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THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

FRANK R. KALMAR

ENTITLED IN SEARCH OF A METHYLTRANSFERASE IN THERMUS AQUATICUS

THAT IS AN ECORI ISOMETHYLOMER

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

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IN SEARCH OF A METHYLTRANSFERASE IN *THERMUS AQUATICUS* THAT IS AN *ECO*RI ISOMETHYLOMER

BY

FRANK R. KALMAR

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LIST OF ABBREVIATIONS

MTases	methyl transferases
R-M	restriction modification
AdoMet	S-adenosylmethionine
M·*Eco*RI	*Eco*RI methylase
M·*Rsr*I	*Rsr*I methylase
T.aquaticus	*Thermus aquaticus*
M·*Taq*III	*Taq*III methylase
HPLC	high pressure liquid chromatography
β-ME	beta-mercaptoethanol
BSA	bovine serum albumin
EDTA	ethylenediaminetetraacetic acid
WCX	weak cation exchange
M·*Taq*I	*Taq*I methylase
E·mpRK	pRK112-8 plasmid DNA premethylated with M·*Eco*RI
T·mpRK	pRK112-8 plasmid DNA premethylated with M·*Taq*I
Ci	Curies
xg	times gravity
cpm	counts per minute
Py	pyrimidine base
Pu	purine base

INTRODUCTION

Methyltransferases (MTases) have several functions in the cell. They are used in methyl-directed mismatch repair, in the restriction-modification (R-M) phenomenon, to affect DNA conformation and in the regulation of gene expression. Specific sequences of DNA are recognized by the methyltransferases of the R-M systems and methyl groups are placed on target nucleotides leaving the sequence altered and resistant to cleavage by the corresponding endonuclease. The MTases use S-adenosylmethionine (AdoMet) as the methyl donor modifying the N^6-amino group of adenine, the N^4-amino group of cytosine, or the C5 carbon of cytosine (7).

Differences in enzyme structure and reaction mechanisms are used to classify the R-M systems into types I, II, and III. Type II R-M MTases are structurally and mechanistically simple enzymes, and therefore provide good systems of protein-DNA interactions for study (7). They are usually monomer proteins and recognize either symmetric (e.g., GGCC), degenerate and symmetric (e.g., GTPyPuAC), or asymmetric (e.g., GACGC) sites (7). They also allow evolutionary and biochemical studies when they are compared to one another. If two MTase genes in different bacteria exist, then the bacteria may be closely linked in evolution. Some MTases from different bacteria recognize the same sequence but are not homologous. Such an example is *Eco*RI methylase (M·*Eco*RI) and *Rsr*I methylase (M·*Rsr*I), which catalyze the same reaction on the same sequence (6).

In order to study such phenomena, one must first verify the presence of the MTase and then purify it. Two methods are used to assay MTases in crude extracts. One method is a protection assay in which DNA is first incubated with the extract, thereby methylating it if the MTase is present then the mixture is incubated with the corresponding endonuclease to see if it had been protected by prior methylation. The DNA species are visualized after electrophoresis on an agarose gel that has been stained with ethidium bromide. The other assay measures incorporation of [^{3}H]AdoMet into plasmid DNA. The DNA is separated from the unreacted AdoMet and the radioactivity incorporated into the DNA is quantified.

Purification of the typeII MTases employs precipitations and chromatography on several types of columns. Most MTases are purified by unique protocols that are devised empirically. Examples of precipitating agents include polyethyleneimine and ammonium sulfate. Many types of matrices exist for chromatographic assays, for example, DEAE-cellulose, phosphocellulose, DNA-cellulose, and hydroxylapatite. During purification, the total activity may decrease significantly but as the purity increases the specific activity is increased dramatically. The degree of purification, i. e., specific activity, is a crucial parameter to monitor throughout the process because it indicates the effectiveness of the purification protocol.

After noting that *T.aquaticus* chromosomal DNA could not be cleaved with *Eco*RI endonuclease, we hypothesized that a methylase might exist in these cells that protected the same sequence (GAATTC) as M·*Eco*RI and M·*Rsr*I uses as a target. These enzymes catalyze the deposition of a methyl group on the second adenine in the recognition sequence and thereby protect the site from cleavage by the endonuclease that recognizes the same site. The putative enzyme from *T.aquaticus* was christened *Taq*III methylase (M·*Taq*III) for purposes of discussion and assays were run to try to find it.

This enzyme (M·*Taq*III) would be of particular interest because of the place of the bacteria on the phylogenic tree. *Thermus aquaticus* (*T.aquaticus*) is an extreme thermophile and is evolutionarily distinct from *E.coli*, *S.sonnei* and *R.sphaeroides*, the known sources of MTase activities recognizing (GAATTC). Homology of *T.aquaticus* proteins with proteins from mesophilic bacteria are of evolutionary interest for the reasons that are discussed above.

In this thesis, I will describe experiments I have performed in order to test the presence or absence of the putative M·*Taq*III in *T.aquaticus* lysates. I have prepared cell extracts and fractionated them by HPLC and affinity chromatography. The interpretations of the experiments were difficult because some of the results were ambiguous. Unfortunately, I have concluded that *T.aquaticus* lacks a MTase with specificity for the GAATTC sequence.

