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Physical Identity of the SV40 Deoxyribonucleic Acid Sequence Recognized by the *Eco* RI Restriction Endonuclease and Modification Methylase†

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ABSTRACT: The *Eco* RI modification methylase introduces two methyl groups into one SV40 DNA molecule. The only base methylated has been identified as N^6-methyladenine. Both of the methyl groups are introduced into the same fragment (designated F, about 400 base pairs long) of a *Hin*$_d$ II endonuclease digest of SV40 DNA. The *Eco* RI endonuclease makes one double-strand cleavage in SV40 DNA. The site of this cleavage is also contained within the F fragment. Analysis of dinucleoside monophosphates, trinucleoside diphosphates, and tetranucleoside triphosphates generated by partial digestion of the methylated DNA with pancreatic DNase I gives the following sequence of nucleotides at the site of methylation by the *Eco* RI methylase: GpApm^{6}ApTpTpC. This sequence (with A in place of m^{6}A) is also found at the site of phosphodiester-bond cleavage by the *Eco* RI restriction endonuclease. Using [γ-^{32}P]rATP and polynucleotide kinase, SV40 DNA has been labeled in each strand with ^{32}P specifically at the phosphodiester bonds cleaved by the *Eco* RI endonuclease. The DNA was polymerized at 4° by hydrogen bonding of the cohesive termini of the *Eco* RI endonuclease break. The labeled 5′-monophosphates at the staggered single-strand breaks were esterified with the adjacent 3′-hydroxyl groups by polynucleotide ligase at low temperature, and the covalently polymerized DNA was methylated by the *Eco* RI modification methylase using *S*-adenosyl-L-[*methyl*-^{3}H]methionine. Analysis of the radioactive labels in the mono- and dinucleotides from a partial digest of this double-labeled DNA identifies physically the same sequence of base pairs in SV40 DNA as the substrate site for the *Eco* RI endonuclease and for the *Eco* RI modification methylase.

Restriction endonucleases and their related modification methylases are enzymes that recognize and react only within well-defined base sequences in double-stranded DNA molecules (Kelly and Smith, 1970; Hedgpeth *et al.*, 1972; Boyer *et al.*, 1973). It has been postulated that the two enzymes react with the same substrate site (Arber and Linn, 1969; Boyer, 1971; Meselson *et al.*, 1972) although there is some suggestion that this might not be true for all restriction enzymes (Horiuchi and Zinder, 1972). Similarities between the DNA sequence cleaved by the *Eco* RII endonuclease[1] and methylated by the *Eco* RII methylase have been reported recently (Boyer *et al.*, 1973).

In SV40 DNA, the *Eco* RI endonuclease makes only one double-stranded break (Morrow and Berg, 1972; Mulder and Delius, 1972) which is staggered in such a way that cohesive termini are formed (Mertz and Davis, 1972). The sequence of DNA base pairs adjacent to the phosphodiester bonds cleaved by this enzyme has been determined (Hedgpeth *et al.*, 1972). Here we present analysis of the sequence of nucleotides adjacent to the adenosine methylated by the *Eco* RI modification methylase. This sequence is found to be chemically identical with the previously determined sequence recognized and cleaved by the *Eco* RI endonuclease (Hedgpeth *et al.*, 1972).

A DNA molecule may be a substrate either for a restriction endonuclease or modification methylase but not for both, since treatment with one enzyme renders the DNA insensitive to the other. Since the single-stranded termini of *Eco* RI restricted SV40 DNA have complementary base sequences, the linear molecules produced by digestion of closed circular SV40 DNA will reassociate under appropriate conditions, forming circular molecules or linear concatemers that will be substrates for polynucleotide ligase, an enzyme that catalyzes the esterification of single-stranded phosphodiester-bond scissions in a bihelical DNA molecule (Weiss *et al.*, 1968a). Using this property, we have produced a DNA molecule that has been used as a substrate by both the *Eco* RI endonuclease and methylase. This DNA was used to demonstrate that the two enzymes recognize a single site in the SV40 genome.

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[1] The *Eco* RI enzymes are controlled by an *fi*$^+$ R factor in *Escherichia coli* and the *Eco* RII enzymes by an *fi*$^-$ R factor. The *Hin*$_d$ II restriction endonuclease is the enzyme from *Hemophilus influenzae* isolated as described by Smith and Wilcox (1970). The abbreviations of species names are those proposed by Danna *et al.* (1973). The symbols A, T, G, and C without a prefix are deoxynucleotides; m^{6}A is N^6-methyldeoxyadenosine. Short DNA sequences containing a radioactive phosphorus will be represented by placing the atomic mass number (32) not at the atomic symbol (P) but rather directly at the symbol (p). For example, pG^{32}pA is a dinucleoside diphosphate having a ^{32}P-labeled phosphate at the internal position.

Experimental Section

Materials. Crystalline pancreatic DNase I, spleen phosphodiesterase, venom phosphodiesterase, and alkaline phosphatase from *Escherichia coli* were purchased from Worthington Biochemical Corp. The alkaline phosphatase was further purified to remove contaminating endonucleases by the method of Weiss *et al.* (1968b). Polynucleotide kinase was prepared by the method of Richardson (1965) and polynucleotide ligase by the method of Modrich and Lehman (1970). The *Eco* RI restriction endonuclease and modification methylase were purified as described by Greene *et al.* (1973). [γ-^{32}P]-rATP (specific activity, 53.6 Ci/mmol) was purchased from ICN, *S*-adenosyl-L-[*methyl*-^{3}H]methionine (specific activity, 8.02 Ci/mmol) from Schwarz/Mann, and pApA from Collaborative Research. The dinucleotide pApm^{6}A was synthesized by Dr. D. V. Santi. In our laboratory it was further purified by the various electrophoretic and chromatographic systems described below, and its structure was proved by identifying the components of digestion by micrococcal nuclease, alkaline phosphatase, spleen phosphodiesterase, and venom phosphodiesterase.

DNA Preparation. Bacteriophage λ DNA uniformly labeled with ^{32}P was isolated from a lysogen of strain RY13 which carries the *Eco* RI restriction and modification genes as previously described (Hedgpeth *et al.*, 1972). Label, [^{32}P]phosphate, was added at a concentration of 3–5 mCi/100 ml of culture medium.

Confluent monolayers of the CV-1 line of African green monkey kidney cells grown in 100-mm plastic petri dishes in Eagle's Minimum Essential Medium (Grand Island Biological Co.), supplemented with 10% fetal calf serum (Pacific Biological Co.), were infected by Simian virus 40 (small plaque variant from Dr. Helene Smith) at a multiplicity of 0.5–2 plaque-forming units/cell. After 3 days at 37°, SV40 DNA was selectively extracted by the Hirt procedure (Hirt, 1967). The bulk of the cell DNA was removed by centrifugation at 23,500*g* for 3 hr at 4° and the supernatant containing the SV40 DNA was extracted with phenol saturated with buffer composed of 20 mM Tris·HCl (pH 7.4), 100 mM NaCl, and 1 mM EDTA. The SV40 DNA was precipitated from the aqueous phase by the addition of two volumes of ethanol. After storage for at least 2 hr at −20° the precipitate was collected by centrifugation at 5000*g* for 30 min at 4° and then resuspended in buffer (0.6 M NaCl–25 mM EDTA, pH 8.0). Nine milliliters of this buffer was used per 40 (100 mm) dishes originally extracted. The DNA solution was titrated to pH 12.35 (Corning Model 12 pH meter, with Corning 476050 combination electrode standardized to pH 10.0) with 2 N NaOH and stirred gently at that pH for 3 min at 25°. It was then neutralized to pH 8.0 (25°) with 2 N HCl, placed on ice, and passed through nitrocellulose filters which retain single-stranded cell DNA. The Selectron B-6 (Schleicher & Schuell) nitrocellulose filters used for the filtration were soaked for 15 min in 30% ethanol and washed with water, and then three of them were stacked on a Millipore filter apparatus without the chimney. When the flow rate slowed down because of clogged pores, the top or all three of the filters were replaced with fresh ones. The filtrate was dialyzed against 10 mM Tris·HCl buffer (pH 7.4)–0.1 mM EDTA for 1 hr, concentrated to about 2 ml by flash evaporation, and then applied to an A50 agarose (100–200 mesh; Bio-Rad) 2 × 50 cm column equilibrated with 50 mM Tris·HCl buffer (pH 7.4), 0.2 M NaCl. The column was eluted with the same buffer. An ISCO uv monitor and recording apparatus were used to monitor the DNA at 260 nm. SV40 DNA elutes in the exclusion volume while the alkali degraded RNA is retarded. The fractions containing DNA were pooled and dialyzed against 4 l. of 10 mM Tris·HCl buffer (pH 8.5), 1 mM EDTA for 1 hr at 25°. The dialyzed solution was concentrated to 3 ml by flash evaporation and redialyzed for 2 hr at 25° against 2 l. of 10 mM Tris·HCl buffer (pH 8.5), 1 mM EDTA. More than 95% of the DNA prepared in this way is in the circular supercoiled form as judged by electron microscopy and agarose gel electrophoresis.

