DNA Cross-Links Hot Paper

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A Simple, High-Yield Synthesis of DNA Duplexes Containing a Covalent, Thermally Cleavable Interstrand Cross-Link at a Defined Location**

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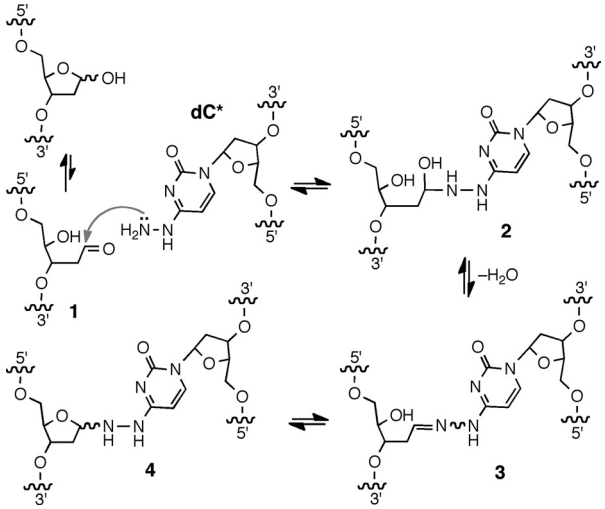
Abstract: *Interstrand DNA–DNA cross-links are highly toxic to cells because these lesions block the extraction of information from the genetic material. The pathways by which cells repair cross-links are important, but not well understood. The preparation of chemically well-defined cross-linked DNA substrates represents a significant challenge in the study of cross-link repair. Here a simple method is reported that employs "post-synthetic" modifications of commercially available 2′-deoxyoligonucleotides to install a single cross-link in high yield at a specified location within a DNA duplex. The cross-linking process exploits the formation of a hydrazone between a non-natural N^4-amino-2′-deoxycytidine nucleobase and the aldehyde residue of an abasic site in duplex DNA. The resulting cross-link is stable under physiological conditions, but can be readily dissociated and re-formed through heating–cooling cycles.*

The sequence of nucleobases on the interior of the DNA double helix constitutes the genetic code that guides all cellular operations.[1,2] Interstrand cross-links (ICLs) are highly toxic to cells, because these lesions prevent the strand separation that is required to extract genetic information from DNA.[3] ICLs are important in diverse fields, including medicinal chemistry, toxicology, and gerontology. For example, ICLs generated by some antineoplastic drugs selectively kill rapidly dividing cancer cells.[3a] ICL formation may contribute to the cytotoxic and carcinogenic effects of various pyrrolizidine alkaloids.[3d] In addition, circumstantial evidence suggests that the formation of some as-yet unidentified endogenous DNA cross-links may drive cellular senescence and aging.[3b,4]

The complex multistep processes by which cells repair cross-links are important, but not well understood.[5] The preparation of chemically well-defined cross-linked DNA substrates represents a significant challenge in the study of cross-link repair. Treatment of duplex DNA with cross-linking agents typically generates mixtures of structurally distinct ICLs in low yields.[3b,6] As a result, laborious purification is required to obtain duplexes containing a single, structurally defined ICL. Alternatively, elegant multistep chemical syntheses of cross-linked duplexes have been developed,[7] but these methods may not be practicable in some biochemistry and biology laboratories, where the study of cross-link repair takes place.

Here we report a very simple method for the generation of cross-linked DNA duplexes in high yields. Our approach employs inexpensive reagents to carry out "post-synthetic" modification of commercially available 2′-deoxyoligonucleotides and installs a single cross-link at a specified location within a DNA duplex. This work was inspired by our recent studies of imine-derived interstrand cross-links generated through the reaction of abasic sites with opposing guanine and adenine residues in duplex DNA.[8] The cross-linking process described here exploits hydrazone formation between the non-natural nucleobase, N^4-amino-2′-deoxycytidine (dC*), and the aldehyde residue of an abasic (Ap) site **1** located on opposing strands of an oligomeric DNA duplex (Scheme 1). Our previous work with the native nucleobases[8] and examination of Ap-containing duplex structures[9] suggested that these reaction partners would be well positioned when embedded within a 5′-ApA/5′-C*A sequence (Figure 1). We elected to mispair the dC* residue, with the expectation that greater dynamic freedom might facilitate cross-linking.

