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Binding of the *Eco*RII methyltransferase to 5-fluorocytosine-containing DNA. Isolation of a bound peptide

Stanley Friedman and Nasrin Ansari
Department of Pharmacology, SUNY Health Science Center at Brooklyn, Box 29, 450 Clarkson Avenue, Brooklyn, NY 11203, USA

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ABSTRACT

The properties of the interaction of 5-fluorocytosine-containing DNA with the *Eco*RII methyltransferase were studied. The DNA used was either a polymer synthesized in vitro, or a 20-mer containing one CCA/TGG sequence. The DNA could be methylated by the enzyme. In the process the enzyme formed a tight binding adduct with the DNA that could be identified by sodium dodecyl sulfate-polyacrylamide gel elctrophoresis. Enzyme activity was inhibited by this interaction. The 20-mer could be used to titrate the active site of the enzyme. The DNA polymer formed a tight binding complex that could be identified following digestion of the DNA with pancreatic deoxyribonuclease or micrococcal nuclease. A peptide-DNA adduct could be isolated after digestion of the *Eco*RII-DNA adduct with staphylococcal protease V8 by high pressure liquid chromatography and polyacrylamide gel electrophoresis. Sequencing of the peptide indicated the DNA bound to a region of the protein that is conserved in all procaryotic DNA(cytosine-5)-methyltransferases. We have previously shown that this region contains a cysteine that can be photomethylated with adenosylmethionine. This region, in addition to forming part of, or being adjacent to, the AdoMet binding site, also forms part of the DNA binding site.

INTRODUCTION

DNA(cytosine-5)methyltransferases are proteins that methylate the 5 position of cytosine in DNA. Several cytosine analogs have been shown to inhibit these enzymes. These analogs must be incorporated into DNA to be effective (1,2).

The catalytic mechanism of these enzymes is believed to be analogous to that of thymidylate synthase (3). This mechanism involves binding of a nucleophile on the enzyme to the 6 carbon of the pyrimidine ring, activating the 5 position, which can then accept a methyl group from the substrate S-adenosylmethionine. The final step is the dissociation of the enzyme from the DNA.

The cytosine analogs that inhibit DNA methylation once incorporated into DNA are 5-azacytosine, 5-fluorocytosine and pseudocytosine (1,2). DNA containing 5-azacytosine is an inhibitor of DNA methylation *in vitro*. Such DNA forms tight binding complexes with DNA(cytosine-5)methyltransferases (4,5). The formation of these complexes is stimulated by, but not dependent on, the presence of AdoMet (6). The replacement of cytosine by azacytosine at the site of methylation is necessary for this tight binding (6). The binding however is not stable and no 5-azacytosine-containing DNA-peptide adduct has been isolated. 5-fluorocytosine in DNA has also been shown to form a tight binding complex with a DNA methyltransferase (7). The formation of this complex is dependent on the presence of AdoMet; no isolatable covalent complex is formed in the absence of this substrate. Methylation of the DNA must occur for the complex to form. It was proposed that the complex that formed once the methyl group was transferred to the 5-position of fluorocytosine would be stable. However attempts to isolate and characterize the adduct have not been reported. This type of mechanism-based inhibition occurs with the enzymes thymidylate synthase (8), tRNA(uracil-5)methyltransferase (9) and dUMP hydroxymethylase (10), with 5-fluorouracil-containing analogs.

In analogous experiments it was found that 5-fluorodeoxycytidylate is a competitive inhibitor of the T4 bacteriophage hydroxymethyldeoxycytidylate synthase, in which methylenetetrahydrofolate is the methylene donor (11). No adduct is formed in this reaction.

In this paper we characterize the adduct formed between 5-fluorocytosine-containing DNA (FCyt-DNA) and the *Eco*RII methyltransferase. An adduct formed between the enzyme and FCyt-DNA can be isolated. The enzyme-DNA adduct can be digested with proteases and a DNA-peptide complex isolated by HPLC and polyacrylamide gel electrophoresis. The sequence of the peptide indicates that the reactive portion of the protein contains cysteine-186, an amino acid present in all DNA(cytosine-5)methyltransferases that has been proposed as the catalytic nucleophile in these proteins (12). We have previously shown that this cysteine undergoes a photomethylation reaction with AdoMet (13). Its presence in the peptide that binds DNA and its reactivity with AdoMet indicates it forms part of the active site of this enzyme.

MATERIALS AND METHODS

Materials

The *Eco*RII methyltransferase was prepared as previously described from an overproducing strain of *E.coli* B WP2 containing the plasmids pGP1 and pRIIM (14). Plasmid pGP1 constructed by Tabor and Richardson confers kanamycin resistance, has the phage T7 RNA polymerase gene cloned downstream to the lambda P_L promoter and contains the gene for the heat sensitive *lambda* repressor (15). Plasmid pRIIM, which confers ampicillin resistance has the *Eco*RII gene cloned downstream to the *phi*10 promoter of the T7 RNA polymerase. 5-fluorodeoxycytidine was purchased from Cal Biochem., Sequenase from U. S. Biochemical, pancreatic DNase and *Staphylococcus aureus* proteinase V8 were purchased from Boehringer Mannheim, TPCK treated trypsin was from Sigma Biochemical Co. Trimethylphosphate and phosphorus oxychloride were purchased from Aldrich and redistilled before use.

