The effect of assay conditions on the fidelity with which *Escherichia coli* DNA polymerase I copies natural DNA has been determined using a modification of the recently developed φx174 fidelity assay system (Weymouth, L. A., and Loeb, L. A. (1978) *Proc. Natl. Acad. Sci. U. S. A.* 75, 1924). In this assay, the error rate which *Escherichia coli* DNA polymerase I copies natural φx174 am3 DNA to wild type DNA. Variations in the divalent metal ion activator used in the copying reaction markedly affect the reversion frequency of copied φx174 am3 DNA. Thus, the calculated error rate of 1 incorrect for every 17,100 correct nucleotides incorporated observed with 5 mM Mg²⁺ can be increased severalfold by the substitution of Mn²⁺ or Co²⁺ for Mg²⁺. The error rate can also be increased by copying in the presence of inhibiting concentrations of Mg²⁺. In selective situations, the reversion frequency is dependent on the relative proportion of the different deoxyribonucleoside triphosphates present in the copying reaction. The error rate increases severalfold when the level of dCTP or dATP is decreased in vitro synthesis. Furthermore, there is a direct proportionality between relative increase in dATP pool size and error rate in Mn²⁺-activated reactions. These results with natural DNA substantiate our previous studies with synthetic polynucleotides and support the concept that the most likely substitutions observed in the φx174 fidelity assay system are a C for T transition and an A for T transversion at position 587, and a C for A transversion at position 586 of the am3 codon.

Until recently, studies on the fidelity of DNA synthesis in *vitro* have involved the use of synthetic polynucleotides. These studies have clearly shown that the frequency of insertion of an incorrect nucleotide is markedly affected by the specific *in vitro* reaction conditions used. Of particular interest are the studies involving the use of mutagenic metals (1-7). For example, substitution of manganese or cobalt, both of which are carcinogenic (8, 9) and mutagenic (10-12), for magnesium as the divalent metal ion activator has been shown to increase the error rate of *in vitro* DNA synthesis by several factors. The effect of divalent metal ion activators and deoxyribonucleoside triphosphate pools on the fidelity of DNA replication is quantitated by measuring the frequency of reversion of copied φx174 am3 DNA containing an amber mutation to wild type DNA. Variations in the divalent metal ion activator used in the copying reaction markedly affect the reversion frequency of copied φx174 am3 DNA. Thus, the calculated error rate of 1 incorrect for every 17,100 correct nucleotides incorporated observed with 5 mM Mg²⁺ can be increased severalfold by the substitution of Mn²⁺ or Co²⁺ for Mg²⁺. The error rate can also be increased by copying in the presence of inhibiting concentrations of Mg²⁺. In selective situations, the reversion frequency is dependent on the relative proportion of the different deoxyribonucleoside triphosphates present in the copying reaction. The error rate increases severalfold when the level of dCTP or dATP is decreased in vitro synthesis. Furthermore, there is a direct proportionality between relative increase in dATP pool size and error rate in Mn²⁺-activated reactions. These results with natural DNA substantiate our previous studies with synthetic polynucleotides and support the concept that the most likely substitutions observed in the φx174 fidelity assay system are a C for T transition and an A for T transversion at position 587, and a C for A transversion at position 586 of the am3 codon.

### Experimental Procedures

#### Materials—Wild type φx174 bacteriophage was provided by M. Edgell, University of North Carolina, φx174 am3 bacteriophage and the bacterial indicator strains *E. coli* HF4714 Su⁺, thy⁻, φx⁰, and *E. coli* HF4704 Su⁺, hcr⁻, thy⁻, φx⁻ were gifts of J. Weiner and A. Kornberg, Stanford University. The pheroplast strain *E. coli* KT1, Su⁺, φx⁻ was provided by M. and M. Hayashi. RNase A, pancreatic DNase I, ethidium bromide, chloramphenicol, protamine sulfate, and bovine serum albumin (Fraction V) were obtained from Sigma Chemical Co. Spermidine and pronase were purchased from Calbiochem. "Povite" albumin (30%) (Serum Biotest Institute, Frankfurt am Main, Germany) was a gift of K. Tartof, Fox Chase Institute for Cancer Research. Lysozyme was obtained from Worthington Biochemical Corp. and unlabeled deoxyribonucleoside triphosphates (dNTPs) from P-L Laboratories. [α-³²P]GTP, [α-³²P]ATP, and [α-³²P]-dATP were purchased from New England Nuclear Corp. Restriction endonuclease *Hae* III was from New England Bio Labs, Beverly, Mass. *E. coli* DNA polymerase I (Pol I)¹ was purified to homogeneity using the procedure of Jovin et al. (17) as modified by Springgate et al. (18).

