Replication bypass and mutagenic effect of α-deoxyadenosine site-specifically incorporated into single-stranded vectors

Hironori Shimizu, Ryohei Yagi, Yoshiharu Kimura, Keisuke Makino, Hiroaki Terato[1], Yoshihiko Ohyama[1] and Hiroshi Ide[1],*

Department of Polymer Science and Engineering, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606, Japan and [1]Graduate Department of Gene Science, Faculty of Science, Hiroshima University, Kagamiyama, Higashi-Hiroshima 739, Japan

ABSTRACT

α-2′-Deoxyadenosine (α) is a major adenine lesion produced by γ-ray irradiation of DNA under anoxic conditions. In this study, single-stranded recombinant M13 vectors containing α were constructed and transfected into *Escherichia coli* to assess lethal and mutagenic effects of this lesion. The data for α were further compared with those obtained with M13 vectors containing normal A or a model abasic site (F) at the same site. The transfection assay revealed that α constituted a moderate block to DNA replication. The *in vivo* replication capacity to pass through α was ~20% relative to normal A, but 20-fold higher than that of F constituting an almost absolute replication block. Similar data were obtained by *in vitro* replication of oligonucleotide templates containing α or F by *E.coli* DNA polymerase I. The mutagenic consequence of replicating M13 DNA containing α was analyzed by direct DNA sequencing of progeny phage. Mutagenesis was totally targeted at the site of α introduced into the vector. Mutation was exclusively a single nucleotide deletion and no base substitutions were detected. The deletion frequency associated α was dependent on the 3′-nearest neighbor base: with the 3′-nearest neighbor base T mutation (deletion) frequency was 26%, whereas 1% with the 3′-nearest neighbor base G. A possible mechanism of the single nucleotide deletion associated with α is discussed on the basis of the misinsertion-strand slippage model.

INTRODUCTION

Cellular DNA is continuously exposed to endogenous and exogenous genotoxic agents that generate a wide variety of structural defects in DNA. These structural defects are restored in cells by multiple pathways such as base or nucleotide excision repair pathways (1). However, recent evidence shows that lesions present in DNA are not repaired at an equal rate, but those in transcribed strands in expressed genes are preferentially repaired (for review, see ref. 2). This raises the possibility that the DNA replication fork encounters DNA lesions before they are restored. In this case, DNA replication could be either aborted due to the lesions or proceed through the sites with a risk of mutation. Accumulated data indicate that the response of DNA polymerases to the encountered lesion is not unique and how they cope with it depends on the structure of the lesion, sequence contexts flanking to the lesion, enzymatic properties of DNA polymerases and accessory proteins (3,4).

We have been focusing our attention on the structural factors of DNA lesions that perturb hydrogen bonding and base stacking interactions, showing that alteration of these interactions exerts differential effects on DNA replication (5–7). In a series of these studies, we have recently shown by *in vitro* experiments that α-2′-deoxyadenosine (α) site-specifically introduced into oligonucleotide templates transiently inhibits DNA synthesis (8). The data also suggest that α is potentially mutagenic. α was originally shown to be produced in γ-irradiation of aqueous deoxyadenosine under anoxic conditions (9) and later this product was found in DNA, poly(dA·dT) and poly(dA) irradiated under similar conditions (10). α has the following unique structural feature. The N-glycosidic bond linking adenine and deoxyribose moieties is flipped due to the abstraction of the H1′ atom by OH radicals, but the base moiety is totally intact. Although basal and induced levels of α in cellular DNA are not known, several lines of evidence imply that this lesion, in particular, or lesions with the α configuration with respect to the N-glycosidic bond might, in general, have some biological relevance. The cell nucleus is a very poorly oxygenated intracellular compartment (11,12), and cell hypoxia is ubiquitously observed in tumor cells and anaerobe. In addition, *Escherichia coli* and yeast cells have repair enzymes such as endonuclease IV (13) and Apn (Ide *et al.*, unpublished data) that recognize this lesion. The nucleoside of 5-formyluracil, resulting from oxidative damage of thymine, readily undergoes base-catalyzed anomerization with respect to the N-glycosidic bond (14).

