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Repair of the Major Lesion Resulting from C5′-Oxidation of DNA

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Abstract

Oxidation of the C5′-position of DNA results in direct strand scission. The 3′-fragments produced contain DNA lesions at their 5′-termini. The major DNA lesion contains an aldehyde at its C5′-position but its nucleobase is unmodified. Excision of the lesion formed from oxidation of thymidine (T-al) is achieved by strand displacement synthesis by DNA polymerase β (Pol β) in the presence or absence of flap endonuclease 1 (FEN1). Pol β displaces T-al and thymidine with comparable efficiency, but less so than a chemically stabilized abasic site analogue (F). FEN1 cleaves the flaps produced during strand displacement synthesis that are two nucleotides or longer. A ternary complex containing T-al is also a substrate for the bacterial UvrABC nucleotide excision repair system. The sites of strand scission are identical in ternary complexes containing T-al, thymidine or F. UvrABC incision efficiency of these ternary complexes is comparable as well, but significantly slower than a duplex substrate containing a bulky substituted thymidine. However, cleavage occurs only on the 5′-fragment and does not remove the lesion. These data suggest that unlike many lesions the redundant nature of base excision and nucleotide excision repair systems does not provide a means for removing the major damage product produced by agents that oxidize the C5′-position. This may contribute to the high cytotoxicity of drugs that oxidize the C5′-position in DNA.

DNA is exposed to a variety of endogenous and exogenous oxidizing agents that produce strand breaks, cross-links, and/or damaged nucleotides (1–3). Damaged DNA can be genotoxic and/or cytotoxic if left unrepaired or misrepaired. Cells contain multiple repair systems, whose employment depends upon the type of damage. Determining which repair pathway(s) act on a particular DNA lesion is important because defective DNA repair and disease are associated with one another (4, 5). For instance, Fanconi anemia is associated with defective repair of interstrand cross-links and defective nucleotide excision repair is associated with Xeroderma pigmentosum (6–9). Base excision repair focuses on modified nucleotides, including various types of abasic sites, whereas bulkier lesions and cross-links are typically excised by nucleotide excision repair (10, 11). However, there are increasing examples of common lesions that are excised by BER and NER. Glycosylases hydrolyze the glycosidic bond of the damaged nucleotide in the first step of BER. These enzymes can selectively recognize lesions via hydrogen bond formation with the modified nucleobases (12). Herein we describe the repair of a lesion that does not contain a modified nucleobase, the major product that results from hydrogen atom abstraction from the C5′-position of DNA.

The C5′-hydrogen atoms are highly accessible to groove binding molecules and diffusible species (13). Hydrogen atom abstraction from the C5′-position of nucleotides occurs when DNA is exposed to hydroxyl radical, which is produced by γ-radiolysis and metal

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Supporting Information Available. Representative plots of LP-BER and NER of ternary complexes containing F and thymidine. Autoradiogram of the reaction of FEN1 with 5′-^{32}P-**4**. This material is available free of charge via the Internet at http://pubs.acs.org.

complexes, such as Fe•EDTA. A number of antitumor agents and metal-oxo species also abstract the C5′-hydrogen atoms upon binding in the minor groove (14, 15). Under anaerobic conditions, purine C5′-radicals add to the C8-position of the nucleobase to form cyclonucleotides (e.g. cdA) that are excised by NER (16–18). The other most common lesions associated with C5′-oxidation are the 5′-aldehyde (e.g. T-al) and dioxobutane (DOB) (Scheme 1). T-al and DOB are unusual in that they are formed concomitantly with single strand breaks.

DOB is highly reactive and chemical synthesis of oligonucleotides containing it has revealed that it is involved in a variety of biochemically interesting processes (19). For instance, it undergoes β-elimination, releasing butene-1,4-dial, which forms exocyclic adducts with dA, dC, and dG (20, 21). Such adducts have altered Watson-Crick faces and are typically mutagenic. DOB also forms interstrand cross-links with the dA that is opposite a 3′-adjacent thymidine (22). Although the fates of these cross-links are unknown, a similar type of lesion formed from C4-AP is misrepaired by NER, resulting in double strand breaks (23). The most striking biochemical effect of DOB is its potent irreversible inhibition of DNA polymerase β an integral component of BER (24, 25). The <15 nM K_I by DOB suggests that this process contributes to the chemical basis for the cytotoxicity of DNA damaging agents that produce this lesion. These same damaging agents produce T-al in higher yields but its repair has not been reported (26).