### Purification of φx174 am3 Viral DNA—φx174 am3 viral DNA was purified by modifications of the method of Iwaya et al. (19). One liter of tryptone broth (containing per liter: 10 g of tryptone, 5 g of KCl, 1 pg/ml of thymine, and 0.1% dextrose) was inoculated with an overnight culture of *E. coli* HF4704 to an A₅₇₀ = 0.050. Growth was at
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37°C with aeration to an AM = 0.58 (4 × 10^11 cells/ml). The culture was made 5 mm in CaCl_2 and ϕX174 am3 bacteriophage (spontaneous reversion frequency 3 × 10^-6) were added at a multiplicity of infection of 5. Ten minutes thereafter, [3H]thymidine was added and incubation continued at 37°C under vigorous aeration. The infected cells were centrifuged at 5,000 × g, washed once with 50 mM sodium borate (pH 9.0), 20 mM EDTA, and resuspended in 4 ml of this same buffer. The cells were lysed by incubating for 20 min at 37°C in the presence of 1 mg/ml of lysozyme. MgCl_2 was added to 30 mM followed by addition of RNase A to 20 μg/ml and pancreatic DNase I to 20 μg/ml. Incubation was continued for 80 min at 37°C and the DNA was harvested by centrifugation. The supernatant was saved and the pellet was resuspended in 10 ml of borate-EDTA and shaken vigorously for 2 h at 0°C in a shaking water bath. The debris was removed as before by centrifugation and the supernatants were combined and stored gently overnight at 4°C.

The debris was once again removed by centrifugation and the supernatant was then centrifuged at 4°C for 3 h at 100,000 × g in a Type 65 fixed angle rotor using a Beckman L2-65B ultracentrifuge. The pelleted phage were resuspended in 50 mM sodium borate. To each milliliter of phage suspension was added 0.625 g of CsCl and the suspension was centrifuged at 140,000 × g for 21 h at 4°C in a Type 65 fixed angle rotor. The phage band was collected and dialyzed overnight against 20 mM changes of 50 mM Tris-HCl (pH 8.1), 1 mM EDTA, 100 mM NaCl. The DNA was extracted by addition of an equal volume of 50 mM Tris-HCl (pH 8.1), 1 mM EDTA, 100 mM NaCl, 1% sodium dodecyl sulfate, and 0.8% 8-hydroxyquinoline. The phenol phases were combined and re-extracted with an equal volume of 50 mM Tris-HCl (pH 8.1), 1 mM EDTA, 100 mM NaCl. All phenol extraction steps were performed at 4°C. The final aqueous DNA fractions were then precipitated by addition of a 3 M sodium acetate, precipitated by addition of 2 volumes of ice-cold 95% ethanol, and kept at −70°C overnight. The DNA was collected by centrifugation, dried under nitrogen, and resuspended in 3 ml of 10 mM Tris-HCl (pH 8.1), 1 mM EDTA, 100 mM NaCl. The sample was divided into two 2.5-ml aliquots which were layered onto two 25-ml 5 to 20% neutral sucrose gradients prepared in the same buffer. Centrifugation at 15°C was for 10 h at 55,000 rpm in a SW 27 rotor. Fractions were collected from the top and the viral DNA peak was identified by measuring radioactivity in aliquots of each fraction. Peak fractions were pooled and ethanol-precipitated as described above. The precipitated DNA was resuspended and stored in a small volume of 10 mM Tris-HCl (pH 8.1), 1 mM EDTA. The concentration of ϕX174 DNA was determined on the basis of 1 AMO unit being equivalent to 36 μg/ml (3.2 × 10^12 molecules/μg) (20). In DNA polymerase reactions, synthesis in the absence of added primer was less than 10% of synthesis using DNA primed with a single restriction fragment.

Purification of ϕX174 am3 RF DNA—ϕX174 am3 RF DNA was prepared using a modification of the technique of Strehlow et al. (21). Two 1-ml aliquots of MT 3XD medium were inoculated to an A600 of 0.05 using an overnight culture of E. coli HF4704 in the same medium and incubated with aeration at 37°C until the A600 was 0.90 (~5 × 10^11 cells/ml). The cells were then infected at a multiplicity of infection of 5 with ϕX174 am3 phage (suspended in MT 3XD medium). Ten minutes later, chloramphenicol was added to 30 μg/ml and 3 min later, [3H]thymidine was added to 0.4 μCi/ml. Incubation was continued for 2 h, and the cells were harvested by centrifugation at 5,000 × g for 10 min, resuspended in 240 ml of 50 mM Tris-HCl (pH 8.1) and distributed into four polyallomer centrifuge tubes (Beckman No. 370624). The tubes were suspended in 50 mM Tris-HCl (pH 8.1) and incubated in a water bath at 37°C for 15 min. The DNA was isolated as above and the A600 was equal to 0.60. The cells were isolated by centrifugation at room temperature for 5 min at 5,000 × g and resuspended in 1.5 ml of 1.5 M sucrose, followed by 0.6 ml of 30% Povite albumin, 0.12 ml freshly prepared lysosome (2 mg/ml in 0.25 M Tris-HCl, pH 8.1) and 0.24 ml of unbuffered 4% EDTA. After 2 min at room temperature, 55 ml of freshly prepared minimal PA medium was added and incubation continued for 12 min at room temperature, without agitation. Finally, 1.2 ml of 10% MgSO_4, 0.15 ml of freshly prepared trisodium phosphate (1%) and 0.1 ml of fresh spermidine (150 mg/ml) were added, and the spheroplast suspension immediately stored on ice.

