On the Fidelity of DNA Replication

CHARACTERIZATION OF POLYNUCLEOTIDES WITH ERRORS IN BASE-PAIRING SYNTHESIZED BY AVIAN MYELOBLASTOSIS VIRUS DEOXYRIBONUCLEIC ACID POLYMERASE*

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SUMMARY

Polynucleotide templates were copied by avian myeloblastosis virus DNA polymerase ("reverse transcriptase") and the frequency and distribution of errors were determined. The error rate with [p(A)]_T(dT)n template-initiator under a variety of conditions was approximately 3600, i.e., one incorrect dCMP incorporated for 600 correct dTMP polymerized. Addition of the metal chelator o-phenanthroline to the reaction inhibited the incorporation of the correct and incorrect nucleotides proportionately. The enzyme exhibited a pH optimum of 8.5 and the error rate remained constant over a range of pH (6.0 to 10.0). The rate of polymerization was greater at higher temperatures and approximately doubled for every 10° increase. The error rate was constant at all temperatures. These results indicate that the purified avian myeloblastosis virus DNA polymerase synthesizes polydeoxynucleotides with an unusually large number of errors in base-pairing.

Velocity sedimentation of the products of the reaction obtained at various times during the course of synthesis indicated that (a) the entire length of the 14 S template was copied, and (b) the incorporation of the incorrect nucleotide did not terminate chain propagation. Isoelectric focusing profile showed that the noncomplementary nucleotides are incorporated into the polynucleotide product. Stepwise degradation and nearest neighbor analysis of the reaction product indicated that (a) the correct and incorrect nucleotides are present in phosphodiester linkages, (b) the errors are not concentrated at either termini; and (c) the errors are uniformly distributed throughout the newly synthesized polydeoxynucleotide.

A universal mechanism for DNA synthesis is suggested by the findings that all DNA polymerases so far described have similar requirements for catalysis (1). Recent studies indicate that DNA polymerases from different sources copy polynucleotide templates with varying degrees of fidelity (2-11). The DNA polymerases purified from prokaryotes (2-6) and eukaryotes (7-9) copy synthetic polynucleotide templates with a high degree of accuracy, whereas the polymerase from an oncogenic RNA tumor virus exhibits an unusually high error rate (10, 11). Theoretical considerations have long suggested that DNA polymerases may play a role in the fidelity of DNA synthesis (12-14). Experimental studies (3, 15, 16) using mutants of bacteriophage T4 provide evidence that DNA polymerase plays a role in base selection. However, studies on the fidelity of purified DNA polymerases from bacteriophages and from prokaryotes are difficult to interpret because these enzymes have an associated exonuclease activity which serves an "editing" function (5, 6, 17-19). Purified DNA polymerase from avian myeloblastosis virus is devoid of deoxynucleoside activity (20, 21) and thus this enzyme is attractive for studying how polymerases function in base selection.

In a previous investigation (10) we have shown that the purified DNA polymerase from avian myeloblastosis virus exhibits an unusually high error rate when copying a variety of polynucleotide templates. Isoelectric focusing profile showed that the correct and incorrect nucleotide incorporation is proportional across the peak, indicating that the same enzyme is catalyzing the incorporation of both the complementary and the noncomplementary nucleotides. The error rate was 3600 with homopolymer templates and 3600 with alternating copolymer templates. The frequency of incorporation of the noncomplementary nucleotide was independent of the concentration of Mg2+, template, initiator, or enzyme. In this study we present an analysis of the pattern and frequency of errors in the product. The evidence indicates that the errors are uniformly distributed.

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 32P-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 sea urchin DNA polymerase (22). Calf thymus DNA, pancreatic deoxyribonu-
cesse, snake venom phosphodiesterase, micrococcal nuclease, and spleen phosphodiesterase were products of Worthington.

Poly(dA-dT)-poly(dA-dT) was prepared by a de novo catalyzed reaction using *E. coli* DNA polymerase (23). Molecules having a size of 14 S were obtained by sedimentation through 5 to 20% linear sucrose gradients containing 0.2 M KCl and 0.02 M Tris-HCl, pH 7.8. [3H]RNA from L cells was prepared as previously described (24) and was a generous gift of D. E. Kelley, The Institute for Cancer Research, Philadelphia, Pa. All other synthetic polynucleotides used were acquired either from Miles Laboratory or P-L Biochemicals.

**Methods**

**Purification of Avian Myeloblastosis Virus DNA Polymerase**—Avian myeloblastosis virus was isolated by sedimentation and equilibrium centrifugation in sucrose gradients (10). The DNA polymerase was purified as previously described (25). Only the most pure fraction of the holoenzyme, PCII, was used in these studies. Upon electrophoresis in sodium dodecyl sulfate polyacrylamide gels the enzyme displays two distinct protein bands of molecular weight 65,000 and 110,000 which have been designated by others (26) as the α and β subunits, respectively.