Molecular mechanics and thermodynamic studies on the duplex DNA containing this lesion have revealed that α paired

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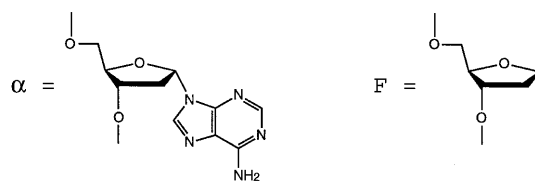| abbreviation	nucleotide sequence
17A	5'-AGCATTC*G*AGACTGGGT
17Gα	5'-AGCATTC*G*αGACTGGGT
17Tα	5'-AGCATTC*G*αTACTGGGT
17F	5'-AGCATTC*G*FGACTGGGT
57scaffold(+)	3'-TAAGCTCGAGCCATGGGCCCTCGTAAG TTCTGACCCACTAGGAGATCTCAGCTGGAC
57scaffold(-)	5'-ATTCGAGCTCGGTACCCGGGAGCATTCAAGACTGGGTGATCCTCTAGAGTCGACCTG
32tempA	5'-AGCATTCG*A*GACTGGGTGATCCTCTAGAGTCG
32tempGα	5'-AGCATTCG*α*GACTGGGTGATCCTCTAGAGTCG
32tempTα	5'-AGCATTCG*α*TACTGGGTGATCCTCTAGAGTCG
32tempF	5'-AGCATTCG*F*GACTGGGTGATCCTCTAGAGTCG
15primer	3'-CTAGGAGATCTCAGC
15linker	5'-GATCCTCTAGAGTCG
16BamHI(+)	5'-CTAGAGGATCCCCGGG
16BamHI(-)	3'-GATCTCCTAGGGGCCC
17primer	3'-AGCACTGACCCTTTTGG

Figure 1. Sequences and abbreviations of oligonucleotides used in this study. α, α-2'-deoxyadenosine; F, a model abasic site (tetrahydrofuran). DNA lesions introduced into oligonucleotides are underlined. The mismatch (G:T) introduced between the 17mer inserts and 57scaffold(+) as a genetic marker is indicated by italic letters.

with pyrimidines generates little distortions in DNA, whereas purines introduce distinct kinks and budges (15). These studies also suggest that the space created in the major groove by the flipped base is key to the specific recognition by endonuclease IV, a repair enzyme from *E.coli* (13).

In this study, we extended our investigations on α to an *in vivo* system. Single-stranded M13 vectors containing α at the defined position were constructed and transfected into *E.coli*. Biological consequences such as lethal and mutagenic effects resulting from this lesion were assessed based on the plaque forming efficiency of transfecting DNA and direct DNA sequencing of progeny phage. The data were further compared with those obtained in parallel experiments for normal A and a model abasic site (F) introduced into the same site of M13 vectors.

MATERIALS AND METHODS

Materials

Four 2'-deoxyribonucleoside 5'-triphosphates (dNTPs, purity >99.5%) and ATP (purity >95%) were purchased from Takara and Boehringer Mannheim, respectively. [α-^{32}P]dATP (110 TBq/mmol) and [γ-^{32}P]ATP (220 TBq/mmol) were from Amersham. *E.coli* DNA polymerase I Klenow fragment (Pol I), T4 DNA ligase and a restriction enzyme *Bam*HI were obtained from New England Biolabs. T4 polynucleotide kinase was from Toyobo. Oligonucleotides used in this study are listed in Figure 1. Oligonucleotides except 32temps A, Gα, Tα and F were synthesized by the standard phosphoramidite chemistry and extensively purified by reversed phase HPLC and polyacrylamide gel electrophoresis if necessary, and desalted as described (16,17). Characterization of DNA lesions (α or F) introduced into oligonucleotides has been

published previously (13). 32mer templates (32tempA, Gα, Tα and F) were constructed by ligation of an appropriate 17mer (17A, 17Gα, 17Tα or 17F) and a 5'-phosphorylated 15mer (15linker) using T4 DNA ligase and 57scaffold(+) (18). The constructed 32mer templates were purified by reversed phase HPLC and desalted (17). Concentrations of oligonucleotides were determined based on UV absorption at 260 nm and calculated molecular absorption coefficients (19).

Construction of single-stranded M13 vectors containing α or F

Single-stranded M13 vectors containing a unique lesion were constructed basically following the reported methods (18,20,21). Single-stranded closed circular M13mp18 DNA was first linearized. M13mp18 DNA (40 µg) and 16*Bam*HI(+) oligonucleotide (108 pmol, ~6-fold excess over M13 DNA), which is complementary to the franking regions of a unique *Bam*HI site present in the multiple cloning site, were heated in 100 µl 150 mM NaCl, 10 mM Tris–HCl (pH 7.9), 10 mM MgCl₂, 1 mM DTT (total volume 100 µl) at 70°C for 5 min and slowly cooled to room temperature. The mixture was incubated with 100 U *Bam*HI at 37°C for 1 h, then 100 U *Bam*HI was further added to the mixture and incubation was continued for another hour. The reaction was terminated by adding EDTA (final concentration 50 mM). Completion of the restriction digestion was confirmed by 1% agarose gel electrophoresis. To remove 16*Bam*HI(+) and digested oligonucleotides, the reaction mixture was subjected to alkaline gel permeation chromatography on a Sephadex G-100 (22). Fractions containing M13 DNA were determined by ethidium bromide staining, neutralized and DNA precipitated by ethanol. Linearized M13 DNA was stored in 10 mM Tris–HCl (pH 7.5).