Preparation of 5-fluorodeoxycytidine triphosphate

5-fluorodeoxycytidine monophosphate (FdCMP) was synthesized from 5-fluorodeoxycytidine (FdCyd) under anhydrous conditions as described by Tanaka et al (16). FdCyd, 9.2 mg, was suspended in 0.15 ml trimethylphosphate and treated with phosporus oxychloride, 5.2 μl. When the 5-FdCyd had dissolved the reaction was incubated at 4°C overnight. The reaction mixture was then added to 1 ml of ice cold 0.2 M triethylammonium bicarbonate. After 30 min the reaction was lyophilized. The reaction products were analyzed by HPLC on a Synchropak 100 anion exchange column (25 cm×4.2 mm) equilibrated with water (solvent A). The nucleotides were eluted with a linear gradient 0−100% B (0.1 M sodium dihydrogen phosphate in 0.8 M sodium acetate, pH 7.3) at a flow rate of 0.5 ml/min. FdCMP eluted at 29 min. The lyophilized product was dissolved in water and applied to an AG-50W-X2 column (20×1 cm) in the H^+ form. The products were eluted with water and the absorbance monitored at 245 nm. The pooled fractions were lyophilized to yield a white powder. Yield of FdCMP, 42%.

The enzymatic synthesis of fluorodeoxycytidine triphosphate (FdCTP) from FdCMP was modified from Bouchard & Momparler (17) and Osterman et al. (7). FdCMP, 15 μmole, was incubated in a 2 ml reaction mixture containing 50 mM Tris-HCl, pH 7.5, 10 mM ATP, 10 mM $MgCl_2$ 2 mM dithiothreitol, 0.2 mg/ml bovine serum albumin, 0.2 ml glycerol, 20 mM phosphoenolpyruvate, 8 units pyruvate kinase, 2 units nucleoside monophosphate kinase, and 10 units nucleoside diphosphate kinase for 1 hr at 37°C and loaded onto a Sephadex DEAE-A-25 column (2×36 cm). The products were eluted with a linear gradient of 0−2 M ammonium formate and lyophilized. The lyophilized product was dissolved in 1 ml water and desalted on a G-10 column (33×1.5 cm) eluted with water. The products were analyzed by HPLC as described above. Elution times were FdCTP, 45 min, ATP, 51 min. The product was lyophilized and dissolved in water. The yield was 57%.

Synthesis of 5-fluorocytosine-containing DNA

A deoxynucleotide polymer containing 5-fluorocytosine was synthesized with CCCA/TGGG as primer. The primer, 6.4 μg, was first phosphorylated with T_4 polynucleotide kinase and ATP in a volume of 10 μl and then ligated with T_4 DNA ligase according to published procedures (18). The reaction was heated to 55°C, dialyzed against Tris-HCl, 10 mM, EDTA, 1 mM, pH 8 (TE buffer), lyophilized, and dissolved in 40 μl TE buffer. The ligated oligonucleotide, 5 μl, was diluted with 19 μl of water, heated to 100°C for 2 min and then slowly cooled to 37°C. The incubation mixture contained the ligated oligonucleotide, 65 mM potassium phosphate, pH 7.5, 6.5 mM $MgCl_2$, 1 mM dithiothreitol, 5 μg/ml bovine serum albumin, 10 nmoles dNTPs, 60 pg pancreatic DNase, 1 μCi [α-^{32}P]dATP and Sequenase, 13 units, in a volume of 40 μl (19). The reaction was incubated at 37°C for 1 hr. The reaction was followed by measuring the incorporation of ^{32}P into trichloroacetic acid insoluble material. The reaction mixture was extracted once with a mixture of chloroform-phenol, twice with chloroform and the DNA was precipitated with ethanol. The DNA from several preparations was dissolved in 0.1 ml TE buffer and passed through a spun column of Sephadex G-50. The yield from 4.8 μg of ligated oligonucleotide, determined by uv spectroscopy (ϵ_{260}=13,000), was 39.4 μg of DNA polymer. The incorporation of fluorocytosine into the DNA polymer was verified by nucleoside analysis. An aliquot of DNA was hydrolyzed to nucleosides with pancreatic DNase, snake venom phosphodiesterase and alkaline phosphatase as described (20) and analyzed by high pressure liquid chromatography on a Beckman ODS column (25×0.46 cm) eluted with 50 mM ammonium phosphate, pH 8, containing 15% methanol.

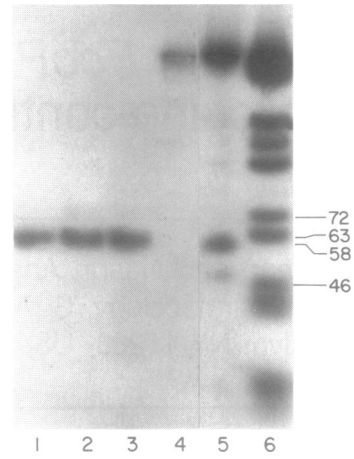

Figure 1. Digestion of the FCyt-DNA polymer with restriction enzymes. The FCyt-DNA polymer, 0.2 μg, was digested with 10 units of the indicated restriction enzyme at 37°C overnight. The products were analyzed on a 10% polyacrylamide gel. Lane 1, *Mbo*I digested, lane 2, *Bst*NI digested, lane 3, *Nla*III digested, lane 4, 58 bp standard, lane 5, standards.

