A novel method for site specific introduction of single model oxidative DNA lesions into oligodeoxyribonucleotides

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ABSTRACT

Calf thymus terminal deoxynucleotidyl transferase was used to incorporate several products of oxidative base damage onto the 3' end of oligodeoxyribonucleotides. Under the defined conditions described in this report, single residues of dihydrothymine, β -ureidobutyric acid, thymine glycol, urea, 7-hydro-8-oxoadenine, 7-hydro-8-oxoguanine, 5-hydroxycytosine and 5-hydroxyuracil were incorporated into oligodeoxyribonucleotides of different lengths. The reaction is both efficient and cost effective. The 3' termini of the reaction products were suitable substrates for ligation by phage T4 DNA ligase, facilitating greatly the construction of oligodeoxyribonucleotides containing unique and site specific oxidative DNA lesions.

INTRODUCTION

Free radicals, biologically active molecules produced by ionizing radiation, chemicals, and cellular metabolic processes (1), have been implicated in mutagenesis, carcinogenesis and aging. A major target of free radical attack are the base moieties in nucleic acids. Since the number of products that free radicals produce in DNA is very large, it is important to establish which of these products contributes to the harmful effects of the agents that produce them. This can be achieved by studying the effects of each of these products on biological endpoints when they are introduced uniquely into nucleic acids.

Two basic approaches, random and site-specific introduction, have been utilized to study the effect of individual types of oxidative damage in DNA. Random introduction generally involves treatment of DNA with chemical modifying agents (2–5), however, this approach suffers from several limitations. For instance, most of the methods described to date do not result in the production of a unique product in DNA, but rather a major product accompanied by several minor products. This precludes making conclusive cause/effect relationships between a particular type of damage and an observed end point. Incorporation of chemically synthesized deoxyribonucleoside triphosphates by DNA polymerase has also been used to randomly introduce

damage into DNA (6–8). This is a cleaner method since minor products are rarely present. Both of these methods have been used to introduce a damage randomly on DNA molecules, thus limiting considerations of the sequence context within which the damage is presented. Site-specific incorporation of DNA lesions has used chemical and enzymological techniques. Chemically synthesized derivatives of lesions were used for incorporation with an oligonucleotide synthesizer (9, 10). However, the preparation of the lesion derivatives is a fairly complicated process, and the deprotection step during synthesis often alters the lesion. RNA ligase has been used to incorporate the 3', 5' diphosphates of several modified nucleosides (11–13). This method is time consuming and costly. DNA polymerase was also used to incorporate nucleoside triphosphates of lesions site-specifically (14); however, only a limited number of these triphosphates are suitable substrates for DNA polymerase.

Here, we demonstrate a method to incorporate a single modified deoxyribonucleotide on the 3' end of an oligodeoxyribonucleotide through the use of terminal deoxynucleotidyl transferase (TdT). This enzyme has been cloned from several mammalian sources and is the subject of several recent reviews (15–18). TdT is a template-independent DNA polymerase which catalyzes the addition of 2'-deoxynucleotides, 3'-deoxynucleotides, 2'-3'-dideoxynucleotides and nucleotides with modifications in either the sugar and base moieties, to the 3' ends of nucleic acids. The substrate (often called initiator) which is extended by TdT must have an hydroxyl group at the 3' end, but otherwise can be either a ribo- or deoxyribonucleic acid of practically any size and can be single or double stranded. TdT also requires a divalent metal ion cofactor. Magnesium has been shown to be the optimal cofactor for the incorporation of purines, while cobalt serves as the best cofactor for the incorporation of pyrimidines (19).

