Interaction of the phage T4 Dam DNA-[N⁶-adenine] methyltransferase with oligonucleotides containing native or modified (defective) recognition sites

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ABSTRACT

The DNA-[N⁶-adenine]-methyltransferase (Dam MTase) of phage T4 catalyzes methyl group transfer from S-adenosyl-L-methionine (AdoMet) to the N⁶-position of adenine in the palindromic sequence, GATC. We have used a gel shift assay to monitor complex formation between T4 Dam and various synthetic duplex oligonucleotides, either native or modified/defective. The results are summarized as follows. (i) T4 Dam bound with ∼100-fold higher affinity to a 20mer specific (GATC-containing) duplex containing the canonical palindromic methylation sequence, GATC, than to a non-specific duplex containing another palindrome, GTAC. (ii) Compared with the unmethylated duplex, the hemi-methylated 20mer specific duplex had a slightly increased (∼2-fold) ability to form complexes with T4 Dam. (iii) No stable complex was formed with a synthetic 12mer specific duplex, although T4 Dam can methylate it. This indicates that there is no relation between formation of a catalytically competent 12mer–Dam complex and one stable to gel electrophoresis. (iv) Formation of a stable complex did not require that both strands be contiguous or completely complementary. Absence of a single internucleotide phosphate strongly reduced complex formation only when missing between the T and C residues. This suggests that if T4 Dam makes critical contact(s) with a backbone phosphate(s), then the one between T and C is the only likely candidate. Having only one half of the recognition site intact on one strand was sufficient for stable complex formation provided that the 5‘ G-C base-pairs be present at both ends of the palindromic, GATC. Since absence of either a G or C abolished T4 Dam binding, we conclude that both strands are recognized by T4 Dam.

INTRODUCTION

The DNA-[N⁶-adenine]-methyltransferases (MTase) of phages (T2 and T4 catalyze methyl group transfer from S-adenosyl-L-methionine (AdoMet) to the N⁶-position of adenine in the palindromic sequence, GATC. The T2 and T4 enzymes belong to the Dam MTase family, which plays varied roles in cellular regulatory processes (1). The catalytic mechanism for methyl transfer is known only for the [C5-cytosine] MTases (2). This involves covalent bonding of a MTase-cysteine sulfur to the C6 ring carbon of the target cytosine residue. This is followed by C5-activation to react with an electrophile, the methyl group of AdoMet. In contrast, because their target is an exocyclic NH₂ group, the [N⁶-adenine] and [N⁴-cytosine] MTases must utilize a different mechanism for methyl transfer; not unexpectedly, amino-MTases are more closely related to one another than to the [C5-cytosine] MTases (3). Therefore, elucidating the mechanism of action of DNA-amino MTases is of great interest.

In the absence of structures from X-ray crystallography, studying complexes between an MTase and its substrates may be of considerable value in elucidating the DNA elements important for recognition by the enzyme. In this regard, use of synthetic oligonucleotide substrates with predetermined modified structures is a widely exploited approach to elucidate the role of individual structural elements in productive complex formation. Such complexes may be detected by a variety of methods, including sucrose gradient centrifugation, gel-filtration and equilibrium dialysis (4). Earlier, we investigated the capability of T4 MTase to form complexes with synthetic oligonucleotide substrates by means of gel-filtration and ultracentrifugation under equilibrium conditions (5). Here, we report studies of complex formation of the T4 Dam MTase with a number of synthetic oligonucleotide duplexes using a gel-shift electrophoresis assay (6–9). Contrary to the common practice of using group substitutions in the recognition site, we have applied the ‘dissected duplex’ principle (10–12), using variant ‘defective’ duplexes lacking a phosphate or nucleotide(s) within the GATC.
target site. In these analyses, self-complementary 12mer and 20mer oligonucleotide duplexes, as well as different methylated variants, were used as ligands; in addition, the effects of adding/omitting substrate, AdoMet, were also examined.

