Effects of sequence contexts on misincorporation of nucleotides opposite 2-hydroxyadenine

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Abstract Twelve oligonucleotides containing 2-hydroxyadenine (2-OH-Ade) with different neighboring bases were used as templates in DNA polymerase reactions, and the effects of the sequence contexts were investigated. DNA polymerases α and β inserted dTMP and dCMP opposite 2-OH-Ade in most of the oligonucleotides tested. The Klenow fragment of DNA polymerase I primarily incorporated dTMP and dGMP. Effects of the 5′-flanking base of 2-OH-Ade was found when the 3′-flanking base of 2-OH-Ade was A or C. Incorporation of dAMP occurred when the oxidized base was located in a 5′ -TA*A- 3′ (A* represents 2-OH-Ade) sequence. These results suggest that the formation of 2-OH-Ade in DNA may induce all the mutations involving A (A → G transition, and A → T and A → C transversions) in cells.

Key words: 2-Hydroxyadenine; Oxidative DNA damage; Misincorporation; DNA polymerase; Sequence context

1. Introduction

Reactive oxygen species, produced spontaneously, by chemicals, X- and γ-rays, and ultraviolet light, attack and oxidize cellular components, including DNA and its precursors. The DNA damage induces mutations, which can lead to cancer. For example, the oxidized purine base, 8-OH-Gua [1] has been proved to be miscoding in vitro [2–4] and to be mutagenic in cells [5–9].

We previously demonstrated that hydroxylation of an adenine base occurred to form 2-hydroxyadenine (2-OH-Ade, 1,2-dihydro-2-oxoadenine or isoguanine) when DNA and dATP were treated with a reactive oxygen species-generating system (Fe^{2+}-EDTA), and that DNA polymerases used 2-OH-dATP as a substrate in vitro [10]. In that study, we showed that mammalian DNA polymerase α (pol α) incorporated 2-OH-dAMP opposite T and C. On the other hand, we have found that dAMP, in addition to dTMP, was inserted opposite 2-OH-Ade in an oligodeoxyribonucleotide containing a 5′ -TA*A- 3′ (A* represents 2-OH-Ade) sequence (template–A1, Table 1) [11]. It is interesting to study whether this discrepancy (incorporations of 2-OH-dAMP opposite C and of dAMP opposite 2-OH-Ade) is due to the employed sequence contexts.

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Abbreviations: 2-OH-Ade, 2-hydroxyadenine; 2-OH-dAMP, 2-hydroxy-2′-deoxyadenosine 5′-monophosphate; 2-OH-dATP, 2-hydroxy-2′-deoxyadenosine 5′-triphosphate; 8-OH-Gua, 8-hydroxyguanine; pol α, DNA polymerase α; pol β, DNA polymerase β; KF^{exo-}, exonuclease-deficient Klenow fragment of DNA polymerase I; KF^{exo+}, exonuclease-proficient Klenow fragment of DNA polymerase I; dNTP, 2′-deoxynucleoside 5′-triphosphate

To evaluate the effects of the sequence contexts on the incorporation of nucleotides opposite 2-OH-Ade, we synthesized 11 new oligodeoxyribonucleotides with the oxidized base and carried out in vitro DNA syntheses using the prepared oligonucleotides as templates. We found that primarily dTMP and dCMP were incorporated opposite 2-OH-Ade by calf thymus DNA pol α and recombinant rat DNA polymerase β (pol β). Moreover, dTMP and dGMP were inserted by the Klenow fragment of DNA polymerase I. Alteration of the 5′-flanking base of 2-OH-Ade in the template DNA affected nucleotide incorporation when the 3′-flanking base was either A or C. We also found that DNA polymerases inserted dAMP opposite 2-OH-Ade in a 5′ -TA*A- 3′ sequence. Therefore, the incorporation of 'incorrect' nucleotides is dependent on the sequence context and the type of DNA polymerase.

2. Materials and methods

2.1. Materials

T4 polynucleotide kinase was purchased from Toyobo Co. Calf thymus DNA polymerase α was obtained from Molecular Biology Resources Inc. The Klenow fragments of DNA polymerase I, with and without exonuclease activity, were from Life Technologies Inc. and United States Biochemical Co., respectively. Recombinant rat DNA polymerase β was a gift from Dr. Akio Matsukage of the Aichi Cancer Center Research Institute of Japan. FPLC-grade dNTPs were from Pharmacia Biotech Inc.

