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Influence of neighbouring base sequences on the mutagenesis induced by 7,8-dihydro-8-oxoguanine in yeast

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We have analysed the influence of neighbouring base sequences on the mutagenesis induced by 7,8-dihydro-8-oxoguanine (8-oxoG or G^o), a typical oxidative lesion of DNA, using the yeast oligonucleotide transformation technique. Two oligonucleotides, oligo-CCG^o and oligo-CGG^o, each possessing a single 8-oxoG residue and represented by the sequences 5′-CCG^o-3′ and 5′-CGG^o-3′, respectively, were introduced into a chromosome of *Saccharomyces cerevisiae* and their mutagenic potentials were compared. In a wild-type strain, 8-oxoG showed very weak mutagenic potential in both cases. However, the lesion in 5′-CCG^o-3′ can cause efficient G-to-T transversion in a strain lacking the *rad30* gene which encodes yeast DNA polymerase η (Ypolη). To explore the properties associated with this translesion synthesis (TLS), the same two oligonucleotides possessing an 8-oxoG were used as templates for a standing-start primer extension assay, and the nucleotide incorporation opposite 8-oxoG was investigated. We found that dATP incorporation opposite 8-oxoG with Ypolη was low for both sequences. In particular, very low dATP incorporation was observed for the 5′-CCG^o-3′ sequence. These results account for the efficient inhibition of mutagenesis by Ypolη. TLS plays an important role in one DNA sequence in terms of avoiding mutagenesis induced by 8-oxoG in yeast. In contrast, human yeast DNA polymerase η showed higher dATP incorporation rates even with the 5′-CCG^o-3′ sequence.

Introduction

Oxidative damage can contribute significantly to the distortion of stable DNA conformations, as well as in disturbing genomic stability. 7,8-Dihydro-8-oxoguanine (8-oxoG or G^o) (1–3) is one of the most widely studied oxidative lesions of DNA that can result from direct intracellular metabolic reactions (4) and oxidative stresses such as ionizing radiation and cigarette smoking (5,6). DNA polymerases can misincorporate adenine *in lieu* of cytosine opposite 8-oxoG residues at different efficiencies (7–9). This misincorporation yields GC-to-AT transversion mutations which appear to be associated with ageing (10), breast cancer (11) and other diseases (12,13). Therefore, suppression of such mutations would be beneficial for the prevention of these disorders. To combat the action of 8-oxoG, cells repair the lesion through base excision repair (14–16) using 8-oxoguanine-DNA glycosylase, OGG1 (17,18), or via the nucleotide excision repair pathway. The lesions that escape the repair processes may be bypassed by translesion synthesis (TLS) during DNA replication (19). The bypass is carried out either error free or error prone, depending on the properties of the polymerases and type of DNA damage. It has been reported that the yeast DNA polymerase η (Ypolη) can bypass 8-oxoG by inserting the correct dCTP opposite the lesion in an efficient and accurate manner (20,21).

Since the 1980s, it has been shown that the potential of DNA lesions to induce mutations is influenced by the sequence context adjacent to the lesion (22,23). Using several repair enzymes (24–26) and replication polymerases (27,28), the effects of nucleotide sequence near the lesion on mutation frequencies have been widely studied, although few rules regarding sequence dependence have been found since the effects vary depending on the adduct, polymerase and other as yet undefined factors. We developed a method to analyse nucleotides incorporated opposite DNA lesions during translesion DNA synthesis (29,30) using a yeast oligonucleotide transformation approach (31). In this assay, transformants can be obtained only if the transforming oligonucleotide is used as a template for translesion DNA synthesis after its incorporation into chromosomal DNA. Therefore, the bases incorporated opposite the lesion can be estimated by sequence analysis of the transformants. In the present study, to investigate whether the translesion ability of the enzymes is affected by the nucleotide at the 5′-flanking position next to the lesion, we examined the mutagenic activity of oligonucleotides containing dGTP or dCTP at the 5′-flanking position (oligo-CGG° or oligo-CCG°) of 8-oxoG (G°). The G-to-T transversion mutation in oligo-CCG° increased significantly in the *rad30*Δ mutant compared to the wild-type strain, while oligo-CGG° did not show this change. In order to determine which nucleotide was incorporated opposite 8-oxoG, a standing-start primer extension assay was performed using Ypolη, human DNA polymerase η (Hpolη) and *Escherichia coli* polymerase I without 5′ to 3′ exonuclease activity (KFexo$^-$). The results showed that the error-free manner of Ypolη during 8-oxoG bypass was influenced by the nucleotide located at the 5′-flanking position next to the lesion.

