Replication Bypass of Interstrand Cross-link Intermediates by *Escherichia coli* DNA Polymerase IV*

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Repair of interstrand DNA cross-links (ICLs) in *Escherichia coli* can occur through a combination of nucleotide excision repair (NER) and homologous recombination. However, an alternative mechanism has been proposed in which repair is initiated by NER followed by translesion DNA synthesis (TLS) and homologous recombination. In *E. coli* incised ICLs, our laboratory has recently explored the biochemical and genetic data supporting such an activity of pol II have subsequently demonstrated that pol II, but not pol IV or V, was functioning in this HR-independent ICL repair pathway. It was hypothesized that pol II could catalyze replication bypass of the incised but still covalently linked DNA (4, 5). However, no biochemical data supporting such an activity of pol II have subsequently appeared.

Using a series of synthetic oligodeoxynucleotide substrates that mimic various intermediates in the processing of NER-incised ICLs, our laboratory has recently explored the biochemical basis of TLS replication (4, 5). The data demonstrated that pol II, but not not pol IV or V, was functioning in this translesion DNA synthesis (TLS) past N2N2-guanine ICL intermediates using human DNA polymerases. These investigations revealed that pol K was able to catalyze replication bypass of ICL lesions in which the nucleotides 3’ to the lesion had been removed (6).

Given a role for *E. coli* pol II in HR-independent ICL repair and that *E. coli* pol IV is an ortholog of human pol K (7, 8), this study was designed to investigate the ability of pol II and pol IV to catalyze TLS past N2N2-guanine ICLs.

**EXPERIMENTAL PROCEDURES**

*Generation of Oligodeoxynucleotides Containing Site-specific ICLs*—Modified oligodeoxynucleotides (ICL1, ICL2, ICL3, and ICL4) (see Fig. 1A) containing various model acrolein-mediated N2N2-guanine cross-links (see Fig. 1B) were synthesized and purified as previously described (6) and were kind gifts of Drs. Carmelo J. Rizzo and Ivan D. Kozekov (Department of Chemistry, Vanderbilt University). A control non-damaged DNA substrate was obtained from the Molecular Microbiology and...
Interstrand Cross-link Bypass by DNA Polymerase IV

TABLE 1
E. coli strains used in this study

| Strain   | Genotype           | Relevant phenotype(s)         | Reference |
|----------|--------------------|-------------------------------|-----------|
| ZK126    | W3110 ΔlacI169    | Wild-type parental strain     | Zambrano et al. (21) |
| SF2003   | ZK126 polB::Spc    | pol II−                      | Yeiser et al. (14)   |
| SF2006   | ZK126 dinE::Kan    | pol IV−                      | Yeiser et al. (14)   |

Immunology Research Core Facility of the Oregon Health & Science University.

Proteins—E. coli pol II and pol IV were purified as described previously (9, 10).

DNA Polymerase Bypass Assays—The primers (see Fig. 1C) were 32P-end-labeled and annealed with the DNA templates as described previously (11). The primer extension assays were conducted in a 10-μl reaction volume containing pol II or pol IV (as indicated in the figure legends), 5 mM primer-template, 5 mM MgCl2, 100 μM dNTPs, 25 mM Tris-HCl (pH 7.5), 25 mM dithiothreitol, 0.5 mg/ml bovine serum, and 10% glycerol. Reactions were carried out at 37 °C for 30 min, followed by termination of the reaction with a solution containing 95% (v/v) formamide, 10 mM EDTA, 0.03% (w/v) xylene cyanol, and 0.03% (w/v) bromphenol blue. DNA replication products were separated through a 15% (w/v) denaturing polyacrylamide gel at 2000 V and later analyzed on a PhosphorImager (GE Healthcare).

Steady-state kinetic assays were performed according to a standard procedure (12, 13). Briefly, reactions were conducted at 22 °C in the same buffer as the primer extension assays with the addition of NaCl (50 mM). The concentration of the primer-template DNA substrates was 10 nM. The concentrations of pol IV and incubation times were adjusted for each particular primer-template combination such that the formation of the product would not exceed 25%. Nucleotide concentrations (dCTP and dGTP) varied. Quantitative analyses were performed using ImageQuant 5.2 software (GE Healthcare). The rates of nucleotide incorporation were plotted as a function of nucleotide concentration, and the kcat and Km parameters were obtained from the best fit of the data to the Michaelis-Menten equation using KaleidaGraph 3.6 software (Synergy Software).