2.2. Oligonucleotide synthesis

Preparation of a phosphoramidite derivative of 2-OH-Ade, 9-[2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)-β-D-*erythro*-pentofuranosyl]-6-[(dimethylamino)methylidene]-9*H*-isoguanine 3′-[(2-cyanoethyl)*N,N*-diisopropylphosphoramidite], and synthesis and purification of oligonucleotides were carried out as described [11,12]. Sequences of the oligonucleotides with 2-OH-Ade were as followings: template–A, 5′-dGCNA*MTATTCCGTCAT-3′; template–B, 5′-dCGTNA*MATTTCTGAT-3′ (NA*M represents 2-OH-Ade and the flanking bases).

2.3. In vitro DNA synthesis

Labeling of primers with ^{33}P was conducted as described previously [11]. Nucleotides incorporated opposite 2-OH-Ade were determined by reactions with a single dNTP [11]. Full-length products were obtained by reactions with the four dNTPs and analyzed as described [11].

3. Results

3.1. Determination of nucleotides incorporated opposite 2-OH-Ade

We previously reported that DNA pol α, pol β, and the exonuclease-deficient Klenow fragment (KF^{exo-}), incorporated dAMP in addition to dTMP opposite 2-OH-Ade in an oligonucleotide (5′ -dGCTA*ATATTCCGTCAT- 3′, template–A1) [11]. We also observed that KF^{exo-} inserted dGMP. On the other hand, pol α incorporates 2-OH-dAMP

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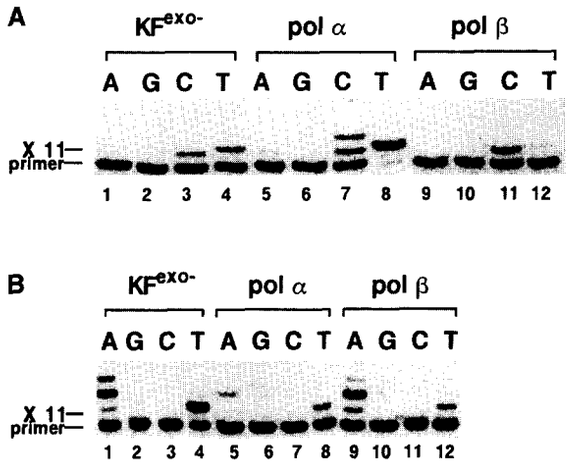

Fig. 1. Incorporation of nucleotides opposite 2-OH-Ade in the presence of a single dNTP with (A) template–B1 and (B) template–B2. Templates–B1 and –B2 were annealed with ^{33}P-labeled primers. The template–primer complex was treated with KF^{exo-}, pol α, or pol β in the presence of a single dNTP. Lanes 1, 5, and 9, in the presence of dATP; lanes 2, 6, and 10, in the presence of dGTP; lanes 3, 7, and 11, in the presence of dCTP; lanes 4, 8, and 12, in the presence of dTTP. Lanes 1–4, KF^{exo-}; lanes 5–8, pol α; lanes 9–12, pol β.

opposite C, but not opposite A, in a template DNA (5′ -AATGCCACG- 3′, C represents the cytosine residue opposite which the 2-OH-dAMP is incorporated) [10]. Since this discrepancy may be due to differences in the sequence contexts, we synthesized an oligonucleotide that has the same sequence as the complementary strand of the template described above (5′ -dCGTGA*CATTTCTGAT- 3′) (template–B1, the underline indicates the complementary portion) and used it as a template DNA for in vitro DNA synthesis.

Template–B1 were annealed with a labeled primer and treated with KF^{exo-} and DNA pol α and pol β. The extensions of the primer by the DNA polymerases were analyzed by denaturing polyacrylamide gel electrophoresis. As shown in Fig. 1A, incorporations of dCMP and dTMP were observed with all of the DNA polymerases tested (lanes 3, 4, 7, 8, 11, and 12). Pol α incorporated a small amount of dAMP (lane 5). The incorporations of dCMP were consistent with the fact that 2-OH-dAMP is inserted opposite C [10], but were in contrast to our previous finding that dAMP is incorporated opposite 2-OH-Ade [11]. We then reanalyzed the incorporation of nucleotides opposite 2-OH-Ade in template–A1, under the same conditions as the experiments with template–B1, and confirmed our previous results (data not shown). Therefore, the incorporation of nucleotides opposite 2-OH-Ade is sequence-dependent.

