Guanine–thymine intrastrand cross-linked lesion containing oligonucleotides: from chemical synthesis to *in vitro* enzymatic replication†

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An intrastrand cross-link lesion, in which two neighboring nucleobases are covalently tethered, has been site-specifically synthesized into defined sequence oligonucleotides in order to perform *in vitro* replication studies using either bacterial replicative or translesional synthesis polymerases. The investigated tandem base lesion that involves a cross-link between the methylene group of thymine and the C8 of an adjacent guanine residue has been prepared by UV-photolysis under anaerobic condition of the photolabile precursor 5-(phenylthiomethyl)-2′-deoxyuridine that has been site-specifically incorporated into a 9-mer oligonucleotide. After ligation, the lesion-containing modified oligonucleotide was used as a DNA template in primer extension reactions catalyzed by several DNA polymerases including the fragment Klenow exo-(Kf-) of *E. coli* polymerase I, the *Thermus aquaticus* polymerase (Taq pol) and the *E. coli* translesional DNA polymerase Pol IV (dinB). It was found that the primer extension reaction was stopped after the incorporation of the correct nucleotide dAMP opposite the 3′-thymine residue of guanine$^{C8–CH2}$ thymine lesion by Kf- and Pol IV; however it was noted that the efficiency of the nucleotide incorporation was reduced. In contrast, the Taq polymerase was totally blocked at the nucleotide preceding the tandem lesion. These results are strongly suggestive that the present intrastrand cross-link lesion, if not repaired, would constitute a blocking lesion for prokaryotic DNA polymerases, being likely lethal for the cell.

Introduction

In the last few years new types of radiation-induced DNA lesions have been shown to be generated between two vicinal bases on the same strand in oxygen-free aqueous solutions.[1–6] Examples of the latter tandem lesions consist of a purine base, either guanine or adenine that is attached through the C8 of the imidazole ring to the methyl group of either thymine or 5-methylcytosine. Interestingly the formation of these cross-links was found to occur as the result of only one-radical hit that could be $^{\bullet}$OH radical or one-electron oxidation leading in both cases to the generation of a methyl radical on the pyrimidine base. This was illustrated initially by the formation of a covalent bond between the methyl group of a thymine and the C8 carbon atom of the vicinal purine as the result of the radiation-induced generation of the 5-(2′-deoxyuridilyl)methyl radical under hypoxic conditions.[7,8] Interestingly the cross-linked tandem base lesions were synthesized in a more specific way within DNA fragments. This was achieved by incorporating a photolabile precursor of the 5-(2′-deoxyuridilyl)methyl radical into oligonucleotides located either 3′ or 5′ to a purine 2′-deoxyribonucleoside. Subsequent UV irradiation in oxygen-free aqueous solutions of the DNA fragment, thus prepared, was found to lead to the formation in a high yield of the cross-link. It was also shown that the four possible vicinal base lesions involving thymine on the one hand and either guanine or adenine on the other hand were induced by $^{\bullet}$OH radical reactions in ODNs and isolated DNA.[3] Moreover, the tandem base lesion with the guanine moiety on 5′-end (G^T) is generated more efficiently in γ-irradiated isolated DNA than the other T^G position isomer and the two related adenine containing cross-links.[3] It has been recently shown by HPLC-MS/MS measurement that G^T is produced in DNA in aerated aqueous solution upon exposure to the oxidizing Cu(II)–H_2O_2–ascorbate system.[6] Even, if the G^T lesion is generated in a very low yield which is about 3 orders of magnitude lower than that of representative and abundant 8-oxo-7,8-dihydro-2′-deoxyguanosine, the observation provides additional support for the biological relevance of the cross-link lesion.

The formation of cross-linked tandem base lesions gives rise to structural changes in DNA, that are likely to affect the synthesis activity of polymerase enzymes during the critical replication step of DNA. Thus, the synthesis of a biopolymer containing a site-specific lesion at a defined site provides a relevant model for studying the biological properties of DNA including mutagenic and toxic features.[9–11]

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† Electronic supplementary information (ESI) available: Electrospray and MALDI-TOF mass spectra of the 9-mer containing the photolabile precursor and the G^T cross link, respectively. ^{1}H NMR and ESI-MS/MS analyses of the d(G^T) tandem base lesion. PAGE analyses of the enzymatic digestion by HpyCH4 III of the unmodified and modified 21-mer. PAGE analysis of the primer extension assays of a 22-mer containing the 5′*S* and 5′*R* diastereomer of 5′,8-cyclodeoxyadenosine, by the polymerase IV enzyme. See DOI: 10.1039/b609460k

