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Genetics

Mutagenesis during *in vitro* DNA synthesis

(error frequency/ϕX174 DNA template/*Escherichia coli* DNA polymerase I)

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ABSTRACT **The error frequency of *in vitro* DNA synthesis using a natural DNA template has been measured with a biological assay for nucleotide substitutions. ϕX174 DNA containing an amber mutation was copied *in vitro* by *Escherichia coli* DNA polymerase I, and the reversion frequency of the progeny DNA was determined by transfection of *E. coli* spheroplasts. *E. coli* polymerase I makes less than 1 mistake at the *am*3 locus for every 7700 nucleotides incorporated under standard reaction conditions. Substitution of Mn^{2+} for Mg^{2+} and unequal concentrations of deoxynucleoside triphosphate substrates raises this mutation frequency to greater than 1 in 1000. Thus, *E. coli* DNA polymerase I can copy natural DNA templates with high fidelity and its accuracy can be affected by alterations in reaction conditions.**

The accuracy of DNA synthesis contributes to the final mutation rate of an organism (1–3). *In vitro* measurements of the fidelity of DNA synthesis have used purified DNA polymerases and synthetic polynucleotide templates (4–6). In most studies, the template has been a homopolymer or alternating copolymer so that a mismatched nucleotide can be identified simply as one not complementary to any of the template nucleotides. However, studies of fidelity using homopolymer and alternating polymer templates may not accurately reflect the events during the synthesis of natural (heteropolymeric) DNA. Indeed, the nature of the template has been found to affect the error frequency of synthesis (3, 5) as well as the kinetics of polymerization (7). Because of their repeating nature, the synthetic templates may tolerate the insertion of incorrect nucleotides at a greater frequency than natural DNA by allowing a slippage of primer and template during synthesis (8) and by allowing mismatched nucleotides to loop out of the nucleic acid helix (9, 10).

To begin to define the biochemical interactions that determine mutation rates within the cell, a method is required for measuring the error frequency of DNA synthesis on a natural DNA template. Because errors in copying are infrequent and occur as single and random nucleotide substitutions (5, 11), they are difficult to detect biochemically. We therefore developed a biological assay to measure such errors. In a defined *in vitro* reaction, DNA polymerase was allowed to copy past a base substitution mutation on a ϕX174 DNA template. The frequency with which back mutations appeared in the daughter strand was then determined by transfection. With this assay we sutdied the error frequency of natural DNA synthesis by *Escherichia coli* DNA polymerase I under different *in vitro* reaction conditions.

MATERIALS AND METHODS

Materials. Wild-type ϕX174 bacteriophage was provided by M. Edgell. *am*3 ϕX174 phage, the bacterial indicator strains *E. coli* HF4704 Su^-, hcr^-, thy^-, ϕX^s and *E. coli* HF4714 Su^+, thy^-, ϕX^s, and single-stranded *am*3 ϕX DNA were provided by J. Weiner and A. Kornberg. The spheroplast strain *E. coli* KT-1, Su^+, was a gift of M. and M. Hayashi. Carrier RNA (ribosomal 18S and 28S RNA from *Drosophila melanogaster*) was prepared by K. Tartof. Double-stranded replicative form ϕX174 DNA, from both *am*3 and wild-type phage, was prepared by a modification of the method of Schekman *et al.* (12). Restriction endonuclease DNA fragments (Z fragments) were prepared by digesting the replicative form DNA with *Hae* III, separating the fragments on a 4% acrylamide tube gel, and isolating the fragments as described by Summers (13).

E. coli DNA polymerase I was purified by the method of Jovin and coworkers (14, 15) and estimated to be free of endonuclease (16) and 99% homogeneous as determined by polyacrylamide gel electrophoresis.

Hybridization of Primer to Template. *am*3 ϕX single-stranded DNA and *am*3 Z-5 fragments were mixed at an estimated primer/template molar ratio of 2.5:1 in 10 mM Tris·HCl, pH 8.0/0.30 M NaCl/0.03 M Na citrate. The DNA fragments were denatured by heating at 100° for 10 min followed by quick cooling to 0°. Annealing was at 65° for 10 min, followed by immediate cooling to 0°. Heteroduplex DNA was prepared as above with wild-type Z-7 fragments which cover the *am*3 mutation site.