MATERIALS

a) Enzymes and proteins

The restriction endonucleases, *Bam*HI, *Bcl*I, *Cla*I, *Dpn*I, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III, *Hpa*I, *Mbo*I, *Nde*I, *Nhe*I, *Pst*I, *Sal*I, *Sau3A*I, *Taq*I, *Xba*I, *Xho*I and DNA ligase were purchased from Bethesda Research Laboratories (BRL). *Taq*I methylase, *Eco*RI methylase and T4 polynucleotide kinase were purchased from New England Biolabs. All enzymes were used according to the specifications of the manufacturer. S-adenosyl-L-[methyl-^{3}H]methionine (11 Ci/mmol) was obtained from ICN, and sinefungin from Sigma.

b) Enzyme buffers

Restriction endonuclease reaction components were supplied with enzymes by the manufacturer (BRL). MTase assay conditions were as follows. The *Taq*I methylase reactions (50 µL) contained 10 mM NaCl, 10 mM Tris-HCl (pH 8.4 @ 25° C.), 600 µM beta-mercaptoethanol (β-ME), 10 µg/ml bovine serum albumin (BSA), and 8 µM AdoMet. Assuming a 0.3 pH unit decrease per 10° C increase in temperature, pH would be 7.2 at 65° C. The *Eco*RI methylase assays contained 100 mM Tris-HCl (pH 8), 5 mM EDTA, 5 mM DTT, 400 µg/ml BSA, and 80 µM AdoMet.

c) DNA

The DNAs of plasmids pBR322 and pUC18 were purified from *E. coli* HB101. Lambda DNA was purchased from BRL, and øX174 DNA from New England Biolabs. A plasmid containing 19 *Eco*RI sites, pRK112-8, was a gift of R. Kim, University of California at Berkeley (1). Plasmid pAN-4 and pBR322ΔRI were gifts from Paul Modrich, Duke University. Plasmid pAN-4 is a 2 kilobase portion of an *E. coli* plasmid that contains the methylase gene, M·*Eco*RI. Plasmid pBR322ΔRI is pBR322 DNA that has been cut with *Eco*RI and the ends filled in by DNA synthesis so that the site no longer exists. The sequence GAATTC is changed to GAATTAATTC by this procedure. Plasmid pRS8-1 is a pUC18 derivative containing a 12 kilobase insert of *Rhodobacter sphaeroides* DNA carrying the *Rsr* I endonuclease and methylase genes. It was produced by Chris Aiken (University of Illinois).

d) Other materials

A random-priming, DNA-labeling system was purchased from BRL. DNA-cellulose was prepared from Whatman CF-11 cellulose and native calf thymus DNA by the method of Alberts and Herrick (2). Other chemicals were reagent grade.

METHODS

a) Growth of cells

A culture of *Thermus aquaticus* YT cells was purchased from American Type Culture Collection. The cells were supplied as a lyophilized powder and were resuspended in the medium described by McClelland (3). They were grown aerobically at 70°C in 1 to 4 liters of the McClelland's medium which conists of 0.5% Bactotryptone (Difco), 0.3% yeast extract (Difco), and 0.2% NaCl. After 16 to 20 hours the cells were harvested by centrifugation, quick-frozen in liquid nitrogen and stored at -70°C. The yield was 2 grams of cell paste per liter of culture medium.

b) Preparation of *T.aquaticus* YT extract

The *T.aquaticus* cells were resuspended in cell-opening buffer, which consisted of 20 mM KH_2PO_4, 1mM EDTA, 100 mM KCl, and 10 mM β-ME. The solution should have had a pH 7.5, although it was not measured in this assay. The cells were lysed by passing them three times through a precooled French press at 16,000-20,000 lb/in^2, with the temperature of the cell extract maintained below 30°C. The lysate was centrifuged at 14,000xg and the supernatant quick-frozen and stored at -70°C. Methylase activity in the lysate was stable at 4°C for at least 2 months.

c) Gel electrophoresis

During the purification, the DEAE-column fractions were assayed qualitatitively for *Taq*I endonuclease activity with lambda DNA using agarose gel electrophoresis. Lambda DNA (1μg) was incubated with enzyme in 50 μl of 1X REact® Buffer 2 (BRL) at 65°C. After 30 minutes, 7 μl of gel-loading dye (0.1% bromphenol blue, 0.1% xylene cyanol, 40% sucrose) were added to all reactions and they were loaded on a 0.8% agarose gel. Electrophoresis was performed in a buffer of 40 mM Tris-acetate (pH 8.3), and 1mM EDTA. DNA was visualized by staining the gel with ethidium bromide, and photographing it under UV light with a

Fotodyne transilluminator and Polaroid MP-4 camera with type 667 film using a Fotodyne filter 3.

d) *Taq*III Methylase Assays

Various amounts of the putative M·*Taq*III from chromatography column fractions were incubated with 20 nM (5µg) pRK112-8 in a total volume of 50 µl containing 100 mM Tris-HCl (pH 8), 5 mM EDTA, 0.4 mg/ml BSA, 2.5 mM DTT, 1.8 mM [^{3}H]AdoMet (11Ci/mmol) for 30 to 60 min. at 65°C. The entire volume (50 µl) was spotted onto DE81 filters (2.3 cm circles; Whatman BioSystems, Inc.), washed 3 times with 0.1 M NH_4HCO_3 to remove unincorporated AdoMet, one time with 95% ethanol, once with diethylether, and dried. Radioactivity was quantified by liquid scintillation counting.

e) Other Methods

Ion-exchange chromatography was performed at room temperature on a Beckman model 420 HPLC system using a 0.75 x 7.5 cm Protein Pak DEAE-5PW column (Waters). Chromosomal DNA from *T.aquaticus* cells was isolated by incubating the cells with lysozyme, followed by phenol extraction and ethanol precipitation according to Silhavy, *et al.* (4).

