On the Fidelity of DNA Replication

LACK OF EXODEOXYRIBONUCLEASE ACTIVITY AND ERROR-CORRECTING FUNCTION IN AVIAN MYELOBLASTOSIS VIRUS DNA POLYMERASE*

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Homogeneous DNA polymerase ("reverse transcriptase") from avian myeloblastosis virus was assayed for exodeoxyribonuclease activity. The substrates were defined template-initiator complexes in which different radioactive nucleotides were present at the 3'-OH termini of the initiator. Even when the number of molecules of enzyme was equal to the number of initiator termini, there was no significant release of radioactivity with any of the template-initiator combinations tested. Under similar conditions, the nuclelease activity associated with either Escherichia coli or T7 DNA polymerases rendered more than 90% of the initiator termini acid-soluble. The ratio of exodeoxyribonuclease activity to protein with avian myeloblastosis DNA polymerase is less than 0.003% of that obtained with E. coli DNA polymerase I. Furthermore, avian myeloblastosis virus DNA polymerase failed to excise mispaired terminal nucleotides in both the presence and absence of polymerization.

Avian myeloblastosis virus DNA polymerase utilizes mispaired initiator termini when complexed to ribohomopolymer and deoxyribohomopolymer templates as functional sites for chain propagation. The efficiency of utilization of the paired and mispaired termini is about equal in that the amount of synthesis and the size of the products formed are similar. The ability of the enzyme to utilize the mismatched termini as starting points for polymerization has been unambiguously demonstrated by transfer of 32P from the newly synthesized product to the mispaired 3'-terminal nucleotide on the initiator.

DNA polymerase ("reverse transcriptase") from RNA tumor viruses have been shown to incorporate large numbers of noncomplementary nucleotides during polymerization. This infidelity has been found from the DNA polymerases from avian myeloblastosis virus (1, 2), Rauscher leukemia virus (3), and Rous sarcoma virus. Detailed analysis of the polydeoxynucleotide products synthesized using avian myeloblastosis virus DNA polymerase showed that the noncomplementary nucleotides are distributed throughout the entire length of the product and are present as single base substitutions (4, 5). This infidelity could lead one to expect that the viral polymerase is able to utilize initiators (primers) terminated in noncomplementary nucleotides and is incapable of exciting the noncomplementary nucleotides. In this communication we present evidence for the ability of avian myeloblastosis virus DNA polymerase to utilize mismatched primer termini for chain elongation and thus substantiate this expectation.

Bacteria and bacteriophages have a "proof-reading" exonuclease activity which is usually associated with their purified DNA polymerase (6). For example, Escherichia coli DNA polymerase I excises noncomplementary bases generated by itself or when presented with mispaired template-initiators. Furthermore, this enzyme cannot extend a mispaired terminus, and if presented with such template-initiators the enzyme excises the noncomplementary portion of the initiator prior to extending the chain. Thus, the 3'→5'-exonuclease activity has been postulated to function in base selection (7) and to guarantee the high fidelity required for DNA replication. On the other hand, 3'-5' exonucleases appear to be absent from extensively purified eukaryotic DNA polymerases (8-10), yet these polymerases make few mistakes in base pairing during DNA synthesis (10-12). Chang has shown that the low molecular weight DNA polymerase from calf thymus is able to carry out polymerization using primers with mismatched 3'-OH termini (12). We have examined avian myeloblastosis virus DNA polymerase for a proof-reading 3'→5'-exonuclease activity using synthetic homopolymer templates hybridized to oligonucleotides with mispaired termini. The 3'-terminal nucleotides of the primers were radioactive and thus serve as highly sensitive substrates for measuring any 3'-exonuclease activity. Our results show that the enzyme lacks the 3'→5'-exonuclease activity.

EXPERIMENTAL PROCEDURE

Materials

Polyribonucleotides, oligodeoxyribonucleotides, and polydeoxythymidylate, primers with H-deoxynucleotides at the 3' termini were synthesized using terminal nucleotidyltransferase (8, 13). To prepare the latter...
compound on an initiator poly(dT) averaging 86 nucleotides in length was reacted with different radioactive nucleotides, the ratio of monomer added to initiator was 1.14, 0.89, 0.75, and 1.55, with dTTP, dCTP, dGTP, and dATP, respectively. Polydeoxy nucleotide-oligo deoxynucleotide complexes and polyribonucleotide-oligo deoxynucleotide complexes were prepared by physical mixing and incubating the appropriate polymers in 0.02 M Tris- HCl at 40° for 10 min and cooling them gradually. The molecular ratio of initiator to template was 1:2 with poly(dA), and 1:1 with poly(dA), as templates. Initiators having mismatched bases at the 3'-OH terminus and poly(dA), were supplied by Dr. F. J. Bollum, University of Kentucky, Lexington, Ky.

