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Extracellular nucleases of Pseudomonas BAL 31. III. Use of the double-strand deoxyriboexonuclease activity as the basis of a convenient method for the mapping of fragments of DNA produced by cleavage with restriction enzymes*

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ABSTRACT

We have previously characterized an extracellular nuclease from Pseudomonas BAL 31 which, in addition to other activities, displays a double-strand exonuclease activity which progressively shortens both strands of linear duplex DNA molecules from both termini. This degradation is accomplished without the introduction of detectable scissions away from the ends of the duplexes. When this nuclease is used to produce a series of progressively shortened samples from a linear duplex DNA, subsequent digestion of these samples with a site-specific restriction endonuclease and analysis of the resulting fragments by gel electrophoresis permits the rapid establishment of the order of the restriction enzyme fragments through the entire genome. This is accomplished by noting from the electropherograms the order in which the various restriction enzyme fragments become noticeably shortened or disappear. Using this method, the five cleavage sites for the endonuclease Hpa I and the single cleavage sites for the nucleases Hpa II and Pst I have been mapped in PM2 bacteriophage DNA. In a more stringent test of the method, 18 of the 24 fragments produced by cleavage of coliphage $\lambda b_2 b_5 c$ DNA with the Pst I nuclease have been mapped, and five of the six remaining fragments have been assigned to small regions of the genome.

INTRODUCTION

Site-specific restriction enzymes are endodeoxyribonucleases which recognize nucleotide sequences in double-stranded DNA and cleave both strands of the duplex. Because of their specificity, sectioning of DNAs with these enzymes has become a powerful tool for both the genetic and physical analysis of nucleic acids. For example, restriction enzymes have been used to fragment larger DNAs for sequence analysis, to isolate genes for molecular cloning, and to study the organization of the eukaryotic chromosome (for a review, see Nathans and Smith[1]). The location of the sites in a given genome at which these enzymes cleave is an important prerequisite for such further studies.

Some of the methods which have been developed for the mapping of restriction enzyme cleavage sites include the analysis of the products

of partial digestions with restriction enzymes,[2-4] the digestion of the fragments resulting from cleavage by one restriction enzyme with a second restriction enzyme, and vice versa,[4,5] the use of deletion and/or substitution mutants of the genome being analyzed,[6,7] and electron microscopy of fragments from restriction enzyme digestion after these have been caused to undergo strand separation and then have been annealed to single strands of the full length genome.[8,9] More recent methods include a procedure in which the identities of the fragments adjacent to a certain fragment are deduced after enzymatic extension, with incorporation of radioactive nucleotides, of one of the strands of the fragment in question, using single strands of the full-length genome as template.[10,11] The analysis of fragments from partial restriction enzyme digestions, after these fragments have been terminally labeled, has been shown to be useful in the mapping of restriction enzyme cleavage sites.[12] A two-dimensional electrophoretic procedure, which establishes the presence or absence of common nucleotide sequences between fragments resulting from digestion of a given genome with one restriction enzyme and those resulting from cleavage with a second restriction enzyme, has been mentioned.[13] Parker et al.[14] have developed a procedure in which the location of cleavage sites in a circular genome can be ascertained by first cleaving the genome under conditions in which the enzyme cleaves at essentially a single one of numerous possible sites in each molecule, then digesting this collection of linear duplexes with a restriction enzyme which cleaves the genome only at a single unique site. Electrophoretic analysis and accurate molecular weight determinations of the resulting fragments allows the deduction of the order of the fragments in most instances.

Some disadvantages of the above techniques are that most are laborious and time-consuming and several require repeated procedures such as the isolation of specific fragments from gels in sufficient quantities for further manipulation. Radioactive DNA, or the introduction of radioactivity into the DNA (such as in terminal labeling) are required in some of the procedures. The methods involving the use of deletion or substitution mutants and electron microscopy of restriction enzyme fragments annealed to the full length genome are restricted to the mapping of relatively large fragments.

Another approach to restriction enzyme cleavage site mapping has been the use of exonucleases to degrade partially a homogeneous sample

of a linear duplex DNA from the ends, after which the partly degraded DNA is digested with a restriction endonuclease and analyzed by gel electrophoresis.[15,16] Those restriction enzyme fragments which contain the ends of the exonuclease-treated DNA will have been partly degraded. Hence, these fragments will no longer migrate to the same positions in the gel, after electrophoresis under a given set of conditions, as will the corresponding fragments which have not been subjected to exonucleolytic degradation. If samples of duplex molecules which have been degraded by exonuclease to varying extents are subjected to restriction enzyme digestion and electrophoresis, it should in principle be possible to determine the order of the fragments produced by a given restriction enzyme. However, this technique has previously been restricted to the mapping of fragments near the ends of the duplex.

Pseudomonas BAL 31 produces extracellularly a highly single strand-specific nuclease which also possesses a potent double-strand exonuclease activity.[17] Digestion of a fully duplex linear DNA with the Ps. nuclease yields progressively shorter duplexes through degradation of both strands from both termini, without the appearance of detectable scissions away from the ends of the molecules. When this nuclease is used to produce a series of samples of progressively degraded linear duplex DNA, subsequent digestion of these samples with a restriction nuclease and their analysis by gel electrophoresis permits the rapid establishment of the order of restriction enzyme fragments through an entire genome.