Methylation of DNA by the Eco RI Methylase. Two milliliters of the reaction mixture contained 1 mg of SV40 DNA (0.30 nmol), 0.1 M Tris·HCl buffer (pH 8.0), 10 mM EDTA (pH 8.0), 18.2 nmol of *S*-adenosyl-L-[*methyl*-^{3}H]methionine (146 μCi; 75.9 × 10^{6} cpm), and 50 units of enzyme. The course of the reaction was followed by withdrawing 2-μl aliquots into 200 μl (80 μg) of an ice-cold calf thymus DNA solution followed immediately by the addition of 2 ml of an ice-cold solution containing 7% $HClO_4$ and 0.1 N $Na_4P_2O_7$. After 30 min at 0° the samples were filtered on glass fiber filters, washed thoroughly with cold 2 N HCl, then with ethanol, dried, and counted in a scintillation counter. The DNA was methylated to saturation within 30–60 min, incorporating a total of 2.44 × 10^{6} cpm (0.585 nmol of [^{3}H]methyl groups) per mg of SV40 DNA. The reaction mixture was extracted with phenol (saturated with 0.05 M Tris·HCl buffer, pH 7.6), and dialyzed against 0.3 M sodium acetate–1 mM EDTA–10 mM Tris·HCl buffer (pH 7.6), and the DNA was precipitated by the addition of two volumes of ethanol and recovered (810 μg) by centrifugation at 20,000*g* for 1 hr at −5°.

Digestion of the DNA to 5′-Mononucleotides. The methylated SV40 DNA was dissolved in 1 ml of 50 mM Tris·HCl buffer (pH 7.6), 10 mM $MgCl_2$. Five microliters of this solution was incubated in a capillary for 1 hr at 37° with about 30 μl of a mixture containing 1 mg/ml each of pancreatic DNase I and venom phosphodiesterase in 50 mM Tris·HCl buffer (pH 7.6), 10 mM $MgCl_2$. The resulting components were separated by electrophoresis at pH 3.5 on Whatman No. 3MM paper (Sanger *et al.*, 1965) in the presence of 5′-mononucleotides that served as uv markers.

Hydrolysis of AMP with Trifluoroacetic Acid. Since the only tritium-labeled component detected in the digest described above migrated in electrophoresis with 5′-AMP, this was eluted with water and after evaporation to dryness hydrolyzed at 175° for 15 min in a sealed tube with 0.3 ml of CF_3COOH in the presence of about 0.2 μmol each of 1-methyladenine, 2-methyladenine, N^6-methyladenine, and N^6-dimethyladenine. Trifluoroacetic acid was removed under reduced pressure, and the residue was dissolved in water and spotted for chromatography (Razin *et al.*, 1970).

Partial Digestion of the DNA. The main portion of the ^{3}H-methylated SV40 DNA (806 μg, 1.96 × 10^{6} cpm in 1 ml of 50 mM Tris·HCl buffer (pH 7.6), 10 mM $MgCl_2$) was mixed with about 5 μg of uniformly labeled λ·RI[^{32}P]DNA (7 × 10^{6} cpm) and digested with 1 mg of crystalline pancreatic DNase I for 2 hr at 37° and then for an additional hour in the presence of 40 μl of alkaline phosphatase (56 units/ml, endonuclease free). The temperature of the reaction mixture was lowered to 20° and the DNA was further digested for 10 min with 20 μl of venom phosphodiesterase (1 mg/ml). The digest was diluted fourfold with 7 M urea in 5 mM Tris·HCl buffer (pH 7.6) and adsorbed onto a DEAE-Sephadex (A-25) column (0.9 × 20 cm), maintained at 65°. Oligonucleotides of increasing chain length were eluted with a linear gradient (0–0.2 M) of NaCl in 7 M urea–5 mM Tris·HCl buffer (pH 7.6) (Junowicz and Spencer, 1970). Fractions containing oligonucleotides of equal chain

length were diluted ten times with water, adsorbed on small (0.5 × 6 cm) QAE-Sephadex columns, washed free of urea with 0.01 M triethylammonium bicarbonate, eluted with 2 M triethylammonium bicarbonate (pH 9), and evaporated to dryness under reduced pressure.

Electrophoretic Separation. Either Whatman No. 3MM and a pyridine–acetic acid buffer (pH 3.5) (Sanger *et al.*, 1965) or DEAE-cellulose paper (DE-81) and a mixture of acetic acid–formic acid (pH 1.9) (Sanger *et al.*, 1965) were used for most separations. Pyridine was removed from the paper in an NH_3 atmosphere if absorbance markers were to be visualized in uv light. Radioactive nucleotides were located by radioautography by placing a sheet of X-ray film (Kodak RP 14) over the electrophoresis paper. Oligonucleotides were eluted either with water from 3MM paper or with 2 M triethylammonium bicarbonate (pH 9) from DE-81 paper. Complex mixtures were separated in both systems. For example, the trinucleoside diphosphate fraction separated into 23 ^{32}P-containing bands, and the tetranucleoside triphosphates into 13 ^{32}P-containing bands on 3MM (pH 3.5) paper. Each band was counted for ^{32}P and ^{3}H, and bands containing both isotopes were eluted and further purified in the second system (DE-81, pH 1.9).

Sequence Determination of the Tri- and Tetranucleotides. Spots containing both ^{32}P and ^{3}H were eluted from the DE-81 paper and evaporated to dryness. They were digested in a capillary for 1 hr at 37° with about 10 μl of spleen phosphodiesterase (10 units/ml in 0.1 M Tris·HCl buffer (pH 7.5), 0.02 M $MgCl_2$) in the presence of 1 μmol of unlabeled N^6-methyldeoxyadenosine[2] and separately with 10 μl of venom phosphodiesterase (1 mg/ml in 0.1 M Tris·HCl buffer (pH 8.5), 0.01 M $MgCl_2$). The digests were separated electrophoretically (Whatman No 3MM, pH 3.5) in the presence of unlabeled 3′-mononucleotides and nucleosides (spleen) or 5′-mononucleotides and nucleosides (venom), that served as uv markers. Areas containing the separated components were cut out and counted for ^{32}P and ^{3}H in a scintillation counter.

Separation of Hin_d II Endonuclease Fragments of SV40 DNA. SV40 DNA was methylated with the *Eco* RI methylase and *S*-adenosyl-L-[*methyl*-^{3}H]methionine or cleaved with the *Eco* RI endonuclease as described below and then, in each case digested with the Hin_d II restriction endonuclease (3 units/mg of SV40 DNA for 2 hr at 37° in 6.6 mM Tris·HCl buffer (pH 7.5), 6.6 mM 2-mercaptoethanol, 6.6 mM $MgCl_2$, and 50 mM NaCl) from *Hemophilus influenzae* (Smith and Wilcox, 1970). The DNA fragments from the digest were separated electrophoretically on 4% slab polyacrylamide gels which also contained 0.5% agarose in a Tris–boric acid–EDTA buffer system (Danna and Nathans, 1971; Peacock and Dingman, 1968). The fragments were located in the gel by staining with ethidium bromide (4 μg/ml for 10–15 min at room temperature) and visualizing them under a long wave uv lamp. In the case of the methylated DNA, the bands were excised with a scalpel and then incubated overnight in sealed scintillation vials at 60° in 10 ml of a mixture of a toluene-based scintillation fluid containing 5% v/v Protosol (New England Nuclear Co.). After cooling, the tritium radioactivity was determined in a scintillation counter.