We have used TdT to incorporate single nucleotides of several oxidative DNA damage products, that are under study in this laboratory, into oligodeoxyribonucleotides. These lesions include dihydrothymine (DHT) and its alkali fragmentation product β -ureidobutyric acid (UBA), thymine glycol (TG) and its alkali fragmentation product urea, 7-hydro-8-oxoadenine (A^{8-oxo}),

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7-hydro-8-oxoguanine (G^{8-oxo}), 5-hydroxycytosine (C^{5-OH}) and 5-hydroxyuracil (U^{5-OH}). The procedure described is simple, efficient and cost-effective. We envision that this method could be used universally for site-specific incorporation of modified nucleotides into DNA.

MATERIALS AND METHODS

Oligodeoxyribonucleotides

Several oligodeoxyribonucleotides varying in size between 8 and 60 nucleotides were prepared on an Applied Biosystems model 380A DNA synthesizer (at the Department of Microbiology and Molecular Genetics, University of Vermont). The oligodeoxyribonucleotides were purified either by high performance liquid chromatography (HPLC) on a Pharmacia Mono-Q anion exchange column following the instructions of the manufacturer or by 20% PAGE containing 8 M urea.

dNTPs and modified dNTPs

2'-Deoxynucleoside triphosphates were obtained from Pharmacia LKB Biotechnology and the Sigma Chemical Co.; 2', 3'-dideoxynucleoside triphosphates were obtained from New England Biolabs; dihydrothymidine triphosphate and thymidine glycol triphosphate were prepared as described (6, 7); triphosphates of β -ureidoisobutyric acid and urea glycoside were prepared from triphosphates of dihydrothymine and thymine glycol, respectively, by hydrolysis in 0.1 M potassium phosphate, pH 12.0 for 4h at 37°C followed by neutralization with 0.1 M potassium phosphate, pH 7.0; 7-hydro-8-oxoadenosine triphosphate was prepared from 7-hydro-8-oxoadenosine monophosphate (synthesized by the method of Ikehara (20, 21)) by the imidazolid activation of the phosphomonoester group followed by treatment with excess tetrabutylammonium pyrophosphate (22). $A^{8-oxo}TP$ was purified by HPLC on a Mono Q anion exchange column followed by desalting on a C 18 column using water as the eluant. The yield of conversion of $A^{8-oxo}MP$ to $A^{8-oxo}TP$ was approximately 40 to 50%. 7Hydro8oxo-guanosine triphosphate was prepared from dGTP using a modification of the method described by Ikehara (20). dGTP was hydroxylated at the position C-8 by ascorbic acid in the presence of O_2 and Fe^{2+} ions in 0.1 M phosphate buffer, pH 6.8, at 37°C in the dark. The nucleoside triphosphates were purified by anion exchange chromatography on DEAE-Sephadex A-25 and eluted with a gradient of 0–1.2 M triethylammonium bicarbonate (TEAB), pH 7.8. $G^{8-oxo}TP$ was separated by HPLC on a C 18 column using 10 mM sodium acetate, 3.5 mM sodium citrate buffer, pH 5.1, containing 5% methanol as the eluant

followed by desalting on the same column using water as the eluant. The yield of $G^{8-oxo}TP$ was approximately 10%. 2'-Deoxy-5-hydroxycytidine-5'-triphosphate ($dC^{5-OH}TP$) was prepared by the bromination of dCTP in aqueous medium followed by 2,4,6-collidine treatment as described (23). The reaction mixture was separated on DEAE-Sephadex A-25 and eluted with a linear gradient of 0–1.5 M TEAB, pH 7.8. The major peak corresponding to $dC^{5-OH}TP$ was desalted by repeated evaporation with ethanol, repurified on a Mono Q anion exchange column and eluted with a linear gradient of 0–0.6 M ammonium bicarbonate. The ammonium bicarbonate was removed by repeated evaporation with ethanol. The final yield was about 95% $dC^{5-OH}TP$ and 5% $dC^{5-OH}DP$. 2'-deoxy-5-hydroxyuridine-5'-triphosphate ($dU^{5-OH}TP$) was synthesized by bromination of dUTP in aqueous medium followed by pyridine treatment as described (24). $dU^{5-OH}TP$ was further purified as described for $dC^{5-OH}TP$ with a final yield of about 95% $dU^{5-OH}TP$ and 5% $dU^{5-OH}DP$.

