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8-(Hydroxymethyl)-3,N<sup>4</sup>-etheno-dC, a Potential Carcinogenic Glycidaldehyde Product,  
Miscodes *In Vitro* Using Mammalian Polymerases†

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Running title: 8-HM-εC replication by mammalian polymerases

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Abbreviations: 8-HM-εC: 8-(hydroxymethyl)-3,N<sup>4</sup>-ethenocytosine; εC: 3,N<sup>4</sup>-ethenocytosine; pol α: polymerase α; pol β: polymerase β; pol η: polymerase η; pol κ: polymerase κ.

**ABSTRACT**

8-(hydroxymethyl)-3,N<sup>4</sup>-etheno-dC (8-HM-εC), is a mutagen and animal carcinogen resulting from the reaction of dC with glycidaldehyde. It was synthesized and its phosphoramidite was incorporated site-specifically into a defined 25-mer oligonucleotide. In this study the mutagenic potential of this compound was investigated in an *in vitro* primer-template extension assay using four mammalian DNA polymerases, and compared to that of the analogous derivative, 3,N<sup>4</sup>-etheno dC (εC). 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. Single nucleotide insertion experiments using pol β showed that dA and dC were incorporated preferentially by both derivatives which resulted in a C → T transition or C → G transversion. Human polymerase η, a product of the XP-V gene, catalyzed significant bypass of the two lesions, with varying amounts of all four bases incorporated opposite the modified bases. Human polymerase κ primarily blocked synthesis at the base prior to the adduct site. 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 similarities in bypass capacity and insertion specificity between the 8-HM-εC and εC bases may be due to the similar planarity and sugar conformations for these two derivatives, as demonstrated by molecular modeling studies.

## INTRODUCTION

Glycidaldehyde is a highly reactive alkylating agent formed by P450 monooxygenase action on glycidyl ethers (1), which are industrial solvents. Glycidaldehyde also occurs in natural sources such as sunflower oil and lard. The compound has produced 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 (4, 5). Glycidaldehyde is classified as an animal carcinogen by International Agency on Research in Cancer (IARC) based on long-term rodent experiments (3, 5). Skin and subcutaneous exposure to glycidaldehyde were also reported to produce squamous-cell carcinomas and local sarcomas in mice and rats (5).

Glycidaldehyde, which has both reactive carbonyl and epoxy functions, was found to form cyclic hydroxymethyl-substituted etheno adducts with dA and dG *in vitro* (6-8). Glycidaldehyde also reacts with non-B DNA to form a dC adduct although the chemical structure of the adduct was not identified (9). In animal experiments, hydroxymethyl-etheno dA has also been detected in the skin of mice exposed to glycidaldehyde (10).

At present, the molecular mechanism of glycidaldehyde mutagenicity is still unknown. Our approach in understanding the mechanism is to examine how this adduct may affect *in vitro* replication processes using mammalian polymerases, which are a key factor in maintaining genomic integrity. There are a number of human DNA-directed polymerases which have been identified as having lesion-bypassing capacity in addition to their various roles in cellular functions (for reviews see refs (11, 12). Polymerase  $\alpha$  (pol  $\alpha$ ) exhibits high fidelity, and is a moderately processive enzyme (13), functioning in both DNA replication and repair. Polymerase  $\beta$  (pol  $\beta$ ) functions primarily in short-patch base excision repair (BER) of DNA as a repair synthesis polymerase (14, 15), and gap-fills DNA following BER and nucleotide excision repair (NER)(14, 15). Polymerase  $\eta$  (pol  $\eta$ ) is coded by the XP-V gene and can bypass *cis-syn* thymine dimers in an error-free

manner. Polymerase  $\kappa$  (pol  $\kappa$ ), the hDINB1 gene product, is a newly identified enzyme with low fidelity and has both error-free and error-prone activities in DNA lesion bypass (20-22). More recently, O-Wang *et al.* (23) reported the overexpression of pol  $\kappa$  in patients with non-small cell lung cancer. This work suggests that pol  $\kappa$  may play a role in the initiation of tumorigenesis.

In this work we report that each mammalian DNA polymerase used has specific bypass capacity and base insertion preferences towards the 8-HM- $\epsilon$ C-containing oligomer. In addition, the base pairing preferences are similar for both the 8-HM- $\epsilon$ C and  $\epsilon$ C-containing oligonucleotides. The latter compound is closely related structurally to 8-HM- $\epsilon$ C and it has been established that it is a mutagenic lesion (24-29). Moreover, by molecular modeling, we showed that little difference exists in the planarity and sugar conformations of the two derivatives in duplex DNA. From data presented in this work, we conclude that, like  $\epsilon$ C, 8-HM- $\epsilon$ C is a potential miscoding lesion when replicated by the mammalian polymerases used.

