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DNA polymerase bypass in vitro and in *E. coli* of a C-nucleotide analogue of Fapy·dG

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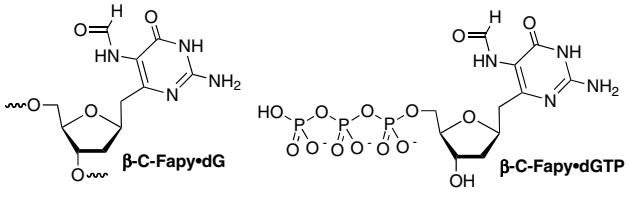
Abstract—Bypass of the configurationally stable analogue (β-C-Fapy·dG) of the formamidopyrimidine lesion derived from 2′-deoxyguanosine oxidation (Fapy·dG) was studied in vitro and in *Escherichia coli*. The exonuclease deficient Klenow fragment of *E. coli* DNA polymerase I (Klenow exo$^-$) misincorporated dA most frequently opposite β-C-Fapy·dG, but its efficiency was <0.2% of dC insertion. Klenow exo$^-$ fidelity was enhanced by the enzyme's high selectivity for extending duplexes only when dC was opposite β-C-Fapy·dG. The expectations raised by these in vitro data were realized when β-C-Fapy·dG replication was studied in *E. coli* by transfecting M13mp7(L2) bacteriophage DNA containing the nucleotide analogue within the *lacZ* gene in 4 local sequence contexts. The bypass efficiency of β-C-Fapy·dG varied between 45% and 70% compared to a genome containing only native nucleotides. Mutation frequencies at the site of the lesions in the originally transfected genomes were determined using the REAP assay [Delaney, J. C.; Essigmann, J. M. *Methods Enzymol.* **2006**, *408*, 1]. The levels of mutations could not be distinguished between those observed when genomes containing native nucleotides were replicated, indicating that the mutagenicity of β-C-Fapy·dG was <1%. These data and previous reports indicate that β-C-Fapy·dG is a good model of Fapy·dG in *E. coli*. In addition, these results and the previous report of β-C-Fapy·dG binding to the base excision repair protein formamidopyrimidine glycosylase suggest that this analogue could be useful as a DNA repair inhibitor.

1. Introduction

Nucleotide analogues are useful mechanistic probes for studying kinetic mechanisms of polymerases and DNA repair enzymes.[1–5] Structural studies of protein–DNA complexes are also made possible by nucleotide based substrate analogues.[6,7] There are also a significant number of examples of modified nucleosides that are successful therapeutic agents.[8,9] Although to our knowledge no modified nucleotide that inhibits a base excision repair protein has been used for therapeutic purposes, the potential for inhibitors of such enzymes to function as drugs is recognized.[4,10,11] C-nucleotides and carbocyclic nucleotides are not substrates for glycosylases due to their lower oxidation state. We describe the bypass of a C-nucleotide analogue (β-C-Fapy·dG) of Fapy·dG (*N*-(2-deoxy-α,β-D-erythropentofuranosyl)-*N*-(2,6-diamino-4-hydroxy-5-formamidopyrimidine)) by the exonuclease deficient Klenow fragment of DNA polymerase I from *Escherichia coli* (Klenow exo$^-$) and its bypass in vivo. The results described herein and those previously reported concerning the properties of β-C-Fapy·dG and its respective nucleotide triphosphate (β-C-Fapy·dGTP) suggest that this nucleotide analogue may be useful as an inhibitor of DNA base excision repair.[5,12]

Structures β-C-Fapy•dG and β-C-Fapy•dGTP



Fapy·dG is produced when DNA is exposed to oxidizing conditions. The lesion is derived from a common intermediate whose reactivity is partitioned between Fapy·dG and 8-hydroxy-7,8-dihydro-2′-deoxyguanosine (OxodG) formation (Scheme 1).[13] Consequently, the chemical and biochemical aspects of Fapy·dG are often

