The Infidelity of Avian Myeloblastosis Virus Deoxyribonucleic Acid Polymerase in Polynucleotide Replication

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SUMMARY

We have studied the accuracy of DNA synthesis in vitro by purified avian myeloblastosis virus (AMV) DNA polymerase ("reverse transcriptase") using synthetic polynucleotide templates. The enzyme catalyzes the incorporation of an exceptionally large number of incorrectly paired bases when copying ribopolyadenylic acid and deoxyribopolyadenylic acid. The frequency of error is approximately 1 in 600 when copying homopolymer templates and 1 in 6000 when copying alternating copolymer templates. The product of the reaction, using a template of polyriboadenylic acid of 2500 nucleotides long hybridized to an initiator of oligodeoxythymidylic acid of 12 to 18 nucleotides long was analyzed by velocity sedimentation and equilibrium density gradient centrifugation. The results indicate that: (a) the entire length of the given template is copied; (b) the incorrectly base-paired nucleotides are an integral part of the polynucleotide product; and (c) these errors are randomly distributed.

Polyacrylamide gel electrophoretic analysis of the purified polymerase showed two subunits, α and β. The incorporation of the correct and incorrect nucleotides catalyzed by the active α subunit appears to be not influenced by the inactive β subunit. The purified polymerase has no detectable exonuclease activity. AMV DNA polymerase exhibits identical requirements for the incorporation of the correct and incorrect nucleotides. The error ratio is not a function of the number of initiator termini, Mg sup+ concentration, time of incubation, or the amount of enzyme. The error rate is, however, dependent on the type of the template and on the ratio of correct to incorrect nucleotides in the reaction mixture.

If this DNA polymerase from avian myeloblastosis virus makes similar errors in vivo this enzyme may be mutagenic.

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The template hypothesis postulated by Watson and Crick (1) implies that the order of addition of deoxynucleotides during DNA replication is guided by the specificity of hydrogen bonding between base pairs. Thus no role in base selection is assigned to the DNA polymerase. However, the difference in free energy between the Watson-Crick base pairs and other base pairs is at most only 2 or 3 Cal per mole (2). This small difference in free energy alone is insufficient to account for the high fidelity of DNA replication and transcription (3, 4). Direct experimental evidence that DNA polymerase participates in base selection was provided by the work of Speyer (5) in which he showed that bacteriophage mutants with an altered DNA polymerase have an increased mutation frequency throughout their genome. Hall and Lehman (6) extended these studies to show that T4 mutant polymerase carries out DNA synthesis in vitro with lower fidelity than does the wild type enzyme. It has been suggested that the exonuclease associated with prokaryotic DNA polymerases may serve to excise mismatched base pairs (7). DNA polymerase of bacteriophage T4 with increased and decreased mutation frequencies have been reported to have decreased and increased ratios of exonuclease to polymerase activity, respectively (8). However, there is evidence that the exonuclease activity of these purified polymerases is unaltered in its ability to remove mismatched nucleotides during DNA synthesis implying that the polymerase function, itself, is responsible for the mutator phenotype (9).

DNA polymerase from animal cells have little if any exonuclease activity (10–12) yet copy polynucleotide templates with few if any mistakes (13–15). We have undertaken to determine the infidelity of DNA synthesis by purified DNA polymerases of animal RNA tumor viruses. In a preliminary communication (16) we reported that DNA polymerase from avian myeloblastosis virus (AMV) makes many mistakes when copying poly(A)-oligo(dT). The frequency of error was higher than that of any other cellular DNA polymerase which has hitherto been studied. We have now investigated in detail the accuracy by which AMV DNA polymerase can copy a variety of synthetic polynucleotide templates. The results presented here indicate that AMV DNA polymerase can catalyze the incorporation of an exceptionally large number of incorrectly base-paired nucleotides.

The abbreviations used are: AMV, avian myeloblastosis virus.
EXPERIMENTAL PROCEDURE

Materials

Plasma from chickens infected with avian myeloblastosis virus was a generous gift of W. J. Beard, Life Sciences Building, St. Petersburg, Fla. The plasma was stored at -70° prior to purification of the virus.

Unlabeled deoxynucleotides were purchased from Calbiochem. Tritium-labeled and 3H-labeled nucleotides were obtained from New England Nuclear. The purity of the labeled compounds was routinely determined by chromatography and by measuring their relative effectiveness as substrates for purified avian DNA polymerase. Calf thymus DNA was obtained from Worthington and "activated" calf thymus DNA was prepared as previously described (17). Whatman phosphocellulose P11 was a product of Reeve Angel Company, New York. Glycerol (spectrograde quality) was obtained from Matheson, Coleman, and Bell. 1.1% Probes provided the ampholines. Homogeneous Escherichia coli DNA polymerase was made by the procedure of Jovin et al. (18) and Springgate et al. (19) and sea urchin DNA polymerase was prepared by the method of Leeb (17). T, DNA polymerase was kindly provided by P. Englund. Johns Hopkins University. Pancreatic DNase and snake venom phosphodiesterase were obtained by Worthington. Poly(dA-dT)·poly(dA-dT), and poly(G)-poly(dC) were prepared by a de novo catalyzed reaction using E. coli DNA polymerase (20). All other synthetic polynucleotides used were acquired either from Miles Laboratories of P-L Biochemicals.

