Nuclear Magnetic Resonance Investigation of Primer−Template Models: Formation of a Pyrimidine Bulge upon Misincorporation[†]

Lai Man Chi and Sik Lok Lam*

Department of Chemistry, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong

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ABSTRACT: Our previous studies have shown that misaligned structures can occur upon misincorporation of a dNTP opposite thymine templates. The formation of misaligned structures during DNA replication, if not repaired properly, can be bypassed and extended by low-fidelity polymerases and ultimately lead to mutations. In this study, the base pair structures at the replicating sites of a set of primer−template models which mimic the situation upon misincorporation of a dNTP opposite cytosine templates have been determined. High-resolution NMR structural results show that misaligned structures with a C-bulge can be formed upon incorporation of dCTP, dTTP, and dATP opposite 5′-GC, 5′-AC, and 5′-TC templates, respectively. The stabilities of misaligned structures depend on the types of terminal base pairs at the replicating sites. Together with the structural findings in thymine templates, we conclude that terminal G•C and C•G base pairs always contribute a larger stabilizing effect to the misaligned structures containing a pyrimidine bulge than terminal A•T and T•A base pairs. Misalignment and thus deletion mutation are more likely to occur if misincorporation of a nucleotide opposite a pyrimidine template can cause template slippage to form a terminal G•C or C•G base pair. Although misalignment also occurs when the newly formed terminal base pair is an A•T base pair or a T•A base pair, both misaligned and mismatched conformers coexist, which can lead to deletion and substitution mutations, respectively.

DNA replication fidelity is known to be associated with the selectivity of DNA polymerase, exonucleolytic proofreading function, and postreplication mismatch repair activity (*1*). To have an accurate genetic information transfer and prevent mutations that can initiate and promote human diseases over generations, highly processive and accurate DNA synthesis is indispensable. Nevertheless, mutations are essential for facilitating translesion synthesis of otherwise replication-blocking lesions and are counterbalanced by the requirement for mutations in evolution, fitness, and immunological diversity (*2*).

Continuous efforts in DNA polymerase research improve our understanding of replication fidelity. The discovery of the proofreading deficient Y-family DNA polymerases provides new mechanistic pathways for evolution by efficiently bypassing damaged templates of bulky DNA adducts and/or replicating undamaged DNA (*3–7*). The Y-family polymerases have no detectable sequence similarity with other family polymerases and lack any intrinsic exonucleolytic activity for proofreading (*8*). Their active sites are open and spacious and allow the accommodation of bulky adducts and wobble or Hoogsteen base pairs (*9–13*). Recently, it has been demonstrated that a single-nucleotide bulge formed at the template terminus could be extended by a low-fidelity polymerase (*14*). In addition, crystal structures of a low-fidelity polymerase containing a misaligned DNA template in active sites have been determined (*12, 15*). These results show the formation and accommodation of a misaligned primer−template motif in the loose active site is possible (*16*), which ultimately can lead to dislocation mutagenesis (*17–19*), yet whether misalignment of the primer−template motif is due to the presence of the polymerase or template sequence remains elusive.

Misaligned intermediates can occur when the polymerase slows or stalls at sites of lesions during DNA replication (*20–26*). However, such effects are much less common when unmodified DNA is replicated. Recently, we performed NMR[1] structural investigations on primer−template oligonucleotide models which mimic the situation in which a dNTP has just been incorporated opposite a thymine template (*27, 28*), revealing the occurrence of misaligned primer−template structures with a T-bulge. Depending on the sequence upstream of the thymine template, further primer extension can lead to realignment and thus the formation of G•T and T•T mismatches. In addition to direct incorporation of a dGTP opposite a thymine template, this provides an alternative mechanistic pathway for G•T wobble pair formation in the loose active center of the human Y-family polymerase Polι (*13*), explaining why insertion of G opposite T is even more efficient than A opposite T (*29–31*).

In this study, we extended our NMR investigations on primer−template models containing a cytosine template. The

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* To whom correspondence should be addressed. Phone: (852) 2609-8126. Fax: (852) 2603-5057. E-mail: lams@cuhk.edu.hk.

[1] Abbreviations: NMR, nuclear magnetic resonance; dNTP, deoxyribonucleotide triphosphate; NOE, nuclear Overhauser effect; 2D NOESY, two-dimensional nuclear Overhauser effect spectroscopy; polι, polymerase ι; DSS, 2,2-dimethyl-2-silapentane-5-sulfonic acid; WATERGATE, water suppression by gradient-tailored excitation.