Experimental Procedures

Materials and General Methods

Oligonucleotides were prepared on an Applied Biosystems Inc. 394 DNA synthesizer. The 50mer containing the fluoresceinylated thymidine was obtained from Sigma-Genosys. Commercially available DNA synthesis reagents, including the 3′-phosphorylation and F reagents were obtained from Glen Research Inc. Oligonucleotides containing the T-al precursor were synthesized as previously described (19). All others were synthesized and deprotected using standard protocols. DNA manipulations were carried out using standard procedures (27). 3′-^{32}P-Labeling of oligonucleotides was carried out on single stranded material prior to hybridization using terminal deoxynucleotide transferase and 5′-α-^{32}P-triphosphate-3′-deoxyadenosine. T4 polynucleotide kinase and terminal deoxynucleotide transferase were obtained from New England Biolabs. FEN1 and Pol β were from Trevigen Inc. Radionuclides were obtained from Amersham Pharmacia. UvrABC was obtained as previously described (28, 29). Analysis of radiolabeled oligonucleotides was carried out using a Storm 840 Phosphorimager and ImageQuant TL software. Each experimental data point consists of 3 replicates and experiments were carried out at least twice.

Preparation of ternary complex containing T-al, F, and T

Hybridization was conducted at 90 °C (5 min) followed by slow cooling to room temperature in the HK buffer [100 mM HEPES-KOH (pH 7.5) and 100 mM KCl]. The radiolabeled oligonucleotide was hybridized with 1.5 equiv of the template strand and the flanking oligonucleotide. Following hybridization, the oligonucleotide complex containing the T-al precursor was activated in 100 mM NaOAc (pH 6.0) with 25 mM $NaIO_4$ at room temperature for 60 min. The ternary complex containing T-al was desalted by passing through a G-25 Sephadex column that was equilibrated with 100 mM KCl. The column was washed with an additional 50 μL of 100 mM KCl. The pH of the combined fractions was adjusted with 1 M HEPES-KOH (pH 7.5).

Strand Displacement Synthesis by Polβ and FEN1

The ternary complex (5′-^{32}P-**1**–**3**) was assembled as described above. Reactions (20 μL) containing Pol β (1 nM) and FEN1 (1 nM or 10 nM) in HM buffer (50 mM HEPES-KOH (pH 7.5) and 5 mM $MgCl_2$), dTTP (50 μM), dCTP (50 μM), and 0.1 mg/mL BSA was performed at 37 °C. Aliquots (2 μL) were removed at 2, 5, 10, 20, 40, 60 min, and reactions were quenched with 1 μL of 250 mM $NaBH_4$. After the sample had been held for 1 h on ice, 10 μL of formamide loading buffer was added and the reactions were analyzed by 20% denaturing PAGE.

Lesion Excision of 3′-^{32}P-labeled substrates by Pol β and FEN1

The ternary complex (3′-^{32}P-**1**–**3**) was reacted (20 μL) with Pol β (1 nM) in HM buffer, along with dTTP (50 μM), dCTP (50 μM), and 0.1 mg/mL BSA at 37 °C in the presence of FEN1 (1 nM or 10 nM). Aliquots (2 μL) were removed at 2, 5, 10, 20, 40, 60 min, and the reactions were quenched with 1 μL of 250 mM $NaBH_4$. After the sample had been held for 1 h on ice, 10 μL of formamide loading buffer was added and the reactions were analyzed by 20% denaturing PAGE.

Flap Excision of 3′-^{32}P-labeled substrates by FEN1

The ternary complex (3′-^{32}P-**4**,**5**) was reacted (20 μL) with FEN1 (1 nM or 10 nM) in HM buffer and 0.1 mg/mL BSA at 37 °C. Aliquots (2 μL) were removed at 2, 5, 10, 20, 40, 60 min, and the reactions were quenched with 1 μL of 250 mM $NaBH_4$. After the sample had been held for 1 h on ice, 10 μL of formamide loading buffer was added and the reactions were analyzed by 20% denaturing PAGE.

