Nucleotide Misincorporation, 3’-Mismatch Extension, and Responses to Abasic Sites and DNA Adducts by the Polymerase Component of Bacterial DNA Ligase D*

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DNA ligase D (LigD) participates in a mutagenic pathway of nonhomologous end joining in bacteria. LigD consists of an ATP-dependent ligase domain fused to a polymerase domain (POL) and a phosphoesterase module. The POL domain performs templated and nontemplated primer extension reactions with either dNTP or rNTP substrates. Here we report that Pseudomonas LigD POL is an unfaithful nucleic acid polymerase. Although the degree of infidelity in nucleotide incorporation varies according to the mispair produced, we find that a correctly paired ribonucleotide is added to the DNA primer terminus more rapidly than the corresponding correct deoxyribonucleotide and incorrect nucleotides are added much more rapidly with rNTP substrates than with dNTPs, no matter what the mispair configuration. We find that 3’ mispairs are extended by LigD POL, albeit more slowly than 3’ paired primer-templates. The magnitude of the rate effect on mismatch extension varies with the identity of the 3’ mispair, but it was generally the case that mispaired ends were extended more rapidly with rNTP substrates than with dNTPs. These results lend credence to the suggestion that LigD POL might fill in short 5’-overhangs with ribonucleotides when repairing double strand breaks in quiescent cells. We report that LigD POL can add a deoxynucleotide opposite an abasic lesion in the template strand, albeit slowly. Ribonucleotides are inserted more rapidly at an abasic lesion than are deoxyxs. LigD POL displays feeble activity in extending a preformed primer terminus opposing an abasic site, but can readily bypass the lesion by slippage of the primer 3’ di- or trinucleotide and realignment to the template sequence distal to the abasic site. Covalent benzo[a]pyrene-dG and benzo[c]phenanthrene-DA adducts in the template strand are durable roadblocks to POL elongation. POL can slowly insert a dNMP opposite the adduct, but is impaired in the subsequent extension step.

Bacterial DNA ligase D (LigD) is distinguished from all other DNA ligases insofar as its enzymatic activity is not limited to sealing DNA strands. Rather, it is a polyfunctional repair enzyme consisting of an ATP-dependent ligase domain fused to a polymerase domain (POL) and a phosphoesterase domain (1–10). Interest in LigD is driven by evidence that it collaborates with a bacterial Ku homolog in a nonhomologous end joining (NHEJ) pathway characterized by a high incidence of frameshift mutations at the sites of double strand break (DSB) repair (1–4, 9, 10). Biochemical characterization of the POL and phosphoesterase components suggests that they provide a means of remodeling the 3’ ends of broken DNA strands prior to sealing by the ligase component (2–10).

The LigD POL domain catalyzes either nontemplated single nucleotide additions to a blunt-ended duplex DNA or fill-in synthesis at a 5’-tailed duplex DNA; these are the molecular signatures of mutagenic mycobacterial NHEJ in vivo at blunt-end and 5’-overhang DSBs, respectively (2, 9, 10). POL activity in vitro is optimal in the presence of manganese (2, 5). rNTPs are preferred over dNTPs as substrates for nontemplated blunt-end addition (2, 5). During templated synthesis in the presence of dNTPs on a DNA primer-template with an 18-nucleotide 5’ tail, the primer is elongated to the end of the template strand and then further extended with a single nontemplated nucleotide (2, 5). LigD POL can also addtemplated ribonucleotides to a DNA primer, but extension is limited to about 4 cycles of rNMP incorporation because the primer-template is rendered progressively less active as ribonucleotides accumulate at the 3’ end (6). These properties suggest that the initial insertions preceding the strand sealing step of NHEJ might involve rNMP incorporation and that the ability of LigD to use rNTPs as substrates might be advantageous for the repair of chromosomal breaks that arise in quiescent cells, insofar as the dNTP pool might be limiting when bacteria are not actively replicating.

The ribonucleotide addition reactions of LigD POL are of particular interest given its structural similarity to the catalytic subunit of archaeal/eukaryal primase (9, 11–14), the enzyme responsible for synthesizing the RNA primers for lagging strand DNA replication in archaeal and eukaryal species. The crystal structures of Pseudomonas LigD POL and Pyrococcus horikoshii primase align with a root mean squared deviation of 3.7 Å at 203 Ca positions (9, 12). LigD POL lacks a distinctive helical domain appended to archaeal primase as an insert to the sequence of the catalytic domain; it also lacks the zinc-binding motif located within the primase catalytic domain (9). Thus, LigD POL is a minimized version of an archaeal primase-polymerase. It is the first example of such a protein in the bacterial domain of life, and one with a novel role in DNA repair rather than priming of DNA replication.

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2 The abbreviations used are: LigD, ligase D; POL, polymerase domain; NHEJ, nonhomologous end joining; DSB, double strand break; PAH, polycyclic aromatic hydrocarbon; BP, benzo[a]pyrene; BPh, benzo[c]phenanthrene.
Eukaryal DNA primase is optimally active in the presence of manganese (15) and displays an exceptionally high rate of nucleotide misincorporation during templated RNA synthesis (16–19). Archaeal DNA primase, too, is optimally active in the presence of manganese (20–22). Archaeal primase has templated DNA and RNA polymerase activities and nontemplated 3′-terminal transferase activity with dNTP or rNTP substrates (22–25). To our knowledge, studies of the fidelity of archaean primase-polymerase during templated synthesis have not been reported. The issue of whether LigD POL is a relatively faithful or unfaithful enzyme during templated synthesis is pertinent, given the mutagenic quality of NHEJ \textit{in vivo}.