RESULTS AND DISCUSSION

We attempted to find a methylase in *T.aquaticus* bacteria that recognizes the *Eco*RI recognition sequence. We searched this organism because its DNA has been reported to be resistant to hydrolysis by *Eco*RI endonuclease (John Trela, U. of Cincinati, personal correspondence) suggesting that it might contain a MTase that methylates the GAATTC site. For the purpose of discussion, I have given the putative enzyme the name, *Taq*III methylase (M·*Taq*III).

a) DNA Protection by M·*Taq*III

Chromosomal DNA (5 µg) from *T.aquaticus* was cut with different enzymes as described below. Incubation with *Eco*RI showed no cutting, while the internal control, plasmid pUC18 DNA mixed with the chromosomal DNA, was cleaved (Fig. 1). This result led us to the hypothesis that there were either no *Eco*RI sites in *T.aquaticus* DNA or there was a methyl group protecting any such sites from cleavage.

Dr. David Gelfand (Cetus Corporation) has sequenced 12 kilobases (out of the approximately 3000 kilobases in the total genome) and found no *Eco*RI sites (personal correspondence). He hypothesized that since the DNA is so rich in guanine and cytosine, which is a feature that is thought to stabilize the DNA at high temperatures of growth, that not enough adenine and thymine existed to form a significant number of sites. *T.aquaticus* DNA contains 68% GC base pairs and according to our calculation, the *Eco*RI site (GAATTC) should be present once every 13 kilobases in the *T.aquaticus* genome. This calculation is based on the probability of each nucleotide being in this particular sequence.

Figure1. Electrophoresis of *T.aquaticus* DNA (lane 1) and *T.aquaticus* DNA and plasmid pUC18 DNA (lane 2) incubated with *Eco*RI endonuclease. No cutting of the DNA is evident.

Therefore, we would expect some fragments to be produced upon *Eco*RI endonuclease digestion. In order to test this hypothesis, *T.aquaticus* DNA was incubated with other enzymes whose recognition sequences contained mostly adenines and thymines, such as those listed in Table 1. All of these

sites, except for *Dra*I, would be expected to occur at the same frequency as do the *Eco*RI site since they have the same base composition.

*Bcl*I	*Hind*III	*Hpa*II	*Dra*I	*Cla*I	*Eco*RV	*Nde*I
TGATCA	AAGCTT	GTTAAC	TTTAAA	ATCGAT	GATATC	CATATG

Table 1. *T.aquaticus* DNA was incubated with these enzymes which have two-thirds of the total base pairs as AT in their recognition site.

None of the enzymes cut, which may mean that the small percentage of AT base pairs accounts for the low frequency of these sites in the DNA. The endonucleases did cut thoroughly internal control DNA, incubated with the *T.aquaticus* DNA and the endonuclease. Therefore the enzymes were active in the assay mixtures.

A double digest of *Taq* DNA with *Xba*I endonuclease followed by EcoRI endonuclease was performed to see if *Eco*RI cuts the smaller and therefore more readily detectable fragments. The single digest had an observable band at around 2 kilobases (Fig. 2) that was not present in the double digest suggesting that cutting by *Eco*RI endonuclease had occurred. To see if the band that was not present in the double digest had been cleaved with *Eco*RI endonuclease, a single digest was carried out and that band was excised from the gel. The DNA was extracted using Geneclean®(Bio101), labeled with $[\gamma\text{-}^{32}P]$ATP and T4 polynucleotide kinase, and incubated with *Eco*RI. Plasmid pUC18 DNA was included as an internal control to make sure the endonuclease was active. The digestion mixture was separated by electrophoresis on an agarose gel containing standards in a separate lane. The gel was stained, photographed, and subjected to autoradiograghy. No cutting was apparent even after exposing the film for six days (Fig. 3). Therefore, it seems that the band that was missing in the double digest was not due to cutting of the DNA by *Eco*RI but to some other phenomenon.

The assays that follow were a direct assay for methyl group transfer to DNA rather than the indirect test of examining DNA cleavage patterns. In order to test whether an extract of *T.aquaticus* is capable of catalyzing methylation of *Eco*RI sites in DNA, lambda DNA was incubated with

T.aquaticus extract, which was obtained from a French press lysate of *T.aquaticus* cells. To quantify the methylation, the lambda DNA was incubated with [^{3}H]AdoMet and the DNA was isolated and counted after incubation with the methylase. After the incubation with *T.aquaticus* extract, the methylated lambda DNA was also incubated with *Eco*RI endonuclease to test for specific methylation at GAATTC sites and a partial digest was observed when compared with unmethylated DNA cut with *Eco*RI endonuclease (Fig. 4). Therefore, these results suggest that either protection of the *Eco*RI recognition sequence occurred, or a nonspecific inhibition of *Eco*RI endonuclease took place. Inhibition could arise from one or more of the many other proteins, such as other MTases, salts, etc. that are present in the *T.aquaticus* extract.

Figure 2. Electrophoresis of *T.aquaticus* DNA incubated with *Xba*I endonuclease (lane 1) and with *Xba*I then *Eco*RI endonuclease (lane 2). A small band appears at 2 kilobases in the single digest that is not present in the double digest.