Keywords: DNA lesions; DNA damage; DNA replication; Modified nucleotides.
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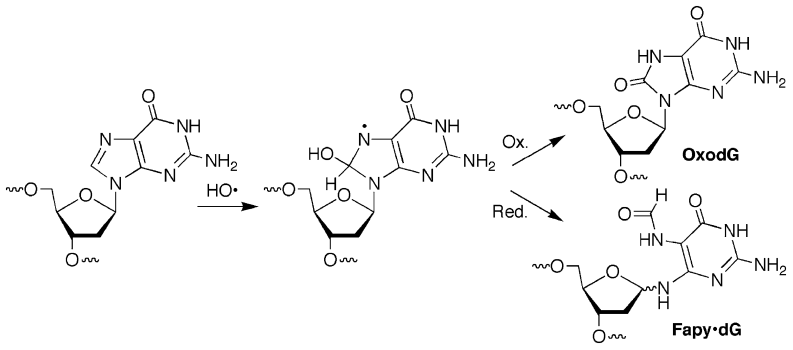
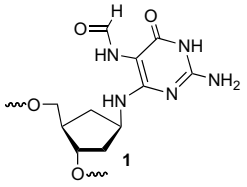


Scheme 1.

compared to the respective properties of OxodG, an extremely well studied DNA lesion.[14] The two lesions are produced in comparable yields when DNA is exposed to γ-radiolysis under aerobic conditions, but the *G*-value for Fapy·dG production is approximately twice that of OxodG under anaerobic conditions.[15,16] OxodG and Fapy·dG share many common biochemical traits as well. For instance, both lesions are excised by the bifunctional formamidopyrimidine DNA glycosylase (Fpg) from *E. coli* selectively when opposite dC, and the repair protein MutY incises dA opposite OxodG or Fapy·dG.[5,17] Furthermore, Fapy·dG and OxodG induce bacterial DNA polymerases to misincorporate dA opposite them in vitro.[18,19] Their effect on DNA polymerase activity is reflected in the outcome of experiments in cells using single stranded vectors containing the lesions at a defined site. Replicative bypass of Fapy·dG in COS-7 cells results in slightly higher G → T transversion frequencies than when genomes containing OxodG are bypassed.[20] In contrast, significantly lower mutation frequencies for both lesions are recorded in *E. coli* using the restriction endonuclease and post-labeling (REAP) assay, but OxodG produces higher G → T transversion frequencies than Fapy·dG in this organism.[21]

Carbocyclic (**1**) and C-nucleotide (β-C-Fapy·dG) analogues of Fapy·dG have been used in structural studies and as biochemical probes.[5,22] The former has proven to be very useful for examining the binding of BER proteins to a formamidopyrimidine via X-ray crystallography.[7] DNA containing β-C-Fapy·dG binds the Fpg repair protein with nanomolar affinity. Although MutY deglycosylates an opposing dA opposite β-C-Fapy·dG much more slowly than when Fapy·dG or OxodG are present in the duplex, DNA containing the analogue binds the enzyme ~7-fold more strongly than does undamaged biopolymer.[5] Studies on β-C-Fapy·dGTP suggest that low levels of this analogue can be incorporated selectively opposite dC.[12] The specificity constant for the incorporation of β-C-Fapy·dG opposite dC is only $\sim 10^{-4}$ that of dG incorporation. These data indicate that β-C-Fapy·dG will replace 1 out of every 10^6 dGs if the concentration of its triphosphate is 1% of dGTP. This incorporation level is comparable to that observed for the production of some lesions in DNA.[23] The incorporation efficiency of β-C-Fapy·dGTP is even more significant because the analogue is an extremely poor substrate for MutT, the enzyme that protects the nucleotide triphosphate pool by hydrolyzing OxodGTP.[12]



