

Methylation by a mutant T2 DNA [*N*⁶-adenine] methyltransferase expands the usage of RecA-assisted endonuclease (RARE) cleavage

Irina Minko, Stanley Hattman¹, R. Stephen Lloyd and Valeri Kossykh*

Sealy Center for Molecular Science, University of Texas Medical Branch, 5.144 Medical Research Building, Galveston, TX 77555-1071, USA and ¹Department of Biology, University of Rochester, Rochester, NY 14627-0211, USA

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ABSTRACT

Properties of a mutant bacteriophage T2 DNA [*N*⁶-adenine] methyltransferase (T2 Dam MTase) have been investigated for its potential utilization in RecA-assisted restriction endonuclease (RARE) cleavage. Steady-state kinetic analyses with oligonucleotide duplexes revealed that, compared to wild-type T4 Dam, both wild-type T2 Dam and mutant T2 Dam P126S had a 1.5-fold higher k_{cat} in methylating canonical GATC sites. Additionally, T2 Dam P126S showed increased efficiencies in methylation of non-canonical GAY sites relative to the wild-type enzymes. In agreement with these steady-state kinetic data, when bacteriophage λ DNA was used as a substrate, maximal protection from restriction nuclease cleavage *in vitro* was achieved on the sequences GATC, GATN and GACY, while protection of GACR sequences was less efficient. Collectively, our data suggest that T2 Dam P126S can modify 28 recognition sequences. The feasibility of using the mutant enzyme in RARE cleavage with *BclI* and *EcoRV* endonucleases has been shown on phage λ DNA and with *BclI* and *DpnII* endonucleases on yeast chromosomal DNA embedded in agarose.

INTRODUCTION

The mapping and subcloning of large genomes could be greatly simplified and accelerated by the development of methods to catalyze sequence-specific cleavage of DNA at a limited number of all the possible site-specific locations within a genome. As currently developed, most methods for sequence-specific cleavage of large DNAs are severely limited because of the high frequency of cleavage sites. However, several excellent proposals have been suggested for decreasing the frequency of restriction and predetermining the sites of cutting by sequence-specific endonucleases (ENases) using cognate DNA methyltransferases (MTases) (1,2).

One such method, called RecA-assisted restriction endonuclease (RARE) cleavage by Ferrin and Camerini-Otero (3) and RecA Achilles cleavage (RecA-AC) by Koob *et al.* (4), is

a simple general method that allows selective DNA cleavage at a single predetermined restriction site. This technique is based on the ability of the RecA protein to bind to an oligonucleotide in the presence of a non-hydrolyzable ATP analog, which results in formation of a stable complex. This preformed nucleoprotein complex binds specifically to homologous duplex DNA to form a triple-stranded DNA-protein complex. The triple helix complex protects DNA from methylation by the cognate MTase, but not the remaining double-helical regions. After complex dissociation and MTase inactivation, DNA cleavage by the cognate ENase is limited to the site that was precluded from methylation.

Originally, RARE cleavage was proposed as a method to simplify and accelerate mapping of large genomes (3,4). Later this approach was used for human genome mapping (5–7) and was also applied to experiments on transgenic expression (8) and site-directed mutagenesis (9,10). However, in practice there are several problems limiting use of RARE cleavage as a general method: (i) the number of commercially available MTases is far lower than that of ENases; (ii) MTases must have a very high activity in order to modify all potential cleavage sites; (iii) MTases should be free of non-specific nuclease contamination, especially for modification of long DNA fragments. In this regard, it was noted that many commercially available MTases are not suitable for RARE cleavage (5,6). At present two adenine-specific MTases associated with cognate ENases (*M.EcoRI/R.EcoRI* and *M.HhaII/R.HinfI*) are applicable to RARE cleavage, but *EcoRI* is the only system that has been used (11).

In order to expand usage of the RARE method, this investigation explores the advantages of using the MTases from the closely related T-even bacteriophages T4 and T2. These MTases, which methylate the tetranucleotide palindrome GATC, differ in only three amino acids (12) and have previously been overexpressed, purified and characterized (13,14). The choice to use adenine-specific MTases in RARE technologies, instead of cytosine-specific MTases, was made because vertebrate DNAs have no methylated adenines (15). This avoids difficulties in interpretation of RARE cleavage results, which can be created using cytosine-specific MTases. At present five recognition sequences overlapping GATC are known in which adenine methylation blocks DNA cleavage by the corresponding ENases: CGATCG (*XorII*), GATC (*MboI*), GGATC(4/5) (*AlwI*), R/GATCY (*MflI*) and T/GATCA (*BclII*)