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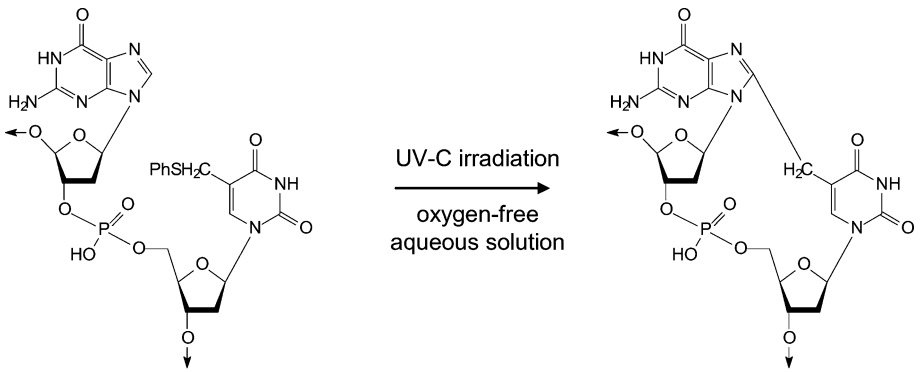

1 **2**

Scheme 1 Structure of the tandem base lesion G^T inserted into oligonucleotide (**2**) and its photoprecursor containing TSPh (**1**).

In this paper, we report the preparation of a DNA template in which the tandem G^T base lesion (**2**) is generated in a selective way at a defined location (Scheme 1). For this purpose the strategy that was applied consisted in the site-specific insertion of the phosphoramidite derivative of 5-(phenylthiomethyl)-2′-deoxyuridine (**1**) into a 9-mer on the 5′-side of a vicinal 2′-deoxyguanosine to prepare a G^T building block. In a subsequent step photolysis in oxygen-free aqueous solution of the modified oligonucleotide thus synthesized allowed the generation of a G^T residue within the 9-mer as an efficient post-synthetic reaction. After purification and characterization, the G^T photoproduct containing nonanucleotide was ligated to a 5′-phosphorylated 12-mer. The resulting 21-mer long modified oligonucleotide was the required template for the *in vitro* replication assays. The ability of three different DNA polymerases, namely the Klenow fragment of *E. coli* polymerase I, *Taq* DNA polymerase, and *E. coli* translesional protein Pol IV,[12,13] to extend a primer annealed with the G^T containing template was investigated.

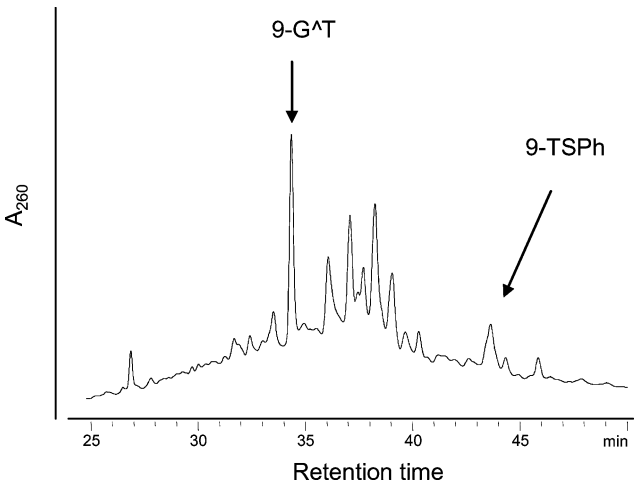

Fig. 1 HPLC elution profile (Hypersil C_{18} 5 μm 250 × 4.6 mm column; acetonitrile gradient in 10 mM TEAA, pH 7) of the 9-mer TSPh upon UV-C irradiation in free-oxygen aqueous solution. Detection by UV absorbance set at 260 nm.

Results

Synthesis and characterization of G^T lesion containing nonanucleotide

A 9-mer oligonucleotide (Table 1) bearing a TSPh residue was synthesized on a solid support using the "Pac chemistry", with a few modifications as previously described.[3] Mass spectrometry measurement by ESI-MS in the negative mode provided support for the integrity and the purity of the radical precursor containing oligonucleotide (data not shown). The pseudo-molecular $[M - 1H]^{1-}$ ion found at $m/z = 2795.4$ is in agreement with the calculated mass of 2797.0. Then, the preparation of a G^T containing 9-mer oligonucleotide was achieved by UV-C irradiation in an oxygen-free aqueous solution of the modified oligomer in which the nucleoside TSPh was inserted. The HPLC elution profile of the photolysis solution, displayed in Fig. 1, shows the presence of a major product which corresponds to the G^T-containing oligonucleotide with a retention time of 34 min under the present analysis conditions. The presence and the integrity of the modification were confirmed by mass measurements (HPLC-MS/MS and MALDI-TOF-MS) coupled with enzymatic digestions (Fig. 2 and see supporting information). It has been previously shown that 3′- and 5′-exonucleases were not able to cleave the phosphodiester bond of the tandem lesion d(G^T).[3] Then, the enzymatic processing by a mixture of nuclease P1 and alkaline phosphatase together with bovine intestinal mucosa phosphodiesterase followed by incubation with calf spleen phosphodiesterase was applied to the modified G^T containing oligonucleotide. HPLC-UV and HPLC-MS/MS analyses of the resulting hydrolysate showed that the dinucleoside monophosphate d(G^T) was quantitatively released (Fig. 2A and B). The ratio of dCyd, dGuo, Thd and [5′-d(G^T)-3′] was found to be 3 : 1 : 2 : 1 in agreement with the structure of G^T containing 9-mer. It may be added that late eluting enzymatically released dAdo was out of the HPLC profile.

