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A Crystallographic Study of the Role of Sequence Context in Thymine Glycol Bypass by a Replicative DNA Polymerase Serendipitously Sheds Light on the Exonuclease Complex

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Thymine glycol (Tg) is the most common oxidation product of thymine and is known to be a strong block to replicative DNA polymerases. A previously solved structure of the bacteriophage RB69 DNA polymerase (RB69 gp43) in complex with Tg in the sequence context 5′-G-Tg-G shed light on how Tg blocks primer elongation: The protruding methyl group of the oxidized thymine displaces the adjacent 5′-G, which can no longer serve as a template for primer elongation [Aller, P., Rould, M. A., Hogg, M, Wallace, S. S. & Doublié S. (2007). A structural rationale for stalling of a replicative DNA polymerase at the most common oxidative thymine lesion, thymine glycol. *Proc. Natl. Acad. Sci. USA*, **104**, 814–818.].

Several studies showed that in the sequence context 5′-C-Tg-purine, Tg is more likely to be bypassed by Klenow fragment, an A-family DNA polymerase. We set out to investigate the role of sequence context in Tg bypass in a B-family polymerase and to solve the crystal structures of the bacteriophage RB69 DNA polymerase in complex with Tg-containing DNA in the three remaining sequence contexts: 5′-A-Tg-G, 5′-T-Tg-G, and 5′-C-Tg-G. A combination of several factors—including the associated exonuclease activity, the nature of the 3′ and 5′ bases surrounding Tg, and the cis–trans interconversion of Tg—influences Tg bypass. We also visualized for the first time the structure of a well-ordered exonuclease complex, allowing us to identify and confirm the role of key residues (Phe123, Met256, and Tyr257) in strand separation and in the stabilization of the primer strand in the exonuclease site.

Introduction

Thymine glycol (Tg; 5,6-dihydroxy-5,6-dihydrothymine) is the most common oxidation product of thymine. Tg is produced endogenously by aerobic metabolism and exogenous factors such as ionizing radiation and chemical oxidants. It has been estimated that, within each cell, about 400 Tg lesions are generated per day.[1–3] Tg lesions in DNA are recognized and processed by DNA glycosylases, such as human NTH1 and NEIL1, from the base excision repair pathway.[4–6] Even though Tg can be repaired, DNA polymerases do encounter unrepaired Tg lesions while replicating DNA. Since replicative DNA polymerases preferentially incorporate an adenine opposite Tg, this lesion is not mutagenic.[7] On the other hand, Tg has been shown

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Abbreviations used: Tg, thymine glycol; 5′-T-Tg-G-ter, 5′-T-Tg-G ternary complex; AcyATP, acyclic ATP; PDB, Protein Data Bank; 5′-T-Tg-G-bin, 5′-T-Tg-G binary complex; PEG, polyethylene glycol.

to constitute a strong block to replication *in vitro*,[8–10] which can be lethal *in vivo* in the absence of translesion synthesis or recombination.[7,11–13] Biochemical and structural studies have shown that the replication block arises not at the insertion step but rather during extension past the Tg·A base pair.[8,10,14–16] Most of the studies of DNA polymerases encountering Tg lesions were performed with the proofreading Klenow fragment from *Escherichia coli* DNA polymerase I, a polymerase of the A family.[8–10,15–18] These studies showed that Klenow fragment can bypass Tg in the specific sequence context 5′-C-Tg-purine in the DNA template strand. In contrast, T4 DNA polymerase, a B-family replicative DNA polymerase, cannot bypass Tg regardless of the sequence context.[15,16] In fact, the ability of a polymerase to bypass replication-blocking lesions, including Tg, is inversely correlated with its exonuclease activity.[8,10,15,16]

