

**ON THE FORMATION OF A NEW FULL-LENGTH DNA
WITH NON-CANONICAL BACKBONE BY SPERMINE
MEDIATED STRAND CLEAVAGE AT ABASIC (AP) SITE
IN DUPLEX DNA**

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In Partial Fulfillment
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Master of Science

By
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December 2024

The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

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Presented by Md Selim Mahbub

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ABSTRACT

Abasic (Ap) sites are among the most prevalent DNA lesions, resulting from the loss of a nucleobase. These sites exhibit chemical reactivity and can lead to strand cleavage via β -elimination, which is catalyzed by various factors, including biological amines. In this study, we investigate the products formed during spermine-mediated strand cleavage at Ap sites. Spermine, a naturally occurring polyamine, facilitates the formation of a reactive α,β -unsaturated iminium ion intermediate (3'ddR-Sp⁺) at the strand break. This intermediate reacts with exocyclic amine groups of nucleobases to produce unique low-molecular-weight interstrand crosslinks (LMW ICLs).

In this thesis, we report the discovery of a novel "re-ligated" product formed through the 1,4-Michael addition of guanine's exocyclic amine group to the iminium ion intermediate. The ligated product exhibits remarkable stability under physiological conditions and resists cleavage by most human DNA repair enzymes, including APE1 and Fpg, but is effectively processed by bacterial endonuclease IV. Our findings highlight the biochemical consequences of spermine-mediated Ap site cleavage and reveal a previously unrecognized DNA lesion with potential implications for genomic stability and cellular repair mechanisms. These results provide critical insights into the reactivity of Ap sites and their repair pathways, advancing our understanding of DNA damage and its biological consequences.

Chapter 1

Cross-links derived from Abasic (Ap) sites in Cellular DNA

1.1 Introduction

DNA is the blueprint of life. It is found in nearly all living organisms and is important for carrying the genetic instructions necessary for the development, functioning, and reproduction of life¹. Structurally, DNA is a double helix, consisting of two intertwined strands that form a ladder-like structure (Figure 1.1). Both strands are made of sugar phosphate backbone and four nitrogenous bases. In the double helix the bases are paired in a specific way². This complementary base pairing allows each strand of the DNA double helix to serve as a template for the creation of a new, identical strand. It guarantees that the genetic code is faithfully passed from parent to offspring, maintaining the integrity of hereditary traits.

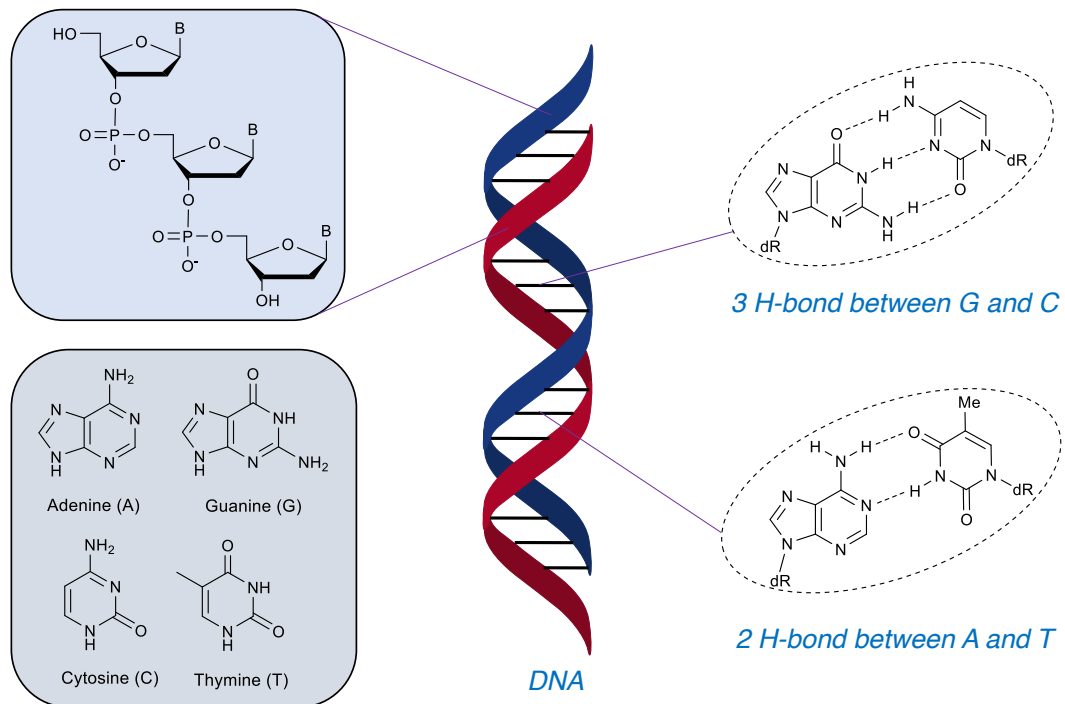


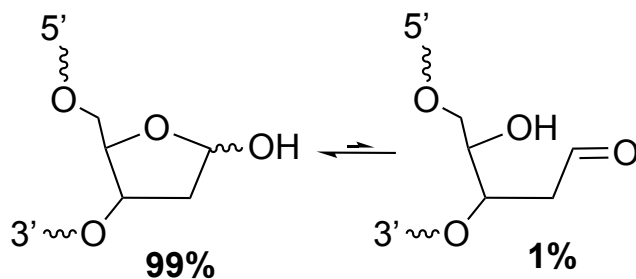
Figure 1.1: DNA double helix

Moreover, base pairing is crucial for DNA repair mechanisms, ensuring that any errors or mutations can be identified and corrected. This molecular blueprint directs the synthesis of proteins, which are essential for cellular structure and activity, making DNA central to the continuity of life. To maintain the genomic stability DNA needs to be stable to the highest degree. Damage to cellular DNA can result in various consequences, such as mutations, cellular dysfunction, cell death (apoptosis), cellular aging (senescence), and increased risk of cancer³⁻⁶. Despite this, DNA is subject to damage by both endogenous and exogenous factors like reactive oxygen species (ROS), lipid peroxidation products, radiation or chemicals⁷.

1.2 Abasic (Ap) site formation in DNA

Abasic sites are the most frequent type of DNA lesion in cells, arising when a nitrogenous base is lost from the DNA backbone⁸. Ap sites can form by spontaneous hydrolysis of N-glycosidic bonds, which link the nitrogenous bases to the DNA, or by the loss of damaged bases generated by endogenous and exogenous factors such as radiation, oxidative stress⁹. Ap sites can also form in base excision repair pathway by DNA glycosylases, in which mis-paired or incorrect nucleobases are removed as a part of DNA damage response(DDR)¹⁰⁻¹². The absence of a base at the Ap site represents loss of coding information in the DNA structure, which can lead to mutations if not properly repaired¹³⁻¹⁵. In addition, Ap sites are chemically reactive and exist as an equilibrium mixture, with the ring-closed hemiacetal form making up 99% and the ring-opened aldehyde form constituting 1% (Scheme 1.1)¹⁶. Ring open aldehyde form of the Ap site can participate in harmful biochemical reactions with number of reagents in the cell because of its high

reactivity¹⁷. Failure to address Ap sites can lead to the formation of inter-strand crosslinks (ICLs) or interfere with DNA replication and transcription, ultimately compromising genomic integrity and contributing to the development of genetic diseases, including cancer¹⁸⁻²¹.

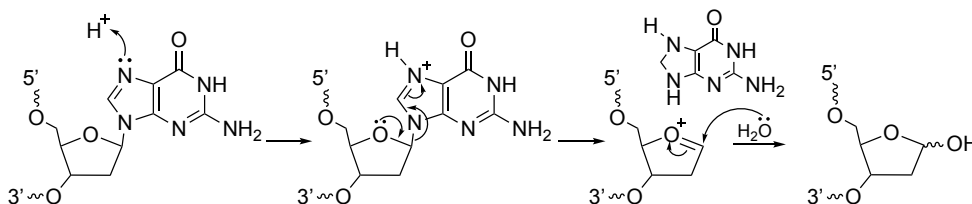


Scheme 1.1: Ring closed and Ring open form of Ap site in DNA

1.2.1 Spontaneous depurination of nucleobase

The N-glycosidic bond, that links the nucleobases to DNA backbone, is the weakest bond in DNA²². Spontaneous hydrolysis of the N-glycosidic bond in DNA is a chemical process of breaking the bond that connects the nitrogenous base to the DNA (Scheme 1.2)^{16, 23}. Although DNA is stable, with the half-lives of N-glycosidic bond hydrolysis in purines and pyrimidines being approximately 730 years and 14,700 years respectively. Despite these low rates, spontaneous hydrolysis still generates around 10,000 abasic sites in each cell each day^{10, 16, 24}. The rate of depurination is approximately 3.0×10^{-11} nucleotides per second, while the rate of depyrimidination is 1.5×10^{-12} nucleotides per second in physiological condition^{25, 26}. Among the purine bases, the depurination of guanine occurs at a rate one and a half times faster than that of adenine²⁷. The rate of spontaneous hydrolysis can increase under certain conditions, such as acidic or oxidative environments,

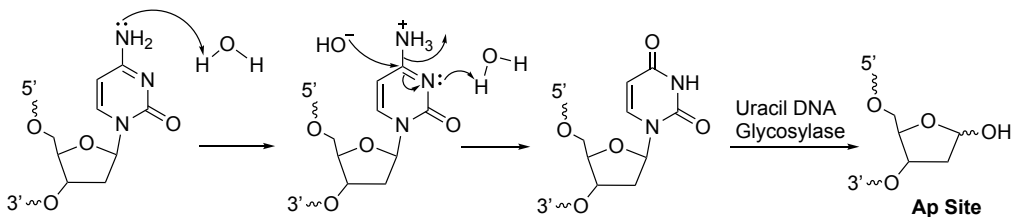
making the process a significant source of endogenous DNA lesion that cell must address to maintain genomic integrity²⁸⁻³⁰. The rate of depurination in single-stranded DNA is four times faster than in double-stranded DNA³¹.



Scheme 1.2: Formation of Ap sites through spontaneous depurination of nucleobase

1.2.2 Ap site formation as an intermediate in Base Excision Repair (BER) Pathway

In base excision repair (BER) pathway, Ap sites form as an intermediate^{11, 32}. In this process, damaged or mis-incorporated bases are recognized and removed enzymatically by specific DNA glycosylases^{11, 33-34}. For example, most common pathway for the formation of abasic sites in cellular DNA involves the removal of mis-incorporated uracil by Uracil DNA glycosylase (UDG) (Scheme 1.3)³⁵. The formation of uracil in DNA occurs primarily through the spontaneous deamination of cytosine³⁶. In this process, the amine group on cytosine is replaced by a carbonyl group, converting cytosine into uracil³⁷. DNA polymerase can also mis-incorporates uracil in place of thymine in DNA³⁸⁻⁴⁹.

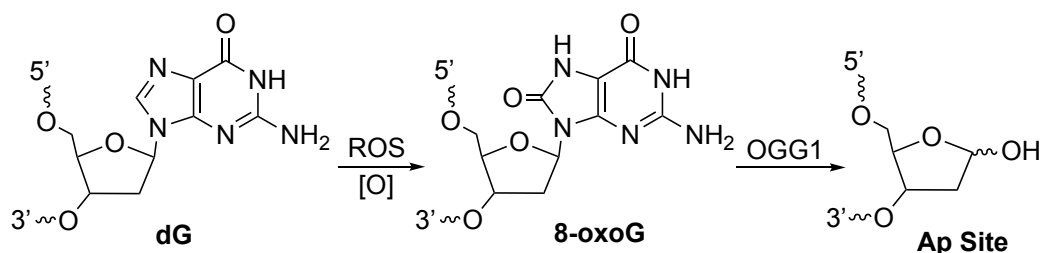


Scheme 1.3: Conversion of Cytosine to Uracil residue and generation of Ap site

Different types of damaged bases are removed by specific DNA glycosylases. For instance, 8-oxoGuanine and FapyGuanine are recognized and removed by OGG1⁴⁰⁻⁴². Additionally, ring opened purines, and oxidized pyrimidines are removed by NTH1 and NEIL1/NEIL2/NEIL3 generating Ap sites⁴³. This controlled enzymatic process is crucial for preventing mutations and maintaining the overall stability of the genome.

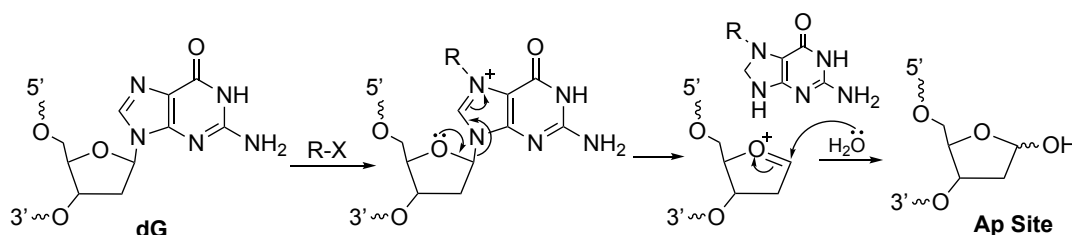
1.2.3 Hydrolysis of damaged bases by Endogenous and Exogenous factors

The hydrolysis of damaged nucleobases in DNA can be triggered by both endogenous and exogenous factors, leading to compromised genomic integrity. Endogenous factors including reactive oxygen species (ROS), spontaneous chemical reactions, and metabolic by-products generated within the cell can damage nucleobase and form Ap site^{22, 44-45}. These factors can lead to the hydrolytic deamination of cytosine into uracil or the oxidation of guanine into 8-oxoguanine (8-oxoG) and FapyG, making base modifications that may result in the formation of abasic sites (Scheme 1.4)⁴⁶.



Scheme 1.4: Formation of 8-oxoG from guanine residue and then Ap site by reactive oxygen species (ROS)

Exogenous factors, including ionizing radiation, UV radiation, and exposure to environmental toxins or chemicals such as alkylating agents can also contribute to the generation of abasic sites through increased rate of hydrolytic loss of nucleobases^{22, 47-49}. For example, methylation at the N7 site of a guanine residue can increase the spontaneous loss of the guanine base from the DNA. Alkylating agents can alkylate specific endocyclic nitrogens of nucleobases. Among all sites, N7-guanine (N7G) is the most vulnerable site for alkylation because N7G is the most nucleophilic site in DNA (Scheme 1.5)⁵⁰.

