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CHARACTERIZATION OF THE DNA SEQUENCE SPECIFICITY OF THE WILDTYPE AND HYPERMETHYLATING MUTANT FORMS OF THE PHAGES T_2 AND T_4 DNA-ADENINE METHYLASE (*DAM*) AND THE *ECO DAM* METHYLASE OF THE HOST BACTERIUM, *ESCHERICHIA COLI*

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Mark M. Doolittle

August 1987

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I dedicate this thesis to the axiom:

"To Err is Human, But to Persist is Diabotical;

To Quit is Not in My Vocabulary."

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ABSTRACT

The use of oligonucleotide nearest neighbor analysis or protection from restriction endonuclease cleavage assays has not proven to be rigorous enough to definitively determine the sequence specificity of the T_2 and T_4^+ dam and dam^h methylases. Therefore, a novel assay was developed to qualitatively and quantitatively determine the specificity of the phage dam methylases and the host methylase, Escherichia coli Eco dam. Plasmid (pBR322) DNA, whose sequence is known, was subjected to methylation in the presence of the radiolabelled methyl donor ^{3}H-S-adenosylmethionine (^{3}H-SAM) then purified using standard nucleic acid techniques. Plasmid restriction fragments, containing one or very few possible methylation sites were isolated from resolving polyacrylamide gels and analyzed by scintillation counting to determine the extent of methylation.

It was established that the Eco dam enzyme only methylated the canonical site (GATC), while the T_2 and T_4 dam enzymes also methylated other GAPyN sites. More specifically, the T_2dam^+ enzyme methylated certain GATG and GACC sites (in addition to the canonical GATC site) more readily than the T_4dam^+ enzyme. The degree to which noncanonical GAPyN sites were methylated by the T_2dam^+ enzyme was shown to be affected by pH and concentration of s-adenosylmethionine (SAM). GAPyN sites, other than the canonical site (GATC) were methylated more readily in methylation reactions containing higher concentrations of SAM. The T_2 and T_4dam^h enzymes differed from their wildtype counterparts in that they methylated GACC sites more readily at lower

SAM concentrations. DNA substrate topology (supercoiling) was shown to alter site preference only slightly.

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GLOSSARY OF TERMS

A	Adenine
AQII	Aquasol II fluor
BPB-XC dye	Bromphenol Blue-Xylene Cyanol Dye
BSA	Bovine Serum Albumin
C	Cytosine
($^{+}CH_3$)	methylated
($^{-}CH_3$)	unmethylated
dam	DNA adenine methylase
dcm	DNA cytosine methylase (mec)
DNA	deoxyribonucleic acid
EDTA	Disodium Ethylenediamine Tetraacetate
EtBr	ethidium bromide
ETOH	ethanol
G	Guanine
gt	glucosylated
hmC	hydroxymethylcytosine
Kb	kilobases
KOH	potassium hydroxide
λ	lambda phage
MEOH	methanol
2-MeOH	2-mercaptoethanol
N	Nucleotide
P.A.	polyacrylamide
PEG	polyethylene glycol

Pu	Purine
Py	Pyrimidine
R.E.	restriction endonuclease (enzyme)
rgl	restriction of unglucosylated hmc DNA
RNA	ribonucleic acid
SAH	s-adenosylhomocysteone
SAM	s-adenosylmethionine
Sevag	25:24:1 phenol:chloroform:isoamyl alcohol
T	Thymine
TCA	trichloroacetic acid
TLC	Thin Layer Chromatography
Tris	Tris(hydroxynethyl)amino methane
UV	ultraviolet light

I. INTRODUCTION

The use of oligonucleotide nearest neighbor analysis or protection from restriction endonuclease cleavage assays has not proven to be rigorous enough to definitively determine the sequence specificity of the T_2 and T_4^+ *dam* and *dam*^{h} methylases. Therefore, a novel assay was developed to qualitatively and quantitatively determine the specificity of the phage *dam* methylases and the host methylase, *Escherichia coli Eco dam*. Plasmid (pBR322) DNA, whose sequence is known, was subjected to methylation in the presence of the radiolabelled methyl donor ^{3}H-S-adenosylmethionine (^{3}H-SAM) then purified using standard nucleic acid techniques. Plasmid restriction fragments, containing one or very few possible methylation sites were isolated from resolving polyacrylamide gels and analyzed by scintillation counting to determine the extent of methylation.

It was established that the *Eco dam* enzyme only methylated the canonical site (GATC), while the T_2 and T_4 *dam* enzymes also methylated other GAPyN sites. More specifically, the T_2*dam*$^+$ enzyme methylated certain GATG and GACC sites (in addition to the canonical GATC site) more readily than the T_4*dam*$^+$ enzyme. The degree to which noncanonical GAPyN sites were methylated by the T_2*dam*$^+$ enzyme was shown to be affected by pH and concentration of s-adenosylmethionine (SAM). GAPyN sites, other than the canonical site (GATC) were methylated more readily in methylation reactions containing higher concentrations of SAM. The T_2 and T_4*dam*^{h} enzymes differed from their wildtype

counterparts in that they methylated GACC sites more readily at lower SAM concentrations. DNA substrate topology (supercoiling) was shown to alter site preference only slightly.

II. LITERATURE REVIEW

A. Sequence Specificity Techniques and Significance

Nearest-neighbor oligonucleotide analysis has enabled researchers to qualitatively determine the identity of sites methylated by the *dam* (*Eco* and phage T_2 and T_4) and P_1 methylases (1-4,7). The technique utilizes *E. coli* or pancreatic DNase and *E. coli* exonuclease I or snake venom phosphodiesterase to generate oligonucleotides from intact methylated DNA. Bacterial alkaline phosphatase is used to remove terminal phosphates and the resulting oligonucleotides are then separated on DEAE cellulose. The eluted oligonucleotides are subsequently either partially or fully digested with snake venom or spleen phosphodiesterase and chromatographically separated to generate a fingerprint of the component bases. Radiolabelled methyl groups on the adenines of intact and digested oligonucleotides are detected by autoradiography.

Several intrinsic features prevent employment of this technique for quantitatively ascertaining methylase specificity. Results are biased due to the preference pancreatic DNase has for cutting on the 5' side of 5hmC and cytosine over the 3' side of the same. This causes one to observe lower than actual levels of AC dinucleotides from digests of oligonucleotides containing NGACCN sites. Venom exonuclease has the property of stopping at the 3' side of 5hmC residues rather than digesting further, resulting in another distortion of the results. A related method in which the methylated DNA is cleaved by a

restriction enzyme not affected by the methylation was used to characterize the sequence specificity of the E. coli dam and dcm (mec) methylases (5,6). The sticky ends are filled in with ^{32}P-labelled nucleotides by E. coli pol I Klenow fragment then degraded by micrococcal nuclease and spleen phosphodiesterase. The resulting labelled oligodeoxynucleoside monophosphates are separated by TLC and autoradiographed. This assay has similar enzyme biases and suffers from the limitation incurred by separating halves of the recognition/cleavage site. The major limitation of this technique is that it lacks the resolution to determine the sequence of oligonucleotides of more than four or five bases. This handicap makes simultaneous determination of the flanking bases on both sides of a tetrameric site extremely difficult at best. The simultaneous determination of bases flanking a methylated hexameric sequence is an order of magnitude more difficult if not impossible. Therefore they are usually determined separately. All such determinations are usually done only after the core recognition sequence of the methylase has been derived by restriction enzyme cleavage studies (7-16).

B. Methylation and Methylases

DNA methylation has some common and different functions among various procaryotic organisms and viruses. Some adenine methylases (i.e., M. Eco RI) and cytosine methylases (i.e., M. Eco RII) of E. coli and other bacteria seem to be principally, if not solely, involved as agents of the restriction/modification systems of their cells (9,11). Methylases which protect a cell's own DNA from cleavage by its own

restriction enzymes are ubiquitous throughout the procaryotes. They are even found in the Thermus (12) and Mycoplasma genera (13). DNA methylases are classified into one of three categories based primarily on whether they are associated with a restriction endonuclease as part of a modification/restriction system and if so on the site of cleavage of their complementary restriction endonuclease which recognizes and binds the same base sequence (14). Methylases within each category have similar numbers of subunits, similar size, molecular weight, and co-factor requirements.

Type I methylases are large three-subunit enzymes isolated in two forms, one form consisting of the recognition and methylase subunits, and the other form having the additional restriction subunit. Both forms recognize and bind to the same DNA recognition sequence. All type I methylases require SAM as a co-substrate and their normally slow rate of methylation is enhanced by the presence of ATP and Mg^{++} (1,2). Type I methylases modify a base within the recognition sequence but their complementary restriction endonuclease may cleave greater or equal to 1,000 bases away from the recognition sequence.

Type II methylases are relatively small in size and are not associated with a restriction enzyme subunit, though a complementary restriction enzyme may or may not exist. Type II methylases methylate one or two bases (i.e., 2"A"s) in a recognition site depending on whether the site is palindromic or not. These methylases do not require ATP or Mg^{++} as cofactors, only SAM and DNA as substrates.

Type II restriction enzymes cleave within or very close to their recognition sites. Cleavage of a Type II restriction enzyme's recognition site may be inhibited by methylation of bases within that site or by the presence of adenine (dam) methylation when a GATC site overlaps with their recognition sequence. Restriction enzymes whose cleavage of sites is unaffected by adenine methylation may be affected by cytosine methylation within or adjacent to the same sites. The latter phenomenon appears to be due to the differences in location of the methyl groups in the DNA helix and their ability to interfere with DNA-protein contacts required for recognition and cleavage of a site. Methylation of the N-6 position of adenine eliminates a hydrogen bond donor site in the major groove of the helix. The methyl group on the C-5 position of cytosine does not interfere with hydrogen bonding but may affect hydrophobic interactions between bases (base stacking), between bases and the enzyme's amino acids, or it may prevent enzyme tracking along the helix via steric hindrance.

Type III modification/restriction systems (i.e., Eco P1) are very similar to type I systems except the former only have one enzyme form with two subunits (14). The enzyme possesses both methylase and restriction endonuclease activity. They require ATP and Mg^{++} for restriction activity which is enhanced by SAM (9,11,15). The methylase modifies a base within the recognition sequence but the restriction endonuclease cleaves 24-27 bases downstream of the recognition sequence, AGACPy (14).

C. Dam Methylases

The Eco dam methylase of E. coli is a Type II methylase whose methylation of adenines at GATC sites seems to be involved in the important process of parental DNA strand recognition during postreplication structural gene (17-19). An extreme abundance (21 sites) of methylatable GATC sites has been discovered to exist at the origins of replication of both E. coli and Salmonella typhimurium (9). The methylation of the adenines of these sites affects the rate of DNA replication initiation, perhaps via secondary structure stabilization or modulation of origin promoter usage, as well as insuring proper replication of origin sequences via efficient mismatch repair (20-23). Dam methylation of sequences upstream of the structural genes encoding dna A (24), the TN 5 (25) and IS 10 (26) transposases, Mu mom (27), and P_1 cre (28) has been shown to have regulatory effects on their expression.

Eco dam's enzyme kinetics have been studied extensively (29). Evidence indicates that it forms a random order ternary complex with SAM and DNA. After catalyzing the transfer of a methyl group from SAM to the adenine of a GATC site, the enzyme releases the products, s-adenosylhomocysteine (SAH) and methylated DNA. The enzyme's turnover rate supports the model that the enzyme binds the DNA and methylates one strand of the target site then releases the site on the DNA molecule. Thus two separate binding and methylation events must occur for a GATC site to become fully methylated. A small amount of cooperativity between the methylation events of the two strands (two

"A"s) of a GATC site indicates that Eco dam might have a slight preference for hemimethylated DNA.

All wildtype T-even phages (T_2, T_4, and T_6) incorporate 5-hydroxymethyl cytosine (5hmC) in place of cytosine into their DNA during replication. After incorporation, these 5hmC residues are glucosylated via α- and β-glucosyl linkages or α-glucosyl and α-β gentibiose linkages (30,31). These modifications serve to protect phage DNA from cleavage by the host hsr and rgl restriction systems (32). In addition, the T_2 and T_4 phages possess dam methylases which modify the adenines within GAPy sequences (33). Hypermethylating mutants (dam^h) of the T_2 and T_4 dam methylase have been isolated from unglycosylated phage (gt^-) which are able to render their DNA resistant to cleavage by the P_1 phage's (lysogen) restriction endonuclease upon infection (7,10,34,35). The P_1 nuclease cleaves 24-27 bases away from its unmodified recognition sequence AGACPy (14). This suggested that the dam^h enzymes methylated AGACPy sites just like the P_1 methylase. However, the P_1 methylase was unable to methylate the hmC-containing DNA of T_2 and T_4 phages (7).

In vitro, T_2dam^h could methylate glucosylated and unglucosylated 5hmC-containing DNA three times as much as the T_2dam^+ methylase suggesting that the mutant enzyme had a broader (more relaxed) specificity than the wildtype enzyme (7). Other in vitro assays showed that while both T_2dam^+ and dam^h could render unmethylated lambda DNA resistant to R. Dpn II digestion and further methylation by Eco dam (the host methylase), only dam^h activity could completely block further

methylation by P_1 methylase (2). T_2dam$^+$ did modify 30% of the lambda sites capable of being methylated by the P_1 methylase. However, prolonged incubation of lambda DNA with T_2dam$^+$ or T_2dam^h enzymes at higher enzyme-to-DNA ratios did yield similar methylation levels and patterns. These results indicated that perhaps both methylases could methylate the same sites but that dam^h had altered activity or K_m for certain species of sites. Analyses of partially methylated lambda DNAs indicated that both enzymes had a specificity for GAPy sites but dam^h methylated GAC sites more readily than dam$^+$ (2,7).

Essential to understanding the biological and evolutionary significance of methylation by the phage and bacterial dam methylases is the ability to quantitatively determine which sequences of DNA are methylated by these enzymes. The ability to analyze the effects of mutations (and the amino acid changes they create) on the sequence specificity of these enzymes in structure-function studies hinges on the ability to precisely quantitate changes in their activity upon DNA substrates. The work described in this thesis combines common, state of the art techniques to quantitate the differences in DNA sequence specificity between the T_2dam$^+$, T_2dam^h, T_4dam$^+$, T_4dam^h, and Eco dam$^+$ methylases.

III. MATERIALS AND METHODS

A. Computer Analysis of Target DNA (pBR322) Sequences

The Korn Queen (Beckman) Microgenie software program (16) on a hard disk was used in an IBM PC computer to analyze pBR322 sequences for putative methylation sites (GAPyN) which could be isolated by themselves on DNA fragments generated by single or double restriction endonuclease digestions. Numerous search files were organized which would analyze the DNA sequences of pBR322 and list the size and base pair boundaries of restriction fragments, in order of size, generated by single or combinations of different restriction enzymes. By visual examination of the computer printout of each digest, a decision could be rendered whether or not a digest could generate a desirable restriction fragment(s), that could be resolved from other fragments using polyacrylamide gel electrophoresis. Adjustments had to be made for the computer software's inability to predict restriction fragment sizes by taking into account different sites of cleavage of the enzyme within or outside of their recognition site. Those restriction enzymes which had recognition sites that overlapped in whole or in part with the species of sequences $\frac{\text{GAPyN}}{\text{CTPuN}}$ were eliminated from our analysis to prevent any conflicts between adenine methylation and restriction activity and minimize nuclease-generated loss of radiolabelled methylated adenines which may occur in overhanging methylated restriction fragment ends.

A double-axis matrix table of restriction enzymes was generated to help determine the fewest enzymes which could be used to generate

several restriction fragments containing one or more of each of the putative methylation sites. Listed on this table, at the intersection of the columns and rows of the same or different restriction enzymes, are the species of putative methylation sites (GAPyN) generated by that enzyme(s). The predicted lengths of fragments are given in parentheses. An enzyme cost per unit versus benefit analysis was performed to ascertain which enzymes to order and use in the methylase specificity determination assay. The enzyme, R. Hha I (36) by itself, generates a series of electrophoretically separable restriction fragments of pBR322, among which two have GATG sites alone, one has two GACC sites, two have GACT sites alone, and three have single GATC sites in conjunction with other GAPyN sites. For this reason, R. Hha I was the principal restriction enzyme used to analyze the methylation specificity of the T_2dam, T_4dam, and Eco dam methylases.

B. Isolation of Methylated and Unmethylated DNAs for Methylation Assays

Unmethylated lambda DNA was obtained from New England Biolabs Company (36). Unmethylated and methylated pBR322 plasmid DNA was procured by transforming GM272 (dam^- dcm^-) and HB101 (dam^+ dcm^+) E. coli strains with plasmid DNA using the standard CaCl method as described in Maniatis et al. (37). Plasmid-bearing transformants were selected on ampicillin-containing LB agar plates then spotted on a master (50 µg/ml) plate and grown up overnight at 37°C. Three ampicillin resistant colonies were analyzed for the presence and size

of plasmids using the alkaline lysis miniprep procedure (John Wills, personal communication). Isolated plasmid DNA was analyzed for size by agarose plasmid gel electrophoresis in parallel with a control lane containing pBR322 DNA. Gels were stained in 500 ml of distilled water containing 0.5 μg Ethidium bromide (EtBr) per ml. DNA-EtBr bands in gel which fluoresced when placed on a long wave UV transilluminator were photographed with Polaroid Type 667 film through a Kodak No. 54 Wratten filter.

A single pBR322-bearing colony was then inoculated into 50 ml broth containing 50 μg/ml ampicillin and grown to stationary phase by shaking overnight at 37°C. Five milliliters of a filter-sterilized 10 mg/ml ampicillin stock and 10 ml of a filter sterilized 100 mg/ml uridine or cytidine stock were added to 1 liter of supplemented M9-glucose medium in a 4 liter flask. Media was prewarmed to 37°C then 50 ml of fresh overnight culture of GM272-pBR322 or HB101-pBR322 was added to media and shaken vigorously for approximately 2.5 hr until a Klett reading of 60 was obtained. Ten milliliters of a 34 mg/ml chloramphenicol (in ethanol) stock was pipetted into each culture to inhibit protein synthesis. Cultures were continuously shaken for an additional 12 hr to maximize plasmid amplification but minimize the incorporation of ribonucleotides into the plasmid DNA (37).

Each culture was put on ice and cells were pelleted by centrifugation at 5,000 rpm for 30 min at 4°C in the GS-3 rotor. Pellets were drained and resuspended in 50 ml 25 mM Tris pH = 8.0; 10 mM EDTA on ice. Cells were repelleted by centrifugation using the

same parameters. Drained pellets were frozen at -20°C and thawed before lysis or used immediately. Upon thawing, plasmid DNA was isolated by the Lysozyme and Triton X-100 Lysis Method (55).

The cell pellet from a 500 ml culture equivalent was suspended in 4.0-ml STE lysis buffer (25% sucrose, 50 mM Tris HCl pH = 8.0, 40 mM EDTA) on ice. Cell suspension was transferred to 30 ml polycarbonate tube. Two and five-tenths milliliters of a 10 mg/ml lysozyme in STE buffer solution were added to each tube followed by gentle vortexing. Following a 15 min incubation on ice, 1.5 ml of a 0.5 M EDTA solution (pH 8.0) was added to stop lysozyme and complete lysis. After a 10 min incubation on ice, 13.5 ml of a 0.1% Triton X-100, 50 mM Tris (pH 8.0) 40 mM EDTA solution was added while vortexing very gently to mix instantaneously. After 10 min on ice, tubes were balanced with 25 mM Tris; 10 mM EDTA buffer and centrifuged in an SW28 rotor at 27,000 rpm for 30 min at 4°C.

The supernatant was poured off, gently, into a sterile plastic tube and kept on ice. One gram of CsCl was added to each tube for each milliliter of supernatant and mixed. To this suspension, 0.08 volumes (0.8 ml per 10 ml) of 10 mg/ml Ethidium Bromide was mixed in. This mixture was then loaded into 39 ml quick seal polyallomer tubes through a 10 ml syringe and 16 gauge needle slowly to prevent shearing. The tubes were balanced with paraffin oil then sealed and centrifuged in a Ti60 rotor at 45,000 rpm for 40-45 hr at 20°C.

Fluorescent bands of plasmid DNA-EtBr were removed from the tubes using a 5 ml syringe with 18 gauge needle using a 16 gauge needle as a

vent in top of tube to prevent turbulence. Plasmid DNA-EtBr solutions were then loaded into waiting 13.9 ml quick seal polyallomer tubes and filled to crush zone with a solution of 10 MM Tris 1 mM EDTA (balanced) buffer; 1.55 gm/ml CsCl; 0.8 mg/ml EtBr and recentrifuged (pH 8.0) in a Ti 50 rotor at 40,000 rpm for 42 hr at 20°C. Again, plasmid DNA-EtBr bands were removed as described in this text previously. EtBr was removed from the DNA solutions by repeated extraction (8-10 times) with equal volumes of sec-butanol saturated with 4.5 M CsCl. DNA solution was diluted with one volume 10 mM Tris (pH = 8.0) 1 mM EDTA buffer followed by one-tenth volume 2 M NaOAc (pH 5.5). Two volumes of ethanol were then added and the mixture was put at -20°C overnight to precipitate the DNA.

Tubes were then centrifuged in SS-34 rotor at 8,000 rpm for 30 min at 4°C to pellet the nucleic acid. Supernatant was decanted off and the pellet washed with 70% ethanol (2 ml) and respun at 8,000 rpm for 1 min to remove salt. Ethanol solution was removed with Pasteur-pipette and the pellets air-dried DNA was resuspended in 1 ml 10 mM Tris (pH 8.0) 1 mM EDTA and transferred to Eppendorf tube for storage. A small volume (1-5 ml) was diluted into 1-1.5 ml 10:1 TE buffer and of DNA sample analyzed spectrographically between 200 and 320 nanometers of light for purity and DNA concentration.

C. Analysis of Maxiprep pBR322 DNA from HB101 and GM272 E. coli Hosts for Methylation and Size

One microgram quantities of HB101-pBR322 and GM272-pBR322 were digested with the restriction enzymes: R. Mbo I, R. Sau IIIa,

R. Dpn I, and R. Eco RI from New England Biolabs, Inc. (36) in separate reactions. Ratios of components of reactions were:

R. Mbo I, R. Sau 3a, R. Dpn I digests

25 µl 2X Enzyme Assay Buffer

5 µl 1 mg/ml BSA

1 µl pBR322 DNA (HB101 or GM272)

18 µl dH_2O

0.5-1.0 µl restriction enzyme (units enzyme)

R. Mbo I--8.7 units/µl

R. Sau IIIa 10 units/µl

R. Dpn I 10 units/µl

R. Eco RI digests

5 µl 10X TA "universal" restriction enzyme buffer

5 µl 1 mg/ml BSA

1 µl pBR322

38 µl dH_2O

2 µl R. Eco RI enzyme

(2 units enzyme) (1 unit/µl)

Digestions were incubated 5 hr at 37°C at which time 10 µl of 1:1 xylene cyanol Ficolle dye: 200 mM EDTA was added to stop reactions. After mixing and microfuging, samples were loaded on 1.5% agarose in 1 XE buffer 15 cm^2 x 4 mm gel along with and electrophoresed till bromphenol blue dye reached 13 cm from top of gel at 3V/cm. ϕX174 Hae III, Hind III, and undigested pBR322 DNA samples were loaded on gels as size markers and controls.

Gels were stained 45 min in 500 ml dH_2O plus 0.5 µg/ml EtBr and photographed after being rinsed with dH_2O.

D. Analysis of Commercial Labelled and Unlabelled S-adenosyl-methionine (SAM) Products for Purity and Concentration

Five milligrams of SAM·HCl (Sigma), SAM·Iodide (Sigma), SAM·HSO_4 (Boehringer Mannaheim), or SAVANT-concentrated SAM·Iodide (Sigma) that had been "purified" over CG-50 amberlite resin were dissolved in 100 µl 0.001 N acetic acid (pH 4.3) to constitute solutions of approximately 100 mM SAM. Five microliters of the solutions and 1.0 µl (0.5 µCi) of tritiated SAM from New England Nuclear were spotted 1.5 centimeters from one end of duplicate 5 x 8 cm silica gel F-254 TLC plates along with adenosine and methionine standards. After spots had air dried, the plates were put (in parallel) in a solvent chamber preequilibrated with the solvent:1-butanol:acetic acid:dH_2O in the ratio 12:3:5 (38). The solvent front was allowed to rise to 6.5 cm from the spot origin at which time plates were removed from the chamber to air dry. The plates were photographed using a Polaroid camera and shortwave UV lighting.

Plate A was dipped in 0.25% Ninhydrin 1% acetic acid in acetone (39) and developed at 70°C under vacuum for 3-5 min. Plate C was stained by dipping in platinum iodide stain then developed in 12 N HCl vapors in sealed jar for 3-5 min (40). Differentially stained colored spots on plates were circled and labelled then photographed with Polaroid camera using flood lighting (visible light). Several drops of ENLIG^{3}HTNING (NEN) fluor was then dripped on lanes of plates where radiolabelled SAM or derivatives would have migrated. Plates were then

dried at 70°C in vacuum oven for 5 min, cooled, covered with plastic wrap and put at -70°C in an exposure holder with a piece of XAR-5 film enclosed. After exposure, film was developed for 5 min in D-5 developer, then put in fixer for 5 min.

Unlabelled SAM·HSO_4 (Boehringer Mannaheim) to be used in enzyme assays was dissolved in 9:1 0.02 N H_2SO_4 (pH = 2.1):ETOH and stored at -20°C. Concentration of original solution and dilutions was ascertained by blanking a Hewlett Packard spectrophotometer with 9:1 0.02 N H_2SO_4:ETOH at 260 nM, then reading the absorbance (260 nM) of 5 µl of SAM solution in 1500 µl 9:1 0.02 N H_2SO_4:ETOH. The SAM concentration was calculated by multiplying the absorbance of solution by the dilution factor--300X--and the SAM molar extinction coefficient (E_m)--14.7 x 10^3--at 260 nM (41).

The fact that the Boehringer Mannaheim SAM·HSO_4 was isolated from crude fractions of SAM and was not analyzed for R or S isomer content could be a source of error. SIGMA Corp. has published that approximately 18% of SAM preparations from yeast may be the unutilized S(+)isomer (s-adenesyl-D-methionine). Experiments were designed to minimize any effects that could be caused by a deviation in effective SAM concentration of this sort.

E. Generation of Purified T_2 and T_4 Phage Stocks

T_2*dam*$^+$, T_2*dam*^{h}, T_4*dam*$^+$, T_4*dam*^{h}, and T_4*dam*^{h}*dam*$^{-x}$ phage (all *agt*$^-$ *bgt*$^-$) were all titered by infecting 0.1 ml of a middle-log phase K803 (*rgl*$^-$) culture with an aliquot dilution of the phage solution in soft-agar then plating on YT agar media. Titers were determined by

multiplying the number of plaques formed after incubation for 10-12 hr at 37°C by the dilution factor. Gt$^+$ (glycosylated) revertants of each phage, needed to infect an rgl$^+$-(restriction of glucosylated DNA) bacteria host, were isolated by infecting 0.1 ml of middle-log-phase BE$^+$ (rgl$^+$) or B834$^+$ (rgl$^+$) E. coli cultures with 10^8 pfus (plaque forming units) in soft agar and plating on YT agar plates. Only αgt$^+$ revertant phage form plaques on these hosts. Single plaques were plucked from the plate with a Pasteur pipette and dispersed in 1 ml of 0.85% NaCl solution to which a drop of $CHCl_3$ was added. These phage solutions were then retitered on a lawn of B834$^+$ (rgl$^+$) cells and B834 rgl$^+$ gal U56 E. coli cells to plaque purify a gt$^+$ revertant. Genuine gt$^+$ revertants formed plaques on the B834$^+$ host but not on the B834 gal U56 host which prevents phage glucosylation. To make phage lysates, single plaques of the gt$^+$ revertants were then plucked from the B834$^+$ lawn and placed in 9.5 ml of M9 media in a 50 ml flask. One half of a milliliter of an overnight cultures of K803 or BE$^+$ cells grown in M9 media was added to each flask and shaken for 6 hr at 37°C then slowly (setting 1-2) for 1 hr (setting 6.5-7.0) to lyse cells. Lysates were refrigerated and several drops of $CHCl_3$ were added. Titers were determined by the method described earlier in this text.

Large scale lysates were made by combining 1 liter of M9-glucose media, 50 ml of a K803 or BE$^+$ overnight culture (in M9 media), and 10^8-10^9 phase (in a broth solution bubbled to remove $CHCl_3$) in a 4 liter flask and shaking vigorously at 37°C for 6 hr then slowly for 1 hr to complete lysis. $CHCl_3$ was added and the lysate put on ice.

DNase and RNase were added to a concentration of 5 μg/ml and 1 μg/ml, respectively, and the lysate left at 4°C overnight. Lysates were then centrifuged for 30 min at 9,000 rpm in GS-3 rotor at 4°C to pellet cell debris. Supernatants were poured into 250 ml GSA rotor bottles and centrifuged for 3 hr at 10,500 rpm in RG5 Sorvall centrifuge at 4°C to pellet phage. Supernatant were drained from pellets and pellets were resuspended in 10 ml M9 plus $MgSO_4$ buffer overnight at 4°C. Phage solutions were transferred to sterile 30 ml polypropylene tubes and centrifuged in SS-34 rotor at 7,000 rpm for 15 min to pellet any contaminating cellular debris. Supernatant was decanted into fresh, sterile 30 ml tube and recentrifuged at 15,000 rpm for 1 hr to pellet phage. Phage pellets were drained and resuspended in 3 ml of M9 plus $MgSO_4$ buffer overnight at 4°C.

CsCl-step gradients were made by layering successive 1/2 ml volumes of 70%, 60%, 50%, 40%, 30% and 20% CsCl in M9 plus $MgSO_4$ buffer solutions into 4.4 ml cellulose nitrate tubes. One milliliter of a phage solution was then layered on top of gradient and the tubes were centrifuged in a SW-60 rotor for 20 min at 35,000 rpm at 20°C. Viable phage particles migrated to the 50%/60% CsCl interface while ghost particles migrated to the 20%/30% CsCl interface. Viable phage and cellular debris were recovered by piercing the bottom of each tube with a 16 gauge syringe and dripping the gradient out, into 1.5 ml Eppendorf tubes.

Viable phage particle fractions were dialyzed gradually against 2 M, 1 M, 0.5 m, 0.25 M, then 0.1 M NaCl and finally two changes of SM

buffer (1 liter each) (37) for 5 hr each to prevent osmotic shock of the phage. The phage were then dripped into sterile glass, capped vials, inoculated with chloroform and stored at 4°C. Titering was performed as described earlier in this text.