Enzymes

Calf thymus terminal deoxynucleotidyl transferase, phage T4 polynucleotide kinase and the exonuclease free version of phage T7 DNA polymerase (Sequenase v.2) were obtained from US Biochemicals; phage T4 DNA ligase was from Gibco-BRL; *E. coli* endonuclease III and formamidopyrimidine (FAPY) DNA glycosylase were prepared as described (24).

Reaction conditions

Oligodeoxyribonucleotides were labeled with ^{32}P using phage T4 polynucleotide kinase following recommendations of the supplier. Terminal deoxynucleotidyl transferase reactions were typically done as follows: 5'- ^{32}P -end-labeled oligodeoxyribonucleotide (5–20 nM) in 0.1 M sodium cacodylate buffer, pH 7.0, 1 mM $CoCl_2$, 0.1 mM dithiothreitol and 50 μ g/ml bovine serum albumin was incubated for 10–45 min at 30°C in the presence of 0.05–100 μ M modified dNTP and 0.3–2.0 units of TdT (18–120 nM). The reaction was stopped by heating for 10 min at 65°C. DNA polymerase reactions were carried out for 15 min at 28°C in 10 mM Tris-HCl, pH 7.5, 8 mM $MgCl_2$, 3.6 mM dithiothreitol and 30 mM NaCl and included 50–100 nM of annealed primer-template, 100 μ M each of dATP, dGTP, dCTP and dTTP and 0.5 unit of phage T7 DNA polymerase. Ligation reactions were performed for 15 min at 28°C in 0.05 M Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM dithiothreitol and 5% (w/v) polyethyleneglycol-8000) and included 50–100 nM of nicked duplex oligodeoxyribonucleotide and 0.1 unit of T4 DNA ligase.

Table 1. Summary of critical parameters required for the construction of oligodeoxyribonucleotides containing modified nucleotides[†]

Modified nucleotide	Nucleotide concentration	Nucleotide initiator (M)	TdT reaction yield [‡]	Extension by DNA polymerase	Ligation by DNA ligase
DHT	50 nM	~ 5:1	40–50%	>95%	>95%
UBA	5 μ M	~ 500:1	50–80%	<10%	20–30%
TG	1 μ M	~ 100:1	50–80%	<10%	>80%
Urea	5 μ M	~ 500:1	50–80%	<10%	20–30%
A^{8-oxo}	2–5 μ M	~ 250:1	>90%	>95%	>95%
G^{8-oxo}	500 nM	~ 50:1	>90%	>95%	>95%
C^{5-OH}	50 nM	~ 5:1	20–40%	>95%	>95%
U^{5-OH}	50 nM	~ 5:1	20–40%	>95%	>95%

[†]The remaining reaction parameters (buffer content, enzyme concentration, temperature and reaction duration are as described in Materials and Methods.

[‡]Numbers represent yields of initiator+1.

Endonuclease III and FAPY DNA glycosylase reactions were done exactly as the ligation reactions except that the incubation period was 5 min and the enzymes were present at a concentration of 150 nM (endo III) and 0.5–5.0 nM (FAPY).

Electrophoresis

Reactions were terminated by adding an equal volume of 95% formamide, 0.03% bromophenol blue and 0.03% xylene cyanol. Reaction products were analyzed by electrophoresis on 0.25 mm thick polyacrylamide gels (19.3% acrylamide, 0.7% bis-acrylamide) containing 8 M urea. The gels were electrophoresed in Tris–borate buffer (89 mM Tris–borate, 2 mM EDTA, pH 8.3) for 3–4 h at 3000 V, dried under vacuum, and exposed to X-ray film.

