**Regular Article**

**Effect of pH on the Misincorporation Rate of DNA Polymerase η**

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Received November 11, 2015; accepted February 24, 2016

The many known eukaryotic DNA polymerases are classified into four families; A, B, X, and Y. Among them, DNA polymerase η, a Y family polymerase, is a low fidelity enzyme that contributes to translesional synthesis and somatic hypermutation. Although a high mutation frequency is observed in immunoglobulin genes, translesional synthesis occurs with a high accuracy. We determined whether the misincorporation rate of DNA polymerase η varies with ambient conditions. It has been reported that DNA polymerase η is unable to exclude water molecules from the active site. This finding suggests that some ions affect hydrophobic bond formation at the active site. We focused on the effect of pH and evaluated the misincorporation rate of deoxyguanosine triphosphate (dGTP) opposite template T by DNA polymerase η at various pH levels with a synthetic template-primer. The misincorporation rate of dGTP by DNA polymerase η drastically increased at pH 8.0–9.0 compared with that at pH 6.5–7.5. Kinetic analysis revealed that the $K_m$ value for dGTP on the misincorporation opposite template T was markedly affected by pH. However, this drastic change was not seen with the low fidelity DNA polymerase α.

**Key words** misincorporation; DNA polymerase; pH; mutation

DNA replication and repair must occur with a high accuracy to maintain the integrity of genomic information. Several eukaryotic DNA polymerases contribute to replication and repair, but with differing fidelity. Eukaryotic DNA polymerases are currently classified into four families; A, B, X, and Y. Among them, Y family polymerases show lower fidelity than other families, lacking the intrinsic 3’ to 5’ exonuclease activity required for proofreading. DNA polymerase η, one of the Y family polymerases, was first found as a responsible gene product of *Xeroderma pigmentosum*. This polymerase contributes to translesional synthesis and somatic hypermutation of immunoglobulin variable genes. Although DNA polymerase η correctly bypasses thymine–thymine dimers resulting from UV-irradiation, it shows a high misincorporation rate of 1 in 28 nucleotides on an undamaged template.

The fidelity of a DNA polymerase depends not only on proofreading activity but also on nucleotide substrate selection at its active site. Nucleotide selection mainly depends on the binding affinity of DNA polymerase to the incoming nucleotide substrate, with the correct binding leading to the conformational change required for the suitable positioning of the active site. Although the hydrogen bond between template and substrate bases governs nucleotide selection, geometric selection is more important rather than the hydrogen bond. Geometric selection is a checking function that determines whether the shape and size of a base pair corresponds to a correct Watson-Crick type. DNA polymerases comprise three structural domains; finger, palm, and thumb; the O-helix in the finger domain governs geometric selection. However, DNA polymerase η is deficient in the O-helix structure compared with polymerases of other families. Accordingly, nucleotide selection by this polymerase depends on the base–base hydrogen bond rather than on geometric selection. Water exclusion is important for the formation of a correct base–base hydrogen bond, since the increase of effective size of DNA bases by hydrogen bonds from bases to water prevents misinsertion of small bases opposite each other. DNA polymerases usually increase nucleotide selectivity by excluding water from the active site, amplifying the difference in free energy between correct and incorrect base pairs. However, both the finger and thumb domains of DNA polymerase η are relatively smaller than those of other family polymerases, so that the active site of DNA polymerase η is unable to exclude water. Thus, the water molecule may affect the nucleotide selection of polymerase η. In contrast, low fidelity DNA polymerase α, belonging to B family, is able to exclude water, and nucleotide selection by this polymerase may be dependent on geometric selection rather than on water exclusion.

One of the unique characteristics of DNA polymerase η, not seen in polymerase α, is its misincorporation of deoxyguanosine triphosphate (dGTP) opposite template T and induction of an A to G transition mutation with a high error rate of $5.7 \times 10^{-2}$. This rate is 5–10-fold higher than that of other types of misincorporation, such as thymidine triphosphate (dTTP) opposite template G. This characteristic is reflected in the mutation spectrum of immunoglobulin gene hypermutation. However, no marked difference in A to G transition mutation frequency except in the immunoglobulin gene has been reported between polymerase η-expressing and deficient cells, even though this polymerase may be strictly restricted to damage sites. Why is hypermutation by DNA polymerase η seen only in the immunoglobulin genes? Does the fidelity of DNA polymerase η vary according to the ambient conditions in living cells? As described above, polymerase η cannot exclude water, suggesting that water or ionized water may affect base-base hydrogen bond formation at the active site of the enzyme. Water molecules are ionized according to pH, but the effect of pH on the fidelity of DNA polymerases η has not yet been investigated. Focusing on the effect of pH, we investigated the misincorporation rate of dGTP opposite template T by DNA polymerases η at various pH levels using a synthetic template-primer. The result showed that the pH markedly af-

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fected the misincorporation rate of dGTP opposite template T by DNA polymerase \( \eta \).

