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The Specificity and Catalytic Properties of Alu I Methylase

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Abstract

The specific methylation site for Alu I methylase was the cytosine nucleotide in Alu I sequence. The position of the methylated cytosine nucleotide was determined by the chemical cleavage reactions of the Maxam-Gilbert DNA sequencing procedure. As expected, the methylated cytosine nucleotide bands were disappeared on C+ T and C lanes on 12% sequencing gels.

Alu I methylase was maximally active at near pH 7.5 in the presence of 50 mM NaCl. The methylase did not require Mg^{++} for activity, and obeyed Michaelis-Menten Kinetics with respect to both AdoMet and DNA. At 37°C, the K_m for AdoMet was 0.44 μ M, that for the Alu I site of pBR 322 DNA was 4.03 nM, and the corresponding turnover numbers were 1.83 methyl transfer per minute per monomer and 1.61 transfers per minute per monomer, respectively.

The importance of restriction-modification enzymes as tools in genetic research is now well established and are the subjects of many review papers. Despite their widespread uses, however, their mechanism of action and the way in which the restriction endonucleases and the cognate methylases recognize and cleave specific nucleotide sequences are not well understood. In order to study their interactions effectively, an experimental system must be established in which both nucleic acid and proteins are present in pure form. Furthermore, it is essential that both nucleic acid and enzymes are thoroughly characterized.

In 1976, Roberts *et al.* (1976) reported that Alu I endonuclease from *Arthrobacter luteus* recognizes base sequence 5'-d(AG \downarrow CT)-3' with cleavage site indicated by the arrow. In 1985, Yoon *et al* (1985) described a detailed procedure for the purification of Alu I methylase and determined the subunit molecular weight of the enzyme.

In 1981, Razin *et al*, (1981) showed that in vitro synthesized hemimethylated ϕ X 174 duplex DNA by using 5-methyl cytosine triphosphate was resistant against Alu I endonuclease. This result implies that Alu I specific methylation site is on the cytosine nucleotide in Alu I site.

However, the possibility that the methylation on the flanked sequences of the Alu I sites could inhibit the Alu I endonuclease activity can not be ruled out.

In this paper, the physical and catalytic properties of Alu I methylase are presented. The specific site for the methylation of Alu I methylase is also described.

Materials and Methods

Materials

S-adenosyl-L- [methyl-³H]-methionine (15 Ci/ mmole) was from New England Nuclear (Boston, Mass., USA). S-adenosylmethionine was from Sigma. α -³²P-dCTP was from Amersham. All other chemicals used during isolation and DNA sequencing procedures are reagent grade. pBR 322 DNA was isolated by the procedure of Clewell and Helinski (1972). Alu I endonuclease and Hinc II endonuclease was purchased from Bethesda Research Laboratories. Alu I methylase was isolated in our laboratory (Yoon *et al*, 1985)

Enzyme assays

The assay for methylase activity measures transfer of ³H-methyl groups from [³H]AdoMet to pBR 322 DNA or Col EI DNA. Reaction mixture (25 μ l) contained 50 mM Tris-HCl, pH 7.5, 2.5 mM EDTA, 50 μ g/ml BSA, 10 mM 2-mercaptoethanol, 0.8 μ M [³H] Ado Met, 40 μ g/ ml of DNA, and the indicated amount of enzyme. After incubation of 15 to 60 minutes at 37°C, 10 μ l samples were spotted on 1 cm² of Whatman DE81 paper and washed five times with a large excess of 0.2M NH₄HCO₃ solution, twice with ethanol, twice with diethyl ether, and dried for 30 minutes at 60°C.

The [³H] CPM was counted with 5 ml of toluene based scintillant (PPO 4g, POPOP 50 mg in 1 ℓ of toluene) by using Beckman scintillation counter (LS-3110 Series). Under these assay conditions, the apparent specific activity of [³H]AdoMet was 9,800 CPM per pmole. One unit of methylase activity transfers 1 pmole of [³H]-methyl group from [³H] AdoMet to pBR 322 DNA in 1 hour at 37°C which binds to DE81 paper.

Polyacrylamide gel electrophoresis

5% polyacrylamide gel electrophoresis was carried out in 100 mM TBE buffer. The DNA bands were visualized by staining with 1 μ g/ml of ethidium bromide for 10 min and the stained gels were photographed on a short-wave ultraviolet light with Polaroid Type 667 film.