Recombinant M13 DNA containing a 17mer insert was constructed as follows. Linearized M13 DNA (10 µg), a 5′-phosphorylated 17mer (17A, 17Gα, 17Tα, 17F, 13.6 pmol, 3-fold excess over M13 DNA), 57scaffold(+) (13.6 pmol) and 16*Bam*HI(–) (22.7 pmol) in 500 µl 50 mM Tris–HCl (pH 7.8), 10 mM MgCl₂, 1 mM DTT, 1 mM ATP and 50 µg/ml BSA were heated at 75°C for 15 min and slowly cooled to room temperature. 16*Bam*HI(–) complementary to 16*Bam*HI(+) was included in the reaction mixture to avoid annealing of trace amounts of remaining 16*Bam*HI(+) to linearized M13 DNA, which would generate original circular M13 DNA without any insert by subsequent ligation. Ligation reaction mixture was incubated with T4 DNA ligase (12000 U) at 14°C for 12 h. DNA was precipitated by ethanol, resuspended in 10 mM Tris–HCl (pH 7.5) with a final concentration 200 ng/µl, and used for transfection. An aliquot of the sample was analyzed by 1% agarose gel electrophoresis. Gel was stained by ethidium bromide, photographed and ligation efficiency and concentration of DNA were determined by densitometric scanning of the picture (see below). Recombinant M13 vectors containing 17mer inserts (17A, 17Gα, 17Tα or 17F) are designated as M1317A, M1317Gα, M1317Tα and M1317F, respectively.

Transfection assay

Recombinant M13 vector (1 µg) and 57scaffold(–) (6.8 pmol) in 6 µl Tris–HCl (pH 7.5) were heated at 90°C for 3 min and quickly cooled on ice. DNA sequencing analysis of progeny phage revealed that, without the oligonucleotide complementary to the 57scaffold(+), a significant portion of progeny phage obtained by transfection contained a 17mer insert derived from 57scaffold(+). This could be readily identified by a G:T mismatch specifically introduced at the 5′ side of the lesion as a genetic marker [see the sequences of the 17mer inserts and 57scaffold(+) in Fig. 1]. Presumably the 17mer gap region in the bridging scaffold was filled *in vivo* and DNA containing this insert was replicated. The progeny phage containing the 17mer insert from 57scaffold(+) was completely eliminated by the addition of complementary 57scaffold(–) in the denaturing step before transfection. Electro-competent JM101 (Δ*lacpro, supE, thi, F′traD36, proAB, laqI-ᵠZΔM15*) prepared by following the instruction of BioRad (0.4 ml) and a recombinant M13 vector (200 ng) were gently mixed and transferred into an electroporation cuvette with a 0.1 cm electrode gap. Electroporation was performed with a time constant ~4.5 µs and at 1.8 kV using a BioRad *E.coli* pulser. After appropriate dilution, the electroporation mixture was mixed with an 1 ml SOC media, 10 µl 100 mM IPTG, 50 µl 2% X-gal, 0.2 ml freshly cultured host JM101 and 3 ml 2× YT top agar, and plated on an L-Broth plate. The plate was incubated overnight at 37°C. Colorless plaques were counted to measure *in vivo* DNA replication capacity to pass through the introduced lesions.

Mutation analysis

For each lesion, progeny phage were randomly isolated from total populations of colorless plaques obtained from four independent transfections. Nucleotide sequences were determined by the dideoxy method (23) using the 17primer, complementary to positions 25–41 downstream of the *Hin*dIII site, and a Sequenase version 2.0 DNA sequencing kit (USB).

