Enzymatic Synthesis of Deoxyribonucleic Acid

XXXVI. A PROOFREADING FUNCTION FOR THE 3' → 5' EXONUCLEASE ACTIVITY IN DEOXYRIBONUCLEIC ACID POLYMERASES*

(Received for publication, July 16, 1971)

DOUGLAS BRUTLAG† AND ARTHUR KORNBERG

From the Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305

SUMMARY

The Escherichia coli and T4 DNA polymerases do not extend chains in which the 3'-terminal nucleotide (primer terminus) is not paired with the template. By using synthetic double-stranded nucleoproteinidic acids, the 3' → 5' exonuclease function of these polymerases was shown to be directed specifically against a mispaired or unpaired primer terminus. Chain extension of such termini begins only after all mispaired nucleotides have been removed and a base-paired terminus is reached. The latter is completely conserved while polymerization is maintained. These results suggest that the function of the 3' → 5' exonuclease activity of DNA polymerases is to remove mispaired nucleotides which have been incorrectly incorporated, thereby increasing the fidelity of template copying. The function of other E. coli exonucleases suggested by their specificities on polynucleotide substrates are trimming loose ends of DNA for exonuclease I and enlarging nicks and gaps within helical regions for exonuclease III.

The studies reported here on the specificity of the 3' → 5' exonuclease support earlier speculations (3, 7, 8) that this activity may be designed to remove mispaired nucleotides which have been incorrectly incorporated at the 3'-hydroxyl end of the double-stranded DNA and thereby serves a proofreading function. By the use of specifically labeled homopolymers and block copolymers as substrates we show in this report that under polymerizing conditions a mispaired primer terminus is quantitatively removed by the DNA polymerase whereas a correctly base-paired primer terminus is completely conserved. It thus appears that the 3' → 5' exonuclease may serve a proofreading role in removing mispaired nucleotides at the growing end of a chain and that the polymerase will not extend a chain until the mispaired 3' terminus is removed.

The availability of these polynucleotide substrates has enabled us also to examine the specificities of the E. coli exonucleases I and III. These nucleases appear to perform complementary roles in trimming single-stranded ends of RNA chains and in enlarging nicks and gaps within helical regions of double-stranded DNA.

MATERIALS

Nucleotides—Unlabeled deoxyribonucleotides were purchased from P-L Biochemicals except for dUTP which was purchased from Calbiochem. [α-32P]dTTP was a gift from Dr. M. Fikus and was prepared by deamination of dATP as described by Inman and Baldwin (9). [3H]dATP and [3H]dUTP were purchased from Schwarz BioResearch. [3H]dTTP was purchased from Amersham-Searle and was purified before use by paper chromatography (Schleicher and Schuell, No. 589 orange ribbon) using an isopropyl alcohol-concentrated NH4OH-water (7:1:2) solvent. [α-32P]dTTP was purchased from ICN and was checked for radiochemical purity by paper electrophoresis (20 mM sodium citrate buffer, pH 3.5). [32P]dTTP was purchased from New England Nuclear. All specific activities were determined by spectral measurement and radioactivity measured on Whatman GF/C glass filters in a Nuclear Chicago scintillation counter. 2',3'-Dideoxythymidine triphosphate and the 6'-deoxy-6'-homothymidine pyrophosphoryl phosphonate were gifts from Dr. J. G. Moffatt. β,γ-d3TP methylene diphosphonate was purchased from Miles Laboratories.

Enzymes—Micrococal nucleases and spleen phosphodiesterase were purchased from Worthington Biochemicals. Alkaline

1 L. E. Orgel and F. H. C. Crick, personal communication.
phosphatase was isolated and purified according to the method of Malamy and Horecker (10). DNA polymerases induced by T4 amN82 and T4 amN82 tsL66, the gifts of Dr. W. M. Huang, had been purified as described (3, 11). E. coli DNA polymerase was purified as described (12). The large fragment obtained by proteolytic cleavage of this enzyme appeared in the purification procedure of the native DNA polymerase and was also purified to homogeneity (6). All DNA polymerases used in this study yielded a single protein band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. E. coli exonuclease I was purified through the hydroxyapatite stage (specific activity of 15,000 units per mg) (13). E. coli exonuclease III had a specific activity of 130,000 units per mg (12). Terminal deoxynucleotidyltransferase (terminal transferase) purified from calf thymus according to homogeneity was purified as described (12). The large fragment obtained by T4 umN82 and T4 umN82 tsL56, the gifts of Dr. W. M. Huang, to Kat0 et al. (14) was a gift from Dr. F. N. Hayes (Los Alamos National Laboratory, Los Alamos, New Mexico).