*To whom correspondence should be addressed. Tel: +1 409 772 2181; Fax: +1 409 772 1790; Email: valkoss@scms.utmb.edu

(2). T4 and T2 Dam MTases also modify some non-canonical sites containing the sequence GAY (GAT or GAC) (16,17), which in turn protects DNA against cleavage not only by ENases having GATC in the target recognition site but also by those that have GAY, e.g. *Clal*, *EcoRV* and *FokI* (14,18). There are 16 ENases known to have a GAY sequence within their recognition site that can be protected against cleavage by adenine methylation (2,19). Additionally, the T2 Dam MTase has been shown to have a 2-fold higher k_{cat} for canonical and non-canonical sites relative to T4 Dam (14).

In order to further expand the utility of the T2 and T4 MTases, strategies were designed to utilize mutants of these enzymes for the RARE technology. In this regard, Revel and Hattman (20) obtained mutants of T4 and T2 phage designated *dam^h* that are able to grow on P1 lysogens, suggesting that methylation protects phage DNA against P1 restriction of the non-canonical sequence AGACC. The nature of the *dam^h* mutation has been elucidated in both T2 and T4; it is a change of Pro126 to Ser (12). In addition, the P126S mutant has an increased ability to protect phage DNA against *HinfI* ENase (GANTC) at enzyme levels at which the parental Dam⁺ enzymes could not (21).

In this study we have investigated the relative abilities of the wild-type T4 and T2 MTases and mutant T2 Dam P126S MTase to methylate synthetic oligonucleotide duplexes containing non-canonical GAY sites. In addition, we have investigated the ability of the mutant MTases to afford protection to phage λ DNA against cleavage by ENases with GAY-containing recognition sequences. Based on the results of these experiments we tested the applicability of T2 Dam P126S for RARE cleavage of λ phage DNA in solution and dissection of yeast chromosomes embedded in agarose.

MATERIALS AND METHODS

Materials

Strains of *Escherichia coli* GM 2971 containing plasmids pSLRT4 *dam⁺*, pSLRT2 *dam⁺* and pINT2*dam*P126S (13,14) were used for enzyme purification.

Oligonucleotides were synthesized by Integrated DNA Technologies (Coraville, IA). RecA, ENases and *N*⁶-methyladenine-free λ DNA were purchased from New England Biolabs (Beverly, MA). *S*-adenosyl-L-methionine (AdoMet) and adenosine 5'-*O*-(3-thiotriphosphate) (ATP- γ -S) were obtained from Sigma. [Methyl-³H]AdoMet was purchased from NEN Life Science Products.

T2 Dam P126S purification

The T4 Dam purification protocol developed by Kossykh *et al.* (13) was modified to obtain T4 Dam, T2 Dam and T2 Dam P126S. Frozen cells (10 g) were suspended in 50 ml of PEM buffer (20 mM phosphate buffer pH 7.4, 1 mM EDTA, 7 mM 2-mercaptoethanol) with 0.1% Nonidet P-40 and 0.4 M NaCl. Lysozyme was added to a concentration of 500 μ g/ml and after a 2 h incubation at 4°C the cells were disrupted by sonication. Insoluble debris was removed by centrifugation at 100 000 *g* for 1 h. The supernatant was diluted 2-fold in PEM buffer and passed through a DEAE-cellulose (DE52) column connected to a P-11 phosphocellulose column. Proteins bound to the P11 phosphocellulose were eluted in a 120 ml NaCl gradient (200–800 mM) in PEM

buffer. Appropriate fractions were pooled, diluted 2-fold in PEM buffer and applied to a 5 ml hydroxyapatite column equilibrated in column buffer (PEM with 0.2 M NaCl). Proteins bound to the matrix were eluted with a 60 ml gradient of 20–500 mM potassium phosphate (pH 7.4) in column buffer. In order to further increase the purity of the enzyme, one additional step in the purification was added. After elution of the proteins from the hydroxyapatite column, appropriate fractions were collected, dialyzed against PEM buffer with 0.1 M NaCl and applied to a 5 ml High S column (Bio-Rad). Proteins that bound to the matrix were eluted with a 40 ml gradient of 0.1–1.0 M NaCl in PEM buffer. The enzyme was dialyzed against PEM with 0.1 M NaCl and 50% glycerol. Protein concentration was measured with the Bio-Rad Dye Reagent and purity of preparations was tested by SDS-PAGE (22).