DNA Synthesis. The standard reaction mixtures (25–100 μl) contained the following: 100 mM Tris·HCl (pH 8.0); 8 mM $MgCl_2$; 5 mM dithiothreitol; *am*3 Z-5-primed *am*3 single-stranded ϕX DNA (3.3 μg/ml or 13.3 μg/ml); 100 μM dATP, dGTP, dCTP, and [α-^{32}P]dTTP (300–2000 cpm/pmol), and DNA polymerase I (at an enzyme/template molar ratio of 115:1). Where indicated, the deoxynucleotide concentrations were changed so that one was increased to 300 μM and the others were decreased to 10 μM each. In certain experiments 0.5 mM $MnCl_2$ was used in place of 8 mM $MgCl_2$. Duplicate aliquots (1 μl) were removed at the indicated times and the acid-precipitable ^{32}P radioactivity was determined (5). The result, expressed as total nucleotides incorporated per template molecule, is simply a calculated average.

Covering Fragments to Template. The efficiency of minus strand expression after transfection was improved by covering the single-stranded ϕX DNA with restriction fragments. Each experimental DNA sample (uncopied, copied, or hybridized to wild-type Z-7) was hybridized to *am*3 Z fragments prior to transfection. First, equal amounts of all the *am*3 Z fragment except Z-5 were combined and denatured by heating. Then the experimental DNA sample was mixed with the combined denatured *am*3 Z fragments (at a ratio of 2.5 mol of each fragment per mol of DNA circles) in 0.30 M NaCl/0.03 M Na citrate. After heating at 65° for 10 min, the DNA was cooled to 0°,

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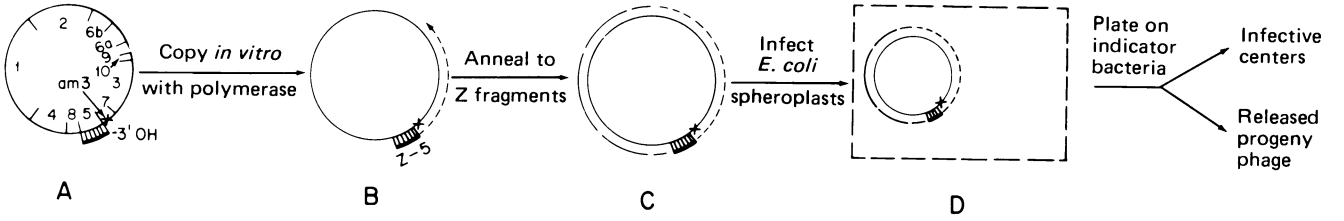

FIG. 1. Experimental plan. The numbers in *A* refer to the *Hae* III restriction fragments Z-1 to Z-10 (18, 19).

diluted in 50 mM Tris·HCl (pH 8.0), and used to infect spheroplasts.

Transfection. Preparation of spheroplasts, infection with DNA, and measurement of infective centers or progeny phage were performed as described by Guthrie and Sinsheimer (17). Indicator bacteria were *E. coli* HF4704 (nonpermissive for *am*3) and HF4714 (which suppresses *am*3 by inserting a serine at the UAG codon). Plaques were counted after 4–6 hr of incubation at 37°.

RESULTS

The experimental plan was to copy past an amber mutation *in vitro* and to determine the frequency of nucleotide substitutions in the daughter strand that reverted the amber mutation (Fig. 1). The template was single-stranded (plus) ϕX174 DNA containing an amber mutation (*am*3 in gene E which codes for host cell lysis). The primer was the *Hae* III restriction fragment Z-5 of *am*3 ϕX174 which, when hybridized to the template (Fig. 1*A*), provided a 3′-OH primer terminus specifically located 83 nucleotides away from the *am*3 mutation (18–20). Synthesis was carried out with *E. coli* DNA polymerase I (Fig. 1*B*) under different *in vitro* conditions. Thereafter, the DNA was hybridized to *am*3 Z fragments (all except Z-5) (Fig. 1*C*) and the covered DNA was used to infect *E. coli* spheroplasts (Fig. 1*D*). The frequency of back mutations that occurred during *in vitro* synthesis was determined by measuring the proportion of infected spheroplasts that yielded revertant (wild-type) phage after plating on the permissive and nonpermissive *E. coli* strains. Two methods of quantitation were used: an infective centers assay that directly measured the proportion of spheroplasts yielding revertant phage, and a progeny phage assay that measured the proportion of revertant phage after a single burst. Analysis of the reversion frequency in the progeny phage assay must take into account the extent of multiplication within the spheroplast.