MATERIALS AND METHODS

Preparation of Bacteriophage DNAs. Covalently closed circular supercoiled (form I) PM2 DNA and $\lambda b_2 b_5 c$ DNA were prepared as previously described.[18,19]

Enzymes. Restriction endodeoxyribonucleases Hpa I and Hpa II from Haemophilus parainfluenzae[20,21] and Pst I from Providencia stuartii[7] were purchased from New England BioLabs. The Eco RI restriction enzyme was purified from Escherichia coli RY13 (New England BioLabs) by the procedure of Modrich and Zabel.[22] The Pseudomonas BAL 31 nuclease used was the highly purified sample described elsewhere.[18] The unit of activity of this enzyme is based upon its ability to render single-stranded DNA acid-soluble[17] and is as defined by Vogt.[23]

Nuclease Digestions. Method A. DNA samples (usually at a concentration of 20 μg/mℓ) were incubated with Ps. nuclease (number of

units as given in Figure legends) at 30°C in a buffer containing 12.5 mM $MgSO_4$, 12.5 mM $CaCl_2$, 0.6 M NaCl, 1 mM EDTA, and 20 mM Tris-HCl (pH 8.1). The reaction was quenched by the addition of 0.1 volume of 0.5 M EDTA (pH 8.1) and the DNA was precipitated by the addition of 2.5 volumes of 95% ethanol. After evaporation of ethanol under a stream of dry nitrogen, the samples were rinsed twice with 70% (v/v) ethanol and resuspended in the appropriate buffer for restriction enzyme digestion. Restriction enzyme digestions were performed according to the information provided by New England BioLabs. Method A proved to be unsatisfactory since the restriction enzymes failed to cleave the DNA in about one out of every four or five samples. This failure did not depend upon the length of the Ps. nuclease digestion. Other investigators[24] have noted similar problems with restriction enzyme digests using DNA substrates which had been treated with the S_1 nuclease of Aspergillus oryzae. Method B was therefore developed to overcome this difficulty.

Method B. DNA samples at concentrations varying from 15 to 150 μg/mℓ were incubated with Ps. nuclease at 30°C in the same buffer as used in Method A except that the concentration of NaCl was reduced to 0.2 M. Aliquots (25 μℓ) of the above mixtures were removed at appropriate times and the reactions were stopped by the addition of 3.5 μℓ of 0.2 M EDTA (pH 8.1). The samples were then adjusted to the appropriate NaCl and $MgSO_4$ concentrations for digestion with Pst I nuclease by dilution with three volumes of 60 mM Tris-HCl, 60 mM $MgSO_4$ (pH 7.5). The restriction enzyme was added and the incubation was continued at 37°C until the reaction was complete. Since chelation of calcium ion by EDTA irreversibly inactivates the Ps. nuclease,[17] no digestion by this enzyme occurs during the restriction enzyme digestion. No random inhibition of the restriction enzyme digests was observed with this method, although higher concentrations of restriction endonuclease [up to 2.3 units/μg of DNA (1 unit is defined as that amount of nuclease required to digest 1 μg of λ DNA to completion in 1 hour in a total volume of 50 μℓ at 37°C)] or longer incubation periods (up to 6 hours) were required to achieve complete digestion as compared to control samples which had not been exposed to the Ps. nuclease. Restriction enzyme digests were stopped by the addition of 0.05 volume of 0.5 M EDTA. For experiments performed with $\lambda b_2 b_5 c$ DNA, samples were heated at 70°C for 10 min to disrupt hydrogen-bonded aggregates of this cohesive-ended DNA immediately prior to digestion with Ps. nuclease.

Samples of $\lambda b_2 b_5 c$ DNA were digested with Eco RI restriction nuclease as described[25] except that the concentration of $MgCl_2$ in the reaction mixture was 10 mM.

The time of incubation of the Ps. nuclease reaction mixtures required to achieve the desired molecular weight reductions were initially estimated with the following equation[26]

$$M_t = M_o - 2M_n V_m t/(K_m + S_o) \qquad (1)$$

where M_o is the original molecular weight of the DNA, M_t is its molecular weight after t minutes of incubation, M_n is the average molecular weight of a mononucleotide (taken to be 330 daltons), V_m is the maximum reaction velocity, S_o is the molar concentration of duplex termini, and K_m is the Michaelis constant. A value of 2×10^{-8} M may satisfactorily be used for K_m under either set of conditions for digestion with the Ps. nuclease given above. Also, a solution of Ps. nuclease containing approximately 28 Vogt[23] units/mℓ will exhibit values of V_m near 2×10^{-6} and 3×10^{-6} mols/ℓ-min for the conditions of method A and method B, respectively.[26]

The Hpa II restriction enzyme has one site of cleavage in PM2 DNA[27] and was used to convert covalently closed circular PM2 DNA to the linear duplex form to provide unique starting termini for the Ps. nuclease. After digestion with Hpa II nuclease, protein in the sample was extracted with distilled phenol. The phenol was removed by three extractions with diethyl ether and dialysis into 0.1 M NaCl, 20 mM Tris-HCl, 1 mM EDTA (pH 8.1).

