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Mapping of closed circular DNAs by cleavage with restriction endonucleases and calibration by agarose gel electrophoresis

(ethidium bromide/DNA of simian virus 40/DNA of bacteriophage PM2)

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ABSTRACT **The cleavage of DNA by restriction endonucleases can be limited by the addition of ethidium bromide. When closed circular DNA is used as a substrate, DNA with one-site cleavages of one or both strands can be made by adding appropriate amounts of dye.**

The singly cleaved DNA is a complete set of full-length permuted linear molecules. Fractionation of the products of a digestion of the permuted linears with a single-hitting restriction endonuclease by gel electrophoresis yields a series of bands that can be used to determine relative molecular weights of the DNA fragments in the gel without the introduction of standards.

It is possible to determine the relative molecular weight of a fragment to within ±2.5%. These molecular weights immediately allow the determination of the *Hin*dIII and *Hpa* I maps of simian virus 40. The *Hin*dIII map of bacteriophage PM2 was determined by this method with one ambiguity that was resolved by using traditional techniques.

Restriction endonucleases are useful for the analysis of the structure of DNA. For a review of the techniques developed for ordering the DNA fragments that result from a DNA/restriction endonuclease digest, see the paper by Nathans and Smith (1). Ideally, a restriction endonuclease map can be determined by knowing the molecular weights of the complete digest products and of many of the partial digest products. At present, it is impossible to determine molecular weights with enough accuracy to reduce mapping to a simple numerical problem.

Slab gel electrophoresis is a powerful tool for the separation of DNA molecules of different molecular weights. Much work has been done in an attempt to describe the electrophoretic mobility of a linear DNA as a function of its molecular weight (2, 3).

Molecular weights have been determined by electron microscopy (4) and radioactivity (4, 5), and by use of synthetic molecules (2). The relationship has been described as electrophoretic mobility being a linear function of the logarithm of the molecular weight. We believe that this analysis is useful in some cases but does not adequately describe the relationship, which is more accurately approximated by a third-order exponential function.

We have developed sets of DNA molecules to study this relationship. They have known molecular weight relationships that are not dependent upon measurement by other techniques. Their inherent relationships also allow for rapid restriction endonuclease mapping of closed circuit DNAs.

Abbreviations: EtdBr, ethidium bromide; Form I DNA, covalently closed circular DNA; Form II DNA, noncovalently closed circular DNA; Form III DNA, linear DNA; *Eco*RI, *Escherichia coli* restriction endonuclease RI; *Hin*dIII *Haemophilus influenzae*, strain d, restriction endonuclease III; *Hpa* I and *Hpa* II, *Haemophilus parainfluenzae* restriction endonucleases I and II; SV40, simian virus 40.

‡ *Deceased.*

MATERIALS AND METHODS

Enzymes and DNA. *Eco*RI endonuclease was a gift from H. Boyer. Other restriction endonucleases were purchased from New England Biolabs. Bacteriophage PM2 DNA was prepared according to Espejo *et al.* (6). Bacteriophage λ DNA was a gift from B. Seed. Simian virus 40 (SV40) DNA was a gift from J. Jordan and H. Kasamatsu and was later prepared as described (7). A unit of enzyme will degrade 1 μg of λ DNA to completion in 60 min at 37° in a volume of 50 μl.

Electrophoresis. A modification of the Aquebogue vertical slab gel electrophoresis apparatus was used, allowing long gels (30 cm) or short gels (15 cm) to be run. A fan was added to cool the gel during the run; tapered combs improved the sample wells. This equipment is available from EPT, Pasadena, Calif.

Agarose (Sea Kem) gels were prepared according to Helling *et al.* (3). They were run at a constant voltage of 3.3 V/cm. The DNA was recovered from preparative gels by the freeze–thaw technique described by Pulleyblank *et al.* (8), who also described conditions for photographing the gels. Electrophoretic mobilities were measured from the photographs.