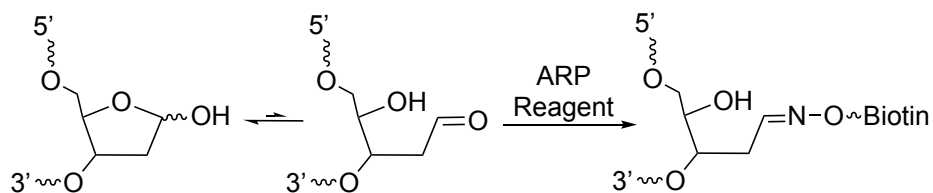


Scheme 1.5: Damage to the nucleobase and formation of Ap sites by alkylating agent

1.3 Detection of Ap site in cellular DNA

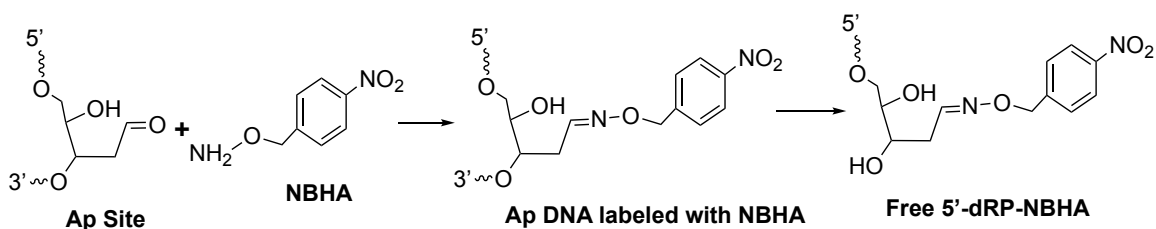
Ap sites, in which a DNA base is missing but the sugar-phosphate backbone remains intact, are the most frequent types of DNA lesion in cell⁵¹⁻⁵². If left unrepaired, Ap site can disrupt DNA replication and transcription, potentially leading to mutations and genomic instability⁵³⁻⁵⁵. Consequently, detecting Ap site is essential to understanding DNA lesions and their repair mechanisms, as well as for assessing the effects of environmental and chemical genotoxins. Multiple methods exist for detecting abasic sites in the cell. For instance, the comet assay allows for detection of Ap sites by reacting with alkali to induce strand cleavage⁵⁶. A widely used technique to detect the lesion in cellular DNA is the

reaction of these sites with hydroxylamine derivatives. ^{14}C -labeled methoxyamine can also be utilized for detection of Ap site⁵⁷⁻⁵⁹. The aldehyde-reactive probes (ARPs) can react covalently with the aldehyde form of abasic site in DNA forming Ap-ARP adducts which then can be detected by several techniques (Scheme 1.6)⁶⁰⁻⁶¹. These probes are often biotin-labeled, allowing for the detection of Ap sites via fluorescence or colorimetric assays⁶¹.



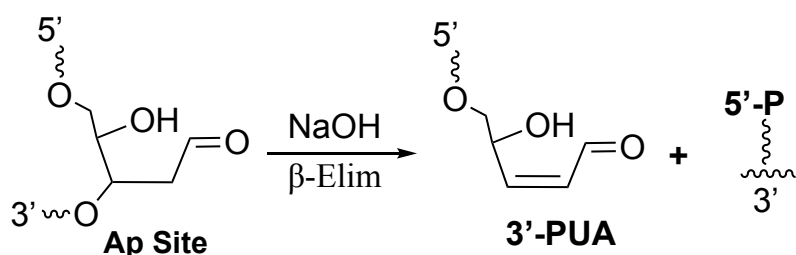
Scheme 1.6: Aldehyde reactive probe (ARP) based detection of Ap site

Although ARP is designed to detect aldehyde form of abasic site in DNA, it can also react covalently to other aldehyde that may present in the system making this technique less accurate⁶². To avoid the issue, Turesky and his colleagues developed an advanced method that utilizes the LC-MS spectrometry to detect O-4-nitrobenzylhydroxylamine (NBHA) pre-labeled Ap site via sophisticated HPLC-ESI-MS/MS instrument (Scheme 1.7)⁶³. This method selectively detects the Ap site in DNA samples.



Scheme 1.7: Detection of Ap sites by LC-MS spectrometry method

Ap sites in DNA can also be detected by treating the Ap-DNA with alkali (a strong base), which induces single-strand break at the Ap site, enabling detection through methods such as gel electrophoresis⁶⁴. For example, Sodium hydroxide (NaOH) can be utilized to react with abasic (Ap) sites in DNA that induces strand break generating a phosphoryl group at the 5'-side (5'-P) and a trans- α,β -unsaturated aldehyde (3'-PUA), or 3'-P or 3'dR at the 3'-side of the strand break, which then can be visualized by polyacrylamide gel electrophoresis (PAGE) analysis (Scheme 1.8)⁶⁵.



Scheme 1.8: Detection of Ap site by alkali in comet assay

1.4 Abasic (Ap) site Repair Pathway

An abasic (Ap) site occurs when a nitrogenous base (purine or pyrimidine) is removed or lost from the DNA backbone, leaving an empty sugar-phosphate backbone⁵¹. The repair of Ap site in DNA is vital to ensure the proper function of cells⁶⁶⁻⁶⁷. Among the

various repair mechanisms, the base excision repair (BER) pathway is the most prominent. The repair of Ap site is further carried out by other pathways, such as nucleotide excision repair (NER) and translesion synthesis (TLS), in cases where BER alone may be insufficient⁶⁸⁻⁶⁹.

1.4.1 Base Excision Repair (BER) Pathway

In duplex DNA, the Base Excision Repair (BER) pathway is one of the most critical repair mechanisms that addresses small damages in DNA⁷⁰⁻⁷³. BER pathway detects and removes DNA lesions in a series of steps. The BER pathway begins with a DNA glycosylase, that detects and eliminates the damaged or mis-incorporated bases in the DNA double helix to generating an Ap site^{11, 74}. Then, the enzyme Ap endonuclease (APE1) hydrolyzes the phosphodiester bond on the 5'-side of the Ap site to generate 3'-OH and 5'-deoxyribosephosphate (5'-dRP) ends with a one nucleotide gap in the DNA³². After the incision, the nicked DNA can be further repaired by either short patch or long patch BER pathway (Figure 1.2)⁷⁵. Short patch BER involves the removal of 5'-dRP end group by the dRP-lyase activity of DNA polymerase β (Pol β), which has separate 5'-dRP lyase activity and polymerase activities⁷⁶. Pol β removes the 5'-dRP group by its dRP-lyase activity, leaving behind a "clean" 5'-Phosphate group that is suitable for ligation⁷⁷. Polymerase β then adds appropriate nucleotide in the 3'OH side based on the complementary strand⁷⁸. Finally, the remaining strand break is stitched-up by the DNA ligase-III in conjunction with the scaffold protein XRCC1⁷⁹. In long patch BER, DNA polymerase β , δ , or ϵ (Pol $\beta/\delta/\epsilon$) incorporate 2-10 nucleobases that leave the 5'dRP end group in an overhanging flap⁸⁰. Then the flap endonuclease 1 (FEN-1) removes the flap,

leaving 3'-OH group that is ready for the ligation⁸¹. In the final step, sealing the nicked DNA is carried out by DNA Ligase-I to get repaired DNA⁸⁰.

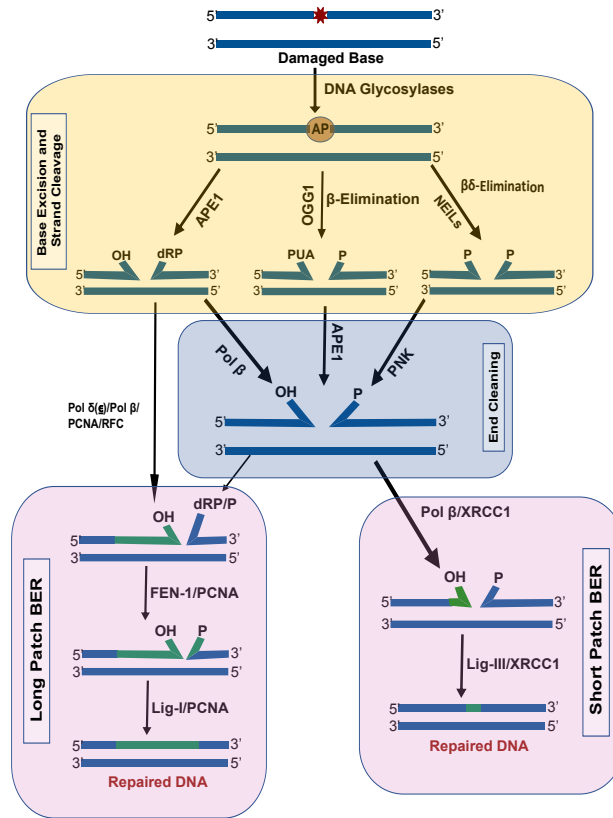


Figure 1.2: Repair of abasic site by BER Pathway

1.4.2 Nucleotide Excision Repair (NER) Pathway

The NER pathway is a critical DNA repair system that addresses bulky lesions that are caused by UV-induced thymine dimers or chemical damage⁸². Unlike other repair mechanisms that focus on specific base alterations, NER removes large, helix-distorting lesions from the DNA⁸³. In this pathway, protein such as XPC recognize the distortions in the DNA double helix⁸⁴. Once the lesion is recognized, a repair complex is brought to the

damage site. This includes the transcription factor TFIIH, which unwinds the DNA strands surrounding the lesion by its helicase activity, which gives DNA repair enzyme the access to the damaged strand⁸². Then endonuclease XPF-ERCC1 and XPG cuts the DNA strand from the either side of the lesion and removed the damaged bases leaving a gap in the DNA strand⁸⁵. Next, the DNA polymerase (Pol δ , ϵ and/or κ) incorporates the correct bases by copying the information from intact complementary strand. In the final step, nicked in the damaged strand is ligated by DNA ligase I-FEN-1 or the ligase III- XRCC1 complex (Figure 1.3)⁸⁶. The steps of NER are similar across all organisms, but it operates through two distinct mechanisms: global genome (GG-NER) repair, which addresses damage throughout the entire genome, on the other hand transcription-coupled (TC-NER) repair, which specifically targets and repairs actively transcribed DNA strands⁸⁷.

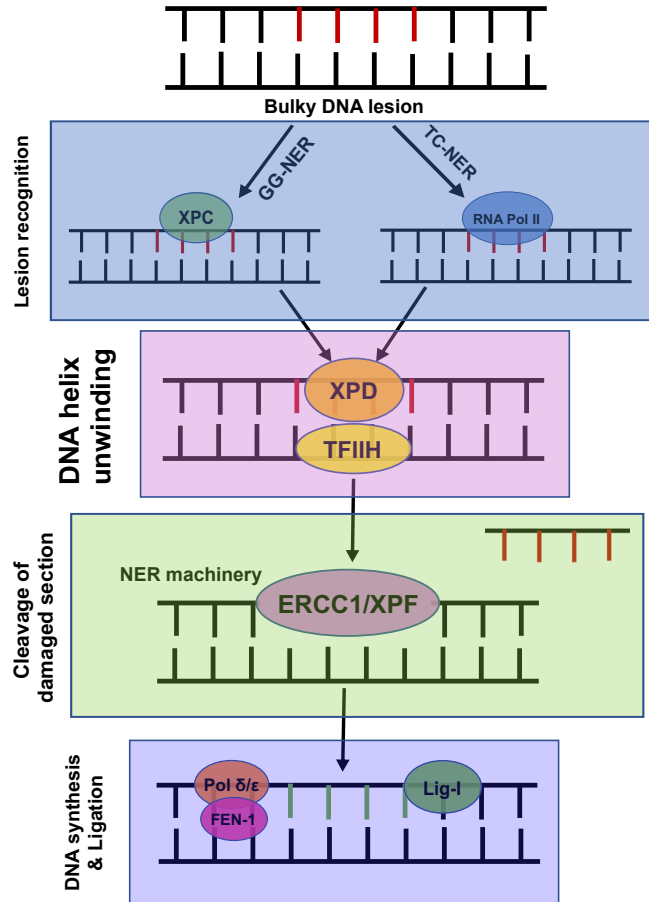


Figure 1.3: Lesion removal in DNA by NER Pathway

1.4.3 Translesion Synthesis (TLS) DNA damage tolerance

Translesion DNA synthesis is a DNA damage tolerance system which involves the DNA replication machinery to bypass lesions or damages that would otherwise stall DNA replication process. At the replication fork DNA exists as single strand⁸⁸. Presence of an abasic site in the single strand poses a problem in the replication process, as the BER pathway can't repair single stranded Ap site because BER machinery and Ap endonucleases can't repair Ap site in the single strand DNA⁸⁹. Furthermore, if an Ap site in the single-stranded DNA at a replication fork undergoes strand cleavage, it generates a

lethal double-strand break. The Ap stabilizing protein HMCES prevents this such strand breakage⁹⁰. During the replication process, if DNA repair polymerases detect damage in the replication fork, the repair system recruit a TLS polymerase (such as Pol $\eta/\iota/\kappa$), which can synthesize DNA across the damaged site⁹¹⁻⁹³. The TLS polymerase bypasses the damaged site by error-prone insertion of a nucleotides opposite the lesion. After the lesion is bypassed by TLS polymerases, the normal replicative polymerase takes over again, continuing the process of DNA replication (Figure 1.4)⁹⁴. TLS polymerase can insert incorrect bases to the DNA as there is no template base (Ap site), or a damaged base, strand to copy the coding information making the repair process highly mutagenic⁹⁵. Despite its mutagenic potential, TLS is an important mechanism for tolerance of lesions that would stall the replication process.

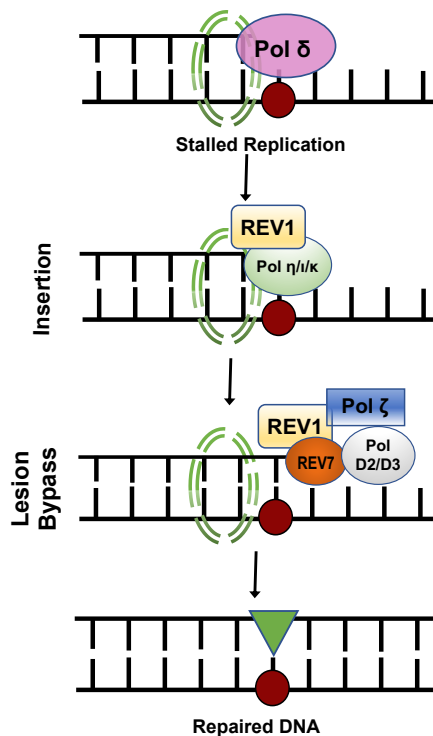


Figure 1.4: Translesion Synthesis DNA Repair Pathway

1.5 Consequences of abasic site in cells

The accumulation of Ap sites in the cell has significant biological consequences. While Ap sites are part of normal cellular processes, their presence in cell without repair disrupts genomic integrity. As a result, Ap lesions are highly cytotoxic and mutagenic because ring open aldehyde form of apurinic sites can reacts with nucleophiles such as exocyclic amino group of nucleobase and forms DNA inter-strand crosslinks⁹⁶. Unrepaired Ap sites can stall the replication fork, as DNA polymerases cannot efficiently bypass these lesions.