Preparation of Spheroplasts—Spheroplasts were prepared as described by Henner et al. (22). Using an overnight culture of E. coli KY-1, 400 ml of fresh M-9 medium, supplemented with 20 μg/ml of adenine and 3 μg/ml of thiamine, was inoculated to an A600 = 0.05 (~5 × 10^11 cells/ml). Growth continued for 15 h at 37°C. The culture was then centrifuged at 10,000 × g for 21 h at 20°C in a type 50 mI fixed angle rotor. The supernatant was saved and the pellet was resuspended in 10 ml of unbuffered 4% EDTA. The yield of each individual fragment from this procedure varied from 50 to 90%.

Hybridization of Primer to Template—A single designated ϕX174 am3 Hae III restriction fragment (primer) and ϕX174 am3 single-stranded DNA (template) were mixed at primer/template ratio of 5:1 in 10 mM Tris-HCl (pH 8.1), 0.3 M NaCl, 0.03 M sodium citrate. The DNA was denatured by heating to 100°C for 10 min, followed by quick cooling to 0°C. Hybridization of primer to template was at 65°C for 10 min, followed by cooling to 0°C. This is the primer-template used in all polymerase reactions described below.

DNA Polymerase Reactions—The DNA polymerase reactions were carried out in 50-μl reaction volumes containing 20 mM Tris-HCl (pH 8.0), 0.2 μg of ϕX174 am3 single-stranded DNA primed at a 5:1 molar ratio with Hae III restriction fragment Zα, Zβ, or Zγ as indicated, E. coli DNA polymerase I at an enzyme/template molar ratio of 25:1, the indicated divalent metal cation and 30 μM dATP, dGTP, dCTP, and [γ-32P]dTTP (500 to 2,000 dpm/pmol). Reactions were incubated for 2 h at 15°C and terminated by addition of EDTA to 1.5-fold molar excess over the divalent metal ion. Duplicate 2-μl aliquots of each reaction mixture were processed for determination of acid-precipitable radioactivity as previously described (14). The results, expressed as total nucleotides incorporated per template molecule, represent a calculated average, assuming all molecules are initiated and copied to the same extent. The remainder of the reaction mixture was used for transfection of spheroplasts.
medium (containing, per liter, 10 g of casamino acids (Difco), 10 g of Nutrient Broth (Difco), 10 g of glucose, 100 g of sucrose, and 0.2% MgSO₄ (added after autoclaving)) was added and the mixture plated by the soft agar overlay technique described by Guthrie and Sinsheimer (23), using PAM agar (1% agar in PAM medium). Indicator base agar with 0.1% of FP4704 (nonpermissive for am3) and FP4714 (which suppresses am3 by inserting a sirene at the UAG codon). For determination of infective center titers on the permissive host, it was necessary to dilute 1 ml of the transfection mixture with PAM medium in order to obtain plates with the appropriate number of plaques. For the nonpermissive host, the entire remainder of the transfection mixture in PAM medium (9 ml) was plated onto multiple plates. Plates were incubated for approximately 6 h at 37°C and plaques scored using a New Brunswick Scientific colony counter.

Improvements in Fidelity Assay—With spheroplasts prepared as described above, we routinely obtain transfection efficiencies of 10⁻² to 10⁻³ for single-stranded φX174 am3 DNA and 10⁻¹ to 10⁻² for copied DNA. This represents an increase in infectivity of 10⁻¹ to 10⁻²-fold over our previous spheroplast preparations. Furthermore, the use of protamine sulfate and spermidine stabilizes the spheroplast preparations such that there is no significant decrease in infectivity for up to 14 days after their preparation (data not shown). Since we observe no difference in reversion frequency between fresh and 14-day-old spheroplasts, we are able to do a large number of comparative experiments using the same spheroplast preparation.

The observation that DNA contaminants can decrease infectivity of φX174 DNA in spheroplast preparations and that single-stranded φX174 DNA is more infective than is double-stranded φX174 DNA (24), prompted us to test the possibility of improving infectivity of our copied DNA by leaving out the secondary annealing of the remaining Hae III restriction fragments (“top annealing”). We previously reported that this step improved the expression of wild type DNA fragments from less than 1% to an average of 13%, in control experiments using Hae III fragments (13). While this result has since been confirmed in numerous experiments for uncopied DNA in a prophage phage assay, the secondary annealing step does not improve wild type expression for the DNA in the infective center assay. The reversion frequency of copied DNA was the same, regardless of whether or not this DNA had been annealed to the remaining fragments (data not shown). Furthermore, as anticipated, elimination of this 5-fold excess of linear DNA restriction fragments from the spheroplast infection mixture improved infectivity of the copied DNA by 2- to 10-fold.