Time Course Analysis of UvrABC incision

UvrA, UvrB, and UvrC were activated separately prior to use by heating at 65 °C for 10 min in NER buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM KCl, 1 mM ATP, and 5 mM dithiothreitol). Reactions (20 μL) were carried out at 55 °C with UvrA (20 nM), UvrB (100 nM), and UvrC (50 nM) in the presence of 2 nM DNA substrates (6–9) in NER buffer. Substrates **7**–**9** were ^{32}P-labeled in one of 3 positions, 5′-terminus of the complementary strand, 5′-terminus of the 5′-fragment, or the 3′-terminus of the 3′-fragment. The fluoresceinylated standard (6) was labeled at the 5′-terminus of the strand containing the modified nucleotide. The typical incubation time was 180 min. Aliquots (2 μL) were removed at 0, 5, 10, 30, 60, 120, and 180 min and were quenched with formamide loading buffer (10 μL; 90% formamide, 10 mM EDTA), followed by heating to 85 °C for 15 min and then cooling on ice. The incision products were separated by 10% denaturing PAGE. A no-enzyme control time course was carried out side by side with these reactions.

Results

Preparation of DNA substrates

Oligonucleotide substrates containing thymidine or F, a stable analogue of an abasic site (AP), were prepared using commercially available reagents (Chart 1). Ternary complexes containing T-al (2, 8) were freshly prepared as needed by treating the chemically stable precursors with sodium periodate as previously described (19). Transformation to T-al was carried out after hybridization in order to minimize heating the lesion at 90 °C. Sodium periodate was removed after the reaction via gel filtration.

T-al removal by long patch base excision repair

When T-al is formed following 5′-oxidation, the 3′-terminus of the accompanying 5′-fragment is phosphorylated (Scheme 1). The 3′-phosphate must be removed before Pol β can

fill-in the gap during BER. Ape1 and polynucleotide kinase are examples of enzymes that dephosphorylate the 3′-termini (30, 31). Consequently, we initiated our studies at the step following dephosphorylation. LP-BER of T-al was first investigated by monitoring strand displacement synthesis using 5′-^{32}P-**2** (Figure 1). Products resulting from the addition of 1 and 3 nucleotides by Pol β (1 nM) were observed when dTTP and dCTP were present, but the 2-nucleotide extension product was not detected. Although the ratio of +3 nucleotide to +1 nucleotide product increased slightly when FEN1 was increased to 10 nM (Figure 1B) from 1 nM (Figure 1A), the overall amount of extended starting material did not increase. For comparison, the respective ternary complexes containing a stable abasic site analogue (F, 5′-^{32}P-**1**) and thymidine (5′-^{32}P-**3**) were examined under the same conditions (See Supporting Information). Strand displacement synthesis of thymidine (5′-^{32}P-**3**) was very similar to that of the T-al lesion, in terms of the overall efficiency and the effect of FEN1 concentration on the ratio of +3 and +1 nucleotide products. In contrast, strand displacement of F (5′-^{32}P-**1**) by Pol β (1 nM) was clearly more efficient. In the presence of 1 nM FEN1 ~99% of the starting material was consumed within 20 min, at which time the +1 and +3 nucleotide extension products were formed in approximately equal amounts. Increasing FEN1 to 10 nM increased the ratio of +3 to +1 nucleotide products to greater than 2:1 but did not significantly increase the overall rate at which starting material disappears.

These observations were complemented by experiments using 3′-^{32}P-labeled substrates in which we measured FEN1 excision of the nucleotides displaced by Pol β extension. FEN1 excision of T-al (3′-^{32}P-**2**, Figure 2) lagged behind the extension of the ternary complex (Figure 1). Unlike the extension reaction, product resulting from loss of a single nucleotide was not observed. In the presence of 1 nM FEN1 the product resulting from loss of 2 nucleotides was the major product (Figure 2A). Significant amounts of the 3-nucleotide excision product were observed at later times. Longer portions of substrate molecules were removed when FEN1 was present in greater concentration (10 nM), and products resulting from loss of 3 and 4 nucleotides were the major products under these conditions. Four nucleotides were excised even though the maximum number added to the 5′-fragment (above) was 3. However, the overall amount of starting material lost was not greater than the amount extended by Pol β. As was the case for Pol β mediated extension, FEN1 excision of the ternary complex containing thymidine (3′-^{32}P-**3**) is virtually indistinguishable from that of T-al (See Supporting Information). FEN1 reactivity with F (3′-^{32}P-**1**) was also correlated with strand displacement of this substrate by Pol β (See Supporting Information). Although the total amount of reaction lagged behind extension of the 5′-fragment of the same ternary complex, FEN1 excision of DNA containing the abasic site (F) was more efficient than for T-al. Loss of 2 nucleotides was the major product in the presence of 1 nM FEN1 but increasing FEN1 to 10 nM resulted in predominant formation of a -4 nucleotide product. Single nucleotide excision product was not observed for the abasic site complex (3′-^{32}P-**1**) under either reaction conditions.