F. Isolation of Phage Methylase Activity (ref.)

Cultures of E. coli strains GM272 (dam$^-$ dcm$^-$), GM119 (dam$^-$ dcm$^-$)/pSSH-1b (T_4dam$^+$), K803 (dam$^-$ dcm$^+$ rgl$^-$) were grown up overnight by inoculation of several bacterial colonies from a streak plate into 50 ml of M9, LB or YT broth. Cultures were shaken at a setting of 6.5 and temperature of 37°C in a New Brunswick Gyratory Water Bath Shaker. One liter volumes of M9, LB or YT broth in 4 liter flasks (prewarmed to 37°C) were inoculated with 5 or 10 ml of a fresh overnight culture of bacteria grown up in the same media to prevent any extended growth lag. Cultures were shaken vigorously for several hours, till the culture reached a Klett absorbance reading of 60-85 indicating the presence of cells at a concentration of 2.5-5.0 x 10^8 cells/ml.

After taking samples of uninfected cells, 1 ml of a sterile 10 mg/ml solution of tryptophan was added to each culture while shaking to insure proper phage adsorption. Five milliliter volumes of the same culture media containing CsCl-step gradient purified phage, previously bubbled to remove $CHCl_3$, were then pipetted into the vigorously shaking cultures to begin the infection (m.o.i. = 5-10). Uninfected cells were poured on ice and processed immediately. A sample of infected cells were taken from the flasks at 5-10 min postinfection for comparison with uninfected samples in plating experiments done to determine the

percent of cells surviving infection. At 17 min postinfection cultures were decanted into GS-3 500 ml centrifuge bottles containing crushed ice to cool rapidly. Bottles were balanced and centrifuged at 8,000 rpm for 70 min at 4°C to pellet the cells. Crude cell extracts devoid of DNA were made by the method of Reuben and Geffter (48). Cell pellets were resuspended in 10 ml sonication buffer 20 mM Tris (pH 7.4) 3M NaCl 5 mM 2-MeOH 10 mM EDTA on ice and transferred by pipette to conical, polystyrene 15 ml tubes. These cell suspensions (often clumpy) were then sonicated (on ice) using three 30 sec bursts of a Branson Sonifier on setting 8.

Sonicated samples were then centrifuged for 10 min on Sorvall GLC-1 preparative table-top centrifuge with adapters at 4°C at 3,000 rpm to pellet cell debris. Sonicate supernatants were transferred to fresh 50 ml polypropylene tubes and the volume was adjusted to 10 ml by adding sonication buffer. Five milliliters of cold 30% PEG:2M NaCl were added to each sonicate; they were mixed by inverting (PEG) tube repeatedly, then put at 4°C overnight (≥4 hr). This step precipitated phage particles and undegraded RNA and DNA. PEG-treated sonicates were spun in Sorvall GLC-2B centrifuge for 10 min at 6,000 rpm at 4°C and the supernatants were decanted to fresh tubes. The supernatants were exhaustively dialyzed to remove SAM, SAH, methionine and other endogenous metabolites in large diameter cellulose tubing against numerous (>5) changes of 1 liter volumes of Tris 20 mM pH = 7.9 EDTA = 10 mM NaCl = 50 mM 2 MeOH = 6 mM over a period of ≥24 hr at 4°C. Sonicate dialysate including fine white precipitates in

tubing were then dripped into sterile 50 ml tubes and stored at 4°C or mixed with an equal volume of sterile glycerol and stored at -20°C.

G. Quantitation of the Methylase Activities from Crude Extracts of Infected Cells

Twenty-five or 50 microliters of dialysates was incubated (1 or 2 µl) in a 50 or 100 µl reaction with 0.5 or 1.0 µg of intact unmethylated DNA and 0.5 or 1.0 µCi ^{3}H-SAM at a final concentration of 825 nM. Components of methylation reactions were added and mixed in a precise order to prevent the DNA or enzyme from being exposed to extreme pH conditions which may be detrimental. The distilled water portion of the reaction volume was mixed with one-tenth of reaction volume of 10X conc. M. Hpa II buffer 50 mM Tris (pH 7.5) 10 mM EDTA 5 mM 2-MeOH or T_2dam$^+$ methylase buffer 50 mM Tris pH = 8.0 10 mM EDTA 50 mM NaCl. Radiolabelled (hot) ^{3}H-SAM 2H, ^{3}H-SAM 3L, and ^{3}H-SAM 4H of 6.5, 41.2, and 7.52 µM, respectively, and unlabelled (cold) SAM were added in their acidic 9:1 0.02 N H_2SO_4:ETOH solutions next.

To neutralize the acid (the buffer didn't seem to offset it) an equal volume (to SAM volume) of 100 mM Tris base was added and all ingredients mixed thoroughly. Next, the DNA was added followed by the addition of Bovine Serum Albumin to a final reaction volume concentration of 200 µg/ml. Finally, the crude dialysate was added and the reaction volume was tapped gently to mix then microfuged momentarily. Methylation reactions were incubated 1-2 hr at 37°C. Upon completion of methylation, 10% SDS and 40 mg/ml proteinase K stock solutions were added to make final concentrations of 0.5% and 50 µg/ml,

respectively. The reactions' contents were then incubated at 65°C for ≥1 hr to digest protein.

In initial experiments, the DNA from the assay was purified by a different series of treatments. The reaction mix was incubated in 0.1 M KOH for 1 hr at 65°C to degrade RNA, neutralized with Tris-HCl then Sevag extracted to remove proteins. The aqueous layer was TCA precipitated by addition of an equal volume of 20% TCA and vacuum filtered through Gelman glass-fibers. The DNA bound to the filters was washed successively with 5 ml volumes of 10%, 5% and 1% TCA then ice cold ethanol. Air dried filters were put in vials with 10 ml OCS fluor (Amersham) and scintillation counted.

In later experiments, the DNA was purified by gel electrophoresis by adding 5 to 10 µl of ficolle:bromophenol blue/xylene cyanol blue dye to the sample and loading the wells of a 0.7%-1.0% agarose 1 X E buffer vertical 15 cm^2 x 4 mm gel. When lambda DNA was the substrate, samples were heated to 65°C for 5 min prior to loading to disrupt λ cos sites. By running gels several hours at 3V/cm till the cyanol dye (top band) reached 6-8 cm from top of gel, the lambda DNA band was sufficiently separated from protein, RNA (mRNA and tRNA), and unincorporated SAM. Gels were stained with EtBr as described previously.

Gel slices containing lambda or pBR322-DNA bands were excised from the gel lanes and placed in preweighed 25 ml scintillation vials. The weight of gel slices was determined and difference between their weight and 4 gm was adjusted by addition of the proper volume of dH_2O. Vials were sealed tightly and placed in a 100°C for 10-15 min to melt the

agar. Samples were swirled to homogenize, then cooled, and 14 ml AQUASOL-II fluor was added. After mixing and cooling at 4°C to form a gel, samples were counted for tritium for 10 min in Beckman LS-7000 counter to achieve less than 5% counting error.

H. Parameters of Methylation Assays Using pBR322 for Sequence Specificity Determinations

Two hundred or 400 microliter volume methylation reactions were used to methylate 10-20 micrograms of (GM272 or HB101 derived) pBR322 DNA, respectively. When radiolabelled SAM-3L (NEN) sp. act = 14.1 Ci/mmole) was used, the addition of 6.0 μCi made [SAM] = 1.036 μM. When 6 μCi of SAM-4H (NEN sp. act = 67.2 Ci/mmole) was added the [SAM] = 150 nM. The concentration and specific activity of SAM in different reactions was altered by the addition of unlabelled-SAM (Boehringer Mannaheim). The pH of different reactions was altered by use of different 10X concentrated stocks of M. Hpa II reaction buffer which had been pH adjusted by the addition of Tris-HCl or Tris Base, closely monitored by a Fisher mini-pH electrode. All volumes of SAM added to a reaction were neutralized by addition of an equal volume of 100 mM Tris Base and Bovine Serum Albumin was added to a final concentration of 200 μg/ml. Half of the volume of every methylation reaction was contributed by the crude sonicate-dialysate added.

Reactions were incubated at 37°C for 2-4 hr, cooled on ice while 20 μl of 10% SDS and 0.1 μl of 40 mg/ml proteinase K were added, then incubated at 65°C for ≥1 hr to digest protein. A sample volume equivalent of 0.25 μg pBR322 DNA of each methylation reaction was mixed

with an equal volume of 1:1 Ficolle dye: 200 mM EDTA and loaded on a 0.7% agarose 1 X E buffer gel. The gel was electrophoresed in a similar fashion to how lambda DNA sample gels were done. Linear pBR322 DNA co-migrated with xylene cyanol blue dye (top) band. The gel was stained as described in the text. Open circular (oc), linear (1), and covalently closed circular (ccc) DNA bands were excised and processed the same as gel slices described earlier in the text.

The remaining volume of each methylation reaction was stored at -20°C. Those reactions, whose DNA was deemed sufficiently methylated were processed further. Suitable DNA samples were purified by phenol:chloroform extraction followed by saturated chloroform and saturated ether extractions. Salt concentration was adjusted to 300 mM with 3 M NH_4COOH or 3 M NaOAc and the DNA was precipitated with 2-2.5 volumes ethanol and incubation on dry ice for 1 hr till frozen solid. DNA was pelleted by microcentrifugation at 12,000 g for 15 min. Drained pellets were washed with 70% ethanol to de-salt pellets. DNA samples resuspended in 10 mM Tris 1 mM EDTA buffer to a concentration of 1 µg/µl were then digested by restriction endonucleases to analyze the methylation pattern.

Digestion volumes were 50-100 µl and were composed of one-tenth volume 10X BRL core buffer or recommended buffer (42), one-fiftieth volume 10 mg/ml BSA, 10-25 µl of 1µg/µl methylated or unmethylated pBR322, 2.5-5.0 µl restriction endonuclease (50% glycerol) and the remaining volume dH_2O. Digestions were usually incubated overnight (10-12 hr) at 37°C. Enzymes were inactivated by incubation at 65°C for

10 min when possible or by addition of one-tenth volume 200 mM EDTA when not. Digested DNA was then precipitated by addition of one-tenth volume 3 M NaOAc and two volumes of ETOH samples were frozen on dry ice, microfuged, and treated as mentioned earlier in text. DNA pellets were either resuspended in 89 mM Tris (pH = 8.0), 178 mM Borate, 2 mM EDTA buffer (TBE) or resuspended directly into a second restriction endonuclease reaction buffer for subsequent digestion. DNA in 1X TBE buffer was mixed with an equal volume Ficolle dye: 1X TBE. After mixing and microfugation to remove air bubbles, DNA-dye samples were loaded in the wells of polyacrylamide gels.

I. Agarose and Polyacrylamide Gel Purification (Electrophoresis) of DNA

DNA-dye samples (10-40 µl) were loaded into the wells of 10-12 lane 15 cm^2 x 1 mm vertical gels of 15% 19:1 acrylamide:N, N:'-methylene bisacrylamide dissolved in 1X TBE buffer. Gels were run at room temperature at 5-10 V/cm until xylene cyanol FF dye (top) band reached 8-11 cm distance from well bottoms. Gels were stained in 500 ml of 0.5 µg/ml EtBr in dH_2O for ≧45 min then drained and rinsed with dH_2O. Gels were photographed on a UV transilluminator with Polaroid camera.

Agarose gel electrophoresis was performed according to Maniatis (37). A 15 cm^2 x 0.4 cm gel was filled with 0.7-1.0% melted Seakem$_{TM}$ agarose in 1 X E buffer. DNA samples were mixed with 1/10 volume BPB-XC Ficolle dye: 200 mM EDTA and loaded in gel wells covered with 1 X E buffer. Electrophoresis toward anode was run at 2-3 V/cm till

the xylene cyanol dye had migrated at least 7 cm from wells. Gels were stained the same as the P.A. gels described above.

J. Isolation of Restriction Fragments from Polyacrylamide and Agarose Gels

Desirable restriction fragments were excised from polyacrylamide gels by cutting around fluorescent bands on UV transilluminator using a one-edged razor blade and spatula. Special attention was given to keeping gel moist with dH_2O, wiping razor blade between samples to prevent cross-contamination, and shielding oneself from the UV-irradiation to prevent "sunburn". Gel slices were deposited in 7 ml scintillation vials for destructive processing or in 1.5 ml Eppendorf tubes if DNA was to be recovered from gel slice.

DNA bands were excised from agarose gels and put into preweighed 20 ml scintillation vials. The difference between the gel slices' weight and 4 gm (4 ml) was compensated by addition of dH_2O. Then the samples were "boiled" in 100°C waterbath for 15-20 min to melt the agarose. After cooling, 11 ml Aquasol II fluor were added and samples were allowed to gel before scintillation counting.

K. Destructive Processing of P.A. Gel Slices of Restriction Fragments

One half milliliter of a 9:1 mixture of Amersham's 0.6 N NCS tissue solubilizer:dH_2O was added to each vial containing a gel slice, being sure to tap the slice to the bottom of the vial (43,45). Samples were incubated $\geq$2 hr at 50°C. Six and one-half milliliters of Amersham's OCS fluor was pumped into each vial, samples were mixed by inversion and counted immediately for 10 min in LS-7000 for tritium.

L. Elution of Restriction Fragment DNA from Polyacrylamide Gel Slices for Nondestructive Analysis

Small DNA fragments (<200 bp) were extracted from the polyacrylamide gel slices by the "Elution by Diffusion Method of Maxam and Gilbert" (44,47). Each DNA-containing polyacrylamide gel slice was placed in its own 1 ml Eppendorf Pipetman tip, which had been stuffed with a wad of glass wool then siliconized and sealed. The gel slice was pulverized using a plastic stirring rod and rinsed off the rod and walls of the tip into the bottom with 0.6 ml of the modified eluting solution: 0.5 M NH_4OAc, 0.01 M EDTA, 0.1% SDS. The tip was then covered with parafilm at both ends and incubated at 37°C overnight (>12 hr). Five µl of 10 mg/ml yeast tRNA was micropipetted into each tip to serve as carrier RNA to minimize losses due to "sticking" and to promote precipitation in subsequent steps. The bottoms of the sealed tips were cut off and each tip was placed in a siliconized 10 x 75 mm test tube then centrifuged at low speed in a bench top centrifuge for 3-5 min to sieve DNA solution through glass wool. The contents of each was rinsed with another 0.2 ml eluted solution and respun. The tip was discarded and DNA solutions were extracted 3-5 times each with equal volumes of unsaturated sec-butanol to extract ethidium bromide and to reduce (partition) volume of samples to 200-300 µl.

Samples were then transferred to Eppendorf tubes (1.5 ml). DNA/RNA was precipitated by addition of one-tenth volume 3 M NaOAc and 2-2.5 volumes ethanol. Samples were put on dry ice till frozen (≥1 hr) then microfuged for 15 min. DNA/RNA pellets were dried and resuspended

in 200 µl 10 mM Tris pH = 8.0 1 mM EDTA by vortexing. Samples were extracted with phenol:chloroform twice then with saturated ether twice. Again DNA/RNA was precipitated by addition of 3 M NaCl and ethanol as before and freezing solid on dry ice. DNA/RNA pellets yielded by centrifugation were desalted with a 70% ethanol rinse, vacuum dried, and resuspended in 20 µl 10 mM Tris pH = 8.0, 1 mM EDTA containing 1100 units RNase Ti/ml and 0.5 mg RNAse A/ml to degrade RNA carrier. Samples were incubated 2 hr at 37°C then put at 4°C. DNA was subsequently analyzed by restriction endonuclease digestions and polyacrylamide electrophoresis as described in earlier text. Gels were stained in 0.5 µg/ml EtBr and UV-fluorescent DNA-bands were excised in gel slices for destructively processing to analyze methylation.

IV. RESULTS

A. Computer-Assisted Selection of R. Hha I Digestion of pBR322 DNA for Methylation Analysis

For determination of the sequence specificity of the dam methylases we used the plasmid pBR322 as a target substrate of its relatively small size (4.4 Kb) and the knowledge of its complete DNA sequence. pBR322 DNA was determined to have numerous possible methylation sites (GAPyN) scattered throughout its genome (Figure 1). The microgenie computer program generated lists of DNA fragment sizes that would be created by digestion of pBR322 with one or several restriction endonucleases. A matrix (Figure 2) was constructed to determine which enzyme or combination of enzymes yielded the most favorable number of fragments containing one or a few GAPyN sites.

By comparing the R. Hha I restriction fragment sizes of pBR322 and their boundary base pair locations with the location of GAPyN sites within pBR322's sequence, we found that many of the smaller R. Hha I fragments contained only one, two or a few of these putative methylation sites (Table 1). Two fragments (28 and 36 bp) contain only one GATG site each, while two others (33 and 54 bp) each contain only one GACT site. Unfortunately, none of the other fragments had only one GATC or GACC site without any other GAPyN sites. The 40 bp fragment has two GACC sites and the fragments that do have palindromic GATC sites (60, 83, and 109 bp) have one or two other species of GAPyN sites, too. Fragments of 30, 67, 93, and 100 bp size each contain two or three species of GAPyN sites but were useful because one or two of

Figure 1. Survey of pBR322 sequence for frequency of GAPyN sites.

Mwt pBR322:

4.4×10^3 bp/molecule x 650 daltons/bp

x 1 bp molecules/mole/daltons/gm

$= 2.86 \times 10^6$ gm/mole

# sites/pBR322 molecule	GAPyN species	[sites] = 25 µg/400 µl
22 (44)	GATC/GATC (2A's/site)	485 nM (970 nM)
48	GATG/CATC	1,060 nM
39	GATA/TATC	860 nM
21	GATT/AATC	462 nM
30	GACC/GGTC	660 nM
22	GACT/AGTC	485 nM
29	GACA/TGTC	640 nM
33	GACG/CGTC	727 nM

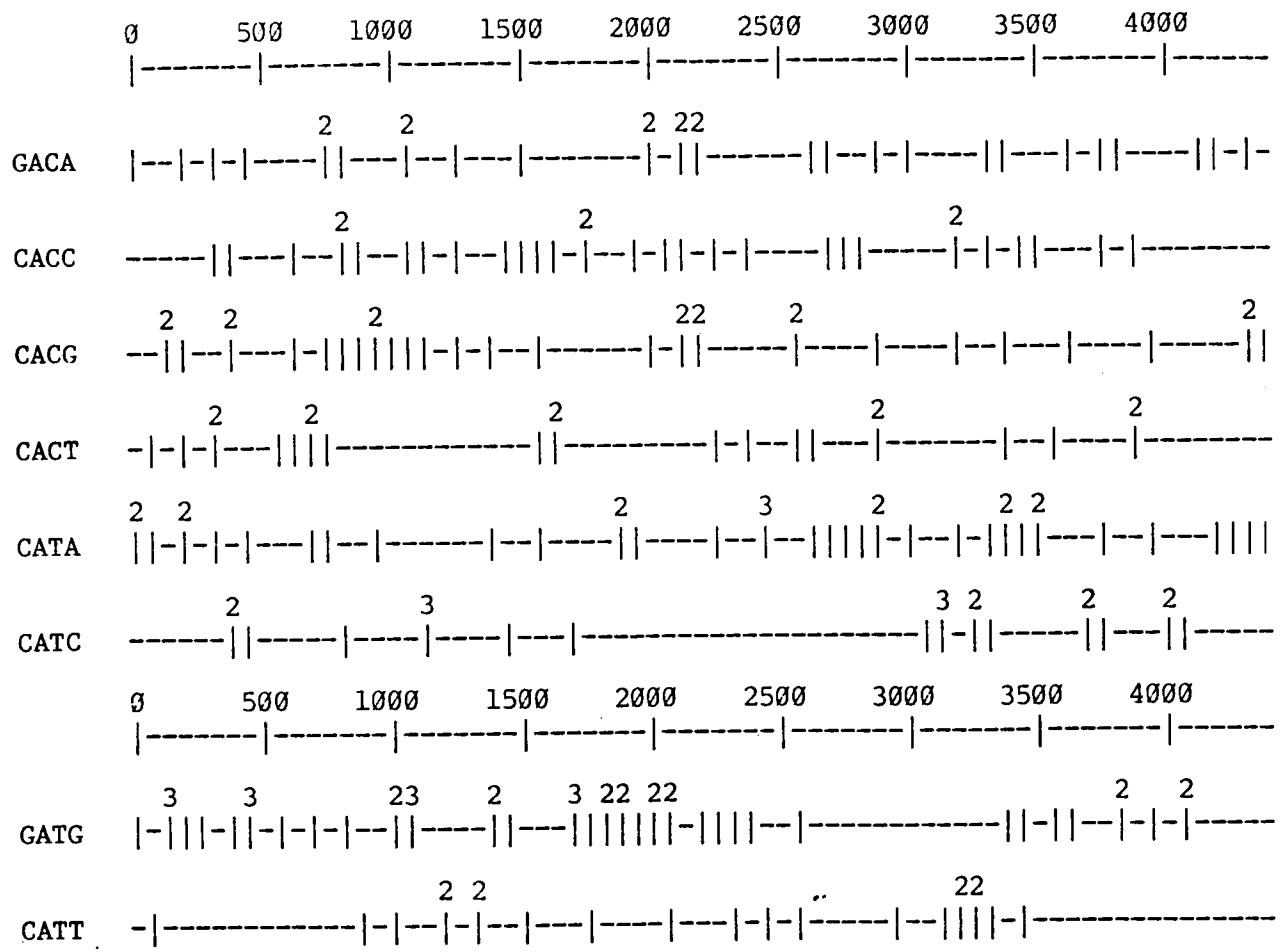

0 500 1000 1500 2000 2500 3000 3500 4000
GACA
CACC
CACG
CACT
CATA
CATC
0 500 1000 1500 2000 2500 3000 3500 4000
GATG
CATT

Figure 1.

pBR322 Fragments Which Contain at Most One GAPyN Site Generated by Single or Double Restriction Enzyme Digestion

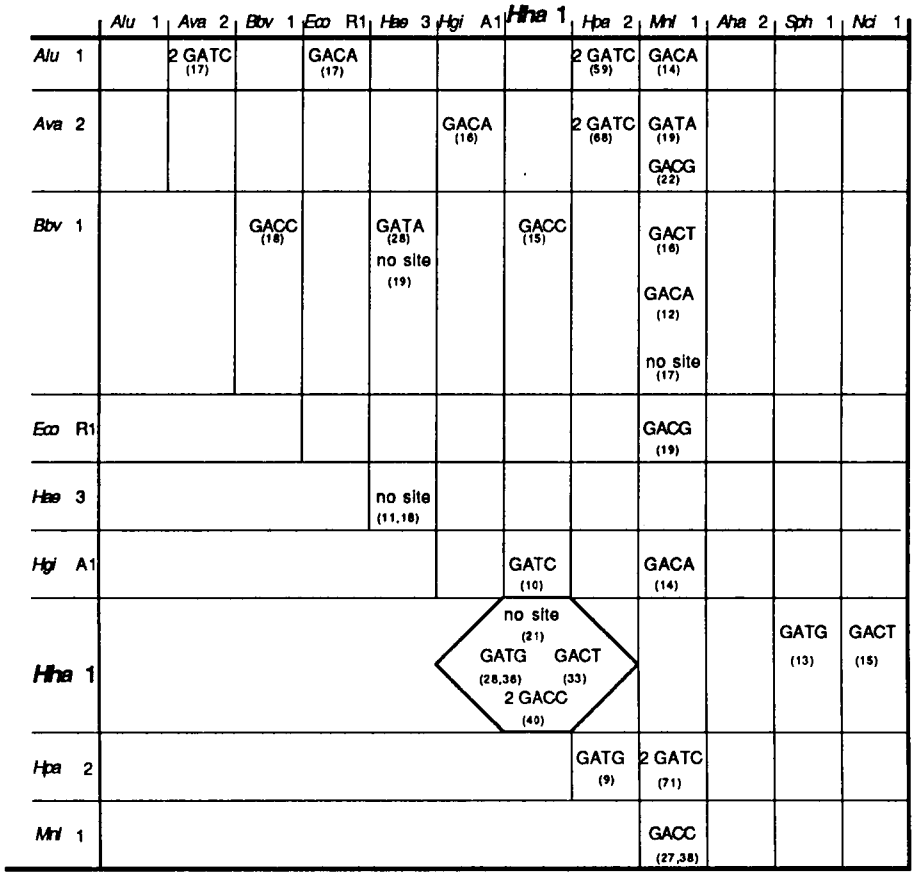


Figure 2. pBR322 fragments which contain at most one GAPyN site generated by single or double restriction enzyme digestion.

Table 1. Hha I fragments of pBR322 (18 of 31).

	Length (bp)	Start (bp)	Stop (bp)	Putative Methylation Sites (GAPyN)
	21	414	435	NONE
TG	28	233	261	tGATGc
	30	2179	2209	cGACAc,tGACGc
	33	2350	2383	tGACTc
TG	36	1419	1455	aGATGc
(2)CC	40	776	816	tGACCc,gGACCg
	54	495	549	gGACTg
TC,(2)TG	60	435	495	cGATAt,gGACAt,tGATGt,gGATGc, aGATCg
(2)TG	62	1357	1419	gGATAt,cGATGg,gGACGc,aGATGg
CC	67	2653	2720	cGACCt,gGATAc,gGACAg
	75	701	776	tGACTa,cGATAg,cGACGa,tGACTc, aGACAg,tGATAa,gGACAg
TC,(3)TG	83	1645	1728	gGATGc,aGATGt,gGATCt,cGATGc
TG	93	3496	3589	gGATAa,gGATGg,aGACTg
	100	2720	2820	aGATAc,cGACCt,cGACCc
	103	2076	2179	tGATGa,tGACGg,tGACAa,aGACGg, tGACCg,aGACAa,gGATGc,aGACAa, tGACGg
TC	109	2994	3103	tGATCc,aGATTa
	131	816	947	cGATGa,tGATCg,etc.
	132	101	233	(4)nGATGn,etc.

these species of sites were on other fragments by themselves. If they were unmethylated when found on a fragment by themselves we concluded that their methylation on a multi-site fragment could be ruled out. This enabled us to quantitate methylation of other sites on these fragments. The 20 bp fragment served as a very good negative control since it did not contain any GAPyN sites. Background scintillation counts were determined using the 21 bp fragment.

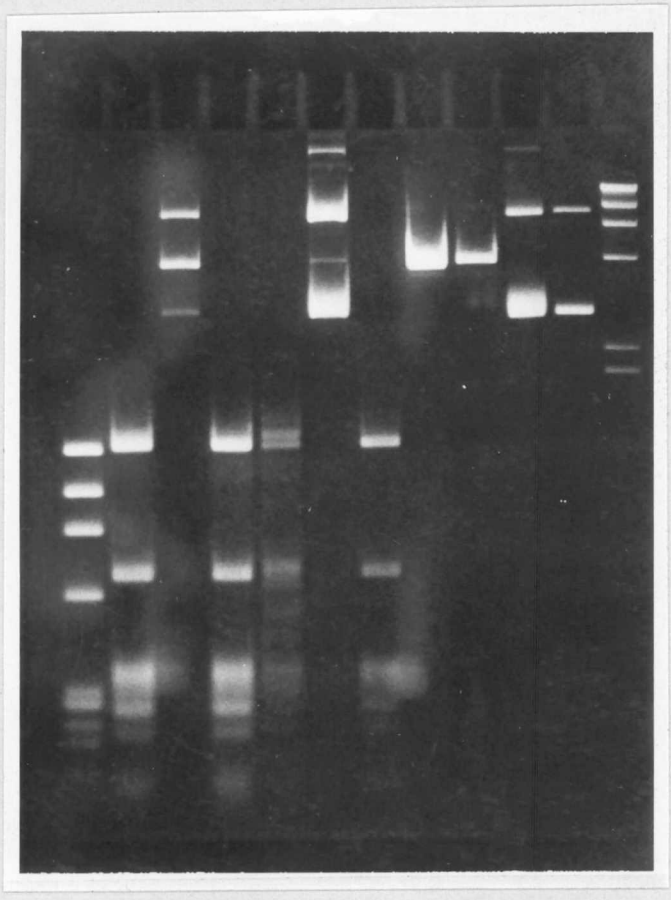
The ability to ascertain the extent of methylation, or percent of molecules methylated at a certain GAPyN site present on all pBR322 molecules, was dependent on the ability to resolve the 18 smallest R. Hha I fragments on a polyacrylamide gel and to be able to carefully remove each band in entirety from the gel for scintillation counting. Usually the bands (21-109 bp) were isolatable as separate entities but the 60/62 and 131/132 fragment doublets would occasionally overlap or co-migrate in the gel so they were isolated and counted together. Therefore, the data was combined for these fragment pairs in all of the tables.

B. Control Assays to Establish the Purity and Concentration of the Methylase Assay Components

In order to measure the specific activity and substrate specificity of the T_2, T_4, and Eco dam methylases, it was necessary to establish the stability and purity of the substrates of the enzymes and to determine the sensitivity of the enzyme assay needed to be ascertained. These intrinsic factors directly affect our ability to interpret the results of our assays.

1. DNA Substrate

The pBR322 DNA used in methylation assays was isolated from a *dam*$^-$ *dcm*$^-$ *r*$^-$*m*$^-$ *E. coli* host (GM272) and a *dam*$^+$ *dcm*$^+$ *r*$^-$*m*$^+$ *E. coli* host (HB101). Unmethylated and methylated plasmid DNAs isolated from strains GM272 and HB101, respectively, were tested for their degree of methylation by digesting them with the restriction enzymes, R. *Mbo* I, R. *Dpn* I, and R. *Sau* IIIA, which are isoschizomers that cleave DNA at GATC sites. As mentioned in the background section of this text, R. *Mbo* I only cleaves GATC sites lacking adenine methylation, R. *Dpn* I cleaves only fully-methylated GATC sites and R. *Sau* IIIA cleaves all GATC sites irrespective of adenine methylation. Results in Figure 3 indicate that pBR322 DNA from strain GM272 is completely unmethylated at GATC sites. This is deduced from the observation that R. *Mbo* I digestion of the latter DNA results in the same restriction pattern (lane 2) as when the same DNA is cut completely by R. *Sau* IIIA (lane 4). The same DNA is not cleaved by R. *Dpn* I (lane 6). The R. *Dpn* I-digested DNA (lane 6) exhibits the same bands as seen in the undigested (no enzyme) DNA (lane 10). pBR322 DNA from strain HB101 is not cut by R. *Mbo* I (lane 3) whose digest DNA bands resemble those of the undigested DNA control (Lane 11). This same DNA cut by R. *Dpn* I (lane 7) yields the same restriction digest pattern as the R. *Sau* IIIA digest pattern (lane 5). Both of these results indicate pBR322 DNA from strain HB101 is fully methylated at all GATC sites in >99% of the molecules since the background in the digest lanes is so low. Contaminating exonucleases, present in enzyme preps are probably



1.5% Agarose Gel (15 cm X 0.4 cm)
Lanes 1 2 3 4 5 6 7 8 9 10 11 12

Figure 3. Restriction enzyme digestions of methylated and unmethylated pBR322 DNA from *E. coli* strains, HB101 and GM272, respectively.