A self-complementary 14-base oligooxyribonucleotide (GAATTCAGGATCCT) was synthesized, phosphorylated with ATP and polynucleotide kinase, self-

annealed, and self-ligated with T4 DNA ligase. The oligooxyribonucleotide was run out on an agarose gel to make sure ligation had occurred. The gel showed that long polymers were formed as a result of ligation. It was supposed to contain the *Eco*RI recognition sequence but all attempts at methylating the oligo with *T.aquaticus* extract (65°C.) and purified *Eco*RI methylase (37°C.) failed. There must be a problem with the sequence, since other investigators in the lab have had problems with the this oligonucleotide preparation. The synthesis of the oligooxyribonucleotide may not have been the correct sequence since the *Eco*RI site seems to be absent.

b) Methyltransferase homologies

We hypothesized that if M·*Taq*III recognized the same site as M·*Eco*RI, there was a chance that they were homologous proteins. If *Eco*RI methylase and the putative M·*Taq* III genes were homologous, hybridization of *T.aquaticus* DNA with the labelled M·*Eco*RI gene might indicate the relationship. The *T.aquaticus* YT chromosomal DNA was cleaved completely with the restriction endonucleases; *Bam*HI, *Xba*I, *Pst*I, *Xho*I, *Hind*III, and *Sal*I. These digestion mixtures were separated by electrophoresis on a 1% agarose gel and a Southern blot was performed to test for cross hybridization using pAN-4 and RS8-1, which contain the methylase gene from *E.coli* and *R.sphaeroides*, respectively, as probes. Hybridization and washing was performed at 42° C according to the procedure of Meinkoth and Wahl (5). They considered this to be a medium stringency hybridization. No hybridization was observed, implying that either the *Taq* III methylase gene does not exist or that it shares little sequence similarity to M·*Eco*RI and M·*Rsr*I genes.

c) Partial Purification of the Putative *Taq*III Methyltransferase HPLC DEAE Chromotography

The French-press lysate of freshly grown *T.aquaticus* cells was equilibrated by dialysis with 750 ml of buffer A for one hour at 4° C. Buffer A (4°C.) consisted of 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 mM β-ME. The sample (1 ml) was applied at a flow rate of 1 ml/min to a Waters Protein-Pak DEAE 5PW column (0.75 cm x 7.5 cm), equilibrated in buffer A. The column was washed for seven minutes and eluted with a 40

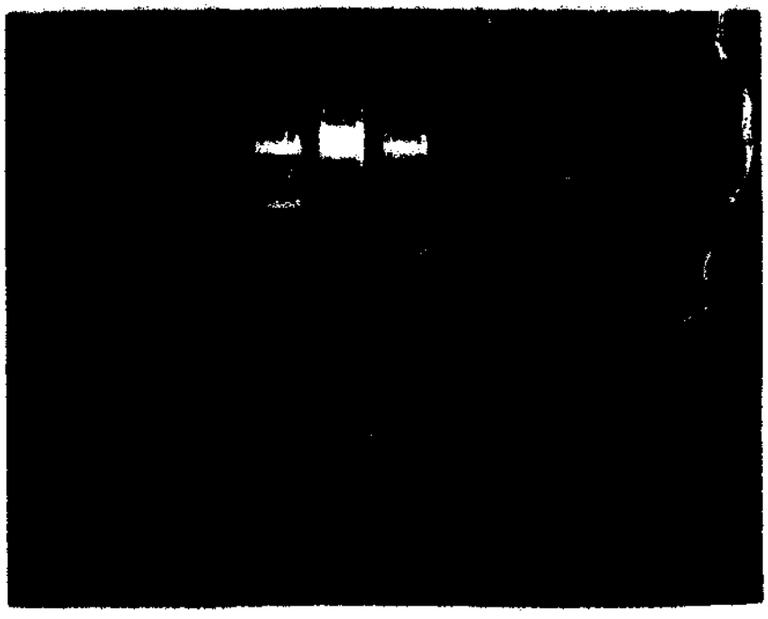

Figure 4. Electrophoresis of lambda DNA incubated with *T.aquaticus* extract and then *Eco*RI endonuclease (lane 2) and incubated with *Eco*RI endonuclease (lane 3). Partial protection is visible in lane 2. Lane 1 is molecular weight markers.

ml linear gradient of 0 to 1 M NaCl in buffer A. The gradient was created with buffer B which contained the same constituents as buffer A plus 1 M NaCl. All fractions were collected, and those that corresponded to peaks on the printout were assayed for activity. A fraction containing M*Eco*RI-like activity, determined by the quantitative assay (described in METHODS), eluted at approximately 0.4 M NaCl (Fig. 5).

DNA-Cellulose Chromotography

The pooled DEAE fractions were dialyzed into 750 ml of buffer A. A 1-ml double-strand DNA-cellulose column was poured into a 2-ml syringe and pre-equilibrated with buffer A at 4 C. The column was washed with five volumes of 20μM sinefungin, an analog of AdoMet. Sinefungin binds to the MTase, presumably allowing it to form ternary complexes (enzyme-sinefungin-DNA). Being bound to the column, the MTase is

