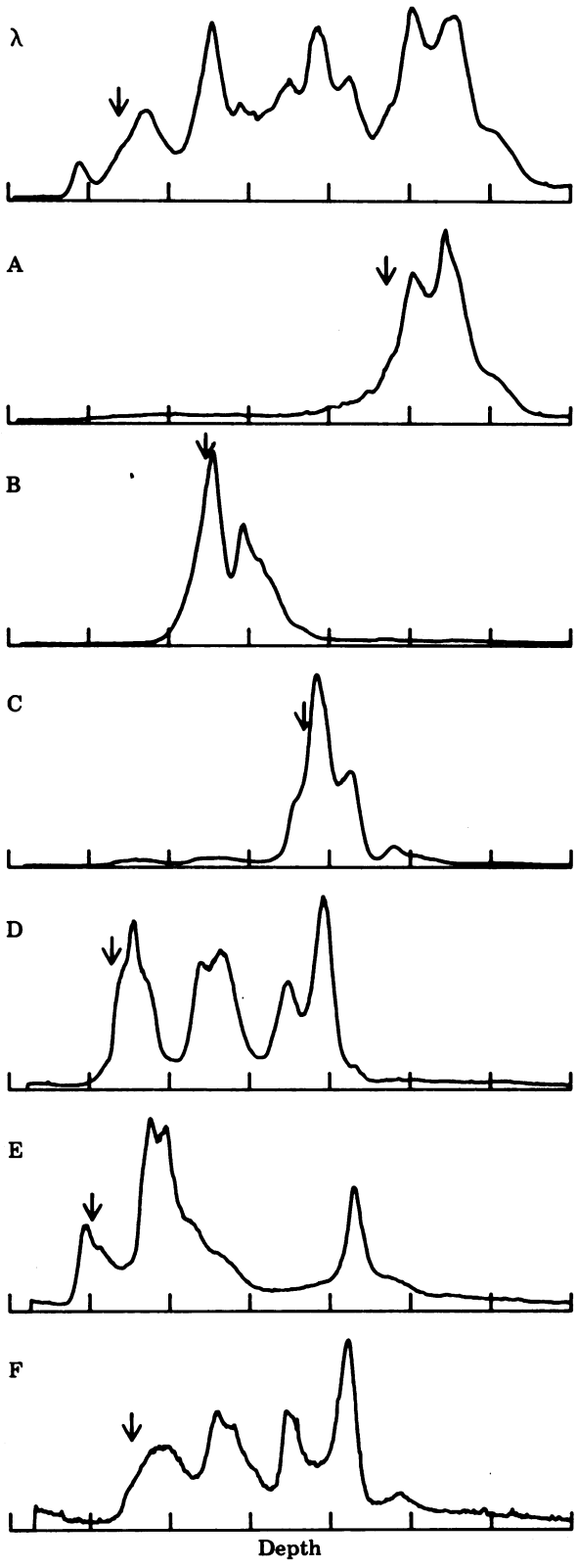

FIG. 3. The distribution of DNA along each channel of the gel. The amount of DNA computed from the photograph shown in Fig. 2 is plotted as a function of distance into the gradient. The scale of each ordinate is adjusted to give an arbitrary uniform maximum. The arrows indicate the relative position of intact *Eco*RI fragments (A–F) when allowed to migrate in a channel adjacent to the sonicate. The curves shown are CalComp plots made directly from the transformed data.