Here we examine the fidelity of the POL component of \textit{Pseudomonas} LigD, focusing on the following questions. How does the rate of incorporation of the correctly base-paired nucleotide compare with the rates of misincorporation of incorrect nucleotides? Is POL fidelity influenced by whether the incoming substrate is a ribonucleotide or a deoxyribonucleotide? How does LigD POL perform in extending a primer containing a preformed 3′ base mismatch? Does its capacity to extend a mismatch depend on whether the enzyme is in DNA polymerase or RNA polymerase mode?

We also address the effects of two kinds of DNA lesions on LigD POL activity: abasic sites and polycyclic aromatic hydrocarbon-DNA adducts. Abasic lesions, which can arise by spontaneous hydrolysis of the N-glycosidic bond or base excision catalyzed by DNA glycosylases, comprise a very common form of DNA damage. Abasic sites can elicit different responses from different DNA polymerases, ranging from arrest of the replication fork to bypass of the lesion. Bypass entails two distinct polymerization steps: insertion of a dNMP across from the abasic site (ostensibly without the benefit of instructional base pairing) and subsequent extension from the 3′-dNMP:abasic terminus (26–28). Here we query how LigD POL behaves when it encounters an abasic site on the template strand at positions opposite from or immediately flanking the 3′ nucleotide of the primer strand.

Polycyclic aromatic hydrocarbons (PAHs) such as benzo[a]pyrene (BP) and benzo[c]phenanthrene (BPh) are potent environmental carcinogens. BP and BPh are converted \textit{in vivo} to bay- or fjord-region diol epoxides, which react with the deoxyadenosine (dA) or deoxyguanosine (dG) bases in DNA to form covalent PAH-DNA adducts. Abasic lesions, which can arise by spontaneous hydrolysis of the N-glycosidic bond or base excision catalyzed by DNA glycosylases, comprise a very common form of DNA damage. Abasic sites can elicit different responses from different DNA polymerases, ranging from arrest of the replication fork to bypass of the lesion. Bypass entails two distinct polymerization steps: insertion of a dNMP across from the abasic site (ostensibly without the benefit of instructional base pairing) and subsequent extension from the 3′-dNMP:abasic terminus (26–28). Here we query how LigD POL behaves when it encounters an abasic site on the template strand at positions opposite from or immediately flanking the 3′ nucleotide of the primer strand.

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Experimental Procedures

LigD POL—His\textsubscript{10}-LigD-POL was purified from a soluble extract of isopropyl 1-thio-β-D-galactopyranoside-induced \textit{Escherichia coli} BL21(DE3) by nickel-agarose chromatography as described (5). The protein concentration was determined with the Bio-Rad dye reagent with bovine serum albumin as the standard.

Primer-Templates—Oligonucleotide primer strands were 5′-[\textsuperscript{32}P]-labeled by reaction with T4 polynucleotide kinase and [\textgamma-\textsuperscript{32}P]ATP, then purified by native gel electrophoresis, and annealed to a 4-fold molar excess of an unlabeled complementary DNA strand to form the primer-templates depicted in the figures. Oligonucleotides containing single tetrahydrufruran abasic sites or single covalent PAH-DNA adducts of known chirality were described previously (33–35).

Assay of Templated Primer Extension—Reaction mixtures containing 50 mM Tris-HCl (pH 7.5), 5 mM dithiothreitol, 5 mM MnCl\textsubscript{2}, 25 mM [\textsuperscript{32}P]-labeled primer-template, 1.4 \mu M LigD POL, and nucleotides as specified were incubated at 37 °C. Aliquots (20 \mu l) were withdrawn at the times specified and quenched immediately by adding an equal volume of 96% formamide, 20 mM EDTA. The [\textsuperscript{32}P]-labeled products were resolved by electrophoresis through a 15-cm 18% polyacrylamide gel containing 7 M urea in TBE (90 mM Tris borate, 2.5 mM EDTA). The products were visualized by autoradiography. Where indicated, primer extension was quantified by scanning the gel with a Fuji BAS-2500 imager. The percentage of input primer extended was plotted as a function of reaction time. The data were then normalized to the end point values for extension (defined as 100%) and the apparent rate constant (k) for the initial addition step was calculated by fitting the normalized data to the equation: 100 − % extension\textsubscript{norm} = 100 e\textsuperscript{-kt}.

Results

Nucleotide Misincorporation by LigD POL—The initial characterization of the templated DNA polymerase activity of \textit{Pseudomonas} LigD POL included a set of dNTP omission experiments, in which the polymerase was expected to arrest primer elongation when it encountered a template position for which the “correct” dNTP was missing from the reaction mixture. Even when the reactions were performed at low dNTP concentrations (1 \mu M), the enzyme was able to elongate past some of the predicted arrest sites (5). These findings raised the prospect that LigD POL misincorporates dNMPs. In light of the recently reported crystal structure of LigD POL revealing its similarity to the archaeal/eukaryal primase-polymerase family (9), and the well documented infidelity of eukaryal primase (16–19), we undertook here to examine the extent to which LigD might also be unfaithful.