Gel Electrophoresis. One quarter volume of 0.1% bromophenol blue in 50% sucrose (w/v) was added to each sample and gel electrophoresis was performed at 3.5 V/cm at approximately 14°C in composite 2% polyacrylamide-0.5% agarose slab gels (0.3 x 8 x 14 cm) prepared as described by Peacock and Dingman[28] using the TEA buffer described by Helling et al.[29] The gels were stained and photographed as described.[18]

Preparative gel electrophoresis in 0.7% agarose slab gels (same dimensions as above) was used to separate the largest fragment produced from $\lambda b_2 b_5 c$ DNA by cleavage with Eco RI endonuclease from the remaining fragments. After electrophoresis of approximately 40 µg DNA per gel in TEA buffer (2 V/cm, 14 hours, 14°C), gels were stained as described[18] and the bands were made visible under long-wave (365 nm) ultraviolet illumination. The DNA was eluted from the appropriate section of the

gels through homogenization of the material as described.[30]

RESULTS

A Test of the method with a simple system. It was previously reported[27] that Hpa I nuclease cleaves the circular PM2 phage genome at four sites, yielding four fragments. However, close inspection of our electropherograms of Hpa I nuclease digests of PM2 DNA (Figure 1, Track 1) reveals that the second band from the top is actually a doublet containing fragments Hpa I-B and Hpa I-C. This result is confirmed by a composite digest with both Hpa I and Hpa II nucleases (Figure 1, Track 2) in which fragment Hpa I-C is cleaved by Hpa II nuclease

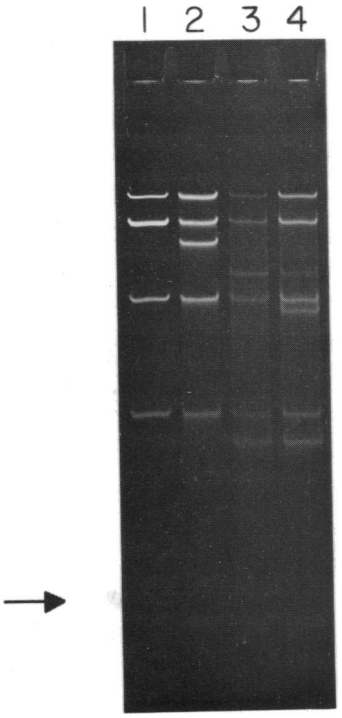


Figure 1. Electropherograms of fragments produced from PM2 form I DNA by various restriction enzymes. Track 1, Hpa I; Track 2, composite digest with Hpa I and Hpa II nucleases; Track 3, composite digest with Hpa I and Pst I nucleases; Track 4, composite digest with Hpa I, Hpa II, and Pst I nucleases. Faint band in Track 4 is result of incomplete digestion by Hpa II nuclease. Arrow indicates position of small fragment Hpa I-C_2 (present in tracks 2 and 4) produced by cleavage of fragment Hpa I-C with Hpa II nuclease (this fragment is visible in original negatives). Electrophoresis, staining of gels, and photography were carried out as described in Materials and Methods. Direction of migration in this and all other electropherograms was from top to bottom.

yielding two new fragments which are designated Hpa I-C_1 and Hpa I-C_2 where fragment Hpa I-C_2 is the shorter of the two. These two new fragments become the terminal fragments when PM2 form I DNA is converted to the linear duplex form by the Hpa II enzyme. This leaves four other unmapped fragments and thus provides a simple system with which to test the feasibility of the Ps. exonuclease mapping method.

Figure 2 shows the initial experiment to map the fragments produced from PM2 form I DNA by the Hpa I nuclease. Track 1 shows all six fragments from the composite Hpa I - Hpa II nuclease digest. These are, starting with the uppermost fragment, fragments A, B, C_1, D, E, and C_2. Track 2 shows the pattern when 15 min of exonuclease digestion of PM2 form III DNA, produced by cleavage with Hpa II nuclease, has preceded the Hpa I nuclease digestion. Inspection of the track shows that frag-

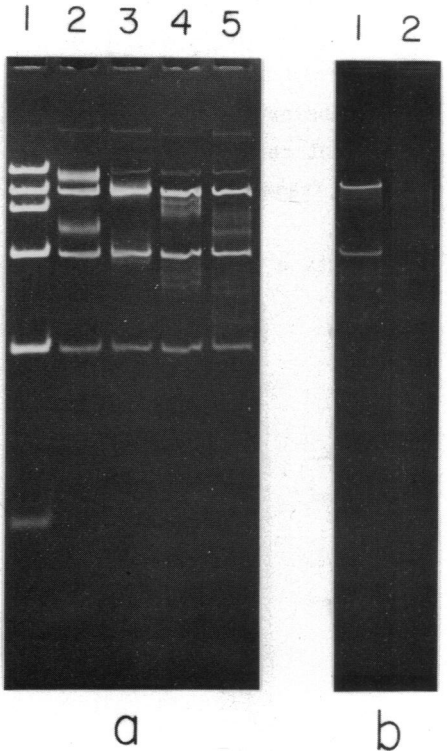


Figure 2. Electropherograms of fragments from a Hpa I nuclease digest of linear duplex PM2 DNA (produced from PM2 form I DNA by cleavage with Hpa II nuclease) which had been digested with Ps. nuclease (87 units/mℓ) according to Method A (Materials and Methods) for the following periods of time. Gel a: Track 1, 0 min; Track 2, 15 min; Track 3, 30 min; Track 4, 45 min; Track 5, 60 min. Gel b: Track 1, 120 min; Track 2, 165 min.

