PCNA-induced DNA synthesis past *cis*-syn and *trans*-syn-I thymine dimers by calf thymus DNA polymerase δ *in vitro*

Christine L.O'Day, Peter M.J.Burgers¹ and John-Stephen Taylor*,

Department of Chemistry, Washington University, One Brookings Drive, St Louis, MO 63130 and

¹Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, 660 South Euclid Avenue, St Louis, MO 63110, USA

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ABSTRACT

Calf thymus proliferating cell nuclear antigen (PCNA) promoted DNA synthesis past *cis*-syn and *trans*-syn-I cyclobutane thymine dimers by calf thymus DNA polymerase δ (Pol δ) *in vitro*. Templates containing site-specific *cis*-syn and *trans*-syn-I thymine dimers were prepared via a combination of solid phase synthesis with photoproduct building blocks and DNA ligation. Extension of a 15-mer primer on the UV dimer-containing templates by Pol δ produced termination and bypass products in a dNTP and PCNA dependent manner. In the absence of PCNA and at dNTP concentrations varying between 1 and 100 μ M, Pol δ could not bypass the *cis*-syn dimer and terminated elongation one nucleotide prior to the 3'-T of the dimer. DNA synthesis past the *trans*-syn-I dimer was even less efficient. In the presence of PCNA, termination occurred primarily one nucleotide prior to the 3'-T of both dimers at 1 μ M dNTPs but opposite the 5'-T of the dimers at 100 μ M dNTPs. In addition, under the latter conditions, bypass of the dimers was observed, to the extent of about 30% of the products for the *cis*-syn dimer and about 15% for the *trans*-syn-I dimer.

INTRODUCTION

During DNA replication, DNA polymerases stall upon encountering ultraviolet induced lesions *in vitro* (1) and *in vivo* (2). If unrepaired by repair systems, bypass DNA replication can be effected in order to complete the DNA synthesis period of the cell. This phenomenon is best understood in *Escherichia coli*. It is mediated by the SOS response in that organism, which presumably modifies the properties of DNA polymerase III holoenzyme to allow error-prone bypass (3).

In eukaryotic cells it is still unclear whether the replicative DNA polymerases can carry out error-prone translesion synthesis, whether these DNA polymerases are modified to carry out translesion synthesis, or whether a separate DNA polymerase is required for this process. Genetic evidence in the yeast *Saccharomyces cerevisiae* indicates that the product of the *REV3*

gene, a putative DNA polymerase, may be involved in error-prone translesion DNA synthesis, but not in regular DNA replication (4).

To understand how accessory factors can modulate the ability of a DNA polymerase to bypass UV dimers, we have studied the effect of the Proliferating Cell Nuclear Antigen (PCNA) on DNA polymerase δ (Pol δ) from calf thymus. Pol δ is required for DNA replication, presumably of the leading strand, during the S phase of the cell cycle (5, 6), and PCNA functions to enhance its processivity (7). Studies in mammalian cells indicate that this DNA polymerase may also be involved in DNA repair of UV lesions. This follows from DNA polymerase inhibitor studies (8), and from immunocytological studies with PCNA antibodies (9). Most recently PCNA has been found to be required for excision repair (10). However, it has been found in *Saccharomyces cerevisiae* that PCNA also interacts with pol ϵ (11), making it unclear which polymerase is actually involved with DNA repair.

Here, we report that calf thymus PCNA dramatically enhances the ability of calf thymus DNA polymerase δ to bypass *in vitro* the UV-induced *cis*-syn and *trans*-syn-I cyclobutane dimers of TpT (Figure 1). As far as we know, this is the first case in which a cellular protein linked to DNA repair has facilitated DNA synthesis past lesions that are normally considered to be absolute blocks to DNA replication.

MATERIALS AND METHODS

Materials

Dimer-containing oligonucleotides and templates were prepared as previously described and estimated to be >95% pure by a variety of assay methods (12–14). The 15-mer primer was labeled by T4 polynucleotide kinase (BRL) catalyzed phosphorylation with [γ -³²P] ATP. DNA polymerase δ from fetal calf thymus was purified as described (15), except that the material from the phosphocellulose column was repurified on a second phosphocellulose column, followed by a hydroxyapatite HPLC column (BioRad) to homogeneity. Calf thymus PCNA was purified as described (7).

* To whom correspondence should be addressed

Effect of dNTP concentration on lesion bypass by Pol δ and PCNA

A solution of 0.1 pmol of annealed cis-syn, trans-syn-I, or non-dimer-containing 41-mer template/15-mer primer was made up to 1, 10, or 100 μ M dNTPs with or without 80 ng of PCNA in 25 μ L assay buffer (40 mM Bis-TrisHCl, pH 6.5, 10% glycerol, 5 mM dithiothreitol, 8 mM Mg(OAc)₂ and 0.1 mg/mL bovine serum albumin). Pol δ (50 ng, approximately 0.2 pmol) was added and, after one hour at 37°C, the reactions were quenched by addition of 5 μ L of 100 mM EDTA. The quenched samples were lyophilized, redissolved in loading buffer, and electrophoresed for six hours at 1400V on a 15%, 0.4 mm thick

8 M urea containing polyacrylamide gel in 130 mM Tris, 44 mM boric acid, and 2.5 mM EDTA. Autoradiography of the wet gel was carried out at -80°C with Kodak SB-5 film.