Steady-state studies

Methyl transfer assays were carried out as described (13). Assay mixtures (50 μ l) contained 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM DDT, 0.2 mg/ml BSA and 0.55 μ M [³H-CH₃]AdoMet. For determination of steady-state kinetic parameters 3–13 nM MTase and various concentrations of substrate were used. All 24mer oligonucleotide duplexes studied had the same general structure



except for individual base substitutions in the target GATC site (Table 1, indicated by italics). Oligonucleotide concentrations were determined spectrophotometrically from molar extinction coefficients of individual oligonucleotides and their sequences. Oligonucleotide duplexes were obtained by heating and annealing mixtures of equimolar amounts of complementary individual oligonucleotides. Kinetic parameters were calculated using the Enzyme Kinetics program from Trinity Software.

Testing the effect of adenine methylation on cleavage by restriction ENases

The methylation mixture (20 μ l) contained 20 mM Tris-HCl pH 8.0, 50 mM NaCl, 7 mM 2-mercaptoethanol, 10 mM EDTA, 0.1 mg/ml BSA, 1 μ g *N*⁶-methyladenine-free λ DNA, T2 Dam P126S MTase and 50 μ M AdoMet. One unit of MTase was defined as that amount of enzyme required to protect all GATC sites in 1 μ g λ DNA in 1 h at 37°C. The amount of enzyme added depended on the DNA sequence to be protected; further details will be addressed in Results. After 1 h incubation at 37°C the reaction was stopped by heat inactivation of the MTase at 65°C for 15 min. Five to ten units of ENase were added. The concentrations of Tris, MgCl₂, NaCl and BSA were pre-adjusted for optimum cleavage conditions and the reaction mixture incubated for an additional 1 h at the temperature recommended by the enzyme supplier. The reaction products were separated by electrophoresis through an agarose gel and visualized by ethidium bromide staining. Control samples of λ DNA were subjected to the same manipulations, but with no MTase added. Unmethylated and undigested λ DNA was used as a molecular length marker. Methylated, undigested λ DNA was used to assay for possible degradation during the methylation reaction with T2 Dam P126S.

Table 1. T4 Dam, T2 Dam and T2 Dam P126S steady-state kinetic parameters determined with synthetic oligonucleotides

Structure of recognition site	T4 Dam			T2 Dam			T2 Dam P126S		
	K_m (nM)	k_{cat} ($s^{-1} 10^{-3}$)	Relative k_{cat} (%)	K_m (nM)	k_{cat} ($s^{-1} 10^{-3}$)	Relative k_{cat} (%)	K_m (nM)	k_{cat} ($s^{-1} 10^{-3}$)	Relative k_{cat} (%)
GATC	10.3 (0.6)	24.80 (4.6)	100	13.9 (1.5)	41.90 (4.3)	169	11.0 (1.6)	39.30 (3.5)	158
GATT	4.4 (1.8)	5.70 (0.4)	23	8.6 (2.4)	9.10 (1.2)	37	16.3 (2.6)	13.90 (1.1)	56
GATA	5.7 (0.9)	5.80 (0.7)	23	7.1 (0.5)	9.10 (0.4)	37	12.2 (0.8)	15.30 (1.8)	62
GATG	5.0 (0.8)	12.40 (0.4)	50	11.7 (4.1)	19.70 (0.3)	79	17.5 (0.6)	26.10 (1.3)	105
GACC	24.6 (4.4)	7.50 (1.0)	30	14.6 (3.9)	7.90 (0.6)	32	13.2 (3.6)	7.30 (2.0)	29
GACT	21.0 (1.3)	1.24 (0.29)	5	61.9 (13.9)	2.10 (0.6)	8	15.5 (2.4)	8.50 (1.5)	34
GACA	3.2 (0.6)	0.13 (0.07)	0.5	15.6 (1.7)	0.11 (0.06)	0.4	10.5 (0.1)	0.46 (0.09)	1.9
GACG	3.2 (0.4)	0.10 (0.02)	0.4	5.9 (1.9)	0.14 (0.03)	0.6	10.0 (2.3)	0.36 (0.04)	1.5

Standard error values are in parentheses.