We exchanged the bases flanking 2-OH-Ade in template–B1 from 5′ -GA*C- 3′ to 5′ -TA*A- 3′ (template–B2, Table 1) and used it for DNA polymerase reactions. As shown in Fig. 1B, the incorporated nucleotides were changed from T and C to T and A (lanes 1, 4, 5, 8, 9, and 12). Thus, the incorporation of nucleotides opposite 2-OH-Ade is affected by 5′- and 3′-flanking bases of 2-OH-Ade.

We then changed the 5′-neighboring base of 2-OH-Ade in template–A1 from T to G (template–A2, Table 1) and to C (template–A3), and studied nucleotide incorporation. With template–A2, incorporations of dCMP were observed by all the polymerases tested merely by altering 5′-flanking base of 2-OH-Ade (Table 1). DNA polymerases inserted dGMP in addition to dTMP with template–A3 (Table 1).

We noted that misincorporation of nucleotides which are complementary to the 5′-flanking position of 2-OH-Ade were 'promoted' with templates–A1 to –A3, and templates–B1 and –B2. Incorporation of dAMP occurred opposite 2-OH-Ade in templates–A1 and –B2, which has T at the 5′-flanking position. Insertion of dCMP occurred opposite the modified base in template–B1, with G at the 5′-adjacent site. DNA pol α and KF^{exo-} inserted dCMP in addition to dTMP, dAMP, and dGMP opposite 2-OH-Ade in template–A2. These results were similar to those obtained using template–A1, except for the incorporation of dCMP. Pol β incorporated dCMP and not dAMP. Conversion of the 5′-flanking base of 2-OH-Ade from T to C (template–A3) accelerated the incorporation of dGMP, the complementary nucleotide of the 5′-flanking base, C.

The 3′-flanking base of 2-OH-Ade was changed from A to other bases, and the effect of the 5′-flanking base was further studied (template–A5 to –A10, Table 1). Although a similar tendency was observed to some extent with pol α and KF^{exo-}, when the 3′-flanking base was C, the effect was not found when the 3′-base was either G or T (Table 1).

Table 1
Nucleotides incorporated opposite 2-OH-Ade in vitro

Template	Sequence[a]	DNA polymerase		
		pol α	pol β	KF^{exo-}
Template–A1	5′ TA*A	T>A	A>T	T>A>G
Template–A2	5′ GA*A	T>C>A~G	T>C	T>A>G>C
Template–A3	5′ CA*A	T>G>C>A	T>G>C	T>G>A>C
Template–A4	5′ AA*A	T>C~A~G	T>C	T>A~G>C
Template–A5	5′ GA*C	T>C>A	T>C	T>G>A~C
Template–A6	5′ TA*C	T>A~C	T>C	T>G>A>C
Template–A7	5′ GA*G	T>C	T>C>A~G	T>G>A>C
Template–A8	5′ TA*G	T>C	T>C>A	T>G>A>C
Template–A9	5′ GA*T	T>C~G~A	T>C>G	T>G>A~C
Template–A10	5′ TA*T	T>C>G	T>C>G	T>G>C>A
Template–B1	5′ CA*G	T>C>A	C>T	T>C
Template–B2	5′ AA*T	T>A	A>T	T>A>G

[a]Sequence around 2-OH-Ade is shown. Whole sequences are 5′ dGCNA*MTATT-CCGTCAT 3′ (template–A) and 5′ dCGTNA*MATTTCT-GAT 3′ (template–B), where NA*M represents 2-OH-Ade and flanking bases.

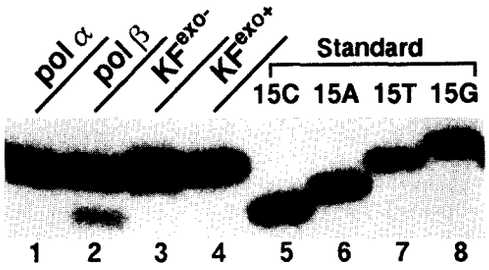

Fig. 2. Analysis of full-length products synthesized by DNA polymerases in vitro. Template–B1 was annealed with a ^{33}P-labeled primer. The template–primer complex was treated with KF^{exo-}, KF^{exo+}, pol α, or pol β in the presence of the four dNTPs. After the addition of a formamide-dye solution and heat denaturation, the reaction mixture was applied to a denaturing 20% polyacrylamide gel (30×80 cm, 0.5 mm thick). 15C, 15A, 15T, and 15G refer to standard oligonucleotides with the indicated base at position 11 (corresponding to the 2-OH-Ade site). Lanes 1–4, full-length products obtained by enzyme reactions. Lane 1, with pol α; lane 2, with pol β; lane 3, with KF^{exo-}; lane 4, with KF^{exo+}. Lanes 5–8, standard oligonucleotides.