Methods

Purification of AMV DNA Polymerase—All operations were carried out at 0-4°. The sucrose solutions used in density gradient centrifugation contained 50 mM Tris-HCl buffer, pH 8.0, 50 mM NaCl, and 0.1 mM EDTA (Buffer A). After preliminary clarification of the plasma by filtration and by low speed centrifugation, the virus was isolated on a discontinuous 20 and 60% sucrose gradient at 110,000X g for 45 min. The virus band collected on the top of the 60% sucrose was recentrifuged to its equilibrium density on a 20 to 70% linear sucrose gradient for 16 hours at 50,000 x g. The visible band of virus that migrated to its characteristic density of 1.16 g cm-3 was collected, diluted with Buffer A, and concentrated by centrifugation at 110,000 X g for 45 min.

The purified virions were disrupted in Buffer A, pH 8.4, containing 0.4% Triton X-100, 5 mM dithiothreitol, and 10% glycerol. After the disruption, the clarified supernatant from the disrupted virus was layered over a 60% sucrose interface in Buffer A. The samples were then centrifuged at 200,000 X g for 17-20 hours and the resulting supernatant was used as the source of the enzyme (21).

The purity of the isolated AMV DNA polymerase was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. AMV PCII enzyme displayed two distinct bands of molecular weight 65,000 and 110,000 designated as the α and β subunits, respectively. These results on homogeneity, molecular weight, and subunit structure are in agreement with the recently published report by Grandgenett et al. (22). The specific activity of AMV PCII with poly(rA)-oligo(dT) as a template was approximately 5000 units of dTMP incorporated per mg of protein per 30 min at 37°. AMV PCII enzyme showed, in addition to a major band coincident with the α subunit, several other minor bands.

Isoelectric Focusing—All operations were carried out at 4°. A minielectrometer isoelectric focusing procedure was developed for the analysis of the subunit composition of AMV PCII and AMV PCII polymerases (22). The apparatus is "U"-shaped. The limbs are made of 5-ml plastic pipettes (Falcon No. 7520) connected by Tygon tubing. One limb contains 1.0 ml of 60% sucrose solution over which 5 ml of 10 to 40% linear glycerol gradient, containing 10 mM dithiothreitol and 0.8% amphotline (pH range 3 to 10), is formed. Then 2% ethylenediamine was gently layered over the glycerol gradient and the limb connected to the electrode. The other limb is filled with 0.2 M phosphoric acid and served as the anode. Platinum electrodes are used at both terminals. The column is run at 600 volts for 1 hour to establish a pH gradient. The polymerase contained in 20% glycerol is delivered through a fine Teflon tubing to the middle of the pH-glycerol gradient. The sample is then electrophoresed for 3 hours at 600 volts after which 0.1-ml fractions are collected from the bottom of the gradient. The polymerase activity and the pH of each fraction is determined.

Polymerase Assay for Fidelity of DNA Synthesis—This assay is designed to measure the concurrent incorporation of the complementary and the noncomplementary nucleotides into an acid-insoluble polynucleotide product. In each assay one of the nucleotides is labeled with 3H and the other with 3H. This was, for example, the standard assay of 0.05 ml using 1.0 μg of the template-initiator [3P-32P]-d(pT)20 (19) contains in addition the following: 50 mM Tris HCl, pH 8.0, 5 mM dithiothreitol, 20 mM KC1, 2 μg of bovine serum albumin; 5 mM MgCl2; 15 μM correct nucleotide, [3P-32P]-dTTP, 25 μM incorrect nucleotide, [3H]-dCTP, and 0.05 to 0.1 μg of AMV DNA polymerase. The assay was incubated at 37° for 60 min. Since a relatively larger amount of the correct nucleotide, as compared to the incorrect nucleotide, was incorporated in these experiments, it was necessary to maintain a low specific activity of [3-32P]-dTTP, 5 to 10 dpm per pmole, and to use the highest available specific activity of [3H]-dCTP, 1 to 50 X 106 dpm per pmole, to detect the minute amounts of the incorporated incorrect nucleotide.

