Reduced *in vivo* mutagenesis by mutant herpes simplex DNA polymerase involves improved nucleotide selection

(antimutator/antiviral drugs/nucleotide analogues/replication fidelity/editing nuclease)

JENNIFER D. HALL*, PHILLIP A. FURMAN†, MARTY H. ST. CLAIR†, AND CHARLES W. KNOPF‡

*Department of Molecular and Cellular Biology, University of Arizona, Tucson, AZ 85721; †Department of Experimental Therapy, Wellcome Research Laboratories, Burroughs Wellcome Company, Research Triangle Park, NC 27709; and ‡Institut für Virusforschung, Deutsches Krebsforschungszentrum, Heidelberg, Federal Republic of Germany

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ABSTRACT We present evidence that mutation frequencies in a mammalian system can vary according to the replication fidelity of the DNA polymerase. We demonstrated previously that several derivatives of herpes simplex virus type 1 that encode polymerases resistant to various antiviral drugs (e.g., nucleotide analogues) also produce reduced numbers of spontaneous mutants. Here we show that the DNA polymerase from one antimutator virus exhibits enhanced replication fidelity. First, the antimutator virus showed a reduced response to known mutagens that promote base mispairing during DNA replication (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, 5-bromo-deoxyuridine). Second, purified DNA polymerase from the antimutator produced fewer replication errors *in vitro*, based on incorporation of mispaired nucleotides or analogues with abnormal sugar rings. We have investigated possible mechanisms for the enhanced fidelity of the antimutator polymerase. We show that the mutant enzyme has altered interactions with nucleoside triphosphates, as indicated by its resistance to nucleotide analogues and elevated K_m values for normal nucleoside triphosphates. We present evidence against increased proofreading by an associated 3',5' exonuclease (as seen for T4 bacteriophage antimutator polymerases), based on nuclease levels in the mutant polymerase. We propose that reduced affinity of the polymerase for nucleoside triphosphates accounts for the antimutator phenotype by accentuating differences in base-pair stability, thus facilitating selection of correct nucleotides.

Accurate DNA replication is a major mechanism for maintaining the integrity of chromosomal information (1). Since the frequency of replication errors is much less than expected from differences in stabilities between correctly and incorrectly paired bases, other components (e.g., replication complex, nucleotide pools, mismatch repair) must contribute to these low frequencies. DNA polymerases in *Escherichia coli* and T4 bacteriophage control replication errors by modulating the selection of nucleoside triphosphates for polymerization and the levels of an associated 3',5' exonuclease able to remove incorrectly inserted nucleotides (2–5).

In mammalian cells, certain evidence suggests that DNA polymerase α (the major replication enzyme) plays a role in controlling mutation frequencies. Such control likely involves changes in specificities of nucleotide selection, since polymerase α lacks a proofreading nuclease. For example, a mutator Chinese hamster cell line produces an altered (aphidicolin-resistant) polymerase α (6), which may contribute to the high cellular mutation rates. Also, polymerases from certain aging and cancer-related cells are error-prone *in vitro* (7–9).

There is genetic proof that the DNA polymerase from herpes simplex virus type 1 (HSV-1) directly controls viral mutation rates. The mutation conferring an antimutator phenotype in one mutant virus (PAA^r-5) maps in the DNA polymerase locus. In addition, other mutator and antimutator strains specify altered polymerases (10, 11).

The herpes polymerase is particularly susceptible to genetic analysis, because it is the target for antiviral drugs, such as nucleotide analogues acycloguanosine (ACG) and 9- β -D-arabinofuranosyladenine (araA) (12, 13). These analogues when phosphorylated inhibit replication by inactivating the viral DNA polymerase (14) or by serving as a poor primer once incorporated.

We have analyzed a derivative of herpes simplex (PAA^r-5) that carries a drug-resistant polymerase and that exhibits a low spontaneous mutation rate due to the altered enzyme (10). We now show that this reduced mutation frequency results from more accurate replication by the drug-resistant polymerase. Since HSV-1 DNA polymerases have an associated 3',5' nuclease (15), we looked for any effect of this activity on mutation rates. In contrast to known antimutators from bacteriophage T4 (3, 4, 16), we find no increase in exonuclease in the herpes antimutator polymerase. Instead, enhanced fidelity appears to result from more stringent selection of nucleoside triphosphates. Based on our observations that this polymerase is resistant to nucleotide analogues and shows elevated K_m values for normal nucleoside triphosphates, we propose that altered nucleoside triphosphate binding may accentuate differences in base-pair stability, thus enhancing fidelity.

