

# Analysis of substrate specificity of the *PaeR7* endonuclease: effect of base methylation on the kinetics of cleavage

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## ABSTRACT

In murine cells expressing the *PaeR7* endonuclease and methylase genes, the recognition sites (CTCGAG) of these enzymes can be methylated at the adenine residue by the *PaeR7* methylase and at the internal cytosine by the mouse DNA methyltransferase. Using nonadecameric duplex deoxyoligonucleotide substrates, the specificity of the *PaeR7* endonuclease for unmethylated, hemi-methylated, and fully methylated N<sup>6</sup>-methyladenine (m<sup>6</sup>A) and C<sup>5</sup>-methylcytosine (m<sup>5</sup>C) versions of these substrates has been studied. The K<sub>m</sub>, k<sub>cat</sub>, and K<sub>i</sub> values for these model substrates have been measured and suggest that fully or hemi-m<sup>6</sup>A-methylated *PaeR7* sites in the murine genome are completely protected. However, the reactivity of fully or hemi-m<sup>5</sup>C-methylated *PaeR7* sites is depressed 2900- and 100-fold respectively, compared to unmodified *PaeR7* sites. The implications of the kinetic constants of the *PaeR7* endonuclease for these methylated recognition sites as they occur in murine cells expressing this endonuclease gene are discussed.

## INTRODUCTION

The introduction of a type II restriction-modification system into mammalian cells to provide a mechanism for the resistance of infection by DNA viruses presents an intriguing possibility. We have pursued such a strategy by introducing and expressing both the *PaeR7* endonuclease and methylase genes in murine host cells (1,2). Using murine cells exhibiting high levels of the *PaeR7* methylase as host cells, 60% of the clones isolated from transfections performed with vectors containing the mouse metallothionein-regulated *PaeR7* endonuclease gene expressed the *PaeR7* endonuclease (2). However, such transfected cells, expressing both the methylase and endonuclease, were incapable of blocking infection by Herpes simplex virus type 1 (strain KOS) or Vaccinia virus (2). Although the cause for this lack of viral restriction has primarily been attributed to insufficient levels of endonuclease expression in the cell lines studied (2), expression of the endonuclease gene was also observed to be unstable.

One cause for this instability of the endonuclease gene may be insufficient levels of protective *PaeR7* N<sup>6</sup>-methyladenine modification (CTCGm<sup>6</sup>AG) in the host murine cells. Although cotransfected DNA and several genomic *PaeR7* sites (CTCGAG) on the mouse chromosome were determined to be fully methylated, it was not possible to check the methylation status of all of the genomic *PaeR7* sites (2). If all *PaeR7* sites were not fully methylated, or became unmethylated as a result of DNA replication, these sites would be susceptible to cleavage by the *PaeR7* endonuclease. In addition to modification by the *PaeR7* methylase, such *PaeR7* sites can be modified by the endogenous mammalian methylase (3,4) at the CpG dinucleotide sites within the hexameric *PaeR7* recognition sequence. However, it is unknown whether such C<sup>5</sup>-methylation of cytosine confers resistance to cleavage in place of the missing *PaeR7* methylation.

In this study, the optimization of the *PaeR7* cleavage reaction and the substrate specificity of the *PaeR7* endonuclease for unmethylated, hemi-methylated, and fully methylated m<sup>6</sup>A and m<sup>5</sup>C versions of the *PaeR7* recognition sequence have been investigated. Using nonadecameric duplex deoxyoligonucleotides, each containing a *PaeR7* recognition site, as model substrates (Figure 1), the effect of N<sup>6</sup>-methyladenine and C<sup>5</sup>-methylcytosine modification on the catalytic and binding properties of the *PaeR7* endonuclease has been measured. The consequences of such methylation patterns in the genomes of cells expressing the *PaeR7* endonuclease are presented.