Excision of one and three nucleotide flaps by FEN1

The absence of single nucleotide excision products in substrates containing F, T, and T-al was inconsistent with a previous study on nicked DNA (32). Consequently, we examined FEN1 excision of independently prepared ternary complexes containing a flap that consisted either of an abasic site (F, 3′-^{32}P-**4**) or a three-nucleotide flap with an abasic site at its 5′-terminus (3′-^{32}P-**5**). Although 3 constructs were examined in this investigation, complexes **4** and **5** contain an abasic site model, which forms less stable duplexes than native nucleotides. This was chosen in order to bias the orientation of the flap to that which is produced transiently during strand displacement synthesis. Reaction of the independently prepared 3-nucleotide flap (3′-^{32}P-**5**) with FEN1 (Figure 3) showed very similar reactivity as observed above for the transiently generated substrate (Figure 2). The 3-nucleotide excision product

was the major product at higher FEN1 concentration (Figure 3B). However, no single nucleotide excision product was detected even when the FEN1 concentration was only 1 nM (Figure 3A). One distinction was that 3′-^{32}P-**5** did not yield any 4-nucleotide excision product when incubated with FEN1. Moreover, no reaction was observed when 3′-^{32}P-**4** was incubated with 1 nM FEN1 for 1 h (See Supporting Information). A few percent of 1- and 2-nucleotide excision products were observed after 40 min when the FEN1 concentration was increased to 10 nM.

T-al removal by nucleotide excision repair

The incision sites generated by reaction of UvrABC with a 51 bp ternary complex containing T-al (8) were determined using material in which the flanking strand was labeled at its 5′-terminus and the T-al containing strand was 3′-^{32}P-labeled in separate experiments. In addition, incision in the opposite strand was examined by labeling its 5′-terminus. No cleavage was detected after 3 h when the 3′-terminus of the strand containing T-al or the complementary strand was labeled. In contrast, time dependent incision at G_{16} and C_{18} (See Chart 1 for positions relative to lesion) was observed in 5′-^{32}P-**8** in which the flanking strand is labeled (Figure 4). These positions correspond to cleavage at the 8th and 10th phosphate diesters to the 5′-side of T-al. The 5′-flanking oligonucleotide in ternary complexes containing F (7) and thymidine (9) were also the only components of these substrates that were cleaved by UvrABC. They too were cleaved at the G_{16} and C_{18} positions (See Supporting Information). However, the preference for cleavage at C_{18} was greater (>3:1) in the abasic site-containing complex (5′-^{32}P-**7**) than when T-al (5′-^{32}P-**8**) or thymidine (5′-^{32}P-**9**) was present (~1–1.4:1). The approximate rates of incision in 5′-^{32}P-**7–9** were similar to one another but were considerably slower than an analogous 50-nucleotide duplex (5′-^{32}P-**6**) containing a C5-fluoresceinylated thymidine (Fl-dT). The latter modification is often used as a standard when determining the susceptibility of lesions to UvrABC (33). In side-by-side reactions ~90% of the fluoresceinylated thymidine standard was incised within 30 min (Figure 5), whereas only 41% of the T-al containing substrate was incised. The amount cleaved was moderately lower than the abasic site but greater than thymidine. No incision was observed on the 3′-side of thymidine, T-al, or F, nor was the complementary cleaved in ternary complexes 3′-^{32}P-**7–9** (Data not shown).

