Mechanism of mutation on DNA templates containing synthetic abasic sites: study with a double strand vector

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ABSTRACT

Mutagenesis at abasic sites was investigated in *E.coli* and simian kidney (COS) cells using a duplex shuttle vector containing synthetic analogs of deoxyribose on the phosphodiester backbone. Lesions were positioned on opposite strands of the vector. When the tetrahydrofuranyl analog was used as the abasic site, AT or TA pairs (65–80%) were introduced at the site of the bistrand lesion. Mutagenesis occurred in the absence of SOS induction. Single base deletions (>80%) dominated the mutational spectra for propenyl and ethenyl analogs of abasic sites lacking a ring structure. For all abasic site analogs, a small proportion of G/C and C/G pairs (6–10%) were observed. dAMP was incorporated predominantly opposite tetrahydrofuranyl sites positioned in the single strand region of a gapped duplex vector. We conclude from these studies that abasic sites positioned in a bistrand configuration are highly mutagenic in *E.coli* and COS cells. Repair DNA synthesis may be involved in this process.

INTRODUCTION

In DNA containing abasic sites (1,2), the deoxyribose moiety of the abasic site is in equilibrium with the open chain aldehyde form of the lesion and exists primarily as a cyclic hemiacetal structure (3,4). Natural abasic sites in DNA are subject to β -elimination, a reaction that leads to strand scission. To avoid this complication, 3-(hydroxy-2-hydroxymethyl)-tetrahydrofuran (F), a cyclic structure that is intrinsically resistant to β -elimination, has been used as a deoxyribose analog for studies of the structural (5–8), thermodynamic (9), and biological (10,11) properties of oligodeoxynucleotides containing abasic sites. Similarly, a propenyl (P) or ethenyl (E) moiety can be incorporated into the phosphodiester backbone to create anucleosidic sites in DNA (9,10). The structure of these synthetic abasic sites is shown in Fig. 1.

Abasic sites introduced into the template strand of duplex DNA act as blocks to DNA polymerase (10,12–14). In *E.coli*, translesional synthesis is facilitated by inducing the SOS response (17,18); during this reaction, dNMPs are incorporated opposite the lesion (15,16). *In vitro*, preferential incorporation of dAMP

at abasic sites was demonstrated in reactions catalyzed by prokaryotic and eukaryotic DNA polymerases (10,12–15,17–19).

Recently, Sarasin and his colleagues reported experiments in which shuttle plasmid vectors containing natural abasic sites were allowed to replicate in simian kidney (COS) cells (20,21). Using single strand (20) or double strand (21,22) vectors, insertion of dNMPs opposite the lesion was relatively non-specific. In experiments using an Epstein–Barr virus-based vector and human cells, Drinkwater *et al.* (22) observed essentially random incorporation of dNMPs opposite abasic sites. If this is correct, the mutagenic properties of abasic sites introduced into mammalian cells would appear to differ from those reported for bacteria.

Bleomycin and neocarzinostatin generate bistrand lesions in DNA (23–24). An abasic site and a single strand nick are created on opposite strands of duplex DNA. Mutagenesis associated with these radiomimetic agents has been attributed to the abasic moiety in the bistrand lesion (25–26).

Experiments reported here were designed to examine mechanisms of abasic site mutagenesis. Single abasic sites in DNA were rapidly repaired in *E.coli* and in COS cells while bistrand lesions were highly mutagenic. Two primary molecular events were observed, reflecting dNTP incorporation opposite an abasic site and deletion at the site of the opposing lesion. In bacterial and mammalian systems, preferential incorporation of dAMP on DNA templates occurs opposite abasic sites containing cyclic structures while deletions predominate at anucleosidic sites. The results of our studies in mammalian cells differ from those reported by other investigators (20–22).

MATERIALS AND METHODS

Plasmids and host cells

Construction of shuttle vectors pBS(–)XME (27) and pGM86 (28) has been described. *E.coli* AB1157 was provided by B.J. Bachman (Yale University). Competent cells were prepared by a modification of the method of Hanahan, as described previously (28). NM522 and DH5 competent cells were purchased from Stratagene. The COS7 simian kidney cell line was developed by Gluzman (29).

