8-(Hydroxymethyl)-3,N^4-etheno-C, a Potential Carcinogenic Glycidaldehyde Product, Miscodes In Vitro Using Mammalian DNA Polymerases†

B. Singer,*,‡ Michael Medina,‡ Yanbin Zhang,§ Zhigang Wang,§ Anton B. Guliaev, and Bo Hang

Donner Laboratory, Lawrence Berkeley National Laboratory, University of California, Berkeley, California 94720, and Graduate Center for Toxicology, University of Kentucky, Lexington, Kentucky 40536

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ABSTRACT: 8-(Hydroxymethyl)-3,N^4-etheno-C (8-HM-ϵC) is an exocyclic adduct resulting from the reaction of dC with glycidaldehyde, a mutagen and animal carcinogen. This compound has now been synthesized and its phosphoramidite incorporated site-specifically into a defined 25-mer oligonucleotide. In this study, the mutagenic potential of this adduct in the 25-mer oligonucleotide was investigated in an in vitro primer-template extension assay using four mammalian DNA polymerases. The miscoding potentials were also compared to those of an analogous derivative, 3,N^4-etheno C (ϵC), in the same sequence. Both adducts primarily blocked replication by calf thymus DNA polymerase α at the modified base, while human polymerase β catalyzed measurable replication synthesis through both adducts. Nucleotide insertion experiments showed that dA and dC were incorporated by pol β opposite either adduct, which would result in a C → T transition or C → G transversion. Human polymerase η, a product of the xeroderma pigmentosum variant (XP-V) gene, catalyzed the most efficient bypass of the two lesions with 25% and 32% for 8-HM-ϵC and ϵC bypassed after 15 min. Varying amounts of all four bases opposite the modified bases resulted with pol η. Human polymerase κ primarily blocked synthesis at the base prior to the adduct. However, some specific misincorporation of dT resulted, forming an ϵC·T or 8-HM-ϵC·T pair. From these data, we conclude that the newly synthesized glycidaldehyde-derived adduct, 8-HM-ϵC, is a miscoding lesion. The bypass efficiency and insertion specificity of 8-HM-ϵC and ϵC were similar for all four polymerases tested, which could be attributed to the similar planarity and sugar conformations for these two derivatives as demonstrated by molecular modeling studies.

Glycidaldehyde is a highly reactive alkylating agent which is an industrial chemical and can be also formed by P450 monooxygenase action on glycidyl ethers (*1*), an important class of industrial solvents. Glycidaldehyde also occurs in natural sources such as sunflower oil and rancid samples of lard (*2*). The compound has been shown to be genotoxic, as evidenced by being able to produce base-pair mutations in two strains of *Salmonella typhimurium* (TA1535 and TA1000) (*2*, *3*) as well as reverse base-pair mutations in *Saccharomyces cerevisiae* strain S211 (*2*). On the basis of long-term rodent experiments (*2*, *4*), glycidaldehyde is classified as an animal carcinogen by the International Agency on Research in Cancer (IARC). In these experiments, skin and subcutaneous exposure to glycidaldehyde were reported to produce squamous cell carcinomas and local sarcomas in mice and rats (*2*, *4*).

Glycidaldehyde, which has both reactive carbonyl and epoxy functions, forms cyclic hydroxymethyl-substituted etheno adducts with dA and dG in vitro (*5*−*7*). Glycidaldehyde was earlier found to react with non-B DNA to form a dC adduct in vitro, although the chemical structure of the adduct was not identified (*8*). Hydroxymethyl-etheno dA has also been detected in the skin of C3H mice 24 h after exposure to glycidaldehyde at 2 or 10 mg/animal (*9*). The same dA adduct was also identified in mouse skin treated with a glycidyl ether (*10*).

At present, the molecular mechanisms of glycidaldehyde mutagenicity and carcinogenicity are still unknown. Our first approach in understanding such mechanisms is to examine how the glycidaldehyde-derived adducts may affect in vitro translesional synthesis catalyzed by mammalian DNA polymerases, the fidelity of which is a critical factor in avoiding mutagenicity. Several human DNA-directed polymerases have recently been identified as having lesion-bypassing capacity in addition to their various roles in cellular functions (*11*−*13*). Therefore, miscoding of an adduct during DNA replication by such enzymes could be attributed to the mutagenicity of the adduct.

In this study, four mammalian DNA polymerases were tested for lesion-bypassing activity. DNA polymerase α (pol α)[1] is primarily involved in normal DNA replication with

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* To whom correspondence should be addressed. Phone: 510-642-0637. Fax: 510-486-6488.

