Replication of DNA Templates Containing the α-Anomer of Deoxyadenosine, a Major Adenine Lesion Produced by Hydroxyl Radicals

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Received November 24, 1993; Revised Manuscript Received March 29, 1994®

ABSTRACT: The α-anomer of deoxyadenosine (α-dA) is a major adenine lesion produced by hydroxyl radicals in DNA. To assess its biochemical effects on DNA replication, α-dA was site-specifically incorporated into oligodeoxyribonucleotide templates using phosphoramidite chemistry. α-dA in the template constituted a transient block to DNA synthesis catalyzed by *Escherichia coli* DNA polymerase I Klenow fragment (Pol I), but translesional synthesis occurred after prolonged incubation. Primer extension assays and Maxam–Gilbert sequencing of newly synthesized products revealed that α-dA directed not only incorporation of the correct nucleotide, dTMP, opposite the lesion but also misincorporation of dAMP and dCMP. dGMP was barely incorporated under these conditions. The order of the incorporation frequency at the α-dA site was affected by the nearest neighbor base pair 3′ to the lesion. T7 and Taq DNA polymerases, as well as RAV-2 reverse transcriptase, showed a selectivity similar to that of Pol I with respect to the nucleotide incorporation opposite α-dA, suggesting that the discrimination of nucleotides associated with α-dA is independent of the origin of DNA polymerases and is an intrinsic feature of the lesion. The mutational spectrum predicted for α-dA (*i.e.*, A → G transitions and A → T transversions) is significantly different from those reported for other hydroxyl radical induced DNA lesions such as abasic sites or 7,8-dihydro-8-oxoguanine, both primarily directing misincorporation of A. Possible biological consequences and the mechanism of dNTP discrimination associated with α-dA are discussed.

Due to their extreme reactivity, hydroxyl radicals, generated by ionizing radiation (Teoule & Cadet, 1978; von Sonntag, 1987) or the Fenton reaction occurring in the vicinity of cellular DNA (Imlay & Linn, 1988), attack numerous target sites on DNA, leaving base and sugar damages. The biological effects of base lesions produced by hydroxyl radicals and their analogues have been extensively studied since unrepaired DNA lesions can result in cell death or mutation, and in the latter case potentially lead to tumor initiation or age-related human diseases (Ames, 1983; Ames et al., 1987; Wallace 1988; Floyd, 1990; Breimer, 1990). The molecular mechanisms of such lethal and mutagenic effects associated with hydroxyl radical induced base lesions and their analogues have been elucidated by a number of laboratories. If unrepaired, abasic sites (Kunkel et al., 1981, 1983; Kunkel, 1984; Sagher & Strauss, 1983, 1985; Lawrence et al., 1990) and thymine ring fragmentation products (Ide et al., 1991; Evans et al., 1993) constitute not only strong blocks to DNA synthesis resulting in cell death but also premutagenic lesions. Thymine glycol is also a blocking lesion in most sequence contexts (Ide et al., 1985; Rouet & Essigmann, 1985; Hayes & LeClerc, 1986; Clark & Beardsley, 1986), but its mutagenic potential is rather weak (Clark & Beardsley, 1987; Hayes et al., 1988). Interestingly, dihydrothymine, which has a structure analogous to that of thymine glycol, is neither a blocking nor a premutagenic lesion (Ide et al., 1991). 7,8-Dihydro-8-oxoguanine and 7,8-dihydro-8-oxoadenine transiently block DNA synthesis, but only the former displays a significant mutagenic potential (Wood et al., 1990, 1992; Moriya et al., 1991; Guschlbauer et al., 1991; Cheng et al., 1992; Shibutani et al., 1991, 1993).

Since hydroxyl radicals react with multiple sites in DNA generating a rather complex spectrum of DNA lesions, with the few exceptions noted above, it has been generally difficult to assess the contribution of the individual DNA lesions to biological end points measured *in vitro* and *in vivo*. However, recent developments in the chemical synthesis of oligonucleotides have made it possible to introduce unique lesions such as model abasic sites (Millican et al., 1984; Randall et al., 1987; Takeshita et al., 1987; Kamiya et al., 1992), 7,8-dihydro-8-oxopurines (Kuchino et al., 1987; Guy et al., 1988; Roelen et al., 1991), and other lesions (Hatahet et al., 1993) into oligonucleotides at desired positions to address the question of whether they have mutagenic or lethal effects.

In the present study, α-deoxyadenosine (α-dA)[1] has been site-specifically incorporated into oligodeoxyribonucleotide templates using phosphoramidite chemistry, and its interaction with DNA polymerases has been examined *in vitro*. α-dA is known to be produced by abstraction of the anomeric hydrogen atom at $C_{1'}$ by hydroxyl radicals (Mariaggi et al., 1979; von Sonntag, 1987) and is a major adenine lesion detected in DNA, poly(dA-dT), and poly(dA) γ-irradiated under anoxic conditions (Lesiak & Wheeler, 1990). Formation of α-dA is more favored in polynucleotides than in the nucleoside or nucleotide. The present results show that α-dA in template DNA constitutes a transient block to DNA synthesis, and more interestingly, it directs novel nucleotide misincorporation during DNA replication.