**Methods**

**Polynucleotides**—The polynucleotides d(T)260 and d(I)260 were prepared with terminal transferase as described (15) except that 20 mM potassium phosphate buffer (pH 7.0), 20 mM potassium cacodylate buffer (pH 7.0), and 8 mM MgCl2 were used for the polymerization of dITTP. d(A)260 was prepared according to Riley, Maling, and Chamberlin (16) and d(C)260 according to Chamberlin and Patterson (17).

**Terminal Labeled Polynucleotides**—Polynucleotides, radioactivity labeled at their 3'-termini with various nucleotides (d(T)260[3H]d(T)260, d(T)260[3H]d(C)11, d(T)260[3H]d(A)9, d(T)260[3H]d(U)1,8, and d(I)260[3H]d(T)1,4)2 were synthesized by incubating labeled triphosphates with unlabeled polynucleotides and terminal transferase. To add a purine nucleotide, dATP for example, to d(T)260, 0.3 mM d(T)260 was incubated with 5 µM [3H]dATP (17.7 Ci per mmole), and 0.15 mg per ml of terminal transferase in 20 mM potassium phosphate buffer (pH 7.0), 20 mM potassium cacodylate buffer (pH 7.0), 8 mM MgCl2, and 1 mM β-mercaptoethanol at 37° for an amount of time (determined by a small scale reaction) sufficient to add about 1 mole of nucleotide per mole of polynucleotide chain. Incorporation was monitored by adsorption to DEAE-paper as described below. To add a pyrimidine nucleotide, 0.1 mM potassium phosphate buffer (pH 7.0), 0.1 mM potassium cacodylate buffer (pH 7.0), and 0.25 mM CoCl2 were used. The reaction was terminated by cooling and adding 50% KOH to a final concentration of 0.3 M. After 10 min at 0°, the mixture was neutralized with 85% H3PO4 and dialyzed extensively against 1 mM NaCl, 10 mM Tris buffer (pH 8.0), and then versus 10 mM Tris buffer (pH 8.0) to remove the salt. The final average number of residues added was determined from the specific activity of the added nucleotide, the length of the polynucleotide, and the radioactivity present in the labeled polymer per mole of total nucleotide as determined by ultraviolet absorption.

**Determination of Distribution of Labeled Residues**—Hayes et al. (18) have shown that limited addition of nucleotides to short oligonucleotide primers by terminal transferase gives a Poisson distribution of added residues. This conclusion was found to apply also to the longer polynucleotide primers used in this study. This determination was made from an independent measure of the distribution of nucleotides as well as the average number of residues added per chain. Degradation of the labeled polymers by micrococcal nuclease and spleen phosphodiesterase converts all the internal nucleotides to 3'-nucleotides and the 3'-terminal residue to a nucleoside. The ratio of label converted to nucleoside to the total label found in the nucleoside and 3'-nucleotide, combined, gives the fraction of the labeled residues at the 3'-terminal position (Pf). For this analysis, 3 nmoles of each of the terminally labeled polynucleotides were degraded with micrococcal nuclease and spleen phosphodiesterase and the products were separated by chromatography as described by Wu and Kaiser (19). The hydrolysis converted >98% of the total label to nucleoside and nucleotide. Table I summarizes the average number of labeled residues and the fraction of these residues which was terminal. The observed fraction of terminal residues was in good agreement with that predicted by a Poisson distribution. It can be demonstrated that this analysis is formally equivalent to showing that the fraction of chains to which no labeled residues were added conforms to the Poisson distribution.

**Determination of Polynucleotide Length**—The number-average length of a polynucleotide was determined by end group labeling analysis as described by Weiss, Live, and Richardson (20).

**Nuclease Assays**—Nuclease assays were performed in 0.2 ml containing 2 nmoles of [3H]d(T)260, with or without 5 nmoles of unlabeled d(A)260, 0.05 M N-hydroxypyridoxylpiperazine-N'-ethanesulfonate buffer (pH 7.4), and 5 mM MgCl2. The mixture was heated to the appropriate temperature and a 20 µl sample was taken and applied to a 1.5-cm square of Whatman DE-81 paper. Then 10 µl of enzyme diluted in bovine serum albumin (1 mg per ml), 0.05 M HEPES2 buffer (pH 7.4), and containing less than 1 pmole of enzyme were added to the reaction; 20 µl samples were removed at appropriate intervals and adsorbed onto 1.5 cm squares of DE-81 paper. The squares were washed three times by gentle agitation for 5 min in 100 ml of ammonium formate, 0.3 M (adjusted to pH 7.8), and dehydrated by two washes in 95% ethanol and one in anhydrous ether. The squares were air dried and the amount of radioactive label remaining in polynucleotide determined in a scintillation counter. (Polymerization was also measured with this technique by using labeled deoxynucleobaseoligonucleotide triphosphates.) Nuclease rates were determined from a least squares fit to initial linear points of nucleotide hydrolyzed plotted against time. Rates were determined from only those assays in which >20% of the polynucleotide had been degraded and in which the rate was linear for at least 3 time points.

