Intrastrand DNA Cross-Links as Tools for Studying DNA Replication and Repair: Two-, Three-, and Four-Carbon Tethers between the N^2 Positions of Adjacent Guanines†

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ABSTRACT: A general protocol for preparation of oligonucleotides containing intrastrand cross-links between the exocyclic amino groups of adjacent deoxyguanosines has been developed. A series of 2, 3, and 4 methylene cross-links was incorporated site-specifically into an 11-mer (5′-GGCAGGTGGTG-3′, cross-linked positions are underlined) via a reaction between oligonucleotide containing 2-fluoro-O^6-trimethylsilylethyl deoxyinosines and the appropriate diamine (ethylenediamine, 1,3-diaminopropane, 1,4-diaminobutane). These cross-linked-oligonucleotides were studied for their ability to bend DNA by the method of Koo and Crothers [Koo, H. S., and Crothers, D. M. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1763−1767] in which the mobility of ligated oligomers in nondenaturing polyacrylamide gels is evaluated. It was found that all cross-links induced bending (2-carbon cross-link, 30.0 ± 4.0 deg/turn; 3-carbon cross-link, 11.7 ± 1.6 deg/turn; 4-carbon cross-link, 7.4 ± 1.0 deg/turn). Despite the differing extent of helical distortion exhibited by the cross-links, all appeared to be equally blocking to replication by the *Escherichia coli* polymerases, pol I, pol II, and pol III. In contrast, when incision of the cross-links by the *E. coli* UvrABC nucleotide incision complex was studied, the extent of incision of the cross-link was found to correlate closely with the degree of bending measured in the gel mobility assay, i.e., the efficiency of incision was 2-carbon ≫ 3-carbon > 4-carbon.

Covalent DNA lesions play important roles both in the etiology and treatment of cancer. Systematic study of their structure and biological effects, made possible by the development of synthetic strategies to prepare oligonucleotides containing adducts with defined structure, provides valuable information about the process of chemical carcinogenesis. One of the most lethal types of DNA lesions is a DNA cross-link, arising from reaction of a bifunctional electrophile with DNA. Attention has focused primarily on interstrand cross-links, since in such cases damage is done to both DNA strands, resulting in a loss of template information for repair enzymes. However, with new concepts correlating bending of the helix axis of duplex DNA induced by a lesion with altered affinity to DNA-binding proteins, intrastrand cross-links are receiving more attention (*1*−*4*). Bends in the helix axis of DNA, either naturally present as a consequence of DNA sequence or formed as a result of complex formation with proteins, are believed to be functionally important (*5*, *6*). DNA bends are involved in the formation of important DNA−protein complexes in events such as transcription, repair, recombination, and replication (*7*). It seems reasonable to assume that modifications of DNA which change the DNA structure might affect all events depending on proper interaction of DNA with proteins. The best-studied DNA lesion that both bends DNA and alters DNA−protein interactions is cisplatin (*3*, *8*). SRY, an HMG (high-mobility group) testis-specific protein involved in transcriptional regulation, binds to an intrastrand cross-link adduct of cisplatin [d(GpG), the major adduct of cisplatin] and a target sequence with comparable affinity, and it has been hypothesized that the exceptional effectiveness of cisplatin against testicular cancer may be attributed to this phenomenon (*4*). Intrastrand cross-link adducts in general should be good candidates for inducing bends in DNA, which, in turn, might serve as recognition sites for certain DNA-binding proteins and ultimately interfere with DNA function. Rink et al. (*9*) reported that the mitomycin C intrastrand cross-link selectively induces bending of duplex B-DNA, while the monoadduct and interstrand cross-link have no effect on DNA global structure. However, in the case of cisplatin, bending induced by the interstrand adduct is larger than bending caused by the intrastrand cross-link (*10*).

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Another important aspect of adduct-induced bending is the role it may play in repair of DNA damage, i.e., is bending a significant recognition factor for damage repair proteins? One of the most widely studied repair systems is the nucleotide excision repair (NER)[1] complex in *Escherichia coli*; in this system, damage recognition and incision of the damaged region is carried out by the UvrABC nuclease (*11*−*15*). NER is the primary route in that organism for repair of bulky adducts, such as those from polycyclic hydrocarbon dihydrodiol epoxides, but it can also repair a wide variety of other DNA lesions including intrastrand cross-links from cisplatin, photoinduced cyclobutane dimers, and mitomycin dG N^2 cross-links. The common feature of the repairable lesions appears to be that they create some kind of distortion in the DNA, such as a bend or flexible hinge or partial unwinding. Existing synthetic strategies for preparing intrastrand cross-links have been limited mainly to direct reactions of electrophiles with oligonucleotides, making the study of these adducts in sequences containing multiple reactive sites quite challenging, if not impossible. Such strategies include hybridization with a second strand to direct the adduction (*9*) or HPLC separation of multiple adducts (*16*).

We thought that it would be of great interest to examine in a systematic fashion the relationship between DNA bending and protein recognition by preparing oligonucleotides containing a homologous series of simple (i.e., alkyl chains with no substituents) cross-links which could be evaluated for bending by electrophoretic mobility and for protein interactions using several of the *E. coli* DNA polymerases and the UvrABC nuclease.

The nonbiomimetic strategy previously employed in our laboratory for the synthesis of interstrand cross-linked oligonucleotides (*17*) was used to prepare oligonucleotides containing intrastrand cross-links between exocyclic amino groups of adjacent deoxyguanosines. A series of 2, 3, and 4-methylene units was incorporated into an 11-mer (5′-GGCAGGTGGTG-3′, cross-linked positions are underlined), generating model intrastrand cross-links of increasing linker length.

To examine the extent of bending introduced by the cross-links, the empirical method of anomalous gel electrophoretic mobility of ligated oligomers originated by Koo and Crothers (*18*) was employed. This method has been widely used for the detection (*8*, *19*−*24*) and quantitation (*18*) of sequence-induced and adduct-induced bending. When bending elements are repeated in phase with the helical turn, the individual bends add up to a large overall bend, which is reflected in progressively decreased electrophoretic mobility of ligated oligomers. The magnitude of the anomaly in gel mobility is a function of the curvature of the DNA molecule. Bend angles for several bent structures have been determined in this way (*9*, *25*−*29*). For the enzymatic studies, a 60-mer template was created by ligating the cross-linked 11-mers with a 5′ 20-mer and a 3′ 29-mer. The template was used in combination with primers of various lengths to evaluate the ability of three *E. coli* polymerases to replicate past the cross-linked lesions. For the studies with UvrABC nuclease, the 60-mer templates were complexed with the complementary strand, and the extent of incision of the lesions was examined by electrophoresis.

[1] Abbreviations: NER, nucleotide excision repair; TMSE, trimethylsilylethyl; DMTr, 4,4′-dimethoxytrityl, DIEA, diisopropylethylamine; TBE, Tris-borate EDTA; DTT, dithiothreitol; SVPD, snake venom phosphodiesterase; AP, alkaline phosphatase; HRMS, high-resolution mass spectometry, FAB, fast-atom bombardment mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; CGE, capillary gel electrophoresis, PAGE, polyacrylamide gel electrophoresis.