In vitro DNA polymerase reaction

5′-³²P labeled 15primer (0.2 pmol) and an appropriate 32temp (1.5 pmol, template excess to ensure complete annealing of the primer) were heated in 10 µl 20 mM Tris–HCl (pH 7.5) and 50 mM NaCl, and slowly cooled to room temperature. Pol I reaction buffer (final composition 10 mM Tris–HCl pH 7.5, 5 mM MgCl₂, 7.5 mM DTT) and four dNTPs (final concentrations 50 µM) were added to the reaction mixture (final volume 19 µl) and preincubated 5 min at 37°C. The replication reaction was initiated by adding 0.1 U Pol I (1 µl) and incubation was continued at 37°C for 5–30 min. The reaction was terminated by the addition of loading buffer consisting of 98% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol and 20 mM EDTA. Reaction products were analyzed by 16% polyacrylamide gel electrophoresis under denaturing conditions (8 M urea). After electrophoresis, the gel was dried and subjected to autoradiography using a Fuji RX film with varying exposure times to ensure linear correlation between radioactivity and band intensity in the autoradiogram. Reaction products obtained with 32tempGα and Tα were isolated from gels as described (8), and full length (32mer) and one nucleotide shorter (31mer) products were separately subjected to Maxam–Gilbert sequencing reactions (24).

Densitometry

Images of autoradiograms and agarose gel pictures were recorded on an Epson GT6000 scanner using the Color Magician III software. Intensity of bands in the recorded images was analyzed by the NIH Image (ver 1.55) using a built-in gel scan macro.

RESULTS

In vitro replication bypass of α

In the previous study, we have shown that α constitutes a transient block to DNA replication catalyzed by Pol I *in vitro* (8). However, *in vitro* replication efficiency of Pol I to pass through this lesion has not been assessed on the quantitative basis. Thus, time course of the replication of oligonucleotide templates containing normal A, α and a model abasic site (F) incorporated at the same position were followed and the relative replication efficiencies were compared. For this purpose, 32temps were primed by 15primer (Fig. 1) and replicated by Pol I as described in Materials and Methods. Reaction products were analyzed by 16% denaturing polyacrylamide gel electrophoresis and the relative band intensities of the reaction products were determined by densitometry of autoradiograms. The position of the band was determined by comparison with standard dideoxy sequencing ladders prepared using an M1317A DNA template containing the same sequence as 32tempA.

Figure 2 shows a typical autoradiogram obtained by 30 min incubation. Replication of the template was completely arrested at F (strong band) or one nucleotide prior to the lesion (weak band) so that bypassed products were not detected within the present detection limit (Fig. 2, lane 6). In contrast to F, significant fractions of the template containing α were replicated to full length (32mer) or one nucleotide shorter products (31mer) at the same incubation time (Fig. 2, lanes 7 and 8). Both full length and one nucleotide shorter products were also produced in the replication of 32tempA containing no lesions (Fig. 2, lane 5). Interestingly, the band intensity of a stalled product appearing one nucleotide prior to α was different for 32tempGα and Tα, indicating a nearest neighbor effect on the replication capacity of

Pol I to pass through α. In Maxam–Gilbert sequencing analysis of the reaction products obtained with 32tempGα and Tα, bands implying incorporation of dTMP, dCMP and dAMP opposite α were present for both full length and one nucleotide shorter products (data not shown). Similar results were obtained in the previous study (8). Thus, one nucleotide shorter products commonly observed for 32tempA, Gα and Tα are likely generated by premature dissociation of Pol I at the end of DNA templates. Figure 3 shows the time course of the replication of 32temps determined by the densitometry. Whether or not the template contained a lesion, the original primer was converted to extended products within 5–10 min, suggesting that initial extension of the primer is independent of the lesions and occurs at similar rates. Replication of control template 32tempA completed in 10 min (Fig. 3A). According to the time course plots of the stalled intermediates, extension of the intermediates for 32tempGα was more efficient than 32tempTα (Fig. 3C and D). For the former, only 4% of the stalled intermediate remained at 20 min, while for the latter, 77% was left unextended at the same incubation time. The stalled products at F were extremely resistant to subsequent elongation by Pol I (Fig. 3B), which is consistent with published data (25).

In vivo replication bypass of α

To evaluate the *in vivo* replication efficiency to pass through lesions, recombinant M13 vectors containing α, F or normal A at the same position were constructed and transfected into JM101 cells. The recombinant M13 vectors contain 17mer inserts in the *Bam*HI site of the multicloning site so that progeny phages resulting from the vectors form colorless plaques due to the (3*n* + 2) frameshift in the lacZ(α) gene. Therefore, progeny phages derived from the recombinant and parental M13 vectors are readily distinguished by the color of plaques, the latter of which forms blue plaques in the presence of X-gal and IPTG. In addition, the potential contamination of colorless plaques derived from replication of the 17mer gap region of the scaffold was ruled out by the single base mismatch serving as a genetic marker (see Materials and Methods).