In order to determine the structure of the polymer it was digested with *Bst*NI. One major band of approximately 60 bp was obtained. The digestion product was gel purified, end filled with the Klenow fragment of DNA polymerase I and cloned into the *Sma*I site of M13mp19 and transfected into *E.coli* HB101. Two isolates were sequenced by the method of Sanger et al. (21).

Synthesis of a deoxyoligonucleotide 20-mer containing 5-fluorocytosine

The template 5'CAAATTTCCTGGATGCGCTC3', 2.3 μg, was annealed with the primer 5'GAGCGCATC3', 1.2 μg, in 0.12 M potassium phosphate, pH 7.5 containing 12 mM $MgCl_2$ in a volume of 70 μl. The mixture was heated to 80°C and slowly

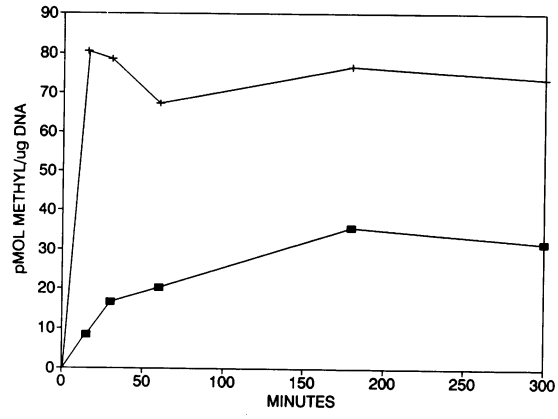

Figure 2. Time course for incorporation of ^{3}H-methyl from AdoMet into DNA. The incubation mixture contained: [methyl-^{3}H]AdoMet, 8 μM, specific activity, 480 Ci/mol, 0.2 μg FCyt-DNA, or 0.1 μg Cyt-DNA, and 6 μg enzyme in a volume of 60 μl. Incubation was performed at 30°C. Cyt-DNA, +—+; FCyt-DNA, ■—■.

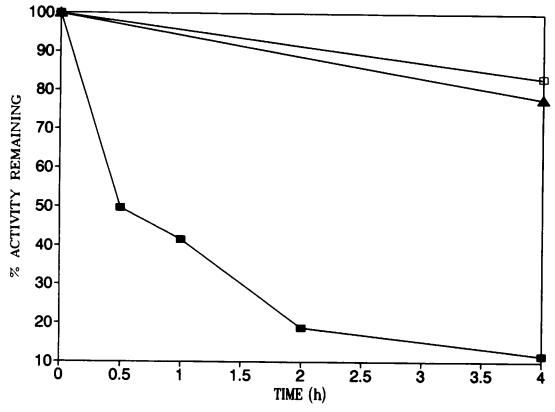

Figure 3. Inhibition of the *Eco*RII methyltransferase by FCyt-DNA. DNA and enzyme were preincubated as described in 'Methods'. Aliquots were taken at the indicated times and assayed for enzyme activity. Control Cyt-DNA, □; FCyt-DNA, without AdoMet, ▲; FCyt-DNA plus 36 μM AdoMet, ■.

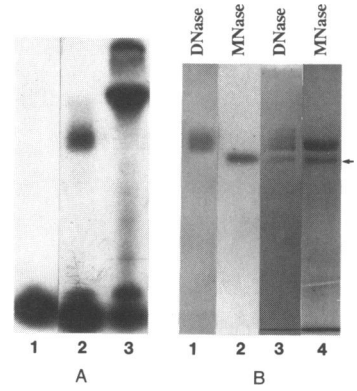

Figure 4. Formation of FCyt-DNA-*Eco*RII methyltransferase adduct. **A.** Cyt-DNA or FCyt-DNA, 0.1 μg, was incubated with 0.7 μg enzyme for 1 h in 10 μl and treated with 1 μg DNase, and 0.1 μmol $MgCl_2$, for 1 h at 37°C. The products were analyzed by SDS-PAGE. Lane 1, Cyt-DNA; lanes 2 & 3, FCyt-DNA; lane 3, without DNase. **B.** Digestion of FCyt-DNA-*Eco*RII methyltransferase adduct with DNase or micrococcal nuclease. FCyt-DNA, 0.1 μg, was incubated with *Eco*RII methyltransferase, 0.25 μg, in a volume of 10 μl for 6 h at 30°C. Either 1 μg DNase and 0.1 μmol $MgCl_2$; or 0.4 μg micrococcal nuclease and 30 nmol $CaCl_2$, were added, the incubation continued for 1 h at 37°C, and the reaction analyzed by SDS-PAGE. Lanes 1 and 3, DNase. Lanes 2 and 4, micrococcal nuclease. Lanes 1 and 2, autoradiogram. Lanes 3 and 4, coommassie blue. The intense band in lane 4 is albumin present in the micrococcal nuclease. The arrow marks the position of the *Eco*RII methyltransferase.

cooled to 30°C. The synthesis was performed as described above except DNase was not added. The products were purified on a Sephadex G-50 column (24×0.6 cm) eluted with 50 mM ammonium bicarbonate. Yield 77%.

