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Replication of 2-hydroxyadenine-containing DNA and recognition by human MutSα

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Abstract

2-Hydroxyadenine (2-OH-A), a product of DNA oxidation, is a potential source of mutations. We investigated how representative DNA polymerases from the A, B and Y families dealt with 2-OH-A in primer extension experiments. A template 2-OH-A reduced the rate of incorporation by DNA polymerase α (Pol α) and Klenow fragment (Kf^{exo-}). Two Y family DNA polymerases, human polymerase η (Pol η) and the archeal Dpo4 polymerase were affected differently. Bypass by Pol η was very inefficient whereas Dpo4 efficiently replicated 2-OH-A. Replication of a template 2-OH-A by both enzymes was mutagenic and caused base substitutions. Dpo4 additionally introduced single base deletions. Thermodynamic analysis showed that 2-OH-A forms stable base pairs with T, C and G, and to a lesser extent with A. Oligonucleotides containing 2-OH-A base pairs, including the preferred 2-OH-A:T, were recognized by the human MutSα mismatch repair (MMR). MutSα also recognized 2-OH-A located in a repeat sequence that mimics a frameshift intermediate.

Keywords

2-Hydroxyadenine; Mismatch repair; Replication; Y family polymerases

1. Introduction

Reactive oxygen species (ROS) can cause oxidative damage to DNA, producing chemical changes to pyrimidine and purine bases, abasic sites and single and double strand breaks. In addition, ROS can react with the DNA precursors in the dNTP pool providing another source of oxidative DNA damage. The relative numbers of different lesions, their intrinsic miscoding potential and the efficacy of dedicated repair systems are all factors that control the final mutational load and the development of oxidation stress-related diseases. Among oxidized

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DNA bases, 8-oxo-7,8-dihydroguanine (8-oxoG) has attracted major attention because of its potent miscoding ability. In vitro studies have shown that template 8-oxoG can direct incorporation by DNA polymerases of either C or A [1]. The latter results in GC>TA transversions [2]. In addition, DNA polymerases can utilise 8-oxodGTP and the resulting incorporation of 8-oxoG opposite A causes AT>CG transversions and contributes to the general oxidative and mutagenic load [3,4].

In addition to 8-oxoG, sources of oxidation such as Fenton-type reagents, γ-rays or antitumor drugs [5–7] all increase the levels of 2-hydroxyadenine (2-OH-A) in DNA. 2-OH-A is also potentially miscoding [8,9]. Replication in bacteria or mammalian cells of shuttle vectors containing a single 2-OH-A produces a broader spectrum of mutations than that produced by DNA 8-oxoG [9,10]. 2-OH-A has received less attention, probably because steady-state levels of 2-OH-A residues in cellular DNA are estimated to be more than one order of magnitude lower than those of 8-oxoG ($1/10^7$ normal nucleotides) [5,6]. Significantly, the main source of DNA 2-OH-A appears to be utilization of 2-OH-dATP during replication whereas in situ oxidation of DNA adenine makes a relatively minor contribution [5]. While no information is available on the excision repair of DNA 2-OH-A, replication-related processing of 2-OH-A, including its incorporation from the oxidized dNTP pool, has been more extensively investigated. Indeed, the major replicative enzyme DNA polymerase α (Pol α) incorporated 2-OH-dATP opposite T and C in the DNA template, while *Escherichia coli* DNA polymerase I Klenow fragment (Kf^{exo-}) incorporated 2-OH-dATP only opposite T [5]. Thus, 2-OH-dATP might have a mutagenic potential for replicative DNA polymerases. It is well established that 2-OH-dATP is a substrate for hydrolysis by the human MutT homolog, hMTH1. This prevents the incorporation of 2-OH-A into DNA [11]. In addition, MYH, the MutY homolog which excises A incorporated opposite DNA 8-oxoG, also removes 2-OH-A from 2-OH-A:G base pairs. This is consistent with a role for MYH in reversing oxidation-related mismatches generated by incorporation of 2-OH-A [12].

Our previous work demonstrated that the post-replicative mismatch repair (MMR) pathway also helps to regulate the steady-state level of DNA 8-oxoG by removing the oxidized base from the nascent DNA strand [13,14]. Furthermore, over-expression of hMTH1 in MMR-defective mouse and human cells reduces the level of DNA 8-oxoG and significantly attenuates their characteristic mutator phenotype [15]. Mutation and microsatellite instability analysis indicated that a significant fraction of the oxidation-related mutations that were subject to correction by MMR occurred at A:T base pairs [15]. In particular, AT>TA, AT>GC mutations and frameshifts in runs of As were all affected. Since hMTH1 acts on both 2-OH-dATP and 8-oxodGTP [16], its expression could influence mutation by either of the oxidized purines, suggesting that DNA 2-OH-A might make a significant contribution to the mutational burden.

As a first step to clarifying the possible involvement of DNA 2-OH-A in oxidation-related mutagenesis, we have investigated some of its biochemical and physical properties. We examined the effect of 2-OH-A on replication in vitro by replicative and translesion synthesis (TLS) DNA polymerases. 2-OH-A miscoding was investigated in two unrelated DNA sequences, in A repeats or in random sequences, in which its effect on the thermal stability of DNA duplexes was quantified. The ability of a purified human MutSα DNA mismatch binding complex to recognize 2-OH-A-containing base pairs was compared in random sequences and in repetitive DNA sequences that represent frameshift intermediates. Our findings indicate that 2-OH-A is a block for replicative DNA polymerases and its bypass by TLS polymerases is mutagenic. In addition the evidence that MutSα can recognize 2-OH-A-containing base pairs, including frameshift intermediates, suggest that MMR might help to counteract the effects of 2-OH-A incorporated from the oxidized pool.

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2. Materials and methods

2.1. Oligonucleotide synthesis

Oligonucleotides were synthesized by MWG-Biotech AG. 6-Carboxyfluorescein (6-FAM) labelled and 2-OH-A containing oligonucleotides were synthesized by the Eurogentec S.A. All oligonucleotides were further purified by denaturing polyacrylamide gels (PAGE).