RESULTS AND DISCUSSION

Incorporation of modified nucleotides with terminal deoxynucleotidyl transferase

In an effort to maximize the efficiency of polymerizing modified deoxynucleotides onto the 3' ends of DNA by terminal transferase, we varied the conditions for all components of the reaction, namely buffer, initiator, nucleotides and enzyme. Table I lists the parameters required for selective incorporation of single modified nucleotides.

Buffers. Previous reports (17) have shown that TdT functions in several buffer systems including Tris–HCl, phosphate and cacodylate. Although all of these buffers work fairly well during the incorporation of natural nucleotides, we found that cacodylate buffer, pH 7.0, at a concentration of 0.1M, worked best for the incorporation of modified nucleotides. Phosphate buffer, 0.05 M, pH 7.0 also worked, but less efficiently (data not shown). Concentrations between 0.05 and 0.2 M as well as pH values between 7.0 and 8.0 were tried for both buffers, but the aforementioned concentration and pH were optimal. Reactions that were buffered with Tris–HCl did not work efficiently for the modified nucleotides.

Metal ions and salts. TdT requires a divalent metal ion as a cofactor (15). Mg^{2+} has been found to be the best cofactor for

addition of purines while Co^{2+} is better for the incorporation of pyrimidines (19). We also observed this purine/pyrimidine cofactor specificity during the incorporation of the modified nucleotides (data not shown). However, the efficiency of incorporation of A^{8-oxo} and G^{8-oxo} when using Mg^{2+} was only slightly enhanced over Co^{2+} , prompting the use of the latter ion as a cofactor in all of our reactions. The concentration of Co^{2+} was varied between 1–5 mM and Mg^{2+} between 1–10 mM. 1 mM Co^{2+} and 4 mM Mg^{2+} were found to be most effective. In an effort to control the number of residues polymerized onto each initiator molecule, we varied the salt concentration from 0.05 to 0.2 M NaCl. However, increasing the salt concentration lowered the overall rate of incorporation rather than the number of nucleotides incorporated (data not shown).

Initiator. The kinetic constants (K_m and V_{max}) for the TdT reaction have been shown to vary by several orders of magnitude depending on both the length of the initiator and the nature of the 3' ultimate nucleotide. In general, the K_m decreases as the size of the initiator increases (15). We have observed similar effects when polymerizing the modified nucleotides onto initiators varying in size between 8 and 60 nucleotides (8mer, 10mer, 17mer, 24mer, 31mer, 35mer, 59mer and 60mer). Product yields were consistently better when the longer initiators were used; however, with longer initiators, a larger number of DHT, C^{5-OH} and U^{5-OH} residues, and predominantly two TG residues were incorporated. Therefore, we elected to use a shorter initiator in order to control the number of residues incorporated. It is interesting to note that even when a 60mer was used as the initiator molecule, incorporation of more than two residues of UBA, TG or urea was not observed. It is not clear whether this limited incorporation is due to an higher initiator K_m value when the modified nucleotides were substrates and whether additional residues might have been incorporated with longer initiators, or if the presence of the modified nucleotide at the 3' terminus rendered the DNA molecule unsuitable as a substrate for TdT.

Duration and temperature of reactions. The incubation temperature was varied between 25°C and 37°C and the duration of the TdT reaction between 10 and 90 min. Longer incubation

Figure 1. Relative incorporation of natural and modified nucleotides by TdT. Each reaction contained 10 nM initiator (10mer), 0.5 μ M natural or modified nucleoside triphosphate, and 60 nM TdT. The reactions were carried out at 30°C for 30 min. The extension products in the C lane were too long to be seen in this photograph.