Binding of FCyt-containing DNA to the *Eco*RII methyltransferase

The reaction mixture contained 33 mM Tris-HCl, pH 8, 6 mM dithiothreitol, 60 mM NaCl, 0.6 mM EDTA, 25 μM S-adenosylmethionine and the indicated amount of DNA polymer or 20-mer and enzyme. Incubation was at 30°C. At the completion of the reaction when the DNA polymer was used as substrate, DNase and $MgCl_2$ were added to a final concentration of 17 μg/ml and 10 mM respectively and the reaction incubated for 1 hr at 37°C. The reaction was then heated to 55°C for 5 min and stored frozen. The reaction was analyzed by electrophoresis on an 8% polyacrylamide gel in a Tris-borate-EDTA buffer (18). Alternatively the reaction was heated to 100°C for two min in SDS gel loading buffer and analyzed by SDS-polyacrylamide gel (SDS-PAGE) electrophoresis as described by Laemmli (22). The gels were either analyzed by autoradiography or cut into slices, digested with 0.4 ml 30% hydrogen peroxide at 70°C overnight and the radioactivity determined by scintillation counting.

Isolation of FCyt-DNA-bound peptide

FCyt-DNA, 30 μg, 100 μg *Eco*RII methyltransferase and 10 μM [methyl-^{3}H]AdoMet, specific activity, 111 Ci/mol, were incubated in a volume of 1.2 ml for 4.5 h at 30°C. $MgCl_2$, 12.5 μmol and 20 μg DNase were added and the incubation continued for 1h at 37°C. An equal volume of SDS gel loading buffer (22) was added and the mixture heated to 100°C for 6 min and dialyzed against 2 changes of 1000 volumes of 0.05 M ammonium bicarbonate. The dialyzed adduct was dried, dissolved in 100 μl 8 M urea and treated with 0.5 μmol DTT and 1 μmol iodoacetamide, as described (23). The sample was desalted by ultrafiltration on a UM 30 centricon filter and washed with 2 ml 0.05 M ammonium bicarbonate. The sample was collected in a volume of 0.1 ml and treated with 10 μg staphylococcal protease V8 at 37°C for 16 h. Aliquots of 30 μl were applied to a Beckman ODS column (0.43×25 cm) and eluted with a gradient of 0—70% acetonitrile in 0.05 M triethylammonium acetate at 0.5 ml/min for 45 min. The fractions containing tritium were concentrated and electrophoresed on a 10% polyacrylamide gel in Tris-borate-EDTA buffer (16) and transblotted to a polyvinylidene difluoride membrane for sequencing. Peptide sequences were determined by Edman degradation on an Applied Biosystems 477A gas-phase protein sequencer. The facility was available under the core program of SUNY Health Science Center at Brooklyn.

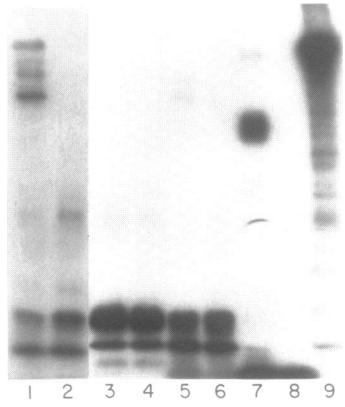

Figure 5. Formation of FCyt-DNA-*Eco*RII methyltransferase adducts with restricted FCyt-DNA. FCyt-DNA was digested as described in the legend to figure 1. The digested DNA was purified by phenol/chlroform extraction. FCyt-DNA, 0.06 μg, was incubated with 1 μg *Eco*RII in the presence or absence of AdoMet as indicated in 'Methods'. The products were analyzed by SDS-PAGE. Lanes 1 & 2, *Nla*III digested, lanes 3 & 4, *Bst*NI digested, lanes 5 & 6, *Mbo*I digested, lanes 7 & 8, digested with DNase after adduct formation, lane 9, undigested DNA without enzyme. Odd lanes, with AdoMet; even lanes, without AdoMet.

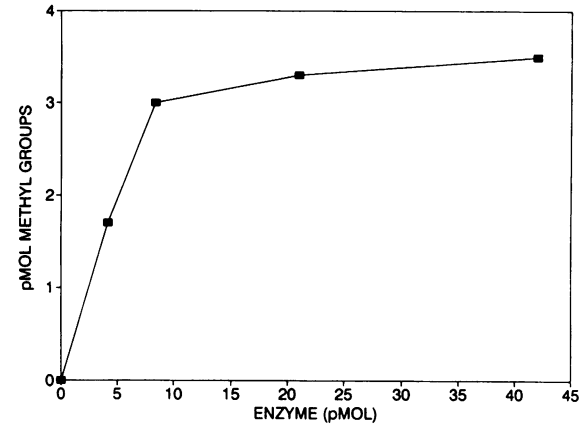

Figure 6. Titration of FCyt-DNA with *Eco*RII methyltransferase. FCyt-DNA, 0.18 μg, and 0.1 mM [methyl-^{3}H]AdoMet, specific activity, 455 Ci/mol, were incubated with increasing amounts of enzyme for 6 h and the products analyzed by SDS-PAGE. The radioactive bands were cut from the gel and the tritium present analyzed by scintillation counting.

Enzyme assays

Assays of methyltransferase activity measured the transfer of ^{3}H methyl groups to *E.coli* B DNA from [methyl-^{3}H]AdoMet as previously described (1). Incorporation of methyl-^{3}H from AdoMet into the 20-mer was determined by applying 15 μl of the reaction mixture to 1.5 cm squares of Whatman DE-81 paper, washing the discs 3 times with 0.2 M ammonium bicarbonate and once each with ethanol and diethylether (24). The discs were dried and counted in a scintillation counter. Enzyme concentration was determined by uv absorbance assuming a molar extinction coefficient at 280 nm of 55,000 determined from the amino acid composition (25).