A set of four primer-templates was prepared that consisted of a 13-mer 5′-[\textsuperscript{32}P]-labeled DNA primer strand annealed to a complementary 18-mer DNA strand to form a 13-bp duplex with a five-nucleotide single-stranded 5′ tail (Figs. 1–4). The primer-templates differed only in the identity of the template deoxyxynucleotide base immediately flanking the 3′-OH primer terminus. We measured the rate of extension of the primer-template (25 nm) by excess POL (1.4 \mu M) in the presence of single dNTP or rNTP substrates (10 \mu M), and thereby probed all possible misincorporation events. A shorter time course was monitored for the reactions containing the correct dNTP and rNTP (e.g. 5 s, 15 s, 30 s, 1 min, and 2 min) than for those containing only mispaired nucleotides (which were sampled at 1, 2, 5, 10, and 20 min in panels A in Figs. 1–4).

Fig. 1A illustrates the selectivity of nucleotide incorporation at a dT in the template strand. When presented with the correct
deoxyribonucleotide dATP, LigD POL sequentially incorporated two dAMP residues in response to the TT sequence in the template strand. Whereas 44% of the input primer was extended by at least one nucleotide in 5 s with dATP, 93% of the input primer was elongated in 5 s with ATP. Extension by two nucleotides was virtually complete within 1 min for both dATP and ATP and there was no further elongation beyond the nth position within the time frame of the experiment. By quantifying the fraction of the input primer extended, we obtained the kinetic profiles for the first adenosine nucleotide addition step shown in Fig. 1B. The correct ribonucleotide was more rapidly utilized by LigD POL (apparent rate constant of −0.6 s⁻¹ for ATP) than was the correctly paired deoxynucleotide (the kobs for dATP being 0.13 s⁻¹). Control experiments showed that the apparent rate constant and the end point of the primer extension reaction in the presence of ATP were unchanged when the concentration of POL was decreased from 1.4 to 0.7 or 0.35 μM, which implied that POL binding to the primer-template was not rate-limiting and that the reaction could be treated as a pseudo-first order process (data not shown). Also, the rate constant for dATP addition did not increase when the POL concentration was doubled to 2.8 μM (data not shown), signifying that enzyme binding to the primer-template was not rate-limiting for the DNA polymerase reaction.

It was remarkable that the nucleotide substrate preference changed during the second cycle of templated dAMP or rAMP addition, as evinced by the fact that the level of the nth intermediate relative to the n+2 end product was much lower in the presence of dATP than it was with ATP (Fig. 1A). By plotting the distributions of the nth and n+2 species (expressed as percent of total label in each) as a function of time and fitting the data to a simple two-step reaction scheme using the pro-
gram CKS (IBM, version 1.0), we estimated rate constants of 0.1 and 0.15 s$^{-1}$ for the first and second dAMP incorporation steps, respectively, and rate constants of 0.5 and 0.055 s$^{-1}$ for the first and second rAMP incorporation steps, respectively (not shown). These results underscore the theme that a ribonucleotide 3’ primer terminus has negative influence on the rate of further primer extension, be it with a dNMP as noted previously (6), or an rNMP as seen here.

We found that LigD POL incorporated a single dTMP residue opposite a T in the template strand when dTTP was the only substrate available; 70% of the input primer was elongated after 20 min (Fig. 1A). The rate of dTMP misincorporation was ~2% of the rate at which the correct dAMP residue was incorporated. LigD POL also misincorporated single dGMP and dCMP residues opposite the template dT, to the extent that 53 and 35% of the input primer was elongated after 20 min in the presence of dGTP and dCTP, respectively (Fig. 1A).

Ribonucleotides were misincorporated more readily by LigD POL than the corresponding deoxynucleotides (Fig. 1A). The addition of single UMP, GMP, and CMP residues to the primer terminus proceeded to completion during the 20-min reaction; the rate constants for addition of UMP (0.02 s$^{-1}$), GMP (0.01 s$^{-1}$), and CMP (0.003 s$^{-1}$) were slower by factors of 30, 60, and 200 than the rate of addition of the correct AMP ribonucleotide (Fig. 1B). However, UMP was incorporated 20-fold faster than dTMP; GMP was added ~12-fold faster than dGMP; and extension with CMP was ~5-fold faster than dCMP.

**Effects of the Templated Base on POL Fidelity**—Fig. 2A shows the kinetics of elongation on a primer-template containing a templated dA base. 5% 32P-labeled primer-template (shown at bottom), 10 μM of the indicated nucleotidetriphosphate, and 1.4 μM LigD POL were incubated at 37 °C for the times specified. B, the percent of the input primer strand that was extended is plotted as a function of reaction time.
with the correct dTTP substrate, such that nearly all of the input primer was extended within 1 min. Note that the POL did not cease after adding the first TMP, but proceeded to add a second dTMP nucleotide opposite the n+2 T on the template strand. This result confirms the finding in Fig. 1A that POL is adept at misincorporating T opposite T. The addition of the correct ribonucleotide UMP was complete in 5 s and the reaction was largely limited to a single step of incorporation. The kinetic profile plotted in Fig. 2B shows that UTP (k = 0.6 s⁻¹) was utilized at least 12-fold faster than dTTTP (k = 0.048 s⁻¹) for the first addition step. Here, dGTP was the best of the misincorporated deoxynucleotides (initial rate 1.6% of dTTTP), followed by dATP (initial rate 1% of dTTTP) and dCTP (0.3% of dTTTP). Note that two nucleotides were added in the presence of dATP, which reflects the correct addition of dAMP opposite the n+2 dT template base despite the presence of a dA:dA mispair at the primer terminus. Ribonucleotide misincorporation was fastest with ATP, where we again observed a second cycle of AMP incorporation opposite the n+2 dT (Fig. 2A). By including measurements of dATP primer extension at shorter time points (Fig. 2B), we calculated an apparent rate constant of 0.02 s⁻¹ for AMP misincorporation opposite dA; this value was ~30-fold less than the rate of correct UMP incorporation. Nonetheless, the initial rate of AMP incorporation was 50-fold faster than the rate of dAMP addition. GTP (k = 0.008 s⁻¹) was utilized at 1.3% the rate of UTP, but 10-fold faster than dGTP. CTP-dependent extension (k = 0.0017 s⁻¹) was 0.3% of the rate with UTP, but still ~10-fold faster than the rate with dCTP.