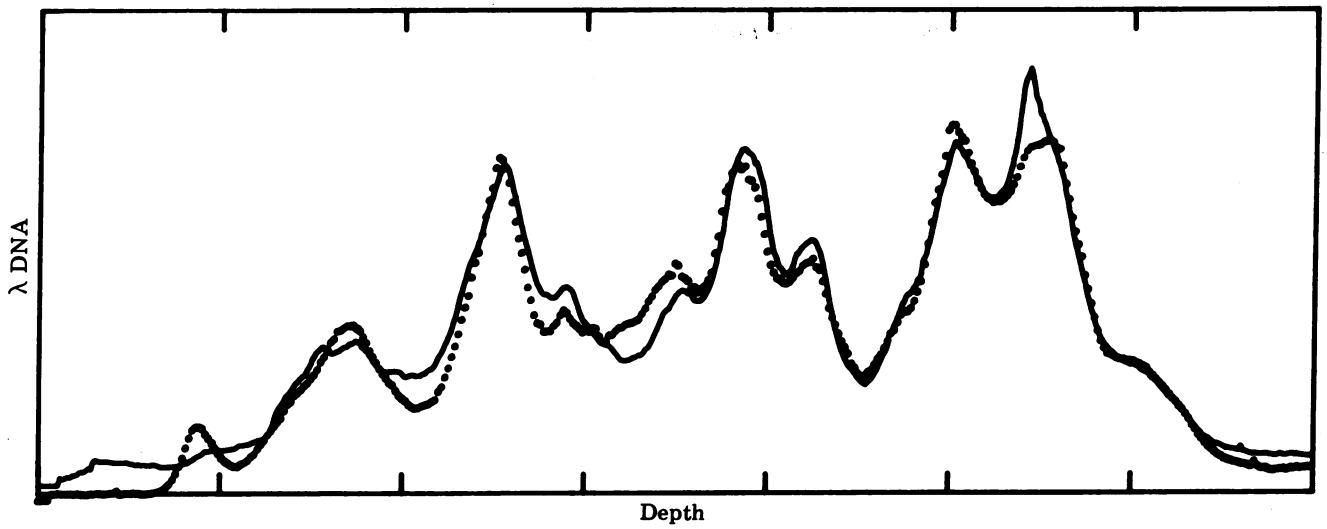

FIG. 4. Comparison of the sonicated λ DNA distribution to a summation of sonicated *Eco*RI fragment distributions. ·····; λ DNA distribution shown in Fig. 3; —, sum of the *Eco*RI patterns A–F in Fig. 3, weighted to give the cumulative least squares deviation from the λ DNA pattern.

the channel containing sonicated whole λ DNA. Many of these were evident in the plot as shoulders on some peaks (Fig. 3). Fewer bands were seen in the channels containing each of the sonicated *Eco*RI fragments; some clearly corresponded to bands in the undigested λ DNA. For example, the uppermost band in the λ DNA channel occurred at the same depth in the gel as did the uppermost band in the channel containing sonicated *Eco*RI fragment E. In addition, the deeply penetrating bands in λ DNA and in the sonicated G+C-rich *Eco*RI fragment A clearly aligned. However, the most deeply penetrating bands from *Eco*RI fragments E and F appeared at about the same depth into the gel. In such cases the corresponding band at that depth in the sonicated λ DNA channel was a composite of both fragment bands.

A pool of all of the DNA produced by sonication of the *Eco*RI fragments is not identical with the sonicate of whole λ DNA; in the former there will be no segments that span the *Eco*RI cleavage sites, and there will be a large number of segments that terminate at the *Eco*RI sites. Because a domain near a terminus may melt more readily than the same sequence bounded at both ends, some bands produced by whole λ DNA might be expected to shift upward or to be lost in the patterns from the *Eco*RI-fragment sonicates. The distribution of DNA in the whole λ DNA sonicate (Fig. 3, λ) was compared with a sum of the sonicated *Eco*RI-fragment distributions (Fig. 3, A–F) in Fig. 4. The close correspondence between the patterns indicated that the most prominent bands were undisturbed by *Eco*RI cleavage, that the fragment sizes among the various sonicates were reasonably similar, and that the transformation of photographic densities into DNA concentration was not grossly in error.

Correlation Between Final Depth of a DNA Molecule and Its Uppermost Band After Random Shearing. A close comparison of the retardation points of the intact and sonicated samples was provided by a gel in which a sample containing λ DNA and an *Eco*RI digest of λ DNA was adjacent to each of the sheared samples. The arrows in Fig. 3 denote the final depth of each of the unsheared DNA molecules in comparison to the DNA distribution of the fragments after random shearing. Unsonicated *Eco*RI fragments traveled only slightly deeper than their uppermost band after shearing, whereas intact λ DNA migrated about 5% deeper into the gel than did its uppermost band after shearing. The slightly greater penetration of unsheared λ DNA indicated that its most easily melted sequence is more stable in the intact molecule than in the sheared fragments containing it.