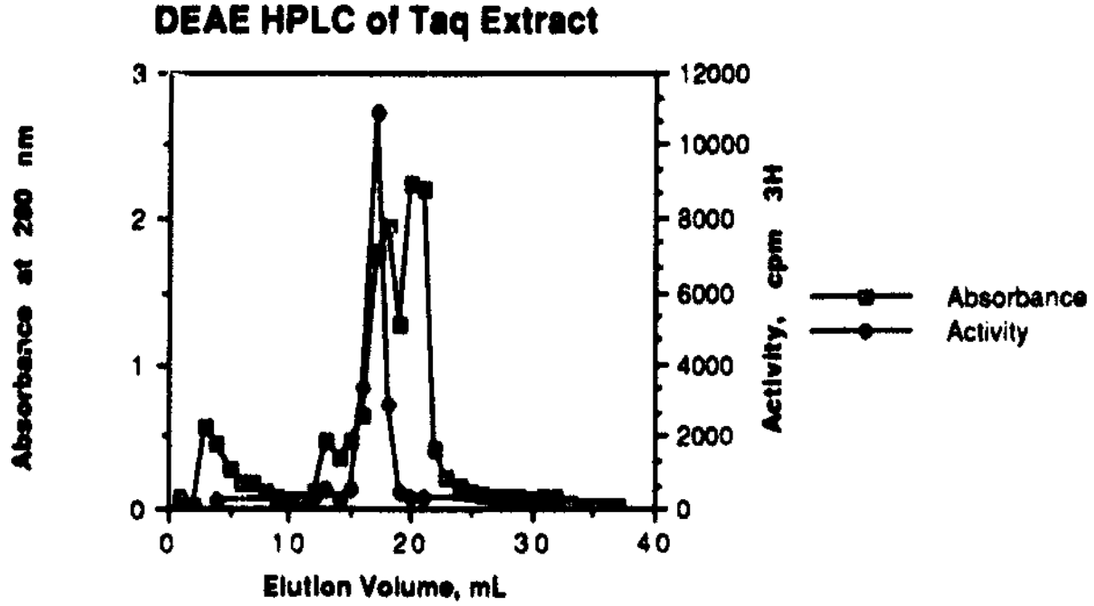

Figure 5. HPLC DEAE-cellulose chromatography of *T.aquaticus* extract showing the fractions that came off and the corresponding activity.

separated from other proteins that wash through without binding. The MTase can then be eluted by increasing the concentration of salt. The dialyzed DEAE fraction containing 20μM sinefungin was filtered and loaded onto the column. After washing for six minutes with buffer A, the column was eluted with a 40 ml linear gradient of 0 to 1 M NaCl in buffer A. Fractions containing M·*Eco*RI-like activity, which eluted between 0.4 and 0.48 M NaCl, were pooled (DNA-cellulose Fraction).

Purification summary

Further purification of the methyltransferase was not attempted because proof of its authenticity was first necessary. The DEAE Fraction contained twice as much total methylase activity as the crude extract, while the DNA-cellulose fraction contained only one-half the total methylase activity of the DEAE fraction. Therefore, most of the activity was lost during the DNA-cellulose chromatography. SDS-polyacrylamide gel electrophoresis of the DNA-cellulose fractions led to no conclusions, other than the need for further purification. When the methylase activities of the fractions were determined, none of these fractions with

high activity correlated to a distinguishable band on the SDS-polyacrylamide gel. HPLC weak cation exchange (WCX) Chromotography was performed on the DEAE fraction but no activity was recovered from this column.

d) Endonuclease activity

Bacterial MTases usually protect DNA in the same organism from cleavage by the corresponding endonuclease that recognizes the same site. Therefore, whenever a MTase is discovered, one searches for an endonuclease that recognizes the same sequence. I tried the same approach after the DEAE Chromatography, each fraction that contained protein was checked for endonuclease activity to see if there was an endonuclease that would cut the GAATTC sequence in lambda DNA. Upon comparing the cleavage patterns to lambda DNA cleaved with *Taq* I endonuclease (NE Biolabs), it was found that the same fraction that contained our methylation activity also contained *Taq*I endonuclease activity, an activity that cleaves the TCGA sequence. No fraction displayed cutting with *Eco*RI endonuclease alone.

e) Varying Reaction Conditions

To prove that methylation was occurring by an enzyme and not by a non-catalytic chemical reaction, we heat-treated the fraction, a denaturant which would denature enzymatic activity but not chemical reactions. Heat-treated (100° C) for ten minutes and SDS- heat-treated (1% and 100° C) DEAE fraction containing the methyltransferase, showed no activity. Therefore, we could assume that methylation of the *Eco*RI site, which we believed was methylated by the putative M·*Taq*III, is not due to a non-catalytic chemical reaction.

[^{3}H]-AdoMet concentrations needed to provide maximum methyltransfer in the quantitative assay were determined by varying the amount of [^{3}H]-AdoMet. AdoMet (2 μM) was adequate to give sufficient activity to detect product and compare the results in a statistically significant manner. From these data we were also able to generate a graph of the extent of reaction versus [AdoMet]. These data fit a hyperbola (Fig. 6), with K_m=1.2 μM and V_{max}=4.6 cpm/min. By running the quantitative assay for varying amounts of time, we found that the product formed was

directly proportional to the time of incubation (Fig. 7). The total concentration of radioactivity was maximized by running the reaction for thirty minutes. An assay varying the amounts of extract added over a fifteen minute incubation was linear to approximately 1 µl of extract (Fig. 8). We used an assay such that an amount of extract transferred up to 400 cpm ^{3}H per minute of reaction, which is equivalent to 33 fmol/min of CH_3 transferred assuming a 50% counting efficiency.

f) Determination of Recognition Site

When the quantitative assays were run, pRK112-8 DNA was utilized as well as pRK112-8 DNA that was pre-methylated with M·*Eco*RI. By pre-methylating the DNA, the *Eco*RI site would not be available for methylation although all other sites would be, except those that overlap with the *Eco*RI site. The latter DNA always showed much less incorporation, and the difference between the two values (i.e. the difference in the amount of DNA methylated) was considered to be the specific methylation of the *Eco*RI recognition sequence. There was however always a small, but significant amount of methylation of pre-methylated pRK112-8, a DNA which lacked the EcoRI site. This was likely due to M·*Taq*I. Plasmid pBR322ΔRI is PBR322 DNA that has been cut with *Eco*RI and filled in so that the site no longer exists. Thus, the sequence GAATTC has been changed to GAATTAATTC. When the quantitative assay was performed with this DNA, high activity was observed using the DEAE and DNA-cellulose fractions. These observations could suggest that the enzyme is methylating another unknown site, or it may not be recognizing the entire *Eco*RI recognition sequence but a part of it such as AATT. Usually type II enzymes recognize a palindromic sequence, a feature of the AATT sequence.