Bacterial Strains—The strains used in this study were derived from E. coli K-12 strain W3110 and are isogenic except for deletion of either dinB or polB (Table 1) (14). All strains were cultured in LB broth supplemented with spectinomycin (100 μg/ml) for the pol II-deficient strain and kanamycin (50 μg/ml) for the pol IV-deficient strain.

Generation of a Vector Construct Carrying a Site-specific N2- -N2’-Guanine Cross-link—The characterization and preparation of a single-stranded pMS2 vector have been reported previously (15, 16). The single-stranded pMS2 DNA (15 pmol), which carries an EcoRV site in the hairpin region, was linearized by digestion with EcoRV (100 units) for 3 h at 37 °C and purified using Amicon 100K centrifugal filter devices according to the manufacturer’s protocol. A 36-mer oligodeoxynucleotide (see Fig. 1D) carrying a site-specific N2- -N2’-guanine ICL was designed in such a way that the single-stranded regions were complementary to the peripheral regions of the linearized pMS2 vector. The 36-mer oligodeoxynucleotide (15 pmol) was phosphorylated using T4 polynucleotide kinase (50 units) for 1 h at 37 °C, added to the linearized pMS2 vector, annealed, and extended using the Klenow fragment of E. coli pol I (25 units). A double-stranded linear product was gel-purified and ligated overnight at 12 °C with T4 DNA ligase (4000 units). The ligated sample was designated pMS2-ICL and further used for transforming E. coli cells.

Transformation of E. coli Strains with pMS2-ICL and pBR322 Plasmids—Initial experiments were conducted using wild-type E. coli cells to determine the amount of pMS2-ICL that had comparable transformation efficiency with 0.5 ng of the reference plasmid, pBR322. For both pMS2-ICL and pBR322, selection of successful transformants was done using resistance to ampicillin. Next, a mixture of plasmids containing pMS2-ICL and pBR322 was prepared at quantities that would provide approximately equal transformation efficiencies, and this mixture was utilized to transform individual E. coli strains. Transformations were done by electroporation as described previously (16).

For further screening, the transformants were individually grown first in LB broth containing ampicillin (100 μg/ml) in 96-well plates at 37 °C for 4–6 h. A 20-μl aliquot from each well was transferred to another 96-well plate containing LB broth with tetracycline (12.5 μg/ml) and grown overnight at 37 °C. Plasmids were isolated from tetracycline-sensitive colonies, thus positive for pMS2-ICL, and subjected to DNA sequencing using an 18-mer oligodeoxynucleotide (5’-AGCA-ACCATTAGTCGCCGCC-3’) as the primer.

RESULTS

Experimental Rationale and Substrate Design—Prior genetic evidence provides strong support for a role of E. coli UvrABC around an ICL site. Furthermore, replication bypass of a 12-mer DNA strand that was still covalently attached to the template strand. This structure would be representative of the product of a dual incision by E. coli UvrABC around an ICL site. Furthermore, replication bypass of N2- -N2’-guanine cross-links has been observed for the human ortholog of E. coli pol IV, pol k (6). Thus, experiments were designed to test the ability of E. coli pol II and pol IV to catalyze TLS on DNA substrates containing a site-specific ICL (Fig. 1A). Specifically, DNA strands in each of the four ICL substrates (ICL1, ICL2, ICL3, and ICL4) are joined via a carbon bridge that models an acrolein-derived ICL (Fig. 1B). ICL1 models the product of incision by the UvrABC complex, whereas ICL2 and ICL3 represent potential repair intermediates in which nucleotides 5’ and 3’ to the ICL, respectively, have been removed. ICL4 contains a residual ICL in which nucleotides both 5’ and 3’ have been removed. The 3’-ends of ICL1 and ICL2 are terminated with a dideoxynucleotide (ddN-3’) to prevent any synthesis from the cross-linked strand. Similarly, ICL3 and ICL4 are 3’-capped with a glycerol (gl-3’) to prevent replication from that site. 32P-Labeled primers (Fig. 1C) were designed to initiate synthesis 1 or 10 nucleotides 5’ to the cross-linked site in the template strand. A 0 primer was used to initiate the replication from C opposite the cross-linked G.
In Vitro Replication Bypass of ICL-containing DNA Substrates—Control non-damaged and ICL-containing DNA substrates were used to analyze replication from a −10 primer by pol II (Fig. 2A) and pol IV (Fig. 2, B and C). Using the non-damaged primer-template substrates, pol II catalyzed a highly processive polymerization to yield full-length primer extension products. However, under identical conditions, pol II was unable to carry out effective strand displacement synthesis on ICL1 and ICL3, whereas on ICL2 and ICL4, it could replicate up to one nucleotide prior to the cross-linked guanine, but no TLS past the lesion was observed (Fig. 2A). ICL bypass by pol II was not observed even when reactions were conducted with increased enzyme concentrations (up to a 1000-fold excess relative to the DNA substrate) (data not shown).