EXPERIMENTAL SECTION

Materials and Methods. Oligonucleotides were prepared on an Expedite 8909 Nucleic Acid Synthesizer using *tert*-butyl-phenoxyacetyl 2-cyanoethyl phosphoramidites and the modified phosphoramidite of 2-fluoro-O^6-trimethylsilylethyl (TMSE)-5′-*O*-dimethoxytrityl (DMTr) 2′-deoxyinosine (*17*, *30*) on a 1 μmol scale. Modified oligonucleotides were cleaved from the solid support and deprotected in 0.1 M NaOH for 16 h at room temperature, followed by neutralization with aqueous acetic acid. Unmodified oligonucleotides were removed from the matrix and deprotected by the standard protocol. Crude material was desalted, purified by reversed-phase HPLC and desalted again. The oligonucleotide of 5′-d(GGCAGGTGGTG)-3′ sequence was purified by 20% denaturing PAGE instead of HPLC. T4 DNA ligase and the pBR322 *Hae* III digest, ATP, snake venom phosphodiesterase (SVPD), and alkaline phosphatase (AP) were obtained from Sigma, and T4 polynucleotide kinase and *Bam*H I linker (10-mer) were from New England Biolabs. Gel loading buffer (6× concentrate) consisted of bromophenol blue (0.25% w/v), xylene cyanole FF (0.25% w/v), and sucrose (40% w/v) in H_2O. Optical density measurements were made on a Hitachi U-3000 spectrophotometer at 260 nm. HPLC purifications were done on a Beckman HPLC (System Gold software, pump module 125, photodiode array detector module 168). Oligonucleotides were desalted on Sephadex G-25 using a Bio-Rad Biologic system. Enzymatic digestion mixtures (0.2−0.5 A_{260} units of oligonucleotide, 20 μL of 0.1 M Tris HCl buffer, pH 9.0, 0.04 unit of SVPD, 0.4 unit of AP) were incubated at 37 °C overnight and subsequently analyzed by HPLC (4.6 × 250 mm YMC ODS-AQ column, Waters Corporation) with the following gradient: (A) 0.1 M ammonium formate, pH 6.4 and (B) CH_3CN, 1−10% B over 15 min, 10−20% B over 5 min, hold for 5 min, and then to 100% B over 10 min at a flow rate of 1.5 mL/min. ^{1}H NMR spectra were recorded at 300.13 and 400.13 MHz on Bruker AC300 and AM400 NMR spectrometers in MeOD-d_4 or DMSO-d_6. Capillary gel electrophoresis was performed on a Beckman P/ACE 5000 system using the manufacturer's ssDNA 100-R columns and Tris-borate-urea buffer; samples were applied at −10 kV and run at −10 kV. MALDI-TOF mass spectra were recorded on a Voyager Elite DE instrument (PerSeptive Biosystems). Negative ion MALDI-TOF mass spectra of adducted oligonucleotides were obtained in a 3-hydroxypicolinic acid (3-HPA) matrix using ammonium hydrogen citrate (7 mg/mL) to suppress multiple sodium and potassium adducts.

*Synthesis and Purification of Intrastrand Cross-linked Oligonucleotides **2a**, **3a**, and **4a***. General procedure: the starting material 5′-d(GGCAXXTGGTG)-3′ (**1**, X = 2-fluoro-O^6-TMSE 2′-deoxyinosine), prepared as previously described (*17*, *30*), was reacted with 1.5 equiv of the appropriate diamine, with the exception of ethylenediamine which required 10 equiv because the adduction reaction was

slow compared to loss of silyl protection, in 0.05 M $Na_2B_4O_7$−NaOH buffer, pH 10 (oligonucleotide concentration 0.8 A_{260} units/μL) for 1−2 days at 45 °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) CH_3CN, 1 to 35% B over 20 min, 35 to 90% 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 20−21 min, uncyclized monoadducts at ∼18 min, and the cross-linked products at 18−20 min.

*2-Carbon Cross-Link (**2a**).* For synthesis of this cross-link, 10 equiv of ethylenediamine were used; the diamine was added in increments over 22 h. At that time 40 additional equiv of ethylenediamine were added, and the reaction mixture was stirred for an additional 3 h. Fully protected cross-linked product **2** was purified on a C8 column (10 × 250 mm, Phenomenex) with the following gradient: (A) 0.1 M ammonium formate and (B) CH_3CN, 10 to 25% B over 5 min, 25 to 30% B over 16 min, 30 to 90% B over 2 min, hold for 4 min, and then to 10% B in 2 min at a flow rate of 3 mL/min; product 2 eluted at 12−13 min. After lyophilization, the TMSE protecting groups were removed by treatment with aqueous acetic acid (pH 3) for 2 h at room temperature. The reaction mixture was then neutralized, lyophilized and repurified by HPLC on a phenylhexyl column (4.6 × 250 mm, Phenomenex) with the following gradient: (A) 0.1 M triethylammonium acetate, pH 7.0, and (B) CH_3OH, 0 to 20% B over 5 min, 20 to 30% B over 30 min, and then 30 to 0% B over 4 min at a flow rate of 1 mL/min; product **2a** eluted at 32−33 min. Mass spectrum: (MALDI-TOF) *m*/*z* calcd for $[M - H]^-$ 3477.6, found 3477.3. Yield: 10 A_{260} units, starting from 47 A_{260} units of the 2-fluorohypoxanthine-containing oligonucleotide **1**.

*3-Carbon Cross-Link (**3a**).* The reaction was completed in 2 days. Fully protected cross-linked product **3** was purified by reversed-phase HPLC as described for **2**; the product **3** eluted at 14−15 min. After lyophilization, the TMSE protecting groups were removed by treatment with aqueous acetic acid (pH 3) for 2 h at room temperature. The reaction mixture was then neutralized, lyophilized, and repurified by HPLC on a C8 column (10 × 250 mm, Phenomenex) with the following gradient: (A) 0.1 M ammonium formate and (B) CH_3CN, 1 to 5% B over 5 min, 5 to 8% B over 20 min, and then 8 to 1% B over 2 min at a flow rate of 3 mL/min, product **3a** eluted at 18−19 min. Mass spectrum: (MALDI-TOF) *m*/*z* calcd for $[M - H]^-$ 3491.6, found 3491.2. Yield: 7 A_{260} units, starting from 47 A_{260} units of the 2-fluorohypoxanthine-containing oligonucleotide **1**.

*4-Carbon Cross-Link (**4a**).* The reaction was completed in 2 days. Fully protected cross-linked product **4** was purified by reversed-phase HPLC as described for **2** except that an analytical C8 column (4.6 × 250 mm, Phenomenex) was used at a flow rate of 1 mL/min; the product **4** eluted at 14 min. After lyophilization, the TMSE protecting groups were removed with aqueous acetic acid (pH 3) for 2 h at room temperature. The reaction mixture was then neutralized, lyophilized, and repurified 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) CH_3CN, 1 to 8% B over 5 min, 8 to 12% B over 15 min, 12 to 90% B over 2 min, hold for 2 min, and then 90 to 1% B over 2 min at a flow rate of 1.5 mL/min; product **4a** eluted at 14−15 min. Mass spectrum: (MALDI-TOF) *m*/*z* calcd for $[M - H]^-$ 3505.6, found 3505.6. Yield: 4 A_{260} units, starting from 31 A_{260} units of the 2-fluorohypoxanthine-containing oligonucleotide **1**.

