EcoRI Cleavage and Methylation of DNAs Containing Modified Pyrimidines in the Recognition Sequence*

Kathleen L. Berkner and William R. Folk
From the Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48109

The effects of substituents at position 5 in the pyrimidine ring of a variety of phage DNAs upon EcoRI endonuclease and methylase activities have been examined. The replacement of cytidine in DNA with glucosylated hydroxymethylcytidine confers resistance to cleavage by the EcoRI endonuclease. Substitution of thymidine in DNA by hydroxymethylcytidine lowers the maximal velocity of endonucleolytic cleavage 20-fold, but has no detectable effect upon the $K_m$. Substitution of thymidine in DNA by uridine (a change in the methyl at position 5 of thymidine for a hydroxymethyl) lowers the maximal velocity of endonucleolytic cleavage 2-fold, but has no detectable effect upon the $K_m$. Substitution of thymidine in DNA by uridine (a change in the methyl at position 5 of thymidine for a hydrogen atom) has no effect upon either the maximal velocity or the $K_m$. The effect of these modifications upon EcoRI methylase activity was markedly different. DNA containing glucosylated hydroxymethylcytidine is methylated as well as normal DNA. DNA containing uridine or hydroxymethyluridine, in place of thymidine, is much more poorly methylated than normal DNA. These different sensitivities of the EcoRI endonuclease and methylase to modifications in the pyrimidine rings of DNA suggest there are significant differences in the manner by which these enzymes recognize and bind to the canonical EcoRI sequence.

Restriction endonucleases and their accompanying methylases are uniquely suited for studying how proteins recognize specific sequences in DNA, since the results of their interactions can be easily measured. However, little is known about the structural features of the binding site(s) of these proteins which confer their remarkable specificity (2–6).

A variety of models have been suggested to account for specific protein-DNA interactions. In several of these models, proteins bind to nucleic acid contacts that are available because of distortions in the DNA double helix (6–8). However, little experimental support is available for these models (9–12). If there is no change in the conformation of the double helix, the contacts that can occur between B form DNA and protein that generate specificity are limited and relatively well defined (5, 13, 14).

* This work was supported by United States Public Health Service Grant 5R01 CA 13979, and in part by American Cancer Society Grant NP172. A preliminary account of these results was given at the 1976 American Society of Biological Chemists Meetings, San Francisco (1).

† Predoctoral trainee supported by United States Public Health Service Grant GM00187.

We have approached one aspect of this problem by studying the effect of modifications of nucleotides within the recognition sequences of DNAs upon the activity of restriction and modification enzymes. The EcoRI restriction nuclease and methylase recognize and act upon the sequence

$$\text{G AATT C} . . . . . . . . . . (5') \text{pG AATT C}$$

$$\text{C TTAA Gp(5')}$$

(The arrows indicate the bond cleaved by the endonuclease. The methylase modifies the DNA through the addition of a methyl group to the six-amino group of the central adenine base (15), indicated in the duplex by an asterisk.) Using bacterial phages DNAs containing modified pyrimidines, together with quantitative assays for methylase activity (16) and nuclease activity (17), we have measured the effect of substitutions at position 5 of the pyrimidine nucleus upon the activities of these enzymes.

EXPERIMENTAL PROCEDURES

Bacteriophages and Their Hosts

Phage PBS2 (ATCC 15575—B. subtilis SB19 (ATCC 15575) was grown to an $A_{	ext{max}}$ of 1.0 in media containing, per liter, 10 g of Bacto-tryptone, 5 g of Difco yeast extract, and 10 g of NaCl supplemented with 0.14% glucose, 10 mM MgSO$_4$, and 100 $\mu$M MnCl$_2$, and infected with PBS2 at a multiplicity of infection (m.o.i.) of two. The culture was agitated at $37^\circ$ for 3 additional h and then lysosome was added to 20 $\mu$g/ml. After stirring for 90 min at $37^\circ$ the culture was chilled and the phage purified as described below.

Phage φ6—Phage φ6 was from D. Roscoe via A. Price (18). $B. subtilis$ 3610 (ATCC 6051) was grown at $37^\circ$ in media containing, per liter, 10 g of Bacto-tryptone, 5 g of Difco yeast extract, and 10 g of NaCl, and 0.9 g of glucose. Upon reaching an $A_{	ext{max}}$ of 0.3, the cells were infected with φ6 at a multiplicity of infection of 0.1, and the culture incubated until it was clear, indicating complete lysis (approximately 5 h.) (19). The culture was chilled, and the phage purified as described below.

Phage φw2—Phage φw2 is a mutant of φ6 that codes for thermosensitive dTTPase and dUTPase activities and hence contains DNA with hydroxymethyluridine and thymidine (20) (from D. Roscoe via A. Price). $B. subtilis$ TLR1 (a low thymine-requiring derivative of $B. subtilis$ 3610, from D. Roscoe via A. Price) was grown at $37^\circ$ in media containing 80 mM NaCl, 20 mM KCl, 20 mM NH$_4$Cl, 1 mM MgCl$_2$, 0.2 mM CaCl$_2$, 150 $\mu$M FeCl$_3$, 2.5 mM Na$_2$SO$_4$, 2 $\mu$M ZnCl$_2$, 0.4% glucose, 1.5 mM K$_2$HPO$_4$, and 120 mM Tris/HCl (pH 7.5). Upon reaching an $A_{	ext{max}}$ of 0.9, the incubation temperature was shifted to $45^\circ$ and 10 min later φw2 was added to a multiplicity of infection of five. Incubation at $45^\circ$ was continued for 2 h until the culture cleared (19).

Phage λ—The preparation of phage λ has been previously described (17).
Phage T₄–Escherichia coli B, was grown in Fraser’s medium (21) at 37°C to an A₅₂₀ of 0.2. Phage T₄, D₂ (22) (from I. Tessenman via G. R. Greenberg) was added at a multiplicity of infection of 0.9 and the culture was shaken for 8 h. CHCl₃ was added to lyse the cells and the phage was purified as described below.