DNA sequencing

Unmethylated and methylated (by Alu I methylase) pBR 322 DNA were cleaved by Hpa II endonuclease and labeled with ³²P at the 3' end by using α -³²P-dCTP and DNA polymerase large fragment. After treatment with Acc I endonuclease, 5% native polyacrylamide gel electrophoresis was performed. The 435 nucleotides long DNA fragment was eluted from each gel by isoelectric elution. The eluted DNA samples were subjected to the DNA sequencing by the method of Maxam-Gilbert (1977). For the sequence analysis, 12% polyacrylamide gels were

used in the presence of 8 M urea.

Results

The methylated nucleotide was 5-methyl cytosine

pBR 322 DNA was methylated with the purified Alu I methylase (Yoon *et al.*, 1985). After Hpa II cleavage reaction, the DNA was labeled with ^{32}P by the procedure described in Methods. As shown in Fig. 1, the methylated cytosine nucleotide was not cleaved by C+T and C reactions of Maxam-Gilbert DNA sequencing procedure. This result indicated that the cytosine nucleotide in Alu I specific DNA sequence was methylated by Alu I methylase.

Determination of the number of [^3H]-methyl groups transferred to one duplex Alu I sequence

For quantitative determination of the number of methyl groups transferred to pBR 322 DNA, which contains 16 Alu I sites, [^3H] AdoMet and two different amounts of the enzyme were used (Table 1). At a low concentration of Alu I methylase (0.2 unit/ μl), no significant increase in the number of methyl groups transferred per Alu I site was observed. These results and the results shown by Yoon *et al.* (1985), indicate that Alu I methylase can methylate Alu I sites completely that have an enzyme to Alu I site ratio of 0.31, the number of methyl groups transferred per Alu I site was 1.9. At a 10 fold higher concentration of the enzyme (2 unit/ μl), no significant increase in the number of methyl groups transferred per Alu I site was observed. These results and the results shown by Yoon *et al.* (1985), indicate that Alu I methylase can methylate Alu I sites completely on both strands of DNA. These results also show that there is no overmethylation by Alu I methylase at reaction conditions used (Table 1). The same results have been observed from Hpa I methylase (Yoo *et al.*, 1982) and Hpa II methylase (Yoo *et al.*, 1980). However, Eco RI methylase showed significant overmethylation (Berkner *et al.*, 1978 ; Woodbury *et al.*, 1980).

Catalytic properties of Alu I methylase

Alu I methylase was maximally active near pH 7.5 (Table 2). Methylation of pBR 322 DNA by Alu I methylase was maximal in the presence of 50 mM NaCl (Table 3). Only 60% of the methylase activity was observed in the absence of NaCl and only 30% of the activity was observed with 200 mM NaCl as compared to the activity observed with 50 mM NaCl (Table 3).

The steady state kinetic studies shown in Fig. 2 and 3 were carried out in the presence of saturating conditions of the invariant substrates. The turnover number determined from Fig. 2 is 1.83 while that obtained from Fig. 4 is similar, being 1.61 per minute per monomer. The corresponding methyl transfers per minute per monomer at 37°C, K_m values are 0.44 μM for AdoMet and 4.03 nM for pBR 322 DNA (in terms of Alu I sequence).

Discussion

To test whether Alu I methylase can methylate native λ DNA was six times higher than that of heat denatured λ DNA (Table 4). This result shows that Alu I methylase prefers double stranded DNA as a substrate which is similar to the results obtained from Hae III and Hpa II methylases (Dobrista, 1980; Mann and Smith, 1979).

In 1978 and 1980, Eco RI methylase has been single stranded DNA as well as double stranded DNA, native DNA and heat denatured DNA were used as substrates. The degree of methylation of found to catalyze methyl group incorporation into DNA at sequences other than the canonical Eco RI sequence (Berkner and Fork, 1978; Woodbury *et al.*, 1980). In contrast to Eco RI methylase, Hpa I methylase transferred only 2 methyl groups per one duplex Hpa I sequence (Yoo *et al.*, 1982) and Hpa II methylase transferred 2.2 methyl groups per one duplex Hpa II sequence (Yoo *et al.*, 1981) and these enzymes did not show any significant overmethylation. Likewise, Alu I methylase transferred 1.9 methyl groups per one duplex Alu I sequence and did not exhibit any significant overmethylation (Table 1).

Dependence of Alu I methylase activity was observed on altered reaction conditions. Alu I methylase is maximally active at near pH 7.5 (Table 2) and in the presence of 50 mM NaCl (Table 3). Alu I methylase did not essentially require magnesium ion, EDTA, sulfhydryl compounds for activity, but was maximally active in the presence of 2.5 mM EDTA and 10 mM 2-mercaptoethanol.