To measure the inhibition of the *Eco*RII methyltransferase by the FCyt-DNA polymer the enzyme was preincubated with 0.27 μg DNA, 0.2 μg enzyme, 35 μM AdoMet, 33 mM Tris-HCl, pH 8.0, 6 mM dithiothreitol, 60 mM NaCl and 0.6 mM EDTA in a total volume of 30 μl. The reaction was incubated at 30°C and aliquots taken at the indicated times and assayed. The assay mixture contained 10 mM Tris-HCl, pH 8, 8 mM 2-mercaptoethanol, 0.8 mM EDTA and 12.5 μg *E.coli* B DNA in a total volume of 0.25 ml. The final concentration of AdoMet was adjusted to 3 μM, specific activity, 362 Ci/Mol. Incubation was for 15 min at 37°C.

RESULTS

Synthesis of FCyt-containing DNA

In order to prepare a substrate enriched in CCA/TGG sequences and containing 5-fluorocytosine, a DNA polymer was synthesized using an oligomer of CCCA/TGGG as template. The septanucleotides were first polymerized with T_4 DNA ligase and the resulting oligonucleotides used as template for the incorporation of nucleotides into a DNA polymer. The polymerization reaction resulted in an 8.2 fold net synthesis based on the amount of input oligonucleotide.

Nucleoside analysis of the polymer by HPLC indicated it was not a repeating polymer of CCCA/TGGG sequences. The relative composition of the DNA was: dCyd, 2; FdCyd, 18; dGuo, 21; Thd, 29; dAdo, 30. The polymer could be hydrolyzed with *Bst*NI endonuclease, which hydrolyzes DNA at CCA/TGG sequences after the 3′ C residue. The polymer was labeled during synthesis with [α-P^{32}]dATP. The digest was analyzed by polyacrylamide gel electrophoresis and autoradiography. Ninety percent of the digest had a mobility of approximately 60 bp. The fragment was cloned and sequenced. The sequence obtained was AGGGAT-CATCATGAAGAACTAATAGCCATTAGTCTTATGAGAC-TAATAGCATACC; a 55 bp oligonucleotide with single *Bst*NI, *Mbo*I and *Nla*III sites. To prove that the original polymer was a repeating polymer containing this sequence it was digested with each of these restriction enzymes. The digests each yielded one fragment having identical mobilities on electrophoresis (figure 1). The basis for the synthesis of this polymer is unclear. Four different batches of Sequenase yielded a product that could be digested with *Bst*NI to yield an oligonucleotide of identical electrophoretic mobility. No polymer was synthesized when DNA polymerase I was used instead of Sequenase. Synthesis with Sequenase only occurred after 15 minutes of incubation and did not occur if the septamer were not first polymerized.

Reaction of FCyt-DNA with the *Eco*RII methyltransferase

The FCyt-containing DNA could be methylated by the *Eco*RII methyltransferase. The time course of methylation was very different when Cyt-DNA polymer and FCyt-DNA polymer were methylated with excess enzyme. The methylation of control DNA was complete within 15 min whereas it took 3 hours for the methylation of FCyt-DNA to be completed. Twice as many methyl groups were incorporated into control DNA as were incorporated into FCyt-DNA (figure 2).

The FCyt-DNA polymer inhibited the enzyme in an irreversible manner (figure 3). The time course of the inhibition was similar to the time course for methylation of the FCyt-DNA by the enzyme. The irreversible inhibition was dependent on the presence of the substrate, AdoMet.

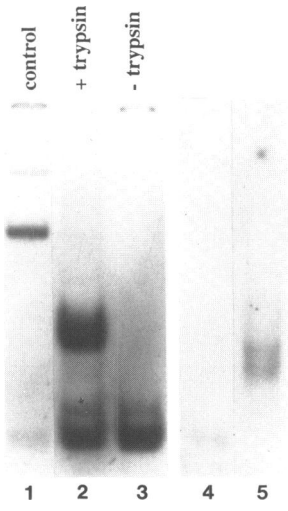

Figure 7. Digestion of FCyt-DNA-*Eco*RII methyltransferase adduct with trypsin or staphylococcal protease V8. FCyt-DNA, 0.36 μg, was incubated with 2.5 μg *Eco*RII methyltransferase, and treated with DNase as described in the legend to figure 4. The incubation mixture was ultrafiltered through a UM10 Centricon filter and washed 3 times with 2 ml TE buffer. The retentate was evaporated to dryness, dissolved in 40 μl 8 M urea containing 0.2 M Tris-HCl, pH 8, and treated with 0.2 μmol DTT and 0.42 μmol iodoacetamide as described (23). The reaction was diluted to 160 μl with water and an 80 μl aliquot was treated with 4 μg trypsin for 16 h at 37°C. The products were analyzed on a nondenaturing gel. Lane 1, adduct prior to ultrafiltration; lane 2, trypsin; lane 3, incubated in the absence of trypsin. Lanes 4 and 5, staphylococcal protease V8 digestion products of the FCyt-DNA-*Eco*RII methyltransferase adduct purified by HPLC as shown in figure 8. Lane 4, fraction lacking tritium. Lane 5, pooled fractions containing tritium.