2.2. Primer extension reactions

In standard primer extension experiments, 6-FAM labelled primers were annealed to the template strands in a 1:1 molar ratio. 6-FAM labelled DNA duplexes (50 nM) were initially pre-incubated with 0.2 pmol of mammalian Pol α in 25mM Tris–HCl (pH 8.0), 0.5mM DTT, 0.25 mg/ml BSA, 10mM $MgCl_2$ buffer for 1min. Nucleotides were then added as specified in the figure legends and the reaction continued for 10 min at 37 °C. Reactions were stopped by addition of gel loading buffer (USB Corporation) (95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), the products were denatured at 95 °C for 5min and separated on denaturing 20% PAGE. Pol α was purified from HeLa cells as described in Ref. [17]. For dNTPs incorporation and extension by Kf^{exo-} (New England Biolabs) 6-FAM labelled DNA substrates (50 nM) were incubated with the enzyme (2.5 nM) and 30μM of each triphosphate at 37 °C in a buffer containing 20mM Tris–HCl (pH 7.7), 2mM $MgCl_2$, 2mM DTT. After 0.5–5 min, an equimolar dNTP mixture was added and incubated for 5min. Primer/template (50 nM) were pre-incubated with 150nM DNA polymerase 4 (Dpo4), purified as described in Gruz et al. [18], at 55 °C in a buffer containing 30mM potassium phosphate, pH 7.4, 7.5mM $MgCl_2$, 1.25mM β-mercaptoethanol and 5% glycerol. After 3min the dNTP (50μM) were added and incubated at 55 °C for 15 min. The subsequent elongation was performed by adding 50μM of dNTPs for further 15 min. Fluorescent bands were visualized by Typhoon 9200 Gel Imager (Amersham Bio-sciences Europe GmbH) and quantitated by ImageQuant TL software.

2.3. Kinetic analysis

Experiments with Pol α, Kf^{exo-} and Dpo4 were performed under the conditions described above using 0.01–100μM dNTP, 0.01–300μM dNTP and 1–300μM dNTP, respectively. Data points were derived from the analysis of the intensities of the products bands. The values of integrated gel band intensities in dependence of the nucleotide substrate concentrations ([dNTP]) were fitted to the equation:

$$I_T^* / I_{T-1} = V_{max}[\text{dNTP}] / \left(K_m + [\text{dNTP}]\right)$$

where T is the target site, the template position of interest; I_T^*= the sum of the integrated intensities at positions T, $T + 1 \cdots T + n$.

Before being inserted in the above equation, the intensities of the single bands of interest were first normalized by dividing for the total intensity of the lane. This reduced the variability due to manual gel loading. An empty portion of the gel was scanned and the resulting value was subtracted as background. The goodness of fit of the interpolated curve was assessed by computer-aided calculation of the sum of squares of errors SSE and the correlation coefficient R^2. Interpolation, SSE, R^2 and standard errors determination were done with the computer programs GraphPadPrism and Kaleidagraph.

2.4. Bypass efficiency assay

Reaction conditions for Dpo4 and polymerase η (Pol η) were the same described previously [19]. Reaction conditions for Kf^{exo-} were as recommended by the manufacturer using 25μM

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dNTPs. A truncated Pol η (residues 1–434) was overexpressed in *E. coli* using a modified pGEX4T3 vector that codes for a GST-Pol η fusion with a TEV cleavage site between the two proteins. Polymerase was purified by batch binding to glutathione Sepharose 4B, on-resin cleavage with TEV and MonoS column chromatography, with similar conditions to those described previously for the full length protein [20]. Dpo4 was purified as previously described [21]. The DNA substrate was a 45-mer template (5′-CCAGCTCGGTACCGGGTTAGCCTTTGGAGTCGACCTGCAGAAATT; underlined A is site of 2-OH-A) annealed to a 24-mer ^{32}P end-labelled primer (5′-AATTTCTGCAGGTCGACTCCAAAG). Reactions were prepared on ice without enzyme, preheated for 30 s to the reaction temperature, and polymerase added to initiate synthesis. Samples were removed at the indicated times, added to an equal volume of formamide loading buffer and processed as described above for analysis by 12% PAGE and scanning densitometry with a Phospholmager. All reactions contained 4 pmol substrate and the substrate:enzyme ratios: Kf^{exo-} = 1000:1, Dpo4 = 1000:1, Pol η = 750:1. Determination of single hit conditions and calculation of bypass efficiency were as described previously [21].

2.5. Bypass fidelity assay

Lesion bypass fidelity assays were performed with a substrate prepared with an unlabelled primer, using instead an internal ^{32}P-dCTP label, as described previously [21,22]. Reaction conditions were the same as for the bypass efficiency reactions, except that a 5:1 substrate:enzyme ratio was used with a 15min incubation for all enzymes. Processing of the full length synthesis products and determination of plaque colour frequency and corresponding error rates were performed as previously described [21,22].

2.6. UV melting

Absorbance versus temperature changes were measured at 260nm by means of a Cary 3 spectrophotometer equipped with a Peltier device for temperature control. Samples resuspended in Tris–HCl 10 mM pH 7.2, $MgCl_2$ 2mM were placed in 1 cm path length quartz cells. The heating rate was 0.5 °C/min and data points were recorded every 0.2 °C. Absorbance values were corrected for the water thermal expansion and normalized at the absorption of 1 OD at 5 °C. Thermodynamic parameters were evaluated by extracting information by single equilibrium transition curves and data analysis was performed according to Breslauer [23].

2.7. MutSα purification and bandshift

MutSα was prepared from approximately 2×10^{10} Raji cells as previously described [24]. The final concentrated *Q* sepharose pool contained approximately 5 pmol MutSα per milliter. For bandshift assays, one unit of MutSα was defined as 5 fmol. Bandshift experiments were carried out with ^{32}P-end labelled oligonucleotide duplexes as previously described. Briefly, MutSα (2–10 units) was pre-incubated (5 min at 20 °C) with 2 pmol non-radioactive matched competitor duplex in 20μl reaction buffer containing 25mM Hepes KOH pH 8.0, 0.5 mM EDTA, 0.1 mM $ZnCl_2$, 10% glycerol, 50μg poly(dI:dC). The 20 fmol substrate duplex was added and incubation continued for a further 20 min. Products were analysed by PAGE on 6% non-denaturing gels.

3. Results

3.1. Replication of 2-OH-A-containing templates by different DNA polymerases

Since miscoding by a DNA lesion can be influenced by the type of DNA polymerase [25,26] and the sequence context, we examined the ability of A, B and Y family DNA polymerases to bypass a template 2-OH-A in two different sequences. Because we identified oxidation-related mutations at A:T base pairs in microsatellites formed by A-runs, we placed a single 2-OH-A

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in the middle of an A run within a 36 mer (6A*, repeated sequence). In the second DNA substrate, a single 2-OH-A replaced the A repeat, and the 15 nt flanking sequence on both 5′ and 3′ sides was retained (A*, random sequence). In control oligonucleotides, A replaced 2-OH-A. The results obtained with each enzyme are detailed in the next sections.

3.1.1. Human DNA polymerase α—The ability of the human B family Pol α to replicate 2-OH-A located in the A* and the 6A* repeat was investigated in primer extension experiments, using primers that terminated one base immediately before the lesion (Fig. 1). Following incubation in the presence of a single dNTP, Pol α incorporated exclusively T opposite 2-OH-A in either sequence (Fig. 1A and B) and there was no detectable incorporation of any of the other three bases. Although 2-OH-A retained the coding specificity of undamaged A, its coding efficiency – the ability to instruct the polymerase to incorporate the complementary base – was significantly reduced (Fig. 1, supplementary data). The apparent incorporation efficiency (k_{cat}/K_m) for T opposite 2-OH-A was 17.5- and 5.5-fold lower than opposite A in the random and in the repeated sequence, respectively (Fig. 1C). Thus, Pol α preferentially incorporates T opposite 2-OH-A, but with lower efficiency than opposite a normal A in both sequence contexts.