Washing Procedure for Fidelity Assays—The assays were terminated by the addition of 2 ml of stopping solution (cold 1.0 N perchloric acid containing 0.05 M sodium pyrophosphate) and 100 μl of heat-denatured calf thymus DNA. After the addition of stopping solution, the samples were allowed to stand 20 min at 0°, then the tubes were centrifuged at 5000 X g for 10 min and the supernatant discarded. The precipitated DNA was solubilized with 0.2 M NaOH containing 50 mM sodium pyrophosphate and then precipitated with 2 ml of 1.0 N perchloric acid containing 50 mM sodium pyrophosphate and centrifuged after 20 min. The solubilization-precipitation centrifugation procedure was repeated three times to reduce soluble nucleotide contamination. The DNA was washed onto Whatman GF/C filter discs of 2.4-cm diameter with aliquots of cold water, stopping solution, and finally ethanol. The disc was then transferred to a glass vial, dried, and covered with 10 ml of scintillation solution containing 5 g of 2,5-diphenyloxazole per liter of toluene. In assays terminated 20 to 30 min after the addition of stopping solution, the radioactivity from the incorparated nucleotide was 160 to 200 dpm and from the correct nucleotide was incorporated prior to incubation the radioactivity from the incorrect nucleotide was 100 to 200 dpm and from the correct nucleotide, as compared to the incorrect nucleotide, was incorporated in these experiments, it was necessary to maintain a low specific activity of [3-32P]-dTTP, 5 to 10 dpm per pmole, and to use the highest available specific activity of [3H]-dCTP, 1 to 50 X 106 dpm per pmole, to detect the minute amounts of the incorporated incorrect nucleotide.

RESULTS

The fidelity of DNA synthesis as a function of the amount of AMV PCII polymerase is shown in Fig. 1. In this experiment the template-initiator [3P-32P]-d(pT)20, was used. The correct complementary nucleotide, dTTP, at a concentration of 15 μM corresponding to its Km value and the incorrect noncomplementary nucleotide, dCTP, at a concentration of 25 μM, were present in the reaction mixture. The product was quantitated by the simultaneous incorporation of the correct nucleotide, [3-32P]-dTTP, and the incorrect nucleotide, [3H]-dCTP. The results show that the utilization of the correct and incorrect nucleotide is proportional to the polymerase concentration. The ratio of the incorrect to correct nucleotide incorporation, i.e. the error rate, was constant irrespective of the quantity of DNA synthesized. The enzyme did not utilize the incorrect nucleotide when used in the absence of the correct nucleotide, thereby suggesting that correct synthesis is necessary for incorrect incorporation. The amount of the incorrect nu-

2 Unpublished results.
FIG. 1. Effect of concentration of AMV PC11 polymerase on the incorporations of dTMP and dCMP using [r(pA250-d(pT)185] as template-initiator. The assay mixture in 0.05 ml consisted of: 50 mM Tris-HCl, pH 8.0; 5 mM MgCl2; 5 mM dithiothreitol; 70 mM KCl; 2 μg of albumin, 15 μM dTTP; 25 μM dCTP; 1 μg of template and 0.00 to 0.10 μg of AMV DNA polymerase. The assays were incubated for 60 min at 37°C.

FIG. 2. Isoelectric focusing. AMV PC11 polymerase was focused on pH stabilized glycerol gradients as described under "Methods." A, "activated" DNA as template. Polymerase activity was determined using a 0.05 ml reaction mixture consisting of: 50 mM Tris-HCl, pH 8.0; 5 mM dithiothreitol; 10 mM MgCl2; 1 μg of albumin; 100 μM dATP; 100 μM dCTP; 2 μCi of [3H]dTTP; 0.15 μM phosphorus of "activated" calf thymus DNA and 10 μl of each fraction. The assays were incubated for 60 min at 37°C. B, [r(pA)G0.d(pT)178] as template. Polymerase activity was determined using a 0.05 ml reaction mixture consisting of: 50 mM Tris-HCl, pH 8.0; 5 mM MgCl2; 5 mM dithiothreitol; 20 mM KCl; 2 μg of albumin; 1 μg of template; 15 μM [α-32P]dTTP; 25 μM [3H]dCTP; and 10 μl of each fraction. The assays were incubated for 60 min at 37°C.

cytosine residue utilized was much less than that of the correct nucleotide, indicating that: (a) in the presence of a template the polymerase discriminates against the incorrect nucleotide; and further (b) the enzyme exercises this capacity during the course of DNA polymerization. A similar analysis, using PC1 polymerase, showed that the incorporation of the correct and incorrect nucleotides is proportional to enzyme concentration (results not shown).

In order to show that the AMV DNA polymerase is indeed catalyzing incorporation of the correct and incorrect nucleotides, we studied the ratio of incorrect to correct incorporation after AMV DNA polymerase was electrofocused on pH stabilized glycerol gradients. Fig. 2A shows the profile of the PC11 DNA polymerase activity using "activated" calf thymus DNA template. Fig. 2B shows the accuracy of DNA synthesis using [r(pA)G0.d(pT)178] template-initiator. The results in Fig. 2, showing the coincident DNA-directed and RNA-directed DNA polymerase activities of the isoelectrofocused AMV polymerase, indicate that the enzyme copies both DNA and RNA templates; this observation is compatible with previously published reports (24-27). Further, the coincidence of the correct and incorrect synthesis across the peak strongly suggests that the infidelity of DNA synthesis is a property of the enzyme itself. The isoelectric point of the polymerase appears to be pH 7.0.