Figure 2. Conditions favoring limited incorporation of modified nucleotides. Each reaction contained 8 nM initiator (24mer), 120 nM TdT and the following concentrations of nucleoside triphosphate: UBA, TG, urea, and A^{8-oxo} , 5 μ M; G^{8-oxo} , 500 nM; U, 200 nM; A, G, C, T, and DHT, 100 nM; C^{5-OH} , and U^{5-OH} , 50 nM. Reactions were carried out at 30°C for 30 min (A, G, C, T, C^{5-OH} , and U^{5-OH}) or 75 min (U, DHT, UBA, TG, urea, A^{8-oxo} , and G^{8-oxo}).

times resulted in higher incorporation levels, however, degradation of a small amount of the initiator (<10%) was also observed during extended incubation times, especially in the absence of any nucleoside triphosphate. This could be due to a contaminant in the TdT preparation. Varying the temperature did not seem to affect the reaction.

Enzyme concentration. TdT was used at several concentrations with TdT: initiator molar ratios ranging between 2:1 and 20:1. As expected, we observed a higher rate of polymerization when TdT was present in large excess. A molar ratio of 5:1 seemed to favor the incorporation of single residues of the modified nucleoside triphosphates. Molar ratios were calculated after obtaining specific activity values for TdT from the manufacturer

(values were obtained for individual preparations), and although most of the work was done with enzyme purchased from US Biochemicals, similar results were obtained with enzyme from Pharmacia.

Nucleotides. As can be seen in Figure 1, at a concentration of 0.5 μ M, the extent to which each of the modified nucleotides was incorporated with TdT varied, reflecting perhaps different K_m values. Two residues of DHT (lanes 8) and many residues of C⁵-OH (lane 14) and U⁵-OH (lane 15) were incorporated under conditions where only one residue of the other modified nucleotides were incorporated. With TG (lane 10), the apparent migration of the extension product suggested that predominantly two residues were incorporated (initiator + 2). Although we were

Figure 3. Panel A. Schematic of internalization of modified nucleotides into oligodeoxyribonucleotides. Panel B. DNA polymerase extension and ligation of oligodeoxyribonucleotides with a 3' terminal G^{8-oxo}. An initiator (24mer, lane 1) was extended with G^{8-oxo} (lane 2) as described in Figure 2, annealed to a complementary sequence of phage f1 where the nucleotide opposite the lesion was C, and then extended by DNA polymerase in the presence of dATP, dGTP, dTTP and ddCTP (lane 3). The DNA polymerase extension product was treated with FAPY DNA glycosylase (5 nM, lane 4 or 0.5 nM, lane 5) to demonstrate specific cleavage of G^{8-oxo}. A second initiator was extended with G^{8-oxo} (lanes 6 and 7) and annealed to a complementary sequence of phagemid SK⁻ where the nucleotide opposite the lesion was A and then ligated (lane 8) to a second oligodeoxyribonucleotide as in panel A. Panel C. Ligation of oligodeoxyribonucleotides with 3' terminal DHT and TG. An initiator (10mer, lane 1 and panel A) was extended with either DHT (lane 2) or TG (lane 4), annealed to a complementary partially double stranded oligodeoxyribonucleotide and ligated (lanes 3 and 5). The TG ligation product was then treated with endonuclease III (lane 6) to demonstrate specific cleavage of the lesion.

able to detect some products with an apparent migration of initiator+3, we were never able to detect a putative initiator+1 product. Furthermore, an initiator+1 product could not be detected when the apparent initiator+2 product was treated with increasing concentrations of piperidine at 95°C (data not shown). This, in addition to results obtained from ligation experiments (see below), indicated that the molecule with an initiator+2 apparent mobility was actually the product of a single residue addition. This does not seem unusual when the apparent mobilities of molecules tailed with T (lane 4) are compared to those tailed with A (lane 1) or U (lane 5). The apparent mobility of the initiator+10 A residues is the same as that of the initiator+9 T residues. Similarly, and not surprisingly, an aberrant migration pattern was observed for the product of extension with UBA and urea where the initiator+1 migrated very closely to the initiator, requiring an extended electrophoresis for the two oligodeoxyribonucleotides to be resolved. The faster mobility of the initiator+1 is probably due to the altered ring structure.