‡ These authors contributed equally to this work.

§ University of Kentucky.

[1] Abbreviations: 8-HM-ϵC, 8-(hydroxymethyl)-3,N^4-ethenocytosine; ϵC, 3,N^4-ethenocytosine; AP site, apurinic/apyrimidinic site; AAF, acetylaminofluorene; pol α, polymerase α; pol β, polymerase β; pol η, polymerase η; pol κ, polymerase κ.

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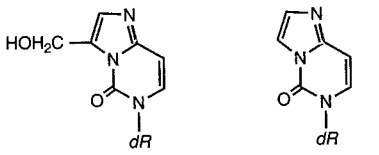

8-(hydroxymethyl)-3,N^4-etheno-dC 3,N^4-etheno-dC

FIGURE 1: Chemical structures of 3,N^4-etheno-dC and 8-(hydroxymethyl)-3,N^4-etheno-dC.

high fidelity (*14*, *15*). Polymerase β (pol β) functions in short-patch base excision repair as a repair synthesis polymerase and gap-fills DNA following base and nucleotide excision (*15*, *16*). Polymerase η (pol η) is coded by the xeroderma pigpentosum variant gene (XP-V) and can bypass *cis*−*syn*-thymine dimers efficiently in an error-free manner (*17*−*20*). However, for most other base lesions tested, pol η catalyzed error-prone bypass (*21*−*25*). Polymerase κ (pol κ), the hDINB1 gene product, is a newly identified human enzyme with low fidelity and has both error-free and error-prone activities in DNA lesion bypass (*26*−*29*). The latter two proteins belong to a group of specialized DNA polymerases (the Y family of DNA polymerases) capable of bypassing certain DNA lesions that usually block replication synthesis by many other polymerases (*11*, *30*).

Given the fact that glycidaldehyde is an animal carcinogen and there is evidence that adducts are formed in vivo, studies on the miscoding potential and repair of these adducts are of importance. For this purpose and also for structural comparisons with related etheno adducts, we recently synthesized 8-(hydroxymethyl)-3,N^4-etheno-dC (8-HM-ϵdC) and its phosphoramidite which has been incorporated site-specifically into a defined 25-mer oligonucleotide (*31*). In the present work, we report that 8-HM-ϵC is a miscoding lesion in replication of the 25-mer adduct-containing template by the mammalian DNA polymerases used. Each of the four DNA polymerases used has varying bypass capacity and base insertion preferences toward the 8-HM-ϵC-containing oligomer. The base pairing preferences were compared with those of ϵC and found to be similar for both the 8-HM-ϵC and ϵC-containing oligonucleotides. ϵC is structurally closely related to 8-HM-ϵC (Figure 1) and is an established mutagenic lesion (*32*−*37*). Such similar miscoding specificity of the two adducts is supported by data from molecular modeling, in which there is apparently little difference in the planarity and sugar conformations of the two derivatives in duplex DNA.

MATERIALS AND METHODS

Oligodeoxynucleotides. 8-(Hydroxymethyl)-3,N^4-etheno-dC (8-HM-ϵdC), 3,N^4-etheno-dC (ϵdC), and their phosphoramidites were synthesized according to Chenna et al. (*31*) and Dosanjh et al. (*38*). All oligonucleotides with site-directed modified nucleotides were synthesized with an Applied Biosystems Model 394 automated DNA synthesizer. The 15-mer primer was synthesized and HPLC-purified by Operon Technologies (Alameda, CA). All adduct-containing oligomers were HPLC-purified and, on enzyme digestion with subsequent HPLC analysis, found to contain the correct modified base. The same 25-mer sequence was used for all templates. As shown below, the modified deoxycytosine is at position 8 from the 5′ end.

5′-CCGCTAG**C**GGGTTAGGAGCTCGAAT-3′

5′-CCGCTAG**εC**GGGTTAGGAGCTCGAAT-3′

5′-CCGCTAG**8-HM-εC**GGGTTAGGAGCTCGAAT-3′

All of these DNA templates were annealed to the same 15-mer primer: 3′-CAATCCTCGAGCTTA-5′, which terminates 2 bases prior to C, ϵC, or 8-HM-ϵC on the 3′ side, thus permitting the same running start for replication of the oligonucleotides (see Figure 2, top).