**Results**

3' → 5' Exonuclease Rates of DNA Polymerases: Influence of Secondary Structure and Temperature—The nuclease rate of the large fragment (a proteolytic fragment of the E. coli enzyme containing only polymerase and 3' → 5' exonuclease activities) on d(T)260 was 4-fold greater with the polymer in a polymer of unlabeled thymidylate to which has been added on the average 1.1 moles of H-labeled deoxycytidylate per mole of polymer.

3' → 5' Exonuclease Rates of DNA Polymerases: Influence of Secondary Structure and Temperature—The nuclease rate of the large fragment (a proteolytic fragment of the E. coli enzyme containing only polymerase and 3' → 5' exonuclease activities) on d(T)260 was 4-fold greater with the polymer in a polymer of unlabeled thymidylate to which has been added on the average 1.1 moles of H-labeled deoxycytidylate per mole of polymer.
TABLE I

Distribution of terminal residues on synthetic polynucleotides

<table>
<thead>
<tr>
<th>Polynucleotide composition</th>
<th>Fraction of labeled residues at the 3' terminus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed</td>
</tr>
<tr>
<td>d(T)125-[3H]d(T), ss</td>
<td>0.54</td>
</tr>
<tr>
<td>d(T)125-[3H]d(C), ss</td>
<td>0.56</td>
</tr>
<tr>
<td>d(T)125-[3H]d(A), ss</td>
<td>0.42</td>
</tr>
<tr>
<td>d(T)125-[3H]d(U), ss</td>
<td>0.50</td>
</tr>
<tr>
<td>d(I)125-[3H]d(T), ss</td>
<td>0.47</td>
</tr>
</tbody>
</table>

* The fraction of labeled residues which are at the 3'-OH terminal position of a chain ($P_1$) was determined by nuclease degradation. Specific activities of the labeled nucleotides were from 7 to 20 Ci per mmole.

† $P_1 = (1 - e^{-r})/r$, for a Poisson distribution of labeled residues where $r$ is the average number of labeled residues per chain.

TABLE II

Influence of temperature on 3' → 5' exonuclease rates of DNA polymerase acting on single- and double-stranded polynucleotides

<table>
<thead>
<tr>
<th>DNA polymerase</th>
<th>Temperature</th>
<th>Substrate</th>
<th>Single-stranded</th>
<th>Double-stranded</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>[dH(dT)]5000</td>
<td>[dH(dC)]5000</td>
</tr>
<tr>
<td>E. coli large fragment</td>
<td>37°C</td>
<td>19</td>
<td>4.3</td>
<td></td>
</tr>
<tr>
<td>T4 wild type*</td>
<td>37°C</td>
<td>1100</td>
<td>350</td>
<td></td>
</tr>
<tr>
<td>T4 wild type*</td>
<td>30°C</td>
<td>850</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>T4 L50*</td>
<td>30°C</td>
<td>790</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

* β-Mercaptoethanol (10 mM) was included in assays of the T4 enzyme.

single-stranded as compared to a double-stranded conformation (d(T)125:d(A)1000) (Table II). A similar preference for single-stranded d(T)125 was observed at 37°C with the T4 enzyme which has a much higher nuclease activity than the E. coli enzyme.

When the nuclease rates of the T4 polymerase on single-stranded d(T)125 at 30°C are compared with the rates at 37°C only a slight reduction in rate (23%) was found. However, there was a 13-fold reduction in rate on the double-stranded substrate (Table II). A similar marked temperature dependence of the nuclease rate of the E. coli large fragment on double-stranded substrates has also been observed.* This temperature dependence indicates a high energy of activation for some rate-limiting step on double-stranded substrates, possibly local denaturation at the 3' terminus. Thus the 3' → 5' exonuclease might require that the terminal nucleotides not be base-paired (i.e. frayed).

Nuclease Action of DNA Polymerases on Primer Terminus—Polynucleotides containing on the average about one radioactive nucleotide residue per chain at the 3' terminus were synthesized employing terminal transferase (Table I). The terminal residue was either the same as the rest of the polymer or distinctive from it. The distribution of labeled residues added to the polynucleotide chains approximates a Poisson distribution. For simplicity, these 3' terminally labeled polymers are referred to with a subscript "1" even though the actual average number of labeled residues only approximates unity.