RARE cleavage of λ DNA

The RARE cleavage protocol was a combination of the original procedure by Ferrin and Camerini-Otero (3) and that developed by Koob *et al.* (4). The oligonucleotide was a 40mer corresponding to the phage λ DNA sequence positions 8816–8856: 5'-ATGAGCGATATCCCGGCACTGTCAGATTTGATCAC-CAGTA-3'. It contains recognition sites (underlined) for *BclI* (TGATCA) and *EcoRV* (GATATC). The solution for formation of the oligonucleotide:RecA complex (9 μ l) contained 25 mM Tris-acetate pH 8.0, 1 mM Mg acetate, 60 ng oligonucleotide and 1.5 μ g RecA. [The working ratio of oligonucleotide:RecA was experimentally determined using the approach described by Koob *et al.* (4).] After incubation of the mixture at 37°C for 1 min, an aliquot of 1 μ l of 0.4 mM ATP- γ -S was added and the reaction was allowed to proceed for another 10 min. To form the oligonucleotide:RecA: λ DNA complex the buffer was adjusted to 25 mM Tris-acetate pH 8.0, 4 mM Mg acetate, 10 mM DDT, 0.1 mg/ml BSA, 0.5 mM spermidine and 1 μ l (0.5 μ g) of λ DNA was added, bringing the final volume of the mixture to 28 μ l. After 30 min at 37°C an aliquot of 1 μ l of T2 Dam P126S and 1 μ l of 1.5 mM AdoMet were added. Conditions of methylation and subsequent ENase cleavage are described above.

RARE cleavage of yeast genomic DNA embedded in agarose

Saccharomyces cerevisiae (strain YPH80, obtained from Dr P. Goldfarb, New England Biolabs) was used to prepare yeast genomic DNA embedded in agarose. For preparation of yeast genomic DNA embedded in 0.7% agarose plugs we used the kit and protocol from Bio-Rad. The oligonucleotide used for RARE cleavage at the overlapping *BclI* and *DpnII* sites (underlined) was 60 nt in length: 5'-GCAAGGAGTTATATT-TTGGAACGATTGATCAATATTTAAAGATTACTTCAA-AGGAAGAT-3'. RARE cleavage was performed on DNA in 50 μ l plugs according to detailed protocols and tips for troubleshooting (23,24). Immediately prior to use the agarose plugs were equilibrated at room temperature in RARE cleavage buffer (25 mM Tris-acetate, pH 8.0, 4 mM Mg acetate, 0.4 mM DTT and 5 mM spermidine). The components added were 15 μ l of 5 \times RARE buffer, 30 μ g RecA protein, 10 μ l of 10 mM ATP- γ -S and oligonucleotide (10–100 ng oligonucleotide/ μ g RecA protein were used to optimize the concentration

for efficient cleavage) and water to a total volume of 200 μ l. After 15 min at 37°C, 50 μ l of agarose plug and 10 μ l of 2 mg/ml acetylated BSA were added and the mixture incubated further at 37°C for 30 min. Then 2 μ l of 4.6 mM AdoMet and 10–50 U MTase were added and incubation continued for 120 min at 37°C. Methylation was stopped by adding 200 μ l of 50 mM EDTA and 2% SDS. After 15 min at 50°C the plugs were washed and equilibrated in restriction buffer with 200 μ l final exchange, then 20 U restriction endonuclease was added for digestion for 1 h at 37°C. The reaction was stopped and the products were examined by pulsed field gel electrophoresis (PFGE), performed in a CHEF-DR II apparatus (Bio-Rad) at 15°C with 200 V applied, switch times of 60 s for 15 h and then 90 s for 9 h. The buffer used was 0.5 \times TBE.

RESULTS AND DISCUSSION

Steady-state kinetics

Standard analyses of the dependence of substrate concentration on velocity were used to obtain the catalytic rate and Michaelis constants and to calculate specific coefficient values from steady-state assays. In preliminary experiments using the canonical duplex the K_m for AdoMet was calculated to be 0.30 μ M. Thus, to determine kinetic parameters for different duplexes (summarized in Table 1) AdoMet was used at a concentration of 0.55 μ M and the kinetics of methyl group transfer were linear under steady-state conditions for T4 Dam, T2 Dam and T2 Dam P126S. As expected, methylation was most efficient on canonical GATC substrates for all enzymes; additionally, sequences containing GATN and GACC were efficiently methylated by all enzymes. In contrast, T2 Dam and T4 Dam poorly methylated GACT, while T2 Dam P126S was very efficient on this sequence. Sequences containing GATR were somewhat more efficient for T2 Dam P126S, but k_{cat} was still reduced ~100-fold from the canonical site.

