

Genetic control of predominantly error-free replication through an acrolein-derived minor-groove DNA adduct

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Acrolein, an α,β -unsaturated aldehyde, is generated *in vivo* as the end product of lipid peroxidation and from metabolic oxidation of polyamines, and it is a ubiquitous environmental pollutant. The reaction of acrolein with the N2 of guanine in DNA leads to the formation of γ -hydroxy-1- N^2 -propano-2' deoxyguanosine (γ -HOPdG), which can exist in DNA in a ring-closed or a ring-opened form. Here, we identified the translesion synthesis (TLS) DNA polymerases (Pols) that conduct replication through the permanently ring-opened reduced form of γ -HOPdG ((r) γ -HOPdG) and show that replication through this adduct is mediated via Rev1/Pol η -, Pol ι /Pol κ -, and Pol θ -dependent pathways, respectively. Based on biochemical and structural studies, we propose a role for Rev1 and Pol ι in inserting a nucleotide (nt) opposite the adduct and for Pols η and κ in extending synthesis from the inserted nt in the respective TLS pathway. Based on genetic analyses and biochemical studies with Pol θ , we infer a role for Pol θ at both the nt insertion and extension steps of TLS. Whereas purified Rev1 and Pol θ primarily incorporate a C opposite (r) γ -HOPdG, Pol ι incorporates a C or a T opposite the adduct; nevertheless, TLS mediated by the Pol ι -dependent pathway as well as by other pathways occurs in a predominantly error-free manner in human cells. We discuss the implications of these observations for the mechanisms that could affect the efficiency and fidelity of TLS Pols.

Acrolein, an α,β -unsaturated aldehyde, is a ubiquitous environmental pollutant formed by incomplete combustion of organic materials, and it occurs in the environment as a component of tobacco smoke and automobile exhaust. Moreover, acrolein is generated endogenously as the end product of lipid peroxidation and during the metabolic oxidation of polyamines (1–5). Acrolein adducts have been detected in DNA from a variety of tissues in rats, mice, and humans, indicating that this DNA adduct is generated *in vivo* from cellular reactions (1–3, 6, 7).

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The reaction of acrolein with the N² of guanine in DNA followed by ring closure results in the formation of the cyclic adduct γ -hydroxy-1- N^2 -propano-2'-deoxyguanosine (γ -HOPdG).² γ -HOPdG can exist in DNA in a ring-closed or ring-opened form (8–10). γ -HOPdG presents a strong block to synthesis by replicative DNA polymerases, and it is also inhibitory to synthesis by yeast and human Pol η , particularly at the nucleotide (nt) incorporation step (11, 12). DNA synthesis opposite γ -HOPdG, however, can be mediated by the sequential action of Pols ι and κ , in which Pol ι incorporates an nt opposite γ -HOPdG and Pol κ performs the subsequent extension step (12). In the presence of a reducing agent, γ -HOPdG can be trapped as the N^2 -(3-hydroxypropyl) 2'-deoxyguanosine adduct, which permanently stays in the ring-opened configuration (Fig. 1A). We refer to this reduced ring-opened form of γ -HOPdG as (r) γ -HOPdG. Biochemical studies with (r) γ -HOPdG have indicated that both yeast and human Pol η can carry out proficient synthesis opposite this adduct by inserting the correct nt and by extending synthesis (11, 13); Pol κ also performs proficient TLS opposite this adduct by inserting the correct nt and by extending synthesis (13). Pol ι incorporates an nt opposite (r) γ -HOPdG; however, it incorporates a T with only an \sim 3-fold reduced catalytic efficiency as compared with the correct C (13).

In this study, we identify the TLS Pols required for replicating through the (r) γ -HOPdG adduct in human cells and show that TLS opposite this adduct is mediated via three independent pathways that involve Rev1 and Pol η in one pathway, Pol ι and Pol κ in another pathway, and Pol θ in the third pathway, and TLS by all of these pathways is mediated in a predominantly error-free manner. We discuss the possible implications of these observations for TLS opposite (r) γ -HOPdG and other minor groove DNA lesions.

Results

Synthesis of (r) γ -HOPdG phosphoramidite

The reduced form of γ -HOPdG needed for these studies was introduced into synthetic DNA sequence during automated solid-phase DNA synthesis via a suitably protected phosphora-

²The abbreviations used are: γ -HOPdG, γ -hydroxy-1- N^2 -propano-2'-deoxyguanosine; (r) γ -HOPdG, permanently ring-opened reduced form of γ -HOPdG; nt, nucleotide(s); NC, negative control; TLC, thin layer chromatography; THF, tetrahydrofuran; DMT, dimethyltrityl; Pol, polymerase; TLS, translesion synthesis; siR, siRNA-resistant.

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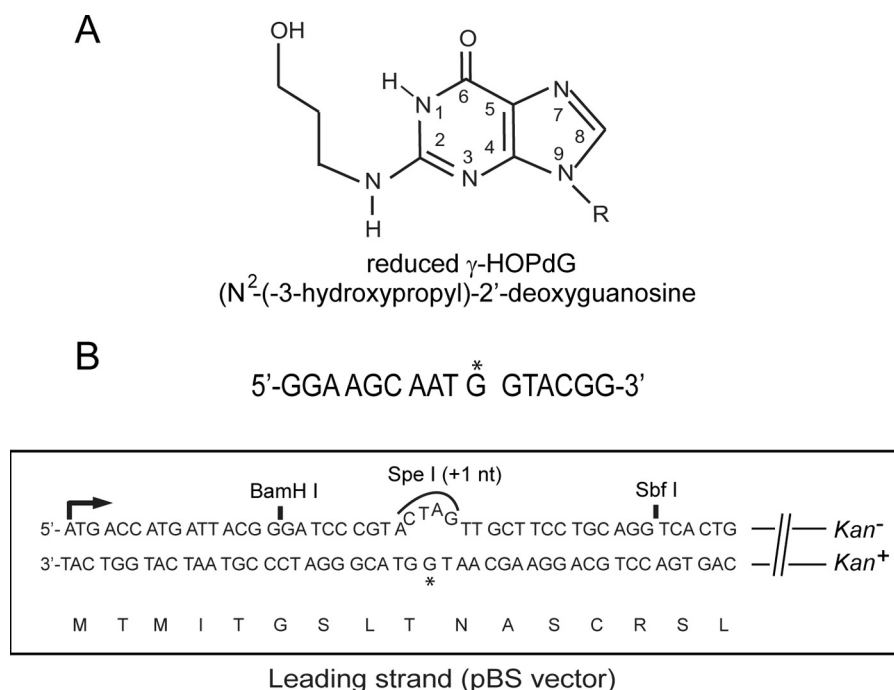


Figure 1. Assay for TLS opposite (r) γ -HOPdG. *A*, structure of reduced γ -HOPdG. *B*, the target 16-mer sequence containing (r) γ -HOPdG nt at G^{*} is shown on top, and the *lacZ'* sequence in the leading strand in the pBS vector containing the adduct at G^{*} is shown below. The (r) γ -HOPdG-containing DNA strand is in frame, and it carries the *Kan*⁺ gene. TLS through the adduct generates *Kan*⁺ blue colonies.