3.1.2. E. coli DNA polymerase I (Kf^{exo-})—In contrast to Pol α, the Klenow fragment (Kf^{exo-}) of *E. coli* DNA polymerase I is able to incorporate various bases opposite a template 2-OH-A [8]. Using the same experimental approach employed for Pol α, we compared the ability of Kf^{exo-} to replicate a template 2-OH-A in the random and in the repeated sequence. The kinetic parameters for correct and incorrect nucleotide insertion by Kf^{exo-} are summarized in Table 1A. Primer extension was observed in the presence of each of the four dNTPs but with very different efficiencies. The degree of dNTP selectivity differed for the random and the 6A* sequence. T was preferentially inserted opposite 2-OH-A in both, although the insertion efficiency was, respectively, 70- and 56-fold lower than opposite A. For the other dNTPs, the preferential order of incorporation in the A* random sequence was T≫G=C≫A with utilization of dATP three orders of magnitude lower than TTP. In the 6A* substrate, the discrimination against A was relaxed and both purines were inserted with similar efficiencies and about three-fold better than C. The preferred order was T≫G = A>C (Table 1A).

We also examined whether Kf^{exo-} could elongate 2-OH-A-containing base pairs. Two-stage reactions were performed. In the first step, Kf^{exo-} was incubated with primer/templates and a single triphosphate to allow incorporation. To monitor the efficiency of elongation from the resulting 3′-terminal 2-OH-A base pair, a second incubation was carried out in the presence of all four dNTPs (Fig. 2, supplementary data). Kf^{exo-} efficiently elongated all terminal 2-OH-A base pairs in the random sequence. In the 6A* sequence, terminal 2-OH-A:C and 2-OH-A:A pairs impeded extension (Fig. 2, supplementary data). Similar results were obtained in an alternative approach, in which the extension of synthetic primers generating different 3′ terminal 2-OH-A-containing base pairs by Kf^{exo-} and all four dNTPs was assayed (Fig. 2A). In the 6A* sequence Kf^{exo-}efficiently elongated a terminal 2-OH-A:T or 2-OH-A:G pair whereas a 2-OH-A:A and, to a more modest extent a 2-OH-A:C pair, prevented elongation by Kf^{exo-}.

We conclude that replication of 2-OH-A by Kf^{exo-}, as with Pol α, is also relatively error-free, and T is the preferentially incorporated base. Unlike Pol α, Kf^{exo-} exhibits a somewhat more relaxed specificity and other base pairs – notably 2-OH-A: G – are also formed. Both the formation and elongation of these promutagenic base pairs is influenced by sequence context and these parameters differ significantly between the random and repeat sequence.

3.1.3. Sulfolobus solfataricus Dpo polymerase 4—*S. solfataricus* Dpo4 is an archeal Y family DNA polymerase [27]. With a single dNTP, Dpo4 inserted T, A or C opposite 2-OH-

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A in the A* random sequence and the preferential order of incorporation was T>C>A (Table 1B). As expected, incorporation by Dpo4 was less selective than Kf^{exo-} and dNTP utilization varied by only 3–5-fold. Surprisingly, however, addition of G was undetectable.

A different result was obtained with the 6A* repeat sequence in which Dpo4 inserted predominantly T or A. Kinetic parameters indicated that T incorporation opposite 2-OH-A is only slightly lower (5.7-fold) than opposite A, while misincorporation of A is further decreased (25-fold) (Table 1B).

In this repeat sequence, Dpo4 efficiently elongated primers creating terminal 2-OH-A:T or 2-OH-A:C pairs, whereas 2-OH-A:G and 2-OH-A:A constituted a significant block (Fig. 2B). In the random sequence, none of the terminal mismatches constituted a significant block to elongation by Dpo4 (Fig. 3, supplementary data).

A template 2-OH-A in a random sequence is therefore quite efficiently bypassed by Dpo4. Bypass occurs with a significantly reduced fidelity via the formation of frequent 2-OH-A:C and 2-OH-A:A mispairs. Once formed, each of these terminal mismatches are easily elongated. Dpo4 is also considerably influenced by the sequence context of the 2-OH-A. In the 6A* repeat sequence, bypass of 2-OH-A by Dpo4 is more efficient. Its inability to extend the frequent terminal 2-OH-A:A mismatches indicates that 2-OH-A bypass in a repeat sequence is also likely to be more error free.

3.2. 2-OH-A differentially affects the bypass efficiency of Dpo4 and human DNA polymerase η

Bypass of 2-OH-A by Y family DNA pols was also investigated by a different approach. We used reaction conditions that reflect a single cycle of DNA synthesis with a primer terminus located such that multiple incorporations are required prior to the lesion being encountered [21]. Short incubation times and large substrate excess are chosen so that each DNA molecule is acted on only once during the time course studied. The advantage to this ‘single hit’ condition is that quantitative measurements of insertion efficiency opposite the lesion, extension efficiency from the damaged primer terminus and bypass efficiency (defined here as incorporation opposite at least one undamaged base beyond the lesion) can be made by comparing synthesis on the damaged and undamaged templates. All reactions described here were confirmed to be under single hit conditions that yield fainter band intensities (Fig. 3) than observed when multiple cycles of synthesis occur (Figs. 1 and supplementary data).

In these assays, bypass efficiency of 2-OH-A by Dpo4 was high, 85% of the efficiency of copying the equivalent undamaged A, and there was no obvious pause at the site of the lesion (Fig. 3, arrows in left panel). The relative insertion efficiency opposite 2-OH-A and the relative extension efficiency from the damaged terminus were 79 and 88%, respectively, indicating that 2-OH-A is only a minor block to normal synthesis.

The same analysis performed with human Pol η, the prototypical Y family polymerase, indicated that bypass of 2-OH-A by this enzyme was only 4% that of undamaged A (Fig. 3, right panel). In addition, insertion opposite the lesion was also very inefficient and was only 22% of the corresponding value for A. Surprisingly, incorporation by Pol η also appeared to be inhibited at the base preceding the 2-OH-A (note the low intensity of the band immediately below the arrow in Fig. 3, right panel), a property not previously reported for this enzyme.