DNA Polymerases. Calf thymus DNA pol α was a generous gift from Dr. Fred Perrino (Wake Forest University, Winston−Salem, NC). The concentration for pol α is 0.05 units/μL (one unit is defined as the amount of enzyme that incorporates 1 nmol of ^{32}P-α-dTMP in 60 min at 37 °C on activated calf thymus DNA). The human pol β was purchased from Trevigen (Gaithersburg, MD). The concentration for pol β is 4 units/μL (One unit is defined as the amount of enzyme required to incorporate 1 nmol of total nucleotide into acid-insoluble form in 60 min at 37 °C). The human pol η and pol κ were purified as previously described by Zhang et al. (*21*, *28*).

Primer Extension Assays

Preparation of DNA Primer-Template. The 15-mer primer was 5′ end-labeled with [γ-^{32}P] ATP (specific activity > 6000 Ci/mmol, Amersham Pharmacia Biotech, Cleveland, OH) as previously described (*39*, *40*). The 5′-^{32}P-labeled primer was then mixed in an equamolar amount with the 25-mer template in a buffer containing 70 mM Tris HCl (pH 7.8) and 10 mM $MgCl_2$. The mixture was heated for 2 min at 100 °C and annealed by slow cooling to room temperature.

Full Replication Assay. The replication mixtures contained 2 nM of a primer-template complex, 200 μM of all four dNTPs (Amersham Pharmacia Biotech) and various concentrations of each DNA polymerase (see figure legends for concentrations) and were incubated in a buffer containing 25 mM KH_2PO_4 (pH 7.0), 5 mM $MgCl_2$, 5 mM DTT, 100 μg BSA/mL, and 10% glycerol. Replication reactions with pol α were incubated at 30 °C, and those with pols β, η, and κ were incubated at 37 °C. Reactions were terminated by adding 2 volumes of a solution containing 90% formamide and 50 mM EDTA (F/E solution). The samples were heated for 3 min at 90−100 °C and then chilled in ice.

Aliquots of 5 μL were loaded onto a 16% polyacrylamide gel containing 8 M urea. Following electrophoresis at 1900 V for 3 h, the gels were dried and autoradiographed. For quantitation, the gels were phosphorimaged on a BioRad FX Molecular Imager and the band intensity quantitated using Quantity One software.

Single Base Insertion and Extension Assay. The replication mixtures contained 2 nM of primer template and 5 μM of dCTP complementary to the two guanine bases 3′ to the adduct in order to initiate a two-base running start (see Figure 3, top scheme). 200 μM of each of four dNTPs was added to each reaction in order to determine which specific dNTP is inserted opposite 8-HM-ϵC or ϵC in the 30 min reaction time. Reactions were terminated by adding 2 volumes of F/E solution. The samples were then heated for 3 min at