midite form of N^2 -hydroxypropanol-2'-deoxyguanosine. Previously, the reduced form of γ -HOPdG has been generated by treatment of γ -HOPdG-adducted DNA with sodium borohydride (11). Here, we describe a new method for the direct synthesis of the reduced form of γ -HOPdG rather than having to first synthesize γ -HOPdG and then converting it to the reduced form. However, site-specific direct alkylation of the N2 position of 2'-deoxyguanosine in DNA is particularly difficult because of multiple competing reactions (14). The method chosen here, although similar to γ -HOPdG modifications synthesized earlier by others (8–10), provides a more direct route for forming site-specific N^2 -(r) γ -HOPdG adduct during solid-phase DNA synthesis. This method is described in detail under “Experimental Procedures.” Briefly, based on the earlier method of Hofmann *et al.* (15) using 2'-deoxy-4-desmethylwyosine for direct N^2 -alkylation and conversion to N^2 -alkylated 2'-deoxyguanosine, this synthesis method was modified for alkylation with (3-bromopropoxy)-*t*-butyldimethylsilyl ether. *t*-butyldimethylsilyl-protected N^2 -(r) γ -HOPdG nucleoside was then converted to *t*-butyldimethylsilyl- N^2 -(r) γ -HOPdG phosphoramidite for use in solid-phase DNA synthesis. The *t*-butyldimethylsilyl protecting group of the propano ether side chain is stable to deprotection conditions during DNA synthesis and is easily converted to the free alcohol by *t*-butylammonium fluoride treatment before final reverse-phase HPLC purification of the (r) γ -HOPdG DNA sequence.

TLS Pols required for replicating through the (r) γ -HOPdG adduct in human cells

To identify the TLS Pols required for replicating through the (r) γ -HOPdG adduct (Fig. 1A), we examined the effects of siRNA depletions of TLS Pols on the frequency of TLS opposite

this lesion carried on the template for leading strand replication in the SV40-based duplex plasmid (Fig. 1B). In this plasmid, bidirectional replication initiates from a replication origin, and TLS through the DNA lesion generates *Kan*⁺ blue colonies (16, 17). The frequency of *Kan*⁺ blue colonies among total *Kan*⁺ colonies gives a highly reliable and repeatable estimate of TLS frequencies (16–18).

In normal human fibroblasts treated with control (NC) siRNA, TLS occurs with a frequency of ~35%, and siRNA depletion of the Rev3 catalytic or Rev7 accessory subunit of Pol ζ confers no reduction in TLS frequencies, indicating that Pol ζ plays no role in TLS opposite (r) γ -HOPdG (Table 1). siRNA depletion of Pol η , Pol ι , Pol κ , or Pol θ reduced the TLS frequency to ~22%, and siRNA depletion of Rev1 reduced the TLS frequency to ~11% (Table 1). To determine which of the Pols function together in the same TLS pathway and which Pols function in different pathways, we examined the effects of their simultaneous depletion on TLS frequency. Our observation that co-depletion of Pols ι and κ conferred a similar reduction in TLS frequency as observed upon their individual depletion indicated that these Pols function together in the same TLS pathway (Table 1). Co-depletion of Pol η with Pol ι or with Pol κ , however, led to a further reduction in TLS frequency to ~12% compared with that seen upon their individual depletion (~22%), indicating that Pol η functions in a TLS pathway independently of Pols ι and κ (Table 1).

To determine whether Pol θ functions together with Pol η or whether it functions in an independent pathway, we examined the effects of co-depletion of Pols η and θ . Our observation that co-depletion of these Pols confers a greater reduction in TLS frequency than that seen upon their individual depletion indi-

Table 1

Effects of siRNA knockdowns of TLS polymerases on the replicative bypass of the (r) γ -HOPdG lesion carried on the leading strand template in human fibroblasts

siRNA	No. of <i>Kan</i> ⁺ colonies	No. of blue colonies among <i>Kan</i> ⁺	TLS
			%
NC	669	235	35.1
Rev3	642	248	38.6
Rev7	486	185	38.1
Pol η	548	130	23.7
Pol ι	623	141	22.6
Pol κ	523	106	20.3
Pol θ	547	123	22.5
Rev1	412	46	11.2
Pol ι + Pol κ	396	96	24.2
Pol η + Pol ι	308	39	12.7
Pol η + Pol κ	326	41	12.6
Pol η + Pol θ	278	32	11.5
Pol ι + Pol θ	317	36	11.4
Pol κ + Pol θ	302	36	11.9

cated that Pol η and Pol θ function in different TLS pathways (Table 1). To verify that Pol ι /Pol κ -mediated TLS operates independently of Pol θ , we examined the effects of co-depletion of Pol ι or Pol κ with Pol θ . The observation that co-depletion of these Pols causes a greater reduction in TLS frequencies than that observed upon their individual depletion (Table 1) indicated that Pol ι /Pol κ and Pol θ function in different TLS pathways. From these observations, we infer that TLS opposite the (r) γ -HOPdG adduct is mediated by three independent pathways dependent on Pol ι /Pol κ , Pol η , and Pol θ , respectively.

To further verify this inference, we examined the effects of depletion of TLS Pols on the frequency of TLS opposite (r) γ -HOPdG in human XPV fibroblasts defective in Pol η (Table 2). In XPV cells treated with control siRNA, TLS opposite (r) γ -HOPdG occurs with a frequency of ~25%. As expected from the involvement of Pol η , Pols ι/κ , and Pol θ , in three independent pathways, respectively, TLS is reduced to ~12% upon depletion of Pol ι , Pol κ , or Pol θ or upon co-depletion of Pols ι and κ , and co-depletion of Pol ι or Pol κ with Pol θ reduced TLS frequencies to ~5% (Table 2). The residual level of TLS that remains in XPV cells co-depleted for Pol ι or Pol κ with Pol θ probably results from the low levels of TLS Pols (~2–3%) that persist in siRNA-treated cells (18, 19) (data not shown). Regardless of this consideration, the requirement of Pol ι /Pol κ and Pol θ for TLS through (r) γ -HOPdG in XPV cells provides further confirmatory evidence for the involvement of three independent pathways for replicating through this minor groove DNA adduct (Fig. 2).

Catalytic and non-catalytic roles of Rev1 in TLS opposite (r) γ -HOPdG in human cells

We have shown previously that Rev1 promotes replication through UV lesions together with Pols η , ι , and κ (18). To verify that for TLS opposite (r) γ -HOPdG also, Rev1 functions together with Y-family Pols, we analyzed the effects of simultaneous depletion of Rev1 with Pol η , Pol ι , or Pol κ on TLS frequencies. TLS frequency in Rev1-depleted cells is reduced to ~11%, a level similar to that in cells co-depleted for Pol η with Pol ι or for Pol η with Pol κ (Table 1). Our finding that simultaneous depletion of Rev1 with Pol η , Pol ι , or Pol κ confers no further reduction in TLS frequency than that observed upon

Table 2

Effects of siRNA knockdowns of TLS polymerases on the replicative bypass of the (r) γ -HOPdG lesion carried on the leading strand template in XPV human fibroblasts

siRNA	No. of <i>Kan</i> ⁺ colonies	No. of blue colonies among <i>Kan</i> ⁺	TLS
			%
NC	238	60	25.2
Pol ι	272	33	12.1
Pol κ	286	30	10.5
Pol θ	236	34	12.8
Pol ι + Pol κ	245	30	12.2
Pol ι + Pol θ	204	10	4.9
Pol κ + Pol θ	196	9	4.6

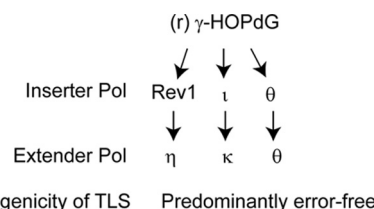


Figure 2. Pathways for replicating through the (r) γ -HOPdG adduct in human cells. Replication through the (r) γ -HOPdG adduct is mediated by three independent pathways in a highly error-free manner. See “Discussion” for roles of the TLS Pols in these pathways.