An additional enzymatic digestion experiment was performed on the modified 9-mer G^T using the bovine intestinal mucosa phosphodiesterase which cleaved the oligonucleotide strand in a stepwise manner from the 3′-end and can be used in combination with matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry to perform sequencing analysis (see supporting information). Firstly, the MALDI mass spectra of 9-mer G^T showed two pseudo-molecular ions $[M - H]^{1-}$ at m/z 2687.2 and $[M - 2H]^{2-}$ at $m/z = 1343.2$ which corresponded

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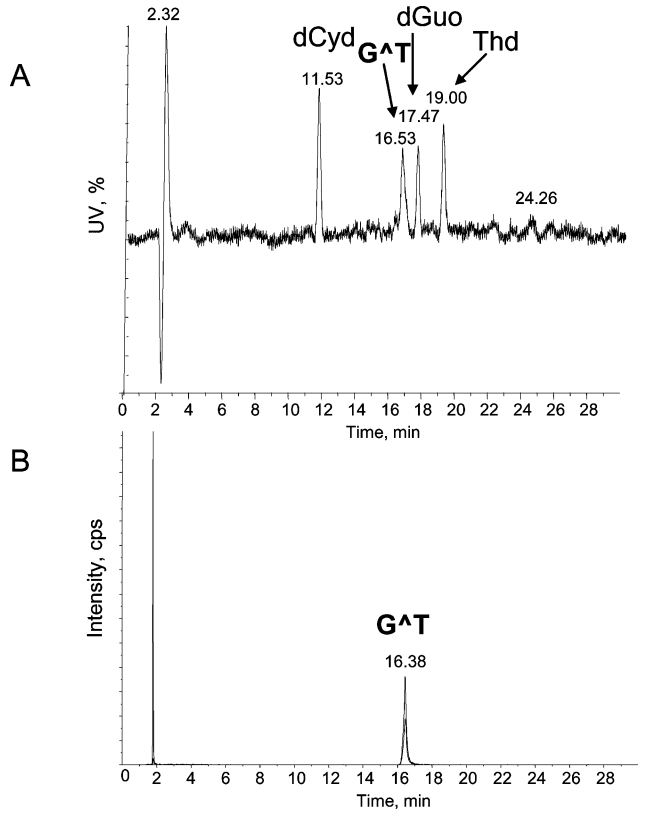


Fig. 2 A) Reversed phase HPLC profile of the enzymatic digestion mixture of 9-mer G^T oligonucleotide by nuclease P1, phosphatase alkaline and exonucleases. B) Mass spectrum of the pseudo-molecular ion of the G^T lesion obtained in the negative electrospray ionization mode (MRM mode, 568 > 470 and 568 > 274 transitions).

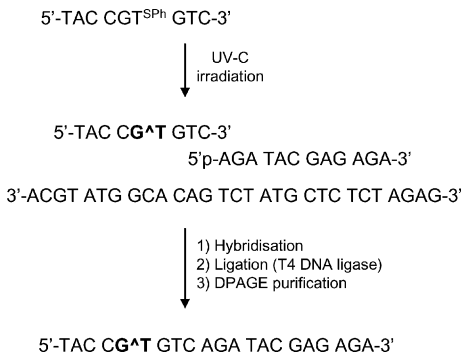
to the calculated mass of 2687.8 in the negative mode. After 1 min digestion, the resulting DNA fragments were analyzed. The different molecular ions observed correspond to digested DNA fragments which differ in mass by successive loss of nucleotides starting from the 3′-end of the DNA fragment. $[M - 1H]^{1-}$ at $m/z = 2093.38$ corresponded to the loss of dCMP + dTMP, and the $[M - 1H]^{1-}$ fragment at $m/z = 1764$ was assigned to the degraded oligonucleotide 5′-d(TAC CG^T)-3′ which was resistant to further cleavage even after a prolonged treatment.

Table 1 Sequences of the oligonucleotides synthesized and used in the present study

Name	Sequences (5′ to 3′)
9-TSPh	TAC CGT^{sPh} GTC
9-G^T	TAC C**G^T** GTC
12-p	pAGA TAC TAC GAG AGA
29-comp	GTG ATC TCT CGT ATC TGA CAC GGT ATG CA
21-GT	TAC CGT GTC AGA TAC TAC GAG AGA
21-G^T	TAC C**G^T** GTC AGA TAC GAG AGA
12 primer	TCT CTC GTA TCT
13 primer	TCT CTC GTA TCT G
14 primer	TCT CTC GTA TCT GA
15 primer	TCT CTC GTA TCT GAC
16 primer	TCT CTC GTA TCT GAC A
17-A primer	TCT CTC GTA TCT GAC AA
17-T primer	TCT CTC GTA TCT GAC AT
17-G primer	TCT CTC GTA TCT GAC AG
17-C primer	TCT CTC GTA TCT GAC AC