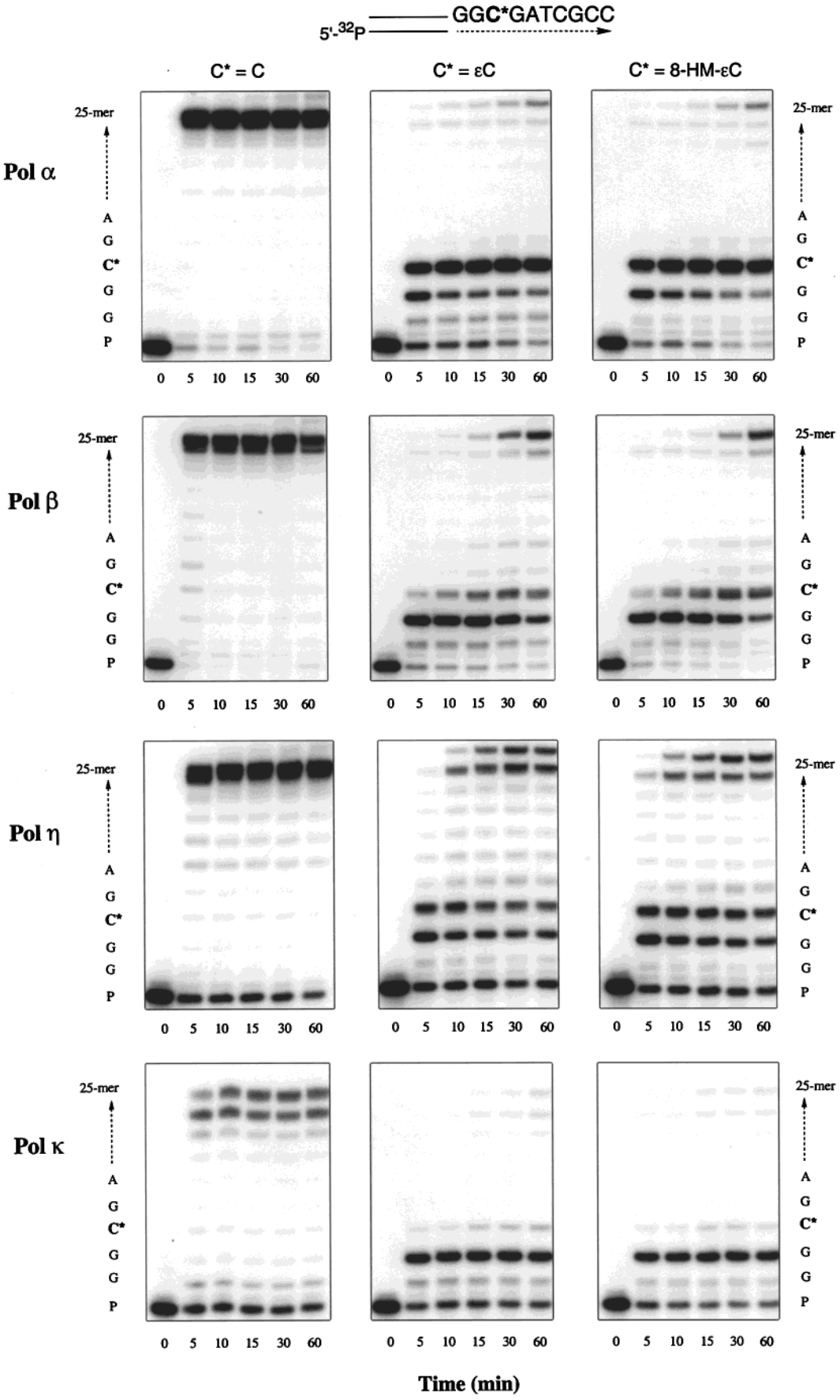

FIGURE 2: Time course of full replication of normal and ϵC- and 8-HM-ϵC-containing oligomers using 200 μM each of all four dNTPs. The enzyme concentrations are 0.03 units of pol α, 3.2 units of pol β, 3.5 ng of pol η, and 25.8 ng of pol κ. Reactions were performed as described in Materials and Methods for the times indicated below each lane. The template primer used is shown on top, and the template sequence is shown vertically on the two sides of the figure.