Calf thymus DNA, pancreatic deoxyribonuclease, snake venom phosphodiesterase, micrococcal nuclease, and spleen phosphodiesterase were products of Worthington. Poly(A), was purchased from Miles Laboratories. Poly(A), template molecules having a mean 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. The source of other materials and reagents is given in the previous papers (5, 15).

**Methods**

**Polymerase Assay.—** Each assay contained the following in a total volume of 0.05 ml: 50 mM Tris-HCl, pH 8.0, 5 mM dithiothreitol; 20 mM KCl; 1 μg of bovine serum albumin; 5 mM MgCl,; 20 μM [3H]dC, or [3H]dG served as mispaired substrates. The nucleotides were then adsorbed onto Norit A and eluted with ammoniacal alcohol (18). The residual Norit was removed by filtration with a 0.35 μm Millipore filter. The filtrate was evaporated to dryness and the nucleotides dissolved in 0.05 ml of 0.01 to 0.05 μg of avian myeloblastosis virus DNA polymerase; and the indicated amount of template-initiator complex. The reaction was carried out at 37° for 60 min and incorporation into an acid-insoluble precipitate was determined as previously described (16). The rate of nucleotide incorporation under these conditions was linear for up to 120 min at 37°.

**Assay for Deoxyribonuclease Activity—** DNase activity was assayed by measuring the amount of 3H radioactivity rendered acid-soluble as previously described (13). The incubation mixture of 0.05 ml was identical with that for the polymerase assay excepting for the presence of the indicated radioactive synthetic nucleotides and the presence or absence of dTTP.

**Nearest Neighbor Analysis—** The purified products of DNA polymerase reactions were degraded to mononucleotides by digestion with micrococcal nuclease and spleen phosphodiesterase in 0.06 M Tris-HCl, pH 8.5, as described (17). The nucleotides were then adsorbed onto Norit A and eluted with ammoniacal alcohol (18). The residual Norit was removed by filtration with a 0.35 μm Millipore filter. The filtrate was evaporated to dryness and the nucleotides dissolved in 0.05 ml of water. The samples were applied to Whatman No. 3 paper along with appropriate markers. Electrophoresis was carried out in 0.05 M acetate buffer, pH 3.5, for 60 min at 4000 volts and 20° using a flat plate 1-cm squares, and the radioactivity determined. The nucleotides in the chromatogram were identified by their relative migration using 3'.5'-exonuclease reactions were degraded to 3'.mononucleotides by digestion with micrococcal nuclease and spleen phosphodiesterase in 0.05 M Tris-HCl, pH 7.5, as described (17). The nucleotides were then adsorbed onto Norit A and eluted with ammoniacal alcohol (18). The residual Norit was removed by filtration with a 0.35 μm Millipore filter. The filtrate was evaporated to dryness and the nucleotides dissolved in 0.05 ml of water. The samples were applied to Whatman No. 3 paper along with appropriate markers. Electrophoresis was carried out in 0.05 M acetate buffer, pH 3.5, for 60 min at 4000 volts and 20° using a flat plate 1-cm squares, and the radioactivity determined. The nucleotides in the chromatogram were identified by their relative migration using 3'.5'-exonuclease activities in different DNA polymerases