The HPLC-purified adducted oligonucleotides **2a**, **3a**, and **4a** were desalted (Sephadex G-25) and analyzed by capillary gel electrophoresis (CGE) and enzyme digestion as well as mass spectroscopy to confirm their purity and composition. Purified **2a**, **3a**, and **4a** were combined with excess oligonucleotide 5′-d(CCCACCACCTG)-3′ containing a nine base complementary region and a 2-base overhang at the 5′ end. The resulting duplexes **2b**, **3b**, and **4b** were separated on a hydroxyapatite column with the following gradient: (A) 10 mM phosphate buffer (pH 7.0) and 0.1 M NaCl, (B) 100 mM phosphate buffer (pH 7.0), 0% B for 9 min, 0 to 100% B over 30 min at a flow rate of 1.5 mL/min and subsequently desalted.

Synthesis of Bisnucleoside Standards. General procedure: a solution of the appropriate diamine, 2-fluoro-O^6-TMSE 2′-deoxyinosine and diisopropylethylamine (DIEA) (molar ratio in order mentioned 1:3:2.5) in DMSO was stirred at 55 °C for 30 h. The solvent was removed in vacuo, and the residue was treated with aqueous acetic acid (pH 3) for 8 h to remove TMSE groups. A small amount of methanol was added to the deprotection reaction in order to improve solubility. The reaction mixture was neutralized, lyophilized, and purified by reverse-phase HPLC.

*1,3-Bis(2′-deoxyguanosin-N^2-yl)ethane (**2c**).* A total of 1.9 mg (0.032 mmol) of ethylenediamine, 35.5 mg (0.096 mmol) of 2-fluoro-O^6-TMSE 2′-deoxyinosine, and 10.9 mg (0.08 mmol) of diisopropylethylamine (DIEA) in DMSO (100 μL) afforded after purification 14.8 mg (82%) of **2c**. The reaction mixture was purified by HPLC on a C18 column (250 × 10 mm, YMC ODS-AQ) with the following gradient: (A) H_2O and (B) CH_3CN, 10−20% B over 10 min, 20−21% B over 4 min, 21−100% B over 2 min, hold for 2 min, and then 100−10% B over 2 min at a flow rate of 3 mL/min, the product **2c** eluted at 10−11 min. ^{1}H NMR (DMSO-d_6, 35 °C) δ 7.87 (s, 2H, H8), 6.11 (m, 2H, H1′), 4.32 (m, 2H, H3′), 3.79 (m, 2H, H4′), 3.51 (m, 6H, H5′, H5″, N−CH_2), 2.54 (m, 2H, H2″), 2.18 (m, 2H, H2′). HRMS (FAB^+) calcd for $C_{22}H_{29}N_{10}O_8$ $(M + H)^+$ 561.2169, found 561.2173.

*1,3-Bis(2′-deoxyguanosin-N^2-yl)propane (**3c**).* A total of 1.6 mg of diaminopropane, 25 mg of 2-fluoro-O^6-TMSE 2′-deoxyinosine, and 7.1 mg of DIEA in DMSO (50 μL) afforded after purification 10.6 mg (84%) of **3c**. The reaction mixture was purified by HPLC on a C18 column (250 × 10 mm, YMC ODS-AQ) with the following gradient: (A) H_2O and (B) CH_3CN, 10 to 21% B over 10 min, 21 to 22% B over 4 min, 21 to 100% B over 2 min, hold for 2 min, and then 100 to 10% B over 2 min at a flow rate of 3 mL/min; the product **3c** eluted at 10−11 min. ^{1}H NMR (MeOD-d_6, 40 °C) δ 7.89 (s, 2H, H8), 6.26 (m, 2H, H1′), 4.52 (m, 2H, H3′), 3.98 (m, 2H, H4′), 3.71 (m, 4H, H5′, H5″), 3.52 (m, 4H, CH_2−N), 2.70 (m, 2H, H2″), 2.36 (m, 2H, H2′), 1.95 (m, 2H, C−CH_2−C). HRMS (FAB^+) calcd for $C_{23}H_{31}$,$N_{10}O_8$ $(M + H)^+$ 575.2326, found 575.2334.

*1,3-Bis(2′-deoxyguanosin-N^2-yl)butane (**4c**).* A total of 2 mg of diaminobutane, 25 mg of 2-fluoro-O^6-TMSE 2′-deoxyinosine, and 7.1 mg of DIEA in 50 μL DMSO afforded after purification 8.9 mg (69%) of **4c**. The reaction mixture was purified by HPLC on a C18 column (250 × 10 mm,

YMC ODS-AQ) with the following gradient: (A) H_2O and (B) CH_3CN, 10 to 22% B over 10 min, 22 to 23% B over 4 min, 21 to 100% B over 2 min, hold for 2 min, and then 100 to 10% B over 2 min at a flow rate of 3 mL/min, the product **4c** eluted at 10−11 min. ^{1}H NMR (DMSO-d_6, 45 °C) δ 10.31 (broad s, 2H, N1*H*), 7.84 (s, 2H, H8), 6.34 (m, 2H, N^2*H*), 6.15 (m, 2H, H1′), 5.11 (d, 2H, 3′-OH, J = 3.7 Hz), 4.67 (t, 2H, 5′-OH, J = 5.3 Hz), 4.35 (m, 2H, H3′), 3.81 (m, 2H, H4′), 3.54 (m, 4H, H5′, H5″), 3.33 (m, 4H, CH_2−N), 2.60 (m, 2H, H2″), 2.21 (m, 2H, H2′), 1.60 (m, 4H, C−CH_2−CH_2−C). HRMS (FAB$^+$) calcd for $C_{24}H_{33}N_{10}O_8$ $(M + H)^+$ 589.2483, found 589.2488.

Ligation. The unmodified duplex and cross-linked duplexes **2b**, **3b**, **4b** (1 A_{260} unit each), and *Bam*H 1 linker duplex ([5′-d(CGGGATCCCG)]$_2$, 0.5 A_{260} unit) were independently phosphorylated at their 5′-ends with T4 polynucleotide kinase (10 units) in 20 μL of kinase buffer [70 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT)] and ATP (0.5 mM final concentration). The samples were incubated at 37 °C overnight. A fraction of each kinase reaction (0.05 A_{260} units) was removed and incubated with 3 units of T4 DNA ligase (with the exception of **2b**, which required 10 units of ligase) in 20 μL of 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1.5 mM ATP, 25 μg/mL BSA at 16 °C overnight. Absolute ethanol (1 mL, −20 °C) was added to each ligation mixture, and the ligation products were collected by centrifugation. Recovered material was washed twice with 90% (aq) ethanol solution (1 mL, −20 °C) and dried under vacuum. The samples were redissolved in water, lyophilized, and then mixed with nondenaturing loading buffer (0.02 A_{260} units of ligated material in 6 μL of nondenaturing buffer per lane).