Rev1 depletion alone indicates that Rev1 functions together with Pols η , ι , and κ for TLS opposite (r) γ -HOPdG (Table 3). Because co-depletion of Rev1 with Pol θ leads to a much greater reduction in TLS frequency than observed upon Rev1 depletion, Pol θ -mediated TLS occurs independently of Rev1 (Table 3).

Among the TLS Pols, Rev1 is highly specialized for incorporating a C opposite template G (20, 21). Crystal structures of yeast and human Rev1 have shown that template G is evicted from the DNA helix into a solvent-filled cavity, and an Arg residue in Rev1 forms hydrogen bonds with the incoming C (22, 23). This protein-template-directed mechanism of nt incorporation is highly suited to allow Rev1 to insert a C opposite the minor groove N2-dG adducts. Therefore, to confirm that Rev1 DNA polymerase activity was required for TLS opposite (r) γ -HOPdG, we expressed the 3 \times FLAG-siRNA-sensitive wildtype Rev1 (Fig. 3A) or the siRNA-resistant (siR) form of either the wildtype or the catalytic mutant D570A/E571A Rev1 in human cells (Fig. 3B) and examined the effects of this mutation on TLS opposite (r) γ -HOPdG. In Rev1 siRNA-treated cells expressing 3 \times FLAG-wildtype Rev1, Rev1 was efficiently depleted (Fig. 3A), and the siRNA-resistant form of wildtype Rev1 or catalytically inactive (AA) Rev1 was not affected (Fig. 3B). As shown in Table 4, TLS in human fibroblast cells treated with Rev1 siRNA and carrying the vector control occurs with a frequency of ~12%, and TLS occurs with a similar frequency in cells expressing the siRNA-sensitive wildtype Rev1 protein. By contrast, in cells expressing the siRNA-resistant WT Rev1, TLS is restored near to WT levels (~32%), indicating that expression of WT Rev1 complements the TLS deficiency caused by Rev1 depletion. Expression of the siRNA-resistant Rev1 D570A/E571A catalytic mutant in cells from which genomic Rev1 has been depleted, however, reduced the TLS frequency to ~22%, which suggested that in addition to its non-catalytic role in TLS in Pol η and Pol ι /Pol κ pathways, Rev1 DNA polymerase

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Table 3

Effects of co-depletion of Rev1 with Y-family Pols or with Pol θ on TLS opposite (r) γ -HOPdG carried on the leading stand template in human fibroblasts

siRNA	No. of <i>Kan</i> ⁺ colonies	No. of blue colonies among <i>Kan</i> ⁺	TLS
NC	669	235	%
Rev1	412	46	35.1
Rev1 + Pol η	381	43	11.2
Rev1 + Pol ι	394	45	11.3
Rev1 + Pol κ	426	47	11.4
Rev1 + Pol θ	468	20	11.0
			4.3

activity is required for TLS opposite (r) γ -HOPdG. To determine whether Rev1 DNA polymerase activity contributes to TLS in the Pol η -dependent pathway, we expressed the siRNA-resistant Rev1 catalytic mutant in cells from which both Rev1 and Pol η have been depleted. Our observation that TLS frequencies remain the same in Pol η -proficient or -deficient cells (~22%) expressing the Rev1 catalytic mutant indicates that Rev1 polymerase activity functions in TLS opposite (r) γ -HOPdG in conjunction with Pol η (Table 4). Altogether, the genetic data in Tables 1–4 support the conclusion that replication through the (r) γ -HOPdG adduct is mediated via three different pathways in which Rev1 and Pol η function in one pathway, Pol ι and Pol κ function in another pathway, and Pol θ mediates the third pathway (Fig. 2).

Low mutagenicity of TLS opposite (r) γ -HOPdG

We find that TLS opposite (r) γ -HOPdG occurs in a highly error-free manner, as only ~1% of TLS products harbor mutations. Among the ~400 TLS products sequenced from cells treated with control siRNA, we observed four mutations wherein an A or T was incorporated opposite the lesion site (Table 5). TLS opposite (r) γ -HOPdG incurred the same low mutagenicity regardless of whether Pol η , Pol ι , Pol κ , or Pol θ was depleted, indicating that all of these Pols function in a predominantly error-free manner opposite this DNA lesion (Table 5).

Roles of TLS Pols in DNA synthesis opposite (r) γ -HOPdG

Rev1 inserts a C opposite the ring-closed form of γ -HOPdG with the same catalytic efficiency and fidelity as it inserts a C opposite undamaged G (24). As expected, in the presence of all four dNTPs, Rev1 inserts a C opposite (r) γ -HOPdG and does not carry out extension of synthesis from the inserted nt (data not shown). For the Rev1 pathway, based on this biochemical observation, genetic data shown in Table 4, and structural observations (22, 23), we suggest a role for Rev1 at the nt insertion step and for Pol η at the extension step of TLS opposite (r) γ -HOPdG. For the Pol ι /Pol κ pathway, we have previously reported biochemical evidence indicating that Pol ι incorporates a C or a T opposite this lesion (13). Based on biochemical (13) and structural considerations (25, 26), we suggest a role for Pol ι at the nt insertion step of TLS opposite (r) γ -HOPdG and for Pol κ in extending synthesis from the nt inserted by Pol ι opposite the lesion site (27). By contrast to the highly error-prone role of Pol ι in incorporating a T nt opposite (r) γ -HOPdG with only an ~3-fold reduced catalytic efficiency compared with a C (13), Pol θ primarily incorporates a C opposite (r) γ -HOPdG (Fig. 4). Although Pol θ can carry out the subsequent

extension of synthesis, it is considerably blocked in extending synthesis from the nt inserted opposite the adduct (Fig. 4).

Discussion

Our genetic observations indicating that replication through the (r) γ -HOPdG adduct is mediated by three independent pathways, composed of Rev1/Pol η , Pol ι /Pol κ , and Pol θ , respectively (Fig. 2), differ strikingly from the roles predicted for TLS Pols from biochemical studies. Thus, from the proficient ability of Pol η and Pol κ to perform TLS opposite (r) γ -HOPdG by both inserting a correct nt opposite the adduct and then by extending synthesis from the inserted nt, one would have expected these two Pols to conduct replication through the adduct via two independent pathways (11, 13). However, rather than acting by themselves in their respective pathway, we find that in human cells TLS opposite (r) γ -HOPdG is performed by Rev1 and Pol η in one pathway and by Pol ι and Pol κ in the other pathway. To explain this discordance in the roles for TLS Pols indicated from biochemical studies *versus* those inferred from genetic studies in human cells, we suggest that replication through DNA lesions in human cells is performed by TLS Pols as components of multiprotein assemblies and that the proficiency and fidelity of TLS Pols are modulated in these assemblies.