EXPERIMENTAL PROCEDURES

Chemicals and Enzymes. Four normal deoxyribonucleotide 5′-triphosphates (dNTPs, purities > 99.5%) and dITP (purity

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® Abstract published in *Advance ACS Abstracts,* May 15, 1994.

[1] Abbreviations: α-dA, α-deoxyadenosine; dITP, deoxyinosine 5′-triphosphate; dNTP, deoxyribonucleoside 5′-triphosphate; PAGE, polyacrylamide gel electrophoresis; Pol I, *E. coli* DNA polymerase I Klenow fragment; Pol I (exo⁻), Pol I lacking a 3′–5′ exonulease activity; RAV-2, Rous-associated virus 2.

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A

Template	1	3′-AGGGTCAGTGCTTACGAαTTTTTTTT
	2	----------Gα--------
	3	----------Cα--------
	4	----------Tα--------
	5	----------TA--------
Primer	1	5′-TCCCAGTCACGAATGCT
	2	----------C
	3	----------G
	4	----------A
	5	TCCCAGTCACGA

B

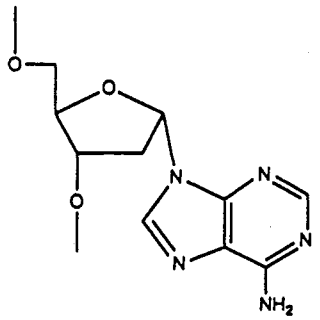


FIGURE 1: Nucleotide sequences of templates and primers used in this study (A) and the structure of α-deoxyadenosine (B). Oligonucleotides without or with modification were synthesized on an automated DNA synthesizer and purified as reported (Ide et al., 1993). The letter α in the sequences denotes α-deoxyadenosine.

96%) were purchased from Takara and Sigma, respectively. [γ-^{32}P]ATP (110 TBq/mmol) was from Amersham.

T4 polynucleotide kinase, *Escherichia coli* DNA polymerase I Klenow fragment (Pol I), Taq DNA polymerase, and Rous-associated virus 2 (RAV-2) reverse transcriptase were obtained from Takara. Pol I lacking 3′–5′ exonuclease [Pol I (exo$^-$)] and genetically engineered T7 DNA polymerase (Sequenase version 2.0) were from USB.

Oligonucleotides. Oligonucleotide templates without or with modification and primers were synthesized using phosphoramidite chemistry on a Milligen/Biosearch Cyclone Plus DNA synthesizer on a 1-μmol scale (Figure 1). The details of the synthesis, purification, and characterization of the phosphoramidite monomer of α-dA and oligonucleotides containing α-dA have been published elsewhere (Ide et al., 1993). The purified oligonucleotides were dissolved in water, and their concentrations were determined by UV measurement using the molecular absorption coefficients (ϵ_{260}) calculated by the method of Puglisi and Tinoco (1989). For convenience, α-dA was treated as a normal deoxyadenosine in the calculation.

DNA Polymerase Reaction. Oligonucleotide primers were 5′-end labeled using T4 polynucleotide kinase and [γ-^{32}P]-ATP and purified by use of a Sep-pack cartridge (Maniatis et al., 1989). An appropriate template and 5′-end-labeled primer (template:primer molar ratio = 3:1) in an annealing buffer consisting of 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, and 0.1 mM DTT were heated to 90 °C for 5 min and cooled to room temperature over a period of 1 h.

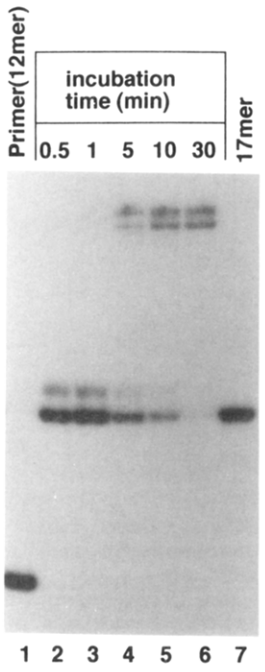
To see the overall effect of α-dA on DNA synthesis, template 4/primer 5 (0.4 pmol as the primer) in 20 μL of a reaction buffer (annealing buffer + 50 μg/mL BSA) was incubated with 0.1 unit of Pol I in the presence of four dNTPs (50 μM) at 25 °C for periods indicated in Figure 2. To determine the nucleotide incorporated opposite α-dA, 0.4 pmol of the complementary template/primer (templates 1–4 and primers 1–4) in 20 μL of the reaction buffer was incubated with a DNA polymerase for the times indicated in Figures 4–6. Unless otherwise noted, the following units of DNA polymerases, dNTP concentrations, and incubation temperatures were used: Pol I (0.1 unit, 50 μM, 25 °C), Sequenase version 2.0 (2 units, 100 μM, 25 °C), Taq DNA polymerase (1 unit, 200 μM, 70 °C), and RAV-2 reverse transcriptase (9 units, 200 μM, 37 °C). Except for Taq DNA polymerase, the composition of the reaction buffer was the same as that for Pol I. The reaction buffer for Taq DNA polymerase consisted of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, and 2 mM $MgCl_2$. The melting temperature (T_m) of a partial duplex of template 2/primer 2 determined by a temperature-dependent UV melting curve was around 68 °C in the presence of Mg^{2+} (data not shown). Thus, at least some fractions of template 2/primer 2 will retain a duplex structure at 70 °C and can serve as template/primer for Taq DNA polymerase. Polymerase reactions were terminated by adding loading buffer consisting of 95% formamide, 0.05% xylene cyanol, 0.05% bromophenol blue, and 20 mM EDTA.