Restriction Endonuclease Digests. Reactions were carried out as previously reported: *Eco*RI (9), *Hin*dIII (4), *Hpa* I and *Hpa* II (5). Reaction temperature, amounts of enzyme and DNA, and concentration of ethidium bromide (EtdBr) varied according to the experiment.

Dialysis. In sequential enzyme reactions the first enzyme was inactivated by the addition of 0.1 volume of 10% sodium dodecyl sulfate. EtdBr was extracted with 1% Sarkosyl in water-saturated butanol. The DNA was dialyzed by a modification of the centrifugation procedure described by Neal and Florini (10). Volumes of 100–500 μl were dialyzed through an SW 50.1 tube containing P-2 gel beads, 100–200 mesh (Bio-Rad) equilibrated in the desired buffer.

RESULTS

In the absence of ethidium bromide, incubation of six units of restriction enzyme with 1 μg of closed-circular DNA overnight at 37° gives a complete digest of the DNA. The presence of ethidium bromide inhibits the reaction. By varying the amount of EtdBr in the reaction it is possible to make many partial digest products, or a mixture that is predominantly open circles (Form II) and full length linear molecules (Form III), or mainly Form II alone.

A cursory examination of temperature effects for *Hin*dIII indicated that 55° gave maximal activity and highest conversion to Form III. At 4° about half of the DNA was converted to Form II; most of the rest remained as Form I during a 16-hr incubation. It was possible to convert greater than 90% of the DNA to Form II at 20°.