MATERIALS AND METHODS

Cell Lines and Virus Strains. Vero and TC7 cells (11) (from African green monkey kidney) were used for genetic experiments. Baby hamster kidney cells (15) (BHK-21, clone 13 Glasgow) or HeLa S3 cells (14) were used to purify DNA polymerase. Virus preparations, plaque assays, and plaque purifications were performed as described (11, 17). PAA^r-5 (10, 18–22), F17, and F18 (23–25) viruses have been described. PAA^r-5 is a spontaneous mutant resistant to phosphonoacetic acid, ACG, and araA (10, 12, 19). These resistances map in the DNA polymerase locus and result from the same (or closely linked) mutation(s) (22). PAA^r-5 exhibits an antimutator phenotype due to its altered DNA polymerase and likely to the same drug-resistance mutation(s) (10).

DNA Polymerase Purifications. DNA polymerase was purified from HeLa cells (K_m and inhibitor studies) as described in ref. 14 and from BHK-21 cells (fidelity and exonuclease

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Abbreviations: HSV-1, herpes simplex virus type 1; ACG, acycloguanosine or 9-[(2-hydroxyethoxy)methyl]guanine; araA, 9- β -D-arabinofuranosyladenine; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

assays) as described here. Infected-cell extracts in buffer A (20 mM Tris-HCl, pH 7.5/2 mM 2-mercaptoethanol) supplemented with 0.5 M potassium phosphate, pH 7.5/0.5% Nonidet P-40/1 mM phenylmethylsulfonyl fluoride were passed through a DE-52 cellulose column. Pooled polymerase (flow-through) fractions were further purified by (i) denatured DNA-cellulose chromatography (20 mg of DNA/g) (26) using a linear NaCl gradient (50–1000 mM) in buffer B (buffer A plus 2 mM EDTA/10% glycerol), (ii) phosphocellulose (Whatman PII) chromatography using a step gradient (50–600 mM NaCl) in buffer B plus 0.5 mg of bovine serum albumin per ml, and (iii) sedimentation through 15–30% glycerol gradients in 20 mM Tris-HCl buffer, pH 7.5/1 mM 2-mercaptoethanol/200 mM NaCl (36,000 rpm, 64 hr, SW 40Ti rotor). Peak polymerase fractions were stored at -20°C in 50 mM Tris-HCl, pH 7.5/1 mM dithiothreitol/0.1 mM EDTA/50% glycerol. Complete separation of viral from cellular polymerases occurred, and no evidence for mycoplasma contamination was found (data not shown).

RESULTS

Previous studies of herpes simplex virus showed that changes in the DNA polymerase could result in both resistance to nucleotide analogues and reduced viral mutation rates (10). Thus, the polymerase response to nucleoside triphosphates may provide clues to the mechanism of the antimutator behavior. Therefore, we studied this response in the drug-resistant virus PAA^r-5. Using biochemical and genetic tests, we have established that nucleoside triphosphate recognition is altered in the mutant, resulting in enhanced replication fidelity and more stringent selection of nucleotides. We use these characteristics to propose a model to explain the antimutator behavior of the mutant polymerase.

Utilization of Nucleoside Triphosphates by Herpes Simplex Virus DNA Polymerases. DNA polymerase from PAA^r-5 has known resistance to two nucleoside triphosphate analogues with abnormal sugar moieties [ACG (20) and araA (19) triphosphates] and has a 4-fold greater K_m for dGTP than wild type (27). Here, we have analyzed the resistance to a third nucleotide analogue with an altered sugar (dideoxyguanosine triphosphate) and have made K_m measurements for other normal nucleoside triphosphates. As shown in Fig. 1, polymerase from PAA^r-5 was more resistant to inhibition of DNA synthesis by dideoxyguanosine triphosphate than wild type (KOS). Also, the antimutator polymerase (PAA^r-5) exhibited 2- to 3-fold higher K_m values for dATP, dCTP, and dTTP than wild type, as shown in Table 1. Based on these and previous observations (19, 20, 27), the mutant polymerase appears to have a lower affinity for nucleoside triphosphates with altered sugar rings and for normal nucleoside triphosphates.