Lane 1. Marker DNA ϕ X 174
2. $+CH_3$ pBR322 DNA X R. *Mbo* I
3. $+CH_3$ pBR322 DNA X R. *Mbo* I
4. $-CH_3$ pBR322 DNA X R. *Sau* IIIA
5. $+CH_3$ pBR322 DNA X R. *Sau* IIIA
6. $-CH_3$ pBR322 DNA X R. *Dpn* I
7. $+CH_3$ pBR322 DNA X R. *Dpn* I
8. $-CH_3$ pBR322 DNA X R. *Eco* RI
9. $+CH_3$ pBR322 DNA X R. *Eco* RI
10. $-CH_3$ pBR322 DNA X undigested
11. $+CH_3$ pBR322 DNA X undigested
12. Marker DNA lambda X R. *Hind* III

responsible for the increased background seen in lanes 2, 4, and 5, which is greater than that of lanes containing undigested (no enzyme) DNAs.

Ribonucleotides are often found in plasmid DNA molecules isolated from cells exposed to chloramphenicol for prolonged periods of time for plasmid amplification purposes. To minimize their polymerization in DNA molecules, amplification with chloramphenicol was limited to 12 hr rather than the usual 16 hr. Though we did not assay for the presence or absence of ribonucleotides in our plasmid DNA, we were of the opinion that if any did exist in our molecules, they would be randomly distributed throughout the sequence of all the molecules and would affect the frequency of methylation of all GAPyN sites equally if at all.

2. Methyl donor substrate: S-adenosyl-L-methionine (SAM)

S-adenosylmethionine, the methyl donor in the in vivo methylation reaction:

$$\underset{\text{(SAM)}}{\text{s-adenosylmethionine}} + \text{DNA (GAPyN)} \xrightarrow{\text{dam methylase}}$$

$$\text{DNA } (\overset{*}{\underset{CH_3}{G}}\text{APyN}) + \text{s-adenosylhomocysteine (SAH)}$$

is added to in vitro methylation reactions as a radiolabelled tracer (^{3}H-SAM), for quantitatively assaying the extent to which different GAPyN sites are methylated in vitro.

It is known that the by-products of SAM degradation and co-purifying (contaminating) compounds, present in some commercial SAM preparations, can interfere with the accurate determination of SAM concentration by spectrophotometric absorption and can act as inhibitors of methylase activity (45). The relative concentration of such extraneous compounds in the preps used in our assay was ascertained by thin layer chromatography.

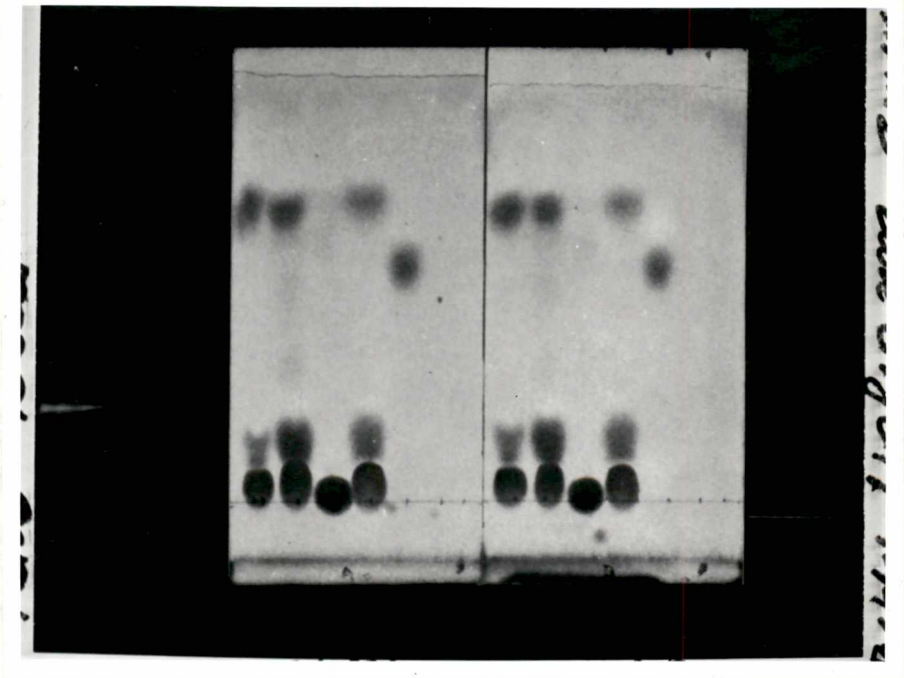
In Figure 4 (panel A) are two duplicate silica gel thin layer chromatography plates spotted with 5 µl (500 nmoles SAM = 2.0×10^{-4} gm = 0.2 mg) of different 100 mM solutions of SAM preparations in lanes 1-4. Lane 4 was spotted with a preparation of SAM-iodide that had been "purified" by binding, washing, and elution from an Amberlite CG-50 column (Hattman, personal communication). As one can see, only the SAM·HSO_4 sample in lane 3 exhibits only one UV-fluorescent spot (SAM) when developed in a butanol: acetic acid: water TLC solvent system. The other three preps, including the so-called purified SAM prep contain two other UV-fluorescent spots besides that of SAM, although they are somewhat reduced in the purified SAM prep. None of the preps seem to contain detectable levels of adenosine which would co-migrate with the spot in lane 5.

Understandably, no UV-fluorescent spots, not even ^{3}H-SAM, were observed in lane 7 due to the low concentration (1 µl of 6.5 µM SAM = 6.5 pmoles SAM) of the sample spotted at the origin. When plate B (Figure 4; panel B) was stained with platinum iodide and baked to develop, spots representing sulfur-containing compounds turned pink (P)

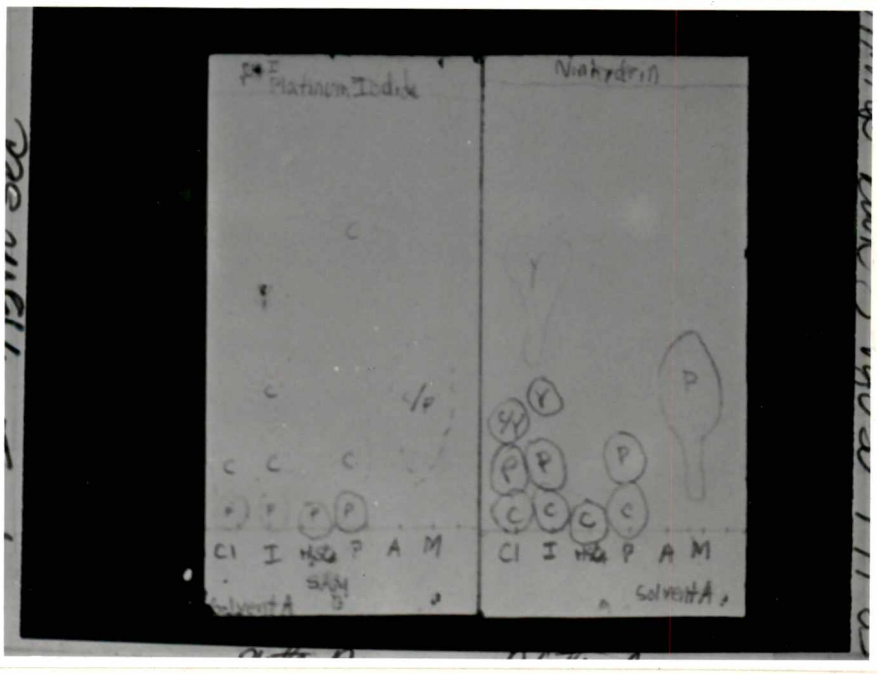
Figure 4. Purity analysis of SAM preps by thin layer chromatography.

Panel A: UV-illumuniation of SAM TLC plates
Column 1. SAM-HCl
2. SAM-Iodide
3. SAM-HSO_4
4. SAM-Iodide (CG-50 purified)
5. Adenosine (10 μg)
6. Methionine (125 μg)
7. H-SAM (1 μCi) NEN tritiated SAM-HSO

Panel B: Platinum iodide or Ninhydrin-stained TLC plates of SAM
Columns 1-7 (same as panel A)



Panel A

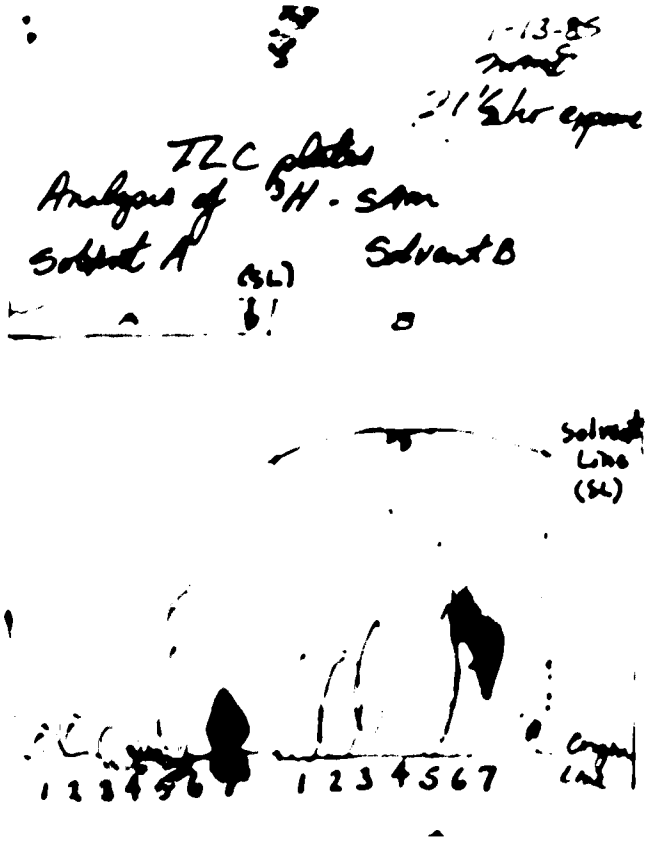


Panel B

Figure 4.

or whitish/clear (C). Again, only the SAM·HSO_4 sample exhibited only one pink spot (SAM) near the origin of the plate. All other SAM samples had additional whitish-clear spots in their lanes. The methionine sample in lane 6 (125 μg) was visible as a large spot (overloaded) of pinkish-white hue. When the duplicate plate A (Figure 4; panel B) was stained with ninhydrin (reacts with α-amino groups of amino acids) and baked to visualize amino acid-like compounds, again only the SAM·HSO_4 sample in lane 3 exhibited only one whitish-clear spot at the origin, whereas all other SAM samples had additional spots in their lanes. The extraneous compounds detected in the SAM·HCl and SAM·iodide preparations are thought to be s-adenosyl-L-homocysteine (RF = 17) and 5'-methylthioadenosine (RF = 66) based on their known mobility constants (R.F.) in solvent A (see Table A-1 in Appendix).

The autoradiogram in Figure 5 depicts the only radioactive spot (SAM) observed in lane 7 of both TLC plates. Although nonradioactive compounds present in the NEN ^{3}H-SAM-HSO_4 sample could not be detected by this method, the presence of ^{3}H-5'methylthioadenosine, ^{3}H-5'methylthioinosine, and S-^{3}H-methyl-L-methionine would be detected. In summary, all of the results discussed above indicated that the HSO_4 forms of SAM were the purest and most stable. In lieu of these results, the SAM·HSO_4 preparations were chosen for use in the methylase assays.



Column 1 2

Figure 5. Autoradiogram of TLC plates treated with ENLIG^{3}HTNING**.

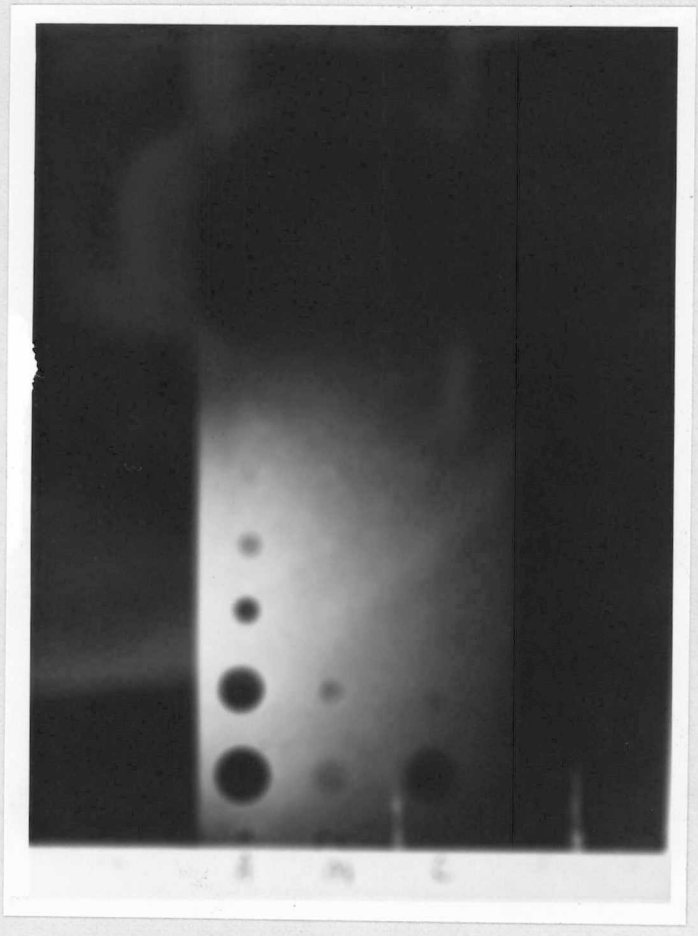
Column 1. ^{3}H-SAM (1 µCi) NEN tritiated SAM-HSO_4 resolved in solvent A.

Column 2. ^{3}H-SAM (1 µCi) NEN tritiated SAM-HSO_4 resolved in solvent B.

Other silica gel plates were spotted with samples of adenosine, methionine, and cysteine from solutions of decreasing concentration to determine the detection limits of TLC analysis of SAM using UV-illumination, platinum iodide/HCl, and ninhydrin staining for visualization. The results of UV-illumination (fluorescence) of the spots on the plate seen in Figure 6 show that the UV detection limits are approximately 100 ng for adenosine and between 10 and 50 μg for methionine and cysteine. After platinum iodide staining and HCl development, only methionine could be detected to the limit of 50 μg. Ninhydrin staining was more sensitive, allowing the detection of as little as 5 μg of methionine per spot. Adenosine and cysteine were undetectable with either stain. If each sample of SAM spotted on the TLC plates was 200 μg, we should have detected any adenosine or other UV-absorbing adenosine derivative composing greater than or equal to 0.05% of the original sample. Somewhat less sensitive, the ninhydrin spray permitted the detection of any reacting compound that composed greater than or equal to 2.5% of the original sample.

C. Methylase Assay Development

Brooks and Hattman (2) showed that crude extracts of phage-infected cells contained levels of *dam* methylase activity sufficient to detect significant differences in extents of methylation of lambda DNA (*in vitro*) by *dam*$^+$ and *dam*^{h} enzymes using 22.8 μM ^{3}H-SAM or ^{14}C-SAM of ≥6 Ci/mmole final specific activity in their reactions. We used crude extracts to assay methylase activity. By using crude extracts, most of the normal population of proteins is present in the assay even though



A M C

Figure 6. Detection limits of adenosine, methionine, and cysteine by UV-illumination.

Column:	(A) Adenosine	(M) Methionine	(C) Cysteine
Spot #1	100 µg	500 µg	500 µg
#2	50 µg	50 µg	50 µg
#3	10 µg	5 µg	5 µg
#4	1 µg	500 ng	500 ng
#5	100 ng	50 ng	50 ng
#6	10 ng	--	--

the concentrations of small molecular weight metabolites, ions, and pH may be different.

1. Determination of Optimal Parameters for the Methylase Assay

Brooks (7) reported that the pH optima for the rate of methylation by T_2dam$^+$ and dam^h enzymes were pH 7.6 to 8.0; Brooks and Hattman (2) used a 50 mM Tris-HCl (pH = 8.0) buffer in their methylase assays. In our hands the dam enzymes had 10-20 fold greater activity (Table 2, filter assay) in M. Hpa II buffer (50 mM Tris pH = 7.5; Biolabs Manual) than in their buffer. This difference might have been due to the temperature at which the pH was determined. The pH of buffers used throughout the experiments reported here were consistently determined at the temperature of use (incubation) due to the changes in pH that Tris buffers are known to undergo with temperature changes. However, other differences between components of buffers (see Methods and Materials section) could also be responsible for the differences in activity observed.

In order to detect site preference and overall sequence specificity of each methylase, it was desirable to methylate target DNAs to different extents of saturation (i.e., half or full). Therefore, assays were performed to determine which incubation conditions and which proportions of crude extract, DNA, and SAM, would yield the proper degree of saturation in methylase reactions. In preliminary saturation studies, the GM119(pSSH-1b)-INF dialysate, whose original assay results are shown in Table 2, was used. The latter dialysate contains methylase activity produced by the T_4D^+ (wildtype)

Table 2. Methylase activity of crude extracts (TCA-ppt and filter binding assay).

Sonicate	Buffer	lambda DNA	cpm
K803 UN*	T_2dam	+	220
K803 UN	M. Hpa II	+	4,721
K803 INF**	T_2dam	+	2,489
K803 INF	M. Hpa II	+	88,246
K803 INF	M. Hpa II	-	226
GM119/pSSH-1b*** INF	T_2dam	+	18,389
GM119/pSSH-1b INF	M. Hpa II	+	227,150
GM119/pSSH-1b UN	T_2dam	+	3,211
GM119/pSSH-1b UN	M. Hpa II	+	45,154
GM119/pSSH-1b UN	T_2dam (no phenol extraction)	+	4,150
M. Hpa II (pure enzyme)	M. Hpa II	+	41,346

*UN = uninfected

**INF = infected by phage T_2dam$^+$

***GM119/pSSH-1b = Eco dam$^-$ dcm$^-$ strain harboring plasmid containing T_4dam$^+$ gene.

Reaction Parameters

100 µl volume; 50 µl dialysate

130 nM ^{3}H-SAM 2H

370 nM lambda DNA GATC site "A"s

Incubation: 4.5 hr at 37°C

infection of a dam$^-$ dcm$^-$ host strain which harbors the T_4dam$^+$ insert-bearing plasmid pSSH-1b (46). The infected host cell dialysate was observed, using the filter-binding purification, to have more than fifty-fold greater methylase activity than the uninfected host dialysate assayed in the same buffer. However, infection of the presumed dam$^+$ dcm$^+$ rgl$^-$ host (K803) by T_4D^+ yielded extracts with only two-fold higher activity. Presumably, as results discussed later in the text will show, the methylase activity in uninfected K803 cells in only dcm methylase activity. All methylase activities were indeed higher in the M. Hpa II buffer than in the T_2dam$^+$ buffer. Calculation of the extent of methylation of the lambda DNA by the dam methylase in the GM119-INF indicates that 14-28% (14% of GATC site "A"s) of the total number of GATC sites of all molecules are either fully or hemimethylated on their adenines, respectively. The concentration of SAM in these assays (130 nM) was significantly below the established K_mSAM value (2.4 μM) (Hattman, pers. comm.) and below the concentration of GATC site "A"s (370 nM) causing the limited degree of methylation.

To ascertain whether complete methylation of GATC sites of lambda DNA was attainable with crude extracts, parallel methylation reactions were incubated with methylase extract or buffer (mock) for intervals of 3, 6, or 9 hr. The 6 and 9 hr incubation reactions had one additional volume of dialysate added at each 3 hr interval for a total of 2 or 3 volumes, respectively.

Each reaction contained 5 μg lambda DNA (370 mM GATC sites at time zero) and 12.5 μM SAM; sp. act. = 0.78 mCi/mmole to optimize assay

conditions. The *in vitro* methylated lambda DNA was then purified by standard techniques and aliquots (1 μg) were digested with R. *Mbo* I, R. *Dpn* I, or R. *Sau* IIIA to determine the extent of methylation at GATC sites. As seen in lanes 10, 14, and 18 of Figure 7, none of the lambda DNA from either the 3, 6, or 9 hr reactions are cleaved by R. *Mbo* I. The unmethylated lambda DNA is cleaved by R. *Mbo* I (lane 26) and R. *Sau* IIIA (lane 27) to the same extent (completion) creating the same restriction patterns. This indicates that all of the GATC sites of the 3, 6, and 9 hr sample DNAs are hemi- or fully-methylated. The observation that more fragments are generated by R. *Dpn* I digestion of DNA samples that were incubated for longer periods of time with methylase suggests that some hemimethylated sites are becoming fully methylated with time. The R. *Dpn* I restriction pattern may never match the R. *Sau* IIIA pattern, even when full methylation is reached, due to the observed effects of flanking bases upon cleavage of GATC sites by R. *Dpn* I (47).

The *in vitro* methylated lambda DNA samples appear to be methylated to a greater extent at GATC sites than *in vivo* (partially) methylated lambda DNA. This was deduced by comparison of the native lambda phage's DNA cut by R. *Mbo* I (lane 30) and R. *Dpn* I (lane 29) with that of the *in vitro* methylated DNA of lambda cut by the same enzymes (lanes 9, 10, 13, 14, 17, 18). The *in vivo* methylated lambda DNA is cleaved more by R. *Mbo* I and less by R. *Dpn* I enzymes than the T_4*dam*$^+$ methylated DNAs. Thus, the GM119-INF dialysate appears to possess sufficient methylase activity to achieve near complete methylation of

Figure 7. Restriction endonuclease digestions of plasmid DNAs and T_4*dam*$^+$-methylated lambda DNAs used to determine the extent of methylation.

Gel #1

Lane 1. pBR322 (HB101) X R. *Dpn* I
2. pBR322 (HB101) X R. *Mbo* I
3. pBR322 (HB101) X R. *Sau* IIIA
4. pBR322 100 ng (HB101) undigested
5. pSSH-1b X R. *Dpn* I
6. pSSH-1b X R. *Mbo* I
7. pSSH-1b X R. *Sau* IIIA
8. pSSH-1b 100 ng undigested
9. T_4*dam*$^+$ triple pump 9 hr lambda X R. *Dpn* I
10. T_4*dam*$^+$ triple pump 9 hr lambda X R. *Mbo* I
11. T_4*dam*$^+$ triple pump 9 hr lambda X R. *Sau* IIIA
12. T_4*dam*$^+$ triple pump 9 hr lambda undigested (250 ng)
13. T_4*dam*$^+$ single pump 3 hr lambda X R. *Dpn* I
14. T_4*dam*$^+$ single pump 3 hr lambda X R. *Mbo* I
15. T_4*dam*$^+$ single pump 3 hr lambda X R. *Sau* IIIA
16. T_4*dam*$^+$ single pump 3 hr lambda undigested plus lambda X R. *Hind* III and ϕX174 X R. *Hae* III Marker DNAs

Gel #2

Lane 17. T_4*dam*$^+$ double pump 6 hr lambda x R. *Dpn* I
18. T_4*dam*$^+$ double pump 6 hr lambda x R. *Mbo* I
19. T_4*dam*$^+$ double pump 6 hr lambda x R. *Sau* IIIA
20. T_4*dam*$^+$ double pump 6 hr lambda (undigested) (250 ng)
21. Dialysis buffer/lambda X R. *Dpn* I
22. Dialysis buffer/lambda X R. *Mbo* I
23. Dialysis buffer/lambda X R. *Sau* IIIA
24. Dialysis buffer/lambda (undigested) (250 ng)
25. Lambda ($-CH_3$) x R. *DpN* I
26. Lambda ($-CH_3$) X R. *Mbo* I
27. Lambda ($-CH_3$) X R. *Sau* IIIA

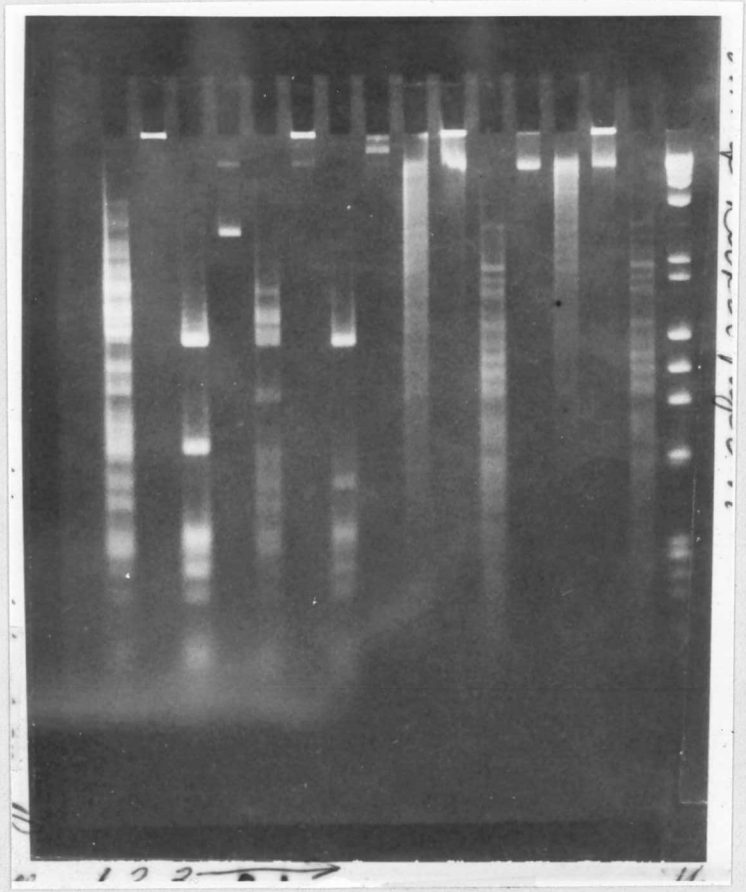
Figure 7 (continued).

28. Lambda ($-CH_3$) undigested (250 ng)
29. Partially methylated lambda X R. Dpn I
30. Partially methylated lambda X R. Mbo I
31. Partially methylated lambda X R. Sau IIIA
32. Partially methylated lambda--undigested (250 ng)

Methylation Reaction Conditions/Parameters:
12.5 μM cold SAM + 130 nM hot SAM; sp. act. of reaction = 780 nCi/nmole
5 μg lambda DNA per 100 μl reaction = 370 nM GATC "A"s at time zero in single dialysate pump reaction

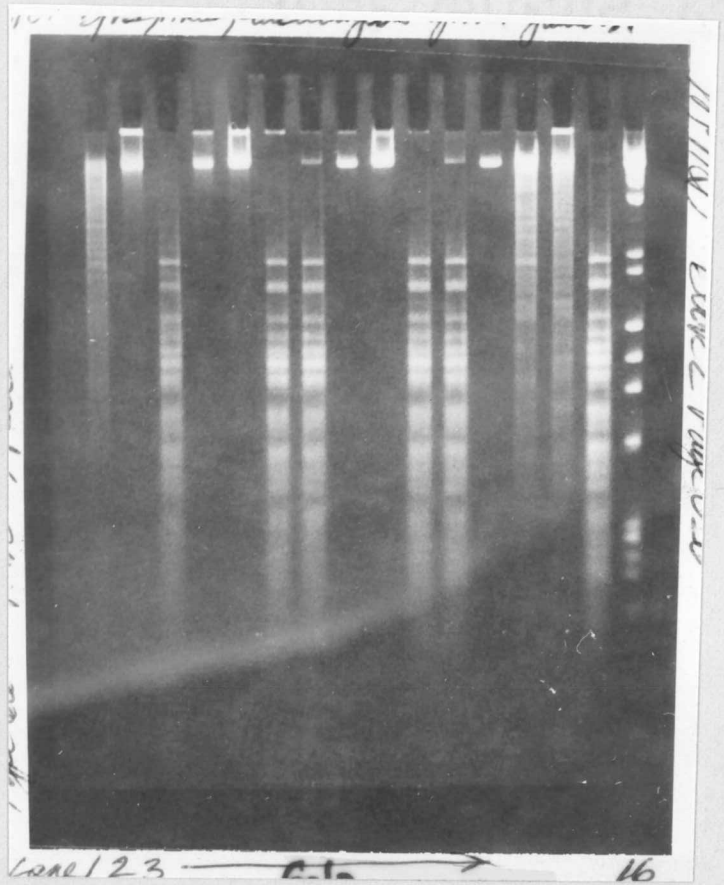
Amount of Crude Extract (GM119-INF)	Incubation Time (hr) at 37°C	Expected cpm/100 μg	cpm/100 ng	% GATC methylated
single pump (50 μl)	3 hr	346 cpm	102	29%
double pump (2 X 50 μl)	2 X 3 = 6 hr	346 cpm	282	81%
triple pump (3 X 50 μl)	3 X 3 = 9 hr	346 cpm	358	103%

Gel 1



Lanes 1 16

Gel 2



Lanes 17 32

Figure 7 (continued)

lambda DNA when the concentration of SAM is optimized. Scintillation counting of 100 ng aliquots of the 3, 6, and 9 hr reactions verified the increase in methyl group transfer to the lambda DNA with increased time of incubation. Analysis of the buffer-control reaction DNA and *in vivo* methylated plasmid DNAs of pBR322 (HB101) and pSSH-1b/T_4*dam*$^+$ (GM119) are shown in adjacent lanes.

There are 7.37 pmoles of GATC sites (two per palindromic site) per microgram (μg) of lambda DNA. Some of these sites are probably still hemimethylated in the 9 hr reaction's DNA even though 7.6 pmoles (103%) of sites (adenines) appear to be methylated. This is probably due to GAPyN sites other than GATC also being methylated.

2. Reproducibility of Methylase Reaction: Gel Purification Technique

To demonstrate the reproducibility of the methylase reactions in combination with the gel purification techniques, two reactions (7-31-84) were incubated (results in Table 3) with two different dialysates, K803INF or GM119/pSSH-1b INF, then were split into five different samples each and loaded on the gel and electrophoresed. Even though the DNA exhibited a significant amount of nuclease degradation (Figure 8), processing and scintillation analysis of the uppermost (concentrated) portions of the band (middle and top) from each lane yielded similar (reproducible) results. The buffer-control and unmethylated DNA (no enzyme or buffer) control bands exhibited only near-background levels of counts created by EtBr-fluorescence.

