Butadiene-induced Intrastrand DNA Cross-links: A Possible Role in Deletion Mutagenesis*

J. Russ Carmical‡‡‡, Agnieszka Kowalczyk, Yue Zou§, Bennett Van Houten‡‡‡, Lubomir V. Nechev, Constance M. Harris, Thomas M. Harris, and R. Stephen Lloyd‡‡‡

From the ‡Department of Preventive Medicine and Community Health, the §Sealy Center for Molecular Science, The University of Texas Medical Branch, Galveston, Texas 77555, and the ¶Department of Chemistry and the Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, Tennessee 37235

To initiate studies designed to identify the mutagenic spectrum associated with butadiene diepoxide-induced N2-N2 guanine intrastrand cross-links, site specifically adducted oligodeoxynucleotides were synthesized in which the added bases were centrally located within the context of the human ras 12 codon. The two stereospecifically modified DNAs and the corresponding unmodified DNA were ligated into a single-stranded M13mp7L2 vector and transfected into Escherichia coli. Both stereoisomeric forms (R,R and S,S) of the DNA cross-links resulted in very severely decreased plaque-forming ability, along with an increased mutagenic frequency for both single base substitutions and deletions compared with unadducted DNAs, with the S,S stereoisomer being the most mutagenic. Consistent with decreased plaque formation, in vitro replication of DNA templates containing the cross-links by the three major E. coli polymerases revealed replication blockage by both stereoisomeric forms of the cross-links. The same DNAs that were used for replication studies were also assembled into duplex DNAs and tested as substrates for the initiation of nucleotide excision repair by the E. coli UvrABC complex. UvrABC incised linear substrates containing these intrastrand cross-links with low efficiency, suggesting that these lesions may be inefficiently repaired by the nucleotide excision repair system.

Metabolic bioactivation of 1,3-butadiene results in a diepoxide. As a bifunctional electrophile, butadiene diepoxide is theoretically capable of producing inter- and intrastrand DNA-DNA cross-links. Cross-linked adducts are thought to be responsible for the observation that the diepoxide is considerably more mutagenic in mice than the monoeoxide under identical conditions (1) and for the fact that butadiene is more genotoxic to mice than rats. The latter observation is attributed to the greater effectiveness of mice at metabolizing butadiene to the diepoxide (2). Both species appear to be equally susceptible to cytogenetic damage inflicted by butadiene diepoxide when the epoxide is introduced directly into isolated rat or mouse lymphocytes (splenic or peripheral blood) (3).

There are a number of studies supporting the existence of butadiene diepoxide-induced interstrand cross-links (4–9). Evidence for such cross-links is based largely on denaturation/renaturation experiments in which interstrand-cross-linked DNA renatures more rapidly than noncross-linked. The only cross-linked species thus far identified, a guanine N7-guanine N7 cross-link, was isolated from salmon sperm DNA by Lawley and Brookes (8). In 1993, Millard and White (10) reported that synthetic oligonucleotide duplexes of varying sequences reacted rather diffusely with butadiene diepoxide but showed preference for interstrand cross-linking at 5′-GNC sites. As expected for guanine N7 cross-links most of the bands that migrated in denaturing gels in the region expected for dimeric structures were cleavable by piperdine at 90 °C. However, some cross-linked material persisted after the alkaline treatment indicating that stable cross-links (of unknown structure) were also formed.