Compared with T4 Dam, the T2 Dam enzyme had an ~2-fold higher k_{cat} for most of the DNA substrates, as well as a slightly higher K_m . With the canonical GATC-containing substrate, T2 Dam P126S had similar kinetic parameters to the wild-type T2 Dam. In contrast, with most non-canonical substrates T2 Dam P126S had a 2- to 4-fold higher k_{cat} and K_m compared to T4 Dam. Based on these data, T2 Dam P126S was chosen for

Table 2. Recognition sequences containing GAY triplets and blocked by overlapping Dam methylation

ENase group	No.	Recognition sequence	Restriction enzymes
I	1	/GATC ^a	<i>Bsp143I</i> ^b , <i>BstEIII</i> ^b , <i>DpnII</i> ^{b,c} , <i>MboI</i> ^b , <i>MmeII</i> ^b , <i>NdeII</i> ^b
	2	CGAT/CG ^a	<i>XorII</i> ^b
	3	T/GATCA ^d	<i>AtuCI</i> ^b , <i>BclII</i> ^{b,c} , <i>BspXII</i> ^b , <i>BstGI</i> ^b , <i>CpeI</i> ^b
	4	GGATC(4/5) ^a	<i>AlwI</i> ^{b,c} , <i>BinI</i> ^b
	5	R/GATCY ^a	<i>MflI</i> ^b
II	6	(5/6)GGATAC ^d	<i>BciVI</i> ^e
	7	GAT/ATC ^d	<i>EcoRV</i> ^{b,c}
	8	(5/4)GACTC ^d	<i>PleI</i> ^e
	9	(5/1)GAGACC ^d	<i>BsaI</i> ^e
	10	AGACC ^a	<i>EcoPI</i> ^b
	11	GACCGA(11/9) ^a	<i>TagII</i> ^b
III	12	GGATG(2/0) ^d	<i>BstF5I</i> ^f
	13	GGATG(9/13) ^d	<i>FokI</i> ^{b,c}
	14	AT/CGAT ^a	<i>BanIII</i> ^b , <i>BseCI</i> ^b , <i>BsiXI</i> ^b , <i>Bsp106I</i> ^b , <i>BspDI</i> ^{b,c} , <i>BspXI</i> ^b , <i>Bsu15I</i> ^b , <i>Clal</i> ^{b,c}
	15	(9/5)GATGC ^a	<i>SfaNI</i> ^{b,e}
	16	GATNN/NNATC ^a	<i>BsaBI</i> ^{b,c} , <i>Bsh1365I</i> ^b , <i>BsiBI</i> ^b , <i>BsrBRI</i> ^g , <i>MamI</i> ^b
	17	GGATG(10/14) ^a	<i>StsI</i> ^b
IV-A	18	GACGT/C ^d	<i>AatII</i> ^e
	19	GACN/NNGTC ^d	<i>PflFI</i> ^e , <i>Tth111I</i> ^e
	20	GACNN/NNGTC ^d	<i>PshAI</i> ^e
	21	GACNNN/NNGTC ^d	<i>AhdI</i> ^e
	22	GACNNNN/NNGTC ^d	<i>DrdI</i> ^e
IV-B	23	GAAGAC(2/6) ^d	<i>BbsI</i> ^e
	24	GACGC(5/10) ^d	<i>HgaI</i> ^e
	25	GGGAC(10/14) ^d	<i>BsmFI</i> ^e
IV-C	26	(5/1)GAGAC ^a	<i>Alw26I</i> ^b
	27	(5/1)GAGACG ^a	<i>Esp3I</i> ^b
	28	G/TCGAC ^a	<i>Rrh4273I</i> ^b , <i>Sall</i> ^b

^aSequences that are predicted to belong to the mentioned group.

^bMcClelland *et al.* (2)

^cSensitivity to adenine methylation was shown using T4 Dam (13).

^dSequences that were tested in this paper.

^eSensitivity to adenine methylation was shown in this paper.

^fDegtyarev *et al.* (19).

^gPromega catalog.

protection of GAY-containing sites against cleavage by restriction ENases and for RARE cleavage.