Size Markers. The length marker used was pB*R*322 *Hae* III digest (0.4 μg/μL, 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). A 1-μL aliquot of the digest mixed with nondenaturing loading buffer was used per marker lane.

Electrophoresis. Electrophoresis was performed using a nondenaturing 8% polyacrylamide gel (mono:bis-acrylamide ratio 29:1, 3.3% catalyst). The gels were polymerized for 5 h at room temperature and equilibrated overnight at 4−8 °C. After loading the samples, the gel was run with constant voltage of 1000 V for 2.5−3 h. The gel was stained with ethidium bromide (300 μg) in 1× TBE buffer (250 mL) for 6 min, followed by destaining in 500 mL of 1× TBE buffer for 20 min. The gel was then imaged and photographed on a UV transilluminator (Alpha Innotech, San Leandro, CA).

*Construction of 60-mer Templates Containing Cross-Linked Oligonucleotides **2a**, **3a**, and **4a**.* The adducted 11-mer deoxyoligonucleotides utilized for the bending studies were also used to construct a 60-mer linear template. The template was constructed such that the adducted guanines were approximately centrally located. To construct the template, the 11-mer, a 20-mer 5′ flank and a 29-mer 3′ flank were annealed to a 45-mer scaffold. The 45-mer was synthesized to be complementary to the 11-mer internally and to the respective ends of the flanks, so as to serve as a bridge to facilitate ligation of the individual oligonucleotides. Prior to the annealing reaction, the adducted 11-mer and 29-mer flanking sequences were phosphorylated at the 5′ end. To visualize the 60-mer ligation product and to aid in the purification, the 20-mer flank was phosphorylated with a 1:10 mix of γ ^{32}P-ATP/ATP. Each component was then added in approximately equal molar concentrations and heated to 70 °C for 5 min. Subsequently, the mixture was incubated in an ice slurry for 15 min. T4 DNA ligase (2000 units) was added and the reaction was allowed to proceed overnight at 16 °C. The 60-mer ligation product was then gel purified in order to remove the 45-mer scaffold, as previously described (*31*−*33*).

In Vitro Replication of 60-mer Templates by E. coli Polymerases. Oligodeoxynucleotides were synthesized to serve as primers for replication of the 60-mer templates. 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 prior to the adduct which would simulate a "standing" start. The second positioned the 3′ hydroxyl five 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 in order to affix a 5′-γ-^{32}P-label. Subsequently, each was diluted to a concentration of 50 fmol/μL and added to the 60-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 slow cooled to room temperature. This reaction was carried out in triplicate for each template/primer combination, to challenge each of the three predominant *E. coli* polymerases. The polymerases assayed and suppliers were as follows: large fragment of polymerase I (Klenow exo$^-$) purchased from New England Biolabs, Beverly, MA, polymerase II provided by Drs. M. F. Goodman and L. Bloom, University of Southern California, Los Angeles, CA, and polymerase III supplied by Dr. Mike O'Donnell, Rockefeller University, New York, NY. Finally, the appropriate salts, 1 μM dNTPs and the supplied 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 2-fold molar excess of enzyme to DNA and the reaction was allowed to proceed at room temperature for 10 min. The reaction was stopped by adding an equal volume of loading buffer, consisting of formamide, xylene cyanol, and bromophenol blue. The extension products were then analyzed by electrophoresis through a 15% polyacrylamide sequencing gel and visualized by exposing an autoradiographic film overnight.

Construction of 60-mer Duplex Template for UvrABC Assays. A 60-mer oligonucleotide complementary to the 60-mer cross-linked sequences used in the polymerase replication studies was synthesized and subsequently gel purified as previously described. Prior to annealing, each template was phosphorylated by T4 polynucleotide kinase incorporating a γ-^{32}P-label on the 5′ end. The labeled 60-mer templates were then annealed to the complement in individual reactions. A reaction mix containing a 5-fold molar excess of complement (500 nmol:2500 nmol) and 10 mM Tris-HCl, pH 7.5, 0.1 μM EDTA was heated to 65 °C and allowed to cool slowly to room temperature. The duplex was then gel purified on a 10% native-polyacrylamide gel.

Nucleotide Incision of the 60-mer Duplexes. The aforementioned, double-stranded 60-mer substrates (5 nM) were

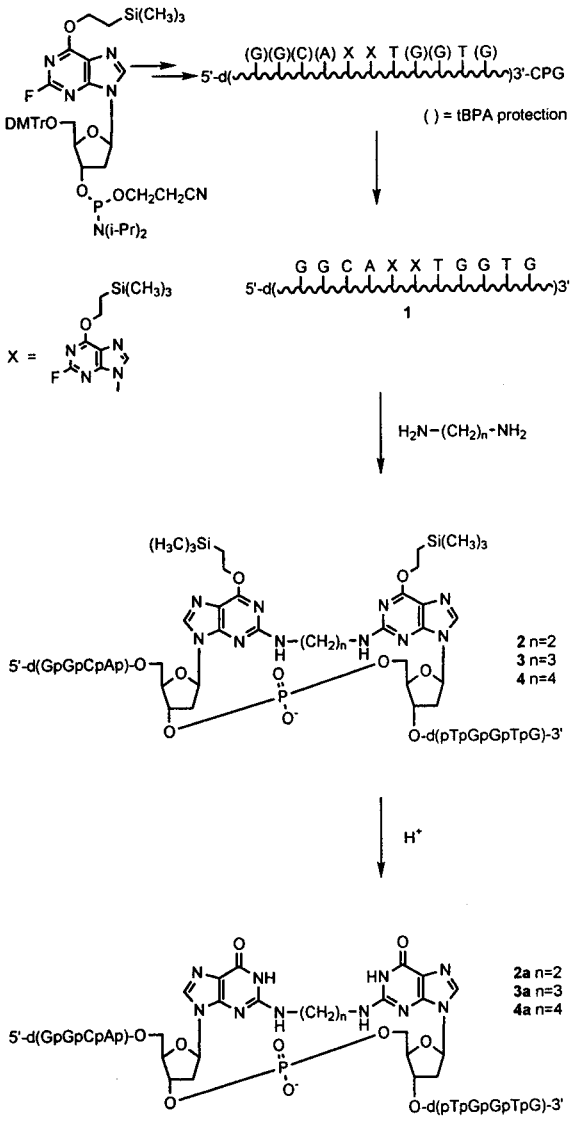

FIGURE 1: Scheme for synthesis of 11-mer oligonucleotides containing 2, 3, and 4-carbon tethers between adjacent guanines.

incubated with UvrABC proteins (UvrA, 10 nM, UvrB, 250 nM, and UvrC, 50 nM) at 37 °C for 30 min in the presence of UvrABC reaction buffer (50 mM Tris-HCl, pH 7.8, 50 mM KCI, 10 mM $MgCl_2$, 5 mM DTT, 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 TBE buffer. The gel was dried and exposed to an X-ray film and a PhosphorImaging screen (Molecular Dynamics) for quantification.