The following assays seemed to suggest that either M·*Taq*III was not methylating the DNA, or that there are other explanations, which are described below, accounting for the previous observations. The following assay seemed to be a positive result for the presence of M·*Taq*III. Plasmid pRK112-8 DNA was premethylated with M·*Eco*RI or M·*Taq*I (denoted E·mpRK and T·mpRK, respectively), and incubated with fractions containing M·*Taq*III activity. Controls were run to ensure that the enzymes actually

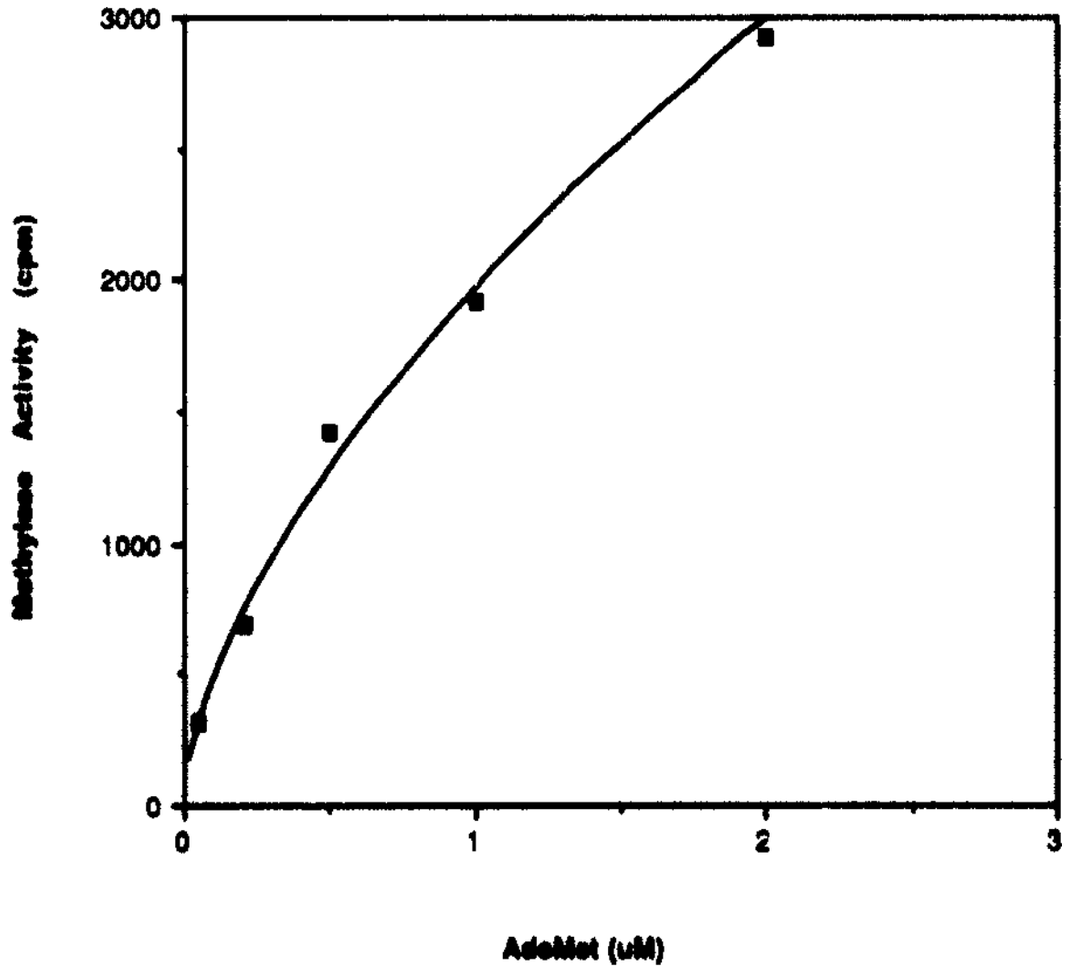

Figure 6. Methylase Activity versus AdoMet concentration. From the graph, K_m= 1.2 mM and V_{max}= 4.6 cpm/min.