The salient themes from the above experiments, that LigD POL is unfaithful and is even more unfaithful than a DNA polymerase, are reinforced by the kinetics of nucleotide addition opposite a templated dC (Fig. 3) and a templated dG (Fig. 4). In brief, dGMP and GMP were both added rapidly opposite the templated dC base (Fig. 3A). From the data in Fig. 3B, we calculated rate constants of 0.31 and 0.57 s⁻¹ for correct dGMP and GMP addition, respectively. All of the incorrect dNTPs were comparatively poor substrates for misincorporation opposite dC (with initial rates of extension on the order of 0.1–0.3% of the initial rate with dGTP). However, rNTPs were readily misincorporated opposite dC (Fig. 3A), with ATP being fastest (k = 0.016 s⁻¹), followed by UTP (k = 0.008 s⁻¹) and CTP (k = 0.003 s⁻¹) (Fig. 3B). The initial rates of AMP, UMP, and CMP addition were 30-, 30-, and 10-fold faster than the rates of dAMP, dTMP, and dCMP, respectively.

dCMP and CMP were incorporated rapidly opposite the templated dG base (Fig. 4A), with rate constants of 0.25 and 0.6 s⁻¹, respectively (Fig. 4B). dTTTP was the best deoxynucleotide substrate for misincorporation, at 0.5% the rate of dCTP utilization. All rNTPs were readily misincorporated opposite dG, with UTP being fastest (k = 0.04 s⁻¹), followed by ATP (k = 0.017 s⁻¹) and GTP (k = 0.006 s⁻¹).

**Elongation of Primer-Templates with a 3’ Base Mispair**—Having established that LigD POL can generate a 3’ mispair by adding an incorrect nucleotide, it was of interest to test how POL behaved when confronted with such mispaired primer termini in the presence of the full complement of dNTP or rNTP substrates. Thus, we prepared four sets of primer-templates (with four primer-templates per set) composed of a 5’-32P-labeled 13-mer primer DNA strand annealed to an 18-mer DNA oligonucleotide to form 13-bp duplexes with five-nucleotide single-stranded 5’ tails. Within each of the four sets, the 3’-nucleoside of the primer was identical (there being a 3’-dT set, a 3’-dA set, etc.) and the four template strands in each set differed only in the identity of the deoxynucleoside base opposite the 3’-nucleotide of the primer strand. In this way, we probed rates of dNTP and rNTP-dependent primer extension at all possible 3’ base mispairs.

Fig. 5A shows the kinetics of extension at a correctly paired 3’-dC:dG primer terminus in the presence of dNTPs (50 μM...
The mispairs were extended with dNMPs at 2 (dT:dT), 7 (dT:dC), and dA:dC (Fig. 6). Ribonucleotides were added 9-fold more rapidly than deoxys at the respective mispaired dA ends, with the dA:dA mispair being the most effective configuration for the RNA polymerase (93% primer utilization in 5 min), followed by dT:dG and dA:dC (Fig. 6B).

Fig. 7 shows similar outcomes for POL activity at paired and mispaired 3’-dT primer termini. The correctly paired dT:dA primer was extended by 5 or 6 dNMPs at the reaction end point, but RNA synthesis was limited to 3 cycles of rNMP addition. The mispairs were extended with dNMPs at 2 (dT:dT), 7 (dT:dG), and 9% (dT:dC) the rate of the correctly paired end (Fig. 7A). dT:dG and dT:dC were the most effective of the mispair configurations for rNMP addition (complete primer utilization within 5 min), followed by dT:dG (Fig. 7B). Ribonucleotides were added 8-, 10-, and 20-fold more rapidly than deoxys at the mispaired dT:dG, dT:dC, and dT:dT ends, respectively.

POL extension of a paired 3’-dT primer terminus with dNTPs resulted in fill-in synthesis (evident at 5 and 10 min) followed by addition of a non templated nucleotide at the blunt DNA end by 10 and 20 min (Fig. 8A). The dG mispairs were poor substrates for the DNA polymerase, with the dG:dT end further elongation occurring slowly at 20 min. The mispairs were extended with dNMPs at 2 (dT:dT), 7 (dT:dG), and 9% (dT:dC) the rate of the correctly paired end (Fig. 7A). dT:dG and dT:dC were the most effective of the mispair configurations for rNMP addition (complete primer utilization within 5 min), followed by dT:dG (Fig. 7B). Ribonucleotides were added 8-, 10-, and 20-fold more rapidly than deoxys at the mispaired dT:dG, dT:dC, and dT:dT ends, respectively.