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Figure 1. Structures of natural and synthetic abasic sites. N: deoxyribofuranosyl; F: 2-methyl-tetrahydrofuran; P: 1,3-propanyl; E: 1,2-ethanyl.

Figure 2. Strategy for site-specific mutagenesis studies. Phosphorylated oligodeoxynucleotides containing single or bistrand lesions are ligated to the vector as shown then used as transforming DNA. Following transformation, mutations in progeny plasmids are determined by colony hybridization and DNA sequence analysis. X represents the abasic site.

Synthesis and purification of oligodeoxynucleotides

Modified oligodeoxynucleotides were prepared on a Dupont Coder 300 automated DNA synthesizer (10) and purified on a reverse phase C_{18} μ Bondapak column (0.39×30 cm), eluted over 60 min at a flow rate of 1.0 ml/min with a linear gradient of 0.05 triethylamine acetate, pH 7.0, containing 10–15% acetonitrile (30). Oligodeoxynucleotides were further purified by electrophoresis on 20% polyacrylamide gels ($15 \times 72 \times 0.2$ cm) in the presence of 7 M urea. Bands were located by UV shadowing; DNA was extracted by suspending the crushed gel overnight in 2.0 ml of distilled water at 4°C. Samples were concentrated using a Centricon 3 filter (Amicon) and rechromatographed on HPLC, as described above, to remove residual urea. To ensure homogeneity, oligodeoxynucleotides were labeled at the 5' terminus (31) then subjected to 20% polyacrylamide gel electrophoresis ($35 \times 42 \times 0.04$ cm) in the presence of 7 M urea. Contaminating oligonucleotides can be detected at a level of 0.003% (32). 32 P-Labeled oligodeoxynucleotides were detected by autoradiography, using Kodak XO-mat XAR film.

Site-specific mutagenesis

Procedures for site-specific mutagenesis studies in *E. coli* and COS cells are described in previous publications from this laboratory (27,28). Oligodeoxynucleotides containing defined DNA lesions were annealed to their complementary strand; the resulting duplex was ligated to the vector to form closed circular DNA. Gapped duplex vectors with a lesion in the single strand region were prepared by ligating a modified oligodeoxynucleotide into a duplex vector (see Fig. 2b). Ligation mixtures were used to transform *E. coli* or transfect COS cells. In COS cells, progeny DNA was recovered by the procedure of Hirt (33). After

Table 1. Sequence of duplex oligonucleotides used in site-specific mutagenesis studies¹

Set A	5' CCTTCXCTAC (Strand 1)
	TCGGAAGXGATGAGCT 5' (Strand 2)
	Sequence 1-----Strand 1, X=C
	Sequence 2-----Strand 2, X=G
	Sequence 3-----Strand 1, X=F
	Sequence 4-----Strand 2, X=F
Set B	5' GTCACCGTCXTACGACTC (Strand 1)
	GCAGXATGC 5' (Strand 2)
	Sequence 5-----Strand 1, X=G
	Sequence 6-----Strand 2, X=C
	Sequence 7-----Strand 1, X=F
	Sequence 8-----Strand 2, X=F
	Sequence 9-----Strand 1, X=P
	Sequence 10-----Strand 2, X=P
	Sequence 11-----Strand 1, X=E
	Sequence 12-----Strand 2, X=E

¹Strand 1 and Strand 2 in Sets A and B contain sequences 1–12 with the DNA base or abasic site indicated at position X. These oligonucleotides were ligated to vectors to form duplexes or as a single strand form.

removing input DNA by treatment with *DpnI*, plasmid DNA was amplified by transforming *E. coli* DH5 cells (28). The nucleotide sequence of progeny plasmids was established by colony hybridization techniques (27,28). Oligodeoxynucleotide probes were designed to identify all base substitutions and the single base deletion targeted to the site of the lesion. In some cases, DNA was subjected to dideoxynucleotide sequence analysis (34) to confirm results of the hybridization procedure. Non-targeted mutations are not included in the mutational analysis.