Maxam–Gilbert Sequencing of Newly Synthesized Products. Template 4 containing α-dA and control template 5 were primed with primer 5 (8 pmol) and replicated by Pol I (2 units) in the presence of four dNTPs for 40 min as described above. The reaction mixture was heated at 100 °C for 5 min and quickly cooled on ice. The products were separated by use of a Sep-pack cartridge followed by 20% denaturing polyacrylamide gel electrophoresis. The bands of extended products were cut out after autoradiography, and the products were extracted from the gel by incubating with 0.5 M triethylammonium acetate and 10 mM magnesium acetate at 37 °C overnight. The extracted products were purified by use of a Sep-pack cartridge and subjected to the base-specific cleavage reactions (Maxam & Gilbert, 1980). The T-specific reaction was performed with OsO_4 (Friedman & Brown, 1978).

Gel Electrophoresis. The samples were heated to 100 °C for 5 min, loaded onto a 20% denaturing polyacrylamide gel, and electrophesed at a constant voltage of 2000 V. The gels were autoradiographed with Fuji RX films at −80 °C overnight.

RESULTS

DNA Synthesis on a Template Containing α-dA. In order to study the effect of α-dA on DNA synthesis, a 26mer template containing an α-dA at the 18th position from the 3′-end (template 4) was primed with a 12mer primer (primer 5) and replicated with Pol I in the presence of four dNTPs. Reaction products were analyzed by denaturing polyacrylamide gel electrophoresis (Figure 2). After 0.5 min of incubation, the band of original primer disappeared almost completely due to its elongation, and strong and weak termination bands attributable to pausing of DNA synthesis were observed in the middle of the gel (lane 2). By comparison with the position of a 17mer marker complementary to template 4 up to one nucleotide prior to the α-dA site (lane 7), the positions of the termination sites were assigned to one nucleotide prior to (strong band) and opposite (weak band) α-dA. After prolonged incubation (5–30 min), two extra bands appeared at the top of the gel (lanes 4–6), indicating that DNA synthesis proceeded past the α-dA site. The relative intensity of the lower top band became faint, and that of the higher one increased in further prolonged incubation. Maxam–Gilbert sequencing analysis revealed that the top two bands cor-

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Template 3'-AGGGTCAGTGCTTACGTαTTTTTTTT
Primer   5'-TCCCAGTCACGA
17mer    5'-TCCCAGTCACGAATGCA
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FIGURE 2: Polyacrylamide gel analysis of the products synthesized by Pol I on a DNA template containing α-dA. Template 4 containing a single α-dA was primed with 5′-^{32}P-labeled primer 5 (0.4 pmol) and replicated by Pol I (0.1 unit) in the presence of four dNTPs (50 μM) at 25 °C for the times indicated. The reaction was terminated by adding loading buffer, and the products were analyzed by 20% denaturing PAGE. Lane 1, primer 5 without incubation; lanes 2–6, products with incubation times of 0.5–30 min; lane 7, 17mer marker (primer 4). The sequences of the oligonucleotides used are shown at the bottom, where α denotes α-deoxyadenosine.