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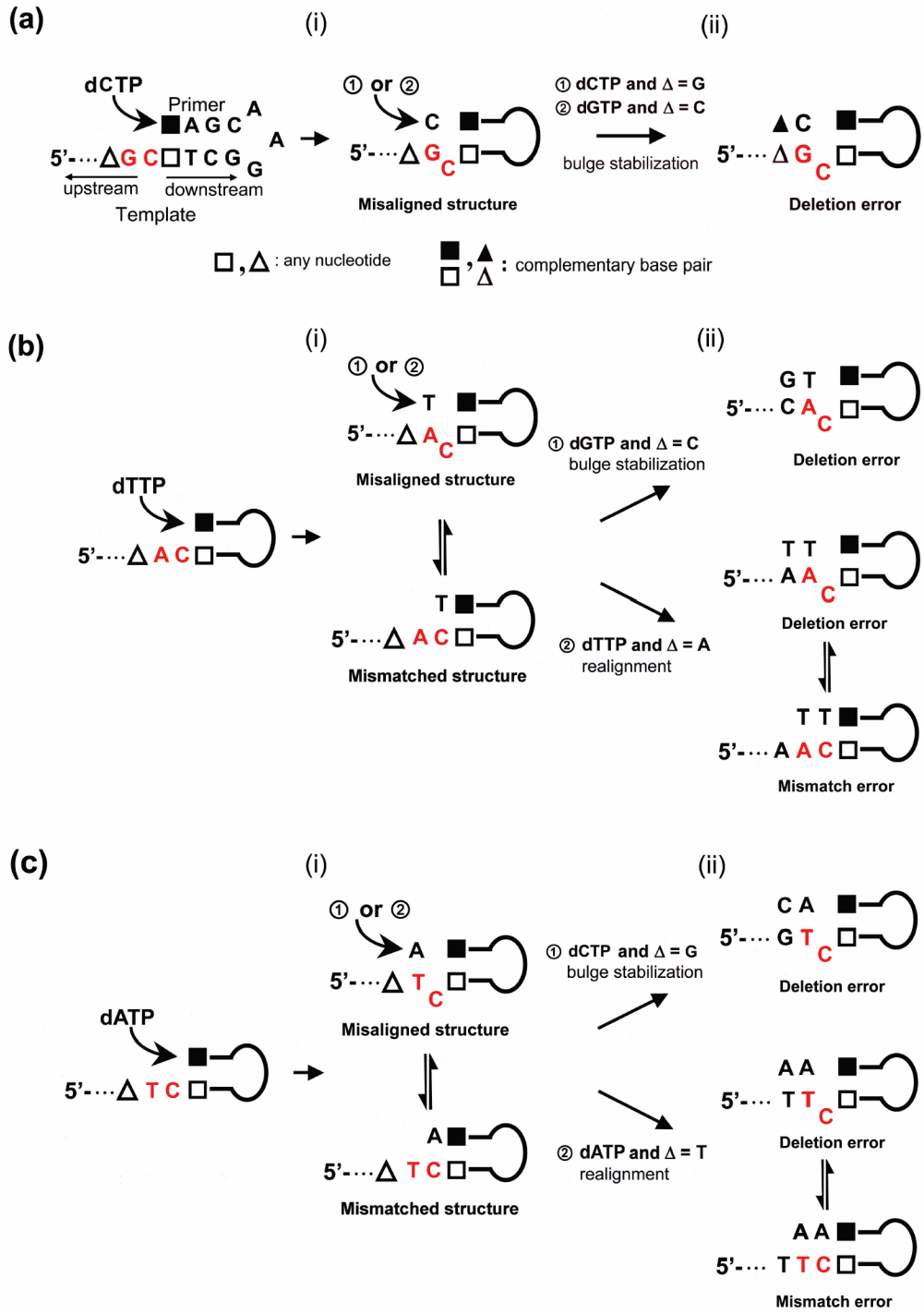

FIGURE 1: DNA primer−template models used in this study. All models were designed to form a hairpin with a 5′-GAA loop. The top and bottom strands mimic the primer and template, respectively. The upstream and downstream directions are from the perspective of the template strand. (a) (i) For a 5′-GC template, misalignment leads to the formation of a C·G base pair and a C-bulge. (ii) Further synthesis on the misaligned sample leads to deletion error. (b) (i) For a 5′-AC template, a misaligned conformer with a T·A base pair and a C-bulge and a mismatched conformer with a T·C mispair were observed. (ii) Further synthesis on the misaligned sample can bring about either deletion or mismatch error. (c) (i) For a 5′-TC template, both misaligned and mismatched conformers are formed upon incorporation of dATP. (ii) Further synthesis can lead to either mismatch or deletion error.

effect of a nucleotide upstream of the templating C was also determined. Upon incorporation of dCTP (Figure 1a), dTTP (Figure 1b), and dATP (Figure 1c) opposite 5′-GC, 5′-AC, and 5′-TC templates, respectively, we found that template slippage occurs and leads to the formation of misaligned structures with a C-bulge. Depending on the type of terminal base pair in these misaligned structures, conformational exchange can occur, leading to the formation of T·C and A·C mismatches in these primer−template models.