DNA polymerase assays

Reaction mixtures containing four dNTPs (100 μ M each), unmodified or modified templates primed with 32 P-labeled 10-mer (0.05 μ M) and varying amounts of the exonuclease-deficient (*exo*⁻) Klenow fragment of DNA polymerase I were incubated for 1 h at 25°C. Samples were heated at 95°C for 3 min in the presence of formamide dye then subjected to 20% polyacrylamide gel electrophoresis ($15 \times 72 \times 0.04$ cm) in the presence of 7 M urea. Bands were identified by autoradiography. The *exo*⁻ Klenow fragment of DNA polymerase was purchased from International Biotechnology, Inc.

RESULTS

Purification of oligodeoxynucleotides containing synthetic abasic sites

Oligodeoxynucleotides used in site-specific mutagenesis studies (Table 1) were purified extensively by HPLC and PAGE, as described in Materials and Methods, to exclude possible contaminants introduced during synthetic procedures. With one exception, oligodeoxynucleotides containing abasic sites could be separated by PAGE from oligomers of similar length containing dG, dA, dT, or dC and from analogous sequences in which the abasic site was deleted. PAGE analysis is shown for sequences listed in Set B (Fig. 3). Seq 8 could not be resolved from the comparable 8-mer containing a single deletion by PAGE (Fig. 3); these oligomers were purified by HPLC (Fig. 4).

Oligodeoxynucleotides listed in Set A, Table 1, were used in conjunction with pGM86 to create shuttle vectors containing

Figure 3. Polyacrylamide gel electrophoresis of standard oligodeoxynucleotides. (a) T, A, C, and G represent X in the 18-mer sequence shown for Strand I (Set B, Table 1). Δ represents the identical sequence in which the abasic site has been deleted. Lanes 1, 2, and 3 represent oligomers with Sequences 7, 9, and 11, respectively. (b) T, A, C, and G represent X in 9-mers with the sequence shown for Strand 2 (Set B, Table 1). Δ represents the comparable sequence lacking the abasic site. Lanes 1, 2, and 3 are Sequences 8, 10, and 12, respectively. All oligodeoxynucleotides were labeled with ^{32}P and subjected to 14% PAGE in the presence of 7 M urea.

either single (F) or opposed bistrand lesions at position 974; these vectors were used to transform *E. coli* AB1157. Only one (C/G) of the four possible base pairs located at this position generates Tet^S phenotype (35). Transformants were screened for the conversion from Tet^S to Tet^R. If bases other than C/G are substituted at the lesion site, progeny would be Tet^R. If the abasic site is repaired or deleted, progeny would display a Tet^S phenotype. All mutations were characterized by colony hybridization.

Initially, transformants were grown on agar plates containing ampicillin or tetracycline and the Tet^R/Amp^R ratio was determined. Abasic sites positioned in a bistrand configuration (F/F) were highly mutagenic while those incorporated in only one strand (F/C and C/F) were not (Table 2, Exp. A). Bistrand lesions strongly inhibited transformation (<10% of control values); single lesions were only slightly inhibitory (Table 2). In Exp. B, Amp^R colonies collected from Exp. A were replica plated on agar containing ampicillin and tetracycline. The mutation frequency of abasic sites determined in this manner is 400-fold higher at bistrand lesions than at a single abasic site (Table 2).

Vectors containing opposed bistrand lesions (F/F) were used to transform *E. coli* and to transfect COS cells (Table 3). This experiment was conducted in duplicate. For *E. coli*, transformation efficiency ranged from 1–9% of control values. For COS cells, the number of transformants was approximately 50% of those recovered in experiments using a vector containing an unmodified oligonucleotide. Mutational analysis (Table 3) indicated that base pairs were introduced at F/F sites. In *E. coli* and COS cells, the sum of A/T and T/A base substitutions was much higher than the sum of C/G and G/C. A/T substitutions predominated in *E. coli*; T/A substitutions predominated in COS cells. There were fewer single base deletions in COS cells than in *E. coli*. Untargeted mutations, particularly frequent in COS cell experiments, were not identified in these experiments.

Figure 4. HPLC separation of oligonucleotides. Seq 8 (GCAGFATGC) was separated from the sequence in which F was deleted (GCAGATGC) on a Bondapak C18 column, using a linear gradient of 0.05 M triethylamine acetate, pH 7.0, containing 7–13% acetonitrile. See Materials and Methods for details.

