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An Assay for the Rates of Cleavage of Specific Sites in DNA by Restriction Endonucleases: Its Use to Study the Cleavage of Phage λ DNA by *Eco*RI and Phage P22 DNA Containing Thymine or 5-Bromouracil by *Hin*dIII

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A method to measure the rates of cleavage of specific sites in DNAs by restriction endonucleases is described. Partial digests are prepared by incubating DNAs with limiting amounts of endonuclease. The termini generated by cleavage are labeled with ^{32}P by the polynucleotide kinase-exchange reaction. The labeled termini are then identified by completing the digestion with the same endonuclease and separating the products by gel electrophoresis. As the products of complete digestion of DNA are often easily separated and can be unequivocally identified, this procedure permits comparison of the rates of cleavage of specific sites in DNAs; furthermore, because detection of the products of cleavage utilizes radioautography and does not depend upon their size, or amount, only small amounts of DNA need to be utilized. This method has been used to examine the cleavage of phage λ DNA by *Eco*RI endonuclease, and to demonstrate that 5-bromouracil substitution in phage P22 DNA reduces the rate of cleavage of most sites by *Hin*dIII endonuclease approximately threefold and the rate of cleavage of one site approximately tenfold.

KEY WORDS: restriction endonucleases; DNAs; *Eco*RI; phage DNAs; polynucleotide kinase.

Studies of the rates of cleavage of specific sites in DNA molecules by sequence-specific endonucleases have largely been limited to genomes with a small number of sites (1–10). These studies have required identification and analysis of intermediates occuring during partial digestion. For genomes with two or more cleavage sites, the rate of appearance of internal fragments depends upon several separate events, making kinetic studies technically difficult. Furthermore, digestion of genomes with many sites often generates intermediates which cannot be separated from each other making analysis by such procedures virtually impossible.

We have devised an alternative method for studying the rates of cleavage of specific sites in complex genomes. Partial digests are prepared by incubating DNAs with a limiting amount of endonuclease. The endonuclease is inactivated, and the termini generated by cleavage are labeled with ^{32}P by polynucleotide kinase exchange (11,12). The labeled termini are then identified by completing the digestion with the same endonuclease and separating the products by gel electrophoresis. As the products of complete digestion of a DNA are often easily separated and can be unequivocally identified, this procedure permits measurement of the cleavage of specific sites in DNAs. Using this method, we have measured the rate of cleavage of *Eco*RI sites in phage λ DNA by *Eco*RI endonuclease.

Previously, we noticed that partial *Hin*dIII digestion of phage P22 DNA substituted with 5-bromouracil gave fragment patterns different than those observed with thymine-con-

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taining P22 DNA (13). This suggested that the rate of cleavage of at least one site in the phage genome was affected by 5-bromouracil substitution. We have confirmed this observation using the method described above.

MATERIALS AND METHODS

Enzymes and DNAs. The preparations of phage T4 polynucleotide kinase. *Eco*RI endonuclease, and *Hin*dIII endonuclease have been previously described (11,13). S_1 nuclease was purified by the procedure of Vogt (14). DNAs from phage λ and phage P22 (with or without 5-bromouracil) were prepared as previously described (13).

Labeling of phage λ DNA by polynucleotide kinase exchange occurs extensively at the naturally occurring cohesive ends. S_1 nuclease was used to make the ends blunt, and thereby greatly reduce their labeling by polynucleotide kinase (12): 53 μg of λ DNA was incubated with S_1 nuclease (sufficient enzyme to degrade single-stranded DNA completely) in 500 μl of 30 mM sodium acetate, pH 4.6, 50 mM NaCl, 1 mM zinc acetate, and 5% glycerol for 30 min at 37°C. The DNA was then extracted with phenol and recovered by ethanol precipitation. *Eco*RI endonuclease digestion gave the expected fragment pattern; the sharpness of the bands suggested that little degradation occurred. The native termini of phage P22 DNA are poorly labeled by polynucleotide kinase exchange (unpublished observations, K. L. Berkner), making S_1 nuclease treatment unnecessary.

Inactivation of enzymes. The procedure for measuring rates of cleavage by labeling partial digests requires that the restriction endonucleases be susceptible to rapid inactivation, and the polynucleotide kinase-exchange labeling be limited to the products of the partial digest and not extend to the products of the second (complete) digestion. Thus, conditions for rapid, reversible inactivation of these enzymes were determined.

Endonuclease *Eco*RI is thermolabile (as are many other sequence-specific endonucleases we have tested). It is readily inactivated by a 1-min exposure to 75°C. Endonuclease *Hin*dIII, however, retains sufficient activity after such treatment that an alternative procedure for its inactivation was developed. It is irreversibly inactivated by treatment with 7 mM diethylpyrocarbonate for 3 min at 4°C in 10 mM KPO_4, pH 7.4. Diethylpyrocarbonate is itself unstable in this buffer at 75°C (15) and subsequent treatment of the solution for 1 min at 75°C is sufficient to inactive it. (In a Tris–HCl buffer, normally used for *Hin*dIII digestions, a tenfold higher concentration of diethylpyrocarbonate is necessary to inactivate the enzyme, and often it is difficult to eliminate residual diethylpyrocarbonate by heating at 75°C.) The phosphate buffer has little effect upon the subsequent polynucleotide kinase-exchange reaction; 50% inhibition occurs at 50 mM KPO_4. However, direct phosphorylation of 5′-hydroxyl termini is more sensitive to KPO_4; 50% inhibition occurs at 10 mM KPO_4, consistent with observations made by Richardson (16).