Purification of Phages

Lysates of all the phage-infected cultures were cleared of debris by centrifugation at 3000 × g for 10 min. After warming the lysates to 20°C, pancreatic DNase (Sigma) and RNase (Sigma) were added to 1 μg/ml. After 2 h incubation at room temperature all lysates but phage T₄ were treated in the following manner: NaCl was added to a final concentration of 40 g/liter for preparation of phage A, to 17.5 g/liter for PBS₂ and to 20 g/liter for T₄ and mmp preparations. Then polyethylene glycol 6000 (Union Carbide) was added: to 69 g/liter for phage PBS₂ and to 20 g/liter for T₄ and mmp. After stirring at 20°C for 1 h, the phage lysates were stored at 4°C overnight, permitting a phase separation. The polyethylene glycol precipitated phage was collected by centrifugation at 8000 × g for 30 min, and resuspended in buffer. The buffer used for φ₄ and φ₄mmp (T₂ buffer) contains, per liter, 5.7 g of Na₂HPO₄·7H₂O, 1.5 g of KH₂PO₄, 0.4 g of NaCl, 5 g of KSO₄, 0.25 of MgSO₄·7H₂O, 11 mg CaCl₂, 10 mg of gelatin (23). For PBS₂, absorption medium (24) contained, per liter, 1 g of yeast extract, 4 g of NaCl, 5 g of KSO₄, 120 mg of MgSO₄·7H₂O, 3 mg of FeCl₃, 1 mg of CaCl₂, 390 mg of Na₂HPO₄, 170 mg of KH₂PO₄, 1.6 mg of MnSO₄·H₂O, 0.5 mg of ZnSO₄·7H₂O, and 1 mg of MgCl₂. For T₄, the resuspension buffer contained 10 mM Tris/HCl, pH 7.5, with 10 mM MgSO₄.

Phage T₄ lysates were sufficiently concentrated so that the polyethylene glycol precipitation was unnecessary. The phage was pelleted by centrifugation at 25,000 rpm at 4°C for 40 min at a type 30 rotor. The pellets were overlaid with resuspension buffer and kept at 4°C for 12 h, to allow resuspension.

All phages were purified by sedimentation through CsCl block gradients. The gradients were prepared by layering 24 ml of concentrated phage onto a 7-ml layer of CsCl (1.4 g/ml) resting on a second 7-ml layer of CsCl (1.6 g/ml). After centrifugation at 20,000 rpm for 2 h at 4°C in a SW 27 rotor, the peak fractions of A₄₂₀ were pooled and dialyzed against two changes of 10 mM Tris/HCl, pH 7.5, 10 mM EDTA, and 10 mM NaCl (Buffer C) at 4°C.

Purification of DNAs

The dialyzed preparations of bacteriophages were lysed in 10 mM Tris/HCl, pH 7.5, 10 mM NaCl, 20 mM EDTA, 0.5% sodium dodecyl sulfate, and 50 μg/ml of Proteinase K (EM Laboratories Inc.). Sterile buffers were used in this and all subsequent steps. After 6 h incubation at 37°C, with gentle rotation, the solutions were extracted twice with phenol (redistilled, and equilibrated with 1 M Tris/HCl to pH 8.0). The phenol layer was back-extracted with 0.1 M Tris/HCl and two 2 ml ethanol rinses.

All DNAs used as substrates for the EcoRI methylase were first sonicated for 30 s to reduce viscosity. The average molecular weight of the sonicated DNAs was 1.6 × 10⁶, as determined by alkaline sucrose gradient sedimentation.

The concentration of each DNA was determined by its A₂₆₀ and by the diphenylamine assay (25), using DAPM as a standard.

Characterization of DNAs

The sizes of the purified phage DNAs were determined from their sedimentation rates in alkaline sucrose gradients. Purified φ₄, φ₄mmp, and PBS₂ DNAs (100 μg, in 0.5 M NaOH) were layered onto 5 to 20% sucrose gradients in 0.5 M NaOH, 1 mM EDTA, and centrifuged at 4°C for 8 h at 25,000 rpm in a SW 27 rotor. Form I polyoma [³²P]DNA marker (provided by D. Anderson) was included in each gradient. Following collection of the fractions from each gradient, the position of the phage DNA and of the polyoma DNA was determined by measuring A₂₆₀ and Cerenkov radiation. The DNAs' sedimentation coefficients relative to polyoma DNA were computed, and using the empirically derived relationship (26):

\[
\frac{MW'}{MW} = D_{20,4}\left(\frac{1500}{D_{20,4}}\right)^{0.67}
\]

the size of each DNA was determined: PBS₂: 2.0 × 10⁹; φ₄mmp: 2.0 × 10⁹; and φ₄: 1.8 × 10⁷. In each case, these sizes indicate that the DNAs have undergone six to eight nicks per chain.

As each of the substituted DNAs is grown in a host containing normal DNA, it is important to show complete separation of the substituted DNAs from their respective host DNAs. To do this, a novel assay employing the EcoRI methylase was developed. Each DNA (75 μg) was sheared by repeated pipetting (10 times) through a 100-μl pipette, and incubated in a final volume of 500 μl containing 2 μM S-adenosyl-L-methyl-³¹³Hmethionine (9.5 Ci/mmol, New England Nuclear), 100 mM Tris/HCl, pH 8.0, 10 mM EDTA, with 8 μg of purified EcoRI methylase at 37°C for 4 h. CsCl was added to a final density of 1.72 g/ml, and T₄ DNA in Buffer C together with marker Form I polyoma [³²P]DNA, and the samples were banded by centrifugation at 40,000 rpm at 20°C for 40 h in a Ti-50 rotor. After fractions were collected from each tube, the refractive index, A₄₂₀, and Cerenkov radiation of each fraction was determined. The DNAs were then precipitated by the addition of 2 ml of 2 N HCl and 2 A₄₂₀ units of salmon sperm DNA. The DNAs were collected on GF/C filters, presoaked in 2 N HCl and 5 μg/ml of S-adenosyl-L-methionine. Following two 10-ml rinses of 2 N HCl and two 2-ml ethanol rinses, the filters were dried and counted in a scintillant containing 2 g of 2,5-diphenyloxazole and 1 liter of toluene. (All scintillation counting was done with this scintillator, unless otherwise specified.)

Fig. 1 shows the CsCl gradient profile for each of the DNAs. PBS₂, φ₄, and φ₄mmp DNAs have buoyant densities sufficiently different from that of their hosts so that contaminating DNAs can be detected with difficulty.
great sensitivity after methylation with [3H]methyl groups. As the specific activity of the S-adenosyl-L-[methyl-3H]methionine is known, the degree of contamination of each phase DNA by bacterial DNA can be determined, assuming that the bacterial DNAs contain randomly distributed methylatable sites (approximately one site per 406 nucleotides) (29). The upper limit of contamination for each preparation is less than 1%. The buoyant density of phase T, DNA is not sufficiently different than that of E. coli DNA to permit a good separation, so for this DNA, the detection of host contamination is not sensitive. However, since pancreatic DNase was employed to digest contaminating DNAs during the phage purification, little host DNA should be present. Furthermore, after EcoRI digestion of DNA, six clean bands were observed after agarose gel electrophoresis, in the presence of ethidium bromide, with no detectable background DNA.