Interstrand cross-links are known to be highly cytotoxic, whereas intrastrand cross-links tend to be more mutagenic (11–13). Although certain types of intrastrand cross-links such as those arising from pyrimidine photodimerization and the chemotherapeutic agents, cisplatin and mitomycin, have been well studied, the question of intrastrand cross-link formation by butadiene diepoxide has not been examined; in theory the guanine N7-guanine N7 cross-link that has been isolated could arise from an intrastrand as well as an interstrand cross-link. We decided to focus on possible stable intrastrand cross-links, because little is known about the replication and repair of aliphatic intrastrand cross-links. Inasmuch as we had data on replication and mutagenicity of butadiene diepoxide monoaducts on guanine N2 for comparison (14), we chose guanine N2-guanine N2 cross-links as our first target. Furthermore, we knew from other experiments1 that a cross-link involving adjacent guanines connected N2 to N2 by an unsubstituted 4-carbon alkyl chain introduces very little distortion into the double helix. Such a lesion might escape detection by repair enzymes and lead to mutations if it were replicated. Hence 8-mer oligonucleotides were synthesized containing site-specific guanine N2-guanine N2 cross-links of (R,R) and (S,S) butadiene diepoxide in the N-ras codon 12 (-GGT-) sequence. To better understand what roles such adducts might play in molecular mechanisms responsible for butadiene-induced carcinogenesis, replication efficiency and mutagenic spectra have been inves-

1 A. Kowalczyk, unpublished data.
tigated, as well as the initiation of repair by the UvABC exinuclease complex. These data suggest that intranuclear butadiene cross-links may contribute significantly to the mutagenic spectrum observed in butadiene-exposed animals.

**EXPERIMENTAL PROCEDURES**

**Syntheses and Characterization of Cross-linked Oligonucleotides**

**Materials and Methods—**Oligonucleotides were prepared on an Expedite® 8909 Nucleic Acid Synthesizer using tert-butyl-phenoxyacetyl 2-cyanethyl phosphoramidites and the modified phosphoramidite of 2-fluoro-O2'-trimethylsilylthiophenyl (TMSE)-5'-O-dimethoxytrityl 2'-deoxycytidine on a 1 mmol scale. Oligonucleotides were deprotected and purified as described previously (15). HPLC purifications were done on a Beckman HPLC (System Gold software, model 125 pump, model 168 photodiode array detector). Oligonucleotides were desalted on Sephadex G-25 using a Bio-Rad FPLC system. Enzymatic digestion mixtures (0.2–0.5 μg of oligonucleotide, 20 μl of buffer (0.01 m Tris-Cl, pH 7.4), and 2.1 units of nuclease P1 (Sigma N-8630) were incubated at 37 °C for 4 h, and then 20 μl of 0.1 m Tris-HCl buffer, pH 9.0, 0.04 unit of snake venom phosphodiesterase. High resolution mass spectra were obtained in positive fast atom bombardment (FAB) mode on a VG ZAB-HF spectrophotometer. 1H NMR spectra were recorded at 194.83 MHz. HRMS (FAB+ m/z) calculated for [M+H]+ 209.1290 found 209.1291 [α]20D was −14.9° (c = 2, ethanol).

**Starting material led to 2.0 g (91%) of the corresponding (2R,3R)-isomer.**

1H NMR (methanol-d4, δ (ppm): 2.87 (m, 4H, 2x CH2), 3.92 (m, 2H, 2x CH), 5.93 (s, 1H, CH-benzyl), 7.37 (m, 3H, m-p, aromatic), 7.49 (m, 2H, o-aromatic). HRMS (FAB+) m/z calculated for [M+H]+ 209.1290 found 209.1291 [α]20D was +16.1° (c = 2, ethanol).

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**SYNTHESIS AND PURIFICATION OF INTRASTRAND CROSS-LINKS (3A AND 3B)—**The general procedure was as follows. The starting material 5'-d(CATXXCTXO)3'-5' (X = 2-fluoro-O2'-TMSE 2'-deoxycytidine) was reacted with the appropriate diaminediethyl in Me2SO in the presence of diisopropylphosphine (DIEA) for 2–3 days at 55 °C. The reactions were monitored by HPLC on a C18 column (4.5 × 250 mm, YMC ODS-AQ) with the following gradient: (A) 0.1 m ammonium formate and (B) CH3CN, 1–10% B over 15 min, hold for 2 min, then to 1% B over 2 min at a flow rate of 1.5 ml/min. 1H NMR (methanol-d4, δ (ppm): 2.71 (t, 1H, CH2), 3.75 (m, 2H, 2x CH), HRMS (FAB+) m/z calculated for [M+H]+ 121.0977 found 121.0987 [α]20D was +15.9° (c = 2, ethanol). A sample of this isomer was converted to its HBr salt. [α]20D +20.1° (c = 2, water) (literature value: [α]20D +20.3° (c = 2, water) (16)).