Preparation and characterization of G^T lesion containing 21-mer ODN

To obtain a G^T-containing oligonucleotide of sufficient length to be used as the substrate for DNA polymerase-mediated elongation assays, enzymatic ligation was carried out (Scheme 2). A 29-mer long complementary strand was used to isolate the ligated 21-mer modified oligomer, which was purified by denaturing PAGE and UV shadowing. Ligation efficiency was low (around 20%), probably because of the structural distortion induced by the tandem base modification in the duplex. Final assessment of the integrity of the vicinal lesion in the oligonucleotide was provided by enzymatic digestion with bovine intestinal mucosa phosphodiesterase and specific restriction enzyme HpyCH4 III from *E. coli* on the 21-mer G^T previously 5′-[^{32}P]-labeled and analyzed by PAGE. The phosphodiesterase-mediated DNA cleavage was prevented by the vicinal damage, leading to the formation of a resistant hexamer, even after long periods of incubation up to 4 h (Fig. 3). In parallel a control experiment carried out with the unmodified oligonucleotide, which contains the normal vicinal thymine and guanine bases in place of the cross-linked lesion, has led to the complete digestion of the DNA fragment. Moreover the restriction enzyme in the presence of the G^T lesion in the close proximity of the cutting site was not able to incise the strand; thus the 4 base oligonucleotide that is released from the control oligonucleotide



Scheme 2 Synthesis of 21-mer G^T by ligation.

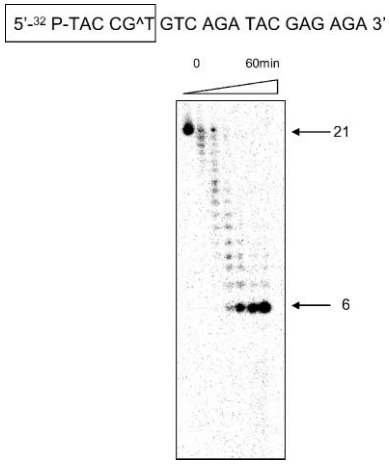


Fig. 3 Enzymatic kinetic hydrolysis of 21-mer G^T purified on denaturing PAGE and 5′-end-labeled, by bovine intestinal mucosa phosphodiesterase, incubation with 10^{-5} enzyme unit, up to 60 min.

was not present in the restriction enzyme digestion solution of the modified 21-mer (data not shown).

In vitro replication studies involving three DNA polymerases

The ability for several polymerases to extend a primer annealed with a 21-mer template bearing the G^T lesion has been assessed. Primers were [^{32}P]-labeled at their 5′-end so that extended DNA fragments upon nucleotide incorporation could be visualized upon sequencing polyacrylamide gel electrophoresis analyses.

Fig. 4 shows the denaturing PAGE bands obtained upon elongation of the 5′-[^{32}P]-labeled 15-mer primer annealed with 21-mer G^T in the presence of the Klenow exo-DNA polymerase. In addition, similar replication assays were performed as control experiments using the unmodified 21-mer in order to assess the activity and the specificity of the latter bacterial polymerase (data not shown). On the one hand, it was found that Kf-polymerase-mediated polymerization exclusively give rise to the incorporation of a dAMP residue opposite the 5′-T residue of the G^T lesion; it should be noted that the synthesis was stopped once the specific incorporation of the nucleotide had occurred. It may be added that the enzyme was not able to extend further the template in the presence of the four dNTPs. On the other hand, it was found that Kf- is not able to extend the primers when a 17-mer primer was used with dAMP opposite the T-moiety and one of the four nucleotides opposite the G residue of the G^T modification (Fig. 5). Moreover, with shorter primers as 12, 13 or 14-mer, Kf- was able to insert dNTP; however, the processing of the enzyme was stopped similarly as observed with the 15-mer primer (Fig. 6), once a nucleotide opposite of the T-residue of the tandem lesion was incorporated.

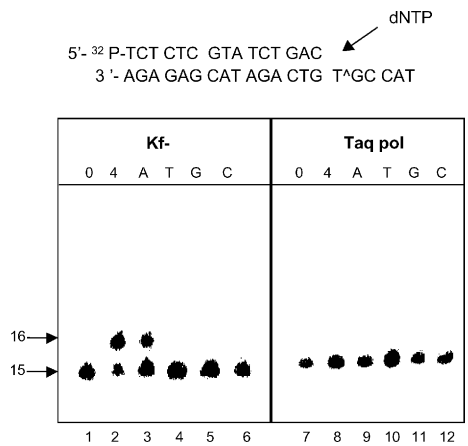


Fig. 4 Modified 21-mer template bearing the G^T lesion annealed with a 5′-end labeled 15-mer. Primer extension reactions were catalyzed by the Klenow fragment (lane 1 to 6) and by Taq DNA polymerase (lane 7 to 12), in the presence of the four dNTP (lane 2 and 8), dATP (lane 3 and 9), dTTP (lane 4 and 10), dGTP (lane 5 and 11), dGTP (lane 6 and 12), as described in the Experimental section. The reaction mixtures were subjected to denaturing 20% PAGE analysis and the extended oligonucleotides were visualized by phosphorimaging with the Image Quan T software.