## MATERIALS AND METHODS

### *Oligodeoxynucleotides*

8-(hydroxymethyl)-3,N<sup>4</sup>-etheno-dC and 3,N<sup>4</sup>-etheno-dC phosphoramidites were synthesized according to Chenna *et al.* (30) and Dosanjh *et al.* (31). Oligonucleotides with site-directed modified nucleotides were synthesized with an Applied Biosystems Model 394 automated DNA synthesizer. The normal 15-mer was purchased from Operon Technologies (Alameda, California). All oligomers were HPLC-purified, and on enzyme digestion, found to contain the expected ratios of nucleosides.

The same sequence of a 25-mer oligonucleotide was used for all templates as shown. The modified cytosine is at position 8 from the 5' end.

5'-CCGCTAGCGGGTTAGGAGCTCGAAT-3'

5'-CCGCTAG $\epsilon$ CGGGTTAGGAGCTCGAAT-3'

5'-CCGCTAG8-HM- $\epsilon$ CGGGTTAGGAGCTCGAAT-3'

All DNA templates were annealed with the same 15-mer primer: 3'-CAATCCTCGAGCTTA-5', which terminates 2 bases prior to C, or  $\epsilon$ C, or 8-HM- $\epsilon$ C on the 3' side, thus permitting the same running start for replication of all the oligonucleotides.

### *DNA Polymerases*

Calf thymus DNA pol  $\alpha$  was a generous gift from Dr. Fred Perrino (Wake Forest University). The polymerase concentration for pol  $\alpha$  is 0.05 units/ $\mu$ l (one unit is defined as the amount of enzyme that incorporates 1 nmole  $^{32}$ P- $\alpha$ -dTTP in 60 min at 37° C on activated calf thymus DNA.) The human pol  $\beta$  was purchased from Trevigen (Gaithersburg, MD). The polymerase concentration for pol  $\beta$  is 4 units/ $\mu$ l. (One unit is the amount of exzyme required to incorporate 1 nmol of total nucleotide into acid-insoluble form in 60 min. at 37° C.) The human pol  $\eta$  and pol  $\kappa$  were purified as previously described by Zhang *et al.* (32).

### *Primer Extension Assays*

Preparation of DNA primer-template: The 15-mer primer was 5' end-labeled with [ $\gamma$ - $^{32}$ P] ATP (specific activity, > 6000 Ci/mmol, Amersham Pharmacia Biotech) as previously described by Singer and colleagues (24, 25). The 5'- $^{32}$ P-labeled primer was then annealed to an equal molar amount of a 25-mer template in 70 mM Tris-HCl (pH 7.8), 10 mM MgCl<sub>2</sub>. The mixture was heated for 2 min at 100°C and slowly cooled to room temperature to ensure annealing.

Full replication assay: The replication mixtures contained 2 nM of primer-template complex, 200  $\mu$ M 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<sub>2</sub>PO<sub>4</sub> (pH 7.0), 5 mM MgCl<sub>2</sub>, 5 mM DTT, 100  $\mu$ g BSA/mL, and 10% glycerol. Replication reactions with pol  $\alpha$  were incubated at 30°C, and those with pols  $\beta$ ,  $\eta$ , and  $\kappa$  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  $\mu$ l were loaded onto a 16% polyacrylamide gel containing 8 M urea. Following electrophoresis at 1900 volts for 3 hours, 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  $\mu$ M 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  $\mu$ M of a single dNTP were also added in order to determine which specific dNTP was inserted opposite  $\epsilon$ C or 8-HM- $\epsilon$ C in the 30 min reaction time. Reactions were terminated after 60 min. by adding 2 volumes of F/E solution. The samples were heated for 3 min at 90°-100°C, then chilled in ice. Gel electrophoresis was performed as described above.

### *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. FA). The  $\epsilon$ C base was constructed by the addition of an exocyclic ring between N3 and N4 of the normal cytosine base. To construct 8-HM- $\epsilon$ C, the hydroxymethyl group was added to the C8 position of  $\epsilon$ C. 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 15mer DNA duplexes, 5'CCGCTAGXGGGTACC3'/5'GGTACCCGCTAGCGG3' (X =  $\epsilon$ C, 8-HM- $\epsilon$ C, or C), which are truncated 25mer form of the DNA oligos used in biochemistry studies in this work. Semi-empirical molecular dynamics calculations with explicit solvent using AMBER 5.0 force field (33) were utilized to explore the conformation of the 8-HM- $\epsilon$ C or  $\epsilon$ C adduct in this DNA duplex (26).