Kf^{exo-} was assayed under the same single hit conditions to provide a comparison (Fig. 3, middle panel). Bypass efficiency was 35% and in this case, insertion opposite the lesion (note the more intense band at the −1 position), rather than subsequent extension was particularly problematic. Both these findings are in agreement with expectations from the kinetic analysis presented in

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Table 1. Thus, under conditions of a single polymerase/template encounter, a template 2-OH-A lesion has a minimal impact of replication by Dpo4 whereas synthesis by human Pol η is strongly inhibited. Its overall effect on replication by Kf^{exo-} is intermediate and, as expected, the major impact is on insertion opposite the lesion.

3.3. Fidelity of 2-OH-A bypass by Dpo4, DNA polymerase η and Kf^{exo-}

In order to measure the error rates for a complete lesion bypass event in the presence of all four dNTPs, we used templates corresponding to the N-terminal region of the *lacZ* α-complementation gene of bacteriophage M13mp2, in which 2-OH-A (or undamaged A) is located in a TAG stop codon. Reactions designed to allow complete synthesis (to the end of the template) were carried out on all substrates and the newly synthesized strand was recovered, hybridized to gapped-duplex M13 molecules, and introduced into the appropriate *E. coli* strain [21]. Errors in synthesis at either the T, A/2-OH-A or G of the stop codon were detected as dark blue plaques and frameshift errors were detected as colourless plaques. The precise change was then identified by sequencing of mutant plaques. The frequencies of the different plaque phenotypes (Table 1, supplementary data) and the corresponding rates of base substitution and insertion/deletion errors at the lesion site are shown in Fig. 4A. Bypass of 2-OH-A by each of the three enzymes induced large increases in base substitutions (5–90 fold) relative to undamaged A, indicating 2-OH-A acts as a miscoding lesion. In addition, replication by Dpo4, but not Pol η or Kf^{exo-}, produced a six-fold increase in frameshifts at 2-OH-A. These were mostly single base deletions at the site of 2-OH-A.

Each polymerase produced a different spectrum of base substitution mutations at the 2-OH-A site (Fig. 4B). Dpo4 bypass of 2-OH-A was associated with both AT>TA transversions and AT>GC transitions (in an approximately 3:2 ratio). By contrast, bypass by Pol η caused predominantly AT>GC transitions, whereas the majority of changes for Kf^{exo-} were AT>CG transversions, with a smaller contribution from AT>TA.

These findings indicate that replication of 2-OH-A by these three polymerases is mutagenic and that the type of mutation is determined by the polymerase. Furthermore, the mutational spectra are in good general agreement with the incorporation preferences of each polymerase inferred from in vitro experiments (Table 1). Interestingly, Dpo4 was the only polymerase that introduced frameshifts during replication of 2-OH-A in a non-repetitive sequence.

3.4. 2-OH-A effect on DNA secondary structure

DNA base unstacking can underlie failure in base extension during replication [28]. To determine the effects of 2-OH-A on DNA base unstacking as well as on hydrogen bond disruption we carried out thermal stability measurements on DNA duplexes containing the lesion in the random and repeated sequences (Fig. 5).

In both sequences, an A-containing mismatch produced the expected decrease in Tm (Fig. 5E and F). Replacement of A:T by 2-OH-A:T produced only minor changes in the thermal and thermodynamic stability of either the repeat or random duplexes (Fig. 5A and B). In the random sequence 2-OH-A caused a slight destabilization (Fig. 5B), whereas it had the opposite effect in the repeat sequence (Fig. 5A). 2-OH-A:C or 2-OH-A:G mismatches were without detectable effect and Tm differences in comparison to 2-OH-A:T were within the limits of experimental error (Fig. 5C and D).

The data indicate that 2-OH-dA can form stable base pairs with T and that the sequence context has a role in stacking optimization with stacking favoured in a repetitive A sequence. In contrast to unmodified A, pairing of 2-OH-A with the other bases, with a single exception of A, are

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relatively favoured. The adoption of a more suitable tautomeric form of the 2-OH-A in base pairs might be responsible for this effect [29].

3.5. Recognition by the hMutSα complex of 2-OH-A containing DNA

Because oxidation-related frameshifts at A repeat sequences are influenced by MMR [15], we examined whether purified MutSα recognizes 2-OH-A-containing base pairs. Duplexes based on the 6A* oligonucleotide in which the 2-OH-A strand was annealed to a complementary strand containing 6, 5 or 7 Ts opposite the A6 sequence were used as substrates for binding by MutSα. These represent frameshift intermediates with an insertion/deletion loop of one base. Band-shift analysis indicated that neither the 2-OH-A:T base pair in the 6A*:6T duplex nor an A:T pair in the control duplex was recognized by MutSα (Fig. 6A). In contrast, we observed significant binding to a duplex containing an extra A in the 2-OH-A-containing strand (6A*:5T). Comparable binding was observed to the duplex in which an extra T was positioned opposite the 2-OH-A-containing strand (6A*:7T) (Fig. 6A). In each case, binding was similar to that observed with duplexes containing either an extra unmodified A or T.

In a second series of experiments, we examined binding to 2-OH-A-containing base pairs in non-repetitive sequences. In this case, 2-OH-A provoked significant recognition by MutSα. Of particular note, binding to a 2-OH-A:T base pair was comparable to that seen with other 2-OH-A-containing mispairs (Fig. 6B). Under the same experimental conditions, there was no detectable binding by MutSα to the control A:T duplex. Recognition of a 2-OH-A:T base pair was also observed with a second series of duplexes based on an unrelated random sequence (data not shown).

These findings indicate that MMR might be engaged at 2-OH-A base pairs. MutSα recognition of 2-OH-A-containing structures resembling slipped-mispaired intermediates suggests a role in counteracting frameshifts caused by these oxidized bases in repetitive sequences. In addition, the ability of MutSα to recognize 2-OH-A containing mispairs is consistent with an involvement in suppressing base substitution mutations caused by oxidatively damaged adenine.

4. Discussion

Our previous analysis of oxidation-related spontaneous mutations in MMR deficient cells identified 2-OH-A as a potentially significant contributor to several classes of mutation [15]. Since these included frameshifts in A runs as well as base substitutions, we investigated some of the structural and biological properties of this oxidized purine in repetitive and non repetitive DNA sequence contexts.

Our findings indicate that incorporation opposite 2-OH-A is difficult for both the A family Kf^{exo-} DNA polymerase and the B family replicative Pol α. This distinguishes 2-OH-A from 8-oxoG, which is easily bypassed by several replicative polymerases via the mutagenic incorporation of an A opposite the lesion [1]. Thus, unlike 8-oxo-G, a template 2-OH-A might cause a permanent or transient replication block thereby provoking recruitment of a TLS polymerase. Among the enzymes we tested, Dpo4 was the least sensitive to 2-OH-A, while human Pol η was inefficient in bypassing the lesion. This raises the possibility that of the human Y family polymerases the Dpo4 homolog, polymerase κ, rather than Pol η, might be better equipped to bypass this oxidized purine. The different efficiencies of 2-OH-A bypass by Dpo4 and Pol η resemble their behaviour with AP sites and may reflect differences in their respective "little finger" subdomains [30,31], probably the critical features that allow lesion bypass [21].