RESULTS AND DISCUSSION

Synthesis of the Adducts. Intrastrand cross-links were synthesized in a direct reaction between modified oligonucleotide **1**, containing two activated nucleosides, and a series of aliphatic diamines as shown in Figure 1 for the synthesis of 11-mer, 5′-GGCA-GG-TGGTG-3′. The progress of the reaction was monitored by HPLC. The diamines reacted with the modified oligonucleotides to form mono-

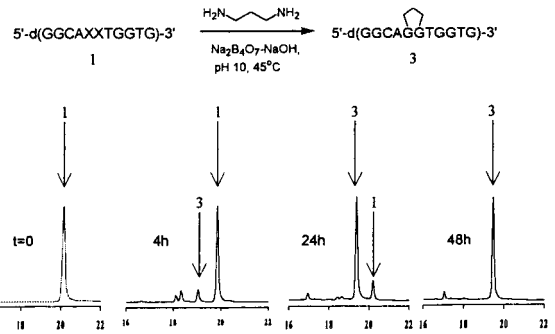

FIGURE 2: Time course of the cross-linking reaction to form the 3-carbon cross-link **3** as followed by HPLC.

adducts, which on further reaction gave cross-linked products. The processes occurred simultaneously until the precursors (unadducted oligonucleotides and monoadducts) were consumed. Fully protected (i.e., O^6-TMSE groups on both guanines), starting material, and products of the reaction were well-separated on HPLC as shown in Figure 2. The portions of the HPLC traces shown contain only fully protected oligonucleotides. However, when one or both of the trimethylsilylethyl protecting groups were lost during the course of reaction, components of the reaction mixture could not be resolved by HPLC; therefore, the cross-linked products had to be purified with both trimethylsilylethyl protecting groups on and then deprotected. Slow loss of the trimethylsilylethyl group occurred during the course of reaction. The unadducted activated nucleoside was more susceptible to this cleavage than the already adducted nucleoside. In the case of shorter oligonucleotides (e.g., an 8-mer) it was possible to use an organic solvent, such as DMSO, as the reaction medium; the trimethylsilylethyl group was more stable under these conditions (results not shown) than in borate buffer. The instability of the TMSE group in the alkaline buffer was not anticipated and future syntheses will use a more stable O^6 protecting group such as *p*-nitrophenethyl (*34*, *35*).

Since formation of the intrastrand cross-link is a two-step event that proceeds via a monoadduct with the second step being an intramolecular process, we initially thought that the ratio of diamine to oligonucleotide was not crucial for successful cross-linking. It was assumed that once the monoadduct was formed, the formation of intrastrand cross-link would be more favorable than adduction of the second diamine molecule at the other activated nucleoside. However, it was found that the outcome of the reaction strongly depended on the ratio of the reactants (Figure 3). If a large excess of diamine was used, each activated nucleoside was adducted independently forming a bis-monoadduct. When 1.2−1.5 equiv of diamine vs oligonucleotide was used, the main product was the desired intrastrand cross-link. Formation of the 2-carbon cross-link required a greater excess of diamine (10 equiv) because the initial adduction reaction was slower than in the case of the more nucleophilic 3-carbon and 4-carbon diamines.

In the case of the 2-carbon cross-link, the cross-linked oligonucleotide eluted at the same retention time as the monoadducts. However, the bis-monoadduct eluted earlier, so a large excess of ethylenediamine was added at the end of the reaction to convert the small amount of remaining monoadducts coeluting with the cross-linked species into the bis-monoadduct byproduct. This procedure allowed cross-

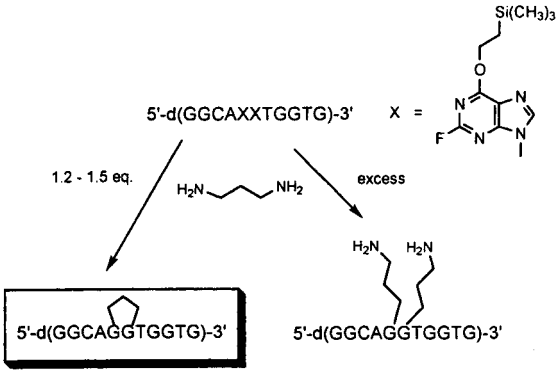

FIGURE 3: Scheme illustrating the requirement for careful control of stoichiometry during synthesis of intrastrand cross-linked oligonucleotides **2a**, **3a**, and **4a**.

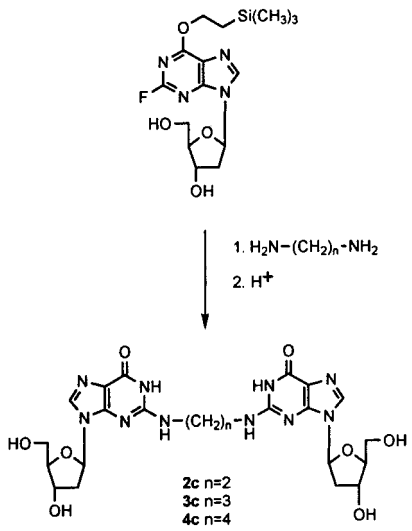

FIGURE 4: Synthesis of bis(2′-deoxyguanosine) nucleosides cross-linked at N^2 through 2, 3, and 4-carbon linkers (**2c**, **3c**, and **4c**).

linked oligonucleotide to be obtained free of monoadduct contamination. It was found that the presence of ammonium formate, used as an HPLC buffer during purification of the starting material, suppressed formation of the cross-link, leading to a mixture of monoadducts and bis-monoadduct. The diamines are stronger bases than ammonia, resulting in a monoprotonation of the diamines in the presence of ammonium ion, which renders the protonated amino group unreactive. For characterization of the adducted oligonucleotides, enzymatic digestion to individual nucleosides was employed, followed by identification of the adducts via HPLC coelution with authentic standards **2c**, **3c**, and **4c**, which were synthesized (Figure 4) and characterized independently. Nucleoside composition was quantified by dividing each peak area from the HPLC profile of the enzyme digest by the appropriate molar extinction coefficient (*36*). The molar extinction coefficients for bisnucleosides **2c**, **3c**, and **4c** were estimated to be twice that of deoxyguanosine. Mass spectra (MALDI-TOF) were obtained to further characterize the adducted oligonucleotides.

Comparison of melting profiles of a series of 8-mers (5′-CAT-GG-TCC-3′), prepared similarly, showed that the 4-carbon cross-linked oligomer did not destabilize the duplex at all (T_m = 40 °C vs 40 °C for unadducted), whereas both the 3-carbon (T_m = 34 °C) and 2-carbon oligomers (T_m = 32 °C) were significantly destabilizing.

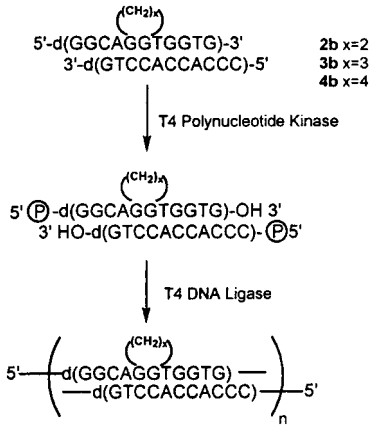

FIGURE 5: Complementary 11-mer duplexes containing 2, 3, and 4-carbon intrastrand cross-links; the duplexes were ligated to form multimers whose bending was evaluated by nondenaturing PAGE.