In performing copying reactions, we routinely observe a large decrease in transfection efficiency for copied φX174 DNA. One possible explanation is that the enzyme preparation itself is inhibiting infectivity. The E. coli DNA polymerase I preparation was previously shown to be free of contaminating nucleases (25). However, DNA polymersases bind to DNA, and our reactions are carried out with a large molar excess of polymerase. Such binding of protein to DNA could potentially decrease infectivity. Several methods of removing the enzyme from the DNA, including salt, heat, phenol, and other protein-denaturing agents were tried. Subjecting the reaction mixture to 65°C for 10 min was the simplest method found to improve infectivity, and increased the number of infective centers observed by 1- to 2-fold.

Thus far, all attempts to increase the number of wild type revertants (“marker rescue”) by using spheroplasts prepared from E. coli strains deficient in one or more nucleases, or DNA repair pathways, or both, have been unsuccessful. This is due to a large decrease in overall infectivity of spheroplasts prepared from these strains.

RESULTS

The basis of the φX174 fidelity assay is the observation that infection of spheroplasts with a mutant φX174 DNA circle hybridized to a wild type DNA fragment which will cover the mutation will result in revertant, wild type, phage are those coding for both functional gene E and functional gene D proteins, it is likely that this assay measures only substitutions at the am3 codon itself.

Dependence of Increase in Reversion Frequency on DNA Synthesis—The in vitro requirements for producing an increased reversion frequency with E. coli DNA polymerase I in a Mn⁺⁺-activated reaction are shown in Table I. Omission of metal activator, DNA polymerase, or any of the four deoxynucleoside triphosphates eliminates the production of revertants above the level obtained with the uninhibited control. These requirements are identical with those needed for DNA synthesis on primed φX174 DNA (Table I) or other templates (28), and suggest that wild type revertants are indeed produced by in vitro DNA synthesis and not by some nongenetic effect of reaction components.

Metal Activation of DNA Synthesis—The ability of Mg⁺⁺, Mn⁺⁺, and Co⁺⁺ to act as divalent metal activators of DNA synthesis using specifically primed φX174 am3 DNA is shown

TABLE I

| Assay conditions       | Reversion frequency (× 10⁻⁴) | Nucleotides incorporated/φX template |
|------------------------|-----------------------------|-------------------------------------|
| Complete, 15°C, 120 min | 1.55                        | 1.620                               |
| - Enzyme               | 0.95                        | <50                                 |
| - Mn⁺⁺                  | 0.96                        | <50                                 |
| - dGTP                  | 0.97                        | 59                                  |
| - dATP                  | 0.96                        | <50                                 |
| - dCTP                  | 0.97                        | <50                                 |
| - Incubation            | 0.95                        | 0                                   |

The reactions were performed as described under “Experimental Procedures,” using 1.0 mM MnCl₂ as the divalent metal activator, 6.4 × 10⁻¹⁰ molecules of Zs-primed φX DNA and 4.8 × 10⁻¹⁰ molecules of E. coli DNA polymerase I. In the experiment in which dTTP was deleted, [α-³²P]dATP was used, at a specific activity of 612 dpm/pmol.

FIG. 1. Metal activation of DNA synthesis with φX174 DNA. DNA polymerase reactions were performed as described under “Experimental Procedures.” Each assay contained 3.2 × 10⁻¹⁰ molecules of φX174 viral DNA, primed at a 5:1 molar ratio with the Hae III restriction fragment Zs, 8 × 10⁻¹⁰ molecules of E. coli DNA polymerase I and the indicated metal ions. The enzyme/template ratio was 25:1. The labeled nucleotide was [α-³²P]dATP (specific activity, 444 dpm/pmol). Incubation was for 120 min at 15°C.
in the copying reaction increases the reversion frequency of the copied DNA when compared to the uncopied control for each concentration of Mg++. This results in an increase in the calculated error frequency from 1/17,100 at 5 mM Mg++ to 1/2,270 at 15 mM Mg++, the highest concentration examined. Substitution of Mn++ for Mg++ increase the calculated error frequency by severalfold over that observed with activating concentrations of Mg++. The error rate of E. coli DNA polymerase I with 1 mM Mn++ is 1/2,060, 8-fold higher than that observed with 5 mM Mg++. Similarly, Co++ increases the frequency of misincorporation by 2- to 4-fold over 5 mM Mg++. The effect of Mn++ on fidelity as measured in this assay system is independent of the concentration of Mn++ used, since the error rate in the presence of suboptimal, optimal, and inhibitor concentrations of Mn++ is approximately the same.