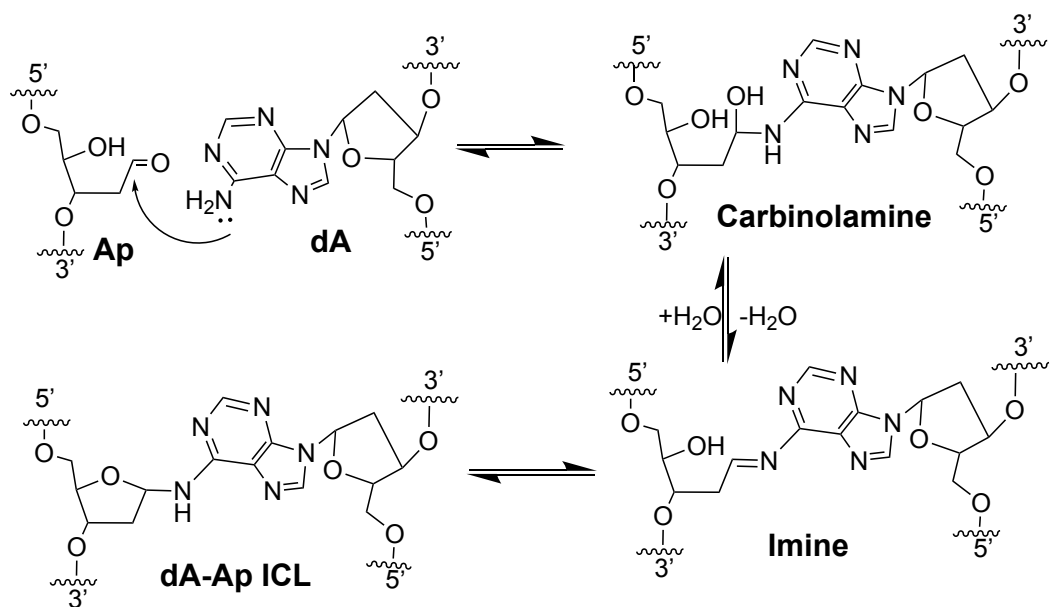
1.5.1 Formation and repair of DNA-DNA inter-strand Cross-links (ICLs) resulting from Ap site in DNA

The presence of Ap sites in cellular DNA is deleterious for cellular integrity and genomic stability as it has highly electrophilic aldehyde moiety⁹⁷. This reactive Ap aldehyde can form imine adducts with various amine species specially with exocyclic amines of heterocyclic nucleobases in DNA. The aldehyde group of the Ap site can undergo nucleophilic attack by amino groups of nucleobases across the template strand forming covalent bond between the two DNA strand resulting DNA inter strand crosslinks (ICLs)⁹⁷. The presence of ICLs poses a serious threat to genomic integrity and cell survival.

1.5.2 Formation of ICL between Adenine and Ap site (dA-Ap ICL)

The electrophilic nature of the Ap aldehyde enables the generation of interstrand cross-links (ICLs) in duplex DNA. The dA-Ap crosslinks formation involves the

nucleophilic attack of N⁶-amino group of adenine to the electrophilic carbonyl group of abasic site forming covalent bond between two DNA strand (Scheme 1.9)⁹⁸. The dA-Ap ICL is reported to be formed in 15-70% yield, depending upon the local sequence⁹⁹. Ap derived ICLs are reported to reach the maximum yield in within 2 to 4d. Once formed, dA-Ap ICL is very stable, with a half-life of 60-90h under physiological conditions. The dA-Ap inter-strand crosslink (ICLs) forms in a DNA sequence where dA is positioned one nucleotide to the 5'-side of the template strand⁹⁹.

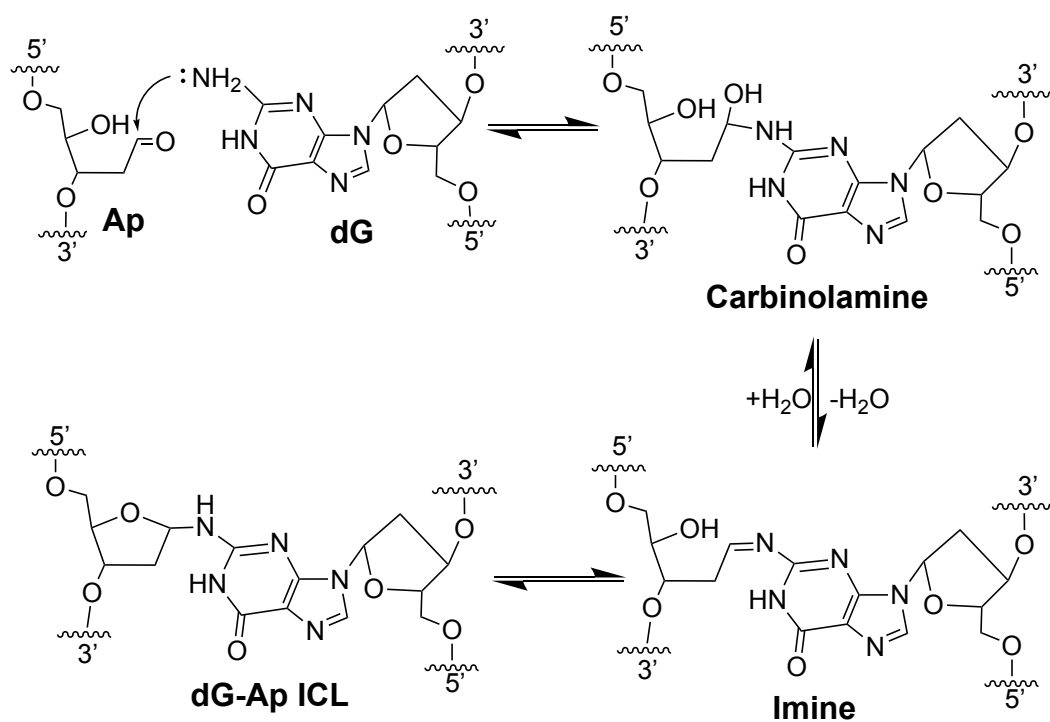


Scheme 1.9: Mechanism for the formation of dA-Ap ICL

1.5.3 Formation of ICL between Guanine and Ap site (dG-Ap ICL)

Cross-links also can be generated by the reaction of Ap sites with guanine residues in duplex DNA¹⁰⁰. The dG-Ap ICL forms when the N²-amino group of nucleobase guanine from the DNA strand covalently binds with the Ap site across the complementary

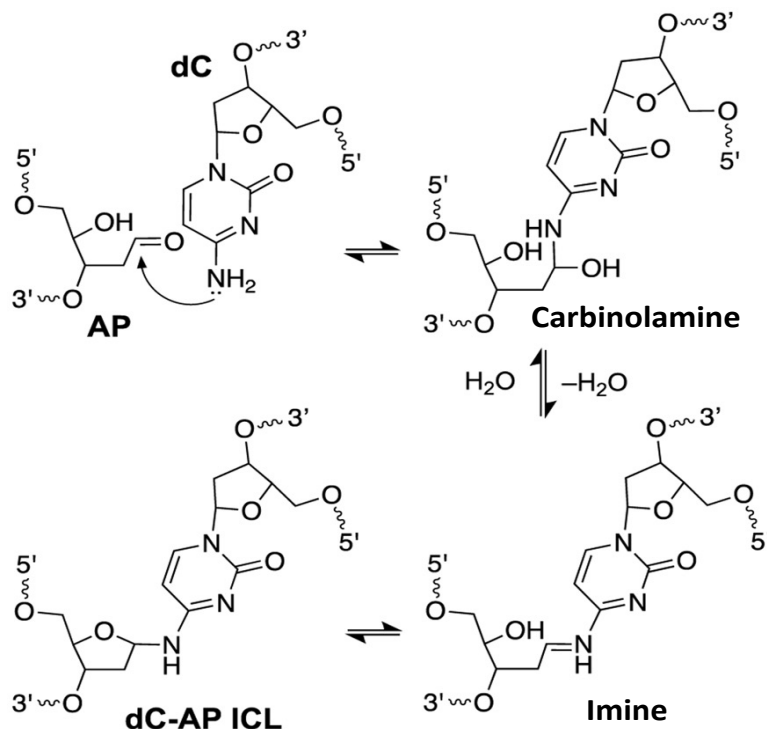
strand(Scheme 1.10)¹⁰⁰. Our research group reported that unreduced dG-Ap ICL formed with the maximum yield of 2-3%, while yield increased up-to 20% when reactions were conducted in the presence of NaBH₃CN¹⁰⁰. Interestingly, the yield of dG-Ap ICL increased up-to 52% when there are mis-paired bases near the abasic site¹⁰¹. In physiological condition half-life of dissociation of native, unreduced dG-Ap ICL was reported to be approximately around ~160h¹⁰⁰.



1.5.4 Formation of ICL between Cyanine and Ap site (dC-Ap ICL)

Inter-strand crosslink between cytosine and Ap site occurs when aldehyde moiety of Ap site covalently reacts with the N⁴-amino group of cytosine from the complementary strand (Scheme 1.11). Our research group has reported the sequence and pH dependent

formation of dC-Ap ICL in double stranded DNA¹⁰². dC-Ap ICL formed with maximum yield of ~50%. But only in a sequence where cytosine is mis-paired on the 3'-side of the abasic site. Under physiological conditions, the dC-Ap ICL is very stable with dissociating only about ~5% after 96h¹⁰³.



Scheme 1.11: Mechanism of formation of dC-Ap ICL

1.6 Repair of DNA dA-Ap Inter-strand crosslinks (ICLs)

Inter-strand crosslinks if formed in cellular DNA believed to be highly cytotoxic lesions because they can stall the replication and transcription process if left unrepaired¹⁰⁴⁻¹⁰⁵. In vertebrates, most important pathway to repair ICL derived from Ap site is the Fanconi Anemia (FA) pathway (Figure1.5)¹⁰⁶. FA is the best characterized pathway for

ICL repair of typical cross-links like cis-platin, N-mustards, etc. Semlow et al. discovered the previously unknown NEIL3 pathway for the repair of dA-Ap ICL¹⁰⁹. The FA pathway activates when the replicative enzymes detect stall at an ICL in the replication fork. The mono-ubiquitination of the FANCI and FANCD2 proteins is the important step in the FA DNA repair pathway¹⁰⁷. The mono-ubiquitination of I-D2 complex leads to the recruitment of FA core complex made up of several proteins (such as FAAP20, FAAP100, FANCA, FANCB, FANCC and FANCE) to the ICL site. Then the DNA endonuclease XPF-ERCC1 incises both side of the damage to unhook the ICL. Such dual incision of endonucleases left a double-strand break (DSB) in DNA. Once the ICL is unhooked, the DSB gets repaired via homologous recombination (HR)¹⁰⁸. Studies shows that dA-Ap ICL can be unhooked by the BER enzyme NEIL3 (Figure 1.6)¹⁰⁹. NEIL3 enzyme unhooks the dA-Ap interstrand crosslink by generating Ap site in one strand and adenine residue on the complementary strand¹¹⁰. Then the Ap site is covalently captured by the SRAP protein HMCES that protects the Ap site from cleavage. Then repair synthesis removes the HMCES-Ap adduct and the Ap site in duplex DNA is repaired by the base excision repair (BER)¹¹⁰.

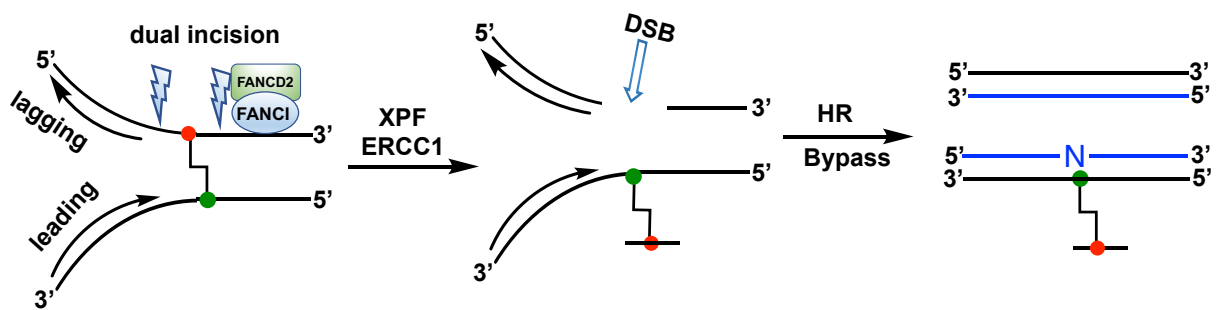


Figure 1.5: Repair of ICL in DNA by Fanconi Anemia (FA) Pathway

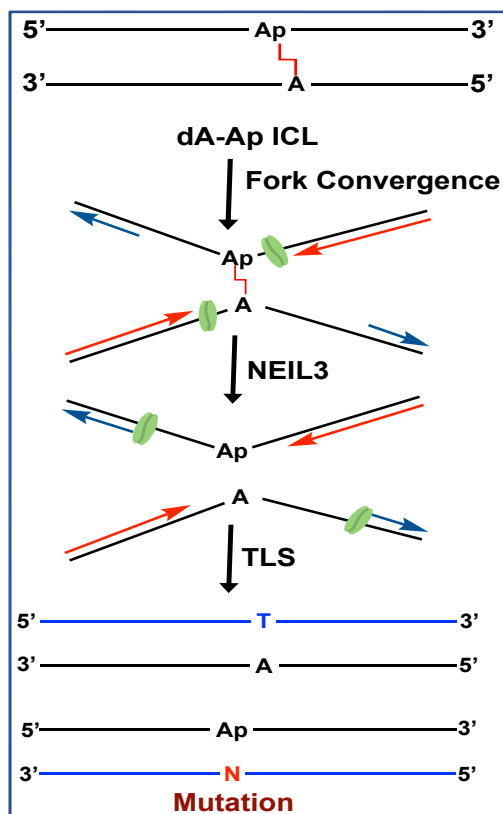
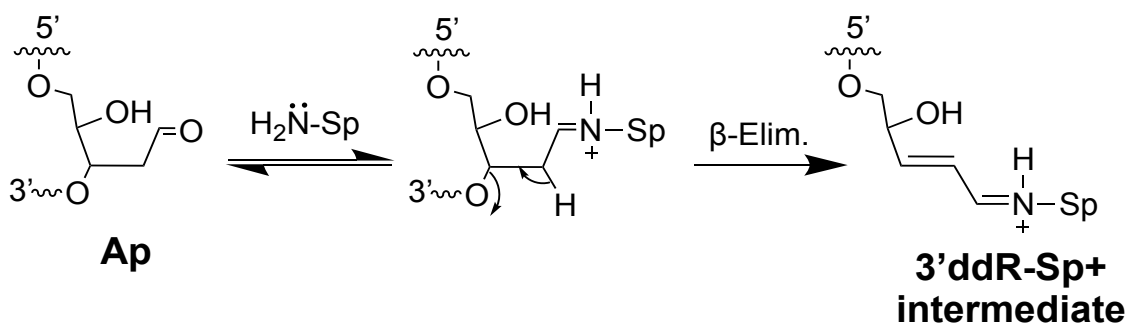


Figure 1.6: Repair of dA-Ap ICL by NEIL3

1.7 Strand Cleavage at Ap Site

Due to the acidic nature of α -proton in the ring opened form of Ap aldehyde residue, these sites in genomic DNA can readily undergo strand cleavage via β -elimination reactions that generate a 2,3-didehydro-2,3-dideoxyribose sugar remnant (3'-ddR) on the 3'side of the break by different factors (Scheme 1.12)¹¹¹. In physiological condition strand breaking at abasic site is a slow process with the half-life of 8 to 80 days. However, higher other factors such as higher temperature, alkaline condition and the presence of amine can substantially increase the rate of strand break¹¹².