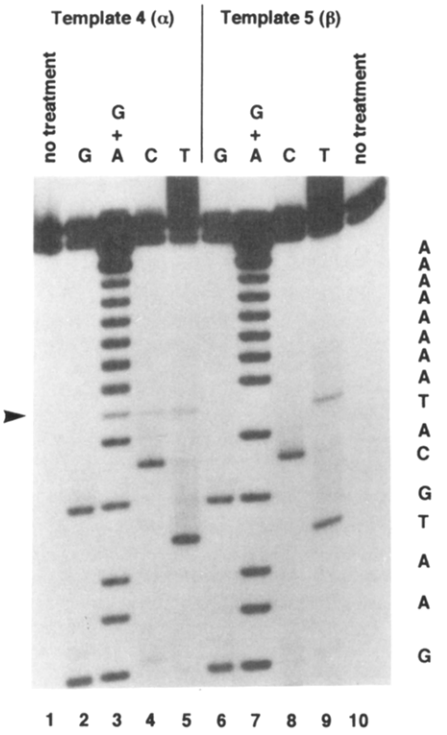

FIGURE 3: Maxam–Gilbert sequencing analysis of the nucleotide incorporated opposite α-dA. Templates 4 and 5 containing an α-dA and a normal β-dA, respectively, were primed with primer 5 and replicated by Pol I in the presence of four dNTPs (50 μM) at 25 °C as described in Experimental Procedures. Replication products (top two bands in lanes 1 and 10) purified by 20% denaturing PAGE were subjected to the base-specific cleavage reactions. Lanes 1 and 10, products from templates 4 and 5, respectively, before the base-specific cleavage reactions; lanes 2–5, products from template 4 containing α-dA after the base-specific cleavage reactions (G, G+A, C, T); lanes 6–9, products from template 5 containing β-dA at the same position. The arrowhead indicates the position of the nucleotides incorporated opposite α-dA.

responded to full-length and one-nucleotide-shorter products (see below). Comparison of the gel mobility of the Pol I reaction products with that of a chemically synthesized marker also showed that the top band corresponded to a full-length product (data not shown). When template 5 containing the normal β-anomer of deoxyadenosine instead of the α-anomer was used in the polymerase reaction, such termination bands were not observed (data not shown). Replication of template 5 was completed within 0.5 min, and only fully replicated and one-nucleotide-shorter products were observed in the product analysis (see also Figure 3, lane 10). These results show that α-dA in template DNA constitutes a transient block to DNA synthesis with the primary termination site being one nucleotide prior to α-dA. However, the block arising from α-dA was not absolute, with the arrested primer terminus being extended beyond the lesion, resulting in translesional synthesis.

Analysis of the Nucleotide Incorporated opposite α-dA. The nucleotide incorporated opposite α-dA due to translesional synthesis was analyzed by both the method of Maxam and Gilbert (1980) and the primer extension method (Moore et al., 1982).

Figure 3 shows Maxam–Gilbert sequencing gels of the bypassed products. With DNA copied from template 4 containing α-dA, bands indicating incorporation of A, C, and T opposite α-dA were present (lanes 3–5). However, no band indicating incorporation of G opposite α-dA was observed (lane 2). With control template 5 containing the normal β-anomer at the same position, only a band derived from incorporation of the complementary nucleotide T was present (lane 9). Since in the bypassed products the exact number of nucleotides added after α-dA was not clearly determined because of the considerable overlapping of original and Maxam–Gilbert bands in the top region (lane 3), we also performed more extensive base-specific reactions to trim these bands (data not shown). The number of ladders obtained after the extensive G+A reaction revealed that 7 or 8 consecutive A's were incorporated beyond the α-dA site. Thus, the two bands visible at the top of the gel were assigned as full-length and one-nucleotide-shorter products. The latter products observed for both templates 4 and 5 (lanes 1 and 10) were probably derived from premature dissociation of Pol I from the templates.

In order to confirm the incorporation of A, C, and T opposite α-dA, the primer extension assay was used (Moore et al., 1982). Here, 5′-end-labeled primers 1–4 were annealed to the complementary templates containing α-dA and extended by Pol I in the presence of a single dNTP. If the added nucleotide is incorporated opposite α-dA, it can be detected by stepping of the original primer band. Figure 4 shows the results of the primer extension assay. In the assay, the nearest neighbor base pair 3′ to the lesion was also varied to clarify its influence on nucleotide incorporation. The stepping of the original primer band occurred in the presence of dATP (panel A, lanes 1, 5, 9, and 13), dCTP (lanes 3, 7, 11, and 15), and dTTP (lanes 4, 8, 12, and 16), but not in the presence of dGTP (lanes 2, 6, 10, and 14). In the presence of dATP (lanes 1, 5, 9, and 13), additional bands were noticeable at the top that derived from translesional synthesis yielding fully replicated and one-nucleotide-shorter products. Since templates 1–4