MATERIALS AND METHODS

Materials

T4 polynucleotide kinase was from SiBeEnzyme (Novosibirsk), [γ-32P]ATP from Biosan (Novosibirsk), and S-adenosyl-L-methionine from Sigma was purified further by chromatography on a C18 reversed-phase column as described previously (13). Oligonucleotides were synthesized on an Applied Biosystems 380A/380B DNA Synthesizer. Oligonucleotide concentrations were determined spectrophotometrically from the molar extinction coefficients of individual oligonucleotides and their known sequences. The T4 Dam MTase was purified to homogeneity as previously described (4,14).

Binding experiments

Binding reactions were carried out in 20 µl mixtures containing 100 mM Tris–HCl (pH 8), 1 mM DTT, 200 µg/ml bovine serum albumin (BSA) and 5% glycerol. Enzyme and AdoMet additions are noted in the figure legends. The reaction tubes were kept on ice until the time of loading on a 10% polyacrylamide gel, which had been pre-electrophoresed for 1.5–2 h. The preliminary experiments showed that oligonucleotide methylation by T4 MTase was very low during the incubation period. Electrophoresis was for 1.5–2 h at 20°C in 100 mM Tris–borate buffer (pH 8.2) containing 2 mM Na2EDTA in a voltage gradient of 8–10 V/cm. A low level of methylation at the start of electrophoresis is not excluded; other workers have also used AdoMet as ligand in similar experiments (7–9,15,16) probably because MTases have a much higher affinity for it than analogs, sinefungin or S-adenosyl-homocysteine. After electrophoresis and drying, the gels were autoradiographed and appropriate slices excised for counting Cerenkov radiation. Although enzyme concentrations were higher than those of the oligonucleotide duplexes (5–10 nM), this method does not permit determination of true equilibrium dissociation constants (8). In some experiments, enzyme concentrations were chosen to give ~50% bound oligonucleotide.

Preparation of synthetic oligonucleotide duplexes

Synthetic duplexes were obtained by heating and annealing individual oligonucleotide chains from 90 to 20°C over 7–12 h (8). Variant duplexes were obtained by combining oligonucleotides that are complementary to a standard 20mer oligonucleotide, I, shown as the upper strand (Table 1). Oligonucleotides I and II containing the canonical MTase recognition site, GATC, in the sequence center, are completely complementary and capable of forming a stable ‘specific’ duplex 1 (Table 2). Oligonucleotides X and XI have base substitutions (relative to I and II) that alter the canonical recognition site to another palindromic sequence, GTAC, they were combined to form the ‘non-specific’ duplex 0 (Table 2). Oligonucleotides III–V and VI–VIII are complementary to portions of the 5′ and 3′-ends, respectively, of oligonucleotide I. Using various combinations of equimolar mixtures of oligonucleotides III–VIII, we obtained duplexes containing a discontinuity in the bottom chain; i.e., lacking either a phosphate or nucleotide(s) at defined positions within the recognition site. Additional duplexes containing a methylated A in the top strand GATC were also prepared (Table 2).

Oligonucleotides were radiolabeled using [γ-32P]ATP and T4 polynucleotide kinase, and then purified by preparative gel electrophoresis. Radioactively labeled strands were annealed with a 1.5-fold higher concentration of unlabeled complementary strand, with the exception of the self-complementary 12mer (Table 1, no. IX). In the initial binding mixtures at 0°C, the complexes were stable; however, in the course of electrophoresis, there was some dissociation, as evidenced by the occasional presence of doublet bands in the vicinity of free oligonucleotide, particularly with the 12mer (see Figs 1B and 6B). A similar pattern has been observed by others (16).