Biological amine can induce strand cleavage at abasic site of duplex DNA by forming iminium ion with the open chain aldehyde group of Ap site¹¹³. Spermine, a polyamine found in cells at millimolar concentrations, is highly efficient at forming iminium ions with the Ap sites in DNA¹¹³. The iminium ion increases the acidity of α -protons that facilitates the β -elimination of phosphate group from the 3'-side of the Ap site generating trans α,β -unsaturated iminium ion(3'ddR-Sp+) at the strand break (Scheme 1.12)¹¹⁴.



Scheme 1.12: Formation mechanism of 3'ddR-Sp+ end group

1.7.1 DNA inter-strand crosslink arising from the strand break at abasic site induced by Spermine

Spermine induced strand break at an abasic site forms a α, β -unsaturated iminium ion (3'ddR-Sp+) at the strand break. This unsaturated iminium ion is very reactive as it can react with the exocyclic amino group of nucleobases specially with dA and dG via 1,4-Michael addition. Spermine facilitated strand break of abasic site can lead to the formation of low molecular inter-strand crosslink (LMW ICL) dG-ddR ICL & dA-ddR ICL at the strand break (Figure 1.7)¹¹¹. This ICL formation proceeds through the reaction of the N²-

amino group of dG & N⁶-amino group of dA from the complementary strand with the electrophilic 3'-ddR-Sp⁺ group formed during the spermine mediated strand break. It was reported that dA-ddR LMW ICL formed faster than as compared to dA-Ap full sized crosslink in duplex DNA¹¹¹. The results also showed that α,β -unsaturated iminium ion intermediate was crucial for the formation of these LMW ICL as the ICL didn't form with normal α,β -unsaturated aldehyde (3'-PUA) group¹¹¹.

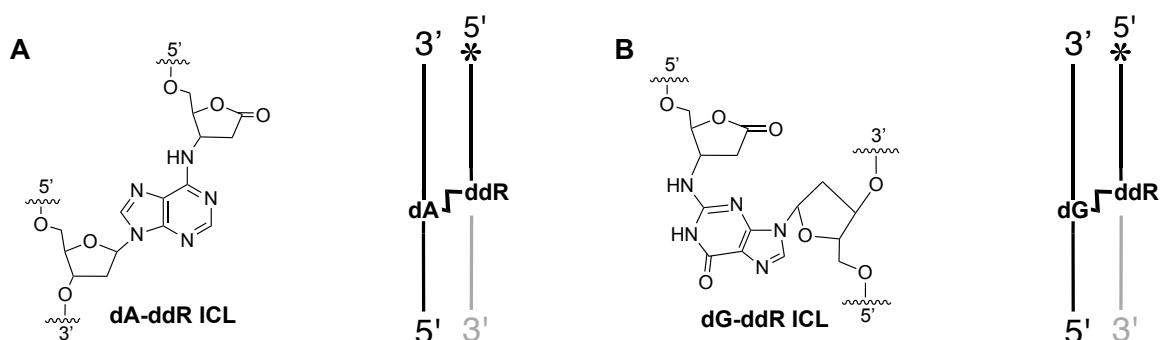


Figure 1.7: Structure of dG-ddR and dA-ddR ICL formed at the Spermine mediated strand cleavage at the Ap site; (A) dA-ddR ICL; (B) dG-ddR ICL

1.8 Conclusion

Ap site is the result of the loss of a purine/pyrimidine base and are common endogenous lesion in cellular DNA. These lesion are highly reactive and unstable, leading to significant consequences if not repaired promptly⁹. Ring open aldehyde form of Ap site is highly reactive as it can reacts with numbers of chemicals in the cell¹¹⁵. Ap site in cellular DNA can lead to the formation of highly mutagenic inter-strand crosslinks (ICLs) in the cell⁹⁶. During DNA transcription and replication, the DNA-DNA inter-strand crosslink can stall the replication process by blocking the action of DNA polymerases. Additionally, Ap

sites can induce single strand breaks (SSB) in DNA, further contributing to genomic instability¹¹⁶. Ap site in DNA can also cause strand break at the abasic site, which can induce the formation of highly cytotoxic DNA inter-strand crosslink. Due to several consequences, detection and repair of abasic sites in the cell is very crucial for the normal function of the cell. Ap site can be repaired by multiple pathways. For example, Ap site in duplex DNA gets repaired by Base Excision Repair (BER) pathway³². Ap sites can also get repaired by NER and TLS repair pathway in the situation where BER might not work.

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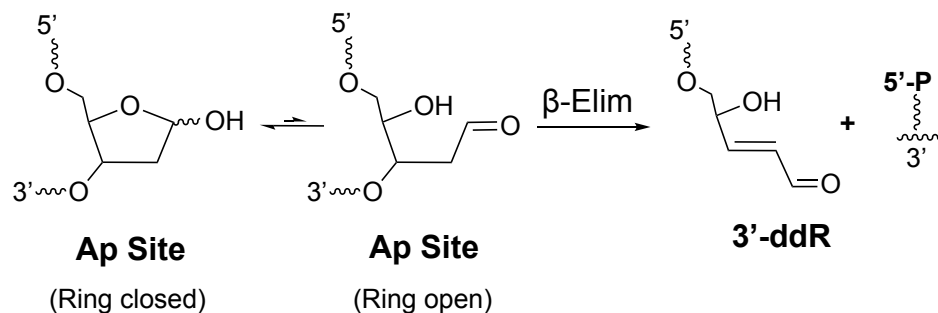
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Chapter 2

Products generated from amine catalyzed strand cleavage at the Abasic (Ap) site

2.1 Introduction

DNA is the storehouse of the genetic information for all living organisms¹⁻². Accurate readout of the genetic information during the replication process is essential to maintain the genomic integrity³. Therefore, any damages to the DNA are deleterious for the survival of the organisms⁴⁻⁶. Despite the fact, DNA is constantly exposed to unavoidable damage from both internal (endogenous) and external (exogenous) factors, such as reactive oxygen species, UV radiation, or chemicals⁷. Abasic (Ap) sites are the most frequent type of DNA lesion, occurring when a nitrogenous base is lost from the DNA backbone⁸⁻⁹. Ap sites are chemically reactive and exist mainly as a ring-closed hemiacetal form (99%) and a ring-opened aldehyde form (1%)¹⁰⁻¹¹. The ring-opened aldehyde form is highly reactive and can participate in harmful biochemical reactions with various cellular reagents¹². Due to the acidic nature of the α -proton in the ring-opened Ap aldehyde, these sites can easily undergo strand cleavage through β -elimination¹³⁻¹⁴. DNA strand cleavage can occur spontaneously under normal physiological conditions or be catalyzed by heat, NaOH, or DNA repair enzymes like Ap endonucleases¹⁵. The β -elimination reaction at Ap sites produces a 2,3-didehydro-2,3-dideoxyribose (3'-ddR) at the 3' end and a 5'-phosphate at the 5' end of the strand break (Scheme 2.1)¹⁶. DNA strand breaks can also generate noncanonical sugar remnants at the 3' end, including 3'-deoxyribose (dR), 3',4'-cyclized deoxyribose, and amine and thiol adducts of alkenals (Figure 2.1)¹⁷. Breaks at Ap sites can lead to mutations and replication blocks if not properly repaired¹⁸⁻¹⁹. DNA strand breaks can also hinder RNA polymerase during transcription, causing interruptions in gene expression and potential cellular dysfunction²⁰⁻²¹.



Scheme 2.1: Strand cleavage at the Ap site in DNA via β -elimination

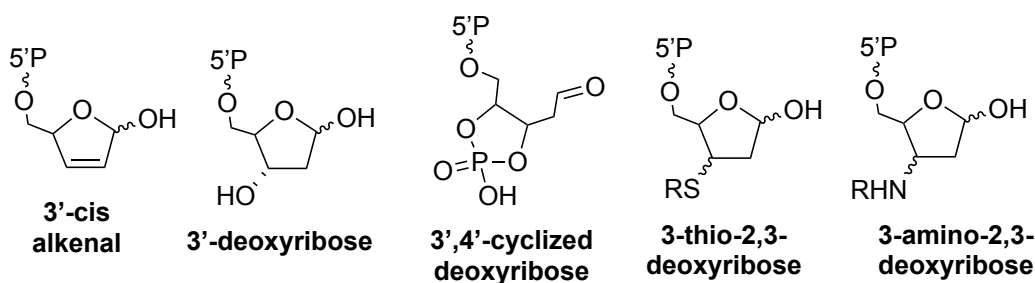
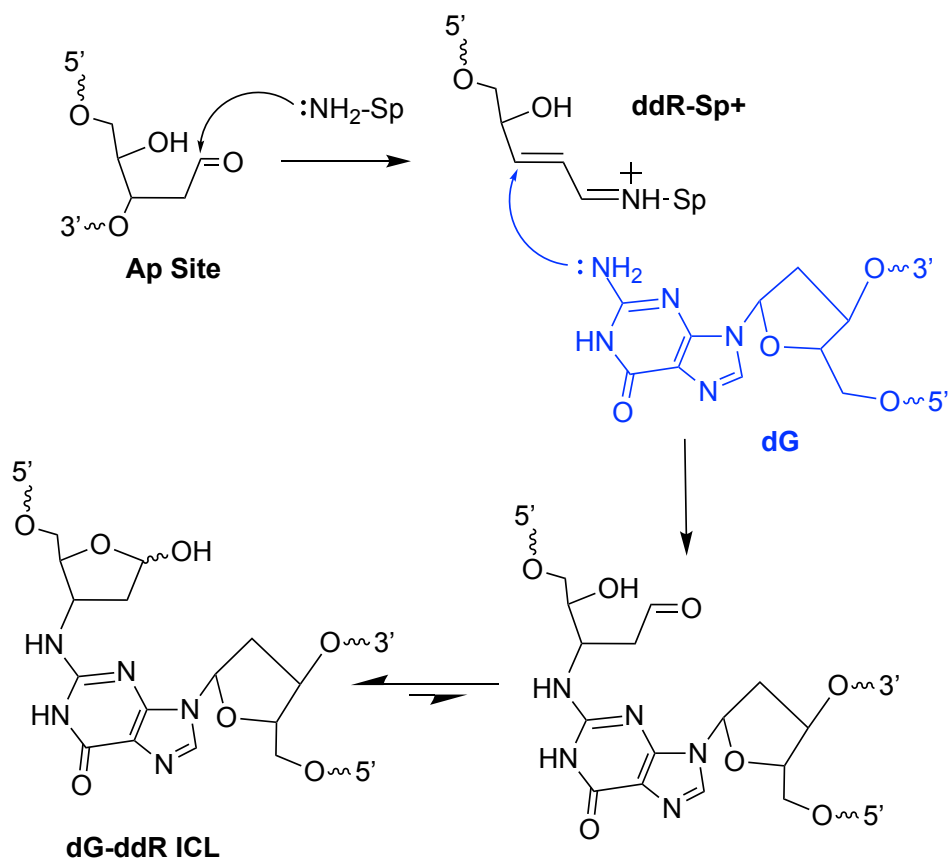


Figure 2.1: Non canonical sugar remnants resulting from strand break at Ap site

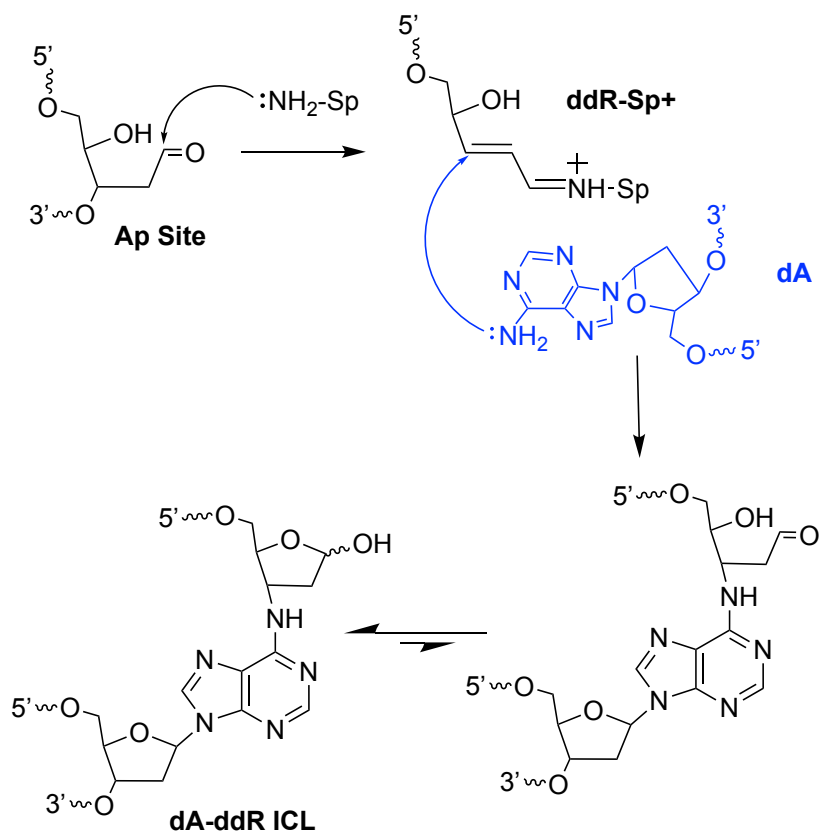
Under physiological conditions, strand breaking at the Ap site in duplex DNA is a slow process, with the half-life of 38h to 54h²². However, factors such as higher temperature, alkaline condition and the presence of amine can substantially increase the rate of strand break at the Ap site²³.