Materials and Methods

Yeast strains

Saccharomyces cerevisiae strain B7528 (*MAT***a** *cyc1-31 cyc7-67 lys5-10 ura3-52*) was obtained from Dr Fred Sherman of Rochester University (31), and the *rad30*Δ (*MAT***a** *cyc1-31 cyc7-67 lys5-10 ura3-52 rad30::kanMX4*) mutant as previously reported (32).

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Oligonucleotides

Oligonucleotides containing 8-oxoG used for yeast transformation were prepared using 8-oxodG amidite obtained from Glen Research Corp. (Sterling, VA) and purified by high-performance liquid chromatography, or purchased from JbioS (Saitama, Japan). The sequences of the oligonucleotides used for transformation and standing-start primer extension are shown in Table I.

Oligonucleotide transformation and sequence analysis of transformants

The transformation was performed as previously described (29,30). Briefly, 300 pmol of 26-meric oligonucleotides were introduced into competent yeast cells by electroporation and treated cells were plated onto YPD plates. Following incubation at 30°C for 5–6 days, $Cyc1^+$ transformants were obtained as overgrown colonies on a lawn of Cyc1-deficient cells and were further selected on YPG plates. The sequences of the reverted *CYC1* gene were determined with DNA extracted from the transformants.

DNA polymerase assays

8-OxoG bypass assays were performed using the exonuclease-deficient Klenow fragment of *E. coli* DNA polymerase I ($KFexo^-$, New England Biolabs, Hitchin, MA) and Ypolη and Hpolη (Enzymax, Lexington, KY). 5′-^{32}P-labelled primer 5′-GAACCGGCCTT-3′ (3 pmole) was annealed to 6 pmole of templates in 10 μl of 100 mM NaCl by heating at 85°C for 5 min, followed by cooling to 25°C for over 2.5 h. In the assay with $KFexo^-$, a reaction mixture (10 μl) containing 25 nM template primer, 75 nM dNTP, 0.075 nM $KFexo^-$, 2.5 mM Tris–HCl (pH 7.4), 0. 01 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM dithiothreitol (DTT) and 5% glycerol was incubated for 10 min after pre-incubation for 2 min at 37°C without dNTP. In the reaction with Ypolη, a template primer (14 nM) was incubated with Ypolη (2.1 nM) in 25 mM potassium phosphate buffer (pH 7) containing 10 μM dNTP, 5 mM $MgCl_2$, 5 mM dithiothreitol and 10 μg/ml bovine serum albumin at 30°C for 30 min. The reaction was initiated by mixing with dNTP. For Hpolη, a 10 μl reaction mixture containing 12.8 nM Hpolη, 14 nM template primer, 25 mM KPO_4 buffer (pH 7), 5 mM $MgCl_2$, 5 mM DTT, 10 μg/ml BSA and 10% glycerol was incubated for 10 min at 37°C after pre-incubation as for the $KFexo^-$ assay. The reactions were terminated by the addition of 10 μl of stop solution (88.25% formamide, 0.05% bromophenol blue and 20 mM EDTA). Samples were heated at 90°C for 3 min, cooled on ice and applied to 15% polyacrylamide gels containing 8 M urea. Following electrophoresis at 1000 V for 3 h and subsequent autoradiography, the extent of incorporation was quantified by measuring the intensity of each band using an Image Analyzer BAS-1800II (Fujifilm, Tokyo, Japan).

Results

Mutation induced by 8-oxodG-oligonucleotide in polη-deficient yeast cells

Using the yeast transformation method, oligonucleotides were introduced into Rad30-proficient and -deficient strains of *S. cerevisiae* by electroporation. Resulting revertants were obtained when these oligonucleotides were replaced with the *cyc1-31* allele of chromosome 10 to revert to wild-type *CYC1*. Any of the five amino acids coded by the CXY triplets at the target site give rise to the $Cyc1^+$ phenotype (30).

Table I. List of 26-meric oligonucleotides used for yeast transformation and standing-start primer extension in the present study

Oligonucleotide	5′ → 3′ sequence	Experiment
Oligo-CGG	ataatgactgaaCGGaaggccggttc	a, b
Oligo-CCG	ataatgactgaaCCGaaggccggttc	b
Oligo-CAG	ataatgactgaaCAGaaggccggttc	b
Oligo-CTG	ataatgactgaaCTGaaggccggttc	b
Oligo-CGG°	ataatgactgaaCGG°aaggccggttc	a, b
Oligo-CCG°	ataatgactgaaCCG°aaggccggttc	a, b
Oligo-CAG°	ataatgactgaaCAG°aaggccggttc	b
Oligo-CTG°	ataatgactgaaCTG°aaggccggttc	b

a = yeast transformation experiments; b = standing-start primer extension experiments.