Examination of the activities of pol IV on the same substrates revealed a less processive synthesis on the non-damaged DNA template and a very poor ability to catalyze strand displacement synthesis on ICL1 and ICL3 following the incorporation of the first nucleotide (Fig. 2B). Using the ICL2 primer-template, in which no strand displacement synthesis is necessary, pol IV was able to synthesize up to one nucleotide prior to the ICL, but was able to catalyze only minimal incorporation opposite the lesion; further synthesis was blocked two nucleotides beyond the cross-linked site. In contrast, pol IV synthesis on the ICL4 primer-template revealed that although multiple pause sites occurred prior to reaching the ICL, there was only modest blockage at the lesion (Fig. 2B). Following incorporation opposite the cross-linked nucleotide, synthesis continued with reduced processivity with full-length DNA products accumulating over time.

Given these data, 30-min reactions were conducted using increasing concentrations of pol IV (Fig. 2C). Again, very poor strand displacement synthesis was observed on ICL1 and ICL3, whereas replication bypass was readily measured on ICL4 and to a lesser extent on ICL2. These data suggest that pol IV can catalyze TLS past N²-guanine ICLs; however, 5′-resection leading up to the lesion and 3′-exonucleolytic processing increase the TLS efficiency.

To determine the identity of nucleotide(s) inserted by pol IV opposite the cross-linked guanine, qualitative single nucleotide incorporation assays were conducted using a −1 primer annealed to ICL4. These data revealed that pol IV faithfully incorporated a dCTP opposite the cross-linked G relative to the control G was reduced by ~50-fold, whereas the efficiency of extension from a C opposite the cross-linked G was reduced by ~2-fold (Table 2).

Replication of ICL-containing Plasmid DNAs in E. coli—To explore a cellular role for E. coli polymerases in the processing
of \(N^2-N^2\)-guanine ICLs, we generated a double-stranded plasmid vector carrying a site-specific \(N^2-N^2\)-guanine cross-link (Fig. 1D) and utilized this modified DNA to transform wild-type and pol II and pol IV deletion \(E. coli\) strains. The efficiency of transformation was evaluated relative to a reference plasmid, unmodified pBR322. The pBR322 plasmid encodes resistance to both ampicillin and tetracycline, whereas pMS2-ICL can be selected only when cells are challenged with ampicillin. This feature allowed us to distinguish between the cells transformed with pBR322 versus the ones transformed with pMS2-ICL. The bacterial cells were electroporated with a mixture of pBR322 and pMS2-ICL and grown on LB agar plates containing ampicillin. For further screening, 192 transformed colonies were selected per strain, and a ratio of transformants carrying pBR322 versus those transformed with pMS2-ICL was determined by growing them first in LB broth with ampicillin, followed by transferring an aliquot of culture to LB broth containing tetracycline.

For the wild-type and pol II-deficient strains, of 192 transformants tested, 76 and 75 transformants, respectively, were found to be tetracycline-negative and thus positive for carrying plasmids that originated from pMS2-ICL. Plasmids were isolated from a subset of pMS2-ICL transformants (10 for the pol II-deficient strain and 52 for the wild-type strain), and the region of modification was analyzed by DNA sequencing. In all these plasmids, the insert sequences were present. Therefore, the relative transformation efficiency measured for the wild-type and pol II-deficient strains (Fig. 4) indicated that pol II has no effect on the intracellular replication of \(N^2-N^2\)-guanine ICL-containing DNA.