In a similar experiment (8-24-84), five methylase reactions were incubated under identical conditions (except the volume was 50 μl

Table 3. Reproducibility of gel electrophoresis purification of methylated DNA.

Sample		cpm ^3H
0.049 µCi ^3H-std.		46,965
Background std.		22
5000 cpm ^3H-SAM std.		5,133
H_2O/fluor control		8
GM119 INF*1	top portion of band	63, 867
	middle portion of band	53,829
	bottom portion of band	36,270
	circular lambda band	758
2		82,629
3		83,585
4		110,520
5		85,208
K803 INF 1		112,650
2		125,600
3		137,025
4		140,212
5		129,756
Dialysis Buffer		228
Unmethylated lambda DNA		218

Methylase Reaction Parameters:

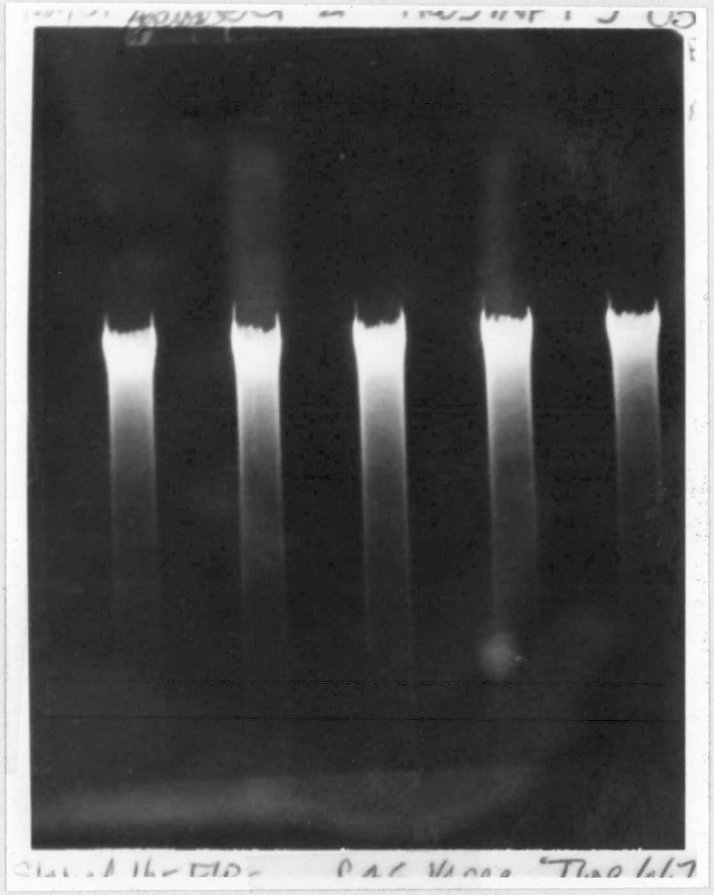
100 µl reaction volume; 50 µl sonicate
130 nM ^3H SAM 2H
370 nM GATC sites "A"s (5 µg lambda DNA)
Incubation time: 2 hr at 37°C

Figure 8. Reproducibility of gel purification of methylated DNA.

Gel 2
Lane 1. K803-INF/lambda DNA-1
3. -2
5. -3
7. -4
9. -5

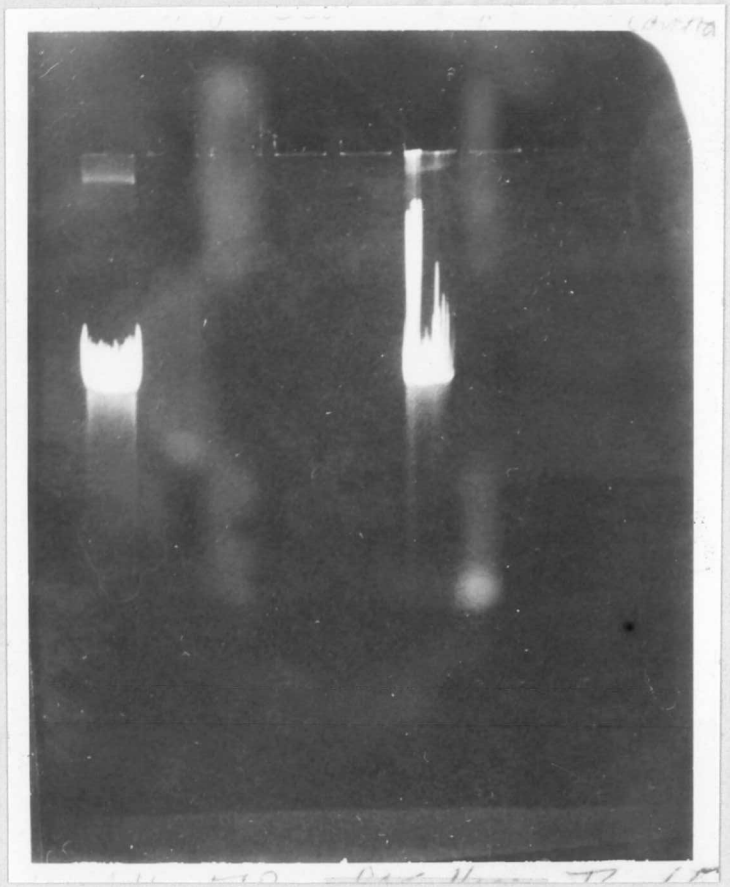
Gel 2
Lane 11. Dialysis buffer/lambda DNA
16. Native unmethylated lambda DNA

Gel 1



Lane 1 2 3 4 5 6 7 8 9 10

Gel 2



Lane 11 12 13 14 15 16 17 18 19 20

Figure 8.

versus 100 µl) using the same GM119-INF dialysate as the methylase source. After processing, 100 and 250 ng samples of each reaction were loaded on an agarose gel and electrophoresed. Results of scintillation analysis are shown in column II of Table 4. One can see the reproducibility between different reactions and the proportionality between samples of the same reaction. Another important observation to be made from these data is that the same crude GM119-INF dialysate used in the prior 6-24-84 assay, still showed significant methylase activity in the 7-31-84 and 8-24-84 assay after one to two months of storage at 4°C. These results indicated that the methylase was very stable when stored in crude extracts for at least two months.

3. Activity of Methylase Preps Used in Sequence Specificity Determinations

The results of scintillation analysis of gel bands of lambda DNA methylated by the dialysates used to methylate pBR322 DNA in the actual DNA sequence specificity assays are depicted in Tables 5 and 6. Conditions of assays are stated below the table. As one can see from the gel picture (Figure 9), the amount of nuclease degradation of the DNA is reduced with respect to earlier assays. The $T_2\underline{dam}^+$, $T_2\underline{dam}^h$, and $T_4\underline{dam}^h$, but not the $T_4\underline{dam}^+$ dialysates have good methylase activity levels. Since the "old" T_4D^+ (GM272) dialysate from an earlier infection still had good activity it was used instead of the newer $T_4\underline{dam}^+$ extract for assays. Dialysates from $T_4D^+(dam^+)$ and $T_2\underline{dam}^+$ infections of GM272 on 3-24-85 were also used in DNA sequence specificity assays and their activity upon unmethylated lambda DNA is

Table 4. Reproducibility of individually methylated DNAs purified by gel electrophoresis.

Sample	Amt. DNA (ng)	cpm ^{3}H	cpm ^{3}H/µg DNA
0.049 µCi ^{3}H Std.	N.A.	47,350	N.A.
Background Std.	N.A.	24	N.A.
5000 cpm ^{3}H-Std.	N.A.	2,897	N.A.
AQII Fluor Ctrl.	N.A.	6	N.A.
GM119 INF*/Lambda DNA-1	100	5,779	58,000
	250	16,172	64,700
GM119 INF*/Lambda DNA-2	100	7,081	70,810
	250	18,258	73,030
GM119 INF*/Lambda DNA-3	100	5,068	50,680
	250	17,166	68,660
GM119 INF*/Lambda DNA-4	100	5,878	58,900
	250	16,668	66,670
GM119 INF*/Lambda DNA-5	100	5,729	57,290
	250	17,051	68,200
Dialysis Buffer	N.A.	28	N.A.

Methylase Reaction Parameters:
50 µl reaction volume; 25 µl sonicate or dialysis buffer
130 nM SAM
370 nM GATC site "A"s (2.5 µg lambda DNA)
Incubation time: 2.5 hr at 37°C

Table 5. Methylase activity of dialyzed sonicates (part I).

Sample	Amt. DNA	cpm ^3H
0.049 µCi ^3H-Std.	N.A.	43,522
Background Std.	N.A.	23
1 µl ^3H-SAM 2H Std.	N.A.	272,486
1 µl ^3H-SAM 2H Std.	N.A.	276,457
1 µl ^3H-SAM 3L Std.	N.A.	342,467
dH_2O Control Std.	N.A.	9
$T_4\underline{dam}^h$ (5 µl)	1 µg lambda DNA	66,938
$T_2\underline{dam}^+$ (5 µl)	1 µg lambda DNA	55,155
$T_2\underline{dam}^h$ (5 µl)	1 µg lambda DNA	119,233
$T_4\underline{dam}^+$ (5 µl)	1 µg lambda DNA	16,401
$T_4\underline{dam}^+$ (10 µl)	1 µg lambda DNA	16,517
$T_4\underline{dam}^+$ (20 µl)	1 µg lambda DNA	13,415
$T_4\underline{dam}^+$ (20 µl + 2 µl SAM)	1 µg lambda DNA	12,333
Dialysis buffer	1 µg lambda DNA	2,492
Gel slice control	N.A.	1,103

Methylase Reaction Parameters:
50 µl reaction volume; 5, 10, or 20 µl sonicate as noted
825 nM SAM (1 µl SAM 3L) or 1.65 µM SAM (2 µl SAM 3L)
148 nM GATC site "A"s (1 µg lambda DNA)
Incubation time: 1 hr at 37°C

Table 6. Methylase activity of dialyzed sonicates (part II).

Sample	Amt. DNA	cpm ^3H
1 µl ^3H-SAM 3 Std.	N.A.	343,450
dH_2O Control (fluor)	N.A.	4
K803 (uninfected)	1 µg lambda DNA	2,595
GM272 (uninfected)	1 µg lambda DNA	647
T_4D^+ (old) 5 µl	1 µg lambda DNA	58,744
T_4D^+ (old) 10 µl	1 µg lambda DNA	83,777
Dialysis buffer	1 µg lambda DNA	1,230
Gel slice control	N.A.	153
T_4D^+ (old) 5 µl	250 ng pBR322 DNA	10,862
T_4D^+ (old) 10 µl	250 ng pBR322 DNA	14,017
$T_2\underline{dam}^+$ 5 µl	250 ng pBR322 DNA	13,004
$T_4\underline{dam}^h$ 5 µl	250 ng pBR322 DNA	3,421
Dialysis buffer	250 ng pBR322 DNA	794
Gel slice control	250 ng pBR322 DNA	214

Methylase Reaction Parameters:
50 µl reaction volume; 5 or 10 µl sonicate as noted
825 nM SAM (1 µl SAM 3L)
148 nM GATC sites (1 µg lambda DNA)
Incubation time: 1 hr at 37°C

Methylase Reaction Parameters Using pBR322 DNA:
200 µl reaction volume; 100 µl sonicate added
1 µM SAM (5 µl SAM 3L)
970 nM GATC site "A"s (12.5 µg pBR322 DNA)
Incubation time: 1 hr at 37°C

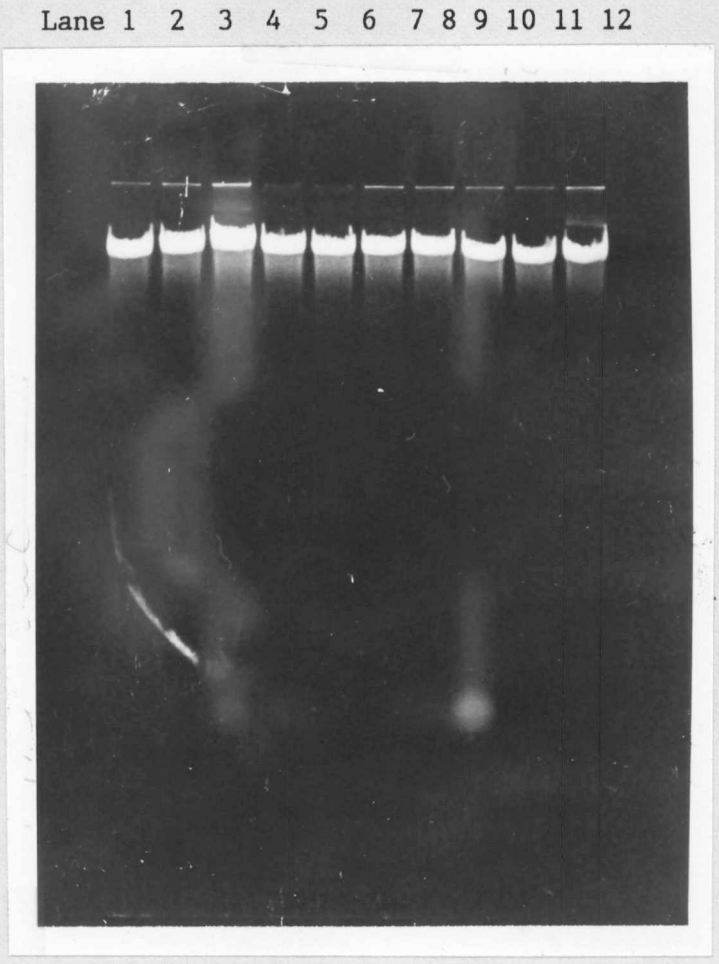


Figure 9. Agarose gel containing <u>in vitro</u> methylated lambda DNA.

Lane 1. No sample
2. K803 (uninfected)/lambda DNA
3. GM272 (uninfected)/lambda DNA
4. $T_4\underline{dam}^h$ (5 µl)/lambda DNA
5. $T_2\underline{dam}^+$ (5 µl)/lambda DNA
6. $T_2\underline{dam}^h$ (5 µl)/lambda DNA
7. $T_2\underline{dam}^+$ (5 µl)/lambda DNA
8. $T_2\underline{dam}^+$ (10 µl)/lambda DNA
9. $T_2\underline{dam}^+$ (20 µl)lambda DNA
10. $T_2\underline{dam}^+$ (20 µl) 2µl SAM 3L/lambda DNA
11. Dialysis Buffer/lambda DNA
12. No sample

shown in Table 7. They possess very high methylase activity and very low nuclease activity as seen in Figure 10.

When the T_2*dam*$^+$ and T_4D^+ (old) dialysates were incubated with *in vivo* methylated or unmethylated pBR322 plasmid DNA for 2 hr at 37°C or overnight at 30°C the results in Table 8 were observed. The SAM and DNA concentrations of the assays were at approximately half the putative K_m values of the T_2*dam*$^+$ methylase, so the reactions should have proceded fairly rapidly. By comparing values of cpm/μg of the DNAs, it is seen that the 2 hr methylation of pBR322 (GM272) DNA by the T_4D^+ dialysate contains approximately three fold more cpm than the pBR322 (GM272) DNA methylated for 2 hr by the T_2*dam*$^+$ dialysate. Both of the latter DNAs have significantly more counts than the same DNAs methylated overnight at 30°C. This difference can probably be attributed to the lower catalytic rate at 30°C together with increased degradation of the pBR322 DNA by nucleases or increased demethylation/depurination with time. The increased background in these sample lanes (not photographed) and the decreased intensity of the DNA bands relative to other samples supports the hypothesis that nucleases have degraded the DNA. The low level of ^{3}H-methyl group transfer from SAM to *in vivo* methylated pBR322 (HB101) DNA indirectly confirms the belief that the T_2*dam*$^+$ and T_4*dam*$^+$ enzymes methylate primarily GATC sites also methylated by *Eco dam*. The fact that the counts are slightly above background does suggest either hemimethylated GATC sites or other GAPyN sites are being methylated under assay conditions.

Table 7. Scintillation data of T_4D^+ and $T_2\underline{dam}^+$ methylated lambda DNA.

Sample	cpm	Gel Lane in Figure 8
2 µl T_4D^+ Dialysate/lambda DNA	62,159	1
10 µl	117,578	3
10 µl	121,878	5
20 µl	105,045	7
20 µl	110,150	9
2 µl $T_2\underline{dam}^+$ Dialysate/lambda DNA	131,833	11
10 µl	144,824	13
10 µl	150,443	15
20 µl	141,963	17
20 µl	143,925	19
10 µl GM119-INF Dialysate ($T_4\underline{dam}^+$)/lambda DNA	109,620	21
20 µl Dialysis Buffer/lambda DNA	617	23
Gel 1 Slice for EtBr Fluor. Ctrl.	208	
Gel 2 Slice for EtBr Fluor. Ctrl.	229	
1 µl ^{3}H-SAM Control (0.5 µCi)	416,600	
dH_2O Control	19	
0.049 µCi ^{3}H Std.	44,289	
Background Std.	16	

Reaction Parameters:

100 µl reaction volume
0.5 µg λ DNA (37 nM GATC sites)
1.0 µl SAM 2H (0.5 µCi) 78 Ci/mmole (65 nM)
Incubation time: 1 hr at 37°C

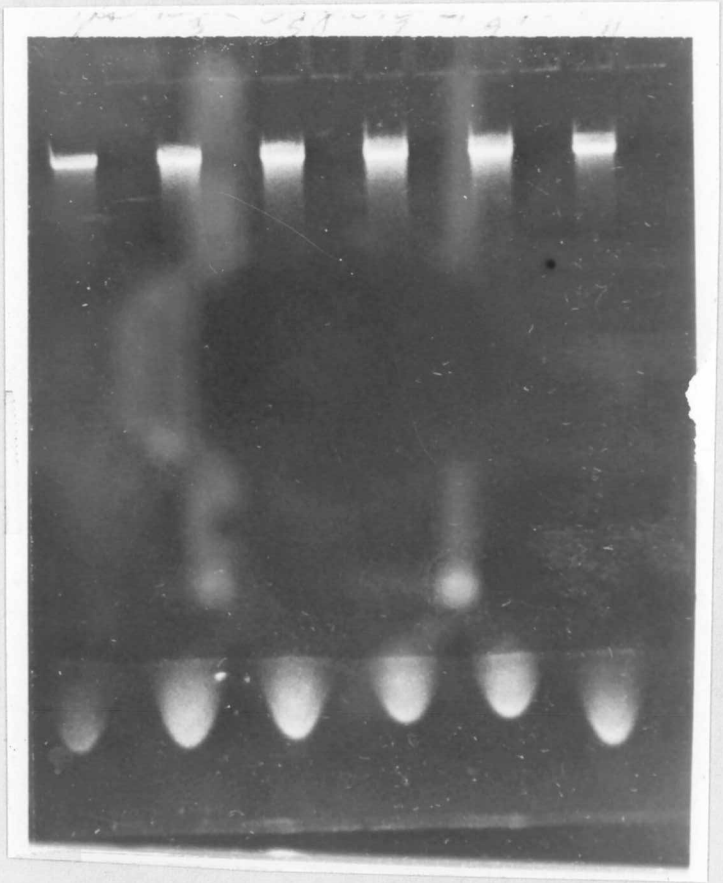
Figure 10. EtBr-stained agarose gels of T_4D^+ and $T_2\underline{dam}^+$ methylated lambda DNA.

Lane 1. 2 µl T_4D^+ dialysate/lambda DNA
3. 10 µl T_4D^+ dialysate/lambda DNA
5. 10 µl T_4D^+ dialysate/lambda DNA
7. 20 µl T_4D^+ dialysate/lambda DNA
9. 20 µl T_4D^+ dialysate/lambda DNA

Lane 11. 2 µl $T_2\underline{dam}^+$ dialysate/lambda DNA
13. 10 µl $T_2\underline{dam}^+$ dialysate/lambda DNA
15. 10 µl $T_2\underline{dam}^+$ dialysate/lambda DNA
17. 20 µl $T_2\underline{dam}^+$ dialysate/lambda DNA
19. 20 µl $T_2\underline{dam}^+$ dialysate/lambda DNA
21. 10 µl GM119 (pSSH-1b) INF/lambda DNA
23. 20 µl Dialysis Buffer/lambda DNA

Gel 1

Lanes 1 12



Gel 2

Lanes 13 24

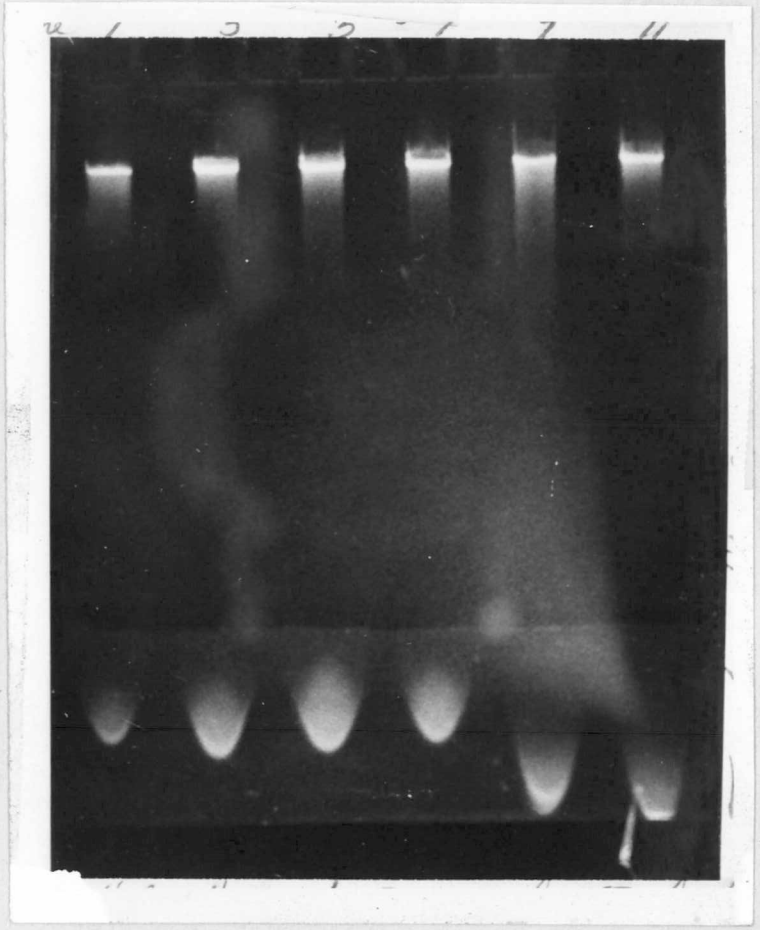


Figure 10.

Table 8. T_4D^+ and $T_2\underline{dam}^+$ methylation of methylated (HB101) and unmethylated (GM272) pBR322 DNA.

Sample	Incubation Time	cpm
1 µl ^{3}H-SAM 3L	N/A	326,915
Background Std.	N/A	7
T_4D^+/pBR322(-CH_3)	2 hr @ 37°C	13,211
T_4D^+/pBR322(+CH_3)	2 hr @ 37°C	214
T_4D^+/pBR322(-CH_3)	0/N @ 30°C	8,661
$T_2\underline{dam}^+$/pBR322(-CH_3)	2 hr @ 37°C	2,537
$T_2\underline{dam}^+$/pBR322(+CH_3)	2 hr @ 37°C	4,689
$T_2\underline{dam}^+$/pBR322(-CH_3)	0/N @ 30°C	1,716
Native pBR322(-CH_3)	0	22
Gel slice EtBr control	N/A	14

Methylate Reaction Parameters:
400 µl reaction volume
10 µl ^{3}H-SAM 3L = 1 µM
25 µg pBR322 (970 nM GATC site "A"s)
Incubation time: as noted for individual sample

D. DNA Sequence Specificity Determinations

The remainder of those in vitro methylated pBR322 DNAs discussed in the last section, which had ample specific activity were purified and processed for isolation of their methylation site-containing R.E. fragments. Additional DNAs were methylated in similar reactions using varied SAM concentrations and specific activities and/or altered pH.

Increases in SAM concentration achieved by dilution of the ^{3}H-SAM with unlabelled SAM lowered the specific activity of SAM in those reaction volumes and accordingly lowered the potential sensitivity of the assay. However, increases in methylase activity resulting from the increased SAM concentration offset the reduced SAM specific activity. The detection limits of the assay which are dependent on methylase activity, DNA (site) concentration, and the specific activity of SAM will be discussed in a subsequent section of the text.

The scintillation counts (cpm) of agarose gel-separated linear (l), open circular (oc), and covalently closed circular (ccc) forms of pBR322 DNA methylated by dialysate of GM272 cells infected by T_2dam$^+$, T_2dam^h, T_4dam$^+$, T_4D^+, T_4dam^h or uninfected GM272 or K803 cells as well as purified Eco dam enzyme are listed in Table 9. The methylated bands of the samples electrophoresed through an agarose gel are seen in Figure 11. The remaining portions of DNAs deemed to be sufficiently methylated were purified, digested with R. Hha I, and run on polyacrylamide gels for subsequent excision of resolved bands (Figure 12; panels A and B). The levels (specific activity) of ^{3}H-methyl incorporated into plasmid DNA incubated with different

Table 9. Scintillation data of agarose gel slices of methylated pBR322 DNA.

	Sample	cpm
1.	T_4D^+/pBR322 (GM272)	7,115
2.	T_2dam$^+$/pBR322 (GM272)	7,522
3.	Eco dam/pBR322 (GM272)	2,971
4.	T_4dam$^+$/pBR322 (GM272)	15,357
5.	T_4dam^h/pBR322(GM272)	11,624
6.	T_2dam$^+$/pBR322 (GM272)	22,273
7.	T_2dam^h/pBR322 (GM272)	34,177
8.	K803 (uninfected)/pBR322 (GM272)	4,784
9.	GM272 (uninfected)pBR322 (GM272)	79
10.	Gel slice fluor. ctrl.	30
11.	0.049 µCi ^{3}H-Std.	39,350
12.	1 µl ^{3}H-SAM 3L Std.	250,989
13.	Background Std.	21
14.	dH_2O Std.	12

Methylase Reaction Parameters (all except Eco dam)

400 µl reaction volume (200 µl dialysate)

25 µg pBR322 DNA (GM272) = 970 nM GATC site "A"s

8 µl ^{3}H-SAM 4H = 150 nM SAM (limiting)

Eco dam Methylation Reaction

100 µl reaction volume

25 µg pBR322 DNA (GM272) = 3.9 µM GATC sites

8 µl ^{3}H-SAM 4H = 600 nM SAM

Eco dam Km(GATC/DNA) = 3.6 nM

Km (SAM) = 12.2 µM

Incubation time for all reactions was 4 hr at 37°C

250 ng DNA was loaded per gel lane from methylase reactions

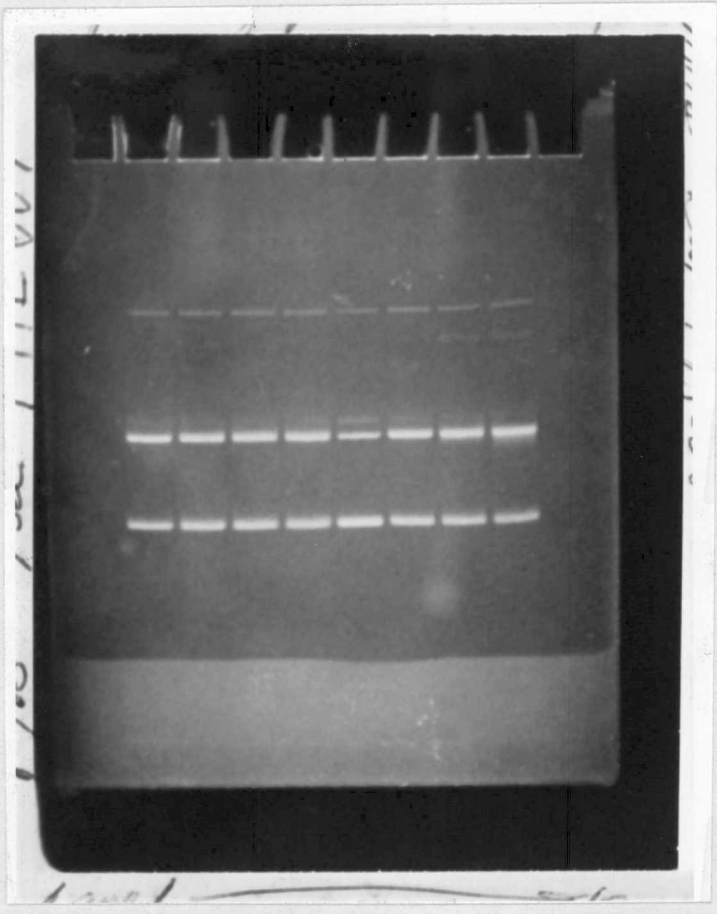


Figure 11. EtBr-stained agarose gel of *in vitro* methylated pBR322 DNA.

Lane 1. No sample
2. T_2*dam*$^+$/pBR322 (GM272)
3. T_2*dam*^{h}/pBR322 (GM272)
4. T_4*dam*$^+$/pBR322 (GM272)
5. T_4D^+/pBR322 (GM272)
6. T_4*dam*^{h}/pBR322 (GM272)
7. K803/pBR322 (GM272)
8. GM272/pBR322 (GM272)
9. Eco *dam* (Biolabs)
10. No sample

Figure 12. EtBr-stained Agarose Gel of R. Hha I digests of methylated pBR322 DNA.