## MATERIALS AND METHODS

### Enzymes and Oligonucleotides

*PaeR7* endonuclease was purified to homogeneity as previously described (5) and was a gift from Drs. J. Brooks and J. Brenner of New England Biolabs. T<sub>4</sub> polynucleotide kinase (EC 2.7.1.78) was obtained from New England Biolabs, and [ $\gamma$ -<sup>32</sup>P]ATP was purchased from ICN. The  $\beta$ -cyanoethyl N,N-diisopropyl phosphoramidite derivatives of 5-methyldeoxycytidine and N<sup>6</sup>-methyldeoxyadenosine were purchased from Applied Biosystems and Pharmacia, respectively. All other reagents for oligonucleotide synthesis were products of Applied Biosystems.

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Oligonucleotides were synthesized on an Applied Biosystems 380A automated DNA synthesizer on a 1- $\mu$ mol scale using  $\beta$ -cyanoethyl phosphoramidites. The methylated deoxynucleotide phosphoramidite derivatives were incorporated during the synthesis into the desired positions of the oligonucleotides, 88-(181–184), following the recommendations of the manufacturers (see Figure 1). Purification of the 5'-tritylated products was carried out according to chromatographic conditions described previously (6). The purified oligonucleotides were resolved on a 20% polyacrylamide gel and observed to be unique bands.

### Kinetic Studies

The concentration of the stock solution of the *PaeR7* endonuclease was determined by the Bradford method (7). Molarities of the *PaeR7* endonuclease are given in terms of the dimeric protein of  $M_r$  58,000. Immediately prior to the kinetic assays, the enzyme stock was diluted in 50% glycerol in Nanopure H<sub>2</sub>O (Barnstead Nanopure II). No loss of activity in a 10-fold dilution of the enzyme was observed over a period of a week when kept at 4°C (data not shown). For the kinetic assays, aliquots of this endonuclease solution were diluted further in 50% glycerol to give the requisite concentrations of the enzyme. The concentrations of the oligonucleotide solutions were calculated from their optical density units and their extinction coefficients using the  $\epsilon$  values at 260 nm of 15,400, 7,300, 11,700 and 8,800 M<sup>-1</sup>cm<sup>-1</sup> for the bases A, C, G and T, respectively (8). Equimolar amounts of complementary oligonucleotide strands were annealed in 0.1 M NaCl, 10 mM Tris, pH 7.4, to form the duplex DNA substrates or competitive inhibitors, and diluted in 10 mM Tris, pH 7.5, 1 mM EDTA, prior to their addition to the reaction digests. Isotopic labelling of the 5' ends of the oligonucleotides was carried out using T4 polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]ATP (9).

### Optimization of Endonuclease Reaction Conditions

All *PaeR7* endonuclease reactions carried out to optimize the reaction conditions were performed in duplicate; each reaction was carried out in a total volume of 11  $\mu$ l using a final concentration of 0.31 nM of the enzyme and 0.81  $\mu$ M [<sup>32</sup>P]-labelled unmodified duplex DNA (6–10 $\times$ 10<sup>6</sup> cpm/pmol). The endonuclease reaction buffer (10 mM MgCl<sub>2</sub>, 10 mM  $\beta$ -mercaptoethanol, 10 mM Tris, pH 8.5, and containing 100  $\mu$ g/ml bovine serum albumin) was systematically altered to determine the optimal concentrations of various buffer components, as well as the optimal pH and temperature conditions. Typically, the reactions were stopped after 15 minutes with the addition of 2  $\mu$ l of 0.25 M EDTA (stop mix) and the reactions were resolved by electrophoresis using a non-denaturing 20% polyacrylamide gel. After autoradiography, the product and unreacted substrate bands were excised, and the Cerenkov counts were determined in a Beckman LS 7800 liquid scintillation system. Approximately 75–90% of the input radioactivity was recovered by this procedure. The extent of the cleavage was calculated from the ratio between the radioactivity in the product band and the sum of the radioactivity in the product and substrate bands. For the temperature profile of the *PaeR7*-catalyzed cleavage reaction, the pH of the buffer was adjusted at each temperature to 8.0, to compensate for the temperature dependence of Tris buffer.