The 2′-deoxyoligonucleotide **B**, which contains a single dC* residue, was prepared by treatment of the corresponding cytosine-containing oligonucleotide **A** with bisulfite (0.3 M)



Scheme 1. Formation of dC*-Ap cross-link in duplex DNA.

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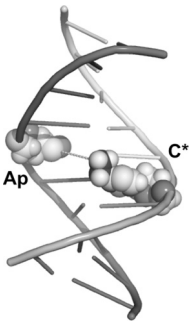
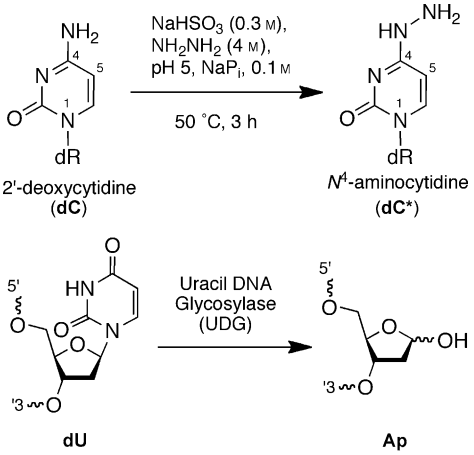


Figure 1. Molecular model illustrating the juxtaposition of the dC* and Ap residues in a DNA duplex containing a 5′-ApA/5′-C*A sequence.

and hydrazine (4 M) in sodium phosphate (0.1 M, pH 5) for 3 h at 50 °C (Scheme 2 A; see also Figure 2). This protocol mirrors the conditions developed by Hayatsu and co-workers for the preparation of the N^4-amino-2′-deoxycytidine nucleoside[10] and adapted by Gao and Orgel for use with 2′-deoxyoligonucleotides.[7c] The 5′-^{32}P-labeled, Ap-containing oligonucleotide **E** was prepared by treatment of the corresponding 5′-^{32}P-labeled 2′-deoxyuridine-containing oligonucleotide **D** with the enzyme uracil DNA glycosylase (UDG, Scheme 2 B).[11] Efficient formation of the Ap-containing oligonucleotide **E** was confirmed by treatment of the DNA with warm piperidine to generate the faster-migrating 3′-phosphate cleavage product **F** (Figure 3, lane 3).[12]

The complementary oligonucleotides **B** and **E** were combined in sodium phosphate (50 mM, pH 5) containing



Scheme 2. Synthesis of dC*- and Ap-containing oligonucleotides.

A 5′-GGTGTGAGGGAGTGAGTGACATTGGAGAGTGTGTT

B 5′-GGTGTGAGGGAGTGAGTGAC*ATTGGAGAGTGTGTT

C 5′-GGTGTGAGGGAGTGAGTGATATTGGAGAGTGTGTT

D 5′-^{32}P-AACACACTCTCCAAdUATCACTCACTCCCTCACACC

E 5′-^{32}P-AACACACTCTCCAAApATCACTCACTCCCTCACACC

F 5′-^{32}P-AACACACTCTCCAA-OPO_3^{2-}

G
5′-^{32}P-AACACACTCTCCAAAp A ATCACTCACTCCCTCACACC
3′-TTGTGTGAGAGGTT A C*TAGTGAGTGAGGGAGTGTGG

H
5′-^{32}P-AACACACTCTCCAAAp A ATCACTCACTCCCTCACACC
3′-AGGAGAATTGTGTGAGAGGTT A C*TAGTGAGTGAGGGAGTGTGG

I
5′-^{32}P-AACACACTCTCCAAAp A ATCACTCACTCCCTCACACCAAGAGGA
3′-TTGTGTGAGAGGTT A C*TAGTGAGTGAGGGAGTGTGG

J
5′-AACATCAAAAp A TTAACACAAA
3′-TTGTAGTTT A C*AATTGTGTTT

K 5′-AACTCAAApAATCCT
3′-TTGAGTT A CTAGGA

L 5′-AACTCAAAp A ATCCT
3′-TTGAGTT A C*TAGGA

Figure 2. DNA sequences used in this study.