Polynucleotide kinase is thermolabile (17). Two sensitive assays were used to demonstrate that exchange labeling could be effectively stopped prior to the second addition of endonuclease. (a) Micrococcal nuclease-digested salmon sperm DNA (37 μg) in 350 μl of buffer containing 70 mM Tris–HCl, pH 7.6, 10 mM $MgCl_2$, 4.5 mM dithiothreitol (DTT),[2] and 67.5 μM ATP was mixed with 6 units of polynucleotide kinase. Samples were then heated at 75°C for 0–2 min and chilled on ice. [γ-^{32}P]ATP (3×10^4 cpm/pmol) was added to each sample and all were incubated at 37°C. At timed intervals, aliquots of 100 μl were withdrawn and incubated at 37°C. Less than 0.5% phosphorylation activity was observed after heating polynucleotide kinase at 75°C for 30 s. (b) Endonuclease *Eco*RI-digested λ DNA (1.5 μg) or P22 DNA (1.5 μg) in 20 μl buffer containing 45 mM imidazole–HCl, pH 6.6, 18 mM $MgCl_2$, 4.5 mM DTT, 45 mM KCl, 0.1 mg/ml autoclaved gel-

[2] Abbreviation used: DTT, dithiothreitol.

atin, 327 μM ADP, and 5.2 μM [γ-^{32}P]ATP were incubated with 10 units of polynucleotide kinase at 37°C for 10 min and then heated at 75°C for 1 min. *Eco*RI-digested λ DNA was then added to the P22 DNA mix, and *Eco*RI-digested P22 DNA to the λ DNA mix. Following incubation at 37°C for 10 min, the DNAs were fractionated by electrophoresis through agarose, stained with ethidium bromide, and then autoradiographed. In each case, only the DNAs incubated with polynucleotide kinase prior to heating incorporated ^{32}P.

Measurement of the relative amounts of labeled termini. Digests to be fractionated on agarose or acrylamide gels were mixed with 1/10 volume of a solution composed of 1% bromphenol blue, 0.1 M EDTA, and 2.5% Ficoll (loading buffer), and subjected to electrophoresis as previously described (12), except that the electrophoresis buffer was made 10 mM in $Na_4P_2O_7$ and 10 mM in KPO_4, pH 7.5. The gels were stained with ethidium bromide and/or dried for autoradiography with Kodak RP/X film. Grain densities of bands were measured by densitometry from duplicate films exposed for different periods of time, to ensure that the range of the film was not exceeded. In some experiments, phosphotungstate intensifier screens were used to increase the sensitivity of detection of labeled DNA fragments. The reliability of these screens for quantitating ^{32}P DNA was checked by fractionating constant amounts of DNA with increasing specific activities, drying the gel, and then exposing it to film with an intensifier screen. The relative grain density of the bands was determined from densitometer tracings both with an Ortec densitometer integrator and by determining 1/2 peak height × width. Both methods gave identical results. As seen in Fig. 1, there is a linear relationship between the amounts of ^{32}P label in DNA and the grain density at low levels of radioactivity,

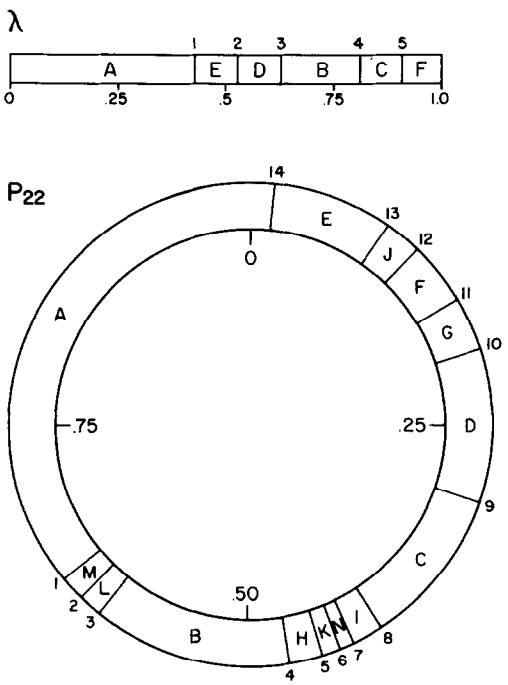

FIG. 2. Cleavage site maps of phage λ and phage P22 DNAs. The *Eco*RI sites in the λ genome (2) and the *Hin*dIII sites in the P22 genome (19) are identified by numbers; fragments are lettered according to decreasing molecular weight. Although the phage P22 genome is linear, it it circularly permuted and hence is drawn here as a circle.