2. Results and discussion

2.1. Nucleotide incorporation by klenow exo$^-$ on a template containing β-C-Fapy·dG

Kinetic studies were carried out under steady-state conditions using a template containing β-C-Fapy·dG.[28] Kinetic data obtained were compared to that obtained using an identical template containing Fapy·dG or dG in place of the nucleotide analogue.[19] Individual incorporation of the four native nucleotide triphosphates opposite β-C-Fapy·dG by Klenow exo$^-$ was examined using a standing start process (Table 1).[24] The apparent K_m for dCTP is only 10 nM higher than when dG is in the template. Part of this increase is compensated for in the specificity constant by a slightly greater V_{max} than is observed for the dG containing template. The net result is that dC is incorporated opposite β-C-Fapy·dG almost as efficiently as it is opposite dG. This is significantly different than Fapy·dG, opposite which dC is incorporated by Klenow exo$^-$ ~50-fold less efficiently than opposite dG in an otherwise identical template.[19]

Of the three native nucleotides whose incorporation opposite β-C-Fapy·dG would give rise to a promutagenic base pair, the misincorporation frequency opposite β-C-Fapy·dG is greatest for dA. This is the same nucleotide most frequently misincorporated opposite Fapy·dG. The specificity constants for dA incorporation opposite β-C-Fapy·dG and Fapy·dG are within experimental error of one another. However, due to the proficient incorporation of dC opposite β-C-Fapy·dG the F_{ins} for dA opposite β-C-Fapy·dG is 30-fold smaller than when Fa-

Table 1. Kinetic parameters for nucleotide incorporation opposite β-C-Fapy·dG and other relevant nucleotides by Klenow exo$^-$[a]

5'-d(AGG CGT TCA ACG TGC AGT **N**AC AGC ACG TCC CAT GGT)
TG TCG TGC AGG GTA CCA)

↓ dN'TP, Klenow exo$^-$

5'-d(AGG CGT TCA ACG TGC AGT **N** AC AGC ACG TCC CAT GGT)
N'TG TCG TGC AGG GTA CCA)

N	dN′TP	V_{max} (% min^{-1})	K_m (μM)	V_{max}/K_m (% min^{-1} μM^{-1})	F_{ins}[b]
β-C-Fapy·dG	C	8.7 ± 2.2	$15.2 \pm 2.3 \times 10^{-3}$	$5.7 \pm 0.9 \times 10^{8}$	1.0
β-C-Fapy·dG	A	32.6 ± 5.1	35.8 ± 2.9	$9.0 \pm 0.7 \times 10^{5}$	1.6×10^{-3}
β-C-Fapy·dG	G	8.3 ± 1.2	96.0 ± 10.6	$8.6 \pm 0.3 \times 10^{4}$	1.5×10^{-4}
β-C-Fapy·dG	T	4.9 ± 0.9	233.9 ± 14.1	$2.1 \pm 0.4 \times 10^{4}$	3.7×10^{-5}
Fapy·dG	C	7.9 ± 0.6	0.4 ± 0.1	$2.0 \pm 0.5 \times 10^{7}$	1.0
Fapy·dG	A	9.9 ± 6.9	10.4 ± 7.3	$9.8 \pm 7.9 \times 10^{5}$	4.8×10^{-2}
Fapy·dG	G	1.0 ± 0.1	26.2 ± 19.5	$3.8 \pm 2.9 \times 10^{4}$	1.9×10^{-3}
Fapy·dG	T	0.3 ± 0.1	103.0 ± 68.0	$2.9 \pm 2.1 \times 10^{3}$	1.5×10^{-4}
dG	C	5.1 ± 1.6	$5.2 \pm 2.7 \times 10^{-3}$	$9.8 \pm 3.6 \times 10^{8}$	1.0
dG	A	14.0 ± 7.9	257.0 ± 135.0	$5.4 \pm 3.2 \times 10^{4}$	5.5×10^{-5}
dG	G	6.1 ± 2.1	74.2 ± 7.5	$8.2 \pm 1.1 \times 10^{4}$	8.4×10^{-5}
dG	T	4.7 ± 2.5	248.0 ± 60.0	$1.9 \pm 0.7 \times 10^{4}$	1.9×10^{-5}