### Determination of $k_{cat}$ and $K_m$

The optimized reaction conditions at 37°C were used to determine the kinetic constants of the cleavage reaction. In all the

experiments, the amount of [<sup>32</sup>P]-labelled duplexed oligonucleotide was kept constant (typically 6–10 $\times$ 10<sup>6</sup> cpm/pmol) and supplemented with unlabelled duplexed oligonucleotide to give the desired concentrations. Initially, time-course experiments were carried out to determine the amount of endonuclease required to effect less than 10–15% cleavage of the substrates at the 15-minute time-point. A zero time-point was used to subtract background cpm from the raw data. Prior to the determination of the kinetic parameters, the approximate  $K_m$  of each substrate was estimated from initial velocities of cleavage at two different substrate concentrations. The approximate  $K_m$  was calculated using a formula described previously (10).

For experiments performed to determine the kinetic parameters, duplex oligonucleotide substrates in 50  $\mu$ l of optimized reaction buffer (10 mM Tris, pH 8.3, 6 mM MgCl<sub>2</sub>, 10 mM  $\beta$ -mercaptoethanol, 100  $\mu$ g/ml BSA) were equilibrated at 37°C, and the cleavage was initiated by the addition of 5  $\mu$ l of diluted endonuclease. The concentrations of the substrates were varied from 0.2–5.0 $\times$  approximate  $K_m$ . The final enzyme concentrations in the reactions with the *PaeR7* recognition site containing substrates I, II, and III (Figure 1) were 23.5 pM, 0.39 nM and 19.5 nM, respectively. The reactions were quenched by adding 5  $\mu$ l of stop mix after 15 minutes for substrates I and II, and after 30 minutes for substrate III. The initial velocities were used to calculate  $k_{cat}$  and  $K_m$  using Lineweaver-Burk plots.

### Determination of $K_i$

The inhibition of the hydrolysis of the unmodified substrate, I, by the methylated oligonucleotide substrates, II–V (Figure 1), was studied according to the method of Dixon (11). Two concentrations of substrate I, 0.085  $\mu$ M and 0.34  $\mu$ M, were used to ascertain the mode of inhibition. A series of assay mixtures in a total volume of 50  $\mu$ l and containing both oligonucleotide I and various concentrations of the inhibitors (0–1.6  $\mu$ M) was prepared in the optimized reaction buffer, and 5  $\mu$ l of endonuclease solution (final concentration, 60 pM) was added to initiate the reaction. The assays were stopped after 15 minutes by addition of the stop mix.

## RESULTS

### Cleavage of Unmodified Substrate (I) by *PaeR7* Endonuclease

Hydrolysis of the [<sup>32</sup>P]-labelled unmodified substrate, I (Figure 1), by the *PaeR7* endonuclease was followed using polyacrylamide gel electrophoresis. Single-stranded oligonucleotide, containing the *PaeR7* recognition sequence, is not a substrate for the enzyme (Figure 2, lane b), indicating the requirement for a double stranded substrate for hydrolysis. Cleavage of the duplex substrate I yielding approximately 10% hydrolysis of substrate (conditions under which kinetic measurements were performed) results in a faster-migrating product (lane d). The extent of the hydrolysis can be quantitated by isolation of the unreacted substrate and product bands from the gel.

The time course of the reaction shows that the rate of hydrolysis is linear with respect to time over the first 20 minutes (Figure 3A) and that the rate of hydrolysis is directly proportional to the concentration of the endonuclease.

### Optimization of the Reaction Conditions

The pH dependence of the cleavage reaction was examined over a range of 7.0–9.5 in Tris·HCl buffer (Figure 3B). Optimal

Substrate	Sequence	Oligo
I	5' CCG GCG C <sup>m</sup> T C G A G ATC TAG A 3' 3' GGC CGC G A G C T C TAG ATC T 5'	88-180 88-179
II	5' CCG GCG C T <sup>m</sup> C G A G ATC TAG A 3' 3' GGC CGC G A G C T C TAG ATC T 5'	88-183 88-179
III	5' CCG GCG C T <sup>m</sup> C G A G ATC TAG A 3' 3' GGC CGC G A G C <sup>m</sup> T C TAG ATC T 5'	88-183 88-184
IV	5' CCG GCG C T C G <sup>m</sup> A G ATC TAG A 3' 3' GGC CGC G A G C T C TAG ATC T 5'	88-181 88-179
V	5' CCG GCG C T C G <sup>m</sup> A G ATC TAG A 3' 3' GGC CGC G <sup>m</sup> A G C T C TAG ATC T 5'	88-181 88-182