Biological amines can induce strand cleavage at the Ap site in duplex DNA by forming iminium ions with the open chain aldehyde group of the Ap site²⁴. Spermine, a polyamine found in cells at millimolar concentrations, is very effective at forming iminium ions with the Ap sites in DNA²⁵⁻²⁶. The iminium ions increase the acidity of α -protons, which facilitates the β -elimination of the phosphate group from the 3'-side of the Ap site, generating trans α,β -unsaturated iminium ion (3'ddR-Sp⁺) at the strand break (Scheme 2.2)²⁷.



Scheme 2.3: Mechanism for the formation of dG-ddR ICL at the strand cleavage site

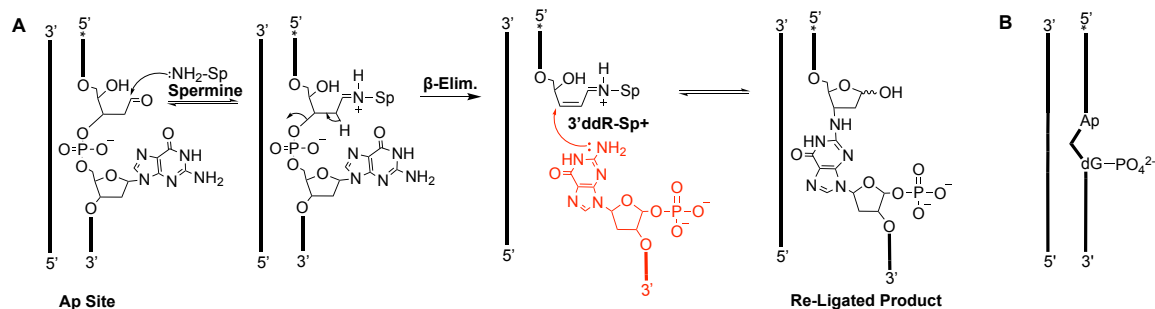
Our group has also demonstrated the formation of LMW ICL between an adenine residue (dA) and the α,β -unsaturated iminium ion intermediates on the opposing strand (Scheme 2.4)²⁹. The dA-ddR crosslinks form through the 1,4-Michael addition of the N⁶-amino group of the adenine residue from the opposite strand to the electrophilic α,β -unsaturated iminium ion intermediate (3'ddR-Sp+)²⁹.



Scheme 2.4: Mechanism for the formation of dA-ddR ICL at the strand cleavage site

This chapter aims to explore the reaction between reactive iminium ion intermediate (3'ddRSp⁺) formed by spermine mediated cleavage of Ap sites and the exocyclic amine group of guanine residue on the 3'-side of the strand break. Herein, we report the formation of previously unrecognized “non-natural” Ap site like product at the spermine catalyzed strand cleavage site (Scheme 2.5). This product will be referred to as the “re-ligated” or “ligated” product in this chapter. The formation of the re-ligated product involves the 1,4-Michael addition of N²-amino group of guanine residue from the 3'-side of the strand break to the electrophilic α,β -unsaturated iminium ion intermediate (3'ddR-Sp⁺). Our results show that once the ligated product forms, it remains stable under physiological conditions. The formation of this previously unrecognized DNA lesion could

be harmful to the cell. With this in mind, we also investigated the possible repair pathways for the ligated product later in this chapter.



Scheme 2.5: Proposed mechanism for the formation mechanism and the structure of the 're-ligated' product. (A) mechanism of formation for the 're-ligated' product; (B) structure of the 're-ligated' product.

2.2 Sequences used in the studies



Figure 2.2: DNA sequences used in this studies

2.3 Gel electrophoresis evidence for spermine catalyzed strand cleavage at Ap site in duplex DNA generating “non-natural” Ap site like re-ligated product in 5'-CApG/5'-GGG sequence

It was previously reported by our group that spermine catalyzes the strand cleavage at the Ap sites in Duplex DNA, generating the reactive 3' ddR-Sp⁺ ion²⁷. This α,β -unsaturated iminium ion intermediate can react with nucleobase guanine (dG) and adenine (dA) on the opposing strand to form low molecular weight interstrand crosslinks, such as dG-ddR ICL ($31 \pm 3\%$) and dA-ddR ICL ($\sim 35\%$) at the strand break²⁷⁻²⁸. To study spermine-induced strand cleavage and the formation of the novel re-ligated product, we used a 46 base pair 5'-Cy5 labeled Ap-containing duplex DNA "A" where guanine (G) on the 3'-side of the Ap site is mis-paired. Ap sites were prepared by treating 2' deoxyuridine containing duplex A with UDG enzyme at 37°C for 2 hour. Successful preparation of the Ap site in duplex DNA was confirmed by treating with NaOH (200 mM, 37°C, 30 min), which induced cleavage of the Ap-containing strand into smaller, fast-migrating products (**Figure 2.3A, Lane 2**).

When the Ap-containing duplex A was incubated with spermine (2mM) in HEPES buffer (50mM, pH 7.4, containing 100mM NaCl) at 37°C, we observed a band corresponding to the formation of the re-ligated product in the gel image (**Figure 2.3A, Lanes 3-9**). The band migrated slightly slower than the native Ap site and appeared just above the Ap site band in the gel electrophoresis image. A time course reaction showed that the formation of the re-ligated product reached a half-maximum yield of 14% at 6 hours and a maximum yield of $28 \pm 2\%$ after 48 hours of incubation at 37°C (**Figure 2.3B**). The formation of the ligated product was accompanied by the slower-migrating low

molecular weight (LMW) dG-ddR ICL previously reported by our group (**Figure 2.3A, Lanes 3-9**)²⁸. We then varied the concentration of spermine from 0.5 mM to 5 mM to observe its effect on the formation of the ligated product. Changes in spermine concentration did not affect the yield of the ligated product, with a maximum yield of $28 \pm 2\%$ formed at 2 mM spermine concentration (**Figure 2.4**).

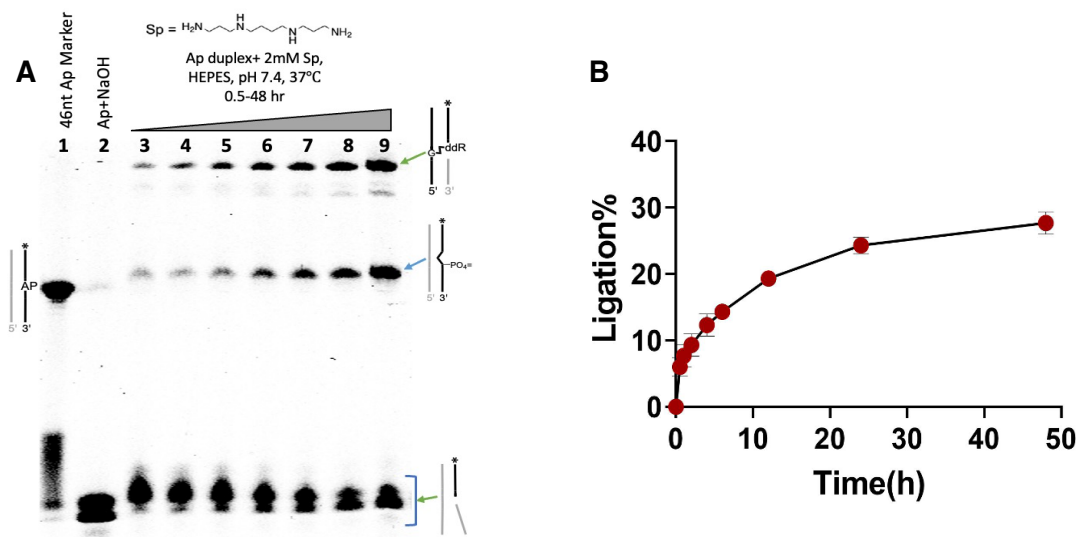


Figure 2.3: **A;** Gel electrophoretic evidence for the formation of ligated product by spermine-catalyzed strand cleavage at the Ap site in duplex DNA. Lane 1: 46mer 5'-Cy5-labeled-Ap-containing duplex **A**. Lane 2: The Ap-containing duplex **A** treated with NaOH (200 mM, 37 °C, 30min) to induce strand cleavage at the Ap site, generating 3'P and 3'ddR cleavage products. Lanes 3–9: Ap-containing duplex **A** was incubated with spermine (2 mM) in HEPES buffer (50 mM, pH 7.4, containing 100 mM NaCl) at 37 °C (Lane 3: 0.5hr; Lane 4: 1hr; Lane 5: 2hr; Lane 6: 6hr; Lane 7: 12hr; Lane 8: 24hr; Lane 9: 48hr). At prescribed time points aliquots were removed and frozen for subsequent gel electrophoretic analysis. The Cy5-labeled oligodeoxynucleotides in the reactions were resolved by

electrophoresis on a 0.4 mm thick 20% denaturing polyacrylamide gel at 500V for 18 hours. **B**; Plot of ligated product formed vs time. The error bar reflects the standard deviation of the measurements.

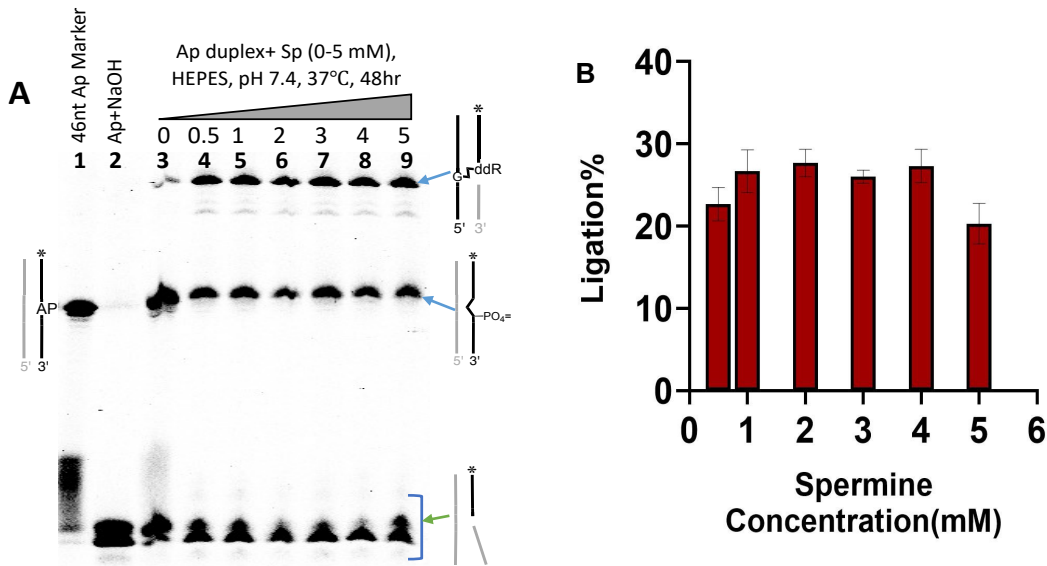


Figure 2.4: A; Ligation product formed in various spermine concentration. Lane 1: 46mer 5'-Cy5-labeled-Ap-containing duplex **A**. Lane 2: The Ap-containing duplex **A** treated with NaOH (200 mM, 37 °C, 30min) to induce strand cleavage at the Ap site, generating 3'P and 3'PUA cleavage products. Lanes 3–9: Ap-containing duplex **A** was incubated with spermine (0-5 mM) in HEPES buffer (50 mM, pH 7.4, containing 100 mM NaCl) at 37 °C; (Lane 3: 0mM; Lane 4: 0.5mM; Lane 5: 1mM; Lane 6: 2mM; Lane 7: 3mM; Lane 8: 4mM). Lane 9: 5mM. The Cy5-labeled oligodeoxynucleotides in the reactions were resolved by electrophoresis on a 0.4 mm thick 20% denaturing polyacrylamide gel at 500V for 18hours.

B; A plot of percent ligated product formed vs Spermine concentrations . The error bar reflects the standard deviation of the measurements.

2.4 Ligated product doesn't form in a sequence (5'-CApT/5'-GGG) where thymine is located on the 3'-side of the Ap site

Formation of ligated product believed to involve the 1,4-Michael addition of exocyclic amine group of nucleobases to the α,β -unsaturated iminium ion end group (3'ddR-Sp+) generated from the spermine catalyzed strand cleavage at Ap site. A ligation band was observed in duplex A, where the nucleobase guanine having an exocyclic amine group is present at the 3'-side of the strand break (Figure 2.5, Lane 5). We replaced the reacting guanine residue with a thymine residue to check if it reacts with the α,β -unsaturated iminium ion intermediate (3'ddR-Sp+). Since the nucleobase thymine does not possess an exocyclic amine group, it is expected that thymine at the ligation site will not form a ligated product. To confirm this, duplex DNA **B** containing a 5'-ApT sequence was incubated for the ligation reaction as described previously. We found that duplex **B** was completely cleaved by spermine, and no ligated product was observed, as indicated by the absence of a slow-moving band in the gel (**Figure 2.5, Lane 4**). Conversely, a ligation band was seen in duplex **A**, where the nucleobase guanine, which has an exocyclic amine group, is present at the 3'-side of the strand break (**Figure 2.5, Lane 5**). These results support the conclusion that the formation of the ligated product requires the presence of nucleobases with exocyclic amine groups at the 3'-side of the strand break.

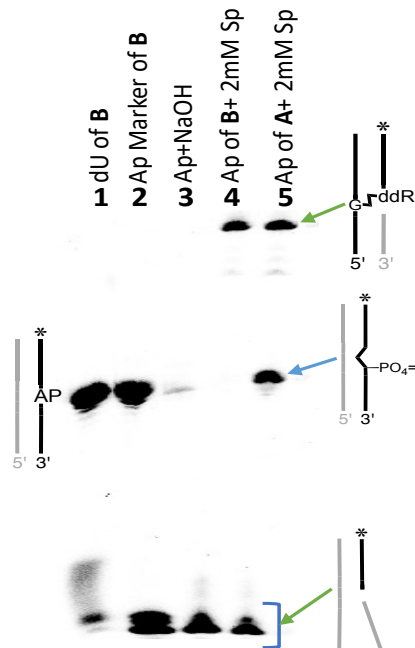


Figure 2.5: Sequence with 5'-ApT gets cleaved in spermine and no ligation product formed after 48hr of incubation Lane 1: dU marker of sequence **B**. Lane 2: 46mer 5'-Cy5-labeled-Ap-containing duplex **B**. Lane 3: The Ap-containing duplex **B** treated with NaOH (200 mM, 37 °C, 30min) to induce strand cleavage at the Ap site, generating 3'P and 3'PUA cleavage products. Lane 4: Ap-containing duplex in **B** was incubated with spermine (2 mM) in HEPES buffer (50 mM, pH 7.4, containing 100 mM NaCl) at 37 °C for 48hr. Lane 5: Ap-containing duplex in **A** was incubated with spermine (2 mM) in HEPES buffer (50 mM, pH 7.4, containing 100 mM NaCl) at 37 °C for 48hr make ligated product marker. The Cy5-labeled oligodeoxynucleotides in the reactions were resolved by electrophoresis on a 0.4 mm thick 20% denaturing polyacrylamide gel at 500V for 18hours.