Effects of Alkylating Agents and Base Analogues on Viral Mutagenesis. We next predicted that the PAA^r-5 polymerase might be less responsive to mutagens that promote base substitution mutations. Consequently, viral mutations were induced by the alkylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). This compound produces *O*⁶-methylguanine residues (in either dGTP or template DNA) that frequently mispair during replication (30–34). Alternatively, viral mutations were induced by 5-bromodeoxyuridine, which promotes transition mutations by either mispairing (35, 36) or creating imbalances in nucleotide pools (37).

To study MNNG-induced mutations, production of 5-iododeoxycytidine-resistant mutants was measured. Spontaneous iododeoxycytidine-resistant mutants are deficient in thymidine kinase activity (10), and most induced mutants also lack kinase activity (data not shown). As described in Fig. 2, virus was grown in the presence or absence of MNNG and the progeny were examined for mutants. Wild-type (KOS) virus produced significantly higher (4- to 25-fold) mutant fractions

in the presence of MNNG than in its absence. PAA^r-5 (the antimutator) produced only 2.2% of the wild-type level of spontaneous mutants (confirming previous data; ref. 10) and failed to exhibit increases in mutant production in concentrations of MNNG up to 3 $\mu\text{g}/\text{ml}$. At higher MNNG concentrations PAA^r-5 did exhibit stimulation of mutant production up to the wild-type level.

These data suggest that DNA polymerase from PAA^r-5 rejects *O*⁶-methyl dGTP more readily than wild type, preventing base substitution mutations. This enzyme may be unable to avoid mutations at high MNNG concentrations because of high levels of *O*⁶-methylguanine in viral DNA or triphosphate pools. We showed previously (10) that viral growth rates in KOS and PAA^r-5 are similar and are unlikely to account for differences in mutation frequencies.

We next examined the effect of the altered DNA polymerase from PAA^r-5 on mutagenesis promoted by 5-bromodeoxyuridine. Reversion of two temperature-sensitive mutations (F17, F18) in a late viral gene was measured by growing the mutant viruses at the permissive temperature in the presence of the drug and analyzing the progeny for temperature resistance. Fig. 3 shows that in strains carrying wild-type polymerase, reversion of both F17 and F18 was strongly stimulated by 5-bromodeoxyuridine. In contrast, strains carrying antimutator polymerases (F17, PAA^r-5 or F18, PAA^r-5) showed little, if any, increased reversion. We suggest that the low production of revertants in strains carrying the altered DNA polymerase results from more discriminating nucleoside triphosphate selection during replication (e.g., rejection of 5-bromodeoxyuridine triphosphates).

Replication Fidelity of Purified DNA Polymerase with Homopolymers *in Vitro*. To test directly whether purified PAA^r-5 DNA polymerase exhibits enhanced replication fidelity, DNA synthesis was measured by incorporation of labeled

FIG. 1. DNA synthesis by purified DNA polymerases from KOS (wild type) (Upper) and PAA^r-5 (antimutator) (Lower) viruses. Assays were performed as described (28, 29) in the presence of 100 μM dATP, dCTP, dGTP, and [³H]dTTP (106 cpm/pmol). ●, Without dideoxyguanosine triphosphate; ○, 200 μM dideoxyguanosine triphosphate.

Table 1. K_m values for nucleoside triphosphates

DNA polymerase	K_m , μM		
	dATP	dCTP	dTTP
KOS (wild type)	0.26 ± 0.05	0.24 ± 0.09	0.22 ± 0.03
PAA ⁻ 5 (antimutator)	0.73 ± 0.16 (2.8)	0.50 ± 0.08 (2.1)	0.50 ± 0.08 (2.3)

K_m values (shown as mean $\pm$ SEM) were determined (28, 29) by using double reciprocal plots of rate versus substrate concentration. Values for PAA⁻5 relative to KOS are shown in parentheses. K_m values for dGTP have been published (27) and were $0.18 \mu M$ for KOS and $0.68 \mu M$ for PAA⁻5.