## **RESULTS**



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

The four mammalian polymerases,  $\alpha$ ,  $\beta$ ,  $\eta$ , and  $\kappa$  were examined for their ability to catalyze extension bypass of the adduct site, as well as dNTP insertion opposite the 8-HM- $\epsilon$ C (see Fig. 1) base in a defined sequence. For comparative studies, the structural analogue,  $\epsilon$ C (Fig. 1), was tested under the same conditions. Both full replication with all four dNTPs (Figure 2) and single base incorporation with each of the four bases (Figure 3) were determined.

Polymerase  $\alpha$  : The 8-HM- $\epsilon$ C and  $\epsilon$ C adducts primarily blocked replication catalyzed by pol  $\alpha$  at the modified base using 200  $\mu$ M of each dNTP (Figure 2). However, a small fraction of the primer was able to bypass the adduct sites after prolonged incubation time 60 min and reached full extension (Fig 2). This enzyme replicated the unmodified oligomer with high efficiency (Figure 2). The single-base insertion assays showed that pol  $\alpha$  inserted T (67%) > A (14%) > C,G opposite 8-HM- $\epsilon$ C, while  $\epsilon$ C preferred A (67%) > T (18%) > C,G (Fig 4).

Polymerase  $\beta$ : The presence of either 8-HM- $\epsilon$ C or  $\epsilon$ C led to a pause in replication by pol  $\beta$  one base prior to the adduct site and at the adduct site (Fig 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  $\beta$  inserted less than 10% A and C in 30 min opposite both 8-HM- $\epsilon$ C and  $\epsilon$ C, while 90% G was incorporated opposite the normal C under the same reaction conditions (Fig 4).

Polymerase  $\eta$ : Pol  $\eta$  extended the primer to one base prior to the adduct site and also at the adduct site (Fig 2). The extent of full replication increased as a function of time (Figs. 3 and 5). Both adducts showed a similar rate of replication catalyzed by pol  $\eta$  (Figure 5). At the 30 minute point, 33% 8-HM- $\epsilon$ C and 39%  $\epsilon$ C containing oligonucleotides had reached full replication. Among the four enzymes tested, pol  $\eta$  was the most efficient. In single base insertion experiments, pol  $\eta$  preferentially inserted A

and G opposite 8-HM- $\epsilon$ C and  $\epsilon$ C, but lesser amounts of C and T were also incorporated (Figs. 3 and 4). In the control panel (Fig 3, left column), pol  $\eta$  also showed incorporation of all four bases opposite the normal C, except that the G incorporation did not lead to a pause at the corresponding C position. This illustrates that pol  $\eta$  is also error prone when acting on unmodified DNA.

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

Taken together, the data using all four DNA polymerases show clear differences in their lesion-bypass efficiency, as well as insertion specificity. However, both 8-HM- $\epsilon$ C and  $\epsilon$ C were similar in the extent of bypass and specificity of base insertion by a given enzyme. It should be noted that the specific activity for each DNA polymerase used for assay differed (Fig. 2).

### *Molecular Modeling*

The molecular modeling in this study showed significant similarity in overall structural conformation between 8-HM- $\epsilon$ C and  $\epsilon$ C (Fig 6). For both adducts, the exocyclic imidazole ring showed planar orientation. The methyl carbon of the 8-HM- $\epsilon$ C 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.*(27), the planar conformation of an adduct, such as  $\epsilon$ C, should aid the stacking interaction of this base during DNA synthesis. Similarly, the planar structure of 8-HM- $\epsilon$ C should also contribute to the stacking interaction of this adduct during synthesis. The OH group of the 8-HM- $\epsilon$ C, similar to the N4 of the  $\epsilon$ C, can be involved in hydrogen bonding to the opposite base. Furthermore, molecular dynamics calculations revealed similar sugar conformations for these adducts. Both 8-HM- $\epsilon$ C and  $\epsilon$ C, 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 (Fig 6). The C3'-endo/C4'-exo sugar conformation of the  $\epsilon$ C adduct shown by modeling was in agreement with the previously reported solution structures of  $\epsilon$ C-containing DNA duplexes, which showed the same conformational range for that sugar (28, 29).