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displaying the best activity (34% of the input primer being extended at 20 min). RNA polymerization at the paired dG:dC primer end was limited to 3 cycles of rNMP addition (Fig. 8B). dG:dT was the most effective of the mispair configurations for rNMP addition (57 and 89% primer utilization at 1 and 5 min, respectively), followed by dG:dG and dG:dA.

**Arrest and Bypass at an Abasic Lesion on the Template DNA Strand**—A set of two primer-templates was prepared that consisted of a 12-mer 5′-32P-labeled DNA primer strand annealed to a complementary 30-mer DNA strand to form a 12-bp duplex with an 18-nucleotide single-stranded 5′ tail. The primer-templates differed only at the template deoxynucleotide immediately flanking the 3′-OH primer terminus, which was either a dT nucleotide or a tetrahydrofuran abasic lesion (Fig. 9A). We measured the rate of extension of the primer-templates (25 nM) by excess POL (1.4 μM) in the presence of all four dNTPs (50 μM). Nearly all of the primer strand of the unmodified control DNA had reacted after 1 min. Elongation to the end of the template strand yielded a 30-mer product that was evident at 5 min (Fig. 9A, left panel). LigD POL was impaired in its ability to add the first dNMP opposite the abasic template lesion, insofar as the input 12-mer primer decayed slowly over 10 min (Fig. 9A, right panel). The apparent rate constant for the first dNMP addition was 0.004 s⁻¹. (This value is 30-fold slower...
than the rate of correct dAMP addition opposite dT calculated from the data in Fig. 1.) In turn, the 13-mer \(n + 1\) product was also extended slowly, implying that a dNMP:abasic pair at the 3'-OH end is itself an impediment to POL elongation.

Nonetheless, a fraction of the primers appeared to be extended to the end of the abasic template strand at \(10 - 20\) min (Fig. 9A, right panel). However, when the extension products formed on the unmodified and abasic templates were analyzed side by side, it was revealed that the fill-in products were \(\sim 3\) nucleotides shorter on the abasic template than the products formed on the unmodified template (Fig. 9B). We surmise that the complete bypass of the abasic site leading to fill-in synthesis entailed a slippage mechanism, whereby the TT dinucleotide at the end of the primer strand slips forward to pair with the AA dinucleotide downstream of the abasic lesion, as illustrated in Fig. 9B. POL extension from this conformation of the primer would yield the 3-nucleotide shorter fill-in product that we observed.

Preferences for Nucleotide Addition Opposite an Abasic Template Lesion—Extension of the 12-mer primer on the unmodified and abasic template strands was analyzed in reaction mixtures containing only a single dNTP or rNTP substrate (10 \(\mu\)M). The unmodified template containing a dT flanking the primer 3'-OH directed rapid incorporation of the correctly paired dAMP, followed by slow misincorporation of a second dAMP opposite the \(n + 2\) dA of the template strand (Fig. 10A). POL catalyzed near quantitative addition of dAMP opposite the abasic lesion (by 20 min), albeit at about one-tenth the rate of the templated dAMP addition reaction (Fig. 9A). Misincorporation of dGMP opposite dT was slower than dAMP addition by \(\sim 40\) fold and only a single \(n + 1\) product was detected at 20 min (Fig. 10D). Yet, the rate and extent of dGMP insertion opposite the abasic site was about the same as its addition opposite the dT nucleotide (Fig. 10D). We estimate that dATP was about 4-fold better than dGTP as a substrate for addition at an abasic template lesion.

As expected, we found that ATP and GTP were better substrates than dATP and dGTP for addition opposite dT on the unmodified 12-mer/30-mer primer template. Yet, ATP and GTP were also far superior to dATP/dGTP (by at least a factor of 20) as substrates for incorporation by POL opposite an abasic site (Fig. 10, A and D). Thus, POL prefers a ribonucleotide whether or not there is an instructive base on the template strand.

The patterns of primer extension on the unmodified 12-mer/30-mer DNA in the presence of dTTP or dCTP revealed further evidence of template slippage. For example, the dCTP reaction resulted in extension by two nucleotides, with scant accumulation of the \(n + 1\) species as an intermediate (Fig. 10C). The likely explanation is that the primer-template adopts the slipped-forward configuration depicted in Fig. 9B, which directs templated incorporation of two consecutive dCMP nucleotides. The same pattern is observed when CTP is the substrate, expect that the overall reaction is faster than with dCTP and the \(n + 1\) intermediate is more evident early on (at 1 min), which reflects slowing of the second ribo incorporation event at a 3'-ribo primer terminus.

In the presence of dTTP, the primer is consumed slowly, but is extended by up to 4 or 5 nucleotides, which is more than what is expected if POL first misincorporates T opposite T and then correctly incorporates two more Ts opposite the AA dinucleotide at \(n + 2/n + 3\). Rather, the predicted \(n + 2\) and \(n + 3\) extension products are underrepresented compared with \(n + 1\) and \(n + 4\). We infer that \(n + 4\) is generated by backward slippage of...
the T-rich primer terminus on the A-rich segment of the template strand, allowing an extra T to be added. Although dTTP, dCTP, UTP, and CTP were as effective (or more so) for primer extension on the abasic primer-template than they were on the unmodified DNA, we cannot unambiguously ascribe these extensions to incorporation opposite the abasic lesion, given the propensity of POL to utilize the slipped conformation of the primer-template when only C or T are provided.