**Purification of Enzymes**

The EcoRI endonuclease and the phase T4 polynucleotide kinase were purified as previously described (17). The EcoRI methylase was purified by a modification of the procedure of Greene et al. (16). Protein (169 mg) from the phosphocellulose column (the final step in the procedure of Greene et al. (16)) was applied to a column of hydroxyapatite (Bio-Rad; 10 ml bed volume) that had been equilibrated with Buffer A (pH 7.0, 7 mM 2-mercaptoethanol, 0.2 M NaCl, and 0.2% NP-40 [Sigma]). The column was washed with 10 ml of this same buffer, and methylase activity was eluted with 0.5 M KPO4, pH 7.0, 0.2% NaCl, 0.2% NP-40, 7 mM 2-mercaptoethanol, and 1 mM EDTA. The fractions with maximum activities were pooled and dialyzed against 20 mM Tris/HCl, pH 7.5, 0.2% NP-40, 0.5 mM 2-mercaptoethanol, 1 mM EDTA, and 50 mM NaCl (Buffer B) made 200 mM in NaCl. The methylase (30 ml) was then chromatographed through a column containing DEAE-Sephadex A-50 (Pharmacia; 15 ml bed volume) equilibrated in Buffer B. Immediately prior to application it was diluted 4-fold with Buffer B to reduce the NaCl concentration. The column was washed with 40 ml of Buffer A and the methylase, which elutes in the column flow-through, was collected. It was then rechromatographed on hydroxyapatite as described above. This protein (11 mg in 15 ml) was dialyzed against a buffer containing 10 mM NaPO4, pH 7.0, 7 mM 2-mercaptoethanol, 1 mM EDTA, and 0.2% NP-40 (Buffer D) made 0.2 M in NaCl. The dialyzed enzyme was applied to a column containing sulfoxipropyl-Sephadex C50 (Pharmacia; 20 ml bed volume) equilibrated in Buffer D. Again, immediately prior to application, the NaCl concentration in the enzyme preparation was reduced with Buffer D. After washing 50 ml of Buffer D containing 50 mM NaCl, a gradient from 50 to 500 mM NaCl in Buffer D was applied to the column (in 800 ml) and 100 fractions were collected. The fractions containing the peak of methylase activities (eluting at approximately 400 mM NaCl) were pooled and dialyzed against Buffer A to reduce the NaCl concentration. The concentrated enzyme (17 mg in 2.5 ml) was passed through a column containing Sephadex G-100 (100 x 1.5 cm) that had been washed with Buffer D containing 0.2 M NaCl. The methylase activity eluted at a position consistent with its reported molecular weight of 36,000 (12). The peak fractions were concentrated by ultrafiltration, and dialyzed against 10 mM NaPO4, pH 7.0, 0.2 mM 2-mercaptoethanol, and 0.2% NP-40, 1 mM EDTA, 1 mM dithiothreitol, and 50% glycerol (v/v).

The methylase has been kept at -70° and has not lost any activity over 18 months. Disc-gel electrophoresis (30) indicated that the methylase was nearly homogeneous with approximately 10% extra- neous protein. The specific activity was found to be 1.1 x 105 units/mg of protein. One unit is the amount of enzyme needed for the incorporation of 1 pmol of S-adenosyl-L-methionine into DNA in 1 min under the conditions described under "Enzyme Assays.'

**Enzyme Assays**

Throughout its purification, EcoRI methylase was assayed by measuring the methylation of sonicated salmon sperm DNA. In a volume of 100 ml, 1.2 A260 units of DNA were incubated with 0.1 M Tris/HCl, pH 8.0, 10 mM EDTA, and 106 pmol S-adenosyl-L-[methyl-3H]methionine. The solution of methylase was incubated for 30 min at 37°, followed by the addition of 2 ml of 7.5% HClO4, and 100 μl of 20 A260/ml of calf thymus DNA. After 30 min on ice, the precipitated DNA was collected by filtration through GF/C filters, and the remaining samples were filtered through nitrocellulose filters. Both sets of samples were rinsed with 2 x 10-m1 rinses of 9 % HCl. The GF/C filters were dried and counted. The nitrocellulose filters were dissolved in 1 ml of ethyl acetate, followed by the addition of 10 ml of aq. sal for (New England Nuclear). The samples were then counted and counted. Aliquots of the reaction mixture were also spotted directly onto either GF/C or nitrocellulose filters, and the latter were dissolved in ethyl acetate and treated as described above. The quenching for the dissolved nitrocellulose filters will be the same for aliquots spotted or precipitated onto the filters. This then allowed us to determine the relative amount of methylated DNA, or precipitated DNA, or precipitated DNA. Samples spotted on a GF/C filter are quenched 1.4 times more than samples precipitated with acid, and filtered. This correction was applied to the determination of the methylation specific activities, and to the determination of the number of methylated sites in a DNA.

EcoRI endonuclease cleavage of phase T4 DNA was measured using the polynucleotide kinase exchange assay described previously (17). DNA, ranging in concentration from 1.5 to 45 μg in 150 ml was incubated in 10 mM Tris/HCl, pH 7.5, 10 mM MgCl2, and 10 mM NaCl (EcoRI endonuclease buffer) at 37° with purified EcoRI endonuclease. Aliquots (25 μl) were removed at intervals and immediately placed at 78° for 60 s to inactivate the enzyme (17). The aliquots were subsequently chilled on ice, and to each tube was added a mixture containing 0.11 mg/ml of autoclaved gelatin, 18 mM MgCl2, 4.5 mM dithiothreitol, 45 mM KCl, 50 mM imidazole/HCl, pH 6.6, 325 μM dNTPs, and 18 μl of [α-32P]dATP (2 x 106 cpm/μl). The sample was incubated at 37° and then 2 units of purified polynucleotide kinase were added to each. After 10 min the reaction was stopped by the addition of 0.5 ml of 0.1 M Na2HPO4. The tube contents were transferred to large vials containing 2.5 ml of 20 mM Na2PO4/0.4 M trichloroacetic acid. Each trichloroacetic acid supernatant was then rinsed with 0.5 ml of 20 mM EDTA and 3.2 μg calf thymus DNA, which was then added to the sample in trichloroacetic acid. After chilling on ice for 30 min, the samples were filtered through GF/F filters as previously described (17).

One unit of EcoRI endonuclease is that amount which cleaves 1 pmol of polyoma Form I DNA to Form III DNA in 1 min at 37°, under the conditions described above.