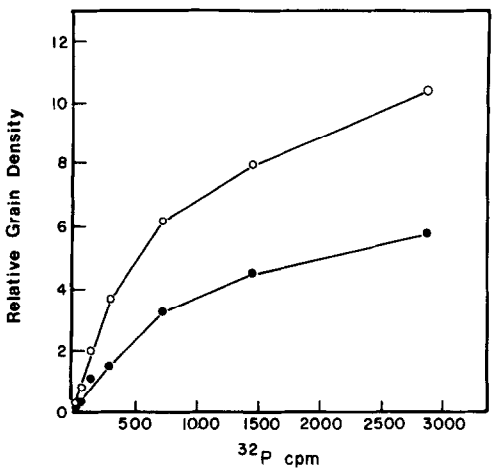

FIG. 1. Use of phosphotungstate intensifier screens for quantitating ^{32}P DNA. Samples of polyoma virus ^{32}P DNA (0.1 μg per sample, from 14 cpm to 2800 cpm) were fractionated by electrophoresis through agarose. Duplicate gels were dried and autoradiographed with an Ilford phosphotungstate intensifier screen at −70°C for 3 (●) or 7 (○) days (18). Grain densities were determined as described under Materials and Methods.

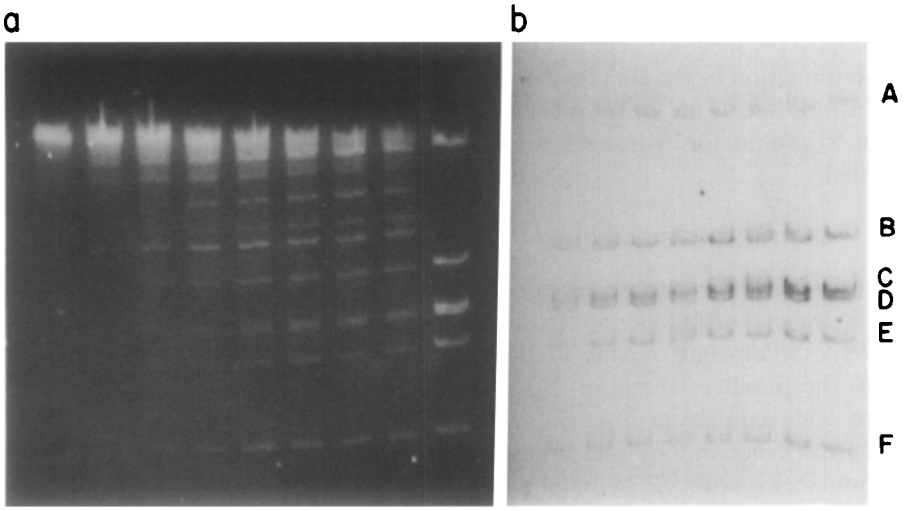

FIG. 3. *Eco*RI digestion of phage λ DNA. S_1 nuclease-treated DNA (10.2 μg) was digested with *Eco*RI endonuclease (8 units) in 100 μl mM Tris–HCl, pH 7.5, 6 mM β-mercaptoethanol, 6 mM $MgCl_2$. Aliquots were withdrawn at 2-min intervals and heated at 78°C for 1 min. An additional aliquot of DNA was digested with 2 units of *Eco*RI for 30 min at 37°C. (a) Part of each aliquot was mixed with loading buffer and fractionated by electrophoresis through 1% agarose; then the gel was stained with ethidium bromide and photographed. (b) An additional part of each aliquot was made up to the following final concentrations in 200 μl: 45 mM imidazole–HCl, pH 6.6; 18 mM $MgCl_2$, 4.5 mM DTT, 0.1 mg/ml autoclaved gelatin, 327 μM ADP, and 16.9 μM [λ-^{32}P]ATP. Polynucleotide kinase (6 units) was added to each, and then they were incubated at 37°C for 10 min, followed by heating at 78°C for 1 min. *Eco*RI endonuclease (2 units) was added to each and the digestion was continued at 37°C for 30 min followed by heating at 75°C for 1 min. Loading buffer was added and the samples were subjected to electrophoresis through agarose and autoradiographed. The DNA samples in (a) and (b) are, left to right: no *Eco*RI (well 1), increasing *Eco*RI (wells 2–8), or complete *Eco*RI digestion prior to polynucleotide kinase exchange (well 9). Letters to the right of part b identify the *Eco*RI fragments of λ DNA.

but leveling off of the grain density occurs as the amount of radioactivity increases. This saturation phenomenon is not solely due to the film's properties, since each amount of radioactivity shows a proportional increase in peak area when exposure is lengthened from 3 to 7 days (Fig. 1). The nonlinear response requires the development of a standard curve. For the studies described here, intensifier screens were used primarily to detect bands having fewer than 500 cpm, which is well within the linear portion of the standard curve.