Effect of abasic site structure on mutagenesis

Bistrand lesions containing F, P, or E, an analog in which the phosphodiester backbone was shortened by one carbon atom, were tested for mutagenicity in pBS(–)XME. The efficiency of the ligation procedure was highest in reactions containing F/F; lowest in those containing E/E (Fig. 5). Aliquots of each ligation mixture were used to transform competent NM522 cells. Results of this experiment are summarized in Table 4. A/T and T/A base substitutions predominate when vectors containing F/F are used; the relative contribution of each abasic site being approximately equal. With vectors containing P/P, the number of progeny containing A/T and T/A pairs decreased significantly and a proportional increase in single base deletions was observed. The number of progeny containing G/C and C/G pairs remained constant. With vectors containing E/E, the proportion of deletions further increased.

The blocking nature of bistrand lesions, revealed by the number of colonies recovered relative to the unmodified control (Table 4), apparently is influenced by the structure of the abasic site. The transformation efficiency of the band labeled OC (Fig. 5) is low while the heavy band, representing a linear dimer (LD) ligated at the *Bst*EII restriction site, has virtually no capacity to transform *E. coli* (data not shown). Assuming that closed circular species (CC) are primarily involved in transformation of bacteria, the number of colonies recovered from CC DNA with different abasic sites was essentially similar.

Mutation spectrum of F determined in a gapped duplex vector

A gapped plasmid vector in which a single F is placed in the single strand region (Fig. 2b) was used to transform competent NM522 cells. T/A (77.6%) and a few C/G pairs (0.7%) and deletions (21.7%) were detected at the site of the lesion (Table 5). The mutation spectrum reveals that A > del > G > T = C.

Incorporation of dAMP opposite E

The ability of DNA polymerase I to incorporate dAMP opposite E during the primer extension reaction is shown in Fig. 6. Fully-extended primers containing different bases are distinguished by

Table 2. Mutagenesis at single and bistrand (opposed) abasic sites¹

Sequence	Lesion	Transformants					
		A			B		
		Amp ^R	Tet ^R	Tet ^R Amp ^R	Amp ^R	Tet ^R	Tet ^R /Amp ^R
Seq 1/Seq 2	C/G	238	0	0	576	0	0
Seq 3/Seq 2	F/G	212	0	0	576	1	0.0017
Seq 1/Seq 4	C/F	139	0	0	576	1	0.0017
Seq 3/Seq 4	F/F	21	16	0.76	144	92	0.64

¹Oligonucleotides in Set A (Table 1) were phosphorylated, annealed, and 2.8 pmol of the duplex were ligated to 0.7 pmol of *BsmI/XhoI*-digested pGM86 overnight at 4°C in 200 µl of a ligation mixture containing 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol, 1 mM ATP and 400 units T4 DNA ligase (Biolab). Aliquots of the ligation mixture, containing 35 ng DNA, were used to transform competent AB1157 cells. After 1 h, 50 µl of the transformation mixture was spread on agar plates containing ampicillin (50 µg/ml) or tetracycline (50 µg/ml), respectively, and the number of colonies recorded (Exp. A). Amp^R colonies, collected from similar experiments, were replica plated on Amp and Tet plates (Exp. B).

Table 3. Base pairs introduced at F/F lesion in *E. coli* strains and COS7 cells¹

Cells	Colonies		Base pair at position 974 (%)				
	Total	With targeted mutation (%)	A/T	T/A	G/C	C/G	Del.
AB1157-1	100	96	55.6	9.1	0	8.2	27.1
AB1157-2	180	87	74.3	3.3	2.0	1.1	19.3
COS7-1	258	41	13.1	68.8	8.3	3.7	6.1
COS7-2	360	46	19.8	58.5	11.0	7.1	3.6

¹Ligation mixtures using pGM86 containing the F/F lesion were prepared as described in Table 2 then used to transform AB1157 and COS7 cells in duplicate sets. Aliquots (50 µl) of the ligation mixtures were used to transfect COS cells. Hirt lysate (10 µl) was used to transform DH5 cells. The probes used for colony hybridization have been described (28).