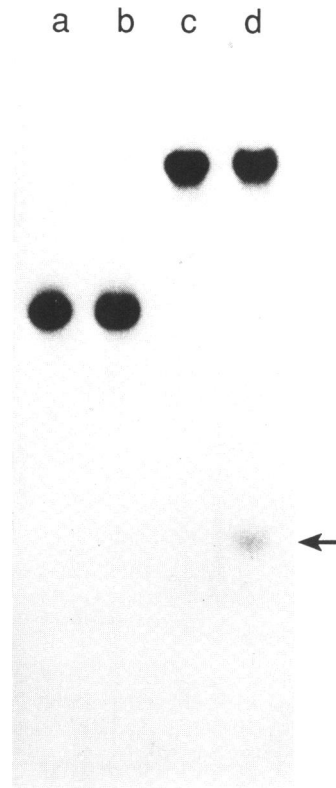
**Figure 1:** Duplex nonadecameric oligonucleotide substrates, containing the *PaeR7* recognition sequence in highlight, are shown with the cleavage sites. The methylated bases, C<sup>5</sup>-methylcytosine (<sup>m</sup>C) and N<sup>6</sup>-methyladenine (<sup>m</sup>A) were incorporated at their designated positions in oligonucleotides 88-(181–184) by solid-phase automated phosphoramidite synthesis. For measurement of the kinetic parameters, the following strands of the duplex substrates (in parentheses) were isotopically labelled at their 5'-termini using T4 polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]ATP: 88–179 (I); 88–183 (II); 88–183 (III); 88–179 (IV) and 88–182 (V).

hydrolysis occurs around pH 8.3, and the extent of cleavage is 2-fold higher than that at pH 7.4, which was used for the earlier endonuclease assays (12). Endonuclease activity is optimal in the absence of added NaCl, and at NaCl concentrations above 100 mM, strong inhibition of the enzyme activity is observed (Figure 3C). Inclusion of  $\beta$ -mercaptoethanol in the reaction buffer enhances the rate of cleavage, with maximal hydrolysis occurring with 10 mM of this reagent (Figure 3D). Mg<sup>2+</sup> ion is an essential requirement for catalysis (Figure 3E), and optimal rates were observed over the range of 5.0–7.5 mM MgCl<sub>2</sub>. Mn<sup>2+</sup> ion is also effective in catalysis. However, the relative rate of hydrolysis is depressed 10-fold when compared to the reaction containing MgCl<sub>2</sub> (data not shown).

The catalytic efficiency of the *PaeR7* endonuclease under optimized buffer conditions is most pronounced at 45°C (Figure 3F). At succeeding higher temperatures, there is a sharp drop of activity. Such loss of activity could conceivably be attributed to the instability of the enzyme or the dissociation of the duplex substrate at these temperatures. The latter possibility however appears unlikely, since the calculated T<sub>m</sub> of 62°C for substrate I (13) would suggest that a significant fraction of the substrate is double stranded.