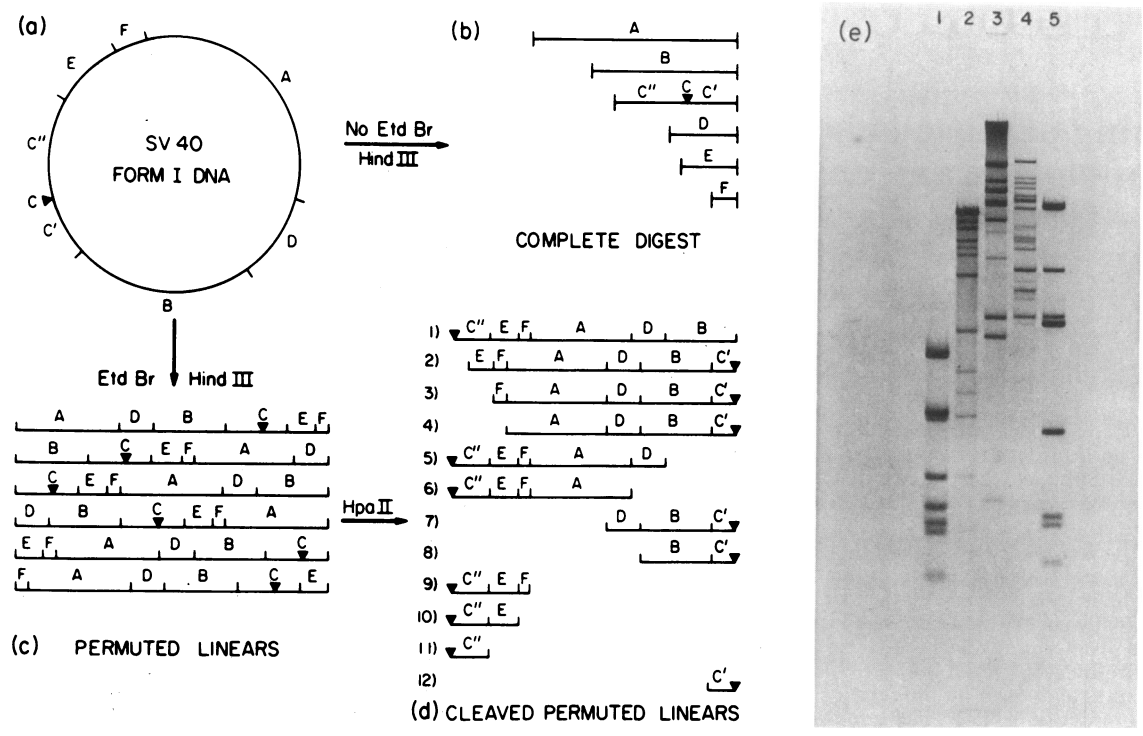

FIG. 1. (a) *Hin*dIII (lines) and *Hpa* II (triangle) restriction enzyme sites on SV40 DNA. (b) Complete digest products from a *Hin*dIII digestion of SV40 DNA. (c) Permuted linears from an EtdBr-limited *Hin*dIII digestion of SV40 DNA. (d) The permuted linears after digestion with *Hpa* II. The 12 fragments are ordered, as they would be resolved in a gel, from largest to smallest. Note that fragment 12 differs from fragment 8 by complete digest product B; similar reasoning yields the map that appears in (a). (e) Gel photograph; migration is from top to bottom. Slot 1: SV40/*Hin*dIII and SV40/*Hin*dIII/*Hpa* II complete digests. Slot 2: SV40/(EtdBr) *Hin*dIII after cleavage of permuted linears with *Hpa* II—the slowest migrating band is SV40 Form III. Slot 3: λ/*Eco*RI and λ/*Hin*dIII complete digests. Slot 4: PM2/(EtdBr) *Hin*dIII after cleavage of permuted linears with *Hpa* II—the slowest migrating band is PM2 Form III. Slot 5: PM2/*Hin*dIII and PM2/*Hin*dIII/*Hpa* II complete digests.

In studying other enzymes we found different temperature optimums. To maximize yields it will be necessary to study each enzyme's activity as a function of temperature. Maximum yields were not a necessity for our work and 37° incubations produced enough Form III DNA. It is possible in the systems we studied to find conditions that convert greater than 50% of the DNA to Form III; these systems utilized *Hin*dIII with PM2 or SV40 DNA and *Hpa* I with SV40 DNA.

Optimum ethidium bromide concentrations also varied in each case. At higher temperatures more EtdBr is needed to achieve the same effect. For PM2 and *Hin*dIII at 55°, 7.8 μg of EtdBr are needed per μg of DNA with 4.7 units of enzyme. For SV40 and *Hin*dIII the corresponding numbers for 1 μg of DNA are 55°, 18.4 μg of EtdBr, 5.5 units of *Hin*dIII. In the SV40–*Hpa* I system 1 μg of DNA was reacted with 20 μg of EtdBr and 4.55 units of *Hpa* I at 37°. All incubations were for at least 8 hr.

Increasing the level of EtdBr lowered the number of molecules receiving more than one cut, as did decreasing the amount of enzyme or DNA in the system. Pre-incubation of the enzyme with EtdBr had no effect on the results.

The Form II DNA was analyzed in an alkaline CsCl velocity experiment (11). It was found to be singly nicked (have one single-strand break). The Form III results from cleavage at any one of the possible restriction endonuclease sites. The sites are cleaved with differing frequencies. Each site is recognized often enough so that a complete set of full length permuted linear molecules is formed (Fig. 1).

The permuted linears upon cleavage with a single-hitting restriction endonuclease make it possible to determine the relationship between molecular weight and electrophoretic mobility, independent of other techniques, assuming that the migration of linear DNA in a constant concentration gel is a smooth function of the molecular weight of the DNA. They also simplify the problem of restriction enzyme mapping.

In an EtdBr-limited digest *Hin*dIII yields seven permuted linears with PM2 DNA and six permuted linears with SV40 DNA; *Hpa* I gives four permuted linears of SV40 DNA. Upon cleavage with a single-hitting enzyme in the absence of EtdBr these systems have 14, 12, and 8 bands. The sets of 14 and 12 bands can be resolved in a 1% agarose slab gel. Only six of the eight bands (12) formed in the SV40/*Hpa* I system can be resolved in 1% agarose because of the closeness of two of the *Hpa* I sites.