The subunit size of the enzyme, as determined by polyacrylamide gel electrophoresis in the presence of 0.1% SDS, was calculated to $56,000 \pm 1,000$ (Yoon *et al.*, 1985). The molecular weight of the native form of Alu I methylase will have to be determined by further sedimentation equilibrium analysis to examine whether Alu I methylase exists as a monomer in solution.

Although the molecular weight of the native form of the Alu I methylase was not determined, it was assumed that Alu I methylase stays as a monomer in solution because all the Type II modification methylases isolated are monomer in solutions (Rubin, 1977) (Yoo *et al.*, 1982) (Dobrista, 1980). The initial velocity of the methylation by Alu I methylase was linear up to the enzyme concentration of 1,000 pM (monomer) (Fig. 4), indicating that the active form of Alu I methylase is the monomeric form and the enzyme do not associate each other during its enzyme reaction. These molecular properties of Alu I methylase are similar to those of Eco RI methylase (Rubin, 1977), Hpa I methylase (Yoo *et al.*, 1982), and Hpa II methylase (Yoo, 1981). Kinetic parameters of Alu I methylation for pBR 322 DNA are similar to those determined for Eco RI methylation of Col EI DNA (K_m for Col EI was 1.3 nM and K_m for AdoMet was 0.26 μ M) and Hpa II methylation of pBR 322 DNA (K_m for pBR 322 was 2.6 nM and K_m for AdoMet was 0.23 μ M) (Rubin, 1977; Yoo *et al.*, 1982).

Recently we have studied the specific interactions between Alu I methylated DNA and Hind III, Pvu II, Sst I and Sac I endonucleases. Since the internal tetranucleotide sequences of these enzymes are overlapped with the Alu I specific sequence, the normal methylation by Alu I methylase gives rise to the introduction of aberrant methylation in the endonuclease sites listed above. Our preliminary data showed that the Alu I specific methylation completely inhibits Pvu

II and Sac I endonuclease activities. These results implies that we can predict the specificity of Pvu II and Sac I methylases which have not been isolated yet.

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- Fig. 2 Steady state kinetics of Alu I methylase. Determination of K_m for AdoMet. Reaction mixture (25 $\mu\ell$) contained 50 mM Tris-HCl, pH 7.5, 2.5 mM EDTA, 50 $\mu\text{g}/\text{ml}$ BSA, 10 mM 2-mercaptoethanol, 0.49 μM Alu I sequence in pBR 322 DNA, 500 pM Alu I methylase and indicated amounts of [^3H]. AdoMet Incubation was at 37°C for 30 minutes
- Fig. 3 Steady state kinetics of Alu I methylase. Determination of K_m for pBR 322 DNA. Reaction mixture (25 $\mu\ell$) contained 50 mM Tris-HCl, pH 7.5, 2.5 mM EDTA, 50 $\mu\text{g}/\text{ml}$ BSA, 10 mM 2-mercaptoethanol, 4.4 μM [^3H] AdoMet, 150 pM Alu I methylase, and indicated amounts of pBR 322 DNA. Incubation was at 37°C for 30 min.
- Fig. 4 Linear relationship between initial rate of methylation and concentration of Alu I methylase. The reaction conditions are described in Methods.

Reaction*	CPM	[³ H]-CH ₃ /Alu I site
A	20,618	1.85
B	22,287	1.99

- a. Reactions (50 *dℓ*) contained 50 mM Tris-HCl, pH 7.5, 2.5 mM EDTA, 0.8 μ M [³H] AdoMet, 50 μ g/ml BSA, 10 mM 2-mercaptoethanol, 50 mM NaCl, and 0.36 pmole (1 μ g) of pBR 322 DNA. Reaction A was incubated with 10 units (1.8 pmole) of Alu I methylase at 37°C. After 1 hour of incubation, 10 μ ℓ (containing 0.0714 pmole of pBR 322 DNA) of sample was used to determine the incorporation of radioactivity in the DNA. Reaction B was incubated with 10 units (1.8 pmole) of Alu I methylase at 37°C. After 1 hour of incubation, the reaction was supplemented to .1.6 μ M [³H] AdoMet and then 100 units (18 pmole) of Alu I methylase was added. After additional 1 hour of incubation, 14 μ ℓ of sample (containing 0.0714 pmole of pBR 322 DNA) was used to determine the incorporation of radioactivity in the DNA.