Labeling of SV40 DNA at the Site Recognized by the Eco RI Restriction Endonuclease and by the Eco RI Modification Methylase (Figure 2). (a) CLEAVAGE BY THE Eco RI ENDONUCLEASE. A 5-ml reaction mixture contained 1.1 mg of SV40 DNA, 0.1 M Tris·HCl buffer (pH 8.0), 5 mM $MgCl_2$, 50 mM NaCl, and 0.2 ml (60 units) of the *Eco* RI endonuclease. The enzyme was added in two 0.1-ml portions at time zero and after 30-min incubation at 37°. Agarose gel electrophoresis (Sharp *et al.*, 1973; Helling, Goodman, and Boyer, unpublished results) and electron microscopy (Davies *et al.*, 1971; Boyer *et al.*, 1973) after 60-min incubation showed that >95% of the original circular form I SV40 DNA was converted to linear unit length molecules.

(b) REMOVAL OF TERMINAL 5′-PHOSPHATES BY ALKALINE PHOSPHATASE. To 5 ml of the above reaction mixture, 0.5 ml of 0.1 M EDTA solution (pH 8.4) was added, followed by two 10-μl portions (0.5 unit each) of alkaline phosphatase added at time zero and after 30-min incubation at 37°. After 1 hr the enzymes were extracted twice with phenol, and the aqueous phase was dialyzed against 50 mM Tris·HCl buffer (pH 7.5), 0.3 M sodium acetate, and 1 mM EDTA. After dialysis, the DNA was recovered (0.88 mg) by precipitation with two volumes of ethanol and centrifugation at 20,000*g* for 1 hr at −5°.

(c) ^{32}P LABELING OF THE 5′-TERMINI BY POLYNUCLEOTIDE KINASE. The DNA (0.88 mg) from step b, containing free 5′-hydroxyl groups (about 0.53 nmol), was dissolved in 1 ml of a mixture containing 10 mM Tris·HCl buffer (pH 7.6), 10 mM $MgCl_2$, 20 mM 2-mercaptoethanol, and 20 nmol of [γ-^{32}P]-rATP (1.1 mCi). Twenty microliters of polynucleotide kinase was added and the mixture was incubated at 37°. One microliter of the enzyme converted between 4 and 130 pmol of ApC into pApC in 30 min when assayed in a reaction mixture of 10 μl containing 10 mM Tris·HCl buffer (pH 7.6), 10 mM $MgCl_2$, 20 mM 2-mercaptoethanol, 10 nmol of ApC, and 0.068–3.9 nmol of [γ-^{32}P]rATP. The reaction mixture was analyzed electrophoretically at pH 3.5 on DE-81 paper to separate ApC and ^{32}pApC from [γ-^{32}P]rATP (Szekely and Sanger, 1969). The course of phosphorylation of the DNA was followed by withdrawing 1-μl aliquots into 200 μl (80 μg) of ice-cold calf thymus DNA followed immediately by the addition of 2 ml of an ice-cold solution containing 7% $HClO_4$ and 0.1 N $Na_4P_2O_7$. After 30 min at 0° the samples were filtered on glass fiber filters, washed thoroughly with cold 2 N HCl, then with ethanol, dried, and counted in a scintillation counter. After 2-, 4-, and 6-hr incubation, additional 20-μl aliquots of polynucleotide kinase were added until a constant level of ^{32}P incorporation (17.9×10^6 cpm total or 0.53 mol of ^{32}P incorporated per mol of DNA substrate) into DNA was reached. To assure complete phosphorylation of the 5′-hydroxyl groups, 0.5 μmol of unlabeled rATP and a last portion of 20 μl of enzyme were added and incubated for an additional hour. rATP was then removed by dialysis first at room temperature, then at 4° against 20 mM Tris·HCl buffer (pH 8.0), 1 mM EDTA, and 0.1 M KCl. The DNA at this stage had the following properties. (1) The [^{32}P]phosphate could be hydrolyzed during a short (30 min) incubation at 37° with alkaline phosphatase, indicating that it was located as phosphomonoesters at either end of the duplex molecule (Weiss *et al.*, 1968b). (2) Electron microscopic inspection revealed that at 4° most of the DNA formed circular molecules and linear polymers (dimers and trimers), whereas at 25° most of it was linear monomers. This indicates that the short cohesive termini produced by the *Eco* RI endonuclease were still intact.

(d) ESTERIFICATION OF THE 5′-LABELED DNA BY POLYNUCLEOTIDE LIGASE. This reaction was carried out in a volume

[2] Commercial spleen phosphodiesterase contains a fairly strong deaminase activity and N^6-methyldeoxyadenosine is one of its substrates (Dugaiczyk, Boyer, and Goodman, to be published). No ^{3}H can be recovered in m^{6}A unless unlabeled N^6-methyldeoxyadenosine is added to the digest in order to provide an excess of substrate for the enzyme.

of 3 ml, containing all of the DNA from step c, and having the following composition (Mertz and Davis, 1972): 20 mM Tris·HCl buffer (pH 8.0), 5 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 100 mM KCl, 100 μM NAD, and 100 μg/ml of bovine serum albumin. The temperature was lowered to 4° to facilitate hydrogen bonding of the cohesive ends which have a T_m of 5–6° (Mertz and Davis, 1972), and 50 μl (25 units) of polynucleotide ligase was added. One unit converts 100 nmol of poly(dA-dT) to a form resistant to exonuclease III in 30 min (Gellert, 1971). The temperature was then increased to 16° and the reaction mixture was incubated for 6 hr. Twice during the incubation the temperature was lowered to 4° for 30 min, 50 μl of enzyme was added, and the reaction mixture was again incubated at 16°. After 10 hr, about 50% of the ^{32}P had become resistant to phosphatase hydrolysis and hence was in internal phosphodiester bonds. The reaction was stopped at this stage by extracting it with phenol and dialyzing the aqueous phase against 100 mM Tris·HCl buffer (pH 8.0), 10 mM EDTA. Examination of the DNA preparation by agarose gel electrophoresis and in the electron microscope revealed that about 90% was in form of linear dimers and trimers (favored over circular molecules because of the high DNA concentration) that were not converted to monomers of unit SV40 DNA length by spreading at room temperature or in the presence of 40% formamide.

(e) METHYLATION OF LIGATED DNA BY *Eco* RI METHYLASE. The DNA, covalently polymerized by the action of polynucleotide ligase into linear dimers and trimers, was methylated with the *Eco* RI methylase under the conditions described above for native, circular SV40 DNA. A total of 323,000 cpm (0.151 nmol of methyl group/mg of SV40 DNA) was incorporated from *S*-adenosyl-L-[*methyl*-^{3}H]methionine into this linearly polymerized SV40 DNA. The DNA (810 μg) also contained 3.6×10^6 cpm of previously incorporated ^{32}P from the polynucleotide kinase reaction (step c).