The rate of removal of the terminal label from d(T)125-[3H]d(T), by the large fragment was too great to permit accurate rate measurements (Fig. 1A). However, the decrease in nuclease rate with the polynucleotide in a double-stranded conformation was large enough to be apparent. Thus the rate of hydrolysis of these terminal residues reflects the preference for single-stranded substrate initially observed with uniformly labeled polynucleotides. The terminal residue of d(T)125-[3H]d(C), which does not base pair with d(A)1000, was removed at a rapid rate both in the single and the double-stranded conformation (Fig. 1B). Although the relative rates of hydrolysis of dCMP in these two cases cannot be determined, the rate of removal of dCMP from d(T)125-[3H]d(C):d(A)1000 was far more rapid than that of dTMP from d(T)125-[3H]d(T):d(A)1000 (compare Fig. 1, a and b). Thus the preference of the nuclease for nucleotides which are not able to base pair with the template is readily apparent.

Removal of Terminal Nucleotides Prior to Polymerization—Polymerization had a dramatic effect on the 3' → 5' exonuclease activity. When the large fragment and the polymer d(T)125-[3H]d(T):d(A)1000 were incubated with dTTP so that polymerization could proceed, there was no detectable loss of terminal la-
bel during the incorporation of dTTP (Fig. 1c). The incorporation of dTTP completely protected the terminal nucleotides from exonuclease action. In a similar experiment in which d(T)₃₅₀·[³²P]d(T)₃₅₀·[³²P]d(C)₃₅₀·d(A)₁₄₂₀. The large fragment (2.4 pmole) was incubated under standard assay conditions at 37°C with the following: α, 0.75 nmole of d(T)₃₅₀·[³²P]d(T)₃₅₀·[³²P]d(T)₃₅₀·[³²P]d(C)₃₅₀·d(A)₁₄₂₀; β, same as α but with 50 nM [³¹P]dTP (10,400 cpm per pmole).

A third requirement for the protection of a terminal residue from exonuclease action is that it be base-paired to the template. When d(T)₃₅₀·[³²P]d(C)₃₅₀·d(A)₁₄₂₀ was incubated with polymerase and dTTP, the terminal dCMP residue was removed rapidly after which polymerization proceeded normally with the primer that remained (Fig. 1d). After extensive polymerization little or no [³²P]dCMP was detectable in the product, indicating that the DNA polymerase does not polymerize on a mispaired 3' terminus; instead the enzyme first removes the non-base-paired nucleotides by 3' → 5' exonuclease action.

In order to investigate further how strict is the base pairing specificity of this exonuclease during polymerization, a copolymer was synthesized containing a block of 5 [α-³²P]dTMP residues subterminal to a block of 16 [³²P]dCMP residues (d(T)₃₅₀·[³²P]d(T)₃₅₀·[³²P]d(T)₃₅₀·[³²P]d(C)₃₅₀·d(A)₁₄₂₀). The fate of the terminal block of 16 non-base-paired dC residues and the subterminal block of base-paired dT residues were both observed. In the absence of dTTP, the enzyme (large fragment) removed the terminal dCMP residues at a maximum rate of 20 nucleotides per min per chain, whereas the dTMP residues were removed only after a significant lag and then only at a maximum rate of 4 residues per min per chain (Fig. 2a). The rate difference is not immediately apparent from Fig. 2a because the plot of the percentage of residues remaining does not reflect that there are initially three times as many dCMP residues as dTMP residues. When dTTP was included so that polymerization could proceed, the terminal dCMP residues were still removed as rapidly but the subterminal, base-paired dTMP residues were retained (Fig. 2b); loss of dTMP was less than 0.5 residue per chain. As expected, the polynucleotide supported synthesis only after a lag, during which the dCMP residues were removed. This experiment indicates not only the specificity of the 3' → 5' exonuclease for a mispaired nucleotide, but also the inability of the polymerase to utilize such a nucleotide as a primer terminus for polymerization.

