Proc. Nat. Acad. Sci. USA
Vol. 72, No. 9, pp. 3310–3314, September 1975
Biochemistry

Specificity of substrate recognition by the *Eco*RI restriction endonuclease

(DNA/plasmid/simian virus 40/modification)

BARRY POLISKY*, PATRICIA GREENE†, DAVID E. GARFIN†, BRIAN J. MCCARTHY*, HOWARD M. GOODMAN*, AND HERBERT W. BOYER†

* Department of Biochemistry and Biophysics and † Department of Microbiology, University of California, San Francisco, San Francisco, Calif. 94143

Communicated by Norman Davidson, May 23, 1975

ABSTRACT **The substrate specificity of the *Eco*RI restriction endonuclease can be varied *in vitro* by changing the pH and the ionic environment of the reaction. Phosphodiester bond cleavage occurs at a DNA hexanucleotide sequence $\frac{\text{d(N-G-A-A-T-T-C-N)}}{\text{d(N-C-T-T-A-A-G-N)}}$ when the ionic strength is high, 100 mM Tris·HCl, 50 mM NaCl, 5 mM $MgCl_2$, and the pH is approximately 7.3. Lowering the ionic strength to 25 mM Tris·HCl, 2 mM $MgCl_2$, and adjusting the pH to 8.5 reduces the recognition specificity of the *Eco*RI endonuclease to the tetranucleotide sequence, $\frac{\text{d(N-A-A-T-T-N)}}{\text{d(N-T-T-A-A-N)}}$. The enzymatic activity responsible for this substrate recognition is referred to as *Eco*RI*. Cleavage of pVH51 plasmid DNA under *Eco*RI* conditions results in a number of partial digest fragments, some of which disappear slowly over a prolonged digestion period. This suggests that different recognition sites are cleaved at different rates. Comparison of DNA fragment patterns of modified and unmodified pVH51 DNA indicates that the canonical *Eco*RI sequence is the most rapidly cleaved site under *Eco*RI* conditions. DNA modified *in vivo* by the *Eco*RI methylase is not cleaved by the *Eco*RI endonuclease under standard conditions, but is cleaved under *Eco*RI* conditions at sites other than the standard *Eco*RI substrate.**

Type II restriction endonucleases and modification methylases are widespread in the microbial world. The substrate specificities of a number of these enzymes have been determined (1–4). The substrates are symmetrical DNA sequences of 4 to 6 nucleotide base pairs. The *Eco*RI restriction endonuclease and modification methylase recognize and enzymatically alter the symmetrical sequence $\frac{\text{d(N-G}\downarrow\text{-A-A}^{*}\text{-T-T-C-N)}}{\text{d(N-C-T-T-A}^{*}\text{-A}\uparrow\text{-G-N)}}$ (5, 6) where the arrows designate the positions of phosphodiester bond cleavage and the asterisks designate methylated nucleotides. These reactions occur at the level of duplex DNA, without involvement of cruciform or "hairpin" structural rearrangements of the polynucleotide strands (Greene *et al.*, manuscript submitted).

Under conditions producing the maximum rate of endonucleolytic cleavage of unmodified DNA the *Eco*RI endonuclease yields limit digests (7–9). Specific alteration of the standard *Eco*RI endonuclease reaction conditions reduces the substrate recognition of the *Eco*RI endonuclease to the tetranucleotide, $\frac{\text{d(N}\downarrow\text{-A-A-T-T-N)}}{\text{d(N-T-T-A-A}\uparrow\text{-N)}}$. In this paper we present a preliminary analysis of this activity, referred to as the *Eco*RI* activity of the *Eco*RI endonuclease.

Abbreviations: dN, any of the four standard deoxyribonucleosides; NP40 is Nonidet P40, a nonionic detergent from Shell Chemical Company; SV40, simian virus 40; col E1, colicin E1 plasmid; r_{RI} and m_{RI}, RI host restriction and modification phenotypes; ASV, avian sarcoma virus.

MATERIALS AND METHODS

Strains and Plasmids. *Escherichia coli* strain HB129 was derived from an endonuclease I deficient *E. coli* 1100 (10, 11). MB100 was derived from HB129 by transformation with the plasmid pMB1 (molecular weight 5.5×10^6 daltons), which is similar to colicin E1 (col E1) (12) except that it carries an additional 1.3×10^6 dalton piece of DNA containing the *Eco*RI restriction and modification genes (M. C. Betlach, unpublished observation). Strain MB101 was derived from HB129 by introduction of the pMB2 plasmid, which was derived from pMB1 by *Hin*dIII endonuclease digestion of pMB1 and removal of a fragment containing the *Eco*RI genes. Strain MV5 is a derivative of *E. coli* C600 and contains the pVH51 plasmid of 2.1×10^6 daltons (13). Strain RY25 contains a mutant derivative of pMB1 which expresses an r^-_{RI} m^+_{RI} (host restriction and modification, respectively) phenotype (14).