(f) DESTRUCTION OF UNMETHYLATED SEQUENCES AND TERMINAL 5′-PHOSPHOMONOESTERS. Although the methylation in step c was carried to saturation, it was difficult to assess whether all of the sequences recognized by the *Eco* RI methylase were indeed methylated because of the numerous previous steps (a–d) in preparing the DNA. In order to destroy any unmethylated sequences, the DNA was treated as in step a with the same amount of *Eco* RI endonuclease used to cleave the original amount of DNA at all the *Eco* RI endonuclease recognition sites. Unligated 5′-phosphomonoesters were then removed in the presence of EDTA with bacterial alkaline phosphatase. Half the preparation was incubated at 37° for 1 hr with 8 units of enzyme; the other half was incubated at 65° for 30 min with the same amount of enzyme added in two portions, the first at time zero and the second after 15-min incubation at 65°. The elevated temperature is required to hydrolyze internal phosphomonoesters located at single-strand breaks (Weiss *et al.*, 1968b). The reaction mixtures were extracted twice with phenol, and the aqueous phase was dialyzed against 0.3 M sodium acetate–100 mM Tris·HCl buffer (pH 7.5)–1 mM EDTA. The DNA from each preparation was precipitated by the addition of two volumes of ethanol and recovered by centrifugation at 20,000*g* for 1 hr at −5°. In the following steps each of the two DNA preparations (phosphatase at 37°, and phosphatase at 65°) was treated separately.

Sequence Analysis of the Doubly Labeled DNA. DIGESTION TO 5′-MONONUCLEOTIDES. The DNA precipitate recovered from step f was dissolved in 0.3 ml of 50 mM Tris·HCl buffer (pH 7.5), containing 10 mM $MgCl_2$. A small aliquot (5 μl) of the DNA solution was digested in a capillary with a mixture of pancreatic DNase I and venom phosphodiesterase as described above for native circular SV40 DNA. The 5′-mononucleotides from the digest were separated electrophoretically at pH 3.5 on Whatman No. 3 MM paper.

PARTIAL DIGESTION. To the remaining DNA solution (295 μl) 0.5 mg of crystalline pancreatic DNase I was added and incubated for 5 hr at 37°. The temperature was then lowered to 22° and the DNA was further digested with 10 μl of venom phosphodiesterase (1 mg/ml for 10 min). The reaction mixture was chilled on ice and immediately applied on Whatman No. 3MM paper for electrophoresis.

ELECTROPHORETIC AND CHROMATOGRAPHIC SEPARATIONS. The first two electrophoretic separations were performed as described above for the separation of sequences derived from methylated circular SV40 DNA: first on Whatman No. 3MM paper (pH 3.5), then on DE-81 paper (pH 1.9). The final separation of pA from pm^{6}A and of pApA from pApm^{6}A was by chromatography on Whatman No. 1 paper which was impregnated by dipping it in a solution made by diluting one volume of saturated $(NH_4)_2SO_4$ with nine volumes of water. After drying the paper in air, the descending chromatogram was developed for 15 hr using a solvent system composed of 80 volumes of 95% ethanol and 20 volumes of water (Singh and Lane, 1964).

IDENTIFICATION OF ^{32}pAp[^{3}H]m^{6}A. The dinucleotide, ^{32}pAp[^{3}H]m^{6}A, obtained from the "doubly-labeled" DNA as detailed above cochromatographed on Whatman No. 1 paper with unlabeled synthetic pApm^{6}A. After elution from the paper with water and desalting by electrophoresis (Whatman No. 3MM, pH 3.5), it was treated with alkaline phosphatase and the products were separated electrophoretically into [^{32}P]P$_i$ and Ap[^{3}H]m^{6}A (containing all the ^{3}H and no ^{32}P). Digestion of Ap[^{3}H]m^{6}A with spleen phosphodiesterase yielded Ap and [^{3}H]m^{6}A, whereas digestion with venom phosphodiesterase yielded A and p[^{3}H]m^{6}A.

Results

Number of Methyl Groups Introduced into SV40 DNA by the Eco RI Methylase. Methylation of SV40 DNA to saturation results in an incorporation of two methyl groups per SV40 genome. For example, in the experiment described in the Experimental Section, 0.585 nmol of methyl group was incorporated into 0.30 nmol of circular SV40 DNA, giving a molar ratio of 1.95. In two other experiments, the values 2.11 and 1.98 were obtained.

Location of the Methyl Groups in the SV40 Genome. The continguous order of the eleven DNA fragments produced by digestion of SV40 DNA by the *Hin*$_d$ II endonuclease has been determined (Danna and Nathans, 1972). Separation of fragments by gel electrophoresis shows that the *Eco* RI endonuclease cleavage occurs within the F fragment produced by the *Hin*$_d$ II enzyme. The same result was obtained by Danna *et al.* (1973). The methyl groups incorporated into SV40 DNA by the *Eco* RI methylase are also located in the F fragment. SV40 was methylated with *S*-adenosyl-L-[*methyl*-^{3}H]methionine and digested with the *Hin*$_d$ II endonuclease and the DNA fragments were separated. In three separate experiments, 90–95% of the [^{3}H]methyl groups was located in fragment F; no other fragment contained more than 3% of the total methyl groups.

Sequence Recognized by the Eco RI Methylase. Digestion to 5′-mononucleotides of SV40 DNA methylated with *S*-adenosyl-L-[*methyl*-^{3}H]methionine produces a tritium-labeled nucleotide which coelectrophoreses at pH 3.5 with adenosine

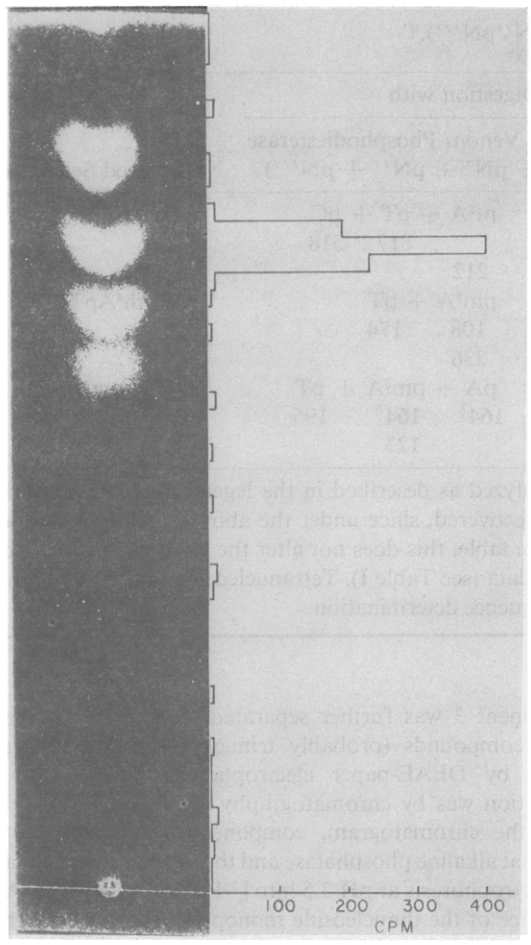

FIGURE 1: Chromatogram (Whatman No. 1) in butanol saturated with water (NH_3 in the vapor phase) of the components of the CF_3COOH hydrolysate of *Eco* RI methylated SV40 DNA. 1-Methyladenine, 2-methyladenine, N^6-methyladenine, and N^6-dimethyladenine separate with increasing R_F values. After photographing the chromatogram in ultraviolet light, the paper was cut into narrow sections and counted for tritium in a scintillation counter as shown in the histogram to the right of the uv photograph.