The PC1 polymerase, presumably devoid of the α subunit (22) was electrofocused on pH stabilized glycerol gradients. The poly(dA-dT)·poly(dA-dT)-directed DNA polymerase activity and the poly(rA)-oligo(dT)-directed DNA polymerase activity coincide across the peak as shown in Fig. 3, A and B, respectively, indicating that the PC1 polymerase can also copy deoxyribonucleotide and ribonucleotide templates. The constancy of the error rate across the electrofocused peak shows that the PC1 enzyme catalyzes the incorporation of both the incorrect and correct nucleotides. Although the PC11 and PC1 polymerases incorporate approximately one incorrect nucleotide of dCMP, for every 600 correct nucleotides of dTTP when [r(pA)G0.d(pT)178] is used as the template-initiator. The results thus suggest that the noncatalytic β subunit (22) does not influence the fidelity of DNA synthesis by the active α subunit.

The requirements for correct and incorrect incorporation by AMV PC11 polymerase are summarized in Table I. For this analysis and most subsequent experiments we have used [r(pA)G0.d(pT)178] as template-initiator. Since both PC11 and PC1 polymerases exhibit errors of the same magnitude, and PC11 polymerase appears to be more homogeneous than PC1 polymerase on dissociating acrylamide gels, we have concentrated on studying the exactness of DNA synthesis using PC11 DNA
polymerase. The enzyme requires the template-initiator, poly (rA) -oligo(dT), and the divalent cation Mg²⁺ for both the correct and incorrect syntheses. There was no significant incorporation either with or without heat-inactivated enzyme. In assays with only the noncomplementary nucleotide, i.e. in the absence of the complementary nucleotide, the incorporation was negligible. Thus the requirements for both the correct as well as for incorrect syntheses are strikingly similar, and are typical of other purified prokaryotic and eukaryotic DNA polymerases (15).

The divalent cation Mg²⁺ is required for AMV DNA polymerase to catalyze the incorporation of the complementary and the noncomplementary nucleotides into the polynucleotide product. As shown in Fig. 4, the enzyme exhibits an optimum concentration of 5 mM for the correct as well as for the incorrect syntheses. The error rate appears to be independent of Mg²⁺ concentration.

Fig. 5 illustrates the kinetics of [r(pA)₂₅₀-d(pT)₁₂₅₁₃] -directed correct dTMP and incorrect dCMP incorporation. The incorporation of the incorrect nucleotide paralleled that of the correct nucleotide suggesting that the incorrect base was inserted as DNA synthesis progressed. The error rate was constant and linear up to about 90 min. Further prolonged incubation for 20 hours did not alter either the amount of the product or the error rate. The product of the reaction under these conditions of assay is equivalent to about 20% of the added template. However, with larger amounts of substrates almost all of the poly (rA)-template is copied. In parallel assays omission of the correct nucleotide reduced the incorrect incorporation to less than 3% of that observed with the complete reaction, suggesting that for faulty incorporation, DNA polymerization is required.

In other studies we found that the error rate did not vary when assays were incubated at temperatures varying from 16 to 46°C (results not shown).

Fig. 6, shows the effect of template concentration on the rate and fidelity of DNA synthesis by AMV DNA polymerase. Both correct and incorrect nucleotide incorporations are totally template-dependent. The error rate remains constant at all template concentrations. In this experiment a large amount of the incorrect nucleotide was present thus permitting nearly 40% of the template to be copied. Again, incorporation of the incorrect nucleotide in the absence of the correct nucleotide was less than 3% of the incorrect incorporation of that obtained using the complete reaction mixture. This incorporation of the incorrect nucleotide alone was negligible even after prolonged incubation up to 20 hours.

The effect of increasing concentration of the incorrect nucleotide, dCTP, on the error rate is documented in Table II. The

### Table I

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>r[pA]₂₅₀-d[pT]₁₂₅₁₃</th>
<th>dTMP</th>
<th>dCMP</th>
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<tbody>
<tr>
<td>Complete</td>
<td></td>
<td>240</td>
<td>0.30</td>
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<tr>
<td>Minus enzyme</td>
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<td>0</td>
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<td>Minus template</td>
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<tr>
<td>Minus Mg²⁺</td>
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<td>0.01</td>
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<tr>
<td>Heated enzyme</td>
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<td>0</td>
</tr>
<tr>
<td>dCTP alone</td>
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<td>0</td>
<td>&lt;0.01</td>
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Table II