Nucleotide concentration during the extension reaction was varied from 0.05 to 100 μ M since it was reported (18), that for the natural nucleotides, it is possible to limit the number of added residues by maintaining a low nucleotide:initiator molar ratio. For each of the modified nucleotides, we obtained concentrations which favor the incorporation of a single residue as demonstrated in Figure 2 and listed in Table I. At a concentration of 50 nM, DHT (Fig. 2, lane 7), C⁵-OH (lane 14) and U⁵-OH (lane 15) were incorporated efficiently, although the yield of initiator+1 was only 30–40%. Such a yield is acceptable when the very low molar ratio of nucleotide:initiator (5:1) and the relative ease of separating short oligodeoxyribonucleotides (<20 nucleotides) by HPLC are considered. A single residue of G⁸-oxo was incorporated with a very high yield (>90%) at a concentration of 0.5 μ M (lane 12). Similarly efficient incorporation of A⁸-oxo was obtained (lane 13), albeit at a higher concentration (2–5 μ M; 5 μ M shown in Figure 2). TG was incorporated at high efficiency and with a yield of initiator +1 of >70% at a concentration of 1 μ M (lane 9). UBA and urea were both incorporated with initiator+1 yields about 50% at a concentration of 5 μ M (lanes 8 and 10). It is interesting to note that the K_m values for incorporation of the nucleoside triphosphates of DHT, C⁵-OH and U⁵-OH by DNA polymerase are 10–50 fold lower than those for A⁸-oxo and G⁸-oxo (26).

Construction of oligodeoxyribonucleotides containing single, internal oxidative lesions

When constructing model systems to study the interactions between individual oxidative DNA lesions and proteins *in vitro* or *in vivo*, it is desirable to position the lesion internally in the DNA molecule rather than at one end. Since TdT leaves the lesion at the 3' terminus of the initiator molecule, two methods were tried to achieve the above goal, extension of the lesion primer terminus with a DNA polymerase or ligating the extended initiator to a second oligodeoxyribonucleotide using DNA ligase. The extended initiator is then used to construct a double stranded DNA molecule which is a suitable substrate for DNA polymerase or DNA ligase as demonstrated by Figure 3A. Since the 3' terminus of the lesion participates in both reactions, it is important to minimize distortions in the structure of the double stranded oligodeoxyribonucleotide in the vicinity of the lesion. Five of the lesions used in this report, DHT, UBA, TG, urea and U⁵-OH should form the most stable structure when A is present on the complementary strand (27, 28). G⁸-oxo should form a stable

structure when either C or A is present on the complementary strand (29, 14) and C⁵-OH should form a stable structure with either G or A on the complementary strand (26). We studied the extension and ligation properties of oligodeoxyribonucleotides with the above seven lesions at the 3' terminus using A as the base complementary to the lesion. We also tested the extension of the 3' terminus of G⁸-oxo opposite C and C⁵-OH opposite G. To study the extension and ligation properties of oligodeoxyribonucleotides with A⁸-oxo at the 3' terminus, we used T, the nucleotide predicted to form the most stable structure opposite A⁸-oxo (30), as the base complementary to this lesion.

As can be seen in Figure 3B, the 3' terminus of G⁸-oxo was extended very efficiently with Sequenase when the complementary base was C (lane 3). The 3' termini of DHT, C⁵-OH and U⁵-OH nucleotides were extended by Sequenase with similar efficiency when the complementary base was properly pairing (see Table I). The 3' termini of UBA, TG and urea nucleotides were not readily extended by Sequenase, however, even when the complementary base was A. These results are not surprising since, in the form of nucleoside triphosphates, they are all poor substrates for DNA polymerases (6, 26). We have also shown that during *in vitro* bypass reactions of UBA, TG or urea residues (31), DNA polymerases were able to insert a base opposite the lesion, but unable to extend the resulting primer terminus.