For any one digest, the resolved electrophoretic bands can be analyzed in pairs. The largest DNA fragment is the slowest migrating band; when paired with the smallest DNA fragment, which is the fastest migrating band in the slot, the result is a full-length molecule. The next to the fastest can be paired to make a full-length molecule with the next to the slowest, etc. We therefore have n equations,

$$MW_i + MW_{(2n+1-i)} = MW_{\text{Form III}} \quad (i = 1, 2, \ldots, n) \qquad [1]$$

where $i = 1$ for the slowest band, etc., MW_i is the molecular weight of band i, and n is the number of restriction endonuclease sites.

We explored various functions in an attempt to relate the molecular weight to mobility. We finally adopted the general form:

$$MW_i = \exp(a_0 + a_1 x_i + a_2 x_i^2 + a_3 x_i^3) = f(x_i) \quad (i = 1, 2, \ldots, n) \qquad [2]$$

in which x_i is the distance migrated by band i and the function

Table 1. Molecular weights of restriction endonuclease products from bacteriophage λ

	Thomas	Wellauer	Our values
*Hin*dIII B	—	5.84	5.97
*Eco*RI B	4.74	—	4.79
*Hin*dIII C	—	4.05	4.22
*Eco*RI C	3.73	—	3.73
*Eco*RI D	3.48	—	3.59
*Eco*RI E	3.02	—	3.07
*Hin*dIII D	—	2.67	2.73
*Eco*RI F	2.13	—	2.18
*Hin*dIII E	—	1.40	1.47

The measurements by Thomas and Davis (9) were done by electron microscopy using phage φX174 DNA as a standard. Wellauer *et al.* (13) also used electron microscopy to determine molecular weights with SV40 DNA as a standard. They used 3.28×10^6 as the molecular weight of SV40; we use 3.27×10^6 (3, 5, 13). Our values are averages from five PM2 calibration curves.

$f(x_i)$ is defined by the equation. In addition, there is the relation

$$MW_{\text{Form III}} = \exp(a_0 + a_1 x_{III} + a_2 x_{III}^2 + a_3 x_{III}^3) = f(x_{III}) \quad [3]$$

The above equations lead to the relations

$$f(x_i) + f(x_{(2n+1-i)}) = f(x_{III}) \; (i = 1, 2, \ldots, n) \quad [4]$$

This provides n equations for determining the four coefficients a_0, a_1, a_2, a_3. These coefficients will vary from experiment to experiment as agarose, buffer, voltage, run length, etc. change.

An additional equation is obtained by the following procedure. A complete digest by the enzyme for which there are multiple sites (sample A) is digested by the second single-hitting enzyme to give sample B. The two samples are run in adjacent slots of the gel used for resolving the cleaved permuted linears and measuring x_i above. One band (p) from sample A will not be present in sample B; instead, there will be two bands, p′ and p″, where p″ is the same as band $2n$ of Eqs. 1 and 2. Fragment p contains the site for the second enzyme. These observations lead to the equation,

$$f(x_p) = f(x_{p'}) + f(x_{p''}) \quad [5]$$

The $(n + 2)$ Eqs. 3, 4, and 5 (with the normalization that $MW_{III} = 1$) were used to determine a_0, a_1, a_2, and a_3. This over-determined nonlinear system of equations was solved with the aid of a computer program, by a least squares technique. The quality of a set of coefficients was determined by using the coefficients to solve the equation $MW_i = \exp(a_0 + a_1 x_i + a_2 x_i^2 + a_3 x_i^3)$ for each of the cleaved permuted linears. The appropriate pairs were then summed. The deviation of the sum of each pair from 1 was then squared and the squares of the deviations were summed. The program then changed the values for $a_0, \ldots, a_3$ until the sum of the deviations squared was minimized.

If it is assumed that the logarithm of the molecular weight is a linear function of the electrophoretic mobility, the best values for relative molecular weights of the recleaved permuted linears give pairs that sum to 1 ± 0.06. This is an error of 6%. By changing the equation, as described, it is possible to obtain molecular weights that upon summing are within ±1.5%. The gel data from the PM2/*Hin*dIII system were used to solve for the coefficients $a_0, \ldots, a_3$. From the general Eq. 2 it was then possible to determine the molecular weights of λ restriction endonuclease fragments run in a parallel slot in the gel (see Table 1). The calibration curve can also be used to map the cleaved permuted linears.