<table>
<thead>
<tr>
<th>Enzyme and template-initiator</th>
<th>Radioactivity rendered acid-soluble cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMV DNA polymerase (0.3 pmol)</td>
<td>&lt;10</td>
</tr>
<tr>
<td>d(A)E.d(T)E.d(PA)E.d(pT)E.d(TH)</td>
<td>&lt;10</td>
</tr>
<tr>
<td>d(A)E.d(T)E.d(PA)E.d(pT)E.d(pC)</td>
<td>&lt;10</td>
</tr>
<tr>
<td>d(A)E.d(T)E.d(PA)E.d(pT)E.d(pG)</td>
<td>&lt;10</td>
</tr>
<tr>
<td>E. coli polymerase I (0.07 pmol)</td>
<td>&lt;10</td>
</tr>
<tr>
<td>d(A)E.d(T)E.d(PA)E.d(pT)E.d(pC)</td>
<td>&lt;10</td>
</tr>
<tr>
<td>T, polymerase (1.5 pmol)</td>
<td>840</td>
</tr>
<tr>
<td>d(A)E.d(T)E.d(pA)E.d(pC)</td>
<td>840</td>
</tr>
<tr>
<td>d(A)E.d(T)E.d(pA)E.d(pG)</td>
<td>840</td>
</tr>
<tr>
<td>d(A)E.d(T)E.d(pA)E.d(pT)</td>
<td>840</td>
</tr>
<tr>
<td>d(A)E.d(T)E.d(pA)E.d(pG)</td>
<td>790</td>
</tr>
<tr>
<td>d(A)E.d(T)E.d(pA)E.d(pT)</td>
<td>820</td>
</tr>
<tr>
<td>d(A)E.d(T)E.d(pA)E.d(pG)</td>
<td>830</td>
</tr>
<tr>
<td>Pancreatic deoxyribonuclease (100 pmol)</td>
<td>2100</td>
</tr>
<tr>
<td>d(A)E.d(T)E.d(pA)E.d(pC)</td>
<td>1030</td>
</tr>
<tr>
<td>d(A)E.d(T)E.d(pA)E.d(pG)</td>
<td>850</td>
</tr>
<tr>
<td>d(A)E.d(T)E.d(pA)E.d(pT)</td>
<td>982</td>
</tr>
</tbody>
</table>

show that both Escherichia coli DNA polymerase I and T, DNA polymerase are able to excise most of the complementary or the noncomplementary nucleotides at the primer terminus. A comparison of the rate of excision of complementary and noncomplementary nucleotides was not carried out with these latter enzymes. In contrast, there was no significant hydrolysis of the primer with homogeneous AMV DNA polymerase. In this experiment the ratio of exonuclease activity to protein with AMV DNA polymerase is less 0.03% of that obtained with E. coli DNA polymerase I (Table I).

Since the viral enzyme preferentially utilizes ribonucleotide templates for synthesis, it is possible that a putative nuclease activity may have similar requirements. The same initiators with radioactively labeled terminal nucleotides were hybridized to poly(rA)E and used as substrates for measuring 3' → 5'-exonuclease activity. Assays were carried out under nonpolymerizing conditions, i.e. in the absence of dTTP. The results presented in Table II show that AMV DNA polymerase did not excise the correct pyrimidine-purine base-paired RA-dT, the pyrimidine-purine RA-dC mispair, the pyrimidine-pyrimidine RA-dA mispair, or pyrimidine-pyrimidine RA-dG mispair situated at the 3' terminus. We have also carried out tests for nuclease activity in both the presence and absence of dTTP as well using 10-fold greater amounts of AMV DNA polymerase than that used in the experiments detailed in Tables I and II. There was no

The abbreviation used is: AMV, avian myeloblastosis virus.

### Table I

**Comparison of 3' → 5'-exonuclease activities in different DNA polymerases**

[Caption: Table I showing the comparison of 3' → 5'-exonuclease activities in different DNA polymerases.]
significant hydrolysis of base-paired or mispaired terminal nucleotides (Table III). Less than 5% of the radioactivity was rendered acid-soluble with any of the template-initiators in the presence or absence of dTTP. In the experiments with the poly(rA) templates the number of molecules of AMV DNA polymerase in each assay was as large as the number of molecules of labeled initiators. A comparison of Tables I and III indicates that AMV DNA polymerase in an amount 43-fold greater than E. coli polymerase I failed to excise detectable amounts of either paired or mispaired terminal nucleotides. These collected results (Tables I to III) clearly show that AMV DNA polymerase lacks a proof-reading 3' 5' exonuclease activity.

**Utilization of Mismatched Primer Termini for Polymerization**—Since AMV DNA polymerase incorporates noncomplementary nucleotides and fails to excise mispaired primer termini it is of interest to determine if the enzyme can utilize initiators with mispaired 3'-OH terminal nucleotides for chain elongation. The complexes used in the nuclease assays as substrates were used as templates to measure polymerization. As expected, the rate of synthesis was greater with poly(A) template than with poly(dA) template. As shown in Table IV the enzyme utilizes the 3' termini containing the standard base pairs, as well as the mismatched base pairs with both poly(rA) and poly(dA) as templates. There was no significant difference in the amount of synthesis observed with the templates containing the correctly paired or incorrectly paired initiator termini.