**Sloff Gel Electrophoresis of DNA Digests**

Phage DNAs (3.5 μg) were incubated in 50 μl of EcoRI endonuclease at 37° for 30 min (sufficient to produce a limit digest). The reactions were stopped by the addition of 5 μl of 75% sucrose, 1% bromphenol blue, and 100 mM EDTA, pH 7.5. Aliquots (20 μl) were applied to a slab gel (1.5 mm x 30 cm x 14 cm) containing 1% agarose (Seakem) in 40 mM Tris/HCl, pH 7.9, 5 mM sodium acetate, and 1 mM MgCl2. After electrophoresis (8, 10 min, 4°C, Buffer E), the gel was stained with 0.2% Coomassie Brilliant Blue R-250 to reduce the background and with 0.5 μg/ml of ethidium bromide (Sigma) in Buffer E for 30 min and then illuminated with a long wavelength ultraviolet light. Pictures were taken with Polaroid 55-P film, using a Wratten No. 21 filter.

To determine the number average molecular weight of the EcoRI endonuclease-generated fragments of PBS2, φ8, and φm, DNAs, the photographic negatives of the digestion patterns were scanned on a Joyce-Loebel densitometer. Using EcoRI endonuclease fragments of λ DNA as molecular weight markers (31), the number average molecular weight of each digest was determined by fitting a Kuhn
complete digestion, as described by Botchan et al. (32).

Poly nucleotide Kinase Labeling of Sheared DNA

Equimolar mixtures of λ DNA plus one of the following DNAs were mixed: λ, PBS2, T, de, or dm. Each mixture was sheared by passing 12 μg of DNA in 75 μl of Buffer C through a syringe equipped with a 30-gauge needle. The average molecular weight of the sheared DNAs was approximately 5 × 10^6 as determined by sedimentation through alkaline sucrose gradients. Each DNA mixture was labeled with ^32P, by incubating 10 μg of DNA in 0.11 mg/ml of autoclaved gelatin, 18 mM MgCl₂, 4.5 mM dithiothreitol, 45 mM KCl, 50 mM imidazole/HCl, pH 6.6, 327 μM ADP, 13 μM [γ-^32P]ATP in a final volume of 200 μl. Polynucleotide kinase (8 units) was added and the reaction proceeded at 37° for 10 min. The labeled DNAs were treated with a 30-gauge needle. The average molecular weight of the sheared DNAs was approximately 5 × 10^6 as determined by sedimentation through alkaline sucrose gradients. Each DNA mixture was labeled and each was incubated with sufficient enzyme to produce a limit digest, and the products were analyzed by electrophoresis through agarose slab gels (Fig. 2). Normal DNA (A, containing methyluridine (+e) were cleaved into fragments that migrated considerably faster than intact DNA. However, even with a large excess of EcoRI endonuclease, cleavage of DNA containing glucosylated hydroxymethylcytidine (T, DNA) was not detected. Although it is possible to determine the number of cleavage sites per genome for λ DNA by simple examination of the limit digest (Fig. 2), it is not possible to do the same for the digests of PBS2, de, or dm DNAs. Digestion of each of these DNAs generates too many fragments of overlapping sizes to be distinguishable in agarose gels. An approximation of the number of cleavage sites can be made by determining the number average molecular weight of the DNA fragments and dividing this into the molecular weight of the DNA. Using the analysis described by Botchan et al. (32), a Kuhn distribution was fit to the densitometer tracing of the agarose gel-separated limit digest, and with these estimates of the average molecular weight, the numbers of cleavage sites for each DNA were calculated (Table I).

To obtain a better picture of the effect of modified nucleotides in the DNAs upon their susceptibility to digestion, each DNA was incubated with several different quantities of EcoRI endonuclease and the production of fragments was measured with the polynucleotide kinase exchange reaction (17). In Fig. 2, the initial velocity of digestion for each DNA is plotted as a function of enzyme concentration. No digestion of GlcHmdCyd DNA (T4) was detected, in agreement with the analysis by agarose gel electrophoresis described above. The lack of incorporation of ^32P into this DNA after incubation with EcoRI endonuclease rules out any significant level of host contamination. E. coli DNA would be expected to be cleaved, and labeled.

DNAs containing hmdUrd in place of thymidine (de and dm) were much poorer substrates than normal DNA (Fig. 3). Their initial rates of cleavage are one-twentieth that of DNA containing thyminid (λ) or uridine (PBS2).

Although there might be a slight difference in the susceptibility of λ DNA and PBS2 DNA, this result is uncertain because of a possible effect of substrate availability upon the initial rate of digestion. The concentration of DNA used in these studies was the same in each case (80 μg/ml). However, since PBS2 DNA contains approximately twice the number of EcoRI sites as λ DNA on a weight basis (Table I), the concentration of EcoRI sites differed for the two DNAs. This difference may explain the increased velocity seen for PBS2 DNA digestion. That the initial velocity of digestion is dependent upon the concentration of sites is demonstrated by experi-

HmdCyd DNA (T4) & HmdUrd DNA (de and dm) were much poorer substrates than normal DNA (Fig. 3). Their initial rates of cleavage are one-twentieth that of DNA containing thyminid (λ) or uridine (PBS2).

Although there might be a slight difference in the susceptibility of λ DNA and PBS2 DNA, this result is uncertain because of a possible effect of substrate availability upon the initial rate of digestion. The concentration of DNA used in these studies was the same in each case (80 μg/ml). However, since PBS2 DNA contains approximately twice the number of EcoRI sites as λ DNA on a weight basis (Table I), the concentration of EcoRI sites differed for the two DNAs. This difference may explain the increased velocity seen for PBS2 DNA digestion. That the initial velocity of digestion is dependent upon the concentration of sites is demonstrated by experi-

The abbreviations used are: GlcHmdCyd, glucosylated hydroxymethylcytidine; hmdUrd, hydroxymethyluridine.
EcoRI Activity on Modified DNAs

Table I

| DNA   | Modified base       | Number of sites estimated<sup>a</sup> | Number of sites methylated<sup>a</sup> | Cleaved sites<sup>a</sup> |
|-------|---------------------|--------------------------------------|---------------------------------------|--------------------------|
| λ     | Glucosylated hydroxymethylcytidine | 53 | 70 | 67 |
| PBS2  | Uridine             | 58 | 53 | 1 |
| T<sub>1</sub> | Glucosylated hydroxymethylcytidine | 52 | 17.1 | 19.4 |
| φ<sub>ε</sub> | Hydroxymethyluridine | 24 | 52 | 16.7 | 16.5 |
| φ<sub>ε</sub> | Hydroxymethyluridine and thymidine | 24 | 52 | 17.1 | 19.4 |

<sup>a</sup> Determined by dividing the number average molecular weight of EcoRI digested DNA into the molecular weight of the DNA. The number average molecular weight was determined by fitting a Kuhn distribution to EcoRI digests fractionated by electrophoresis through agarose gels as described in the text.

