

# Novel Enzymatic Function of DNA Polymerase $\nu$ in Translesion DNA Synthesis Past Major Groove DNA–Peptide and DNA–DNA Cross-Links

Kirin Yamanaka,<sup>†,‡</sup> Irina G. Minko,<sup>†</sup> Kei-ichi Takata,<sup>||</sup> Alexander Kolbanovskiy,<sup>⊥</sup>  
Ivan D. Kozekov,<sup>#</sup> Richard D. Wood,<sup>||</sup> Carmelo J. Rizzo,<sup>#</sup> and R. Stephen Lloyd<sup>\*,†,§</sup>

Center for Research on Occupational and Environmental Toxicology, Department of Physiology and Pharmacology, and Department of Molecular and Medical Genetics, Oregon Health & Science University, L606, 3181 SW Sam Jackson Park Road, Portland, Oregon 97239, Department of Carcinogenesis, The University of Texas Graduate School of Biomedical Sciences at Houston, The University of Texas M. D. Anderson Cancer Center, Science Park-Research Division, Smithville, Texas 78957, Department of Chemistry, New York University, New York, New York 10003, and Department of Chemistry, Center in Molecular Toxicology, VU Station B, 351822, Vanderbilt University, Nashville, Tennessee 37235

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DNA polymerase  $\nu$  (POLN or pol  $\nu$ ) is a newly discovered A family polymerase that generates a high error rate when incorporating nucleotides opposite dG; its translesion DNA synthesis (TLS) capability has only been demonstrated for high fidelity replication bypass of thymine glycol lesions. In the current investigation, we describe a novel TLS substrate specificity of pol  $\nu$ , demonstrating that it is able to bypass exceptionally large DNA lesions whose linkages are through the DNA major groove. Specifically, pol  $\nu$  catalyzed efficient and high fidelity TLS past peptides linked to  $N^6$ -dA via a reduced Schiff base linkage with a  $\gamma$ -hydroxypropano-dA. Additionally, pol  $\nu$  could bypass DNA interstrand cross-links with linkage between  $N^6$ -dAs in complementary DNA strands. However, the chemically identical DNA–peptide and DNA interstrand cross-links completely blocked pol  $\nu$  when they were located in the minor groove via a  $N^2$ -dG linkage. Furthermore, we showed that pol  $\nu$  incorporated a nucleotide opposite the 1, $N^6$ -etheno-dA ( $\epsilon$ dA) in an error-free manner and (+)-*trans-anti*-benzo[*a*]pyrene-7,8-dihydrodiol 9,10-epoxide-dA [(+)-BPDE-dA] in an error-prone manner, albeit with a greatly reduced capability. Collectively, these data suggest that although pol  $\nu$  bypass capacity cannot be generalized to all major groove DNA adducts, this polymerase could be involved in TLS when genomic replication is blocked by extremely large major groove DNA lesions. In view of the recent observation that pol  $\nu$  may have a role in cellular tolerance to DNA cross-linking agents, our findings provide biochemical evidence for the potential functioning of this polymerase in the bypass of some DNA–protein and DNA–DNA cross-links.

## Introduction

Translesion DNA synthesis (TLS)<sup>1</sup> is a significant DNA damage tolerance mechanism to overcome replication blocks caused by DNA lesions or to seal gaps opposite lesions (1, 2). In the past decade, numerous DNA polymerases (pols) have been discovered that possess lesion bypass activity. Recently, a new human DNA pol has been identified, POLN or pol  $\nu$ , and falls in the A-family of DNA pils (3). This family includes *Escherichia coli* (*E. coli*) pol I and human pol  $\theta$ . Pol  $\nu$  is a moderately processive enzyme and is able to carry out strand displacement DNA synthesis with much higher efficiency than

the Klenow fragment, the pol-proficient portion of *E. coli* pol I. It also replicates nondamaged (ND) DNA with low fidelity; specifically, it frequently misincorporates dT opposite template dG with about half the efficiency of incorporation of the correct nucleotide dC opposite template dG (4, 5). Because low fidelity of DNA synthesis is common for TLS pils and pol  $\nu$  lacks 3'→5' exonuclease proofreading activity, a role of pol  $\nu$  in the bypass of specific DNA lesions has been proposed (4, 5). Indeed, this pol can carry out TLS in vitro, being specifically proficient in accurate bypass of thymine glycols, major groove DNA lesions (5). However, it is completely blocked by a number of other DNA modifications, including a cisplatin-induced GpG intrastrand cross-link, an abasic site, a cyclobutane pyrimidine dimer, and a thymine–thymine 6-4 photoproduct (5).

A function of pol  $\nu$  in DNA cross-link repair in mammalian cells has been proposed (6). In particular, the data suggested that pol  $\nu$  is involved in homologous recombination in response to various DNA cross-linking agents and potentially interacts with multiple proteins in the Fanconi anemia pathway, which are relevant to DNA cross-link repair. However, a cellular role for pol  $\nu$  in homologous recombination-independent, TLS-dependent repair of DNA cross-links has not yet been addressed. Additionally, as already mentioned, thymine glycol is the only lesion that pol  $\nu$  has been reported to be able to bypass in vitro

\* To whom correspondence should be addressed. Tel: 503-494-9957. Fax: 503-494-6831. E-mail: lloydst@ohsu.edu.

<sup>†</sup> Center for Research on Occupational and Environmental Toxicology, Oregon Health & Science University.

<sup>‡</sup> Department of Physiology and Pharmacology, Oregon Health & Science University.

<sup>§</sup> Department of Molecular and Medical Genetics, Oregon Health & Science University.

<sup>||</sup> The University of Texas M. D. Anderson Cancer Center.

<sup>⊥</sup> New York University.

<sup>#</sup> Vanderbilt University.

<sup>1</sup> Abbreviations: TLS, translesion DNA synthesis; pol, polymerase; *E. coli*, *Escherichia coli*; ND, nondamaged; BPDE, benzo[*a*]pyrene-7,8-dihydrodiol 9,10-epoxide; PCL, DNA–peptide cross-link; ICL, interstrand DNA cross-link;  $\epsilon$ dA, 1, $N^6$ -etheno-dA.