[a] Data for Fapy·dG and dG are taken from Ref. 19.
[b] F_{ins} = (V_{max}/K_m, N = dG, Fapy·dG, or β-C-Fapy·dG, dN′TP = T, A, G, or C)/(V_{max}/K_m, N = dG, Fapy·dG, or β-C-Fapy·dG, dN′TP = C).

py·dG is present in the template. In addition, the F_{ins} for dA opposite β-C-Fapy·dG is ~28-times greater than when dG is in the template, and is mostly attributable to a ~20-fold increase in the specificity constant for the former.

The specificity constants for misincorporation of dG and dT opposite β-C-Fapy·dG are very similar to those measured when dG is in the template. The kinetic parameters result in a very modest increase (<3-fold) in misincorporation frequencies of dG and dT opposite β-C-Fapy·dG relative to those opposite dG. Comparisons to misincorporation of dT and dG opposite Fapy·dG are less uniform. However, the misinsertion frequency of dG and dT opposite β-C-Fapy·dG is decreased when compared to the F_{ins} of these nucleotides opposite Fapy·dG.

2.2. Bypass of β-C-Fapy·dG by Klenow exo$^-$

The very efficient incorporation of dC opposite β-C-Fapy·dG indicates that the model compound will not induce significant levels of mutations, provided the primer is readily extended. Indeed, the specificity constant for primer extension past β-C-Fapy·dG when the lesion is opposed by dC is within experimental error of that for a template containing dG (Table 2). In contrast, extension past a Fapy·dG:dC base pair is ~100-fold less efficient than past a dG:dC base pair.[19] Klenow exo$^-$ also bypasses β-C-Fapy·dG more selectively than it does Fapy·dG. Although Fapy·dG is bypassed approximately as efficiently when dA is opposite the lesion as when it is opposed by dC, extension of a primer containing dA opposite β-C-Fapy·dG is so inefficient that kinetic parameters could not be extracted (data not shown).

Table 2. Kinetic parameters for extension past β-C-Fapy·dG and other relevant nucleotides by Klenow exo$^-$[a]

5'-d(AGG CGT TCA ACG TGC AGT **N** AC AGC ACG TCC CAT GGT)
N'TG TCG TGC AGG GTA CCA)

↓ dATP, Klenow exo$^-$

5'-d(AGG CGT TCA ACG TGC AGT **N** AC AGC ACG TCC CAT GGT)
A **N'**TG TCG TGC AGG GTA CCA)

N	N′	V_{max} (% min^{-1})	K_m (μM)	V_{max}/K_m (% min^{-1} μM^{-1})	F_{ext}[b]
β-C-Fapy·dG	C	5.1 ± 2.0	6.9 ± 1.7	$7.2 \pm 1.7 \times 10^{8}$	1.0
β-C-Fapy·dG	G	0.9 ± 0.1	53.8 ± 25.8	$1.8 \pm 0.7 \times 10^{4}$	2.5×10^{-5}
Fapy·dG	C	6.3 ± 1.9	0.8 ± 0.4	$7.9 \pm 4.6 \times 10^{6}$	1.0
Fapy·dG	A	11.0 ± 3.0	3.4 ± 0.9	$3.2 \pm 1.2 \times 10^{6}$	0.4
dG	C	1.1 ± 0.3	$2.0 \pm 0.8 \times 10^{-3}$	$5.5 \pm 2.7 \times 10^{8}$	1.0
dG	A	0.9 ± 0.1	374.0 ± 40.0	$2.4 \pm 0.4 \times 10^{3}$	3.7×10^{-5}

[a] Data for Fapy·dG and dG are taken from Ref. 19.
[b] F_{ext} = (V_{max}/K_m, N = dG, Fapy·dG or β-C-Fapy·dG, N′ = A, G, or C)/(V_{max}/K_m, N = dG, Fapy·dG or β-C-Fapy·dG, N′ = C).