The time course with which DNA polymerase I copied ϕX174 DNA is shown in Fig. 2. The extent and direction of DNA synthesis were determined by analyzing the fragments obtained from digesting copied DNA with *Hae* III restriction endonuclease. Even at early time points, copying had occurred past the Z-7 region of the genome, which contains the *am*3 mutation (18, 20).

Biological Assay. When a specific wild-type restriction fragment is hybridized to a mutant ϕX DNA plus strand and the heteroduplex DNA is used to infect spheroplasts, the genotype of the minus strand is expressed in the progeny phage (the marker rescue technique) (16, 21). For the *am*3 mutation, we found that the maximum efficiency of expression of the minus strand genotype in marker rescue experiments was quite low ($<10^{-2}$). In order to increase the efficiency of expression, a second step was required in which the partially duplex DNA was made almost totally duplex by hybridization to the remaining *Hae* III fragments. These covering *Hae* III fragments were derived from *am*3 DNA so that they would not be contaminated with the wild-type sequence at the *am*3 locus. Titration experiments indicated that maximum expression of the minus strand genotype occurred at a molar ratio of wild-type fragments to circles of about 5:1. In a total of eight determinations measuring progeny phage, the penetrance of the minus strand genotype averaged 0.13 ± 0.06; with infective centers it was essentially the same.

In six experiments, the background reversion frequency of *am*3 DNA annealed to an *am*3 primer fragment measured by progeny phage averaged $(1.7 \pm 0.6) \times 10^{-6}$ (Table 1; unpublished results). Because the number of wild-type progeny phage observed was less than that expected from a single burst (40–100), they presumably arose during multiplication within the spheroplasts. With infective centers, revertants were not detected, the spontaneous reversion frequency being $<3 \times 10^{-5}$. The biological assay for minus strand revertants therefore ex-

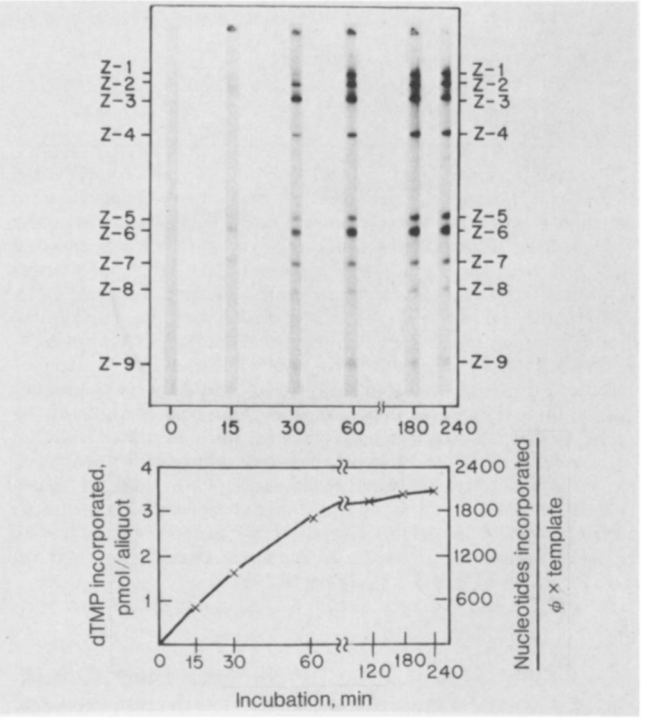

FIG. 2. Time course of *in vitro* DNA synthesis. DNA was synthesized by DNA polymerase I at 15°. At the times indicated, 1-μl samples were taken to measure incorporation and 3-μl samples were withdrawn for autoradiography. The latter samples were pipetted into stopping solution (10 mM Tris, pH 7.5/10 μM EDTA/0.5% Sarkosyl/100 mM NaCl) and then 10 μg of carrier RNA was added. The DNA was precipitated with 95% ethanol and digested with *Hae* III, and the fragments were separated on a 5% acrylamide slab gel by electrophoresis at 100 V for 4 hr. An x-ray film was exposed to the dried gel for 72 hr; the resulting autoradiogram is shown. Label migrating in the region of Z-5 and Z-6 results partly from nicked-translation of unhybridized primer fragments, as indicated by electrophoresis of DNA not digested by *Hae* III (results not shown). After incubation for 60 min, this corresponds to 3% of the total DNA synthesized.