MATERIALS AND METHODS

Sample Design. All DNA samples were designed to form a hairpin with the top and bottom strands mimicking the primer and template, respectively (Figure 1). The 5′-GAA hairpin loop connects the primer and template strands to simplify the sample preparative work (*32*). These samples mimic the situation in which a dNTP has just been incorporated into the templates. The 5′-overhang region of the samples represents the template

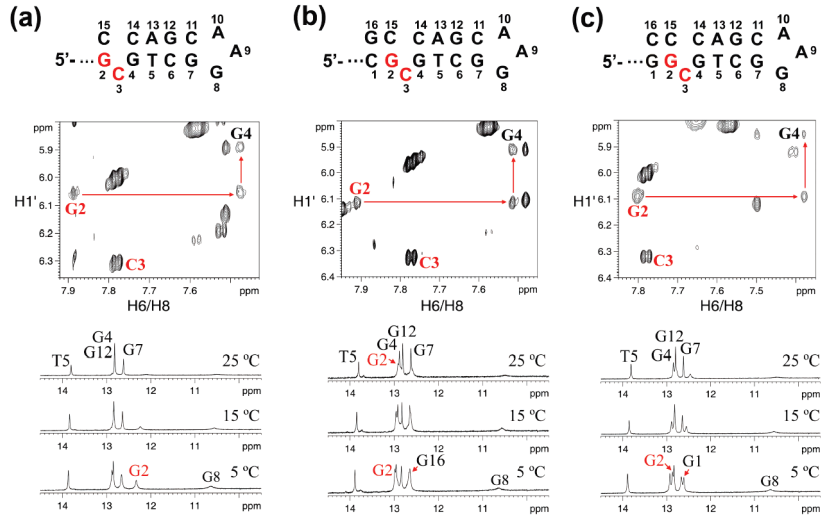

FIGURE 2: NMR evidence for misaligned structures of 5′-GC templates. (a) Misaligned structure formed after incorporation of a dCTP opposite a 5′-GC template. The NOESY H6/H8−H1′ fingerprint region at 25 °C shows an unusual cross-peak between G2 and G4. The G2 imino signal was clearly observed at 5 °C. The small imino signal at 10.7 ppm corresponds to the G8 imino signal of a sheared G8·A10 mispair in the GAA loop. (b) The same NOESY region of the primer−template model at 25 °C shows the G2−G4 NOE after further extension with a G opposite C upstream of the 5′-GC template. Both G16 and G2 imino signals were clearly observed at 5 °C. (c) Further extension with a C opposite G upstream of the 5′-GC template. A G2−G4 NOE was also observed at 25 °C. Both G1 and G2 imino signals were clearly observed at 5 °C.

sequence, whereas the 3′-terminal nucleotide represents the newly incorporated dNTP at the primer terminus.

Sample Preparation. All DNA samples were synthesized using an Applied Biosystems model 392 DNA synthesizer and purified using denaturing polyacrylamide gel electrophoresis and diethylaminoethyl Sephacel anion exchange column chromatography. NMR samples were prepared by dissolving 0.5 μmol of purified DNA samples into 500 μL of buffer solution containing 150 mM sodium chloride, 10 mM sodium phosphate (pH 7.0), and 0.1 mM DSS.

NMR Analysis. All NMR experiments were performed using either a Bruker ARX-500 or AV-500 spectrometer operating at 500.13 MHz. For studying labile proton resonance signals, the samples were prepared in a 90% H_2O/10% D_2O buffer solution. One-dimensional (1D) imino proton spectra were acquired using the WATERGATE pulse sequence (*33, 34*), and two-dimensional (2D) WATERGATE-NOESY experiments were performed with a mixing time of 300 ms. For studying nonlabile proton signals, the solvent was exchanged with a 100% D_2O solution. 2D NOESY experiments were also performed with a mixing time of 300 ms, and 4K × 512 data sets were collected. The acquired data were zero-filled to give 4K × 4K spectra with a cosine window function applied to both dimensions. In general, these experiments were conducted at 25 °C. To better observe and resolve the labile and nonlabile signals, we also repeated some of these experiments at lower temperatures.