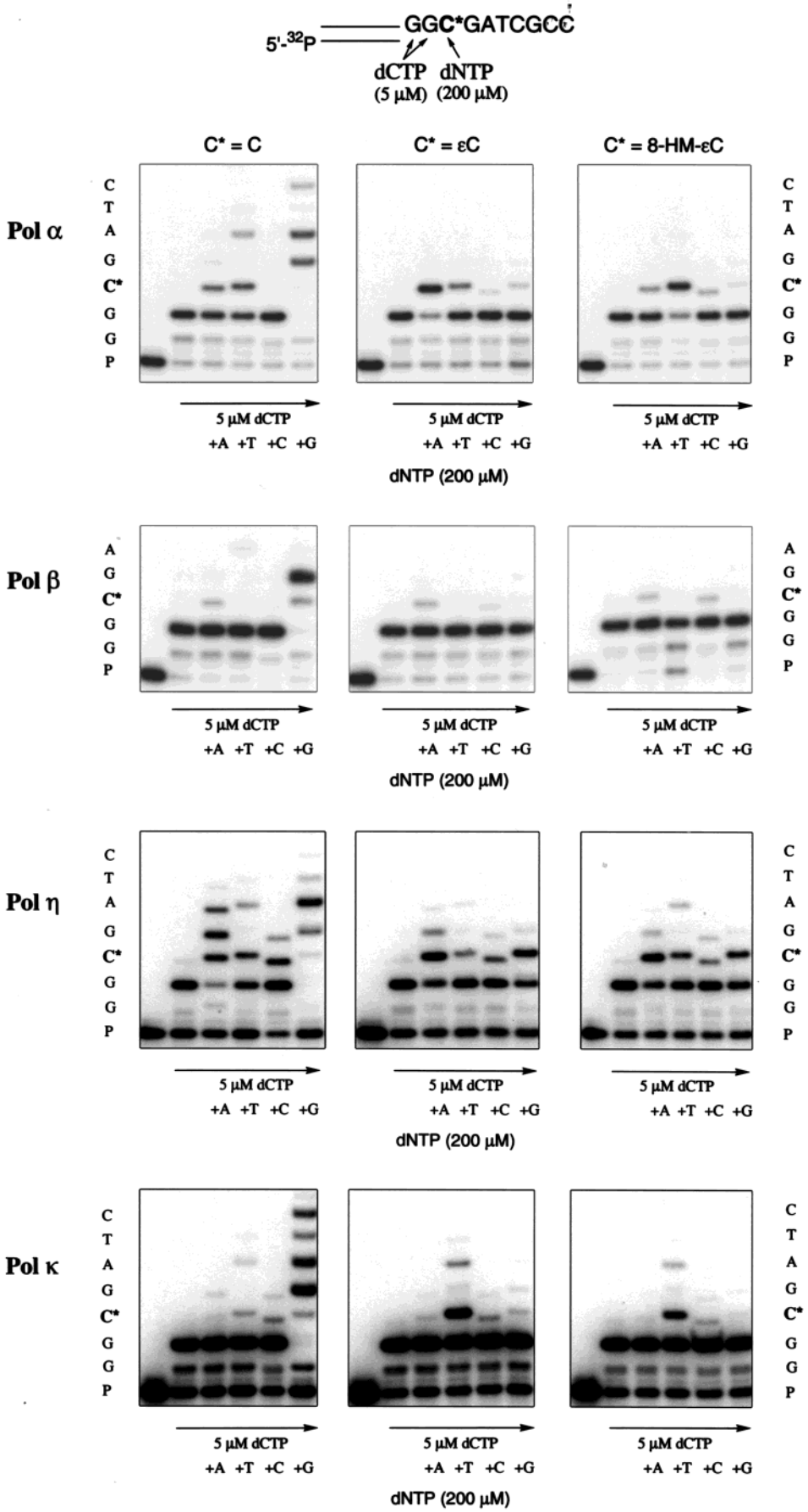

FIGURE 3: Single base insertion of normal and ϵC- and 8-HM-ϵC-containing oligomers using 200 μM of a single dNTP. dCTP (5 μM) was also added to initiate a running start one base before the C* sites. Reactions containing pols β, η, and κ were incubated for 30 min at 37 °C, and the reactions containing pol α were incubated for 30 min at 30 °C.

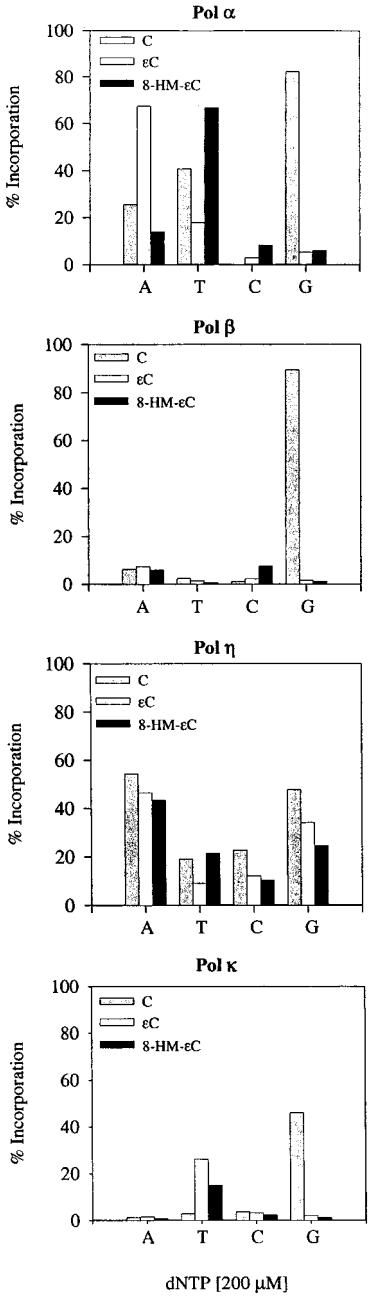

FIGURE 4: Bar graph quantitation of data from single base insertion experiments in Figure 3. The bars were calculated as the percent of primer in each lane extended to the C* site and beyond as a result of dNTP insertion. Note that, in the case of pol η, although the percent of incorporation of A and G is similar, the G bypass is extended more readily than that for A as shown in Figure 2.

90−100 °C and then chilled in ice. Gel electrophoresis was performed as described previously. After the gels were phosphorimaged, the percent incorporation of each dNTP was calculated as the percent of primer in each lane extended to the C* site and beyond (see Figure 4).