Table 1. Replication efficiency of recombinant M13 vectors containing a single lesion α or F[a]

Experiment No.	Plaque forming efficiency (%) relative to control 17A			
	17A[b]	17Gα[b]	17Tα[b]	17F[b]
1	100	10.0	10.0	1.4
2	100	22.6	18.9	2.2
3	100	36.7	18.1	0.8
4	100	20.8	16.7	0.9
Average (±SD)[c]	100	22.5 (±11.0)	15.9 (±4.0)	1.3 (±0.6)

[a]Average numbers of colorless plaques per 1 μg of closed circular recombinant M13 DNA were 8900 (17A), 1600 (17Gα), 1400 (Tα) and 130 (F). Blue plaques were excluded from data since they did not contain the 17mer insert. Blue plaques consisted of <2% of total plaques obtained with control M1317A.
[b]17mer inserts introduced into the *Bam*HI site in M13 vector.
[c]Average of four experiments with a standard deviation in the parenthesis.
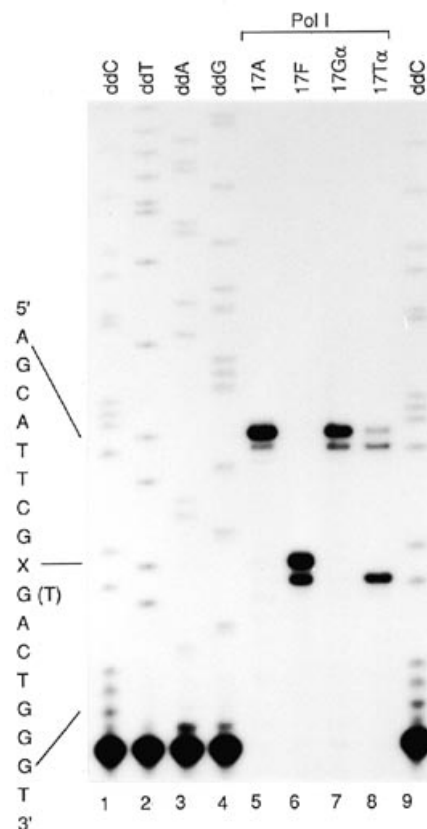

Figure 2. *In vitro* replication of oligonucleotide templates containing α, F and control A by Pol I. 32mer templates [32tempA (lane 5), 32tempF (lane 6), 32tempGα (lane 7) and 32tempTα (lane 8), 0.2 pmol each] were primed with 5′-³²P labeled 15primer and replicated by 0.1 U *E.coli* DNA polymerase I Klenow fragment at 37°C for 30 min as described in Materials and Methods. Standard sequencing ladders [ddC (lanes 1 and 9), ddT (lane 2), ddA (lane 3) and ddG (lane 4)] were generated using 15primer and M1317A phage DNA containing the same sequence as 32tempA. Products were separated by 16% denaturing polyacrylamide gel electrophoresis. A part of the sequence of 32mer templates is shown on the left, where X denotes α, F or A, and (T) indicates the sequence for 32tempTα.

Table 1 shows the replication efficiencies of recombinant M13 vectors containing α, F or normal A. The data are based on the number of colorless plaques. Typically, blue plaques excluded from the data consisted of <2% of total plaques obtained by the transfection of M1317A. M1317Gα and M1317Tα containing α were replicated with 22.5 and 15.9% efficiencies, respectively, relative to control M1317A. These transfection data indicate that α allows appreciable translesion bypass when DNA templates containing this lesion are replicated in *E.coli*. In contrast, M1317F containing a model abasic site was replicated with much lower efficiency (1.3%) than M1317Gα and M1317Tα. The replication efficiency obtained for M1317F is in good agreement with those (0.3–0.6%) for single-stranded recombinant M13 DNA containing a single natural abasic site (21).