Panel A

Lane 4. T_4D^+ pBR322 (GM272) (24 µg) X R. Hha I

6. K803 (UN) pBR322 (GM 272) (12 µg) X R. Hha I

8. T_2dam^+ pBR322 (GM 272) (24 µg) X R. Hha I

Panel B

Lane 2. Eco dam (SAM 3L) pBR322 (GM272) x R. Hha I (18 µg)

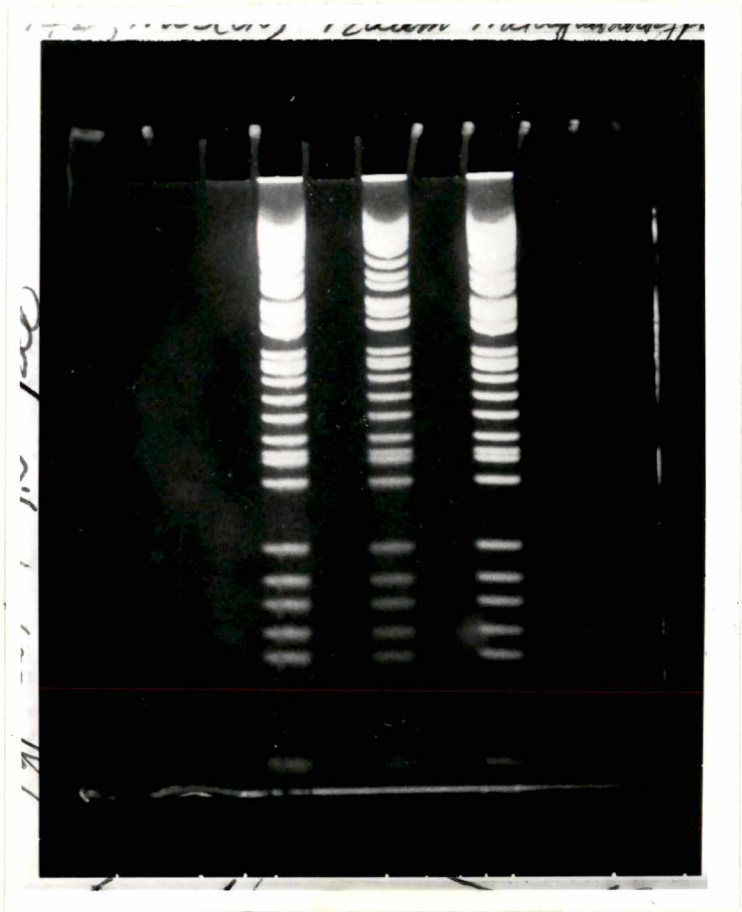
4. T_4dam^h (SAM 3L) pBR322 (GM272) x R. Hha I (12 µg)

6. T_4dam^h (SAM 4H) pBR322 (GM272) x R. Hha I (12 µg)

8. T_2DC-6 (dam^+) (SAM 4H) pBR322 (GM272) x R. Hha I (12 µg)

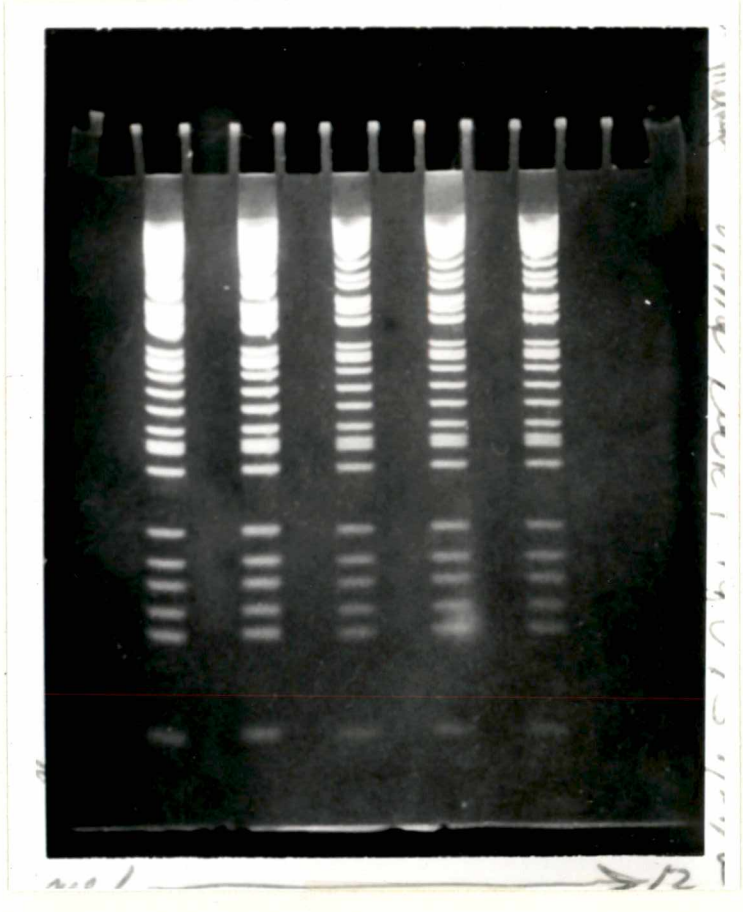
10. T_4DC-1 (dam^+) (SAM 4H) pBR322 (GM272) x R. Hha I (12 µg)

Panel A



Lane 1 2 3 4 5 6 7 8 9 10

Panel B



Lane 1 2 3 4 5 6 7 8 9 10 11 12

Figure 12.

dialysates or pure enzyme and different SAM concentrations, used in analyses discussed in this text, are shown in Table 10. The time, temperature and amount of digested DNA loaded on the polyacrylamide gels are listed in these tables for comparison.

To make comparison of the results of different assays easier, the data were normalized by subtracting the background cpm of R. Hha I fragment 21 (bp) of the same assay from each fragment's cpm then dividing by the number of counts in R. Hha I fragment 109 (bp) in the same assay. The data are expressed as a percentage of the counts in fragment 109 (bp). The data were normalized to fragment 109 because it contains one GATC site along with only one other possible methylation site (GATT). Additionally, the greatest levels of incorporation in the majority of assays were found in fragment 109 (bp). Evidence discussed later suggests the GATT site is not methylated on this fragment.

Due to our inability to resolve fragments 60 (bp) and 62 (bp) in some gels they were excised and processed together. Therefore the counts from fragments 60 and 62 were summed for assays in which they were separable on gels to facilitate comparison between assays. In assays in which the latter fragments were separable, fragment 60 exhibited considerably more counts than fragment 62 on account of its GATC site. The relative percentages of fragment-molecule sites methylated (% Sat) has also been determined for fragments in each methylation reaction. This was done by subtracting background counts ("21" bp counts) and dividing by a "saturation" number equaling the expected cpm for 100% methylation of all that specie's sites on all the

Table 10. Scintillation counts of methylated pBR322 and reaction parameters of methylase sequence specificty assays.

Methylase	Table Ref.	Date		SAM (Ci/mmole)			pBR322		Time of Incubation	µg/gel lane**
		Methylase	RxN	conc.	sp. act.	pH	sp. act. cpm/µg	DNA conc./ GATC "A" conc.		
T_2dam^+	11	5/21	6/27	150	67.2	7.5	75,000	22 nM/970 nM	4 hr	4-5 µg
	11	5/21	6/20	150	67.2	7.5	89,100	22 nM/970 nM	4 hr	20-24 µg
*	11	5/21	6/26	1000	14.1	7.5	92,424	22 nM/970 nM	4 hr	4-5 µg
	11	5/21	6/15	1000	14.1	7.5	47,800	22 nM/970 nM	2 hr	10-12 µg
T_2dam^+	13			150	67.2	7.5	20,600	22 nM/970 nM	4 hr	10-12 µg
	13			1000	14.1	7.5	8,240	22 nM/970 nM	4 hr	10-12 µg
	13			3000	4.7	7.5	11,040	22 nM/970 nM	4 hr	10-12 µg
	13			15,000	0.94	7.5	1,200	22 nM/970 nM	4 hr	10-12 µg
T_2dam^+	14			1000	14.1	6.5	6,560	22 nM/970 nM	4 hr	10-12 µg
	14			1000	14.1	7.5	8,240	22 nM/970 nM	4 hr	10-12 µg
	14			1000	14.1	8.5	3,560	22 nM/970 nM	4 hr	10-12 µg
T_2dam^+ SUP-1A	15			1000	14.1	7.5	18,756	22 nM/970 nM	2 hr	4-5 µg
SUP-1B	15			1000	14.1	7.5	18,756	22 nM/970 nM	2 hr	4-5 µg
LIN-2A	15			1000	14.1	7.5	N.D.‡	22 nM/970 nM	2 hr	4-5 µg
LIN-2B	15			1000	14.1	7.5	N.D.	22 nM/970 nM	2 hr	10-12 µg
T_2dam^+	16	5/21	6/20	150	67.2	7.5	89,100	22 nM/970 nM	4 hr	20-24 µg
	16	5/21	6/26	1000	14.1	7.5	92,424	22 nM/970 nM	4 hr	4-5 µg
T_4dam^+	16			150	67.2	7.5	71,000	22 nM/970 nM	4 hr	20-24 µg
	16			1000	14.1	7.5	52,400	22 nM/970 nM	2 hr	4-15 µg
T_4dam^h	17			150	67.2	7.5	46,500	22 nM/970 nM	4 hr	10-12 µg
	17			1000	14.1	7.5	N.D.	22 nM/970 nM	4 hr	10-12 µg
T_2dam^h	17			150	67.2	7.5	56,708	22 nM/970 nM	4 hr	10-12 µg
	17			1000	14.1	7.5	43,700	22 nM/970 nM	4 hr	10-12 µg
Eco dam	18			600	67.2	7.5	12,000	84 nM/3100 nM	4 hr	10-12 µg
	18			4000	14.1	7.5	N.D.	126 nM/4650 nM	16 hr	15-18 µg
K803 (uninfected)	19			160	67.2	7.5	19,200	22 nM/970 nM	4 hr	10-12 µg
GM272 (uninfected	19			1000	14.1	7.5	716	22 nM/970 nM	4 hr	10-12 µg

*40 bp analysis reaction.

**Approximate amount of DNA due to varying recoveries, etc.

‡N.D. means not determined.

molecules. The "saturation" number was derived by taking into account the amount of DNA loaded on the gel, the specific activity of the SAM, the number of methylatable "A"s per site, and the scintillation counting efficiency. See the Appendix to observe calculations used in the normalization of scintillation data.

1. DNA Sequence Specificity of T_2dam$^+$

a. Methylation of Specific Species of GAPyN Sites

Table 11 depicts the scintillation counts of R. Hha I restriction fragments of pBR322 methylated by T_2dam$^+$ dialysate. In the four separate methylation assays, the reaction contained either 150 or 1,000 nM (1 μM) SAM and was incubated for 2 or 4 hr at 37°C. Although the amounts of digested DNA from each assay that were loaded on the polyacrylamide gels differed, the T_2dam$^+$ pattern of methylation was the same among the assays done at the identical SAM concentrations.

In all four of the T_2dam$^+$ assays whose results are shown in Table 11, fragments 60/62, 83, and 109 contain significantly more counts than any of the other fragments. We attributed this to the presence of a methylatable GATC site (2 "A"s) in each of these fragments. As will be shown later, when the methylated 83 and 109 bp fragments are isolated and digested with R. Sau IIIA, the adenines of the complementary GATC sites on opposite strands of each fragment are separated. Separation of the resulting fragments by electrophoresis and subsequent counting of the bands revealed that the counts were almost equally split between the half-sites.

Table 11. The methylation pattern of T_2*dam*$^+$.

Hha I Frag.	GAPyN Sites	150 nM cpm	150 nM % Sat	150 nM Norm %	150 nM cpm	150 nM % Sat	150 nM Norm %	1 μM cpm*	1 μM % Sat	1 μM Norm %	1 μM cpm	1 μM % Sat	1 μM Norm %
21	None	19		0.	24		0.	21		0.	18		0.
28	GATG	20		0.017	43		0.052	105	1.1%	2.5	126	0.5%	2.8
30	GACA,GACG	18		-0.017	34		0.028	23		0.060	20		0.052
33	GACT	23		0.068	49		0.069	40		0.57	29		0.29
36	GATG	28		0.15	67	0.02%	0.12	87	0.8%	2.0	93	0.4%	2.0
40	GACC(2)	49	0.08%	0.51	170	0.06%	0.40	636	7.7%	18.	739	3.6%	19.
54	GACT	50	0.08%	0.53	128	0.05%	0.29	53	0.4%	0.95	48	0.2%	0.78
60/62	GATC,GATC(4),GACA GATA(2),GACG	2052	2.7%	35.	9864	2.1%	27.	4177	26%	124.	6413	16%	166.
67	GACC,GATA,GACA GACT(2),GATA(2)	62	0.1%	0.73	189	0.07%	0.46	118	1.2%	2.9	124	0.5%	2.8
75	GACA(2),GACG	77	0.2%	0.99	190	0.07%	0.46	78	0.7%	1.7	74	0.3%	1.5
83	GATC,GATC(3)	4529	6%	77.	27,204	5.7%	75.	4402	28%	131.	4707	12%	122.
93	GATG,GATA,GACT	100	0.1%	1.4	349	0.14%	0.90	168	1.9%	4.4	100	0.4%	2.1
109	GATC,GATT	5887	7.7%	100.	36,247	7.7%	100.	3624	23%	100.	3860	9.7%	100.
	SAM sp. act. and conc. Ci/mmole	67.2; [150 nM]			67.2; [150 nM]			14.1; [1 μM]			14.1; [1 μM]		
cpm expected	GATC site (2As)	76,000			475,000			15,880			39,700		
cpm expected	1/2 site	38,000			237,500			7940			19.850		
Amt. of DNA		4-5 μg			20-24 μg			4-5 μg			10-12 μg		

*Methylated used for analysis of methylation of GACC sites on 40 bp R. *Hha* I fragment.

In the two assays containing 150 nM SAM, the GATC site of the 109 bp fragment is methylated more than that of the 83 bp fragment which is in turn methylated more than the 60 bp fragment's GATC site. Calculations taking into account the specific activity of the SAM in the assays and scintillation counting efficiencies indicate that the GATC sites of the 60, 83, and 109 bp fragments are methylated to 2.4, 5.9, and 7.7% of their maximum, respectively (methylation of sites on both strands of all molecules). These values represent the percent of GATC sites which could be fully methylated. It is more likely that 4.8, 11.8, and 15.4% of their GATC sites, respectively, are hemimethylated, though our assay can not definitively determine this.

Of the other fragments in these two assays, only the 75 and 93 bp fragments are methylated to greater than or equal to 1% the extent of the 109 bp fragment though others exhibit a considerable number of cpm above background. Which of their (fragments 75 and 93) GAPyN sites are methylated can not be determined conclusively from these results. When the concentration of SAM is increased to 1 μM (but the specific activity decreases) in parallel $T_2\underline{dam}^+$ methylations of pBR322 DNA, the methylation pattern changes. Now the 109 bp fragment is observed to be methylated to a lesser degree than 60/62 and 83 bp fragments. Based on the number of GATC sites (molecules) methylatable in these fragments, the specific activity of the SAM, and scintillation counting efficiencies, the 60/62, 83, and 109 bp fragment GATC sites were calculated to be methylated to 26, 28, and 23% of their maximum (saturation) for one of the 1 μM SAM assays and 16, 12, and 9.7% for

the other 1 μM SAM assay. The difference in saturation levels is probably due to the difference in incubation time.

The observation that fragments 28 and 36, which contain only one GAPyN site (GATG), are methylated to a level 2-3% that of the fragment 109 suggests that the reason levels of methylation of the 83 and 60/62 bp fragments are greater than that of fragment 109 may be due to the methylation of their 3 or 4 GATG sites, respectively. The GATG-containing 93 bp fragment also exhibits an increased level (2-4 fold) of methylation. In ascertaining the extent of methylation of a GAPyN site with respect to a GATC site, it must be remembered that all GAPyN sites other than the palindromic GATC site contain the concensus recognition site (GAPyN) only on one strand and thus are methylatable to only one half the extent (50%) of a GATC site. Accordingly, their relative methylation values should be doubled.

Even more evident than the methylation of the GATG-containing fragments in the assays using 1 μM SAM is the methylation of the 40 bp fragment, which contains two GACC sites, to 18% the level of the 109 bp fragment. The 67 bp fragment, which contains a GACC site as well as a GATA and GACA site is also methylated in these reactions but only to 3% of the level of the 109 bp fragment.

b. Determination of the Extent of Methylation of the GACC Sites in the 40 bp R. Hha I Fragment by T_2dam^+

The discovery that the 40 bp fragment was methylated to such a high level prompted further inquiry into whether both GACC sites on

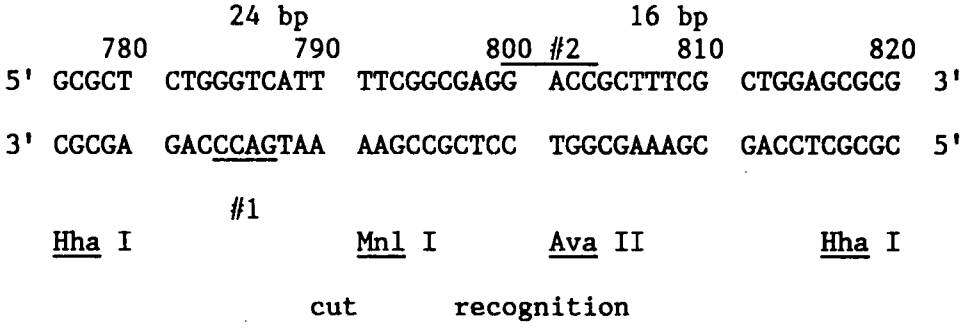
this fragment are methylated and if so was one preferred over the other. Examination of the DNA sequence of the 40 bp fragment revealed the presence of an R. Ava II and an R. Mnl I site (Figure 13 legend) whose cleavage would result in the separation of the adenines of the two GACC sites (located on opposite strands). Twenty-four micrograms of pBR322 (GM272) were methylated by T_2dam$^+$ in the presence of 1000 nM. SAM (sp. act. 14.1 Ci/mmole). Following determination of the specific activity, the DNA was purified, digested with R. Hha I, and loaded in parallel lanes of a polyacrylamide gel in 4 and 20 µg quantities. After electrophoretic separation (Figure 14), the bands from the 4 µg lanes were excised and processed destructively to determine the methylation pattern. The results are shown in Table 11. The 40, 83, and 109 bp DNA fragments were isolated from the 20 µg lane (lane 3) of the gel. Their DNA was extracted and subsequently subjected to restriction and scintillation analyses.

Assuming 100% recovery of each R. Hha I fragment, approximately 182 ng of the 40 bp, 365 ng of the 83 bp and 570 ng of the 109 bp fragments would have been recovered. The 40 bp DNA sample was split into four aliquots and three were digested with one of the three restriction enzymes: R. Ava II, R. Mnl I, or R. Sau IIIA. The fourth was kept as an undigested control. Two samples, one containing one-third of the 83 bp DNA sample, the other containing one-third of the 109 bp fragment DNA sample, 120 ng and 190 ng, respectively, were digested with R. Sau IIIA. Equivalent samples were kept as undigested control. Digested and undigested samples were electrophoresed through

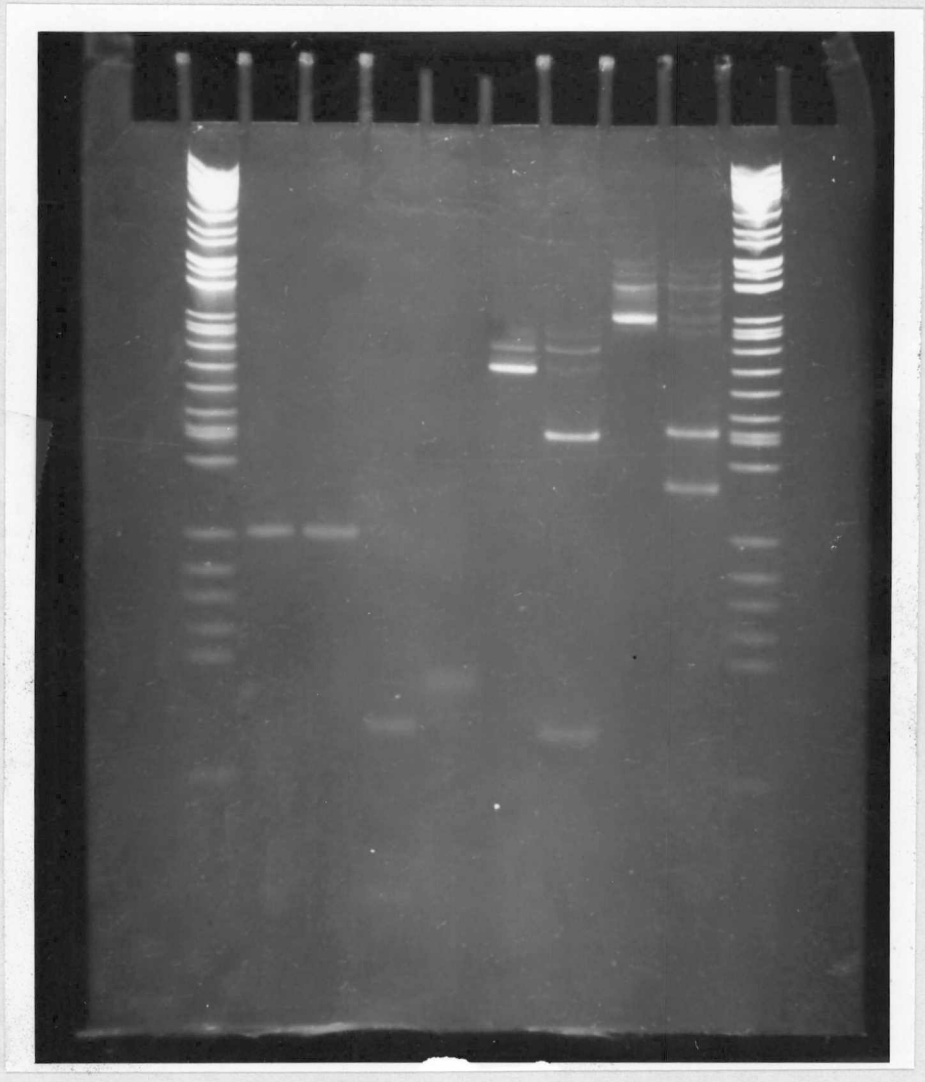
Figure 13. Restriction analysis of 40 bp Hha I fragment of pBR322 to determine the $T_2\underline{dam}^+$ methylation site (SAM 3L; 1 μM).

Gel Samples

Lane 2. 5 μg pBR322 X R. Hha I
3. R. Hha I 40 bp undigested
4. R. Hha I 40 bp X R. Sau 3A
5. R. Hha I 40 bp X R. Ava I
6. R. Hha I 40 bp X R. Mnl I
7. R. Hha I 83 bp undigested
8. R. Hha I 83 bp X R. Sau 3A
9. R. Hha I 109 bp undigested
10. R. Hha I 109 bp X R. Sau 3A
11. 5 μg pBR322 X R. Hha I

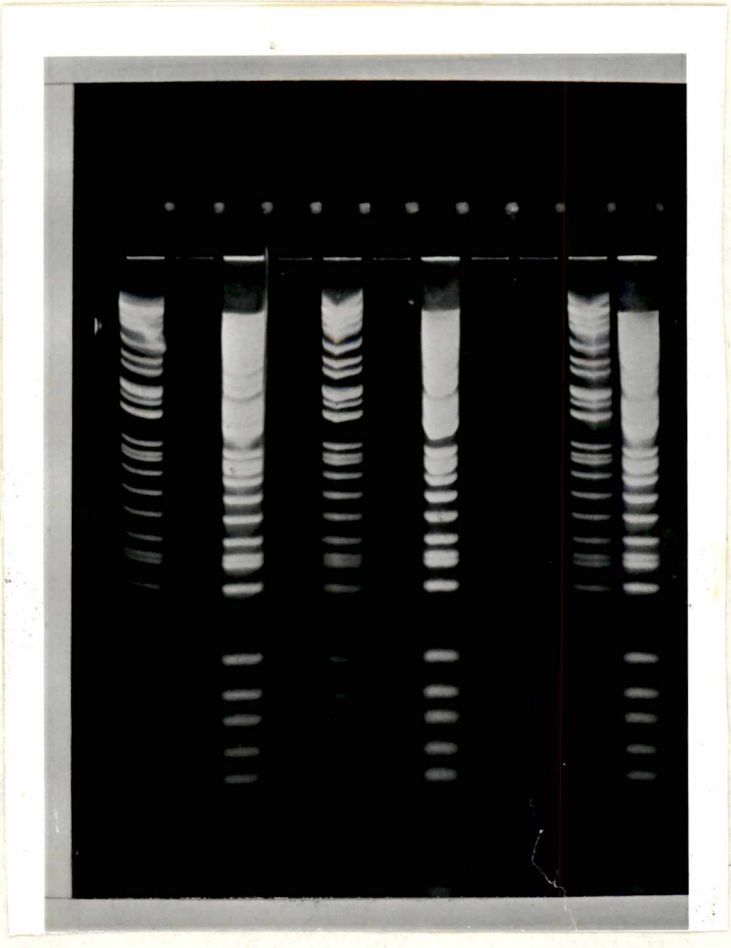


40 bp (38 bp + 4 b) Hha I fragment



Lanes 1 2 3 4 5 6 7 8 9 10 11 12

Figure 13.



Lanes 1 2 3 4 5 6 7 8 9 10 11 12

Figure 14. R. Hha I digest of T_2dam$^+$-methylated pBR322 DNA (SAM = 1000 nM or 150 nM).

Gel Samples

Lane 1. 4 µg T_2dam$^+$ SAM 3L (1 µM) X R. Hha I

3. 20 µg T_2dam$^+$ SAM 3L (1 µM) X R. Hha I

5. 4 µg T_2dam$^+$ SAM 4H + cold SAM (1 µM) X R. Hha I

7. 20 µg T_2dam$^+$ SAM 4H + cold SAM (1 µM) X R. Hha I

10. 4 µg T_2dam$^+$ SAM 4H (150 nM) X R. Hha I

11. 20 µg T_2dam$^+$ SAM 4H (150 nM) X R. Hha I

Mehtylase Reaction Parameters

400 µl volume reactions (200 µl dialysates)

10 µl ^{3}H-SAM 3L (5.9 µCi; 1 µM) or 12 µl ^{3}H-SAM 4H (6.0 µCi; 150 nM) T

4.5 µl 70.125 µM cold SAM

25 µg pBR322 (GM272) DNA = 970 nM GATC site "A"s

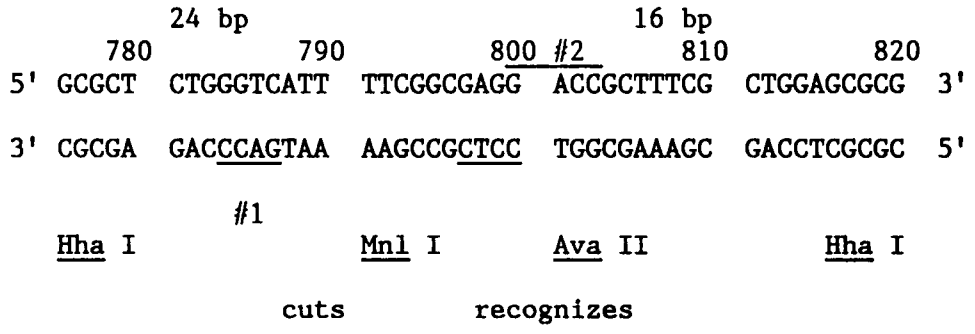
Incubation time = 4 hr at 37°C

a 15% polyacrylamide gel along with two R. Hha I-digested pBR322 (5 μg each) samples for size and percent recovery determinations (see gel Figure 13). Comparison of the 40 bp fragment bands in lanes 3 and 4, and the 83 and 109 bp fragment banks in lanes 7 and 9, respectively, with their counterparts in adjacent marker lanes, suggest that recovery of each fragment from the prior gel (Figure 14) was between 75 and 100%.

Results of scintillation analysis of gel bands from the digested and undigested samples are shown in Table 12. For the R. Ava II digestion, all of the detectable counts (467) are contained in the 24 bp fragment which contains the tGACCc (site 1) whereas the gGACCg (site 2) of the 16 bp fragment is not methylated to a detectable level (≤10% of the 24 bp level). The 11 bp and 27 bp fragments generated by R. Mnl I digestion exhibited 60 cpm and 21 cpm, respectively. These results agree with those of the R. Ava II digestion in that only the fragment (11 bp) containing the tGACCc site was detectably methylated but the fragments detectable counts were significantly lower than the 467 cpm of the 24 bp R. Ava II fragment. This might have been due to some of the diffuse DNA band (11 bp) running off the bottom of the gel or a large number of the 11 bp molecules may have denatured during digestion at 37°C or during electrophoresis resulting in anomalous migration.

Table 12. Restriction analysis of 40 bp Hha I fragment of pBR322 to determine the T_2dam$^+$ methylation site.

Methyl. Site	Size of R.E. Fragment	cpm	Size of R.E. Fragment	cpm
both	40 bp (undigested)	379	83 bp (undigested)	4087
both	40 bp (uncleaved Sau IIIa)	474	83 bp (uncleaved Sau IIIa)	207
			23 bp (Sau IIIa) gGATCt	931
tGACCc	24 bp (Ava II)	467	60 bp (Sau IIIa) aGATCc	1570
gGACCg	16 bp (Ava II)	21	and (3) GATG sites	
			109 bp (undigested)	2564
tGACCc	11 bp (Mn1I)	60	109 bp (uncleaved Sau IIIa)	215
gGACCg	27 bp (Mn1I)	21	48 bp (Sau IIIa) gGATCa	1319
			61 bp (Sau IIIa) tGATCc aGATTa	1031



c. Methylation of 40 bp Fragment Is Not Due to Mutation of GACC to GATC

One-fourth of the 40 bp DNA sample was digested with R. Sau IIIA to eliminate the possibility that the methylation of the 40 bp R. Hha I fragment was due to the mutation (i.e., deamination of cytosine) of the GACC site to a GATC site and subsequent propagation (expansion) in the population of pBR322 molecules, while still in the E. coli cells. The results on the gel (Figure 13) and in the table (cpm) (Table 12) support the belief that none of the 40 bp DNA digested with R. Sau IIIA was lost from the 40 bp band due to cleavage by the restriction enzyme. Neither 24 and 16 nor 32 and 8 bp fragments were visible in lane 4 (Figure 13) which would have resulted from R. Sau IIIA cleavage at sites 2 or 1, respectively.

The observation that the undigested 40 bp sample only gave 379 cpm (Table 12) can be explained by underloading of the gel. The coincidence between the values of the cpm exhibited by the R. Sau IIIA-digested 40 bp fragment (474 cpm) with that of the cpm emitted by the 24 bp R. Ava II fragment strongly suggests this explanation is correct and that in fact ≥90% of all the methylation detected on the 40 bp fragment is at the tGACCc site. R. Sau IIIA digestions of the 83 bp and 109 bp R. Hha I fragments were done as positive controls for enzyme activity and to observe any asymmetry of the methylation at the palindromic GATC sites of these fragments. These results will be discussed later.

d. The Effect of SAM Concentration on T_2dam$^+$ Sequence Specificity

To ascertain whether any increases in the SAM concentration above 1 μM caused other GAPyN sites to be methylated (or their methylation to be detected) additional quantities of unlabelled SAM were added to reactions containing labelled SAM to raise their SAM concentrations to 3 μM and 15 μM. While increasing the "cold" SAM concentration lowers the specific activity of the SAM in a reaction and hence the detection limits of the assay, increases in methylase activity upon certain species of GAPyN sites might possibly offset these decreases.