Table 1: Curvature of Cross-linked Oligonucleotides **2a**, **3a**, and **4a** Calculated from Electrophoretic Mobility of Ligated Double-stranded Oligomers

cross-linked DNA	curvature[a] (deg/lesion)
2a	30.0 ± 4.0
3a	11.7 ± 1.6
4a	7.4 ± 1.0

[a] Bend angles are reported as per lesion in an 11-mer repeat.

Bending Studies. The initial goal of synthesizing oligonucleotides with defined intrastrand cross-links having been accomplished, evaluation of the bending induced in duplexes containing these linkers was undertaken by the method of Koo and Crothers (*18*). This method is based on the principle that linear DNA migrates faster upon electrophoresis in a nondenaturing gel than does rigidly curved DNA. The DNA samples for electrophoresis were prepared by annealing cross-linked 11-mers **2a**, **3a**, and **4a** (in individual experiments) with an 11-mer containing a nine nucleotide complementary region and a 2-base overhang; the duplexes were phosphorylated on the 5′ end with T4 polynucleotide kinase followed by ligation with T4 DNA ligase to yield a mixture containing oligomers of different lengths, 22 bp, 33 bp, etc. (Figure 5). The length of the oligonucleotides, 11 bases, was chosen so that in the multimers there would be one adduct per helical turn [10.5 bp for B-DNA (*27*)], i.e., the adducts would be in identical spatial contexts such that if bending occurred it would be additive or in phase. The mobility of the ligated oligomers was analyzed by nondenaturing polyacrylamide gel electrophoresis (Figure 6). Multimers formed by ligation of a 10-bp *Bam*H 1 linker, known to display normal electrophoretic mobility, were used to calculate the apparent length of the adducted oligomers using an equation relating the migration distance of the unknown versus that of the *Bam*H 1 multimers. The length of *Bam*H 1 oligomers was calibrated using enzyme digests of plasmid pBR322 DNA which contain fragments of known composition. Relative mobilities (R_L) were calculated as a ratio of apparent length and actual length and plotted against the actual length of multimer (Figure 6). Unmodified oligonucleotide was also ligated and analyzed to exclude any sequence-directed bending. It exhibited normal mobility (R_L = 1). The angle of absolute curvature (Table 1) was estimated using the empirical relationship derived by Koo and Crothers which

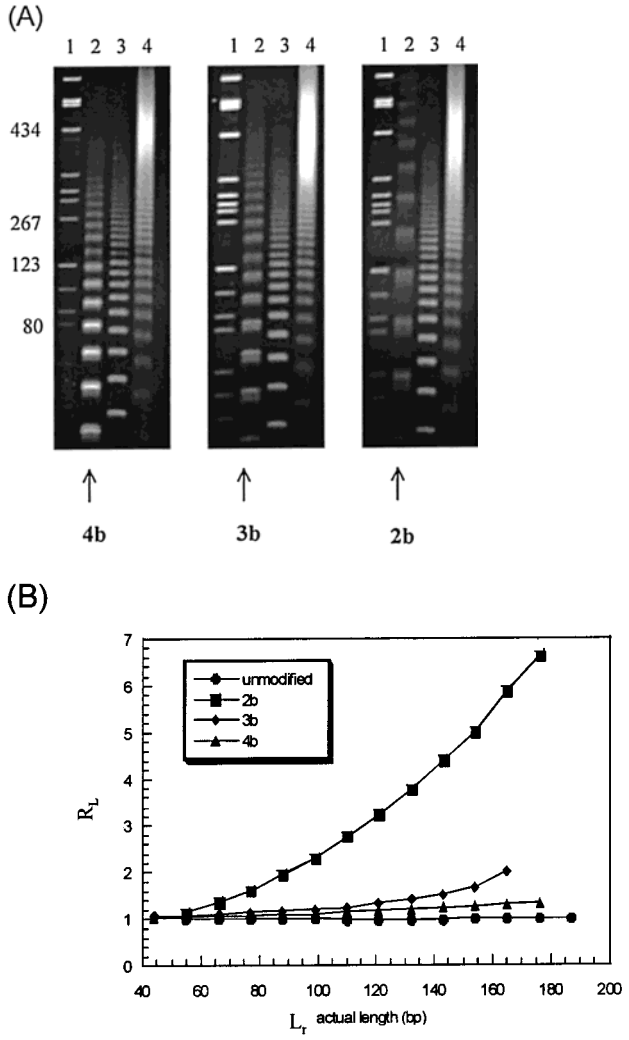

FIGURE 6: (A) Ligation ladders. Lane 1, pBR322 *Hae* III digest; lane 2, cross-linked multimer; lane 3, *Bam*H 1 linker; lane 4, unmodified multimer. (B) Relative mobility of cross-linked DNA as a function of actual length.

is considered valid only where $R_L \geq 1.2$ in the region $120 \leq L_r \leq 170$ bp (R_L is the ratio of apparent to actual length and L_r is the actual length of the multimer). Even though our experiment shows that the relative mobilities increase with the length of the multimers, which suggests that the bends are being amplified as a consequence of coherent in-phase addition, the phase relation between the sequence length and helical repeat may not be perfect, resulting in less than maximum retardation. Therefore, the measured curvature values should be considered minima.

A reasonable correlation can be made between the curvature determined for the duplex containing the 4-carbon tether (**4b**) and the mitomycin C intrastrand cross-link studied by Rink et al. (*9*). Both cross-links have the same number of carbon atoms (4) between the N^2 positions of adjacent deoxyguanosines; the curvature induced by the mitomycin C cross-link ($14.6 \pm 2.0°$/lesion) is greater than the $7.4 \pm 1.0°$/lesion reported in this paper for **4b**; however, both deformations are relatively small. It is not surprising that mitomycin causes a little more bending because its tether is conformationally constrained. This comparison supports the hypothesis that easily synthesized site-specific and stable cross-links with methylene linkers can be used as models of more complex cross-links, at least with respect to bending ability. The 3-carbon tether may be an even better model for the intrastrand N^2 dG-dG cross-links of acrolein detected by Kawanishi et al. (*37*); such cross-links were postulated to be responsible for the high level (12%) of tandem base substitutions observed at G−G sites in the *supF* gene when fibroblast cells were transfected with acrolein-treated *supF* shuttle vector plasmids.