RESULTS

In this study, we performed high-resolution NMR spectroscopic investigations focusing on 1D imino proton and 2D NOESY analyses to probe the replicating site structures of 5′-GC, 5′-AC, and 5′-TC templates after the incorporation of dCTP, dTTP, and dATP, respectively. Sequential proton resonance assignments were made by studying the fingerprint regions in 2D NOESY and WATERGATE-NOESY spectra (Supporting Information, S1−S11).

Incorporation of a dCTP Opposite a 5′-GC Template. When dCTP was incorporated opposite a 5′-GC template sequence, a C·G Watson–Crick base pair and a C-bulge were formed due to misalignment (Figure 1a, i). This was supported by the presence of an unusual G2 H1′−G4 H8 NOE (Figure 2a), which indicates G2 and G4 are close in space. Sequential NOE connectivities between consecutive nucleotides would be present in the 2D NOESY spectrum if there were no misalignment (*35*). However, no sequential G2 H1′−C3 H6 and C3 H1′−G4 H8 NOEs were observed in this case. In addition, the presence of the G2 imino signal (Figure 2a) and a NOE between G2 imino and C15 amino protons (Supporting Information, S1b) at lower temperatures reveal the formation of C15·G2 Watson–Crick base pair, confirming the primer−template model was misaligned.

When the primer with a G opposite C upstream of the 5′-GC template was further extended, the C-bulge of the misaligned structure was stabilized as supported by a G2−G4 NOE and a G2 imino signal at 25 °C (Figure 2b). This is further supported by the appearance of a G16 imino signal, G16 imino−C1 amino, and G2 imino−C15 amino NOEs (Supporting Information, S2b) at lower temperatures, confirming the presence of G16·C1 and C15·G2 base pairs. Similarly, when the nucleotide upstream of a 5′-GC template was a G, the misaligned structure was also formed upon extension with a C. No realignment leading to the formation of a C15·C3 mispair and a C16·G2 base pair was observed, as evidenced by the G2−G4 NOE and the presence of G1 and G2 imino signals (Figure 2c).

To investigate if the base pair downstream of the 5′-GC template affects the misalignment process, we substituted the C14·G4 base pair with A14·T4, T14·A4, and G14·C4 base pairs. In all three cases, the template strands were also slipped, leading to C-bulge formation as supported by their