Elongation of Primer-templates with a 3′-OH dNMP Opposite an Abasic Lesion—Given that LigD POL can generate a 3′-dNMP:abasic terminus by adding a (purine) nucleotide across from an abasic site, we queried how well POL can extend dNMP:abasic termini in the presence of all four dNTPs. A set of 5′-32P-labeled 13-mer primers, differing only in the 3′-terminal deoxynucleotide base, was annealed to a 30-mer DNA containing a single abasic site opposite the 3′-terminal deoxynucleotide base, was annealed to a 30-mer DNA containing a single abasic site opposite the 3′ nucleotide of the primer strand (Fig. 11). As controls, we annealed the labeled 13-mer primers to an unmodified 30-mer template with a dT opposite the 3′-terminal deoxynucleotide base, was annealed to a 30-mer DNA containing a single abasic site opposite the 3′-terminal deoxynucleotide base, was annealed to a 30-mer DNA containing a single abasic site opposite the 3′-terminal deoxynucleotide base, was annealed to a 30-mer DNA containing a single abasic site opposite the 3′-terminal deoxynucleotide base.

FIGURE 10. Kinetics of nucleotide insertion at an abasic site. Reaction mixtures containing 25 nM 5′-32P-labeled primer-template (shown at bottom), 1.4 μM LigD POL, and 10 μM of the indicated nucleoside triphosphate were incubated at 37 °C for the times specified.

The situation was quite different for the extension reaction at a 3′-dT:abasic primer terminus depicted in Fig. 11B, in which virtually all of the primer strand was extended to the end of the template and there was no accumulation of a paused n + 1 intermediate. The mispaired dT:dT control was extended with a...
similar kinetic profile (Fig. 11B). The relatively facile extension of these two 3'-dT primer strands annealed to the 30-mer template contrasts with the much feeble reactivity of LigD POL at the mispaired dT:dT primer-template used in Fig. 7A. The key difference is that the template strand of the 13-mer/30-mer primer template allows for slippage of the 3'-TTT trinucleotide over the flanking mispaired dT or abasic site on the template so that the T-rich primer end can pair with the AA dinucleotide at \( n+1/n+2 \) on the template strand.

The 3'-dC:abasic primer-template was also filled in with high efficiency and with no accumulation of a paused \( n+1 \) intermediate (Fig. 11C). The 3-dC:dT mispaired primer was consumed at a slightly slower rate, and the ladder of elongated strands extended to \( n+14 \) at 10 min, rather than the \( n+17 \) species expected if POL simply copied the template strand from the site of the mispair. The extension to \( n+14 \) is consistent with forward slippage of the primer 3'-TTC trinucleotide to anneal with the AAG triplet on the template strand, as depicted in Fig.

**FIGURE 11.** Kinetics of extension of a primer 3' end at an abasic site. Reaction mixtures containing 25 nM 5'\(^{32}\)P-labeled primer-template (shown below the autoradiographs), 50 \( \mu \)M each of dNTPs, and 1.4 \( \mu \)M LigD POL were incubated at 37 °C for the times specified. The identity of the 3' primer nucleotide was varied as follows: 3'-dA (A), 3'-dT (B), 3'-dC (C), and 3'-dG (D). The 3' primer terminus was positioned across from a dT or an abasic site in the template strand (the tetrahydrofuran abasic lesion is indicated by \( - \)). The proposed conformation of the primer-template after slippage of the primer strand 3'-TTC end is shown in panel C next to the autoradiograph of a side by side analysis of the products of a 20-min reaction of LigD POL with the indicated primer(3'-dC)-templates.
C. POL apparently prefers the slippage path to direct extension of the dC:dT mispaired end, which we found to be an inefficient reaction when there was no potential for realignment of the primer strand terminus (see Fig. 4A). Side by side product analysis showed that the distribution of fill-in synthetic products on the dC:abasic template strand overlapped with the longest species formed by extension at the dC:dT mispair, but also included slightly longer products consistent with copying the template without slippage (Fig. 11C). We surmise that POL can choose between direct extension of the dC across from the abasic lesion or elongating the realigned primer that has slipped past the abasic site.

Arrest of LigD POL by a Benzo[c]phenanthrene Adduct on the Template Strand—Benzo[c]phenanthrene exemplifies the sterically hindered, nonplanar “fjord region” class of PAHs. Activated BPh diol epoxides react at the benzylic C1 position by trans addition of adenine N6 in DNA to form covalent S and R BPhdA adducts. In duplex DNA, the BPh intercalates from the major groove, such that the hydrocarbon portion of the S diastereomer is on the 3′ side of the modified dA base and that of the R diastereomer is on the 5′ side (36,37). In the experiments shown in Fig. 12, we probed the effects of R and S BPhdA adducts on LigD POL. The BPhdA lesions were situated 5 nucleotides from the 5′ end of an 18-mer template DNA strand, whereas the length of the primer strand was varied so that its 3′-terminal nucleotide was poised 2 nucleotides or 1 nucleotide upstream of the adduct (Fig. 12, A and B), directly opposite the adduct (Fig. 12C) or 1 nucleotide past the adduct (Fig. 12D).