Table 4. Effects of the structure of abasic sites on the introduction of base pairs¹

Sequence ²	Lesions	Colonies		Bases pairs introduced at abasic sites (%)						
		Total	With targeted mutation (%)	A/T	T/A	A/T+T/A	G/C	C/G	G/C+C/G	Del.
Seq 7/Seq 8	F/F	345	94.3	40.4	41.5	81.9	5.5	0	5.5	12.6
Seq 9/Seq 10	P/P	210	83.9	6.6	2.0	8.6	8.6	1.3	9.9	81.5
Seq 11/Seq 12	E/E	187	77.6	0.8	0	0.8	2.3	3.9	6.2	93.0

¹*Bst*XI/*Bst*EII-digested pBS(-)XME, 0.7 pmol, was incubated overnight at 4°C with 2.8 pmol of the phosphorylated and annealed oligonucleotides listed in Table 1, Set B, in reaction mixtures containing 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol, 1 mM ATP and 400 units of T4 DNA ligase in a final volume of 200 µl. An aliquot of this ligation mixture (40 ng DNA) was used to transform NM522, and plated on Amp plates after 2 h incubation. The oligonucleotide sequences used for colony hybridization were ACCGTCXTACGACTC, where X = G, A, C, or T, and CACCGTCTACGACTC.

²When unmodified sequences (Seq 5/Seq 6) were used for this experiment, 2200 colonies were recovered with no mutations.

Table 5. Mutational specificity of a single F placed in a gapped duplex vector¹

Colonies	Base incorporated opposite abasic site (%)					
	Total (No.)	With targeted mutation (No.)	T	A	C	G
217	137	0	77.6	0	0.7	21.7

¹Seq 7 was ligated to pBS(-)XME as described in Table 4 to form a gapped duplex.

their differential migration on PAGE. Primer extension proceeds rapidly on unmodified templates, producing an 18-mer reaction product with the expected base sequence (Lane 2). When the template contains E at position 13, DNA polymerase is blocked at position 12 (Lane 3). Translesional synthesis is observed only when a 100-fold excess of enzyme is added (Lane 5); under these conditions, dAMP is incorporated opposite the lesion, shown by comparing migration of the reaction product with a standard

18-mer containing A at position 13. Deletions were not analyzed in this experiment.

DISCUSSION

In this paper, we report that synthetic abasic sites, positioned as a bistrand lesion in a duplex shuttle vector, are mutagenic when introduced into *E. coli* and COS cells. In these experiments, the

Figure 5. Analysis of ligation mixtures of duplex plasmid vectors containing bistrand lesions. Aliquots of ligation mixtures described in footnote to Table 4, each containing 75 ng DNA, were subjected to electrophoresis on 1% agarose in the presence of ethidium bromide (0.5 μ g/ml). CC: closed circular DNA; OC: open circular DNA; L: linear DNA; LD: linear dimer. Lane 1: Oligonucleotides omitted from the ligation mixture; Lane 2: Seq 5/Seq 6; Lane 3: Seq 7/Seq 8; Lane 4: Seq 9/Seq 10; Lane 5: Seq 11/Seq 12.

Figure 6. Incorporation of dNMP opposite E during translesion synthesis catalyzed by an *exo*⁻ DNA polymerase. An 18-mer template containing G or E at position 13 (CCTTCECTACTTTCCTCT), was primed with a ³²P-labeled 10-mer (A-GAGGAAAGT) and used in primer extension reactions catalyzed by DNA polymerase, as described in Materials and Methods. Lane 1: No enzyme added. Lane 2: unmodified template, 0.005 units enzyme; Lane 3–5: templates contained E and 0.005, 0.05 and 0.5 units polymerase, respectively. Lanes C, A, G and T: standard markers containing these bases opposite E. The position of standard markers is shown on the left of the figure: T10, primer; G12, 12-mer; A13 and G13, 13-mers in which G or A is incorporated opposite E; A18 and C18, 18-mers containing A or C opposite E.

total number of mutational events remained relatively constant; the relative proportion of base substitutions targeted to the lesion site and deletions was influenced by the structure of the abasic analog used. The predominant base substitutions observed were A/T and T/A. Preferential incorporation of dAMP also was observed during translesional synthesis past a single strand region of a gapped duplex vector. These results are consistent with reports of primer extension experiments catalyzed by purified

DNA polymerases (10,12,15,36) and with previous studies of abasic site mutagenesis in *E.coli* (16) but are in conflict with recent observations that all four dNMPs are incorporated at comparable frequency opposite abasic sites in mammalian cells (20–22).