Kinetics of lesion bypass by Pol δ and PCNA

A solution of 0.4 pmol of annealed template/primer was made up to 100 μ M dNTP's in 100 μ L assay buffer in the presence or absence of 0.32 μ g of PCNA. Pol δ (200 ng) was added to the solution and 25 μ L aliquots were quenched at 3, 9, 30 and 90 min and processed as described above.

RESULTS**Primer-extension opposite undamaged DNA by Pol δ**

At 1 μ M in each dNTP, primer-extension opposite the undamaged template proceeded poorly in the absence of PCNA (Figure 2) and terminated primarily at a site with a potential hairpin structure. Higher levels of dNTP's, i.e. 10–100 μ M, were required to completely extend the primer. In agreement with previous observations, the addition of PCNA increased the processivity of Pol δ to such an extent that complete extension of the primer was observed, even with only 1 μ M dNTP's present (7, 16). At higher levels of dNTP's, the non-templated addition of an extra nucleotide was observed, but only in the presence of PCNA. The nature of this extra addition was not further investigated, though it has also been observed for other DNA polymerases (17). Similar results were obtained when primer-extension opposite the undamaged DNA was followed as a function of time (Figure 3). Whereas complete extension of the primer by Pol δ alone was only observed after 9 minutes of reaction, upon addition of PCNA complete extension had already occurred at the first time point of 3 minutes.

Figure 1. Structures of the cis-syn and trans-syn-I dimers of TpT and of the dimer containing template/primers used in this study.

Figure 2. Effect of dNTP concentration on primer-extension by Pol δ in the presence and absence of PCNA. See Materials and Methods for details. The four lanes to the right are sequencing lanes of the non-dimer template. The label 41 refers to a labelled 41-mer complementary to the sequence containing a dimer. The printed sequence on the right side corresponds to that of the primer-extended fragment and on the left side to that of the template containing the dimer. The location of the dimer is indicated by the bracket.

Bypass of thymine dimers

In the absence of PCNA, Pol δ extended the primer no further than one base prior to the dimer site, regardless of the dNTP concentration. This termination site was the same for the cis-syn and trans-syn-I dimers (Figure 2). Primer-extension opposite the template containing the trans-syn-I dimer was not as efficient, however, as that of the other templates. Although the reaction was repeated several times, there appeared to be significantly more termination bands prior to the trans-syn-I dimer than the cis-syn dimer, especially at low dNTP concentrations (Figure 2). There was some variability at 100 μ M dNTPs, as evidenced by the lack of a significant difference in the efficiency of primer-extension on the cis-syn and trans-syn-I dimer-containing templates in the kinetic study (Figure 3). It is possible that inhibition of DNA synthesis on the trans-syn-I 41-mer was due to an impurity, though somewhat unlikely considering that both the trans-syn-I and cis-syn dimer-containing templates were prepared and purified at the same time and in the same fashion. Furthermore, there was no evidence of inhibition of DNA synthesis on the trans-syn-I template with pol δ at low dNTPs in the presence of PCNA. Another possible explanation is that the trans-syn-I dimer, which is known to distort DNA to a much greater extent than the cis-syn dimer (18), causes premature

termination of synthesis and/or enhances 3'-5' exonucleolytic degradation of the primer terminus.

When PCNA was added to Pol δ , DNA synthesis proceeded opposite both dimer sites, even at 1 μ M dNTP's. At increasing levels of dNTP's DNA synthesis proceeded further into the dimer site and bypass DNA synthesis was also observed, to the extent of 4% and 33% on the cis-syn dimer template at 10 and 100 μ M dNTP's, respectively, and 15% on the trans-syn-I dimer at 100 μ M dNTP's (Figure 2, Table 1). On the cis-syn dimer template polymerization terminated primarily opposite the 5'-thymine of the dimer at 10 as well as at 100 μ M dNTP's. On the trans-syn-I dimer template polymerization terminated predominantly opposite the 3' thymine at 10 μ M dNTPs and opposite the 5' thymine at 100 μ M dNTPs. Surprisingly, at 100 μ M dNTPs, 15% bypass occurred, producing three different length products. One band appears to be a full length product, and the other two are probably 37-mers and 36-mers (Figure 2).

A kinetic study of the reaction in the absence of PCNA showed that though most of the primer had been extended up to both dimer sites after 9 minutes, no further synthesis opposite the dimers was observed upon continued incubation (Figure 3). In sharp contrast, however, when PCNA was present limited incorporation opposite both thymines of the dimer was already apparent after 3 minutes, and this incorporation increased and shifted to longer product lengths when incubation was continued up to 90 minutes (Figure 3).

Table 1. Products of Pol δ and PCNA action on the cis-syn and trans-syn-I thymine dimer-containing templates as a function of dNTP concentration (37°C, 1hr.)