nucleoside triphosphates into poly(dC)-oligo(dG) templates. Since the PAA⁻5 enzyme is resistant to nucleotides with abnormalities in either the sugar or base moieties, we examined the incorporation of either a correctly base-paired nucleotide with an abnormal sugar (dideoxyguanosine triphosphate) or a mispaired nucleotide with a normal sugar (dATP). The ratios of incorporation of dGTP (correct nucleotide) to dATP or dideoxyguanosine triphosphate are shown in Table 2. The PAA⁻5 (antimutator) enzyme showed 1.5- to >50-fold more accurate synthesis than wild type (KOS). Large differences were observed when the nucleotide contained the incorrect sugar (dideoxyguanosine triphosphate). Smaller but reproducible differences occurred for incorporation of the mispaired base (dATP). These results suggest that the antimutator phenotype of PAA⁻5 is due to more accurate replication by its DNA polymerase.

Polymerase-Associated Exonuclease. Finally, we wished to know whether alterations in the 3', 5' exonuclease associated with the herpes DNA polymerase might account for enhanced replication fidelity. We measured this activity by using two assays. In assay A, exonuclease activity (release of nucleoside monophosphates from DNA) was measured in the absence of concurrent DNA replication and was compared to DNA polymerase activity in a parallel reaction. In assay B, exonuclease and polymerase activities were measured in the

same reaction mixtures. In general, the antimutator polymerase (PAA⁻5) showed similar or slightly lower levels of nuclease than wild type (KOS) (see Table 3). We conclude that elevated levels of editing nuclease cannot account for the enhanced replication fidelity of the PAA⁻5 DNA polymerase.

DISCUSSION

We provide evidence that the accuracy of DNA replication by a mammalian DNA polymerase directly controls mutation rates. Polymerase control over production of mutants has been shown previously in prokaryotic systems (2-5) and is shown here for HSV-1 by comparing wild-type virus (KOS) with a derivative (PAA⁻5) exhibiting reduced spontaneous mutation rates. We conclude that replication accuracy of the herpes polymerase affects mutation frequencies based on

FIG. 2. Fraction of iododeoxycytidine-resistant viruses from infections grown in the presence of MNNG. Mutagenesis assays were conducted as described (10). Briefly, Vero cells were infected at 3×10^{-5} plaque-forming units per cell and incubated at $37^\circ C$ for 4-6 days until cytopathic effects were observed. MNNG ($5 \times 10^3 \mu g/ml$, in acetone) was diluted as indicated in the growth medium. For a given drug concentration, wild-type and PAA⁻5-infected cultures were harvested at the same time and gave comparable progeny yields ($\approx 10^8$ plaque-forming units). To determine the resistant fractions, progeny virus was plated onto TC7 cells in the presence or absence of $150 \mu g$ of iododeoxycytidine per ml. The data represent the average of two independent experiments. ●, KOS (wild type); ○, PAA⁻5 (antimutator).

FIG. 3. Fractions of temperature-resistant (ts^+) revertants in viral infections by temperature-sensitive mutants (F17 or F18) and by recombinant viruses carrying the same temperature-sensitive allele and phosphonoacetic acid resistance from antimutator strain PAA⁻5. Such recombinants were deemed to have acquired the altered polymerase from PAA⁻5, since the resistance mutation maps in the DNA polymerase locus and is closely linked to (possibly identical with) the mutation that confers the antimutator phenotype (10). F17, PAA⁻5 and F18, PAA⁻5 recombinants were obtained by infecting Vero cells with 2 plaque-forming units per cell of each parental virus, incubating 27 hr at $33^\circ C$, and identifying progeny virus exhibiting both phosphonoacetic acid resistance and temperature sensitivity. Recombinants were produced at 2-4%, a level significantly higher than the spontaneous production ($<0.1\%$) of drug-resistant derivatives. To measure production of temperature-resistant revertants, Vero cells were inoculated at $33^\circ C$ (permissive temperature) with one of the above viruses as described in the legend to Fig. 2. Where indicated, 5-bromodeoxyuridine was diluted into the medium to $1 \mu g/ml$. Progeny viruses were harvested and the fractions of revertants were determined by titrating on Vero cells at $33^\circ C$ and $39^\circ C$ (nonpermissive temperature). Each point represents an independent infection. ○, Infections with 5-bromodeoxyuridine; ●, infections without drug.