**Synthesis of butadiene cross-links (3a and 3b)—**For synthesis of this cross-link, 18 A250 units of (1), 1 drop of DIEA, and 67 μl (1.5 equivalents) of a solution of 200 units of 2S,3S-1,4-Diamino-2,3-Butanediol—(2S,3S)-2,3-O-Benzylidene-1,4-Diamino-2,3-butane was protected with the appropriate diaminediethyl in Me2SO and, in the presence of diisopropylphosphine (DIEA) for 2–3 days at 55 °C (19). The reactions were monitored by HPLC on a C18 column (250 × 10 mm, YMC ODS-AQ) with the following gradient: (A) 0.1 m ammonium formate and (B) CH3CN, 1–15% B over 20 min, 15–35% B over 3 min, hold for 2 min, and then to 1% B over 2 min at a flow rate of 1.5 ml/min. Starting material eluted at 21–22 min, and cross-linked products 2a and 2b eluted at ~18–19 min (fully TMSE-protected), partially TMSE-protected products at ~12–13 min, and final products 3a and 3b at ~9–10 min (fully deprotected).

**3b-dimer observed from 3a.** The reaction mixture was then neutralized and combined with 3a isolated previously.

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3% B over 2 min at a flow rate of 3 ml/min; the product (3b) eluted at 22 min. The HPLC-purified cross-linked oligonucleotides 3a and 3b were desalted (Sephadex G-25) and analyzed by mass spectroscopy; electrospray ionization-mass spectroscopy: [3a] calculated M of 2471.7, measured mass based on [M + Na-H2]2+: 2471.7 ([M + Na-H2]2+: 3950.6); [3b] calculated M of 2471.7, measured mass based on [M + Na-H2]2+: 2471.7 ([M + Na-H2]2+: 3950.6). The resulting solution was acidified with aqueous acetic acid to pH 4 to remove the TMSE groups and stirred at room temperature for 8 h. The reaction mixture was neutralized, lyophilized, and purified by reverse-phase HPLC on a C8(250 mm × 10 mm, Phenomenex) column with the following gradient: (A) H2O and (B) CH3CN, 5–8% B over 5 min, 8–11% B over 11 min, 11–90% B over 2 min, hold for 3 min, and then 90–5% B over 2 min at a flow rate of 3 ml/min, both products eluted at 11.5–12.5 min.

1.4-Butanediol Formation—Synthesis of Bisnucleoside Standards—A solution of the appropriate diamine, 2-fluoro-0-0-TMSE 2-deoxyinosine, and DIEA (molar ratio in order mentioned 1:3:5) in Me2SO was stirred at 55 °C for 30 h. The reaction solution was acidified with aqueous acetic acid to pH 4 to remove the TMSE groups and stirred at room temperature for 8 h. The reaction mixture was neutralized, lyophilized, and purified by reverse-phase HPLC on a C8(125 mm × 2.1 mm, Phenomenex) column with the following gradient: (A) H2O and (B) CH3CN, 5–8% B over 5 min, 8–11% B over 11 min, 11–90% B over 2 min, hold for 3 min, and then 90–5% B over 2 min at a flow rate of 3 ml/min, both products eluted at 11.5–12.5 min.