**Inhibition of 3' → 5' Exonuclease by Deoxyribonucleoside Triphosphates**—The previous experiments demonstrated that dTTP in an exonuclease assay with the base-paired template primer d(T)₃₅₀·[³²P]d(T)₃₅₀·[³²P]d(T)₃₅₀·[³²P]d(C)₃₅₀·d(A)₁₄₂₀ completely inhibited the 3' → 5' exonuclease activity of the DNA polymerase on terminal dTMP residues. Incorporation of nucleotides on a terminally labeled polynucleotide covers the labeled residue and protects it from exonuclease action. In order to determine whether incorporation of the triphosphate is required for protection of a terminal nucleotide, other deoxyribonucleoside triphosphates were tested for their ability to inhibit exonuclease (Table III). Nucleotides not complementary to the template d(A)₁₄₂₀ (i.e. dATP, dGTP, and dCTP) were not incorporated and also failed to inhibit exonuclease. The phosphonates, dTMPNPCP and 6'-deoxy-6'-homothymidine pyrophosphoryl phosphate, are analogues of dTTP, which bind to the triphosphate-binding site on the enzyme (21), and have the same base pairing specificity as dTTP. Neither analogue is incorporated by DNA polymerase nor does either analogue inhibit exonuclease. Three nucleotides which were incorporated (dTTP, ddTTP, and ddTTP) all inhibited exonuclease (Table III).

The behavior of ddTTP is of interest in two respects. Since ddTTP lacks a 3'-hydroxyl group only 1 residue is incorporated per chain (22). Nevertheless, this limited incorporation was sufficient to protect essentially all of the terminal nucleotides from exonuclease. Secondly, ddTTP is incorporated at a rate

![Fig. 2. 3' → 5' exonuclease action of the large fragment on the terminal residues of d(T)₃₅₀·[³²P]d(T)₃₅₀·[³²P]d(C)₃₅₀·d(A)₁₄₂₀.](image-url)

The deoxyribonucleoside triphosphate, 50 μM, was incubated with 0.6 nmole of d(T)₃₅₀·[³²P]d(T)₃₅₀, 2.0 nmole of d(A)₁₄₂₀, and 5.4 pmole of large fragment at 37°C under standard assay conditions for 4 min. Incorporation was followed using ³¹P- or ³²P-labeled triphosphate (10,000 cpm per pmole) except where noted.

<table>
<thead>
<tr>
<th>Nucleotide triphosphate added</th>
<th>Triphosphate incorporation*</th>
<th>% of initial labeled primer remaining*</th>
</tr>
</thead>
<tbody>
<tr>
<td>dATP</td>
<td>None</td>
<td>&lt;5</td>
</tr>
<tr>
<td>dGTP</td>
<td>None</td>
<td>&lt;5</td>
</tr>
<tr>
<td>dCTP</td>
<td>None</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Homophosphonate*</td>
<td>None</td>
<td>&lt;5</td>
</tr>
<tr>
<td>dTMPNPCP</td>
<td>Normal</td>
<td>100</td>
</tr>
<tr>
<td>dTTP</td>
<td>Normal</td>
<td>95</td>
</tr>
<tr>
<td>ddTTP</td>
<td>One</td>
<td>97</td>
</tr>
</tbody>
</table>

* "None" indicates no incorporation detectable (less than residue per chain); "normal" indicates rapid chain extension; "one" indicates a single residue incorporated per chain (22).

* Values of <5% indicate that both the rate and the extent of hydrolysis were the same as a control without any deoxyribonucleoside triphosphate added.

* 6'-Deoxy-6'-homothymidine pyrophosphoryl phosphate.

The inability of DNA polymerase to incorporate these analogues was shown by their inability to replace dTTP in a poly[d(A-T)] primed reaction (Brutlag and Kornberg, unpublished results).
with 0.6 nmole of primer polynucleotide d(T)$_{14}$ (first three columns) or d(C)$_{1400}$ (fourth column) labeled at the 3' terminus with the designated (*) nucleotide and annealed to 5 pmole of a template (d(A)$_{1400}$ or d(C)$_{1400}$). dTTP or dGTP of the T4 polymerase. The temperature was as designated except that 10 mM p-mercaptoethanol was included in assays.

<table>
<thead>
<tr>
<th>DNA polymerase</th>
<th>% of initial labeled primer terminus remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli large fragment</td>
<td>98</td>
</tr>
<tr>
<td>T4 wild type</td>
<td>97</td>
</tr>
<tr>
<td>T4 L56</td>
<td>100</td>
</tr>
</tbody>
</table>

* These assays were performed at 30°.

which is a 1000-fold less than that of dTTP (22). Although this rate of dTTP incorporation (0.4 nucleotide per chain per min) is 10-fold slower than that of exonuclease action on the terminal base-paired residues in the absence of a triphosphate, the presence of dTTP completely inhibited the exonuclease. Thus the ability of a nucleoside triphosphate to bind to the enzyme and to base pair with the template strand is not sufficient to inhibit exonuclease. It is required that the triphosphate establish and maintain an effective polymerization complex with the template and primer terminus, even if the completion of the phosphodiester bond is exceedingly slow.