Enzymes and DNA. The *Eco*RI methylase was purified as described elsewhere (15). The *Eco*RI endonuclease was purified by a modification of a published procedure (16) in which the DEAE-cellulose chromatography step is replaced by chromatography on CM-cellulose. The CM-cellulose chromatography was carried out as described for the *Eco*RI methylase purification (15). *Eco*RI endonuclease was stored in 500 mM K_2HPO_4–KH_2PO_4, pH 7.0, 200 mM NaCl, 7 mM 2-mercaptoethanol, and 0.2% of Nonidet P40 (NP40). *E. coli* polynucleotide ligase was purified according to the method of Modrich *et al.* (17). RNA-directed DNA polymerase from avian sarcoma virus (ASV polymerase) was prepared (18) from virions of the Prague C strain of ASV provided by J. M. Bishop. Polynucleotide kinase was purchased from P-L Biochemicals, Inc. All other enzymes were obtained from the Worthington Biochemical Corp. [^{3}H]dTTP was from Schwarz/Mann and α-^{32}P-labeled dATP and dTTP were from New England Nuclear. The preparation of [γ-^{32}P]rATP has been described (5). Plasmid DNA was purified from cleared lysates (19) by centrifugation to equilibrium in CsCl–propidium diiodide gradients. Simian virus 40 (SV40) DNA was purified as described previously (6).

Other Procedures. Electrophoresis of DNA in agarose gels has been described (9, 16). Gels were soaked in dilute ethidium bromide (1 μg/ml) and the fluorescing DNA was pho-