Roles of TLS Pols in Rev1/Pol η and Pol ι /Pol κ pathways

Rev1/Pol η pathway—The protein-template-directed mechanism of nt incorporation provides Rev1 the ability to insert a C opposite *N*²-dG adducts that protrude into the DNA minor groove (22, 23). We find that in the presence of all four dNTPs, Rev1 selectively incorporates a C opposite (r) γ -HOPdG, but it fails to extend synthesis from the inserted nt (data not shown). Based on the ability of Rev1 for nt insertion and on the ability of Pol η to extend synthesis from the inserted nt, we suggest that TLS in the Rev1/Pol η pathway is mediated by the sequential action of Rev1 and Pol η , in which following the incorporation of a C opposite (r) γ -HOPdG by Rev1, Pol η would extend synthesis from the inserted nt. As judged from the proficient ability of Rev1 for incorporating a C and of Pol η for extending synthesis from it, Rev1/Pol η -mediated TLS opposite (r) γ -HOPdG would occur in an error-free manner in human cells.

Pol ι /Pol κ pathway—Based on biochemical and structural considerations, we suggest a role for Pol ι at the nt incorporation step and for Pol κ at the extension step of TLS. Pol ι differs greatly from other DNA Pols in the ways it synthesizes DNA opposite template purines and pyrimidines (28–31). Pol ι incorporates nt with a much higher catalytic efficiency and fidelity opposite template purines than opposite template pyrimidines, and among template purines, Pol ι shows a higher efficiency and fidelity opposite template A than opposite template G (28–31). Pol ι incorporates nt opposite purine templates by pushing the template A or G into a *syn* conformation, which then forms a Hoogsteen base pair with the incoming T or C, respectively (25, 26, 32). The ability of Pol ι to push the (r) γ -HOPdG adduct into a *syn* conformation would allow the adduct to form a Hoogsteen base pair with the correct C or the incorrect T nt, and our previous biochemical studies with Pol ι have shown that it proficiently incorporates a C or a T opposite (r) γ -HOPdG (13).



Figure 3. Stable expression of wildtype Rev1 and catalytic mutant of Rev1 in human fibroblasts. Normal human fibroblasts (GM637) expressing 3 \times FLAG-siRNA-sensitive WT Rev1 (A) or 3 \times FLAG-siRNA-resistant (siR) WT Rev1 or 3 \times FLAG-siR D570A/E571A (AA) mutant Rev1 (B) were treated with siRNA for 48 h. The efficiencies of Rev1 depletion and Rev1 expression were determined by Western blot analysis with anti-FLAG antibody. Actin was used as the loading control.

Table 4

Effect of siRNA knockdown of Rev1 alone or together with Pol η on TLS opposite (r) γ -HOPdG present on the leading strand template in wildtype human fibroblasts carrying a vector expressing the siRNA-resistant (siR) form of wildtype or catalytically inactive Rev1

siRNA	Vector-expressing	No. of <i>Kan</i> ⁺ colonies	No. of blue colonies among <i>Kan</i> ⁺	TLS %
Rev1	Vector control	325	39	12.0
Rev1	FLAG-WT-Rev1	384	45	11.7
Rev1	FLAG-WT-siR-Rev1	326	104	31.9
Rev1	FLAG-D570A/E571A-siR-Rev1	358	79	22.1
Pol η + Rev1	FLAG-WT-Rev1	348	40	11.5
Pol η + Rev1	FLAG-WT-siR-Rev1	316	75	23.7
Pol η + Rev1	FLAG-D570A/E571A-siR-Rev1	372	81	21.8

Table 5

Effects of siRNA knockdowns of TLS polymerases on the frequencies of nucleotides inserted opposite (r) γ -HOPdG carried on the leading strand template in human fibroblasts

siRNA	No. of <i>kan</i> ⁺ blue colonies sequenced	Nucleotide inserted				Mutation frequency %
		A	G	C	T	
NC siRNA	386	2	0	382	2	1.0
Pol η	384	2	0	381	1	0.8
Pol ι	288	2	0	284	2	1.4
Pol κ	196	1	0	194	1	1.0
Pol θ	196	0	0	194	2	1.0

Biochemical and structural studies have shown that Pol κ is highly adapted for extending synthesis opposite from minor groove DNA lesions (13, 27); hence, Pol κ could extend synthesis from the nt inserted by Pol ι .

The propensity of Pol ι for incorporating a T opposite (r) γ -HOPdG (13) would suggest that TLS mediated by the sequential action of Pols ι and κ would occur in a highly error-prone manner. However, TLS opposite this adduct by this pathway as well as by the other two pathways operates in a predominantly error-free manner in human cells. Thus, the inference derived from biochemical studies for the highly error-prone role of Pol ι in TLS opposite (r) γ -HOPdG does not extend to TLS that occurs in human cells.

Non-catalytic role of Rev1 in TLS—Previously, we showed that for TLS opposite UV-induced CPDs and 6-4 photoproducts, Rev1 functions as an indispensable scaffolding component of Y-family Pols η , ι , and κ and that it does not function together with Pol ζ (18). For TLS opposite (r) γ -HOPdG, we find that Rev1 functions together with Y-family Pols η , ι , and κ and not with Pol θ . Presumably, Rev1 bound to Pol η , Pol ι , or Pol κ plays a crucial role in the formation of multiprotein assembly. It would be of great interest to identify the components of such assemblies, to determine the roles they play in the efficiency

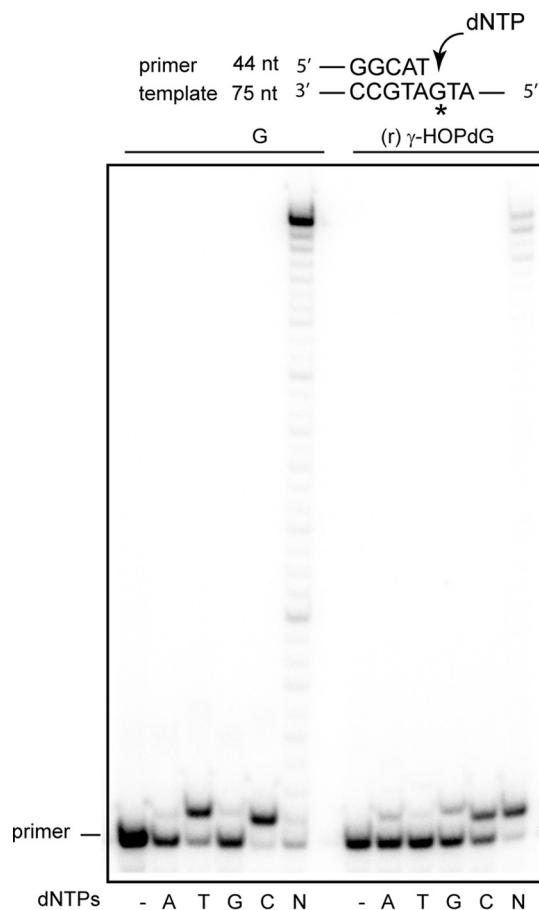


Figure 4. Deoxynucleotide incorporation opposite undamaged G and (r) γ -HOPdG by Pol θ . Pol θ (1 nM) was incubated with DNA substrate (10 nM) and 10 μ M each of a single deoxynucleotide dATP, dTTP, dGTP, or dCTP, or all four deoxynucleotides (N) for 10 min at 37 $^{\circ}$ C. DNA substrate is shown above the gel where an asterisk denotes an undamaged G or an (r) γ -HOPdG template base.