(5). To understand the cellular function of pol  $\nu$  in TLS, it is crucial to identify types of DNA lesions that this pol can bypass. Because it has been previously established that both the  $\text{exo}^+$  and the  $\text{exo}^-$  Klenow fragment can catalyze TLS past certain major groove DNA adducts (7, 8), we hypothesized that in addition to thymine glycols, pol  $\nu$  may catalyze replication bypass of additional major groove DNA lesions. This study was designed to test this hypothesis to facilitate insight into the cellular function of pol  $\nu$  in TLS.

### Experimental Procedures

**Materials.** [ $\gamma\text{-}^{32}\text{P}$ ]ATP was obtained from PerkinElmer Life Sciences (Waltham, MA). P-6 Bio-Spin columns were obtained from Bio-Rad (Hercules, CA). Uracil DNA glycosylase, T4 DNA ligase, and T4 polynucleotide kinase were purchased from New England BioLabs (Beverly, MA). Sodium cyanoborohydride was obtained from Sigma (St. Louis, MO). Slide-A-Lyzer dialysis cassettes with a molecular weight cutoff of 10000 were purchased from Thermo Scientific (Rockford, IL). Human pol  $\nu$  was purified as described (5). Yeast pol  $\delta$  and PCNA were purified as previously described (9) and were generous gifts from Dr. Peter M. J. Burgers (Washington University, St. Louis, MO). The Lys-Trp-Lys-Lys and Lys-Phe-His-Glu-Lys-His-His-Ser-His-Arg-Gly-Tyr peptides were obtained from Sigma-Genosys (St. Louis, MO).

**Oligodeoxynucleotide Synthesis.** (a) All ND oligodeoxynucleotides were synthesized by the Molecular Microbiology and Immunology Research Core Facility at Oregon Health & Science University (Portland, OR).

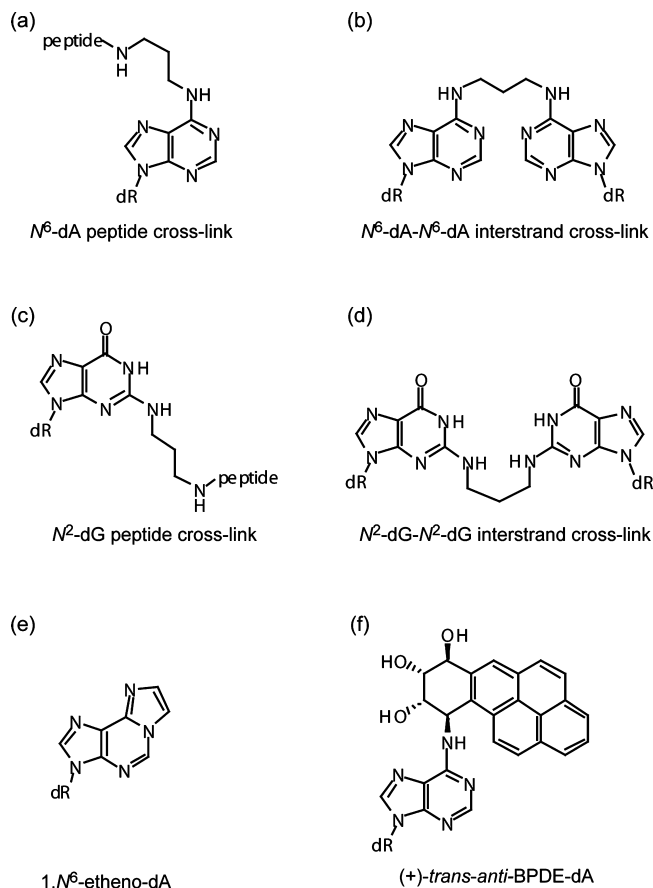
(b) Oligodeoxynucleotides containing  $\gamma$ -hydroxypropano-dA or  $\gamma$ -hydroxypropano-dG were synthesized as previously described (10). DNAs containing DNA-peptide cross-links (PCLs) (Figure 1a,c) were prepared by the reaction of the  $\gamma$ -hydroxypropano-dA- or  $\gamma$ -hydroxypropano-dG-containing oligodeoxynucleotides with either 20 nmol of Lys-Trp-Lys-Lys or 4 nmol of Lys-Phe-His-Glu-Lys-His-His-Ser-His-Arg-Gly-Tyr in the presence of 50 mM sodium cyanoborohydride according to the previously published procedure (11). The sequences of oligodeoxynucleotides containing a  $N^6$ -dA PCL and a  $N^2$ -dG PCL are shown in Table 1, column 2, rows 1 and 2, respectively.

(c) The synthesis of the oligodeoxynucleotides containing interstrand DNA cross-link (ICL) between  $N^6$ -dAs of complementary strands (Figure 1b) is described in the Supporting Information. The sequences of a  $N^6$ -dA ICL1 and a  $N^6$ -dA ICL2 are shown in Table 1, column 2, rows 3 and 5, respectively. The  $N^6$ -dA ICL2 oligodeoxynucleotide was generated from a precursor oligodeoxynucleotide (pre- $N^6$ -dA ICL2, Table 1, column 2, row 4) as described previously (12).

(d) Oligodeoxynucleotides containing an ICL between  $N^2$ -dGs (Figure 1d) were synthesized as previously described (12–14), and the sequences of the oligodeoxynucleotides containing each  $N^2$ -dG- $N^2$ -dG ICL ( $N^2$ -dG ICL1,  $N^2$ -dG ICL2,  $N^2$ -dG ICL3, and  $N^2$ -dG ICL4) are shown in Table 1, column 2, rows 6–9. A detailed description of each type of  $N^2$ -dG ICLs has been reported previously (12).

(e) An oligodeoxynucleotide containing 1, $N^6$ -etheno-dA ( $\epsilon$ dA) (Figure 1e and Table 1, column 2, row 10) was synthesized by the Molecular Microbiology and Immunology Research Core Facility at Oregon Health & Science University (Portland, OR).