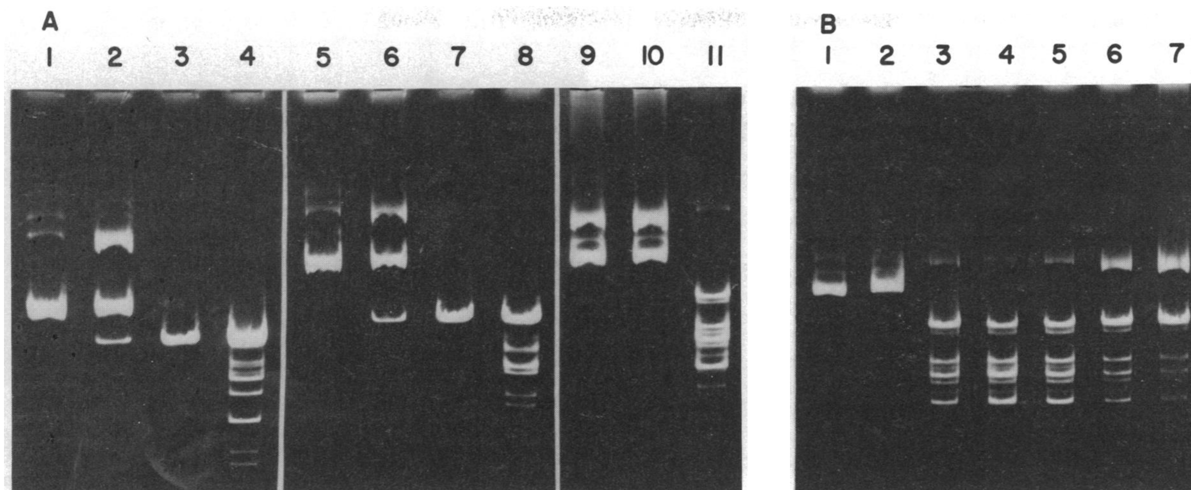


FIG. 1. (A) *Eco*RI endonuclease reaction under standard and *Eco*RI* conditions. Lanes 1–4 contain 0.2 μg of SV40 DNA, 5–8 contain 0.22 μg of col E1 DNA (both of these substances contain one unmethylated *Eco*RI substrate sequence). Lanes 9–11 contain 0.2 μg of pMB1 DNA (this substrate is methylated *in vivo* at the *Eco*RI sequences). Reactions were carried out in 20 μl. Samples run in lanes 1–3, 5–7, 9, and 10 were from reactions incubated in 0.1 M Tris·HCl, pH 7.2, 0.05 M NaCl, 5 mM $MgCl_2$ (standard reaction conditions) at 37° for 5 min. Samples run in lanes 4, 8, and 11 were from reactions incubated in 25 mM Tris·HCl, pH 8.5, 2 mM $MgCl_2$ (*Eco*RI* conditions) at 37° for 15 min. Samples run in lanes 1, 5, and 9 were from reactions with no enzymes. The SV40 and col E1 DNA in lanes 1 and 5 is predominantly supercoil. The pMB1 DNA is a mixture of supercoil and open circle. Lanes 2 and 6 were from reactions incubated with 0.005 units of *Eco*RI and under conditions described. Unit length, linear DNA migrates fastest, then supercoiled DNA and open circular DNA. Lanes 3, 4, 7, 8, 10, and 11 were from reactions with one unit of *Eco*RI endonuclease. Under standard *Eco*RI conditions SV40 DNA (lane 3) and col E1 DNA (lane 7) are converted entirely to unit length linears; while pMB1 DNA (lane 10) is uncleaved. Under *Eco*RI* conditions (lanes 4, 8, and 11) all three DNAs are cleaved at several sites. *Eco*RI endonuclease cannot be stored or diluted into low ionic strength buffer. Thus, the addition of enzyme adds the following components (final concentration) to the reaction mixture: 12.5 mM KPO_4, 5 mM NaCl, 0.005% NP40, 0.175 mM 2-mercaptoethanol, and 2.5 μM EDTA. The Tris–borate buffer used for gel electrophoresis, staining, and ultraviolet photography has been described previously (16). SV40 (1–4) and col E1 (5–8) DNAs were separated on 1% gels run at 18 V/cm for 1.5 hr. pMB1 DNA was separated on 0.8% gels run at 14 V/cm for 1.5 hr. (B) Magnesium ion optimum for *Eco*RI* endonuclease activity. pMB1 DNA (0.2 μg) plus one unit *Eco*RI endonuclease were incubated in 20 μl of 25 mM Tris·HCl, pH 8.5, and varying concentrations of $MgCl_2$ for 5 min at 37°. Lanes 1–7 have: no enzyme, no $MgCl_2$, 1 mM $MgCl_2$, 2 mM $MgCl_2$, 3 mM $MgCl_2$, 4 mM $MgCl_2$, 5 mM $MgCl_2$, in that order.

tographed while illuminated by a short wave ultraviolet light source (9, 16). Procedures for nucleotide sequence analyses have been described (4). All pH values refer to the pH at 37°. Trizma-base was titrated with HCl at room temperature (24–26°) to the desired pH, which is 0.3 of a pH unit higher than at 37°.

RESULTS

Reaction Conditions for *Eco*RI and *Eco*RI* Endonucleolytic Activities. Incubation of supercoiled col E1 or SV40 DNA with the *Eco*RI endonuclease in the presence of 100 mM Tris·HCl, pH 7.2, 5 mM $MgCl_2$, 50 mM NaCl at 37° results in the production of unique linear molecules as a limit product (Fig. 1A, lanes 3 and 7). Under the same conditions with excess *Eco*RI endonuclease, pMB1 plasmid DNA, modified *in vivo* by the *Eco*RI methylase, remains intact (Fig. 1A, lane 10). However, in the absence of NaCl and at a reduced concentration (25 mM) of Tris·HCl, pH 8.5, SV40, col E1, and the *Eco*RI-modified pMB1 plasmid DNAs are cleaved into a number of small fragments (Fig. 1A, lanes 4, 8 and 11).

The optimum conditions for this endonucleolytic activity, referred to as *Eco*RI*, were determined by qualitatively estimating the extent of cleavage of pMB1 DNA on agarose gels (e.g., see Fig. 1B). The *Eco*RI* activity was found to be optimal at 37°, pH 8.5, 25 mM Tris·HCl, and 2 mM $MgCl_2$. No other buffer [glycine–NaOH, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) and *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Tes)] examined was more effective than Tris·HCl in the same pH range. Maximum *Eco*RI endonucleolytic activity is limited to the narrow pH range of 7.0–7.5, while the *Eco*RI* endonucleolytic activity is maximal between 8 and 9.5. At a pH of 10.0 the *Eco*RI* activity is noticeably reduced. The *Eco*RI endonucleolytic activity is maximal over a broad range of ionic strength; 3–10 mM $MgCl_2$, 25–100 mM Tris·HCl, and 50–120 mM NaCl, while the *Eco*RI* endonucleolytic activity is evident over a narrow range of ionic strength, being maximal at 2 mM $MgCl_2$ (Fig. 1B) and 25 mM Tris·HCl. The *Eco*RI* activity is reduced by increasing the concentration of Tris·HCl and/or NaCl to 25–50 mM. *Eco*RI* activity is not detectable under the standard *Eco*RI cleavage conditions (Fig. 1A, lane 10).

Correlation of the *Eco*RI* Endonucleolytic Activity with the *Eco*RI Endonuclease. Several observations suggest that the *Eco*RI endonuclease is responsible for both the *Eco*RI and *Eco*RI* activities. The most convincing evidence is provided by examination of extracts of strains with genetically altered plasmids defective in *Eco*RI restriction activity. Extracts of strains MB101 (r^-_{RI} m^-_{RI}) and RY 25 (r^-_{RI} m^+_{RI}) purified through the phosphocellulose chromatographic step exhibit neither *Eco*RI nor *Eco*RI* activity. Both activities copurify on phosphocellulose from an extract of MB100 (r^+_{RI} m^+_{RI}) and the most purified *Eco*RI endonuclease preparations (greater than 99% pure) have *Eco*RI* activity. These observations suggest that one gene is responsible for both the *Eco*RI and *Eco*RI* endonucleolytic activities.