Less than 5% of the primer was extended, even after a 30 min reaction in the presence of 750 μM dATP (the next nucleotide to be incorporated). Of the 3 mispairs, only β-C-Fapy·dG:dG was extended efficiently enough for kinetic analysis to be carried out. Even in this instance, the F_{ext} was so low that when combined with the low F_{ins} for dG opposite β-C-Fapy·dG, it is highly improbable that dG would be incorporated opposite the C-nucleotide analogue.

5'-d(GAA GAC CTX YGC GTC C)
2a-d X = β-C-Fapy•dG
3a-d X = Fapy•dG
4 X = G, Y = G
a Y = A, **b** Y = C, **c** Y = G, **d** Y = T

2.3. Bypass of a single stranded genome containing β-C-Fapy·dG in *E. coli*

Experiments using Klenow exo$^-$ indicate that promutagenic base pairs will form very infrequently due to unfaithful bypass of β-C-Fapy·dG. In addition, the Klenow exo$^-$ data indicate that the fidelity of nucleotide incorporation opposite β-C-Fapy·dG will be reinforced by inefficient extension of mispairs. The Klenow exo$^-$ data also suggest that β-C-Fapy·dG should be readily bypassed and will not be a blocking lesion, as nonnative nucleotides and analogues sometimes are.[3,25] Bypass and mutagenicity of β-C-Fapy·dG were determined in wild type *E. coli* using single stranded genomes that were prepared from 16-nucleotide synthetic oligonucleotides. Aside from the presence of β-C-Fapy·dG, the sequences of the oligonucleotides (**2a**–**d**) were the same as those used in studies on Fapy·dG and OxodG.[12]

The bypass efficiency of β-C-Fapy·dG (**2a**–**d**) showed little dependence on the flanking sequence and ranged from ~45 to 70% in *E. coli* (Fig. 1). Overall, bypass efficiency was approximately one-half that of dG, but within experimental error of Fapy·dG (**3a**–**d**) in the same sequence contexts (Fig. 1). Although the bypass of β-C-Fapy·dG was not greater than that of Fapy·dG, which was predicted by the Klenow exo$^-$ experiments, it was higher than other nucleotide analogues and abasic sites.[3,25] Abasic sites are potent blocks to replication in *E. coli*.[26] Importantly, the data indicate that β-C-Fapy·dG is not a significant block of replication in *E. coli*.

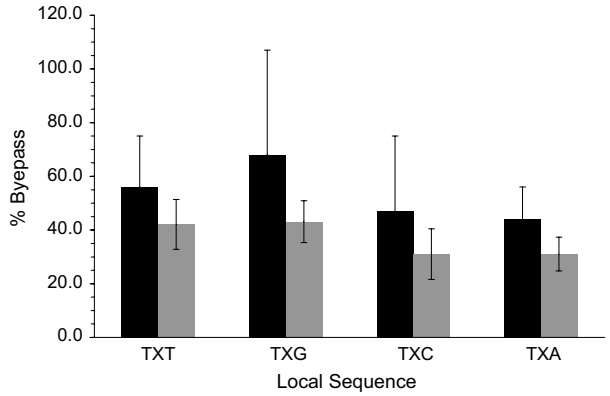


Figure 1. Percent bypass of β-C-Fapy·dG (**2a**–**d**, black) and Fapy·dG (**3a**–**d**, gray) in wild-type *E. coli* as a function of flanking sequence.