The results of these experiments are presented in Table 13. For each of the GATC site-containing fragments (60/62, 83, and 109 bp) the extent of methylation (% Sat) of these sites increases with increasing SAM concentration and plateaus (reaches saturation) between 1 μM and 3 μM SAM (see gel in Figure 15). The GACC site-containing fragments (40, 67 bp) as well as the GATG and GACT site-containing fragments also show significant increases in their extent of methylation as the SAM concentration is increased. Even at the highest SAM concentrations, the T_2dam$^+$ enzyme exhibits a preference of GATC>GACC>GACT≥GATG but the margins of preference (specificity) are narrowed. This order of preference appears to be influenced somewhat by species of nucleotides flanking the GAPyN sites which cause intraspecies differences in methylatability (i.e., the GACCs of the 40 bp fragment versus the GACC of the 67 bp fragment). The effects of flanking bases on site preference will be discussed later.

Table 13. The effect of (SAM) on T_2*dam*$^+$ sequence specificity.

		[SAM] =	150 nM			1 μM			3 μM			15 μM		
Hha I Frag.	GAPyN Sites		cpm	% Sat	Norm %	cpm	% Sat	Norm %	cpm	% Sat	Norm %	cpm	% Sat	Norm %
21	None		27		0.	33		0.	43		0.	78		0.
28	GATG		69	0.05%	0.098	157	0.6%	0.92	251	3.2%	1.4	450	28%	30.
36	GATG		87	0.07%	0.14	131	0.5%	0.72	175	2.0%	0.91	224	11%	12.
40	GACC(2)		268	0.25%	0.56	897	4.4%	6.4	1207	17.6%	8.0	571	38%	39.
54	GACT		383	0.37%	0.83	268	1.2%	1.7	316	4.1%	1.9	57	--	-1.7
60/62	GATC,GATC(4)		12,576	6.6%	29.	14,316	36%	110.	11,273	85%	77.	1426	51%	107.
67	GACC		368	0.36%	0.79	365	7.7%	2.5	383	5.2%	2.3	255	13.4%	14.
83	GATC,GATC(3)		27,483	15%	54.	10,771	27%	79.	14,048	106%	96.	1331	47%	100.
109	GATC		42,997	23%	100.	13,553	34%	100.	14,584	100%	100.	1333	47%	100.
SAM sp. act. and conc. (Ci/mmole)			67.2; [150 nM]			14.1; [1 μM]			4.7; [3 μM]			0.94; [15 μM]		
cpm expected	GATC site (2As)		190,000			39,700			13,200			2640		
cpm expected	1/2 site		95,000			19,850			6,600			1320		
Amt. of DNA			10-12 μg			10-12 μg			10-12 μg			10-12 μg		

Lanes 1 2 3 4 5 6 7 8 9 10 11 12

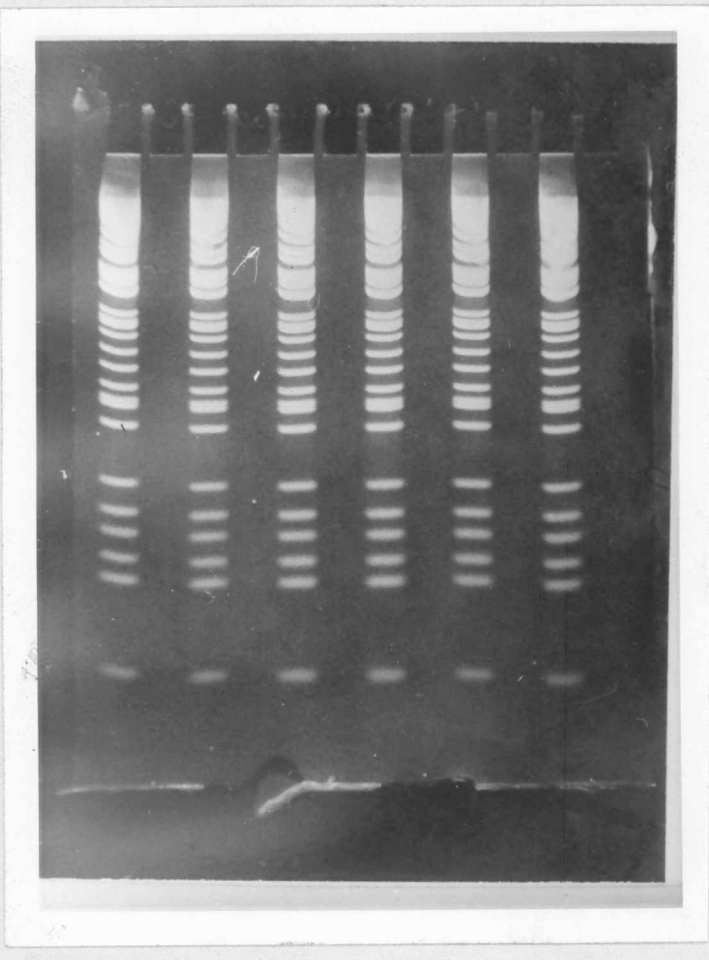


Figure 15. Fifteen percent PAGE of R. Hha I digest of pBR322 DNA methylated by T_2dam$^+$ in reactions with different [SAM] or pH.

Lane 1. T_2dam$^+$ pH 6.5 [SAM] = 1 μM (12 μg)
3. T_2dam$^+$ pH 7.5 [SAM] = 1 μM (12 μg)
5. T_2dam$^+$ pH 8.5 [SAM] = 1 μM (12 μg)
7. T_2dam$^+$ pH 7.5 [SAM] = 150 μM (12 μg)
9. T_2dam$^+$ pH 7.5 [SAM] = 3 μM (12 μg)
11. T_2dam$^+$ pH 7.5 [SAM] = 15 μM (12 μg)

e. The Effect of pH on T_2dam$^+$ Sequence Specificity

When pBR322 DNA was methylated by T_2dam$^+$ *in vitro* under different conditions of pH (6.5, 7.5, and 8.5), changes in the level of methylation of different GAPyN species occurred. The most noticeable change (Table 14) was the decrease in methylation of the GATC sites (and probably GATG sites) in fragments 60 and 83 at pH = 8.5. However methylation of the GATC site in the 109 bp fragment was similar level at all pH values tested. T_2dam$^+$ exhibited very low specificity for the GATG sites of fragments 28 and 36 as well as the GACC(s) on the 40 bp fragment at pH = 8.5. Methylation of the 67 bp fragment's GACC site was only decreased by one-half.

By comparison, pBR322 DNA methylated at pH = 6.5 retained almost the same methylation pattern as DNA methylated at pH = 7.5. The major difference appears to be that methylation of the GATG sites is significantly reduced and may be responsible for lowering the counts incorporated into the 60 and 83 bp fragments which contain GATC and GATG sites.

f. The Effect of Template Topology (Form) on T_2dam$^+$ Sequence Specificity

Experiments were performed to determine what effect, if any, DNA supercoiling had on the ability of T_2dam$^+$ to methylate any of the GAPyN sites. In two parallel assays, 20 micrograms of pBR322 DNA were methylated for 2 hr by T_2dam$^+$ crude extract in the presence of radiolabelled SAM (1 μM). In one assay, the DNA was cut with R. Hha I before methylation and in the other assay after methylation. In this

Table 14. The effect of pH on T_2*dam*$^+$ sequence specificity.

		pH	6.5			7.5			8.5	
Hha I Frag.	GAPyN Sites	cpm	% Sat	Norm %	cpm	% Sat	Norm %	cpm	% Sat	Norm %
21	None	81		0.	33		0.	22		0.
28	GATG	100	0.1%	0.13	157	0.6%	0.92	37	0.06%	0.12
36	GATG	111	0.15%	0.20	131	0.5%	0.72	37	0.06%	0.12
40	GACC(2)	670	3.0%	4.0	897	4.4%	6.4	87	0.32%	0.54
54	GACT	385	1.5%	2.1	268	1.2%	1.7	137	0.58%	0.95
60/62	GATC,GATC(4)	14,316	36%	96.	13,087	33%	96%	4332	11%	36.
67	GACC	400	1.6%	2.2	365	1.6%	2.5	163	0.7%	1.2
83	GATC,GATC(3)	13,763	34%	92.	10,771	27%	79.	5954	15%	49.
109	GATC	14,878	37%	100.	13,553	34%	100.	12,104	30%	100.
	SAM sp. act. and conc. (Ci/mmole)		14.1; [1 μM]			14.1; [1 μM]			14.1; [1 μM]	
cpm expected	GATC site (2As)	39,700			39,700			39,700		
cpm expected	1/2 site	19,850			19,850			19,850		
Amt. of DNA		10-12 μg			10-12 μg			10-12 μg		

way, the ability of the T_2dam$^+$ enzyme to methylate the linear R. Hha I fragments was compared with its ability to methylate sites on the negatively supercoiled plasmid DNA. Supercoiled covalently-closed-circular (CCC) plasmid DNA was the predominant species present in our native pBR322 plasmid sample as seen in lane 10 of Figure 3, p. 37.

Scintillation analysis of the gel bands from two lanes loaded with 10 μg of DNA of each assay yielded the data in Table 15. The normalized data from the duplicates of each assay were averaged. Upon comparison of the mean supercoiled values (SUP-1*) with the mean linear values (Lin-2*), it is apparent that the methylation patterns are very similar. No extreme differences in methylation are evident, disregarding those of the 60/62 bands of both assays which were totally unreproducible for some unknown reason. Differences of approximately two-fold observed between values for fragments 67, 75, and 93 bp of the two assays are significant because the Lin-2* values are higher than Sup-1* values in every instance. The latter trend is also seen between the values of the two assays for fragments 28, 30, 33 and 36.

2. The DNA Sequence Specificity of the T_4dam$^+$ Methylase

Comparison of the scintillation counts of the pBR322 DNA methylated by T_4dam$^+$ digested with R. Hha I with those of T_2dam$^+$ assays reveals striking similarities and differences (see Table 16). T_4dam$^+$ like T_2dam$^+$ shows a strong preference for the GATC sites of the 60, 83, and 109 bp fragments. Both methylases seem to have a preference for the site(s) on the 83 and 109 bp fragments over the one(s) on the 60/62 bp fragments though each fragment contains only one GATC site in

Table 15. The effect of template topology (form) on T_2*dam*$^+$ sequence specificity [SAM] = 1000 nM (1 μM).

Hha I Frag.	GAPyN Sites	SUP-1A			SUP-1B			LIN-2A			LIN-2B			SUP-1	LIN-2
		cpm	% Sat	Norm %	cpm	% Sat	Norm %	cpm	% Sat	Norm %	cpm	% Sat	Norm %	Norm	Norm
21	None	15	--	0	21	--	0	32	--	0	35	--	0	0	0
28	GATG	93	1.0%	2.0	176	2%	3.2	221	2.4%	3.6	302	1.3%	2.2	2.6	2.9
30	GACA,GACG	13	--	0	31	0.1%	0.2	50	0.2%	0.34	145	0.54%	0.92	0.1	0.6
33	GACT	4	--	0	41	0.2%	0.4	57	0.3%	0.48	102	0.34%	0.56	0.2	0.52
36	GATG	60	0.5%	1.0	168	1.8%	3.0	206	2.2%	3.3	321	1.4%	2.4	2.0	2.9
40	GACC(2)	706	8.5%	18.0	1041	12.8%	21	1269	15.6%	24	2001	10%	16	19.5	20
54	GACT	15	--	0	74	0.7%	1.0	153	15.2%	2.3	202	0.8%	1.4	0.5	1.9
60/62	GATC,GATG(4),GACA GATA(2),GACG	6261	40%	160	3479	22%	71	6352	40%	120	9864	25%	82	116	101
67	GACC,GATA,GACA GACT(2),GATA(2)	91	1.0%	2.0	168	1.8%	2.4	251	2.8%	4.2	506	2.4%	3.9	2.2	4.1
75	GACA(2),GACG	41	0.3%	1.0	100	1.0%	1.6	266	3%	4.5	421	2%	3.2	1.3	3.9
83	GATC,GATG(3)	4674	30%	122	5486	34%	112	5803	36%	110	12,677	32%	106	117	108
92	GATG,GATA,GACT	67	0.3%	1.0	174	2%	3.1	347	4%	6.0	600	2.8%	4.7	2.1	5.4
109	GATC,GATT	3827	25%	100	4913	31%	100	5269	33%	100	11,984	30%	100	100	100
SAM sp. act. and conc.		14.1; [1 μM]			14.1; [1 μM]			14.1; [1 μM]			14.1; [1 μM]				
cpm Expected GATC site (2As)		15,880			15,880			15,880			39,700				
cpm Expected 1/2 site		7,940			7,940			7,940			19,850				
Amt. of DNA		4-5 μg			4-5 μg			4-5 μg			10-12 μg				

Table 16. Comparison of T_4*dam*$^+$ and T_2*dam*$^+$ sequence specificities.

Hha I Frag.	GAPyN Sites	T_4*dam*$^+$ cpm	T_4*dam*$^+$ % Sat	T_4*dam*$^+$ Norm %	T_4*dam*$^+$ cpm	T_4*dam*$^+$ % Sat	T_4*dam*$^+$ Norm %	T_2*dam*$^+$ cpm	T_2*dam*$^+$ % Sat	T_2*dam*$^+$ Norm %	T_2*dam*$^+$ cpm	T_2*dam*$^+$ % Sat	T_2*dam*$^+$ Norm %
21	None	19		0.	29		0.	24		0.	21		0.
28	GATG	50	0.02%	0.11	48	0.22%	0.47	43		0.052	105	1.1%	2.5
30	GACA,GACG	46	0.01%	0.010	24	--	-0.12	34		0.028	23		0.060
33	GACT	44	0.01%	0.009	24	--	-0.12	49		0.070	40		0.57
36	GATG	84	0.04%	0.24	40	0.14%	0.27	67	0.02%	0.12	87	0.8%	2.0
40	GACC(2)	218	0.1%	0.73	166	1.7%	3.4	170	0.06%	0.40	636	7.7%	18.
54	GACT	172	0.08%	0.56	172	1.8%	3.6	128	0.05%	0.29	53	0.4%	0.95
60/62	GATC,GATC(4),GACA GATA(2),GACG	7819	4.1%	29.	4019	25%	99.	9864	2.1%	27.	4177	26%	124.
67	GACC,GATA,GACA	180	0.08%	0.59	53	0.3%	0.60	189	0.07%	0.46	118	1.2%	2.9
75	GACT(2),GATA(2) GACA(2),GACG	237	0.11%	0.80	46	0.21%	0.42	190	0.07%	0.46	78	0.7%	1.7
83	GATC,GATC(3)	27,324	7.2%	100.	3293	21%	81.	27,204	5.7%	75.	4402	28%	130.
93	GATG,GATA,GACT	409	0.2%	1.4	94	0.8%	1.6	349	0.14%	0.90	168	1.9%	4.4
109	GATC,GATT	27,122	7.1%	100.	4048	25%	100.	36,247	7.7%	100.	3624	23%	100.
	SAM sp. act. and conc. Ci/mmole	67.2; [150 nM]			14.1; [1 μM]			67.2; [150 nM]			14.1; [1 μM]		
cpm expected	GATC site (2As)	380,000			15,880			475,000			15,880		
cpm expected	1/2 site	190,000			7,940			237,500			7,940		
Amt. of DNA		20 μg			4-5 μg			24 μg			4-5 μg		

addition to other GAPyN sites. $T_4\underline{dam}^+$ methylates the GACC site of the 67 bp R. Hha I fragment to the same relative extent at 150 and 1000 nM SAM as $T_2\underline{dam}^+$ does at 150 nM SAM. Methylation of this fragment's GACC site by $T_2\underline{dam}^+$ at 1 μM SAM is six fold greater than that at 150 nM. Similarly, $T_2\underline{dam}^+$ methylates the tGACCc site of the 40 bp fragment at a much higher frequency at 1 μM SAM than at 150 nM SAM. By contrast, $T_4\underline{dam}^+$ only methylates one or both of the 40 bp fragment's GACC sites (undetermined) at a slightly higher frequency in reactions of 1 μM SAM than those of 150 nM SAM. $T_2\underline{dam}^+$ and $T_4\underline{dam}^+$, whose K_mSAMs are reportedly of similar magnitude (2.4 μM; Hattman, pers. comm.) methylate the 40 bp fragment's site(s) to the same extent at 150 nM SAM, but, $T_4\underline{dam}^+$ does not exhibit as great an increase in methylation of GACC sites as $T_2\underline{dam}^+$ does when the SAM concentration is increased.

$T_4\underline{dam}^+$ also seems to have weak specificity for the GATG sites of the 28, 36, 60/62, 83, and 93 bp fragments. Methylation of the GACT sites of the 54, 75, and 93 bp fragment seems to be evident in $T_4\underline{dam}^+$ assays containing 150 nM or 1 μM SAM concentrations. However the GACT in the 33 bp fragment is not methylated to any detectable level. Again flanking nucleotides appear to have a significant effect on the methylatability of each GAPyN species. Because the 93 bp fragment has both a GATG and a GACT site, one can not conclude that one or the other and not both are methylated since both species of sites are methylated to some extent on other fragments.

3. The DNA Sequence Specificity of T_4*dam*^{h} and T_2*dam*^{h} Methylases

Analysis of the R. Hha I fragments from pBR322 molecules modified by the hypermethylating mutant forms of the *dam* enzymes of T_4 and T_2 revealed (Table 17) that both T_4 and T_2*dam*^{h} methylate GACC sites at high frequency in addition to the canonical GATC sites, even at low SAM concentrations (150 nM). T_2 and T_4*dam*^{h} are able to methylate the GACC sites) of the 40 bp fragment at 150 nM SAM to levels equivalent to the T_2*dam*$^+$ enzyme at 1 μM SAM. Frequency of methylation of the 40 bp fragment site(s) by T_2 and T_4*dam*^{h} was even higher at 1 μM SAM than at 150 nM SAM. The GACC site(s) of the 40 bp fragment was modified at a significantly higher rate than the GACC site within the 67 bp fragment similar to the preference observed for the *dam*$^+$ enzymes.

A striking difference observed in the T_4*dam*^{h} methylation pattern is that the GATG-containing fragments are modified to significantly higher extents (relative to the 109 bp fragment) than those by the T_4*dam*$^+$ methylase. GACT site-containing fragments are also methylated but at a level equivalent to or less than those modified by T_4*dam*$^+$. T_2*dam*^{h} doesn't appear to methylate GACT or GATG sites at higher levels (relative to GATC sites) than the T_2*dam*$^+$ enzyme at 1 μM SAM. It does methylate them more frequently than the wildtype enzyme at comparatively low SAM levels (150 nM). Interestingly, Brooks (7) reported that T_2*dam*^{h} has the same K_mSAM as T_2*dam*$^+$ yet the increased methylatability of the GACC, GATG, and GACT sites at low SAM concentrations suggests that the T_2*dam*^{h} enzyme's K_mSAM might differ. If Brooks' measurements are indeed accurate, then the results presented here would

Table 17. Comparison of T_4dam^h and T_2dam^h sequence specificities.

Hha I Frag.	GAPyN Sites	T_4dam^h cpm	T_4dam^h % Sat	T_4dam^h Norm %	T_4dam^h cpm	T_4dam^h % Sat	T_4dam^h Norm %	T_2dam^h cpm	T_2dam^h % Sat	T_2dam^h Norm %	T_2dam^h cpm	T_2dam^h % Sat	T_2dam^h Norm %
21	None	23		0.	19		0.	21		0.	18		0.
28	GATG	57	0.035%	0.45	114	0.49%	1.5	88	0.07%	0.73	75	0.28%	1.0
30	GACA,GACG	31		0.11	31		0.19	33		0.13	30		0.22
33	GACT	94	0.05%	0.93	41	0.06%	0.36	47	0.03%	0.28	67	0.24%	0.90
36	GATG	170	0.16%	1.9	347	1.6%	5.3	215	0.21%	2.1	144	0.63%	2.3
40	GACC(2)	1531	1.6%	20.	2687	13.3%	43.	2138	2.2%	23.	1873	9.2%	34.
54	GACT	77	0.05%	0.71	84	0.33%	1.0	98	0.09%	0.84	97	0.4%	1.4
60/62	GATC,GATC(4),GACA GATA(2),GACG	4625	2.5%	60.	6335	16%	100.	5469	2.8%	60.	5828	15%	110.
67	GACC,GATA,GACA	302	0.3%	3.7	506	2.45%	7.9	212	0.19%	2.1	314	1.5%	5.4
75	GACT(2),GATA(2) GACA(2),GACG	92	0.05%	0.91	173	0.77%	2.5	131	0.12%	1.2	132	0.6%	2.1
83	GATC,GATC(3)	7142	3.7%	94.	8001	20%	130.	5287	2.8%	58.	7217	18%	130.
93	GATG,GATA,GACT	233	0.23%	2.8	291	1.4%	4.4	264	0.26%	2.7	255	1.2%	4.3
109	GATC,GATT	7632	4.0%	100.	6212	16%	100.	9152	4.7%	100.	5490	14%	100.
	SAM sp. act. and conc. Ci/mmole	67.2; [150 nM]			14.1; [1 M]			67.2; [150 nM]			14.1; [1 μM]		
cpm expected	GATC site (2As)	190,000			39,700			190,000			39,700		
cpm expected	1/2 site	95,000			19,850			95,000			19,850		
Amt. of DNA		10-12 μg			10-12 μg			10-12 μg			10-12 μg		

indicate that the DNA sequence specificity (preference) of the dam^h enzymes has been altered. Attempts by the author to measure the K_m SAM of the dam enzymes in crude extracts (data not shown) were not definitive due to other SAM binding/utilizing proteins.

4. The Methylation Specificity of the Host (E. coli) Eco dam Enzyme

Inspection of the methylation pattern (scintillation counts) of the R. Hha I fragments of pBR322 (GM272) DNA methylated by the Eco dam enzyme reveals (Table 18) significant similarities and differences with respect to those of the T_4 and T_2dam enzymes. Like the T_4 and T_2dam$^+$ enzymes, Eco dam predominantly methylates the GATC-containing fragments (60, 83, and 109 bp) as expected from results of other researchers' published experiments (3,8,29). However, it apparently does not methylate the GATG, GACT, nor GACC sites of the 28 to 40 bp fragments to any extent detectable by our assay. The GACC or GACA sites of the 67 bp fragment may be methylated though as the results in Table 18 indicate. The scintillation counts of methyl groups incorporated into the 54 bp fragment indicates that perhaps the GACT site within its sequence is also methylated. The 75 and 93 bp fragments exhibit counts significantly above background which could represent methylation of their GACT sites and/or other sites. Determination of the actual site(s) methylated on the 67 bp or other fragments has not been done.

5. Methylation Patterns of the Uninfected Host (E. coli) Cell Extracts

Cell extracts of uninfected E. coli host strains K803 (dam$^+$ dcm$^+$) and GM272 (dam$^-$ dcm$^-$) were made in the same manner as those of infected

Table 18. The methylation pattern of Eco dam.

Hha I Frag.	GAPyN Sites	SAM (nM) =	cpm 600 nM	cpm 4000 nM	% Saturation 600 nM	% Saturation 4000 nM	Norm % 600 nM	Norm % 4000 nM
bp 21	None		28	65			0.	0.
bp 28	GATG		37	45			0.021	-0.11
bp 30	GACA,GACG		38	39			0.023	-0.14
bp 33	GACT		35	45			0.016	-0.11
bp 36	GATG		41	72		0.02%	0.030	0.039
bp 40	GACC(2)		59	70	0.03%	0.02%	0.071	0.028
bp 54	GACT		118	138	0.1%	1.4%	0.21	0.40
bp 60/62	GATC,GATG(4),GACA GATA(2),GACG		13,516	17,973	8%	29%	31.	99.
bp 67	GACC,GATA,GACA		181	183	0.16%	0.4%	0.35	0.65
bp 75	GACT(2),GATA(2), GACA(2),GACG		258	224	0.02%	0.5%	0.53	0.88
bp 83	GATC,GATG(3)		27,472	19,206	14%	31%	63.	110.
bp 93	GATG,GATA,GACT		497	416	0.4%	1.1%	1.1	1.9
bp 109	GATC,GATT?		43,826	18,226	22%	29%	100.	100.
SAM sp. act. and conc. Ci/mmole			67.2;	[600 nM]14.1;	[4 µM]			
cpm Expected	GACT site (2As)		190,000	59,500				
cpm Expected	1/2 site		95,000	29,750				
Amt. of DNA			10-12 µg	15-18 µg				
Amt. of Eco dam			40 units	60 units				

cells and used to assay for the presence of host methylase activity. All of the R. Hha I fragments of pBR322 DNA methylated by the GM272 extracts exhibited only scintillation counts equivalent to background levels (Table 19). These results confirmed the lack of leakiness of the null dam$^-$ and dcm$^-$ alleles of this strain. However, the K803 strain's methylation pattern was somewhat of a surprise. None of the GATC-containing fragments (60, 83, and 109 bp) exhibited counts above background indicating that no dam activity was present in the extract. The 36 and 131/132 bp fragments, which contain the dcm methylase target sequence CCA/TGG, were methylated to 1-2% of saturation indicating the presence of the dcm methylase in the K803 cell extract. Dcm methylates the inner cytosine of the target site on both strands of the DNA site. Experiments to confirm the actual site of the methylation by assaying for resistance to R. Eco RII digestion were not done due to the low percentage of sites methylated. In summary, these results indicate that E. coli strain K803 has the genotype dam$^-$ dcm$^+$.

E. Determination of the Degree of DNA Methylation by R. Mbo I Digestion

It is desirable to know what degree of site methylation (saturation) has been attained in an assay in order to make statements about an enzyme's overall site-specificity versus its preference for one species of sites over another. Generally, saturation of methylatable sites is desired for the determination of overall sequence specificity (GAPyN species capable of being methylated) while partial methylation of sites is desired for the determination of an enzyme's site preference (i.e., GAPyN #1 > GAPyN #2).

Table 19. The methylation patterns of cell extracts of *E. coli* strains K803 (*dam*$^-$ *dcm*$^+$) and GM272 (*dam*$^-$ *dcm*$^-$).

Hha I Frag.	K803 [SAM (nM) = 150]	GM272 [SAM (nM) = 1000]
bp 21	20	15
bp 28	19	17
bp 30	25	11
bp 33	37	13
bp 37 (CCA/TGG)	3461 (0.9%)	28
bp 40	30	27
bp 54	34	14
bp 60/62*	76	26
bp 67	42	13
bp 75	43	12
bp 83	49	14
bp 93	50 (.02%)	39
bp 100/103	104	33
bp 109	61	21
bp 131/132* (CCA/TGG)	3806 (1%)	33
	(10-12 µg)	(10-12 µg)
SAM sp. act. and conc. Ci/mmole	67.2; [150 nM]	14.1; [1 µm]
cpm expected		
CCA/TGG site (2 Cs)	190,000	39,700
1/2 site	95,000	19,850
Amt. of DNA	10-12 µg	10-12 µg

*Values (cpm) of doublet fragments equal the addition of their separate values together; thus their values should equal at least double the background value (bp 21).

For each of the assays shown in the data tables of this text, the percent methylation of a fragment's site(s) has been determined by dividing the observed counts incorporated by the expected counts incorporated for each fragment. The value of the expected counts incorporated was determined by calculating the molarity of plasmid molecules (and a single plasmid GAPyN site) in the amount of methylated plasmid DNA loaded in a gel lane and multiplying by the specific activity of the radiolabelled SAM present in the assay. Corrections were made in the expected cpm per site (fragment) value for the presence of one or two methylatable adenines (i.e., GATC/CTAG has 2 methylatable A's; GACC/CTGG has only one) in the sites. The implications are that when the methylation of a specific GATC site has a value of 50% then the majority, if not all, of the molecules containing this GATC site have one of the site's two adenines methylated since they are probably methylated independently (noncooperatively) as observed for the Eco dam methylase (29). The 50% value is the minimum value which would be exhibited by a GATC-containing fragment or plasmid molecule in order for it to be rendered resistant to R. Mbo I cleavage. If only one of the two adenines of a GATC site are methylated, it is R. Mbo I resistant.

To test whether our calculations of degree of DNA methylation derived from scintillation data are accurate, portions of several methylated DNA samples used on sequence specificity determinations were tested for susceptibility to R. Mbo I cleavage. In Panel A of Figure 16 is a photograph of the agarose mini-gel in which 1 microgram

Figure 16. Methylated DNAs before (A) and after (B) R. Mbo I digestion.