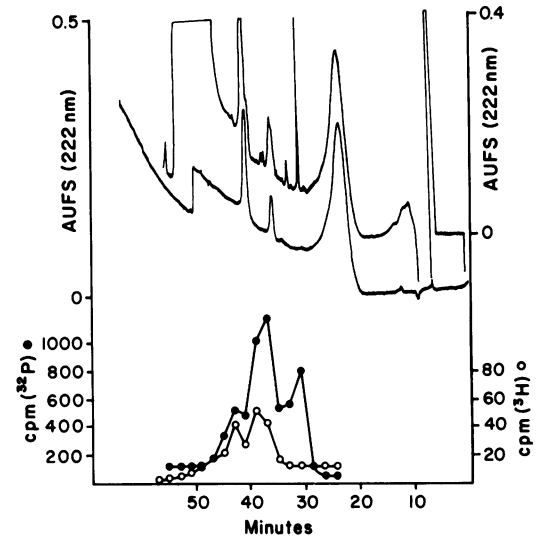

Figure 8. HPLC of a staphylococcal protease V8 digest of the FCyt-DNA-*Eco*RII methyltransferase adduct. The FCyt-DNA peptide adduct was prepared as described in 'Methods'. The staphylococcal protease V8 digestion product was purified by HPLC. Aliquots were removed for tritium determination by scintillation counting. Fractions eluting between 37 and 40 min were pooled and further purified by electrophoresis as described in 'Methods'. Recovery of tritium was 30%. Upper two tracings, absorbance from experimental (upper) and control (lower) chromatograms. Lower two tracings, Cerenkov radiation, •; tritium, ○.

Adduct formation of *Eco*RII methyltransferase with FCyt-containing DNA

When FCyt-containing DNA that was synthesized with [α-^{32}P]ATP was incubated with the *Eco*RII methyltransferase, and the mixture treated with pancreatic DNase an adduct could be detected by SDS-PAGE (figure 4A). This adduct did not form with the control DNA polymer. The appearance of the adduct was dependent on the presence of AdoMet in the incubation mixture. Digestion with micrococcal nuclease yielded a unique radioactive band on SDS-PAGE which coincided with a coommassie blue staining band that had the mobility of the intact enzyme (figure 4B, lanes 2 & 4, marked by an arrow).

The adduct that was present after digestion with DNase gave a broad radioactive band on SDS-PAGE (figure 4B, lanes 1 & 3). The appropriate amount of DNase used had to be titrated for each experiment. No adduct formed if the AdoMet analogs S-adenosylhomocysteine or sinefungin were used in place of AdoMet (data not shown).

If the enzyme were binding to the CCA/TGG sequence in the DNA it should not bind to the FCyt-DNA if the DNA were first digested with *Bst*NI, but should bind to DNA digested with *Nla*III (recognizes CATG). Binding should also be decreased if the DNA is hydrolyzed with *Mbo*I (recognizes GATC) since this site occurs adjacent to the CCA/TGG sequence. As shown in figure 5 the enzyme bound to the *Nla*III digested polymer, bound weakly to the *Mbo*I digested polymer but did not bind to the *Bst*NI digested polymer.

To determine if tritium from [methyl-^{3}H]AdoMet were incorporated into the adduct the ^{32}P-containing band obtained after DNase digestion was cut from the gel and the tritium present determined by scintillation counting. Tritium from [methyl-^{3}H]AdoMet was incorporated into the adduct. However, when ^{35}S-AdoMet was used as substrate no ^{35}S was incorporated into the adduct (data not shown). The DNA must therefore undergo methylation for a stable adduct to be formed.

The amount of ^{3}H-methyl incorporated into the adduct was determined in the presence of excess enzyme. Radioactive bands were cut from the gel and the amount of tritium present was determined by scintillation counting. 3.5 pmoles of methyl group were incorporated per 560 pmoles of DNA nucleotide (figure 6). Assuming the frequency of *Eco*RII sites as one per 55 bp of DNA, as determined from the sequence, then 69% of the sites could accept one methyl group from the *Eco*RII methyltransferase.

The stability of the adduct was studied. Overnight storage at pH 5 or lower caused complete loss of the adduct, whereas the adduct was stable for 5 days at room temperature after heating at 60°C for 5 min.

Digestion of the methyltransferase-FCyt-DNA adduct with protease

The reaction mixture, after DNase treatment, was ultrafiltered through UM30 membranes or chromatographed on Sephadex G-50 to remove excess substrate and digested DNA. The adduct was then reduced with dithiothreitol and alkylated with iodoacetamide in 8 M urea prior to digestion with trypsin. The products were analyzed by gel electrophoresis in the Tris-borate buffer system. As shown in figure 7 the adduct could be digested by trypsin to yield a stable intermediate. However in the control reaction, one that was not treated with trypsin, the adduct dissociated. Similar results were obtained when staphylococcal

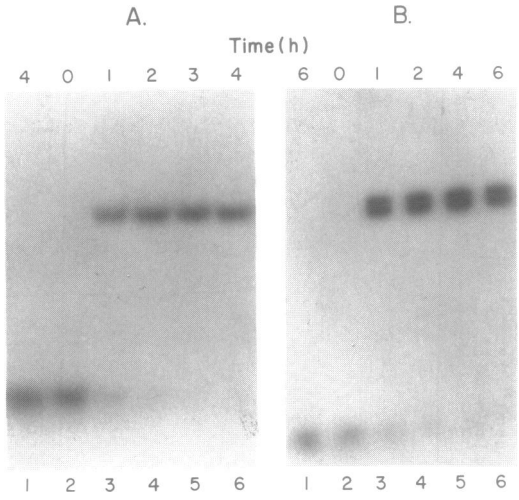

Figure 9. Time course for binding of FCyt-20-mer to the *Eco*RII methyltransferase. The 20-mer, 3 pmol, was incubated at 30°C in a final volume of 30 μl with 36 μM AdoMet and 27 pmol enzyme. Panel A, aliquots were taken at the indicated times, mixed with 10 μl loading buffer containing 30% glycerol, 0.25% bromphenol blue and 0.25% xylene cyanol, heated to 55°C for 5 min and analyzed by electrophoresis on an 8% polyacrylamide gel in Tris-borate-EDTA buffer. Lane 1, Cyt-20-mer; lanes 2–6, FCyt-20-mer. Panel B, samples were analyzed by SDS-PAGE; lane 1, Cyt-20-mer; lanes 2–6, FCyt-20-mer.