TABLE I: Analysis of Methylated Trinucleoside Diphosphates (NpN'pN'').[a]

Spot No.	Isotope	Cpm in the Products of Digestion with: Spleen Phosphodiesterase (Np + N'p + N'')	Cpm in the Products of Digestion with: Snake Venom Phosphodiesterase (N + pN' + pN'')	Deduced Sequence
1		Gp + Ap + m⁶A	pA + pm⁶A	GpApm⁶A
	³²P	120 118	134[b] 134[b]	
	³H	215	240	
2		m⁶Ap + Tp	m⁶A + pT	m⁶ApTpT
	³²P	950 840	1214	
	³H	145	116	
3				Apm⁶ApT[c]

[a] SV40 DNA was methylated with the *Eco* RI modification methylase and *S*-adenosyl-L-[*methyl*-³H]methionine, λ·RI-[³²P]DNA added, the mixture digested with pancreatic DNase I and bacterial alkaline phosphatase, and the various isostichs were separated by column chromatography. After further separation of trinucleoside diphosphates by paper electrophoresis each was analyzed by digestion with spleen and venom phosphodiesterase. [b] The ³²P cpm listed here are one-half the total recovered, since under the above conditions pA and pm⁶A do not separate. However, as can be seen from the table, this does not alter the analysis. [c] This sequence was not detected in this experiment. However, in separate experiments, complete digestion of [³H]methyl-labeled DNA to dinucleoside monophosphates (NpN') with micrococcal nuclease plus alkaline phosphatase or pancreatic DNase I, *E. coli* exonuclease I, and alkaline phosphatase gave only two labeled dinucleoside monophosphates: Apm⁶A and m⁶ApT. Therefore the trinucleoside sequence around the methylated base is Apm⁶ApT.

monophosphate. This was further identified as N^6-[³H]methyladenine by hydrolysis with CF_3COOH and comparison with several methylated adenine derivatives by chromatography (Figure 1). The nucleotide sequence containing the N^6-[³H]-methyladenine was determined by partial digestion of the methylated SV40 DNA (mixed with uniformly labeled λ·RI-[³²P]DNA, *i.e.*, DNA containing methylated RI sites) with pancreatic DNase I, isolation of the isostichs by column chromatography, separation of the isomers of each size class by high-voltage paper electrophoresis in two systems, and final analysis by snake venom phosphodiesterase and spleen phosphodiesterase digestion. It is concluded from the combined data of the analysis of the trinucleoside diphosphates (Table I) and tetranucleoside triphosphates (Table II) that the specific methyl groups added to SV40 DNA by the *Eco* RI methylase occur in only one sequence: GpApm⁶ApTpTpC.

Common Sequence Recognized by the Eco RI Endonuclease and Eco RI Methylase. We prepared SV40 DNA molecules specifically labeled with ³²P at the bond in each strand cleaved by *Eco* RI restriction endonuclease and at the same time specifically labeled with [³H]methyl groups at the site methylated by the related *Eco* RI modification methylase (Figure 2; Experimental Section). Closed circular SV40 DNA was cleaved with *Eco* RI endonuclease and after removal of the 5'-phosphates with bacterial alkaline phosphatase the 5' termini of the breaks were rephosphorylated with ³²P using polynucleotide kinase and [γ-³²P]rATP. These linear SV40 DNA molecules could not serve as substrates for polynucleotide ligase. However, after polymerization into circular molecules and linear concatemers at 4° by annealing of the cohesive termini of the *Eco* RI endonuclease break, the single-strand nicks at the joining points could be ligated with polynucleotide ligase. Ligation re-forms the substrate sequence since this DNA is again a substrate for the *Eco* RI methylase (Figure 3) and the only base methylated was N^6-methyladenine. The annealed ligated DNA also regains its ability to be a substrate for the endonuclease because treatment with the endonuclease destroys all sites for the methylase (Figure 3).

Next, the ³²P-labeled ligated DNA was methylated with [³H]methyl groups using the *Eco* RI methylase and was again digested with an excess of endonuclease. This step cleaves any substrate sites that are not methylated. Subsequent treatment with bacterial alkaline phosphatase at 37° removed all terminal phosphates while treatment at 65° also removed any unligated internal phosphates. This final double-labeled DNA preparation was partially digested with pancreatic DNase I and venom phosphodiesterase and the products of the digest (mainly mononucleotides and dinucleotides) were separated sequentially in three different systems: (1) electrophoresis on

TABLE II: Analysis of Methylated Tetranucleoside Triphosphates (NpN'pN''pN''').[a]

Spot No.	Isotope	Cpm in the Products of Digestion with: Spleen Phosphodiesterase (Np + N'p + N''p + N''')	Snake Venom Phosphodiesterase (N + pN' + pN'' + pN''')	Deduced Sequence
1		m^{6}Ap + Tp	m^{6}A + pT + pC	m^{6}ApTpTpC
	^{32}P	560 990	817 518	
	^{3}H	255	212	
2		Ap + m^{6}Ap + Tp	pm^{6}A + pT	Apm^{6}ApTpT[c]
	^{32}P	147[b] 147[b] 168	108 174	
	^{3}H	557	336	
3		Gp + Ap + m^{6}Ap	pA + pm^{6}A + pT	GpApm^{6}ApT[c]
	^{32}P	154 170[b] 170[b]	164[b] 164[b] 196	
	^{3}H	108	123	

[a] The methylated tetranucleoside triphosphates were isolated and analyzed as described in the legend to Table I and in the Experimental Section. [b] The ^{32}P cpm listed here are one-half the total recovered, since under the above conditions neither Ap and m^{6}Ap nor pA and pm^{6}A separate. However, as can be seen from the table, this does not alter the analysis. [c] Other isomers are theoretically possible, but they are incompatible with the rest of the data (see Table I). Tetranucleoside triphosphates having m^{6}A at the 3' end were detected but were not sufficiently pure to permit sequence determination.

paper at pH 3.5 (Figure 4a,b), (2) electrophoresis on DEAE-cellulose paper at pH 1.9 (Figure 5), and (3) paper chromatography (Figures 6 and 7).

The proof of the identity of the site cleaved by the *Eco* RI endonuclease and methylated by the methylase is based on the identification of component 3 (Figures 4b, 5, 6, and 7). After separation of the DNA digest, previously treated with phosphatase at 65° (Figure 2, step f), by electrophoresis (Figure 4b), component 3 was further separated from trace amounts of other compounds (probably trinucleotides and higher isostichs) by DEAE-paper electrophoresis (Figure 5). Final separation was by chromatography (Figure 6). After elution from the chromatogram, component 3 was digested with bacterial alkaline phosphatase and the products were separated by electrophoresis at pH 3.5 into [^{32}P]P$_i$ and Ap[^{3}H]m^{6}A. The sequence of the dinucleoside monophosphate was determined