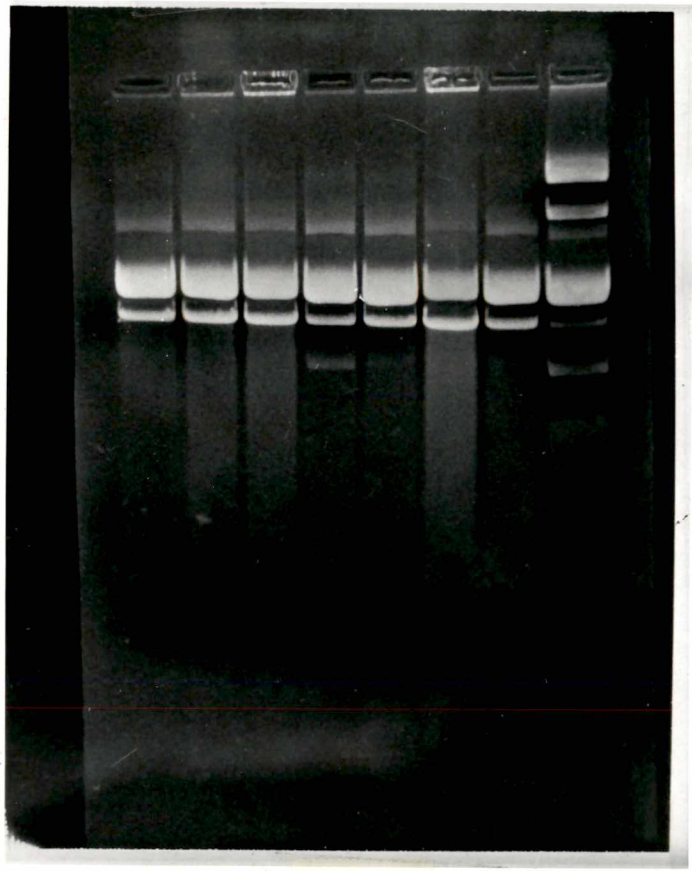
Panel A--1% agarose minigel in 1 X E buffer plus 0.5 μg/ml EtBr

Lane 1. Eco dam/SAM 3L (4 μM)
2. T_2dam$^+$/pH = 6.5 SAM 3L (1 μM)
3. T_2dam$^+$/pH = 7.5 SAM 3L (1 μM)
4. T_2dam$^+$/pH = 8.5 SAM 3L (1 μM)
5. T_4dam$^+$/SAM 4H 150 nM
6. T_2dam$^+$/SAM 4H 150 nM
7. T_2dam$^+$/SAM 3L + cold SAM 3 μM
8. T_2dam$^+$/SAM 3L + cold SAM 15 μM + pSSH-1b (internal control)

Panel B--1% agarose gel 15 cm x 0.4 cm in 1 X E buffer

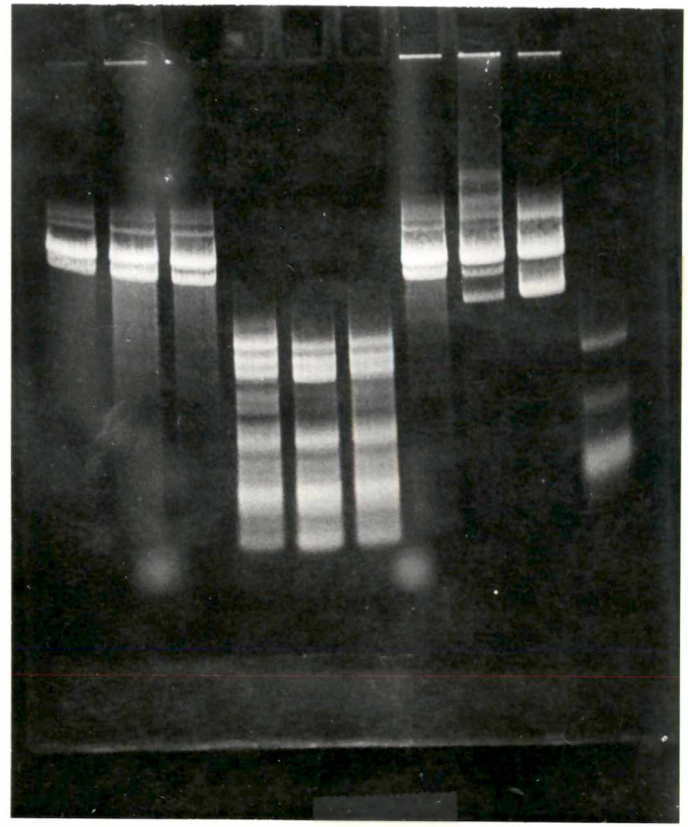
Lane 1. Sample 1 (Panel A) X R. Mbo I
2. Sample 2 (Panel A) X R. Mbo I
3. Sample 3 (Panel A) X R. Mbo I
4. Sample 4 (Panel A) X R. Mbo I
5. Sample 5 (Panel A) X R. Mbo I
6. Sample 6 (Panel A) X R. Mbo I
7. Sample 7 (Panel A) X R. Mbo I
8. Sample 8 (Panel A) X R. Mbo I
9. pSSH-1b undigested
10. Sample 7 (Panel A) X R. Sau IIIA

Panel A (Before)



Lane 1 2 3 4 5 6 7 8

Panel B (After)



Lane 1 2 3 4 5 6 7 8 9 10

Figure 16.

(1 μg) of each DNA to be R. Mbo I digested was electrophoresed. As one can see, the majority of the DNA is in the covalently closed circular (ccc) form which migrates slower than the linear (l) form in an EtBr-containing gel. Most of the DNAs exhibit only minor degradation seen as a fluorescent blur extending through the lane below the intact bands.

In Panel B of Figure 16 is a photograph of the agarose gel containing the electrophoresed DNA samples (2 μg) after a 5X R. Mbo I digestion, and one sample in duplicate digested with R. Sau IIIA to give a pattern of fragments resulting from cleavage at all GATC sites (regardless of adenine methylation) to use for comparison. Interestingly, neither Eco dam ([SAM] = 4 μM) methylated DNA (lane 1) nor the T_2dam$^+$ methylated DNAs of the pH = 6.5, [SAM] = 1 μM; pH = 7.5, [SAM] = 1 μM; pH = 7.5, [SAM] = 3 μM; pH = 7.5, [SAM] = 15 μM reactions (lanes 2, 3, 7, and 8, respectively) were cleaved appreciably by R. Mbo I. The reason for the extra bands or anomalous migration profile of the T_2dam$^+$ 3 μM and 15 μM SAM samples in lanes 7 and 8 is unknown. DNAs methylated by T_2dam$^+$ (pH = 8.5), T_4dam$^+$ [SAM] = 150 nM, or T_2dam$^+$ [SAM] = 150 nM, in lanes 4, 5, and 6, respectively, were cleaved by R. Mbo to a similar, but nonidentical, extent. These results indicated the latter DNA's GATC sites were not all methylated. They were not totally unmethylated either as would be suggested if their digest patterns resembled that of the R. Sau IIIA-digested DNA in lane 10. Most likely some GATC sites were methylated on some molecules, and not others. The 150 nM SAM reactions could not possibly methylate 100% of their DNA's GATC sites since there were only 62 pmoles SAM to donate methyl groups to 308 pmoles GATC "A"s.

V. DISCUSSION OF RESULTS

A. T_2*dam*$^+$ Methylation Site Preference and Factors Affecting It

The DNA sequence specificity data for the T_2, T_4, and *Eco dam* methylases presented in the results section of this text support the findings of Brooks' and Hattman's (1,2,7) sequence specificity experiments. Results of the T_2*dam*$^+$ sequence specificity analyses reveal that the methylase modifies adenines of cytosine-containing DNA to a substantial extent with a site (specificity) preference of GATC>GACC>GACT≥GATG.

Analysis of scintillation count data of R. *Hha* I restriction fragments methylated by T_2*dam*$^+$ indicates that the methylase has preferences among GATC, GACC, GACT and GATG sites flanked by different nucleotides. While further analysis of the 40 bp R. *Hha* I fragment showed that the preference of T_2*dam*$^+$ for tGACCc sites over gGACCg sites at 1 μM SAM, no definite order of preference has been determinable for the GATC sites due to their palindromic nature and the probability that the two half sites of each $\frac{\text{GATC}}{\text{CTAG}}$ site are methylated independently. The data do support the model that tGATGc sites are preferred over aGATGc sites.

The discovery that as the concentration of SAM in methylase reactions is raised (even beyond the K_m[SAM] value), the percent of plasmid molecules methylated at the "minor" methylation sites (GACC and GATG) rises disproportionately to the percent of molecules methylated at the GATC sites and to the relative increase in the methylase's specific activity suggested to us that several phenomenon could be

occurring. It suggested that the increased concentrations of SAM facilitate T_2*dam*'s methylation of GAPyN sites which normally form less stable interactions with the enzyme than GATC sites. The scintillation data for the 15 μM and 3 μM SAM reaction's DNAs (Table 14, p. 89) indicate that GACC sites of the former DNA get methylated at the expense of the GATC sites which are hemi-methylated rather than fully methylated in this DNA.

The productive binding and catalysis of methyl transfer at these minor sites probably competitively inhibits methylation of GATC sites by the T_2*dam*$^+$ enzyme at these higher SAM concentrations. This hypothesis is supported by the methylation data for the 15 μM SAM assays in which one would expect GATC sites to be saturated or at least to exceed the level of saturation of the GATC site methylated in the 3 μM SAM reactions. Contrarily, they are methylated less and the 40 bp GACC site(s) are methylated substantially. In effect the T_2*dam*$^+$ enzyme has a different K_mSAM or SAM threshold for each methylatable species of GAPyN site for which it has any specificity at all.

Alternatively, one could speculate that a proofreading function of T_2*dam*$^+$ may be inhibited or altered by high SAM concentrations or that SAM acts as both a positive allosteric effector and substrate of T_2*dam*$^+$. If one plotted the velocity (v) of the methylation reaction versus SAM concentration in the presence of excess DNA using purified methylase one could expect to see a sigmoidal curve as opposed to a hyperbolic curve if SAM was a positive allosteric effector.

It has also been demonstrated in this text that changes in pH greatly alter the sequence specificity of the $T_2\underline{dam}^+$ enzyme. One possible explanation for the conspicuous alteration in the enzyme's sequence specificity (activity) under basic conditions could be that the labile SAM molecules are being degraded at a faster rate resulting in reduction of the effective concentration of SAM present during the methylase assay. Adding greater excesses of SAM in reactions run at pH = 8.5 could eliminate this degradation from having an effect on the enzyme's sequence specificity. Alternatively, enzyme structure and/or enzyme-substrate interactions could be altered under the basic conditions. A change in the degree of protonation of different amino acids in the active site of the $T_2\underline{dam}^+$ enzyme could be affecting its specificity/activity.

The results of the methylase assays done at different pHs indicate that the increase in methylation of GATG and GACC-containing fragments in the assays containing 3 μM and 15 μM SAM were indeed due to the changes in SAM concentrations rather than any changes in pH caused by the addition of the acidic SAM solution or the neutralizing tris base solution.

It appears that supercoiling may slightly inhibit methylation of some of the minor methylation sites (i.e., GATG, GACT) but does not seem to affect methylation of GATC or GACC sites to any measurable extent. The actual magnitude of differences in site methylatability caused by DNA supercoiling (topology) may not be as detectable using crude extracts as with purified enzyme because of nucleases in the

crude extracts which relax and linearize the covalently-closed-circular DNA during incubation. Results of the experiments (LIN-1) that demonstrated T_2*dam*$^+$'s ability to methylate linear R. *Hha* I fragments of pBR322 also showed T_2*dam*$^+$ could methylate GAPyN site "A"s as close as four bases from the end of the fragment (i.e., 36 bp GATG site).

B. *Differences Between T_4dam$^+$'s and T_2dam$^+$'s Site Preferences*

In comparison with T_2*dam*$^+$ results, T_4*dam*$^+$ methylase exhibits much less plasticity in its sequence specificity. While T_4*dam*$^+$ readily methylates GATC sites with flanking sequence preferences similar to T_2*dam*$^+$, it does not substantially methylate GATG, nor GACT sites contained within the pBR322 R. *Hha* I fragments analyzed. This lack of methylation of these minor sites was observed for DNAs methylated in the presence of 150 nM and 1 μM SAM. Neither of these minor site species were methylated in excess of 1% of the expected saturation value. One or both of the GACC sites (not determined) of the 40 bp fragment were methylated significantly above background but these values only reached the 1-2% methylation level at 1 μM SAM.

C. *Alterations in Site Preference Characteristic of the dam^h Phenotype*

Assays of T_2*dam*^{h} and T_4*dam*^{h} enzymes' activities revealed that these mutants had very similar sequence specificity to T_2*dam*$^+$ and each other. They were both able to methylate minor sites such as GATG, GACT, and GACC on pBR322 similar to proportions and levels seen for T_2*dam*$^+$. Surprisingly, the *dam*^{h} enzymes had the ability (specificity) to methylate these minor sites even at low SAM concentrations (i.e.,

150 nM). The order of site preference is homologous to that of T_2dam$^+$: GATC>GACC>GACT≥GATG. However, the dam^h enzymes' preference for GATC over GACC sites seems to be much less than that of T_2dam$^+$ at both concentrations of SAM (150 nM and 1 μM). Apparently, the mutation causing the dam^h phenotype of enhanced methylation of P_1-endonuclease recognition sites (AGACC) has caused an amino acid change that has either directly affected DNA sequence specificity (preference) of the enzyme, its affinity for SAM, or perhaps a proofreading function. One could envision the amino acid change altering a combination of the latter properties of the enzymes. Unfortunately the amino acid change has not been identified to date (Hattman and Schlagman, pers. comm.). Perhaps the change has increased the dam^h enzymes' affinity for SAM and reduced its catalysis rate but left the K_mSAM value the same as wildtype. While Brooks (178) reported the K_mSAM for the T_2dam$^+$ and T_2dam^h enzymes as being 2.4 μM, one could conceptualize that the methylase has different K_mSAM values for each species of GAPyN sites representing differences in the stability of binding of the methylase to these sites. One can not rule out the possibility that the methylase's binding of SAM doesn't somehow affect its ability to bind stably to certain sites.

The possibility that enhanced methylation of GACC sites is due to an increase in catalytic activity of dam^h over dam$^+$ can be excluded due to the lack of saturation of GATC sites observed in dam^h assays compared with dam$^+$ assays. In fact overall methylation levels of DNAs subjected to dam^h extracts were often lower than those of dam$^+$. This

was probably due to the increased lability of the $\underline{dam}^h$ enzymes at 37°C observed by Brooks (7) and their lower shelf life at 4°C with respect to the $\underline{dam}^+$ enzyme. However, the presence of SAM has been reported by Heihlman and Hattman (47) to reduce the heat-inactivation of the enzyme. The finding that $T_2\underline{dam}^h$ does not methylate any species of GAPyN different from $T_2\underline{dam}^+$ but merely has a different preference supports similar findings by Brooks and Hattman (2) that prolonged incubation of lambda DNA with either $T_2\underline{dam}^+$ or $T_2\underline{dam}^h$ yielded similar extents of methylation of GAT and GAC sequences. That is to say, they saw no new species of labelled oligonucleotides among DNA sequences methylated by $T_2\underline{dam}^h$ versus $T_2\underline{dam}^+$.

D. Eco dam Has the Most Stringent Specificity of the dam Enzymes

Eco dam methylase seems to differ significantly from those of the T_2 and T_4dam methylases. Eco dam seems to have a very rigid specificity for GATC sites. The data do suggest though that it may also methylate some GACT sites and a GACC or GACA site. Eco dam does not appear to have any specificity for GATG or GACC sites typical of $T_2\underline{dam}^+$ and $T_4\underline{dam}^+$ (at 1 μM SAM). Its site specificity did not change significantly with an increase in the SAM concentration from 600 nM to 4 μM although the extent of methylation of the DNA increased. These findings agree with those of Schlagman and Hattman (pers. comm.) who found that Eco dam and T_4dam methylate the adenines of the E. coli genome to 1.3 mole% and 1.7 mole% as determined by HPLC analysis.

Originally, it was our desire to use our assay to compare the specificity of the purified Eco dam (Biolabs) enzyme with that of the

Eco dam enzyme present in the crude extract of uninfected E. coli (K803) cells to test the effects of native cell proteins and extract buffer versus BSA on sequence specificity. Unfortunately, no dam methylase activity was detected in the K803 cell extract much to our surprise (chagrin) although dcm methylase activity was present. To determine any effects cellular proteins (components) or the dialysis buffer might have on Eco dam's activity (specificity) one could assay Eco dam's sequence specificity when added to an uninfected K803 or GM272 cell dialysate.

E. Importance of Flanking Sequences

The importance of flanking sequences can even be shown for the canonical GATC sequence. Throughout this subsection, we will represent sites as 6-base sequences with the outer bases as lower case letters as in Table 2, p. 47, to aid visualization of the inner 4-base sequences. The cpm data of the R. Sau IIIA-cleaved 109 bp fragment (Table 12, p. 83) show that the aGATTa site, which is on the 61 bp fragment after Sau 3A digestion, is essentially not methylated. Since some GATT sites have been shown by others to be highly methylated (9), this lack of methylation is indirect evidence for the importance of flanking sequences in determining the methylatability of a GAPyN site. More direct evidence is provided by the data presented in Table 1 (p. 34) for both the T_2 and T_4dam$^+$ enzyme reactions containing limiting (150 nM) SAM concentration. The methylation of the 109 bp Hha I fragment is only at its canonical (GATC) site. However, it is

methylated much more than the combined 60 and 62 bp Hha I fragments, which have other sites in addition to the canonical site.

This is direct evidence that, the $\frac{\text{gGATCa}}{\text{cCTAGt}}$ site on the 109 bp fragment is methylated in preference to the $\frac{\text{aGATCg}}{\text{tCTAGc}}$ site on the 60 bp fragment. However, it appears from the data in Table 12 (p. 83) that the gGATCa half site (48 bp R. Sau IIIA subfragment of 109 bp R. Hha I fragment) is methylated in preference to the tGATCc half-site (61 bp R. Sau IIIA subfragment of 109 bp R. Hha I fragment). The Eco dam enzyme showed similar results (Table 18, p. 97) with about the same relative preference for different GATC-containing R. Hha I fragments as the T_2dam$^+$ enzyme.

The importance of flanking sequences on site methylatability are supported by the data in Table 12 showing that the tGACCc site on the 40 bp fragment is methylated but the gGACCg site is essentially unmethylated by the T_2dam$^+$ gene product. T_2dam$^+$'s preference for tGACCc of the 40 bp R. Hha I fragment over the cGACCt site of the 67 bp R. Hha I fragment as seen in Table 11, p. 75, indicates it has a preference for tGAPyCc over cGAPyCt sites.

As a final example, Table 17 (p. 95) shows that the dam^h enzymes methylated the aGATGc of the 36 bp fragment in preference to the tGATGc of the 28 bp fragment. All of these comparisons imply that the ability of different GAPyN sites to be methylated by the T_2dam$^+$ enzyme may be affected differently by the presence of the same flanking bases.

That is to say that T_2dam$^+$ has a preference for different flanking sequences associated with different GAPyN sites that have different bases at the 3rd or 4th position.

F. Relative Methylation At Minor Sites

From the above discussion of the importance of flanking sequences, it is clear that one must be careful about overgeneralizing. However, as shown by the data of Table 11 (p. 75), the cGACAc, tGACTc, and tGACGc sequences at the sites examined are not methylated to any detectable amount, if at all (see the data from the 30 and 33 bp fragments) by T_2dam$^+$. In contrast, both of the fragments containing GATG sequences did not have other possible GAPyN sequences and were slightly methylated by the T_2dam$^+$ enzyme, and more by the dam^h enzymes. The dam^h enzymes methylated the aGATGc in preference to the tGATGc whereas T_2dam$^+$ preferred tGATGc over aGATGc.

Previously it was observed (1) that no additional oligonucleotide sequences were methylated by the dam^h than the dam$^+$ alleles. In all the cases where we have compared specific single possible GAPyN sequences, we also find that the sequences slightly methylated by dam$^+$, are the sequences highly methylated by dam^h.

G. Significance of in vitro dam Methylation Results to in vivo dam Methylation of Phage DNA

The actual biological relevance of the presented sequence specificity results is not clearly understood. The SAM concentration in bacterial cells has not been determined but is thought to be very low which would favor methylation of primarily GATC sites.

Additionally, our determinations have been on C-containing not glucosylated, hmC-containing DNA of the wildtype phage. Differences between specificity on glucosylated hmC-DNA and C-containing DNA could be substantial due to steric interference the modified C residues of GATC and GACC sites could have upon the active sites of the methylases. Whereas hmC is made from C prior to DNA polymerization, methylation and glucosylation are postreplicative events. Their order of occurrence upon newly replicated phage DNA has not been determined but glucosylation could have an important bearing on methylase sequence specificity. Analysis of hmC DNA plus or minus glucosylation has been greatly hindered by the rarity of restriction enzymes which can cleave it. Whereas plaque size and morphology of T_4dam gt^+ and T_4dam^h gt^- phage-infected cells differ from those of their T_4dam$^+$ counterparts, no measurable difference in their DNA replication rates have been detected using ^{3}H-thymidine incorporation assays (data not shown). All results to date indicate that dam^h methylation has no serious effect on phage processes and that it only serves to protect gt^+ phage from restriction by P_1 prophage.

Hattman and Schlagman (19) have shown there is a significant functional difference between Eco dam and T_4dam$^+$ enzymes in that T_4dam$^+$ enzymes seems not to be able to interact in place of Eco dam in E. coli's replicative machinery complex to suppress the hyper-recombination phenotype of the dam$^-$ host. Biological significances pertaining to their differences in sequence specificity have not be elucidated to date.

H. Future Studies

More work needs to be done to fully and definitively ascertain the DNA sequence specificity of the dam methylases. It would be of value to study the effects of pH and higher [SAM] on the specificity of T_4dam$^+$ and the T_2 and T_4dam^h enzymes. Analysis of differential methylation of the two GACC sites within the 40 bp R. Hha I fragment of pBR322 DNA, methylated by the dam$^+$ and dam^h enzymes at low and high [SAM]s, might uncover further information about the differences in their sequence preference. Actual verification of the methylation of AGACC (P_1 restriction site) sites of pBR322 by the dam^h enzymes using our assay still remains to be done, though a R. Dde I/R. Hpa II double restriction enzyme digest pattern of pBR322 has been discovered to yield such an AGACC site by itself on an isolatable 84 bp fragment.

Once the latter experiments are done and the sequence specificities of these enzymes are better defined, more in-depth studies of the effects of salt, gycerol, and DMSO on dam sequence specificity can be done similar to those of Woodbury et al. (48) and Halford et al. (49) conducted on M. Eco RI and M. Eco RV, respectively. Additionally, mutants of dam or other methylases having altered sequence specificity could be generated and characterized quantitatively.

The question of whether the differences in sequence specificity between T_2 and T_4dam$^+$ methylases is due to other phage proteins present in infected cells could be answered by adding purified T_2 or T_4dam$^+$ enzyme to either sonicates of T_4dam$^-$ or T_2dam$^-$ phage-infected cells,

respectively. The effects of other proteins on methylation could be tested by adding them to crude extracts or purified enzyme preps used in reactions. Ligases or topoisomerases in the dialysates were of no consequence to our assay since no exogenous ATP was added to methylase reactions. However, the effects of these proteins on methylation in the presence of ATP and Mg^{++} may be worth studying.

I. Correlation Between Counts Observed for GATC-Containing Fragments, Extent of Methylation (Percent Saturation), and Susceptibility to Cleavage by R. Mbo I

When the R. Mbo I restriction analysis data (Figure 11, p. 69) and the scintillation analysis (of the R. Hha I fragments) of the same methylated DNAs are compared an obvious correlation is observed. The R. Mbo I-cleavable DNAs exhibit lower cpm for GATC-containing R. Hha I fragments in comparison with those of DNAs resistant to R. Mbo I cleavage. The puzzling observation that must be reckoned with, though, is that the four DNAs methylated by Eco dam, [SAM] = 4 μM; T_2dam$^+$; pH = 6.5, [SAM] = 1 μM; T_2dam$^+$ pH 7.5, [SAM] = 1 μM],; T_2dam$^+$ pH 7.5, [SAM] = 3 μM (lanes 1, 2, 3, and 7, respectively), are not cleaved by R. Mbo I presumably due to methylation of one or both of their GATC site adenines. Hemimethylation of a GATC site is sufficient to prevent R. Mbo I cleavage. The scintillation data however (Tables 14, 15, 16 and 18, pp. 89, 91, 92 and 97) indicate that only between 50-75% of the GATC sites of all the plasmid molecules are hemimethylated, if the three GATC sites examined are indeed representative of all pBR322 GATC sites. Lanes 1, 2, 3, and 7 do show background below the predominant plasmid band which could represent cleavage of one or two sites not

methylated on some molecules. This background is absent from the T_2dam$^+$ 15 μM SAM reaction lane 8. The lack of R. Mbo I cleavage of T_2dam$^+$ 15 μM SAM reaction's DNA (lane 8) suggests that all of the DNA molecules of these samples have at least one methyl group on 95-100% of their GATC sites. Scintillation analyses indicated that 85-100% of the GATC site "A"s are fully methylated on the 3 μM SAM reaction's DNA whereas 74-51% of the 15 μM SAM's DNA's GATC sites "A"s are methylated. It is the author's opinion that the conflicting sets of data can be reconciled by invoking that errors in estimating DNA recoveries from ETOH precipitation or when gel loading could have created these discrepancies when calculating the percent saturation of sites.

Another reasonable explanation for the discrepancies could be that the radiolabelled SAM is perhaps not homogeneously labelled with tritium on the methyl group that is transferred to DNA. The exhaustively dialyzed enzyme preps may also contain some residual amount of endogenous SAM bound by the methylase or other protein molecules. Unfortunately, R. Mbo I restriction digestions were not performed on DNA samples from assays in which only enzyme and DNA (no exogenous SAM) were added to methylase reactions as a control for the latter.

Even though the amount of DNA per gel lane might have varied somewhat due to difference in ETOH precipitation recoveries, the intensity of bands in each lane appeared to vary in a linear fashion in accordance with their size which would imply that there was no differential ethanol precipitation of restriction fragments of

different size after restriction digestion. Digestion of methylated DNA samples with R. Dpn I, which cuts only GATC sites with both adenines methylated, might have given some insight into the degree of GATC site methylation by comparison with R. Sau IIIa digests of the same DNA.

An important observation came from the R. Sau IIIA digests of the 83 and 109 bp R. Hha I fragments originally isolated from pBR322 (GM272) DNA methylated by T_2dam$^+$ in the presence of 1 μM SAM. The two fragments generated from digestion of the 83 and 109 bp fragments each had a single GATC half site on them. They were analyzed by scintillation counting after separation by gel electrophoresis. The cpm detected for each fragment is shown in Table 13 (p. 86). The amount of DNA in samples digested with R. Sau IIIA or used as undigested controls for both the 83 and 109 bp fragments was a 6 μg equivalent or 140 ng and 200 ng, respectively. The 83 and 109 bp fragments of the 4 μg DNA sample analyzed in column 3 of Table 13 gave scintillation counts of 4402 (28-55 % Sat) and 3624 (23-45 % Sat). Their values indicate that 6 μg samples equivalent of the 83 and 109 bp fragments should yield 6603 and 5436 cpm. The observation that a 6 μg equivalent of 83 and 109 bp fragment undigested by R. Sau IIIA gives 4087 and 2564 cpm, respectively, indicates that DNA recovery from gel slices was approximately 50-62%. The most important observation though is that the two NGATCN species (half sites) located on either strand of a palindromic GATC site are methylated at approximately the same frequency throughout the population of plasmid molecules in the T_2dam$^+$

assay but that slight differences (a preference) do occur. Neither R. Mbo I nor R. Dpn I digestions of the 83 and 109 bp fragment DNA were done, unfortunately, so definitive statements about the degree of methylation of these fragments can not be made.

The observation that the majority of the cpm (1319) resulting from the methylation of the "109" bp fragment's GATC are associated with the "48" bp's GATC half-site strongly indicates that the GATT site location on the "61" bp fragment, which contains the other GATC half-site, is not methylated. If this is true, then cpm data normalized by dividing by the "109" bp's cpm data was not skewed by any non-GATC (GATT) associated methylation. Methylation of the 109 bp Hha I fragment's GATT site could be definitively determined by digesting the methylated fragment with R. Dpn I (which cuts only fully methylated GATC sites) and seeing if the cpm for the resultant 48 and 61 bp fragments are equal. If the cpm of the 61 bp fragment significantly exceeds that of the 41 bp fragment this would spuport the GATT site being methylated.

One problem with the assay's data which has not been resolved is the nonstoichiometric ratio of polyacrylamide gel fragment total cpm to that of agarose gel total DNA cpm. The observed ratios do not correspond closely to the expected values based on the ratio of base pairs in the R. Hha I fragments versus intact pBR322 molecules (0.18) or based on the ratio of GATC sites in the R. Hha I fragments versus intact pBR322 molecules (0.13). Ratios were corrected for differences in the amounts of DNA in gels and scintillation counting efficiencies. Values determined for different assays varied from 0.05 to as much as

1.0, which span a twenty-fold range (see Table A-3 of Appendix). Differences in recovery of DNAs from ethanol precipitations after R. Hha I digestion do not vary by more than a few percent as can be seen by comparing fluorescent band intensities of EtBr-stained polyacrylamide gel lanes loaded with equivalent volumes of different samples expected to have the same DNA concentration. A potential source of error that may be contributing to the observed discrepancy could be that the DNA electrophoresed through the agarose gels was not phenol extracted or purified in any manner prior to electrophoresis. It was treated with SDS and proteinase K for 1 hr at 65°K subsequent to methylation but the efficiency of this treatment was not tested by Coomassie Blue staining of the gels or soaking them in ampholytes and doing autoradiography to detect proteins co-migrating with the DNA.

An event which could be contributing to this puzzling discrepancy is that in some assays the proportion of linear to supercoiled or open circular pBR322 DNA generated during incubation with crude extract is greatly increased. Subsequent digestion of this linear DNA with R. Hha I will result in the loss or anomalous migration of resultant fragments which had the original linearizing double-stranded break in them. The anomalous migration of these broken fragments also creates the problem of increased nonspecific background in a gel lane. The resolving power of the 15% polyacrylamide gels causes the separation of small fragments from 20-100 bp. Larger fragments form bands higher in the gel that compacted tighter together. Anomalously migrating DNA molecules of ever greater size will not be as well resolved either,

resulting in an increase in background counts in gel slices from upper portions versus lower portions of a gel lane.

VI. CONCLUSIONS

1. The sequence specificity of DNA methylases can be reproducibly quantitated by the technique described.
2. Under certain *in vitro* conditions, the T_2*dam*$^+$ enzyme methylates a *tGACCc* site and *GATG* sites in pBR322 but less frequently than *GATC* sites.
3. S-adenosylmethionine concentration and pH influence the ability of the T_2*dam*$^+$ enzyme to methylate different *GAPyN* sites.
4. The T_4*dam*$^+$ enzyme has greater specificity for the canonical sequence, *GATC*, than the T_2*dam*$^+$ enzyme.
5. The T_2*dam*^{h} and T_4*dam*^{h} (mutant) enzymes methylate the same noncanonical sites, *GATG* and *GACC*, as the T_2*dam*$^+$ methylase, but do so at a lower concentration of S-adenosylmethionine.
6. The Eco *dam*, T_2*dam*$^+$, and T_4*dam*$^+$ enzymes preferentially methylate $\begin{matrix}\text{tGATCc}\\\text{aCTAGg}\end{matrix} \geq \begin{matrix}\text{gGATCT}\\\text{cCTAGa}\end{matrix} > \begin{matrix}\text{aGATCg}\\\text{tCTAGc}\end{matrix}$ sites in pBR322.

VII. EPILOGUE

A. Limitations and Possible Modifications of the Methylase Sequence (Site) Specificity (Preference) Assay

This novel assay for ascertaining a methylase's DNA sequence specificity, using the already established technology of restriction enzyme digestion, gel electrophoresis, and scintillation counting, has produced reproducible results in studies of the T_2, T_4, and Eco dam methylases. While the extent of methylation of DNAs from repetitive assays of the same methylase run under similar conditions has varied, the ratio (proportionality of methylation among different species of GAPyN sites) has remained relatively constant. This is evident from making comparisons of the normalized (NORM) data.

One limitation of this technique is that not every species of GAPyN site with each of the 16 possible combinations of flanking base pairs on either side of a site can be isolated by itself on resolvable fragments generated by a single or double restriction digest of pBR322 DNA. Various combinations of single and double digests used in parallel could possibly yield such a full set of sites but verification of this possibility was not within the scope of this project. Other target DNAs (i.e., SV40 DNA) isolated in unmethylated form may serve as better assay substrates for observing the methylation of certain sites (sequences).