Table 2. DNA synthesis with purified DNA polymerases and poly(dC)-oligo(dG) templates

	Error rate	
	KOS (wild type)	PAA ^r -5 (antimutator)
Assay A: 100 μ M dGTP		
+ 100 μ M dATP	1/2385 (2.6)	1/6223 (1.0)
+ 200 μ M dATP	1/1700 (2.0)	1/3307 (1.0)
+ 500 μ M dATP	1/905 (2.6)	1/2318 (1.0)
Assay B: 50 μ M dGTP		
+ 50 μ M dATP	1/4400 (1.5)	1/6500 (1.0)
+ 100 μ M dATP	1/1850 (4.2)	1/7750 (1.0)
Assay B: 50 μ M dGTP		
+ 50 μ M Dideoxy-GTP	1/30,000 (>3.3)	<1/100,000 (1.0)
+ 100 μ M Dideoxy-GTP	1/1850 (>54)	<1/100,000 (1.0)

Replication fidelity assays were conducted under reaction conditions described in ref. 15 using poly(dC)-oligo(dG) templates (7:3, wt/wt) (50 μ g/ml) and the indicated concentrations of dGTP (correct nucleotide) and dATP or dideoxyguanosine triphosphate (dideoxy-GTP) (incorrect nucleotide). Incubations were for 30 min at 37°C. Samples were spotted onto gf/c filters (presoaked in 1 M potassium phosphate/1% pyrophosphate) and washed six times with 50 ml each of 5% trichloroacetic acid. More than 300 pmol of correct nucleotide occurred for each assay. Fidelity was determined by two assays. In assay A (single label), parallel reaction mixtures were compared, containing [³²P]dGTP (0.25 μ Ci; 1 Ci = 37 GBq) or [³²P]dATP (12.5 μ Ci) plus 100 μ M dGTP and the indicated concentrations of dATP. Assay B employed a double-label technique in which both correct and incorrect triphosphates were labeled in the same reaction mixtures with different isotopes (either 50 μ M [³H]dGTP (250 cpm/pmol) and the indicated amounts of [³²P]dATP (5000 cpm/pmol) or 50 μ M [³²P]dGTP (50 cpm/pmol) and the stated amounts of dideoxy-[³H]GTP (1100 cpm/pmol)). For each reaction, blank values (without template) were subtracted. Misincorporation values were ≥ 6 -fold above these blanks. Values were corrected for channel overlap where appropriate. Backgrounds were 180–260 cpm. Ratios of incorrect-to-correct incorporation are presented as error rates. Error rates for the KOS polymerase relative to that for PAA^r-5 are presented in parentheses.

observations that (i) the mutation conferring the antimutator phenotype maps in the DNA polymerase gene (10), (ii) viruses carrying this altered polymerase are resistant to mutagenesis by base analogues (e.g., *O*⁶methylguanine, 5-bromouracil), and (iii) the purified mutant polymerase shows reduced nucleotide misincorporation during *in vitro* replication.

An indication of the mechanism of enhanced fidelity comes from an altered and apparently weaker interaction between the antimutator (PAA^r-5) polymerase and nucleoside

triphosphates. This polymerase exhibits higher K_m values for nucleoside triphosphates than wild type (Table 1, ref. 27). It is hypersensitive to aphidicolin (38), a compound thought to compete for dCTP binding (39), it is resistant to nucleotide analogues with abnormal sugar rings (Fig. 1; refs. 19 and 20). Analogue resistance appears related to the antimutator behavior, since both phenotypes result from closely linked or identical mutations in PAA^r-5 (10) and since several other strains resistant to nucleotide analogues also exhibit antimutator phenotypes (10).