Limit Digests. The *Eco*RI* endonucleolytic digestion of SV40 or pMB1 DNA results in a complex fragment pattern. To examine the time course of *Eco*RI* digestion, a smaller supercoiled DNA molecule, pVH51 plasmid DNA (2.1×10^6 daltons), was used as a substrate. The *Eco*RI* digestion products of pVH51 DNA were analyzed by agarose gel electrophoresis (Fig. 2, Table 1). Complete conversion of the cir-

Table 1. *Eco*RI* digestion of pVH51 plasmid DNA

Fragment molecular weight × 10^{-6}	% of total DNA after digestion for:				
	5 min	20 min	1 hr	3 hr	16 hr
2.1	77	40	5.8	—	—
1.9	20	27	21	2.4	—
1.45	1.8	8.5	18	14	4.5
1.3	0.4	3.9	15	42	31
0.95	—	1.7	0.8	3.6	24
0.86	1.2	6.5	2.1	—	—
0.75	—	0.9	4.1	—	—
0.61	—	2.6	11	18	18
0.50	—	8.4	15	17	19
0.35	—	—	—	—	1
0.29	—	—	—	—	1
0.23	—	—	—	1.9	1.7

Negatives of lanes 2–7 of Fig. 2 were scanned at 500 nm in a Beckman Acta CIII spectrophotometer. Mobilities relative to SV40 and lambda DNA fragment markers were used to determine molecular weights of pVH51 fragments. The area of each pVH51 fragment in the densitometric scan is presented as a percentage of the total area.

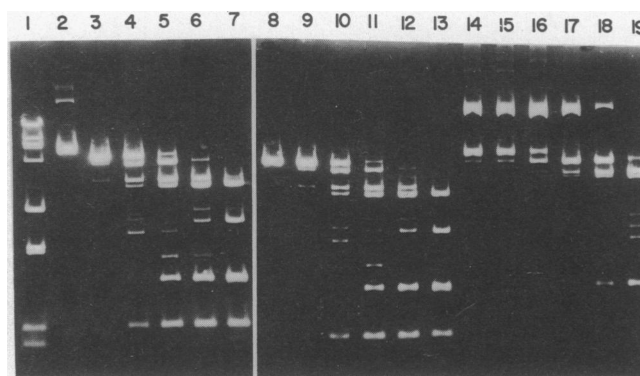


FIG. 2. *Eco*RI* digestion of pVH51 DNA. pVH51 DNA (10 μg) plus 10 units of *Eco*RI endonuclease were incubated in 250 μl of 25 mM Tris·HCl, pH 8.5, 2 mM $MgCl_2$ at 37°. Similarly, 10 μg of pVH51 DNA cleaved at the *Eco*RI site and 10 μg of *Eco*RI methylated pVH51 DNA were digested under *Eco*RI* conditions. Aliquots were removed from each of the three reactions at 5 min, 20 min, 1 hr, 3 hr, and 16 hr and the fragments were separated on 1.2% agarose gels in Tris–borate buffer at 15 V/cm for 1 ¾ hr. Lane 1 molecular weight markers: *Eco*RI digest of lambda DNA plus *Hin*dIII digest of SV40 DNA. Lanes 2–7: *Eco*RI* digest starting with supercoiled pVH51 DNA: 2, no enzyme; 3, 5 min; 4, 20 min; 5, 1 hr; 6, 3 hr; 7, 16 hr. Lanes 8–13: *Eco*RI* digest starting with *Eco*RI linear pVH51; 8, no enzyme; 9, 5 min; 10, 20 min; 11, 1 hr; 12, 3 hr; 13, 16 hr. Lanes 14–19: *Eco*RI* digest starting with *Eco*RI methylated pVH51 DNA; 14, no enzyme: (about half of the supercoiled DNA has been converted to open circles and a small amount to linears during the *in vitro* *Eco*RI methylation), 15, 5 min; 16, 20 min; 17, 1 hr; 18, 3 hr; 19, 16 hr.

cular DNA to a linear form occurs within 5 min, and this partial digestion product persists for one hour. Rapid conversion to linear molecules under *Eco*RI* conditions occurs at the canonical *Eco*RI site. This was demonstrated by using as a substrate DNA modified by the *Eco*RI methylase. Modified DNA is slowly converted to a linear form (Fig. 2 lane 18). Smaller fragments appear before the remaining circular DNA is converted to the linear form. Furthermore, if pVH51 DNA is first cleaved under standard *Eco*RI conditions and then used as a substrate for *Eco*RI*, the time course of appearance of fragments is identical to that of supercoiled pVH51 (Fig. 2, lanes 2–13).

It is evident that some fragments are intermediate products while others accumulate and probably represent limit products (Table 1). We have been unable to obtain a limit digest of this DNA under optimum *Eco*RI* conditions, presumably because some of the *Eco*RI* cleavage sites are much more resistant to cleavage than others.