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and fidelity of TLS Pols opposite DNA lesions, and to see whether the composition of multiprotein assemblies for Y-family Pols and for other TLS Pols differs for different types of DNA lesions. For elucidating the highly error-free role of Pol ι in TLS opposite (r) γ -HOPdG, it would be important to decipher how Rev1 together with other components of the multiprotein assembly modulates the Pol ι active site opposite this lesion such that its proficiency for the insertion of the incorrect nt T is greatly reduced and its ability to insert the correct nt C is highly enhanced.

Possible roles of multiprotein assemblies in activation or inhibition of TLS Pols in the Rev1/Pol η and Pol ι /Pol κ pathways—We surmise that in the Rev1/Pol η and Pol ι /Pol κ pathways, in which Rev1 and Pol ι would act at the nt incorporation step of TLS and Pol η and Pol κ would carry out the extension step in the respective pathway, the proficient ability of Pol η or Pol κ to insert an nt opposite (r) γ -HOPdG would be inhibited in the multiprotein ensemble of these Pols. Such inhibitory effects of multiprotein assemblies on TLS Pols, such as Pol η and Pol κ , which can replicate through (r) γ -HOPdG with a high catalytic efficiency and fidelity (11, 13), raise the intriguing possibility that multiprotein assemblies of TLS Pols have evolved to become highly specialized for replicating through particular types of DNA lesions. Thus, for replication through the large variety of minor groove N^2 -dG adducts, the Y-family Pols may employ an identical or a very similar multiprotein assembly in which the various components coordinate TLS in a highly efficient and relatively error-free manner.

Role of Pol θ in TLS

By contrast to the requirement of different inserter and extender Pols for TLS mediated by Rev1/Pol η and Pol ι /Pol κ opposite (r) γ -HOPdG, our genetic studies indicate that Pol θ would act at both the nt insertion and the subsequent extension step of TLS opposite this DNA lesion. How could Pol θ incorporate the correct nt opposite this minor groove adduct? One possibility is that in the Pol θ active site, the N^2 -dG adduct stays in the *anti* conformation and forms a Watson–Crick base pair with the incoming C, and Pol θ then extends synthesis from this base pair. An alternative possibility is that this minor groove DNA adduct is accommodated in a *syn* conformation in the Pol θ active site, and it forms a Hoogsteen base pair with the incoming C residue. Such a mode of accommodating minor groove DNA lesions in its active site could provide Pol θ a greater latitude in its ability to replicate through the diverse array of DNA adducts that form at the highly reactive N2 group of a deoxyguanine.

The high fidelity of TLS in human cells

In human cells, TLS opposite (r) γ -HOPdG occurs with a high fidelity, generating only \sim 1% of mutations, similar to that observed for TLS opposite DNA lesions, such as *cis-syn* TT dimer (16), 6-4 TT photoproduct (17), thymine glycol (33), N^3 -methyl adenine (34), and N^3 -methyl adenine (35), where at most \sim 1–2% of the TLS products harbor mutations. This is despite the fact that TLS Pols synthesize DNA opposite DNA lesions with a poor fidelity. For example, although Pol ι incorporates a T opposite the (r) γ -HOPdG adduct with only a some-

what reduced catalytic efficiency as compared with a C (13), TLS opposite this adduct occurs in a predominantly error-free manner in human cells. The evidence for predominantly error-free TLS opposite a number of DNA lesions in human cells strongly suggests that TLS mechanisms have been adapted to act in highly specialized and predominantly error-free ways.

Experimental procedures

Synthesis of (r) γ -HOPdG phosphoramidite

All solvents and reagents were purchased from either Fisher or Sigma-Aldrich with the exception of bromine, which was purchased from Fluka. Anhydrous solvents were additionally treated with molecular sieves and measured to be less than 50 ppm water content by Karl–Fischer titration before use.

All NMR data were obtained on a Bruker Ultrashield 300, Avance II, 300 MHz. Positive-mode mass spectroscopy data were obtained on a Sciex 5800 MALDI TOF/TOF.

The method of Hofmann *et al.* (15) via 2'-deoxy-4-desmethylwytosine as the protected intermediate is preferred for the direct alkylation of the N2 position of guanosine by 3-bromo-1-(*t*-butyldimethylsilyl)-propanoether. For this synthesis, bromoacetone was freshly prepared using the method of Levene *et al.* (36). 3-Bromo-1-(*t*-butyldimethylsilyl)-propanoether was prepared as described (37). Synthesis of (r) γ -HOPdG phosphoramidite comprised the six steps, 1–6, described below and outlined in Fig. 5.

Bromoacetone (1)—Following the method of Levene (36), acetone (50 ml, 0.676 mol) was added to a three-neck flask containing water (160 ml, 8.855 mol). Acetic acid (38 ml, 0.661 mol) was added, and the mixture was heated to 70–80 °C. Bromine (37.4 ml, 0.730 mol) was added dropwise over 1–1.5 h. When the solution was completely decolorized, it was diluted with cold water (80 ml). The mixture was cooled to 10 °C and neutralized with sodium carbonate (which turns yellow/orange; there are two layers). The oil was separated and dried with anhydrous calcium chloride. The product was distilled using a house vacuum; the fraction at 65–70 °C (brown) was the main fraction (25.88 g). ^1H NMR showed mostly product, with some dibromo side products present. A second distillation gave pure product as a pale yellow liquid (22.54 g, 24.34%). ^1H NMR (CDCl_3), δ (ppm): 3.87 (CH_2); 2.34 (CH_3).

2'-Deoxy-4-desmethylwytosine (2)—Using the protection method of Hofmann *et al.* (15), 2'-deoxyguanosine monohydrate (1) (10 g, 35.1 mmol) was coevaporated with 20 ml of anhydrous pyridine twice and then dissolved in anhydrous dimethyl sulfoxide (120 ml) under argon with stirring. Sodium hydride (60% suspension) (1.472 g, 36.8 mmol) was added, and the mixture was stirred at room temperature under argon for 1 h. Bromoacetone (5.04 g, 36.8 mmol) was added, and stirring continued under argon for 1 h (the solution immediately turned brown). Ammonium hydroxide (60 ml, 889 mmol) was added, and the mixture was stirred an additional 2 h at room temperature and then evaporated to \sim 60 ml. Acetone (60 ml) was added, and the mixture was poured into a solution of acetone (800 ml) and ether (200 ml). The mixture was stirred at 0 °C for 3 h (monitored by TLC using 10% MeOH/ CH_2Cl_2 , and developed using 1% anisaldehyde/ethanol spray with heating). When