MATERIALS AND METHODS

**Template-Primer** Synthetic oligonucleotides were used as a template-primer. The sequences of template and primer are shown in Fig. 1A. The 5‘-end of the primer was labeled with \(^{32}\)P.

**DNA Polymerases** The budding yeast DNA polymerase \( \eta \) was prepared as described previously. \(^{23}\) DNA polymerase \( \alpha \) was purified from calf thymus. \(^{24}\)

**Primer Extension Reaction** The primer extension reaction was performed with a reaction mixture (5 \( \mu \)L) containing 40 mM Tris–HCl (pH 6.5–9.0), 0.4 pmol template-\(^{32}\)P-primer, 100 \( \mu \)M of each of the 4 dNTPs, 10 \( \mu \)M dithiothreitol, 100 \( \mu \)M KCl (for DNA polymerase \( \eta \)), 5 \( \mu \)M MgCl\(_2\), and 0.01 unit DNA polymerase \( \eta \) or 0.003 unit DNA polymerase \( \alpha \). The mixture was incubated at 37°C for 30 min. After the reaction, DNA was purified and separated by 7 M urea–20% polyacrylamide gel electrophoresis. The \(^{32}\)P-labeled products were detected and analyzed with a Fuji BAS 1500 phosphor imager.

**Quantification and Kinetic Analysis** The amounts of incorporated nucleotides were determined as the ratio of the intensity of the elongated band to that of remaining primer band based on the phosphor imager analysis. The \( K_m \) and \( V_{\text{max}} \) values of incorporated nucleotides were obtained from the double reciprocal plots (Lineweaver–Burk plots).

RESULTS

**Effect of pH on the Incorporation of Nucleotides into a Synthetic Template-Primer by DNA Polymerase \( \eta \)** The primer extension assay with DNA polymerase \( \eta \) was performed using the synthetic template-primer at various pH levels. The sequence of the template-primer is shown in Fig. 1A. The X in the template varied several bases according to the combination of substrates. Four combinations of template base and substrate were investigated: thymine at X in the template and dATP substrate (designated as T : dATP), cytosine at X and dGTP (C : dGTP), thymine at X and dGTP (T : dGTP), and guanine at X and dTTP (G : dTTP). Products were separated by 7 M urea–20% polyacrylamide gel electrophoresis and detected with a Fuji BAS1500 phosphor imager. Typical results are shown in Fig. 1B. The amounts of products (16 mer) at each pH were determined and plotted (Fig. 1C). In the case of T : dATP and C : dGTP, a standard Watson–Crick type base pair, the 16mer products were detected at each pH, and the optimal pH was 8.5. In contrast, the mismatched products of T : dGTP were clearly detected at pH 8.0–9.0 whereas those at pH 6.5–7.5 were rare. This result indicates that DNA polymerase \( \eta \) efficiently incorporated dGTP opposite template T at pH 8.0–9.0 but not at pH 6.5–7.5. Thus, the misincorporation rate of dGTP opposite template T by DNA polymerase \( \eta \) may be markedly affected by pH. Interestingly, the mismatched products of G : dTTP, an opposite combination of substrate and template base, were rare at each pH (Figs. 1B, C). This result suggests that pH does not affect the incorporation of dTTP opposite template G by DNA polymerase \( \eta \).

**Concentration-Dependent Misincorporation by DNA Polymerase \( \eta \)** To confirm the effect of pH on the misincorporation by DNA polymerase \( \eta \), the same reaction was performed again with different concentrations of dGTP from 0 to 1000 \( \mu \)M at various pH levels. The products were analyzed by 7 M urea–20% polyacrylamide gel electrophoresis followed by phosphor imaging. The results are shown in Fig. 2. The
amounts of incorporated products were gradually increased with the concentration of dGTP when the reaction was performed at pH 7.5–9.0. In contrast, few products were detected at pH 6.5–7.0 even at a high concentration of dGTP. This result confirms that the high rate of misincorporation of dGTP opposite template T by DNA polymerase η is pH-specific and independent of the concentration of dGTP.