**Effect of Metal Activators on Fidelity of DNA Synthesis—**The fidelity of DNA synthesis on specifically primed φX174 am3 DNA in reactions containing various concentrations of Mg++, Mn++, and Co++ was determined. In these and all subsequent experiments, whenever metal activator concentrations above the optimum were employed, the enzyme/template ratio was increased to the extent necessary to achieve approximately the same level of synthesis observed at the optimum metal concentration. Control experiments indicate that the error rate with equal concentrations of all four dNTPs, calculated as described previously (13), does not change significantly with enzyme concentration or primer utilized (Zs, Zn, or Z0), provided synthesis has proceeded beyond the am3 site (data not shown).

As shown in Table II, increasing the concentration of Mg++

### Table II

| Metal Ion | Concentration (mM) | Nucleotides incorporater-template | Reversion frequency (x 10^-4) | Error rate | No. of experiments |
|-----------|--------------------|----------------------------------|-------------------------------|------------|-------------------|
| Mg++      |                     |                                  |                               |            |                   |
| 1.0       | 1,800              | 0.38                             | 1/22,400                      | 1          |                   |
| 5.0       | 1,690              | 0.84                             | 1/17,100                      | 11         |                   |
| 7.5       | 1,720              | 1.00                             | 1/7,800                       | 6          |                   |
| 10        | 2,240              | 1.21                             | 1/3,090                       | 1          |                   |
| 15        | 2,150              | 1.15                             | 1/2,270                       | 2          |                   |
| Mn++      |                     |                                  |                               |            |                   |
| 0.25      | 780                | 1.66                             | 1/2,240                       | 2          |                   |
| 0.50      | 1,390              | 1.77                             | 1/2,520                       | 5          |                   |
| 1.0       | 1,650              | 1.66                             | 1/2,060                       | 3          |                   |
| 1.75      | 1,840              | 1.57                             | 1/2,680                       | 2          |                   |
| 2.5       | 1,300              | 1.33                             | 1/1,750                       | 1          |                   |
| Co++      |                     |                                  |                               |            |                   |
| 2.5       | 3,150              | 1.24                             | 1/4,150                       | 2          |                   |
| 3.75      | 2,800              | 1.57                             | 1/5,980                       | 1          |                   |
| 7.5       | 797                | 0.01                             | 1/3,850                       | 1          |                   |

### Table III

Effect of deoxynucleoside triphosphate pool size on fidelity

The reactions were performed as described in the legend to Table II. The designation “equal dNTP” indicates all four deoxynucleoside triphosphates (dNTPs) present in the in vitro DNA synthesis reaction (13). An analysis of this phenomenon using an infective centers assay is presented in Table III. In reactions activated with 1 mM Mn++, the error rate is dependent on the relative concentrations of dNTPs in the DNA polymerase reaction. The reversion frequency of DNA copied in the presence of a 10-fold excess of dATP was 13.6 x 10^-4 as compared to 1.45 x 10^-4 for DNA copied in the presence of equimolar concentrations of all four dNTPs. This calculates to approximately a 14-fold increase in error rate of DNA synthesis. The effect is similar, although not as marked, with dCTP. A 5- to 10-fold excess of dCTP yields a 1- to 3-fold increase in the calculated error rate (see also Table VI). In contrast to these results, similar increases in either dGTP or dTTP concentrations during DNA synthesis yield a decreased production of wild type phage. For example, a 10-fold excess
the direct proportionality of error rate to relative increase in dATP concentration (Table IV). When dATP is present during DNA synthesis in 50-fold greater quantity than the other three dNTPs, the error rate, as calculated from reversion of the am3 locus, increases 45-fold. It was not possible to test higher concentrations of dATP, since synthesis was greatly reduced, presumably due to chelation of the divalent metal ion by the deoxynucleoside triphosphates. The direct proportionality of error rate to relative dATP concentration described here was observed only with Mn\(^{2+}\) and dATP. Use of increased dCTP with Mn\(^{2+}\) or Co\(^{2+}\) increased with Mg\(^{2+}\) or Co\(^{2+}\) did not result in this proportionality.

### Reversal of Pool Size Effects

To further delineate the types of nucleotide substitutions that were occurring at the am3 locus when altered pools were present, the relative amounts of two different dNTPs were altered at the same time (Tables V and VI). In Mn\(^{2+}\)-activated reactions containing a 5-fold excess of dATP, in which the error rate increased 5-fold from 1/2,600 to 1/489, the relative concentrations of each of the other three dNTPs were varied independently in graded amounts. Under these conditions, dGTP had essentially no effect on the error rate, since a 10-fold increase in dGTP yielded an error rate of 1/423, similar to the 1/489 seen with 5 \times dATP alone. Increasing the concentration of dCTP progressively reversed the effect of excess dATP, such that the "5 \times dATP + 10 \times dCTP" condition exhibited a higher fidelity than when all four dNTPs were equal. In contrast, increasing dCTP concentrations enhanced the error rate approximately 2-fold over that observed with "5 \times dATP" only. A similar experiment using a 5-fold excess of dCTP as baseline is shown in Table VI. Once again, a combination of increased dATP and dCTP gave enhancement of infidelity over the excess dCTP only. However, both dGTP and dTTP showed a marked ability to reverse the effect of increased dGTP or dTTP in the copying reaction produced a reversal frequency less than that of the uncopied control. Uncopied control reactions containing increased concentrations of each dNTP independently yield phage with a reversal frequency identical with that obtained with the uncopied control. These controls indicate that increased dNTP concentrations alone were not responsible for the observed increase in error rate with copied DNA.