The mutation spectra of transformants are shown in Figure 1. In the wild type (Rad30 proficient), both oligonucleotides containing 8-oxoG showed similar low G-to-T mutation frequencies. In the polη-deficient strain (*rad30*Δ), 8-oxoG in oligo-CGG° showed a mutagenic potential similar to that of the wild type, however, with oligo-CCG° the mutation rate increased significantly. These results indicate that the A to C ratio of the incorporation opposite 8-oxoG may be affected by the 5′-flanking nucleotide next to the lesion in cells lacking polη. Mutations other than G-to-T were also observed with oligo-CCG° in both wild-type and *rad30*Δ strains, but was not observed with oligo-CGG°. From the above results, the translesion ability of Ypolη clearly plays a very important role and it would be of interest to investigate the effects of 5′-flanking nucleotides on Ypolη activities.

Incorporation and extension of 8-oxoG by $KFexo^-$ DNA polymerase

In order to investigate how the 5′-flanking nucleotide next to the lesion influences the fidelity of DNA polymerases, we carried out a standing-start primer extension assay using templates containing different nucleotides 5′ to 8-oxoG. $KFexo^-$, a typical DNA polymerase without 5′-to-3′ exonuclease or proofreading activities, was used to assess the effects of 5′ neighbours on mutagenesis. The pattern of deoxyribonucleotide incorporated by $KFexo^-$ opposite unmodified guanine and 8-oxoG is shown in (Figure 2a). Only dCTP was incorporated efficiently opposite guanine to produce

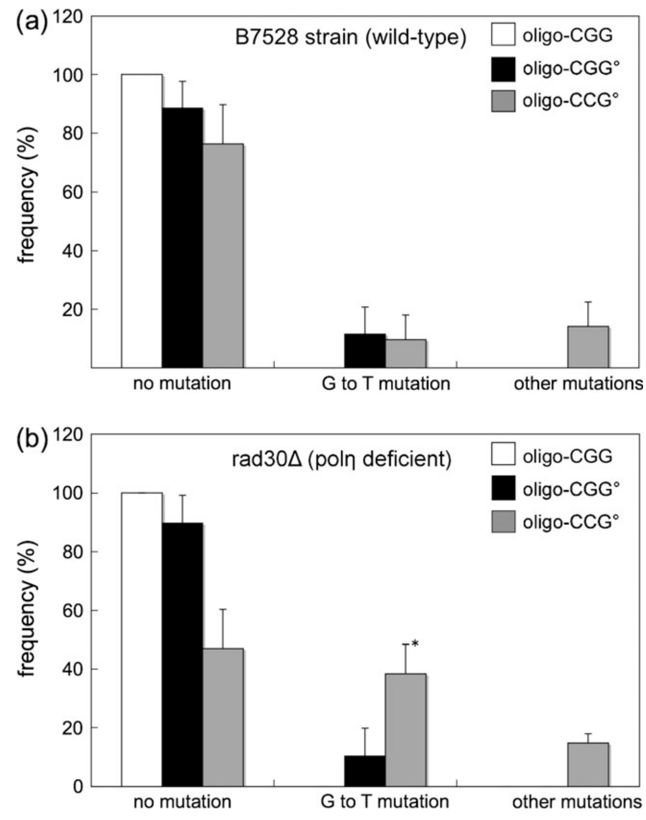

Fig. 1. Mutation spectrum induced by 8-oxoG in (**a**) wild-type and (**b**) *rad30*Δ strains. Bars show the mutation types of oligo-CGG and -CCG (open bars), oligo-CGG° (solid bars) and oligo-CCG° (grey bars) induced by 8-oxoG. *$P < 0.05$ (versus wild type). Other mutations include large deletion, base changes other than G-to-T at the 8-oxoG position; one or two base deletions/insertions or frameshifts which are undetectable in this assay.