2.5 Evidence that re-ligation doesn't occur in single stranded DNA

We next examined whether the ligated product forms in single-stranded DNA. To do this, the 5'-Cy5 labeled strand in sequence **A** was treated with UDG in HEPES buffer (50 mM, pH 7.4, containing 100 mM NaCl) at 37°C for 2 hours to generate a single-stranded Ap site. The resulting single-stranded (SS) Ap site was then incubated with spermine (2mM) in HEPES buffer (50 mM, pH 7.4, containing 100 mM NaCl) at 37°C to induce the formation of the re-ligated product. The gel electrophoresis results showed no bands for re-ligated products after 48 hours of incubation (**Figure 2.6, Lanes 3-9**). In contrast, a ligation band was present in the case of duplex DNA **A** (**Figure 2.6, Lane 10**). These results indicate that ligated product formation occurs only when the template strand is present in the system.

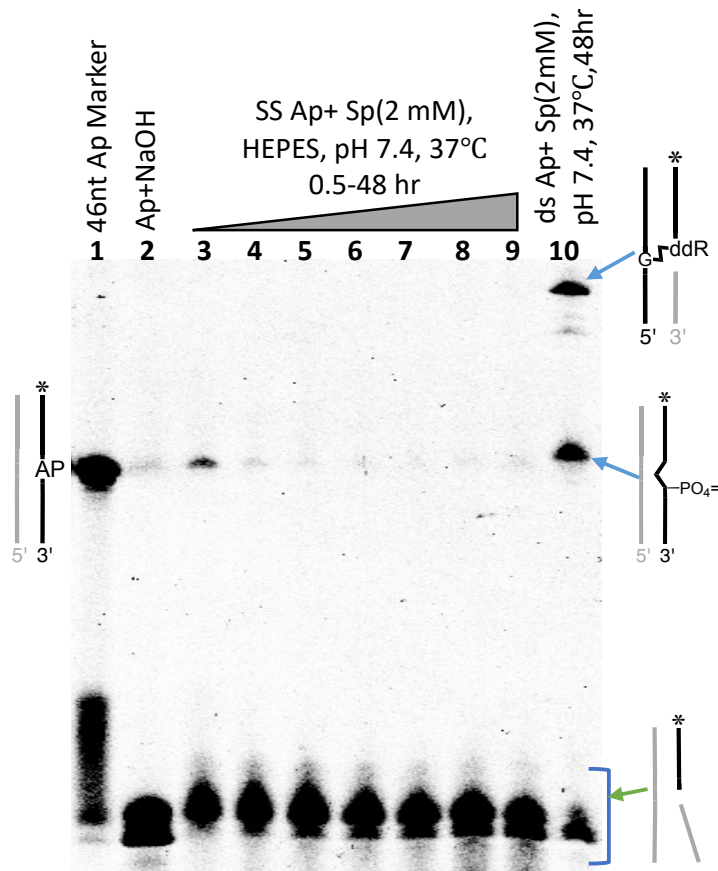


Figure 2.6: Gel electrophoretic evidence that ligation only occurs in double stranded DNA. Lane 1: 46mer 5'-Cy5-labeled-Ap-containing duplex A Lane 2: The Ap-containing duplex A treated with NaOH (200 mM, 37 °C, 30min) to induce strand cleavage at the Ap site, generating 3'P and 3'PUA cleavage products. Lanes 3–9: Single stranded Ap site was incubated with spermine (2 mM) in HEPES buffer (50 mM, pH 7.4, containing 100 mM NaCl) at 37 °C; Lane 3: 0.5hr; Lane 4: 1hr; Lane 5: 2hr; Lane 6: 6hr; Lane 7: 12hr; Lane 8: 24hr; Lane 9: 48hr. Lane 10: Double stranded Ap site in duplex A was incubated with spermine (2 mM) in HEPES buffer (50 mM, pH 7.4, containing 100 mM NaCl) at 37 °C for 48hr. At prescribed time points aliquots were removed and frozen for gel electrophoretic analysis. The Cy5-labeled oligodeoxynucleotides in the reactions were

resolved by electrophoresis on a 0.4 mm thick 20% denaturing polyacrylamide gel at 500V for 18hours.

2.6 Formation of ligated product in the 5'-AApG/5'-CTT sequence where guanine (G) is paired with cytosine (C) on the opposing strand

We previously found a ligation product in duplex A (5'AApG/GGG) where G is unpaired since it has a G on the opposing strand. We wanted to check if the same product forms when G is paired with a C on the opposing strand. For this, Ap-containing normal DNA duplex C was prepared and incubated with spermine (2 mM) in HEPES buffer (50 mM, pH 7.4, containing 100 mM NaCl) at 37°C for 48 hours. The results showed that spermine-mediated strand cleavage in normal duplex C led to the formation of slow-migrating ligation bands in the gel (**Figure 2.7**). A time course analysis demonstrated that the formation of the ligated product reached its maximum yield of 3% after 48 hours of incubation (**Figure 2.7, Lanes 4-11**). The ligation band appeared just above the Ap site in the gel, and its mobility matched that of the standard ligation band observed in duplex A (**Figure 2.7, Lane 12**). The yield is significantly lower compared to the yield in sequence A. This suggested that the ligation product is favored when there is a mismatch and reacting G is more in flexible condition to react with the reactive ddR-iminium ion.

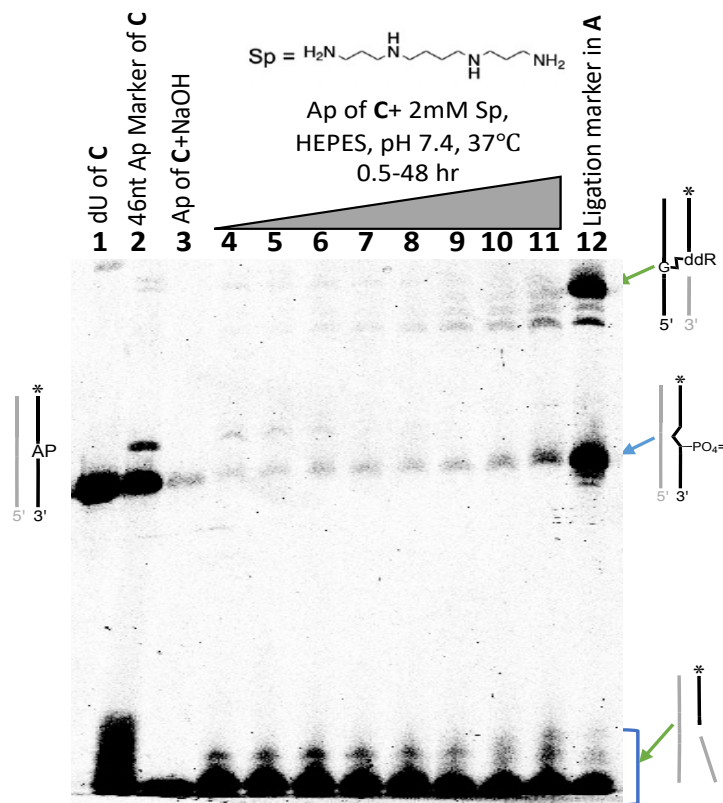
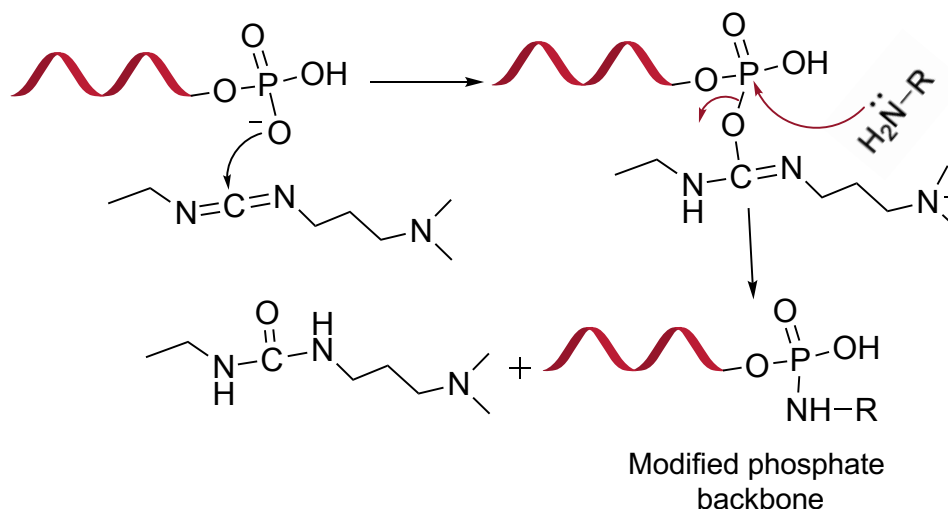


Figure 2.7: Formation of ligated product in the 5'-AApG/5'-CTT sequence where guanine (G) is paired with cytosine (C) on the opposing strand Lane 1: dU oligo of duplex C. Lane 2: 46mer 5'-Cy5-labeled-Ap-containing duplex C. Lane 3: The Ap-containing duplex C treated with NaOH (200 mM, 37 °C, 30min) to induce strand cleavage at the Ap site, generating 3'P and 3'PUA cleavage products. Lanes 4–11: Ap-containing duplex C was incubated with spermine (2 mM) in HEPES buffer (50 mM, pH 7.4, containing 100 mM NaCl) at 37 °C; (Lane 4: 0.5hr; Lane 5: 1hr; Lane 6: 2hr; Lane 7: 4hr; Lane 8: 6hr; Lane 9: 12hr; Lane 10:24hr; lane 11: 48hr). Lane 12: Double stranded Ap site in duplex A was incubated with spermine (2 mM) in HEPES buffer (50 mM, pH 7.4, containing 100 mM NaCl) at 37 °C for 48hr. The Cy5-labeled oligodeoxynucleotides in the reactions were resolved by electrophoresis on a 0.4 mm thick 20% denaturing polyacrylamide gel at 500V for 18hours.

2.7 Modification of the ligated product with EDCI to confirm the presence of a non-canonical backbone in the re-ligated product

EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) (EDCI) is a coupling reagent commonly used to modify the 5'-free phosphate group of unlabeled DNA oligonucleotides³⁶. EDCI achieves this by first activating the phosphate group to form an active ester intermediate, which can then be replaced by a nucleophilic molecule, such as an amine, to form a phosphoramidate linkage, thereby modifying the phosphate backbone of the DNA (Scheme 2.6)³⁷.



Scheme 2.6: EDCI modifications to the DNA phosphate backbone

In our study, the ligated product contains a flanking phosphate group at its center. To explore whether this structure could undergo similar EDCI modification, we treated the ligated product, formed as described previously, with EDCI and spermine under physiological conditions. The ligated product was treated with EDCI (250mM) and

spermine (2mM) in HEPES buffer (50 mM, pH 7.4, containing 100 mM NaCl) at 37°C for 24hr. Successful modification of the flanking phosphate group was monitored by gel electrophoresis, where a gel shift would indicate a change in the electrophoretic mobility of the modified product. After treatment with EDCI and spermine, a new band appeared in the gel, which migrated slightly slower than the original ligated product, indicating the formation of a modified product (**Figure 2.8, Lanes 10-13**). A time-course analysis revealed that approximately 50% of the ligated product was converted to the modified form after 12 hours of incubation (**Figure 2.8, Lane 13**). In contrast, no modification was observed in a dU oligonucleotide under identical conditions, as no gel shift was observed. These results provide strong evidence that the ligated product possesses a non-canonical DNA backbone structure. The modification of the flanking phosphate group by EDCI confirms the presence of an altered backbone in the ligated product, which is consistent with the hypothesis that the product contains non-natural phosphate linkages.

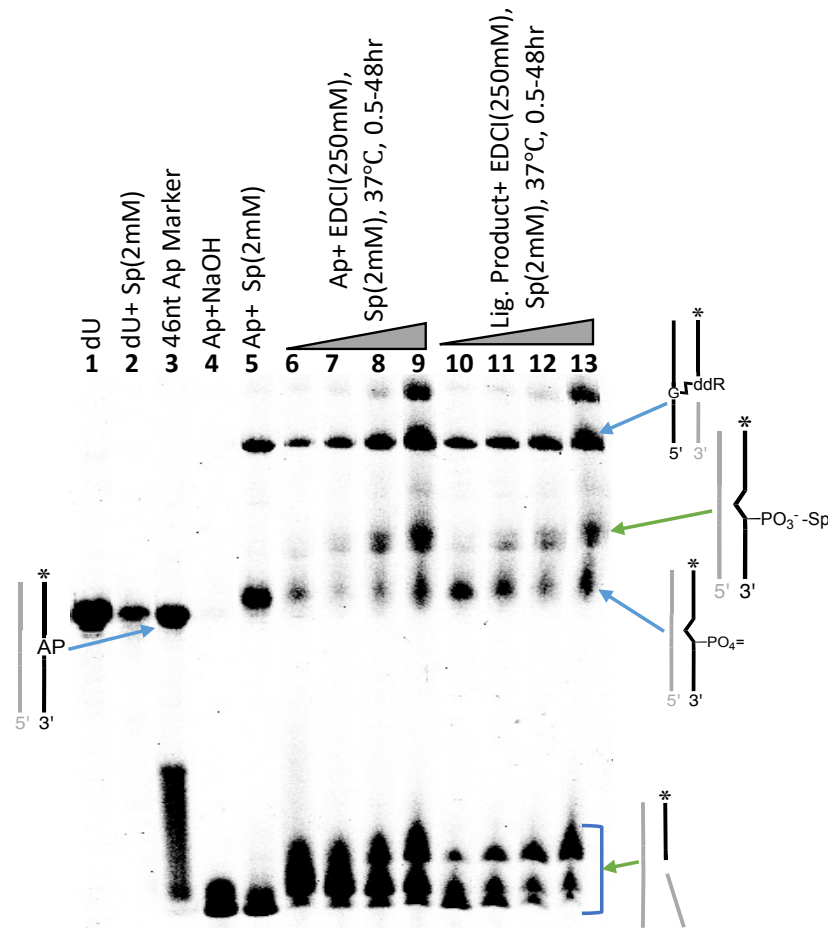


Figure 2.8: EDCI-spermine work-up formed an additional band with ligated product Lane 1: dU oligo in A. Lane 2: dU oligo treated with EDCI (250mM) and spermine (2 mM) in HEPES buffer (50 mM, pH 7.4, containing 100 mM NaCl) at 37°C for 48hr. Lane 3: 46mer 5'-Cy5-labeled-Ap-containing duplex A Lane 4: Ap-containing duplex A treated with NaOH (200 mM, 37°C, 30min) to induce strand cleavage at the Ap site, generating 3'P and 3'PUA cleavage products. Lane 5: Ap-containing duplex A was incubated with spermine (2 mM) in HEPES buffer (50 mM, pH 7.4, containing 100 mM NaCl) at 37 °C for 48hr to make ligated product marker. Lanes 6-9: Ap duplex A treated with EDCI (250mM) and 2mM Spermine in HEPES buffer (50 mM, pH 7.4, containing 100 mM NaCl) and incubated at 37°C for 24hr (Lane 6; 0.5hr, Lane 7; 1hr, Lane 8; 12hr, Lane 9; 24hr). Lanes

10-13: Ligated product (in Lane 5) was ethanol precipitated and re-dissolved in HPLC water and incubated with EDCI (250mM) and 2mM Spermine in HEPES buffer (50 mM, pH 7.4, containing 100 mM NaCl) and incubated at 37 °C for 24hr (Lane 10; 0.5hr; Lane 11; 1hr, Lane 12; 12hr, Lane 13; 24hr). The Cy5-labeled oligodeoxynucleotides in the reactions were resolved by electrophoresis on a 0.4 mm thick 20% denaturing polyacrylamide gel at 500V for 18hours.