Through studies with 10 oligonucleotides containing different 5′- and 3′-flanking bases (templates–A1 to –A10, Table 1), it was concluded that dCMP was incorporated most frequently opposite 2-OH-Ade, except for dTMP, by pol α and pol β. In the case of KF^{exo-}, dGMP appeared to be inserted more efficiently than dCMP and dAMP. Interestingly, DNA polymerases inserted dAMP opposite 2-OH-Ade in the 5′ -TA*A- 3′ sequence (templates–A1 and –B2). Therefore, the incorporation of dAMP opposite 2-OH-Ade, which we found in the previous study [11] was not a general phenomenon, but may be an event that occurs with a 5′ -TA*A- 3′ sequence.

We also studied the effects of neighboring base(s) on the incorporation of nucleotides opposite unmodified adenine residues. We used templates which have the same sequences as templates–A1, –A2, and –B1, respectively, except that adenine replaces the 2-OH-Ade. Under the same conditions as those used with the templates with 2-OH-Ade, misincorporation opposite adenine was very rare, and we did not observe incorporation that was dependent on neighboring base(s) (data not shown).

3.2. *Analysis of full-length products*

Template–B1 annealed with a primer was treated with KF^{exo+}, KF^{exo-}, pol α, and pol β in the presence of the four dNTPs, and the full-length products that were synthesized in the polymerase reactions were analyzed by denaturing 20% polyacrylamide gel electrophoresis. The oligonucleotides (15mer) containing C, A, T, or G opposite the 2-OH-Ade position (15C, 15A, 15T, and 15G) can be distinguished by their relative mobilities in the gel (Fig. 2, lanes 5–8) although the separations of 15C and 15A, and of 15T and 15G were not perfect. However, we did not detect the incorporation of dGMP opposite 2-OH-Ade in template–B1 in the absence of other nucleotides (Fig. 1A), indicating that the incorporation of dGMP in the presence of the three other nucleotides would be negligible. For the same reason, the incorporation of dAMP with KF^{exo-} and pol β would not occur. Moreover, we did not think that incorporation of dGMP or dAMP occurs with KF^{exo+}, because the proof-reading activity of the enzyme does not appear to affect the incorporation of nucleotides qualitatively.

The products with pol β showed the same mobilities as those of the 15T and 15C standards (Fig. 2, lane 2). This means that pol β inserted dCMP opposite 2-OH-Ade, even in the presence of dTTP. The ratio of dTMP to dCMP incorporated was 7:1. The major products with pol α, KF^{exo-}, and KF^{exo+} had the same mobility as the standard 15T (Fig. 2, lanes 1, 3, and 4). With KF^{exo+} and KF^{exo-}, the dCMP incorporation that was found in the experiment in the presence of a single dNTP (Fig. 1A, lane 3) was not observed. On the other hand, the incorporation of dCMP was detected in the case of pol α. The ratio of dTMP to dCMP incorporated by pol α was 200:1. Although the separation of the oligonucleotides with C and A was not perfect, we concluded that no dAMP was incorporated because (1) the insertion of dAMP was much less than that of dCMP (Fig. 1A, lanes 5 and 7) and (2) we detected no radioactivity in the area between the '15T' and '15C' areas, which would have been detectable if incorporation of dAMP occurred. These results indicate that dCMP was inserted opposite 2-OH-Ade by DNA pol α and pol β in the presence of the four nucleotides. No incorporation of dCMP was observed when the unmodified template was used (data not shown).

The results described above indicate that the 2-OH-Ade residues present in DNA strands are miscoding, although the normal and mutant Klenow enzymes did not insert an 'incorrect' nucleotide. Moreover, the results were different from those we obtained with template–A1 (incorporation of dAMP) under similar conditions [11], indicating that sequence-dependent incorporations of nucleotides opposite 2-OH-Ade occur in the presence of the four nucleotides.

4. Discussion

With a total of 12 oligonucleotide templates containing 2-OH-Ade, we found that DNA polymerases incorporated various nucleotides opposite 2-OH-Ade in the presence of a single dNTP (Table 1). We also observed that dCMP was incorporated most frequently (except dTMP) by DNA pol α and pol β. dTMP and dGMP were inserted most frequently by KF^{exo-}. Alteration of the 5′- and/or 3′-flanking base(s) of the oxidized adenine affected the incorporation of nucleotides (Table 1). Moreover, incorporations of dAMP opposite 2-OH-Ade, which we reported previously [11], in particular occurred when the base was located in a 5′ -TA*A- 3′ sequence. Therefore, the incorporation of nucleotides opposite 2-OH-Ade was dependent on its neighboring sequence.