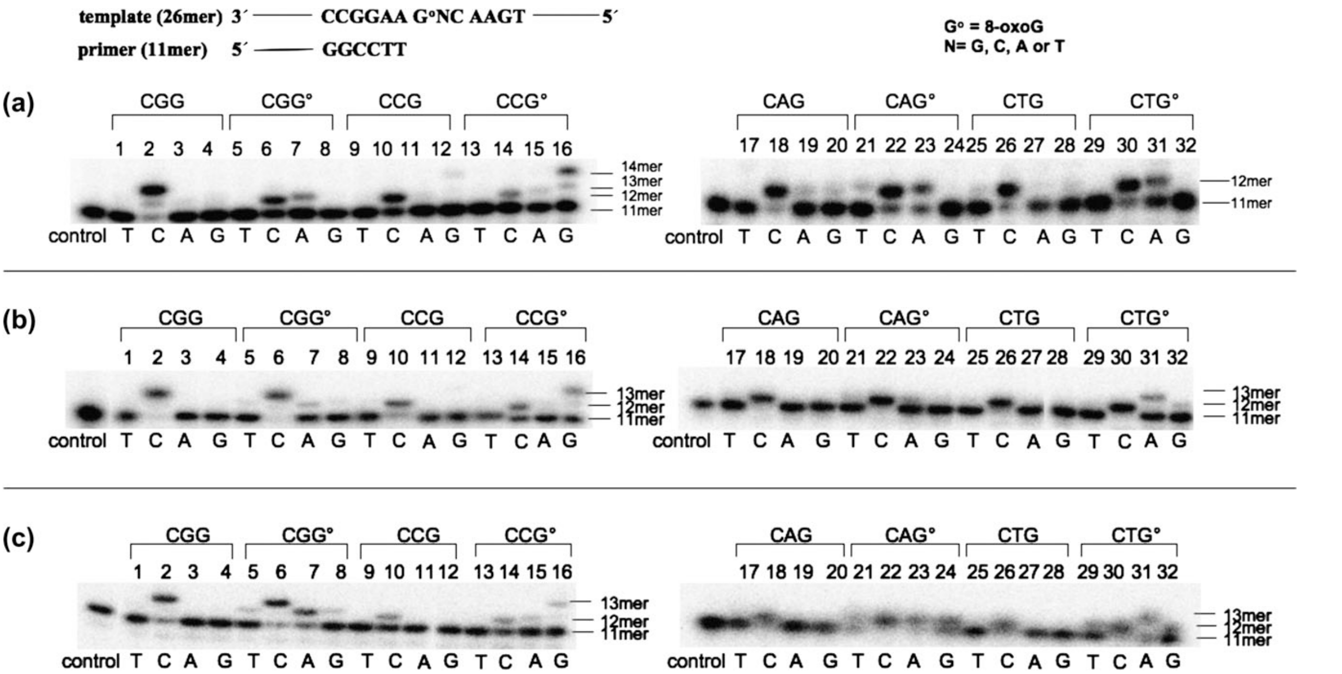

Fig. 2. Primer extension of unmodified (lanes 1–4, 9–12, 17–20 and 25–28) and 8-oxoG-containing (lanes 5–8, 13–16, 21–24 and 29–32) 26mer templates primed with 11mer using (**a**) KFexo^{-}, (**b**) Ypolη or (**c**) Hpolη. The 5′-end of the primer was ^{32}P labelled and reactions were carried out in the presence of single deoxyribonucleoside triphosphate dTTP (T), dCTP (C), dATP (A), dGTP (G) or in the absence of dNTP (control) as indicated. DNA size markers are indicated on the right.

a 13mer with oligo-CGG (lane 2) and a 12mer with the other templates (oligo-CCG, -CAG and -CTG) (lanes 10, 18 and 26) as expected, while with all 8-oxoG-containing templates, dATP was also inserted opposite 8-oxoG and terminated at the 12mer position. We also unexpectedly found that dGTP was incorporated opposite 8-oxoG more efficiently than dATP with oligo-CCG° and that the primer was extended to 13mer or 14mer (lane 16).

Translesion of 8-oxoG by Ypolη

The nucleotide insertion specificity of Ypolη was examined against unmodified G and 8-oxoG in templates. While undamaged templates were correctly replicated by Ypolη as with KFexo^{-}, dATP as well as dCTP were incorporated opposite 8-oxoG in both modified oligonucleotides (Figure 2b). The template oligo-CCG^{o} showed low nucleotide insertion ability compared to other sequences as shown by the KFexo^{-} experiments. Use of template oligo-CGG^{o} with dCTP and oligo-CTG^{o} with dATP resulted in the primer being extended to the 13mer [(Figure 2b), lanes 6 and 31] as two nucleotide molecules were incorporated, while with other oligonucleotide primer extension terminated at the 12mer.