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Each polymerase showed a strong preference for insertion of T opposite 2-OH-A although our incorporation data suggested a range of alternative permissible base pairings that were affected by the sequence context of the oxidized base. These findings are consistent with the known ability of DNA 2-OH-A to adopt multiple tautomeric forms that are influenced by temperature, solvent polarity and neighbouring bases [32–34]. In particular, a shift from the prevailing keto tautomer (N1-H) towards the enol form (O2H) is likely to affect the probability that the lesion is accommodated as 2-OH-A:T or with other partners through classical W–C bonding or wobble base pairs. They are also consistent with our Tm measurements, which indicated that 2-OH-A forms stable base pairs not only with T, but also with C or G. Thus, the different abilities of DNA polymerases to accommodate otherwise unfavourable 2-OH-A tautomers within their active sites might influence replication fidelity [35].

A previous investigation of mutational spectra in MMR-defective cells indicated that AT>GC and AT>TA base substitutions (and to a minor extent AT>CG) might derive from miscoding by an oxidized purine [15]. These were the most frequent mutations observed following replication of 2-OH-A by Dpo4 and Pol η under conditions of limited polymerase engagement. The same analysis also revealed that frameshifts, with deletion of the 2-OH-A adduct, were significantly increased following replication by Dpo4. This polymerase has a very low frameshift fidelity, which is consistent with its ability to accommodate unpaired nucleotides in the active site [30]. It is also known to generate deletions at noniterated nucleotides [36]. Together with the apparent difficulties experienced by replicative polymerases at a template 2-OH-A, these findings strengthen the likelihood that Y polymerase-mediated TLS occurs at this lesion.

Little or no information is available on the repair of 2-OH-A formed by in situ oxidation of DNA adenine. We show here that the major human MMR recognition complex, MutSα, recognizes 2-OH-A-containing oligonucleotides. Recognition by MutSα was context dependent. MutSα efficiently recognized oligonucleotide duplexes containing 2-OH-A in structures mimicking insertion/deletion loop (IDL) within a 6A repeat. In this regard, the behaviour of the oxidized adenine was indistinguishable from its normal homolog. Within the same type of IDL structure, the behaviour of 2-OH-A differs from DNA 8-oxoG, which appears to assume a conformation that renders it invisible to MutSα [37]. The ability of MutSα to recognize 2-OH-A within an IDL context may have relevance in vivo as it is consistent with the apparent contribution of the oxidized base to microsatellite instability at the A_{26} *BAT26* sequence in MMR-defective cells [15].

Although duplexes containing 8-oxoG:C base pairs are not recognized [38,39], MutSα bound each of the four 2-OH-A-containing base pairs to a similar extent. Alterations in base geometry and local flexibility are likely to be major determinants of mismatch recognition [28,40]. Recognition of 2-OH-A:T pairs might again reflect the unique ability of 2-OH-A to adopt multiple tautomeric forms, some of which are associated with wobble base pairs and local distortion [33]. A 2-OH-A:T pair in a fully paired A6 repeat sequence was not recognized by MutSα, however. A_n repeats of this kind are known to assume unusual conformations in which the A:T base pairs show large propeller twist angles and form bifurcated hydrogen bonds involving the N6 amino group of A and the O4 atoms of two adjacent Ts in the opposite strand. We speculate that this particular structural arrangement might confer a greater resistance to deformation and thereby prevent DNA from adopting the structural changes needed to trigger MutSα recognition.

Notwithstanding the precise mechanism by which MutSα recognizes 2-OH-A-containing base pairs, our findings clearly indicate how MMR might help control the steady-state levels of DNA 2-OH-A. The major source of DNA 2-OH-A is acknowledged to be the oxidized dNTP pool [5]. MMR would reverse this incorporation and prevent the accumulation of 2-OH-A in

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DNA. This role is consistent with the higher levels of DNA 2-OH-A observed in msh2$^{-/-}$ ES cells in comparison to wild type ES cells [41].

In summary, we have shown that replication fork block is the likely outcome of a replicative DNA polymerase encountering a template 2-OH-A. Our findings indicate that a specialized bypass polymerase might overcome the block at the expense of replication fidelity. In addition, MutSα recognition of 2-OH-A-containing substrates indicates that MMR might contribute to mutation avoidance by acting on 2-OH-A-containing base pairs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.dnarep.2006.11.002.

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Abbreviations

ROS
: Reactive oxygen species

8-oxoG
: 8-oxo-7,8-dihydroguanine

2-OH-A
: 2-hydroxyadenine

MMR
: Mismatch repair

6-FAM
: 6-carboxyfluorescein

Pol α
: Polymerase α

Kf^{exo-}
: Klenow fragment

Dpo4
: DNA polymerase 4

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Pol η

Polymerase η

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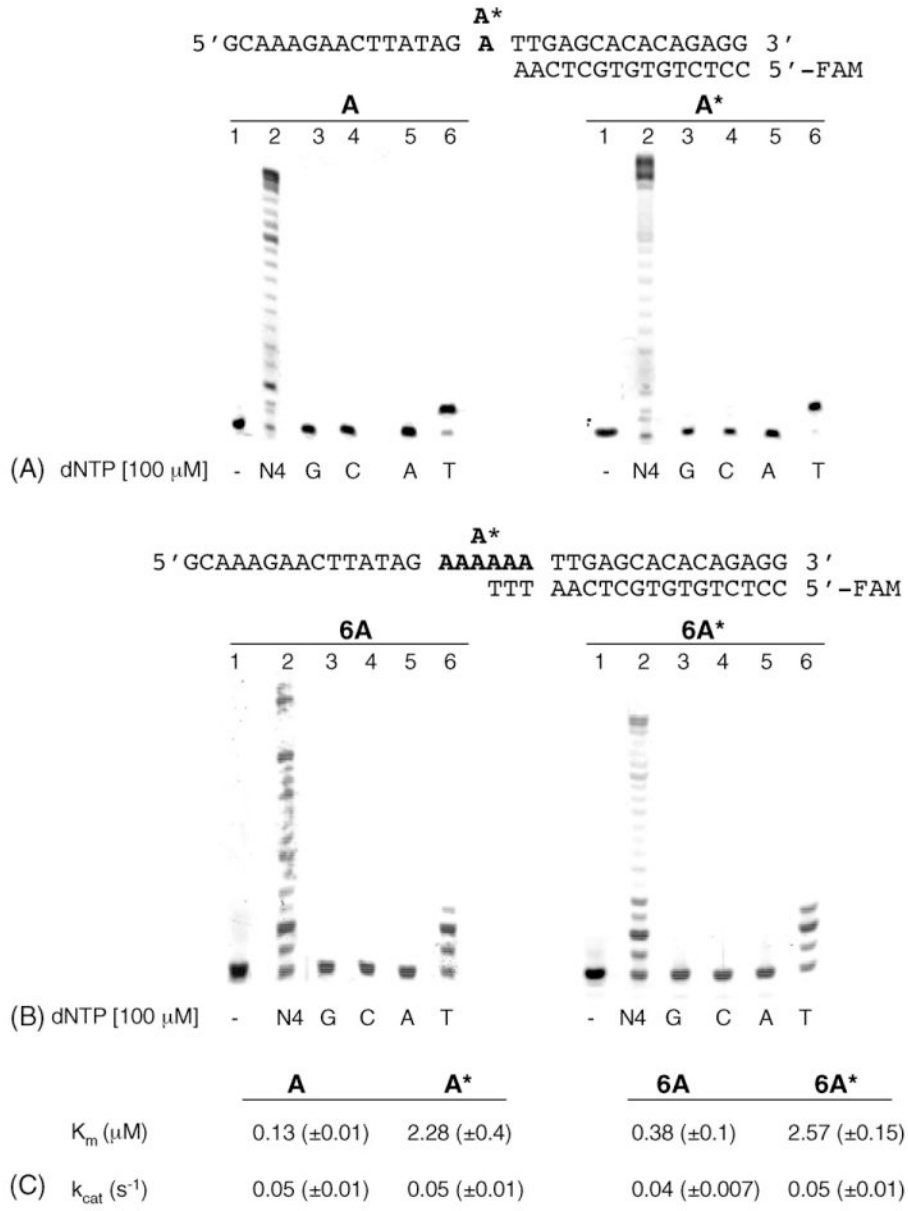