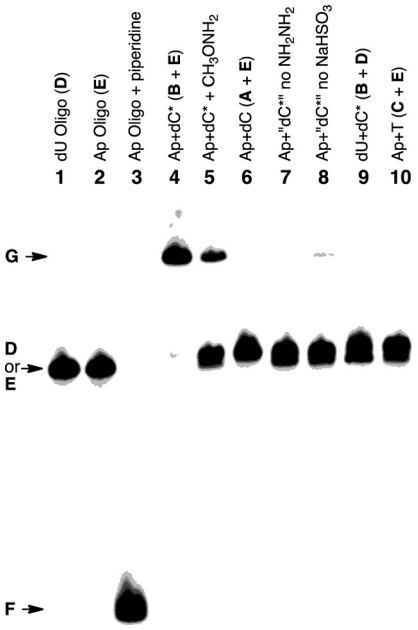


Figure 3. Generation of an interstrand dC*-Ap cross-link in duplex **G**. Complementary oligonucleotides in sodium phosphate buffer (50 mM, pH 5) containing NaCl (100 mM) were briefly warmed to 50 °C and then incubated at room temperature (23 °C) for 5 h. The samples were mixed with formamide loading buffer and subjected to electrophoretic analysis on a 20% denaturing polyacrylamide gel (0.4 mm thick). The labeled fragments in the gel were visualized and quantitatively measured by phosphorimager analysis. Lane 1: size marker consisting of the ^{32}P-labeled dU-containing oligonucleotide **D**; Lane 2: the Ap-containing 2′-deoxyoligonucleotide **E**; Lane 3: the Ap-containing oligonucleotide **E** treated with piperidine (1 M, 95 °C, 30 min); Lane 4: generation of cross-linked duplex **G** from the complementary oligonucleotides **D** + **E**; Lane 5: **D** + **E** in the presence of CH_3ONH_2·HCl (2 mM); Lane 6: **A** + **E**; Lane 7: **A** + **E**, when hydrazine was omitted from the C* protocol; Lane 8: **A** + **E**, when bisulfite was omitted from C* the protocol; Lane 9: **B** + **D**; Lane 10: **C** + **E**, where oligo **C** was subjected to the C*-forming protocol.

NaCl (100 mM) at 50 °C, and allowed to stand at room temperature for 5 h. Electrophoretic analysis of the resulting labeled DNA on a 20% denaturing polyacrylamide gel showed a very high yield (90 ± 2%) of a slowly migrating band in the region of the gel where the cross-linked duplex **G** would be expected[8a–c] to appear (Figure 3, lane 4). Inclusion of the aldehyde-capping reagent methoxyamine (2 mM)[13] in the reaction significantly inhibited the formation of the slowly migrating band (85% decrease in yield), consistent with involvement of the Ap aldehyde residue in the generation of this product (Figure 3, lane 5). No slowly migrating band was generated when the ^{32}P-labeled Ap-containing oligonucleotide **E** was incubated with the native dC-containing oligonucleotide **A** (Figure 3, lane 6) or when hydrazine was omitted from the protocol used for preparation of the dC*-containing oligonucleotide **B** (Figure 3, lane 7). When bisulfite was omitted from the dC* preparation protocol, a weak slowly migrating band (≈8%) was generated (Figure 3, lane 8). Combination of the dC*-containing oligonucleotide **B** with the dU-containing oligonucleotide **D** did not produce a slowly migrating band (Figure 3, lane 9). Likewise, treatment of a thymine-containing control oligonucleotide **C** (lacking the

critical C residue) with bisulfite and hydrazine, followed by combination with the Ap-containing oligonucleotide **E**, did not produce a slowly migrating band (Figure 3, lane 10).