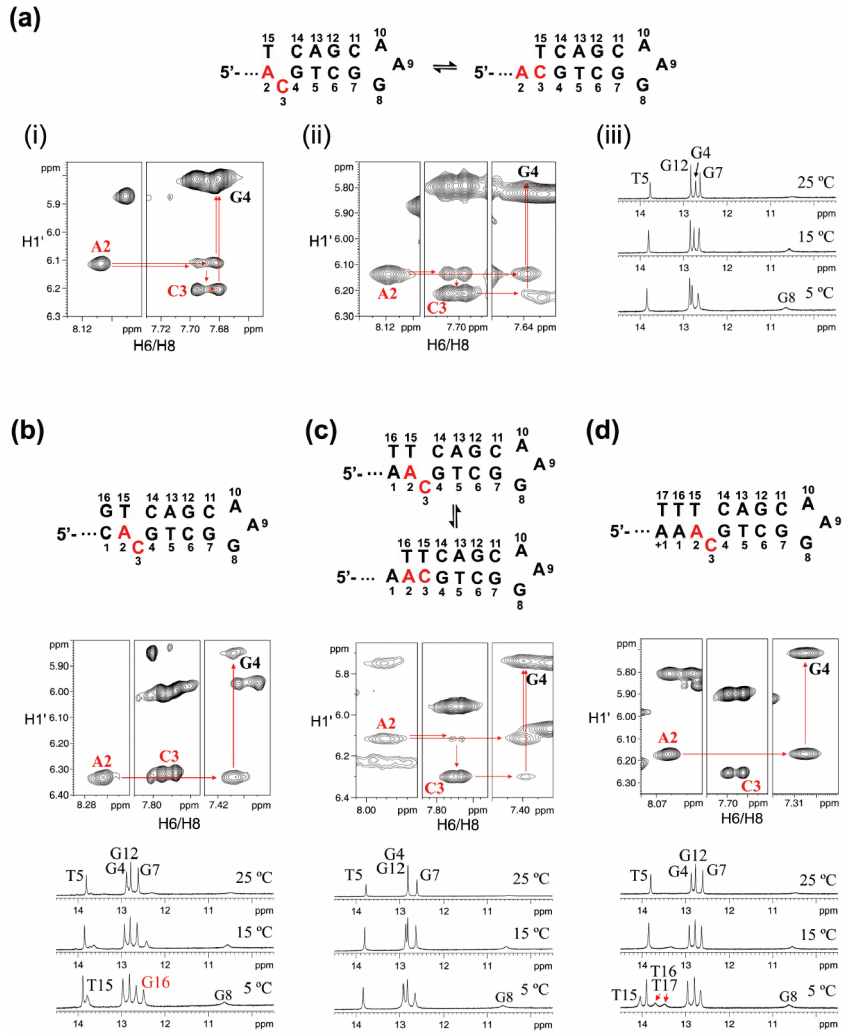

FIGURE 3: (a) Both misaligned and mismatched conformers are present upon incorporation of a dTTP opposite a 5′-AC template. The A2 H1′−G4 H8 and A2 H1′−C3 H6, A2 H1′−C3 H6, and C3 H1′−G4 H8 NOEs were (i) partially overlapped at 25 °C but (ii) resolved at 15 °C. (iii) No T15 imino signal from either conformer is present due to end fraying and rapid conformational exchange. (b) Addition of a G•C Watson–Crick base pair stabilizes the misaligned conformer and hampers the exchange with the mismatched conformer. An A2−G4 NOE was observed at 25 °C. G16 and T15 imino signals were also observed at lower temperatures. (c) Addition of a T•A base pair does not hamper the conformational exchange. The A2−G4, A2−C3, and C3−G4 NOEs were observed at 5 °C. (d) Further addition of another T•A base pair hampers the formation of the mismatched conformer. An A2−G4 NOE was observed at 25 °C. T15, T16, and T17 imino signals were also observed at 5 °C.

NOESY and imino proton spectra (Supporting Information, S12). The presence of a NOE between the second and fourth nucleotides at 25 °C and G2 imino signals at lower temperatures confirmed these misaligned structures. Unlike the T-bulge with a downstream A14•T4 base pair that resulted in bulge rearrangement (*28*), no C-bulge rearrangement was observed when the C14•G4 base pair was substituted with a G14•C4 base pair, the template of which contains two successive cytosines (Supporting Information, S12c).

Incorporation of a dTTP Opposite a 5′-AC Template. When dTTP was incorporated opposite a 5′-AC template, an averaged structure of a misaligned conformer with a C-bulge and a mismatched conformer with a T•C mispair was observed (Figure 1b, i). The presence of the misaligned conformer was supported by an unusual A2 H1′−G4 H8 NOE, which was partially overlapped with an A2 H1′−C3 H6 NOE at 25 °C (Figure 3a, i). For the mismatched conformer, A2 H1′−C3 H6 and C3 H1′−G4 H8 NOEs were observed, the latter of which was also partially overlapped with the C3 H1′−C3 H6 NOE. These partially overlapped NOEs were resolved when the temperature was lowered to 15 °C (Figure 3a, ii). From the imino spectra, only a single set of imino signals was observed (Figure 3a, iii), suggesting the misaligned and mismatched conformers undergo rapid exchange. Because of this exchange process and the end fraying effect, no T15 imino signal was observed even when the temperature was lowered to 5 °C. Similar results were observed when the C14•G4 base pair was substituted with A14•T4, T14•A4, and G14•C4 base pairs (Supporting Information, S13). In all three cases, rapid conformational exchange between the misaligned and mismatched conformers was observed. The presence of the C-bulge misaligned conformers was supported by the NOEs between the second and fourth nucleotides, whereas the NOEs between the second and third nucleotides and between the third and fourth nucleotides at 5 °C support the mismatched conformer. Again, unlike the T-bulge (*28*), no C-bulge rearrangement