In the presence of Taq polymerase the denaturing PAGE bands obtained showed that the enzyme progression was blocked in front of the lesion and could not even insert dAMP opposite the 3′-thymine moiety as Kf- did (Fig. 4). The other sequences of primers

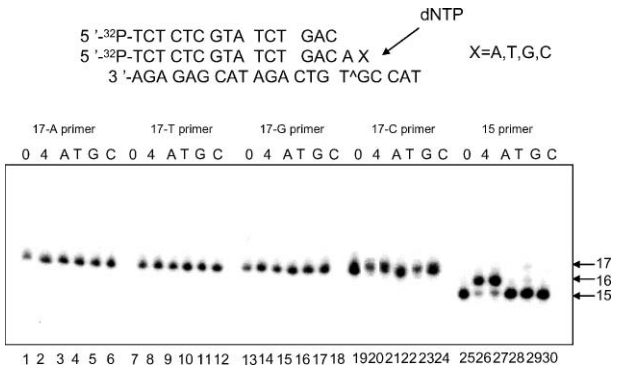


Fig. 5 Modified 21-mer template annealed with 5′-end labeled 15-mer and four different endings 17-mer primers. Primer extension reactions catalyzed by the Klenow fragment, in the presence of 5 μM of the four dNTP (lane 2, 8, 14, 20, 26), dATP (lane 3, 9, 15, 21, 27), dTTP (lane 4, 10, 16, 22, 28), dGTP (lane 5, 11, 17, 23, 29), dCTP (lane 6, 12, 18, 24, 30). The reaction mixtures were subjected to denaturing 20% PAGE analyses and the extended products were visualized by phosphorimaging with the Image Quan T software.

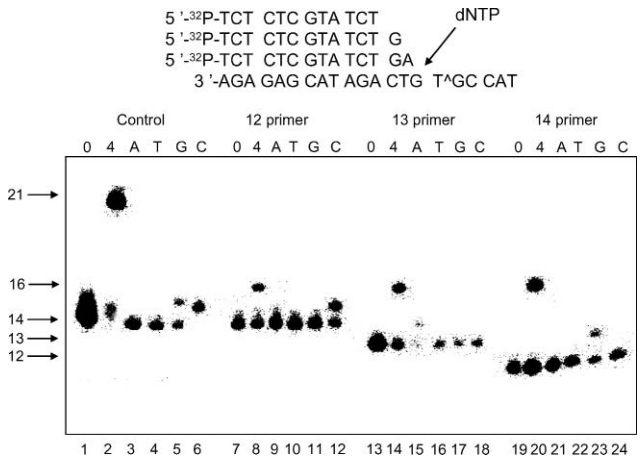


Fig. 6 Unmodified (lane 1 to 6) annealed with 5′-end labeled 14-mer and modified 21-mer template (lane 6 to 24) annealed with 5′-end labeled 12, 13 and 14-mer. Primer extension reactions catalyzed by the Klenow fragment, in the presence of 5 μM of the four dNTP (lane 2, 8, 14, 20), dATP (lane 3, 9, 15, 21), dTTP (lane 4, 10, 16, 22), dGTP (lane 5, 11, 17, 23), dCTP (lane 6, 12, 18, 24). The reaction mixtures were subjected to denaturing 20% PAGE analysis and the extended oligonucleotides were visualized by phosphorimaging with the Image Quan T software.

have been tested and in all cases the modification did not allow the enzyme to pass the cross-link lesion (data not shown).

We next examined the mutagenic properties of G^T by carrying out primer extension assays with a bacterial translesional polymerase. Thus the ability for the *E. coli* translesional DNA polymerase Pol IV (dinB) to bypass the cross-link lesion has been assessed using the same constructs that were previously involved in the other DNA polymerase studies. The obtained data reported in Fig. 7 showed that even with a large amount of enzyme as previously described,[12] no bypass of the lesion was found to occur. As shown for Kf-, the elongation is stopped after the incorporation of the first nucleotide dAMP opposite the 3′-T residue from the G^T lesion has taken place. Using primers that contained the complementary base pair of the G^T damage, namely AC, no

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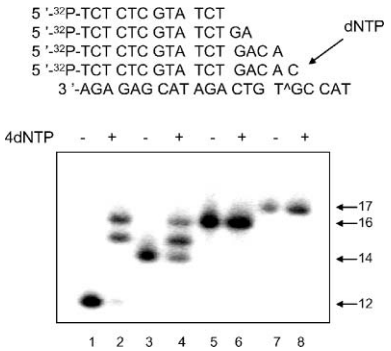

Fig. 7 Modified 21-mer G^T template annealed with 5′-end labeled 12, 14 and 16 and 17-mer. Primer extension reactions catalyzed by the translesional DNA polymerase pol IV, 800 fmol, in the presence of 100 μM of the four dNTP (lane 2, 4, 6, 8). The reaction mixtures were subjected to denaturing 20% PAGE analysis and the extended DNA fragments were visualized by phosphorimaging with the Image Quan T software.

detectable extension by this polymerase was found to take place (Fig. 7).