RESULTS AND DISCUSSION

Binding of T4 Dam MTase to synthetic oligonucleotide duplexes

Figure 1A and B shows the results of the gel shift assay for complexes formed between T4 Dam and specific 20mer duplex 1 (Table 2) in the absence or presence of 5 µM AdoMet, a concentration sufficient to saturate T4 Dam (4). In a control experiment, complex formation was shown to be weak between T4 Dam and non-specific duplex 0 (Fig. 1C), which contains another palindrome, GTAC, in place of GATC (Table 2); this was independent of the presence or absence of AdoMet. Figure 1D shows a plot of the fraction of 32P bound in the complex for duplexes 0 and 1 (± AdoMet) versus T4 Dam concentration. Similar curves were generated for other duplexes, and the apparent KD derived from [E]1/2, the concentration of enzyme needed to complex 50% of the oligonucleotide, are shown in Table 2. Since the gel shift assay is not an equilibrium method, the true KD of enzyme–DNA complexes were not determined by this procedure. Still, [E]1/2 may be a good approximation of KD for stable complexes, but will be an overestimate if dissociation is too fast for a ‘caging effect’ (7). It can be seen that presence of AdoMet enhanced MTase-binding 2-fold (Fig. 1D, Table 2); however, the magnitude of this stimulation was not as large as has been observed with other MTases using AdoMet (or its analogs) (7–9,15). In contrast to the results...
obtained with 20mer specific duplex 1, no complex was detected with a 12mer specific (GATC-containing) duplex, even when AdoMet was present (Fig. 2). It should be noted, however, that the 12mer duplex actually serves as a good substrate for methylation by T4 Dam (unpublished observation). Thus, it appears that certain catalytically competent MTase–duplex complexes may be too labile to be detected under the conditions of this experiment. The largest fraction of complex formed was barely 10%, even at the maximal T4 Dam concentration used. The appearance of a slower migrating complex may indicate formation of T4 Dam aggregates.

Table 2. Recognition sequence of oligonucleotide duplexes with a native or defective recognition site for T4 Dam and apparent $K_d$ values for binding duplexes

<table>
<thead>
<tr>
<th>Duplex no.</th>
<th>Combination of oligonucleotides</th>
<th>Structure of recognition site</th>
<th>$K_d$, nM in absence of AdoMet</th>
<th>$K_d$, nM in presence of AdoMet</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>X + XI</td>
<td>None*</td>
<td>&gt;&gt;&gt;1460</td>
<td>&gt;&gt;&gt;1460</td>
</tr>
<tr>
<td>1</td>
<td>I + II</td>
<td>5′-G-A-T-C-</td>
<td>43 (±6.4)</td>
<td>17 (±4.1)</td>
</tr>
<tr>
<td>1m</td>
<td>Im + II</td>
<td>5′-G-M-T-C-</td>
<td>23 (±2.9)</td>
<td>8.5 (±1.8)</td>
</tr>
<tr>
<td>2</td>
<td>I + IV + VII</td>
<td>5′-G-A-T-C-</td>
<td>&gt;1000 [500]</td>
<td>&gt;1000 [70]</td>
</tr>
<tr>
<td>2m</td>
<td>Im + IV + VII</td>
<td>5′-G-M-T-C-</td>
<td>&gt;1000 [230]</td>
<td>&gt;1000 [100]</td>
</tr>
<tr>
<td>3</td>
<td>I + III + VIII</td>
<td>5′-G-A-T-C-</td>
<td>28 (±5.9)</td>
<td>9 (±2.3)</td>
</tr>
<tr>
<td>3m</td>
<td>Im + III + VIII</td>
<td>5′-G-M-T-C-</td>
<td>96 (±40.4)</td>
<td>47 (±10.6)</td>
</tr>
<tr>
<td>4</td>
<td>I + V + VI</td>
<td>5′-G-A-T-C-</td>
<td>18 (±5.1)</td>
<td>5 (±2.2)</td>
</tr>
<tr>
<td>4m</td>
<td>Im + V + VI</td>
<td>5′-G-M-T-C-</td>
<td>16 (±2.9)</td>
<td>5 (±0.6)</td>
</tr>
<tr>
<td>5</td>
<td>I + IV + VIII</td>
<td>5′-G-A-T-C-</td>
<td>35 (±17)</td>
<td>12 (±10)</td>
</tr>
<tr>
<td>5m</td>
<td>Im + IV + VIII</td>
<td>5′-G-M-T-C-</td>
<td>23 (±6)</td>
<td>7 (±5)</td>
</tr>
<tr>
<td>6</td>
<td>I + IV + VI</td>
<td>5′-G-A-T-C-</td>
<td>16 (±5)</td>
<td>2.7 (±0.5)</td>
</tr>
<tr>
<td>6m</td>
<td>Im + IV + VI</td>
<td>5′-G-M-T-C-</td>
<td>12 (±4)</td>
<td>2.4 (±0.5)</td>
</tr>
<tr>
<td>7</td>
<td>I + VII</td>
<td>5′-G-A-T-C-</td>
<td>&gt;1450 [1000]</td>
<td>&gt;1450</td>
</tr>
<tr>
<td>7m</td>
<td>Im + VII</td>
<td>5′-G-M-T-C-</td>
<td>&gt;1000 [230]</td>
<td>&gt;1000 [100]</td>
</tr>
<tr>
<td>8</td>
<td>I + VIII</td>
<td>5′-G-A-T-C-</td>
<td>&gt;1450 [1000]</td>
<td>&gt;900 [200]</td>
</tr>
<tr>
<td>8m</td>
<td>Im + VIII</td>
<td>5′-G-M-T-C-</td>
<td>600 [200]</td>
<td>&gt;150 [72]</td>
</tr>
<tr>
<td>9</td>
<td>I + V</td>
<td>5′-G-A-T-C-</td>
<td>&gt;&gt;1450</td>
<td>&gt;&gt;1450</td>
</tr>
<tr>
<td>9m</td>
<td>Im + V</td>
<td>5′-G-M-T-C-</td>
<td>&gt;&gt;1450</td>
<td>&gt;&gt;1450</td>
</tr>
<tr>
<td>10</td>
<td>II + X</td>
<td>5′-G-A-T-C-</td>
<td>156 (±45)</td>
<td>3.3 (±1.7)</td>
</tr>
</tbody>
</table>