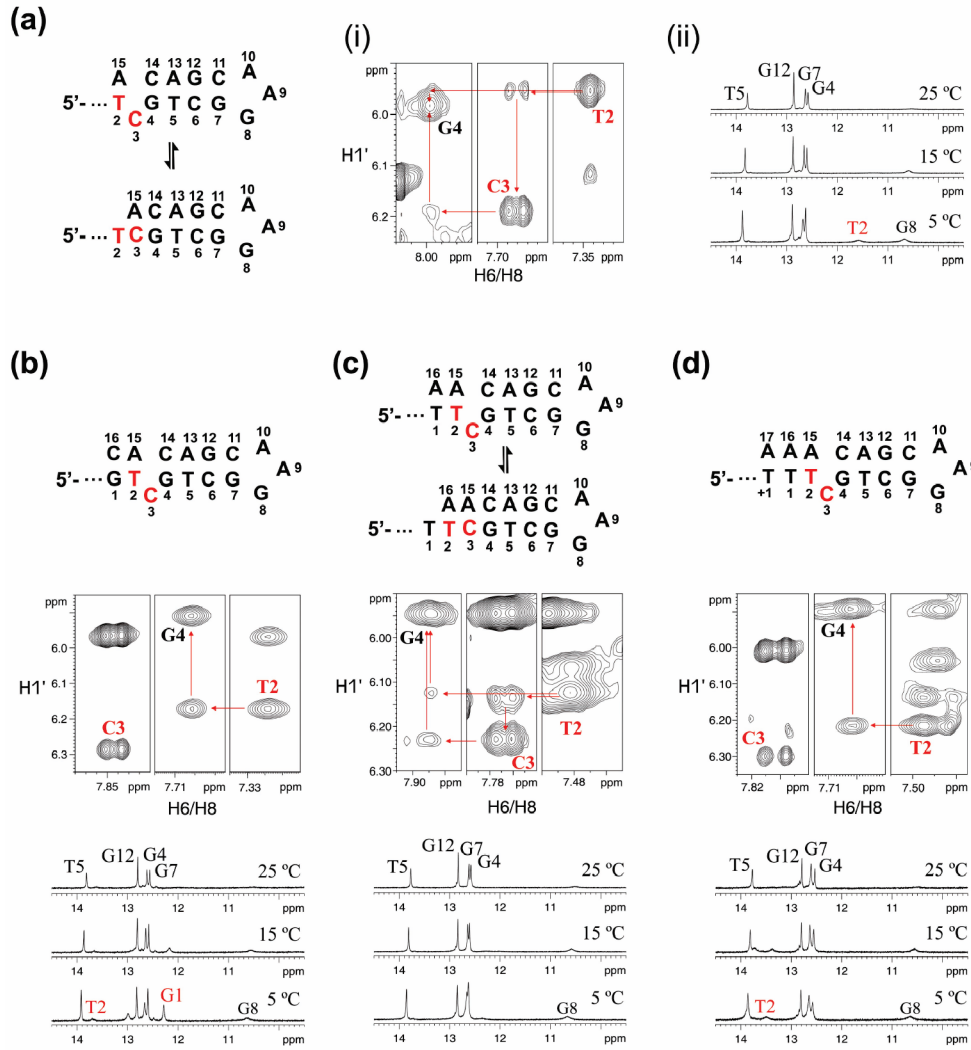

FIGURE 4: (a) Both misaligned and mismatched conformers are present upon incorporation of a dATP opposite a 5′-TC template. (i) The T2−G4, T2−C3, and C3−G4 NOEs were observed at 5 °C. (ii) A single set of imino signals indicates rapid conformational exchange between the two conformers. (b) Addition of a C•G Watson–Crick base pair stabilizes the misaligned conformer and hampers the formation of the mismatched conformer. A T2−G4 NOE was observed at 25 °C. G1 and T2 imino signals were also observed at lower temperatures. (c) Addition of an A•T base pair does not hamper the conformational exchange. The T2−G4, T2−C3, and C3−G4 NOEs were observed at 5 °C. (d) Further addition of another A•T base pair hampers the formation of the mismatched conformer. A T2−G4 NOE was observed at 25 °C.

was observed when the C14•G4 base pair was substituted with the G14•C4 base pair (Supporting Information, S13c).

When the primer with a G opposite C upstream of the 5′-AC template was further extended, the formation of the mismatched conformer was hampered as evidenced by the disappearance of the A2−C3 and C3−G4 NOEs (Figure 3b). Only the misaligned conformer was observed, as supported by the presence of the A2−G4 NOE at 25 °C, G16 and T15 imino signals (Figure 3b), and G16 imino−C1 amino and T15 imino−A2 H2 NOEs (Supporting Information, S5b) at lower temperatures, confirming the presence of G16•C1 and T15•A2 base pairs. On the other hand, upon further primer extension with an A upstream of the 5′-AC template, the conformational exchange process remained rapid at 5 °C as supported by the presence of A2−G4, A2−C3, and C3−G4 NOEs (Figure 3c). The more intense A2−G4 NOE, however, suggests the averaged structure is biased toward the misaligned conformer. With addition of another T17•A+1 base pair (Figure 3d), the conformational exchange process was hampered and only the C-bulge misaligned conformer was observed. This was evidenced by the presence of the A2−G4 NOE and T15, T16, and T17 imino signals that come from T15•A2, T16•A1, and T17•A+1 base pairs, respectively.