Methylation by T2 Dam P126 protects GAY-containing sequences against cleavage by restriction ENases

It is known that methylation of *N*⁶-adenine in 16 different GAY-containing recognition sequences inhibits DNA cleavage by certain ENases (2,19). A list of these sequences is presented in Table 2. Additionally, a search of the Rebase Enzyme Data Base (New England Biolabs) revealed 12 commercially available enzymes with recognition sequences that overlap GAY and different from those cited above. Using

T2 Dam P126S we tested the sensitivity to adenine methylation of this group, namely *AatII*, *AhdI*, *BbsI*, *BciVI*, *BsaI*, *BsmFI*, *DrdI*, *HgaI*, *PflFI*, *PleI*, *PshAI* and *Tth111I* (an isoschizomer of *PflFI*). To verify that the conditions of T2 Dam P126S methylation could afford protection, ENases *BclII*, *BstF5I*, *EcoRV* and *FokI* were included, since they have been shown previously to be inhibited by the presence of *N*⁶-methyladenine in the cleavage site. The results in Figure 1 are representative of the analyses for methylation protection against cleavage by some ENases. All the ENases tested in this particular experiment had a restricted cleavage of DNA that had been methylated by T2 Dam P126S. After a series of

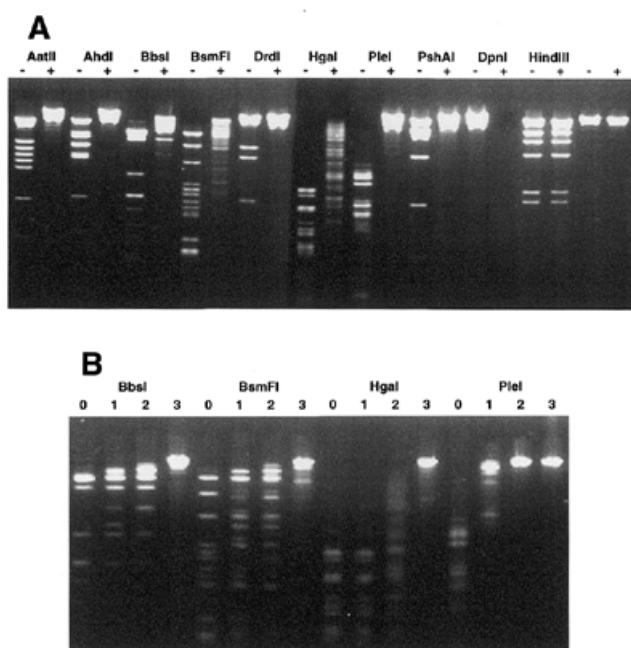


Figure 1. Methylation by T2 Dam P126S MTase alters the sensitivity of phage λ DNA to restriction ENase. Methylation and the cleavage assay were performed under the conditions described in Materials and Methods. ENases are designated at the top. (A) Phage λ DNA was methylated (+) or not (-) with 400 U T2 Dam P126S MTase and then incubated with the designated ENases. (B) The amounts of T2 Dam P126S MTase in the incubation mixture were as follows: lane 0, 0 U; lane 1, 6 U; lane 2, 60 U; lane 3, 600 U.

experiments varying the MTase concentration it was determined that the ENases tested could be divided into five distinct groups according to the level of MTase required to block cleavage completely. (I) This group has GATC in its recognition sequence, so it was not surprising that loss of DNA cleavage capability was achieved at a relatively low concentration (1–5 U) of MTase for *DpnII* and *BclI*. (II) For non-canonical sequences GATA or GACY ~40 U T2 Dam P126S were required to protect against cleavage by *BciVI*, *BsaI*, *EcoRV* and *PfiI*. These results are also consistent with steady-state kinetic analyses of T2 Dam P126S methylation of synthetic duplexes, although the k_{cat} values with the GATA- and GACY-containing substrates were 2–5 times lower than with the canonical GATC-containing substrate (Table 1). (III) *BstF51* and *FokI*. About three times more T2 Dam P126S (~120 U) was needed to fully protect non-canonical GATG sites in λ DNA relative to the amount needed to protect GATA sites. According to steady-state kinetic analysis the k_{cat} for the GATG-containing duplex was comparable with the k_{cat} for the GATA-containing substrate. However, the apparent difference can be explained by the fact that these two sequences are present in λ DNA at different frequencies. (IV) This group of enzymes contain GACX in their recognition sequence. Full protection of GACR or GACC sequences against *AatII*, *AhdI*, *DrdI*, *PfiI*, *PshAI* and *Tth111I* required ~400 U T2 Dam P126S. Poor protection against cleavage by *AatII* suggests that GACR sites were poor substrates for T2 Dam P126S methylation. This notion was confirmed by steady-state kinetic analysis of

GACR-containing synthetic duplexes, namely the k_{cat} values were ~100 times lower than k_{cat} for a duplex with a canonical GATC site. Five ENases (*AhdI*, *DrdI*, *PfiI*, *PshAI* and *Tth111I*) mentioned in this group have a GACX sequence in their recognition site. Obviously, efficacy of DNA protection by T2 Dam P126S against cleavage by these ENases depends on which base, Y or R, is in the fourth position. (V) *BbsI*, *BsmFI* and *HgaI*. Methylation of λ DNA with up to 1000 U T2 Dam P126S was sufficient to fully block the action of *BsmFI* and *HgaI* ENases, which recognize sequences containing GACG or some subset of GACX.