**Polymerase Assay for Fidelity of DNA Synthesis**—This assay is designed to measure the concurrent incorporation of the complementary and the noncomplementary nucleotides. In each assay one of the labeled nucleotides contains 3H and the other α-32P.

The standard assay of 0.05 ml containing 1.0 µg of the template-initiator (r[pA]300-d(pT)200) (27) contains in addition the following: 50 mM Tris-HCl, pH 8.0; 5 mM dithiothreitol; 20 mM KCl; 2 µg of bovine serum albumin; 5 mM MgCl2; 15 µm correct nucleotide, α-32PdTTP; 25 µm incorrect nucleotide, [3H]dCTP; and 0.03 to 0.1 pg of avian myeloblastosis virus DNA polymerase. The assays were incubated at 37° for 60 min. 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 α-32PdTTP, 5 to 10 dpm/pmol, and to use the highest available specific activity of [3H]dCTP, 1.0 to 3.0 X 106 dpm/pmol, to enable detection of minute amounts of the incorporated incorrect nucleotide. Incorporation of the radioactive nucleotides into an acid-insoluble precipitate was determined after repeatedly precipitating the polynucleotide product with 1.0 n perchloric acid and solubilizing with 0.2 M NaOH. The error rate is defined as the ratio of the noncomplementary to the complementary nucleotide incorporation.

**Analysis**—After 2 hours of incubation the polymerase reaction was stopped by adding NaCl and Na dodecyl sulfate to a final concentration of 0.2 M and 1%, respectively. Ten micromoles of unlabeled dCTP and dTTP were added and the reaction mixture was extracted with an equal volume of phenol containing 0.1% 8-hydroxyquinoline by shaking at room temperature. After centrifuging at 5000 X g for 10 min at 20° the aqueous phase was dialyzed against two liters of 0.1 M Tris-HCl buffer, pH 7.4, containing 0.5 M sodium chloride. The dialysis buffer was changed six times over a period of 3 days. The dialyzed product was shown to be free of contaminating nucleotides by electrophoresis. Greater than 90% of the radioactivity in the product was acid-insoluble. The dialyzed products were concentrated by evaporation using a stream of nitrogen gas at room temperature and then degraded to 3'-mononucleotides by digestion with micrococcal nuclease followed by spleen phosphodiesterase, essentially following the procedure of Josse et al. (28). The 3'-nucleotides in the clear supernatant digest were separated by electrophoresis (29). Samples were applied to Whatman No. 3 paper along with appropriate controls. The electrophoresis was carried out in 0.05 M citrate buffer, pH 3.5, for 40 min at 8000 volts and 5° using a flat plate electrophoresis unit, Savant Instruments, Inc. The chromatogram was dried, cut into 1-cm strips, and the radioactivity determined in a liquid scintillation counter. The nucleotides in the chromatogram were identified by their relative migration using 3'-nucleotide monophosphates as standards.

**RESULTS**

**Effect of 1,10-Phenanthroline on Error Rate**—Avian myeloblastosis virus DNA polymerase has been shown to contain zinc (30-32). Inhibition of polymerase activity by the chelator 1,10-phenanthroline suggests that the zinc functions in catalysis (30-32).

In order to study the influence of 1,10-phenanthroline on the fidelity of DNA synthesis we have measured the incorporation of the correct and incorrect nucleotides at varying concentrations of 1,10-phenanthroline. The results in Fig. 1 show that the incorporation of the correct and incorrect nucleotides is inhibited proportionately. The error rate was constant at all concentrations of 1,10-phenanthroline, further indicating that the avian myeloblastosis virus DNA polymerase catalyzes the incorporation of both the correct and the incorrect nucleotides.

**Effects of pH and Temperature on Error Rate**—In order to gain information as to how the structure of the polynucleotide template affects the error rate, we investigated the influence of the hydrogen ion concentration and temperature. The results are presented in Figs. 2 and 3. In these experiments the template-initiator (r[pA]300-d(pT)200) was used. The products were quantitated by the simultaneous incorporation of the correct nucleotide [α-32P]dATP and the incorrect nucleotide [3H]dCTP. The results in Fig. 2 show that the enzyme has a broad pH optimum between 7.5 and 9.5 with a maximum activity around pH 8.5 (33). Within the range indicated the error rate is constant across the peak irrespective of the hydrogen ion concentration in the assay and the amount of DNA synthesized.