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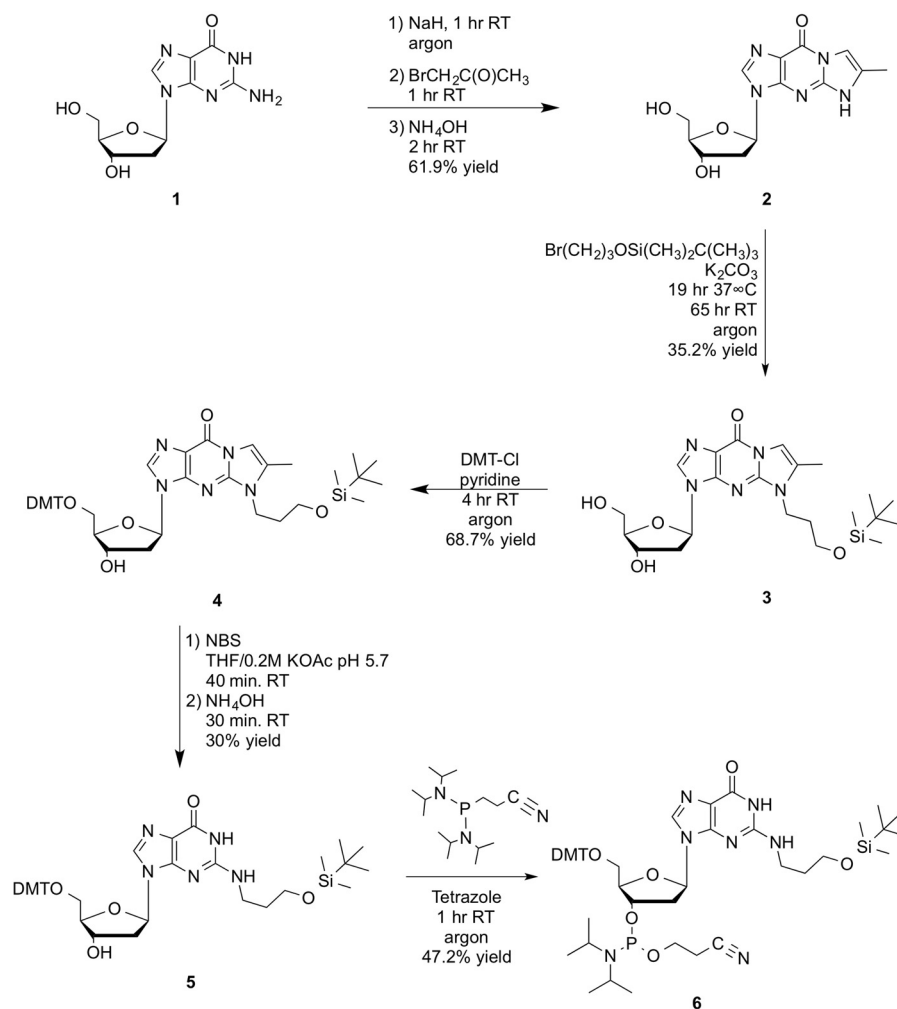


Figure 5. Synthesis of (*r*) γ -HOPdG phosphoramidite. Synthesis of (*r*) γ -HOPdG phosphoramidite comprised six steps beginning with protection of the N1-N2 positions of deoxyguanosine (1) with bromoacetone to give 2'-deoxy-4-desmethylwyosine (2). Alkylation of 2 with (3-bromopropoxy)-*t*-butyldimethylsilyl ether gave 3-(*t*-butyldimethylsilyloxy)propyl-2'-deoxy-4-desmethylwyosine (3). Protection of the 5'-hydroxyl group of 3 using dimethoxytrityl chloride gave 5'-DMT-(3-(*t*-butyldimethylsilyloxy)propyl)-2'-deoxy-4-desmethylwyosine (4). Deprotection of the N1-N2 positions of 4 gave 5'-DMT-(3-(*t*-butyldimethylsilyloxy)propyl)-2'-deoxyguanosine (5). Phosphoramidite (6) was prepared using the bis-*N,N,N',N'*-diisopropyl- β -cyanoethyl phosphitylation method.

the reaction showed no further progress, the mixture was transferred to a 2-liter separatory funnel, and the layers were separated. The resulting lower layer of oil was washed further with ether (2 \times 500 ml), separated, and evaporated. Water (900 ml) was added, and the crude product was preabsorbed onto silica gel (10 g) by evaporation followed by coevaporation three times with 200 ml of ethanol. The 2'-deoxy-4-desmethylwyosine absorbed silica was added to a silica gel column (200 g) and eluted using a step gradient elution of 100% CH₂Cl₂ to 90% CH₂Cl₂/MeOH to yield pure product (6.62 g, 21.68 mmol, 61.9% yield). ¹H NMR (DMSO-*d*₆) δ (ppm): 2.23 (d, *J* = 1.2 Hz, 3H, CH₃); 2.57 (m, 2H, H2'); 3.53 (m, 2H, H5'); 3.83 (m, 1H, H4'); 4.35 (m, 1H, H3'); 4.93 (t, *J* = 5.4 Hz, 1H, 5'-OH); 5.27 (dd, *J* = 4.2, 2.1 Hz, 1H, 3'-OH); 6.21 (t, *J* = 6 Hz, 1H, H1'); 7.30 (s, 1H, H9); 8.07 (s, 1H, H2); 12.30 (s, 1H, NH). ES-MS+ 306.0 g/mol, exact mass = 305.11 g/mol.

(3-Bromopropoxy)-*t*-butyldimethylsilyl ether—Following the preparation method of Choi *et al.* (37), fresh 3-bromo-1-propanol (2 ml, 22.12 mmol) was dissolved in dry CH₂Cl₂ (8 ml). Triethylamine (3.39 ml, 24.33 mmol) and 4-(dimethylamino)pyridine (0.29 g, 2.433 mmol) were added under argon with

stirring. The solution was cooled to 0 °C under argon, and *t*-butyldimethylsilyl chloride (3.33 g, 22.12 mmol) was added. The solution was allowed to come to room temperature while stirring for 3 h under argon (monitored by TLC using 15% ethyl acetate/hexane with anisaldehyde detection). The reaction was quenched with water (5 ml) and allowed to stir for 15 min. The crude product was extracted from the aqueous solution with CH₂Cl₂, (3 \times 10 ml). The combined organic extracts were dried over Na₂SO₄, filtered, and evaporated. Crude (3-bromopropoxy)-*t*-butyldimethylsilyl ether was purified on a silica gel column using 15% ethyl acetate/hexane to yield 4.52 g, 17.85 mmol, 81% yield, as a yellow oil. ¹H NMR (CDCl₃) δ (ppm): 0.089 (s, 3H, SiCH₃); 0.094 (s, 3H, SiCH₃); 0.91 (s, 9H, *t*-Bu); 2.06 (m, 2H, BrCH₂CH₂CH₂O); 3.53 (t, *J* = 5.7 Hz, 2H, BrCH₂CH₂CH₂O); 3.75 (t, *J* = 5.7 Hz, 2H, BrCH₂CH₂CH₂O).