ment C_2 has disappeared and that fragments C_1 and A have been shortened, as these now migrate faster than the corresponding full-length fragments. This confirms that fragments C_1 and C_2 are the terminal fragments and also shows that fragment A is next to fragment C_2. The remaining tracks in the gel of Figure 2a show the continuing shortening of fragments C_1 and A. Figure 2b (Track 1) shows that fragment E is the next fragment to disappear and must therefore map after C_1, as the shorter fragment C_1 will be completely degraded before fragment A is completely degraded. Figure 2b (Track 2) shows that the exonuclease encounters fragments B and D at approximately the same time and thus the relative locations of these final two fragments cannot be determined from this experiment. This situation illustrates a disadvantage of this technique, which is that ambiguities may arise due to the fact that the enzyme is removing fragments simultaneously from both ends. In this instance the map can readily be completed with the aid of a partial Hpa I nuclease digest of PM2 form I DNA (Figure 3). An intermediate digest product migrates directly below the B-C doublet and must therefore be a composite fragment containing fragments D and E. Fragment D is therefore next to fragment E, which provides sufficient information to complete the map.

Figure 1, Track 3, represents a composite digest of PM2 form I DNA

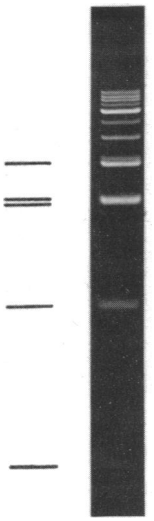


Figure 3. Electropherogram of fragments from a partial Hpa I nuclease digest of PM2 form I DNA. The horizontal lines to the left of the electropherogram indicate the positions of the fragments which result from complete digestion.

with Hpa I and Pst I nucleases and shows that Pst I nuclease cleaves PM2 DNA at one site. This cleavage occurs in fragment Hpa I-C, which is the same fragment in which the Hpa II endonuclease cleavage site is located. Track 4 of Figure 1, which shows a composite digest of viral PM2 DNA with Hpa I, Hpa II, and Pst I enzymes, indicates that the Pst I nuclease cleavage occurs in fragment Hpa I-C_1.

The molecular weights of the various Hpa I, Hpa II, and Pst I nuclease fragments are given in Table 1. These values were determined

TABLE I: Molecular Weights of Fragments of PM2 DNA Produced by Cleavage with Hpa I, Hpa II, and Pst I Restriction Endonucleases.

DNA Fragment	Molecular Weight x 10^{-6}, daltons[a]
Hpa I-A	2.12
Hpa I-B	1.64
Hpa I-C	1.59
Hpa I-D	0.90
Hpa I-E	0.49
Hpa I-C_2[b]	1.36
Hpa I-C_2[b]	0.24
Hpa I-C_1'[c]	1.09
Hpa I-C_2'[c]	0.43

[a]The products of cleavage of PM2 form I DNA with Hpa I nuclease, with both Hpa I and Hpa II nucleases, and with both Hpa I and Pst I nucleases were subjected to electrophoresis in a single composite polyacrylamide-agarose slab gel as described in Materials and Methods. The same gel contained, in separate tracks, the fragments produced by cleavage of phage ϕX174 replicating form I DNA (prepared according to the procedure of Godson and Vapnek[31] as modified by Gray et al.[17] and by Legerski and Gray[32] with Hpa I and with Hpa II nucleases. Using the molecular weights for the ϕX174 DNA fragments given by Lee and Sinsheimer,[4] a plot of log(molecular weight) vs. distance migrated was found to be linear and was used as a calibration curve to obtain the molecular weights of the fragments of PM2 DNA.

[b]Fragments produced by cleavage of fragment Hpa I-C by Hpa II nuclease.

[c]Fragments produced by cleavage of fragment Hpa I-C by Pst I nuclease.

by calibration of the gels with restriction enzyme fragments of known molecular weight as described in the footnote to Table 1. Figure 4 shows the completed PM2-Hpa I cleavage map with the Hpa II and Pst I nuclease sites also indicated.

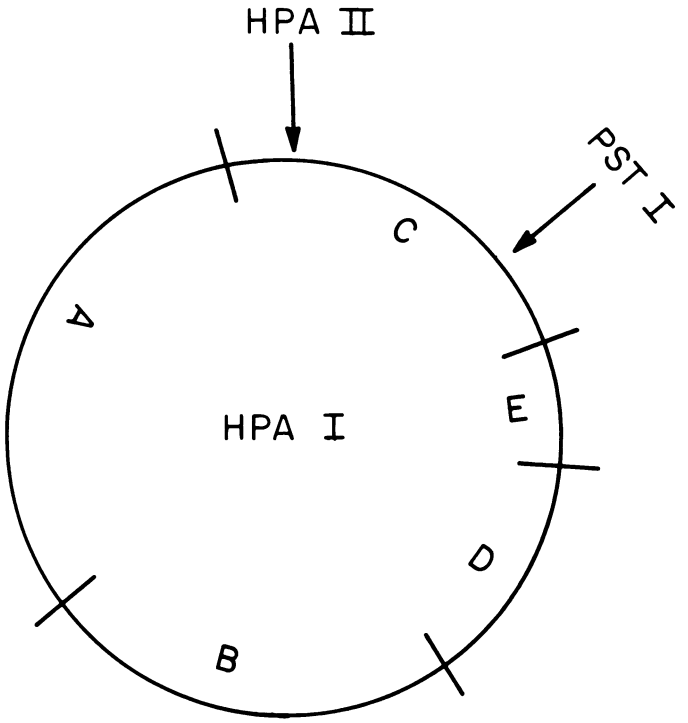


Figure 4. Map of the sites of cleavage by Hpa I, Hpa II, and Pst I restriction enzymes in PM2 DNA.