The results presented in Fig. 3 show that the rate of polymerization is linear with time. As expected, the rate of polymerization is greater at higher temperatures and approximately doubles for every 10° rise in temperature. At all temperatures the rate of incorporation of the noncomplementary nucleotide parallels that of the complementary.
myeloblastosis virus DNA polymerase. The assays were incubated for 2 hours of synthesis was not observed in repeated experiments. The slight change in proportionality between 3H and 32P after 2 hours at 20°C was not observed in repeated experiments. After the indicated periods of incubation, the product of the reaction was sedimented through a gradient of alkaline sucrose. The sucrose gradient profiles indicate that the size of the product increases with time. By 30 min the predominant species is nearly 14 S and corresponds to the size of the poly(rA) template used in the assay.

The ratio of complementary to noncomplementary nucleotide incorporation is constant across the peaks and does not vary as the concentration of the complementary nucleotide increases. Thus, the error rate is independent of the temperatures of the assay (16-39°C) and of the amount of DNA synthesized.

Distribution of Errors during Chain Elongation—An analysis of the size of the product of the reaction and the distribution of incorporated noncomplementary nucleotides is seen in Fig. 4. After the indicated periods of incubation, the product of the reaction was sedimented through a gradient of alkaline sucrose. In this experiment, the molar ratio of template (r(pA)m.d(pT)m) to initiator (d(pT)m) to polymerase was 25:5:1. Thus, less than 2% of template would be expected to have more than one initiator and only about 2% of the template-initiator complexes would be associated with more than one polymerase molecule at the start of the reaction. The reaction mixture contained the complementary nucleotide, dTTP, at a concentration of 15 μM (Km) and the noncomplementary nucleotide, dCTP, at 25 μM. The reaction was carried out at 25°C to slow down the rate of polymerization (see Fig. 4). The sucrose gradient profiles indicate that the size of the product increases with time. By 30 min the predominant species is nearly 14 S and corresponds to the size of the poly(rA) template used in the assay.

The ratio of complementary to noncomplementary nucleotide incorporation is constant across the peaks and does not vary as the concentration of the complementary nucleotide increases. Thus, the error rate is independent of the temperatures of the assay (16-39°C) and of the amount of DNA synthesized.

Density Gradient Analysis of Product—The product of the reaction was sedimented to equilibrium on a neutral cesium sulfate density gradient (Fig. 5A). The template-initiator, [r(pA)m.d(pT)m], would be expected to have a density corresponding to that of poly(rA). After synthesis, the density of the product corresponds to that of a 1:1 hybrid of poly(rA)-poly(dT), suggesting that the entire length of the poly(rA) template is copied. The ratio of H:32P radioactivity is constant across the range of densities of the product.
peaks, indicating that the dCMP is indeed incorporated into the polynucleotide and that the pattern of distribution of dCMP is not related to the size of the product.

Hydrolysis of the product in alkali resulted in a shift in density (Fig. 5B) so that after centrifugation in alkaline cesium sulfate the density of the product was similar to that of a polydeoxy-
nucleotide marker, poly[d(A-T)]. These results are consistent with hydrolysis of the ribonucleotide template by alkaline leaving an intact single-stranded polydeoxynucleotide product into which both dTMP and dCMP are incorporated.

Kinetics of Product Degradation — In order to confirm further the incorporation of the incorrect nucleotides into polynucleotide product and to ascertain the distribution and frequency of errors, we have carried out a stepwise degradative analysis of the product. The double labeled 14S polydeoxynucleotide product made on 14S poly(rA)-oligo(dT) template-initiator was subjected to hydrolysis by saturating amounts of snake venom exonuclease. The results in Fig. 6 illustrate that the ratio of the release of incorrect nucleotide to correct nucleotide was constant. The results show that: (a) the correct and incorrect nucleotides are present in phospho-
diester linkage; (b) the errors are not concentrated at either terminus; and (c) the errors are uniformly distributed over the entire length of the product.

In another experiment, the product containing [α-32P]dTMP and [3H]dCMP was hydrolyzed by snake venom exonuclease. The hydrolysate was subjected to high voltage paper electrophoresis along with dCMP and dTMP markers. The 32P label of the hydrolysate migrated with dTMP and 3H with dCMP, showing that the 32P radioactivity in the product was derived from the substrate [α-32P]dTMP and was present as dTMP. Most importantly, the 3H radioactivity in the product was derived from the substrate [3H]dCMP and was present as dCMP.