<table>
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<tr>
<th>Ratio of dCTP:dTTP</th>
<th>dTMP incorporated</th>
<th>dCMP incorporated</th>
<th>Error rate</th>
</tr>
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<tbody>
<tr>
<td>0.30</td>
<td>65.5</td>
<td>0.020</td>
<td>1/3200</td>
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<tr>
<td>0.50</td>
<td>50.0</td>
<td>0.036</td>
<td>1/1610</td>
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<td>0.80</td>
<td>63.0</td>
<td>0.046</td>
<td>1/1370</td>
</tr>
<tr>
<td>1.20</td>
<td>63.0</td>
<td>0.092</td>
<td>1/685</td>
</tr>
<tr>
<td>1.66</td>
<td>65.5</td>
<td>0.107</td>
<td>1/607</td>
</tr>
<tr>
<td>2.46</td>
<td>64.0</td>
<td>0.154</td>
<td>1/420</td>
</tr>
</tbody>
</table>

**Fig. 4 (left).** Effect of Mg²⁺ concentration on the activity of AMV DNA polymerase using poly (rA) -oligo(dT) as template-initiator. The reaction mixture of 0.05 ml consisted of the following: 0.05 M Tris-HCl, pH 8.0; 5 mM dithiothreitol; 2 μg of albumin; 20 mM KCl; 100 μM dTTP; 50 μM dCTP; 1 μg of template; the indicated concentrations of Mg²⁺; and 0.05 μg of AMV polymerase. The assays were incubated for 60 min at 37°C.

**Fig. 5 (center).** Time course of incorporation of dTMP and dCMP with [r[pA]₂₅₀-d[pT]₁₂₅₁₃] as template-initiator. The assay conditions are as given under “Methods” except for the indicated period of incubation.

**Fig. 6 (right).** Effect of [r[pA]₂₅₀-d[pT]₁₂₅₁₃] template-initiator concentration on the incorporation of dTTP and dCMP. The assay mixture in 0.05 ml consisted of: 50 mM Tris-HCl, pH 8.0; 5 mM dithiothreitol; 20 mM KCl; 2 μg of albumin; 5 mM MgCl₂; 120 μM dTTP; 50 μM dCTP; 0.05 μg of AMV DNA polymerase; and the indicated concentrations of [r[pA]₂₅₀-d[pT]₁₂₅₁₃] as template initiator. The incubation was for 60 min at 37°C.
results show that increasing the concentration of dCTP and thus varying the ratio of dCTP to dTTTP over a 10-fold range in this reaction mixture did not alter the quantity of DNA synthesized. The error rate is greater at higher concentrations of the incorrect substrate and appear to be proportional to its concentration.

The results in Table III show that AMV DNA polymerase does barely utilize either single-stranded ribohomopolymers or single-stranded deoxyribohomopolymers as templates for either correct or incorrect DNA syntheses (Table III). However, if the single-stranded polymers are complexed with their complementary oligonucleotides or polynucleotides to serve as initiator, the single-stranded polymers serve as effective templates for both correct and incorrect incorporation. The enzyme appears to prefer ribohomopolynucleotides in that with poly(rA)·oligo(dT) template about 300 pmoles of nucleotides are incorporated, whereas with poly(dA)·oligo(dT) only 8.0 pmoles of nucleotides are incorporated. When the ratio of template to initiator varied from 1:1 to 1:100 there was no change either in the amount of product synthesized or in the error rate. Thus the error rate is not a function of the number of initiator termini.

The accuracy of copying ribohomopolymers and deoxyribohomopolymers as well as their relative efficiency as templates under comparable conditions are documented in Table IV. The concentration of both the correct and incorrect nucleotides used in these experiments is 20 µM. In agreement with the results of others (24, 26, 27) we found that the ribohomopolymer templates, poly(rA)·oligo(dT) and poly(rC)·oligo(dG), are more efficient than are the deoxyribohomopolymers, poly(dA)·oligo(dT) and poly(dG)·poly(dC). However, the error rate is similar in magnitude, and does not appear to be related to the sugar moiety in the polynucleotide templates. With poly(rA)·oligo(dT) as template, the error rate with dCTP was 1 in 600 whereas with dGTP no significant number of errors were detected. The limit of detection in these experiments was 1 in 20,000.