Incorporation of a dATP Opposite a 5′-TC Template. When dATP was incorporated opposite a 5′-TC template, an averaged structure of a misaligned conformer with a C-bulge and a mismatched conformer with an A15•C3 mispair was observed. This was evidenced by the presence of T2 H1′−G4 H8, T2 H1′−C3 H6, and C3 H1′−G4 H8 NOEs at 5 °C (Figure 4a, i) as well as a single set of imino signals (Figure 4a, ii). Because of the higher spectral resolution in the F_2 dimension of the NOESY spectrum, T2 H1′−G4 H8 and G4 H1′−G4 H8 NOEs were better resolved when we analyzed the H1′ signals from the F_2 dimension (Supporting Information, S14a, ii). Because of the weak terminal A•T base pair of the misaligned conformer, a significant end fraying effect resulted. Thus, the small imino peak at 11.6 ppm in Figure 4a (ii) was mainly contributed by the overhang T2 imino signal of the mismatched conformer, as supported by the corresponding overhang T2

imino signals of reference samples at 11.5 ppm (Supporting Information, S14b, ii and iii).

When the downstream C14•G4 base pair of the 5′-TC template was substituted with A14•T4, T14•A4, and G14•C4 base pairs, similar results were found. NOEs between the second and fourth, second and third, and third and fourth nucleotides were observed at 5 °C, suggesting rapid conformational exchange between the misaligned and mismatched conformers (Supporting Information, S15). For the sample with a downstream G14•C4 base pair, no C-bulge rearrangement was observed in the misaligned conformer as evidenced by the presence of T2−C4, T2−C3, and C3−C4 NOEs (Supporting Information, S15c). Interestingly, the misaligned conformer was found to be more populated when compared to the other three cases, as supported by the more intense T2−C4 NOE and the more downfield T2 imino signal (Supporting Information, S15).

Further extending the primer with a C opposite G upstream of the 5′-TC template hampered the conformational exchange process between the misaligned and mismatched conformers, resulting in a single misaligned conformer (Figure 1c, ii). The presence of a T2−G4 NOE at 25 °C supports the formation of a C-bulge, whereas the presence of G1 and T2 imino signals (Figure 4b) and G1 amino−C16 amino and T2 imino−A15 H2 NOEs suggests the formation of C16•G1 and A15•T2 base pairs (Supporting Information, S9b). In addition, A2−T3 and T3−G4 NOEs disappeared (Figure 4b), indicating there is no mismatched conformer. On the other hand, adding an A•T Watson–Crick base pair did not hamper the formation of the mismatched conformer (Figure 4c), as supported by the presence of T2−G4, T2−C3, and C3−G4 NOEs at 5 °C. Further addition of another A•T Watson–Crick base pair resulted in a single misaligned conformer, as evidenced by the presence of the T2−G4 NOE and the disappearance of T2−C3 and C3−G4 NOEs at 25 °C (Figure 4d). Because of a serious end fraying effect, only the T2 imino signal was observed at 5 °C.

DISCUSSION

Significance of the Terminal Base Pair in the Misaligned Conformer. When a misincorporation of a dNTP opposite cytosine templates resulted in a terminal G•C or C•G base pair, only the misaligned conformer was observed. On the other hand, when a terminal A•T or T•A base pair was formed in the misaligned conformer, it did not outweigh the destabilizing effect of the C-bulge which exaggerates end fraying. Thereby, both of the misaligned and mismatched conformers were formed and underwent rapid exchange. The formation of the mismatched conformer was hindered when the terminus of the misaligned conformer was further stabilized by the addition of Watson–Crick base pairs, revealing the conformational exchange process depends on the relative stabilities of the two conformers. If the misaligned conformer is more stable than the mismatched one, it is likely that only the misaligned conformer will be present. On the other hand, if both conformers have similar stabilities, coexistence of the misaligned and mismatched conformers will occur. Similar findings were also observed in our previous studies on thymine templates (*27, 28*), suggesting that terminal G•C and C•G base pairs always contribute a larger stabilizing effect to the misaligned conformer than terminal A•T and T•A base pairs.

