Studies on the Mechanism of Escherichia coli DNA Polymerase I Large Fragment

EFFECT OF TEMPLATE SEQUENCE AND SUBSTRATE VARIATION ON TERMINATION OF SYNTHESIS*

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Termination of Escherichia coli DNA polymerase I large fragment after processive synthesis on natural and other well-defined template-primer systems has been examined. We found that after any given deoxynucleoside monophosphate incorporation termination occurs in a nonrandom manner with φX174 DNA as template. Termination is much more likely at some nucleotide residues along the template than at others. Analysis of these stronger termination sites indicates that the template base:incoming nucleotide combination influences termination. Introduction of a double-stranded region along the φX174 template induces termination, and reducing dNTP concentrations or substituting 2'-deoxynucleoside 5'-O-(1-thio)triphosphate substrates also increases termination.

Observations with the φX174 DNA template system were extended with a defined template containing 1 inosine residue in an otherwise d(T), homopolymer. Termination at the I residue is modulated by dCTP and decreases as dCTP concentration increases. A similar relationship is seen with the dCTP (1-thio) derivative, but termination is higher at given concentrations of this derivative than with dCTP. Pyrophosphate decreases general processivity in this system, but does not counteract the effect of increasing dCTP. Hill plot analysis of the dCTP effect in the inosine-containing template system gave a linear plot with Hill coefficient of 0.34, suggesting that dCTP influences termination at several steps in the polymerase reaction scheme. Substituting a methylated template base for I also increased termination, producing very strong blocks to processive synthesis. The results are consistent with a model in which termination occurs with several enzyme forms that are in equilibrium in an ordered catalytic mechanism.

The model DNA polymerase, Escherichia coli DNA polymerase I large fragment (Pol II), normally binds to a template primer and incorporates many dNMP residues before dissociating from the template-nascent chain complex. The mechanism of the dissociation event (i.e., chain termination) after this so-called "processive" DNA synthesis is the subject of current investigation. Analysis of the mechanism of product release and chain termination after processive synthesis has lagged behind analysis of the initiation phase of synthesis. For example, insight into whether termination is ordered versus random or to the number of enzyme forms capable of terminating is minimal. This stems both from lack of appropriate assays for termination and lack of a theoretical framework within which to study termination. In the present study, we address both of these problems in that several ways of experimentally modulating chain termination are described, and the results are discussed in the context of a hypothetical scheme involving an ordered mechanism with four enzyme forms capable of termination.

Earlier reports on DNA polymerase termination involved homopolymer replication systems and incubation conditions where each product molecule represented only one chain termination event (1, 2). Measurement of the number of product molecules, and hence chain termination events, corresponding to each dNMP addition along a template revealed that termination is influenced by a complicated array of variables. It was possible, however, to identify general features about Pol I if termination. First, there is a finite chance for termination after any given dNMP addition (1, 2). Second, the chance for termination increases with higher concentrations of template-primer (2) or lower concentrations of dNTP (1, 2). Third, the chance for termination is higher after addition of the first several dNTP residues to a primer than after addition of more than 7 residues, suggesting some change in the enzymatic mechanism as synthesis proceeds from initiation to processive elongation (2).

In the homopolymer replication systems tested earlier, it was unclear whether differences in base composition of the template changed termination after processive synthesis. In the present study, we use a natural DNA template-primer system to examine the effect of template base composition. The results are in accord with the points noted above from earlier work and information is extended by the demonstration that template base composition influences termination probability. We also find that lowering levels of dNTPs, substituting dNTPs substrates, or interfering with normal hydrogen bonding increases the chance for termination. This information with a natural DNA template is applied for detailed study of the effects of substrate concentration and other parameters on termination at one specific site in a defined synthetic template-primer system. The results are discussed in the context of kinetic models for the enzyme.
EXPERIMENTAL PROCEDURES