Table. 1

pH								
5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0
Radioactivity(CPM) ^a								
76	159	1,046	1,307	1,686	1,929	934	830	101

- a. 50 mM sodium phosphate (pH 5.0-6.5), 50 mM Tris-HCl (pH 7.0-9.0) buffers were used. Each reaction mixture contained 2.5 mM EDTA, 10 mM 2-mercaptoethanol, 50 µg/ml BSA 1 µg of pBR 322 DNA, 0.8 µM [³H] AdoMet, and 3.6 units of Alu I methylase. Incubation was for 20 min at 37°C, 5 µℓ of reaction mixture was used to determine radioactivity.

Table. 2

Ionic strength (NaCl)					
0 mM	10 mM	20 mM	50 mM	100 mM	200 mM
Radioactivity (CPM) ^a					
877	1,241	1,261	1,520	1,134	488

- a. Each reaction mixture contained 50 mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol, 2.5 mM EDTA, 50 µg/ml BSA, 0.8 µM [³H] AdoMet, 1 µg of pBR 322 DNA, and 4 units of Alu I methylase. Incubation was for 15 minutes at 37°C. 5 µl of reaction mixture was used to determine radioactivity.

Table. 3

Reaction ^a	DNA	Alu I methylase [³ H]-CPM ^b
A	λ DNA	— 46
B	Heat denatured λ DNA	+ 4,620
C	λ DNA	+ 24,200

- a. Reaction A and C contained 1 μ g of native DNA, and reaction B contained 1 μ g of heat denatured λDNA (heated at 100°C for 5 min and then quickly cooled in ice-bath). Each of reactions was performed under the conditions described in "Methods" and was incubated at 37°C for 30 min either with 300 units of Alu I methylase (reaction B and C) or without Alu I methylase (reaction A).
- b. 10 μ l of reaction mixture was used to determine the incorporation of radioactivity in the DNA. Methylase assay was carried out as described in "Methods"

