5-methylcytosine enhance the substrate activity of DNA polymerase

Tian Tian, Shuang Peng, Heng Xiao, Yuelin Long, Boshi Fu, Xiaoe Zhang, Shan Guo, Shaoru Wang*, Xiang Zhou*, Songmei Liu, Xin Zhou

Here, we first demonstrated that 5-MedCTP could be incorporated into diverse synthetic DNA templates by the exonuclease deficient Klenow Fragment with a much higher efficiency than dCTP and 5-Hydroxymethyl-dCTP. And the efficiency gap of incorporation could be increased in presence of different amounts of Ag¹. Further, we first conducted a comparable study of primer extension reaction using templates containing deoxycytidine (dC) or 5-methyldeoxycytidine (5-mdC) for incorporating different triphosphates of dCTP, 5-MedCTP and 5-Hydroxymethyl-dCTP. Based on our finding, 5-methyldeoxycytidine could enhance the substrate activity of Klenow Fragment (exo-) and this feature could be potentially used in DNA methylation analysis.
Supporting Information

5-methylcytosine enhance the substrate activity of DNA polymerase

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General information: The oligonucleotides and dCTPs were purchased from Invitrogen Technology(Shanghai, China). Klenow Fragment(exo-), Klenow Fragment, T4 DNA polymerase, Taq DNA polymerase and 5-MedCTPs were purchased from New England Biolabs, Inc. 5-Hydroxymethyl-dCTPs were purchased from Bioline, Inc. Tris base, MgCl₂, KCl, NaCl, formamide(deionized) and dithiothreitol(DTT) were purchased from Sigma Inc. PAGE analyses were performed with denaturing 20%(19:1) polyacrylamide gel in presence of 8 M urea.

DNA polymerization by polymerase: The reaction mixtures (20 µL) containing 100 nM primer, 150 nM template, 20 µM dNTPs, 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂, 1 mM Dithiothreitol, 0.6 units of polymerase and 1 mM dithiothreitol(DTT), in the presence or absence of various concentrations of metal ions were incubated at 37 °C for 1 h. The reactions were quenched by adding 100 µL 80% aqueous formamide(deionized), and the solutions were immediately heated at 90 °C for 10 min. After cooling down to 4 °C, the solutions were analyzed by denaturing 20% PAGE. FAM labeled 15-primer, 19-mer and 24-mer were used as the markers in gel analysis.

Kinetic studies of 5-MedCTP or dCTP incorporation by Klenow Fragment (exo-). The reaction mixtures (20 µL) containing 500 nM primer, 1.0 µM template, 20 µM dATP, 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂, 1 mM Dithiothreitol, 1.0 unit of Klenow Fragment (exo-) and 1 mM dithiothreitol(DTT), in the presence of various concentrations of 5-MedCTP or dCTP were incubated at 37 °C for 6 mins. The reactions were quenched by adding 100 µL 80% aqueous formamide(deionized), and the solutions were immediately heated at 90 °C for 10 min. After cooling down to 4 °C, the solutions were analyzed by denaturing 20% PAGE. FAM labeled 15-primer, 19-mer and 24-mer were used as the markers in gel analysis. Kinetic data were derived as described in known literature[1].
**Table S1** Sequences of oligomers used for polymerization.

<table>
<thead>
<tr>
<th>Oligomer</th>
<th>Sequence (from 5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template</td>
<td>GGTGCGTGGCTATAGTGAGTCGTA</td>
</tr>
<tr>
<td>Template Me</td>
<td>GGTG (MeC) GTGGCTATAGTGAGTCGTA</td>
</tr>
<tr>
<td>FAM-15mer</td>
<td>5’-FAM-TACGACTCACTATAG</td>
</tr>
<tr>
<td>FAM-19mer</td>
<td>5’-FAM-TACGACTCACTATAGCCACA</td>
</tr>
<tr>
<td>FAM-24mer</td>
<td>5’-FAM-TACGACTCACTATAGCCACACACC</td>
</tr>
</tbody>
</table>

**Table S2** Kinetic parameters of 5-MedCTP or dCTP incorporation by Klenow Fragment (exo-).

<table>
<thead>
<tr>
<th>Incorporation</th>
<th>K_M (µM)</th>
<th>kcat (min⁻¹)</th>
<th>kcat/K_M (min⁻¹ µM⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-MedCTP</td>
<td>0.95</td>
<td>3.9</td>
<td>4.10</td>
</tr>
<tr>
<td>dCTP</td>
<td>8.9</td>
<td>2.7</td>
<td>0.30</td>
</tr>
</tbody>
</table>
Figure S1. Illustration of different metal ion-base complex. a, C-Ag⁺-C; b, T-Hg²⁺-T, c, A-Ag⁺-C.

Figure S2. The polymerization reaction by the Klenow Fragment(exo-) in the absence or presence of Ag⁺ ions. The reaction was performed in a buffer consisting of 100 nM primer, 150 nM template, 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂, 1 mM Dithiothreitol, 0.6 units of Klenow Fragment(exo-), 1 mM dithiothreitol(DTT) and 20 µM dNTPs (dATP + dCTP or dATP + 5-MedCTP), in the presence or absence of different amounts of AgNO₃. M: mixtures of FAM-15mer, FAM-19mer and FAM-24mer; 1-4: 20 µM dCTP together with dATP was used; 5-8: 20 µM 5-MedCTP together with dATP was used.
**Figure S3.** The reaction mixtures (20 µL) containing 500 nM primer, 1.0 µM template, 20 µM dATP, 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂, 1 mM Dithiothreitol, 1.0 unit of Klenow Fragment (exo-) and 1 mM dithiothreitol(DTT), in the presence of various concentrations of dCTP were incubated at 37 °C for 6 mins. The reactions were quenched by adding 100 µL 80% aqueous formamide(deionized), and the solutions were immediately heated at 90 °C for 10 min. After cooling down to 4 °C, the solutions were analyzed by denaturing 20% PAGE. FAM labeled 15-mer, 19-mer and 24-mer were used as the markers in gel analysis.