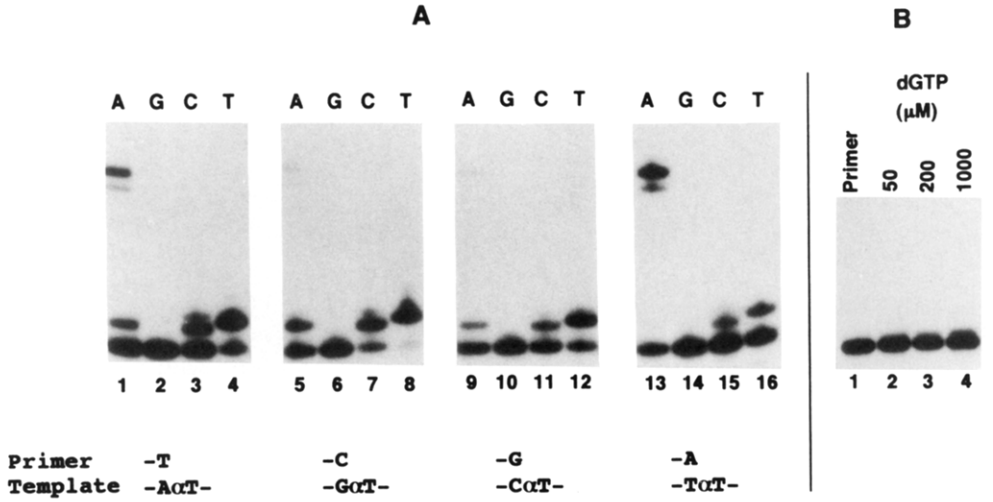

FIGURE 4: Primer extension assay to determine the nucleotide incorporated opposite α-dA by Pol I. Panel A: Primers 1–4 (0.4 pmol) which are one nucleotide shorter than the α-dA site were annealed to the complementary templates 1–4, respectively, and extended by Pol I (0.1 unit) in the presence of a single dNTP (50 μM), indicated at the top, at 25 °C for 10 min. Products were analyzed by 20% denaturing PAGE. The bottom bands correspond to the original primers. The nearest neighbor base pairs at the original primer terminus are shown below the autoradiograms. Panel B: The same sample as in lane 6 in panel A except that the incubation period was extended to 60 min and dGTP concentration was varied (50, 200, or 1000 μM) as indicated at the top.

contain a track of T's 5′ to the α-dA site, the polymerase could extend the primer terminus beyond the lesion in the presence of the complementary nucleotide dATP.

These data obtained by use of the primer extension reactions are consistent with those of the Maxam–Gilbert sequencing (Figure 3) and show that α-dA in template DNA directs misincorporation of incorrect nucleotides A and C as well as incorporation of the correct nucleotide T opposite the lesion. On the basis of the band intensities of the extended products, it appears that the frequency of nucleotide insertion opposite α-dA and the efficiency of translesional synthesis observed in the presence of dATP are dependent on the nearest neighbor base pair at the primer terminus. The order of the insertion frequency opposite α-dA was T > C ≥ A for nearest neighbors of A(template)·T(primer), G·C, and C·G, while it was A > T ≥ C for T·A. The efficiency of the translesional synthesis (lanes 1, 5, 9, and 13) decreased in the following order: T(template)·A(primer) > A·T > G·C ≥ C·G.

The primer extension reaction was also performed to check the possibility of incorporation of G opposite α-dA under the conditions where incorporation of G was favored, *i.e.*, increased dGTP concentration and incubation time (60 min). A faint band showing the incorporation of G appeared when the concentration of dGTP was 1000 μM (Figure 4, panel B, lane 4). Since substantial incorporation of the other three nucleotides (A, C, and T) occurred with 50 μM dNTP and 10 min of incubation, it is roughly estimated that the incorporation of G opposite α-dA is 2 or 3 orders of magnitude less favored than incorporation of A, C, and T.

Effect of 3′–5′ Exonuclease on Nucleotide Incorporation opposite α-dA. Primer 2 annealed to template 2 was extended by Pol I or Pol I lacking 3′–5′ exonuclease (proofreading) activity [Pol I (exo⁻)] in the presence of a single dNTP. Figure 5 shows the time course of the primer extension reaction obtained with Pol I (panel A) and Pol I (exo⁻) (panel B). The relative rate and frequency of nucleotide incorporation were essentially identical for Pol I and Pol I (exo⁻). These results suggest that excision of the nucleotide incorporated opposite α-dA by exonucleolytic proofreading plays a minor role in the nucleotide discrimination. Instead, binding of dNTP to the template/primer/polymerase complex or formation of a new phosphodiester bond after proper alignment of dNTP appears

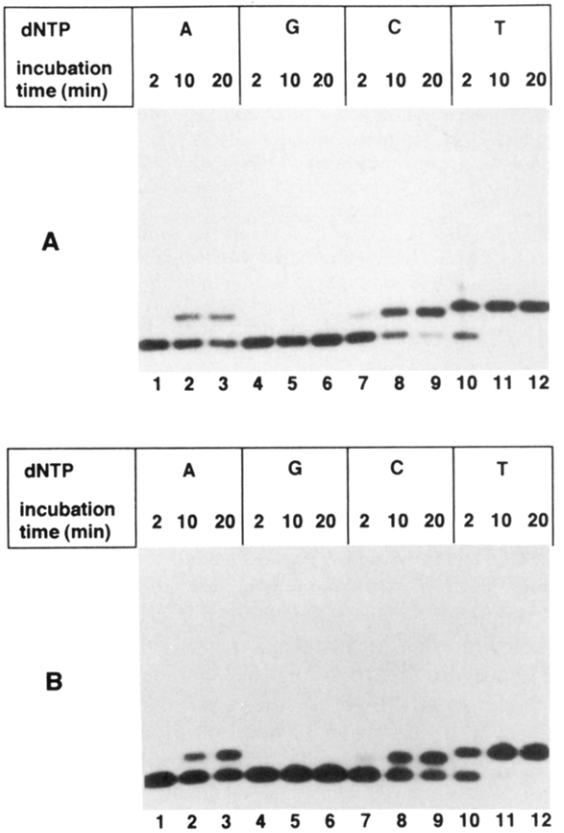

FIGURE 5: Time course of primer extension reactions with (A) Pol I and (B) Pol I (exo⁻). Template 2/primer 2 (0.4 nmol) was incubated with 0.1 unit of Pol I or Pol I (exo⁻) in the presence of a single dNTP (50 μM), indicated at the top, at 25 °C. After appropriate incubation periods as indicated, the reactions were terminated by adding loading buffer and the products were analyzed by 20% denaturing PAGE. The bottom bands correspond to original primer 2.