Materials

Homopolymous Pol I If was a generous gift from Dr. W. E. Brown (Carnegie-Mellon University). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis and Coomasie Blue staining revealed that the only visible protein in the preparation was the Mr = 68,200 enzyme polyepptide. The enzyme was stored at −20 °C in 100 mM potassium phosphate buffer, pH 7.0, 1 mM 2-mercaptoethanol, and 50% glycerol. Pol I If used in sequencing reactions was from New England Biolabs. T4 polynucleotide kinase and dNTPs were obtained from Pharmacia LKB Biotechnology Inc. (γ-32P)ATP was obtained from Du Pont-New England Nuclear or ICN Radiochemicals. dATPaS and dCTPaS were generously donated from Dr. Fritz Eckstein (Max Planck Institut, Gottingen). dCTPaS and dGTPaS were obtained from Pharmacia. dATPaS was the A isomer or Sg configuration (3). The other thiol derivatives were mixtures of the A and B isomers. For experiments with dNTPaS substrates, concentrations are expressed as the amount of A isomer present.

DNA plus strand DNA, formamide, and urea were obtained from Bio-Rad.

Methods

Preparation of Labeled DNA Primers—Synthetic DNA primers were 5'-end-labeled with 32P in accordance with the procedure of Maxam and Gilbert (4). DNA was extracted with phenol, residual phenol was removed with ether, and the DNA was precipitated with ethanol.

In Vivo DNA Synthesis—Primers were hybridized to 32P DNA at a 5:1 molar ratio of primer/template by heating to 100 °C and slowly cooling to room temperature. DNA synthesis reactions (20 μl) contained 20 mM Tris-HCl, pH 7.5; 10 mM NaCl; 5 mM MgCl2; 1 mM dithiothreitol; 400 μg/ml bovine serum albumin; 20% glycerol; 15 μM each dATP, dCTP, dGTP, and dTTP; 5 fmol of Pol I If; and 200 fmol of DNA plus strand primer-template. Reaction mixtures were incubated at 35 °C for 200 s, and reactions were stopped by adding EDTA to a final concentration of 18 mM. A dye mixture in deionized formamide was then added to a total volume of 45 μl. 5- to 15-μl portions were loaded for gel electrophoresis.

In Vitro DNA Synthesis—DNA synthesis reactions were also carried out with the synthetic template-primers described in Figs. 4 and 6. These templates consisted of 16 nucleotides complementary to Primer-A, followed by 13 dT residues, a residue with an altered base, and 10 more dT residues. The altered base at position 30 was either inosine, w-Me-dT, or w-Me-dA. Primers were hybridized to template as described above at a primer/template molar ratio of 2.5:1. DNA synthesis reactions were described above but with 15 μM dATP and additional dNTPs as described in individual experiments.

Analysis of Products of DNA Synthesis—Products were analyzed by gel electrophoresis as previously described (2). Electrophoresis was conducted on a 12% polyacrylamide, 7 M urea gel (40 × 30 cm × 0.8 mm). The gel was prerun for 1 h at 40–45 watts without cooling and electrophoresis was performed at 40–45 watts. After electrophoresis, the gel was fixed in acetic acid/methanol, transferred to Whatman 3MM paper, and covered with Saran Wrap. Products were visualized by autoradiography with Kodak XAR-5 film at room temperature. Some exposed films were evaluated for band intensity with a Zeineh soft laser scanning densitometer (Biomed Instruments). Areas under peaks were determined by cutting and weighing. Termination probability at a specific site is defined as the ratio: number of product molecules at a given site divided by this same number plus the number of all longer product molecules. Termination probability will thus vary between 0 for no termination and 1 for termination of all product molecules. DNA sequencing reactions, to verify the positions of identified termination sites, were carried out by the dideoxynucleotide method using 32P-labeled DNA as template, and either synthetic oligomer as 3′-labeled primer.