Mutagenic effects of α

The mutagenic consequences of replicating M13 DNA containing α were examined by sequencing total 201 progeny phage DNA. In control experiments, M13 phage DNA containing a
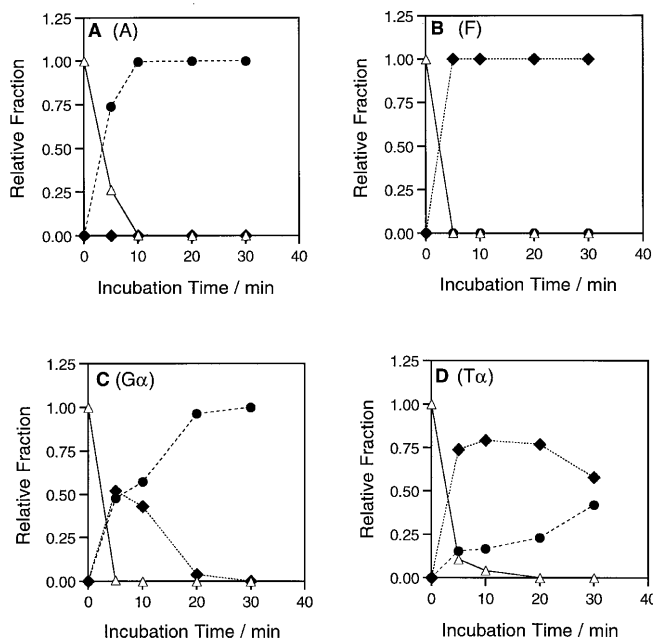
Figure 3. *In vitro* replication bypass of α and F by Pol I. 32mer templates (0.2 pmol) containing control A (32tempA, **A**), a model abasic site (32tempF, **B**) or α (32tempGα, **C**; 32tempTα, **D**) were primed with 5′-³²P labeled 15primer and replicated by 0.1 U *E.coli* DNA polymerase I Klenow fragment at 37°C for the periods indicated in the figure. Products were separated by 16% denaturing polyacrylamide gel electrophoresis. Band intensities of autoradiograms were analyzed by densitometric scanning as described in Materials and Methods. Relative amounts of products were calculated as a fraction of total band intensities and plotted as a function of incubation time. △, original primer (15primer); ◆, products arrested by lesions; ●, bypass products including full length and one nucleotide shorter products.

model abasic site (F) or normal A was also transfected and progeny phage DNA was sequenced. The mutagenic spectra and frequency associated with abasic sites have been extensively studied using transfecting DNA (for example, 21,26–28), thereby providing an appropriate standard in comparing mutagenic effects of α and other damages. Nucleotide sequences of all isolated phages were analyzed for 30 nucleotides (nt) on the 3′ side of the 17mer insert and ≥50 nt on the 5′ side. Sequence analysis showed that except for the lesion site, the nucleotide sequence of all isolated phage was normal. In addition, none of DNA randomly isolated from blue plaques contained the 17mer insert and all had the same sequence of original M13mp18 (data not shown). Potential –2 or +1 frameshift mutation that restores the reading frame and gives rise to blue plaques and extensive deletions that could occur during the construction of vectors and give rise to colorless plaques were practically eliminated by these data.

Table 2 summarizes the mutation spectra and frequency determined for recombinant M13 vectors. Out of 100 progeny phages derived from M1317Gα, where the lesion α is flanked by G on the 3′ side, 99% contained A at the site of α. The rest (1%) was a single nucleotide deletion at the lesion. However, with M1317Tα, where α is flanked by 3′ T, the deletion frequency markedly increased up to 26% in total 101 phage sequenced. The rest (74%) contained A at α. In both vectors containing α, the deletion was totally targeted at the lesion and no base substitutions

such as α→G transitions or α→T, α→C transversions were detected. These results indicate that template α primarily directs incorporation of T *in vivo*, which does not result in mutation since α is derived from A by the anomerization of the N-glycosidic bond. However, a single nucleotide deletion becomes a major consequence depending on the 3′ nearest neighbor base.

Table 2. Numbers and spectrum of mutations derived from replication of DNA lesions (X = α, F or A) site-specifically introduced into M13 vectors[a]

	Lesion (X)			
Mutation	A	Gα[b]	Tα[b]	F[b]
X→A	20 (100%)	99 (99%)	75 (74%)	11 (39%)[c]
X→T	0	0	0	13 (46%)
X→C	0	0	0	1 (4%)
X→G	0	0	0	0
1 deletion	0	1 (1%)	26 (26%)	3 (11%)
total[d]	20	100	101	28

[a]Numbers in the parentheses indicate percentage of the total number of sequences analyzed.
[b]Gα:α with the 3′ nearest neighbor base G; Tα:α with the 3′ nearest neighbor base T; F:abasic site.
[c]Two of them were tandem mutation (3′-GF→3′-AA).
[d]Total number of plaque sequenced.