Table 2. Molecular weights of cleaved permuted linears and complete digest products

PM2/(EtdBr) *Hin*dIII/*Hpa* II	PM2/ *Hin*dIII	SV40/ (EtdBr) *Hpa* I/*Eco*RI	SV40/ *Hpa* I	SV40/ *Hpa* I*
III 6.27	A 3.53	III 3.22	A 1.33	1.353
1 4.95	A′ 1.98	1 2.77	B 1.24	1.261
2 4.65	A″ 1.48	2 2.53	A′ 0.75	0.785
3 4.35	B 1.42	3 2.08	C 0.63	0.656
4 3.99	C 0.61	4 1.20	A″ 0.53	0.572
5 3.80	D 0.265	5 0.75		
6 3.74	E 0.245	6 0.53		
7 3.45	F 0.18			
8 2.86	G —			
9 2.59				
10 2.52				
11 2.34				
12 1.98				
13 1.72				
14 1.48				

All data have been converted to molecular weights $\times 10^{-6}$. We determined from our curves that SV40 = 51.6% of PM2. The Form III values in the table are less than 100% because of the mathematical function used. All fragments smaller than 1.42×10^6 were measured from SV40 calibration curves. The SV40 curves slightly overestimated molecular weights of fragments greater than 50% of SV40 and slightly underestimated smaller fragments. The PM2 data are accurate to within ±2.5%. It is not possible to determine the molecular weights of the smallest *Hin*dIII complete digest products because they are smaller than any of the cleaved permuted linears. Columns 1 and 3 are cleaved permuted linears; 2, 4, and 5 are complete digests.
* These values for the complete digest products are from ref. 5.

Each of the $2n$ cleaved permuted linears has one end of the molecule in common—the site for the second, single-hitting restriction endonuclease (Fig. 1). The other end of these molecules is a restriction site for the first, EtdBr-limited enzyme. All possible permutations are represented. Each molecule differs in size from one of the other molecules by the size of a complete digest product of the DNA with the first enzyme.

By calculating the differences in the relative molecular weights of the cleaved permuted linears and by knowing approximate molecular weights of the complete digest products (these can often be determined from Eq. 2) it is possible to orient the complete digest fragments with respect to the single-hitting enzyme. There may be some ambiguity when some complete digest products are very similar in size. It may be necessary to orient those final few pieces by more traditional methods (14). It was necessary to resolve one ambiguity in the *Hin*dIII map of PM2 by cutting out a PM2/*Hin*dIII partial digest fragment and redigesting it with *Hin*dIII. The map that had already been developed from the cleaved permuted linears clearly indicated which partial digest fragment had to be isolated from a gel and cleaved.

The relative sizes of the cleaved permuted linears and the maps this information leads to are shown in Table 2 and Fig. 2. The data presented in Table 2 are averages from many gels. Each gel contained the cleaved permuted linears, the double enzyme digest, and Form III DNA. For brevity, the SV40/*Hin*dIII data are not presented in Table 2. The map and the fragment sizes that the data imply are shown in Fig. 2.