UV measurements were taken at 1-min intervals with a 1 °C/min temperature gradient. At the end of the experiment, the mixture was cooled to room temperature and then incubated in ice slurry for 15 min. T4 DNA ligase (2000 units) was added, and the reaction was allowed to proceed overnight at 16 °C. The 50-mer ligation product was then gel-purified to remove the 45-mer scaffold, as described previously. Four oligodeoxynucleotide probes (17-mers) were incorporated. These 17-mer probes were radioactively labeled by incubating 1 μg of DNA with 0.50 mCi of [γ-32P]ATP and 20 units of T4 DNA ligase (2000 units). Each of the four nicked nucleotides was labeled accordingly and subsequently hybridized with the radioactively labeled probes overnight. The hybridization conditions were such that only the perfectly hybridized complement would anneal. Radiolabeled probes were washed as described previously, and filters were exposed to autoradiographic film overnight.

In Vitro Replication of 1,3-Butadiene-modified Linear Template

Oligodeoxynucleotides were synthesized to serve as primers for replication of the 50-mer template. Three primers were designed such that they would anneal to specific sites on the templates, thus providing a 3'-hydroxyl at various distances relative to the adduct. In effect, the primers would simulate scenarios that a polymerase might encounter in vivo. The first positioned the 3'-hydroxyl one base pair to the adduct, which would simulate a "standing" start. The second positioned the 3'-hydroxyl four bases prior to the adduct, which would simulate a "running" start. Finally, the third primer placed the 3'-hydroxyl five bases beyond the adduct to determine any downstream effects. Each primer was phosphorylated by T4 polynucleotide kinase to affix a 5'-γ-32P label. Subsequently, each was diluted to a concentration of 50 fmol/μl and added to the 50-mer template in a ratio of 1:3 in the presence of the appropriate reaction salts. To promote proper annealing, the mixture was heated to 90 °C for 2 min and slowly cooled to room temperature. This reaction was carried out in triplicate for each template/primer combination. The polymerases assayed and suppliers were as follows: large fragment of polymerase I (Klenow exo-) was purchased from New England Biolabs, Beverly, MA; polymerase II was supplied by Drs. M. F. Goodman and L. Bloom, University of Southern California, Los Angeles, CA; and polymerase III was supplied by Dr. Mike O'Donnell, Rockefeller University, New York, NY. Finally, the appropriate salts, 1 μM DNTPs, and the buffer specific for the polymerase being assayed were added to the template/primer complex in a total reaction volume of 9 μl. Individually, the polymerases were added at the following concentration: 2-fold molar excess of enzyme to DNA and allowed to proceed at room temperature for 10 min. The reaction was stopped by adding an equal volume of stopping buffer, consisting of formamide, xylene cyanol, and bromphenol blue. The extended products were then analyzed by electrophoresis through a 15% polyacrylamide sequencing gel and visualized by exposing an autoradiographic film overnight.

In Vivo Replication of 1,3-Butadiene-modified Circular DNA

Modified M13mp17L2 vector was used to transfect repair-deficient AB2480 (avrA+, recA-). Escherichia coli cells via electroporation as previously reported (14, 21). Subsequently, the electroporation mixture was plated on precured LB broth agarose plates in the presence of 500 μl of AB2480 E. coli and 5 ml of top agar (LB + 0.7% agarose). Each plate was inverted and incubated overnight at 37 °C. The resulting plaques were then transferred to nitrocellulose filters in four successive lifts for each plate. These filters were then processed as described previously (14, 21). The plaques were subsequently screened for positive base substitutions at position 2 of the ras-12 codon via differential hybridization techniques (18–21). Four oligodeoxynucleotide probes (17-mers) were synthesized as described in the section order mentioned 1:3:5:3 and 3’ to the DNA surrounding and including the 8-nucleotide insert. The 17-mers were varied in the sequence identity of the nucleotide opposite the 3'-guanine of the cross-link, where each of the four possible nucleotides were incorporated. These 17-mer probes were radioactively labeled by incubating 1 μg of DNA with 0.50 mCi of [γ-32P]ATP and 20 units of T4 DNA ligase (2000 units). Each of the four nicked nucleotides was labeled accordingly and subsequently hybridized with the radioactively labeled probes overnight. The hybridization conditions were such that only the perfectly hybridized complement would anneal. Radiolabeled probes were washed as described previously, and filters were exposed to autoradiographic film overnight.