An optional modification of the assay, which may enhance reproducibility and sensitivity, might entail using HPLC analysis to measure the ratio of methylated bases (i.e., 6-methyladenine) to

unmethylated bases in the DNA of a restriction fragment isolated from the polyacrylamide gel. This technique would be favorable for assaying the extent of methylation of adenines or opposite strands of a fragment, which could be isolated from a denaturing gel. I has a detection limit of approximately 50 pmoles (17.5 ng) of methyladenines. Identification of the strands would be possible due their differences in base composition (molar ratios). In this way, the effects of flanking bases on the methylation of one adenine of a GATC site over the adenine on the opposite strand may be examined. This method could also facilitate the determination of methylation of the adenines (bases) of two nonpalindromic sites, situated on opposite strands of a fragment, which may not be separated by another restriction site whose cleaveage would permit the separation of the two sites on two separable fragments. The use of radiolabelled SAM and scintillation counting could also be used but strand identification would require other adjustments or modifications to be made.

B. The Use of Alternative Target DNAs

The use of individually purified restriction fragments or double-stranded oligonucleotides containing one putative methylation site would be the ideal target substrate for assays. The use of synthesized double-stranded oligonucleotides or purified restriction fragments containing one or at most two GAPyN (if on opposite strands) sites for assay substrates would refine sequence specificity determinations and allow direct determination of $K_m$site and K_mSAM values for each GAPyN species. One complication of using these target

DNAs is that some methylases can not methylate sites close to the end of molecules (5). In these cases, the restriction fragments or oligonucleotides would have to be ligated together prior to methylation. The T_2dam$^+$ enzyme does not have this trait as demonstrated by the results in Table 15 (p. 91) that shows R. Hha I fragments (LIN-2) are methylated by T_2dam$^+$. The specificity of M. Hpa II and M. Hae III methylases has been studied using oligonucleotide target DNAs (50) and Bolden et al. (51) have used oligonucleotide substrates to uncover which DNA sequences influence maintenance and de novo methylation by mammalian DNA methylases.

Isolation of a methylase enzyme preparation of high specific activity may be important for achieving high levels (near 100%) of methylation of low preference (minor) sites in comparison with major sites of methylation. Equally important is the consideration of competing methylatable sites of a different DNA sequence species. Catalytic activity of the enzyme upon these competing sites will affect the ability of the enzyme to methylate the primary site of interest. The frequency of each GAPyN species is roughly equivalent in pBR322 molecules (Figure 1, p. 31). The latter minimizes any inherent bias in the target DNA, although proportions of each subspecies with different flanking bases may differ.

Although intact or R. Hha I-digested pBR322 is more readily available than oligonucleotide sources of target site DNA, the frequency of a site per microgram of DNA is much lower. The low concentration of one site within the intact DNA compromises the

detectability of its methylation in two portions of the assay: (1) the site is in lower concentration in the methylation reaction; (2) only a limited amount of DNA can be loaded in a gel lane (25 μg) before the small DNA fragments containing the sites are no longer resolvable and subsequently separable by excision from the gel. It may be possible to separate methylated R.E. fragments by HPLC (53,54).

C. Detection Limits

All parameters considered, the bottom line in detectability of methylation of a site lies in the specific activity of the radiolabelled tracers and one's ability to detect their incorporation. Incorporation is limited by scintillation counting efficiencies or the sensitivity of the X-ray film emulsion used during autoradiographic exposure, not to mention the time you can afford to wait. Intrinsic differences in the K_m(SAM) parameter for different methylases will affect the detection limits of this assay. At the high specific activity and low concentration end of the scale for SAM, in an assay with (SAM) = 150 nM; sp. act. = 67.2 Ci/mmole, one could expect to observe counts of 16,625 cpm per μg of DNA for a nonpalindromic site methylated to the extent of 100% on only one strand. If complete methylation of a site is attainable under these conditions, using 25 μg of DNA in a gel lane, one could theoretically observe 415,625 cpm for such a site. One could then detect (discriminate) as little as 0.05% methylation (210 cpm) at such a site with an ample quantity of cpm detectable above background (20-80 cpm).

At the other end of the scale, in an assay using (SAM) = 15 μM; sp. act. = 0.94 Ci/mmole, 25 μg of a pBR322 that is methylated to saturation at the one nonpalindromic site would yield 5775 cpm for the specific site-containing restriction fragment. Theoretically, one could detect as little as 2% methylation (115 cpm) of this site in the population of molecules. Therefore, it is necessary to adjust the assay to the intrinsic parameters (i.e., K_m) of one's methylase enzyme to optimize enzyme activity and detectability. Most of the values discussed herein are given assuming ideal assay conditions and optimal DNA recovery frequencies. Variance will alter the detection limits slightly from assay to assay.

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APPENDIX

Table A-1. Rf Values of SAM-Prep Components (Spots) Separated by TLC.

Solvent A	Plate	Solvent Line = 8.1 cm	Solvent B	Plate	Solvent Line = 6.5 cm
Lane 1 SAM·HCl	1st Spot 2nd Spot 3rd Spot	RF = 4/81 x 100 = 5 = 11/81 x 100 = 13.6 = 52/81 x 100 = 64.2	Lane 1 SAM·Hcl	1st Spot 2nd Spot	RF = 6/65 x 100 = 9.2 = 16/65 x 100 = 24.6
Lane 2 SAM·Iodide	1st Spot 2nd Spot 3rd Spot 4th Spot	Rf = 4/81 x 100 = 5 = 11/81 x 100 = 13.6 = 24/81 x 100 = 29.6 = 52/81 x 100 = 64.2	Lane 2 SAM·Iodide	1st Spot 2nd Spot ext. of 2nd Spot	Rf = 6/65 x 100 = 9.2 = 16/65 x 100 = 24.6 = 21/65 x 100 = 32.3
Lane 3 SAM·HSO_4	1st Spot Blur 1 Blur 2	Rf = 3/81 x 100 = 3.7 = 46/81 x 100 = 56.8 = 52/81 x 100 = 64.2	Lane 3 SAM·HSO_4	1st Spot 2nd Spot	RF = 6/65 x 100 = 9.2 Rf = 21/65 x 100 = 32.3
Lane 4 Adenosine	One Spot	Rf = 42/81 x 100 = 51.9	Lane 4 Adenosine	Only Spot	Rf = 26/65 x 100 = 40
Lane 5 Methionine	No Spots (too dilute)		Lane 5 Methionine	Slight Blue *Artifact--Spot at top, far right is where silica gel is missing	0-30/65
Lane 6 ^{3}H-SAM·HSO_4	No Spots		Lane 6 ^{3}H-SAM·HSO_4	No Spots	
1st Spot in lanes 1-3 is SAM			2nd Spot in lanes 1-3 is SAM		

*Samples were not neutralized before spotting silica gel plates. Samples migrated with Rf values more like paper chromatography.

**Spots on Thin Layer Chromatography (TLC) plates observed by UV-illumination.

Rf values for paper chromatography	Solvent A	Solvent B	Suspected impurities in SAM·Hcl and ·iodide
s-inosyl-L-butyric acid	15	60	--
s-inosyl-L-methionine	6	65	--
s-methyl-L-methionine	17	96	--
s-adenosyl-L-homocysteine	17	15	+
5' methyl thio-adenosine	66	7	+
s-adenosylmethionine	5	37	(SAM)
5' methyl thio-inosine	56	24	+

Table A-2. ^{3}H-SAM Activities and Counting Efficiencies.

Fluor	Sample	sp. act.	Sample Size	Actual 1 µCi vol	Expected cpm	Observed	c.e.
NCS/OCS	^{3}H-SAM3L	14.1 Ci/mmole	1.1 µl	1.7 µl	1,294,116	457,933	35.4%
NCS/OCS	^{3}H-SAM4H	67.2 Ci/mmole	1.0 µl	2.0 µl	1,000,000	396,767	39.7%
AQII	^{3}H-SAM2H	78.0 Ci/mmole	1.0 µl	2.0 µl	1,000,000	276,250	27.6%
AQII	^{3}H-SAM3L	14.1 Ci/mmole	1 µl	1.7 µl	1,176,471	342,467	29%

Control Reactions

400 µl reactions (200 µl T_2dam^+ dialysate)

25 µg pBR322 (GM272) = 970 nM GATC site "A"s

A. 10 µl ^{3}H-SAM 3L = 5.9 µCi[1 µM] or B. 12 µl ^{3}H-SAM 4H = 6.0 µCi + 4.5 µl 70.125 µM cold SAM = [1.014 µM]

Conversion: 1 µCi = 2×10^6 cpm

Scintillation Counts of Agarose Gel Slices of ≈250 ng DNA (Minigel)

T_2dam^+/SAM 3L (1 µM)	23,106 = 92,424 cpm/µg ± 28 + cpm
T_2dam^+/SAM 4H + cold SAM (1.014 µM)	25,004 = 100,016 cpm/µg ± 28 + cpm
Gel Slice EtBr Fluor. Ctrl.	71

Table A-3. Counting Efficiencies of DNA in Polyacrylamide.

	Nick Translated ^{3}H-DNA	15% Polyactylamide	NCS Solubilizer +50°C - 2 hrs.	Fluor	cpm	cf x cpm = Actual dpm
1.	+	-	-	AQII	14,300	1/0.28 = 3.6 x 14,300 = 51,00
2.	+	-	+	OCS	11,700	
3.	+	+ (25 µl)	+	OCS	10,600	10,600 ÷ 51,000 = 0.21; 21% ce
4.	+	+ (50 µl)	+	OCS	5,700	5,700 ÷ 51,100 = 0.11; 11% ce

Table A-4. Counting Efficienies of DNA in Agarose Gel Slices.

	^{3}H-CH_2-DNA (pBR322)	Electrophoresis in 1% Agarose	Boiling at 100°C to Melt Agarose Slice	cpm in AQII Flour	ce %
After phenol and ETOH ppt	(+) 250 ng	-	-	21,820	28
Before phenol and ETOH ppt	(+) 250 ng	+	+	23,106	29.6

Table A-5. The Correlation of Specific Activities of Methylated DNA Analyzed in Argarose and Polyacrylamide Gel Slices.

Table Ref.	Specific Activity DNA cpm/μg	Total cpm in DNA	Total cpm in Gel Bands	Ratio of C/B x CF*
13	20,600	206,000	84,250	$\frac{4.2 \times 10^5}{6.8 \times 10^5} = 0.62$
13	8,240	82,400	40,490	$\frac{2.0 \times 10^5}{2.7 \times 10^5} = 0.75$
13	11,040	110,400	42,280	$\frac{2.1 \times 10^5}{3.6 \times 10^5} = 0.58$
13	1,200	12,000	5,725	$\frac{28,625}{39,600} = 0.72$
16	71,000	1,420,000	63,724	$\frac{318,620}{4,686,000} = 0.07$
16	52,400	209,600	12,056	$\frac{60,280}{691,680} = 0.09$
16	89,100	2,138,400	74,558	$\frac{3.7 \times 10^5}{7.1 \times 10^6} = 0.05$
16	92,424	369,696	13,281	$\frac{66,405}{1.2 \times 10^6} = 0.06$

Table A-5 (continued).

Table Ref.	Specific Activity DNA cpm/μg	Total cpm in DNA	Total cpm in Gel Bands	Ratio of C/B x CF*
17	46,500	465,000	22,009	$\frac{110,045}{1.5 \times 10^6} = 0.07$
17	56,708	567,080	23,155	$\frac{115,775}{1.87 \times 10^6} = 0.06$
17	43,700	437,000	21,340	$\frac{106,700}{1.44 \times 10^6} = 0.07$
14	6560	65,600	43,475	$\frac{217,375}{216,480} = 1.0$
14	8240	82,400	40,490	$\frac{202,455}{271,920} = 0.74$
14	3560	35,600	22,873	$\frac{114,365}{117,480} = 0.98$
11	75,000	300,000	12,914	$\frac{64,570}{1,000,000} = 0.07$
11	89,100	2,138,400	74,558	$\frac{372,790}{7.12 \times 10^6} = 0.05$

Table A-5 (continued).

Table Ref.	Specific Activity DNA cpm/μg	Total cpm in DNA	Total cpm in Gel Bands	Ratio of C/B x CF*
11	92,424	369,696	13,281	$\frac{66,405}{1.2 \times 10^6} = 0.06$
11	47,000	470,000	16,351	$\frac{81,755}{1.55 \times 10^6} = 0.05$

*CF = scintillation counting correction factor =

$$\frac{\text{PAGE c.e. factor}}{\text{Agarose c.e. factor}} \text{ or } \frac{\text{NCS/OCS c.e.}}{\text{AQII c.e.}} = \frac{5}{3.3} = 1.52$$

Table A-6. Source and Phenotypes of Bacteria, Bacteriophage, and Plasmid DNA.

Bacteria/ Phage/ Plasmid	Genotype	Methylation	Host Strain	Phenotype	Source
Bacteria					
GM272				RGL^+, DAM^-, DCM^-	S. Hattmann
GM119/pssH-1b				RGL^+, DAM^-, DCM^+, (T_4*dam*$^+$ inserted pBR322 plasmid)	S. Hattman
Be				RGL^+	L. Snyder
HB101				DAM^+, DCM^+	L. Snyder
K803*				DAM^-, DCM^+	L. Snyder
Phage					
T2	αgt^-, βgt^-			RGL^S	S. Hattman
T2	αgt^+, βgt^-			RGL^R	This paper
Plasmid					
pBR322		($+CH_3$)	HB101		
pBR322		($-CH_3$)	GM272		
pSSH-1b		($+CH_3$)	GM119		

*Phenotypes determined during the course of this study (data not shown).

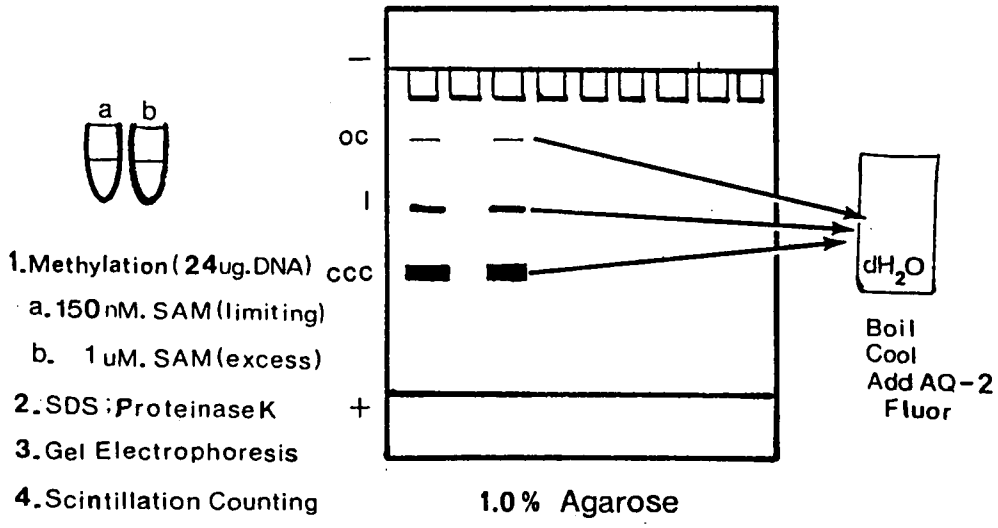
Table A-7. Analysis of Methylation Pattern.

Fragment Length (BP)	Boundaries (BP)	Nucleotide Sequence (top strand only) and Sites
21	414-435	420 430 CGCCAC AGGTGCGGTT GCTGG NH AH RA 11
28	233-261	240 250 260 CGCTATA TGCGTTGATG CAATTTCTAT G % M H % S H t T A g 1 1
30	2179-2209	2180 2190 2200 2210 C GCGTCAGCGG GTGTTGCCGG GTGTCGGGG F % H % H N % S % H U c P c A 2 g 2 a 1
33	2350-2383	2360 2370 2380 CGCTCTTCCG CTTCCTCGCT CACTGACTGC CTG M % B H N % B H L c V A 1 t 1 1
36	1419-1455	1420 1430 1440 1450 C GCATCTCGGG CAGCGTTGGG TCCTGGCCAC GGGTG % A B A B BH MH % V B V S AA SH t A V A T LE TA g 1 1 2 1 13 11
40	776-816	780 790 800 810 CGCT CTGGGTCATT TTCGGCGAGG ACCGCTTTCG CTGGAG % M A% H % N V% H c L Ac A c 1 2c 1
54	495-549	500 510 520 530 540 CGCTT GTTTCGGCGT GGGTATGGTG GCAGGCCCCG TGGCCGGGGG ACTGTTGGG S GH N % NH A DA C % AH U IE I c RA 1 23 1 t 11
60	435-495	440 450 460 470 480 490 CGCCT ATATCGCCGA CATCACCGAT GGGGAAGATC GGGCTCGCCA CTTCGGGCTC ATGAG % % % % % H H HH % % % % % G G AH t c t t t I I EA a a g g c 2 2 21
62	1357-1419	1360 1370 1380 1390 1400 1410 CGC AAACCAACCC TTGGCAGAAC ATATCCATCG CGTCCGCCAT CTCCAGCAGC CGCACGCGG % % F % % B F FF H % % N % % B N NN H t t U c t V U UU A a g 2 g R 1 1 21 1

Table A-7 (continued)

Fragment Length (BP)	Boundaries (BP)	Nucleotide Sequence (top strand only) and Sites
67	2653-2720	2660 2670 2680 2690 2700 2710 2720 CGCTCTC CTGTTCCGAC CCTCCCCCTT ACCGGATACC TGTCCGGCTT TCTCCCTTCG GGAAGCGTGG % F H % % HH % N P % % AH c U A t c EA c 1 2 a a 21
75	701-776	710 720 730 740 750 760 770 CGCGGGGCA TGACTATCGT CCCCCGCACTT ATGACTGTCT TCTTTATCAT GCAACTCGTA GCACAGGTGC CGGCAG F % % % F % % % % % H NH B HH N % % % N % % % % G AP B AH U c t c U c c t c I EA V EA 2 t q g 1 t a a a 1 12 1 21
83	1645-1728	1650 1660 1670 1680 1690 1700 1710 1720 CGCCC TGCACCATTA TGTTCCGGAT CTGCATCGCA GGATGCTGCT GGCTACCCTG TGGAACACCT ACATCTGTAT TAACGAAG H % % % % B % HH O % % % % B % AC A t t t t V t EA 2 c g g 1 g 21
93	3496-3589	3500 3510 3520 3530 3540 3550 3560 3570 3580 CGCA GAAGTGGTCC TGCAACTTTA TCCGCCTCCA TCCAGTCTAT TAATTGTTGC CGGGAAGCTA GAGTAAGTAG TTCGCCAGTT AATAGTTTG A % M % % N A MH V % N % % C L SH A t L t c I U TA 2 a 1 g t 1 1 11
100	2720-2820	2730 2740 2750 2760 2770 2780 2790 2800 2810 2820 CGCTTTCTCA ATGCTCACGC TGTAGGTATC TCAGTTCGGT GTAGGTCGTT CGCTCCAAGC TGGGCTGTGT GCACGAACCC CCCGTTCAGC CCGACCGCTG % D % H % N B H % D % G % S B H t E c I c P V A a 1 c 1 c 2 1 1
103	2076-2179	2080 2090 2100 2110 2120 2130 2140 2150 2160 2170 CGCG TTTCGGTGAT GACGGTGAAA ACCTCTGACA CATGCAGCTC CCGGAGACGC TCACAGCTTG TCTGTAAGCG GATGCCGGGA GCAGACAAGC CCGTCAGGG F % % M % N B A N H % % A % % N H N % % N % $ B L C P % % L % % C H U t c L c P V U I A C C U C + I c g c A 2 g g 1 a 1 1 1 1 2 G C 1 a g 1 a g 1
109	2994-3103	3000 3010 3020 3030 3040 3050 3060 3070 3080 3090 3100 CGCTCT GCTGAAGCCA GTTACCTTCG GAAAAAGAGT TGGTAGCTCT TGATCCGGCA AACAAACCAC CGCTGGTAGC GGTGGTTTTT TTGTTTGCAA GCAGCAGATT ACG A % H B % FH L % P B % NH U t A V t UA 1 c 2 P 2 1 t 21
131	816-947	820 830 840 850 860 870 880 890 900 910 920 930 940 CGCG ACGATGATCG GCCTGTCCCT TGCGGTATTC GGAATCTTGC ACGCCCTCGC TCAAGCCTTC GTCACTGGTC CCGCCACCAA ACGTTTCGGC GAGAAGCAGG CCATTATCGC CGGCATGGCG GCCGACG F % % % H % % % % % A HH % BH FXH % FH N % % % A % % % % % F AA % GP NMA % NH U c t t E c t L c A EE t LA UAE c UA 2 g g c 3 a t 1 g 2 13 1 12 133 g 21
132	101-233	110 120 130 140 150 160 170 180 190 200 210 220 230 CGCTCATCG TCATCCTCGG CACCGTCACC CTGGATGCTG TAGGCATAGG CTTGGTTATG CCGGTACTGC CGGGCCTCTT GCGGGATATC GTCCATTCCG ACAGCATCGC CAGTCACTAT GGCGTGCTGC TAG % % % N H % B % H R N SH M % % % % % % % B HH % % % N G I c S T P S C AA N % % % % % % B AH t c t L I c T 1 A A I UE L t t c c t c V EA g g g 1 1 g 1 g 2 1 1 13 1 a a g a g t 1 21

METHYLASE SEQUENCE SPECIFICITY AND SITE PREFERENCE DETERMINATION



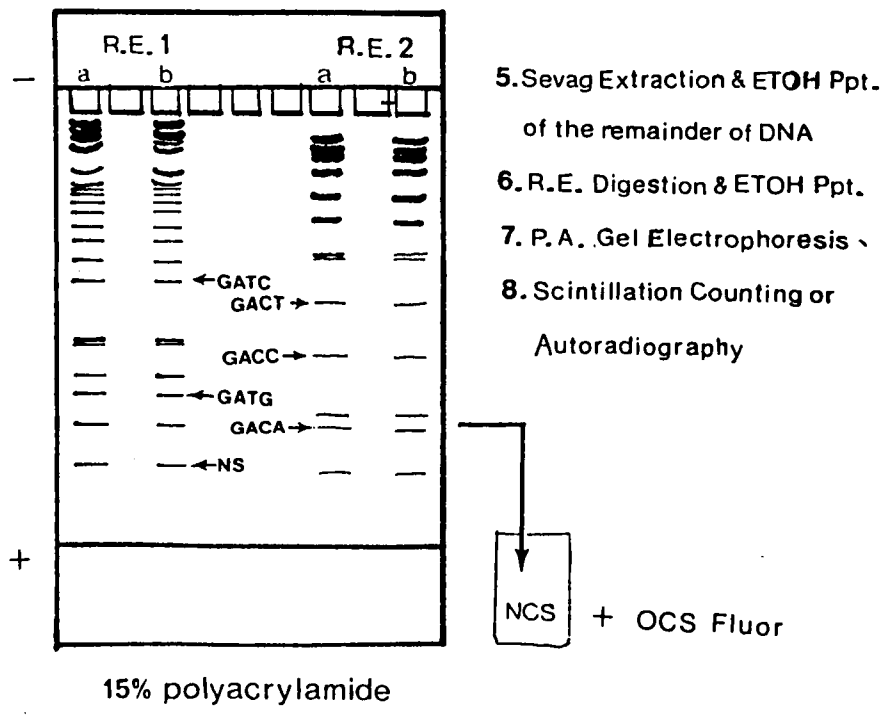


Figure A-1. Methylase sequence specificity and site preference determination.

[DNA or GATC site "A"s] and [SAM] CALCULATIONS

λ genome = 116 GATC sites/λ molecule

48,502 bp/λ genome x 650 dalton (gm)/mole-bp = 31,526,300 (31.5 Mdal)

$$\frac{1 \times 10^{-6} \text{ gm (1 } \mu\text{g) } \lambda \text{ DNA}}{31.5 \times 10^{6} \text{ gm/mole } \lambda \text{ DNA}} = 3.17 \times 10^{-14} \text{ moles/}\mu\text{g } \lambda$$

3.17×10^{-14} x 116 GATC sites/λ molecule = 3.7×10^{-12} moles-sites/μg

3.7×10^{-12} moles-sites x 2"A"s/GATC site = 7.4×10^{-12} moles-"A"s/μg

7.4 x 10 pmoles-"A"s/μg λ x 78.0×10^{-9} Ci/pmole

= 5.75×10^{-7} Ci/μg λ x 2×10^{12} dpm/Ci

= 1.15×10^{6} dpm/μg λ = 1,150,000 dpm/μg λ

5 μg λ/100 μl rxn = 18.5 pmoles GATC sites/100 μl

$$= \frac{18.5 \text{ pmoles sites}}{100 \text{ } \mu\text{l}} \times \frac{10^{4} - 100 \text{ } \mu\text{l}}{\text{liter}} = \frac{185 \text{ nmoles}}{\text{liter}}$$

= 185 nM x 2"A"s/GATC site = 370 nM "A"s

Actually 37 pmoles "A"s/5 μg λ = 740 fpmoles (10^{-15} moles)/100 ng

740 fpmoles x 780 fpCi/fpmole x 2×10^{-3} dpm/fpCi = 1054 dpm

1054 dpm x 0.3 (c.e.) = 346 cpm

pBR322 = 22 GATC sites/molecule

4,362 bp/molecules x 650 dalton (gm)/mole-bp

= 2.84×10^{6} dalton/mole-plasmid molecules

$$\frac{1 \times 10^{-6} \text{ gm (1 } \mu\text{g) pBR322 DNA}}{2.84 \times 10^{6} \text{ gm/mole-plasmid molecules}} = 3.52 \times 10^{-13} \text{ moles/}\mu\text{g pBR322}$$

3.52×10^{-13} moles/μg pBR322 x 22 GATC sites/pBR322 molecule

$= 77.5 \times 10^{-13}$ moles GATC sites/μg pBR322 molecules

77.5×10^{-13} moles GATC sites/μg x 2"A"s/symetric GATC site

$= 155 \times 10^{-13}$ moles GATC "A"s/μg pBR322

15.5 pmoles "A"s/μg x 78.0×10^{-9} Ci (SAM-2H) /pmole

$= 1.2 \times 10^{-6}$ Ci/μg pBR322 x 2×10^{12} dpm/Ci

$= 2.4 \times 10^{6}$ dpm/μg = 2,400,000 dpm/μg pBR322

2.5 μg pBR322 x 155×10^{-13} moles GATC "A"s/μg = 3.875×10^{-10} moles GATC "A"s

3.875×10^{-10} moles GATC "A"s/400 μl x 2.5×10^{3} (400 μl)/liter

$= 9.68 \times 10^{-7}$ moles GATC "A"s/liter = 968 nM GATC "A"s

10 μg pBR322 x 77.5×10^{-13} moles GATC sites/μg pBR322

$= 7.75 \times 10^{-11}$ moles GATC sites/10 μg

$$\frac{77.5 \text{ pmoles GATC sites/10 } \mu\text{g}}{22 \text{ sites/molecule}}$$

= 3.52 pmoles of one GATC sites/10 μgx 2"A"s/site

= 7.04 pmoles of methyl "A"s (GATC)/10 μg site

7.04 pmoles x 67.2 nCi/pmole (SAM 4H = 67.2 Ci/mmole)

$= 474$ nCi x 2×10^{3} dpm/nCi = 9.5×10^{5} dpm/10 μg site

= 950,000 dpm x 0.20 c.e. = 190,000 cpm/site "A"s

= 95,000 cpm/½ site/10 μg

7.04 pmoles x 14.1 nCi/pmole (SAM3L = 14.1 Ci/mmole)

= 99.3 nCi x 2 x 10^3 dpm/nCi = 19.85 x 10^4 dpm/10 µg site

= 198,500 dpm x 0.20 c.e. = 39,700 cpm/site

= 19,850 cpm/site

7.04 pmoles x 4.7 nCi/pmole (sp. act 3 µM SAM rxn)

= 33.0 nCi x 2 x 10^3 dpm/nCi = 66.0 x 10^3 dpm

= 66,000 dpm x 0.20 c.e. = 13,200 dpm/10 µg GATC site

= 6,600 dpm/½ site/10 µg

7.04 pmoles x 0.94 nCi/pmole (sp. act. 15 µM SAM rxn)

= 6.6 nCi x 2 x 10^3 dpm/nCi = 13.2 x 10^3 dpm

= 13,200 dpm x 0.20 c.e. = 2640 dpm/10 µg site "A"s

= 1320 dpm/½ site/10 µg

pBR322 has 6 CCA/TGG sites/molecule; thus expect:

$\frac{6 \text{ CCA/TGG sites}}{22 \text{ GATC sites}}$ (0.27) x 3.52 pmoles of GATC site (one)/10 µg DNA

= 0.95 pmoles dcm sites/10 µg pBR322 DNA

*Note dcm methylates both internal cytosines (one/strand)

0.95 pmoles of a site x 14.1 nCi/pmole (SAM 3L) = 13.4 nCi

13.4 nCi x 2 x 10^3 dpm/nCi = 26.8 x 10^3 dpm

= 26,800 dpm x 0.20 c.e. = 5,360 cpm x 2"C"s/site = 10,720

0.95 pmoles of a site x 67.2 nCi/pmole (SAM 4H)

= 63.8 nCi x 2 x 10^3 dpm/nCi

= 1.28 x 10^5

128,000 x 0.20 = 25,600 cpm x 2 "C"s/site = 51,200

Problems/Differences may be attributed to endogenous SAM or 18% wrong isomer or lower c.e. (SIGMA Manual catalog)

VITA

Mark M. Doolittle was born in Norwood, Massachusetts, on January 21, 1956, to Mr. Milton A. Doolittle, an industrial engineer in corporate management and Mrs. Olga H. (Freeman) Doolittle, a housewife and mother. He graduated from the Advanced Sciences Program of Simsbury High School in Connecticut in 1973. Attending Southeastern Massachusetts University (SMU) in Dartmouth, Massachusetts, he graduated with a Bachelor of Science Degree with distinction in 1977 having majored in Marine Biology. Subsequently he worked as a Research Associate in the Department of Biology at Holy Cross College in Worcester, Massachusetts, where he collaborated in the study of yeast disomes. He then attended the Department of Microbiology at the University of Tennessee, Knxoville, where the research was conducted leading to this Master's thesis. At this time he is currently enrolled in the Ph.D. program of the Molecular Biology Department of Vanderbilt University. He plans to use his education to eventually study molecular interactions in marine organisms and the effects of marine pollutants.