Using the pol IV-deficient strain, the relative efficiency of transformation with pMS2-ICL was, in contrast, extremely low; of 192 transformants, only five were tetracycline-negative. When plasmids isolated from these transformants were subjected to DNA sequencing, three of five contained the insert sequences, whereas two others were homologous to religated pMS2 vectors without inserts. Thus, relative to the wild type, the efficiency of transformation with plasmids containing \(N^2-N^2\)-guanine ICL was reduced by \(~40\)-fold in the pol IV-deficient strain (Fig. 4). These data strongly support our hypothesis that pol IV is essential for cellular processing of \(N^2-N^2\)-guanine ICLs. It is important to emphasize that the yield of non-adducted pBR322-transformed progenies remained comparable for the wild-type and pol II- and pol IV-deficient strains.

Interestingly, for all the strains, sequence analysis of the screened progenies originating from pMS2-ICL did not reveal any deletions or base substitutions at the adducted site or the neighboring bases. Thus, recombination-independent repair of \(N^2-N^2\)-guanine ICLs in \(E. coli\) is essentially non-mutagenic. Given an accurate bypass of these ICLs by pol IV \textit{in vitro} and on the basis of our results from plasmid-based assays, we speculate that pol IV would be primarily responsible for the non-mutagenic TLS past \(N^2-N^2\)-guanine cross-links \textit{in vivo}.

**DISCUSSION**

For over a decade, genetic evidence in \(E. coli\) has demonstrated that repair of ICLs can proceed via an HR-independent, pol II-dependent pathway. A model to account for these data hypothesized that following an NER-mediated unhooking of one of the strands associated with the ICL by the activity of the UvrABC complex, pol II could catalyze TLS past the intact

| DNA substrate | Primer | dNTP | \(k_{\text{cat}}\) \(\mu\text{m}^{-1}\text{min}^{-1}\) | \(k_{\text{m}}\) \(\mu\text{m}\) | \(k_{\text{cat}}/k_{\text{m}}\) | Relative efficiency |
|---------------|--------|------|----------------|---------|----------------|-------------------|
| ND | -1 | dCTP | 0.90 ± 0.08 | 168 ± 30 | 5.4 × 10^{-3} | 1 |
| ICL4 | -1 | dCTP | 0.020 ± 0.001 | 183 ± 18 | 0.11 × 10^{-3} | 0.02 |
| ND | 0 | dGTP | 3.9 ± 0.2 | 85 ± 8 | 46 × 10^{-3} | 1 |
| ICL4 | 0 | dGTP | 0.75 ± 0.02 | 36 ± 3 | 21 × 10^{-3} | 0.46 |
12-mer still attached to the complementary DNA strand. In this investigation, we sought to obtain biochemical data to support a role for pol IV in catalyzing this reaction. However, our data revealed that at least in the case of N²-N²-guanine ICLs, pol IV (but not pol II) could catalyze TLS on ICL-containing substrate that had been designed to model the product of exonucleolytic processing of the eight nucleotides 5’ to the ICL site. Additionally, the efficiency of TLS could be enhanced by removal of nucleotides 3’ to the ICL, implying that an exonuclease activity on both ends of the incised strand may be required to stimulate the bypass activity of pol IV at an ICL site.

The biochemical observations suggesting a role for pol IV in TLS past N²-N²-guanine ICLs were further substantiated by plasmid reactivation assays, indicating that the pol IV-deficient strain is significantly impaired in its ability to replicate DNA. This linkage is within the minor groove of DNA, and this difference may strongly dictate which polymerase might be capable of catalyzing TLS past specific ICLs. Thus, these data suggest that the repair of cross-linked DNAs via a mechanism not requiring HR may rely on different polymerases or combinations of polymerases to carry out TLS and that the conformational flexibility of both the unhooked or incised strand and the active site of the polymerase will dictate the efficiency and fidelity of the bypass reaction.

In summary, we propose a model in which error-free TLS-assisted repair of ICL-containing DNA (with an N²-N²-guanine linkage) is mediated by pol IV. Our model is based on the following observations: 1) an efficient and accurate bypass of N²-N²-guanine ICLs by pol IV in vitro and 2) an indispensable role of pol IV in replication of N²-N²-guanine ICL-containing plasmids.

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