*The $K_d$ values for unstable complexes correspond to $[E]_{1/2}$ values; and values for more stable complexes were obtained from binding curve fitting to the simplest hyperbola, $P = P_{\text{max}}/(1 + [E]/K_d)$. This was achieved using a computer program for non-linear regression analysis (developed by A.N. Naumochkin, Novosibirsk), with the minimization of $\chi^2$ determining the fit; standard error values are presented in parenthesis. Values in brackets were calculated using the ratios of radioactivity in [upper band + smear]/lower band fit to the best possible hyperbola.

In duplex 0, the canonical GATC site has been altered to GTAC, which is also palindromic; duplex 10 has two mismatched bases due to the presence of canonical GATC and non-canonical GTAC. Hemimethylated duplexes (2m–9m) were formed using the methylated upper strand, Im. Duplexes 2–4 and 2m–4m each lacked a single phosphate, denoted by ^. Duplexes 5–9 and 5m–9m contain one or two nucleotide gaps, denoted by (...).
**Figure 1.** Binding of T4 Dam to 20mer specific duplex 1 containing the canonical palindromic methylation sequence, GATC. In this and subsequent figures, the binding mixture contained oligonucleotide duplex at 5 nM. Binding in the absence (A) or presence (B) of AdoMet (5 µM). Samples contained T4 Dam as follows. Sample nos 1–12 had concentrations of 5, 8, 13, 21, 34, 55, 87, 139, 223, 360, 570 and 920 nM T4 Dam, respectively. (C) Binding of T4 Dam to 20mer non-specific duplex 0 containing the palindrome, GTAC, in place of GATC. Binding in the absence (samples 1–6) or presence (samples 7–12) of AdoMet (5 µM), and T4 Dam as follows. Sample nos 1–12 had concentrations of 49, 98, 195, 390, 780, 1560, 49, 98, 195, 390, 790 and 1560 nM T4 Dam, respectively. (D) Binding of T4 Dam with native specific 20mer duplex 1 and non-specific duplex 0 in the absence and presence of AdoMet.

**Binding of T4 Dam to duplexes with a defective DNA target site**

In the previous section we showed that T4 Dam forms a stable complex with a specific 20mer oligonucleotide duplex containing a native GATC recognition site. This analysis was extended to investigating the influence of introducing defects in the recognition site (Table 2). Figures 3–5 show the results of gel shift assays for complexes of T4 MTase with various defective duplexes formed in the presence/absence of AdoMet. In all these duplexes, the upper strand (by definition) was intact and either unmodified or N6-methylated on the adenine residue in GATC. The results are summarized below.