In vivo replication of M1317F containing a model abasic site resulted in the nucleotide insertion (89%) or a single nucleotide deletion (11%). The nucleotide insertion or deletion was targeted at the lesion except for two cases, which were tandem changes at the sites of the lesion and the immediately flanking 3′ nucleotide. The progeny phage DNA contained the following nucleotides at the lesion: T (46%) > A (39%) > C (4%) > G (0%), indicating that nucleotides were incorporated opposite template F in the following order of the frequency: A > T > G > C. The preferential incorporation of A at non-instructive lesions such as abasic sites, called A-rule, has been well established *in vitro* (25,29,30), and *E.coli* (21,26,27) although there are exceptions in eukaryotic cells (28,31,32). Furthermore, the present results on the relative order of nucleotide insertion frequencies as well as the nucleotide deletion frequency agree fairly well with the those obtained by the transfection of M13 DNA containing natural abasic sites (21,27).

DISCUSSION

In this study, single-stranded recombinant M13 vectors containing an α lesion at the defined position were constructed and transfected into *E.coli* to assess *in vivo* replication capacity to pass through this lesion and its mutagenic potential. The data obtained for α were further compared with those obtained with M13 vectors containing normal A or a model abasic site (F) at the same site. Biological consequences associated with DNA lesions are generally combined outcome of DNA replication and repair (1). However, the latter contribution is virtually eliminated (or at least minimized) in this study since α and F lesions incorporated in single-stranded DNA are poor substrates of *E.coli* endonuclease IV and exonuclease III that are responsible for the repair of these lesions (13,33,34). Thus, lethal and mutagenic effects of the introduced lesions in this study are primarily correlated with the events occurring in DNA replication.

The transfection data show that α constitutes a moderate block to DNA replication *in vivo* (Table 1). The *in vivo* replication capacity of α was ~20% relative to normal A, but 20-fold higher than that of the abasic site that was an almost absolute replication block. These data are consistent with the time course study obtained by *in vitro* replication of templates containing α or F (Fig. 3). The *in vivo* replication efficiency for α is comparable to those obtained for etheno-deoxycytidine (35,36), etheno-deoxyadenosine (35) and a *cis*-diamminedichloroplatinum adduct of guanine (37) using uninduced *E.coli* hosts. These lesions including α maintain base ring structures, hence retaining potential base stacking interactions. Interestingly, the efficiency of replication bypass of α was higher when this lesion is flanked by the nearest neighbor base G than T at the 3′ position both *in vitro* and *in vivo*, although *in vivo* data are not totally conclusive because of the small difference in the efficiencies and relatively large standard deviations.

The mutagenic consequences of replicating M13 DNA containing α were analyzed by direct DNA sequencing of progeny phage (Table 2). The mutagenesis was totally targeted at the site of α introduced into the vector. Mutation was exclusively a single nucleotide deletion and no base substitutions were detected. This result is rather interesting and suggests some differences in the responses of *E.coli* DNA polymerases I (Pol I) and III holoenzyme (Pol III). Pol I incorporated C and A in addition to T opposite α although the frequencies for C and A were lower than that of T (8). However, base substitution mutations resulting from incorporation of C or A opposite α were not observed in the transfection of M13 vectors containing α, where the replicating enzyme was Pol III. Differential processing of template lesions by Pol I and Pol III has been implicated in transfection studies (36,38), but the molecular mechanism of the differential processing remains to be clarified. The frequency of the deletion was dependent on the 3′ nearest neighbor base of α. With the 3′ nearest neighbor base T, mutation frequency was 26%, whereas 1% with the 3′ nearest neighbor base G. These data suggest that although α primarily directs incorporation of the correct nucleotide T during DNA replication *in vivo*, a single nucleotide deletion will be a significant event depending on the flanking sequences. It is also noted that the mutation spectrum for α is unique. Several DNA lesions site-specifically introduced into vectors are known to generate a single nucleotide deletion *in vivo* (21,35,39–41). However, unlike α in this study, deletions account for a minor part of the mutation spectra (for an exception, see ref. 42).