The effects of altered dNTP pools on fidelity of Mg\(^{2+}\)- and Co\(^{2+}\)-activated reactions, while qualitatively similar to those found with Mn\(^{2+}\), were not nearly so pronounced. Thus, a 10-fold increase in dATP pool size gave little or no increase in error rate with 7.5 mM or 15 mM Mg\(^{2+}\) and only a 3-fold increase with 3.75 mM Co\(^{2+}\) (Table III).

### Proportionality between Increase in dATP Concentration and Increase in Error Rate

The decrease in error rate of E. coli DNA polymerase I when copying primed \(\phi X174\) DNA in the presence of 1.0 mM Mn\(^{2+}\) is directly proportional to the relative increase in dATP concentration (Table IV). When dATP is present during DNA synthesis in 50-fold greater quantity than the other three dNTPs, the error rate, as calculated from reversion of the am3 locus, increases 45-fold. It was not possible to test higher concentrations of dATP, since synthesis was greatly reduced, presumably due to chelation of the divalent metal ion by the deoxynucleoside triphosphates. The direct proportionality of error rate to relative dATP concentration described here was observed only with Mn\(^{2+}\) and dATP. Use of increased dCTP with Mn\(^{2+}\) or Co\(^{2+}\) or increased dATP with Mg\(^{2+}\) or Co\(^{2+}\) did not result in this proportionality.

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To further delineate the types of nucleotide substitutions that were occurring at the am3 locus when altered pools were present, the relative amounts of two different dNTPs were altered at the same time (Tables V and VI). In Mn\(^{2+}\)-activated reactions containing a 5-fold excess of dATP, in which the error rate increased 5-fold from 1/2,600 to 1/489, the relative concentrations of each of the other three dNTPs were varied independently in graded amounts. Under these conditions, dGTP had essentially no effect on the error rate, since a 10-fold increase in dGTP yielded an error rate of 1/423, similar to the 1/489 seen with 5 \times dATP alone. Increasing the concentration of dCTP progressively reversed the effect of excess dATP, such that the "5 \times dATP + 10 \times dCTP" condition exhibited a higher fidelity than when all four dNTPs were equal. In contrast, increasing dCTP concentrations enhanced the error rate approximately 2-fold over that observed with "5 \times dATP" only. A similar experiment using a 5-fold excess of dCTP as baseline is shown in Table VI. Once again, a combination of increased dATP and dCTP gave enhancement of infidelity over the excess dCTP only. However, both dGTP and dTTP showed a marked ability to reverse the effect of increased dGTP or dTTP in the copying reaction produced a reversal frequency less than that of the uncopied control. Uncopied control reactions containing increased concentrations of each dNTP independently yield phage with a reversal frequency identical with that obtained with the uncopied control. These controls indicate that increased dNTP concentrations alone were not responsible for the observed increase in error rate with copied DNA.
errors with dCTP, as for example, with “5 × dCTP + 10 × dTTP” or “5 × dCTP + 10 × dGTP”.

**Discussion**

The modified biological assay of fidelity of DNA synthesis described here has achieved approximately a 50-fold increase in infectivity of copied φX174 DNA molecules over that previously published (13). The use of protamine sulfate in spheroplast preparations provided the most dramatic increase in transfection efficiency, while the combination of protamine sulfate and spermidine stabilized the spheroplasts for up to 14 days. This latter phenomenon is particularly valuable when performing direct comparative experiments. The transfection efficiencies obtained in our experiments (10^-4 to 10^-6) are somewhat lower than those obtained by Henner et al. (22) for untreated φX174 DNA (10^-2 to 10^-3). We attribute this difference in part to the fact that we are infecting spheroplasts with molecules that are partially double-stranded due to the copying reactions, and are thus intermediate between viral DNA and RF DNA. Also, manipulation of the DNA in the copying reactions, different suppression efficiency of the spheroplast strain and subtle differences in preparation of spheroplasts (e.g., use of Sigma Fraction V bovine serum albumin in some instances instead of “Povite” albumin) could account for the somewhat lower transfection efficiency. Control of these variables could therefore potentially increase transfection efficiencies even more.

Our modified assay allows one to perform a large number of experiments using relatively small quantities of primer and template DNA. More importantly, the assay can now be performed entirely as an infective center assay and still provide a significant number of revertants. This is an advantage over studies involving progeny phage, which require a large number of controls to determine possible differences in the extent of multiplication of wild type and mutant DNA.