With double-strand polynucleotides as templates (poly(dG)·poly(dC) and poly(rI)·poly(rC)) we have carried out two reactions. In each reaction only one of the complementary nucleotides was present so that only one of the templates would be copied. Thus, we can compare the efficiency of each of the polynucleotides as templates and the ability of each to be copied accurately by the polymerase. The results in Table IV with matched polynucleotides show that poly(rC) and poly(dC) are superior templates in comparison to poly(rI) and poly(dG). However, there was no significant difference in the magnitude of misincorporation using poly(rC) and poly(dC) as templates (1 in 600), again indicating that the sugar moiety of the template does not influence the error synthesis by AMV DNA polymerase. Table V shows the results of studies with alternating copolymers. In this limited study the double-stranded deoxyribocopolymers appear to be more effective as templates for AMV DNA polymerase than are the double-stranded ribocopolymers.

| Table III |

Infidelity of DNA synthesis using AMV DNA polymerase

The polymer-oligomer combinations were made by mixing the indicated proportions of each in 0.01 M Tris-HCl, pH 7.4 containing 0.1 M KCl and incubating each mixture at 50 for 15 min and allowing it to cool slowly at room temperature. The accuracy of DNA synthesis was measured by the procedure described under "Methods." The assay mixture in 0.05 ml consisted of: 50 µM Tris-HCl, pH 8.0; 20 mM KCl; 2 µg of albumin; 5 mM MgCl2; 40 µM dCTP; 100 µM dTTP; 1 µg of template; and 0.10 µg of AMV DNA polymerase. The reaction mixtures were incubated for 60 min at 37°C.

<table>
<thead>
<tr>
<th>Template</th>
<th>Correct nucleotide incorporation [pmoles]</th>
<th>Incorrect nucleotide incorporation [pmoles]</th>
<th>Error rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>poly(rA)·oligo(dT)12-18, 1:1.5</td>
<td>300</td>
<td>0.46</td>
<td>1/650</td>
</tr>
<tr>
<td>poly(rA)·oligo(dT)12-18, 1:1</td>
<td>300</td>
<td>0.38</td>
<td>1/778</td>
</tr>
<tr>
<td>poly(rA)·oligo(dT)12-18, 1:2</td>
<td>300</td>
<td>0.45</td>
<td>1/600</td>
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<tr>
<td>poly(rA)·oligo(dT)12-18, 1:100</td>
<td>330</td>
<td>0.48</td>
<td>1/128</td>
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<tr>
<td>poly(rA)·oligo(dT)12-18, 1:100</td>
<td>18</td>
<td>0.62</td>
<td>1/620</td>
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<td>1/670</td>
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<td>poly(rA)·oligo(dT)12-18, 1:100</td>
<td>2.0</td>
<td>0.01</td>
<td>1/620</td>
</tr>
<tr>
<td>poly(rA)·oligo(dT)12-18, 1:100</td>
<td>0</td>
<td>0.01</td>
<td>1/670</td>
</tr>
<tr>
<td>poly(rA)·oligo(dT)12-18, 1:100</td>
<td>0</td>
<td>0.01</td>
<td>1/670</td>
</tr>
</tbody>
</table>

* This complex was obtained from Miles Laboratories, and contained equimolar base ratio of A to 1, 1:1.

| Table IV |

Fidelity of DNA replication with ribohomopolymer and deoxyribohomopolymer templates

DNA synthesis was measured by the procedure described under "Methods." The assay mixture of 0.05 ml is made up of the following: 0.1 M Tris-HCl, pH 8.0; 5 mM dithiothreitol; 5 mM MgCl2; 20 mM KCl; 40 µg of bovine serum albumin; 1 µg of the respective template; and 20 µM each of the correct and incorrect nucleotides. The ratio of template to initiator was approximately 1:1. Wherever possible the correct and incorrect nucleotides were labeled with different radioactive isotopes and present in the same assay mixture. However, where the use of double labels was not feasible separate assays were run for correct and incorrect synthesis.

<table>
<thead>
<tr>
<th>Template</th>
<th>Correct nucleotide incorporation [pmoles]</th>
<th>Incorrect nucleotide incorporation [pmoles]</th>
<th>Error rate</th>
</tr>
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<td>poly(rA)·oligo(dT)12-18, 1:1.5</td>
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<td>0.48</td>
<td>1/128</td>
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<td>0.62</td>
<td>1/620</td>
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<tr>
<td>poly(rA)·oligo(dT)12-18, 1:100</td>
<td>0</td>
<td>0.01</td>
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<td>poly(rA)·oligo(dT)12-18, 1:100</td>
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<td>poly(rA)·oligo(dT)12-18, 1:100</td>
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</tr>
</tbody>
</table>

| Table V |

Infidelity of DNA replication using alternating ribopolymer and deoxyribopolymer templates

See legend to Table IV.