In parallel to this work, other modified templates have been used as references. They involved oligonucleotides in which the 5′*R* and 5′*S* diastereomers of 5′,8-cyclo-2′-deoxyadenosine (5′,8-cyclodA), synthesized earlier in the laboratory, have been site-specifically inserted.[14] Previously, the translesional synthesis activity of human DNA polymerase η towards 5′,8-cyclodA diastereoisomers has been studied.[15] Thus, it was found that pol η could catalyze the incorporation of one nucleotide opposite (5′*S*)-5′,8-cyclodA but could not extend the elongation. As a striking feature, pol η preferentially incorporated dAMP opposite the *R* diastereomer; interestingly the elongation was found to occur to completion, however only once the correct nucleotide dTMP was incorporated in front of the lesion. Here, the ability for pol IV to catalyze translesional synthesis has been investigated. A 22-mer oligonucleotide bearing a single lesion was employed as the template and annealed to a 5′-[^{32}P] labeled 12-mer oligonucleotide primer (see supporting information). In the presence of the four nucleotides Pol IV was found to synthesize DNA products up to 22 nucleotides in length using the control undamaged template. When the template DNA contained either the (5′*R*)-5′,8-cyclo-dA or (5′*S*)-5′,8-cyclodA residues, pol IV is able to bypass the lesion. However it could be seen on the gel that the synthesis efficiency was lower at the site of the lesion as inferred from the persistence of the 14 base bands.

Discussion

Several studies performed by Box, Wang and our group demonstrated that intrastrand cross-link lesions involving a purine base and an adjacent thymine, cytosine or 5-methylcytosine can be produced upon exposure of aqueous solution of DNA to ionizing radiation and Fenton type oxidizing system.[1–6,16] Some of the latter tandem base lesions can be site-specifically incorporated into defined sequence DNA fragments in order to prepare probes for the assessment of the biological properties of the tandem damage. We report herein the synthesis, purification and structural assignment of a G^T cross-link lesion containing oligonucleotide. The latter synthetic modified template was used to assess the mutagenic properties of such damage if not repaired in the cell. *In vitro* replication studies showed that Taq polymerase stops synthesis before the 3′-T residue. However the processing of Kf-replicative polymerase and pol IV translesional polymerase from *E. coli* was stopped after the insertion of the first nucleotide opposite the 3′-T nucleotide of the lesion. It should also be emphasized that the insertion was incomplete as the result of the presence of the cross-link. Available results from the literature show that the 3′-cytosine moiety of the d(G^^{Me}C) cross-link lesion does not affect the efficiency and the fidelity of nucleotide insertion by yeast pol η.[4,17] However, the efficiency for the incorporation of the correct nucleotide dCMP was reduced in front of the 5′-guanine residue. Moreover error-prone synthesis was found to take place as inferred from the insertion of dGMP or dAMP opposite the guanine moiety. Interestingly the d(G^T) and the d(G^^{Me}C) cross-link lesions exhibit distinct behavior upon nucleotide insertion by *E. coli* pol IV and the yeast pol η respectively, two enzymes belonging to the "Y" polymerase superfamily involved in translesion synthesis. Possible explanations of the polymerase specificity on each lesion can be the capability of Watson–Crick (or Hoogsteen) base pairing, location, shape, size, hydrophobicity and stacking of the lesion in the active site of each enzyme.

Structural studies performed using semi-empirical approaches on d(A^T), d(T^A) dinucleoside monophosphate by our group (Berri *et al.*, in press) and on d(G^^{Me}C) by Wang *et al.*[4] are indicative of the induction of pronounced conformational changes that could be also present in duplex DNA. It was proposed that a purine nucleotide can be incorporated opposite the 3′-pyrimidine residue of the cross-link lesion due to the occurrence of a stronger stacking interaction with the 3′-nucleotide of the primer rather than the second base moiety of the tandem lesion. In *E. coli* three SOS-controlled DNA namely polymerases II, IV, V are involved in TLS and mutagenesis.[18] It has been reported that pol IV and V exhibit low fidelity when copying undamaged DNA,[19] whereas in the presence of DNA lesions the two latter polymerases show different features. When pol V exhibits the correct *in vivo* specificity copying TT *cis-syn* cyclobutane dimers and TT(6–4) photoproducts, pol IV fails to copy the latter thymine photoproducts.[20] It may be pointed out that pol IV is able to copy bulky adducts as benzo[*a*]pyrene diol epoxide.[18] In a similar way we wanted to know if pol IV may be able to bypass the bulky tandem base lesion containing probe synthesis described herein.