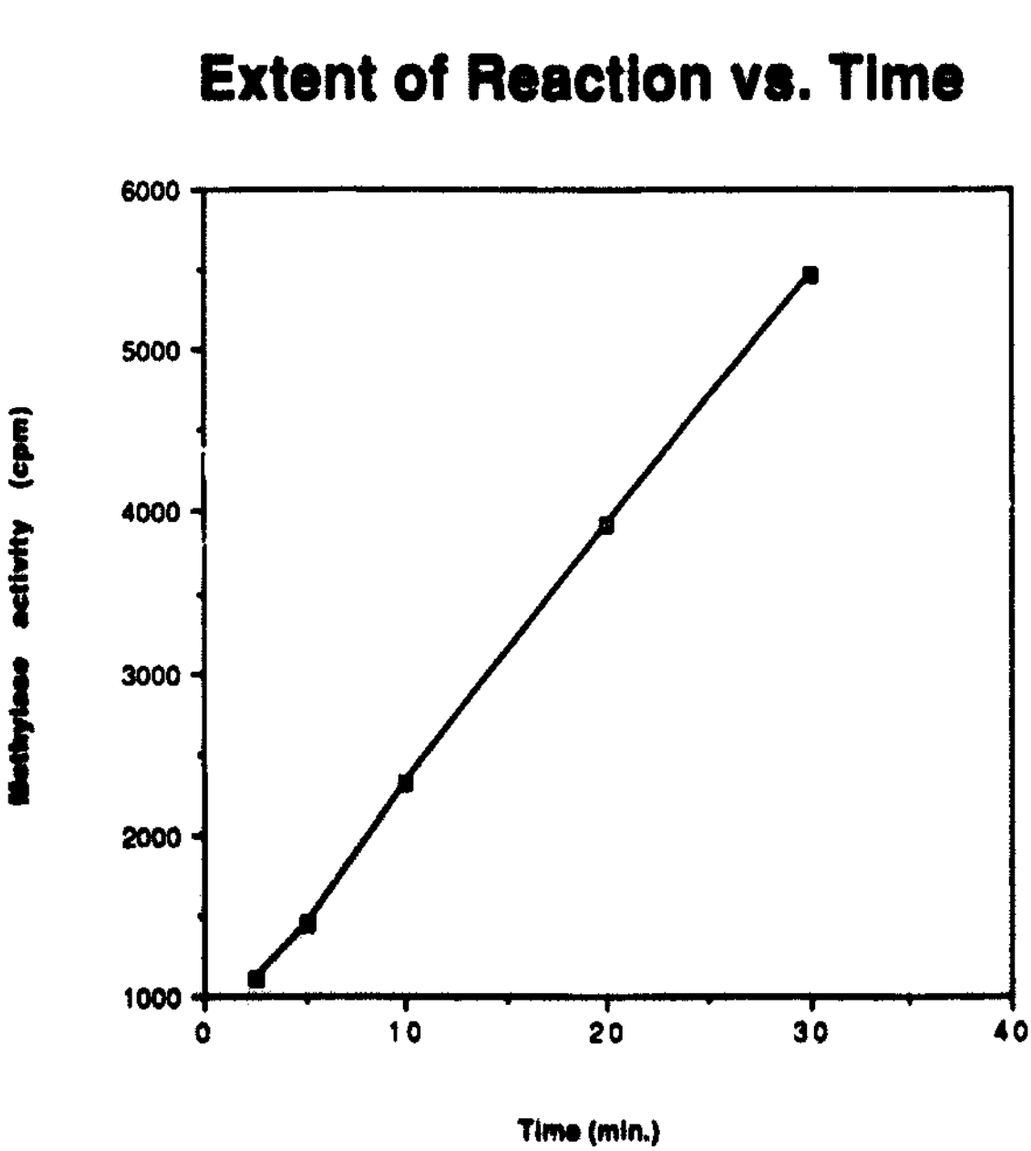

Figure 7. Extent of Reaction versus Time. The line indicates that the longer a reaction is run, the more methylation will occur. The curve would have leveled off if there was a long enough reaction time