DISCUSSION

Whereas we have no direct evidence that melting is the basis of mobility retardation, local melting of λ DNA at temperature and denaturant conditions similar to those in the gels has been demonstrated by electron microscopy (5, 6). The order of *Eco*RI fragments in the gel correlates with the order in which denaturation bubbles appear—first in the region of the E fragments, next in the regions of the B, D, and F fragments (in undetermined order), and last in the A fragment region, after most of the right half of the molecule is melted. Correspondingly, only a minor amount of the sonicated pieces of the *Eco*RI fragments from the right half of λ DNA move as deeply into the gradient as does the DNA from *Eco*RI A (Figs. 2 and 3). The identification of the early retardation of fragment *Eco*RI E with the first hyperchromic subtransition is also in agreement with the disappearance of that subtransition in a deletion mutant in which the E region is absent (7–11).

The distribution of DNA in the denaturing gradient after all fragments have undergone their retarding transition will depend on fragment size. If the fragments are smaller than the domains that correspond to hyperchromic melting subtransitions, then complete separation of the strands and their rapid migration out of the gel might be expected, instead of retardation. If the fragments are long enough to contain more than one domain, they will be retarded at the level of the domain that is most easily melted, and there will be less or no representation of DNA at the level corresponding to the melting of the more resistant domain. The spread in concentration of urea/formamide between the first and last peak in the λ DNA channel of Fig. 3 corresponds to a temperature interval of about 16°C according to the equivalences proposed by Hutton (12)—about the same as the temperature interval between the first and last thermal hyperchromic subtransitions (3). Also, the average densities at the top and bottom of the DNA distribution are similar to the distribution of hyperchromicity in the thermal pattern. We infer therefore that the average fragment size used

in these experiments is not very far above the length sufficient to contain one domain.

Close comparison of the retardation positions of intact *Eco*RI fragments with the distribution of pieces in the sonicates is somewhat difficult because of the very large size differences. The small molecules in the sonicates reach retardation positions more quickly and continue slow migration while the *Eco*RI fragments approach retardation. Nevertheless the positions of the *Eco*RI fragments, after the same electrophoresis time as with the sonicates (Fig. 3) and after much longer time (20-hr electrophoresis; figure 6 of ref. 1), correlate unambiguously with the region of the first peak in the sonicates. Since intact *Eco*RI fragments are retarded at a depth in the gradient at which some or most of their constituent sequences are not yet melted, as judged by the deeper retardation of some of their sonicated subfragments, we infer that partial melting is sufficient to effect retardation.

If the denaturant concentration capable of retarding an intact *Eco*RI fragment can be identified with the concentration sufficient to melt a definable part of the molecule [which might be either a particular fraction of the total length (or a particular number of nucleotides) or some fraction of melting of the most sensitive domain], then a uniform criterion should apply to all fragments. We have compared the depth of retardation for each of the intact *Eco*RI fragments after 20-hr electrophoresis (ref. 1, figure 6) with the points on the distribution of sheared fragments (Fig. 3) corresponding to each of these hypotheses. If the entire distribution is identified with the number of nucleotides in the intact fragment, the integrals of the electrophoretic distributions in Fig. 3, starting from the left, permit calculations of the depths at which a certain number of nucleotides have accumulated. The closest correspondence to a linear relationship, correlation coefficient of 0.996, is between the depth of retardation of the intact fragment and the depth for the accumulation of 500 nucleotides. The correlation suggests that the retardation of *Eco*RI fragments is effected by melting of less than one-fifth of the smallest fragment.

The anomalous retardation of *Eco*RI E earlier than λ DNA is understandable if the domain controlling the retardation of *Eco*RI E lies near the *Eco*RI cleavage site and if melting proceeds from the cut end of the fragment rather than formation of an interior loop, as in intact λ DNA. The melting temperature of that domain in the fragment will then be decreased.

Denaturing gradient gel electrophoresis already has been applied in two-dimensional separation of restriction endonuclease fragments (1). Understanding which properties of the fragments determine their depth in the gradient should expand the usefulness of the gels. For example, the *Sma* I cleavage map of λ DNA could have been predicted by comparing the coordinates of the *Sma* I and *Eco*RI fragments in Fig. 1. In addition, fragments produced by partial restriction endonuclease digestion can be identified with at least one of their completely digested products by their agreement in retardation depth. The electrophoresis of randomly sheared DNA may be useful in the analysis of complex DNA. Fragments which may differ in base composition but share least-stable sequences will accumulate at the same gel depth. Thus, for those genomic functions that are characterized by their thermal stability, fragments containing these sequences may be enriched at a characteristic depth.

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