### Steady-State Kinetics

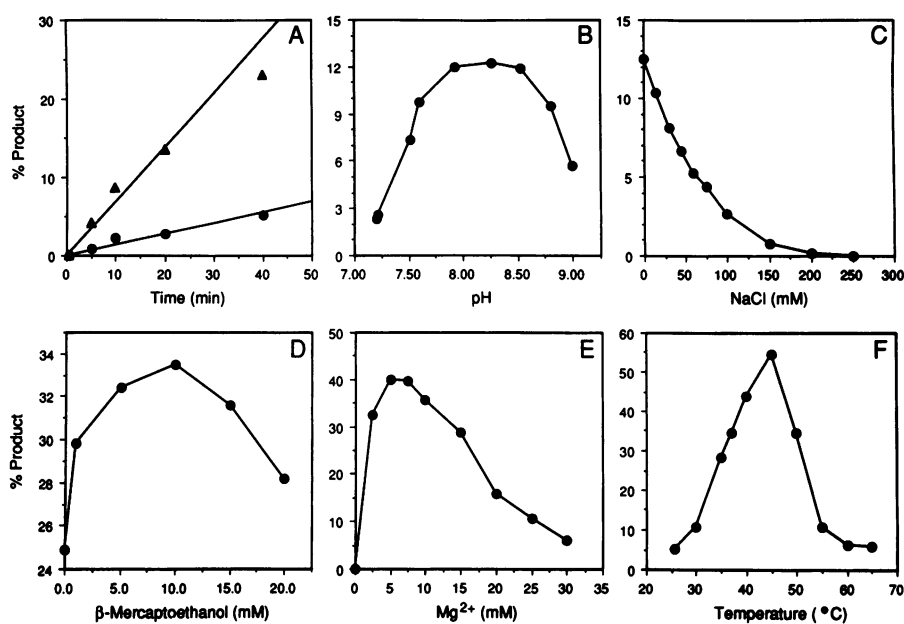
The steady-state kinetics of the duplexed oligonucleotides, I-V, were carried out at 37°C, primarily because of our interest in the activity of *PaeR7* restriction endonuclease at ambient temperatures of growing cells in culture. In all cases, one strand was end-labelled, and thus the polyacrylamide gel analysis employed shows only the kinetics of hydrolysis of this particular strand. As incorporation of methylated base analogues in the substrates gave rise to very different reactivities, the endonuclease concentration was adjusted accordingly for each substrate to obtain initial velocities. The second-order rate constant,  $k_{cat}/K_m$ , which is a measure of the selectivity of an enzyme for its substrate (14), was used as the parameter for the reactivity of *PaeR7*



**Figure 2:** Autoradiogram showing *PaeR7* endonuclease specificity for duplex substrate, I. Endonuclease (78 pM dimer) was incubated with either 65 nM single stranded oligonucleotide 88–179 and 33 nM unmodified substrate, I (see Figure 1 for sequences). The reaction was stopped after 15 min with addition of EDTA as described under 'Materials and Methods'. Lane (a) oligonucleotide 88–179; lane (b) oligonucleotide 88–179 with *PaeR7* endonuclease showing lack of reaction; lane (c) duplex substrate I; lane (d) substrate I and endonuclease resulting in ~10% cleavage and a faster migrating product (shown by arrow).

endonuclease towards these substrates. The endonuclease obeyed Michaelis-Menten kinetics with oligonucleotide substrates I, II and III, and Figure 4 shows a representative Lineweaver-Burk plot for the unmodified substrate, I. In contrast, no significant hydrolysis was observed with the hemi-A or the fully-A methylated oligonucleotide substrates, IV or V, even when elevated concentrations of the *PaeR7* endonuclease (20–40 nM) were utilized.

The kinetic parameters for the cleavage of the oligonucleotide duplexes, I-III, are summarized in Table I. Methylation of the cytosines in the CpG dinucleotide in the *PaeR7* recognition sequence results in a 100-fold decrease in  $k_{cat}/K_m$  for substrate II. When the cytosine in the complementary strand is also methylated (as in substrate III), the specificity of the enzyme for the substrate drops 2900-fold relative to substrate I. This difference in the reactivity of the substrates with the endonuclease is better understood when one compares their  $k_{cat}$  and  $K_m$  values. A comparison of the catalytic binding constant,  $K_m$ , for the three substrates reveals a 7-fold increase upon hemi-methylation of substrate I and a 24-fold increase for the fully methylated substrate III. Cytosine methylation of the substrate, however, has a more pronounced influence on the turnover number. There is a 15-fold decrease in the  $k_{cat}$  for the hemi-methylated substrate, II. The effect on  $k_{cat}$  is magnified further in the case of the fully methylated III, where greater than a 100-fold decrease in the turnover number is observed.