Taken together, the results provided evidence for the central roles of both the dC* and Ap residues in the generation of the slowly migrating band observed on the gel. Furthermore, combination of noncomplementary oligonucleotides containing the dC* and Ap residues did not produce detectable amounts of a slowly migrating band (Figure S1, Supporting Information). This provided evidence that an enforced proximity of the dC* and Ap residues within the DNA duplex was required for the generation of the slowly migrating band seen in Figure 3. Importantly, gel electrophoretic analysis showed that the putative cross-linked duplex **G** was completely stable under physiological conditions (37 °C, 16 h, pH values of 5, 7, or 8, Figure S2, Supporting Information).

We next conducted experiments to establish that the slowly migrating band seen in Figure 3 was, in fact, the cross-linked duplex **G**. First, addition of a seven-nucleotide overhang to the 3′ end of either oligonucleotide **B** or **E** resulted in further retardation in the gel mobility of the slowly migrating band (Figure 4). These results provided evidence that the slowly migrating bands observed in Figure 4 were, indeed, cross-linked duplexes **G**, **H**, and **I** containing two full-length strands. Second, ESI-TOF mass spectrometric analysis of the cross-linked duplex **J** (Figure S3) showed strong signals matching the isotope envelope expected for the isobaric hydrazone cross-links **3** or **4** (molecular formula $C_{410}H_{517}N_{151}O_{245}P_{40}$).[14]

We also examined the ability of the dC*-Ap cross-link to stabilize a 15-nucleotide DNA duplex against thermal melting. Toward this end, we first characterized melting of the Ap-containing duplex **K** using UV/Vis spectrometry. This duplex displayed a melting temperature of 32 ± 0.6 °C (1 °C min^{-1}, 1 μM duplex, in 50 mM sodium phosphate, pH 5, 100 mM NaCl, Figure 5A). The cooling curve for **K** closely matched the melting curve, as expected for a reversible DNA melting–hybridization process. Duplex **L**, containing the dC*-Ap cross-link, showed a marked increase in the melting transition, with an apparent T_m of 64 ± 1 °C at a heating rate of 1 °C min^{-1} (Figure 5A). Interestingly, the cooling curve for duplex **L** did not mirror the melting curve and instead closely resembled the annealing curve for the Ap-containing duplex

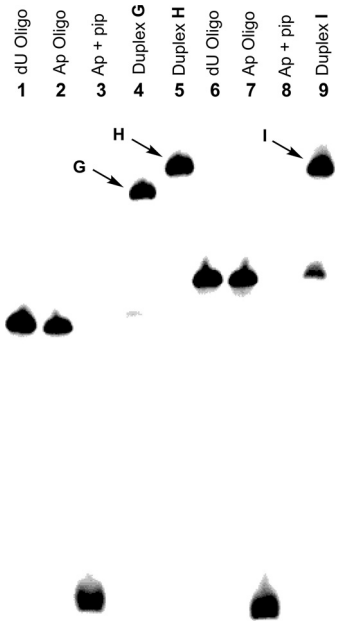


Figure 4. Evidence that the slowly migrating band is an interstrand DNA cross-link containing both full-length oligonucleotides. Complementary oligonucleotides in sodium phosphate buffer (50 mM, pH 5) containing NaCl (100 mM) were briefly warmed to 50 °C and then incubated at room temperature (23 °C) for 5 h. The samples were mixed with formamide loading buffer and subjected to electrophoretic analysis on a 20% denaturing polyacrylamide gel (0.4 mm thick). The labeled fragments in the gel were visualized and quantitatively measured by phosphorimager analysis. Lane 1: dU-containing oligonucleotide **D**; Lane 2: Ap-containing oligonucleotide **E**; Lane 3: Ap-containing oligonucleotide **E** treated with piperidine (1 M, 95 °C, 30 min); Lane 4: cross-linked duplex **G**; Lane 5: cross-linked duplex **H**; Lane 6: size marker consisting of the ^{32}P-labeled, dU-containing oligonucleotide precursor component of duplex **I**; Lane 7: Ap-containing oligonucleotide component of duplex **I**; Lane 8: Ap-containing oligonucleotide component of duplex **I** treated with piperidine (1 M, 95 °C, 30 min); Lane 9: cross-linked duplex **I**.