Construction of 1,3-Butadiene-modified Duplex Template for UvrABC Assays

The 50-mer templates containing the butadiene cross-linked DNAs were constructed as above except the 5'-end was not labeled until immediately prior to use in incision and binding assays. A complemen-
tary 50-mer oligodeoxynucleotide was synthesized and subsequently gel-purified as described previously and used as the substrate for UvrABC binding and incision assays. Prior to annealing, each template was phosphorylated by T4 polynucleotide kinase incorporating a \( \gamma \)\(^{32}\)P label on the 5′-end. The labeled 50-mer templates were then annealed to the complement in individual reactions. A reaction mixture containing a 5-fold molar excess of complement (500 nmol:2500 nmol) and 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA was heated to 85 °C and allowed to slowly cool to room temperature. The duplex formation was then gel-purified on a 10% native polyacrylamide gel.

Nucleotide Incision of 1,3-Butadiene-modified Linear Template

The aforementioned, double-stranded 50-mer substrates (5 nM) were incubated with \( E. \ coli \) UvrABC proteins (10 nM UvrA, 250 nM UvrB, and 50 nM UvrC) at 37 °C for 30 min in the presence of UvrABC reaction buffer (50 mM Tris-HCl, pH 7.8, 50 mM KCl, 10 mM MgCl\(_2\), 5 mM dithiothreitol, and 1 mM ATP). Prior to the addition of the DNA substrate, the Uvr subunits were diluted with storage buffer. Finally, the reactions were terminated by adding EDTA (20 mM) or heating to 90 °C for 3 min. The samples were denatured with formamide and heated to 90 °C for 5 min and then quick-chilled on ice. The digested products were analyzed by electrophoresis through a 12% polyacrylamide sequencing gel under denaturing conditions with UvrABC buffer. The gel was dried and exposed to an x-ray film and a PhosphorImaging screen (Molecular Dynamics) for quantification.

Gel Mobility Shift Assays

Binding of the DNA substrates by the \( E. \ coli \) UvrA protein was determined by gel mobility shift assays. Typically, the substrate (4 nM) was incubated with the UvrA with the indicated concentrations in 20 μl of UvrABC buffer without ATP at 37 °C for 15 min. After the incubation, 2 μl of 80% (v/v) glycerol was added, and the mixture was immediately loaded onto a 3.5% native polyacrylamide gel in TBE running buffer and separated by electrophoresis at room temperature.

RESULTS

Synthesis and Characterization of Butadiene Diepoxide Cross-linked Oligonucleotides 3a and 3b—The oligonucleotides utilized in these studies were synthesized by a novel application of the postoligomerization strategy developed by Harris et al. (15, 22, 23) (Fig. 1). For the intrastrand cross-link, two adjacent halopurines were introduced into the 8-mer oligonucleotide using the phosphoramidite of 2-F-\( O\)\(^{6}\)-trimethylsilyl-ethyl 2′-deoxyinosine. The halopurine-containing oligonucleotide was removed from the matrix, deprotected, and purified before reaction with the chiral diaminobutanediol. The stoichiometry of the reaction was carefully controlled to avoid the risk of introduction of two diaminodiol residues. Oligonucleotides 3a and 3b were purified by HPLC and characterized by electrospray mass spectrometry, which showed them to be of the expected mass. Enzymatic digestion showed the constituent nucleosides in the expected ratios (3dC, 2T, 1dA, and 1 bisnucleoside). To rigorously minimize the possibility of contaminants that could alter the mutagenic spectrum, the ras 12\(^{2}\)-N\(^{2}\)-N\(^{2}\)guanine 1,3-butadiene-modified M13mp7L2 in AB2480 (uvrA\(^{-}\), recA\(^{-}\)) E. coli