## DISCUSSION

The primary aim of this work was to investigate the *in vitro* miscoding of the newly synthesized derivative, 8-HM- $\epsilon$ C (30), a potential product from the carcinogen, glycidaldehyde. In addition to 8-HM- $\epsilon$ C, a structural analogue,  $\epsilon$ C, was also tested in the same manner. An *in vitro* replication system was used to measure the extent of lesion bypass and all possible base substitutions opposite the damaged base (25, 34). For this purpose, four mammalian polymerases were used, including two well-characterized mammalian DNA polymerases, pol  $\alpha$  and pol  $\beta$ , and two recently described translesional human DNA polymerases, pol  $\eta$  and pol  $\kappa$ . 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 (, 29). .

In this work, lesion bypass of both 8-HM- $\epsilon$ C and  $\epsilon$ C templates using the primer extension assay was found for all four mammalian polymerases tested, but varied significantly in extent (Fig 2). Pol  $\alpha$  and pol  $\kappa$  showed a minimal amount of bypass synthesis under the conditions used, which included high concentrations of all four dNTPs at 200  $\mu$ M each. However, both adducts primarily blocked replication catalyzed by these two enzymes. The difference between the two enzymes is that the extension catalyzed by pol  $\alpha$  was mainly blocked at the adduct site, while the extension by pol  $\kappa$  was blocked one base prior to the adduct (Fig 2). In contrast, pol  $\eta$  catalyzed the most efficient bypass of 8-HM- $\epsilon$ C and  $\epsilon$ C, 33% and 39% respectively at the 30-minute time point (Fig 4). Pol  $\beta$  also showed bypass of both

adducts, but to a lesser extent. All four mammalian enzymes replicated the unmodified template much more efficiently than the adduct-containing templates.

In order to determine which bases were inserted, single base incorporation experiments were used. The miscoding specificities of 8-HM- $\epsilon$ C or  $\epsilon$ C varied depending on the DNA polymerase used, as shown in Figs. 3 and 4. All base substitutions detected were mismatches, except for replication by pol  $\eta$  which inserted 20-30% G, the correct base, opposite both C adducts, thus leading to both error-free and error-prone synthesis (Figure 4). These two adducts showed similar patterns of base incorporation for all four polymerases tested. The similarities in bypass efficiencies and miscoding specificities between 8-HM- $\epsilon$ C and  $\epsilon$ C are likely due to the structural similarities between these adducts, which were observed by molecular modeling in this work (Fig 6). Similar planar and sugar conformations of  $\epsilon$ C and 8-HM- $\epsilon$ C can lead to the similar stacking potential of the modified base with the neighboring bases, and the ability to form a stable pair with the incorporated base, thus affecting replication efficiency and specificity. In another study on the thermodynamic stability of 15-mer duplexes with an  $\epsilon$ C or 8-HM- $\epsilon$ C paired with G, both adducts showed similar destabilization of the double helix (35).

The miscoding properties of  $\epsilon$ C have been the subject of a number of studies since the early 1980's when three different laboratories first reported its miscoding potential when present in chloroacetaldehyde-modified DNA.  $\epsilon$ C•T or  $\epsilon$ C•A were the main mispairs when *E. coli* polymerases were used (36-38). A later study using M13 viral DNA showed that 30% of progeny phage obtained by transfecting  $\epsilon$ C-DNA had a base substitution mutation at the lesion site (39). In agreement with the first reports, all these mutations were either C  $\rightarrow$  T transitions or C  $\rightarrow$  A transversions. Another *in vivo* assay using simian kidney (COS) cells revealed that  $\epsilon$ C produced very high mutation frequency (81%) with predominantly C  $\rightarrow$  A and C  $\rightarrow$  T base substitutions (40). Most recently, Shibutani *et al* studied the miscoding of  $\epsilon$ C by mammalian pol  $\alpha$ ,  $\beta$  and  $\delta$  using a similar primer/template extension assay (41). Our current results of  $\epsilon$ C replication by pol  $\alpha$  and  $\beta$  were basically in agreement with those of Shibutani *et al.*, in that pol  $\alpha$  inserted A and T opposite  $\epsilon$ C, and pol  $\beta$  incorporated A and C (Figure 3). This present study

on pol  $\eta$  and pol  $\kappa$  adds further evidence that  $\epsilon$ C is a miscoding lesion for mammalian replication enzymes.