<table>
<thead>
<tr>
<th>Template</th>
<th>Correct nucleotide incorporation [pmoles]</th>
<th>Incorrect nucleotide incorporation [pmoles]</th>
<th>Error rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>poly(dI-dC)·poly(dI-dC)</td>
<td>100</td>
<td>3.0</td>
<td>1/670</td>
</tr>
<tr>
<td>poly(dI-dC)·poly(dI-dC)</td>
<td>56.0</td>
<td>0.01</td>
<td>1/600</td>
</tr>
<tr>
<td>poly(dI-dC)·poly(dI-dC)</td>
<td>56.0</td>
<td>0.01</td>
<td>1/600</td>
</tr>
<tr>
<td>poly(dI-dC)·poly(dI-dC)</td>
<td>6.0</td>
<td>0.01</td>
<td>1/600</td>
</tr>
<tr>
<td>poly(dI-dC)·poly(dI-dC)</td>
<td>1.0</td>
<td>0.01</td>
<td>1/600</td>
</tr>
</tbody>
</table>

With double-strand polynucleotides as templates (poly(dG)·poly(dC) and poly(rI)·poly(rC)) we have carried out two reactions. In each reaction only one of the complementary nucleotides was present so that only one of the templates would be copied. Thus, we can compare the efficiency of each of the polynucleotides as templates and the ability of each to be copied accurately by the polymerase. The results in Table IV with matched polynucleotides show that poly(rC) and poly(dC) are superior templates in comparison to poly(rI) and poly(dG). However, there was no significant difference in the magnitude of misincorporation using poly(rC) and poly(dC) as templates (1 in 600), again indicating that the sugar moiety of the template does not influence the error synthesis by AMV DNA polymerase. Table V shows the results of studies with alternating copolymers. In this limited study the double-stranded deoxyribocopolymers appear to be more effective as templates for AMV DNA polymerase than are the double-stranded ribocopolymers.
The alternating copolymers appear to be copied with 10-fold greater precision than are the homopolymer templates. The approximate error rate with double-stranded alternating copolymer (Table V) is 1 in 6000 and with homopolymers (Table IV) the frequency of error is around 1 in 600. Thus the accuracy of copying may be greater as the polynucleotide serving as template gets more heterogeneous in base composition, or when the adjacent nucleotides are different or both.

In order to study the nature of the product and the pattern of distribution of errors we analyzed the template-initiator [r(pA)1300-d(pT)1500]-directed product on a neutral cesium sulfate equilibrium gradient. The results show that the product bands at a density corresponding to a 1:1 hybrid of poly(rA)-poly(dT) (Fig. 7). The fact that the level of fidelity across the polynucleotide peak is constant, suggests that the pattern of distribution of errors is not related to the size of the product.

The product of extensive synthesis using 14 S [r(pA)1500-d(pT)1500] template-initiator by AMV DNA polymerase was analyzed on alkaline sucrose gradients, in order to determine the size of product formed relative to its 14 S template. The results in Fig. 8 show that the product formed is alkali-resistant and sediments at 14 S, suggesting that the entire length of the 14 S poly(rA) template was copied. The constant error rate across the peak indicates that the distribution of errors in the product with respect to size is random.

The kinetic experiments described earlier (Fig. 5) suggest that the product of the reaction is not degraded upon prolonged incubation (20 hours at 37°C). There was no change in the ratio of the incorrect to correct nucleotide incorporated into the product. We have also designed specific experiments to assay for possible exonuclease and endonuclease activities of AMV DNA polymerase which might selectively degrade the products of the reaction. The 14 S DNA product made on 14 S poly(rA)-oligo(dT) template-initiator was incubated for 5 hours at 37°C with 10 times the quantity of the enzyme that was used initially to synthesize the product. A portion of the product before and after incubation along with size markers was banded for size in 5 to 20% alkaline sucrose gradients. The incubated and the nonincubated products co-banded in a region corresponding to 14 S, indicating the absence of detectable endonuclease activity.

In order to test for possible exonuclease activity, a second portion of the incubated, and nonincubated products, along with dCMP and dTMP markers were analyzed by paper electrophoresis (28). Less than 2% of the applied radioactivity was detected in the unincubated and nonincubated control product was recovered at the point of application on the chromatographic paper, thus indicating negligible hydrolysis of the product. The results suggest that AMV DNA polymerase has no detectable DNA exonuclease or endonuclease activity which hydrolyzes the poly(rA)·oligo(dT)-directed product. Thus our results confirm and extend earlier published work (29, 30) on the lack of DNase activity in purified AMV DNA polymerase.