Table II shows the dNTP/dCTP ratios for each template. Ypolη has a higher bypass accuracy than KFexo^{-} as dATP insertion by Ypolη was much lower. Of the two modified oligonucleotides used in the *in vivo* assay, oligo-CCG° showed the lowest dATP/dCTP ratio, 0.12, which is consistent with the results found in the *in vivo* mutation assay, namely that Ypolη effectively suppressed misincorporation in the template that has dC at the 5′-flanking position next to 8-oxoG. In order to further examine the effect of sequence on the TLS properties of Ypolη, two additional templates (oligo-CAG^{o} and oligo-CTG^{o}) were employed in the *in vitro* experiments. The extent to which 5′-flanking nucleotides affected dATP incorporation

Table II. Relative ratio of the amount of elongated products with dATP, dGTP or dTTP to that of dCTP

Polymerase	Template sequence	RE_{dATP}/RE_{dCTP}	RE_{dGTP}/RE_{dCTP}	RE_{dTTP}/RE_{dCTP}
KFexo^{-}	5′-CGG°-3′	0.58	N/A	N/A
	5′-CCG°-3′	0.49	1.19	N/A
	5′-CAG°-3′	0.62	N/A	0.14
	5′-CTG°-3′	0.61	N/A	N/A
Ypolη	5′-CGG°-3′	0.20	0.07	0.05
	5′-CCG°-3′	0.12	0.74	N/A
	5′-CAG°-3′	0.14	N/A	0.06
	5′-CTG°-3′	0.16	0.05	N/A
Hpolη	5′-CGG°-3′	0.69	N/A	0.16
	5′-CCG°-3′	0.33	0.40	N/A
	5′-CAG°-3′	0.97	0.50	0.07
	5′-CTG°-3′	0.81	0.34	0.18

The ratio, RE_{dNTP}, = El/(El + Pr), where Pr is the band intensity of the 11-meric primer and El is the sum of the bands longer than the primer. The values were calculated from the data shown in (Figures 2a, b and c). The mean values were shown from three to five independent experiments. N/A: insertion of dATP, dGTP or dCTP opposite 8-oxoG was undetectable or the ratio was smaller than 0.05.

was in the order G > T > A > C. These results indicated that the mutagenicity of 8-oxoG was affected by 5′-flanking nucleotide sequences.

A high rate of dGTP insertion was observed with oligo-CCG° similar to the case with KFexo^{-} [(Figure 2a), lane 16]. dGTP insertion was more frequent than dCTP or dTTP, although dGTP was hardly incorporated opposite the lesion with other sequences (20).

Translesion of 8-oxoG by human polη

We also analysed the sequence dependence of Hpolη. We previously reported a brief summary of the Hpolη data (33). As shown in (Figure 2c), the lesion was mainly bypassed by incorporation of dCTP and dATP (lanes 6, 7, 14, 15, 22, 23, 30 and 31). Besides these two nucleotides, dGTP (lanes 8, 16, 24 and 32) and dTTP (lanes 5, 21 and 29) insertions were also found. Hpolη showed greater variation in the dNTP/dCTP ratio than Ypolη (Table II). In oligo-CGG^{o}, -CAG^{o} and -CTG^{o}, the order of incorporation efficiencies opposite 8-oxoG was $C > A >> G > T$, which corresponds to the results reported by Zhang *et al.* (34). However, compared with other oligonucleotides, oligo-CCG^{o} had a different character in that the bypass was very inefficient and the dATP/dCTP ratio (0.38 in oligo-CCG^{o}) was much lower than that of the other oligonucleotides, being 0.68, 0.93 and 0.81 in oligo-CGG^{o}, oligo-CAG^{o} and oligo-CTG^{o}, respectively.

Discussion

In this study, we analysed the effects of sequence contexts on the mutagenesis induced by 8-oxoG *in vivo* using the oligonucleotide transformation assay and *in vitro* using the polymerase primer extension assay. The base excision repair system attacks 8-oxoguanine residues produced in living cells and remove it from the DNA. The nucleotide excision and mismatch repair systems are also involved in the process. When the lesion escapes the repair process, TLS may act to avoid mutations (20,21). In the present study, we set out to investigate the details of this process with a focus on the effects of neighbouring nucleotides or sequences. We have shown that TLS plays an important role in one DNA sequence in terms of avoiding mutagenesis induced by 8-oxoG in yeast. The relationship between nucleotide sequence and TLS properties had hitherto not been investigated, although a great deal of literature has shown that sequence context effects near the lesion can influence the rate of enzymatic repair efficiencies (23,25,35,36). Two oligonucleotides oligo-CGG° and oligo-CCG°, containing 5′-CGG°-3′ and 5′-CCG°-3′ sequences, respectively, were used for both assays. The oligonucleotide transformation assay showed that in the *rad30*Δ strain the G-to-T mutation frequencies of oligo-CCG° were significantly higher than that for oligo-CGG°. Thus, Ypolη appears to suppress the incorporation of dATP opposite 8-oxoG in the 5′-CCG°-3′ sequence.