Comparison of Specificity of E. coli and T4 DNA Polymerases in 3' → 5' Hydrolysis of Various Mismatched Primer Terminals—
The T4 polymerase showed the same capacity as the E. coli enzyme to preserve the terminal dTMP residue of d(T)$_{260}$-[H]d(C)$_{1}$; d(A)$_{1000}$ during polymerization and to remove the terminal dCMP residue of d(T)$_{260}$-[H]d(C)$_{1}$; d(A)$_{1000}$ (Table IV).

These enzymes also effectively removed a purine nucleoside from a purine-purine mismatch (d(T)$_{260}$-[H]d(A)$_{1}$; d(A)$_{1000}$) and a pyrimidine nucleoside from a pyrimidine-pyrimidine mismatch (d(T)$_{260}$-[H]d(T)$_{1}$; d(C)$_{1000}$). With the latter polymer incubated in the presence of dGTP, there was efficient removal of the terminal dTMP residue (Table IV). This primer then supported incorporation of dGTP. The polymer (d(T)$_{260}$-[H]d(U)$_{1}$; d(A)$_{1000}$, in which the terminal dUMP residue base pairs with the template, was also tested with the E. coli large fragment. The result, as expected, was that more than 96% of the dUMP residue was retained in the presence of either dUTP or dGTP.

It is of interest to compare the base pairing specificity of the 3' → 5' exonuclease activity of the mutant T4-induced DNA polymerase (L56) with that of the wild type enzyme. A mutator role has been proposed for the enzyme in T4 L56, a temperature-sensitive mutant in gene 43, the gene which codes for the DNA polymerase (23, 24). The exonucleases of both T4 DNA polymerases had approximately the same rate at 30° (Table I) and both showed base pairing specificity identical with the E. coli large fragment (Table IV). Terminal base-paired residues were retained during polymerization whereas mispaired residues were removed. The base pairing specificity of the mutator DNA polymerase appears at this level of analysis not to be markedly different from the wild type enzyme. However, a 1% failure by the mutant polymerase to remove a mispaired primer terminus would not have been detected above the background in our assay.

Pyrophosphorylation Is Reversal of Polymerization and Is Distinct from 3' → 5' Exonuclease—Suggestions that the 3' → 5' exonuclease activity of the E. coli DNA polymerase is analogous to pyrophosphorylase (28, 26) appeared unlikely on the basis of later evidence (27). The requirement for all the components of the polymerization reaction argued that pyrophosphorylase is the reversal of polymerization; the ability of the 3' → 5' exonuclease activity to degrade single-stranded DNA was taken as
evidence that this hydrolytic reaction was distinct from pyrophosphorolysis which requires a double-stranded DNA. The availability of primer templates in which only the terminal nucleotide of the primer is mispaired provides an even more exacting test of the requirement of pyrophosphorolysis for a base-paired 3' terminus.

When 1 mM pyrophosphate was present, the sole radioactive products of degradation of single-stranded d(T)$_{260}$-[H]d(T)$_1$, and d(T)$_{260}$[H]d(C)$_1$ by the large fragment were still monophosphates: [H]dTMP and [H]dCMP, respectively (Table V). This result verifies that 3'-terminal nucleotides of single-stranded and d(T)$_{260}$-[H]d(C)$_1$ by the large fragment were still mono- double-stranded polynucleotides d(T)$_{260}$-[H]d(T)$_1$ chains are not removed by pyrophosphorolysis. With the detectable pyrophosphorolysis of the non-base-paired [3H]dC residues were released by pyrophosphorolysis but there was no d(T)$_{260}$-[H]d(C)$_1$:d(A)$_4000$ residues (Table V). These results establish that pyrophosphorolysis of this enzyme was further analyzed with terminally labeled polynucleotides. With enzyme present in excess over those of [aH]dT and Exonuclease I degrades double-stranded DNA at a 40,000-fold slower rate than denatured DNA (13). The specificity of this enzyme was further analyzed with terminally labeled polynucleotides. With enzyme present in excess over the available 3' termini, the terminal nucleotide was removed rapidly from d(T)$_{260}$-[H]d(T)$_1$ but only slowly (if at all) from the same polymer annealed to d(A)$_{4000}$ (Fig. 3a). Attack on the mispaired terminal nucleotide of d(T)$_{260}$-[H]d(C)$_1$:d(A)$_{4000}$ was similar (Fig. 3b). Whereas, removal of the dCMP residues from the single-strand was rapid, removal of d(T)$_{260}$-[H]d(C)$_1$:d(A)$_{4000}$ was very slow. When the number of terminal dCMP residues was increased to 16, exonuclease I removed up to 60% of the dC residues of d(T)$_{260}$-[H]d(C)$_1$:d(A)$_{4000}$ rapidly, but the remaining nucleotides were removed very slowly. These data indicate that exonuclease I removes unpaired nucleotides that are within 6 to 8 residues of a base-paired region very slowly compared with nucleotides in a single-stranded chain. Thus exonuclease I shows a general specificity for extended single-stranded structures.