Calculation of the relative rates of cleavage at individual sites. Since polynucleotide kinase-exchange labeling of single-stranded breaks is inefficient (11), primarily double-stranded cleavages are measured by the assay. The rate is defined by $dP/dT = k^1_{cat}[ES]_1 + k^2_{cat}[ES]_2$, where P is the concentration of fragment generated during the initial part of the reaction and k^1_{cat} and k^2_{cat} are rate constants for the cleavage of flanking sites which generate fragment P. Application of steady state theory lends to a derivation of the following expressions for the initial velocities of reaction with two competing substrates (sites 1 and 2) at identical initial concentrations, as in the case of two sites in a DNA,

$$V^1_0 = k^1_{cat}[ES]_1 = \frac{k^1_{cat}}{K^1_m}[E][DNA],$$

and

$$V^2_0 = k^2_{cat}[ES]_2 = \frac{k^2_{cat}}{K^2_m}[E][DNA].$$

Since [E][DNA] is constant, it can be elimi-

nated when rates are compared, giving relative measures of the specificity constants (k_{cat}/K_m) or discrimination by the enzyme for competing sites in DNA. If the K_m's for each site are known, k_{cat} values for hydrolysis can then be computed.

RESULTS

The approach we have taken to measure the rate of cleavage of individual restriction sites in DNA circumvents having to isolate or identify the initial products of digestion (which are often composite fragments). Instead, termini produced by endonuclease cleavage are labeled with ^{32}P by polynucleotide kinase exchange and then identified by completing the cleavage of the DNA with additional endonuclease. In such a procedure it is important to ensure that the initial incubation with endonuclease is limited (to permit measurement of initial rates), that the endonuclease is inactived prior to labeling the termini with ^{32}P, and that inactivation of polynucleotide kinase is accomplished prior to the second endonuclease digestion. In initial experiments, we studied these aspects of the procedure, using endonucleases *Eco*RI, *Hin*dIII, *Hin*dII, and *Hha*I. We confirmed that rapid, complete inactivation could be achieved by heating at 75°C (*Eco*RI, *Hin*dII, *Hha*I) or exposure to diethyl pyrocarbonate (*Hin*dIII). Similarly, polynucleotide kinase activity can be eliminated by heating at 75°C for 1 min. Once conditions giving only limited digestion (for the first incubation with endonuclease) or complete digestion (for the second incubation with endonuclease) were determined, and knowing the conditions required for polynucleotide kinase exchange labeling (12), we used the procedure to measure the rates of cleavage of sites in phage λ DNA by *Eco*RI, and the cleavage of sites in phage P22 DNA by *Hin*dIII.

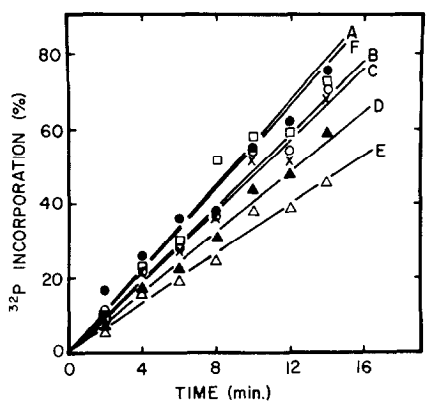

FIG. 4. *Eco*RI digestion of individual sites in phage λ DNA. Duplicate autoradiographs like that illustrated in Fig. 3 were scanned and the area under each peak measured. Bacteriophage λ DNA completely digested with *Eco*RI prior to polynucleotide kinase exchange was quantitated in the same manner. The area for each fragment at each time point is expressed relative to the area for each fragment in the complete digest.

TABLE 1

RELATIVE SPECIFICITY CONSTANTS FOR CLEAVAGE OF INDIVIDUAL *Eco*RI SITES IN λ DNA

Site	k_{cat}/K_m Low salt, 37°C	k_{cat}/K_m High salt, 25°C
1	1.8	1.3
2	0.4	0.3
3	2.2	2.5
4	1.0	1.1
5	2.2	3.1

Note. From the rate determined in Figs. 4 and 5, knowing the order of the *Eco*RI sites in λ DNA (2), $(k_{cat}/K_m)_{1-5}$ were determined as follows: fragments B, C, D, and E have two *Eco*RI termini, while fragments A and F have one. Thus, $dA/dt = k^1_{cat}/K^1_m$ [Enzyme] [DNA], $d[Enzyme]/dT = (k^2_{cat}/K^2_m + k^1_{cat}/K^1_m)$ [Enzyme] [DNA], etc. As the enzyme and DNA concentrations are equivalent for all reactions, they are eliminated from the expressions when values are normalized.

Digestion of Phage λDNA by EcoRI. Previously, Thomas and Davis noted that *Eco*RI sites toward the right end of the λ genome were cleaved preferentially by *Eco*RI endonuclease (2). This prompted us to measure the rate constants for cleavage of individual *Eco*RI sites by polynucleotide kinase-exchange labeling. Because the naturally occurring λ DNA termini are readily labeled by polynucleotide kinase exchange, S_1 nuclease was used to first remove their cohesive ends. The termini re-