It may be added that attempts were made to investigate the reparability of the G^T cross-link lesion by purified DNA glycosylases and AP-endonucleases (unpublished data). Thus, *E. coli* Fpg, endo III, endo IV and exo III or yOgg1 and Ntg1 from *S. cerevisiae* were incubated with the G^T containing 21-mer. Subsequently the DNA fragment was subjected to hot piperidine treatment in order to reveal putative abasic sites that may have been generated. However, the latter enzymatic and chemical treatment was not found to induce any DNA cleavage at the site of the lesion. Such a bulky and distorting lesion in DNA duplex is expected to be removed by the enzymes of the nucleotide excision repair (NER) pathway. In this respect, bacterial UvrABC enzymatic complex was incubated with a potential DNA substrate bearing the d(G^T) cross-link.[21] The main conclusion of the latter study reported by Zou *et al.*, was that the intrastrand cross-link which is more resistant to nucleotide excision repair than other bulky adducts is likely to persist in cells. Further studies should be

aimed at assessing the ability for NER enzymes to excise the other purine^thymine cross-links by comparison with known bulky substrates such a cyclobutadithymine and 5′,8-cyclodA diastereomers. Shuttle vectors studies involving the use of site-specifically modified oligonucleotides for tranfection purposes represent a suitable approach to assess the toxic and lethal potential of G^T adduct during cellular repair and replication processes.

Experimental

Preparation of a site-specific guanine–thymine bridged lesion containing oligodeoxynucleotide

Oligodeoxynucleotide bearing the 5-(phenylthiomethyl)-2′-deoxyuridine (T^{SPh}) was prepared on solid support, at the 1 μmol-scale, using the "Pac" phosphoramidite chemistry as previously described.[2,3] The photoreactive precursor X was site-specifically incorporated into the 5′-TACCGT^{SPh}GTC-3′ DNA fragment. The resulting modified oligonucleotide was cleaved from the solid support and subsequently deprotected by incubation with 30% ammonia at room temperature for 16 h. The released 5′-tritylated-oligomer was purified by RP-HPLC on a polymeric column using an on-line detritylation–purification procedure.[22] After UV-C irradiation under hypoxic conditions, the tandem base lesion containing oligonucleotide 5′-TACCG^TGTC-3′, was isolated by reversed-phase HPLC on a Hypersil octadecylsilyl silica gel column (5 μm, 250 × 4.6 mm) using an acetonitrile gradient in 10 mM triethyl ammonium acetate (pH 7) buffer with the following eluting conditions: flow-rate of 1 mL min^{-1}; from 3% to 10% acetonitrile in 50 min.

Enzymatic digestion of 9-mer G^T containing oligonucleotide

Typically 10 pmol (10 μL) of 9-mer G^T ODN was incubated for 2 h at 37 °C with 5 μL of nuclease P1 (1 unit μL^{-1}), 1 μL of calf spleen phosphodiesterase (0.004 unit), and 10 μL of enzymatic buffer (200 mM succinic acid, 100 mM $CaCl_2$, 0.2 M ammonium citrate, pH 5). Then, 10 μL of alkaline phosphatase buffer (500 mM Tris, 1 mM EDTA, pH 8.5) was added together with 5 units of alkaline phosphatase and 0.003 unit of bovine intestinal mucosa phosphodiesterase and the incubation was resumed for 2 h. The enzymatic reaction was quenched by addition of 10 μL of HCl 0.1 M. The resulting solution was centrifuged, and the content was analyzed by HPLC-MS/MS.

Enzymatic digestion of 9-mer G^T oligonucleotide by bovine intestinal mucosa phosphodiesterase

10 pmol of 9-mer G^T ODN in 20 μL of 0.02 M ammonium citrate buffer (pH 9) was digested at 37 °C by incubation with 10^{-5} units of bovine intestinal mucosa phosphodiesterase (3′→5′ exonuclease activity). At increasing periods of time, aliquots from the digestion mixture were collected and analyzed by MALDI-TOF-MS.

Enzymatic assay using denaturing PAGE analysis

Single-stranded oligonucleotide was 5′-end-labeled with [γ-^{32}P]-ATP. Typically, 10 pmol of the oligonucleotide was mixed with [γ-^{32}P]-ATP and 3 units of T4 polynucleotide kinase (Pharmacia) in 10 μL of the supplied buffer. The reaction was held for 30 min at 37 °C and the oligonucleotide was purified on a Microspin G-25 column (Pharmacia).

All reactions were terminated by the addition of 5 μL of solution containing 99% formamide, 0.025% bromophenol blue and 0.025% xylene cyanol. Oligonucleotide fragments were separated by electrophoresis on a 8 M urea denaturing 20% polyacrylamide gel (0.4 mm thickness). The gels were subjected to electrophoresis with 1 × TBE (50 mM Tris borate and 1 mM EDTA buffer, pH 8). The radioactive bands on the gel were located and quantified using the Biorad Quantity One v4.2.2 software.