Table 1. Effect of different *in vitro* copying conditions on reversion frequency of DNA* synthesized by DNA polymerase I

Conditions	Exp.	Infective centers PFU $\times 10^{-3}$	Infective centers wt PFU	Infective centers Rev. freq. $\times 10^5$	Progeny phage PFU/ml $\times 10^5$	Progeny phage wt PFU/ml	Progeny phage Rev. freq. $\times 10^5$
			Copied DNA				
Standard	2	28.8	<1	<3.5	3.6	0.5	0.1
	3	4.8	<1	<21.0	0.7	<0.3	—
	4	14.8	<1	<6.7			
	5	12.0	<1	<8.3			
Mg^{2+}, dCTP	6	97.0	<1	<1.0			
Mg^{2+}, dUTP	6	124.0	<1	<0.8			
Mn^{2+}, dCTP	1				3.4	230	68.0
	2				5.3	250	48.0
	3	10.8	2	19.0	1.1	25	23.0
	4	4.8	3	63.0			
	5	6.4	3	47.0			
Mn^{2+}, dGTP	1				3.8	<0.3	—
	3				0.7	<0.1	—
Mn^{2+}, dUTP	6	127.0	<1	<0.8	51.0	25	<0.5
			Uncopied DNA				
Standard	2				6.2	<0.3	—
Mg^{2+}, dGTP	1				57.0	13	0.2
Mn^{2+}, dCTP	2	32.8	<1	<3.0	6.2	1	0.2
	3	25.0	<1	<4.0	4.9	0.5	0.1
Mn^{2+}, dGTP	1				83.0	20	0.2

*am*3 single-stranded DNA, primed with *am*3 Z-5, was copied. After a second hybridization in which the DNA circles were annealed to *am*3 Z-fragments to improve the efficiency of minus strand expression, the DNA was used to infect spheroplasts. Uncopied DNA was exposed to identical conditions as copied DNA except that DNA polymerase was omitted from the reaction mixture and, in Exp. 1, uncopied DNA was not subjected to the second hybridization reaction. In a typical spheroplast infection, 4×10^{10} molecules of copied DNA were incubated with 8×10^8 spheroplasts in a final volume of 8 ml. For the infective center assay, the total number of wild-type (wt) plaque-forming units (PFU) was determined on four 1-ml aliquots of the infected spheroplast suspension. Total PFU were calculated from the results of serial dilutions. Progeny phage were obtained by incubating 4 ml of the infected spheroplast suspension for 3 hr, and the values represent plaques per ml. In all experiments performed, multiplication from infective centers to progeny phage ranged from 40 to 100. The dNTP named was present at an increased concentration, 300 μM for dCTP and dGTP and 100 μM for dUTP.

* DNA synthesized averaged (±SD) 670 ± 78 nucleotides per template.

tended over a range of about 10^4 per assay, from the background reversion frequency ($<3 \times 10^{-5}$) to the reversion frequency of heteroduplex DNA (1.3×10^{-1}).

The ability of the marker rescue technique combined with the second covering hybridization to detect the presence of a small proportion of wild-type minus strands among many mutant minus strands was tested. Heteroduplex wild-type/*am*3 DNA was serially diluted with homoduplex *am*3/*am*3 DNA. Each DNA mixture was used to infect *E. coli* spheroplasts (Fig. 3). The ratio of minus strand genotypes in the infecting DNA was accurately reflected in the ratio of genotypes in the phage, measured either as infective centers or as progeny phage. This demonstrates that the multiplication from infective centers to progeny phage was the same for wild-type and mutant phage. Thus, reversion frequency can be measured as progeny phage