Exonuclease III (a 3' → 5' exonuclease which attacks native DNA preferentially (28)) shows a specificity for double-stranded structure. The terminal residues of d(T)$_{260}$-[H]d(T)$_1$ were hydrolyzed very rapidly by exonuclease III only when the polymer was annealed to d(A)$_{4000}$ (Fig. 4a). Likewise, most of the residues of d(T)$_{260}$-[H]d(C)$_1$ were sensitive to exonuclease III only when the polymer was in the double-stranded conformation (Fig. 4b). In experiments with polymers that contained blocks of dCMP residues, resistance to hydrolysis increased in proportion to the length of the sequence that did not anneal to the d(A)$_{4000}$. From these results it appeared that exonuclease III hydrolyzes double-stranded polymers which contain one, two, or even three mispaired terminal nucleotides at a rapid rate. About 90% of the terminal residues were removed from d(T)$_{260}$-[H]d(C)$_1$:d(A)$_{4000}$ in 4 min (Fig. 4b). But polymers with 5 or more residues were attacked very slowly, if at all, as 10% of the terminal residues of d(T)$_{260}$-[H]d(C)$_1$:d(A)$_{4000}$ remained and a Poisson distribution predicts that 10% of the total label resides in chains with 5 or more labeled residues.

DISCUSSION

Specificity of 3' → 5' Exonuclease Associated with DNA Polymersases—The 3' → 5' exonuclease activity of the E. coli and the T4 DNA polymerases degrades both single- and double-stranded DNA. Temperature had no more than the usual influence on degradation of single strands. However, with double-stranded DNA, the influence of temperature was profound. An increase of only 7° (from 30° to 37°) enhances the rate more than 10-fold. This behavior suggests that it is the frayed end of a helix which is required for action of the nuclease, as does an alkaline pH optimum (1), at which melting of the helix is favored. These suggestions, indicating a requirement of the nuclease for an unpaired 3'-hydroxy terminus, have been confirmed by our studies with synthetic DNA polymers.

In the studies reported here, homopolymer pairs were used as nuclease substrates in which the 3'-hydroxy end of the primer was either a proper base pair with the template or any of the several kinds of possible mispairings. When the triphosphates appropriate for polymerization were present, then two things were found. A properly base-paired terminus was extended without measurable nuclease action, whereas a mispaired terminus was quantitatively removed before polymerization was initiated. Mispaired termini included purine-purine and pyrimidine-pyrimidine pairs as well as purine-pyrimidine mismatches.

Suppression of 3' → 5' Exonuclease by Polymerization—What components of the polymerization reaction are required to suppress the 3' → 5' exonuclease? We have found that only deoxyribonucleoside triphosphates which match the template and can be incorporated into polymers suppress nuclease action. Phosphonate analogues of dTTP which bind to the triphosphate site and are suitable for base pairing with a poly(dA) template do not serve as substrates and are correspondingly ineffective in suppressing nuclease. Of special interest is the dioxyribonucleotide analogue (ddTTP) which is incorporated at a rate 1000-fold slower than that of dTTP and to the extent of only 1 nucleotide per chain. Nevertheless ddTTP inhibits exonuclease activity as completely as does dTTP. These results indicate that the primer terminus in its complex with template and triphosphate is invariable to nuclease even though the rate-limiting step in polymerization is exceedingly slow.
Pyrophosphorolysis as Reversal of Polymerization—Template-directed chain extension as catalyzed by DNA polymerase results in a properly base-paired 3' terminus which is utilized for subsequent polymerization. Pyrophosphorolysis, as the reversal of polymerization, would be expected to show specificity for the product of polymerization, i.e. a base-paired 3' terminus. The experiments described show that only such a terminus is removed by pyrophosphorolysis. A mispaired primer terminus was not attacked by pyrophosphatase. If such a terminus can be removed by pyrophosphorolysis, then it must be at a rate very much slower than that of hydrolysis. This specificity clearly distinguishes pyrophosphorolysis which attacks only base-paired termini from hydrolysis in which attack is only on unpaired termini. Thus there may be two forms of an enzyme-DNA complex. One form, with the primer terminus base-paired, would allow incorporation of a triphosphate complex. One form, with the primer terminus base-paired, would allow incorporation of a triphosphate or attack by pyrophosphatase, and the other form, in which the 3' terminus is frayed would be available only for hydrolysis.