The mechanism of the single nucleotide deletion by α is likely to be explained by the 'misinsertion-strand slippage model' (Fig. 4) (43–47) proposed based on the original model for undamaged DNA (48). In a previous primer extension assay using Pol I, we have shown that nucleotide insertion frequency opposite α was T > C ≥ A >> G (8). We have also shown by the molecular modeling that pyrimidine nucleotides (T and C) placed opposite α fit into B form DNA without introducing any significant distortion, but purine nucleotides (A and G) produce distinct distortions due to the steric hindrance between α and purine bases (15). In addition, an α:T pair forms two stable hydrogen bondings, one of which is not canonical and is locating in the minor groove. T_m measurements have also revealed that thermodynamic stability of 9mer duplexes containing a central α:N base pair (N = A, G, C, T) is α:T > α:C ≈ α:A > α:G (15). The duplex containing an α:T pair is as stable as that contains a normal A:T pair at the same site. In light of these structural and thermodynamic data, it is likely that
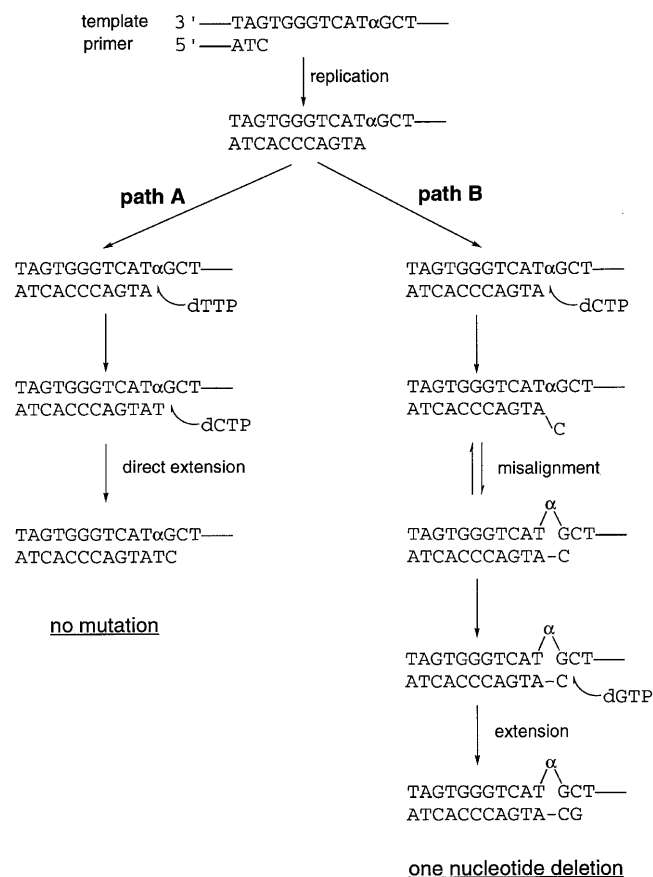

Figure 4. A possible mechanism for a single nucleotide deletion at α.

when encounters a template α lesion, DNA polymerase III holoenzyme (Pol III) incorporates T most frequently opposite template α, followed by C. When T is incorporated opposite α, the resulting primer terminus mimics canonical one in geometry and stability as described above. Thus, this will allow Pol III for subsequent polymerization, but no mutations result in this case (Fig. 4, path A). On the contrary, incorporation of C opposite α will produce a breathing primer terminus since C fits in B geometry but can not form proper hydrogen bondings with α (11). Accordingly, while Pol III is stalling at this site, transient misalignment between C at the primer terminus and template G located at 5′ to α occurs, and extension of the transiently misaligned primer terminus fixes a single nucleotide deletion (Fig. 4, path B). Such a mechanism involving misinsertion followed by misalignment of template–primer has been proposed to explain frame shifts observed in iterated nucleotide sequences (44,48) and a single nucleotide deletion at DNA lesions such as abasic sites (46,49) or other DNA adducts (45–47).

Compared with the role of the 5′ nearest neighbor nucleotide of α in translesion bypass and deletion frequency, that of the 3′ nucleotide is not clear. Napolitano *et al.* recently showed that the frequency of a single nucleotide deletion induced by an N-acetyl-aminofluorene (AFF) adduct of guanine is affected by the 3′ flanking nucleotide of the lesion (47). Our results indicate that 3′ nearest neighbor G that permits relatively efficient translesion bypass results in low deletion frequency (1%). In contrast, 3′

nearest neighbor T inhibiting translesion bypass generates a high level of the deletion (27%). These data imply that stalling time of Pol III at the lesion is key to generation of the deletion. As discussed above, if Pol III stalls at the lesion long enough, this may permit transient misalignment of the template–primer, thereby generating a deletion (46).

In summary, it has been shown that α constitutes a moderate replication block both *in vitro* and *in vivo*, and generates a single nucleotide deletion exclusively *in vivo*. This mutation spectrum is characteristic of α. The flanking sequence of α affects the deletion frequency and bypass efficiency. The mechanism of the deletion associated with α is best explained by the misinsertion-strand slippage model (43–48), which is further supported by molecular mechanics and thermodynamic data (15).

ACKNOWLEDGEMENT

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