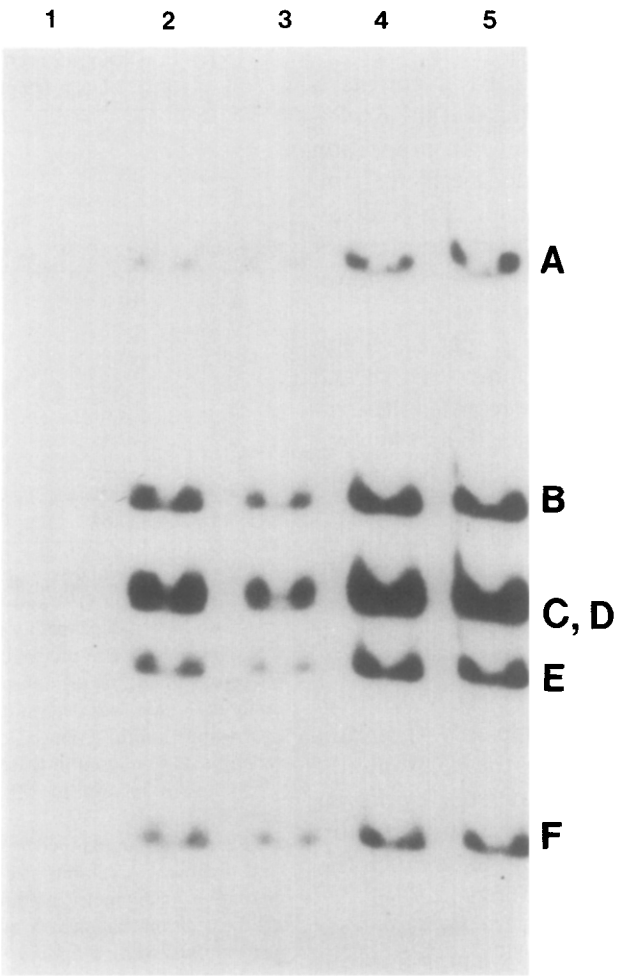

FIG. 5. *Eco*RI digestion of phage λ DNA in high ionic strength buffers. λ DNA (0.5 μg) was incubated with *Eco*RI at 25°C for 1 min in 40 μl of buffer containing S_1-treated DNA, 100 mM Tris–HCl, pH 7.5, 100 mM KCl, then $MgCl_2$ (to 10 mM) was added, and the samples were incubated for 10 or 30 min, followed by heating at 78°C for 1 min. A complete digest was also prepared by treating the λ DNA (0.5 μg) in the same buffer with 4 units *Eco*RI for 30 min at 37°C. The samples were made to the final concentrations in 20 μl: 45 mM imidazole HCl, pH 6.6, 18 mM $MgCl_2$, 4.5 mM DTT, 0.1 mg/ml autoclaved gelatin, 327 μ*M* ADP and 12.4 μ*M* [λ-^{32}P]ATP and incubated with 6 units polynucleotide kinase for 10 min at 37°C, and then heated at 78°C for 1 min. *Eco*RI endonuclease (3 units) was added and the digestion was continued at 37°C for 30 min, followed by heating at 75°C for 1 min. The samples were fractionated by electrophoresis and subjected to autoradiography. The DNAs, from left to right, were incubated with (1) 1 unit *Eco*RI, 1 min; (2) 1 unit *Eco*RI, 10 min; (3) 2 units *Eco*RI, 1 min; (4) 2 units *Eco*RI, 30 min; (5) 10 units *Eco*RI, 30 min.

sulting from this treatment are inefficiently labeled; thus, the two terminal *Eco*RI fragments (A and F, Fig. 2) incorporate ^{32}P primarily at their *Eco*RI termini.

To study *Eco*RI cleavage of λ DNA, partial digests were prepared, labeled by polynucleotide kinase exchange and then digested to completion. In early stages of digestion, as in late stages, we observed no large difference in the rate of appearance of any of the *Eco*RI fragments (Fig. 3). The only significant difference that is consistently observed is a slower

than average cleavage of the site(s) bordering fragments D and E (Fig. 4).

As the order of the *Eco*RI fragments is shown (Fig. 2), and assuming that the *Eco*RI-generated termini are labeled in proportion to their concentration, the specificity constants for cleavage of each site can be determined (Table 1). The specificity constant for *Eco*RI cleavage of site 2 is considerably lower than that for the remaining sites.

Since the activity of the *Eco*RI appears to be dependent upon both ionic strength and temperature (2,7,20,21), we repeated these experiments at high ionic strength and lowered temperature. With a digest that is approximately 5% complete, there is no preferential appearance of any individual fragment (Fig. 5). As fragments A and F are labeled primarily at their *Eco*RI termini, and do not require other cleavages to appear as end products of digestion, preferential cleavage of either site 1 or site 5 (Fig. 2) would be easily detected. With high ionic strength, the only significant difference that is observed is the small amount of fragments A and E generated by *Eco*RI (Fig. 5). When specificity constants were compared, site 2 was again found to be much less prefered than the others (Table 1).