Construction of the G^T lesion-containing 21-mer ssDNA fragment

The 21-mer long oligonucleotide that contained the G^T lesion was obtained by enzymatic ligation of a 12-mer 5′-phosphorylated ODN with the 9-mer G^T ODN. Hybridization with a longer 29-mer complementary strand was achieved by heating 100 pmol of each of the oligonucleotides at 80 °C for 5 min in 100 μL of ligation buffer that contained 50 mM Tris-HCl (pH 7.6), 10 mM dithiothreitol, bovine serum albumin 500 μg mL^{-1}. This was followed by a slow cooling down to 4 °C. The ligation reaction was carried out overnight at 4 °C in the same buffer with 2 units of T4 DNA ligase (Boehringer-Mannheim). The resulting double strand DNA was then purified using denaturing PAGE conditions. The 21-mer corresponding band was visualized by UV shadowing prior to be excised from the gel. The oligonucleotide was subsequently eluted with water for 20 h at room temperature and then desalted on a NAP-5 column (Amersham Biosciences).

Enzymatic digestion of G^T-containing 21-mer ODN by bovine intestinal mucosa phosphodiesterase

Typically 1 pmol of 5′-[^{32}P]-labeled G^T-containing 21-mer ODN in 10 μL of 0.02 M ammonium citrate buffer (pH 9) was digested at 37 °C by incubation with 10^{-5} unit of bovine intestinal mucosa phosphodiesterase (Sigma). At increasing periods of time, aliquots of the digestion mixture were withdrawn and the reaction was stopped by addition of loading buffer. After heating at 85 °C, oligonucleotide fragments were analyzed by 20% PAGE.

Enzymatic digestion of G^T-containing 21-mer ODN by HpyCH4 III restriction enzyme

Typically 0.1 pmol of 5′-[^{32}P]-labeled control 21-mer or G^T-containing 21-mer ODN in 50 mL of Tris-acetate, 10 mM magnesium acetate, 1 mM dithiotreitol, pH 7.9 was incubated at 37 °C with 5 units of HpyCH4 III from *E. coli.* At increasing periods of time, aliquots of the digestion mixture were withdrawn and the reaction was stopped by addition of the loading buffer. After heating at 85 °C, oligonucleotide fragments were analyzed by 20% PAGE.

Primer extension reactions catalyzed by the Klenow exo-fragment, Taq polymerase and Pol IV

Buffered solutions that contained the oligonucleotide templates (1.5 pmol) modified or not, and the 5′-[^{32}P]-labeled primer (1 pmol) were heated at 85 °C for 10 min prior to being cooled and

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maintained at 4 °C overnight. For each enzymatic reaction 0.1 pmol of the latter duplex was used.

Reactions catalyzed by the Klenow exo-fragment were carried out in 10 μL solution of Tris-HCl (50 mM, pH 7.5), $MgCl_2$ (10 mM), bovine serum albumin (0.05 mg mL^{-1}) and dithiotreitol (1 mM). Polymerization reactions were performed with 5 μM solutions of either a single dNTP or a mixture of all four dNTPs, in the presence of 0.002 U Klenow exo-fragment, at 37 °C during 30 min.

Primer extension experiments by Taq polymerase were conducted in 10 μL solution of Tris-HCl (10 mM, pH 8.3), $MgCl_2$ (1.5 mM), and KCl (50 mM). Either 20 μM dNTP or the four dNTPs were added together with 0.05 U of Taq polymerase and then the resulting mixture was incubated at 37 °C for 30 min.

The replication assay with the translesional enzyme Pol IV was carried out in 10 μL EDB buffer (50 mM HEPES pH 7.5, 100 mM potassium glutamate, 0.02 mg mL^{-1} bovine serum albumine, 20% glycerol, 0.02% NP-40, 10 mM DTT) supplemented with 300 μM ATP, 7.5 mM $MgCl_2$. Firstly with the 21-mer G^T containing template, 800 fmol of enzyme was used at 37 °C during 1 h 30 min with 100 μM dNTPs, and secondly with the cyclodA template 25, 50 and 100 fmol of enzyme were used for 30 min. Reactions were stopped by addition of loading buffer (5 μL) and samples were then loaded onto a 20% denaturing PAGE. The products of the reaction were finally visualized by phosphorimaging.

Abbreviations

d(G^T): 2′-deoxy-8-[[1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-1,2,3,4-tetrahydro-2,4-dioxo-5-pyrimidinyl]methyl]-5′-guanylic acid intramol. 5′,3‴-ester; ODN: oligodeoxyribonucleotide; TSPh: 5-(phenylthiomethyl)-2′-deoxyuridine; Taq pol: *Thermus aquaticus* polymerase; Pol IV: *E. coli* DNA polymerase IV; Kf-: Klenow exo-fragment of *E. coli* polymerase I; PAGE: polyacrylamide gel electrophoresis; MALDI-TOF MS: matrix assisted laser desorption ionization time of flight mass spectrometry; HPLC-ESI-MS/MS: high-performance liquid chromatography-electrospray ionization tandem mass spectrometry.

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