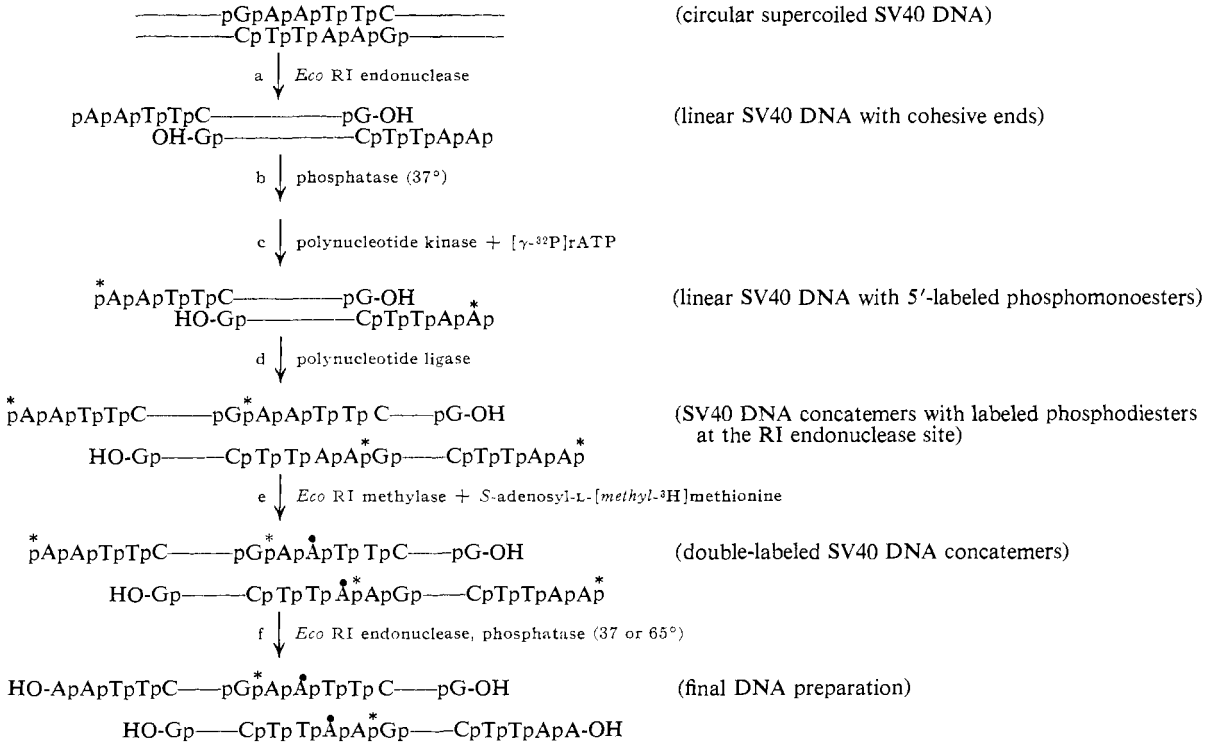

FIGURE 2: Scheme for the preparation of SV40 DNA, containing ^{32}P-labeled phosphodiesters at the site cleaved by the *Eco* RI endonuclease, and also containing ^{3}H-labeled methyl groups at the site modified by the *Eco* RI methylase. The two strands of an SV40 DNA duplex are schematically presented by two parallel lines and only the sequences recognized by the enzymes are designated. The sequence of the *Eco* RI endonuclease site is from Hedgpeth *et al.* (1972). The external A·T base pairs considered to be part of the site for λDNA are omitted here since it has not yet been proven whether they constitute part of the SV40 DNA *Eco* RI endonuclease site. Enzymatic steps a to f are described in detail in the Experimental Section. The asterisks denote the position of the ^{32}P label and the dots the position of the [^{3}H]methyl label.

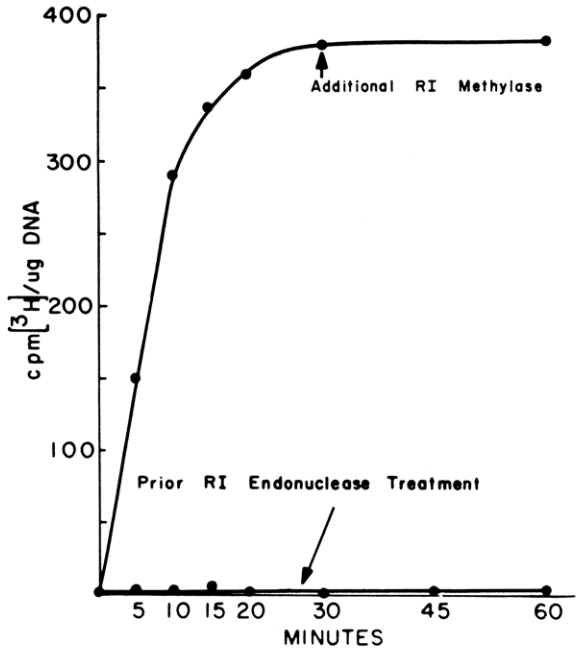

FIGURE 3: Kinetics of methylation of linearly polymerized SV40 DNA by the *Eco* RI methylase. Ten milliliters of the reaction mixture contained 810 μg of DNA, 0.1 M Tris·HCl buffer (pH 8.0), 10 mM EDTA (pH 8.0), 18.2 nmol of *S*-adenosyl-L-[*methyl*-^{3}H]methionine (50 μCi), and 50 units of methylase. Aliquots (5-μl) were withdrawn at the indicated times into 200 μl of an ice-cold solution of calf thymus DNA (80 μg) and the acid-precipitable radioactivity was determined. An additional 25 units of methylase was added at 30 min. The lower curve indicates methylation after pre-treatment with 50 units of *Eco* RI endonuclease.

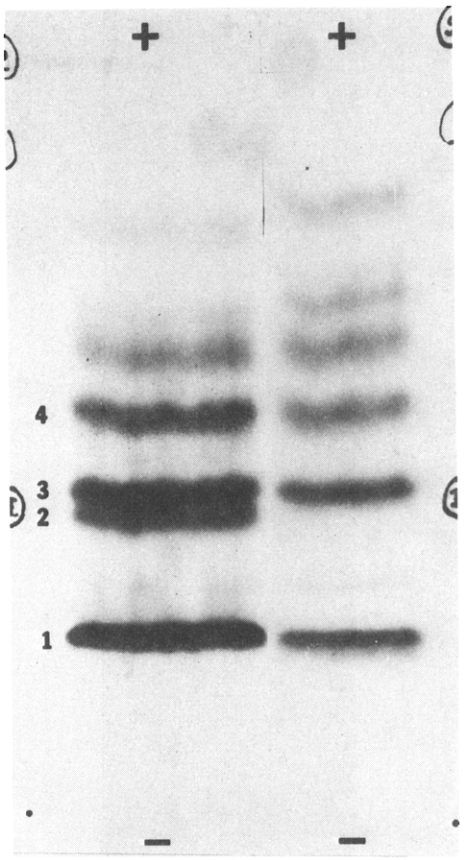

FIGURE 4: Autoradiogram, showing the electrophoretic separation (Whatman No. 3MM, 2 hr, 35 V/cm) in a pyridine–acetic acid buffer (pH 3.5) of the partial DNA digest. (a, left) Digest of the DNA treated in step f with phosphatase at 37°; (b, right) digest of the DNA treated at 65° with phosphatase. "B" represents the position of the blue dye (Xylene Cyanole FF, Sanger *et al.*, 1965); "Y," that of the yellow dye (Orange C, Sanger *et al.*, 1965).

by digestion with spleen phosphodiesterase (yielding Ap and [^{3}H]m^{6}A) and snake venom phosphodiesterase (yielding A and p[^{3}H]m^{6}A). This unambiguously identified component 3 as ^{32}pAp[^{3}H]m^{6}A.

The following other components have been identified from the digest and were expected on the basis of the sequence of the endonuclease and methylase site: components 1a and 1b, ^{32}pA and p[^{3}H]m^{6}A, respectively; component 2, ^{32}pApA; and component 4, pG^{32}pA.

Components 1a and b (Figure 4a, b), purified from trace impurities by electrophoresis on DEAE paper (Figure 5), was separated by chromatography (Figures 6 and 7) into ^{32}pA (component 1a, containing all the ^{32}P and no ^{3}H) and p[^{3}H]-m^{6}A (component 1b, containing no ^{32}P and all the ^{3}H). This result proves that the adenosine that is methylated by the *Eco* RI methylase is not the adenosine that is adjacent to the phosphodiester bond cleaved by the *Eco* RI endonuclease.

Component 2 is the only one that does not appear in the partial digest (Figure 4b) when DNA preparation included treatment with phosphatase at 65° (Figure 2, step f). This fragment therefore originates from an internal ^{32}P-labeled phosphomonoester located at a single-strand break and resistant to alkaline phosphatase at 37° (Weiss *et al.*, 1968b) and must, therefore, have resulted from incomplete ligation of some sites in our DNA preparation (Figure 2, step d). On DEAE-cellulose electrophoresis (Figure 5) component 2 migrates the same distance as does component 3, which is unaltered by prior treatment of the DNA preparation with alkaline phosphatase at 65° (Figure 4a,b). However, the two components are very well distinguished by chromatography (Figure 7).

Component 2 contains ^{32}P and no ^{3}H and migrates with synthetic pApA (Figure 7). It was identified after elution from the chromatogram in the same way as component 3. Treatment with phosphatase resulted in two products, [^{32}P]P$_i$ and ApA (identified by its mobility and further digestion of the uv marker). Therefore, component 2 is ^{32}pApA which is not present in the partial digest (Figures 4b and 6) when the DNA (Figure 2, step f) has been previously treated with phosphatase at elevated temperature (65°).

Component 4 (Figure 4a, b), after a second electrophoretic separation (Figure 5), was identified as pG^{32}pA. The major radioactive spot was eluted from the DEAE paper (Figure 5). Treatment with bacterial alkaline phosphatase produced a dinucleoside monophosphate (^{32}P labeled), which was separated from P$_i$ (unlabeled) by electrophoresis. The dinucleoside monophosphate (NpN′) had the expected mobility of GpA. Its identity was confirmed by digestion with spleen phosphodiesterase (yielding G^{32}p) and snake venom phosphodiesterase (yielding ^{32}pA). Therefore, the original spot was a dinucleotide, pG^{32}pA (with ^{32}P only at the phosphodiester position).