The order of the Pst I nuclease cleavage fragments of $\lambda b_2 b_5 c$ DNA. Pst I nuclease cleavage of coliphage $\lambda b_2 b_5 c$ DNA produces 24 fragments ranging in molecular weight from 10×10^6 to 6.1×10^4 daltons. Figure 5 shows the initial exonuclease mapping experiment to determine the order of the $\lambda b_2 b_5 c$-Pst I fragments. Rather than discuss the gels track by track, the information obtained in this first experiment is summarized by Figure 6. Fragment A is found to be a terminal fragment which agrees with the work of Smith et al.[7] in which they found that the largest fragment of a Pst I nuclease digest of wild type λ DNA contains the right hand cohesive end. Starting from the left hand end, the fragments through fragment J can be ordered except for the relative positions of fragments P and K (Figure 5a,b). Since fragments B and G

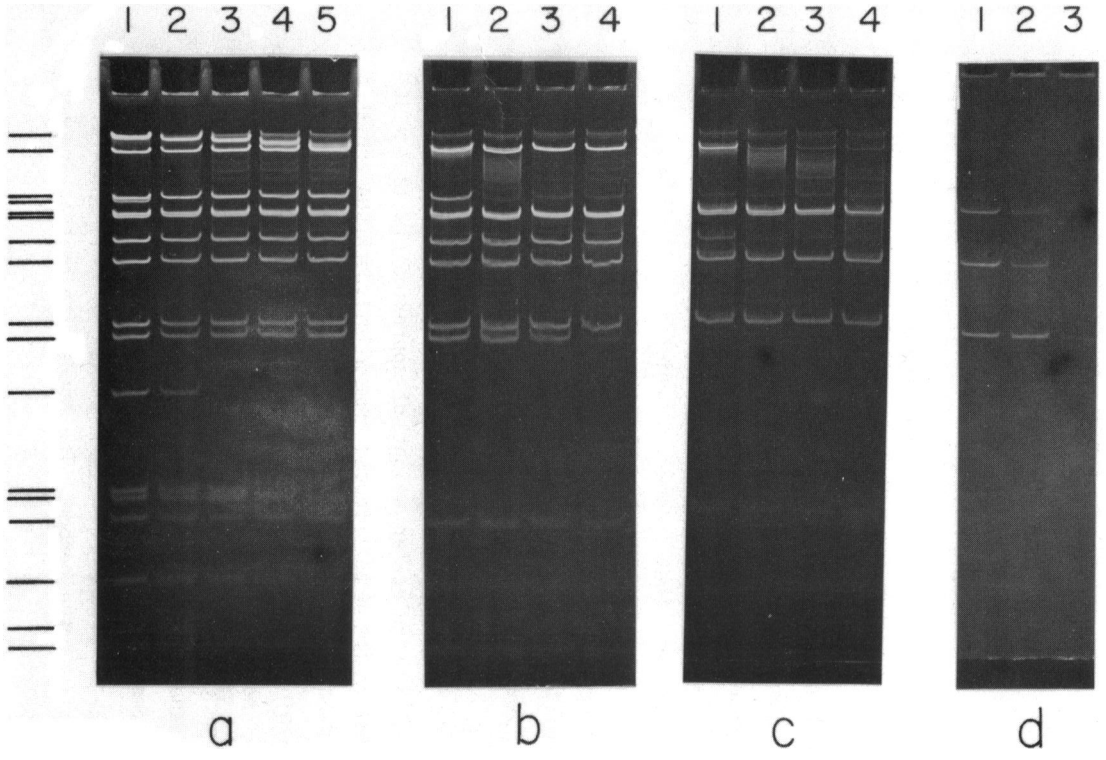
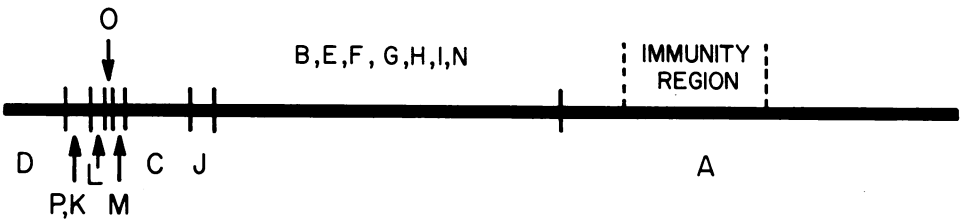


Figure 5. Electropherograms of the 17 largest fragments from Pst I nuclease cleavage of λb2b5c DNA which had been digested with Ps. nuclease (260 units/ml) according to Method B (Materials and Methods) for the following periods of time. Gel a: Track 1, 0 min; Track 2, 10 min; Track 3, 31 min; Track 4, 45 min; Track 5, 55 min. Gel b: Track 1, 63 min; Track 2, 86 min; Track 3, 110 min; Track 4, 124 min. Gel c: Track 1, 149 min; Track 2, 173 min; Track 3, 188 min; Track 4, 211 min. Gel d: Track 1, 225 min; Track 2, 251 min; Track 3, 290 min. The horizontal lines to the left of gel a are for the purpose of clearly indicating the positions of the fragments produced from full-length λb2b5c DNA.