Cohesive Nature of the Termini Generated by the *Eco*RI* Endonucleolytic Activity. It was of considerable interest to determine if the termini generated by the *Eco*RI* endonucleolytic activity were cohesive, as is the case with the *Eco*RI endonuclease. Fragments of the pMB1 plasmid DNA generated by the *Eco*RI* activity were covalently circularized after treatment for 24 hr at 4° with polynucleotide ligase. Comparison of ligase-treated and untreated control DNA fragments by agarose gel electrophoresis indicated that a large number of the fragment molecules were covalently linked (data not shown). Similar observations were obtained with *Eco*RI*-derived fragments of SV40 DNA.

An independent verification of the cohesive nature of *Eco*RI* termini was provided by ASV-polymerase-mediated repair synthesis of *Eco*RI*-cleaved DNA. The template-primer requirement of the polymerase can only be satisfied if the endonuclease makes staggered breaks with internal 3′-hydroxyl ends and protruding 5′-single-strand ends. Both dATP and dTTP can be incorporated by ASV polymerase into acid-insoluble products using *Eco*RI*-cleaved pMB1 DNA as template-primer (Table 2). This result indicates that the termini at the *Eco*RI* cleavage sites are single-stranded and together with the sequence analysis of the cleavage site (see below) also shows that the ends are complementary. The same type of analysis was previously used (5) to demonstrate the "sticky ends" at the *Eco*RI sites.

The Sequence at the *Eco*RI* Cleavage Site. One explanation for the greater frequency of *Eco*RI* cleavage sites per DNA molecule compared to *Eco*RI sites is that the sequence recognized by *Eco*RI* is shorter, e.g., a tetranucleotide. Among tetranucleotide sequences, an obvious candidate is the central tetranucleotide of the canonical *Eco*RI* substrate, d(pA-A-T-T). That this tetramer is in fact recognized under *Eco*RI* conditions is shown by sequence data obtained from two types of experiments.

The first approach utilized 5′-end labeling and two-dimensional homochromatography procedures. SV40 DNA was cleaved under *Eco*RI* or *Eco*RI conditions and pMB1 DNA was cleaved under *Eco*RI* conditions. The terminal phosphate residues were removed by alkaline phosphatase and replaced with ^{32}P by treatment with polynucleotide kinase and [γ-^{32}P]rATP. Electrophoretic separation of a complete digest of the 5′-terminally labeled DNA to nucleoside 5-monophosphates with pancreatic DNase and snake venom phosphodiesterase indicated that 99% of the radioactivity was in pdA after *Eco*RI* digestion. [pMB1: pdA, 286,550 cpm (99.2%); pdC, 767 cpm (0.3%); pdG, 526 cpm (0.2%); pdT, 800 cpm, (0.3%)]. One- and two-dimensional separations of small terminally-labeled oligonucleotides generated by pancreatic DNase digestion were performed to determine the 5′-terminal sequence at the *Eco*RI* cleavage site. These data (Fig. 3) show that the *Eco*RI* recognition site has the unique sequence d(pA-A-T-T) followed by degeneracy in the fifth position.

A minor related sequence is observed in the homochomatograms of *Eco*RI*-cleaved pMB1 DNA (Fig. 3c). This sequence may be d(pA-T-T-N). For technical reasons, it has been difficult to quantitate this sequence with respect to the principal recognition sequence, d(pA-A-T-T-N). However, it