(C)

	A	A*	6A	6A*
K_m (μM)	0.13 (±0.01)	2.28 (±0.4)	0.38 (±0.1)	2.57 (±0.15)
k_{cat} (s^{-1})	0.05 (±0.01)	0.05 (±0.01)	0.04 (±0.007)	0.05 (±0.01)

Fig. 1.

DNA polymerase α: incorporation of dNTP opposite 2-OH-A. (A) Random sequence. Primer/templates (50 nM) were pre-incubated with Pol α. After 1min, reactions were initiated by the addition of a single triphosphate (100μM) and continued for 10 min. Reactions products were analysed by denaturing PAGE as described in Section 2. Controls (lanes 1) were incubated without enzyme or dNTP. A^* = 2-OH-A; (B) 6A or $6A^*$ repeat sequence. The experimental conditions were the same used for the random sequence. (C) Kinetic parameters for dTTP incorporation opposite A or 2-OH-A by Pol α in the random and in the repeated sequence. K_m and k_{cat} values were evaluated as described in Section 2.

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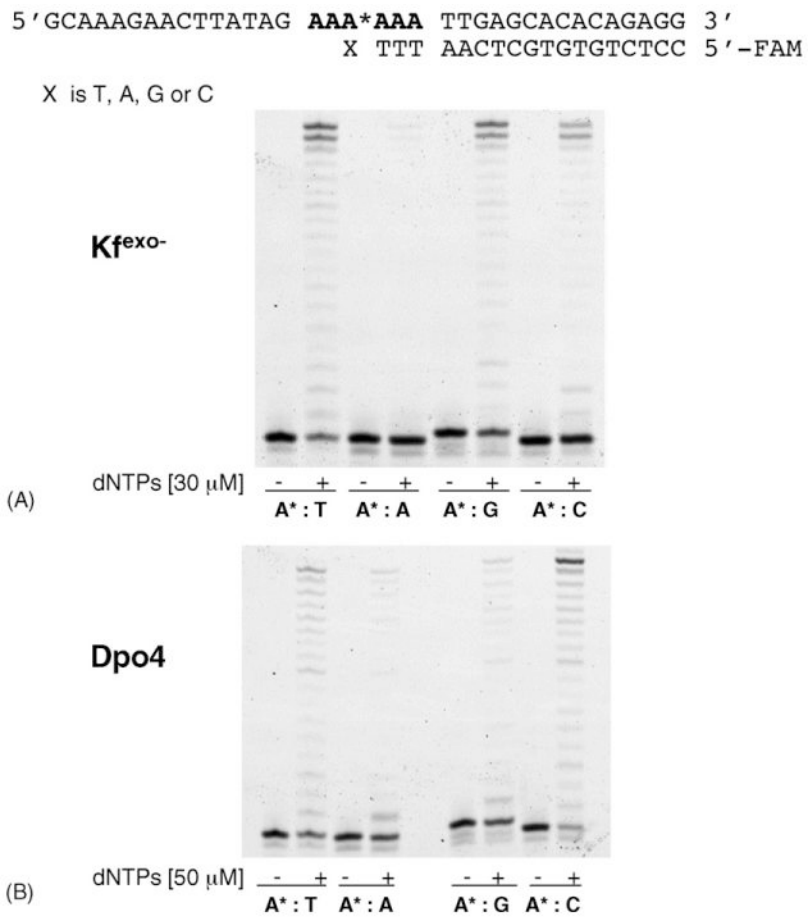

Fig. 2.

Elongation of terminal 2-OH-A:T, 2-OH-A: A, 2-OH-A:G and 2-OH-A:C base pairs. (A) Elongation of 2-OH-A containing terminal mismatches in the 6A* sequence by Kf^{exo-}. Reactions contained 50nM primer/template and 2.5nM Kf^{exo-} supplemented with an equimolar mixture of dNTPs (30μM). Controls were incubated without enzyme or dNTP. (B) Elongation of 2-OH-A containing terminal mismatches in the 6A* sequence by Dpo4. Primer/template (50 nM) were pre-incubated with 150nM Dpo4 enzyme at 55 °C for 3min. The elongation was performed by adding 50μM of dNTPs for 15 min.

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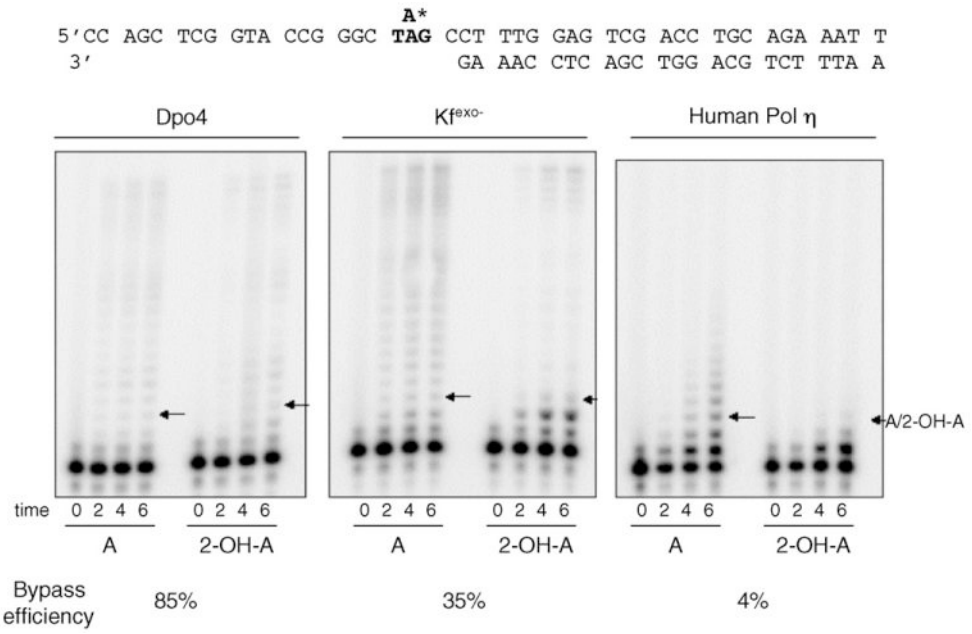

Fig. 3.