protease V8 was used instead of trypsin to digest the alkylated adduct.

Isolation of a peptide-DNA adduct

The reaction mixture containing the methyltransferase-FCyt-DNA adduct was treated with DNase and heated in 1% SDS. The adduct was purified by ultrafiltration, reduced with dithiothreitol and alkylated with iodoacetamide as desribed in the 'Methods' section. It was then digested with staphylococcal protease V8 and the products purified by HPLC. Two radioactive peaks containing ^{32}P were eluted from the column. The peak that eluted first did not contain any tritium. The second peak contained tritium in addition to ^{32}P (figure 8). When these peaks were analyzed by gel electrophoresis in a Tris-borate buffer system and autoradiography, the second peak contained the digestion product whereas the mobility of the first peak corresponded to the DNA released from the adduct if no protease were added (figure 7, lanes 4 & 5).

The gel was electroblotted onto a polyvinylidene difluoride membrane and the radioactive band was subjected to peptide sequencing on a gas phase Applied Biosystems sequencer. Data were obtained from twenty cycles. The sequence obtained was:

__VPD__DVLLAGFP__QPFSLA

Predicted Sequence: (E)HVPDHDVLLAGFPCQPFSLA-GVSKKNSLGRAHGFE. The first cycle yielded several amino acids including histidine. No amino acid was obtained at the 5th and 14th cycle. Cysteine, the expected amino at cycle 14, is not detected by the sequencing procedure. If the cysteine were present as carboxamidomethylcysteine, the expected product if cysteine is not bound covalently to the DNA, it can sometimes be detected; but failure to detect it is not definitive evidence for its absence. The ^{32}P radioactivity remained bound to the membrane after the sequencing procedure was terminated.

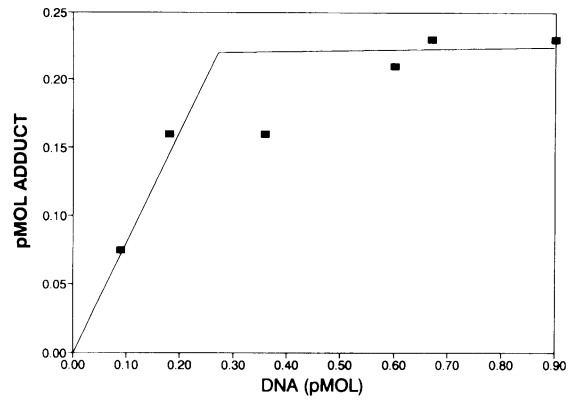

Figure 10. Titration of enzyme with 20-mer. FCyt-20-mer was incubated with 0.33 pmol *Eco*RII methyltransferase as indicated in the legend to figure 9 for 4 h and analyzed on a nondenaturing gel. The radioactive adduct was cut from the gel and counted in a scintillation counter.

This sequence was consistent with the staphylococcal protease V8 digestion product expected for amino acids 173 to 192. This product contains the amino acid sequence G__PC_____S____G present in all DNA(cytosine-5)methyltransferases sequenced to date.

Formation of an adduct between the enzyme and FCyt-containing 20-mer

A defined deoxyribooligonucleotide 20-mer, containing a single 5-fluorocytosine residue, was prepared by enzymatic synthesis with the template 5'CAAATTTCCTGGATGCGCTC3' and the primer 5'GAGCGCATC3'. This oligonucleotide contains one CCA/TGG site. The cytosine in the synthesized strand that normally undergoes methylation is replaced by 5-fluorocytosine.

The 20-mer could also be methylated with [methyl-^{3}H]AdoMet. With this oligonucleotide an equal amount of methyl groups was incorporated in both the control and FCyt-containing oligomer, 2.1 and 1.9 moles per mole 20-mer methylated, respectively. This would be the expected result if the Cyt-containing strand were methylated much faster then the FCyt-containing strand.

Incubation of the oligomer with the *Eco*RII methyltransferase and AdoMet resulted in the formation of an adduct which could readily be detected by electrophoresis at pH 8 (figure 9A). An adduct could also be detected on SDS-PAGE after heating at 100°C in the presence of 1% SDS (figure 9B). Formation of the adduct was dependent on the presence of AdoMet in the incubation mixture.

The enzyme could be titrated with the FCyt-containing oligomer (figure 10). To quantitate the amount of adduct formed the bands were cut out of the gel and the Cerenkov radiation determined in a scintillation counter. The titration shown in figure 9 yielded 0.23 pmole of adduct. This was 70% of the theoretical yield based on the amount of enzyme added as determined by uv spectrometry. This adduct behaved similarly to the adduct prepared with the FCyt-DNA containing polymer. It dissociated after ultrafiltration and incubation in 8 M urea. However if it were first boiled in SDS, purified by ultrafiltration, reduced and alkylated in 8 M urea and digested with staphylococcal protease, a peptide adduct could be identified by gel electrophoresis in Tris-borate-EDTA buffer (data not shown). This adduct was not analyzed further.