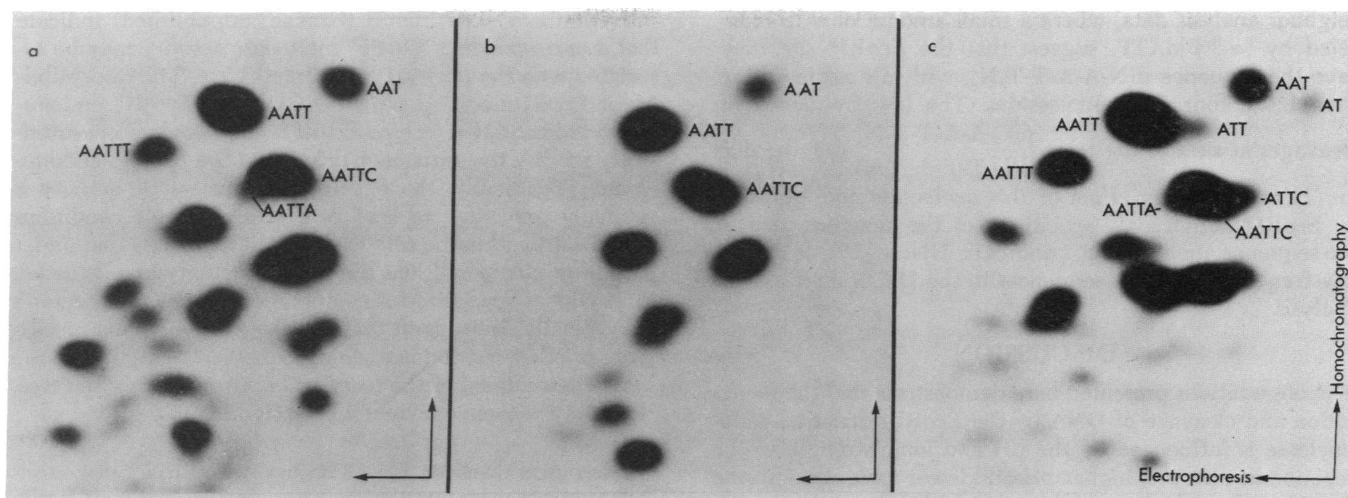


FIG. 3. Pancreatic DNase fingerprints of 5′-^{32}P-labeled *Eco*RI* and *Eco*RI fragments. SV40 DNA (a) and pMB1 DNA (c) were digested under *Eco*RI* conditions as described in the *text*. For purposes of comparison, SV40 DNA was also digested under *Eco*RI conditions (b). Cleavage products were dephosphorylated with alkaline phosphatase and rephosphorylated with [^{32}P]phosphate by enzymatic treatment with polynucleotide kinase and [γ-^{32}P]rATP. The labeled products were digested for 1 hr at 37° with pancreatic DNase at 0.1 mg/ml in 0.01 M Tris·HCl, pH 7.4, 0.01 M $MgCl_2$. Digestion products were fractionated in two dimensions by electrophoresis on cellulose acetate in 7 M urea, pH 3.5, and homochromatography in homomixture C consisting of a 3% solution of 30-min-hydrolyzed RNA. The labeled oligonucleotides were located by radioautography and identified by their positions on the chromatograms. Complete details of the procedure are presented elsewhere together with the methods for verifying the sequences and extension of the nucleotide sequence at the *Eco*RI site of SV40 DNA (Garfin, Boyer, and Goodman, manuscript in preparation). We identify only those oligonucleotides pertinent to our discussion to simplify the figure. All labeled oligonucleotides have 5′-[^{32}P]phosphate groups.

constitutes approximately 10–20% of the cleavages in pMB1 DNA but is virtually undetectable in SV40 DNA.

The second approach to the sequence determination of the *Eco*RI* cleavage site was by analysis of nearest neighbor data obtained from ASV polymerase repair synthesis of *Eco*RI* termini (Table 2). [^{3}H]dTTP incorporation is dependent on the presence of dATP in the reaction mixture (Table 2, lines 1 and 2). This result, together with the nearest neighbor labeling of dAp and dTp by [α-^{32}P]dTTP and of dAp, dGp, and dTp by [α-^{32}P]dATP (Table 2, lines 3 and 4), confirms the tetranucleotide sequence obtained by 5′-end labeling (see above) and also determines the nucleotides on the 5′-side of the phosphodiester bond cleavage; dN in the sequence d(N-A-A-T-T) is 59.2% dG, 26.1% dA, 14.2% dT, and 0.55% dC.

We have not obtained conclusive evidence that the sequence d(pA-A-T-T-G) (from 5′-terminal labeling analysis) occurs at an *Eco*RI* cleavage site. However, weak spots in the fingerprints of *Eco*RI*-treated pMB1 and SV40 DNAs may be d(pA-A-T-T-G), and together with the nearest

Table 2. Nearest neighbor analysis of nucleotides incorporated into *Eco*RI*-endonuclease-treated pMB1 DNA by ASV DNA polymerase

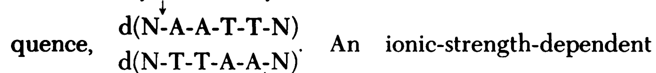
Labeled substrate	Unlabeled substrate	pmol label incorporated per pmol DNA	Relative distribution of radioactivity in nearest neighbor analysis: dCp	dAp	dGp	dTp	Nearest neighbor
1. [^{3}H]dTTP	—	0.1	Not applicable				
2. [^{3}H]dTTP	dATP	7	Not applicable				
3. [α-^{32}P]dTTP	dATP	10	0.008	1	0.006	0.15	dAp[dT], dTp[dT]
4. [α-^{32}P]dATP	dTTP	25	0.04	8.9	4.2	1	dAp[dA], dGp[dA], dTp[dA]