Products of 2-OH-A bypass reactions. Reactions shown are for bypass reactions with Dpo4, Kf^{exo-} and human DNA Pol η (left, middle and right panel, respectively). Each panel shows bypass of an undamaged A and 2-OH-A within a 5′-TAG substrate (as indicated in the sequence). An arrow indicates the location of the lesion.

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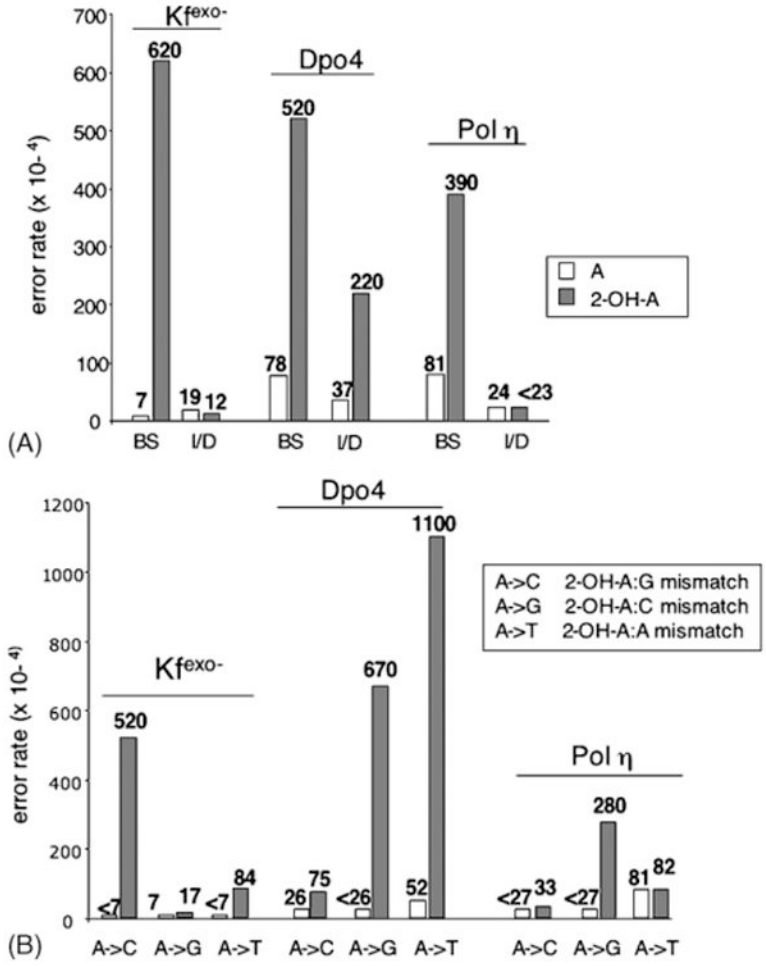

Fig. 4.

(A) Error rates for undamaged A and 2-OH-A by Dpo4, Kf^{exo-} and human DNA Pol η. The error rates of the different polymerases for undamaged A (open bars) and 2-OH-A (filled bars) are shown. BS, base substitution; I/D, insertion/deletion. (B) Base substitution error rates for Kf^{exo-}, Dpo4 and human DNA Pol η for undamaged A (open bars) and 2-OH-A (filled bars). In the box are indicated the possible 2-OH-A containing mismatches causing the observed mutations.

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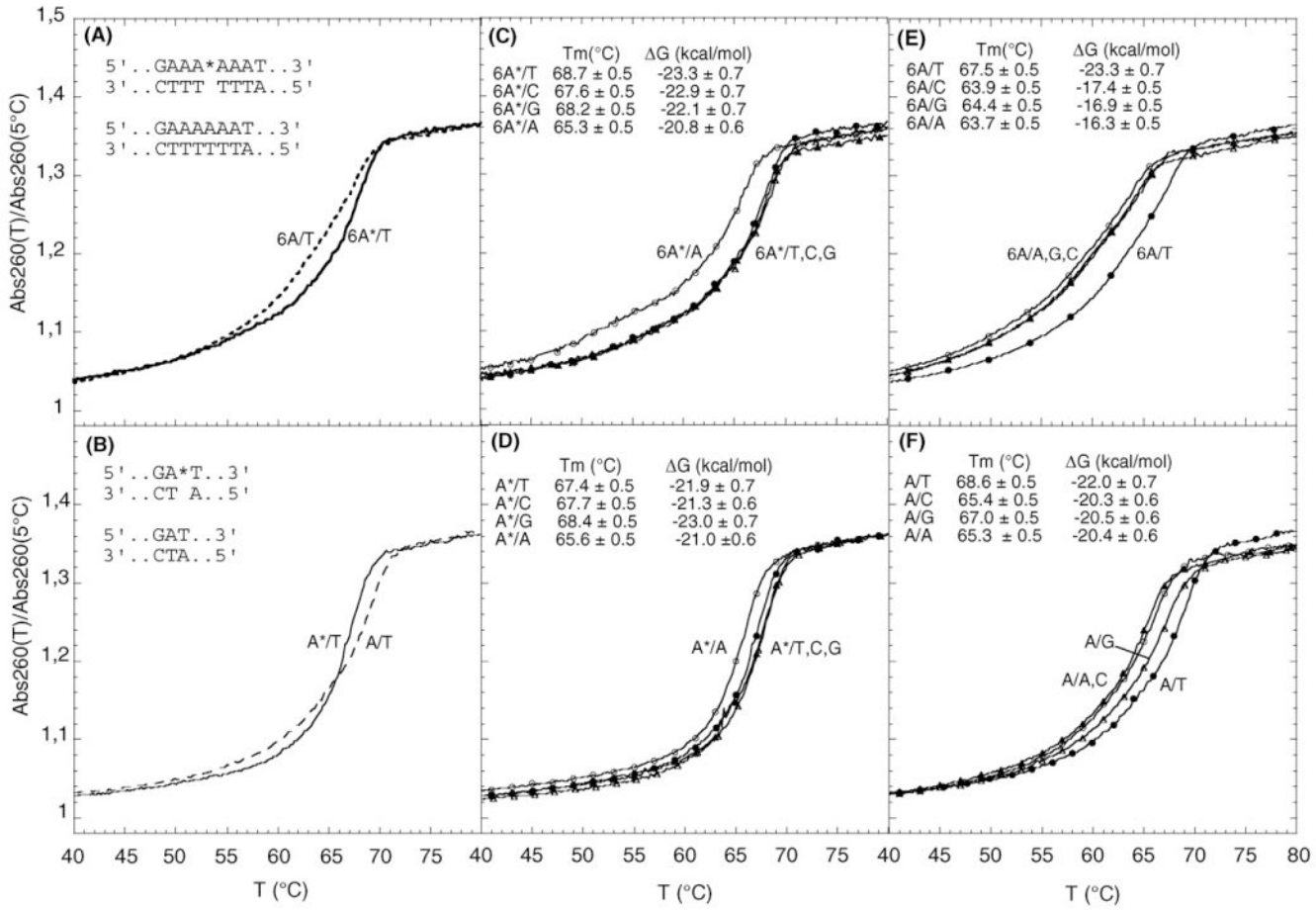