(i) Absence of a single internucleotide phosphate in any of the three bottom strand internal positions of the GATC site had variable effects (Figs 3 and 4, Table 2). For example, absence of a phosphate between the T and C residues (duplexes 2 and 2m), strongly reduced complex formation/stability relative to duplex 1. In contrast, absence of a phosphate between the G and A residues (duplexes 4 and 4m) or the central A and T residues (duplexes 3 and 3m) increased complex formation, and binding was improved further by the presence of AdoMet; however, duplex 3 complexes tended to dissociate during electrophoresis, as evidenced by the smear of radioactivity between the bound and free oligonucleotides (Fig. 3). These results suggest either that T4 Dam makes a critical contact with the backbone phosphate between T and C, or that absence of this phosphate indirectly affects duplex conformation and, consequently, the ability to recognize the adjacent G.C base pair. Contacts with backbone phosphate(s) can be investigated by ethyl nitrosourea-ethylation interference footprinting analysis using an appropriate macromolecular DNA substrate.

(ii) Single nucleotide gaps in the bottom strand of the recognition site had variable effects on complex formation. Duplex 5, lacking T, formed complexes as well as specific duplex 1 (Fig. 3). In contrast, weak complexes were produced with duplexes 7 and 9 (Figs 3 and 5), in which the nucleotide gap was the bottom strand C or G, respectively. A strong complex was
observed with duplex 6, which has an internal AT-dinucleotide gap (Figs 3 and 4), and binding was improved further by the presence of AdoMet. In contrast, a very weak complex was formed with duplex 8, having a TC-dinucleotide gap. Thus, having only one half of the recognition site intact on one strand is sufficient for stable complex formation provided that the 5′G·C base pairs be present at both ends of the palindromic, GATC. Since absence of either a G or C abolished T4 Dam binding, we conclude that both strands are recognized by T4 Dam.

(iii) We have also tested binding of T4 Dam to the imperfect duplex 10, in which there is a double mismatch at the middle two bases (Table 2). In the absence of cofactor AdoMet, affinity for duplex 10 was reduced compared with duplex 1; however, surprisingly, in the presence of AdoMet duplex 10 formed a more tight complex (Fig. 6, Table 2). These results are consistent with a two strand recognition where the terminal G·C base pairs are critical players.

Taken together our results demonstrate that formation of a stable T4 Dam–synthetic DNA duplex complex does not require that both strands be contiguous or completely complementary. Thus, certain missense (duplex 10) or absence of internal base pairs (duplexes 5 and 6) still allows binding (although duplex 10 required AdoMet for tight binding). Likewise, absence of the phosphate between G and A (duplex 4) or A and T (duplex 3) promoted complex formation. This suggests that absence of certain H-bonds or internucleotide phosphates in the interior of the methylation site can promote T4 Dam binding. It is not clear if this is related to ‘the proposed flipping’ of the target A residue out of the double helix, as has been suggested for the TaqI (17), EcoRI (18) and EcoRV (19) [N6-adenine] DNA MTases. However, to date, target base flipping has been observed directly only with the [C5-cytosine] MTases, M.HhaI and M.HaeIII (20,21). In this regard, the 5′ and 3′ phosphates of the ‘flipped’ C in both targets are contacted by protein extensively. These contacts are important to increase the interstrand phosphate–phosphate distance, disrupt target base-pairing and promote base flipping out.
Figure 5. Binding of T4 Dam with oligonucleotide duplexes (see Table 2). (A) 7 (lanes 1–6) and 7m (lanes 7–12); (B) 8 (lanes 1–6) and 8m (lanes 7–12); (C) 9 (lanes 1–6) and 9m (lanes 7–12); in the absence of AdoMet (lanes 1–3 and 7–9) and in the presence of AdoMet (5 µM) (lanes 4–6 and 10–12). The concentrations of T4 Dam in lanes 1, 4, 7 and 10, 2, 5, 8 and 11, 3, 6, 9 and 12 were 72, 290, and 1450 nM T4 Dam, respectively.

of the double helix (22). Thus, lacking the 5′ or 3′ phosphate to A could make T4 Dam base-flipping easier, assuming it is still stacked inside the helix, and stabilize the complex.