| TABLE I | Plaque-forming efficiency |
|---------|---------------------------|
| DNA Input DNA Average Plaques/Plate Efficiency |
| Unadducted 8 × 10\(^{6}\) 1566 2 × 10\(^{8}\) |
| GG R R 0.025 250 1 × 10\(^{7}\) |
| GG S S 0.025 198 8 × 10\(^{7}\) |

| TABLE II | In vivo replication of ras 12\(^{2}\)-N\(^{2}\)-N\(^{2}\)guanine 1,3-butadiene-modified M13mp7L2 |
|-----------|-----------------|-----------------|-----------------|-----------------|
| Adduct     | G → G | G → A | G → T | G → C |
| Nonadducted | 32,000 | 0 | 0 | 0 |
| GG R R      | 14,000 | 4 | 10 | 1 |
| GG S S      | 15,000 | 41 | 25 | 25 |

FIG. 1. Structures of 1,3-butadiene-induced N\(^{2}\)-N\(^{2}\) guanine stereoisomers.

FIG. 2. End-labeled 8-mer oligodeoxynucleotides (nonadducted and adducted). The purity of the adducted oligodeoxynucleotides was determined by end-labeling and polyacrylamide gel electrophoresis. 1, nonadducted; 2, R R N\(^{2}\)-N\(^{2}\) guanine; 3, S S N\(^{2}\)-N\(^{2}\) guanine.
to the unmodified 8-mer (Fig. 2). In an attempt to visualize even the minutest contaminant, the autoradiographic film was overexposed. However, no contaminants were detected. These data reinforce that a single species was present in each of the adducted samples and that the correct chemical composition was obtained. Ultimately, capillary gel electrophoresis analysis of the synthesis confirmed the purity of each sample.

In Vivo Replication of 1,3-Butadiene-modified Circular DNA—The in vivo replication fate for each of the R,R and S,S stereo-specific butadiene-induced guanine cross-link-containing DNAs (Fig. 1), as well as for unmodified counterpart, was determined by inserting the individual oligodeoxynucleotides into the EcoRI site of a single-stranded M13 vector (see “Experimental Procedures”). Each 8-mer was ligated into the EcoRI cloning site of M13mp7L2 and quantitated. The modified M13 vectors were replicated in E. coli and evaluated for the inhibition of phage replication and mutagenesis (Tables I and II, respectively). Using the M13mp7L2 single-stranded DNA system, the E. coli replicative polymerases are forced to synthesize past the cross-linked site to generate an intact minus strand from which to catalyze rolling circle replication. Thus, the efficiency of plaque formation is indicative of the efficiency of lesion bypass. The data shown in Table I reveal that both R,R and S,S N²-guanine cross-links were extremely inhibitory to replication bypass, with plaque formation decreased by more than four orders of magnitude in both cases. These data are suggestive that in the absence of an efficient DNA repair mechanism, these butadiene lesions could produce severe or permanent blocks to replicative synthesis.

Even though replication efficiencies were severely compromised, the plaques that were formed were subsequently analyzed for point mutations and deletions (Table II and III). These experiments were carried out as three separate and independent processes from construction of the adducted M13mp7L2 vector through the screening of plaques for mutations. The data represented in Tables I–III are the culmination of these experiments. As expected, screening of the unmodified DNAs resulted in no mutations of any type. In contrast, analyses of the plaques that were formed using the modified DNAs revealed a multitude of mutations (Table II). Each putative mutant was screened a second time to confirm it as a positive, and subsequently, the sequences of a subset of plaques were determined. Representatives of each type of base substitution are depicted in Fig. 3. On average, the mutation frequencies corresponding to the cross-links exceeded those of the mono-base guanine adducts by an order of magnitude (14, 21). However, it should be emphasized that the plaque-forming ability was down by more than four orders of magnitude relative to the unadducted DNAs. The mutation frequencies were calculated
and determined to be statistically significant by calculating the binomial confidence intervals to a 0.995 degree of certainty. The confidence intervals for each stereo-specific adduct were then plotted against those for the unmodified DNAs (Fig. 4).