Table. 4

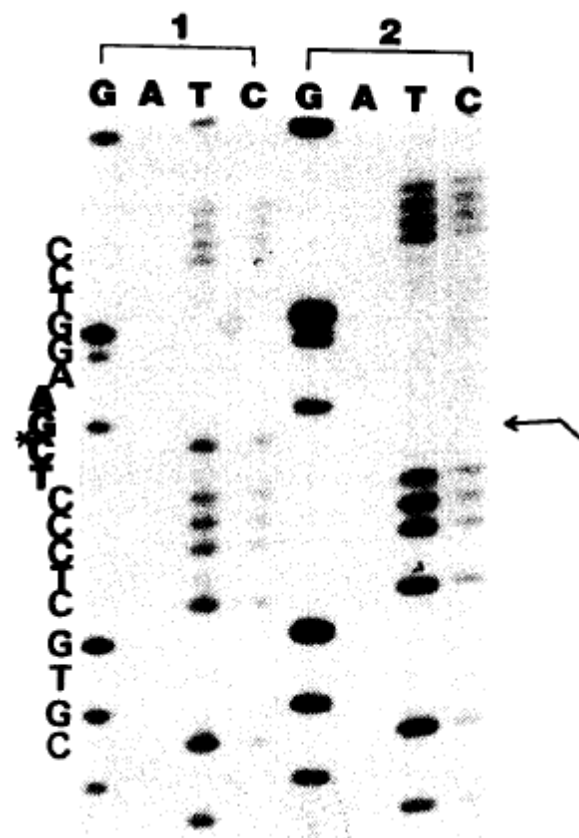


Fig. 1

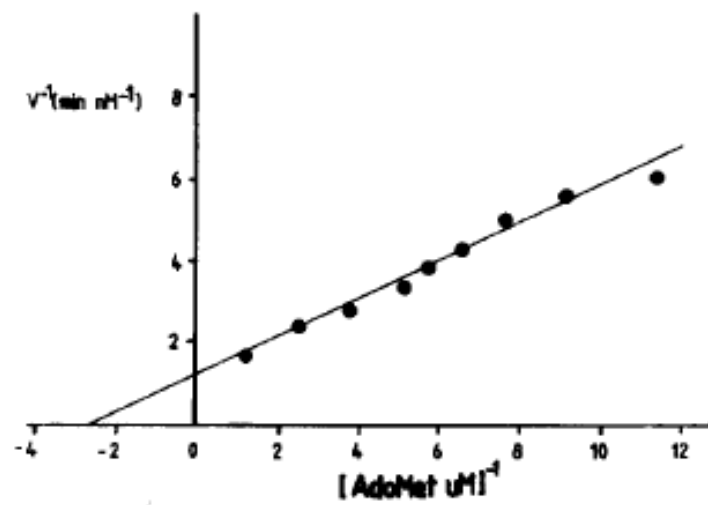


Fig. 2

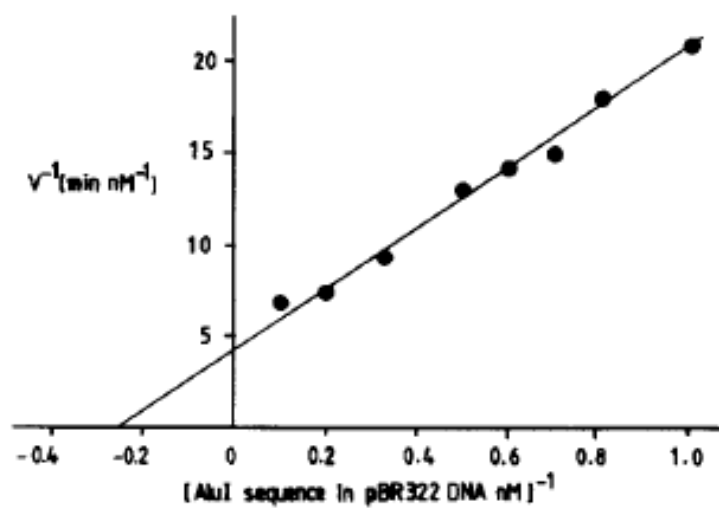


Fig. 3

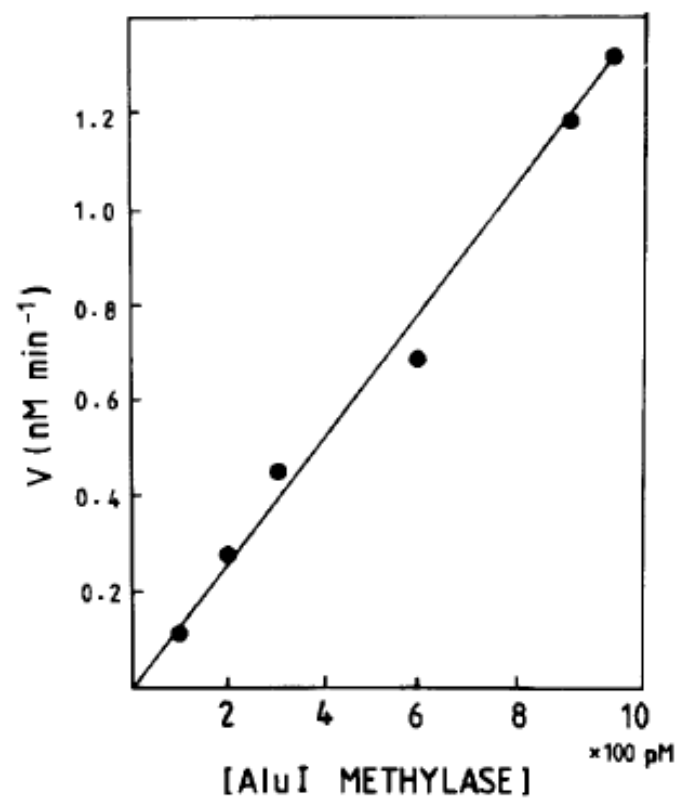


Fig. 4

초록 : Alu I methylase의 효소적 특성과 작용 부위

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순수 정제된 Alu I methylase (Yoon *et al.*, 1985)의 specificity와 그 효소적 특성을 연구하였다. 이 효소는 DNA의 염기 배열 5'-d (AG⁺CT)-3'을 인식하였으며 그 중 cytosine nucleotide에 methylation을 시키고 있음이 Maxam-Gilbert의 방법에 의한 DNA sequencing을 통하여 밝혀졌다. Alu I methylase는 Alu I site 당 두 개의 methyl group을 붙여 주었으며 Eco RI에서와 같은 overmethylation현상은 보여주지 않았다. 효소반응은 pH 7.4에서 7.6사이, NaCl은 50 mM에서 최적 이었다.

Alu I methylase는 Michaelis-Menten kinetics를 따랐으며 S-Adenosyl-methionine에 대한 K_m 값은 $0.44 \mu M$ 이었고 pBR 322 DNA를 사용 하였을때의 DNA에 대한 K_m 의 값은 $4.03 \mu M$ 이었다. 이 K_m 값들로부터 얻어진 turnover number는 각각 1.83/min과 1.61/min이었다.