Figure 6. Binding of T4 Dam with imperfect duplex 10 (see Table 2) in the absence (lanes 1–6) or presence (lanes 7–12) of AdoMet. Samples contained concentrations of T4 Dam as follows. Sample nos 1–12 had concentrations of 49, 98, 195, 390, 780, 1560, 49, 98, 195, 390, 790 and 1560 nM T4 Dam, respectively.

Binding of T4 Dam to duplexes with a hemimethylated DNA target site

Since certain MTases prefer hemimethylated substrates, we compared complex formation of synthetic duplexes and their hemimethylated variants. The level of complex formation to specific 20mer duplex 1 increased with T4 Dam concentration (Fig. 1A) and reached a maximum above 200 nM. Duplex 1m bound T4 Dam (in the absence of AdoMet) with ~2-fold higher affinity than the unmethylated duplex (Fig. 7). An analogous result has been observed for the EcoRV DNA MTase (8). In contrast to these results, methylation of the upper strand GATC-adenine residue or presence of AdoMet did not always lead to complex stabilization with the duplexes 2–9 or 2m–9m (Figs 3–5; the results are summarized in Table 2), where we observed variable effects on complex formation. Thus, for example, while hemimethylation decreased T4 Dam binding with duplexes 2 and 3, it enhanced complex formation with duplexes 1, 5 and 8, and exerted no influence with duplexes 4, 6, 7 and 9. Hence, there was no correlation between the effects of hemimethylation and recognition site defect nature/location. Lack of additivity due to chemical or structural modification has been observed previously with the EcoRI MTase (23) and EcoRI restriction endonuclease (24).

Final comments

Recently, the ‘dissected molecular method’ was used for a study of interaction between (C5-cytosine) HhaI MTase and oligonucleotide duplexes containing native GCGC site or its modifications (16). Interestingly, the elimination of certain internucleotide phosphates produced qualitatively the same effects as for T4 Dam MTase, despite the fact that the two enzymes have different recognition sites and mechanisms of action. As in our experiments, a sharp decrease HhaI binding was shown when a single internucleotide phosphate was absent between the 3rd G and 4th C residues of 5′-GCGC (this is analogous to our duplex 2 in Table 2); the effect of a missing
neutral or increased affinity for the enzyme. abolished T4 Dam binding. Other target defects were either ends of the palindromic, GA TC; absence of either G or C phosphate at other positions was minor. Introduction of a single nucleotide gap in one strand did not decrease HhaI Mtase-binding; with T4 Dam, the position of the gap was an important factor determining complex formation. Dinucleotide gaps destroyed HhaI Mtase-binding capability; in contrast, T4 MTase effectively bound duplex 6 having an internal A T-dinucleotide gap. Thus, the HhaI and T4 Dam MTases exhibit different behaviours with duplexes containing some strand discontinuity. The results of our investigation are summarized as follows. (i) T4 Dam MTase bound with ∼100-fold higher affinity to a 20mer specific duplex containing the canonical palindromic methylation sequence, GATC, compared with a non-specific duplex in which GATC was replaced by another palindrome, GTAC. No stable complex was formed with a 12mer specific duplex. (ii) Compared with the unmethylated duplex, the hemimethylated specific 20mer duplex had a slightly increased (∼2-fold) ability to form complexes with T4 Dam. (iii) Studies with duplexes containing defects in the methylation site showed that the formation of a stable complex with T4 Dam did not require that both strands be contiguous nor completely complementary. Having only one half of the recognition site intact was sufficient for stable complex formation provided that the 5' GC base-pairs be present at both ends of the palindromic, GATC; absence of either G or C abolished T4 Dam binding. Other target defects were either neutral or increased affinity for the enzyme.

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REFERENCES