DNA Polymerase II (*polB*) Is Involved in a New DNA Repair Pathway for DNA Interstrand Cross-Links in *Escherichia coli*

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DNA-DNA interstrand cross-links are the cytotoxic lesions for many chemotherapeutic agents. A plasmid with a single nitrogen mustard (HN2) interstrand cross-link (inter-HN2-pTZSV28) was constructed and transformed into *Escherichia coli*, and its replication efficiency (RE = [number of transformants from inter-HN2-pTZSV28]/[number of transformants from control]) was determined to be ~0.6. Previous work showed that RE was high because the cross-link was repaired by a pathway involving nucleotide excision repair (NER) but not recombination. (In fact, recombination was precluded because the cells do not receive lesion-free homologous DNA.) Herein, DNA polymerase II is shown to be in this new pathway, since the replication efficiency (RE) is higher in a *polB*+ (~0.6) than in a ΔpolB (~0.1) strain. Complementation with a *polB*+-containing plasmid restores RE to wild-type levels, which corroborates this conclusion. In separate experiments, *E. coli* was treated with HN2, and the relative sensitivity to killing was found to be as follows: wild type < *polB* < *recA* < *polB recA* ~ *uvrA*. Because cells deficient in either recombination (*recA*) or DNA polymerase II (*polB*) are hypersensitive to nitrogen mustard killing, *E. coli* appears to have two pathways for cross-link repair: an NER/recombination pathway (which is possible when the cross-links are formed in cells where recombination can occur because there are multiple copies of the genome) and an NER/DNA polymerase II pathway. Furthermore, these results show that some cross-links are uniquely repaired by each pathway. This represents one of the first clearly defined pathway in which DNA polymerase II plays a role in *E. coli*. It remains to be determined why this new pathway prefers DNA polymerase II and why there are two pathways to repair cross-links.

Interstrand cross-links are likely to be the crucial cytotoxic lesion formed by a variety of classes of effective antitumor agents, including the mustards, of which nitrogen mustard was the first member used and cyclophosphamide is currently the most commonly used (13, 22, 26, 31, 40). Factors that modulate the levels of cytotoxic interstrand cross-links, such as DNA repair, are expected to influence the effectiveness of such agents. It has been known for many years that interstrand cross-links may be repaired by a combination of nucleotide excision repair (NER) and recombination, which has been most extensively studied with psoralens (45, 46, 49). In special cases, other DNA repair pathways may also be important for the repair of intermediates that give rise to interstrand cross-links, for example, with BCNU [1,3-*bis*-(2-chloroethyl)-1-nitrosourea] (8, 16, 17, 42, 50).

Although some progress on assessing a role for interstrand cross-links in causing cytotoxicity has been made, for example, with HN2 (1, 36, 37), much is still not known, in part because, when cross-linking agents react with DNA, many lesions form, including interstrand, intrastrand, and protein-DNA cross-links, as well monoadducts. This and other factors have made it difficult to dissect definitively the details about how these various lesions are processed and their unique biological effects (30, 43).

To overcome such complications, we developed a general procedure to construct a plasmid with a single DNA-DNA interstrand cross-link at a defined genomic position (21, 38). By these methods, inter-HN2-pTZSV28, a plasmid which contains a single nitrogen mustard interstrand cross-link, was constructed and characterized as described previously (4, 21, 38). Importantly, we showed that more than ~98% of the plasmids constructed contain a single, intact cross-link. We have shown that the nitrogen mustard moiety is attached to N7-dG in both strands of a 5′-GNC-3′ target sequence in a unique *Acc*I/*Sal*I site (38). The problem of the inherent chemical lability of N7-dG adducts was circumvented by converting them to their corresponding ring-opened, N7-FAPY adducts, which are stable (38).

Cells face a difficult logistical problem in the repair of interstrand cross-links, since both DNA strands are damaged. The commonly accepted pathway to repair interstrand cross-links involves NER to nick the first strand, which is then replaced with DNA from a lesion-free, homologous, sister chromosome by recombination, as demonstrated most clearly for psoralens (45, 46, 49). The second strand is then presumably repaired as a monoadduct by NER.

Previously, we showed that a high yield of progeny plasmids could be derived from inter-HN2-pTZSV28 transformed into *Escherichia coli*, and this process was dependent on a DNA repair pathway for the interstrand cross-link in inter-HN2-pTZSV28 that included NER (4). However, three lines of evidence argued against a role for recombination in this repair pathway, notably that the yield of progeny plasmids was unaffected in a Δ*recA* strain of *E. coli*. In fact, our experimental approach actually precluded a recombination-dependent repair pathway, since the cells did not receive a lesion-free copy of the pTZSV28 plasmid. We also showed that this recombination-independent pathway did not involve base excision repair (BER) or several 5′→3′ exonuclease activities in *E. coli* (4). (In previous work we provided arguments for why we

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TABLE 1. Strains and plasmids

Strain or plasmid	Relevant genotype	Source and/or reference
Strains		
FC40	Wild type	10
C600	Wild type	CSHL[a] (2)
AB1157	Wild type	S. Boiteux (5)
PFB60	FC40 but with $\Delta polB1$	18
SC301	C600 but with $\Delta polB1$	M. Goodman (18)
PFB50	FC40 but with $uvrA6$	This study
BH200	AB1157 but with $uvrA$::Tn10	S. Boiteux (15)
PFB175	FC40 but with $uvrA6$ $\Delta polB1$	This study
FC348	FC40 but with $\Delta recA$	This study
PFB98	FC40 but with $\Delta recA$ $\Delta polB1$	This study
Plasmids		
pBIP3	None	46
pHC206	pBIP carrying $polB^+$	M. Goodman (9)

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concluded that the effects that we have been investigating are by necessity attributable to the nitrogen mustard interstrand cross-link located in inter-HN2-pTZSV28 [4].)

We show here that DNA polymerase II (DNA Pol II) is another component of the recombination-independent, DNA-DNA interstrand cross-link repair pathway. To test the physiological relevance of this new pathway, we describe the treatment of *E. coli* with nitrogen mustard and conclude that it is likely the NER/DNA Pol II pathway functions in parallel with the NER/recombination pathway in *E. coli* (i.e., in circumstances where there is a lesion-free homologous chromosome) and that these two pathways are not completely functionally redundant.

MATERIALS AND METHODS

Bacterial strains and plasmids are summarized in Table 1, including several new strains. The $uvrA6$ derivatives were constructed by transducing strains to mal::Tn10 and then to mal^+ $uvrA6$ by using AB1886 (24) as a donor. $\Delta recA$

derivatives were made by transducing the strains to $\Delta(recA\ srlR301$::Tn$10)$ (14). Standard genetic techniques were used (32).

All materials and methods were identical to those described previously (4, 21, 38) except as noted below.

In brief, inter-HN2-pTZSV28, which contains a single nitrogen mustard interstrand cross-link, was constructed as follows. A nitrogen mustard interstrand cross-linked duplex oligonucleotide was synthesized (38) and incorporated into the parent plasmid pTZSV28 in a five-step procedure, and closed circular material was isolated by cesium chloride density gradient centrifugation (21). pTZSV28 itself was constructed from pTZ19R (ColE1 and f1 origins, *bla* for ampicillin resistance and a *lacZ'* gene with a polylinker) and a portion of simian virus 40 (simian virus 40 origin and the large T antigen gene). The cross-link is located in a unique *AccI/SalI* site in the polylinker. Inter-HN2-pTZSV28 was extensively characterized, notably to show that >98% of the material contains a single, intact cross-link (4). Inter-HN2-pTZSV28 or its non-cross-link-containing control (C-pTZSV28) was transformed (via electroporation) in parallel in triplicate into each of the indicated strains (Table 2) and plated in duplicate on ampicillin-containing plates. One day later the numbers of ampicillin-resistant colonies were determined, and the average of the six plates is reported (Table 2).

The survival curves after exposure to nitrogen mustard (see Fig. 2) were generated as follows. To 1.0 ml of the indicated *E. coli* strains (from an overnight culture grown in Luria broth [LB]), nitrogen mustard (stored as the hydrochloride at −80°C in a 1 M stock solution) was added (along with an identical volume of 1 M NaOH) to give the desired final concentrations. After incubation for 1 h at 37°C, the samples were diluted (by a factor of 10^3 or greater) and plated (in triplicate) on LB plates. After incubation at 37°C for ∼18 h, the colonies were counted to determine the levels of surviving colonies.

RESULTS

As we had done previously (4), a bacterial transformation assay was used to study the extent to which a single nitrogen mustard interstrand cross-link might inhibit DNA replication. Equal amounts (∼25 pg) of either inter-HN2-pTZSV28, which contains a single cross-link, or C-pTZSV28, which is an identically constructed control plasmid that contains no cross-link, were transformed into cells, and the number of ampicillin-resistant colonies was determined (4). The replication efficiency (RE), i.e., the ratio of colonies from (inter-HN2-pTZSV28/C-pTZSV28), was determined to be ca. 0.3 to 0.6 for cells proficient in all known DNA repair pathways (reference 4 and Table 2). The RE was shown to be significantly lower in several *uvrA* and *uvrB* strains compared to corresponding *uvr*$^+$ strains (reference 4 and Table 2), implicating NER in the repair of the cross-link in HN2-inter-pTZSV28. To simplify

TABLE 2. Comparison of the RE values of inter-HN2-pTZSV28 and C-pTZSV28 in different *E. coli* strains to determine a role for DNA Pol II in interstrand cross-link repair[a]

Strain[b]	Genotype[c]	Plasmid[d]	RE in expt no.: 1	2	3	4	5	6	RRE (avg)[e]
FC40	Wild type		0.64	0.51	0.57	0.60	0.59	0.67	1.00
C600	Wild type		0.56	0.58					1.00
AB1157	Wild type				0.71				1.00
PFB60	$\Delta polB$		0.094	0.126	0.11	0.10	0.087	0.13	0.18
SC301	$\Delta polB$		0.100	0.096					0.17
PFB50	$uvrA6$					0.043	0.022		0.055
BH200	$uvrA$::Tn10			0.024					0.034
PFB175	$\Delta polB$ $uvrA6$						0.017		0.028
FC40	Wild type	pBIP				0.56		0.58	1.00
PFB60	$\Delta polB$	pBIP				0.12		0.17	0.26
FC40	Wild type	pHC206 ($polB^+$)				0.66		0.65	1.00
PFB60	$\Delta polB$	pHC206 ($polB^+$)				0.57		0.71	0.98

[a] Inter-HN2-pTZSV28 contains a single nitrogen mustard interstrand cross-link. Inter-HN2-pTZSV28 or its non-cross-link-containing control (C-pTZSV28) was transformed (electroporation) in parallel in triplicate into each of the indicated strains and then plated in duplicate on ampicillin-containing plates. One day later the numbers of ampicillin-resistant colonies were determined, and the average of the six plates for each point was calculated. The RE is defined as the ratio of ampicillin-resistant colonies: [inter-HN2-pTZSV28]/[C-pTZSV28].
[b] Strain name (see Table 1).
[c] Relevant genotype (see Table 1). Mutant strains are paired as follows: FC40 and PFB60, PFB50, or PFB175; C600 and SC301; and AB1157 and BH200.
[d] Plasmid name (see Table 1).
[e] RRE is defined as follows: (RE in a repair-deficient strain)/(RE in a repair-proficient strain) (see text). Values for the RRE are based on the average for all of the relevant experiments.
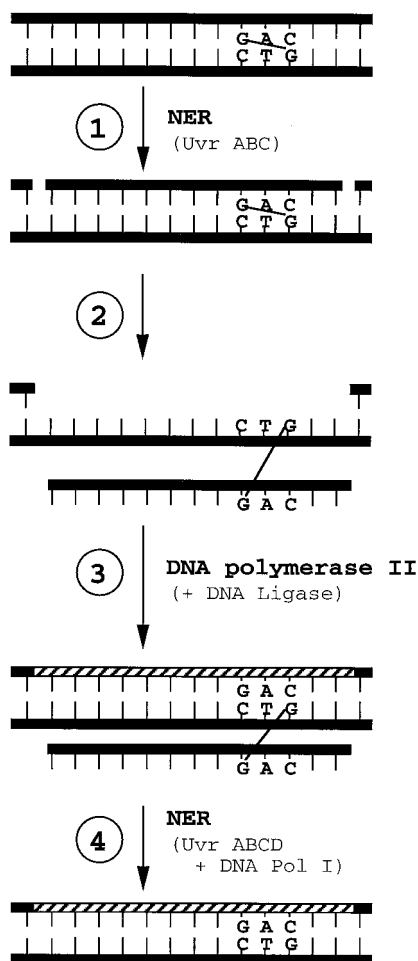
FIG. 1. The NER/DNA Pol II pathway for the repair of a nitrogen mustard interstrand cross-link. Repair of the top strand is proposed to involve NER (step 1), followed by DNA Pol II filling of the gap, including bypass of the lesion and ligation (steps 2 and 3) and finally NER of the resulting monoadduct in the bottom strand (step 4).
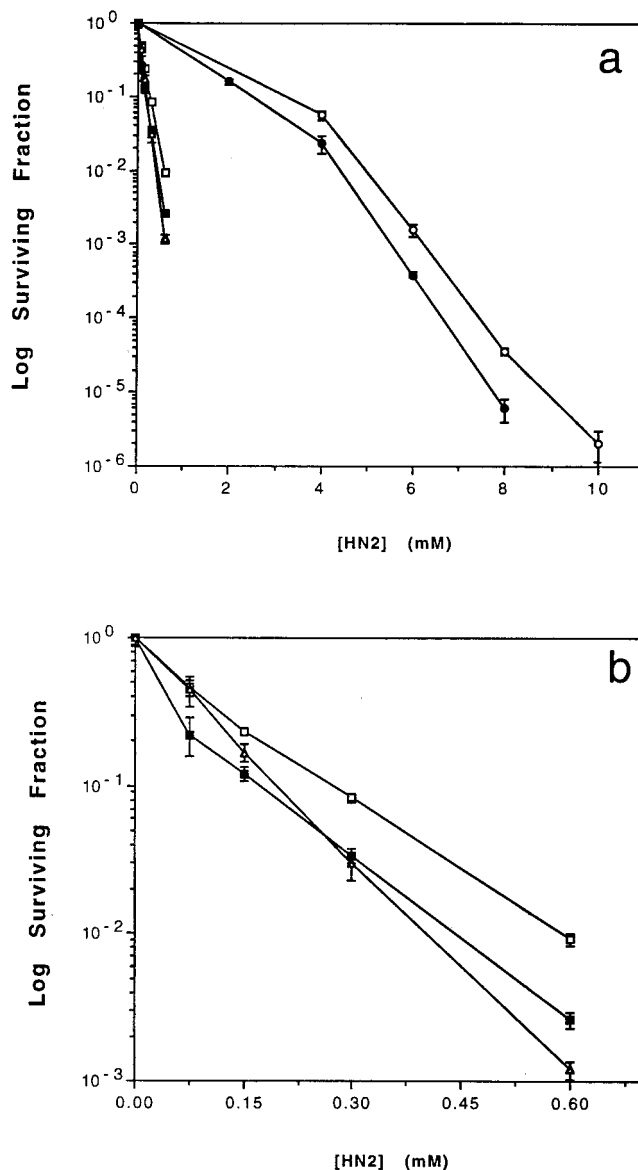

FIG. 2. Survival curves for various strains of *E. coli* treated with nitrogen mustard (see Materials and Methods). Strains: FC40 (wild type [○]), PFB60 (Δ*polB* [●]), FC348 (Δ*recA* [□]), B98 (Δ*polB* Δ*recA* [■]), and PFB50 (*uvrA6* [△]). Panel a shows all of the data, whereas panel b shows the data for the latter three strains with an expanded abscissa. Strain FC348 is strain FC40 with a Δ*recA* allele. Strain B98 is strain FC40 with both Δ*polB* and Δ*recA* alleles. Strain PFB50 is strain FC40 with a *uvrA6* allele. Strains are described in Table 1.

comparisons, it is useful to define the relative replication efficiency (RRE) as the ratio of RE for any repair-deficient strain of *E. coli* versus the RE for its corresponding wild-type strain; e.g., the RRE is 0.055 for *uvrA6* versus *uvrA*⁺ (Table 2), which is similar to results we obtained previously (4). In previous work, analogous experiments excluded a role for recombination, BER, and several 5′→3′ exonuclease activities in this DNA repair pathway (4).

This recombination-independent repair was hypothesized to proceed by UvrABC nicking one strand near the cross-link followed by the action of a DNA polymerase (Fig. 1). A role for DNA Pol II was evaluated, and evidence for its involvement was obtained in that the RRE was ~0.18 (Table 2) in two different strains of *E. coli* that contained a deletion of a *polB* gene, which was originally developed in the laboratory of Myron Goodman (18). The repair defect in the Δ*polB* strain was complemented by the cloned *polB*⁺ gene (Table 2, compare PFB/pBIP [RRE = 0.26] with PFB/pHC206 [RRE = 0.98]), showing that the deficiency was truly due to the *polB* allele and not to some other gratuitous genetic change in the cells. No complementation was observed with the plasmid not containing the *polB*⁺ gene. In experiment 5 (Table 2), the RE was similar for a *uvrA6* (0.022) versus a *uvrA* Δ*polB* (0.017) strain.

To assess whether the NER/DNA Pol II pathway is physiologically significant, the survival of *E. coli* cells exposed to nitrogen mustard was determined (Fig. 2). Cells deficient in DNA Pol II (Δ*polB*) appear to be slightly more sensitive to killing by nitrogen mustard than were wild-type cells. In addition, a Δ*polB* Δ*recA* strain was more sensitive than a Δ*recA* strain.

DISCUSSION

NER and DNA Pol II can be involved in nitrogen mustard interstrand cross-link repair in *E. coli* based on the RRE values (Table 2) in *uvr* (0.055) and *polB* (0.18) strains, respectively,

compared to wild-type uvr^+ $polB^+$ (1.0) strains. The hypersensitivity of $polB$ E. coli to killing by nitrogen mustard (Fig. 2) suggests that this pathway is physiologically relevant. The fact that the RRE is similar for both uvr and uvr $polB$ strains (Table 2) suggests that NER and DNA Pol II are in the same pathway.

We found evidence for this NER/DNA Pol II pathway only because the commonly accepted pathway for the repair of interstrand cross-links, which involves NER and recombination (45, 46, 49), was precluded since the cells receiving inter-HN2-pTZSV28 in our experiments do not receive a lesion-free piece of DNA that is homologous to the region around the cross-link, thus making recombination impossible. These two pathways for the repair of interstrand cross-links are likely to work in parallel and are not functionally redundant, since cells deficient in either the NER/recombination pathway alone ($\Delta recA$) or the NER/DNA Pol II pathway alone ($\Delta polB$) were each hypersensitive to the killing effects of nitrogen mustard (Fig. 2). (It seems most likely that these results are attributable to killing by cross-links rather than to killing by monoadducts, although our results do not definitively distinguish between them.) The NER/Pol II pathway for coping with interstrand cross-links probably serves a role analogous to the role of SOS-dependent, translesion synthesis of bulky monoadducts (20). These pathways are likely to be necessary when lesion tolerance mechanisms (notably, those involving recombinational repair) are impossible, such as when there are closely spaced lesions in both sister chromosomes or when a cell does not have a homologous, sister chromosome (e.g., in late-replicating DNA).

The $\Delta recA$ strain is more sensitive to the killing effects of nitrogen mustard than the $\Delta polB$ strain, suggesting that the NER/recombination pathway probably repairs a greater fraction of cross-links than does the NER/DNA Pol II pathway. While it is difficult to make a rigorous quantitative comparison, we estimate that the NER/DNA Pol II pathway appears to be responsible for repairing ~12% of the cross-links, based upon the differences in the dose of nitrogen mustard required to kill a particular fraction of wild-type versus $polB$ versus $recA$ cells (Fig. 2). {The 12% value was calculated as follows. The number of cross-links formed in the cells is assumed to be approximately proportional to the dose of nitrogen mustard. It is also assumed that the same number of cross-links are responsible for reducing cell survival to the 1% level in wild-type, $polB$, and $recA$ cells, which occurs at nitrogen mustard concentrations of 5.0, 4.4, and 0.6 mM, respectively. The relative fraction of lesions removed by the $polB$-dependent pathway is estimated as follows: (wild type − $polB$)/([wild type − $recA$] + [wild type − $polB$]) = 0.12.} The fact that the survival curve in the $\Delta polB$ $\Delta recA$ strain is virtually superimposable on the curve for the $uvrA$ strain (Fig. 2) suggests that NER is common to both pathways and that these are the only two NER-dependent pathways that repair interstrand cross-links.

The NER/DNA Pol II pathways appears to be active on other cross-links, such as that with mitomycin C (unpublished observation), which may provide an explanation for an old observation that mutagenesis by certain cross-linking agents (e.g., mitomycin C, as well as malondialdehyde) actually decreases in NER-deficient strains (27, 33, 34), implying that the mutations occur during DNA repair of a lesion, probably a cross-link, perhaps via the NER/DNA Pol II pathway. Interestingly, the NER/DNA Pol II pathway does not appear to be able to act on all cross-links, notably those from psoralens (as discussed in reference 4).

The involvement of DNA Pol II in this pathway is of interest for several reasons. Although the $polB$ gene for DNA Pol II was first identified in 1972 (11, 23), the pathway depicted in

Fig. 1 represents one of the few cases where there is good evidence of a concrete role for DNA Pol II in E. coli. It has been known for some time that DNA Pol II is damage inducible as part of the SOS response, implying that it plays some role in damage management and DNA repair (6, 7, 25). However, reports have indicated that DNA Pol II appears not to participate in UV resistance (25), UV mutagenesis (25), cyclobutane dimer mutagenesis (28), repair gap UV mutagenesis in cells (12) (although it can function in vitro [48]), Weigle reactivation (28), thymine glycol mutagenesis (28), mismatch repair (28), and UVM mutagenesis (39). There is indirect evidence for DNA Pol II involvement in abasic site mutagenesis (47), although direct evidence suggests otherwise (28). Studies have shown that DNA Pol II can substitute for DNA Pol III in some aspects of E. coli replication (41), that it is important for adaptive mutagenesis (18, 19), and that it is involved in protecting cells from H_2O_2 toxicity (18), although its exact role in each of these processes is unclear. In summary, DNA Pol II seems not to play a role in a variety of replication and repair processes, and where there is some evidence for its involvement, the components and the details of the pathway have not been delineated.

The results presented in Table 2 clearly indicate that DNA Pol II can be involved in the pathway depicted in Fig. 1. However, the RRE in $polB$ cells (~0.18) is higher than in uvr cells (~0.055). This implies that some polymerase in addition to DNA Pol II either is responsible for a minor fraction of the repair or can substitute for DNA Pol II, albeit less efficiently (i.e., <20%). Nevertheless, it does appear that DNA Pol II is preferred, raising the question: why does this pathway not use either of E. coli's other two DNA polymerases (i.e., Pol I or Pol III), which participate in other DNA repair pathways (20). Perhaps DNA Pol I is too error prone in the bypass of lesions (as discussed in references 29 and 30), whereas DNA Pol III, which is able to bypass lesions reasonably accurately, at least in some cases (e.g., see references 3 and 35), is too large a complex to bypass the extremely bulky lesion depicted in step 3 in Fig. 1.

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