$\lambda b_2 b_5 c$ DNA

Figure 6. Partial map of the sites of cleavage by Pst I nuclease in $\lambda b_2 b_5 c$ DNA as determined from the gel electrophoresis experiments of Figure 5.

disappear at approximately the same time (Figure 5c), fragments mapping after this point cannot be unambiguously ordered. However, it is apparent that fragment I is the last fragment to be reached by the enzyme (Figure 5d), that fragments E and H lie on the immediate opposite sides of I (Figure 5d), and that fragment G, which is followed by fragment F, lies on the opposite end to fragment B (Figure 5c). Finally, fragment Q maps either between fragments A and B or between fragments J and G and fragment N lies next to either fragment E or H.

In order to complete the ordering of the seventeen largest fragments the following experiment was performed. The restriction enzyme Eco RI cleaves $\lambda b_2 b_5 c$ DNA at four sites to produce five fragments. The largest fragment, with a molecular weight of approximately 13.7×10^6 daltons, is the same fragment as produced from wild-type λ DNA and thus contains the left hand half of the molecule.[29] This fragment was purified from the remaining fragments (Materials and Methods) and subjected to the exonuclease mapping procedure. Figure 7 shows the results of this experiment. Track 1 represents a Pst I nuclease digest of full length $\lambda b_2 b_5 c$ DNA and Track 2 shows a Pst I nuclease digest of the Eco RI fragment. Comparison of gel Tracks 1 and 2 shows that fragments B and Q are not present in the Eco RI fragment and therefore lie in the right hand half of the genome. The non-stoichiometric fragment present at the top of Track 2 is the Eco RI nuclease fragment containing the right hand cohesive end which apparently copurified with the left hand fragment due to the joining of the complementary single-stranded ends of this DNA. This fragment contains only part of fragment A of the Pst I nuclease digestion and thus is not cleaved by Pst I nuclease. The

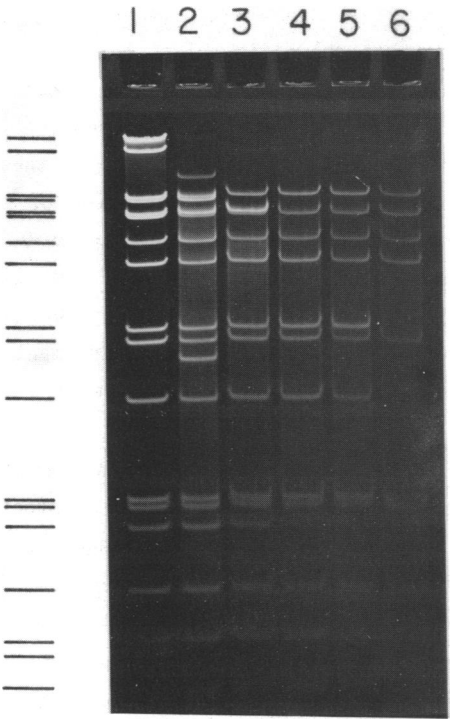


Figure 7. Electropherograms of the fragments produced by cleavage with Pst I nuclease of the largest fragment produced by Eco RI nuclease cleavage of $\lambda b_2 b_5 c$ DNA. Prior to digestion with Pst I nuclease the fragment was digested with Ps. nuclease (130 units/mℓ) according to Method B (Materials and Methods) for the following periods of time. Track 2, 0 min; Track 3, 15 min; Track 4, 35 min; Track 5, 60 min; Track 6, 100 min. Track 1 contains the fragments produced by cleavage with Pst I nuclease of full length $\lambda b_2 b_5 c$ DNA. The horizontal lines to the left of the electropherograms indicate the positions of the fragments produced from full-length $\lambda b_2 b_5 c$ DNA.

new fragment occurring below fragment J in Track 2 is the left hand section of Pst I nuclease fragment B produced by the Eco RI nuclease cleavage. This is confirmed by the fact that it is the first fragment to be degraded by the exonuclease (Track 3). Moving across the gel, it can be seen that fragments N and E disappear at about the same time, which places fragment N next to fragment E. Fragments E and I are degraded in that order which completes the mapping of the right hand end. Tracks 5 and 6 indicate that fragment P maps before fragment K which completes the map of the seventeen largest fragments.

The lower portion of Track I of Figure 7 indicates that there are more than seventeen fragments produced by Pst I nuclease cleavage of

$\lambda b_2 b_5 c$ DNA. This is confirmed in experiments carried out at shorter times of electrophoresis (data not shown) in which at least 24 fragments can be counted. This includes two fragments (R_1 and R_2) assigned to one band which clearly contains too much material to represent only a single fragment. We have not been able to map precisely all of these smaller seven fragments primarily due to the lack of intensity of the corresponding bands (fragment W, for example, represents only 0.2% of the $\lambda b_2 b_5 c$ genome). It was possible, however, to use the exonuclease mapping procedure, applied to both the full length DNA and to the largest Eco RI nuclease fragment, to map precisely fragment T and to assign all but fragment W to small regions of the genome. The final map is shown in Figure 8.