Digestion of Phage P22 by Endonuclease HindIII. Phage P22 DNA contains fourteen sites cleaved by *Hin*dIII endonuclease (Fig. 2). We had previously noticed that *Hin*dIII cleavage of P22 DNA substituted with 5-bromouracil generates a pattern of fragments slightly different than that observed with thymine-containing DNA (12). A high-molecular-weight fragment appears, and seems to replace two low-molecular-weight fragments, suggesting that one site in 5-bromouracil-substituted P22 DNA is particularly resistant to *Hin*dIII digestion (Table 2, Figs. 6, 7). Digestion of the DNA with high concentrations of *Hin*dIII reduces the amount of this fragment (D-G) and increases the amounts of fragments D and G; moreover, the size of fragment D-G is approximately the sum of the sizes of two smaller fragments. Thus, 5-bromouracil substitution may unequally alter the susceptibility of sites in P22 DNA to *Hin*dIII digestion. Quantitative measurements of the rate of cleavage of individual sites in the P22 genome should determine whether this is correct.

TABLE 2

EXTENT OF POLYNUCLEOTIDE KINASE LABELING OF P22 DNA *Hin*dIII FRAGMENTS

Fragment	M_r ($\times 10^{-6}$)	^{32}P Incorporation into P22 DNA[a]	
		Thymine-containing	5-bromouracil-substituted
A	10.3[b]	0.36	0.59
B	4.2	1.00	1.00
D-G	3.8[c]	0	0.69
C	2.8	0.84	0.86
D	2.6	0.97	0.31
E	2.4	0.81	0.81
F	1.2	0.80	0.77
G	1.0	0.57	0.05
H + I	0.64	0.68	0.52

Note. The molecular weights of P22 *Hin*dIII are from Deans and Jackson (19) and agree well with measurements we have independently made. Terminally labeled DNA fragments were prepared, fractionated by electrophoresis, and autoradiographed. After scanning, the area under each peak was determined by measuring the half height times width. Averages from at least four different experiments were used in these determinations.

[a] Expressed relative to ^{32}P incorporation into fragment B.

[b] Because of the manner by which P22 DNA is packaged, fragment A exhibits size heterogeneity and is not present in stoichiometric amounts in enzyme digests (19, 22). In addition, fragment A incorporates less ^{32}P due to one terminus being a mature P22 end, which is labeled poorly even in intact DNA (unpublished results, K. L. Berkner).

[c] The size is determined from its mobility in agarose gels, using a standard curve prepared from *Hin*dIII-digested P22 and λ DNAs.

To analyze the rate of digestion of individual sites, a series of partial *Hin*dIII digests of P22 DNA and 5-bromouracil-substituted P22 DNA were prepared (Fig. 6), and their termini were labeled with ^{32}P by polynucleotide kinase exchange. The partial digests were then digested to completion with additional *Hin*dIII

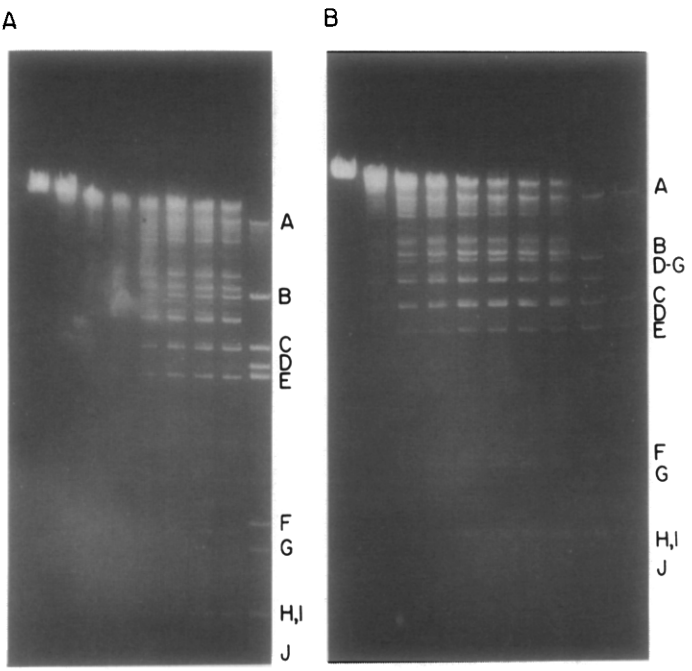

FIG. 6. *Hin*dIII digests of P22 DNA and 5-bromouracil-substituted P22 DNA detected by eithidium bromide fluorescence. Phage 22 DNA (8.8 μg) was incubated in 100 μl of 5 mM KPO_4, pH 7.5, 5 mM KCl, and 10 mM $MgCl_2$ with 5 units *Hin*dIII (P22 DNA) or 20 units *Hin*dIII (5-bromouracil DNA) at 37°C. At time intervals, aliquots (10 μg) were withdrawn and the reaction stopped with gel loading buffer. The samples were fractionated by electrophoresis through agarose gels, stained with ethidium bromide, and then photographed. The digestion pattern for P22 DNA (A), and for 5-bromouracil substituted P22 DNA (B), extended from 0 to 15 min (slots 1–8, left to right) with 1 h digest (slot 9), and a 1-h digest with three times greater enzyme (slot 10, B).

and fractionated by electrophoresis through agarose gels. Autoradiograms of two typical series of partial digests are given in Fig. 7. It is apparent that not all termini are generated at the same time. At early stages of digestion, for example, termini of fragment G are underrepresented in both preparations, and the termini of fragment C are generated at a faster rate in the 5-bromouracil-substituted DNA.