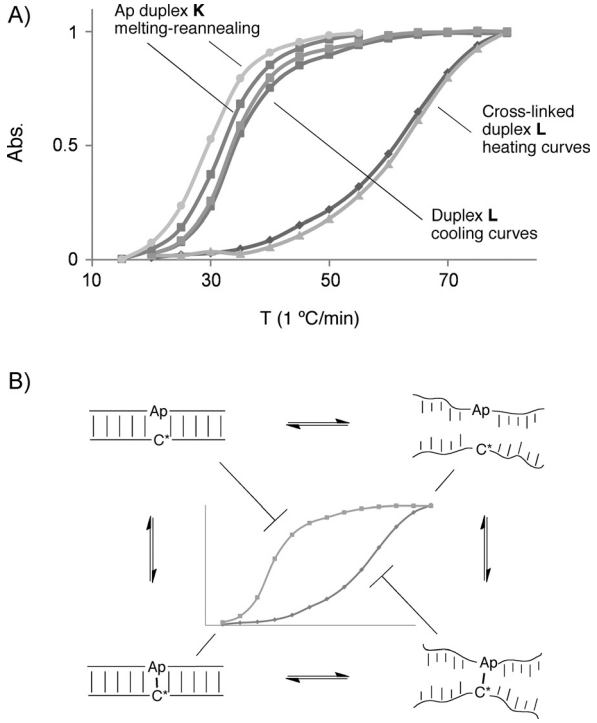


Figure 5. Thermal cycling of the dC*-Ap cross-link. A) Melting–annealing cycles of the cross-linked duplex **L** and the Ap-containing duplex **K**. The DNA duplexes (≈1 μM) were heated in sodium phosphate buffer (50 mM, pH 5) containing NaCl (100 mM) at a rate of 1 °C min^{-1} and absorbance was monitored at 260 nm. The melting curve of the Ap-containing duplex **K** is the one second from left (T_m 32 ± 0.6 °C) and the annealing curve for **K** is the one farthest left (T_m 28 ± 0.6 °C). The first heating curve for cross-linked duplex **L** is fifth from left (T_m 64 ± 1 °C), the first cooling curve for **L** is fourth from left (T_m 33 ± 1 °C), the second heating curve is sixth from left, and the second cooling curve is third from left. B) Schematic diagram of species proposed to be present at various times during the melting and annealing of duplex **L**.

K. This suggested that the hydrazone cross-link in duplex **L** hydrolyzed during the melting process (Figure 5B). Re-melting the sample after cooling produced a melting curve that closely matched the original melting curve, indicating that the cross-link in duplex **L** was regenerated during the cooling/annealing process. A second round of cooling gave a curve that again closely matched the annealing curve of the Ap-containing duplex **K**. Consistent with the notion that the heating curve observed for duplex **L** (Figure 5) reflects cross-link hydrolysis alongside duplex melting, the rate of heating exerted a significant effect on the apparent melting temperature, with T_m values of 56 ± 2 °C and 64 ± 1 °C, observed at heating rates of 0.5 °C min^{-1} and 1 °C min^{-1}, respectively. Taken together, the data are consistent with the presence of a covalent, but thermally cleavable cross-link in duplex **L** (Figure 5B). The covalent, cleavable nature of the dC*-Ap cross-link is strongly reminiscent of the "post-it type" interstrand cross-links, such as the one that employs reversible imine formation, recently described by Carell and co-workers.[7a–c,o,n]

In conclusion, we have developed a fast, simple, and inexpensive protocol for the installation of a single interstrand cross-link at a well-defined location in duplex DNA. The cross-link possesses sufficient chemical stability under physiologically relevant conditions for use in biochemical assays and structural studies.[15] In addition, we provided evidence that the dC*-Ap cross-link stabilizes duplex DNA against thermal melting and can reversibly traverse a thermal cycle involving hydrolysis of the hydrazone linkage during heating/melting, followed by regeneration of the cross-link during cooling/annealing. This type of covalent, thermally cleavable cross-link may find applications in both biochemistry and materials science.[7a,15,16]

Keywords: abasic sites · DNA cross-links · hydrazones · nucleic acids

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