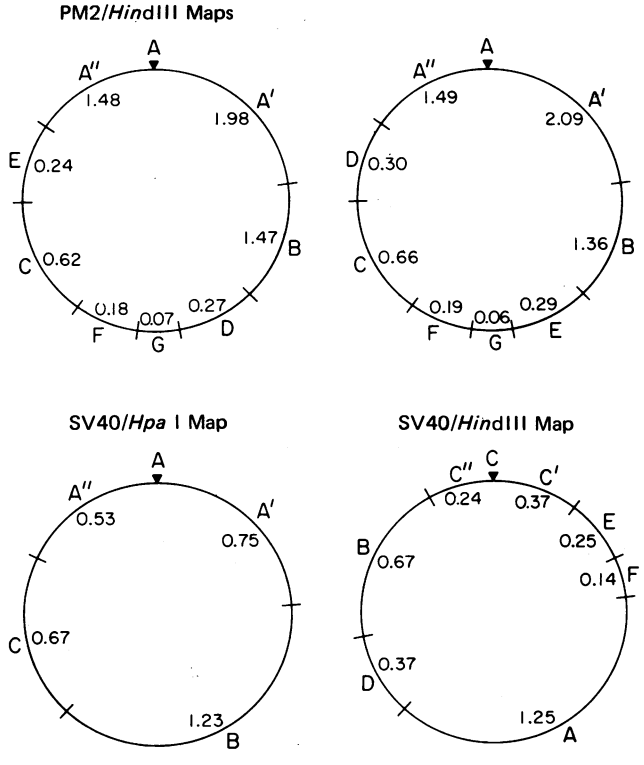

FIG. 2. *Upper left.* PM2/*Hin*dIII map from the information in the sizes of the smaller half of the cleaved permuted linears. *Upper right.* PM2/*Hin*dIII map from the information in the sizes of the larger half of the cleaved permuted linears—note that fragments D and E are reversed compared to the first map. *Lower right.* SV40/*Hin*dIII map from the information in the sizes of the smaller half of the cleaved permuted linears—the information in the larger half gives the same map. *Lower left. SV40/Hpa* I map from the information in the sizes of the smaller half of the cleaved permuted linears—the information in the larger half gives the same map.

In order to map the DNA, the molecular weight data describing the cleaved permuted linears can be analyzed by determining the differences in the molecular weights of either the smaller half of the molecules or the larger half. These differences should be sizes of complete digest products and, in principle, these approaches should give the same map.

Digestion of PM2 DNA with *Hin*dIII gives two pieces (fragments D and E) (Table 2) that are very similar in size. When the smaller half of the cleaved permuted linears is analyzed the resulting map places E, the smaller of D and E, beside the largest fragment in the complete digest, A, and fragment D beside fragment B. The larger half of the cleaved permuted linears indicates that fragment D is connected to fragment A and E is connected to B (Fig. 2).

To clarify the situation we partially digested PM2 with *Hin*dIII and isolated the fragment containing A and its neighbor and the fragment containing B and its neighbor. Upon digestion of the partials with *Hin*dIII the map derived from the larger half of the cleaved permuted linears, which placed D beside A and E beside B, was proven to be correct.

While this manuscript was in preparation Brack *et al.* published a map of the *Hin*dIII and *Hpa* II sites of PM2 DNA (15). Our map is in agreement with theirs. The SV40/*Hin*dIII and SV40/*Hpa* I maps confirm data published elsewhere (16, 17).

It is possible to map the sizes of DNA fragments from the calibration curve because the electrophoretic mobility of a linear DNA does not change from one slot of the gel to the next when certain precautions are taken. Mapping and gel calibration are dependent upon being able to compare samples in one gel slot with those in other slots. We minimized slot-to-slot variation in mobility as a function of molecular weight by modifying the equipment, which reduced the uneven heating in the gel that leads to DNA in the center slots running faster than in the side slots. Sample concentration is also important. If the mass of an individual sample of DNA is too great, the band that it forms in a gel will run faster than a smaller amount of the same material. For a sample run halfway into our 4 mm thick, 1% agarose short gels (running distance $\approx$7.5 cm) the band preferably should contain less than 50 ng of DNA. We found that band shape after the run was a function of the volume of the sample applied. Our sample wells had a minimal cross-sectional area of 10 mm^2, so we limited our sample volumes to 25 μl.

DISCUSSION

The function we have used to relate electrophoretic mobility and molecular weight was arbitrarily chosen. A linear logarithmic function was used with less satisfactory results. A cubic polynomial was almost as successful as the cubic exponential function.