3-(*t*-Butyldimethylsilyloxy)propyl-2'-deoxy-4-desmethylwyosine (3)—Using the alkylation method of Hofmann *et al.* (15), 2'-deoxy-4-desmethylwyosine (1 g, 3.28 mmol) was dissolved in anhydrous *N,N*-dimethylformamide (13 ml) under an argon atmosphere. Anhydrous potassium carbonate (0.475 g, 3.44 mmol) and (3-bromopropoxy)-*t*-butyldimethylsilyl ether

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(2.074 g, 8.19 mmol) were added. The mixture was stirred at 37 °C for 19 h (turned dark reddish brown) and followed by TLC (10% MeOH/CH₂Cl₂, anisaldehyde detection). A second addition of (3-bromopropoxy)-*t*-butyldimethylsilyl ether (0.83 g, 3.28 mmol) was added, and the reaction was stirred at room temperature for an additional 65 h (followed by TLC using 15% MeOH/CHCl₃). The reaction mixture was filtered through celite, and the celite pad was washed with additional warm dimethylformamide until the washings showed no UV absorbance at 254 nm. The filtrates and washings were combined and evaporated to give crude product that was purified on a silica gel column using an elution gradient of chloroform with 0.2% triethylamine to 30% methanol/chloroform with 0.2% triethylamine to give pure 3-(*t*-butyldimethylsilyloxy)propyl-2'-deoxy-4-desmethylwyosine (550 mg, 1.152 mmol, 35.2% yield). ¹H NMR (CDCl₃) δ (ppm): .03 (s, 6H, Si-CH₃); .87 (s, 9H, *t*-Bu); 1.96 (m, 2H, NHCH₂CH₂CH₂O); 2.33 (s, 3H, CH₃); 2.44 (m, 1H, H-2'); 2.81 (m, 1H, H-2'); 3.55 (m, 2H, NHCH₂CH₂CH₂O); 3.64 (m, 2H, H-5'); 3.71 (m, 2H, NHCH₂CH₂CH₂O); 3.87 (m, 1H, H4'); 4.15 (m, 2H, NHCH₂CH₂CH₂O); 4.77 (m, 1H, H-3'); 6.37 (t, *J* = 6.6 Hz, 1H, H-1'); 7.32 (s, 1H, H9); 8.04 (s, 1H, H-2). ES-MS+: 478.2/mol, exact mass = 477.24 g/mol.

5'-DMT-(3-(*t*-butyldimethylsilyloxy)propyl)-2'-deoxy-4-desmethylwyosine (**4**)—3-(*t*-Butyldimethylsilyloxy)propyl-2'-deoxy-4-desmethylwyosine (700 mg, 1.47 mmol, from two prepared batches) was coevaporated twice with anhydrous pyridine (5 ml) and then redissolved in anhydrous pyridine (15 ml) under argon with stirring. Dimethoxytrityl chloride (0.546 g, 1.61 mmol) was added, and the reaction was stirred at room temperature for 3 h under argon (monitored by TLC 10% MeOH/CH₂Cl₂ containing 0.1% triethylamine, anisaldehyde detection). A second addition of fresh dimethoxytrityl chloride (0.25 g, 0.74 mmol) was added, and after an additional 1-h reaction, TLC showed completion. The reaction was evaporated of solvent and redissolved in 15 ml of CH₂Cl₂. The organic solution was washed with saturated NaHCO₃ (10 ml), followed by water (10 ml), and the organic layer was dried over Na₂SO₄, filtered, and evaporated. The 5'-DMT-(3-(*tert*-butyldimethylsilyloxy)propyl)-2'-deoxy-4-desmethylwyosine was purified on a silica gel column using a step gradient of CH₂Cl₂ with 0.1% triethylamine to 10% MeOH/CH₂Cl₂ with 0.1% triethylamine to give pure product (0.794 g, 1.01 mmol, 68.7% yield). ¹H NMR (CDCl₃) δ (ppm): 0.08 (s, 6H, SiCH₃); 0.92 (s, 9H, *t*-Bu); 1.86 (m, 2H, NHCH₂CH₂CH₂O); 2.45 (s, 3H, CH₃); 2.5 (m, 1H, H-2'); 2.68 (m, 1H, H-2'); 3.29 (m, 1H, NHCH₂CH₂CH₂O); 3.4 (m, 1H, NHCH₂CH₂CH₂O); 3.62 (t, *J* = 5.7 Hz, 2H, H5'); 3.72 (m, 1H, H4'); 3.77 (s, 6H, OCH₃); 4.1 (m, 2H, NHCH₂CH₂CH₂O); 4.64 (m, 1H, H-3'); 6.39 (t, *J* = 6.3 Hz, 1H, H-1'); 6.78 (dd, *J* = 2.4, 4.5 Hz, 4H, DMT-meta H's); 7.26 (m, 7H, DMT-ortho H's, Bz and H9); 7.38 (m, 2H, DMT-ortho H's); 7.7 (s, 1H, H-2). ES-MS+: 780.2 g/mol, exact mass = 779.37 g/mol.

5'-DMT-(3-(*t*-butyldimethylsilyloxy)propyl)-2'-deoxyguanosine (**5**)—Deprotection was performed following the methods of Casale and McLaughlin (38), Hofmann *et al.* (15), and Boryski and Ueda (39). 5'-DMT-(3-(*t*-butyldimethylsilyloxy)propyl)-2'-deoxy-4-desmethylwyosine (0.79 g, 0.99 mmol) was dissolved in a mixture of THF (27 ml) and 0.2 M aqueous potassium acetate, pH 5.7 (11 ml). *N*-Bromosuccinimide (0.216 g,

1.19 mmol) was added, and the mixture was stirred at room temperature for 40 min. (the solution turns light blue and then pale yellow as the reaction proceeds). Ammonium hydroxide (5.7 ml) was added, and stirring was continued for 30 min (monitored by TLC using 5% MeOH/CH₂Cl₂ with 0.1% triethylamine). When the reaction was complete, solvent was evaporated, and the crude product was purified by silica gel column using a step gradient of 100% CH₂Cl₂ to 5% MeOH/CH₂Cl₂ with a 1% solution of triethylamine in H₂O to give pure 5'-DMT-(3-(*t*-butyldimethylsilyloxy)propyl)-2'-deoxyguanosine (226 mg, 0.305 mmol, 30% yield). ¹H NMR (CDCl₃) δ (ppm): 0.010 (s, 6H, Si-CH₃); 0.85 (s, 9H, *t*-Bu); 1.78 (m, 2H, NHCH₂CH₂CH₂O); 2.51 (m, 1H, H2'); 2.70 (m, 1H, H2'); 3.33 (m, 2H, NHCH₂CH₂CH₂O); 3.61 (t, *J* = 5.7 Hz, 2H, H5'); 3.67 (m, 1H, H4'); 3.70 (s, 6H, OCH₃); 4.08 (m, 2H, NHCH₂CH₂CH₂O); 4.68 (m, 1H, H3'); 6.23 (t, *J* = 6 Hz, 1H, H1'), 6.73 (m, 4H, DMT meta H's); 7.16 (m, 7H, DMT-ortho H's and Benzyl H's); 7.33 (m, 2H, DMT-ortho H's); 7.64 (s, 1H, H2) ES-MS+: 742.2 g/mol; exact mass 741.36 g/mol.