The fidelity of β-C-Fapy·dG replication was determined using the restriction enzyme and postlabeling (REAP) method developed by Essigmann.[34] REAP measurements indicated that dC was incorporated opposite β-C-Fapy·dG >99% of the time. The levels of misincorporation of the other 3 native nucleotides opposite β-C-Fapy·dG were indistinguishable from control experiments in which dG was present in the template (**4**). The extent of nucleotide misincorporation opposite β-C-Fapy·dG correlates with the results of the Klenow exo$^-$ experiments, which indicated that the C-nucleotide analogue would be bypassed with greater fidelity than Fapy·dG. In contrast, Fapy·dG gives rise to low levels of G to T transversions in *E. coli*. However, it is worth noting that the highest level of dA misincorporation opposite Fapy·dG was less than 2%, which was considerably lower than mutation frequencies observed when otherwise identical genomes containing OxodG were replicated.[21] These data indicate that the mutagenicity of β-C-Fapy·dG is below the level of detection of the REAP assay. We cannot rule out the possibility that a more sensitive assay, such as one that utilizes hybridization would detect very low (<1%) levels of mutations.

3. Conclusion

These experiments reveal that the C-nucleotide analogue of Fapy·dG, β-C-Fapy·dG, exhibits no detectable mutagenicity in *E. coli*. Fapy·dG is weakly mutagenic in *E. coli* indicating that in this respect β-C-Fapy·dG is different than the lesion it was designed to model. Experiments using the model *E. coli* DNA polymerase, Klenow exo$^-$, provide a rationalization for the lack of an effect on replication in *E. coli* by β-C-Fapy·dG. The misinsertion frequency of dA, the nucleotide that is most frequently misincorporated opposite β-C-Fapy·dG is ~30-fold lower than that for Fapy·dG but less than 30-fold higher than when an undamaged dG is present in a comparable template. The Klenow exo$^-$ experiments suggest that formation of the promutagenic base pair (β-C-Fapy·dG:dA) is further limited because extension past the lesion is facile for the 'correct' base pair (β-C-Fapy·dG:dC) but is negligible for the promutagenic β-C-Fapy·dG:dA base pair.

Consideration of these results in the context of previous studies on β-C-Fapy·dG and its triphosphate raise the interesting possibility that the C-nucleotide analogue could be useful as an inhibitor of DNA repair.[5,12] Molecules that inhibit DNA repair and that are incorporated into the biopolymer would be useful as mechanistic probes and possibly even as therapeutic agents. β-C-Fapy·dG is not a substrate for the Fpg repair protein, but binds strongly to it. In addition, the respective nucleoside triphosphate is a poor substrate for the OxodGTPase, MutT but is incorporated opposite dC by Klenow exo$^-$.[12] Furthermore, the results presented above show that β-C-Fapy·dG is faithfully replicated in *E. coli*. If cells efficiently incorporate β-C-Fapy·dG, these data suggest the

molecule will inhibit DNA repair by Fpg, but will not induce high levels of mutations. These are desirable properties for a molecule whose purpose is to inhibit DNA repair in cells and indicate that future in vivo studies are warranted to determine whether this nucleotide analogue is a viable DNA repair inhibitor.

4. Experimental

4.1. General methods

Oligonucleotides containing native nucleotides were synthesized on an Applied Biosystems Incorporated 394 DNA synthesizer using standard protocols. Oligonucleotides containing β-C-Fapy·dG or Fapy·dG were synthesized as previously described.[27–30] DNA synthesis reagents were purchased from Glen Research. DNA was purified by 20% polyacrylamide denaturing gel electrophoresis (5% cross-link, 45% urea (by weight)). DNA manipulation, including enzymatic labeling, was carried out using standard procedures. T4 polynucleotide kinase, Klenow exo$^-$, inorganic pyrophosphatase, and dNTPs were obtained from New England Biolabs. [γ-^{32}P]-ATP was purchased from Amersham Pharmacia Biotech. Quantification of radiolabeled oligonucleotides was carried out using a Molecular Dynamics Storm 840 Phosphorimager equipped with ImageQuant Version 5.1 software.