Separate aliquots of *Eco*RI*-digested pMB1 DNA were incubated at 37° with ASV polymerase in 0.1 M Tris-HCl, pH 8, 0.01 M $MgCl_2$, 0.2% 2-mercaptoethanol containing the deoxyribonucleoside trisphophates listed in columns 2 and 3 at 4–10 μM. Reactions were allowed to proceed 2–3.5 hr. The 50 μl ^{32}P-reactions (Nos. 3 and 4) were sampled to test for acid-insoluble products and then passed through 0.8 × 18 cm columns of Sephadex G-75 in 0.1 M NaCl. Incorporation of label (column 4) was estimated from measurements of acid-insoluble radioactivity. Labeled DNA was precipitated twice from 70% ethanol and then digested to nucleoside 3′-monophosphates with micrococcal nuclease followed by spleen phosphodiesterase. The resultant mononucleotides were separated by electrophoresis at pH 3.5 (5% acetic acid, 0.5% pyridine) on Whatman 3MM paper and identified by radioautography. Transfers of [^{32}P]phosphate from input α-labeled nucleoside triphosphates to the growing ends of the repaired DNA strands were determined by liquid scintillation counting of the radioactivity in the 3′-mononucleotides. The relative amounts of the nucleotides labeled in the repair reactions are shown in columns 5–8. The radioactivity obtained from reaction 3 has been normalized to the radioactivity in dAp for comparison purposes. Similarly, the data from reaction 4 have been normalized to dTp. Nearest neighbors are displayed in column 9, where brackets designate the input nucleotides. The information in this table is compatible with the interpretation that 5′-single-stranded DNA "tails" with sequences d(A-A-T-T-N. . .) have been repaired. With input [α-^{32}P]-dTTP (row 3) we would expect equal labeling of dAp and dTp. The 6-fold disparity in radioactivities demonstrates that the second dT-residue has not been completely incorporated. Some of this disparity may be due to the generation by *Eco*RI* of d(A-T-T-N. . .) sequences from pMB1 DNA as mentioned in the *text*. Input [α-^{32}P]dATP (row 4) is expected to transfer label equally to dAp and total (four) dNp. Accounting for this by assuming that half of the total radioactivity results from transfer of label to the first dA-residue incorporated gives the relative proportions of the four dNp's. These, normalized to dTp, are: dCp, 0.04; dAp, 1.8; dGp, 4.2; dTp, 1.0.

neighbor analysis data, where a small amount of dCp is labeled by [α-^{32}P]dATP, suggest that the *Eco*RI* site may have the sequence d(N-A-A-T-T-N), with dN representing any of the four deoxynucleosides. The low frequency of cleavages at sites of the form $\frac{\text{d(C-A-A-T-T-N)}}{\text{d(G-T-T-A-A-N)}}$ may be due to: (*1*) an inhibitory effect of this nucleotide configuration on *Eco*RI* activity; (*2*) specificity of the enzymes used in the sequence analysis, e.g., pancreatic DNase (20); or (*3*) the low frequency of these sequences in the DNAs used for the analysis.

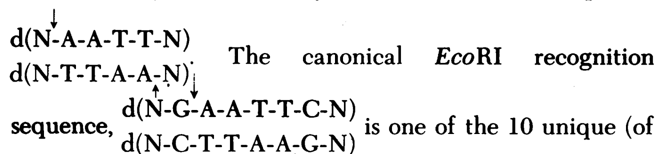
DISCUSSION

The observations presented here demonstrate that the recognition and cleavage of DNA by the *Eco*RI restriction endonuclease is influenced by the pH and ionic strength of the reaction *in vitro*. At higher pH and lower ionic strength the *Eco*RI endonuclease recognizes and cleaves a shorter sequence of base pairs than under the conditions usually employed for the reaction. This activity, designated the *Eco*RI* endonucleolytic activity, cleaves the tetranucleotide sequence,



$\frac{\text{d(N}\downarrow\text{A-A-T-T-N)}}{\text{d(N-T-T-A-A}\uparrow\text{N)}}$. An ionic-strength-dependent endonucleolytic activity in *Eco*RI preparations has been noticed previously (21, 22). The observations presented here suggest that this activity was that now referred to as *Eco*RI*.

The set of sequences recognized by the *Eco*RI* endonuclease activity is defined by the tetranucleotide sequence



$\frac{\text{d(N}\downarrow\text{A-A-T-T-N)}}{\text{d(N-T-T-A-A}\uparrow\text{N)}}$. The canonical *Eco*RI recognition sequence, $\frac{\text{d(N-G}\downarrow\text{A-A-T-T-C-N)}}{\text{d(N-C-T-T-A-A}\uparrow\text{G-N)}}$ is one of the 10 unique (of 16 possible) hexanucleotide sequences containing the core tetranucleotide and the most rapidly cleaved under all conditions examined. The propensity of the *Eco*RI* activity to cleave certain sites in DNA faster than others is indicated by the rate of appearance and disappearance of DNA fragments during a digestion (see Fig. 2). Thomas and Davis (23) have observed that the rates of *Eco*RI endonuclease cleavage of the standard *Eco*RI site in phage lambda DNA also vary. This suggests that nucleotides beyond the hexanucleotide sequence have a moderate influence on the probability of cleavage by the *Eco*RI endonuclease. It will be interesting to determine whether the hierarchy of recognition under *Eco*RI* conditions proceeds first through the other symmetrical members of the set of hexamers or if preference exists for certain arrangements of pyrimidines or purines at the outside positions. Examination of 5′ terminal sequences resulting from *Eco*RI* cleavage of pMB1 DNA demonstrates that recognition can occur at sequences which are nonsymmetrical beyond the tetranucleotide sequence. This conclusion is based on finding the 5′ terminal sequence d(pA-A-T-T-C) in *Eco*RI* digest of pMB1 DNA. Since the canonical *Eco*RI sequence is methylated in this DNA, d(pA-A-T-T-C) must be generated from a sequence which is nonsymmetrical beyond the central tetranucleotide.