(f) An oligodeoxynucleotide (5'-CTCTCACTTCC-3') containing (+)-*trans-anti*-benzo[*a*]pyrene-7,8-dihydrodiol 9,10-epoxide-dA [(+)-BPDE-dA] (Figure 1f) at the underlined nucleotide was synthesized as previously described (15) and was a generous gift from Dr. Nicholas E. Geacintov (New York University, NY). This oligodeoxynucleotide was ligated to 16-mer oligodeoxynucleotide (5'-ATTCCGTATCCATTTT-3') using 800 units of T4 DNA ligase at 12 °C overnight in the presence of 23-mer scaffold oligodeoxynucleotide (5'-TGGATACGGAATGGAAGTGAGAG-3'). The ligation reaction was terminated by the addition of an equal volume of formamide dye solution [95% (v/v) formamide, 10 mM EDTA,



**Figure 1.** Structures of DNA adducts. The PCLs consist of either a tetra (Lys-Trp-Lys-Lys) or dodecyl (Lys-Phe-His-Glu-Lys-His-His-Ser-His-Arg-Gly-Tyr) peptide attached via an acrolein moiety at the  $N^6$  position of dA (a) or  $N^2$  position of dG (c). The resulting PCLs are referred to as  $N^6$ -dA PCL4,  $N^6$ -dA PCL12,  $N^2$ -dG PCL4, and  $N^2$ -dG PCL12. (b) Structure of  $N^6$ -dA- $N^6$ -dA interstrand cross-link. (d) Structure of  $N^2$ -dG- $N^2$ -dG interstrand cross-link. (e) Structure of 1, $N^6$ -etheno-dA. (f) Structure of (+)-*trans-anti*-BPDE-dA.

0.03% (w/v) xylene cyanol, and 0.03% (w/v) bromphenol blue], and the products were separated on a 10% acrylamide gel containing 8 M urea. DNA was eluted from the gel with 0.5 M ammonium acetate/10 mM magnesium acetate solution, dialyzed against 10 mM Tris-HCl (pH 7.4) buffer containing 1 mM EDTA in Slide-A-Lyzer dialysis cassette (MWCO of 10000), and further purified through a P-6 Bio-Spin column. The resulting 27-mer was used for DNA replication assays (Table 1, column 2, row 11).

**DNA Replication Assays.** For replication assays, primers were designed for reactions under a running or standing start condition. In the former case, the primer 3'-OH was positioned several nucleotides upstream of the lesion site. In latter case, the primer 3'-OH was positioned immediately prior to the lesion site (−1 primer). The sequences of primers are listed in Table 1, column 3. DNA replication assays were carried out in the following buffers: For pol  $\nu$ , the reaction mixture contained 25 mM Tris-HCl (pH 7.5), 8 mM  $\text{MgCl}_2$ , 10% glycerol, 100  $\mu\text{g}/\text{mL}$  bovine serum albumin, and 5 mM dithiothreitol. For reaction with pol  $\nu$  with  $N^2$ -dG- $N^2$ -dG ICLs, Mg-acetate was used instead of  $\text{MgCl}_2$ . For pol  $\delta$ , the reaction mixture contained 40 mM HEPES-KOH (pH 6.8), 10% glycerol, 200  $\mu\text{g}/\text{mL}$  bovine serum albumin, 1 mM dithiothreitol, and 8 mM  $\text{MgCl}_2$ . All reactions were carried out at 37 °C. DNA substrate, pol, and dNTP(s) concentrations, as well as reaction times, are given in figure legends. Reaction products were resolved through 15% acrylamide gel containing 8 M urea and visualized using a PhosphorImager screen. The percentage of primer extended in extension reactions was determined by taking the ratio of extended primer beyond the adducted base to the total amount of primer (unextended primer + extended primer up to and opposite the lesion + extended primer beyond the lesion). The percentage

Table 1. Sequences of DNA Primers and Lesion-Containing Templates<sup>a</sup>

	Adducted Template	Template Sequence	Primer Sequence
1	N <sup>6</sup> -dA PCLs	5'-GCTAGTACTCGTCG <u>CA</u> AATCCGTATCCAT-3'	(-1) 5'-AAAATGGATACGGAATTG-3' (-3) 5'-AAAATGGATACGGAAT-3'
2	N <sup>2</sup> -dG PCLs	5'-GCTAGTACTCGTCG <u>CA</u> AATCCGTATCCAT-3'	(-4) 5'-AAAATGGATACGGAAT-3'
3	N <sup>6</sup> -dA ICL1	3'-gl-AUTATCGTA-5' 5'-AGCGATAGACAT <u>A</u> TAGCATCGCTGGTACCGACTCG-3'	(-9) 5'-AAACGAGTTCGGTACCAGC-3'
4	pre-N <sup>6</sup> -dA ICL2	3'-gl-AUTATCGTAGCGACCA-5' 5'-AGCGATAGACAT <u>A</u> TAGCATCGCTGGTACCGACTCG-3'	N/A
5	N <sup>6</sup> -dA ICL2	3'-gl-A-5' 5'-AGCGATAGACAT <u>A</u> TAGCATCGCTGGTACCGACTCG-3'	(-1) 5'-CGAGTCGGTACCAGCGATGCTAT-3' (-9) 5'-AAACGAGTTCGGTACCAGC-3'
6	N <sup>2</sup> -dG ICL1	3'-(dd)CTGTGCTATCGTAG-5' 5'-AGCGATAGACAC <u>G</u> ATAGCATCGCTGGTACCGACTCG-3'	(-10) 5'-CGAGTCGGTACCAG-3'
7	N <sup>2</sup> -dG ICL2	3'-(dd)CTGTGC-5' 5'-AGCGATAGACAC <u>G</u> ATAGCATCGCTGGTACCGACTCG-3'	(-10) 5'-CGAGTCGGTACCAG-3'
8	N <sup>2</sup> -dG ICL3	3'-gl-GCTATCGTAG-5' 5'-AGCGATAGACAC <u>G</u> ATAGCATCGCTGGTACCGACTCG-3'	(-10) 5'-CGAGTCGGTACCAG-3'
9	N <sup>2</sup> -dG ICL4	3'-gl-GC-5' 5'-AGCGATAGACAC <u>G</u> ATAGCATCGCTGGTACCGACTCG-3'	(-10) 5'-CGAGTCGGTACCAG-3'
10	edA	5'-GCTAGTACTCGTCG <u>CA</u> AATCCGTATCCAT-3'	(-3) 5'-AAAATGGATACGGAAT-3'
11	(+)-BPDE-dA	5'-CTCTC <u>A</u> CTCCATTCGGTATCCATTTT-3'	(-1) 5'-ATGGATACGGAATGGAAG-3' (-6) 5'-AAAATGGATACGGAAT-3'