Calculations of error rates of the DNA polymerase depend on three assumptions (13). The first is that nucleotide substitutions at other sites do not mimic mutations at the am3 locus. This is reasonable since am3 is an amber mutation in gene E which overlaps gene D (29). Thus, only nucleotide substitutions which code for functional E and D proteins will produce revertant infective centers. The second assumption is that the efficiency of minus strand expression, or penetration, is the same for copied DNA as for control heteroduplex DNA. The presence of 0.13 ± 0.06 was determined using heteroduplex DNA formed from intact φX174 am3 viral DNA annealed to Hae III Fragment Z (13). If frequency of marker rescue is indeed determined by a competition of replication on three assumptions (13). The first is that nucleotide substitutions at other sites do not mimic mutations at the am3 locus. This is reasonable since am3 is an amber mutation in gene E which overlaps gene D (29). Thus, only nucleotide substitutions which code for functional E and D proteins will produce revertant infective centers. The second assumption is that the efficiency of minus strand expression, or penetration, is the same for copied DNA as for control heteroduplex DNA. The presence of 0.13 ± 0.06 was determined using heteroduplex DNA formed from intact φX174 am3 viral DNA annealed to Hae III Fragment Z (13). If frequency of marker rescue is indeed determined by a competition of replication

**Effect of Metal Activators on Fidelity**—Both Mn2+ and Co2+ are known to be mutagenic and carcinogenic (32). We have previously shown that both metals substitute quite well for Mg2+ as divalent metal ion activators for DNA synthesis by several DNA polymerases (2, 5, 7). Furthermore, the fidelity of DNA synthesis has been shown to be dependent on the nature and concentration of the metal activator used. Thus, both Mn2+ and Co2+ have been found to decrease fidelity when compared to Mg2+-activated DNA synthesis on synthetic polynucleotides for Pol I (2), avian myeloblastosis virus DNA polymerase (5), and human placenta DNA polymerases α and β (7). The data in Fig. 1 and Table II on metal ion activation and fidelity confirm these findings and extend them to DNA synthesis using a natural template. The error rates observed here are in fact quite similar to those found when Pol I utilizes poly(dA-T) as a template (2). This represents an important confirmation that studies of fidelity utilizing synthetic polynucleotides are real and that valid conclusions can be made from such studies.

The data obtained here with natural DNA differ from data obtained using synthetic polynucleotides in two aspects. The first is the extent of the decrease in fidelity of DNA synthesis in reactions activated with optimal versus inhibiting concentrations of Mn2+. This decrease is much more pronounced with natural DNA (10-fold at 1.0 mM versus 15 μM Mn2+). Table II) than with poly(dA-T), where the decrease is typically at most 2-fold with Pol I.2 The second is that the error rate in copying natural DNA is relatively constant at all concentrations of Mn2+ examined (Table II), contrasted with the large decrease in fidelity observed utilizing poly(dA-T) with increasing Mn2+ concentrations (2). While the exact reasons for these differences are not clear, such effects could be explained on the basis of metal-template interactions. Physical studies have shown that divalent metal ions interact with both phosphates and bases on polynucleotides (33). Thus, metal-template interactions may be crucial in determining the fidelity of DNA synthesis. Differences between synthetic polynucleotides and natural DNA with respect to metal ion binding constants could account for the differences in fidelity observed. The suggestion that metal-template interactions may be crucial to fidelity is supported by these studies, in which the error rate of DNA synthesis was invariant over a range of enzyme concentrations (from an enzyme/template ratio of 20:1 to 500:1), when the amount of template used was kept constant. Furthermore, we have recently demonstrated that the fidelity with which Pol I copies poly(dA-T) is dependent on the concentration of template in the reaction mixture.3

**Effect of Alterations in dNTP Pool Sizes**—The fidelity of DNA synthesis on synthetic polynucleotide templates has previously been shown to be dependent on the ratio of incorrect to correct nucleotides in the reaction mixture. T4 DNA polymerase (6, 15), avian myeloblastosis virus DNA polymerase (16, 29), and Pol I (25) all make more mistakes when the incorrect nucleotide concentration is increased relative to the correct one. Our results confirm this trend in the case of DNA synthesis utilizing dNTPs. However, we have observed the opposite effect with reaction mixtures utilizing dCTP, as for example, with “5 × dCTP + 10 × dTTP” or “5 × dCTP + 10 × dGTP”. This effect is particularly pronounced when the concentration of dCTP is increased. We have observed that the fidelity of DNA synthesis is decreased when the concentration of dCTP is increased, even with the 5:1 primer/template ratio used here. This, however, all molecules may not have been copied and the error rate may be greater than calculated. From such considerations, it is obvious that the error rates presented here represent initial estimations, and are subject to change. This does not, however, change the error rates relative to one another, and allows conclusions to be made from these direct comparative studies.