Thus, all the ENases were inhibited by T2 Dam P126S methylation of their DNA recognition sequences. Based on these results, it is possible to add 12 more members to the list of the GAY-containing recognition sequences in which adenine methylation blocks ENase cleavage. These data also show that any GAY site is an appropriate *in vitro* substrate for T2 Dam P126S methylation, although GACR sites are methylated very slowly. Finally, these results allow one to select ENases for RARE cleavage experiments.

RARE cleavage of phage λ DNA

In order to demonstrate the utility of T2 Dam P126S for use in a RARE cleavage assay, a 40mer synthetic oligonucleotide (corresponding to the phage λ DNA sequence 8816–8856) was used for strand invasion. This DNA contains both a *BclI* site and an *EcoRV* site, thus it provides an opportunity for a direct comparison of two modification systems, the canonical T2 Dam P126S/*BclI* and non-canonical T2 Dam P126S/*EcoRV*.

To develop the T2 Dam P126S/*BclI* RARE cleavage protocol, a number of parameters were tested: concentrations of MTase and RecA proteins, the RecA:oligonucleotide ratio and duration of the methylation reaction. In our hands the optimal amount of RecA was shown to be 1.5 μ g/60 ng oligonucleotide. Increasing the RecA concentration resulted in incomplete methylation (data not shown), presumably because of non-specific RecA binding to λ DNA. Determination of the optimal RecA:oligonucleotide ratio is a standard procedure required for optimal RARE cleavage. To determine this ratio we carried out the titration reaction according to Koob *et al.* (4) with 1.5 μ g RecA protein and 30, 60, 120, 180 and 240 ng oligonucleotide. Sixty nanograms of the oligonucleotide was shown to be optimal both to provide protection of the targeted restriction site and to avoid non-specific protection of other sites (data not shown). Experiments to determine the amount of T2 Dam P126S sufficient for full methylation under RARE cleavage conditions showed that the MTase had the same high activity and stability as under standard optimal conditions (data not shown). Three units of the enzyme and a 30 min reaction time is recommended for methylation of canonical GATC sites. These results clearly demonstrate that T2 Dam P126S, in combination with methyladenine-sensitive, GATC-recognizing ENases, was perfectly suited for the RARE cleavage technique.

Figure 2 shows a test of the applicability of T2 Dam P126S to RARE cleavage using *EcoRV*, which has a non-canonical Dam methylation site within the ENase cleavage sequence. Having experimentally determined the optimal RecA:oligonucleotide ratio for the canonical GATC sequence, a time course T2 Dam P126S/*EcoRV* RARE cleavage experiment was performed with varying MTase concentrations (Fig. 2). Again, the amount of T2 Dam P126S sufficient for complete

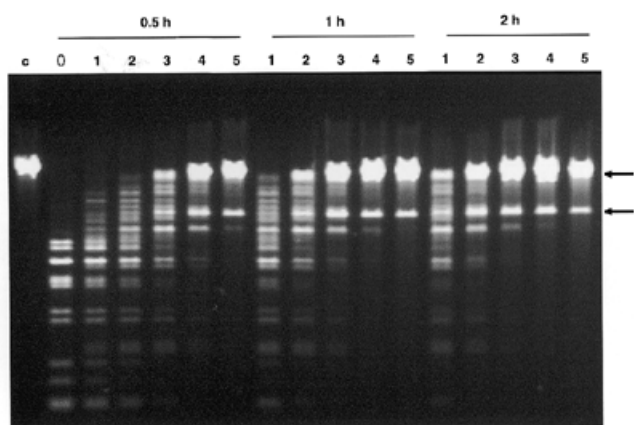


Figure 2. RARE cleavage of phage λ DNA with *EcoRV*. The conditions are described in Materials and Methods. The RecA:oligonucleotide (40mer) duplex ratio was 1.5 μ g:60 ng. Duration of the methylation reactions was 0.5, 1 or 2 h as shown. The amounts of T2 Dam P126S MTase in the incubation mixture were as follows: lane 0, no MTase; lane 1, 2.5 U; lane 2, 5 U; lane 3, 10 U; lane 4, 20 U; lane 5, 40 U. Lane c, λ DNA. Arrows show localization of products of RARE cleavage of λ DNA.

protection was close to that found in preliminary experiments on methylation (namely 40 U in a 60 min reaction). These data indicate that a combination of T2 Dam P126S with the non-canonical site-recognizing ENase *EcoRV* can be successfully applied to RARE cleavage.