*In Vitro Replication of 60-mer Templates Containing **2a**, **3a**, and **4a**.* DNA templates (60-mers) containing the lesions or an unadducted site were constructed as shown in Figure 7 and primed for synthesis using −1, −5, and +5 primer DNAs. As expected, the −1, −5, and +5 primers were successfully extended by all three *E. coli* polymerases to full-length product when utilizing the unadducted 60-mer template (Figure 8, lane 2). However, when the templates containing the guanine-guanine cross-links were utilized and primed by the −1 primer, no synthesis could be detected using any of the major *E. coli* polymerases (Figure 8, lanes 3−5 for Pol I, II, or III). Extension assays using the −5 primer showed major blockage of all three polymerases one nucleotide prior to the first guanine of the cross-link and that only Pol I was able to incorporate a base opposite the first guanine (Figure 8, lanes 8−10); no incorporation was seen opposite the second guanine. In the case of the +5 primer, where the 3′ hydroxyl is beyond the site of the adduct, all three polymerases were able to extend the primers to full-length products (Figure 8, lanes 13−15). Because these adducts did not allow for incorporation of a nucleotide off the −1 primer, we were unable to determine which base was incorporated adjacent to the adduct. However, at higher than physiological concentrations of dNTPs, Klenow fragment of polymerase I was able to incorporate a guanine opposite the first adducted guanine (data not shown).

It is perhaps not surprising that the polymerases cannot successfully bypass these lesions since the methylene tethers, although quite flexible, may severely inhibit the ability of the attached nucleosides to achieve the correct conformation at the active site of the polymerase. For many polymerases, interactions with the minor groove of the template DNA, in which these cross-links lie, appear to be critical for proper replication (*38*).

UvrABC Incision of 2, 3, and 4-Carbon Intrastrand Cross-Linked Substrates. Nucleotide excision repair is one of the major cellular defense systems which removes a large variety of structurally diverse lesions. DNA repair is an important modulating factor in mutagenesis, since a rapid removal of DNA lesions in cells could lead to the reduction of their mutagenic potentials. Individuals with genetic defects in excision repair are well-known to be at increased risk for certain types of cancer. The question of how the enzymes involved in repair recognize damaged DNA has intrigued scientists for some time (*11*−*15*). It has been suggested that there are at least two stages to the damage recognition process for the UvrABC complex (*39*, *40*). Initial recognition is by $UvrA_2$/$UvrA_2B$ which binds to the damaged DNA; however, binding to the UvrA protein does not necessarily correlate with efficiency of incision (*41*). A second stage of damage recognition occurs after DNA strand opening and is performed by UvrB/UvrBC (*39*). As the DNA is unwound at the damage site, UvrB binds and kinks the single-stranded region toward the major groove. The $UvrA_2$ dimer is released, UvrC is bound to the UvrB−DNA complex and

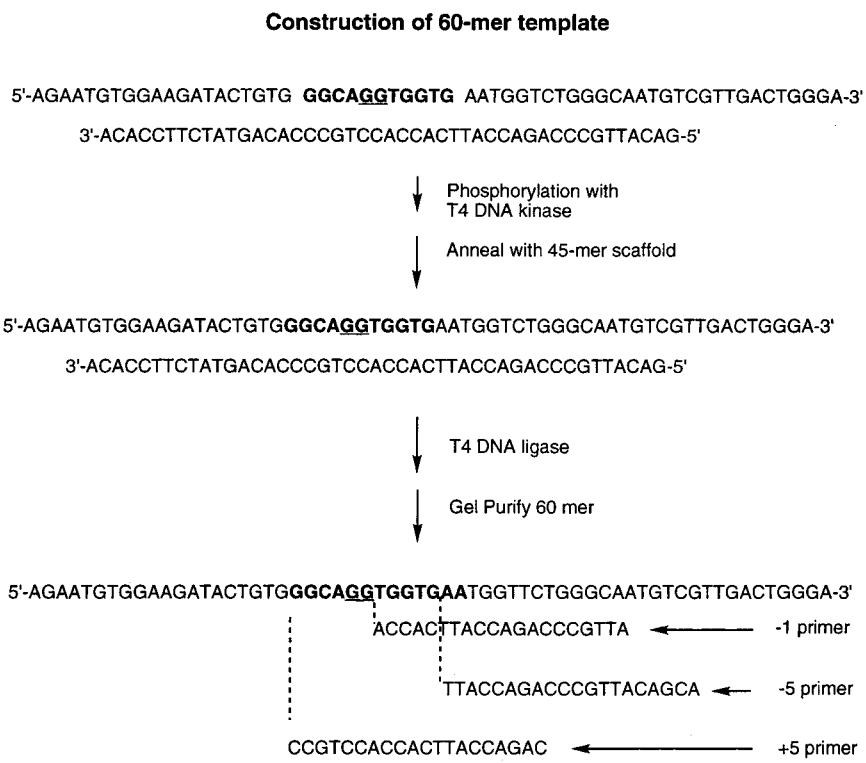

FIGURE 7: Construction of the 60-mer templates containing 2-carbon, 3-carbon, and 4-carbon cross-linked 11-mers, **2a**, **3a**, and **4a**.