**DISCUSSION**

The results presented in this report suggest that purified DNA polymerase ("reverse transcriptase") from an avian RNA virus permits errors in base pairing while copying synthetic polynucleotides in vitro. That this enzyme indeed catalyzes the incorporation of an unusually large number of incorrectly base-paired nucleotides during polymerization has been unambiguously established by a number of criteria. (a) In order to show that the error-prone enzyme was part of the virion, AMV DNA polymerase was isolated from virions which were purified by both velocity and equilibrium sedimentation. The coincidence of DNA polymerase activity with both correct and incorrect nucleotide incorporations after subjecting the purified enzyme to isoelectric focusing, indicates that the polymerase catalyzes the incorporation of the incorrect nucleotide. (b) The requirements for both correct and incorrect nucleotide incorporation are identical, and are typical of that of other DNA polymerases studied. (c) The incorporation of the incorrect nucleotide is dependent on the incorporation of the correct nucleotide. (d) Changing other factors in the reaction mixture, such as temperature, Mg²⁺ concentration, the number of initiator termini, or time of incubation which altered the amount of incorporation by as much as 2 orders of magnitude did not affect the frequency of errors.
The possibility that the misincorporation could be due to the contamination of nucleotides, templates, or the reagents, is unlikely because of the following. (a) In the experiments using poly(dA-dT) poly(dA-dT) template, a set of parallel assays were carried out with the same reagents, substituting E. coli DNA polymerase I for AMV DNA polymerase. The rate of misincorporation with E. coli polymerase was less than one incorrect nucleotide in 100,000 correct nucleotides polymerized, while with AMV polymerase the error rate was 1 in 6,000. This high fidelity with E. coli DNA polymerase I was also found using poly(dG-dC).poly(dG-dC) as template. Moreover, when homogeneous Tt DNA polymerase was substituted with the same pool using poly(dA-dT).poly(dA-dT) templates, the level of infidelity was less than one incorrect in 10,000 correct nucleotides polymerized. This finding is consistent with the published report of Hall and Lehman (6). (b) When the correct nucleotide was omitted from the assays less than 3% incorrect nucleotide of that of the complete assays was incorporated. (c) The error rate appears to be dependent on the ratio of incorrect to correct nucleotides in the reaction mixture and is thus not due to possible contamination of the templates.

That the misincorporated nucleotides are an integral part of the polynucleotide product and are randomly distributed has been suggested by the following findings. The [p(pA)poly(dG)poly(dT)] template-initiator-directed product synthesized using [α-32P]dCTTP and [3H]dTTP, was hydrolyzed by snake venom exonuclease. The hydrolysate was subjected to high voltage paper electrophoresis along with dCMP and dTMP markers. The 3H label of the hydrolysate migrated with dCMP and [32P]dTMP, showing that the 3H radioactivity in the product was derived from the substrate [α-32P]dCTTP and was present as dTMP. Most importantly, the 3H radioactivity in the product derived from the substrate [3H]dTTP was present as dCMP in phosphodiester linkages. The [p(pA)poly(dG)poly(dT)] directed product when centrifuged to equilibrium density in neutral cesium sulfate was found at a density corresponding to a 1:1 hybrid of poly(rA)-poly(dT). The constancy of error rate across the putative hybrid peak suggests that the pattern of distribution of errors is random. The polynucleotide product synthesized on a sized 14 S, poly(rA) template sedimented in an alkaline sucrose gradient at 14 S indicating that the product is alkali-resistant and of similar size to that of the template. Moreover, the error rate was invariant across the peak, again suggesting that the errors in the alkali-resistant product are randomly distributed.

While the DNA polymerases of Rous sarcoma virus and other mammalian viruses (31, 32) have been reported to consist of a single polypeptide, the polymerases of AMV (22, 33) and of Rous sarcoma virus appear to be composed of two polypeptide chains, α and β (34). The smaller α subunit of AMV polymerase was reported to possess both the DNA polymerase and nuclease activity specific for ribostrands of RNA-DNA hybrids (RNase H). No function has been attributed to the larger 6 subunit. Our results and earlier published reports (29, 30), indicate there to be no DNase activity associated with AMV DNA polymerase. The AMV polymerase copied the entire length of the provided 14 S poly(rA) template, and did not degrade the polynucleotide product after prolonged incubation.

In recent times evidence has been accumulating for the presence of viral information (35, 36) and viral polymerases in human acute lymphatic cells (37, 38). We have reported (14) that nucleic acid free extracts of human acute leukemic lymphocytes make many more mistakes in copying synthetic templates than do similar extracts from normal phytohemagglutinin-stimulated lymphocytes. It will be of interest to determine if the “RNA-dependent” polymerase reported to be present in these cells is faulty in base selection.

The role of viral polymerase in cell transformation is not understood. There are indications in the literature that DNA polymerase mutants of Rous sarcoma virus (39) and mouse sarcoma virus (40) fail to transform cells in culture. Gallo et al. (11) showed a direct relationship between the inhibition of viral polymerase and transforming ability of Rauscher leukemia virus again suggesting that viral polymerase is necessary for transformation. There are temperature sensitive mutants known (42) of Rous sarcoma viruses that replicate at higher temperature but do not transform. It should be of interest to investigate the exactness of the DNA synthesis by the temperature sensitive mutant polymerases of oncogenic RNA viruses.

DNA polymerases from various normal cells copy DNA templates exactly in vitro (16). They exhibit little, if any specificity for a particular DNA. It is conceivable that the introduction of a faulty DNA polymerase, i.e. a polymerase from an oncogenic virus into a normal cell could cause mutations to arise by inaccurately copying host cell DNA. Such mutations could lead to malignant transformation and to tumor progression.

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