An unmodified primer-template control was analyzed in parallel for each series of POL extension reactions, which were conducted in the presence of a full complement of 4 dNTPs. When the 12-mer primer terminus was located two nucleotides upstream of the BPhdA adduct, POL catalyzed rapid and efficient incorporation of the first templated dNMP to form a 13-mer (Fig. 12A).

The next step of dNMP insertion across from BPhdA occurred slowly; the 14-mer extension product accumulated steadily over 20 min and few primers were elongated beyond that position. In contrast, POL extended the control primer by 6 nucleotides to fill in the unmodified template, then added a nontemplated nucleotide to the blunt end.

When the primer end was poised immediately prior to BPhdA, POL slowly added a single dNMP opposite the lesion (Fig. 12B). Few of the primers on the BPhdA templates were extended past the lesion. Adjusting the primer 3′ end so that it was directly opposite BPhdA strongly inhibited addition of the next nucleotide (Fig. 12C). Yet, activity was regained when the
primer terminus was moved 1 nucleotide past the BPhdA lesion (Fig. 12D).

The effects of BPhdA on LigD POL appear to be tightly focused on the translesion insertion and first translesion extension steps. We noted a subtle difference between the R and S diastereomers of BPhdA in their ability to slow the insertion step opposite BPhdA and block the extension step of translesion synthesis by LigD POL, whereby the S diastereomer had a slightly less severe effect. This difference was more clear at the level of restoration of POL activity when the primer was moved 1 nucleotide past the lesion (Fig. 12D). In this setting, POL was more reactive with the S BPhdA primer-template, extending all primers within 1 min, whereas POL reacted about 10-fold slower with the primer on the R BPhdA template. This makes sense, insofar as the S adduct is predicted to intercalate on the 3' side of the modified dA of the primer-template substrate in Fig. 12D, thereby placing it farther away from the reactive primer 3'-OH than the R adduct, which should intercalate on the 5' side.

BPdG lesion did not restore POL activity (Fig. 13C). Thus, the “footprint” of BPdG adduct interference with LigD POL extends further than that of the BPhdA adduct. We infer that the POL makes important interactions with the minor groove of the DNA duplex segment at the primer terminus.

DISCUSSION

LigD is revealed in the present study to be an unfaithful nucleic acid polymerase. Although the degree of infidelity in nucleotide incorporation varies according to the nature of the mispair produced, the following general themes stand out: (i) a correctly paired ribonucleotide is added to the DNA primer terminus more rapidly than the corresponding correct deoxyribonucleotide; (ii) incorrect nucleotides are added much more rapidly with rNTP substrates than with dNTPs, no matter what the mispair configuration. These findings lend impetus to the suggestion that LigD POL might fill in short 5'-overhang DSBs with ribonucleotides during its NHEJ function in vivo. The ability to switch-hit as a templated RNA or DNA polymerase might...
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be particularly advantageous when repairing DSBs by NHEJ during periods of quiescence, insofar as dNTP pools are likely to be scant compared with rNTPs (38).

The properties of LigD POL in vitro are most consistent with a short-tract synthetic function during DNA repair, given that its rate of templated nucleotide addition is much slower than that of replicative bacterial DNA polymerases. In the present study we see that it takes LigD POL between 1 and 5 min to completely fill-in with dNMPs a 5-nucleotide overhang attached to a 13-bp duplex; POL takes 5 min to fill in an 18-nucleotide 5′ overhang to a 12-bp duplex. As noted here and previously (6), the RNA polymerase activity of LigD POL decays as successive rNMPs are incorporated at the 3′ primer terminus, which argues that rNTPs would yield an even shorter repair patch than dNTPs. Yet, these properties do not augur badly for the role of LigD in bacterial DSBR repair, which is entirely dependent on Ku (2), a protein that has a DNA end-binding function (3), and interacts physically with LigD via the POL domain (2,8). It is assumed that Ku recruits LigD to DSBs where its action is needed. It seems unlikely that LigD POL is called upon to add long tracts of nucleotides in vivo, given that DSBR formation in response to DNA damaging agents likely entails closely staggered breaks on the two strands of the duplex. The critical issue with respect to the biological plausibility of a rNMP addition function for LigD POL is whether or not the ligase component of LigD can effectively seal a strand containing one or more ribonucleotides at the reactive 3′-OH terminus. We find that *Pseudomonas* LigD is adept at this sealing reaction.³

The crystal structure of LigD in complex with Mn-ATP and Mn-dATP substrates provides clues to its preference for rNMP additions (9). The divalent cations and triphosphate moieties are bound identically to the enzyme in the Mn-ATP versus Mn-dATP cocrystals, but the nucleosides adopt different conformations. The adenosine of ATP extends away from the triphosphate group and is engaged to the enzyme via stacking of the adenine base on Phe⁶⁰⁴ and a network of direct and water-mediated contacts to the O2′ and O3′ atom of the ribose sugar. In contrast, the deoxyadenosine of dATP is folded back over the triphosphate so that the adenine base stacks on the guanidinium moiety of the Arg⁷⁷⁸ side chain; in this conformation, there are no apparent protein contacts to the O3′ of the deoxyribose sugar. Changing Phe⁶⁰⁴ to alanine suppressed both rNMP and dNMP incorporation, whereas changing Arg⁷⁷⁸ to alanine had minimal impact on POL activity (9). These results suggest that, although ribonucleotides and deoxyribonucleotides can assume different conformations in the substrate-binding cleft, the ATP mode is the productive one for templated nucleotide addition. Conceivably, the two binding modes are in equilibrium, in which case rNTPs would more readily adopt the productive mode by virtue of the protein contacts to the ribose O2′ that favor the extended conformation with stacking on Phe⁶⁰⁴.