Discussion

Unlike all other lesions except 2′-deoxyuridine, T-al's nucleobase is unmodified. T-al is distinguished from 2′-deoxyuridine because it is produced opposite its cognate nucleotide. It is also unusual because ternary complexes containing T-al melt at higher temperatures than the respective molecules containing thymidine (19). Hence, there is no reason to expect that this unique product of oxidative stress will be a substrate for a glycosylase, the first step in short patch BER. These data indicate that LP-BER provides an alternative means for removing T-al. Strand displacement synthesis of the tetrahydrofuran abasic site model (F) by Pol β has been reported and was used as a benchmark for T-al (32). FEN1 stimulates LP-BER involving Pol β mediated strand displacement synthesis to varying extents for a variety of other abasic sites and a tandem lesion that contains an oxidized abasic lesion (34–36). However, it was recently shown that the two enzymes do not coordinate the passing of the DNA substrate to one another (37). During LP-BER of T-al increasing FEN1 from 1 nM to 10 nM did not increase the overall level of repair but did alter the product distribution. The 4 nt excision product was the major one at the higher FEN1 concentration when the ternary complex contained T-al, F, or thymidine even though a comparable length extension product was not formed. Excision of 4 nucleotides is consistent with previous studies (32, 38). However, other observations presented above are inconsistent with these previous studies, including the hit and run mechanism of Pol β/FEN1 LP-BER (32). Reaction of any of the 3

ternary complexes with Pol β and FEN1 failed to produce any single nucleotide incision product. Because this is inconsistent with previous study on ternary complexes containing F we examined the reaction of FEN1 with independently prepared ternary complexes (32). Although a three nucleotide flap was incised, FEN1 slowly incised small amounts of a single nucleotide flap containing F.

Although LP-BER provides a means to remove T-al, organisms employ redundant DNA repair pathways to protect their genomes (39–41). Consequently, we examined T-al repair by the UvrABC nucleotide excision repair system. UvrABC recognized the ternary complex containing T-al, as well as those containing F or thymidine at the 5′-terminus of their 3′-fragments. Although the 3 ternary complexes were substrates for the NER system, the uncleaved complementary strand was not incised. Hence, unlike recently studied interstrand cross-links, double strand breaks do not result from T-al misrepair (23, 42, 43). UvrABC incises the 5′-fragments of ternary complexes containing T-al, F, and thymidine, but the protein complex does not cleave any of the 3′-fragments. This cleavage pattern is consistent with previous reports of UvrABC action on nicked, ternary complexes (44, 45). However, failure to incise the fragment of the ternary complex that contains T-al means that this lesion is not repaired by nucleotide excision repair.

Summary

The major lesion resulting from 5′-oxidation of duplex DNA, a strand break containing a 5′-terminal aldehyde on the 3′-fragment is ineffectively repaired by bacterial nucleotide excision repair. The UvrABC complex incises a ternary complex containing a 5′-aldehyde (e.g. T-al) but not the strand that contains the lesion, indicating that the cleavage is a non-repair event. T-al is removed during strand displacement synthesis by FEN1 with comparable efficiency as an abasic site. Strand displacement synthesis by Pol β and FEN1 is the only method thus far identified for repair of T-al. The reliance on Pol β for T-al removal is potentially significant. Pol β is over expressed in some tumor cell lines and is a potential target of inhibitors that are designed to be adjuvants to DNA damaging agents (46–48). Recently, it was reported that another lesion (DOB, Scheme 1) that forms in competition with T-al following C5′-oxidation irreversibly inhibits Pol β (24, 25). T-al and DOB may have a synergistic effect on cells. Irreversible inhibition of Pol β by DOB will give rise to a more potent effect of T-al on the well being of a cell in the absence of an alternative repair pathway for the latter. The effects of these lesions on DNA repair may help explain why some of the agents that produce them are such potent cytotoxins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AP	abasic site
F	tetrahydrofuran abasic site
BER	base excision repair
LP-BER	long patch-base excision repair

NER	nucleotide excision repair
APE1	apurinic/apyrimidinic endonuclease 1
Pol β	DNA polymerase β
FEN1	flap endonuclease 1