[dNTP], μ M	cis-syn dimer			trans-syn-I dimer		
	1	10	100	1	10	100
prior	79	29	10	77	33	8
opposite 3'	18	22	6	23	55	25
opposite 5'	3	45	51	—	12	52
bypass	—	4	33	—	—	15

DISCUSSION

In this paper we have shown that a processivity factor for a DNA polymerase enables that polymerase to replicate past DNA lesions. Although it is a reasonable assumption that an increase in polymerase processivity may enable translesion synthesis, this is the first experimental evidence that we know of where this

Figure 3. Kinetics of primer-extension by Pol δ in the presence or absence of PCNA. For details, see Materials and Methods and the legend to Figure 2.

has actually been observed. Pol δ alone was not able to incorporate nucleotides opposite the 3'-thymine of either the cis-syn or trans-syn-I dimer, but addition of PCNA enabled Pol δ to incorporate nucleotides opposite both the 5'-T and 3'-T of the dimer and proceed to full length product. Polymerization with PCNA and Pol δ also displayed the time and dNTP dependence expected for trans-lesion synthesis.

PCNA-induced translesion synthesis past the cis-syn dimer by Pol δ produced the same size products as observed before for DNA polymerase I, Klenow fragment (13). A direct sequence analysis of the bypass products made by the Klenow enzyme showed that exclusively dAMP residues were inserted opposite the dimer thymine positions (13). Bypass of the cis-syn dimer by Pol δ with PCNA probably occurred in a similar fashion, though this remains to be determined. Primer-extension with PCNA proceeded fairly easily into the dimer positions, but elongation of these products was the slowest step in bypass synthesis (Figure 3). In addition, a faint termination band one nucleotide past the 5'-thymine of the cis-syn dimer was observed, indicating that distortion of the template-primer caused by the UV dimer extends at least one base beyond the dimer site (Figure 3).

Until now, trans-syn-I dimers have been absolute blocks for all DNA polymerases tested, including *E. coli* DNA polymerases I and III holoenzyme, *Thermus aquaticus* DNA polymerase I, phage T4 DNA polymerase, Sequenase 1.0, and AMV reverse transcriptase (14). None of these enzymes was even able to synthesize opposite the 5'-thymine of the trans-syn-I dimer. Unexpectedly, PCNA-induced bypass of the trans-syn-I dimer was readily observed. Bypass not only produced a full length product, but also two smaller fragments, 36 and 37 nucleotides in length (Figure 3). The appearance of the full-length product is indicative of normal bypass synthesis by Pol δ and PCNA. Unfortunately, not enough bypass product was made to enable us to isolate it and confirm its identity by direct sequence analysis. The smaller bypass products may be indicative of a type of bypass synthesis where the trans-syn-I dimer had been extruded and 4 or 5 nucleotides of the template were not replicated at all, resulting in deletion products. It is known that the trans-syn-I dimer causes single base deletion mutations in vivo, although 4 and 5 base deletions as indicated by this assay were not observed (19). Alternatively, the distortion caused by the trans-syn-I dimer may have caused premature termination, or, because of this distortion, full length products may more easily have been degraded by the 3'-5' exonuclease activity of Pol δ . In support of this explanation, the trans-syn-I dimer has been found to be much more disruptive to DNA structure than the cis-syn dimer (18), and has been found to bend duplex DNA by 22° (Wang and Taylor, submitted), whereas the cis-syn dimer only causes a 7° bend (20).

Accommodation of a lesion in the enzyme is probably where sequence context plays its largest role. DNA polymerases contain a cleft through which the DNA strand containing a bulky lesion must pass. Some enzymes may be able to accommodate certain lesions to a larger extent than others. Strauss observed DNA synthesis past putative dimer sites in the presence of Mn²⁺, which is proposed to relax the fidelity of *E. coli* DNA polymerase I, but only at certain sites (21). By relaxing fidelity, inhibiting exonuclease activity and/or increasing processivity, certain sites or lesions may be selectively bypassed. Other templates containing isolated cyclobutane dimers are needed to determine

if the effect of PCNA on bypass is a general phenomena or is isolated to certain sequences and lesions.

The role of PCNA and its activity on UV irradiated DNA may be strictly coincidental. Because it is required in excision repair synthesis (10), it is possible that PCNA does play some role in a mammalian type 'SOS' response and that this may have implications for mutagenesis induced by repair of closely opposed lesions. In this regard it is interesting to note that the *umuD* protein, required for error-prone translesion synthesis by *E. coli* DNA polymerase III holoenzyme shows sequence similarity with phage T4 gene 45 protein, which in turn has sequence similarity with PCNA (22-24). The functions of T4 gene 45 protein and PCNA are to increase the processivity of their respective DNA polymerase. It has been proposed that the *UmuD* protein has a similar function, but only during the SOS response, to increase the processivity of Pol III holoenzyme specifically across DNA lesions. In this paper, we have shown that PCNA can fulfill this role for DNA Pol δ .

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