4.2. Kinetics of Klenow exo$^-$ nucleotide incorporation

Prior to forming duplex-enzyme cocktails (2×), Klenow exo$^-$ (3.68 μM, 5 U/μL, 0.340 μL, 1.25 pmol) was treated with inorganic pyrophosphatase (40 U/mL, 8.5 μL, 0.34 U) at room temperature for 10 min. A 2× DNA-enzyme solution was then prepared containing hybridized DNA (100 nM), 10 nM Klenow exo$^-$, and 0.02% w/v BSA in 2× Klenow exo$^-$ buffer (20 mM Tris, pH 7.5, 10 mM $MgCl_2$, 15 mM DTT). The reactions were initiated by adding 5 μL of the 2× DNA-enzyme solution to 5 μL of a 2× dNTP solution. The reactions were carried out for fixed periods of time at room temperature and then quenched with 20 μL 95% formamide loading buffer containing EDTA. The samples were denatured by heating at 90 °C for 3 min and cooled immediately by placing on ice. The samples (4 μL) were loaded on a 20% denaturing PAGE gel. Kinetic parameters for the incorporation of nucleotides across β-C-Fapy·dG by Klenow exo$^-$ (Table 1) were determined under the following conditions: (dNTP, concentration range, reaction time) dCTP, 5–50 nM, 3.0 min; dGTP, 10–800 μM, 4.5 min; dTTP, 0.2–1.2 mM, 9.0 min; dATP, 5–100 μM, 1.0 min. Kinetic parameters for the extension of a primer one nucleotide past a β-C-Fapy·dG base pair (Table 2) were determined under the following conditions: (nucleotide opposite β-C-Fapy·dG, dATP concentration range, reaction time) dC, 10–1000 nM, 3.0 min; dG, 50–750 μM, 15.0 min, dA, 50–750 μM, 15.0 min, dT, 50–750 μM, 15.0 min. Kinetic parameters were calculated by a nonlinear fitting of plots of velocity versus [dNTP]. Velocities were calculated by using the equation $[100\ I_1/(I_0 + 0.5\ I_1)]t$, where t is the time of the reaction, I_1 is the amount of product formed (18 mer), I_0 is the amount of starting material, and I_1 is less then 25% of I_0.[31,32] The maximum magnitude of I_1 was controlled by adjusting the concentrations of dNTPs in the reactions, and the reaction times. All values are the averages of three independent experiments run in triplicate.

4.3. M13 genome construction and replication in *E. coli*

Each insert (**1**–**3**) was cloned into the M13mp7(L2) vector in triplicate as previously described.[33,34] Briefly, the insert (10 pmol) was phosphorylated and ligated into 6 pmol EcoRI-digested M13mp7(L2) using complementary scaffolds. After digestion of the scaffolds with T4 DNA polymerase, the vectors were purified by phenol extraction and Centricon 100 membrane filtration. AB1157 cells were pelleted, washed with ice-cold water, and finally resuspended in 10% glycerol. Competent AB1157 cells (100 μL) were electroporated with 1 pmol of the vector (~2.64 kV, 4.74 ms) and a portion was immediately plated with X-Gal and IPTG to assess lesion bypass, by comparing the number of blue transformants from the vector containing the lesion of interest to the number of transformants of a control vector containing dG at the appropriate position.

4.4. REAP assay to determine mutation frequency

Mutation analysis was carried out using the restriction endonuclease and postlabeling (REAP) assay which has previously been described.[33,34] Briefly, viral DNA was recovered from the growth medium and PCR amplified. Following digestion with BbsI and shrimp alkaline phosphatase, the DNA was ^{32}P-labeled and further digested with HaeIII. The desired 18mer product was purified using 20% denaturing PAGE and desalted using a G25 Sephadex column. Finally, the samples were digested with nuclease P1 and nucleotides were separated on a PEI cellulose TLC plate which was run with saturated $(NH_4)_2HPO_4$ and H_3PO_4, pH 5.8.

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