The molecular weights of the $\lambda b_2 b_5 c$-Pst I fragments are listed in Table 2. These were calibrated as described in the footnote to the table, using several sets of restriction enzyme fragments and full-length linear duplex DNAs for which molecular weights have been reported. Fragment I is the last remaining fragment in the exonuclease digestions, from which it might be inferred that it is equidistant from the two ends. However, if the molecular weights on either side of fragment I are totaled, it is found that the region to the right of this fragment has a mass of 15.8×10^6 daltons while the region to the left has a mass of 10.7×10^6 daltons. Since the estimates of the molecular weights of the fragments are not likely to be sufficiently in error to produce such a result adventitiously, this difference is taken to indicate that there is a difference in the rate of exonucleolytic digestion between the two regions. The left half of $\lambda b_2 b_5 c$ DNA contains approxi-

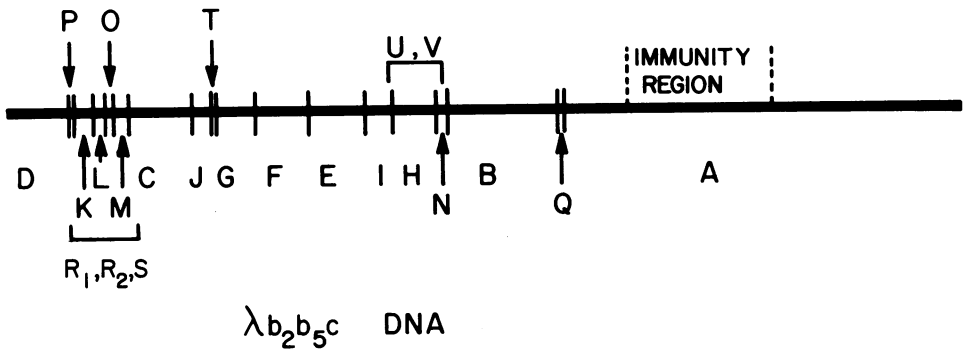


Figure 8. Map of the sites of cleavage by Pst I nuclease in $\lambda b_2 b_5 c$ DNA. Fragments R_1, R_2, S, U, and V map within the regions indicated by the brackets.

Table II: Molecular Weights of Fragments of $\lambda b_2 b_5 c$ DNA Produced by Cleavage with Pst I Restriction Endonuclease.

DNA Fragment	Molecular Weight x 10^{-6}, daltons[a]
A	10.02
B	3.51
C, D[b]	1.76
E, F[b]	1.48
G	1.20
H	1.05
I	0.73
J	0.68
K	0.53
L	0.33
M	0.32
N	0.29
O	0.22
P	0.18
Q	0.17
R_1, R_2	0.14
S	0.13
T	0.11
U	0.10
V	0.071
W	0.061

[a]In order to obtain the molecular weights of the larger fragments, the fragments generated by cleavage of λcI857S7 phage DNA (prepared using a procedure similar to that of Hedgpeth et al.[33]) with Eco RI restriction nuclease, and PM2 form III and φX174 replicating form III DNAs, produced by cleavage of the respective form I DNAs with Pst I nuclease, were subjected to electrophoresis in a single 1% agarose slab gel under the conditions used for 0.7% agarose gels (Materials and Methods). A separate track of the same gel contained the Pst I nuclease cleavage products of $\lambda b_2 b_5 c$ DNA. The molecular weights given for the Eco RI fragments of the λ DNA species, PM2 DNA, and φX174 replicating form DNA were those reported by Helling et al.,[29] Kriegstein and Hogness,[34] and Sinsheimer[35] (by doubling his molecular weight for the single-strand viral DNA), respectively. The plot of log(molecular weight) vs. distance migrated constructed from the above known molecular weights was used to obtain the molecular weights of fragments A and B. For the remaining fragments, the molecular weight calibration was based on the electrophoretic pattern, in composite polyacrylamide-agarose slab gels in which electrophoresis was carried out as described in Materials and Methods, of the fragments produced by cleavage of PM2 form I DNA with Haemophilus influenzae c (Hinc) II restriction enzyme (New England BioLabs) and the fragments produced from φX174 replicating form I DNA by Hpa I and by Hpa II nucleases. The molecular weights of the Hinc II nuclease fragments of PM2 DNA were those given by Streeck et al.[36] and the molecular weights of the fragments of φX174 replicating form DNA were taken from Lee and Sinsheimer.[4] As before, the fragments of cleavage of $\lambda b_2 b_5 c$ DNA by Pst I nuclease were subjected to electrophoresis in a separate track of the same gel as the fragments of known molecular weight.

[b]These fragments do not resolve when electrophoresis is carried out under conditions for which all fragments remain in the gel.

mately 55% G·C base pairs while the right half contains approximately 45% G·C base pairs[37] which means that the left half has a greater thermal stability. This implies that the base pairs at or near the ends of the duplex must be at least transiently unstacked before a phosphodiester bond can be cleaved by the enzyme. This result is consistent with the known single strand specificity of the enzyme. It should also be noted that this result does not in any way invalidate the restriction fragment maps determined above.

The bands in the gels representing the shortened restriction enzyme fragments resulting from sequential exonuclease treatment and restriction enzyme cleavage of the DNAs of this study broaden markedly with increasing extent of exonuclease digestion. Such broadening is presumably the result of increasing heterogeneity in the molecular weight distribution of the shortened fragments with decreasing average molecular weight.[17] This feature is advantageous as it serves to prevent the confusion of a shortened fragment with a full-length fragment in cases where these migrate to approximately the same position in the gel. It is also noted that in some cases the broadened bands representing shortened fragments contain multiple discrete species (i.e., Figures 2a, 5c). Such species could arise, superimposed upon a continuous background of shortened duplexes, if there are specific regions in the genome where the rate of exonucleolytic degradation is slow compared to the average rate (e.g., regions of high G·C content). Alternatively, the enzyme may be acting in a processive or quasi-processive manner such that it may stop or release from the DNA molecule at specific sites. It is emphasized, however, that the weight-average molecular weight of the sample decreases linearly with the time of exposure to the nuclease[17] so that estimation of the time required to accomplish a desired amount of degradation from Equation 1 is valid.