The predicted number of sites is a function of DNA molecular weight and base composition assuming that the sites are distributed randomly. The molecular weight of a DNA is 3.2 × 10<sup>6</sup>, of PBS2 DNA 1.5 × 10<sup>6</sup>, of T<sub>1</sub> DNA 1.2 × 10<sup>6</sup>, and of φ<sub>ε</sub> DNA and φ<sub>ε</sub> DNA 1.2 × 10<sup>6</sup>.

To determine the number of methylated sites per DNA molecule, 16 µg of each DNA were incubated in 150 µl of 10 mM Tris/HCl, pH 8.0, 10 mM EDTA, and 0.95 µM S-adenosyl-L-[methyl-<sup>3</sup>H]methionine with 2 µg of methylase. After 2 h at 37°C, half of the reaction mixture was precipitated with 3.5% HClO<sub>4</sub>, and 1 µg of additional methylase was added to the remaining mixture. Following a 2-h incubation at 37°C, this DNA was precipitated. The DNA samples were filtered on GF/C filters and counted as described under "Experimental Procedures." Usually no increased methylation was detected after the initial incubation. Under some conditions, however, we have observed amounts of methylation over those described here.

The number of endonuclease sites per molecule was determined by incubating 12 µg of DNA in 10 mM Tris/HCl, pH 7.4, 10 mM MgCl<sub>2</sub>, 50 mM NaCl, and 2 µg of EcoRI endonuclease in a total volume of 150 µl. Aliquots were withdrawn at different times to ensure that the reaction went to completion. DNA 5'-phosphoryl termini were labeled with ³²P, by polynucleotide kinase exchange, and the DNA was precipitated and counted as described under "Experimental Procedures."

Fig. 4. Kinetics of EcoRI digestion of DNAs. To reaction mixtures (150 µl) containing 21.0 µg (λ DNA), 16.5 µg (PBS2 DNA), 14.0 µg (φ<sub>ε</sub> DNA), or 20.1 µg (φ<sub>ε</sub> DNA) DNA, 10 mM Tris/HCl, pH 7.4, 10 mM MgCl<sub>2</sub>, and 50 µM NaCl was added EcoRI endonuclease. For φ<sub>ε</sub> DNA and φ<sub>ε</sub> DNA, 0.6 µg of enzyme was used, for PBS2 DNA 0.3 µg, and for λ DNA 0.06 µg. Aliquots were withdrawn at different times, the enzyme was inactivated by heating at 78°C, and EcoRI endonuclease-generated 5'-phosphoryl termini were quantitated by the polynucleotide kinase exchange reaction, as described under "Experimental Procedures." ○ ○ ○, PBS2 DNA; x --- x, λ DNA; ○ --- ○, φ<sub>ε</sub> DNA; ● --- ●, φ<sub>ε</sub> DNA.

Fig. 5. Quantitation of EcoRI termini by polynucleotide kinase exchange. EcoRI-digested λ DNA (17), ranging in concentration from 0.16 to 3.5 µg/50 µl, was incubated with 0.11 mg/ml of autoclaved gelatin, 18 mM MgCl<sub>2</sub>, 4.5 mM dithiothreitol, 45 mM KCl, 50 mM imidazole/HCl, pH 6.6, 327 µM ADP, 13 µM [γ-³²P]ATP, and 2 units of polynucleotide kinase for 10 min at 37°C. The DNA was precipitated, filtered, and counted as described under "Experimental Procedures."

The initial rates of cleavage of φ<sub>ε</sub> DNA and φ<sub>ε</sub> DNA are plotted as a function of DNA concentration in Fig. 6a. The two DNAs provide very similar kinetic data, K₉ values of 110 µM DNA-phosphate and 150 µM DNA-phosphate, respectively, and maximal rates of 1.05 pmol of DNA-phosphate/s/µg. These maximal rates are much lower than for PBS2 DNA (49.5 pmol of DNA-phosphate/s/µg) or for λ DNA (43.5 pmol of DNA-phosphate/s/µg) (Fig. 6b). However, the K₉ values for each of

mente described under "Effect of Concentration of Cleavage Sites upon EcoRI Endonuclease Digestion of DNAs."

Effect of Concentration of Cleavage Sites upon EcoRI Endonuclease Digestion of DNAs - To examine the dependence of the initial rate of EcoRI endonuclease digestion upon the concentration of cleavage sites, λ DNA, φ<sub>ε</sub> DNA, φ<sub>ε</sub> DNA, and PBS2 DNA were each incubated at different DNA concentrations with a constant amount of EcoRI endonuclease. The quantities of 5'-phosphoryl termini generated were measured using the polynucleotide kinase exchange assay. A sample time course digestion for each DNA is shown in Fig. 4. In preliminary experiments, we demonstrated that the exchange assay provides a reliable means to measure variable numbers of 5'-phosphoryl termini at varying DNA concentrations, since the extent of exchange of γ-³²P with 5'-phosphoryl termini is independent of the DNA concentration over the concentration range used here (Fig. 5).
EcoRI Activity on Modified DNAs

FIG. 6. Dependence of EcoRI endonuclease cleavage upon DNA concentration. In 150 μl of 10 mM Tris/HCl, pH 7.4, 10 mM MgCl₂, and 50 mM NaCl, from 0.5 to 25 μg of DNA was incubated with EcoRI endonuclease. For φe DNA and φm DNA, 0.6 μg of enzyme was used, for PBS2 DNA 0.3 μg and for λ DNA 0.06 μg. All rates were normalized to a constant amount of EcoRI endonuclease (0.03 μg).

Aliquots were withdrawn at timed intervals and the digestion was stopped by heating at 72°C. The number of 5'-phosphoryl termini in each aliquot was quantified by the polynucleotide kinase exchange reaction. In a, the DNAs are ○ ○, φe DNA; △ △, φm DNA; and in b, they are △ △, λ DNA; ○ ○, PBS2 DNA.

FIG. 7. Isopycnic banding of [³²P]DNAs labeled by the polynucleotide kinase exchange reaction. An equimolar mixture of λ DNA and φe DNA (5 μg each) was sheared, labeled with [³²P], by polynucleotide kinase exchange and then banded in CsCl, as described under "Experimental Procedures." A density standard of λ [¹H]DNA was included in the gradient. Similar results were obtained with λ DNA mixed with φm DNA, PBS2 DNA, and T₄ DNA, although in the latter case the DNAs were not as well resolved in CsCl.

hydrogen atom does not dramatically affect either the $K_{eq}$ or $V_{max}$. Modification of cytosine to glucosylated hydroxymethylcytosine renders the DNA insensitive to the endonuclease.