to be a key step for the discrimination of nucleotides at the α-dA site.

Primer Extension Assay with DNA Polymerases from Various Origins. α-dA was found to direct incorporation of A, C, or T opposite the lesion during the DNA synthesis catalyzed by Pol I or Pol I (exo⁻). However, G was barely incorporated under these conditions. To determine whether this is a universal feature associated with α-dA or a

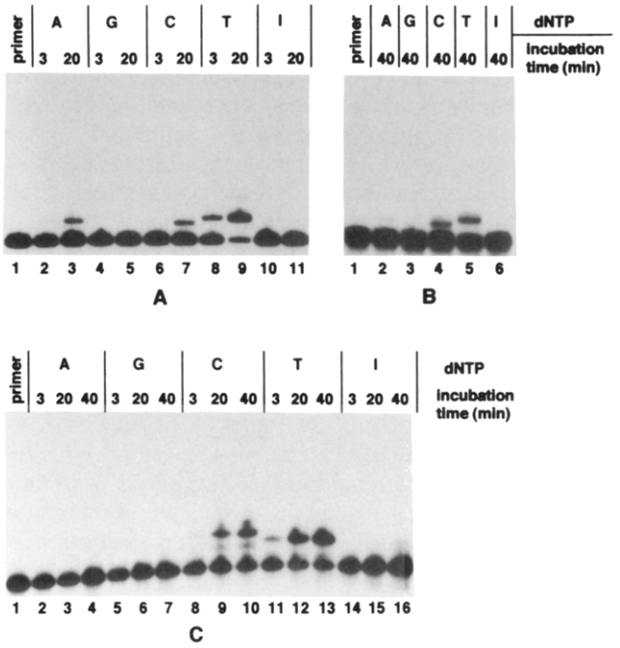

FIGURE 6: Primer extension assay to elucidate the effect of different DNA polymerases on the nucleotide incorporation opposite α-dA. Primer 2 (0.4 pmol) annealed to template 2 was extended by (A) T7 DNA polymerase (Sequenase version 2.0, 2 units), (B) Taq DNA polymerase (1 unit), or (C) RAV-2 reverse transcriptase (9 units) in the presence of a single dNTP (100 μM for panel A and 200 μM for panels B and C). The added nucleotide (A, G, C, T, or I) and incubation time are shown at the top, where I denotes deoxyinosine 5′-triphosphate. Reaction products were analyzed by 20% denaturing PAGE.

phenomenon dependent on the DNA polymerases used, the primer extension assay was performed using DNA polymerases from various origins. Figure 6 shows the results of the primer extension assay, where template 2/primer 2 was incubated with T7 DNA polymerase (Sequenase version 2.0) (panel A), Taq DNA polymerase (panel B), or RAV-2 reverse transcriptase (panel C). For all polymerases tested, the primer was extended in the presence of dATP, dCTP, and dTTP, with the frequencies of their incorporation being T > C ≥ A, but not in the presence of dGTP. It is noted that for unknown reasons RAV-2 reverse transcriptase, which has a low fidelity of DNA synthesis (Kornberg & Baker, 1992), incorporated two consecutive C's opposite and beyond the lesion (panel C, lanes 9 and 10). The data on dITP is discussed in a later section (see Discussion).

The results obtained for the DNA polymerases above are in good agreement with those for Pol I and Pol I (exo⁻) using the same template/primer (Figure 5), implying that the spectra and frequencies of nucleotides incorporated opposite α-dA are basically independent of the DNA polymerase involved in the processing of the lesion.