DISCUSSION

This study has shown that the exonuclease activity associated with the Ps. nuclease can be used as the basis for a method to determine the order of the fragments produced by restriction enzyme cleavage of a moderately large DNA, such as that of coliphage $\lambda b_2 b_5 c$, through the entire genome. The use of exonucleases as an aid to establishing the order of restriction enzyme fragments near the ends of various genomes has previously been described;[15,16] however, no attempts to determine the order of the fragments through an entire genome were reported in

those studies. The λ exonuclease, which degrades one strand of linear duplex DNA from each 5' terminus so as to leave protruding 3' ends[38,39] was used by Hayward et al.[15] to identify the terminal fragments from Eco RI and Hin III nuclease digests of herpes simplex virus DNA. McDonell et al.[16] employed a combination of exonucleases III and VII of E. coli to confirm their assignments of the fragments near the ends of coliphage T7 DNA produced by nucleases Hpa I and Mbo I (or Dpn II). As E. coli exonuclease III is well-known to degrade linear duplex DNA from each 3' terminus, leaving protruding 5' ends, and E. coli exonuclease VII removes protruding single-stranded regions from duplex DNA independently of whether these contain 3' or 5' termini,[40,41] the combined effect of these enzymes is to shorten the duplex without leaving long protruding single-stranded regions.

The technique described in this study has a number of practical advantages over the use of other exonucleases for determining the order of restriction enzyme fragments. It degrades both strands of DNA from both ends, whereas the λ exonuclease leaves long protruding single-stranded ends which may interfere with the interpretation of the gel electrophoresis patterns in extended digests. With Method B (Materials and Methods), it was possible to carry out the restriction enzyme reactions after dilution of the irreversibly quenched Ps. nuclease reaction mixtures and addition of an appropriate buffer, and then to subject these samples directly to electrophoresis. The conditions of reaction for the λ exonuclease are not compatible with those for restriction enzymes, so that Hayward et al.[15] found it necessary to dialyze their samples before carrying out the restriction enzyme reactions. McDonell et al.[16] performed extractions with phenol on their samples treated with the combined E. coli exonucleases before digestion with restriction enzymes, an apparent requirement which would become tedious with a large number of samples. The exonucleases used in the previous work must be extensively purified, whereas the concentrated culture fluid of Ps. BAL 31 may be used directly as a double-strand exonuclease preparation in 0.6 M NaCl without the appearance of contaminating activities.[17] This has also been shown to be true for conditions of digestion at lower ionic strengths comparable to that used in Method B (Materials and Methods) (R. J. Legerski and H. B. Gray, Jr., unpublished results).

The enzyme is also highly stable (an unpurified sample of culture fluid supernatant has now retained its activity for over 3 years at 4°C) and is remarkably resistant to denaturing agents such as sodium dodecyl

sulfate[17] and urea (T. P. Winston, J. L. Hodnett, and H. B. Gray, Jr., unpublished results). It is also active over a wide range of ionic strengths (i.e., up to 4 M NaCl) although the exonuclease activity is decreased somewhat at very elevated salt concentrations. The Ps. nuclease thus can be used under a very wide variety of conditions, including those compatible with the subsequent use of various restriction endonucleases.

The exonuclease-dependent restriction enzyme fragment mapping procedure described in this study appears to be less laborious and somewhat more rapid than the other present methods of such mapping. Its major potential disadvantage is that, because it degrades both ends of a linear duplex DNA molecule, the enzyme may initiate the degradation of two fragments at or near the same time, preventing the unambiguous assignment of the positions of the fragments past this point without additional information. As is done when ambiguities arise in connection with other methods of restriction enzyme fragment mapping, the limited amount of additional information required to resolve the ambiguity may be obtained using an alternate method for such mapping [for example, the PM2-Hpa I map of this study (Figure 4) was completed with the aid of one piece of information from a partial restriction enzyme digest]. Alternatively, as was done in this study in the case of the map of the seventeen largest Pst I nuclease fragments of $\lambda b_2 b_5 c$ DNA (Figure 8), a second restriction enzyme can be used to create a new terminus (or termini). After isolation of the desired fragment or fragments, the exonucleolytic method can be used to complete the map.

The complexity of the restriction fragment patterns which can readily be solved with this method is believed to be fairly illustrated by the map of Figure 8. It is difficult to establish the exact positions of very small fragments because of their low intensities, relative to the larger fragments, in the ethidium bromide-stained gels. Additionally, if two very small fragments prove to be adjacent, it could be difficult to ascertain their order because of the molecular weight heterogeneity which arises in the course of an extensive digest. It is evident, however, that the method described here both complements and extends the existing methods for restriction enzyme fragment mapping.

Finally, it should be noted that an enzyme which removes both strands of duplex DNA in an exonucleolytic manner has other utility than the application described here. It could, for example, be used to remove unwanted sequences from the ends of genes which are to be cloned,

or to reduce the length of DNA fragments until genetic functionality is lost, thus establishing the positions of critical sequences. Additionally, as has been done with other nucleases, it could be used to isolate sequences of DNA protected by bound proteins.

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