Discussion

It has been reported that the *Eco* RII endonuclease and methylase interact within the same (chemically defined) base sequence in double-stranded DNA molecules (Boyer *et al.*, 1973). In the present work the sequence containing the site methylated by the *Eco* RI methylase has been determined as

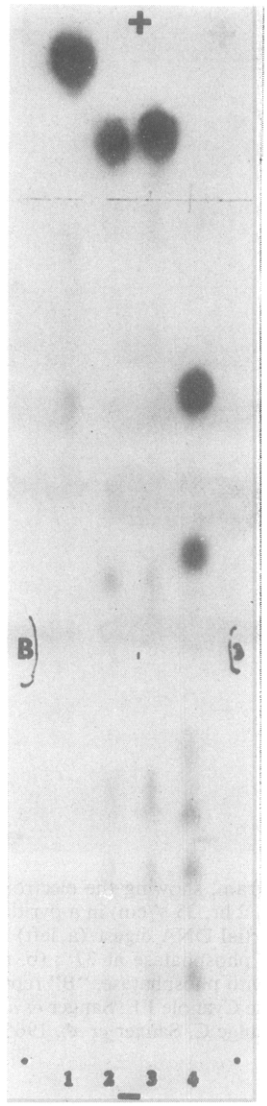

FIGURE 5: Autoradiogram, showing the electrophoretic separation (10 hr, 20 V/cm) on DEAE-cellulose (Whatman DE-81) paper in a CH_3COOH–HCOOH system (pH 1.9). The four main components of the partial digest were eluted with water from the first electropherogram (Figure 4a) and subjected to further purification on DE-81 paper pH 1.9. The figure shows the separation for the DNA treated with phosphatase at 37° (Figure 4a). Identical results were obtained for components 1, 3, and 4 from Figure 4b. Component 4 separates into two major ^{32}P-containing spots. The main component is pG^{32}pA (see text) and the minor component with the lower mobility contains ^{32}P but no ^{3}H. This component has not been completely identified, but it is probably pNpG^{32}pA or a higher isostich.

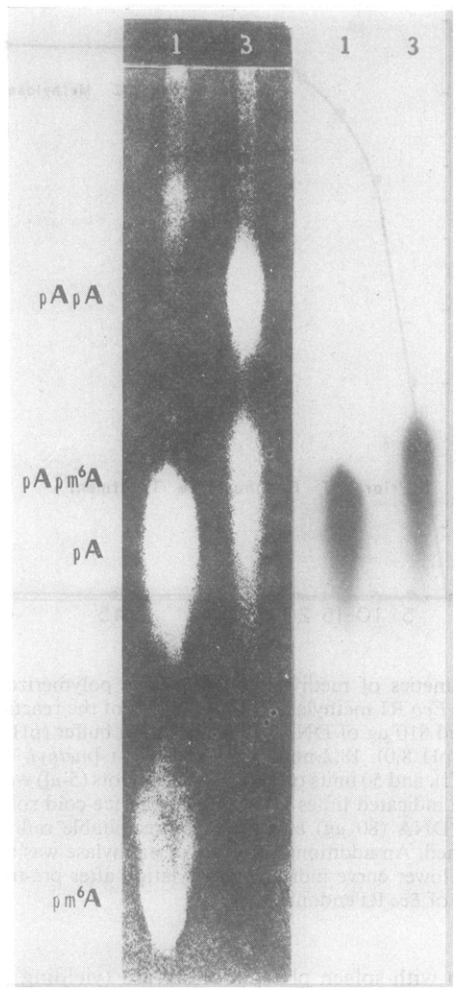

FIGURE 6: Autoradiogram and ultraviolet photograph showing the final chromatographic separation of components 1 and 3 obtained from the partial digest of DNA, after it was treated with phosphatase at 65°. Components 1 and 3 shown in Figure 4b were eluted, separated on DE-81 paper at pH 1.9 (data not shown, but identical to Figure 5), eluted, and separated by chromatography on Whatman No. 1 paper which was previously impregnated by dipping it into a solution made by diluting one volume of saturated $(NH_4)_2SO_4$ with 9 volumes of water. After drying the paper in air, it was developed by descending chromatography for 15 hr using a solvent system composed of 80 volumes of 95% ethanol and 20 volumes of water (Singh and Lane, 1964). The tank had been previously saturated for at least 12 hr with the vapors of the solvent by placing it at the bottom of the tank. The mono- and dinucleotides had the following isotopic composition (cpm): component 1 from Figure 4b, for pA, ^{32}P (9423) and ^{3}H (0); for pm^{6}A, ^{32}P (72) and ^{3}H (1512); components 3 from Figure 4b, for pApA, ^{32}P (0) and ^{3}H (0); for pApm^{6}A; ^{32}P (8856) and ^{3}H (3540).

GpApm^{6}ApTpTpC. Therefore, the sequence of the double-stranded DNA, which is the substrate site for the methylase, is the same as the sequence at which the *Eco* RI endonuclease-specific phosphodiester bonds are cleaved (Hedgpeth *et al.*, 1972). We find that there are two methyl groups, one on each strand of the double-helical DNA, added to each SV40 DNA molecule by the *Eco* RI modification methylase. As only double-stranded and not single-stranded DNA is a substrate for the methylase and since the methylated sequence is its own complement, we conclude that both methyl groups occur at the same site, and that SV40 DNA has only one site that is methylated by the *Eco* RI modification methylase. SV40 DNA also has one *Eco* RI endonuclease site (Morrow and Berg, 1972; Mulder and Delius, 1972). Digestion of SV40 DNA with the *Hin*$_d$ II endonuclease from *H. influenzae* (Smith and Wilcox, 1970), after it is first restricted with the *Eco* RI endonuclease or labeled with [^{3}H]methyl groups by the *Eco* RI methylase, shows that both sites are contained within the same DNA fragment (present results; Danna *et al.*, 1973) which is 7.5% of the length of SV40 DNA (Danna and Nathans, 1972) or approximately 400 base pairs long. All of these results suggest very strongly but do not prove that the substrate site for the two enzymes is physically the same.

The evidence for the physical identity of the *Eco* RI restriction and modification sites in SV40 DNA depends on the separation of two crucial fragments (pApA and pApm^{6}A) derived from partial digestion of an SV40 DNA molecule that was substrate for both the *Eco* RI endonuclease and the *Eco* RI methylase. This DNA was specifically labeled with