The misincorporated products of G:dTTP also increased with the concentration of dTTP (Fig. 3). However, the drastic change in misincorporation rate was not seen at all pH levels.
tested, although the product amounts were slightly increased at pH 8.0–9.0.

Effect of pH on the Incorporation of Nucleotides into Template-Primer by DNA Polymerase α As described above, the misincorporation rate of dGTP opposite template T by DNA polymerase η was markedly affected by pH. We then determined whether this pH effect was shared by another low fidelity DNA polymerase. The same reaction was performed with DNA polymerase α. Figure 4A shows the result of the primer extension assay with various combinations of template base and nucleotide substrate at pH 6.5–9.0 using the same template-primer. Similarly to the case of DNA polymerase η, the products of Watson–Crick type base pairs (T:dATP and C:dGTP) were detected at each pH, and the optimal pH was 7.0. In contrast, the amounts of mismatch products (T:dGTP and G:dTTP) were much lower than those of matched products, and the amounts of neither of the mismatch products were drastically changed by pH (Fig. 4B). We also investigated the effect of dGTP concentration on mismatch formation by DNA polymerase α at various pH levels (Fig. 5). The amounts of products gradually increased with dGTP concentration and pH, but no drastic change in the misincorporation rate of dGTP opposite template T was observed (Fig. 5B). This result strongly indicates that the effect of pH on the mis-

**Fig. 4. Primer Extension Assay with DNA Polymerase α**

(A) Effect of pH on the incorporation of dNTP into synthetic template-primer. Combination of template base and nucleotide substrate is shown in a–d. Position of primer (15 mer) and product (16 mer) are indicated at the left of each panel. (B) Amount of products shown in (A) were determined and represented as a graph. ○: template T:dATP, ▲: template C:dGTP, △: template T:dGTP, ■: template G:dTTP.

**Fig. 5. Concentration-Dependent Misincorporation of dGTP Opposite Template T by DNA Polymerase α**

(A) Primer extension assay was performed with various concentration of dGTP at pH 6.5–9.0 using the T template (X=T). The pH is indicated in a–f. Concentration of dGTP is shown at the bottom of each panel. Position of primer (15 mer) and product (16 mer) are indicated at the left of each panel. (B) Amount of products shown in (A) were determined and represented as a graph. ▲: pH 6.5, ○: pH 7.0, ■: pH 7.5, △: pH 8.0, △: pH 8.5, ○: pH 9.0.
polymerase and different effects of pH on the misincorporation rates of DNA. In the present study and η of dGTP opposite template T between DNA polymerases the differing effects of pH on the misincorporation mechanism to dGTP. These differences in kinetic parameters may reflect reaction velocity of DNA polymerase rather than the affinity value gradually increased with pH. Thus, pH may affect the template T was drastically decreased at pH 7.5–8.0, and the m value for dGTP at pH 8.0 to 9.0 was that at pH 7.5. The K value for dGTP was drastically decreased at pH 7.5–8.0 (Table 1). This result suggests that the affinity of DNA polymerase η to dGTP for mismatch formation with template T increases at pH 8.0–9.0. The active site of η polymerase is unable to exclude water. Thus, ions such as the OH− ion may affect base pair formation at the active site of DNA polymerase η, and the OH− ion may stabilize the wobble type base pair between thymine on the template and guanine on the substrate. A crystal structure study of Bacillus DNA polymerase I fragment with the G-T mismatched base pair revealed that an additional water-mediated hydrogen bond between H−N2 of guanine and O7 of thymine was observed. This stabilization effect may increase the affinity of DNA polymerase η to dGTP that is paired with thymine. In contrast, no strong effect of pH on the misincorporation rate of dTTP opposite template G was seen (Fig. 3). A crystal structure study of Bacillus DNA polymerase I fragment suggested that the geometrical position of template and substrate bases at the active site of the polymerase differs between T:dGTP and G:dTTP. One of the reasons for the differing effect of pH on T:dGTP and G:dTTP mismatch formation may be this geometrical difference. However, the further analysis is required.