Methylation of Phage DNAs by EcoRI Methylase — We examined the capacity of the methylase to act on DNAs containing modified pyrimidines, to determine whether the effect of each modification was the same upon methylase activity as

the four DNAs differ only slightly (200 μM DNA-phosphate for λ DNA, and 400 μM DNA-phosphate for PBS2 DNA).

Labeling of Modified DNAs by Polynucleotide Kinase Exchange — In the previous experiments in which polynucleotide kinase exchange was employed to label the 5'-phosphoryl termini of EcoRI endonuclease-digested DNAs, an assumption was made that the extent of labeling was unaffected by the modifications at the pyrimidine ring within the DNA. Several studies using polynucleotide kinase phosphorylation support this assumption (34, 35), since fingerprinting of DNAs and RNAs labeled either by polynucleotide kinase phosphorylation in vitro or by in vivo incorporation provide identical results. Furthermore, the actual nucleoside which accepts the phosphate from [³²P]ATP is adenosine, rather than a modified nucleoside (36).

To provide further support for the notion that the differences in exchange described previously reflect the concentrations of 5'-phosphoryl termini, and not the reactivity of the substrate with polynucleotide kinase, experiments were performed to show that polynucleotide kinase labels PBS2, φe, φm, and T₄ DNAs equally as well as λ DNA. Equimolar DNA-phosphate mixtures of each DNA were mixed with λ DNA and each was sheared by repeated passages through a syringe. The DNAs were then labeled with polynucleotide kinase, using the exchange reaction, and divided into two parts. One-half of each mixture was acid-precipitated, and the total amount of [³²P] incorporation into termini was measured (Table II). In each case, the polynucleotide kinase exchange reaction labeled the DNAs with modified bases equally as well as normal DNA. The other half of each sample was band in CsCl and the extent of incorporation into each DNA measured after separation by its buoyant density. Again, all the modified DNAs were labeled as efficiently as the DNA containing thymidine (Fig. 7). Although the termini produced by shearing do not have the sequence of the EcoRI termini, these results argue that the polynucleotide kinase does not differentiate between DNA containing a pyrimidine other than thymine and normal DNA.

In summary, these and the previous experiments indicate that the EcoRI endonuclease differentiates among DNAs that contain modifications at position 5 of the thymine and cytosine rings. The substitution of the methyl group of thymidine by a

TABLE II

| DNA mixture | cpm incorporated/μg total DNA |
|-------------|-----------------------------|
| λ/λ         | 934                         |
| λ/PBS2      | 1011                        |
| λ/φe        | 1104                        |
| λ/T₄        | 1115                        |
| λ/φm        | 1039                        |

In summary, these and the previous experiments indicate that the EcoRI endonuclease differentiates among DNAs that contain modifications at position 5 of the thymine and cytosine rings. The substitution of the methyl group of thymidine by a
The EcoRI methylase, it is possible to obtain an independent measure of the number of EcoRI recognition sites in every DNA that is susceptible to methylation. In those instances where a comparison is possible (T, and λ DNA), the results of this analysis (Table I) agree with the estimates obtained by exchange of 32P into 5'-phosphoryl termini, or by statistical analysis of the number average molecular weight distribution of fragments. Occasionally, however, we have observed that greater numbers of methyl groups can be introduced by increasing the enzyme concentration. The significance of this overmethylation is being studied.

Both T, DNA and λ DNA possess less than one-half the number of EcoRI recognition sites per molecule that would be predicted for their size and base composition. This difference may result from selection of T, or λ DNA against sequences that sensitize them to restriction (29, 37). Bacteriophage λ DNA has only five EcoRI sites, yet 12 would be predicted on the basis of its size.

**DISCUSSION**

The manner by which proteins recognize specific sites in DNA is a fundamental, unsolved problem in molecular biology. Restriction endonucleases and methylases which act on distinct nucleotide sequences provide an excellent opportunity to explore DNA-protein interactions, since they produce an easily quantifiable event. We have studied the effect of modifications at position 5 of pyrimidines in DNA upon the activities of the EcoRI endonuclease and methylase, both of which recognize and act upon the sequence (5’p)GAATTTC in duplex DNA. It is clear from our studies that modifications of the pyrimidines in this sequence affect cleavage and methylation. Because the modifications have different effects upon the rate of cleavage and methylation, each modification will be treated independently in this discussion.

The EcoRI endonuclease does not cleave DNA with complete substitution of cytosine by hydroxymethylcytosine, that is esterified with glucose (38). This is in agreement with the observations of Kaplan and Nierlich (39).

Our data indicate that DNA containing hydroxymethyluracil is a considerably poorer substrate than DNA containing either uracil or 5-methyluracil (thymine). The rate of cleavage is diminished, as demonstrated by a 20-fold reduction in $V_{max}$. Itos et al. (40) have noted the susceptibility to cleavage of a similarly modified DNA (from phage SP01). The method that they used (agarose gel electrophoresis) was qualitative and thus the relative susceptibility of SP01 DNA and normal DNA to cleavage could not be evaluated. Our results, then, are not inconsistent with theirs.

For both modified pyrimidines (glucosylated hydroxymethylcytidine and hydroxymethyluridine), the modifications considerably increase the van der Waals radius of the group at position 5 in the pyrimidine ring, and may alter catalytic activity and/or binding simply by steric hindrance. These groups lie in the major groove of the DNA double helix, and therefore may make contact with an opposing group in the protein. The EcoRI endonuclease is already known to be extremely sensitive to the replacement of a small hydrogen atom in the major groove by a methyl group, as methylation of the central adenosine in the recognition sequence prevents cleavage (15).

Substitution of the methyl group in thymidine by a hydroxymethyl group, as in PBS2 DNA, has little effect upon the susceptibility of DNA to cleavage. As the number of sites cleaved is approximately what one might predict for the size of the PBS2 genome, it is not likely that the EcoRI endonuclease recognizes a sequence in this DNA that is significantly different than the canonical sequence. From these observations, it can be argued that the methyl group per se of thymidine is not an important contact point that the enzyme utilizes for sequence specificity.

The susceptibility to cleavage of each of the DNAs containing modifications at position 5 of the uracil ring (either hydroxymethyl, CH₂ or CH₂OH) varies with the electronegativity of the substituents. Thus, a possible explanation for the differential susceptibility of each of these DNAs would have the interaction between protein and DNA be dependent upon the electronegativity of the atom at position 5 of the uracil ring. A polarizable group, such as a hydroxymethyl might not interact with a hydrophobic pocket on the enzyme in the manner required for activity.