Molecular Modeling

To explore the conformational space for the adducts used in this study, ab initio quantum mechanical calculations were employed using HyperChem 4.5 (Hypercube, Inc., Gainesville, FL). The ϵC base was constructed by the addition of an exocyclic ring between the N3 and N4 of the normal cytosine base. To construct 8-HM-ϵC, the hydroxymethyl group was added to the C8 position of ϵC. The geometry of each base was optimized using Hartree−Fock ab initio methods at the 6-21G* basis set level. The geometry optimized adducts were inserted into 15-mer DNA duplexes, which is the truncated form of the 25-mer used for replication work

5′CCGCTAGXGGGTACC3′/5′GGTACCCGCTAGCGG3′
(X = ϵC, 8-HM-ϵC, or C)

Semiempirical molecular dynamics calculations with explicit solvent using AMBER 5.0 force field (*41*) were utilized to explore the conformation of the 8-HM-ϵC or ϵC adduct in this DNA duplex (*42*).

RESULTS

Insertion of dNTPs Opposite 8-HM-ϵC or ϵC and Further Extension

The four mammalian DNA polymerases, α, β, η, and κ, were tested for their ability to catalyze extension bypass of 8-HM-ϵC as well as their specificity of dNTP insertion opposite the adduct using the 15-mer primer/25-mer template complex (see Materials and Methods). For comparative studies, a structural analogue, ϵC (Figure 1), was also tested under the same conditions. The results of full replication with all four dNTPs and single base incorporation with each of the four bases are shown in Figures 2 and 3, respectively. The quantitative results on single base insertion are shown in a bar graph figure for each enzyme used (Figure 4).

Pol α. The 8-HM-ϵC and ϵC adducts primarily blocked replication catalyzed by pol α at the modified base when 200 μM of each dNTP was used (Figure 2). A small fraction of the primer was able to bypass the adduct sites after prolonged incubation time (60 min) and reached full extension (Figure 2). The single-base insertion experiments showed that pol α inserted T and A greater than C and G opposite both adducts (Figures 3 and 4).

Pol β. The presence of either 8-HM-ϵC or ϵC led to a pause in replication by pol β one base prior to the adduct site and at the adduct site (Figure 2). With time, the amounts of primer reaching full extension increased. A significant amount of replication is seen after 60 min. The single-base insertion assays showed that pol β inserted less than 10% of A and C in 30 min opposite both 8-HM-ϵC and ϵC, while approximately 90% of G was incorporated opposite the normal C under the same reaction conditions (Figures 3 and 4).

Pol η. Of the four enzymes tested, pol η was the most efficient polymerase in bypassing the two adducts (Figure 2). Pol η extended the primer to one base prior to the adduct site and also at the adduct site (Figure 2). Both adducts showed a similar rate of replication catalyzed by pol η. At the 15 min point, 25% of 8-HM-ϵC and 32% of ϵC containing oligonucleotides were almost fully replicated, as measured by scanning results (data not shown). After 30 min, both bypass reactions reached saturation (~40%). In single nucleotide insertion experiments, pol η preferentially inserted A and G opposite 8-HM-ϵC and ϵC, but lesser amounts of C and T were also incorporated (Figures 3 and 4). In the control panel (Figure 3, left column), pol η also incorporated all four bases opposite the normal C except that the G incorporation did not lead to a pause at the corresponding C