<sup>a</sup> Modified bases are underlined. A 3'-glycerol unit (gl) or a dideoxycytidine (dd) were incorporated to prevent DNA synthesis off of the shorter strand of the ICL-containing oligodeoxynucleotides. The number in the parentheses in front of the primer sequence indicates the position of the primer 3'-OH.

of primer extended in single nucleotide incorporations was determined by taking the ratio of extended primer to the total amount of primer (unextended primer + extended primer). Quantifications were done using ImageQuant 5.2 software.

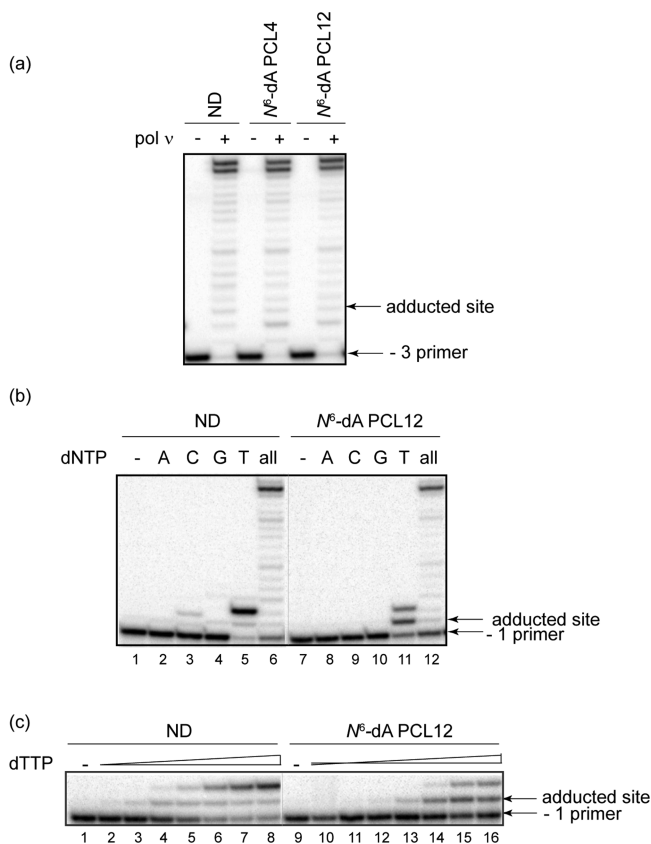
## Results

**Replication Bypass of N<sup>6</sup>-dA Peptide Cross-Links by Pol  $\nu$ .** To address the hypothesis that pol  $\nu$  can catalyze replication bypass of additional major groove DNA lesions, we utilized N<sup>6</sup>-dA acrolein-mediated PCLs as a model for the bulky major groove DNA adducts (11). Specifically, tetra- and dodecylpeptide cross-links (Figure 1a, N<sup>6</sup>-dA PCL4 and N<sup>6</sup>-dA PCL12, respectively) were used. The molecular masses of these PCL-modified bases were 763 Da for N<sup>6</sup>-dA PCL4 and 1738 Da for N<sup>6</sup>-dA PCL12 versus 135 Da for unmodified adenine. These lesions were positioned 15 nucleotides from the 5' end of the 30-mer template and annealed with a 16-mer radiolabeled primer in which the 3'-OH was three nucleotides upstream of the lesion site.

Data in Figure 2a revealed that pol  $\nu$  could fully extend primers annealed to these adducted templates; under the conditions used, very minimal blockage to DNA synthesis was observed one nucleotide prior to the lesion. To assess the identity of the nucleotides incorporated opposite the lesion, single nucleotide incorporations were conducted. Because N<sup>6</sup>-dA PCL12 is bulkier than N<sup>6</sup>-dA PCL4, further assays used only substrate containing this lesion. Data showed that pol  $\nu$  incorporated only the correct nucleotide, dT, opposite N<sup>6</sup>-dA PCL12 (Figure 2b), suggesting that it catalyzed error-free bypass of this adduct. It is of interest to note that two nucleotides were incorporated for both the ND and the N<sup>6</sup>-dA PCL12 substrates when only dTTP was present. It has been reported that pol  $\nu$  catalyzes error-prone insertion of dT opposite dG (4, 5). Because the templating nucleotide immediately downstream of the adducted base is a dG, this specific sequence context likely accounts for the observed incorporation of the second dT. To quantitatively evaluate the extent of the blockage posed by N<sup>6</sup>-

dA PCL12 for the pol  $\nu$ -catalyzed nucleotide insertion, dTTP titration assays (6.4 nM to 100  $\mu$ M dTTP at 5-fold incrementally increased concentrations, left to right) were performed (Figure 2c). These results demonstrated that pol  $\nu$  could incorporate approximately an equal amount of dT opposite the adducted base at concentrations only 5-fold higher relative to ND dA; specifically, ~20% of primers were extended at 800 nM dTTP using ND substrate and at 4  $\mu$ M dTTP using damaged substrate.