The results of modifications at position 5 in the pyrimidine ring of DNAs upon the activity of the EcoRI endonuclease is made particularly interesting by comparison with their effect upon the activity of the EcoRI methylase. The EcoRI methylase acts as readily upon T, DNA containing glucosyl hydroxymethylcytosine as on unmodified λ DNA. Here, it appears, the presence of a large bulky group in the major groove has no effect upon the acceptability of a DNA as a substrate. In contrast, at the neighboring thymine residues substitution of methyl by hydroxymethyl alters susceptibility. Replacement
by a hydrogen atom renders the DNA insusceptible to methylation. These effects are almost the reverse of those observed with the endonuclease. It appears then that the EcoRI methylase has a strong requirement for some substituent on position 5 of the uracil ring. In a comparison of the phage φX174 and φM13, it was found that the two DNAs do not show much difference either in rates of digestion or methylation. Although with both enzymes, DNA appears to react slightly faster than φX DNA, the difference is within the limits of experimental error and therefore may not be significant. The degree of substitution of thymine for hydroxymethyl uracil in φM13 is not precisely known. Marcus and Newlon (20), using [methyl-3H]thymine incorporation into phage particles as a criteria, calculated that this substitution is 20%. A value of 7 to 11% was determined by Dunham, using physical techniques (19). In a preparation of [methyl-3H]thymine-labeled φM13 DNA, we obtained DNA with about 10% thymine incorporation, not correcting for endogenous thymine pool dilutions (data not shown). If a substitution value of at least 10% is assumed, it is interesting that φX DNA and φM13 DNA react similarly in both their digestion and methylation.

If φM13 DNA consisted of fast reacting (i.e. thymine in the EcoRI sequence) and slow reacting (i.e. only hydroxymethyluracil in the EcoRI sequence) fractions, this would have been detected for either reaction. If the thymine bases in φM13 DNA are distributed randomly in the DNA sequence, then it is likely that no 4 thymine residues are present in the same EcoRI recognition sequence. Thus it appears that hybrid EcoRI sites made up of both thymine and hydroxymethyluracil are poor substrates for the methylase and endonuclease as positions completely substituted with hydroxymethyluracil.

It can be argued that the substitution of an unusual base in DNA might affect the EcoRI endonuclease or methylase specificity such that the size of the recognition site is altered. This does not appear to be the case here. The number of EcoRI sites could be determined from the amount of [3H]methyl incorporated, from the amount of 32P, exchanged with endonuclease generated 5'-phosphoryl termini and from the number average molecular weight of the digestion products. For each DNA the number of calculated sites agreed fairly well with the number of predicted sites. The DNAs from φX and φM13 DNA were distributed randomly in the DNA sequence, and it is likely that no 4 thymine residues are present in the same EcoRI recognition sequence. Thus it appears that hybrid EcoRI sites made up of both thymine and hydroxymethyluracil are poor substrates for the methylase and endonuclease as sites completely substituted with hydroxymethyluracil.

The EcoRI endonuclease has been found by Polinsky et al. (41) to be capable of recognizing and cleaving the duplex sequence

\[(5')pAATT\]
\[\ldots\]
\[TTAAp(5')\]

under modified conditions of pH, ionic strength, and enzyme concentration. They have designated this new activity EcoRI* and maintain, both by genetic and biochemical arguments, that it is a property of the EcoRI endonuclease rather than a contaminant activity. However, the EcoRI* activity is approximately 1% that of the EcoRI activity. Furthermore, even though the EcoRI sequence is only 1 of 16 sequences cleaved by the EcoRI endonuclease, it is the most rapidly cleaved under all conditions examined. It is not likely that the EcoRI* activity is a significant factor in our studies.

Based on the observations that restriction enzymes are highly discriminatory and upon the palindromic nature of the cleavage sites, it has been suggested that the nucleotide sequence of the recognition site may facilitate intrachain base-pairing to produce a cruciform structure that can be recognized by the restriction enzyme. One important feature of the cruciform model is that distinguishing features of the bases at a recognition-cleavage site are very accessible to specific interactions with enzymes, possibly, it has been argued, more than they would be in the bihelical structure. Thus, in the EcoRI endonuclease site a cruciform rearrangement, according to the model of Meselson et al. (6), would result in the central A/T and T/A base pairs being exposed in a way free of basepairing constraints, with their side groups more accessible to probing by the EcoRI endonuclease.

Greene et al. (12), using a synthesized self-complementary octanucleotide containing the EcoRI endonuclease cleavage site, showed by computer analysis of initial reaction rate data that the octanucleotide must be present as a dimer to be a substrate. They interpret these kinetic data to argue against a cruciform rearrangement. However, they also found that the K_0.5 for the octamer was 200 times higher than for SV40 DNA. This value reflects the affinity of the enzyme for each substrate, since the K_0.5 values for each were the same. Thus, it may still be the case that the usual substrate for the EcoRI endonuclease is a looped out structure, and that the synthetic bihelical octamer is a poor substrate with a low affinity for the enzyme, due to its inability to form a stable cruciform.

Alternatively, a number of models have been proposed to describe how proteins can recognize specific nucleotide sequences of bihelical DNA (6, 10, 14, 42). If the DNA maintains a bihelical structure as it binds to the enzyme, the central A/T and T/A base pairs in the EcoRI site are likely to be important. Interactions of proteins with the thymine methyl group are one of the contact points considered to be important in models which describe protein interactions with DNA in a rigid double helical form (5, 13). From our studies it is apparent that the substituent at position 5 of the uracil ring is one of the features that the EcoRI endonuclease probes, since a replacement of the thymine by a hydroxymethyluracil can decrease the enzyme's activity.

The observed preference of the EcoRI endonuclease for DNAs containing thymine or uracil, rather than hydroxymethyluracil is apparently unrelated to the thermal stability of the DNAs with these modified nucleosides. Alternating deoxyribocopolymers of d(A-hmU) and d(A-T) all have similar T_m values (43). Thus, whether the bihelical DNA sequence alone or a structural change in the duplex DNA of a given sequence is responsible for the specific recognition and interaction of enzymes with DNA is not yet known. Both possibilities permit interactions between a protein and position 5 of the pyrimidine ring.

It would be of interest to look at more DNAs with different base substitutions, but the number of naturally occurring DNAs which contain unusual bases is limited. We are presently examining the kinetics of cleavage and methylation of DNAs containing halogenated pyrimidines. An alternative approach which has proven useful is to prepare synthetic oligonucleotides with different base substitutions. Greene et al. (12) have prepared a self-complementary oligonucleotide with a uracil substituted for a cytosine within the canonical EcoRI endonuclease sequence. This oligonucleotide could not serve as a substrate for the EcoRI endonuclease, due to its destabilizing effect on the duplex structure of the substrate.