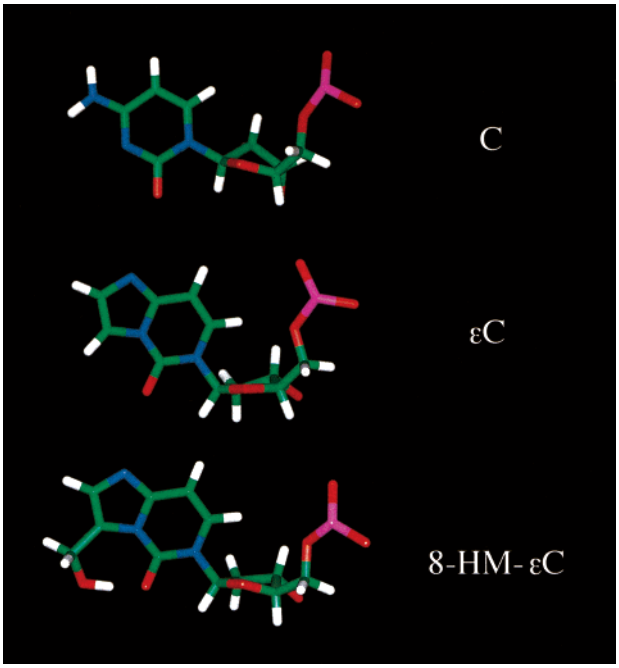

FIGURE 5: Structures of C, ϵC, and 8-HM-ϵC in duplex DNA as shown by molecular modeling. The 8-HM-ϵC has a similar planar and sugar conformation as the ϵC. The OH group of 8-HM-ϵC is displaced 15° away from the exocyclic ring plane. The similarity in planar and sugar conformations of the 8-HM-ϵC and ϵC could contribute to the similar rate of replication synthesis as well as insertion specificity.

position. This illustrates that pol η is also error prone when acting on unmodified DNA. A second band seen below the full extended band (Figure 2) could be due to deletions as a result of slippage at or around the modified base. This possibility is currrently under investigation.

Pol κ. 8-HM-ϵC and ϵC primarily blocked replication synthesis by this enzyme (Figure 2). However, the same enzyme replicated the unmodified oligomer template efficiently (Figure 2). In the single base insertion assays, pol κ predominantly paired T opposite both 8-HM-ϵC and ϵC, with very little other incorporation observed, while the normal C paired primarily with G (Figures 3 and 4).

In summary, the data using all four DNA polymerases show clear differences in lesion-bypass efficiency as well as insertion specificity. While all enzymes showed misincorporation of nucleotides opposite the modified base, only pol η and pol β were able to carry out significant bypass. Both 8-HM-ϵC and ϵC were similar in the extent of bypass and specificity of base insertion by a given enzyme (Figures 2 and 3).

Molecular Modeling

The molecular modeling in this study showed significant similarity in overall structural conformation between 8-HM-ϵC and ϵC (Figure 5). For both adducts, the exocyclic imidazole ring showed planar orientation. The methyl carbon of the 8-HM-ϵC remained in the same plane with the exocyclic ring, and hydroxyl group was displaced by only 15° from the exocyclic ring plane. As proposed earlier by Zhang et al. (*43*), the planar conformation of an adduct, such as ϵC, should aid the stacking interaction of this base during DNA synthesis. Similarly, the planar structure of 8-HM-ϵC should also contribute to the stacking interaction of this adduct during synthesis. The OH group of the 8-HM-ϵC, similar to the N4 of the ϵC, can be involved in hydrogen bonding to the opposite base. Furthermore, molecular dynamics calculations showed similar sugar conformations for these adducts. Both 8-HM-ϵC and ϵC, when incorporated into the DNA duplex, have a sugar pucker in the C3′-*endo*/C4′-*exo* region, while the rest of the residues, including unmodified C, are in the C2′-*endo*/C3′-*exo* range (Figure 5). The C3′-*endo*/C4′-*exo* sugar conformation of the ϵC adduct shown by modeling in this work was in agreement with the previously reported solution structures of ϵC-containing DNA duplexes which showed the same conformational range for the same sugar (*44*, *45*).

DISCUSSION

The primary aim of this work was to investigate the in vitro miscoding potential of the newly synthesized derivative, 8-HM-ϵC (*31*), an expected product from the mutagen/animal carcinogen, glycidaldehyde, on the basis of the chemical studies as discussed in the introduction. In addition to 8-HM-ϵC, a structural and well-studied analogue, ϵC, was also tested in parallel in order to assess mutagenic specificity as a function of adduct structure. For this purpose, an in vitro replication system (*40*, *46*) was used to measure the extent of lesion bypass and the four possible base substitutions opposite the damaged base.

In this work, lesion bypass of both 8-HM-ϵC and ϵC was found for all four mammalian polymerases tested in 25-mer templates using a primer extension assay (*39*, *40*). However, the extent varied significantly (Figure 2). Pol α and pol κ showed a minimal amount of bypass synthesis when 200 μM of each dNTs was used. These experiments indicated that both adducts primarily blocked replication catalyzed by pol α and pol κ. The difference in blockage between the two enzymes is that the extension catalyzed by pol α was mainly at the adduct site, while the extension by pol κ was blocked one base prior to the adduct (Figure 2). In contrast, pol η catalyzed the most efficient bypass of 8-HM-ϵC and ϵC (25% and 32%, respectively) at the 15-min time point. Pol β also showed measurable amount of bypass of both adducts but to a lesser extent than pol η (Figure 2). All four mammalian enzymes replicated the unmodified template much more efficiently than the adduct-containing templates (Figure 2, left panels).