Regardless of which function is chosen, the methodology will only yield reliable data if mobility is a smooth function of molecular weight. This precludes the possibility that different DNAs within the sample may have greatly varying G+C composition if, as has been reported (9, 18), G+C bias alters mobility in gel electrophoresis. Assuming that mobility is a smooth function of molecular weight, the experiments presented here offer a simple method for gel calibration without the introduction of standards and provide a rapid mapping technique for circular DNAs.

It is possible to derive molecular weights of fragments only within the range determined by the mobility of Form III and the mobility of the smallest cleaved permuted linear. An additional point, as noted in the legend to Table 2, is that our SV40 curves slightly underestimate the size of fragments smaller than 50% of the DNA and overestimate the larger fragments. The complementary nature of the problem is due to the constraint imposed by Eq. 4, demanding that pairs sum to 100%. In our experience so far, the error is never greater than 2% of the total size of the DNA.

A partial remedy for this problem is provided by using the added constraint $MW_{p'} + MW_{p''} = MW_p$ (Eq. 5). The usefulness of this constraint is dependent upon the size of the "p" fragment. In the SV40 *Hin*dIII system "p" equals the C fragment, which is approximately 20% of the genome. The calibration curve in this system deviates from expected values by as much as 2% of the full length of SV40 DNA. In the cases where the "p" fragment was either 40% or 55% of the genome, as in the SV40/*Hpa* I or PM2/*Hin*dIII systems, respectively, the resulting calibration curves were very good. They give SV40/*Hpa* I complete digest product sizes that are less than 1% smaller than accepted molecular weights. Additional constraints may further improve the mathematics. One is readily available: the sum of the molecular weights of complete digest products equals 1; another, the sum of two products equals a partial, requires knowing the map of the calibrating DNA. SV40 DNA is so well mapped this need not be a problem.

The calibration curve from the PM2/*Hin*dIII system is also very good. All of the λ restriction endonuclease fragment sizes presented in Table 1 were determined from PM2 calibration

curves. Our values, which span the range of the calibration curve, are greater than or equal to previously published values; but do not deviate greatly from those values. If the curves were failing in the overestimating/underestimating manner, some of our values would be higher than literature values while others would be lower.

There is further evidence for the accuracy of the PM2 calibration curve. The six largest cleaved permuted linears from the SV40/*Hin*dIII system and the three largest cleaved permuted linears from the SV40/*Hpa* I system fall within the range defined by the PM2/*Hin*dIII system. Therefore, it is possible to determine the molecular weights of those nine SV40 fragments from SV40 calibration curves and from PM2 calibration curves. This is done by using Eq. **2.**

These nine fragments are all greater than 50% of SV40. Their sizes are slightly overestimated by the SV40 calibration curves. The PM2 calibration curves give lower values for all of the fragments. All of these lower values, when compared to expected values, are within the 1.5% error margin that is inherent in the best fit for Eq. **2.** This error margin is much smaller than the normal standard deviation in sizes of large fragments measured by other methods. Therefore, we recommend the use of cleaved permuted linears for the mapping of closed circular DNAs and molecular weight determinations of linear DNA molecules. Our 1% agarose gel only resolved six of the 8 SV40/*Hpa* I-cleaved permuted linears. Those numbered 2 and 5 in Table 2 are doublets that did not resolve. We treated them accordingly and therefore our *Hpa* I map of SV40 contains only three fragments. The fourth fragment is located between the A and C fragments.

Even with all mathematical problems solved, DNA with many restriction enzyme sites will not be easy to analyze with this technique until gels with better resolution are developed. A gradient agarose gel system may expand the permissible size range for cleaved permuted linears.

While this manuscript was in preparation Nosikov *et al.* reported that distamycin A and actinomycin D can inhibit restriction endonuclease activity (19). We have also used propidium diiodide and actinomycin D. Site preference, but not exclusion, is observed in the EtdBr-limited systems at the dye levels we have explored. We have not determined whether this differs from the site specificity that often occurs in the absence of EtdBr.

This research was done under the leadership of Prof. Jerome Vinograd. Without his contributions, direction, and inspiration this paper would not exist. While this manuscript was in its early stages of preparation his unfortunate death occurred.

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