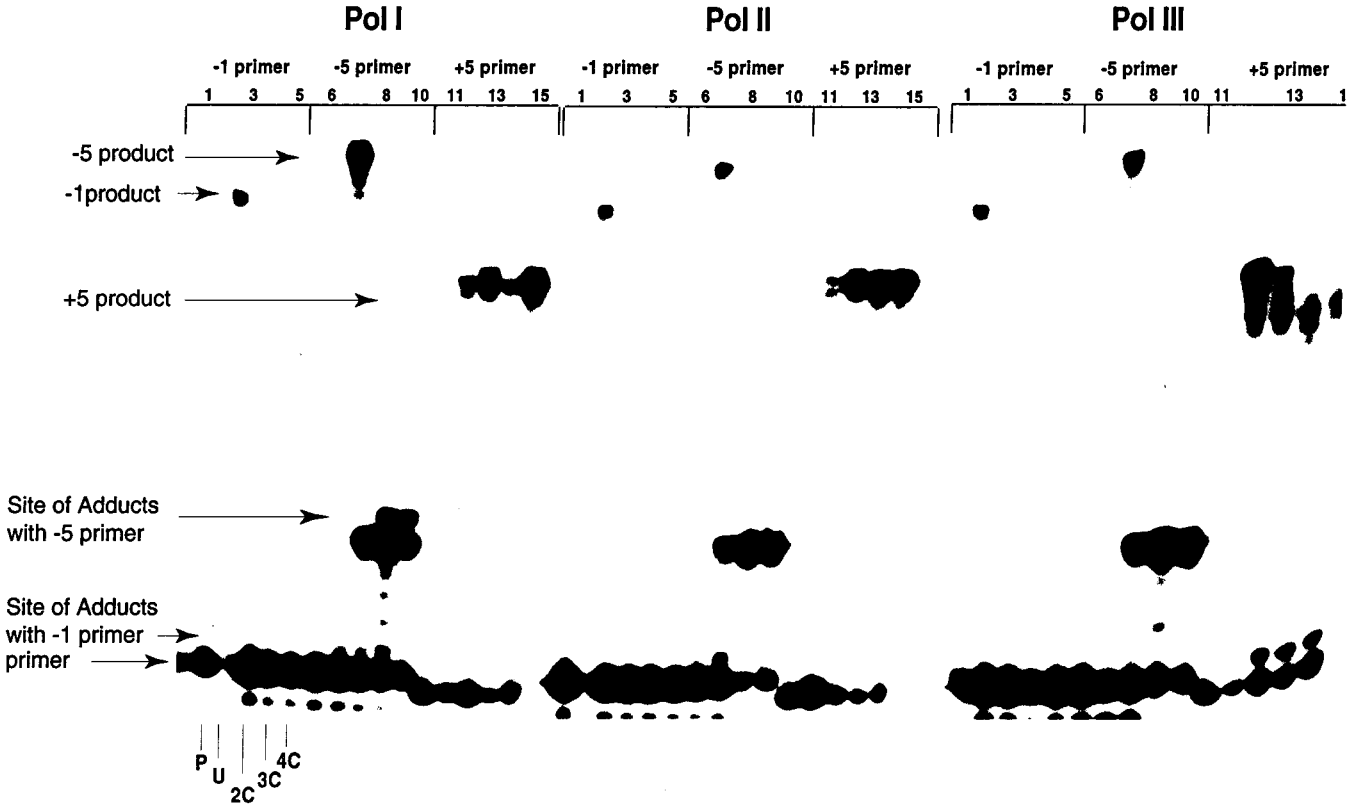

FIGURE 8: Primer extensions of the cross-linked-containing templates. Each of the templates (unadducted, 2-C, 3-C, and 4-C cross-links) was annealed to one of 3 primers (−1 primer, −5 primer, and +5 primer). Each possible template-primer combination was extended with each of three polymerases *(E. coli* Pol I, II, and III) and the products electrophoresed through a polyacrylamide gel. **−1 primer** (lanes 1−5): 1, primer alone; 2, nonadducted; 3, 2-C cross-link; 4, 3-C cross-link; 5, 4-C cross-link. **−5 primer** (lanes 6−10): 6, primer alone; 7, nonadducted; 8, 2-C cross-link; 9, 3-C cross-link; 10, 4-C cross-link. **+5 primer** (lanes 11−15): 11, primer alone; 12, nonadducted; 13, 2-C cross-link; 14, 3-C cross-link; 15, 4-C cross-link.

the two proteins carry out excision at the fourth, fifth, or sixth bond 3′ to the adduct and at the eighth bond 5′ to the lesion, releasing 12−14-mer oligonucleotides.

It is generally agreed that the UvrABC complex recognizes helical distortion. To examine the question of how sensitive the nuclease is to minor changes in structure and degree of

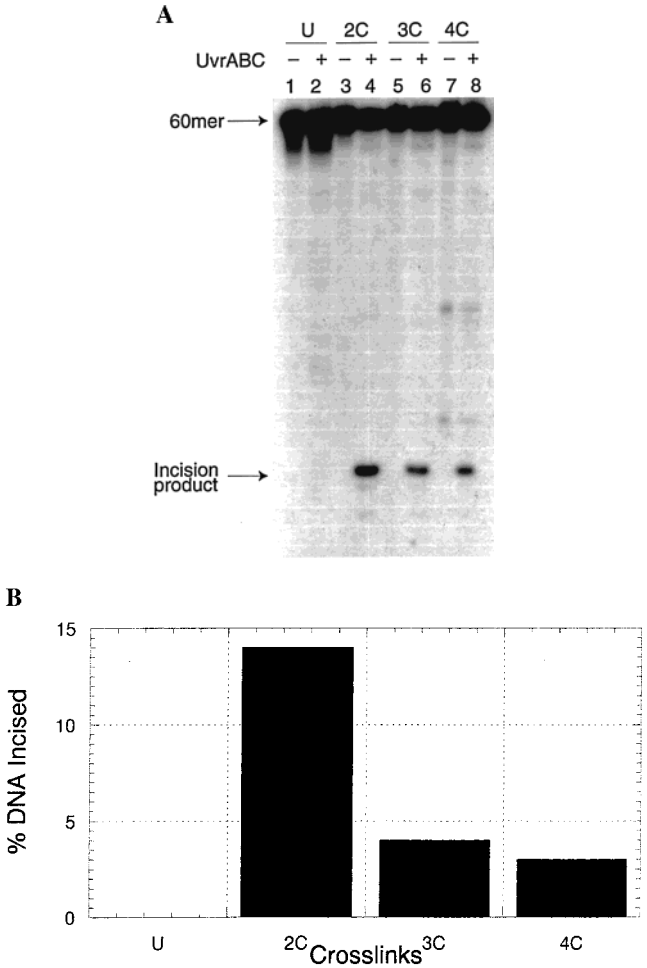

FIGURE 9: (A) UvrABC incision assay. Lane (1) 60-mer duplex, (2) 60-mer duplex reacted with UvrABC, (3) 2-C 60-mer, (4) 2-C 60-mer reacted with UvrABC, (5) 3-C 60-mer, (6) 3-C 60-mer reacted with UvrABC, (7) 4-C 60-mer, (8) 4-C 60-mer reacted with UvrABC. (B) Percentage of DNA substrate incised versus substrate.

bending, the 60-mer templates used for the experiments with the *E. coli* polymerases were annealed with the complementary strand and incubated with the exinuclease. The extent of incision was evaluated by polyacrylamide gel electrophoresis. As shown in Figure 9, the ability of UvrABC nuclease to recognize and incise the lesions is directly related to the degree of bending induced by the linker. Although these lesions were not incised as well as a control BPDE-damaged DNA, it is evident that they are potential, albeit poor, substrates for nucleotide excision repair. Incidental to a recent study of the mutagenicity of two other 4-carbon intrastrand N^2 dG-dG cross-links it was found that they also were poorly incised by UvrABC (*43*); however, nothing is known about bending induced by these tethers. In the present study the incision efficiency was found to follow the order: 2-C $\gg$ 3-C > 4-C lesions with the incision of the 2-carbon cross-link being ~5-fold more efficient than the 4-carbon cross-link (Figure 9) indicating that the amount of helical perturbation dictates the efficiency of adduct incision and recognition by UvrABC nuclease. These findings concur with previous reports on the mechanisms responsible for UvrABC-substrate recognition (*40*, *42*).

Although the present study focused on the relationship of DNA bending to polymerase bypass and excision repair, such cross-linked oligonucleotides should be equally useful for investigating other repair pathways, such as recombination, which are known to be operative in intrastrand cross-link repair (*43*). It would also be interesting to see if the simple cross-linked oligonucleotides can interact with other DNA-binding proteins in such a way as to impair their normal function, such as the cisplatin intrastrand cross-links do with the SRY protein or with the human 3-methyladenine DNA glycosylase (*44*). In the latter case the cross-linked adducts bind but are not cleaved, thus tying up the protein and preventing it from repairing its normal targets, such as 3-methyladenine, 7-methylguanine, and 1,N^6-ethenoadenine.

The synthetic strategy described herein can be easily extended to a wide repertoire of intrastrand cross-links, varying in size, shape, rigidity, and functionality. These tailor-made molecules should be valuable tools for studying DNA structure and DNA interactions with other biological molecules.

SUPPORTING INFORMATION AVAILABLE

HPLC traces of enzyme digestion mixtures of **2a**, **3a**, and **4a**; ^{1}H NMR spectra of **2c**, **3c**, and **4c**; CGE electropherograms of **2a**, **3a**, and **4a**; MALDI-TOF spectra of **2a**, **3a**, and **4a**. This material is available free of charge via the Internet at http://pubs.acs.org.

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