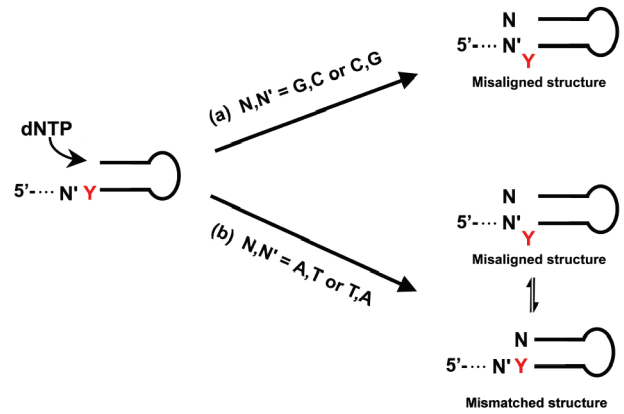

FIGURE 5: Consequences of misincorporation of a dNTP opposite a pyrimidine template. (a) A single misaligned structure will be formed if the primer−template slippage results in a terminal G•C or C•G base pair. (b) The misaligned structure will undergo conformational exchange with a mismatched structure if the slippage results in a terminal A•T or T•A base pair.

The Downstream Base Pair Determines Bulge Rearrangement. Because of improved base pair stacking which leads to a gain in the level of stabilization, rearrangement of the T-bulge was observed in the 5′-GT template with a downstream T but not in 5′-CT and 5′-AT templates (*27, 28*). However, such bulge rearrangement was not observed in the misaligned structures of all cytosine templates with a downstream C. The difference in the behavior of the T- and C-bulges can be rationalized by the strength and stability of the base pair downstream of these bulges, in which the G•C base pair in the C-bulge misaligned structures is significantly more stable than the A•T base pair in the T-bulge misaligned structures (*36, 37*).

Biological Implications of Pyrimidine Bulge Formation. In this work, we have investigated the base pair structures at replicating sites of primer−template models and demonstrated that C-bulge formation is possible upon misincorporation of a dNTP opposite a cytosine template. Together with our previous findings in thymine templates (*27, 28*), we conclude that misincorporation of a dNTP opposite a pyrimidine template can lead to misaligned structures. On one hand, if the primer−template slippage results in a terminal G•C or C•G base pair, a single misaligned structure will be formed (Figure 5a). On the other hand, if the slippage results in a terminal A•T or T•A base pair, the misaligned structure will undergo conformational exchange with a mismatched structure (Figure 5b). Upon further primer extension, the misaligned structure can (i) further be stabilized, (ii) realign to form a mismatched conformer, or (iii) exchange with a mismatched conformer. Depending on the template sequence further upstream, subsequent incorporation of dNTPs can lead to either mismatch or deletion error.

As crystallographic studies have shown the spacious active site of low-fidelity polymerase is capable of accommodating a misaligned DNA template (*12, 15*) and enzymatic assays have shown that primer extension by low-fidelity polymerase on a single-nucleotide bulge can occur at the template terminus (*14*), it is possible that errors resulting from low-fidelity DNA replication are related to the formation propensity of misaligned structures in different templates. To gain a thorough picture of intrinsic structural features possessed by different primer−template models, we are in

the process of investigating the structural features of purine templates. Since individual DNA polymerases are likely to interact with primer−template models differently, the extent to which these structures occur in DNA polymerases may also depend upon the specific polymerase in question. To investigate the mechanistic pathways for the occurrence of mutations during low-fidelity DNA replication, further investigation is needed to determine whether misalignment occurs on a time scale faster than the rate at which a second nucleotide is incorporated.

CONCLUSIONS

Combining the results of this study with our previous findings, we conclude that formation of a misaligned structure with a pyrimidine bulge is possible upon misincorporation of a dNTP opposite a pyrimidine template. Terminal G•C and C•G base pairs always contribute a larger stabilizing effect to the misaligned conformer than terminal A•T and T•A base pairs, leading to a single misaligned conformer. Conformational exchange between misaligned and mismatched conformers occurs in all cases with a terminal A•T or T•A base pair, indicating A•T and T•A base pairs are less sustainable to the destabilizing pyrimidine bulge than G•C and C•G base pairs. Our results suggest alternative pathways for base substitution and frameshift mutations, as well as the involvement of DNA primer−template motifs during low-fidelity DNA replication.

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SUPPORTING INFORMATION AVAILABLE

Figures showing sequential assignment, NOESY, and imino proton spectra of the primer−template models. This material is available free of charge via the Internet at http://pubs.acs.org.

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