Mutations at abasic sites in *E.coli* generally are reported to be SOS dependent. Cells were not treated with DNA-damaging agents in our study. Nevertheless, the possibility that SOS functions are partially induced during cell growth or by procedures used for transformation cannot be excluded. Under conditions used in this study, DNA containing synthetic abasic sites was found to generate similar mutagenic spectra in RecA proficient and RecA deficient strains of *E.coli* (unpublished studies).

The use of abasic site analogs in this study permits the structure of these lesions to be correlated with mutagenic events. Duplex DNA containing the tetrahydrofuran analog serves as substrate for AP endonucleases (10,11) and as a template for *in vitro* studies of translesional synthesis (10,12). In this respect, the biological properties of this synthetic analog resemble closely those associated with natural abasic sites. Base substitutions predominate when the tetrahydrofuran structure was used for mutagenesis studies. Presumably, the ring structure of the deoxyribose analog facilitates extension of the phosphodiester backbone during translesional synthesis. Such structures have been demonstrated for DNA duplexes when dAMP is positioned opposite the abasic site (5,6).

Deletions are the predominant event observed in our studies of propenyl and ethanyl abasic sites. These analogs do not contain a ring structure. A currently-favored model for deletion mutagenesis involves misalignment of the DNA template (30,37). 2D-NMR studies of duplexes containing a pyrimidine positioned opposite an abasic site suggest the presence of a misaligned structure in which the base opposite the lesion is positioned extrahelically (7,8). The ethanyl moiety shortens the backbone, increasing further the tendency for extrahelical rotation of bonds at the abasic site; the collapsed structure is stabilized by stacking of bases flanking the lesion.

We show in this paper that preferential incorporation of dAMP opposite abasic sites occurs both in *E.coli* and simian kidney cells. This so-called 'A rule' was established in *E.coli* (38); non-specific incorporation of DNA bases opposite abasic sites has been observed in studies with mammalian cells (20–22). There is no obvious explanation for the discrepancy between these reports and the studies reported here; however, there are certain differences in the experimental approaches used. We utilize DNA containing a pre-synthesized abasic site of demonstrated high purity while Drinkwater and Sarasin used natural abasic sites for their studies. In some of their reports (21,22), abasic sites were introduced as single lesions in double strand vectors. Synthetic abasic sites are rapidly repaired under such conditions (Table 2); thus, mutations observed when single abasic sites are present represent rare events. In other studies reported by Drinkwater and by Sarasin, abasic sites were introduced in a random fashion. Site-specific mutagenesis, utilized in our experiments, permits direct correlation between lesions and mutagenic events; furthermore, the same vector preparation was used to transform *E.coli* and to transfect COS cells, providing an internal control. Since A/T mutations were observed in *E.coli* and T/A mutations in COS cells, contamination of the abasic lesion seems unlikely. This species difference reflects different mechanism of processing for bistrand lesions.

Bistrand lesions were shown to be highly mutagenic in this study. This observation is immediately relevant to mutagenesis produced by bleomycin and neocarzinostatin, drugs that form bistrand lesions in DNA (25,26). Loss of a base destabilizes duplex DNA (9) and alters its reactivity to chemical agents (39). Thus, one might envision that damage could concentrate at or around an abasic site (or nick), increasing the number of spontaneously generated bistrand lesions in genomic DNA. Bistrand synthetic abasic sites are readily incised by AP endonucleases (unpublished results) and a single abasic site is repaired in extracts of eukaryotic cells (40). Thus, mutagenesis observed with bistrand lesions in our study may involve short patch repair, as observed by Dianov and Lindahl (41). DNA polymerase I (41) or DNA polymerase I* (42) may be involved in this reaction in *E.coli*.

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