Hill Plot Analysis—Hill (6) analyzed the experimental data for the binding of oxygen to hemoglobin and found that the data fit the equation log w = n log x + C, where w is the percentage saturation of hemoglobin with oxygen, x the partial pressure of oxygen, and the slope n (experimentally determined) is a combination of the number of reactive sites and the extent of interaction between them.

This analysis can be extended to the general situation where a ligand S can bind to an enzyme E at any of n binding sites on the enzyme:

\[ E + nS \rightleftharpoons ES_n \]

If one defines \( \bar{x} \) as the average number of molecules of S bound per molecule of E, then

\[ \bar{x} = \frac{[S]}{[E] + [S]} \]

where \([S] \) represents bound substrate, and \([E] \) represents the concentration of all forms of the enzyme. If one now defines \( \gamma = \bar{x}/(1 - \bar{x}) \), then it can be derived that log \( \gamma = n \log [S] + \log K \), where K is the equilibrium constant for Reaction 1. This is the general expression for the Hill equation. Plotting the log of \( \gamma \) versus log of substrate concentration will allow the determination of \( n \) as slope of the line. If \( n \) has a value of 1, binding is noncooperative and binding sites are identical. If \( n = 1 \), binding is negatively cooperative, or the enzyme has more than one class of sites (7).

Hill plot analysis can be extended to other conditions of enzyme activity and can be used to determine the molecular order of participation of activators or inhibitors in a variety of biological systems (8, 9). It was also possible to extend this analysis to the experimental system depicted in Fig. 4, where termination of synthesis on the insosine-containing template was dependent on concentration of dCTP; the incoming substrate at template position 30. Any polymerase molecule which has extended the primer to position 29 has two alternative modes of action: It can terminate synthesis, or it can extend the primer by incorporating dCTP. The probability of termination, \( P_o \), at position 30 will be the ratio of (DNA product molecules 29 nucleotides long) to (DNA product molecules 29 nucleotides or longer). The probability, \( P_o \), at position 30, will be 1 – \( P_t \). Thus, the total enzyme population, \([E]\), which reaches position 29, will consist of enzymes which terminate at position 30, or enzymes which extend synthesis by incorporating dCTP at position 30.

One can thus plot the data from this system in a manner analogous to the Hill plot, by defining \( x = P_t/(1 - P_t) = P_t/P_o \). Plotting log \( x \) versus log[dCTP] generated the curves shown in Fig. 6, and the resultant equation is then

\[ \log x = \log[dCTP] + k \]

At the dCTP concentration where the termination probability is 50%, log \( x \) vanishes and at that point \( x = -\log[dCTP] \).

For Pol I If, which is a single subunit enzyme with one dNTP binding site (28), Hill plot analysis can provide information on the cooperativity of the catalytic reaction, rather than for ligand binding (9). Such analysis of termination probability is capable of revealing noncooperativity, positive cooperativity, or negative cooperativity, depending on the amount of change in log \( x \) as a function of log[dCTP] in Equation 2.

RESULTS

In the first part of the description to follow, termination in a natural heteropolymer template system is surveyed, and several techniques for modulating termination are identified. These descriptive studies then are followed in the second portion with experiments to evaluate details of termination at a precise locus after processive synthesis.

Survey of Termination with a Natural DNA Template

Preferential Termination Sites in a Heteropolymer—It is well established that Pol I If has difficulty copying a template region involved in double-stranded structure, such as a hairpin stem or long-range interaction between strands. When the enzyme encounters such a structure it fails to reinitiate, leading to accumulation of product molecules corresponding in chain length to the precise location of the double-stranded structure. In order to examine the termination event by Pol I
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If as a function of template nucleotide sequence, instead of secondary structure, we conducted experiments where the template was a region of φX174 DNA, residue 652 to residue 550 of the plus strand, that is secondary structure-free on the basis of computer-derived predictions and previous enzymatic studies (10). DNA synthesis was initiated at residue 652 or residue 617 on a oligonucleotide primer 32P-labeled at the 5' end. This primer was annealed to the template prior to the start of the polymerase reaction, and reaction conditions, such as enzyme and substrate levels, ratio of enzyme to template-primer, and incubation time were adjusted so that each product molecule was formed by processive synthesis and represented only one chain termination event (1, 2). The relative number of product molecules at each chain length along the template was determined by high resolution polyacrylamide gel electrophoresis.