2.8 Chemical stability of the ligated product under physiological conditions

A time-course analysis of ligated product formation demonstrated that the reaction reached half of its maximum yield within approximately 6 hours (**Figure 2.3B**). Following this observation, we sought to determine whether the ligated product remained stable under physiological conditions. The ligated product was synthesized using duplex **A** as described previously, followed by ethanol precipitation to remove the excess spermine. The sample was then resuspended in HPLC-grade water. The sample was then incubated at 37°C in a buffer consisting of 50 mM HEPES (pH 7.4) and 100 mM NaCl for 120 hours. Under these conditions, the ligated product exhibited significant chemical stability, undergoing only about 8% decomposition after 120 hours of incubation (**Figure 2.9, Lanes 3-11**). This minimal level of degradation indicates that the ligated product maintains its structural integrity under physiological conditions over extended periods.

To assess the susceptibility of the ligated product to harsher conditions, we investigated its behavior under acidic, high-temperature treatment. Literature reports

indicate that guanine (G) residues can be fully depurinated when DNA is heated at 65°C in 10 mM HCl with 1 mM EDTA for 1 hour³⁰. In alignment with this approach, the pre-formed ligated product in duplex A was treated with 30 mM HCl in the presence of 1 mM EDTA and incubated at 65°C for 2 hours. Electrophoretic analysis revealed that the ligated product completely degraded after 2 hours of incubation under these conditions (**Figure 2.10, Lane 5**). These findings indicate that while the ligated product is chemically stable under physiological conditions, it is susceptible to depurination and degradation when exposed to more rigorous environments, such as acidic conditions combined with elevated temperatures.

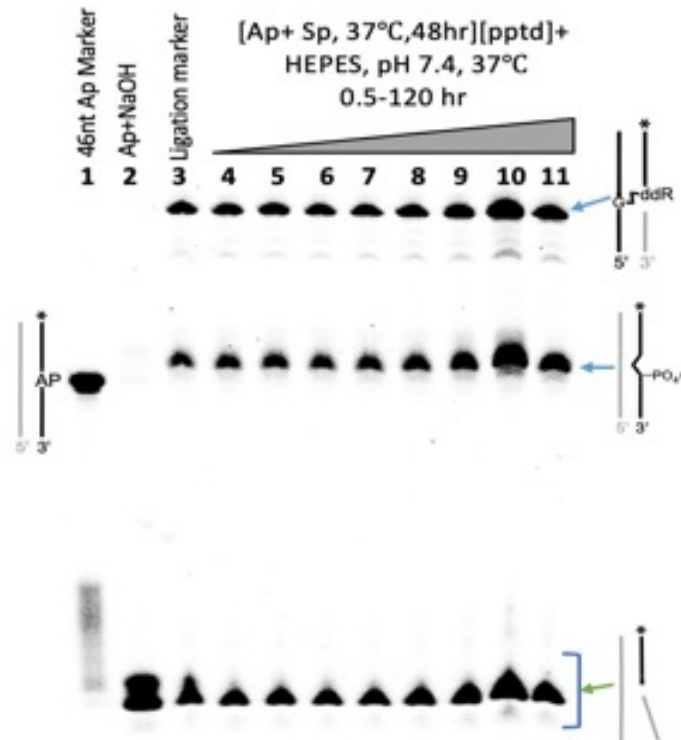


Figure 2.9: A time course dissociation of ligated product in HEPES buffer. Lane 1: 46mer 5'-Cy5-labeled-Ap-containing duplex A. Lane 2: The Ap-containing duplex A treated with NaOH (200 mM, 37 °C, 30min) to induce strand cleavage at the Ap site, generating 3'P

and 3'PUA cleavage products. Lanes 3: Ap-containing duplex A was incubated with spermine (2 mM) in HEPES buffer (50 mM, pH 7.4, containing 100 mM NaCl) at 37 °C for 48hr to make ligated product marker. Lanes 4-11: Ap-containing duplex A was incubated with spermine (2 mM) in HEPES buffer (50 mM, pH 7.4, containing 100 mM NaCl) at 37 °C for 48hr to make ligated product, then the ligated DNA was ethanol precipitated and re-dissolved in HEPES buffer (50 mM, pH 7.4, containing 100 mM NaCl) and incubated at 37°C for 120hr; (Lane 4: 0.5hr; Lane 5: 1hr; Lane 6: 2hr; Lane 7: 6hr; Lane 8: 12hr; Lane 9: 24hr; Lane 10: 48hr; Lane 11: 120hr). At prescribed time points aliquots were removed and frozen for subsequent gel electrophoretic analysis. The Cy5-labeled oligodeoxynucleotides in the reactions were resolved by electrophoresis on a 0.4 mm thick 20% denaturing polyacrylamide gel at 500V for 18hours.

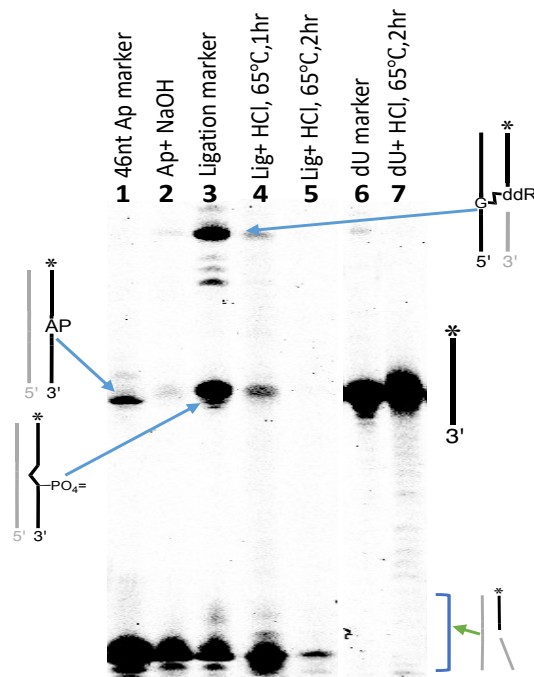


Figure 2.10: HCl depurination of ligated product in 1mM EDTA at 65°C. Lane 1: 46mer 5'-Cy5-labeled-Ap-containing duplex A. Lane 2: Ap-containing duplex A treated with NaOH (200 mM, 37°C, 30min). Lane 3: Ap-containing duplex A was incubated with spermine (2 mM) in HEPES buffer (50 mM, pH 7.4, containing 100 mM NaCl) at 37°C for 48hr to make ligation marker. Lanes 4-5: Ligated product (in Lane 3) incubated with 30mM HCl in EDTA (1mM) at 65°C for 2hr; (Lane 4: 1hr; Lane 5: 2hr). Lane 6: dU oligo of duplex A. Lane 7: dU oligo treated with 30mM HCl in HEPES buffer (50 mM, pH 7.4, containing 100 mM NaCl) at 65°C for 2hr. The Cy5-labeled DNA in the reactions were resolved by electrophoresis on a 0.4 mm thick 20% denaturing polyacrylamide gel at 500V for 18hours.

2.9 Repair of re-ligated product by Bacterial Endonuclease IV (Endo IV)

Our next objective was to determine whether base excision repair enzymes, such as human APE1 (hAPE1), Endo IV, and formamidopyrimidine DNA glycosylase (Fpg), could cleave the non-natural Ap-like ligated product.

APE1 is a human Ap endonuclease enzyme, which plays a central role in the Base Excision Repair (BER) pathway³³. APE1 enzyme hydrolyzes the phosphodiester backbone immediately 5' to an Ap site, producing a one-nucleotide gap with a 3'-hydroxyl group and a 5'-deoxyribose phosphate end³⁴. Our gel electrophoresis analysis demonstrated that a freshly prepared Ap site in duplex **A** was completely cleaved by 30nM APE1 within 30 minutes at 37°C (**Figure 2.11; Lanes 4-6**). However, when the pre-synthesized ligated product in duplex **A** was incubated with 30nM APE1 under identical conditions in a buffer containing 20mM Tris (pH 7.9), 20 mM MgCl₂, and 1 mM DTT at 37°C, no cleavage was observed even after 48 hours incubation period (**Figure 2.11; Lanes 7-12**). Conversely, APE1 effectively cleaved the dG-ddR ICL, as previously shown by our group, with approximately 75% dissociation occurring after 48 hours at 37°C (**Figure 2.11; Lanes 7-12**). These results provide evidence that the ligated product has a non-natural Ap site that resists cleavage by APE1, unlike natural Ap DNA.

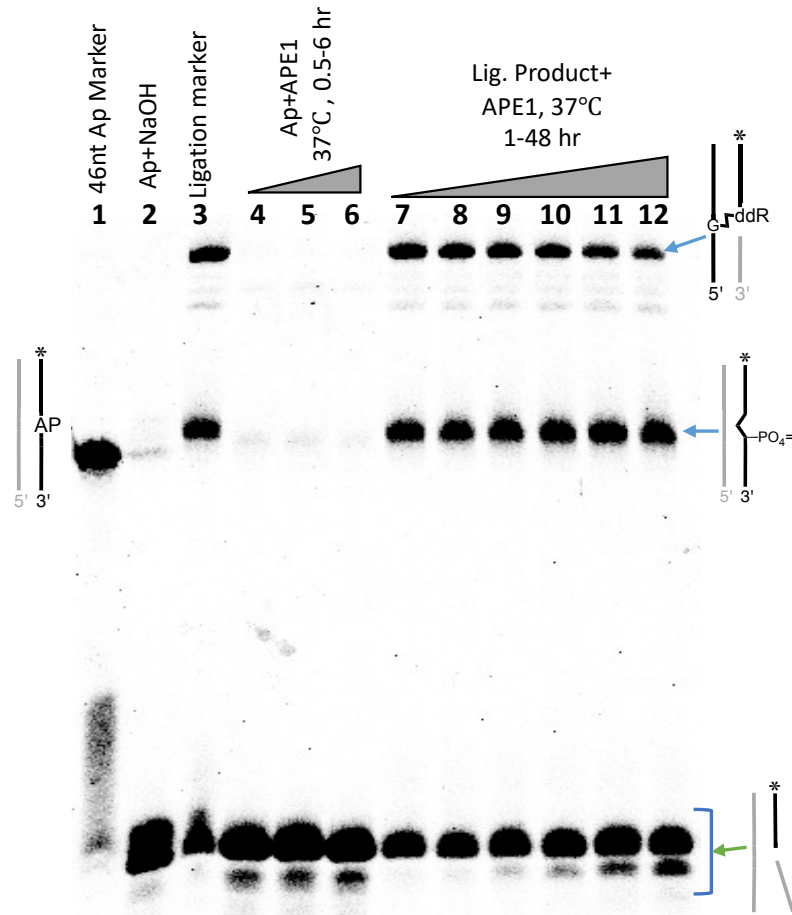


Figure 2.11: DNA repair enzyme hAPE1 can't cleave ligated product. Lane 1: 46mer 5'-Cy5-labeled-Ap-containing duplex A. Lane 2: The Ap-containing duplex A treated with NaOH (200 mM, 37 °C, 30min) to induce strand cleavage at the Ap site, generating 3'P and 3'PUA cleavage products. Lanes 3: Ap-containing duplex A was incubated with spermine (2 mM) in HEPES buffer (50 mM, pH 7.4, containing 100 mM NaCl) at 37°C for 48hr to make ligated product marker. Lanes 4-6: Ap duplex A incubated with hAPE1 (30nM) in endo buffer composed of 20mM Tris, pH 7.9 containing 20mM MgCl₂ and 1 mM DTT at 37 °C for 6hr (Lane 4; 1hr, Lane 5; 2hr, Lane 6; 6hr). Lanes 7-12: Ligated product (in lane 3) was ethanol precipitated and re-dissolved in HPLC water and incubated with hAPE1 (30nM) in endo buffer composed of 20mM Tris, pH 7.9 containing 20mM

MgCl₂ and 1 mM DTT at 37°C for 48hr (Lane 7; 1hr; Lane 8; 2hr, Lane 9; 6hr, Lane 10; 12hr, Lane 11; 24hr, Lane 12; 48hr). The Cy5-labeled oligodeoxynucleotides in the reactions were resolved by electrophoresis on a 0.4 mm thick 20% denaturing polyacrylamide gel at 500V for 18hours.

We next evaluated whether bacterial Ap endonuclease enzyme IV (Endo IV) could cleave the ligated product. According to the literature, bacterial Ap endonuclease IV (Endo IV) can cleave DNA at native Ap sites, generating a 3'-deoxyribose hydroxyl (3'-OH) and a 5'-deoxyribose phosphate (5'-dRP) end group³¹⁻³². We observed that Endo IV efficiently cleaves native Ap DNA to form a 3'-OH end within 30 minutes of incubation at 37°C (**Figure 2.12; Lanes 4-6**). When the pre-synthesized ligated product in duplex **A** was incubated with Endo IV (9.6nM) in tris buffer (50mM, pH 7.9) containing NaCl (100mM), MgCl₂ (10mM), DTT (1mM) at 37°C, it was nearly completely cleaved within 6 hours (**Figure 2.12; Lane 7**). A time-course study revealed that the half-time for cleavage was approximately 2hours, with complete degradation occurring by 12hours (**Figure 2.12; Lanes 7-12**). The gel mobility of the cleavage product is also consistent with the 3'-OH end group.