Proofreading Role for 3' → 5' Exonuclease—The observed specificity of the 3' → 5' exonuclease during polymerization suggests that this nuclease may act as a proofreading mechanism to remove a mismatched nucleotide. Inasmuch as the polymerase cannot extend a mispaired terminus, its exonuclease would remove a mismatched nucleotide generated by itself as well as those in the synthetic polymers studied here. The fidelity of template copying would be far greater as the result of having two base pairing selection steps: first the selection of a nucleotide for polymerization, and second, a determination that the primer terminus is properly paired before adding the next nucleotide. The presence of the same base pairing specificity in the exonuclease activity of two different DNA polymerases (T4 and E. coli) points to the fundamental importance of the exonuclease for accuracy in a template-directed polymerization process. England (8) has also suggested a role for the exonuclease of the T4 DNA polymerase in fidelity of template copying based on removal of unpaired nucleotides by this enzyme prior to polymerization. We have recently investigated yet a third DNA polymerase, DNA polymerase II isolated from E. coli by Kornberg and Gefter (29), and have shown that this enzyme may also contain a 3' exonuclease activity with the same specificity as greater than 80% of a base-paired terminus was retained during polymerization, while a mismatched terminus was quantitatively removed.

Defect in 3' → 5' Exonuclease May Be Mutagenic—Gene 43 of T4 codes for the DNA polymerase and certain lesions in the gene are known to have a profound effect on the spontaneous mutation rate (24, 30, 31). Allen et al. (32) have suggested on genetic grounds that the exonuclease activity of the T4 polymerase is involved in the fidelity of replication. If the proofreading exonuclease function of the polymerase is important for replication accuracy, then an alteration in exonuclease might cause mutagenic effects. A known mutagenic polymerase has been purified, and Hall and Lehman (11) have demonstrated in an in vitro experiment with the polymerase induced by T4 L56, a 4-fold increase in misincorporation of dTTP with a poly(dC) template as compared with the wild type T4 polymerase. We tested the L56 polymerase to determine whether it is defective in its ability to remove a mispaired nucleotide. In none of the cases tested was the mutator polymerase significantly different from the wild type polymerase in its exonuclease specificity (Table IV). In an experiment with polyinosinic acid, terminally labeled with dTMP residues and annealed to d(C)100 (the case most closely resembling the conditions of Hall and Lehman), there was less removal of the mismatched primer dT terminus by the mutator polymerase. However, more rigorous and sensitive assays, such as measuring direct transfer of dGTP to the terminus, will have to be performed to establish differences. Studies with other mutator DNA polymerases, as well as more extensive evaluation of the base pairing specificity, including all 12 possible nucleotide mismatches, will help in providing insights into the function of this nuclease.

Coordinated Roles of Exonuclease I and Exonuclease III—The specificities of the DNA polymerase and the other exonucleases of E. coli demonstrate that they are capable of coordinated hydrolytic action on a variety of structures at the 3' terminus (Table VI). Displaced single-stranded 3' termini which might result from various processes in repair, recombination, and replication of DNA would be rapidly degraded by either exonuclease I or the 3' → 5' exonuclease of DNA polymerase. Once the length of the displaced strand is reduced to 5 or 6 nucleotides, exonuclease I action becomes very slow, but the specificity of exonuclease III (or again, the DNA polymerase) would allow degradation into the base-paired region (Table VI). The nuclease activity of the DNA polymerase on displaced single strands is similar to the combined action of exonuclease I and exonuclease III. In a double-stranded region of DNA, the DNA polymerase would begin chain extension, whereas exonuclease III would continue hydrolysis leaving a single-stranded region or a gap. Gaps might also be produced by DNA polymerases if the amount of DNA precursors was severely limited. Such gaps might be important in the metabolism of DNA. The redundant action of these enzymes, and also their multifunctional nature indicates that each may be involved in more than one physiological process. Their redundant nature would ensure the integrity of the metabolism of DNA and their multifunctional

### Table VI

<table>
<thead>
<tr>
<th>Structure</th>
<th>Action on 3' primer terminus</th>
<th>Chain extension by DNA polymerase</th>
<th>3' → 5' Hydrolysis by Exonuclease I</th>
<th>3' → 5' Hydrolysis by Exonuclease III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Base-paired terminus</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Mismatched terminus</td>
<td>No</td>
<td>Yes</td>
<td>No‡</td>
<td>Yes</td>
</tr>
<tr>
<td>Displaced strand</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No‡</td>
</tr>
</tbody>
</table>

*In the presence of triphosphates.

†Hydrolsis is very slow.
character would insure proper coordination of these vital molecular events.

REFERENCES