The amount of ^{32}P in each fragment was quantitated by densitometer tracings of autoradiograms such as those shown in Fig. 7. The incorporation of ^{32}P into the termini of each fragment in the partial digest was measured as a fraction of the total observed in that fragment in a complete digest (Fig. 8). It is apparent from such an analysis that *Hin*dIII sites are cleaved at different rates. With thymine-containing P22 DNA, termini of fragments A, F, and G appear at nearly threefold lower rates than termini of fragments B, D, E, and H + I, which in turn appear 1.5 times more slowly than termini of fragment C. With 5-bromouracil substituted DNA, the overall rate of digestion is three- to fourfold slower. Individuallly, an approximate tenfold decrease in the rate of appearance of the termini for fragments G is observed, a three- to fourfold decrease is observed for the termini of fragments B, D, E, and H + I, and the rate of appearance of termini of fragment C is reduced only twofold. Thus, it is clear that not all *Hin*dIII sites in the P22 genome are cleaved equally well, and that 5-bromouracil substitution can alter the relative sensitivity to endonuclease digestion of individual sites. The appearance of fragment D-G in digests of 5-bromouracil-substituted DNA, in particular,

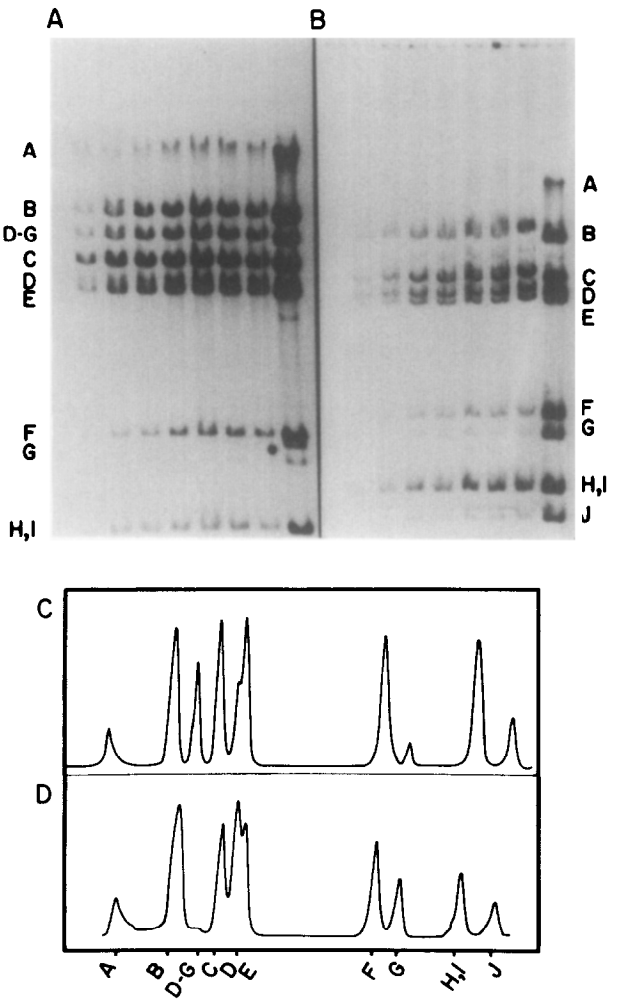

FIG. 7. *Hin*dIII digests of P22 DNA and 5-bromouracil-substituted P22 DNA labeled by polynucleotide kinase exchange. Phage P22 DNA were digested with *Hin*dIII as described in the legend to Fig. 6. Aliquots (10 μl) were removed and mixed with 1 μl 70 mM diethylpyrocarbonate and chilled on ice for 5 min. Digests with excess enzyme were obtained by incubating 1 μg of each DNA with 2 units of *Hin*dIII in the same buffer for 60 min at 37°C. All samples were subsequently heated at 75°C for 2 min and mixed with additional buffer to these final concentrations: 45 mM imidazole–HCl, ph 6.6, 18 mM $MgCl_2$, 4.5 mM DTT, 0.1 mg/ml autoclaved gelatin, 327 μM ADP, and 10.4 μM [λ-^{32}P]ATP. Six units of polynucleotide were added to each. After a 10-min incubation at 37°C, each sample was heated to 75°C for 1 min and then mixed with 5 units of *Hin*dIII. The samples were digested for 60 min and then fractionated by electrophoresis on agarose gels. Autoradiographs of gels of 5-bromouracil substituted DNA (A) and thymine-containing DNA (B) contain from left to right: undigested DNA (slot 1); samples taken at increasing intervals of time (slots 2–8); digests made with excess *Hin*dIII (slot 9). Densitometer tracings of digests made with excess enzyme are shown in (C) (5-bromouracil-substituted DNA) and (D) (thymine-containing DNA).

appears to be due to both an intrinsic resistance to *Hin*dIII cleavage of sites surrounding the D-G fragment in P22 DNA and to a selective increase in resistance when the DNA is substituted with 5-bromouracil. Relative specificity constants for sites 8–12 (Fig. 2) were determined from the rate of appearance of the three fragments D-G, D, and G. Not surpris-