**Figure S4.** The reaction mixtures (20 µL) containing 500 nM primer, 1.0 µM template, 20 µM dATP, 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂, 1 mM Dithiothreitol, 1.0 unit of Klenow Fragment (exo-) and 1 mM dithiothreitol(DTT), in the presence of various concentrations of 5-MedCTP were incubated at 37 °C for 6 mins. The reactions were quenched by adding 100 µL 80% aqueous formamide(deionized), and the solutions were immediately heated at 90 °C for 10 min. After cooling down to 4 °C, the solutions were analyzed by denaturing 20% PAGE. FAM labeled 15-mer, 19-mer and 24-mer were used as the markers in gel analysis.
Figure S5. Effects of Ag\textsuperscript{I} ions on the polymerization reaction by the Klenow fragment(exo-). The reaction was performed in a buffer consisting of 100 nM primer, 150 nM template, 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl\textsubscript{2}, 1 mM Dithiothreitol, 0.6 units of Klenow Fragment (exo-), 1 mM dithiothreitol (DTT) and 20 µM dNTPs, in the presence or absence of different amounts of AgNO\textsubscript{3}. And dATP + dCTP was used in the assay. Bars showed the relative amounts of the full-length product of Ag\textsuperscript{I} mediated reactions over the stopped product of the reactions compared with control reaction without Ag\textsuperscript{I} ions. Values are averages and the standard deviation determined by three independent experiments.

Figure S6. Effects of Ag\textsuperscript{I} ions on the polymerization reaction by the Klenow fragment(exo-). The reaction was performed in a buffer consisting of 100 nM primer, 150 nM template, 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl\textsubscript{2}, 1 mM Dithiothreitol, 0.6 units of Klenow Fragment (exo-), 1 mM dithiothreitol (DTT) and 20 µM dNTPs, in the presence or absence of different amounts of AgNO\textsubscript{3}. And dATP + 5-Hydroxymethyl-dCTP was used in the assay. Bars showed the relative amounts of the full-length product of Ag\textsuperscript{I} mediated reactions over the stopped product of the reactions compared with control reaction without Ag\textsuperscript{I} ions. Values are averages and the standard deviation determined by three independent experiments.
**Figure S7.** Effects of Ag\(^{I}\) ions on the polymerization reaction by the Klenow fragment (exo-). The reaction was performed in a buffer consisting of 100 nM primer, 150 nM template, 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl\(_2\), 1 mM Dithiothreitol, 0.6 units of Klenow Fragment (exo-), 1 mM dithiothreitol (DTT) and 20 µM dNTPs, in the presence or absence of different amounts of AgNO\(_3\). And dATP + 5-MedCTP was used in the assay. Bars showed the relative amounts of the full-length product of Ag\(^{I}\) mediated reactions over the stopped product of the reactions compared with control reaction without Ag\(^{I}\) ions. Values are averages and the standard deviation determined by three independent experiments.

**Figure S8.** Effects of Ag\(^{I}\) ion concentrations on the polymerization reaction by the Klenow Fragment with exonuclease activity. The reaction was performed in a buffer consisting of 100 nM primer, 150 nM template, 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl\(_2\), 1 mM Dithiothreitol, 0.6 units of Klenow Fragment, 1 mM dithiothreitol(DTT) and 20 µM dNTPs (dATP + dCTP), in the presence or absence of different amounts of AgNO\(_3\). M: mixtures of FAM-15mer, FAM-19mer and FAM-24mer; 1-3: template without 5-methyldeoxycytidine was used; 4-6: template with one 5-methyldeoxycytidine was used.
Figure S9. Effects of Ag⁺ ion concentrations on the polymerization reaction by T4 DNA polymerase. The reaction was performed in a buffer consisting of 100 nM primer, 150 nM template, 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂, 1 mM Dithiothreitol, 0.6 units of T4 DNA polymerase, 1 mM dithiothreitol(DTT) and 20 μM dNTPs (dATP + dCTP), in the presence or absence of different amounts of AgNO₃. M: mixtures of FAM-15mer, FAM-19mer and FAM-24mer; 1-4: template without 5-methyldeoxycytodine was used; 5-8: template with one 5-methyldeoxycytodine was used.

Figure S10. Effects of Ag⁺ ion concentrations on the polymerization reaction by Taq DNA polymerase. The reaction was performed in a buffer consisting of 100 nM primer, 150 nM template, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 4 mM MgCl₂, 0.08% (v/v) Nonidet P40, 0.6 units of Taq DNA polymerase, 1 mM dithiothreitol(DTT) and 20 μM dNTPs (dATP + dCTP), in the presence or absence of different amounts of AgNO₃. M: mixtures of FAM-15mer, FAM-19mer and FAM-24mer; 1-4: template without 5-methyldeoxycytodine was used; 5-8: template with one 5-methyldeoxycytodine was used.
Figure S11. Effects of different metal ions on the polymerization reaction by the Klenow fragment (exo-). Bars showed the relative amounts of the full-length product over the stopped product of the reactions compared with Ag⁺.

References: