Evidence for a Recombination-Independent Pathway for the Repair of DNA Interstrand Cross-Links Based on a Site-Specific Study with Nitrogen Mustard†

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ABSTRACT: DNA−DNA interstrand cross-links are thought to be important for the cytotoxicity of many chemotherapeutic agents. To study this more definitively, adduct site-specific methods are used to construct a plasmid with a single nitrogen mustard interstrand cross-link (inter-HN2-pTZSV28). Replication efficiency (RE = [colonies from (inter-HN2-pTZSV28)/(control with no cross-link)]) is ∼0.3 following transformation into *Escherichia coli*, implying that the cross-link is repaired. The commonly accepted pathway for cross-link repair, which involves both nucleotide excision repair (NER) and recombination, is ruled out since RE is ∼0.3 in a Δ*recA* strain. Non-RecA-directed recombination such as copy-choice is also unlikely. However, NER is involved since RE was ∼0.02 in strains deficient in NER. Base excision repair is not important since RE is ∼0.3 in strains deficient in 3-methyladenine DNA glycosylases I and II, FAPY DNA glycosylase, both known apurinic/apyrimidinic endonucleases, or DNA deoxyribophosphodiesterase. Another hypothetical repair pathway hinging on a 5′ → 3′ exonuclease activity is unlikely since RE is ∼0.3 in cells deficient in either the 5′ → 3′ exonuclease activities of DNA polymerase I, exonuclease VII, or RecJ. Thus, aside from NER, it is unclear what else participates in this recombination-independent repair pathway, although a pathway involing NER followed by replicative bypass of the lesion is the current working hypothesis. Psoralen interstrand cross-links appear *not* to be repairable by this second pathway, which may have implications for the relative cytotoxicity of interstrand cross-links from different agents.

A variety of anticancer, chemotherapeutic agents are bifunctionally reactive and, therefore, can cross-link biological macromolecules, notably DNA. The ability of these DNA cross-links to interfere with DNA replication and, ultimately, to cause cytotoxicity appears to be essential to this anticancer activity (Colvin, 1982; Ludlum, 1986; Kohn & Gibson, 1986; Hemminki & Ludlum, 1984; Pratt et al., 1994). DNA−DNA interstrand and intrastrand as well as DNA−protein cross-links each form, raising the questions of which cross-link is important for cytotoxicity and chemotherapeutic efficacy, and why? Where it has been studied, the formation of DNA−protein cross-links does not appear to correlate with cytotoxicity (Erickson et al., 1980). Interstrand cross-links have been implicated in the case of nitrosoureas (Erickson et al., 1980; Zlotogorski & Erickson, 1984; Dolan et al., 1986, Samson et al., 1986; Brennard et al., 1986) and are likely to be relevant for the psoralens [Liu et al., 1984; Piette et al. (1988) and references therein]. In contrast, intrastrand cross-links have been implicated for *cis*-diamminedichloroplatinum(II) (*cis*-DDP)[1] and its derivatives (Roberts & Thompson, 1979; Zamble & Lippard, 1995). Cytotoxicity from a variety of mustards correlates with the formation of interstrand cross-links in many (O'Conner & Kohn, 1990; Aida & Bodell, 1987), but not all (O'Conner et al., 1991), cases. Older studies on sulfur mustard seemed to imply a dominant role for intrastrand cross-links in a simple model system (Lawley et al., 1969).

The discussion in the previous paragraph raises two interrelated issues. (1) It is not obvious why different kinds of cross-links should be responsible for cytotoxity in the case of different agents (e.g., BCNU vs *cis*-DDP). (2) When a particular cross-linking agent is reacted with DNA, invariably all of these cross-links, as well as other adducts, are formed more or less randomly around the genome, which makes it difficult to determine what adduct is responsible for what biological end point and why. Both of these issues can be addressed if the biological consequences of individual DNA adducts can be studied, e.g., by using adduct site-specific techniques, which permit the construction of vectors that contain adducts of defined chemical structure at known genome locations (Singer & Essigmann, 1991; Loechler, 1996). A variety of approaches have been used to study monoadducts and DNA−DNA intrastrand cross-links, which both have modifications in a single strand of DNA. In addition, we developed a general strategy to do adduct site-specific work with interstrand cross-links (Ojwang et al., 1989; Grueneberg et al., 1991).

We chose to begin our studies with nitrogen mustard, because it and its derivatives (e.g., cyclophosphamide and melphalan) are used as widely as any anticancer drugs, although less is known about their precise mechanism of

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[1] Abbreviations: HN2, nitrogen mustard; 1-M1-HN2-X1, a partially duplex oligonucleotide covalently linked by a single nitrogen mustard interstrand cross-link (for details see Materials and Methods); inter-HN2-pTZSV28, a plasmid containing a single nitrogen mustard interstrand cross-link; C-pTZSV28, a plasmid constructed identically to inter-HN2-pTZSV28 but lacking the cross-link; *cis*-DDP, *cis*-diamminedichloroplatinum(II); BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; Kf, Klenow fragment of DNA polymerase I; ss, single stranded; ds, double stranded; SD, standard deviation; RE, replication efficiency (see text and footnote *b* in Table 1); RRE, relative replication efficiency (see text and footnote *c* in Table 1); PAGE, polyacrylamide gel electrophoresis; PAG, polyacrylamide gel; AP, apurinic/apyrimidinic.

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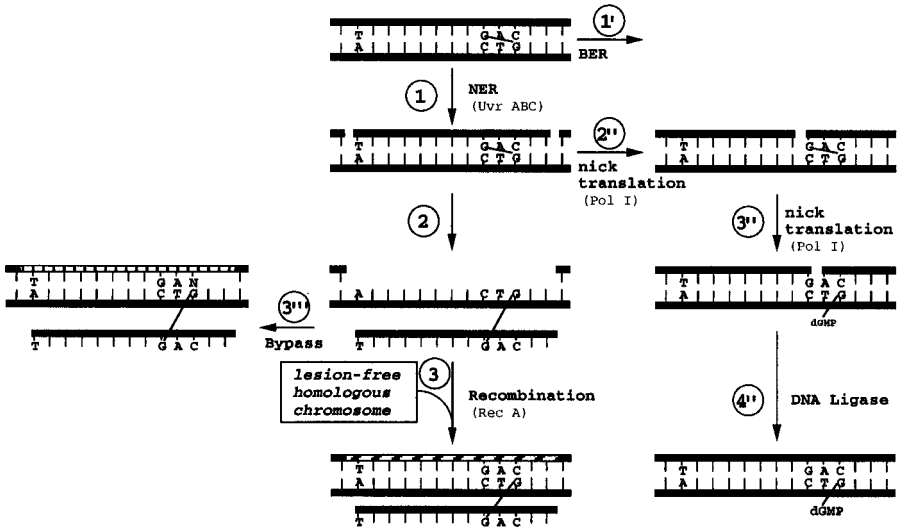

FIGURE 1: Four potential pathways to repair the first strand of a nitrogen mustard DNA−DNA interstrand cross-link in inter-HN2-pTZSV28. (1) The commonly accepted pathway for the repair of interstrand cross-links requires NER (step 1), followed by RecA-mediated recombination (steps 2 and 3), which involves replacement of the nicked fragment with a lesion-free, homologous region from another chromosome (slashed line). [Steps 1−3 are oversimplified (Sladek et al., 1989b).] (2) The base excision repair (BER) pathway is not elaborated (step 1′). (3) A nick translation mechanism (steps 1, 2″, and 4″) is considered (see text). (4) Polymerase bypass of the lesion is also possible (steps 1, 2, and 3‴). In each case following repair of the first strand, the second strand can be repaired by NER, since a monoadduct remains.

action at the level of their DNA adducts than some other less clinically relevant agents. We (Ojwang et al., 1989) and others (Millard et al., 1990; Rink et al., 1993) showed that nitrogen mustard preferentially forms interstrand cross-links in 5′-GNC-3′ sequences, where the cross-link is between the N7 position of the guanines in the opposing strands. It was of interest to learn what the biological consequence of this interstrand cross-link is in cells, and we have begun this by investigating their ability to block DNA replication as influenced by DNA repair. Interstrand cross-links pose a particularly difficult logistical problem: by what strategy can a cell break the cross-link in the repair of the first strand of the DNA−DNA cross-link and, yet, retain the sequence integrity of this DNA strand? The commonly accepted pathway to accomplish this begins with the nucleotide excision repair (NER) machinery making a nick (or nicks) near the interstrand cross-link in one strand (Friedberg et al., 1995; Van Houten et al., 1986; Sladek et al., 1989a,b). This nicked strand is then replaced with DNA from a lesion-free homologous chromosome by recombination. This is depicted as steps 1−3 in Figure 1, although this is an oversimplification of the actual process (Sladek et al., 1989b). Repair of the second strand is completed by NER. In the experiments reported herein, such a recombination-dependent mechanism of repair is not possible, because no lesion-free homologous DNA is present.

Results described herein show that the presence of a single nitrogen mustard interstrand cross-link in a plasmid does not interfere dramatically with replication, at least based upon the criteria that—following transformation into *Escherichia coli*—the yield of progeny plasmids from a plasmid containing a single nitrogen mustard interstrand cross-link is ∼0.3 when compared to a control plasmid containing no cross-link. This implies that repair is occurring, since the presence of the cross-link is expected to completely block replication. We show that NER is involved, but not recombination, not base excision repair (BER), nor several other hypothetical pathways that hinge on 5′ → 3′ exonuclease activity. These results suggest that in circumstances when recombination-dependent repair is not possible, a second, recombination-independent pathway is available to some, although perhaps not all, DNA−DNA interstrand cross-links.

EXPERIMENTAL PROCEDURES

Plasmid vectors were described previously (Grueneberg et al., 1991). Strains are listed in Table 1 (Boiteux & Huisman, 1989; Palejwala et al., 1991; Joyce & Grindley, 1984; Czeczot et al., 1991; Howard-Flanders et al., 1966; Matijasevic et al., 1993; Cunningham et al., 1986; Lovett & Clark, 1984; Razaby et al., 1996; Joyce et al., 1985). Stains WM101 (AB1157 + *uvrA::Tn10 alkA1 tag-1*) and WM103 (AB1157 + *uvrB::Tn10 alkA1 tag-1*) were constructed by P1 transduction of the *uvrA::Tn10* allele in BH200 and the *uvrB5* allele in AB1885, which is closely linked to a Tn10 insertion, respectively, into strain MV1932 and selection on tetracycline. The phenotypes of WM101 and WM103 were confirmed in that both strains were hypersensitive to killing by both UV light (thus, *uvr*$^-$) and MMS (thus, *alkA1*$^-$/*tag-1*$^-$). The handling of nitrogen mustard was as described previously (Ojwang et al., 1989). All reagents whose sources are not given explicitly have been described previously (Ojwang et al., 1989; Grueneberg et al., 1991).

The construction of inter-HN2-pTZSV28 from 1-M1-HN2-X1 and pTZSV28 is described in Ojwang et al. (1989) and Grueneberg et al. (1991) and is briefly outlined below. 1-M1-HN2-X1 was prepared by reacting nitrogen mustard with a mixture of 5′-CGCGT***GTCGAC***T-3′ and 5′-CTA-GA***GTCGAC***A-3′. 1-M1-HN2-X1 has both an *Mlu*I and an *Xba*I overhang (underlined) and a single nitrogen mustard interstrand cross-link bound at N7-Gua in the opposing strands of a 5′-GNC-3′ sequence. The target sequence is in a unique *Acc*I/*Sal*I site (italics). Although there are two 5′-GNC-3′ sequences in this pair of oligonucleotides, only one is adducted: the ratio of cross-link in the 5′-TGTCG-3′ vs the 5′-AGTCG-3′ sequence was estimated to be ∼80:20 (Ojwang et al., 1989). (In spite of several attempts, we have been unable to purify these two species from each other or

Table 1: Comparisons of the Replication Efficiency (RE) of Inter-HN2-pTZSV28 vs C-pTZSV28 in *E. coli* Strains both Wild-Type and Deficient in Various Known DNA Repair Pathways

		RE[b]				RRE[c]	
strain[a]	genotype	expt 1	expt 2	expt 3	expt 4	expts 1−4[d]	other[e]
wild-type strains							
AB1157[f]	wt	0.26		0.39	0.38	1.0	1.0 (6)
KH2[g]	wt		0.26			1.0	1.0 (2)
CM4722[h]	wt		0.35			1.0	1.0 (1)
mutant strains with an effect							
BH200[i]	*uvrA::TN10*	0.013				0.049	0.053 (5)
AB1885[j]	*uvrB5*	0.018				0.068	0.091 (1)
mutant strains with no effect							
KH2R[k]	Δ*recA*		0.25			0.98	1.0 (2)
BH20[l]	*fpg-1::kan*		0.32		0.40	1.05[m]	1.22 (1)
MV1932[n]	*alkA1/tag-1*	0.21				0.78	0.94 (2)
BW528[o]	*xth/nfo::kan*		0.22			0.68[m]	0.85 (2)
JC13031[p]	*recJ*			0.34		0.87	0.77 (2)
SZ784[q]	*xseA*			0.42		1.09	0.82 (2)
CM5411[r]	*polA107*		0.24			0.71	1.0 (1)
other strains							
BH190[s]	*fpg/uvrA*		0.016		0.018	0.049[m]	0.12 (1)
WM101[t]	*alkA/tag/uvrA*	0.007				0.027	0.067 (1)
WM103[t]	*alkA/tag/uvrB*	0.008				0.030	0.050 (1)

[a] Strain name. The first three rows are wild-type strains, while the remaining strains are deficient in some known DNA repair component. A deficient strain has the same genotype as its corresponding wild-type strain as follows: AB1157 is wild-type to all mutant strains, except KH2R (wt: KH2, footnote *g*) and CM5411 (wt: CM4722, footnote *h*). [b] RE (replication efficiency) is defined as the ratio of ampicillin-resistant colonies from [inter-HN2-pTZSV28]/[C-pTZSV28], where the former contains a single nitrogen mustard interstrand cross-link and the latter is identical but without the cross-link. [c] RRE (relative replication efficiency) is defined as [RE in a repair-deficient strain]/[RE in a repair-proficient strain] (see text). [d] RRE determined from experiments 1−4. [e] RRE from other experiments where RRE, but not RE, could be determined (see text). The average value is given with the number of experiments in parentheses. [f] AB1157 [*xyl-5, mtl-1, galK2,* Δ*rac, rpsl31, kdgk51,* Δ*(gpt-proA) 62, lacY1, tsx-33, supE44, thi-1, leuB6, hisG4, mgl-51, arg-3, rfbD1, ara-14, thr-1*] from S. Boiteux (Boiteux & Huisman, 1989) as a companion to BH200, BH20, and BH190. [g] KH2 [*SupO,* Δ*lac-pro, trpE9777,* F′*LacI*^{q}*Z*Δ*M15Pro*$^+$] from Z. Humayun (Palejwala et al., 1991). [h] CM4722 [F$^+$ Δ*(gal-bio), thi-1, relA1, spoT1*] from C. Joyce (Joyce & Grindley, 1984). [i] Czecot et al., 1991. [j] Howard-Flanders et al., 1966. [k] Palejwala et al., 1991. [l] Boiteux & Huisman, 1989. [m] In experiment 2, the data for AB1157, which is the parent of BH20, BW528, and BH190, did not emerge. Values of RRE for BH20 and BH190 cells were obtained from RE for BH20 and BH190 cells in experiments 2 and 4 in comparison to RE for AB1157 in experiments 1, 3, and 4. Values of RRE for BW528 cells were obtained from RE for BW528 cells in experiment 2 compared to RE for AB1157 in experiments 1, 3, and 4. [n] Matijasevic et al., 1993. [o] Cunningham et al., 1986. [p] Lovett & Clark, 1984. [q] Razaby et al., 1996. [r] Joyce et al., 1985. [s] Czecot et al., 1991. [t] This study (Experimental Procedures).

to develop a workable procedure to incorporate a cross-link into a single 5′-GNC-3′ target sequence in a unique restriction endonuclease recognition site.) 1-M1-HN2-X1 was treated in base such that the N7-guanine adducts would be converted to their corresponding ring-opened, FAPY adducts, which are more stable chemically (Ojwang et al., 1989). 1-M1-HN2-X1 was incorporated into pTZSV28 using a five-step procedure. pTZSV28 was linearized with *Mlu*I (step 1), and 1-M1-HN2-X1, which has an *Mlu*I overhang, was ligated onto both ends (step 2). 1-M1-HN2-X1 was isolated with a phosphate on its *Mlu*I, but not its *Xba*I, overhang, so that only a single 1-M1-HN2-X1 was incorporated per end. One of these oligonucleotides was removed by cleavage with *Xba*I (step 3), which removed ∼30 bp from one end and exposed a unique, internal *Xba*I site in the vector itself. Phosphorylation (step 4) of the *Xba*I site originally associated with the oligonucleotide generated a species that was recircularized with DNA ligase (step 5), giving inter-HN2-pTZSV28. Following treatment with *Acc*I and *Pst*I (see below) to linearize unwanted contaminants, closed circular material from step 5 was purified by cesium chloride density gradient centrifugation. Inter-HN2-pTZSV28 was subjected to a variety of characterizations, the details of which are given in Grueneberg et al. (1991).

Competent bacteria were prepared according to Rodriguez et al. (1992). In brief, 250 mL of LB medium was inoculated with 2.5 mL of a 10-mL overnight culture and grown to $OD_{550nm} = 0.6$. The cell culture was placed into two 250-mL spin bottles (100 mL of culture/bottle) and kept on ice for 10 min prior to centrifugation at 4000*g* in a Sorvall GSA rotor at 4 °C for 10 min. The supernatant was decanted and each cell pellet was resuspended in 200 mL of ice-cold distilled, deionized water and centrifuged at 8000*g* for 10 min at 4 °C. This step was repeated. The liquid was decanted and the last traces of supernatant were removed by aspiration. Following addition of 0.2 mL of 10% aqueous glycerol, each cell pellet was resuspended and transferred to a 1.5-mL microfuge tube and kept on ice. These competent bacteria (50 μL) were mixed with either C-pTZSV28 or inter-HN2-pTZSV28 (∼25pg in 2 μL unless noted otherwise in TE containing 20% sterile glycerol) and transferred to an electroporation cuvette (0.1-cm gap) on ice. Samples were electroporated (1.8 kV/200 Ω/25 μF) using a Bio-Rad *E. coli* gene pulser, immediately resuspended in 1 mL of SOC medium, and incubated for 1 h at 37 °C with shaking. Following the recovery period, 100 μL was plated on LB agar containing 0.1 mg/mL ampicillin and incubated for 14−16 hours at 37 °C.

In parallel, inter-HN2-pTZSV28 and C-pTZSV28 were quantitated as follows. Each construct was digested with the same restriction endonuclease (see below), followed by 3′-end labeling with DNA polymerase I (Klenow fragment), [α-^{32}P]dATP, and other appropriate dNTPs. Following agarose gel electrophoresis and gel drying, each sample was analyzed densitometrically (data not shown) by phosphorimaging (Molecular Dynamics Phosphorimager Model SF with the software ImageQuant version 3.3).

The following method was used to determine whether progeny plasmids from inter-HN2-pTZSV28 had mutations at or near the original site of the cross-link. Individual ampicillin-resistant colonies from transformations of inter-HN2-pTZSV28 into either AB1157 or BH200 (*uvrA*$^-$) cells were grown overnight in 10 mL of LB containing 0.1 mg/mL ampicillin. Plasmids were prepared with the aid of a Pharmacia Easy Prep apparatus and purified according to the manufacturer's protocol. A mutation within several base pairs of the original site of the cross-link would give a progeny plasmid that was *Sal*I^r. (For most procedures, *Acc*I is a better enzyme than *Sal*I; however, the recognition sequence for *Acc*I is 4-fold degenerate, so *Sal*I, which has a unique recognition sequence, is preferred for mutant characterization.) Progeny plasmids were also tested with *Pst*I, because pTZSV28 starting material has a unique *Pst*I site that is removed during construction of inter-HN2-pTZSV28 (Grueneberg et al., 1991).

RESULTS

Construction and Characterization of Inter-HN2-pTZSV28. A plasmid containing a single nitrogen mustard DNA−DNA interstrand cross-link at a specific genomic position was constructed and characterized as described previously by us (Ojwang et al., 1989; Grueneberg et al., 1991; Experimental Procedures). In summary, nitrogen mustard was reacted with a partially duplex oligonucleotide containing the preferred target sequence (5′-GNC) for the formation of a nitrogen mustard DNA−DNA interstrand cross-link. The cross-linked oligonucleotide was purified by denaturing polyacrylamide gel electrophoresis and was shown to contain a cross-link at the expected site (Ojwang et al., 1989). A single, purified cross-linked oligonucleotide was subsequently incorporated into the shuttle plasmid pTZSV28 in a five-step procedure (Grueneberg et al., 1991) to give inter-HN2-pTZSV28, which was purified as closed circular material. A control vector (C-pTZSV28), which lacked the interstrand cross-link, was constructed in parallel.

Inter-HN2-pTZSV28 was characterized. The cross-link was located in a unique *Acc*I/*Sal*I restriction endonuclease recognition site in inter-HN2-pTZSV28, and this blocked cleavage by *Acc*I as expected (Figure 2, lane 4). Twelve base pairs, including a *Pst*I site, must be lost from starting material pTZSV28 in the construction of inter-HN2-pTZSV28 and C-pTZSV28 given the construction strategy (Grueneberg et al., 1991); we confirmed that inter-HN2-pTZSV28 and C-pTZSV28 were resistant to cleavage by *Pst*I as expected (data not shown). The presence of the cross-link was confirmed by two approaches. (1) Inter-HN2-pTZSV28 was cleaved to give linear ds-DNA (Figure 3, lane 3) and then denatured in alkali and renatured, in which case it also migrated as if it were linear ds-DNA (Figure 3, lane 4). In contrast the control vector, C-pTZSV28, migrates as linear ss-DNA following this same denaturation/renaturation protocol (Figure 3, lane 2). This demonstrates that inter-HN2-pTZSV28 renatures more rapidly than C-pTZSV28, which we take as presumptive evidence for the presence of an interstrand cross-link that provided a site of nucleation for the renaturation process. (2) C-pTZSV28 and inter-HN2-pTZSV28, as well as starting material pTZSV28 itself, were treated with the restriction endonucleases *Eco*RI and *Hin*dIII, which cleave all three plasmids 5 times, importantly resulting in the cross-linked region being in the smallest fragment in each case. Following, 3′-[^{32}P]-radiolabeling, the material was separated by *denaturing* PAGE. The smallest fragment from C-pTZSV28 (Figure 4, lane 2) migrated more rapidly than the smallest fragment from inter-HN2-pTZSV28 (Figure 4, lane 3), which is attributed to the fact that the complementary strands from the latter cannot be separated since they are connected together by the cross-link. The smallest fragment from C-pTZSV28 (45 nucleotides) is expected to migrate more rapidly than the smallest fragment from starting material pTZSV28 (57 nucleotides), which was observed (Figure 4; compare lanes 2 and 1, respectively). Densitometric analysis of the results in lane 3 of Figure 4 indicate that inter-HN2-pTZSV28 contains contamination from non-cross-linked material at the ∼1.0% level and contains <∼2% of starting material pTZSV28 (see below).

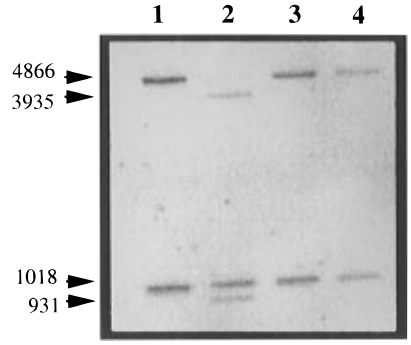

FIGURE 2: Purified closed circular inter-HN2-pTZSV28 contains a lesion (presumably the nitrogen mustard interstrand cross-link) in its unique *Acc*I/*Sal*I site, based on its insensitivity to cleavage by *Acc*I. Cesium chloride purified C-pTZSV28 and inter-HN2-pTZSV28 were treated with *Nde*I, which cleaves both plasmids twice to give fragments of 4866 and 1018 bp, and then ^{32}P-radiolabeled with Kf, [α-^{32}P]dATP, and dTTP. Subsequently, C-pTZSV28 was either not treated (lane 1) or treated (lane 2), and inter-HN2-pTZSV28 was either not treated (lane 3) or treated (lane 4) with *Acc*I. *Acc*I cleaves the larger *Nde*I fragment into 3935- and 931-bp fragments in the case of C-pTZSV28 (lane 2) but not inter-HN2-pTZSV28 (lane 4). In all cases, ∼25 ng (∼1000 cpm) of DNA was electrophoresed through an agarose gel, which was subsequently dried and autoradiographed.

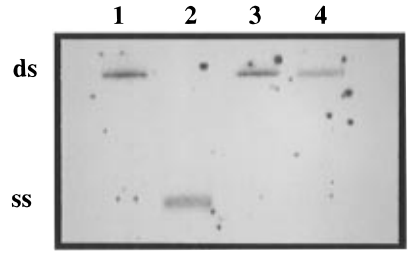

FIGURE 3: Inter-HN2-pTZSV28 contains an interstrand cross-link based upon its ability to renature relatively rapidly after denaturation. Purified C-pTZSV28 and inter-HN2-pTZSV28 were first cleaved with *Eco*RI and then ^{32}P-radiolabeled with Kf and [α-^{32}P]dATP. C-pTZSV28 (lane 1) migrated with a mobility characteristic of double-stranded pTZSV28, while following denaturation/renaturation (lane 2) it migrated as if it were single-stranded. Purified inter-HN2-pTZSV28, before (lane 3) and after (lane 4) denaturation/renaturation, migrated with a mobility characteristic of ds pTZSV28.

Biological Studies with Inter-HN2-pTZSV28. A bacterial transformation assay was used to study the extent to which this single nitrogen mustard interstrand cross-link might inhibit DNA replication. Equal amounts of inter-HN2-pTZSV28 and C-pTZSV28 (∼25 pg; see Experimental Procedures) were transformed in triplicate into AB1157 cells,

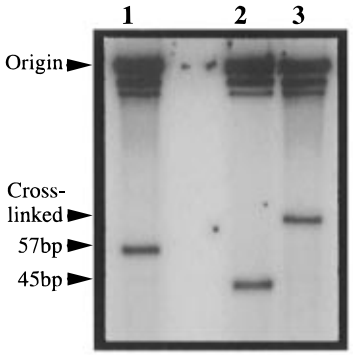

FIGURE 4: Inter-HN2-pTZSV28 contains an interstrand cross-link based on an analysis using denaturing PAGE. Purified C-pTZSV28 and inter-HN2-pTZSV28 (as well as starting material pTZSV28) were first cleaved with both *Eco*RI and *Hin*dIII, which gave fragments of length 3579, 1169, 553, 526, and 45 bp (57 bp for pTZSV28). The cross-link in inter-HN2-pTZSV28 is located in the 45-bp fragment. This material was ^{32}P-radiolabeled with Kf and [α-^{32}P]dATP and separated on a 15% denaturing (7 M urea) PAG. Starting material pTZSV28 (lane 1) and C-pTZSV28 (lane 2) gave fragments that migrated at the expected positions, notably with the smallest fragments appearing at 57 and 45 nucleotides, respectively. In contrast, the smallest fragment from inter-HN2-pTZSV28 migrated more slowly than expected for a 45-nucleotide fragment (lane 3), presumably since the presence of the cross-link prevented strand separation.

which are wild-type for all known DNA repair functions. Totals of 1335, 1830, and 1890 colonies were obtained from inter-HN2-pTZSV28 and 8140, 5695, and 5430 colonies from C-pTZSV28. Each value represents an independent transformation and is the average of two platings. We define replication efficiency (RE) as the ratio of colonies from [inter-HN2-pTZSV28/C-pTZSV28], which was ~0.26 from these results (Table 1, experiment 1). [Typically, a fraction of progeny in adduct site-specific studies are due to genetic engineering side reactions. However, in this study such progeny were present at a very low level (<~0.4%; see below), so RE does not have to be corrected for this problem.] Based on the results from four determinations in AB1157 and two other strains that are wild-type for DNA repair, values of RE ranged from 0.26 to 0.39 with an average of 0.31 (Table 1). Similar values for RE have been reported by us in several preliminary experiments (Ojwang et al., 1990).

Values of RE are subject to the uncertainty associated with our ability to estimate the relative amounts of inter-HN2-pTZSV28 vs C-pTZSV28, which was determined by quantitating both species following [^{32}P]-radiolabeling in parallel (Experimental Procedures). For the material used in experiments 1−4 in Table 1, quantitation was repeated seven times following initial cleavage with the following restriction enzymes, where the ratio (C-pTZSV28/inter-HN2-pTZSV28) is given in parentheses: *Eco*RI (6.15, 7.80, 7.74, 8.05), *Bam*HI (7.14), *Nde*I (5.80), and *Nco*I (5.97). From these results the standard deviation is ±14%.

Since an intact interstrand cross-link is expected to completely block DNA replication, a value of RE ~0.3 suggested that DNA repair was occurring. To define the components of this repair pathway(s), we did similar transformations into strains of *E. coli* that were deficient in various components of known DNA repair pathways. RE was reduced to 0.013 and 0.018 in *uvrA*- and *uvrB*-deficient strains (Table 1), respectively, suggesting a role for the UvrABC endonuclease complex and nucleotide excision repair (NER) in the repair of the cross-link.

We also define the relative replication efficiency (RRE) as the ratio of RE for any repair-deficient strain of *E. coli* vs the RE for its corresponding wild-type strain: e.g., RRE for *uvrA*$^-$ vs *uvrA*$^+$ is 0.049 (= 0.013/0.263) based on the results in Table 1. We note that values for RRE are not subject to the uncertainty associated with our ability to estimate the relative amounts of inter-HN2-pTZSV28 and C-pTZSV28, since both strains received the same relative amounts of each of these vectors. The one assumption in the previous statement is that RE must vary proportionally to the amount of plasmid used in the transformation, which was confirmed (data not shown). Table 1 also includes data from an additional set of experiments where we were able to determine RRE reliably but not RE; these values agree.

We tested for participation of other pathways in the repair of the cross-link in inter-HN2-pTZSV28 (Table 1). RRE was unaffected in studies with *E. coli* containing a *recA* deletion, suggesting no role for RecA-mediated recombination. (See discussion for arguments against a role for non-RecA-mediated recombination.) Base excision repair (BER) also does not seem to be involved, since RRE was unaffected in cells deficient in (i) FAPY DNA glycosylase (*fpg*), (ii) 3-methyladenine DNA glycosylases I and II (*tag*/*alkA* double mutant), (iii) both known AP endonucleases (*xth*/*nfo* double mutant), and (iv) DNA deoxyribophophodiesterase (dRpase = RecJ [*recJ*]), which nicks on the 3′-side of AP sites. Finally, 5′ → 3′-exonuclease activity does not appear to be critical for repair since RRE was unaffected in cells deficient in the 5′ → 3′ exo activity of DNA polymerase I (*polA* [5′ → 3′ exo$^-$]) or exonuclease VII (*xseA*). Furthermore, RecJ also has a 5′ → 3′ exonuclease activity, which is probably not involved given the results in the *recJ* strain.

The results in Table 1 suggest a role for the UvrABC complex; however, RE in *uvrA* and *uvrB* cells was greater than 0. We sought to determine if this residual could be attributed to another minor repair pathway working in parallel. The fact that a value for RE in *uvrA*/*fpg* and *uvrA*/*tag*/*alkA* strains is approximately the same as in a *uvrA* strain alone suggests that—if there is a pathway in parallel to the UvrABC system—it does not involve either the FAPY DNA glycosylase or 3-methyladenine DNA glycosylases I or II.

Is DNA Repair of the Cross-Link Accurate? We determined whether DNA repair was accurate. The cross-link in inter-HN2-pTZSV28 is located in a polylinker region of a *lacZ*′ fragment, which restores β-galactosidase activity via α-complementation to certain *E. coli* (e.g., DH5-α). β-Galactosidase activity is readily monitored on plates using a substrate that turns colonies blue if it is active (Experimental Procedures). A batch preparation of progeny plasmids from a transformation into wild-type AB1157 cells was isolated and retransformed into DH5-α cells, and the ratio [blue colonies/white colonies] was determined to be 899/1. This result suggests that very few frameshift mutations were generated, since they usually give rise to white colonies because α-complementation is lost. [In addition, this suggests that genetic engineering-derived mutations, which usually appear as white colonies and often represent a significant fraction of progeny in adduct site-specific studies, were not significantly present in this study.] Progeny vectors from 100 colonies from AB1157 cells were isolated, and all were sensitive to cleavage by *Sal*I. Because the cross-link

was originally located in the unique *Acc*I/*Sal*I site of inter-HN2-pTZSV28, these results demonstrated that repair was accurate since a mutation would have generated a *Sal*I^r progeny plasmid. These same 100 colonies were also resistant to cleavage by *Pst*I, showing that progeny vectors could not be derived from contaminating starting material, pTZSV28 itself, which is *Pst*I^s. Plasmids from 100 colonies from experiments in a *uvrA* strain were also isolated: 97 appeared not to contain mutations in the vicinity of the cross-link because they were *Sal*I^s/*Pst*I^r. The remaining three colonies were *Sal*I^s/*Pst*I^s, which suggests that starting material, pTZSV28, which is *Pst*I^s, does contaminate inter-HN2-pTZSV28. However, because these results were obtained in cells (i.e., *uvrA*$^-$) where the yield of progeny from inter-HN2-pTZSV28 was ~5%, the estimated level of contamination of inter-HN2-pTZSV28 by starting material is only 0.0015 (= 3/100 × 5/100).

DISCUSSION

The data reported in Table 1 indicate a role for UvrABC, and thus NER, in the repair of the nitrogen mustard DNA−DNA interstrand cross-link present in inter-HN2-pTZSV28. The fact that the yield of progeny plasmids from inter-HN2-pTZSV28 (i.e., RE) decreases substantially in either *uvrA* or *uvrB* cells also establishes that the high yield of progeny from inter-HN2-pTZSV28 in wild-type (and other) strains cannot be attributed to the presence of a substantial amount of a non-lesion-containing contaminant.

Furthermore, it is virtually certain that these progeny arose from a plasmid species that originally did indeed contain the interstrand cross-link and not some other lesion. Figure 3 shows that the overwhelming majority (>95%) of the material designated inter-HN2-pTZSV28 is cross-linked, and Figure 4 shows that the contamination is no more than ~1%. In fact, the data in Figure 4 was obtained ~16 weeks after inter-HN2-pTZSV28 construction, whereas most of the results in Table 1 (all except experiment 4) were completed within 3 weeks of the construction. The stability of the cross-link in inter-HN2-pTZSV28 can be attributed in part to our having purposely converted the initially formed, nitrogen mustard N7-Gua adducts into the corresponding, more stable, FAPY adducts (Ojwang et al., 1989; Experimental Procedures). While FAPY adduct formation was done to improve the feasibility of our studies, a significant fraction (~30%) of nitrogen mustard N7-Gua adducts appear to be converted to FAPY in cells (Chetsanga et al., 1982).

Evidence for a Recombination-Independent DNA Repair Pathway. The commonly accepted pathway for the repair of interstrand cross-links involves initial nicking of one of the two DNA strands in the vicinity of the cross-link by UvrABC and subsequent replacement of the nicked strand from a lesion-free homologous chromosome in a RecA-mediated recombinational event (steps 1−3 in Figure 1; Van Houten et al., 1986; Sladek et al., 1989a,b). Three results argue against a role for recombination in the repair of the cross-link in inter-HN2-pTZSV28 in our studies. First, transformation of inter-HN2-pTZSV28 into a *recA* deletion strain did not affect progeny plasmid yield (Table 1). Second, low concentrations of inter-HN2-pTZSV28 were used during transformation (~25 pg), which ensures that most cells received only a single copy of the plasmid genome[2] and precludes recombination from a homologous chromosome. Third, even if a sizable fraction of cells did pick up a second copy of inter-HN2-pTZSV28, it is not lesion-free since no cross-link-free material was present in the mixture (Figure 3). Finally, we note that all recombinational pathways in *E. coli* require RecA with the exception of copy-choice recombination and the RecE pathway (Friedberg et al., 1995), neither of which is possible without a lesion-free homologous chromosome. The RecE pathway is also not possible since all strains employed, notably AB1157, are Δ*recE* (Kaiser & Murray, 1979).

We wish to emphasize that we believe that interstrand cross-links *can* be repaired by a recombination-dependent pathway in some circumstances (e.g.) as has been established most definitively in the case of psoralen interstrand cross-links both in cells (Sladek et al., 1986a) and *in vitro* (Sladek et al., 1986b). However, the nature of our experiments precluded such a pathway, because no lesion-free copy of the plasmid genome was provided from which recombination could occur. In fact, it was probably these unique circumstance that allowed us to find evidence for what appears to be a second pathway for the repair of interstrand cross-links that is recombination-independent. The fact that progeny yield in UvrABC-deficient cells is nonzero suggests that there may be yet a third, NER-independent DNA repair pathway, although this is less certain.

BER and 5′ → 3′ Exonuclease Activities Are Not Involved in Repair. It is not obvious how both strands of an interstrand cross-link would be repaired in a recombination-independent pathway. The major logistical problem is the error-free repair of the first strand in any such pathway. One possibility is that a DNA glycosylase might initiate base excision repair (BER; step 1′, Figure 1) in the first strand, as has been proposed on several occasions (Zhen et al., 1986; Kohn, 1981). This is plausible given that glycosylase-initiated repair can involve a DNA repair track of as little as a single nucleotide (Friedberg et al., 1995) and that the base complementary to both modified guanines in the nitrogen mustard interstrand cross-link is a lesion-free cytosine. Two glycosylases, namely, FAPY DNA glycosylase and 3-methyladenine DNA glycosylase II (as well as possibly 3-methyladenine DNA glycosylase I), have been reported to have activity on some adducts formed at the N7 position of purines (Friedberg et al., 1995). The results in Table 1 using *fpg*-, *tag*-, and *alkA*-deficient strains of E. coli argue against a role for these glycoslyases in the repair of the nitrogen mustard interstrand cross-link. Substrate specificity of glycosylases is often inexplicable. Thus, to test further whether BER might be involved following the action of some other unidentified glycosylase, inter-HN2-pTZSV28 was transformed into cells deficient in the second step of BER, which involves an AP endonuclease (*xth*/*nfo* double mutant), and the third step of BER, which involves dRpase (*recJ*). RRE was unaffected in either of these cases (Table 1), suggesting that BER is not involved in a major way in

[2] The fraction of transformed cells picking up two copies of a plasmid was assessed as follows. Equal amounts of two plasmids with different antibiotic resistance markers [pTZSV28 (ampicillin) and pSV-neo (neomycin)] were transformed by electroporation into AB1157 cells. Even when both plasmids were present at ~5 ng each in a transformation mixture with 50 μL of cells, only ~1 in 10^4 colonies was resistant to both ampicillin and neomycin. This indicates that very few cells picked up two plasmids in our transformation protocol, in which ~25 pg of plasmid was used.

the repair of the interstrand cross-link in inter-HN2-pTZSV28.