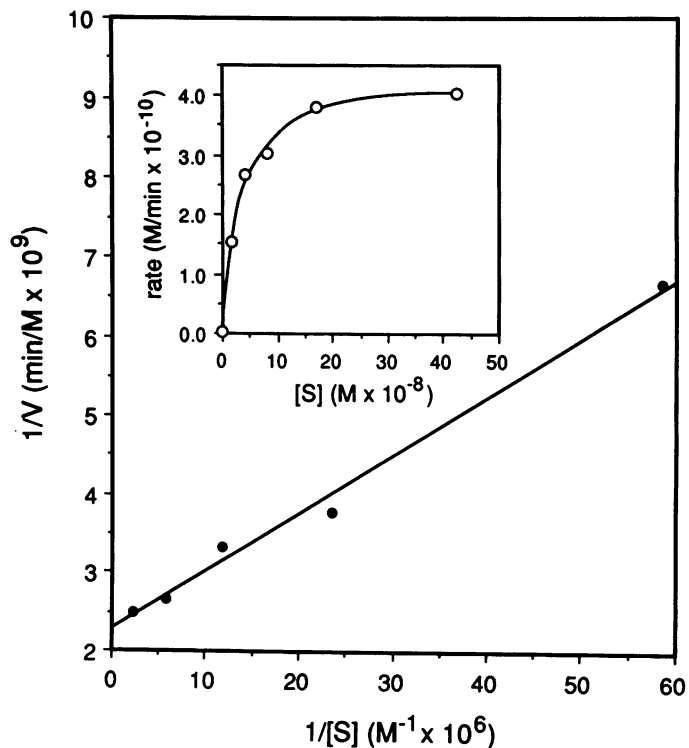
**Figure 3:** Optimization of the reaction conditions of *PaeR7* endonuclease using duplex I as substrate. Panel A: Time course of the endonuclease cleavage of I (1.1  $\mu\text{M}$ ) with (●) 16 pM and (▲) 78 pM of the enzyme. Panel (B-F): effect of the pH, buffer components, and temperature on the *PaeR7* catalyzed cleavage of I. Reactions were carried out in 10 mM Tris.HCl buffer at the indicated pH values at 37°C (panel B). Subsequently, each component of the original *PaeR7* endonuclease buffer (11) and the temperature was systematically varied keeping the other variables constant (panel C–F).

### Competitive Inhibition by Methylated Duplexes

To further understand the observed discrimination of the *PaeR7* endonuclease in the hydrolysis of the duplexes represented in substrates I–V, we attempted to study the binding affinity of the enzyme for the sequences in the absence of  $\text{Mg}^{2+}$  ion. In no case was any complex formation observed, using either a gel shift (15,16) or nitrocellulose binding assay (17–19). In contrast, *HhaI*, *TaqI*, *SmaI*, and *EcoRI* endonucleases were found to bind fragments of DNA containing their recognition sequences under our assay conditions (data not shown). While instability of an initially formed *PaeR7*-DNA complex under the experimental conditions cannot be ruled out, an implication of this lack of interaction may be that  $\text{Mg}^{2+}$  cation is an absolute requirement for binding.