DISCUSSION

Santi et al. proposed that a nucleophile on the enzyme added to the 6 position of cytosine to activate the 5 position to accept a methyl group from AdoMet (3). This mechanism has been substantiated for thymidylate synthase. The latter enzyme forms an adduct with 5-FdUMP in the presence of the methyl donor methylene tetrahydrofolic acid. A peptide bound to 5-FdUMP was obtained but the peptide adduct was sensitive to the acid conditions of Edman degradation and a single amino acid-5-FdUMP complex was never obtained (26), even though the nucleophile has been shown to be cysteine by X-ray crystallography (8).

In order to determine if a similar mechanism were used by the DNA(cytosine-5)methyltransferases the interaction of the *Eco*RII methyltransferase with DNA containing 5-fluorocytosine was studied. Two species of FCyt-DNA were synthesized. A defined oligomer containing 5-fluorocytosine in one strand and a polymer containing 5-fluorocytosine in both strands.

No enzyme oligomer adduct could be detected by electrophoresis with cytosine incorporated into the oligomer. No free FCyt-containing oligomer could be detected when this oligomer was incubated with excess enzyme. Therefore no cytosine was present in the synthesized strand of the latter oligomer and the 5-fluorocytosine present in the oligomer was stable during synthesis, storage, and under the conditions of incubation.

The oligomer was methylated by the enzyme and in the process formed an adduct that could withstand boiling in 1% SDS. The DNA could be used to titrate the active site of the enzyme and gave results consistent with one active site per monomer.

When the 20-mer was used as substrate with excess enzyme the number of methyl groups incorporated into the control oligonucleotide and the oligonucleotide containing 5-fluorocytosine in one strand were the same; two moles of methyl group were incorporated per mole oligomer. However, with the FCyt-DNA polymer there were approximately twice as many radioactive methyl groups incorporated into the control than the FCyt-DNA polymer; 2.3 moles per mole 55 bp repeat for the control vs. 0.9 moles per mole 55 bp repeat for the FCyt-DNA polymer. These results are consistent if, 1: the methylation of cytosine in the oligomer occurs faster then the methylation of 5-fluorocytosine and, 2: methylation of 5-fluorocytosine causes binding of the enzyme to the FCyt-DNA, preventing methylation of the opposite strand. Both these conditions are satisfied since DNA containing cytosine is methylated much faster than DNA containing 5-fluorocytosine and tight binding to FCyt-DNA does occur.

A peptide-FCyt-DNA adduct could be isolated from the FCyt-DNA enzyme complex following reduction and treatment with iodoacetamide. The dialyzed, reduced and alkylated adduct was labile. However, if the adduct were first treated with SDS prior to reduction, alkylation and protease digestion, a more stable product was obtained. This adduct was purified by taking advantage of the characteristics of the attached oligonucleotide. Due to the acid lability of the adduct, purification had to be performed using neutral solvents. The staphylococcal protease V8 digestion product was first purified by reverse phase HPLC. Those fractions containing both ^{32}P from the DNA and ^{3}H-methyl from AdoMet were pooled. The product was then subject to electrophoresis at pH 8, conditions that take advantage of the electronegativity of the bound DNA. The region of the transblot containing ^{32}P was then subjected to sequencing. In view of the sensitivity of the adduct to acid conditions the adduct would not be expected to remain intact throughout the sequencing procedure since the cleavage reaction uses trifluoroacetic acid. We therefore cannot ascertain which amino acid the DNA is attached to by the failure to recover it from the sequencer.

Our failure to recover cysteine is not meaningful since it is not detected by the procedure used. However, failure to detect carboxamidomethylcysteine may be significant, indicating that this cysteine is protected from alkylation. This product is not always detected by the gas phase sequencing procedure. The only conclusion to be drawn from the sequence obtained is that it is consistent with Cys 186 playing a role as the proposed nucleophile.

With both the FCyt-DNA polymer and the FCyt-DNA oligomer the adduct was not stable to purification unless it were first boiled in SDS before reduction and alkylation in urea. This is unexpected if the adduct involves a covalent bond between a cysteine in the enzyme and the 6 position of the methylated 5-fluorocytosine in DNA. The lability of the adduct precludes its unambiguous identification. We are attempting to identify the products of the reaction after dissociation of the adduct.

The data indicate that the region of the protein containing the conserved cysteine 186 forms part of the binding site for DNA. Since it can be photomethylated by AdoMet, it also forms part of, or is adjacent to, the binding site for AdoMet. If cysteine 186 is the catalytic nucleophile binding to C6 of cytosine their would have to be juxtaposition of the methyl group of AdoMet and the sulfur of cysteine 186. The steric placement of AdoMet in the binding site would explain the preferential photomethylation of this cysteine in the protein.

NOTE ADDED IN PROOF

L.Chen et al. (*Biochemistry* **30,** 1108−11025, 1991) have done work similar to that presented in this paper on the *Hae*III methylase and M.Wyszynski et al. (*Nucleic Acids Res.* **20,** 319−326, 1992) have presented genetic data suggesting an essential role for the conserved cysteine in M.*Eco*RII catalysis.

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