DISCUSSION

Biological Effects of α-dA in DNA. α-Oligonucleotides with all *N*-glycosidic bonds in an α-configuration have been designed and synthesized for regulation of gene expression by the antisense method since they exhibit nuclease resistance and yet retain the ability to base pair with complementary β-strands to form parallel strands [for a review, see Rayner et al. (1989)]. However, the potential biological effects of an isolated α-nucleoside in β-DNA have not been studied in association with DNA damages. α-dA is a major adenine lesion produced by hydroxyl radicals, and its formation is more favored in polynucleotides than in the free nucleosides or nucleotides (Lesiak & Wheeler, 1990). In the present study, α-dA has been site-specifically incorporated into DNA templates and its effect on DNA replication has been assessed *in vitro*. Although copying the template by Pol I was transiently arrested due to the lesion, translesional synthesis occurred in the subsequent incubation. In this case, either the correct or an incorrect nucleotide (*i.e.*, other than T) could be incorporated opposite α-dA. If the latter occurs *in vivo*, mutation will result. To assess the premutagenic potential of α-dA, the nucleotide incorporated opposite α-dA was determined by Maxam–Gilbert sequencing and the primer extension assay. Both analyses gave consistent results, showing that either A, C, or T is incorporated opposite α-dA, but incorporation of G is 100–1000-fold less favored than incorporation of the other nucleotides. The order of nucleotide insertion frequency at α-dA varied depending on the nearest neighbor base pair, with orders of T > C ≥ A for A(template)·T(primer), G·C, and C·G and A > T ≥ C for T·A. The insertion frequency at abasic sites by *Drosophila* Pol α has also been shown to be dependent on the nearest neighbors of these sites (Randall et al., 1987). In contrast, the spectrum of nucleotides incorporated opposite α-dA was essentially independent of the origin of the DNA polymerases used whether or not these polymerases had different 3′–5′ exonuclease activities, processivities, or nucleotide turnover numbers (Kornberg & Baker, 1992). If the correct nucleotide T is incorporated opposite α-dA during the translesional DNA synthesis in the cell, mutation will not result. However, if C or A is incorporated, A → G transitions or A → T transversions will occur. In these cases, the mutational spectrum will be potentially sequence context dependent since the nucleotide insertion frequency opposite α-dA was affected by the nearest neighbor base pair.

The predicted mutational spectrum for α-dA is significantly different from those reported for other hydroxyl radical induced DNA lesions: Abasic sites (Kunkel et al., 1981,1983; Kunkel, 1984; Sagher & Strauss, 1983, 1985; Randall et al., 1987; Takeshita et al., 1987; Lawrence et al., 1990) and thymine ring fragmentation products (Ide et al., 1991; Evans et al., 1993) direct misincorporation of purine nucleotides (primarily A), and 7,8-dihydro-8-oxoguanine directs A as well as the correct C (Wood et al., 1990, 1992; Moriya et al., 1991; Shibutani et al., 1991, 1993; Cheng et al., 1992; Moriya, 1993). Thymine glycol (Clark & Beardsley, 1987; Hayes et al., 1988) and 7,8-dihydro-8-oxoadenine (Guschlauer et al., 1991; Wood et al., 1992) are not primary premutagenic lesions, although incorporation of G opposite thymine glycol or 7,8-dihydro-8-oxoadenine at a very low frequency has been reported (Basu et al., 1989; Shibutani et al., 1993). Dihydrothymine, a lesion analogous to thymine glycol, appears to be innocuous to cells (Ide et al., 1991).

DNA lesions result in mutation if (i) they direct misincorporation of an incorrect nucleotide and subsequently (ii) the newly formed primer terminus is extended beyond the lesion by translesional synthesis yielding a fully replicated product. Concerning the second step, the primer terminus containing an α-dA·A pair formed by incorporation of A opposite α-dA was further extended by Pol I (Figure 4). We have also confirmed by a separate experiment that primer termini containing α-dA·C or α-dA·T, formed by incorporation of a single C or T opposite α-dA, could be extended (data not shown). These data further support the predicted premutagenic potential of α-dA *in vivo*. Interestingly, the data shown in Figure 4 imply that the efficiency of translesional synthesis at α-dA is nearest neighbor dependent. If this is true *in vivo*,

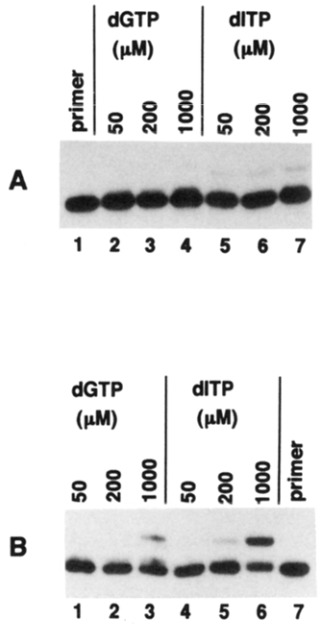

FIGURE 7: Comparison of the incorporation frequencies of dGMP and dIMP opposite α-dA by (A) Pol I and (B) T7 DNA polymerase (Sequenase version 2.0). Template 2/primer 2 (0.4 pmol) was incubated with Pol I (0.1 unit) or T7 DNA polymerase (2 units) in the presence of dGTP or dITP (concentrations indicated at the top) at 25 °C for 60 min. Reaction products were analyzed by 20% denaturing PAGE.

the biological consequences of α-dA, such as lethality and mutagenicity, need to be assessed in association with the nearest neighbor influence.