Even though the rate of synthesis on matched and mismatched initiator termini from homopolymer templates was about the same (Table IV) the results do not indicate that the products are of equal size. Products were made using poly(A) template hybridized to oligo(dT) primer containing at its 3'-OH end complementary (dT) or noncomplementary (dC). The polynucleotide products synthesized on these matched and mismatched template-primer complexes were then analyzed for size by paper electrophoresis and their radioactive content determined. The size of products as analyzed by alkaline sucrose gradients was also the same (results not shown).

**Transfer of Radioactive Label to Demonstrate Initiation on Mispaired Primer Termini**—In order to show unambiguously that AMV DNA polymerase does indeed catalyze initiation on mispaired primer termini from homopolymer templates, we have carried out a label transfer experiment. The design of the experiment is presented in Fig. 2. Products were made using poly(A) template hybridized to oligo(dT) initiator containing mispaired 3'-OH dC at its 3' terminus, and [a-32P]dTTP was used as substrate. The assay conditions were the same as those used in the experiments measuring rates of synthesis with matched and mismatched primer termini (Table IV). The products of the reaction were enzymatically hydrolyzed with micrococcal nuclease and spleen phosphodiesterase. The nucleotides were separated by paper electrophoresis and their radioactive content determined. In this procedure, the [a-32P] radioactivity present in the nucleotide substrate is found in the adjacent nucleotide on the 5' end following hydrolysis. Thus,
Figs. 1 and 2. Schematic representation of the transfer of \( ^{32}P \) label from position 5' of dTMP to \([^{3}H]dC\) at the 3'-OH terminus of the initiator.

prior to synthesis the terminal labeled nucleotide would be found after hydrolysis as dC; after polymerization it would be recovered as \([^{3}H]dC\). The data in Table V shows that prior to synthesis 53% of the radioactivity in the initiator was recovered with deoxycytidine. Thus, 53% of the label is present in the terminal 3'-OH nucleotide. By equating the fraction of molecules contains one or more mismatched terminal nucleotides. This calculation indicates that 34% of the labeled initiators contain one mispaired terminus at the 3' end and that 24% of the initiator molecules lack dC at the 3' terminus. This result is in agreement with an estimate of 37% for one dCMP calculated from a Poisson distribution based on the ratio of monomer to initiator in the reaction mixture. Since there is a class of molecules without mismatched termini, it is important to show that initiation actually takes place on the mismatched termini.

A similar analysis of the distribution of radioactivity was carried out after synthesis. The results show that the \( ^{32}P \) originally present in the \([^{32}P]dTTP\) was transferred onto the mispaired terminus to yield \([^{32}P]dCMP\). Moreover, all of the \( ^{3}H \) radioactivity originally present as deoxycytidine was recovered after synthesis as dCMP indicating that the enzyme has initiated polymerization on all the mismatched termini. The ratio of counts per min recovered in dCMP (termini) and dTMP (chain) is approximately 1:1000 and is in reasonable agreement with the size of the product. Thus, avian myeloblastosis virus DNA polymerase is able to catalyze covalent initiation on mispaired initiator termini.

**Discussion**

The results presented in this paper clearly demonstrate that the AMV DNA polymerase lacks a 3' 5' proofreading exonuclease activity. Using initiators with correctly and incorrectly base-paired termini, excision of 3' terminal nucleotides was undetectable. This lack of 3' 5' exonuclease activity was documented with ribo- and deoxyribonucleotide templates. Under similar conditions both *Escherichia coli* and T, DNA polymerases excise about 90% of the terminal 3'-OH nucleotides. A correctly base-paired initiator terminus is absolutely required (6, 7) for initiation of polymerization by *E. coli* polymerase I. In contrast, both the correctly base-paired and mispaired primer termini serve for polymerization using AMV DNA polymerase. Moreover, with AMV DNA polymerase activity was documented with ribo- and deoxyribonucleotide templates. Under similar conditions both *Escherichia coli* and T, DNA polymerases excise about 90% of the terminal 3'-OH nucleotides. A correctly base-paired initiator terminus is absolutely required (6, 7) for initiation of polymerization by *E. coli* polymerase I. In contrast, both the correctly base-paired and mispaired primer termini serve for polymerization using AMV DNA polymerase. Moreover, with AMV DNA polymerase.