DMT-5-(3-(*tert*-butyldimethylsilyloxy)propyl)-2'-deoxyguanosine-3'-(*N,N*,-diisopropyl- β -cyanoethyl-phosphoramidite, [(r) γ -HOPdG phosphoramidite] (**6**))—5'-DMT-(3-(*t*-butyldimethylsilyloxy)propyl)-2'-deoxyguanosine (0.30 g, 0.40 mmol) was dissolved in dry methylene chloride (11 ml) in an argon atmosphere in a sealed round-bottom flask with rubber stopper. 2-Cyanoethyl-*N,N,N',N'*-tetraisopropylphosphine (0.177 ml, 0.55 mmol) and tetrazole/THF solution (1.2 ml, 0.51 mmol tetrazole) were added simultaneously via syringes under argon. The solution was stirred at room temperature for 1 h (monitored by TLC using prerun ethyl acetate, 0.1% triethylamine TLC plates, dried, spotted, and eluted with the same solvent and detected with anisaldehyde). A second addition of 2-cyanoethyl-*N,N,N',N'*-tetraisopropylphosphine (0.128 ml, 0.40 mmol) and tetrazole solution (0.938 ml, 0.40 mmol) were added, again simultaneously with stirring, and the reaction was continued for 30 min. The reaction was diluted with CH₂Cl₂ (11 ml), and the product was washed with 5% aqueous sodium bicarbonate (10 ml) followed by washing with an aqueous brine solution (10 ml). The organic layer was separated, dried over sodium sulfate, and filtered. Triethylamine (20 μ l) was added to the filtrate, and the solution was evaporated. The crude 5'-DMT-(3-(*t*-butyldimethylsilyloxy)propyl)-2'-deoxyguanosine phosphoramidite mixture was purified on a silica gel column using a step gradient of 100% ethyl acetate with 0.1% triethylamine to 10% MeOH/ethyl acetate with 0.1% triethylamine as eluant. TLC using prerun ethyl acetate, 0.1% triethylamine TLC plates, dried, spotted, and eluted with the same solvent gave two isomeric phosphoramidite products that were detected with anisaldehyde. Dittmer reagent was utilized to detect any excess 2-cyanoethyl-*N,N,N',N'*-tetraisopropylphosphine agent separated from the product isomers. The phosphoramidite products were combined to yield 120 mg (0.127 mmol, 47.2% yield) ¹H NMR (CD₃CN) δ (ppm): 0.04 (s, 6H, SiCH₃); 0.89 (s, 9H, *t*-Bu); 1.20 (m, 12H, NCH(CH₃)₂); 1.74 (m, 2H, NHCH₂CH₂CH₂O); 2.52 (m, 3H, H2' and CH₂CH₂CN); 2.63 (t, *J* = 6 Hz, 2H, NCH(CH₃)₂); 2.94 (m, 1H, H2'); 3.30 (m, 4H, H5' and NHCH₂CH₂CH₂O); 3.67 (m, 4H, CH₂CH₂CN and NHCH₂CH₂CH₂O); 3.73 (s, 6H, OCH₃);

4.16 m, 1H, H4'); 4.74 (m, 1H, H3'); 6.21 (t, $J = 6.6$ Hz, 1H, H1'); 6.78 (m, 4H, DMT meta H's); 7.24 (m, 7H, DMT ortho H's and bz H's); 7.39 (m, 2H, DMT ortho H's); 7.66 (s, 1H, H2). ^{31}P NMR (CD_3CN) δ (ppm): 148.79; 148.88.

Oligonucleotide syntheses

Oligonucleotides containing (r) γ -HOPdG were synthesized on a model 8909 Expedite DNA synthesizer using standard DNA synthesis chemistry. The (r) γ -HOPdG phosphoramidite was incorporated using an offline coupling method to conserve reagent. The oligonucleotides were deprotected using standard concentration ammonia deprotection, with an additional deprotection step using 0.1 M *t*-butylammonium fluoride, THF for removal of the *t*-butyldimethylsilyl chloride group from the propanohydroxyl side chain. Oligonucleotides were purified and analyzed by reverse-phase HPLC on a Beckman System Gold HPLC. Purified oligonucleotides were analyzed and confirmed by MALDI-MS on a Bruker Autoflex MALDI mass spectrophotometer.

Construction of plasmid vectors containing (r) γ -HOPdG

The in-frame target sequence of the *lacZ'* gene containing (r) γ -HOPdG is shown in Fig. 1B. Because the *lacZ'* sequence in the (r) γ -HOPdG-containing DNA strand is in-frame, it encodes functional β -gal; the opposite DNA strand harbors a SpeI restriction site containing a +1 frameshift, which makes it non-functional for β -gal. The (r) γ -HOPdG-containing strand carries the kanamycin gene (*Kan*⁺), whereas the other DNA strand has the *kan*⁻ gene (Fig. 1B). The detailed methods for the construction of lesion-containing SV40-based duplex plasmids have been published previously (16, 17).

Assays for translesion synthesis and mutation analyses of TLS products in human cells

The detailed methods for TLS assays have been described previously (16, 17). Briefly, human fibroblast GM637 cells are transfected with the particular siRNA, and after 48 h of incubation, the target vector DNA and siRNA (second transfection) are co-transfected. After 30 h of incubation, plasmid DNA is transfected into *E. coli* XL1-Blue supercompetent cells (Stratagene), and cells are plated on LB/Kan plates containing isopropyl-1-thio- β -D-galactopyranoside (GenDEPOT) and 100 $\mu\text{g}/\text{ml}$ X-Gal (GenDEPOT). TLS frequency is determined from the number of blue colonies among total colonies growing on LB/Kan plates, and mutation frequencies and mutational changes are analyzed by DNA sequencing.

DNA polymerase assays

DNA substrates consisted of a radiolabeled oligonucleotide primer annealed to a 75-nt oligonucleotide DNA template by heating a mixture of primer/template at a 1:1.5 molar ratio to 95 °C and allowing it to cool to room temperature for several hours. The template 75-mer oligonucleotide contained the sequence 5'-AGC AAG TCA CCA ATG TCT AAG AGT TCG TAT GAT GCC TAC ACT GGA GTA CCG GAG CAT CGT CGT GAC TGG GAA AAC-3' and was either undamaged G or harbored an (r) γ -HOPdG at the underlined position. For examining the incorporation of dATP, dTTP, dCTP, or dGTP

nucleotides individually or of all four dNTPs, a 44-mer primer, 5'-GTT TTC CCA GTC ACG ACG ATG CTC CGG TAC TCC AGT GTA GGC AT-3' was annealed to the abovementioned 75-mer template.

The standard DNA polymerase reaction (5 μl) contained 25 mM Tris-HCl (pH 7.5), 5 mM MgCl_2 , 1 mM dithiothreitol, 100 $\mu\text{g}/\text{ml}$ BSA, 10% glycerol, 10 nM DNA substrate, and 1 nM Pol θ . For nucleotide incorporation assays, 10 μM dATP, dTTP, dCTP, or dGTP (Roche Applied Science) were used, and for examining synthesis through the (r) γ -HOPdG lesion, all four dNTPs (10 μM each) were used. Reactions were carried out for 10 min at 37 °C. Reaction products were resolved on a 12% polyacrylamide gel containing 8 M urea and analyzed by a PhosphorImager.

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