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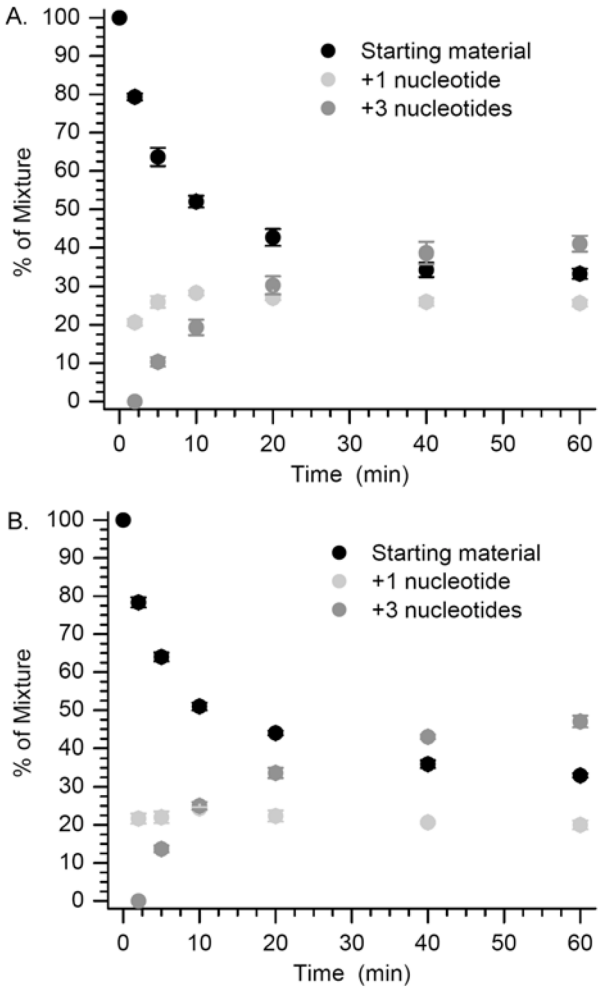


Figure 1.
Strand displacement synthesis of T-al (5′-^{32}P-**2**) by Pol β (1 nM) in the presence of (A) 1 nM FEN1 (B) 10 nM FEN1.

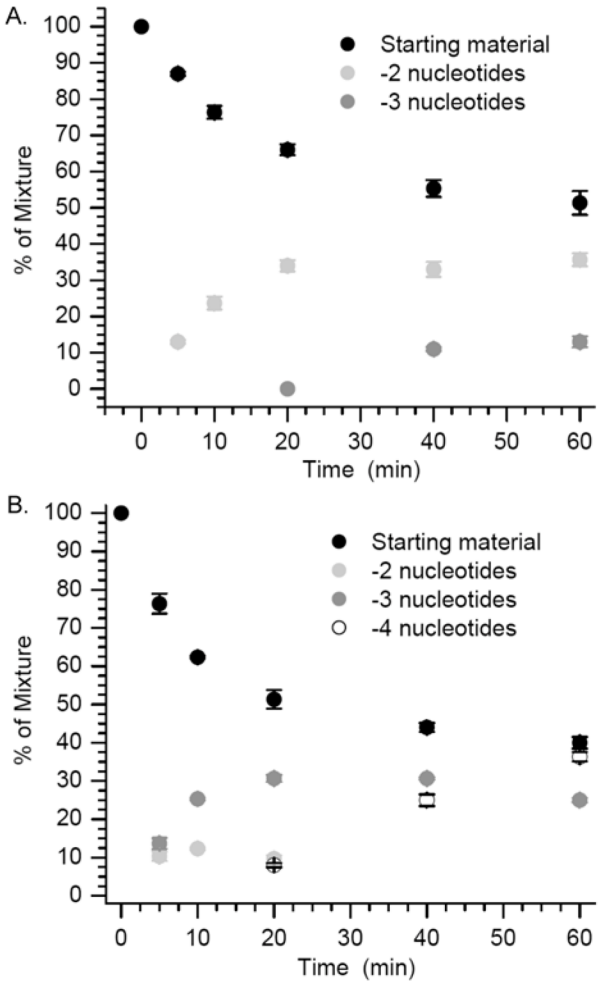


Figure 2.
Cleavage of T-al (3′-^{32}P-**2**) in the presence of Pol β (1 nM) and (A) 1 nM FEN1 (B) 10 nM FEN1.