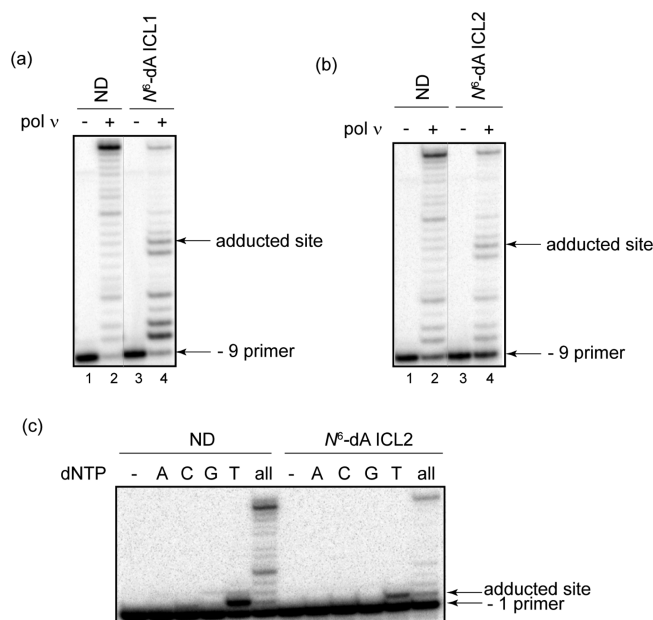
**Replication Bypass of N<sup>6</sup>-dA–N<sup>6</sup>-dA DNA Interstrand Cross-Links by Pol  $\nu$ .** Having demonstrated that pol  $\nu$  can readily bypass N<sup>6</sup>-dA PCLs, we hypothesized that it may also be able to carry out TLS past other N<sup>6</sup>-dA major groove adducts, including N<sup>6</sup>-dA–N<sup>6</sup>-dA ICLs (Figure 1b). Although ICLs had been previously thought to pose an insurmountable block to replication, our laboratory has recently demonstrated that human DNA pol  $\kappa$  and *E. coli* DNA pol IV can catalyze replication bypass of an ICL between the N<sup>2</sup>-positions of dGs, which models the ICL formed by acrolein in a CpG sequence context (12, 16). Related ICLs were constructed between the N<sup>6</sup>-positions of dA placing the cross-link in the major groove (Figure 1b and Table 1, rows 3 and 5). An N<sup>6</sup>-dA ICL1 was designed to model for a potential intermediate of ICL processing that could be generated by exo/endonucleolytic removal of DNA patch 3' to the ICL site. Even though N<sup>6</sup>-dA ICL1 contains an eight nucleotide duplex region 5' to the ICL site, it was anticipated that pol  $\nu$  would efficiently catalyze strand displacement DNA synthesis due to its intrinsic high strand displacement activity (5), a property that is useful for replicating this type of DNA lesion. A N<sup>6</sup>-dA ICL2 was constructed to model another potential intermediate of ICL processing; this could be generated by removal of DNA patches both 3' and 5' to the ICL site. When these major groove ICL-containing DNAs were used in primer extension reactions, pol  $\nu$  could extend primers and generate full-length products (Figure 3a,b). For both types of ICLs, the amount of primers extended beyond the adducted site was less as compared to ND substrate; specifically, a 10- and 4-fold decrease was observed for N<sup>6</sup>-dA-ICL1 and N<sup>6</sup>-dA-ICL2,



**Figure 2.** Replication bypass of  $N^6$ -dA peptide cross-links by human pol  $\nu$ . (a) Primer extensions were catalyzed by 50 nM human pol  $\nu$  under running start conditions (-3 primer) in the presence of 5 nM primer template containing ND,  $N^6$ -dA PCL4, or  $N^6$ -dA PCL12 for 30 min at 37 °C. (b) Single nucleotide incorporations and primer extensions were catalyzed by 5 nM human pol  $\nu$  under standing start conditions (-1 primer) in the presence of 2 nM primer template containing ND or  $N^6$ -dA PCL12 for 15 min at 37 °C. Reactions shown in lanes 1–6 and lanes 7–12 were conducted side-by-side, and products were separated on the same gel. The reactions shown in panels a and b were carried out in the presence of 100  $\mu$ M individual or all dNTPs. (c) dTTP titration assays with 2 nM primer template were catalyzed by 5 nM human pol  $\nu$  under standing start conditions (-1 primer) for 10 min at 37 °C using the same substrates as described in panel b. dTTP concentration ranges from 6.4 nM to 100  $\mu$ M (5-fold incremental increase). Reactions shown in lanes 1–8 and lanes 9–16 were conducted side-by-side, and products were separated on the same gel.

respectively. As expected, pol  $\nu$  catalyzed strand displacement synthesis on the  $N^6$ -dA ICL1 template (Figure 3a). When the identity of nucleotides incorporated opposite the cross-linked site was examined by single nucleotide incorporations using the  $N^6$ -dA ICL2 template, the data indicated that pol  $\nu$  preferentially incorporated the correct nucleotide, dT, opposite the  $N^6$ -modified dA (Figure 3c).

**Pol  $\nu$ -Catalyzed Replication Is Blocked by  $N^2$ -dG Peptide and DNA Interstrand Cross-Links.** Given that pol  $\nu$  was able to bypass the extremely bulky major groove  $N^6$ -dA PCLs and ICLs, we tested whether it could replicate past chemically identical PCL and ICL lesions except situated in the minor groove through a linkage with the  $N^2$  position of dG (Figure 1c,d). Primer extensions using these  $N^2$ -dG linked PCLs revealed that pol  $\nu$  was strongly blocked one nucleotide prior to both PCLs and was completely blocked at the site opposite the lesion, with no further primer extension observed (Figure 4a). The identity of the nucleotide(s) incorporated opposite the PCLs was not determined due to the limited nucleotide incorporation. Even though pol  $\nu$  could not replicate past the