1. M. Koplitz and L. A. Loeb, unpublished results.
2. M. Koplitz and E. Katz, unpublished results.
the correct nucleotide. Conversely, Pol I is reportedly much more faithful when the correct nucleotide is proportionately higher (25). Based on these observations, we determined which nucleotide substitutions are most likely to occur at the am3 locus under precisely defined reaction conditions.

The genetic consequences of various potential nucleotide substitutions at the am3 locus are shown in Table VII. Measured here as infective centers, increased reversion frequencies were observed in the presence of increased levels of dATP or dCTP, while the use of increased dGTP or dTTP resulted in reversion frequencies less than control values, copied at equimolar dNTP concentrations. Qualitatively, these data confirm our previous findings measured with the progeny phage assay (13). However, measured as progeny phage, increased dCTP yielded a much greater effect in Mn**+-activated reactions than did increased dATP. The reasons for this difference are not clear.

The most dramatic decrease in fidelity observed here was with increased dATP levels in Mn**+-activated reactions. The only probable direct substitution of A at the am3 codon is for T at position 587. The fact that this transversion is not nearly as frequent in Mg**- or Co**+-activated reactions (Table III) suggests that Mn**+ promotes transversions more effectively than Mg**- or Co**+. This possibility is supported by the report of Orgel and Orgel (11) of a class of Mn**+-induced T mutants which had the properties expected of transversion mutants. The findings here are in contrast to studies involving synthetic polynucleotides, which suggest that >95% of errors are transitions rather than transversions (25). Conceivably the nature of the metal activator, or the neighboring nucleotide sequences, or both, could play a major role in determining the nature of the substitution. Further studies to test this possibility could utilize other amber mutations having different neighboring nucleotides.

The increased reversion frequency of DNA copied in the presence of high C (Table III and V) can be explained by a C for T transition at position 587, which would result in the original wild type DNA sequence (Table VII). A second possibility is a C for an A transition at position 586, a possibility supported by the fact that the combination of high A and high C increases the reversion frequency over that observed when either is increased by itself (Tables V and VI).

The effects of increased dTTP and dGTP suggest that the substitutions of these nucleotides which could potentially produce increased revertants do not occur at detectable levels. This is even more striking when one realizes that one possible substitution, a G for T transversion at position 587, codes for an amino acid known to be functional (serine, which suppresses am3 in E. coli HF 4/14), and would therefore produce wild type phage. Potentially, the inability to insert a G at this position could be a restriction by the neighboring nucleotides.

The observation that both T and G actually decrease the reversion frequency below that of DNA copied at equimolar concentrations of all four dNTPs suggested that T and G were competing with C, or A, or both. This possibility is strongly supported by the data in Tables V and VI. T competes with both A and C, since the reversion frequency in the presence of increased A or C decreases as the level of T is increased. G competes with C, but does not compete with A. Interestingly, if G competes with C at position 587, it may do so without being incorporated, since a G substitution at 587 should be functional and would therefore produce an increased reversion frequency, which is not observed (Tables III and VI). Alternatively, it is possible that the G substitution at position 587 could be repaired in vivo and thus not be observed.

Taken together, the data in Tables III through VI suggest that there are three substitutions in the am3 codon which produce detectable wild type phage in our assay system. These are an A for T transversion or C for T transition at position 587, or a C for A transversion at position 586. These conclusions do not take into account the possibility of specific in vivo repair processes in the spheroplast strain which may limit the detectable substitutions at the am3 locus. The use of several spheroplast strains with different repair capabilities and the use of amber mutations at other loci in the φX genome will be required to detect the range of possible nucleotide substitutions which may occur.

It is important to note that the error rate was proportional to the relative concentration of incorrect to correct nucleotides (biased pools) in only one situation, Mn**+, and high dATP. A proportional change was not observed with Mg**- or Co**+. Thus, measurements of fidelity which depend on extrapolation from biased pools could be invalid. In every case controls are required to demonstrate that the change in error rate is proportional to the relative concentrations of incorrect to correct nucleotides.

The observation that the error rate of DNA synthesis in vitro is dependent on the relative concentrations of each of the four nucleotide substrates is of particular interest in light of the recent report of Peterson et al. (34) that in vivo mutagenesis of Chinese hamster cells is increased 3- to 10-fold in the presence of biased thymidine and deoxyctydine pools.

Highly accurate copying conditions produce copied DNA having a reversion frequency very similar to or less than uncopied control values (Tables II, III, V, VI). This sensitivity limitation would make any systematic study of factors which improve fidelity difficult. In order to circumvent this problem, we desired to define a set of error-prone conditions which could potentially be used as a model system to study factors which improve fidelity. The data in Table IV represent our initial attempts in this regard. The extremely error-prone copying conditions shown here allow one a wide range of detectable improvement in fidelity by added exogenous factors such as any of the numerous proteins thought to be involved in DNA replication, or repair, or both (28, 35). The φX174 fidelity assay system described here thus allows one to address problems involving both increased and decreased fidelity. In addition, by choice of mutant DNA templates containing different neighboring sequences, one can address questions of specificity.

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