To investigate how the accuracy and efficiency of Ypolη-mediated TLS through 8-oxoG was influenced by the 5′-flanking nucleotides, a standing-start primer extension assay was performed. As shown in Table II, Ypolη showed low dNTP/dCTP ratios, which indicates high fidelity of Ypolη bypassing the lesion. These results are in agreement with previous reports showing that Ypolη bypasses 8-oxoG efficiently and accurately (20,37).

Oligo-CCG° had the lowest dATP/dCTP ratio (0.12). This low mutagenic potential is consistent with the efficient suppression of mutagenesis by the *rad30* gene *in vivo*. In the case of oligo-CGG°, the low mutation frequencies seem to be driven in the absence of polη-dependent TLS. Replication by an enzyme other than polη may be sufficient to keep the mutation rates low. Thus, yeast cells may employ different strategies to avoid mutations induced by 8-oxoG residues in 5′-CGG°-3′ and 5′-CCG°-3′ sequences.

In mammalian systems, different sequence contexts induce different mutation spectra (38,39). 8-OxoG can induce mutations not only at the modified position but also in the 5′-flanking position, mainly due to hydrogen bonding,

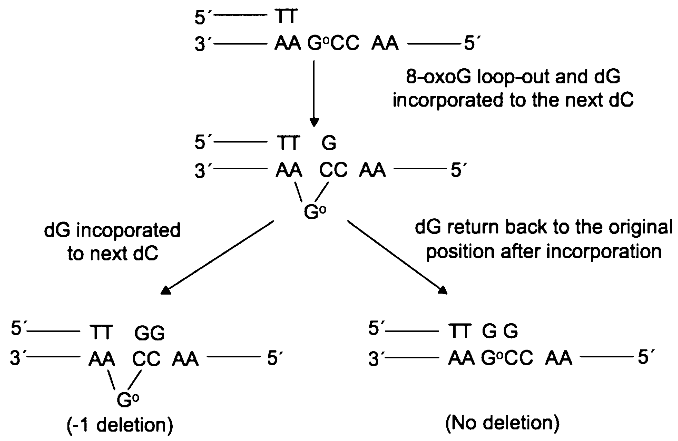

Fig. 3. Proposed mechanisms of dGTP bypass of 8-oxoG during replication.

mobility and electrostatic charge near the modified region (40–42). The present study suggests that the mutation spectrum was affected by the 5′-flanking nucleotide next to 8-oxoG if the polymerase is involved. We showed that this effect was more conspicuous with Hpolη, where 5′ nucleotides have effects similar to Ypolη, although the polymerase showed a higher dNTP/dCTP ratio in bypassing 8-oxoG consistent with a previous report (21).

Both KFexo^{-} and Ypolη showed high dGTP/dCTP ratios with the oligo-CCG° sequence and efficient dGTP incorporation opposite 8-oxoG. This observation appears inconsistent with the report by Haracska *et al.* (20), where dGTP insertion catalysed by Ypolη was much slower than dCTP and dATP. We presumed that 8-oxoG might loop-out from the template and that dGTP was paired with the adjacent cytosine on the 13mer or 14mer positions. These results suggest that for oligo-CCG^{o}, dGTP incorporation opposite dCTP placed 5′ to 8-oxoG occurred preferentially as shown in Figure 3.

The effect of the 5′-flanking nucleotide on frameshift mutations induced by various lesions has previously been investigated (43,44). Investigations are currently in progress to gain further insight into the frameshift due to the mispairing of 8-oxoG or sequence effects. In order to determine whether the insertion spectrum was affected by the reaction time, we analysed the insertion rate of each dNTP with CGG° and CCG° at different time intervals. The dNTP/dCTP ratios were found not to change significantly, which indicates that the mutation spectrum induced by each nucleotide is independent of the reaction time. In this study, we demonstrated that yeast polη was responsible for accurate TLS for the template containing 8-oxoG and that TLS accuracy is influenced by the 5′-flanking nucleotide next to the lesion.

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