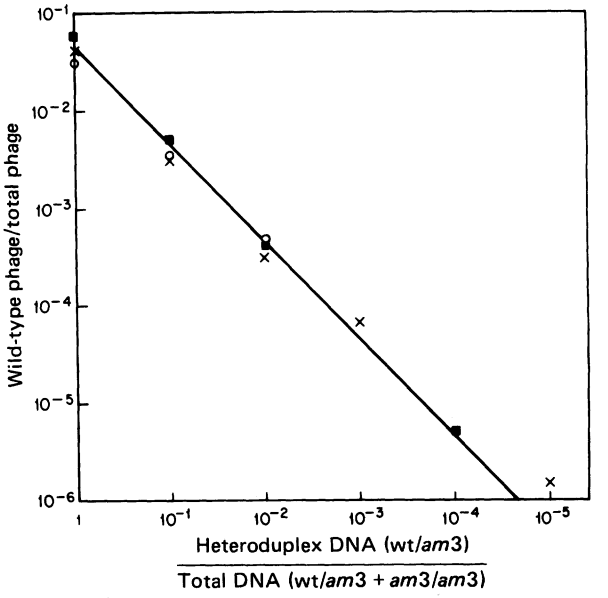

FIG. 3. Relationship between genotype ratio of infecting DNA and genotype ratio of phage after transfection. Heteroduplex DNA was formed by hybridizing wild-type (wt) Z-7 fragments to *am*3 single-stranded DNA circles at a 5:1 molar ratio. Homoduplex DNA was formed similarly by using *am*3 Z-7 fragments. The heteroduplex DNA was serially diluted with homoduplex DNA to the ratios shown on the abscissa. Each DNA mixture was then annealed in a second hybridization to denatured *am*3 Z-fragments and used to infect spheroplasts. Results of two experiments are plotted. In one, the genotype ratio of the phage was measured in progeny phage after release from spheroplasts (■). In the other experiment, the genotype ratio was measured in both infective centers (○) and in progeny phage (×). The straight line (slope = 1) represents the theoretical dilution obtained if the genotype ratio of the phage was proportional to the genotype ratio of the infecting DNA. The points for 10^{-5} and 10^{-4} dilutions could have been derived from the multiplication of less than one infectious center and therefore may not be statistically valid. In different experiments the penetrance ranged from 0.04 to 0.2.

if the number of sampling events (i.e., infective centers) is calculated to be significant.

Reaction Conditions Mutagenic for DNA Replication. *am*3 ϕX DNA primed with an *am*3 Z-5 fragment was copied by DNA polymerase I under different *in vitro* conditions. The extent of DNA synthesis under the different conditions was kept to approximately the same level within each experiment by varying the time of incubation. The copied DNA was used to infect spheroplasts, and the reversion frequency of the resulting phage was determined either as infective centers or as progeny phage (Table 1). No revertant infective centers were detected with uncopied DNA ($<3 \times 10^{-5}$). With DNA copied under "standard" conditions (Mg, 8 mM; equimolar saturating concentrations of dNTPs, 100 μM each) there were no detectable revertants, except in one measurement of progeny phage in which the revertants appeared at the background level of 1.4×10^{-6}

ϕX DNA copied by DNA polymerase I in the presence of $MnCl_2$ and a high dCTP concentration (300 μM dCTP compared to 10 μM of each of the other nucleotide substrates) consistently resulted in phage with a reversion frequency of $1.9\text{–}6.8 \times 10^{-4}$. This level of revertants was detected in both infective centers and in progeny phage and was therefore not due to selective multiplication. This observed reversion frequency was approximately 10 times greater than the reversion frequency of DNA copied under other reaction conditions or of DNA incubated with the same reaction mixture (Mn^{2+}, high

Table 2. Effect of relative nucleotide concentrations on reversion frequency of DNA* copied with Mn^{2+}

Incubation conditions	Progeny phage PFU/ml $\times 10^{-5}$	wt PFU/ml	Reversion freq. $\times 10^5$
	Copied DNA		
Mn^{2+}, dCTP	1.1	25	23.0
Mn^{2+}, dTTP	1.0	0.2	0.2
Mn^{2+}, dATP	1.3	8.2	6.3
Mn^{2+}, dGTP	0.7	<0.1	
	Uncopied DNA		
Mn^{2+}, dCTP	4.9	0.5	0.1