We proposed that a DNA repair pathway for an interstrand cross-link could also potentially involve the nick-translating ability of DNA polymerase I (Ojwang et al., 1990). Following the action of UvrABC (Figure 1, step 1), pol I could conceivably begin nick translation from a nick on the 5′-side of the cross-link (Figure 1, step 2″). If nick translation could proceed and the 5′ → 3′ exonuclease activity could remove the adducted guanine in the cross-link, then presumably a guanine could be incorporated in its place, since the complementary cytosine is lesion-free (Figure 1, step 3″). Accurate repair of the top strand would be completed following ligation (Figure 1, step 4″). The fact that progeny plasmid yield is not affected following transformation into an *E. coli* deficient in the 5′ → 3′ exonuclease activity of pol I (Table 1) appears to rule out a pathway involving nick translation. Although we investigated this pathway as a formal possibility, the fact that it does not appear to operate was expected given what is known about pol I. Pol I is involved in NER with monoadducts, but nick translation does not occur, and in fact pol I can only work when coupled with the UvrD helicase, which releases an adduct-containing oligonucleotide (Friedberg et al., 1995). Although there is evidence that pol I 5′ → 3′ exonuclease functions in the repair of psoralen interstrand cross-links, it is responsible for removing DNA downstream of the nick on the 3′-side of the cross-link as a prelude to RecA-mediated homologous chromosome exchange (Sladek et al., 1989b).

A variation on this pathway involving DNA polymerase III and another *E. coli* 5′ → 3′ exonuclease, such as exonuclease VII or RecJ, which are thought to function during mismatch repair (Friedberg et al., 1995), is also unlikely on the basis of the fact that RE was unaffected in either an *xseA* or a *recJ E. coli* (Table 1). However, we cannot rule out a pathway by which DNA polymerase III can act with either exonuclease VII or RecJ, which would require a study in a *xse*/*recJ* double mutant. This possibility is currently under investigation.

Could Repair Involve DNA Polymerase Bypass? DNA repair might also involve a DNA polymerase simply synthesizing a replacement strand following the action of UvrABC (Figure 1, steps 1, 2, and 3‴). The drawback of such a pathway is that repair would require translesion synthesis of the adduct in the strand opposite to the one in which UvrABC made its initial nick, and this might result in replication errors. Our finding that all 100 progeny plasmids analyzed did not have a mutation at the genome position originally occupied by the cross-link (i.e., the *Acc*I/*Sal*I site) suggests that MF is reasonably low by whatever pathway repair occurred. The question is, if DNA repair did involve translesion synthesis, what MF is expected in the progeny plasmids? Recent evidence from a variety of adduct site-specific studies involving the adducts of bulky carcinogens suggest that MF might in fact be quite low; e.g., it is ∼0.1% or lower for several bulky polycyclic aromatic hydrocarbon (PAH) adducts and *cis*-DDP intrastrand cross-linked adducts under comparable cellular conditions [Singer & Essigmann, 1991; Loechler, 1996; reviewed in Loechler (1994)]. Such bypass would have to be SOS-independent since RE is unaffected by working in a Δ*recA* strain (Table 1). Both the *cis*-DDP and nitrogen mustard adducts are attached at N7-Gua, and adduction does not directly affect the hydrogen-bonding potential of the base moiety of these, as well as the PAH adducts. Preliminary experiments suggest that MF is ∼0.1% with inter-HN2-pTZSV28 (data not shown), which is not unreasonable for a repair pathway involving translesion synthesis. The investigation of a repair pathway involving polymerase bypass is more complicated since simply determining RE in DNA polymerase I- or III-deficient strains is not possible; however, this mechanism is currently under investigation using other approaches.

There is some evidence that interstrand cross-link repair can involve excision repair and polymerase bypass. Some cross-linking agents, such as mitomycin C (Kondo et al., 1970; Murayama & Otsuji, 1973) and malondialdehyde (Mukai & Goldstein, 1976), are more mutagenic in excision repair-proficient than -deficient strains of *E. coli*. These results are most easily rationalized if an error-prone repair pathway is initiated by the action of NER (e.g., Figure 1, steps 1, 2, 3‴). While this interpretation is certainly sensible, the results do not prove that an interstrand cross-link is necessarily involved and do not address whether such a pathway either can also be nonmutagenic or is quantitatively significant.

Conclusions and Speculations. In conclusion, our results suggest that there is a recombination-independent pathway available to repair nitrogen mustard interstrand cross-links. In addition, we believe that, in circumstances where a lesion-free homologous chromosome is available, a second, recombination-dependent pathway is also likely to operate (Sladek et al., 1989a,b). In contrast, both of these DNA repair pathways may not be able to operate on all interstrand cross-links depending upon exact chemical structure. Such a conjecture might explain why—in an analogous adduct site-specific study (Piette et al., 1988)—the yield of progeny plasmids from a psoralen interstrand cross-link gave a much lower RE of ∼0.02 even though the cells (AB1157) were identical to those in which we obtained RE ∼0.3 for the nitrogen mustard interstrand cross-link. Furthermore, the yield of progeny plasmids from the psoralen interstrand cross-linked vector did not change in going from *uvr*$^+$ to *uvr*$^-$ *E. coli* (RE ∼0.02 in both cases), which contrasts with the decrease obtained herein for the nitrogen mustard interstrand cross-link. Taken together, these comparisons imply that the psoralen interstrand cross-link cannot be repaired by the recombination-independent pathway that apparently can operate on the nitrogen mustard interstrand cross-link.

It is premature to speculate on a possible reason for this difference, but we note that the nitrogen mustard interstrand cross-link is more flexible than the rigid psoralen interstrand cross-link, which fixes the two strands of DNA with respect to each other due to the cyclobutane adduct linkages. This raises the intriguing possibility that not all interstrand cross-links are repaired comparably, which might have an influence on the relative ability of each to be repaired and contribute to genome inactivation and cytotoxicity.

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