Table 3. Exonuclease activities of herpes simplex virus DNA polymerase

DNA polymerase	Template	Labeled nucleotide	Activity, pmol/min per μ l		Ratio*
			Polymerase	Exonuclease	
Assay A					
KOS	Activated DNA	dTTP	17.2 $\pm$ 1.1	4.2 $\pm$ 0.4	0.24 (1.0)
PAA ^r -5			3.6 $\pm$ 0.3	0.8 $\pm$ 0.05	0.22 (0.92)
KOS	Poly(dC)-oligo(dG)	dGTP	26.7 $\pm$ 2.0	4.5 $\pm$ 0.3	0.17 (1.0)
PAA ^r -5			5.3 $\pm$ 0.5	1.2 $\pm$ 0.1	0.23 (1.3)
KOS	Poly(dA)-oligo(dT)	dTTP	43.6 $\pm$ 3.0	0.7 $\pm$ 0.05	0.016 (1.0)
PAA ^r -5			5.5 $\pm$ 0.5	0.1 $\pm$ 0.01	0.018 (1.1)
Assay B					
KOS	Poly(dC)-oligo(dG)	dGTP (50 μ M)	6.9 $\pm$ 0.5	2.7 $\pm$ 0.5	0.39 (1.0)
PAA ^r -5			3.5 $\pm$ 0.3	0.83 $\pm$ 0.25	0.24 (0.62)
KOS	Poly(dC)-oligo(dG)	dGTP (20 μ M)	1.0 $\pm$ 0.1	2.9 $\pm$ 0.2	2.9 (1.0)
PAA ^r -5			0.57 $\pm$ 0.05	1.4 $\pm$ 0.1	2.5 (0.86)

Exonuclease was quantitated by using standard reaction conditions (15). In assay A, nuclease was measured as release of nucleoside monophosphates from labeled templates [activated DNA (250 μ g/ml) or synthetic templates (50 μ g/ml) labeled with [³²P]dTTP or [³²P]dGMP (2200 cpm/pmol) by *E. coli* DNA polymerase I] in the absence of nucleoside triphosphates by polyethylenimine-cellulose thin-layer chromatography. DNA polymerase activities were determined in parallel reactions in the presence of labeled nucleoside triphosphates and unlabeled templates (15). Exonuclease or polymerase velocities were calculated from initial slopes in plots of activity versus incubation time. In assay B, nucleoside triphosphate incorporation (polymerase activity) and nucleoside monophosphate generation (exonuclease activity) were determined in the same reaction mixtures in the presence of 20 or 50 μ M [³²P]dGTP (820 cpm/pmol) and 50 μ g of poly(dC)-oligo(dG) per ml by thin-layer chromatography after 30-min incubations. Reactions were linear for ≥ 60 min. Data are the average (mean $\pm$ SEM) of three independent determinations. Activities for the antimutator (PAA^r-5) polymerase relative to wild type (KOS) are presented in parentheses.

*Exonuclease/polymerase ratio.

Based on these altered nucleotide interactions, we propose a model to explain the mutant (PAA⁻-5) DNA polymerase behavior. We suggest that replication fidelity depends upon the relative strength of two types of interactions: (i) binding of the DNA polymerase to the sugar ring of nucleoside triphosphates and (ii) base-pairing interactions with template DNA. In wild-type polymerase, binding to sugar rings is strong, making base-pairing interactions less significant. Thus, misincorporation of incorrectly paired nucleotides occurs. Wild-type polymerase is sensitive to nucleotide analogues with altered sugar rings, since strong interactions with the remaining normal features of the nucleotides stabilize the complex. In the antimutator polymerase nucleotide interactions are weaker. This reduced affinity results in higher fidelity, since only correctly paired nucleotides acquire the necessary additional template interactions to stabilize the complex. Nucleoside triphosphate analogue resistance occurs because the nucleotide-polymerase complex is destabilized by loss of normal interactions with sugar groups in the analogue.

The DNA polymerase of HSV-1 has an associated 3',5' exonuclease. A similar activity in T4 bacteriophage is increased in several antimutator strains (3, 4, 16), thus improving replication fidelity. In contrast, the level of exonuclease in the herpes antimutator polymerase was similar to or slightly less than wild type (Table 3). This result suggests that enhanced fidelity in the herpes system does not result from altered proofreading, although we cannot rule out altered nuclease specificity in the mutant enzyme.

The DNA polymerase from herpes simplex virus provides a unique opportunity to study the effect of altered polymerases on mutation rates in mammalian cells and to analyze these enzymes for altered biochemical properties. Correlations between *in vitro* and *in vivo* properties have been crucial for suggesting molecular models of mutagenesis in prokaryotic systems (2-5).

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