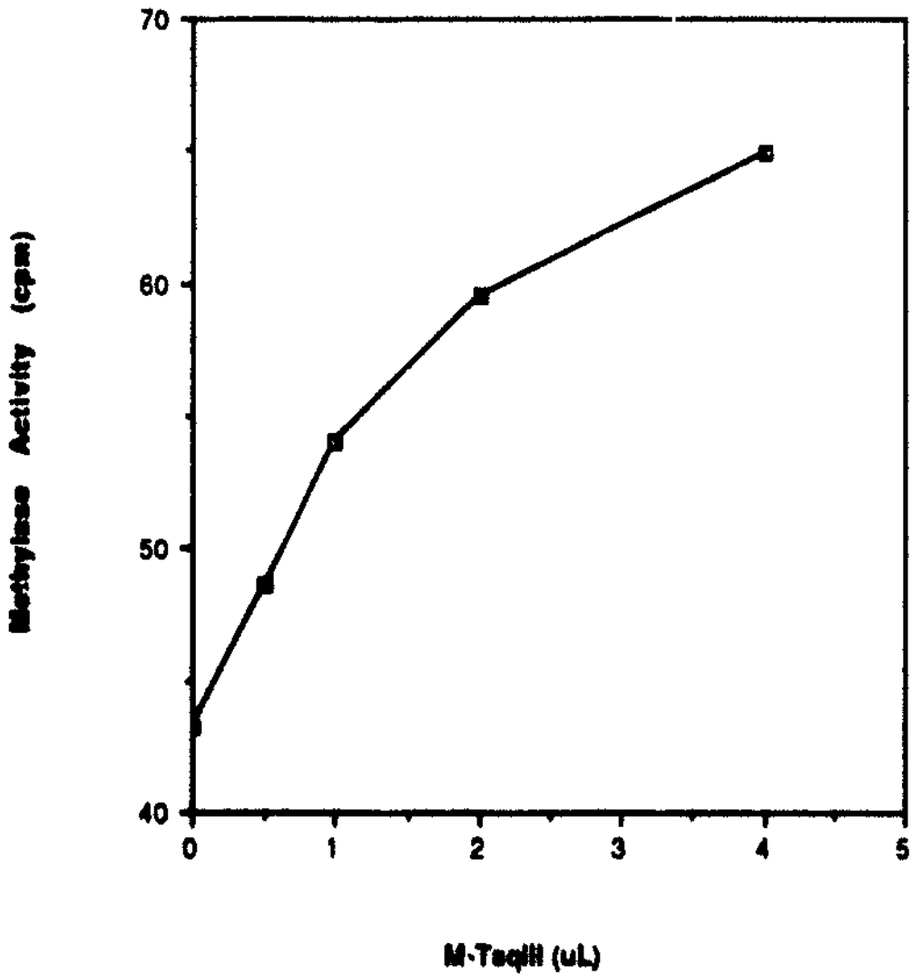

Figure 8. Methylase Activity versus M·*Taq*III Concentration. The graph is linear up to 1 µl which corresponds to 33 fmol/min of CH_3 transferred.

methylated DNA before they were submitted to further methylation. The E·mpRK DNA had the *Eco*RI recognition sequence methylated and that of *Taq*I unmethylated. This DNA showed very little methylase activity when it was incubated with the DNA-cellulose fraction. Conversely, the T·mpRK DNA had the *Eco*RI site available for methylation and showed three times as much activity. This result suggests that an enzyme is methylating a site other than the *Taq*I site and possibly a site that is contained within the *Eco*RI site or on its periphery. The GA in the beginning of the GAATTC recognition sequence may overlap with the TCGA which a *Taq*I methylase would recognize. Therefore, M·*Eco*RI may inhibit the M·*Taq*I activity that seems to be present in some other assays, although this would not account for such a large difference since a majority of *Eco*RI and *Taq*I sites do not overlap.

Assays using DNA that was premethylated with M·*Eco*RI or M·*Taq*I and then incubated with fractions containing the putative M·*Taq*III activity were not significantly methylated. Also, pre-methylation was sometimes incomplete as proven by the controls. For these reasons, a quantitative methylase activity assay was performed on DNA in which the DNA was cleaved at the potential sites of methylation and then incubated with the column fractions to see if methylation occurs at the sites that were not cleaved. If the DNA were to be cut at the putative site of methylation, then the corresponding MTase could not methylate at that site because the site had been destroyed but other MTases whose sites were still available could methylate them. It is easier to verify complete digestion by a restriction endonuclease than to verify complete methylation by the corresponding methylase. Therefore, plasmid pBR322 and plasmid pRK112-8 DNAs were both cleaved with *Taq*I or *Eco*RI endonucleases and then incubated with both the DEAE and DNA-cellulose fractions. DNA (plasmid pBR322) cut with *Eco*RI left the *Taq*I site available for methylation and when incubated with the DEAE fraction showed twice as much methylation activity as the DNA cut with *Taq*I, which left the *Eco*RI site available for methylation. When plasmid pRK112-8 DNA was run with the DEAE fraction the same result was observed. The same phenomenon was observed when the DNAs were incubated with the DNA-

cellulose fraction, although the radioactivity was much lower (four times less) due to the lower activity in the fractions from that column (see Table 2 for the actual cpm's). This suggests that there is M·*Taq*I in predominant amounts but that M·*Taq*III may also be present. There was still considerable incorporation of methyl groups into the DNA that had been cut with *Taq*I endonuclease, which has the *Eco*RI site open; therefore methylation may be due to M·*Taq*III. On the other hand, it is possible that not all the *Taq*I sites were cut and that the methylation was due to mere residual sites.

DNA Incubated with DEAE Fraction		
DNA	Endonuclease	Radioactivity (cpm)
pBR322	*Taq*I	5113
pBR322	*Eco*RI	11,253
pRK112-8	*Taq*I	1604
pRK112-8	*Eco*RI	5720

DNA Incubated with DNA-Cellulose Fraction		
DNA	Endonuclease	Radioactivity (cpm)
pBR322	*Taq*I	726
pBR322	*Eco*RI	3017
pRK112-8	*Taq*I	411
pRK112-8	*Eco*RI	1513

Table 2. Plasmid DNA cleaved with *Taq*I and *Eco*RI endonucleases and incubated with fractions.

Several of my results lead to the conclusion that a methylase that recognizes the *Eco*RI site may exist. The hypothesis that very few *Eco*RI sites are present in *Taq* DNA remains a plausible one, particularly since other enzymes, that have the same base composition, show no cleavage (Table 2.). The activity that appeared to come from the putative M·*Taq*III could be from protein or possibly from RNA methylases. These methylases were likely present because the DNA isolation did not include a cesium

chloride gradient, which would have separated the DNA from other proteins and nucleic acids. The RNA MTases can bind to the DNA just as DNA MTases do and therefore inhibit binding of DNA MTases. Further experiments that need to be carried out include further purification of the putative M·*Taq*III. A purification step that separates M·*Taq*I and the putative M·*Taq*III is crucial to remove the activity that may obscure a possible M·*Taq*III activity. Inhibiting M·*Taq*I activity with antibodies and then searching for the M·*Taq*III activity could help solve the problem, although the antibodies may be difficult to obtain. A new oligooxyribonucleotide that contains an *Eco*RI site but not a *Taq*I site could be synthesized to see if it could be methylated, since the oligooxyribonucleotide in our assays was not synthesized correctly, lacking the *Eco*RI site. Therefore we might see the actions of the putative M·*Taq*III without M·*Taq*I activity.

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