RARE cleavage of yeast genomic DNA

To determine whether large DNA molecules methylated with T2 Dam 126S MTase can be effectively analyzed by RARE cleavage whole chromosomes of the yeast *S.cerevisiae* (16 linear chromosomes and a total genome size of 11 Mb) were used as a model system. To protect a specific site on chromosome XVI we added a 60mer oligonucleotide (containing *BclII* and *DpnII* sites) that was homologous to the sequence 531716–531776. Methylation of yeast chromosomes in the presence of oligonucleotide and RecA, and subsequent digestion with *BclII* or with *DpnII*, resulted in cleavage of chromosome XVI at a single site to produce two fragments (Fig. 3). Preliminary RARE experiments were performed to optimize the oligo:RecA ratio to form a complex, to protect the targeted restriction sites and to eliminate non-specific protection of other sites. We found that efficient cleavage occurred at a molar stoichiometry of 3–5 oligonucleotide bases per RecA protein. This is close to what we had observed for RARE cleavage of phage λ DNA. In these experiments the ratio of RecA to oligonucleotides is very important. RecA has to saturate the oligonucleotides, but there should be no excess RecA remaining, as that could promote non-specific protection against MTase, leading to non-specific cuts. Non-specific binding of RecA to DNA can be eliminated either by titrating the oligonucleotide with RecA protein (23,24) or by adding a non-specific oligonucleotide, such as oligo(dT) (4). These aspects of the procedure were stressed by Szybalski in his review (25). In this regard, Koob *et al.* (4) found that an additional back titration with oligo(dT) can be a more predictable and convenient

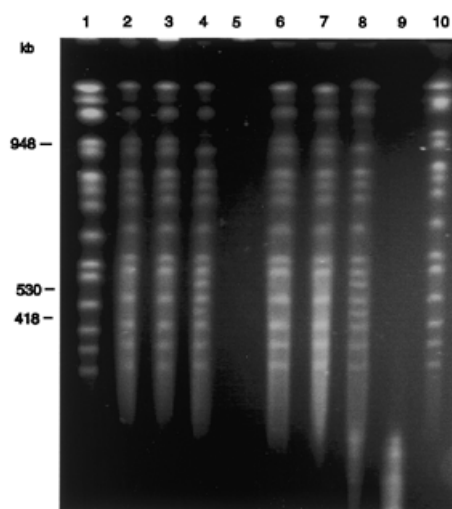


Figure 3. RARE cleavage of yeast genomic DNA. DNA in agarose plugs from yeast strain YHP80 was subjected to RARE cleavage at *BclII* and *DpnII* sites corresponding to positions 531742 and 531741 on yeast chromosome XVI. Details are given in Materials and Methods. Aliquots of 30 μ g RecA and 1150 ng 60mer oligonucleotide were used in these reactions. Treated chromosomes were analyzed by PFGE. Sizes of yeast chromosomes were from public databases (NCBI). The two RARE cleavage products have molecular sizes of 530 and 418 kb. The yeast chromosomes and their products were visualized by staining with ethidium bromide. Lanes 1 and 10 show control chromosomes without treatment. Chromosomes were incubated with the following enzyme(s)/oligo: lane 2, RecA/oligo; lane 3, RecA/oligo, T2 Dam P126S MTase; lane 4, RecA/oligo, T2 Dam P126S MTase and *DpnII*; lane 5, *DpnII*; lane 6, RecA/oligo; lane 7, RecA/oligo, T2 Dam P126S MTase; lane 8, T2 Dam P126S MTase and *BclII*; lane 9, *BclII*.

procedure to remove excess RecA, especially where spurious DNA bands appeared after electrophoresis.

In this study we have exploited the ability of T2 Dam P126S to methylate non-canonical GAY sites and investigated the sensitivity to adenine methylation of several ENases using λ DNA as a substrate. We also tested the applicability of T2 Dam P126S MTase to RARE cleavage, modifying the canonical sequence GATC, as well as non-canonical GAY sites. Additionally, we showed the feasibility of performing RARE cleavage, exploiting the advantage of methylation by bacteriophage T2 Dam P126S MTase. The approach of utilization of mutant MTases with extended methylation capabilities allows use of a broader range of restriction enzymes in the RARE method and can create new tools for precise physical dissection of small and large DNA molecules.

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