In addition to those plaques that hybridized to one of the specific mutant probes (Table II), a portion of resulting plaques either did not hybridize or only lightly hybridize (Table III) to any of the four probes, an observation that could be indicative of deletions. To address this issue, a subset of both “nonhybridizing” and “lightly hybridizing” plaques was selected and sequenced in the appropriate portion of the M13L2 genome to screen for possible deletions. DNA sequence analysis of those plaques that did not hybridize to any of the mutant probes revealed that these were M13mp7L2 molecules that had not been linearized at the EcoRI site. Although the EcoRI-restricted M13L2 DNAs were analyzed by electrophoresis through an agarose gel and visualized by ethidium bromide staining, an undetectable amount of circular M13 may have been present in the input DNA. These plaques were not included in the final analysis. Conversely, the plaques that only lightly hybridized to the four probes were sequenced and revealed various mutations at other sites within the probed region (Table III). Interestingly, the majority of those sequenced (R,R cross-link, 83% and S,S cross-link, 92%) contained a G→C transversion 8 base pairs downstream from the cross-link. The remainder of the plaques contained a variety of deletions, insertions, and base substitutions (Table III). These deletions accounted for about the same frequency of changes as did the

FIG. 5. Construction of 1,3-butadiene cross-link-adducted 50-mer template.

![Construction of 50-mer Template](image)

FIG. 6. Primer extensions of the butadiene-cross-link adducted templates. Each of the templates (unadducted, R,R N²-N² guanine, and S,S N²-N² guanine) was annealed to 1 of 3 primers (−1 primer, −4 primer, and +5 primer). Each possible template-primer combination was extended with each of three polymerases (E. coli Pol I, II, and III), and the products were separated by electrophoresis through a polyacrylamide gel. −1 primer (lanes 1–4) lane 1, primer (P) alone; lane 2, nonadducted (U); lane 3, R,R N²-N² guanine (GGR); lane 4, S,S N²-N² guanine (GGS); −5 primer (lanes 5–8) lane 5, primer alone; lane 6, nonadducted; lane 7, R,R N²-N² guanine; lane 8, S,S N²-N² guanine; +5 primer (lanes 9–12) lane 9, primer alone; lane 10, nonadducted; lane 11, R,R N²-N² guanine; lane 12, S,S N²-N² guanine.

| Modified DNA | DNA sequence | Sequence alteration | % |
|--------------|--------------|-------------------|---|
| GGR          | GAATGGCCATGCCTGAACTCAT | bs               | 83 |
|              | GAATGGCCATGCCTGAACTCAT | d, i             | 17 |
|              | CAATGGCCATGCCTGAACTCAT | bs               | 92 |
| GGS          | GAATGGCCATGCCTGAACTCAT | d                | 8  |

* bs, base substitution (underline); d, deletion (space), i, insert (lower case).
BPDE (0.31 pmol DNA incised/min).

3.5% native polyacrylamide gel. The UvrA-DNA complex formed as the indicated concentrations of protein in the UvrABC buffer in the DNA substrates was conducted by incubating the substrates with gives the order of incision efficiencies: R(0.024) < S,S(0.044) < BPDE (0.31 pmol DNA incised/min). B, binding of the UvrA protein to the DNA substrates was conducted by incubating the substrates with the indicated concentrations of protein in the UvrABC buffer in the absence of ATP at 37 °C for 15 min and then subjected to analysis on a 3.5% native polyacrylamide gel. The UvrA-DNA complex formed as indicated. At 50 nM of UvrA concentration, the binding follows the order: R,R(1.0) < S,S(1.4) < BPDE (14.2% DNA bound).