To determine which nucleotide was inserted opposite the modified base, single nucleotide incorporation experiments were performed in which only one of the four nucleotides per reaction was added at 200 μM to test for the relative affinity for incorporation (*40*). The miscoding specificities of 8-HM-ϵC or ϵC varied depending on the DNA polymerase used, as shown in Figures 3 and 4. All base substitutions opposite both adducts detected were mismatches except for replication by pol η which inserted 20−30% of the correct base, G, opposite both C adducts, thus leading to both error-free and error-prone synthesis (Figure 4). The two adducts showed similar patterns of base incorporation for all four polymerases tested. The similarities in bypass efficiencies and miscoding specificities between 8-HM-ϵC and ϵC are likely due to their structural resemblance, which was observed by molecular modeling in this work (Figure 5). Similar planar and sugar conformations of ϵC and 8-HM-

ϵC could lead to the similar stacking potential of the modified base with the neighboring bases as well as the ability to form a stable pair with the incorporated base, thus affecting replication efficiency and specificity. In a related study on the thermodynamic stability of 15-mer duplexes with an ϵC or 8-HM-ϵC paired with G, both adducts showed similar destabilization of the double helix (*47*). These results support the similar structural impact of the two exocyclic adducts.

The miscoding properties of ϵC have been the subject of many studies since the early 1980s (*32*−*36*). More recently, Shibutani et al. studied the miscoding of ϵC by mammalian pol α, β, and δ using a primer/template extension assay (*37*). Our current results of ϵC replication by pol α and β are basically in agreement with those of Shibutani et al. in that, preferentially, pol α inserted A and T opposite ϵC and that pol β incorporated A and C (Figure 3). In addition, the data on ϵC in this study using pol η add further support for the mutagenic potential of ϵC because this enzyme is shown to perform a significant amount of bypass synthesis with misincorporations at the ϵC site (Figures 2 and 3).

As discussed previously, of the four enzymes used in the present study, pol η facilitated the most efficient bypass synthesis of 8-HM-ϵC or ϵC, with all possible base substitutions occurring opposite the modified base (Figures 3 and 4). Except for error-free bypass of T−T dimers (*48*, *49*), human pol η has also been shown to perform error-prone translesional syntheses toward such DNA lesions as 8-oxoguanine, an AP site, and (+)-*trans*-*anti*-benzo[α]pyrene-N^2-dG, a bulky adduct (*21*). The present finding that pol η also efficiently bypasses two other exocyclic adducts, 8-HM-ϵC and ϵC, suggests a broad substrate range for the enzyme. Pol η also replicated the unmodified template, forming all four base substitutions (Figures 3 and 4). This highly error-prone nucleotide incorporation supports previous findings that the enzyme replicates normal DNA with low fidelity (*50*−*52*).

Human pol κ is reported to synthesize undamaged DNA with very low fidelity (*26*, *27*). Using DNA containing various different lesions, Zhang et al. (*27*) showed that pol κ has a broad substrate affinity in its error-free and error-prone bypass of 8-oxo-dG, AP site, AAF-dG, and (+)-*trans*-*anti*-benzo[α]pyrene-N^2-dG (*27*). In the present work, pol κ showed a highly specific insertion preference for both 8-HM-ϵC and ϵC, primarily incorporating T opposite the adduct, which would direct a C → A transversion (Figure 3).

It is not known how various DNA polymerases are recruited to sites of DNA damage for translesion synthesis. It now appears that when highly processive, semiconservative DNA replication is blocked by a DNA lesion, the replicative machinery is displaced from the replication fork and replaced by these lesion-specific DNA polymerases (*13*). These enzymes may facilitate lesion bypass either in an error-free or error-prone manner, depending on the type of lesion and the particular polymerase used by the cell. Following lesion bypass, the polymerase displacement/replacement process is reversed, and the normal replication machinery continues.

In conclusion, the in vitro experiments in this study show that, like ϵC, 8-HM-ϵC is miscoding in replication by the four mammalian DNA polymerases used, although these enzymes exhibit different base incorporation specificities and bypass capacity. On this basis, if any of these polymerases is involved in 8-HM-ϵC translesional synthesis in vivo, unrepaired 8-HM-ϵC adducts would be expected to be mutagenic.

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