Among the four enzymes used, pol  $\eta$  facilitated the most efficient bypass synthesis of 8-HM- $\epsilon$ C or  $\epsilon$ C with all possible base substitutions (Figs. 3 and 4). This enzyme is encoded by the XP-V gene and characterized by its ability to catalyze error-free bypass of UV-induced DNA damage by incorporating two A's opposite a cis-syn T-T dimer (42, 43). However, human pol  $\eta$  has also been shown to perform error-prone translesional syntheses toward such DNA lesions as 8-oxoguanine, an AP site and (+)-trans-anti-benzo[ $\alpha$ ]pyrene-N<sup>2</sup>-dG bulky adduct (44). The fact that this enzyme also efficiently bypasses two other exocyclic adducts, 8-HM- $\epsilon$ C and  $\epsilon$ C, suggests a broad substrate range for the enzyme. Pol  $\eta$  also replicated the unmodified template, forming all four base substitutions (Figs 3 and 4). This highly error-prone nucleotide incorporation supports previous findings that the enzyme replicates normal DNA with low fidelity (45-47). Some of this may be explained by a loose requirement of the polymerase for correct Watson-Crick base-pairing geometry.

In a previous report, pol  $\kappa$  showed diverse substrate affinity in its error-free and error-prone bypass of 8-oxo-dG, AP site, AAF-dG and (+)-trans-anti-benzo[ $\alpha$ ]pyrene-N<sup>2</sup>-dG (22). In the present work, pol  $\kappa$  showed a highly specific insertion preference for both 8-HM- $\epsilon$ C and  $\epsilon$ C, mainly incorporating T opposite the adduct, which would direct a C  $\rightarrow$  A transversion (Fig 4). Human pol  $\kappa$  synthesizes undamaged DNA with very low fidelity (21,22). A recent lung tumor study implicates the overexpression of pol  $\kappa$  in tumorigenesis (23), which may account for the accumulation of mutations as the enzyme has very low fidelity.

It is not known how various DNA polymerases are recruited to sites of DNA damage for translesion synthesis. A current view holds 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 (for review, see (12)). 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, these four mammalian polymerases exhibit differential base incorporation specificities and bypass capacity. These *in vitro* experiments showing miscoding and/or bypass can be useful in evaluating *in vivo* mispairing as a function of the presence of specific replicating enzymes.

## FIGURE LEGENDS

Fig 1: Chemical structures of dC, 3,N<sup>4</sup>-etheno-dC, and 8-(hydroxymethyl)-3,N<sup>4</sup>-etheno-dC.

Fig 2: Time course of full replication using 200  $\mu$ M of all four dNTPs of normal,  $\epsilon$ C, and 8-HM- $\epsilon$ C containing oligomers by 0.03 Units of pol  $\alpha$ , 3.20 Units of pol  $\beta$ , 3.46 ng of pol  $\eta$ , and 25.83 ng of pol  $\kappa$ . Reactions were performed as described in Materials and Methods for the times indicated below each lane. The template sequence is shown center-top, and vertically on the two sides of the figure.

Fig 3: Single base insertion assays of normal,  $\epsilon$ C, and 8-HM- $\epsilon$ C containing oligomers by pol  $\alpha$ , pol  $\beta$ , pol  $\eta$ , and pol  $\kappa$  using 200  $\mu$ M of a single dNTP and 5 $\mu$ M dCTP to initiate a running start one base before the C\* sites. Reactions containing pols  $\beta$ ,  $\eta$ , and  $\kappa$  were incubated for 30 min at 37°C, and the reactions containing pol  $\alpha$  were incubated for 30 min at 30°C.

Fig 4: Bar graph quantitation of data from single base insertion experiments in Fig 2. Graphs were calculated as the percent of primer in each lane extended to the C\* site and beyond as a result of dNTP insertion.

Fig 5: Comparison of the rates of replication synthesis from Fig 2, as a representative plot to show the extent of full replication of 8-HM- $\epsilon$ C and  $\epsilon$ C containing oligomers by pol  $\eta$ .

Fig 6: Structures of C,  $\epsilon$ C and 8-HM- $\epsilon$ C produced by molecular modeling. The 8-HM- $\epsilon$ C has similar planar and sugar conformation to the  $\epsilon$ C. The OH group of 8-HM- $\epsilon$ C is displaced 15° away from the exocyclic ring plane. The similarity in planar and sugar conformations of the 8-HM- $\epsilon$ C and  $\epsilon$ C could contribute to the similar rate of replication synthesis as well as insertion specificity.

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