**Table V**

<table>
<thead>
<tr>
<th>Initiator</th>
<th>Deoxycytidine</th>
<th>dCMP</th>
<th>dTMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>dpT(TP)H</td>
<td>cpm</td>
<td>cpm (pmol)</td>
<td></td>
</tr>
<tr>
<td>dpT(TP)H</td>
<td>320</td>
<td>280</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 1.** Alkaline sucrose sedimentation analysis of products. The assay mixture of 0.2 ml consisted of: 50 mM Tris-HCl, pH 8.0, 5 mM dithiothreitol, 5 mM MgCl₂, 20 mM KCl, 0.1 mM of the template-initiator, 60 \( \mu \)M \([^{32}P]dTTP\), and 40 \( \mu \)g of AMV DNA polymerase. The assay was incubated for 60 min at 37°. The products were extracted with phenol and dialyzed extensively against four changes of 2 liters each of 50 mM Tris-HCl, pH 8.0, containing 0.2 \( M \) NaCl. The salt concentration was reduced by final dialysis against distilled water. Nearest neighbor analysis was carried out as described under "Methods."
corporates an unusually large number of noncomplementary bases are frequently incorporated by DNA polymerases and only the prokaryotic enzymes are able to discriminate between these termini. That the enzyme fails to distinguish between these termini. The lack of 3'-exonuclease activity in the viral DNA polymerase reported herein further indicates that this exonuclease is not present on all DNA polymerases (8) and suggests that the mechanism of catalysis by the viral enzyme may be similar to that of DNA polymerases from animal cells (10).

Purified avian myeloblastosis virus DNA polymerase has been demonstrated to contain an associated RNase H activity which specifically degrades the RNA moiety of the RNA-DNA hybrid molecules (19-21). The manner of degradation has been shown to be processive and exoribonucleolytic (22). Moreover, holoenzyme containing the α and β enzyme subunits appears to hydrolyze processively while the isolated α subunit appears to possess a random mode of action (23). In these studies the deoxyribo portion of the hybrid was not hydrolyzed, and thus the results are not in conflict with those reported in this paper.

It has been demonstrated that the AMV DNA polymerase incorporates an unusually large number of noncomplementary nucleotides while copying synthetic polynucleotide templates (1, 2, 4). It remains to be demonstrated whether or not a similar infidelity is observed with natural DNA and RNA templates and that frequent mispairings occur during viral replication. When examined, DNA polymerases from prokaryotes have been shown to contain an associated 3'-5' exonuclease (6). Evidence suggests that this exonuclease functions in base selection; it specifically excises noncomplementary nucleotides during polymerization (7, 24). It can be argued that the infidelity of DNA synthesis by AMV DNA polymerase is a direct consequence of the lack of exonuclease activity. Thus noncomplementary bases are frequently incorporated by DNA polymerases and only the prokaryotic enzymes are able to remove them during polymerization. However, it should be noted that DNA polymerases from animal cells are highly accurate in base selection (11, 12) and yet also lack a 3'-5' exonuclease activity. For example, the DNA polymerase extensively purified from nuclei of sea urchin lack a 3'-exonuclease (20), but when copying poly(dA-T) it incorporates less than one dCMP or dGMP into the product per 10,000 or 150,000 nucleotides polymerized (1). Similarly, the low molecular weight DNA polymerase from calf thymus has been shown to lack any exonuclease activity (8) but the frequency of misincorporated nucleotides appears to be less than 1 in 100,000 (12). Thus, these DNA polymerases from animal cells must have other means for guaranteeing the accuracy of DNA synthesis.

Acknowledgments—We thank Dr. P. J. Dollum for the gift of oligonucleotides with different radioactive nucleotides at the 3'-terminus which made this study possible. We thank Dr. J. Beard and M. A. Chirigos for generously supplying avian myeloblastosis virus and Dr. S. Litwin from this Institute, who derived the equation for determining the distribution of mismatched termini.

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On the fidelity of DNA replication. Lack of exodeoxyribonuclease activity and error-correcting function in avian myeloblastosis virus DNA polymerase.

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