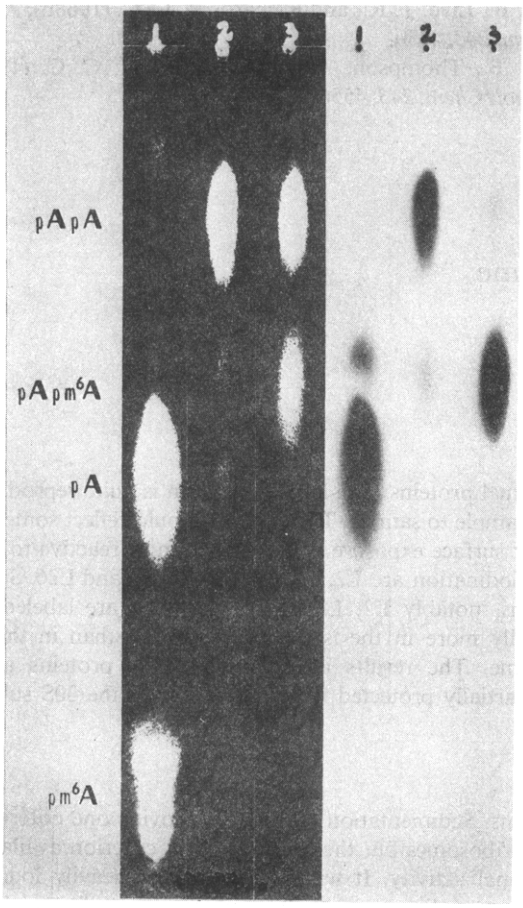

FIGURE 7: Autoradiogram and ultraviolet photograph showing the final chromatographic separation of components 1, 2, and 3 obtained from the partial digest of DNA, after it was treated with phosphatase at 37°. Components 1, 2, and 3 shown in Figure 4a were eluted, separated on DE-81 paper at pH 1.9 (Figure 5), eluted and separated by chromatography under the same conditions as those in Figure 6. The mono- and dinucleotides had the following isotopic composition (cpm): component 1 from Figure 4a, for pA, ^{32}P (11,656) and ^{3}H (0); for pm^6A, ^{32}P (89) and ^{3}H (1744); component 2 from Figure 4a, for pApA, ^{32}P (5363) and ^{3}H (0); for $pApm^6A$; ^{32}P (651) and ^{3}H (219); component 3 from Figure 4a, for pApA, ^{32}P (668) and ^{3}H (0); for $pApm^6A$, ^{32}P (9599) and ^{3}H (3812). The ^{32}P (957)-containing spot above pA is of unknown composition.

^{32}P at the endonuclease breaks and with ^{3}H at the site methylated by the modification methylase. If the two sites were physically distinct, that is, located at different points on the SV40 DNA, then all the ^{32}P should be found in $^{32}pApA$ (with no ^{3}H), whereas all the ^{3}H should be encountered in $pApm^6A$ (with no ^{32}P). Analysis showed that there was no $^{32}pApA$ in internal bihelical sequences (after phosphatase treatment at 65°). The fact that all of the ^{32}P and ^{3}H were found in the same dinucleotide, $^{32}pAp[^{3}H]m^6A$, proves that both enzymes act within (physically) the same sequence of nucleotides.

The question remains why is there $^{32}pApA$ which can be eliminated by phosphatase treatment at 65° but not at 37°. This dinucleotide presumably originates from an internal ^{32}P-labeled phosphomonoester as a result of incomplete ligation of the DNA preparation. It is conceivable that when ligase sealed one strand, the other had its 3′ terminus digested by a contaminating exonuclease. Polynucleotide ligase has polymerase I activity throughout most of the purification procedure (Modrich, personal communication) and this has a 3′-exonuclease activity (Lehman and Richardson, 1964). The absence of one or a few nucleotides in the sequence could obviate the ligation and consequent methylation of this sequence.

Although the identification of the doubly labeled dinucleotide ($^{32}pAp[^{3}H]m^6A$) proves that all the sites that are restricted are also methylated, it does not prove the converse, because a singly labeled dinucleotide ($pAp[^{3}H]m^6A$) resulting from a putative site that was methylated but not restricted would not separate from the double-labeled ($^{32}pAp[^{3}H]m^6A$) fragment and would have escaped detection. However, our quantitative data on the number of methyl groups introduced into SV40 DNA eliminate a second substrate site for the *Eco* RI methylase on this DNA. Therefore, there is one and only one site for the *Eco* RI endonuclease and methylase in SV40 DNA that serves as a convenient reference point on the circular molecule to which other features of the genome can be related.

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Surface Topography of the *Escherichia coli* Ribosome Enzymatic Iodination of the 50S Subunit†

David Jay Litman and Charles R. Cantor*

ABSTRACT: The surface topography of the 50S subunit of the *Escherichia coli* ribosome has been investigated by lactoperoxidase iodination. Heavily iodinated ribosomes (40 iodines/70S particle) retain substantial protein synthesis activity. The free 50S subunit incorporates about 15% more ^{125}I than the 50S moiety of the 70S ribosome under comparable conditions of labeling. The pattern of protein reactivity was determined by two-dimensional gel electrophoresis analysis of iodinated ribosomal proteins. The labeling pattern is quite reproducible from sample to sample. This pattern should reflect some measure of surface exposure. The proteins most reactive to enzymatic iodination are L2, L5, L6, L10, L11, and L26. Several proteins, notably L2, L26, L28, and L18, are labeled substantially more in the isolated 50S particle than in the 70S ribosome. The results indicate that these proteins are at least partially protected from iodination by the 30S subunit.

More than 50 different proteins from the 70S *Escherichia coli* ribosome have been identified (Garret and Wittmann, 1973). The location of these proteins is not yet known in any detail. One of the simplest approaches is to explore the surface structure of the ribosome. A technique that allows one to compare exposed proteins of the 70S ribosomes with those of the individual subunits is especially useful.

This paper describes the use of lactoperoxidase to investigate proteins on the surface of the 50S subunit of the 70S ribosome and on the surface of the isolated 50S particle. Lactoperoxidase enzymatically iodinates tyrosines (Morrison *et al.*, 1970). The large size of this enzyme (78,000) allows it to selectively label surface tyrosines. For example, a class of exposed proteins on the intact human erythrocyte was investigated with this enzymatic iodination system (Phillips and Morrison, 1971). While our studies on iodination of ribosomes were in progress, a preliminary report appeared comparing the one-dimensional gel patterns of iodinated native and unfolded 50S particles (Michalski *et al.*, 1973). This work showed that only a few proteins were highly modified. A one-dimensional gel, however, does not permit an unequivocal identification of these proteins.

A number of previous studies of ribosomal protein exposure have been reported (Huang and Cantor, 1972; Craven and Gupta, 1970; Kahan and Kaltschmidt, 1972; Crichton and Wittmann, 1971; Chang and Flaks, 1971; Hsiung and Cantor, 1973). What would be especially useful is a technique which allows greater resolution and quantitation. We shall show results to indicate that lactoperoxidase iodination monitored by two-dimensional gels offers significant advantages.

One problem with any chemical modification approach to structure is the risk of distortion of the particle by covalent reactions. Sedimentation coefficients provide one criterion of native ribosomes but the most stringent criterion available is functional activity. It will be shown that heavily iodinated ribosomes sediment normally and show significant protein synthesis activity. This lends considerable weight to any structural conclusions which can be drawn from the pattern of protein iodination.

Materials and Methods

Ribosomes. 70S ribosomes were prepared from mid-logarithmic phase *Escherichia coli* A19 as previously described (Traub *et al.*, 1971). The following buffers were used: A [10 mM Tris (pH 7.6)–30 mM NH_4Cl–10 mM $Mg(OAc)_2$]; B [A plus 6 mM β-mercaptoethanol]; C [10 mM Tris (pH 7.6)–30 mM NH_4Cl–0.3 mM $Mg(OAc)_2$–6 mM β-mercaptoethanol]. 30S and 50S ribosomal subunits were prepared from 70S ribosomes by dialysis against buffer C for 48 hr at 4° and were separated on 10 → 30% 38 ml sucrose gradients in an SW 27 rotor at 25K for 16 hr. Analytical gradients (5 ml) were centrifuged in an SW 50.1 rotor at 45K for 1–3 hr.

Iodination of Ribosomes. Lactoperoxidase was obtained from Sigma Chemical Co. and purified as described by Morrison and Hultquist (1963); its concentration was determined by absorbance at 412 nm using an extinction coefficient of 114 mM^{-1} (Morrison *et al.*, 1957).

70S or 50S ribosomes were dialyzed against buffer A for 36 hr. A typical 30-ml reaction mixture contained 150 mg of 70S or 50S ribosomes, 300 μg of lactoperoxidase, 1.13 ml of 9.75 mM H_2O_2, and 3 ml of 2×10^{-3} M $Na^{125}I$. The specific activity of the $Na^{125}I$ was usually 10 Ci/M. The reaction mixture was stirred for 30 min. The reaction was stopped by adding 25 μl of β-mercaptoethanol (375 μmol). Ribosomes were pelleted, resuspended, and dialyzed against buffer B.

Extraction of Ribosomal Proteins. 50S ribosomes in buffer C were stripped of proteins either by the acetic acid method (Hardy *et al.*, 1969) or by a modification of LiCl and urea

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