**DISCUSSION**

Previously, we reported the mutagenic frequencies of butadiene-induced N²-guanine and N²-adenine adducts using the same system described herein (14, 21). Whereas the monoadduct guanine lesions were much more blocking to replication and an order of magnitude more mutagenic, both adducts resulted in a <1% mutagenic frequency with no evidence for deletions. However, this degree of mutagenic potency does not account for the mutagenic frequencies observed in rodent studies (24–29).

Like the guanine monobase adducts, the N²-N² guanine cross-links served as a major block to DNA replication (Fig. 6). Each of the three E. coli polymerases was blocked one base prior to the first adducted guanine. These data are consistent with the in vivo replication studies, which showed a very large decrease in plaque-forming ability (Table I). The severity of replication blockage in vitro corresponds well with that observed in vivo. The fact that some replication bypass can occur in vivo but could not be visualized in vitro may suggest that the intracellular environment possesses other accessory factors lacking in the in vitro reaction, which may aid in replication bypass. Bypass of these lesions may also result in a more error prone replication. Alternatively, the in vitro assays may simply not be sufficiently robust to detect this minor bypass mechanism.

Of the few DNA molecules that could be replicated, the screening of these plaques revealed a mutagenic frequency that was on average an order of magnitude higher than that produced by the guanine monobase adducts. Consistent with the monobase adducts, the S,S stereoisomer was more mutagenic than the R,R stereoisomer. Interestingly, the melting temperatures determined for the adducted 8-mers were somewhat lower for the S stereoisomers compared with the corresponding R stereoisomers for both the monobase adducts and cross-links. These data are indicative of the S stereoisomer being more disruptive to the DNA helix compared with the R stereoisomers.
and may result in a lesion more prone to mutagenic bypass. The mutagenic spectrum for both stereoisomeric forms of the cross-link consists of an array of base substitutions at position 2 of ras codon 12. Each of the possible base substitutions was well represented, and these data are generally consistent with the mono- and diolepoxide N2-guanine adducts. However, the severity of the replication blockage is likely to create in vivo lesions such as paired via a mechanism that repairs other intrastrand cross-links; that is, results in genomic deletions. Previous studies in human lymphocytes and Drosophila indicate that butadiene-induced deletions may be responsible for the carcinogenesis associated with butadiene exposure (30–32). Thus, these data may suggest that the intranstrand G-G cross-links could be a primary butadiene lesion that results in genomic deletions.

In cells that are exposed to activated forms of butadiene, it might be predicted that intranstrand cross-links would be repaired via a mechanism that repairs other intranstrand cross-links such as cis,syn-cyclobutane dimers, 6–4 photoproducts, and bifunctional psoralen cross-linking agents, that system is the nucleotide excision repair pathway. To test whether the cross-linked lesions were subject to this type of repair, these lesions were assayed for both binding and incision by E. coli UvrABC. The R,R and S,S guanine-guanine intranstrand cross-linked DNAs were inefficiently recognized and incised by UvrABC (Fig. 7). These data may imply that intranstrand DNA cross-links arising from butadiene exposure may elude or be poorly detected by the nucleotide excision pathway, thus allowing them to persist in the cell. This may then lead to increased problems for the cell, because neither of the lesions could be bypassed, as measured in vitro. In addition, the in vitro assays revealed a bypass efficiency of less than one in ten thousand. Thus, an increased cellular half-life may ultimately result in an increased mutagenic potential, both for point mutations and deletions, for these lesions.

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J. Russ Carmical, Agnieszka Kowalczyk, Yue Zou, Bennett Van Houten, Lubomir V. Nechev, Constance M. Harris, Thomas M. Harris and R. Stephen Lloyd

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