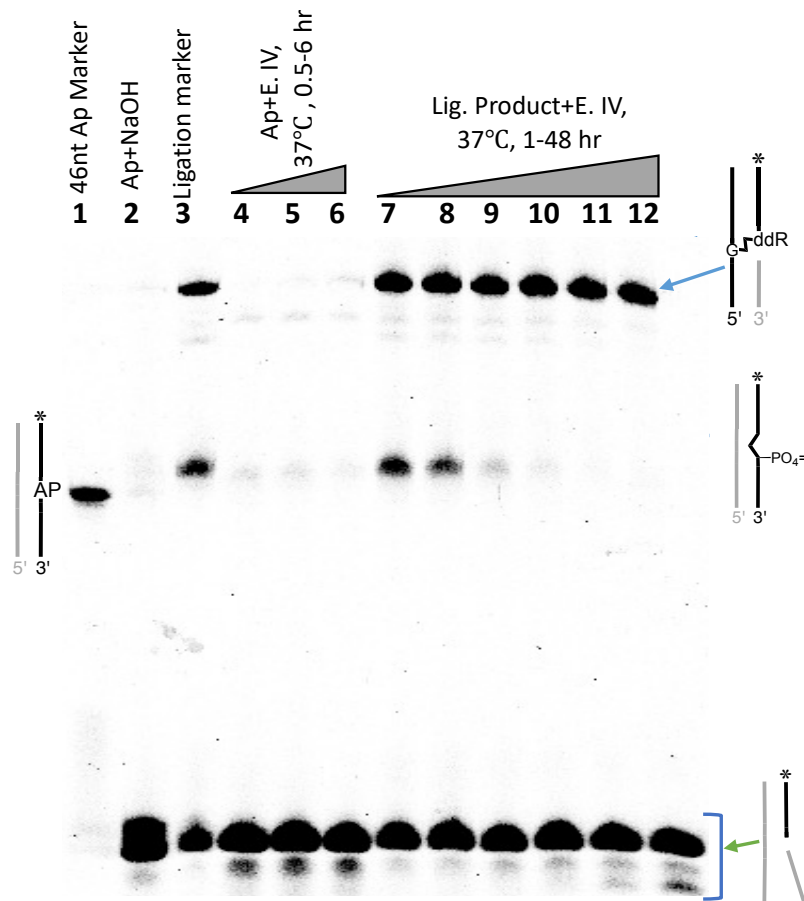


Figure 2.12: Repair of ligated product containing “non-canonical” DNA backbone by Ap endonuclease IV. Lane 1: 46mer 5'-Cy5-labeled-Ap-containing duplex **A** Lane 2: The Ap-containing duplex **A** treated with NaOH (200 mM, 37 °C, 30min) to induce strand cleavage at the Ap site, generating 3'P and 3'PUA cleavage products. Lanes 3: Ap-containing duplex **A** was incubated with spermine (2 mM) in HEPES buffer (50 mM, pH 7.4, containing 100 mM NaCl) at 37 °C for 48hr to make ligated product marker. Lanes 4-6: Ap duplex **A** incubated with Endo IV (9.6nM) in tris buffer (50mM, pH 7.9) containing NaCl (100mM), MgCl₂ (10mM), DTT (1mM) at 37°C for 6hr (Lane 4; 1hr, Lane 5; 2hr, Lane 6; 6hr). Lanes 7-12: Ligated product (in lane 3) was ethanol precipitated and re-dissolved in HPLC water and incubated with Endo IV (9.6nM) in tris buffer (50mM, pH 7.9) containing NaCl

(100mM), MgCl₂ (10mM), DTT (1mM) at 37°C for 48hr (Lane 7; 1hr; Lane 8; 2hr, Lane 9; 6hr, Lane 10; 12hr, Lane 11; 24hr, Lane 12; 48hr). The Cy5-labeled oligodeoxynucleotides in the reactions were resolved by electrophoresis on a 0.4 mm thick 20% denaturing polyacrylamide gel at 500V for 18hours.

Finally, we investigated whether Fpg, a bifunctional enzyme could process the ligated product. Fpg is a bifunctional enzyme, possesses both glycosylase activity as well as the Ap lyase activity³⁵⁻³⁶. Freshly prepared Ap site in duplex **A** was incubated with enzyme Fpg (2μM) in in 40 mM HEPES-KOH, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8.0 at 37°C. The results showed that Fpg completely cleaved the Ap site within 30 minutes (**Figure 2.13, Lanes 4-6**). However, when the ligated product was treated under similar conditions, no cleavage was detected, and the product remained intact even after 48 hours (**Figure 2.13, Lanes 7-12**).

Overall, these results demonstrate that while the ligated product is resistant to cleavage by human APE1 and Fpg, it can be effectively cleaved by bacterial Endo IV. This suggests that the ligated product possesses a “non-natural” Ap site in its structure, conferring resistance to typical human base excision repair enzymes but allowing cleavage by bacterial Endo IV.

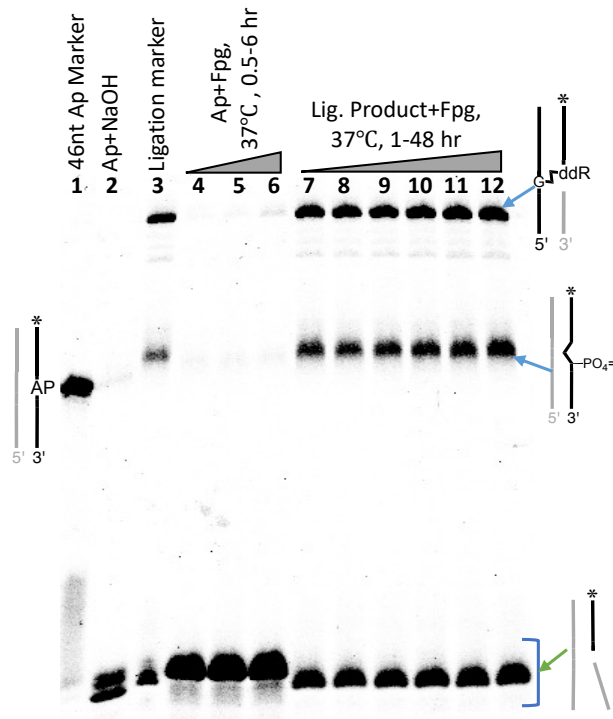


Figure 2.13: Ligated product is stable against bifunctional enzyme Fpg. Lane 1: 46mer 5'-Cy5-labeled-Ap-containing duplex A Lane 2: The Ap-containing duplex A treated with NaOH (200 mM, 37 °C, 30min) to induce strand cleavage at the Ap site, generating 3'P and 3'PUA cleavage products. Lanes 3: Ap-containing duplex A was incubated with spermine (2 mM) in HEPES buffer (50 mM, pH 7.4, containing 100 mM NaCl) at 37 °C for 48hr to make ligated product marker. Lanes 4-6: Ap duplex A treated with FPG (2 μ M) in 40 mM HEPES-KOH, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8.0 at 37°C for 6hr (Lane 4; 1hr, Lane 5; 2hr, Lane 6; 6hr). Lanes 7-12: Ligated product (in Lane 3) was ethanol precipitated and re-dissolved in HPLC water and incubated with FPG (2 μ M) in 40 mM HEPES-KOH, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8.0 at 37°C for 48hr (Lane 7; 1hr; Lane 8; 2hr, Lane 9; 6hr, Lane 10; 12hr, Lane 11; 24hr, Lane 12; 48hr). The Cy5-labeled oligodeoxynucleotides in the reactions were resolved by electrophoresis on a 0.4 mm thick 20% denaturing polyacrylamide gel at 500V for 18hours.

2.10 Conclusions

Ap sites are the result of the loss of nucleobases and are common endogenous lesions in cellular DNA³⁸. The aldehyde moiety of the ring open Ap site can react with various reagents forming different adducts³⁹. Ap site can also undergo strand cleavage by several factors including higher temperature and in the presence of amines²³. This chapter focuses on the products generated by amine-catalyzed strand cleavage at Ap sites in DNA. We specially utilized spermine to induce strand cleavage at the Ap site. Spermine is a polyamine that is found in the cell at millimolar concentrations⁴⁰. Spermine can catalyze the strand cleavage at the Ap sites via β -elimination reaction leading to the formation of reactive α,β -unsaturated iminium ion intermediate (3'ddR-Sp+)²⁷. The electrophilic α,β -unsaturated iminium ion intermediate can covalently react with the exocyclic amine group of nucleobase to form low molecular weight ICL (LMW ICL)²⁷⁻²⁸. In this chapter we have characterized previously unknown novel re-ligated product resulting from the spermine mediated strand cleavage at the Ap site. The product we characterized forms quickly in presence of spermine and is quite stable in physiological conditions. The ligated product having non-natural DNA backbone resists cleavage by most DNA repair enzymes but can be processed by bacterial endonuclease IV.

2.11 Experimental

2.11.1 Materials

46-mer Cy5 labeled oligonucleotides, Urea and Spermine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Uracil DNA glycosylase (UDG), human Ap endonuclease 1 (hAPE1), Endonuclease IV (Endo IV) were purchased from New England Biolabs (Ipswich, MA, USA). Acrylamide/ bis-acrylamide 19:1 (40% solution/electrophoresis) were obtained from Fisher Scientific (Waltham, MA). All other chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.11.2 Preparation of Ligated Products in duplex

46-mer 5'-Cy5 labeled single stranded oligonucleotides containing 2'-deoxyuridine were annealed to their respective complementary strands in a buffer composed of HEPES (50mM, pH 7.4) containing NaCl (100mM). The resulting duplex DNA **A/B/C** was treated with uracil DNA glycosylase (UDG) enzyme (10 μ L, final concentrations, 50units/mL) to generate Ap site and samples were ethanol precipitated . Resulting Ap duplex **A/B/C** was dissolved in HPLC water and then treated with spermine (final concentrations, 2mM) in a buffer consists of HEPES (50mM, pH 7.4) containing NaCl (100mM) and then incubated at 37°C for 48hours. Aliquots (20pmoles, 2.5 μ L) were removed from the reaction mixture at specific time points and frozen at -20°C immediately until gel electrophoretic analysis. The DNA in the reactions were then combined with formamide loading buffer and resolved by gel electrophoresis on a 0.4mm thick, 20% denaturing polyacrylamide/ 7M urea gel in 1X TBE buffer (45mM Tris-base, 45mM boric acid, 1mM EDTA pH 8.0) and

electrophoresed at 500V for 18 hours. Fluorescence activity of each band in polyacrylamide gels was visualized and quantified using Fujifilm FLA 3000 (GE healthcare) with Image Gauge (v 1.6) and Image reader (v 1.4) software respectively.

2.11.3 Formation of Ligated product in single stranded (SS) DNA

5'-Cy5 labeled 2'deoxyuridine containing strand in sequence **A** was treated with uracil DNA glycosylase (UDG) enzyme (10 μ L, final concentrations, 50units/mL) in HEPES buffer (50mM, pH 7.4) containing NaCl (100mM) and incubated at 37°C to make SS Ap site. The resulting SS Ap site was ethanol precipitated and redissolved in HPLC water and then treated with spermine (final concentrations, 2mM) in a buffer consists of HEPES (50mM, pH 7.4) containing NaCl (100mM) and then incubated at 37°C for 48hours. At prescribed time points aliquots (20pmoles, 2.5 μ L) were removed from the reaction mixture at specific time points and frozen at -20°C immediately until gel electrophoretic analysis. The resulting DNA samples were then analyzed using gel electrophoresis as described above.

2.11.4 Dissociation of re-ligated product

Formation of re-ligated product in duplex **A** was carried out in the method described above. The resulting ligated product was incubated at 37°C in a buffer consists of HEPES (50mM, pH 7.4) containing NaCl (100mM) for 120 hours. In a separate tube, the ligated product was treated with 30mM HCl in 1mM EDTA and incubated at 60°C for 12hours. Aliquots (20pmoles, 2.5 μ L) were removed from the reaction mixture at specific time points

and frozen at -20°C immediately. The DNA in the reaction mixtures was then combined with formamide loading buffer and analyzed using gel electrophoresis as described above.

2.11.5 Treatment of Ligated product with Ap endonuclease 1 (hAPE1), Endo IV and Fpg

The ligated products in duplex **A** were prepared using method described above. To study the endonuclease activity provided by human Ap endonuclease APE1 on the ligated product, the ligated product was treated with APE1 (30nM) in 20mM Tris buffer, pH 7.9 containing 20mM MgCl₂ and 1mM DTT and incubated at 37°C for 48hours. Ap duplex in **A** was subjected to the identical conditions as a control. At prescribed time points aliquots (20pmoles, 2.5µL) were removed from the reaction mixture at specific time points and frozen at -20°C immediately until gel electrophoretic analysis.

To check the stability of ligated product against bacterial enzyme endo IV, the prepared ligated samples were treated with Endo IV (9.6nM) in a buffer consisting of tris (50mM, pH 7.9) containing NaCl (100mM), MgCl₂ (10mM), DTT (1mM) and incubated at 37°C for 48hours. Ap duplex in **A** was subjected to the identical conditions as a control. At prescribed time points aliquots (20pmoles, 2.5µL) were removed from the reaction mixture at specific time points and frozen at -20°C immediately until gel electrophoretic analysis.

In an individual study, the ligated products were also treated with Fpg (2 µM) in a buffer composed of 40 mM HEPES-KOH, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8.0, incubated at 37 °C for 48hours. Ap duplex in **A** was subjected to the identical conditions as a control. At prescribed time points aliquots (20pmoles, 2.5µL) were removed

from the reaction mixture at specific time points and frozen at -20°C immediately until gel electrophoretic analysis. The DNA in the reactions were then combined with formamide loading buffer and resolved by gel electrophoresis on a 0.4mm thick, 20% denaturing polyacrylamide/ 7M urea gel in 1X TBE buffer (45mM Trisbase, 45mM boric acid, 1mM EDTA pH 8.0) and electrophoresed at 500V for 18 hours. Fluorescence activity of each band in polyacrylamide gels was visualized and quantified using Fujifilm FLA 3000 (GE healthcare) with Image Gauge (v 1.6) and Image reader (v 1.4) software respectively.

2.11.6 EDCI modifications to the re-ligated products

46-mer 5'-Cy5 labeled Ap site containing duplex A and the ligated product in the duplex A was prepared in the method described above. To modify the phosphate in ligated product, the product was treated with EDCI (250mM) and 2mM Spermine in HEPES buffer (50 mM, pH 7.4, containing 100 mM NaCl) and incubated at 37 °C for 24hr. In a different tube, Ap containing duplex A was with EDCI (250mM) and 2mM Spermine in HEPES buffer (50 mM, pH 7.4, containing 100 mM NaCl) and incubated at 37 °C for 24hr. In addition, dU oligo (labeled strand) from duplex A was also with EDCI (250mM) and 2mM Spermine in HEPES buffer (50 mM, pH 7.4, containing 100 mM NaCl) and incubated at 37 °C for 24hr. At prescribed time points aliquots (20pmoles, 2.5µL) were removed from the reaction mixture at specific time points and frozen at -20°C immediately until gel electrophoretic analysis. The DNA in the reaction mixtures was then combined with formamide loading buffer and analyzed using gel electrophoresis as described above.

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