Fig. 5.

UV denaturation profiles of DNA duplexes with and without 2-OH-A. Effect of 2-OH-A:T pairing in the 6A repeat sequence (panel A) and in a random sequence (panel B); continuous line, 2-OH-A:T; dashed line, A:T. Thermal stability of DNA duplexes with A or 2-OH-A paired with T, G, C or A in the A-repeat sequence (panels E and C, respectively) and in the random sequence (panels F and D). Filled circle, A:T and 2-OH-A:T; empty circle, A:A and 2-OH-A:A; filled triangle, A:C and 2-OH-A:C; empty triangle, A:G and 2-OH-A:G.

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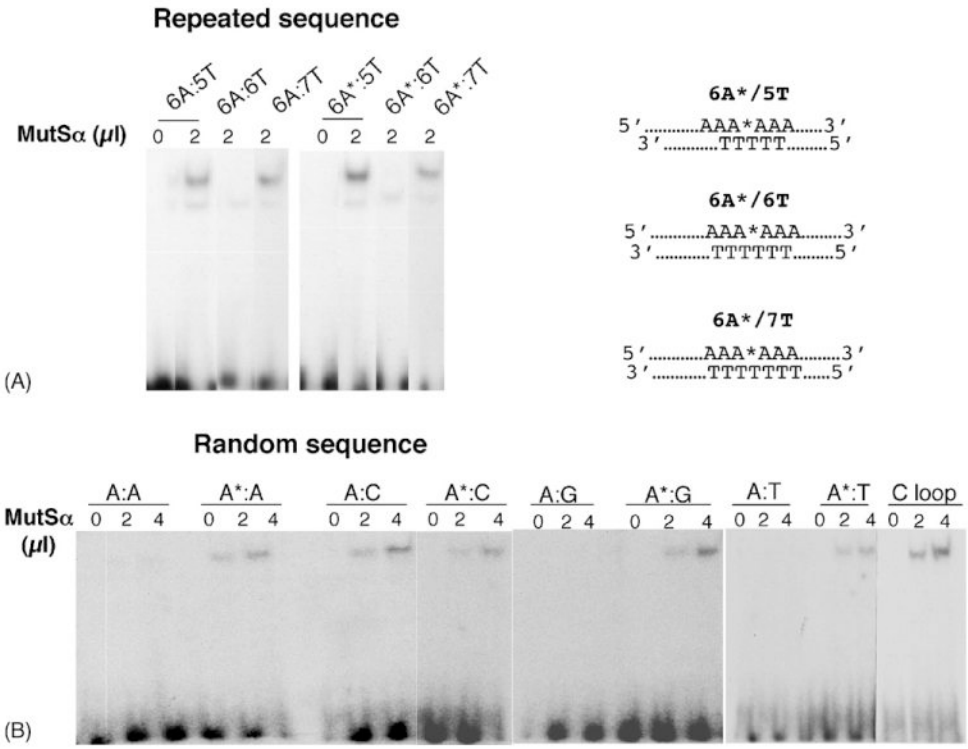

Fig. 6.

MutSα binding to 2-OH-A. End-labelled oligonucleotide duplexes of the repeated sequences shown in panel A were incubated with MutSα. In panel B is shown MutSα binding to 2-OH-A in random sequences. Products were analysed by non-denaturing PAGE as described in Section 2.

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Table 1

Kinetic parameters for dNTP incorporation opposite adenine or 2-OH-adenine by Kf^{exo-} (A) and Dpo4 (B) in a random and in a repeated sequence

A

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1} × 10^5)	Selectivity[a]
Kf^{exo-}				
A				
dTTP	0.0042(±0.0004)	0.6(±0.006)	1428(±14.8)	1
A*				
dTTP	0.21(±0.04)	0.43(±0.02)	20.5(±0.49)	69.7
dATP	18.1(±3.6)	0.049(±0.002)	0.03	47600
dCTP	27.7(±4.5)	0.19(±0.01)	0.67	2131
dGTP	2.1(±0.2)	0.141(±0.003)	0.7	2040
6A				
dTTP	0.06(±0.007)	1.89(±0.04)	315(±3.68)	1
6A*				
dTTP	0.55(±0.11)	0.31(±0.01)	5.6(±0.1)	56.2
dATP	4.93(±0.97)	0.04(±0.002)	0.081(±0.002)	3888.9
dCTP	13.9(±1.7)	0.039(±0.001)	0.028	11250
dGTP	3.7(±0.7)	0.036(±0.001)	0.09(±0.002)	3500

B

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1} × 10^4)	Selectivity[a]
Dpo4				
A				
dTTP	0.79(±0.1)	0.035	4.43	1
A*				
dTTP	11.3(±1.7)	0.017(±0.001)	0.15	29.5
dATP	85.4(±13)	0.031(±0.003)	0.03	147.7
dCTP	69.2(±11)	0.038(±0.005)	0.05	88.6
dGTP	N.D.			
6A				
dTTP	8.8(±1.4)	0.11(±0.005)	1.25	1
6A*				
dTTP	32.9(±4.5)	0.073(±0.006)	0.22	5.7
dATP	41.7(±6.5)	0.023(±0.002)	0.05	25
dCTP	N.D.[b]			
dGTP	N.D.			

[a] Selectivity is defined as $(k_{cat}/K_m)_{TTP}/(k_{cat}/K_m)_{dXTP}$, where X is A, G or C. The value represents the number of correct (TTP) incorporation events for each incorrect (dXTP) incorporation.

[b] Not detectable.