Analysis of Product for Frequency of Nearest Neighbor Base Pairs — Two double labeled products were made using high specific activity 32P and 3H nucleotides. One product was made using [α-32P]dTTP and [3H]dCTP (Product 1) and another product (Product 2) was made with the radioactive labels reversed, i.e. [3H]dTTP and [α-32P]dCTP. The products were then enzymatically degraded with micrococcal nuclease and spleen phosphodi-
esterase by the procedure of Josse et al. (28). The nucleotides were separated by paper electrophoresis and the radioactivity deter-
mined. In this procedure, the 32P radioactivity in the present in the nucleotide substrate is to be found in the adjacent nucleotide following hydrolysis of the products and conversely the 3H radioactivity present in the original substrate would remain in the same nucleotide. Thus, the 3H radioactivity serves as a marker for identification of the original substrate, whereas the 32P radioactivity which does change nucleotide position serves to quantitate the nearest neighbors.

The results of nearest neighbor analysis are summarized in Table I. Hydrolysis of Product 2 shows that 99% of the original 32P radioactivity present in the noncomplementary nucleotide dCTP was recovered with dTMP and the remaining 1% was recovered with dCMP. The 3H radioactivity, as expected, remained with the same nucleotide base following hydrolysis. In other experiments the transfer of 32P to dTMP ranged from 70% to 100%. These collected results indicated that the noncomple-
mentary dCTP is incorporated throughout the entire length of the newly synthesized polynucleotide.

**Discussion**

We have previously reported that homogeneous avian myeloblastosis virus DNA polymerase incorporates noncomplementary base-paired nucleotides while copying a variety of synthetic polynucleotides in vitro (10). A number of criteria presented in this investigation show that the errors are in the DNA product and are uniformly distributed over the entire length of the newly synthesized polynucleotide. (a) The product of the reaction using poly(rA) template banded at a density corresponding to a 1:1 hybrid of poly(rA)-poly(dT) after centrifugation to equilibrium in cesium sulfate. When the RNA portion of the hybrid was hydrolyzed and the product was rebanded in alkaline cesium sulfate, it moved to a density corresponding to that of poly(dT). Most importantly, the constant error rate across the peaks in both neutral and alkaline cesium sulfate equilibrium gradients shows that the errors are an integral part of polydeoxyx nucleotide product. (b) Alkaline sucrose gradient analysis of 14S poly(rA)·oligo(dT)-directed products show that the errors are present in all polymer size classes. The error rate is invariant with respect to size. Thus, the polymerase can copy the total length of the template, and the incorporation of the noncomplementary nucleotide does not terminate chain growth. (c) When the product...
was degraded sequentially by snake venom exonuclease, the complementary and noncomplementary nucleotides were released in constant ratio. (d) Nearest neighbor analysis of double labeled products indicates that the incorrect nucleotides are connected by phosphodiester bonds predominantly as single base substitutions.

Purified DNA polymerases from diverse sources have been shown to have tightly bound zinc in stoichiometric amounts (34) and the zinc is implicated in the mechanism of polymerization (1, 13, 35). Avian myeloblastosis virus DNA polymerase contains tightly bound zinc in stoichiometry of approximately 1 g atom/mol of enzyme (30). Exposure of avian myeloblastosis virus DNA polymerase to the chelator, o-phenanthroline, results in an immediate inhibition of polymerase activity, whereas m-phenanthroline, a nonchelating analogue, at a 10-fold higher concentration is without effect (30, 32). The results in Fig. 1 show that both the complementary and the noncomplementary nucleotide incorporation are equally inhibited by o-phenanthroline. This implies that the avian myeloblastosis virus polymerase catalyzes the incorporation of both the correct and incorrect nucleotides and that zinc is involved in these catalytic processes.

The ordered structure of the polynucleotide template and the extent of protonation of the nucleotides base pair dependent on the conditions of the reaction (36). If errors incorporated by the polymerase were a function of the conformation of the template or the degree of tautomerization of the nucleotides, appropriate changes in the reaction conditions should affect error rate. We find that alterations in the hydrogen ion concentration (Fig. 2) did not influence the error rate, even though the amount of DNA synthesized varied. Changes in the temperature of the reaction affect the rate of polymerization but do not change the ratio of complementary to noncomplementary nucleotide incorporation.

Our studies indicate that avian myeloblastosis virus DNA polymerase incorporates a large number of noncomplementary base-paired nucleotides during polymerization. The homogeneous enzyme is devoid of deoxyribonuclease activity and does not excise terminally mismatched base pairs. It has been reported (1, 9) that homogeneous DNA polymerases from eukaryotes with little or no exonuclease activity copy polynucleotide templates with a high degree of accuracy. The high error rate of avian myeloblastosis virus DNA polymerase thus appears to be a property of the enzyme. With this enzyme it should be possible to gain insight into the mechanism of base selection by kinetic analysis of the incorporation of complementary and noncomplementary nucleotides.

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