This conclusion was confirmed by comparison of the full-length products obtained by in vitro DNA synthesis with template–B1 (Fig. 2) and template–A1 [11]. Although the actual mechanism of the context effect is not clear, it is noteworthy to mention that with DNA polymerases, particularly pol α, the nucleotide appears to be incorporated opposite 2-OH-Ade by recognizing the 5′-flanking base (Table 1). Among the results of the 12 templates in Table 1, 10 cases are explained by this incorporation mechanism.

Some investigators have studied the influence of neighboring base(s) on the incorporation of nucleotides opposite a DNA lesion. Singer et al. reported that the 3′-flanking bases of O^6-methylguanine affected the incorporation of dTMP in vitro [13]. The 5′- and 3′-flanking bases influenced the extension of the methylated base paired with T or C [14]. The nucleotide incorporations opposite O^6-methylguanine and 8-

OH-Gua residues in codon 12 (5′ GGC 3′) of a *ras* proto-oncogene were compared, and different ratios of T/C and of A/C, respectively, were described [4]. Distinct mutation spectra of 8-OH-Gua in different positions in NIH3T3 cells were found [8]. Misincorporation opposite 8-hydroxyadenine was found in a *ras* sequence [15], while no misincorporation was detected in another oligonucleotide [16]. Although limited kinds of oligonucleotides with a DNA lesion were employed in these studies, we used a total of 12 oligonucleotides with 2-OH-Ade in the present study, and analyzed the effects of the 5′- and/or 3′-flanking base(s) on the frequencies and kinds of misincorporations. Therefore, this is the first detailed study of the influences of the flanking bases of a DNA lesion on the kind of nucleotides misincorporated.

We have studied the effects of sequence contexts on the incorporation of 2-OH-dAMP and have found no differences. Specifically, we used oligonucleotides with the same sequence as the complementary strand of template–A1, with which the insertion of dAMP opposite 2-OH-Ade was found, but 2-OH-dAMP was incorporated opposite T and C, but not opposite A, in the template [10]. Therefore, it appeared that the sequence contexts affect the incorporation of nucleotides opposite 2-OH-Ade, but not 2-OH-dAMP.

Loeb's group has investigated the mutation spectra of oxygen radicals produced by the autooxidation of Fe^{2+} [17] and by phorbol ester-stimulated human leukemia cells [18] with single-stranded M13 DNA. They observed that G → C, G → T, and C → T mutations are frequently induced. They also detected point mutations involving A (A → G, etc.) with approximately 50% frequency of the G → T transversion [17,18]. As shown in Table 1, 2-OH-Ade may have a broad mutation spectrum, and the mutations of A may have been possibly induced by 2-OH-Ade.

2-OH-Ade is formed in monomers at a similar level to 8-OH-Gua, while its formation in DNA is 40-fold less than that of 8-OH-Gua [10]. This fact implies that the incorporation of 2-OH-dATP by DNA polymerases is a major pathway of the formation of 2-OH-Ade in DNA. In the case of 8-OH-Gua, it was shown that the incorporation of 8-hydroxy-2′-deoxyguanosine 5′-triphosphate by DNA polymerases is as important as the direct oxidation of guanine residues [19]. The incorporation of 2-OH-dATP by DNA polymerases is as efficient as that of 8-hydroxy-2′-deoxyguanosine 5′-triphosphate [10]. Thus, the oxidation of dATP and its precursors, and the incorporation of 2-OH-dATP appear to be major contributors to the formation of 2-OH-Ade in DNA. Furthermore, we determined that the oxidized nucleotide is incorporated opposite T and C residues in the template DNA in the ratio of about 4.5:1, calculated from the kinetic data [10]. On the other hand, we showed that dTMP was incorporated most frequently opposite 2-OH-Ade by DNA polymerases in vitro (Table 1). Therefore, the formation of 2-OH-dATP by oxygen radicals may induce a C·G → T·A transition, which was most frequently observed in spontaneous mutations (47% of base substitutions) [20] and in superoxide-induced substitution mutations [21] in the *lacI* gene of wild-type *Escherichia coli*.

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