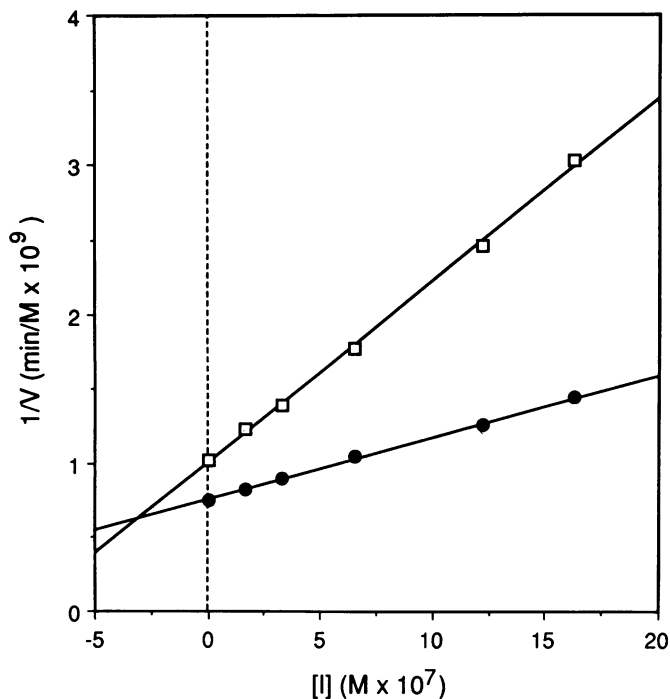
It was possible, however, to investigate the binding of the methylated duplexes to the *PaeR7* endonuclease in the presence of  $\text{Mg}^{2+}$  ion by determining their inhibition constants. As discussed in an earlier section, the efficiency of cleavage of substrates II and III is substantially reduced relative to substrate I, and substrates IV and V do not function as substrates. This permitted us to follow the *PaeR7*-catalyzed cleavage of the unmodified substrate I in the presence of methylated substrates, since insignificant or no hydrolysis of these methylated sequences should occur during the course of the assay.

A pattern consistent with competitive inhibition involving the methylated substrates was revealed using Dixon plots and is exemplified in Figure 5, where the hemi-methylated duplex, II, was used to inhibit the *PaeR7*-catalyzed hydrolysis of I. As seen in Table I, there is approximately a 2-fold difference in the  $K_i$  values for the hemi-C and fully-C methylated sequences II and III. The inhibition constants for methylated-A duplexes, IV and V, were somewhat higher than the  $K_i$  values for II and III, suggesting a greater steric hindrance for binding for these duplexes. Further, the nearly identical  $K_i$  values for IV and V



**Figure 4:** Lineweaver Burk plot of *PaeR7* endonuclease cleavage of unmodified substrate, I. The endonuclease (24 pM) was incubated with 0.017–0.43  $\mu\text{M}$  substrate, and the initial rates were determined as described under 'Materials and Methods'. The kinetic parameters were obtained from a least squares line fit of the data. Inset shows saturation kinetics with increasing concentration of substrate.

implies that impairment of binding is not additive with additional  $\text{N}^6$ -methyladenine substitution in the recognition sequence of the complementary strand.



**Figure 5:** Dixon plot showing competitive inhibition of *PaeR7* endonuclease cleavage of the unmodified substrate, I, by the hemi-C methylated duplex, II. The inhibitor concentration was varied between 0–1.6  $\mu\text{M}$  for substrate concentrations of (□) 0.085 and (●) 0.34  $\mu\text{M}$  respectively.

**TABLE I** Kinetic parameters<sup>a</sup> for the *PaeR7* endonuclease

Substrate	$k_{\text{cat}}^b$ ( $\text{min}^{-1}$ )	$K_m$ (nM)	$k_{\text{cat}}/K_m$ ( $\text{min}^{-1}\text{M}^{-1} \times 10^5$ )	$K_i$ (nM)
I	$18.4 \pm 1.7$	$30.6 \pm 4.4$	$6050 \pm 500$	not applicable
II	$1.21 \pm 0.06$	$211 \pm 56$	$60.2 \pm 14$	$329 \pm 80$
III	$0.15 \pm 0.05$	$730 \pm 298$	$2.11 \pm 0.16$	$701 \pm 103$
IV	no cleavage			$991 \pm 150$
V	no cleavage			$976 \pm 270$

<sup>a</sup> Kinetic constants are reported as the mean value from 4 independent measurements  $\pm 1$  standard deviation.

<sup>b</sup>  $k_{\text{cat}}$  expressed as mole labelled strand cleaved per mole of the *PaeR7* endonuclease dimer.