At this time we can conclude that both the *Eco*RI and *Eco*RI* activities are controlled by a single gene. However, we do not have conclusive evidence that both activities are associated with the same protein molecule, since the primary gene product might be modified to forms with differing substrate affinities.

Preliminary experiments (Greene, unpublished) indicate that a corresponding *Eco*RI* methylase activity may be associated with the purified *Eco*RI methylase. The susceptibility of *Eco*RI-modified pMB1 DNA to the *Eco*RI* endonuclease indicates that *in vivo* *Eco*RI methylase does not extensively modify the sequences cleaved by the *Eco*RI* endonuclease. Presumably, the *Eco*RI* endonucleolytic activity is normally prevented *in vivo* as a result of ionic conditions which exist within the cell. However, it is possible that under certain *in vivo* conditions the *Eco*RI* activity may function to provide a specialized recombination pathway in bacteria (15). Finally, we suggest that the range of sequence specificities of other restriction endonucleases can be altered by the ionic environment of the reaction *in vitro*. If this is the case, the utility of these enzymes will be significantly extended.

The authors thank Ms. F. DeNoto and M. Betlach for their excellent assistance. This investigation was supported by Public Health Service Grants GM 14378, CA 14026, and AI 00299, and a grant from the National Science Foundation, GB 35704. B.P. is a fellow of the Leukemia Society of America.

1. Boyer, H. W. (1974) *Fed. Proc.*, **33**, 1125–1127.
2. Arber, W. (1974) in *Progress in Nucleic Acid Research and Molecular Biology* (Academic Press, Inc., New York), Vol. 14, pp. 1–38.
3. Old, R., Murray, K. & Roizes, G. (1975) *J. Mol. Biol.* **92**, 331–340.
4. Garfin, D. E. & Goodman, H. M. (1974) *Biochem. Biophys. Res. Commun.* **59**, 108–116.
5. Hedgpeth, J., Goodman, H. M. & Boyer, H. W. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 3448–3452.
6. Dugaiczyk, A., Hedgpeth, J. & Boyer, H. W. & Goodman, H. M. (1974) *Biochemistry* **13**, 503–511.
7. Morrow, J. & Berg, P. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 3365–3369.
8. Mulder, C. & Delius, H. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 3215–3219.
9. Helling, R. B., Goodman, H. M. & Boyer, H. W. (1974) *J. Virol.* **14**, 1235–1244.
10. Durwald, H. & Hoffman-Berling, H. (1968) *J. Mol. Biol.* **34**, 331–346.
11. Roulland-Dussoix, D. & Boyer, H. W. (1969) *Biochim. Biophys. Acta* **195**, 219–229.
12. Bazaral, M. & Helinski, D. R. (1968) *J. Mol. Biol.* **36**, 185–194.
13. Hershfield, V., Boyer, H. W., Lovett, M., Yanofsky, C. & Helinski, D. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 3455–3459.
14. Yoshimori, R. N., Roulland-Dussoix, D. M. & Boyer, H. W. (1972) *J. Bacteriol.* **112**, 1275–1279.
15. Roulland-Dussoix, D., Yoshimori, R., Greene, P., Betlach, M. Goodman, H. M. & Boyer, H. W. (1974) in *Microbiology—1974, American Society for Microbiology Conference on Bacterial Plasmids*, ed., Schlessinger, D. (ASM Press, Washington, D. C.), Vol. 1, pp. 187–198.
16. Greene, P. J., Betlach, M., Goodman, H. M. & Boyer, H. (1974) in *Methods in Molecular Biology Series: DNA Replication and Biosynthesis*, ed. Wickner, R. B. (Publisher, Mercel Dekker, New York), Vol. 7, pp. 87–111.
17. Modrich, P., Anraku, Y. & Lehman, I. R. (1973) *J. Biol. Chem.* **248**, 7495–7511.
18. Kacian, D. L., Watson, K. F., Burny, A. & Spiegelman, S. (1971) *Biochim. Biophys. Acta* **246**, 365–383.
19. Guerry, P., LeBlanc, D. J. & Falkow, S. (1973) *J. Bacteriol.* **116**, 1064–1066.
20. Ehrlich, S. D., Bertazzoni, U. & Bernardi, G. (1973) *Eur. J. Biochem*, **40**, 143–147.
21. Griffin, B., Fried, M. & Cowie, A. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 2077–2081.
22. Robberson, D. & Fried, M. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 3497–3501.
23. Thomas, M. & Davis, R. W. (1975) *J. Mol. Biol.* **91**, 315–328.