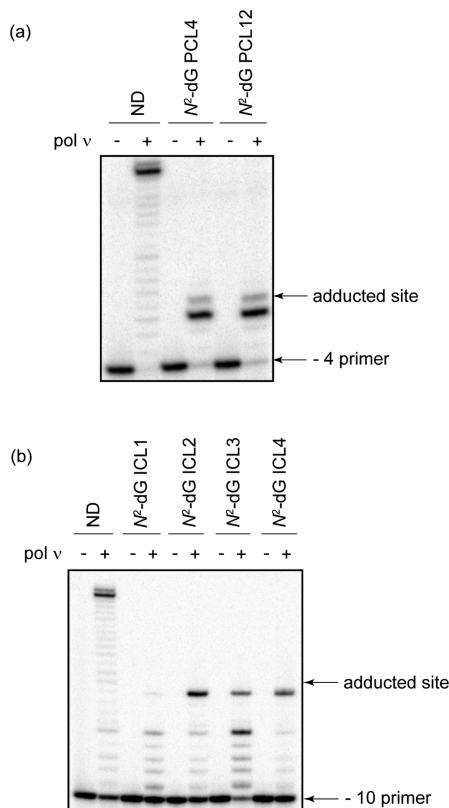


**Figure 3.** Replication bypass of  $N^6$ -dA- $N^6$ -dA interstrand cross-links by human pol  $\nu$ . Primer extensions were catalyzed by 10 nM human pol  $\nu$  under running start conditions (-9 primer) using ND,  $N^6$ -dA ICL1 (a), or  $N^6$ -dA ICL2 (b). Reactions shown in lanes 1–2 and lanes 3–4 were conducted side-by-side, and products were separated on the same gel. (c) Single nucleotide incorporations and primer extensions were catalyzed by 5 nM human pol  $\nu$  under standing start conditions (-1 primer) using ND and  $N^6$ -dA ICL2. All reactions were carried out in the presence of 5 nM primer template and 100  $\mu$ M individual or all dNTPs for 15 min at 37 °C.

$N^2$ -dG PCLs, suggesting an inability to replicate past minor groove DNA adducts, we tested whether pol  $\nu$  could catalyze TLS past  $N^2$ -dG- $N^2$ -dG ICLs (Figure 1d and Table 1, rows 6–9). No incorporation opposite the lesion site was observed for these ICL-containing DNAs (Figure 4b). Thus, similar to the observations made using PCL-containing DNAs, pol  $\nu$  has much higher tolerance for the major groove  $N^6$ -dA ICLs than the minor groove  $N^2$ -dG ICLs.

**Substrate Limitations of Pol  $\nu$ -Catalyzed Bypass of Major Groove-Linked DNA Lesions.** Because pol  $\nu$  was able to accurately replicate past major groove  $N^6$ -dA PCL and ICL lesions, we wanted to further explore the substrate range of pol  $\nu$  and address the question concerning pol  $\nu$  ability to bypass dA adducts in which both N1 and  $N^6$  sites are blocked by modification. Thus, the ability of pol  $\nu$  to bypass an  $\epsilon$ dA (Figure 1e) was examined.  $\epsilon$ dA is a promutagenic lesion that can be formed by exposure to industrial toxicants such as vinyl chloride and by the reaction of DNA with bis-electrophiles produced by lipid peroxidation (17). The running start primer extensions demonstrated that pol  $\nu$  was severely blocked one nucleotide prior to the  $\epsilon$ dA adduct, with only a very small amount of full-length product formed (Figure 5a). However, the nucleotide incorporation opposite the lesion appeared to be quite accurate. Under the conditions used, the primers were extended in the presence of dTTP but not other dNTPs (Figure 5b). On the basis of these data, we conclude that relatively nonbulky modifications at both N1 and  $N^6$  of dA are sufficient to inhibit pol  $\nu$ -catalyzed TLS.

We also investigated the ability of pol  $\nu$  to carry out TLS past (+)-BPDE-dA (Figure 1f), one of the adducts formed by the active metabolite of the prevalent environmental pollutant, benzo[*a*]pyrene (18). To construct an oligodeoxynucleotide of sufficient length for TLS analyses, an 11-mer oligodeoxynucleotide containing the (+)-BPDE-dA was  $^{32}$ P-labeled, ligated to



**Figure 4.** Replication bypass of  $N^2$ -dG peptide and interstrand cross-links by human pol  $\nu$ . (a) Primer extensions were catalyzed by 10 nM human pol  $\nu$  under running start conditions ( $-4$  primer) in the presence of 5 nM primer template containing ND,  $N^2$ -dG PCL4, or  $N^2$ -dG PCL12 for 30 min at 37 °C. (b) Primer extensions were catalyzed by 25 nM human pol  $\nu$  under running start conditions ( $-10$  primer) in the presence of 7.5 nM primer template containing ND,  $N^2$ -dG ICL1,  $N^2$ -dG ICL2,  $N^2$ -dG ICL3, or  $N^2$ -dG ICL4 for 10 min at 37 °C. All reactions were carried out in the presence of 100  $\mu$ M dNTPs.

a 16-mer oligodeoxynucleotide, and gel-purified. Residual radioactive signals of the resulting 27-mer template are evident in all lanes [Figure 5c,d, (+)-BPDE-dA-27-mer template]. Primer extensions revealed that pol  $\nu$  could not fully extend the  $-6$  primer and was severely blocked one nucleotide prior to the adducted site (Figure 5c). In addition, pol  $\nu$  displayed error-prone patterns of nucleotide incorporation opposite the lesion (Figure 5d); in the presence of dATP,  $\sim 16\%$  of primers were extended, while only  $\sim 1\%$  of primers were extended in the presence of the correct dTTP. A limited primer extension ( $<1\%$ ) was also observed in the presence of dGTP. Overall, pol  $\nu$ -catalyzed TLS past (+)-BPDE-dA was inefficient, incomplete, and error-prone.