To overcome the limitation of poor extension by DNA polymerase, we asked whether the 3' termini of the modified nucleotides could serve as substrates for phage T4 DNA ligase. Figure 3C shows the ligation products of oligodeoxyribonucleotides extended with DHT (lane 3) and TG (lane 5). It is important to note that only the initiator+1 molecules ligated properly, especially in the case of TG where we suggested above that the migration of the initiator+1 was aberrant. The migration of the ligated products containing DHT and TG (lanes 3 and 5) were similar and of the predicted size as judged by dideoxynucleotide sequence ladders (data not shown), supporting this suggestion. Similarly, efficient ligation reactions were obtained with A⁸-oxo, G⁸-oxo, C⁵-OH and U⁵-OH when the complementary nucleotide paired (Table I). However, the 3' termini of UBA and urea were poor substrates for DNA ligase. This, however, does not represent a major setback, since these two lesions can be introduced site specifically into DNA simply by incorporating DHT or TG first and then converting these to UBA and urea by alkaline hydrolysis.

By using DNA ligase, we were also able to introduce lesions into oligodeoxyribonucleotides where the base opposite the lesion is mismatched. Figure 3B, lane 7 shows the efficient ligation of G⁸-oxo opposite A. C⁵-OH was also ligated opposite A but the efficiency of the reaction was poorer (data not shown). In both cases, however, the lesions partially pair with A (29, 26) and we would not expect efficient ligation when the lesion and the base on the opposite strand result in a distorted DNA structure. Ligation reactions were also performed using *E. coli* DNA ligase and phage T4 RNA ligase, and similar results were obtained (data not shown). However, the efficiency and lower cost of T4 DNA ligase make it the enzyme of choice for this application.

To confirm that site-specific incorporation of a single oxidative DNA lesion into an oligodeoxyribonucleotide was successful, we treated the ligation products with *E. coli* endonuclease III and FAPY DNA glycosylase. Endonuclease III has a glycosylase activity which recognizes DHT, UBA, TG and urea but not A⁸-oxo or G⁸-oxo (32, 33) while FAPY DNA glycosylase

recognizes G^{8-oxo} but not the other lesions (10). Both glycosylase activities are coupled to a DNA lyase activity which leaves a DNA strand break on the 3' side of the removed base. In Figure 3B, lanes 3 and 4 show the cleavage of oligodeoxyribonucleotides containing G^{8-oxo} by FAPY DNA glycosylase. Similarly, endonuclease III cleaved the oligodeoxyribonucleotide containing TG. The endonuclease reactions were specific and no cleavage of TG ligation products was observed by FAPY DNA glycosylase or vice versa (data not shown).

In this report we have presented a simple and efficient method to incorporate oxidative DNA lesions site-specifically into DNA. The reaction yields of both the incorporation step with TdT as well as the internalization of the lesion in an oligodeoxyribonucleotide ranged between 30 and >90%. The lesions studied represent a wide spectrum of oxidative DNA damages; they are derived from A, G, C, T and U and they vary structurally from pyrimidine ring saturation to 'open ring' products. This flexibility leads us to believe that this method could be utilized to introduce other types of modified nucleotides site-specifically into DNA. Furthermore, the concentrations at which these nucleotides were incorporated (nM) are orders of magnitude lower than those required for incorporation by DNA polymerases or RNA ligase. Coupled with the relative low cost of TdT, this method is more cost-effective in view of the time and effort required to synthesize the modified nucleoside triphosphates. An added advantage is the template-independent nature of TdT. This feature facilitates the construction of double stranded DNA molecules with the modified nucleotide opposite several nucleotides of choice. The availability of such DNA molecules containing unique oxidative DNA lesions is an important step necessary for elucidating the biological roles of each of these lesions. Such DNA molecules can be used both *in vitro* and *in vivo* in studies involving interactions between DNA lesions and cellular components such as DNA polymerases, RNA polymerases and repair enzymes.

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