We recently solved the crystal structure of a homolog of the T4 replicative DNA polymerase, bacteriophage RB69 gp43, in complex with Tg-containing DNA in the sequence context 5′-G-Tg-G.[14] The structure revealed how the nonplanar Tg displaces its 5′-neighbor guanine and thereby blocks elongation after the incorporation of an adenine opposite the lesion. In order to further investigate the molecular mechanism underlying Tg bypass by a B-family DNA polymerase, we performed elongation assays of RB69 gp43 lacking exonuclease activity in four different template sequence contexts (5′-N-Tg-G, with N = A, G, T, or C) and solved the crystal structures of polymerase/DNA complexes in the same contexts. A comparison of the different structures suggests that both the 5′-neighbor base and the 3′-neighbor base of Tg influence bypass. In addition, the 5′-T-Tg-G ternary complex (5′-T-Tg-G-ter) structure, which contains four polymerase/DNA complexes per asymmetric unit, revealed a polymerizing complex (with the fingers domain opened) and an editing complex (in which the primer and the lesion-containing template are, for the first time, visualized in their entirety interacting with the β-hairpin structure of the exonuclease domain).

Results

RB69 gp43 exo⁻ is able to bypass Tg *in vitro*

We investigated the ability of a processive DNA polymerase of the B family to bypass Tg in different sequence contexts (5′-N-Tg-G, with N = A, T, G, or C) using primer extension assays. With an exonuclease-deficient variant of the bacteriophage RB69 DNA polymerase (RB69 exo⁻), we found that, like other DNA polymerases,[8–10,15,16] RB69 exo⁻ incorporates an adenine opposite Tg (Fig. 1, lane +dATP), independently of the sequence context. In the sequence context 5′-T-Tg-G and in the presence of dATP, two adenines are added.

When all four deoxynucleoside triphosphates were added to the reaction mix (Fig. 1, lane +dNTP), RB69 exo⁻ extended the primer to completion (17-mer). However, for the 5′-G-Tg-G sequence (Fig. 1, lane +dNTP, 5′-G-Tg-G), the polymerase stalled for a longer time after the incorporation of presumably an adenine opposite Tg (14-mer on the gel; Fig. 1) than for the other three sequences. The previously solved crystal structure of RB69 exo⁻ in complex with DNA containing this sequence context showed how the methyl group of

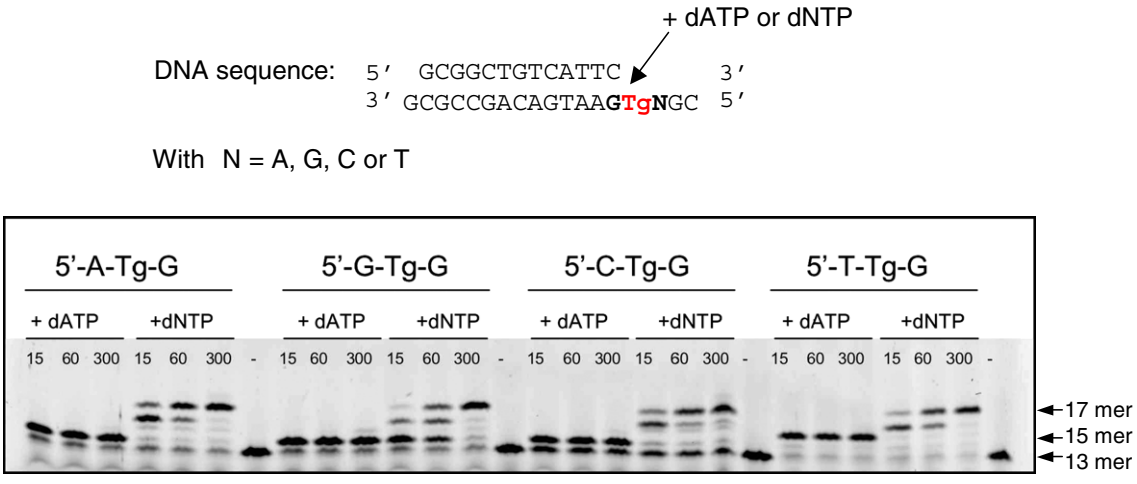

Fig. 1. RB69 gp43 exo⁻ can bypass Tg. Primer extension assays were performed for the four sequence contexts by adding either dATP only or a mix of the four deoxyribonucleotides (dNTP). The incubation times were varied: 15 s, 60 s, and 300 s. The initial length of the primer before the reaction is 13-mer. When dNTP is added, DNA synthesis goes to completion (17-mer).