## Discussion

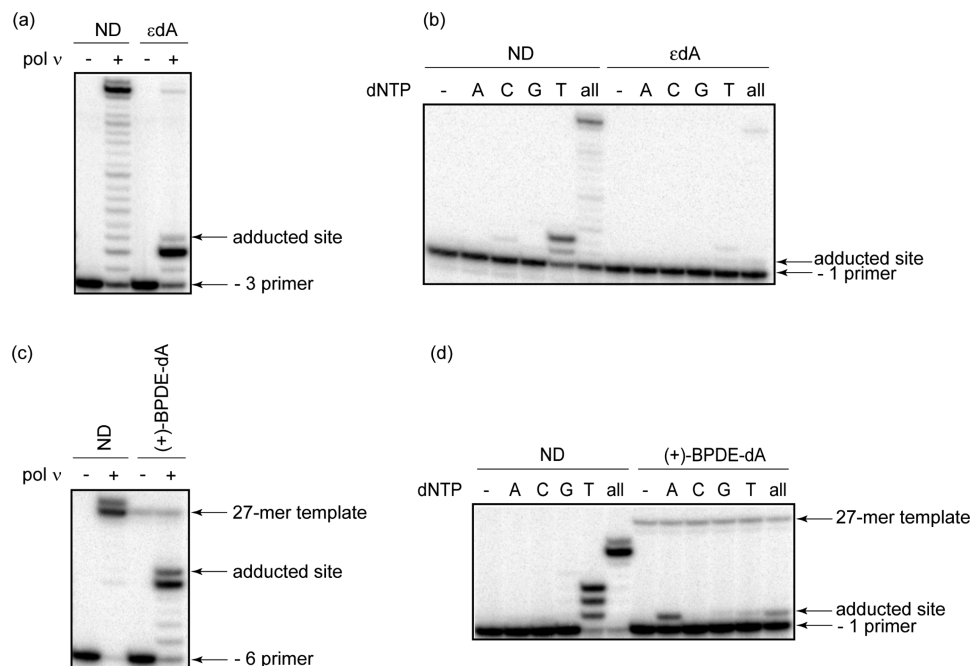
Although very significant progress has been made in both structural analyses of many of the TLS pols and the range of damaged DNA substrates that these pols can bypass, in the majority of cases, the biological role of these enzymes can only be inferred by changes in efficiency and mutagenic rates of TLS in their absence. Little data have been gathered concerning the role of these enzymes in developmental biology, their tissue-specific expression and steady-state distribution in adult organisms, the mechanism(s) for recruitment to sites of DNA damage, and the processes that regulate their expressions in response to environmental toxicant exposures and/or endogenous stresses. In the case of pol  $\nu$ , the initial characterizations have demonstrated a very limited repertoire of lesions that pol  $\nu$  can

efficiently bypass (5). Although a cellular role of pol  $\nu$  in TLS has not yet been established, its high frequency of misincorporation of dT opposite dG suggests that its expression would be tightly regulated. Thus, understanding the potential substrate specificity of pol  $\nu$  may help to guide future investigations of its biological role in TLS and circumstances for induction or activation. In this regard, we demonstrated that although pol  $\nu$  could carry out TLS past bulky major groove DNA lesions such as  $N^6$ -dA PCLs and  $N^6$ -dA- $N^6$ -dA ICLs, it failed to replicate DNAs containing chemically similar minor groove DNA lesions. Thus, the location of the lesion within DNA is one important structural determinant that influences the ability of pol  $\nu$  to bypass the lesion.

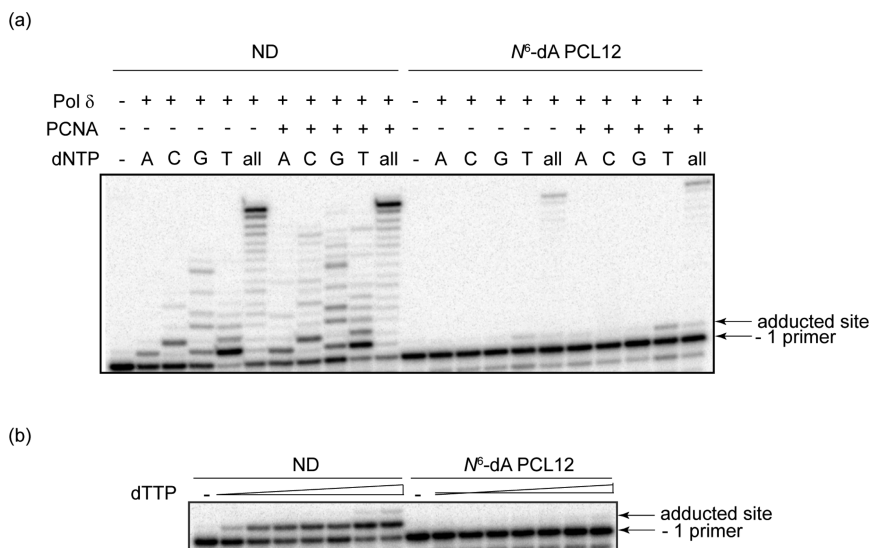
We also discovered that pol  $\nu$  could not efficiently bypass  $\epsilon$ dA, and we speculate that this may be either due to pol  $\nu$  requirement for the Watson-Crick edge for efficient dA templating or structural heterogeneity of the lesion within the active site. Regarding the former possibility, pol  $\nu$  may fail to catalyze efficient TLS past  $\epsilon$ dA because the Watson-Crick edge of this adduct is blocked by the modification. The majority of DNA pols utilize the geometry of the canonical Watson-Crick pairs for the nucleotide selection (19), but some TLS pols utilize alternative mechanisms. For example, pol  $\iota$  induces a *syn* conformation on template purines, which results in a Hoogsteen base pairing with the correctly matched incoming nucleotide and enables bypass of lesions that disrupt the Watson-Crick edge but not the Hoogsteen edge (20). The diminished capability of pol  $\nu$  to bypass  $\epsilon$ dA strongly suggests that the accessibility of the Watson-Crick edge of the template dA is important for the efficient replication by this pol; thus, it is likely that pol  $\nu$ -catalyzed polymerization involves the recognition of the Watson-Crick geometry.

With regard to structural heterogeneity of  $\epsilon$ dA adduct, NMR analyses have revealed that orientation of the base around the glycosidic bond is affected by identity of nucleotide opposite the lesion (21, 22). When paired with dT, the  $\epsilon$ dA is in a normal *anti* conformation; however, the  $\epsilon$ dA base prefers the *syn* conformation when placed opposite a mispaired dG. Additionally, the cocrystal structure of DNA containing an  $\epsilon$ dA bound to pol  $\iota$  also revealed that the lesion adopted a *syn* conformation (20). Thus,  $\epsilon$ dA can adopt a *syn* or *anti* conformation depending on the base it is paired with, as well as interactions with the pol. There is a possibility that similar to pol  $\iota$ , the adducted base is in a *syn* conformation in the pol  $\nu$  active site. However, it is unclear whether either orientation of the  $\epsilon$ -modified base can be efficiently utilized for nucleotide insertion.