Mechanism of Nucleotide Discrimination at α-dA. α-dA directed incorporation of A, C, and T opposite the lesion, but incorporation of G was extremely unfavored. Our preliminary molecular modeling study using an energy minimization program suggests that the adenine moiety of α-dA in duplex DNA maintains an *anti* position as in the normal β-anomer and that the base moiety resides deep in the minor groove relative to the corresponding β-anomer. The NOE measurement of a free α-dA in D_2O supports the *anti* conformation (Ide et al., 1993). If G is placed opposite the α-dA in the model, the 2-amino group of the G projecting into the minor groove comes in close contact with the adenine moiety of α-dA, creating steric repulsion between the two bases. To test whether steric hindrance plays a role in the observed discrimination of G during DNA synthesis, a primer extension reaction was performed in the presence of deoxyinosine 5′-triphosphate (dITP), which lacks the corresponding 2-amino group of dGTP. Incorporation of dIMP by DNA polymerases was still less favored than incorporation of A, C, and T under standard assay concentrations of dITP [Figure 6, panel A, lanes 10 and 11 (Sequenase Version 2.0), panel B, lane 6 (Taq DNA polymerase), and panel C, lanes 14–16 (RAV-2 reverse transcriptase); Figure 7, panel A lane 5 (Pol I), and panel B, lane 4 (Sequenase Version 2.0)]. However, incorporation of dIMP by Pol I and Sequenase Version 2.0 occurred when the concentration of dITP was increased (Figure 7, panel A, lanes 5–7, and panel B, lanes 4–6). By comparing the band intensities of the extended products for dGTP and dITP (Figure 7), it is evident that the frequency of inosine incorporation opposite α-dA is several fold greater than that of G. Thus, we assume that the steric repulsion between the bases α-dA and G in the minor groove is partly responsible for the discrimination of dGTP, but that it is not an exclusive mechanism and additional factor(s) need to be considered.

In relation to the order of incorporation frequency at α-dA, based on melting temperature (T_m) measurements, we found that an oligodeoxynucleotide duplex containing an α-dA·T pair is thermally as stable as one containing a normal A·T pair at the same position, whereas those containing an α-dA·A or α-dA·C pair were less stable than one containing an α-dA·T pair, and a duplex containing an α-dA·G pair was least stable (Ide et al., unpublished results). This order parallels that of nucleotide incorporation frequency at α-dA (T > C ≥ A ≫ G) observed for the nearest neighbors of A·T, G·C, and C·T, suggesting that thermal stability of the base pairs containing α-dA plays an important role in the discrimination. However, for a full account of the nucleotide discrimination at α-dA, including nearest neighbor influences, further study is necessary.

The following should be noted in association with the response of a DNA polymerase that encounters α-dA in a template. In the Pol I reaction with template 4 containing α-dA, full-length and one-nucleotide-shorter products were observed (Figure 2, lanes 4–6, and Figure 3, lane 1). There are two ways to produce the latter product: (i) incomplete replication of the template due to premature dissociation of the polymerase from the template and (ii) generation of a one-base deletion opposite the lesion that loops out the template (Shibutani & Grollman, 1993, and references cited therein). Although the second possibility cannot be fully ruled out, we believe the first one is more likely in the present study on the basis of the following observations. (1) The one-nucleotide-shorter product was also produced with control template 5 without α-dA (Figure 3, lane 10), indicating that this product is not characteristic of the template containing α-dA. (2) If α-dA looped out the template to form a bulge upon replication, Pol I would exclusively insert A using the template base T on the 5′ side of the α-dA. After realignment of the template and primer strands, this would show up as preferential incorporation of A opposite α-dA in the product analysis. However, this was not the case in the present study. (3) Even if Pol I uses the 5′-flanking T as a template, incorporation of T giving rise to a T·T mismatch is most unfavored among possible mismatches (T·T, T·C, and T·G) (Joyce et al., 1992). But we consistently saw favored incorporation of T.

Finally, the present data suggest that α-dA produced in DNA constitutes a genotoxic lesion by inhibiting DNA synthesis or directing misincorporation of the incorrect nucleotides. Genotoxic DNA lesions are generally removed by cellular DNA repair enzymes (Friedberg, 1985; Wallace 1988; Doetsch & Cunningham, 1990). We suspect that this is also the case for α-dA, but so far no repair activity has been reported that specifically recognizes this lesion. Thus, a search for a possible cellular repair activity that restores α-dA lesions is an essential subject for future study, together with confirmation of the predicted biological effects of α-dA *in vivo*. In addition, no information is currently available on the basal and induced levels of α-dA present in cellular DNA. In light of the fact that α-dA is formed by ionizing radiation under anoxic conditions, absence of oxygen or low concentrations of oxygen appear to be crucial for the formation of α-dA. In this regard, recent arguments by Joenje (1989) and Lindahl (1993) that the cell nucleus is a very poorly oxgenated intracellular compartment are worth mentioning. One can then speculate that α-dA may be formed preferentially in nuclear DNA but not in the DNA of mitochondria, which are proficient in oxygen metabolism in eukaryotic cells.

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