At present, it is unknown whether pH affects the misincorporation rate of DNA polymerase η in the living cells. As a role of polymerase η, high accuracy is required for the translesional synthesis whereas a high misincorporation rate is necessary for somatic hypermutation. Our results strongly suggest that the fidelity of polymerase η varies with ambient conditions. DNA polymerase η is recruited to UV-damaged sites or immunoglobulin genes by the ubiquitylated proliferating cell nuclear antigen (PCNA) and interacts with several proteins such as other translesional polymerases. The possibility that these protein–protein interactions affect the fidelity of DNA polymerase η cannot be ruled out, although it is reported that the non-ubiquitylated PCNA has little effect on the fidelity of the polymerase. In contrast to DNA polymerase η, DNA polymerase α did not show a drastic change in the misincorporation rate of dGTP opposite template T with pH (Figs. 4, 5). The K value for dGTP was almost the same at pH 7.0–9.0 (Table 1). DNA polymerase α is able to exclude water from the active site so that ionized water may not affect the base pair formation at the active site of this polymerase. These structural differences between DNA polymerases η and α may account for their differing behavior in response to pH. It would be of interest to determine whether the other Y family polymerases, such as DNA polymerase i or κ show the same behavior as polymerase η.

**Acknowledgments** We thank Dr. Thomas A Kunkel at the National Institute of Environmental Health Sciences for the critical reading of this manuscript. This work was performed, in part, at the Kurokami RI, Institute of Resource Development and Analysis Radio Isotope Center, Kumamoto University.

| pH   | K (µM) ± | V max (fmmol) | V max/Km (fmmol/µM) | f (a) |
|------|---------|---------------|----------------------|------|
| 7.0  | 750±15  | 32±1.1        | 0.043                | 0.046|
| 7.5  | 780±10  | 72±5.1        | 0.092                | 0.10 |
| 8.0  | 360±8.0 | 230±16        | 0.64                 | 0.69 |
| 8.5  | 260±10  | 230±20        | 0.88                 | 0.96 |
| 9.0  | 250±7.0 | 230±12        | 0.92                 | 1.0  |

(a) Relative ratio of V max/Km value.

**DISCUSSION**

A high misincorporation rate of dGTP opposite template T is a unique characteristic of DNA polymerase η that is not seen in other low fidelity polymerases. In the present study we investigated the effect of pH on the misincorporation rate of DNA polymerase η. Results showed that pH strongly affected the misincorporation rate of dGTP opposite template T by DNA polymerase η (Figs. 1, 2). The crystal structure of Bacillus DNA polymerase I fragment with DNA containing a mismatched base pair has been reported. According to the authors’ analysis, the mismatched base pair of thymine on template and guanine on substrate is of a wobble type. The geometrical position of a wobble type base pair is different from that of a Watson–Crick type base pair at the active site of a polymerase. DNA polymerase η has a weak geometric selection ability owing to its deficient O-helix structure in the finger domain. Thus, a wobble type base pair is more readily formed on η polymerase than on DNA polymerases of other families that have O-helix structures such as α polymerase. Current crystal structure studies of DNA polymerase η in ternary complex with a cis-syn thymine dimer and with undamaged DNA showed that an active site cleft of polymerase η was much more open than other polymerases. The K value for dGTP was drastically decreased at pH 7.5–8.0 (Table 1). This result suggests that the affinity of DNA polymerase η to dGTP for mismatch formation with template T increases at pH 8.0–9.0. The active site of η polymerase is unable to exclude water. Thus, ions such as the OH− ion may affect base pair formation at the active site of DNA polymerase η, and the OH− ion may stabilize the wobble type base pair between thymine on the template and guanine on the substrate. A crystal structure study of Bacillus DNA polymerase I fragment with the G-T mismatched base pair revealed that an additional water-mediated hydrogen bond between H−N2 of guanine and O7 of thymine was observed. This stabilization effect may increase the affinity of DNA polymerase η to dGTP that is paired with thymine. In contrast, no strong effect of pH on the misincorporation rate of dTTP opposite template G was seen (Fig. 3). A crystal structure study of Bacillus DNA polymerase I fragment suggested that the geometrical position of template and substrate bases at the active site of the polymerase differs between T:dGTP and G:dTTP. One of the reasons for the differing effect of pH on T:dGTP and G:dTTP mismatch formation may be this geometrical difference. However, the further analysis is required.

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Conflict of Interest  The authors declare no conflict of interest.

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