Intriguingly, pol  $\nu$  also could not fully bypass a (+)-BPDE-dA. The NMR solution structure of this lesion opposite dT in duplex DNA showed complex conformational heterogeneity (23). Computational studies using an NMR structure of duplex DNA where dT was placed opposite the modified dA have revealed that the glycosidic bond of (+)-BPDE-dA was in *syn-anti* equilibrium; therefore, the modified dA has a diminished capability to participate in Watson-Crick base pairing (24). It is possible that the glycosidic bond of modified dA rotates and adopts a *syn* conformation at the primer-template junction and therefore cannot participate in Watson-Crick base pairing. An alternative explanation may be that the structure of the active site of pol  $\nu$  is significantly distorted by bulky aromatic ring system of this lesion. It has been shown that the BPDE moiety of this lesion is intercalated into the double helix, on the 3' side of the adducted dA, and thus causes significant structural distortions at the active site of T7 pol (25). Because the T7 pol is also a member of the A-family pols, the inability of pol  $\nu$  to



**Figure 5.** Replication bypass of 1,*N*<sup>6</sup>-etheno-dA and (+)-*trans-anti*-BPDE-dA by human pol  $\nu$ . (a) Primer extensions were catalyzed by 10 nM human pol  $\nu$  under running start conditions (-3 primer) in the presence of 5 nM primer template containing ND or  $\epsilon$ dA. (b) Single nucleotide incorporations and primer extensions were catalyzed by 5 nM human pol  $\nu$  under standing start conditions (-1 primer) in the presence of 5 nM primer template using the same substrates as described in panel a. (c) Primer extensions were catalyzed by 10 nM human pol  $\nu$  under running start conditions (-6 primer) in the presence of 2 nM primer template containing ND or (+)-BPDE-dA. (d) Single nucleotide incorporations and primer extensions were catalyzed by 5 nM human pol  $\nu$  under standing start conditions (-1 primer) in the presence of 2 nM primer template using the same substrates as described in panel c. All reactions were carried out in the presence of 100  $\mu$ M individual or all dNTPs for 15 min at 37  $^{\circ}$ C.



**Figure 6.** Replication bypass of *N*<sup>6</sup>-dA peptide cross-link by yeast pol  $\delta$ . (a) Single nucleotide incorporations and primer extensions were catalyzed by 0.5 nM yeast pol  $\delta$  with 2 nM primer template containing ND or *N*<sup>6</sup>-dA PCL12 under standing start conditions (-1 primer) in the presence or absence of 5 nM PCNA for 20 min at 37  $^{\circ}$ C. Reactions were carried out in the presence of 100  $\mu$ M individual or all dNTPs. (b) dTTP titration assays with 2 nM primer template were catalyzed by 0.5 nM yeast pol  $\delta$  under standing start conditions (-1 primer) in the absence of PCNA for 10 min at 37  $^{\circ}$ C using the same substrates as described in panel a. dTTP concentration ranges from 6.4 nM to 100  $\mu$ M (5-fold incremental increase).

bypass this lesion may be due to the active site distortions, similar to those shown for T7 pol. With regard to the low fidelity synthesis past this lesion by pol  $\nu$ , it has been shown that the adducted dA is displaced from the active site of T7 pol as a result of steric hindrance between the BPDE moiety and the primer template. Among the four dNTPs, dATP fits best into the dNTP binding pocket that is enlarged due to the displacement of adducted dA (25). This structural change provides a likely explanation for the predominant misincorporation of dA by pol  $\nu$ .

Recently, we reported a comparative study of the mutagenic consequences of identical lesions linked in the major groove via *N*<sup>6</sup>-dA versus the minor groove linkage via *N*<sup>2</sup>-dG during the replication of single-stranded pMS2 shuttle vectors in COS-7 cells (11); these lesions were the same site-specific PCL4s substrates used in this investigation. These data revealed that DNA containing either lesion could be replicated, with the *N*<sup>2</sup>-dG PCL4 being moderately mutagenic, while the *N*<sup>6</sup>-dA PCL4 showed very low mutagenic potential. Although it is anticipated that the extremely bulky nature of the *N*<sup>6</sup>-dA PCLs lesions would

block replicative pols, we carried out in vitro replication assays using pol  $\delta$  (Figure 6). The primer extensions and single nucleotide incorporations showed some limited bypass of  $N^6$ -dA PCL12 at very high dNTP concentrations (100  $\mu$ M) (Figure 6a), while the titration experiment with varying concentrations of dTTP (from 6.4 nM to 100  $\mu$ M) revealed the severity of the blockage posed by this lesion for the pol  $\delta$ -catalyzed nucleotide insertion (Figure 6b). Specifically, pol  $\delta$  could incorporate dT opposite the ND dA at concentrations as low as 6.4 nM with  $\sim$ 8% of primers being extended, while even at 100  $\mu$ M dTTP, only  $\sim$ 2% of primers were extended on the  $N^6$ -dA PCL12-containing substrate. These results suggest that although in vitro conditions could be manipulated to observe bypass of  $N^6$ -dA PCLs by pol  $\delta$ , the block to replication is likely to occur when pol  $\delta$  encounters such bulky lesions; therefore, specialized pols may be recruited to carry out TLS. The data presented herein suggest that pol  $\nu$  is a likely candidate to perform TLS past a variety of major groove lesions. At present, the structural basis is unknown why pol  $\nu$  could efficiently bypass PCL and ICL as large as 3025 Da but fails to bypass less bulky BPDE adduct. However, we speculate that the lesions that are readily bypassed by pol  $\nu$  may have significant conformational flexibility and thus, can be accommodated without distorting DNA–enzyme interactions within the pol active site. Overall, our data suggest that since pol  $\nu$  is capable of bypassing extremely large major (but not minor) groove lesions, it may play a role in TLS following toxicant challenges that result in the formation of such lesions in the cells.

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**Supporting Information Available:** Scheme and methods for the syntheses of oligodeoxynucleotides containing  $N^6$ -dA– $N^6$ -dA interstrand cross-links. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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