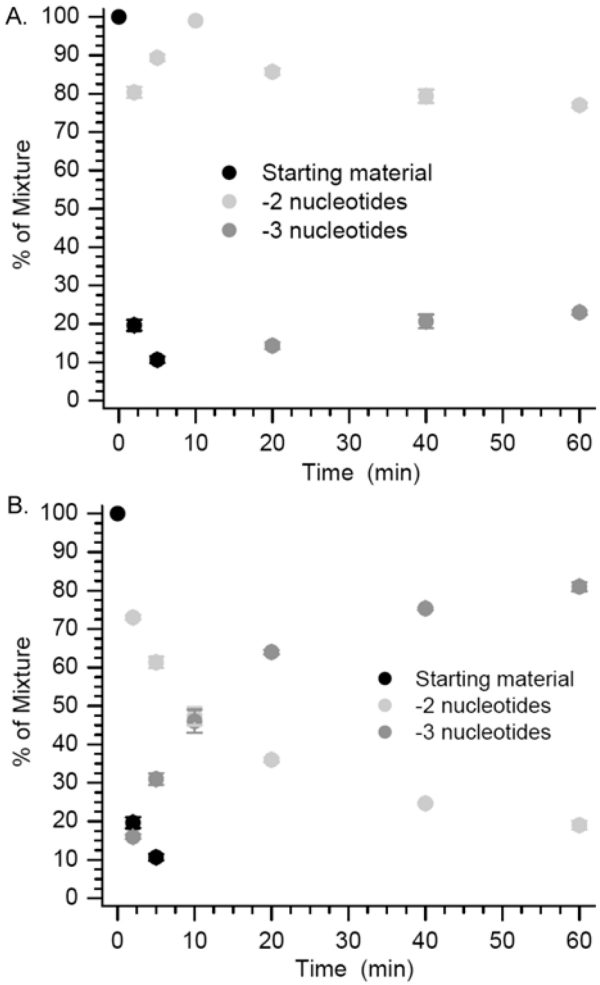


Figure 3.
FEN1 incision of F (3′-^{32}P-**5**). (A) 1 nM FEN1 (B) 10 nM FEN1.

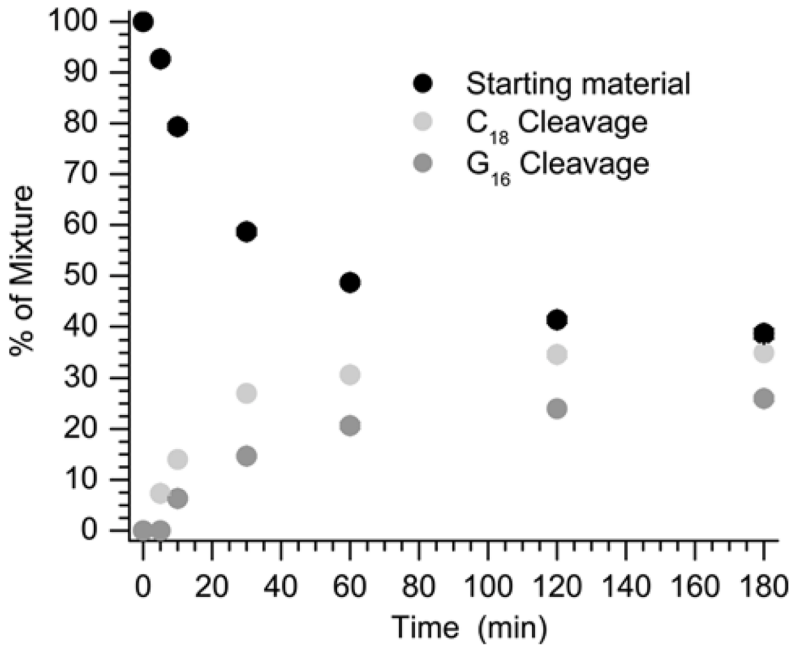


Figure 4.
Incision of ternary complex containing T-al (5′-^{32}P-**8**) by UvrABC.