Whether or not it would affect the endonuclease's activity in some other way is not known. In a high molecular weight DNA duplex structure this effect of an unusual base on the
melting temperature of the molecule would not be as significant.

We observed, as have Greene et al. (16) that the EcoRI methylase does not show a linear relationship between enzyme concentration and initial velocity. This was seen with $\lambda$ DNA and T, DNA. However, $\phi$ DNA and $\phi$ m, DNA were both methylated at rates that are directly proportional to the amount of methylase added. It has been suggested that the methylase, which exists as a monomer in solution, needs to dimerize in order to modify its DNA substrate (12). With increasing enzyme concentrations, then, dimerization of the enzyme is enhanced. If this is so, the linearity of $\phi$ DNA and $\phi$ m, DNA can be explained, since at the very high concentrations of methylase needed to modify them, the enzyme is already maximally dimerized. Contrary to this observation by Jane Bancuk, for the advice and space provided by Dr. Al Berkner, K. L., and Folk, W. R. (1976) Fed. Proc. 35, 1680-1686.

An Acknowledgments—We are grateful for assistance provided by Jane Bancuk, for the advice and space provided by Dr. Al Berkner, K. L., and Folk, W. R. (1976) Fed. Proc. 35, 1680-1686.

REFERENCES

1. Berkner, K. L., and Folk, W. R. (1976) Fed. Proc. 35, 1680
2. Arber, W. (1974) Prog. Nucleic Acid Res. Mol. Biol. 14, 1-37
3. Boyer, H. W. (1971) Annu. Rev. Microbiol. 25, 153-176
4. Nathans, D., and Smith, H. O. (1975) Annu. Rev. Biochem. 44, 273-293
5. Von Hippel, P. H., and McGhee, J. D. (1972) Annu. Rev. Biochem. 41, 231-300
6. Meselson, M., Yuan, R., and Heywood, J. (1972) Annu. Rev. Biochem. 41, 447-466
7. Gierer, A. (1969) Nature 221, 1480-1481
8. Sobell, H. M. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 2483-2487
9. Wang, J. C., Barkley, M. D., and Bourgeois, S. (1974) Nature 251, 247-249
10. Richmond, T. J., and Steitz, T. A. (1976) J. Mol. Biol. 101, 25-38
11. Maniatis, T., and Ptashne, M. (1973) Proc. Natl. Acad. Sci. U. S. A. 70, 1531-1535
12. Greene, P. J., Poonian, M. S., Nussbaum, A. L., Tobias, L., Garfin, D. E., Boyer, H. W., and Goodman, H. M. (1975) J. Mol. Biol. 99, 237-261
13. Seeman, N. C., Rosenberg, J. M., and Rich, A. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 804-808
14. Adler, K., Beyreuther, K., Panning, E., Geisler, N., Gronenberg, B., Klemm, A., Müller-Hill, B., Pfahl, M., and Schmitz, A. (1973) Nature 247, 322-327
15. Dugas, L., and Hedgcock, J., Boyer, H. W., and Goodman, H. M. (1974) Biochemistry 13, 500-512
16. Greene, P. J., Betlach, M. C., Boyer, H. W., and Goodman, H. M. (1974) Methods Mol. Biol. 7, 87-111
17. Berkner, K. L., and Folk, W. R. (1977) J. Biol. Chem. 252, 3176-3184
18. Roscoe, D. H., and Tucker, R. G. (1966) Virology 29, 465-466
19. Dunham, L. (1973) Ph.D thesis. University of Michigan. Ann Arbor, Mich.
20. Marcus, M., and Newlon, M. C. (1971) Virology 44, 83-93
21. Prasher, D. and Jerrel, E. A. (1973) J. Biol. Chem. 248, 2411-2415
22. Hall, D. H., Tevlin, T., and Karlström, O. (1967) Virology 31, 442-448
23. Sonenshein, A. L., and Roscoe, D. H. (1969) Virology 39, 265-276
24. Price, A. R., and Cook, S. J. (1972) J. Virol. 9, 602-610
25. Burton, K. (1956) Biochem. J. 62, 315-323
26. Lovin, D., and Hutchinson, F. (1972) J. Mol. Biol. 75, 495-509
27. Takahashi, I., and Marr, J. (1963) Nature 197, 794-795
28. Sober, H. A., ed (1973) CRC Handbook of Biochemistry, Molecular Biology, H-9
29. Mertz, E. J., and Davis, R. W. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 3370-3374
30. Lauter, U. K. (1970) Nature 227, 690-695
31. Thomas, M., and Davis, R. W. (1975) J. Mol. Biol. 91, 315-328
32. Botchan, M., McKenna, G., and Sharp, P. A. (1973) Cold Spring Harbor Symp. Quant. Biol. 38, 385-390
33. Thomas, M., and Davis, R. W. (1975) J. Mol. Biol. 75, 258-278
34. Lauer, G. D., and Klotz, L. C. (1976) J. Virol. 18, 1163-1164
35. Sanger, F. (1977) J. Mol. Biol. 30, 704-710
36. Murray, K. (1973) Biochem. J. 131, 569-583
37. Arber, W., and Linn, S. (1969) Annu. Rev. Biochem. 38, 467-500
38. Lehman, I. R., and Pratt, E. A. (1969) J. Biol. Chem. 245, 3254-3259
39. Kaplan, D. A., and Nierlich, D. P. (1975) J. Biol. Chem. 250, 2395-2397
40. Ro, J., Kawamura, F., and Duffy, J. J. (1975) FEBS Lett. 55, 278-281
41. Price, A. R., and Cook, S. J. (1973) J. Mol. Biol. 101, 25-38
42. Polinsky, B., Greene, P., Garfin, D. E., McCarthy, B. J., Goodman, H. M., and Boyer, H. W. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 3105-3114
43. Gilbert, W., Maizels, N., and Maxam, A. (1973) Cold Spring Harbor Symp. Quant. Biol. 38, 845-855
44. Sober, H. A., ed (1973) CRC Handbook of Biochemistry, Molecular Biology, H-22
EcoRI cleavage and methylation of DNAs containing modified pyrimidines in the recognition sequence.
K L Berkner and W R Folk

J. Biol. Chem. 1977, 252:3185-3193.

Access the most updated version of this article at http://www.jbc.org/content/252/10/3185

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/252/10/3185.full.html#ref-list-1