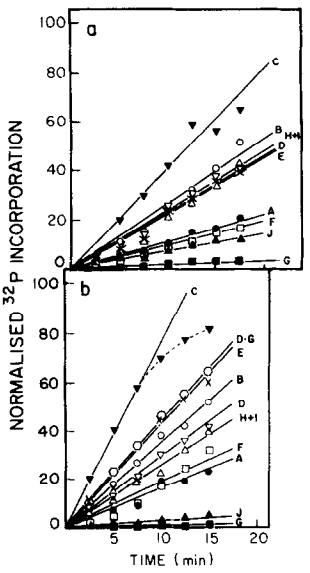

FIG. 8. Rate of appearance of individual *Hin*dIII fragments of P22 DNA. Triplicate autoradiographs like those illustrated in Fig. 7 were scanned and the peak areas were quantitated by densitometry. These were normalized to the areas of analogous peaks from digests of P22 DNA made with excess enzyme. Overall, the rate of digestion of 5-bromouracil-substituted P22 DNA is three to four times slower than that of thymine-containing P22 DNA (12). The rate of appearance of thymine-containing P22 DNA fragments is shown in (a) of bromouracil-substituted P22 DNA fragments, in (b). (Note that the digestion of 5-bromouracil-substituted DNA was performed with a fourfold higher concentration of *Hin*dIII.)

ingly, the specificity constant for site 10 (Fig. 2) is approximately 50-fold lower than that for sites 9 and 11.

DISCUSSION

Studies of the susceptibility to endonuclease cleavage of different sites in a DNA molecule is providing insights into the manner by which these enzymes recognize and cleave DNAs, as well as information about the structure of DNA in solution. In preliminary experiments not reported here, we have examined the rates of cleavage of polyoma DNA by endonuclease *Hpa*II, *Hin*dII, *Hin*dIII, and *Hha*I. Although these studies are incomplete, together with one described above, they confirm that the procedure we have developed for studying the rates of cleavage of individual sites is relatively simple and can be applied to a wide variety of DNAs.

While studying the digestion of phage λ DNA by endonuclease *Eco*RI, we found that one of the sites (site 2) was more refractory to cleavage than the others. The resistance of site 2 is in accord with the observation that *in vivo* is the least susceptible to host restriction (23). Site 2 is also the most strongly protected against cleavage by binding of distamycin-A or 6,4′-diamidine-2-phenylindole (4,6). Protection of this site by dyes may be a reflection of its intrinsic resistance as well as its location in an A-T rich region of the λ genome (where these dyes bind preferentially).

Although we have been unable to confirm the dramatic difference in susceptibility between the site at the right end of the λ genome (site 5) and interior sites reported by Thomas and Davis (2), our data are in accord with the gradient of susceptibility that they noted, in which at high ionic strength sites 1 and 2 are cleaved at lower rates than sites 3, 4, and 5.

Others have noted differences in the cleavage of individual *Eco*RI sites in DNAs (3,7,8,21,24–26). Using DNAs with single *Eco*RI sites that differ in their flanking sequences, two distinct mechanisms of action have been observed. In one, the enzyme remains bound to the DNA between the first and second strand scission, while in the other, DNA is released after the first scission. Observations such as these underscore the significance of elements outside the canonical recognition sequence for the recognition and cleavage of DNA's by site-specific endonucleases (27).

Cleavage of phage P22 DNA by *Hin*dIII is kinetically heterogeneous, with a group of four sites cut approximately threefold more slowly, and one site approximately 1.5 times more rapidly than the remaining four sites examined. When substituted with 5-bromouracil, the overall rate of cleavage is reduced threefold and the differences in rates of cleavage of several sites are accentuated. One site in

particular is very resistant to *Hin*dIII cleavage, and because its resistance is selectively increased by 5-bromouracil substitution, it results in the formation of a new fragment which is not normally observed in *Hin*dIII digests of thymine-containing P22 DNA. As *Hin*dIII acts only upon the sequence (5')AAGCTT (28), this difference in the rate of cleavage must reflect properties of the DNA molecule surrounding the cleavage site. Other studies of *Hin*dIII cleavage of DNAs, particularly of the inhibitory effect of dyes which bind to DNA (5), indicate that neighboring sequences can influence the rate of cleavage of DNAs by *Hin*dIII. We looked for a correlation between the sensitivity to cleavage and the base composition of particular fragments of P22 DNA but could find no obvious relationship (data not shown).

Differential susceptibility of 5-bromouracil substituted DNA to endonuclease cleavage has also been reported by Marchionni and Roufa (29). In their studies, an effect by sequences outside the canonical recognition sequence is indicated by the resistance of sites in 5-bromouracil-substituted DNA to endonuclease *Sma*I, an enzyme whose recognition sequence lacks thymine.

At present, there are only meager data indicating how sequence-specific endonucleases interact specifically with their recognition sequences, and how adjoining stretches of DNA affect such interactions. The method we have developed for measuring rates of cleavage of sites in DNAs should be of use in studying these problems.

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