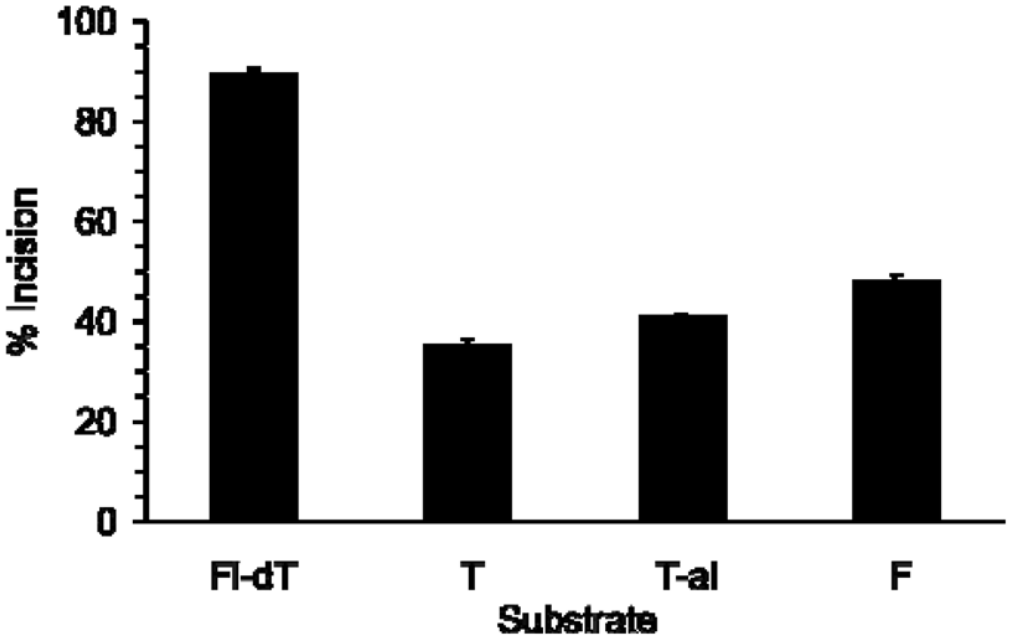
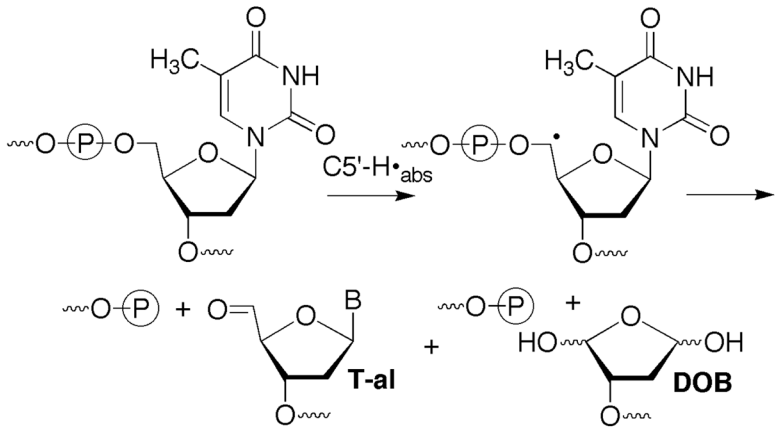


Figure 5.
Comparison of UvrABC incision of DNA containing Fl-dT (6), thymidine (9), T-al (8), and F (7) in 30 min.



Scheme 1.
Formation of T-al and DOB from a C5′-radical.

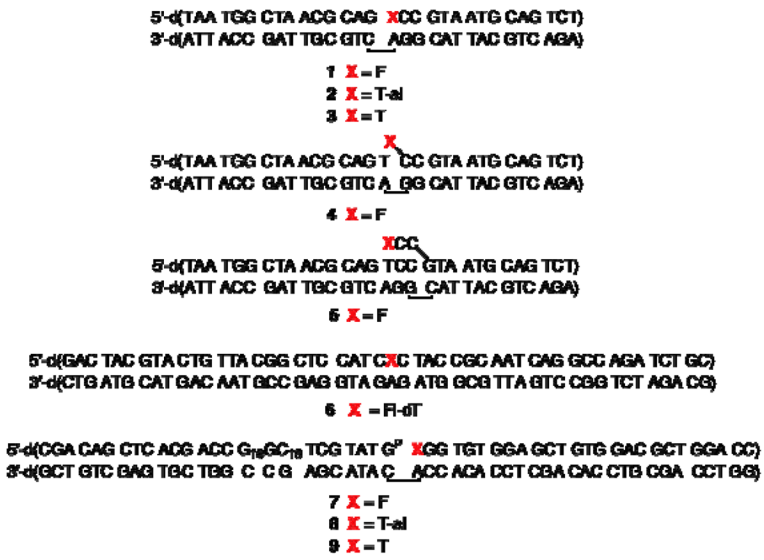


Chart 1.
Nucleic acid complexes used in this study.