Experimental procedures and notations identical to those in Table 1; all data were from Exp. 2. Each reaction mixture contained 500 μM $MnCl_2$, 300 μM dNTP, and the remaining three dNTPs at 10 μM each. Total volume of the infected spheroplast suspension used to measure progeny phage was 4 ml for the DNA copied with Mn^{2+}, high dCTP and for the uncopied DNA, and 8 ml for each of the others.
* DNA copied averaged (±SD) 929 ± 136 nucleotides/template.

dCTP) in the absence of DNA polymerase. DNA copied in the presence of Mn^{2+} and high dGTP resulted in no detectable revertants. The results suggest that, when DNA polymerase I copied *am*3 ϕX DNA under these *in vitro* conditions of Mn^{2+} and high dCTP, it made an increased number of nucleotide substitutions, detectable as an increased number of daughter-strand revertants at the *am*3 locus. Because dCTP contained 0.11% dUTP it was possible that the observed reversions with high dCTP (30 μM) and Mn^{2+} resulted from the preferential incorporation of dUTP during Mn^{2+} activated *in vitro* synthesis followed by an error-prone excision process. However, the direct addition of 100 μM dUTP did not increase reversion frequency with either Mg^{2+} or Mn^{2+}. In other experiments it was observed that the substitution of Mn^{2+} for Mg^{2+} during DNA synthesis increased the number of mutant progeny when the dNTPs were present at equal concentrations, whereas increasing the concentration of dCTP 300-fold (3 mM) did not cause revertants if the metal activator was Mg^{2+} (data not shown).

The effect of increasing the concentration of each of the nucleotides individually with Mn^{2+} as an activator is shown in Table 2. Only with high dCTP concentrations were significant numbers of revertant progeny phage detected. High dTTP concentrations resulted in progeny phage with a reversion frequency of 2.0×10^{-6}, equal to the background level. High dATP yielded phage with a reversion frequency of 6.3×10^{-5}, which is just within the detectable limits of the assay. High dGTP produced no detectable revertants; the reversion frequency was estimated as less than 6.6×10^{-5} (Table 1).

DISCUSSION

The biological assay used in these experiments is designed to detect nucleotide substitutions at the *am*3 locus in the minus strand of ϕX DNA. It is likely that revertant phage result only from nucleotide substitutions located within the *am*3 codon that prevent chain termination of gene E protein. Because gene E overlaps gene D in phage ϕX (19), the only nucleotide substitutions that will result in revertant phage are those coding for both functional E and functional D proteins.

The reversion frequencies of phage resulting from infection by copied DNA may be used to estimate the error frequency with which the DNA was copied. Fig. 3 shows that the ratio of genotypes in the minus strands of the infecting DNA molecules is proportional to the ratio of genotypes in the progeny phage.

Incorrect nucleotide (minus strand)	Correct nucleotide (minus strand)	Position of mispair	Amino acid change incurred: Gene E	Gene D
C	T	587	am→Trp (wt)	Val→Val (wt)
	A	586	am→Glu	Val→Gly
T	C	588	am→ochre	Gly→Arg
	A	586	am→Lys	Val→Glu
A	T	587	am→Leu	Val→Val
	C	588	am→Tyr	Gly→opal
G	A	586	am→Gln	Val→Ala
	T	587	am→Ser	Val→Val
	C	588	am→Tyr	Gly→Arg

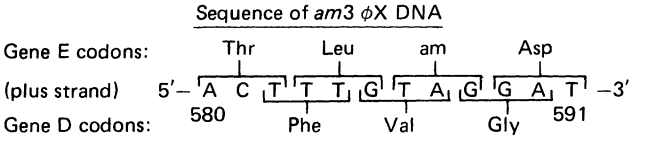

FIG. 4. Nucleotide substitutions possible at *am*3 codon, based on the *am*3 ϕX plus strand sequence published by Sanger *et al.* (18) and the wild-type (wt) ϕX plus strand sequence published by Barrell *et al.* (19). The codons amber, ochre, and opal cause termination of the polypeptide chain in nonpermissive hosts. The direction of minus strand synthesis is from right to left in the sequence depicted at the bottom (20).