## DISCUSSION

The model nonadecameric duplex oligonucleotide substrate I, which contains an internal *PaeR7* recognition site, CTCGAG, was studied as a substrate for the enzyme. Saturation kinetics were observed with increasing concentrations of substrate I, and  $k_{\text{cat}}$  and  $K_m$  values of  $18 \text{ min}^{-1}$  and  $31 \text{ nM}$ , respectively, were obtained from Lineweaver-Burk plots. The turnover number for substrate I is typical of the  $k_{\text{cat}}$  values for oligonucleotide substrates of other type II restriction endonucleases, such as *EcoRI* (20,21) and *BamHI* (22). In comparison, the  $K_m$  value of substrate I is 7- to 230-fold lower than the  $K_m$  values reported for oligonucleotide substrates of these enzymes. The synthetic substrates used in several endonuclease studies have been short (8–10 bases in length) (20,23,24) and their  $K_m$  values have typically been found to be 200- to 8000-fold higher than larger duplex DNA substrates. From these studies, it has been suggested that sequences external to the recognition site play a role in stabilizing the DNA-enzyme complex. The low  $K_m$  value of

substrate I may therefore be reflective of favorable interactions of its flanking sequences with the enzyme.

We studied the effect of substituting methylated analogues in the hexameric sequence recognized by the *PaeR7* endonuclease. Circular dichroism (25), NMR (26) and thermal stability data (25,27) indicate that such replacements do not interfere with Watson-Crick base pairing, and the conformation of the helices are right handed and in the B-form. An  $\text{N}^6$ -methyladenine substitution in one or both strands (IV or V) resulted in duplexes which were inert to hydrolysis by the enzyme. The duplexes, IV and V, are the expected products of the methylation of I by the *PaeR7* methylase (12). Their lack of cleavage by the *PaeR7* endonuclease is in accord with the observation that *PaeR7* methylase modification of its recognition site protects the sequence from cleavage by its cognate endonuclease. This inability of the *PaeR7* endonuclease to cleave duplexes IV or V may be due to the steric interference of the  $\text{N}^6$ -methyl group in the major groove, thereby preventing access of the enzyme to the scissile phosphodiester bond. Alternatively, the methylation of the amino group results in the loss of a critical hydrogen-bond interaction with the enzyme, which could result in an altered and catalytically unproductive mode of binding. The competitive inhibition constant ( $K_i$ ) of  $1 \mu\text{M}$  for IV as well as V is indicative of such an interaction with the enzyme.

The substitution of 5-methylcytosine for the internal cytosine of the recognition sequence (Figure 1) results in poor substrates for the endonuclease. From van der Waal's diagrams, the methyl group of methylated-C sequences in B-form DNA projects into the solvent region of the major groove (28). If the *PaeR7* endonuclease, like the *EcoRI* enzyme (29), is presumed to make hydrogen bonding contacts in the major groove, it was anticipated that the placement of the methyl group there would introduce unfavorable steric interactions. From our kinetic studies, the influence of the methyl substituent is evident from the increase in the  $K_m$  values of substrates II and III. However, the magnitude of the  $K_m$  and  $K_i$  values of substrates II and III is still indicative of a tight association with the *PaeR7* enzyme. Interestingly, the decreased specificity of the *PaeR7* endonuclease towards II and III is manifested much more significantly in the turnover numbers than in the  $K_m$  values of the substrates. Besides invoking steric hindrance for this observation, yet another consequence of cytosine methylation may be subtle changes in the conformation of the duplex, which can adversely affect its interaction with the enzyme during catalysis.

Not surprisingly, the results of this study predict that the fully or hemi-methylated *PaeR7* sites in the murine genome which are modified with  $\text{N}^6$ -methyladenine should be completely protected. The presence of  $\text{C}^5$ -methylcytosine in the CpG dinucleotide of the *PaeR7* recognition sites also partially protects these sites from cleavage in the absence of  $\text{N}^6$ -methyladenine. The  $k_{\text{cat}}/K_m$  values indicate that cleavage of *PaeR7* sites fully and hemi-methylated with  $\text{C}^5$ -methylcytosine by the endonuclease are 2900- and 100-fold slower, respectively, than an unmodified *PaeR7* site. Consequently, we expect that in cells in which *PaeR7* sites lose  $\text{N}^6$ -methyladenine modification, partial protection from cleavage by the coexpressed *PaeR7* endonuclease should be afforded by cellular DNA methyltransferase.

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