The POL structure also provides clues to its infidelity, insofar as the π stack on Phe⁶⁰⁴ is the only contact between the enzyme and the adenine base of the ATP substrate. Similar stacking of the pyrimidine base on Phe⁶⁰⁴ was seen in cocrystals of LigD Pol with Mn-CTP and Mn-UTP (9). Given that the nucleotide and metal can clearly bind to the POL domain in the absence of instructions from a primer-template, that the NTP base is exposed on the surface of the enzyme and makes no base-edge contacts to the protein, and that there are no obvious mobile structural elements that could close around the NTP base, it is unlikely that LigD POL could adopt a tight substrate fit mechanism (39) to ensure high fidelity. A simple view would be that the faster incorporation of the correct nucleotide versus the “next best” of the incorrect nucleotides (which is on the order of 15–35-fold for the rNTP substrates) reflects the contributions of Watson-Crick base pairing. This model can ultimately be tested by examining the utilization of apolar base isosteres of the template bases and the rNTP/dNTP substrates (40–42).

The introduction of a base mispair at the 3′-OH terminus of a primer-template can either: (i) impede further elongation by the polymerase that created the mismatch, or (ii) allow further elongation by that polymerase, thereby embedding the error within the newly synthesized polynucleotide. Whereas many DNA polymerases come equipped with a proofreading 3′-exo-nuclease function that can remove 3′ mispaired nucleotides, the POL domain of LigD evidently does not have such an activity, insofar as we detected no transient shortening of any of the 3′ mispaired primer strands during the extension reactions studied in Figs. 5–8. Our experiments show that 3′ mispairs can be extended with high yield by LigD POL, albeit at diminished rates compared with 3′ paired primer-templates. The magnitude of the rate decrement in subsequent nucleotide incorporation varies according to the nature of the 3′ mispair, but it was generally true that the mispaired ends were extended more rapidly with rNTP substrates than with dNTPs, no matter what the mispair configuration. These findings suggest that LigD POL is itself capable of embedding mispairs within repair tracts (particularly RNA repair patches). This contrasts with the division of labor observed in some error-prone lesion bypass polymerase systems, where one polymerase is responsible for incorporating a nucleotide opposite the lesion on the template strand, but cannot then extend the terminus it created. Instead, a second DNA polymerase specializes in extending the aberrant 3′ end of the primer-template (26).

The nucleotide incorporation and mismatch extension properties observed here for LigD POL resonate with the prior studies concerning the infidelity of the homologous eukaryal primase, including the capacity of primase to embed incorrectly paired rNMPs (16). The fact that LigD POL can ignore template instructions when provided with the wrong nucleotide is in line with the fact that it is adept at adding nontemplated ribo- or deoxyribonucleotides at a blunt duplex DNA end.

There has been little attention paid to how members of the archaeal/eukaryal primase enzyme family respond to DNA damage in the template strand. This issue is relevant now that LigD POL is clearly identified as a member of this family with a role in DNA repair rather than primer synthesis. The present characterization of LigD POL shows that it is not adept by itself at bypassing abasic sites, which are among the most common types of DNA damage. POL is capable of catalyzing the trans-

³ H. Zhu and S. Shuman, unpublished data.
lesion insertion step with high yield across from a tetrahydrofuran abasic site. Ribonucleotides are again superior to the cognate deoxynucleotides as substrates for this reaction. Yet, POL is impaired in the subsequent extension step. These properties suggest that were LigD confronted with an abasic site in vivo, then successful translesion synthesis (not involving a frameshift) would require the participation of another polymerase that performs the extension step (26). Alternatively, LigD POL might acquire improved translesion synthesis activity under the influence of an accessory factor.

Our experiments highlight an alternative pathway of lesion bypass by LigD POL, whereby the enzyme allows slippage of the primer terminus across an abasic site (or a 3’ mismatch) to pair with downstream bases of the template strand. Bypass via this route results in a shorter fill-in product, indicative of a microdeletion of the looped out template segment. One might question the wisdom of a lesion bypass mechanism that generates frameshift mutations. However, this is a minor concern in the setting of bacterial NHEJ, which is already highly mutagenic, because the frameshifts generated by LigD when it “correctly” fills in 5’ overhangs or adds nontemplated nucleotides at broken DNA ends prior to the sealing step (2, 9). The slippage response to an abasic lesion that we invoke for LigD POL has been described previously for the Y-family polymerase Dpo4, especially the high yields of the insertion product, albeit slowly, but is impeded to a greater degree at the extension step. The deletion of the looped out template segment. One might question the influence of an accessory factor.

Our inference from these results is that LigD POL is more acutely reliant on contacts with the minor groove of the DNA terminus than with the major groove. Indeed, we propose that the reason POL activity decays when ribonucleotides are added at the 3’ primer terminus is because the resulting RNA-DNA hybrid adopts an A-like secondary structure with altered minor groove dimensions compared with B-form DNA.

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