The proportionality factor may be called the penetrance, P, because it is the efficiency with which the minus strand genome is expressed.
Thus,

$$\frac{\text{(no. of revertant phage)}}{\text{(total no. of phage)}} = \frac{\text{P (no. of revertant DNA (}-\text{) strands)}}{\text{(total no. of DNA (}-\text{) strands)}}.$$

Because, by definition, the error frequency of DNA synthesis is the ratio,

$$\frac{\text{(no. of revertant DNA (}-\text{) strands)}}{\text{(total no. of DNA (}-\text{) strands)}},$$

it follows that,

error frequency of DNA synthesis

$$= \frac{\text{(no. of revertant phage)}}{\text{P (total no. of phage)}}.$$

The mean (±SD) value for P is 0.13 ± 0.06 in our experiments. This calculation rests on three assumptions: (*i*) every DNA molecule was copied past the *am*3 mutation; (*ii*) the efficiency of minus strand expression for copied DNA was as high as that for heteroduplex DNA; and (*iii*) nucleotide substitution at other sites did not mimic or interfere with mutations at the *am*3 locus. If either of the first two assumptions were wrong, the true error frequency would be higher than that calculated; if the third were wrong, the true error frequency may be either higher or lower than that calculated.

For DNA synthesis by DNA polymerase I in our experiments under standard conditions, no revertant infective centers were detected after transfection and were estimated at less than 1 revertant in a total number of infective centers assayed in four experiments—i.e., less than 1 in 60,400. With this reversion frequency, the error rate of ϕX DNA synthesis was estimated to be less than 1/7700. At least 10 times more infective events would have to be sampled to measure an actual error frequency

for DNA polymerase I, whose error frequency has been reported to be less than 1/80,000 with alternating copolymers (22). When DNA polymerase I synthesized ϕX DNA in the presence of Mn^{2+} and a 30-fold excess of dCTP, the error frequency of DNA synthesis ranged from 1/667 to 1/192. Because nucleotide sequence data suggest that substitutions occur at one position (see below), the error rate calculated is the frequency of nucleotide substitution per base pair.

The error frequency of ϕX DNA synthesis in the presence of Mn^{2+} appeared to be affected by the relative concentrations of the nucleotide substrates because an increase in the concentration of dCTP caused a significantly increased phage reversion frequency whereas an increase in the concentration of any of the other deoxynucleoside triphosphates did not (Table 2). This observation corroborates studies on fidelity with homopolymers (2, 5, 6, 11). The effects of changing the relative nucleotide substrate concentrations (Table 2) can be interpreted in terms of the genetic consequences of the possible nucleotide substitutions (Fig. 4). Increased dCTP during synthesis led to an increased level of revertant phage. Fig. 4 shows that a transition substitution of C for T at position 587 leads, in fact, to the original wild-type DNA sequence. Increased dTTP caused no reversions. This is consistent with the fact that a transition substitution with T as the incorrect nucleotide would cause another nonsense codon to replace *am*3. Increased dATP resulted in a small number of revertants. If these are significant and are due to dATP substitution, then the most probable change is an A-for-T transversion at position 587. Increased dGTP caused no detectable revertants, although one possible nucleotide substitution would definitely code for functional protein. (At position 587, G-for-T transversion would code in the D protein for valine, the wild-type amino acid, and in the E protein for serine, the amino acid known to suppress *am*3 in *E. coli* HF 4714.) The lack of detectable revertants with increased dGTP and the low level with dATP suggests that during *in vitro* synthesis of ϕX DNA, transversions may have occurred less frequently than transitions, as found during copying of homopolymers (unpublished data).

The observation that the substitution of $MnCl_2$ for $MgCl_2$ increased the error frequency with which DNA polymerase I copied ϕX DNA *in vitro* is in accord with reports that Mn^{2+} increases the *in vitro* error frequency of homopolymer synthesis (2, 6, 23, 24) and is mutagenic *in vivo* for *E. coli* (25) and bacteriophage T4 (26).

With this method, it is now possible to measure the mutation rate of natural DNA synthesis in a biochemically defined *in vitro* system. The assay may be used in its simplest form—DNA polymerase plus nucleic acid—to study the error frequencies of different DNA polymerases, the K_m of different dNTPs as correct and incorrect substrates, and the effect of mutagens on the fidelity of DNA synthesis. Its greatest potential, however, lies in the possibility it provides for using nucleotide selection as a probe to investigate the interaction between replicative proteins and nucleic acids in a biochemically defined system. An immediate possibility is the investigation of protein interactions in an *E. coli* or T4 replicating complex (27–29).

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