Typical results of this analysis with φX174 plus strand as template are shown in Fig. 1. The turnover number of the enzyme in these experiments was 2 nucleotides/s, and in the 200-s incubation, the average enzyme molecule could have formed 8 product molecules 50 residues long. As seen in the autoradiography lanes in Fig. 1b, product molecules representing additions of 70 dNMP or more to the primer formed a step-ladder pattern in the gel. This indicated that a certain amount of termination occurred after incorporation of each residue along the template, and this was consistent with earlier work with homopolymer templates (1, 2). However, in contrast to the homopolymer systems, the number of product molecules at some positions along the template was much higher than at other positions, indicating that the chance for termination was not identical after each dNMP addition to the nascent DNA chain.

Examination of the products of DNA synthesis shows a generalized termination for about the first 8 to 10 nucleotides added to either primer. This is consistent with earlier observations with Pol I if and homopolymer templates, where the first few nucleotides showed a higher termination probability than later positions (2). After these regions of generalized termination, however, one observes visually and with densitometry tracing that several positions stand out in comparison with neighboring sequence positions as strong termination sites. These sites, in addition, do not appear to be blocked for reinitiation: The strong bands disappear with longer incubation times or when enzyme concentration is increased 10-fold after a short incubation (data not shown).

Positions were identified as strong termination sites by comparing their densitometry traces with those of neighboring sequence positions. Since the proportion of product molecules decreases generally with larger product size, this comparative analysis, rather than an identification of absolute band intensity, was necessary to designate strong termination sites. Fig. 1a indicates 18 positions which are strong termination sites for DNA synthesis from either Primer-A or Primer-B. Most of the strong termination sites identified for Primer-B are also strong sites for Primer-A, although there are a few exceptions: Termination site 9 is relatively stronger with extension of Primer-A, and sites 11 and 14 are relatively stronger with Primer-B. In numerous experiments, we observed the patterns in Fig. 1b reproducibly.

Sequence Analysis of Strong Termination Sites—The irregular spacing of strong termination sites off either primer and the fact that many sites are common to both primers suggests that position effects (such as turns in the DNA helix) are less important than sequence for causing termination. Table I analyzes all 18 strong sites for correlation with the incoming nucleotide (which is not inserted at a termination site because the polymerase leaves the template before incorporation) and with the 3'-nucleotide (which is newly incorporated just before termination). The table compares the frequency of strong termination sites with the occurrence of sequence combinations from φX position 644 (discounting the region of generalized termination off Primer-A) to position 556 (the incoming nucleotide after the last clearly distinguishable termination site). The 18 sites over these 88 nucleotides represent a strong site frequency of 20%. In Table I, the highest correlation with termination occurs when dTTP is the incoming dNTP; and all three cases where dGMP is the 3'-nucleotide and dTTTP is incoming substrate are strong termination sites. The lowest correlations occur when dATP is the incoming substrate, and when dTMP is the newly inserted base. One also sees a moderately higher strong termination frequency when dGMP is the 3' base, but most of these termination sites occur when the incoming substrate is a pyrimidine. Compressing the data to purine/pyrimidine combinations, Table I shows that the highest frequency of strong termination sites occurs with a pyrimidine as newly inserted nucleotide and a pyrimidine as the incoming substrate. We find that there is no significant correlation of strong termination site frequency with the penultimate nucleotide (inserted just prior to the 3'-nucleotide) (data not shown).

When three-nucleotide sequences are analyzed, one finds that strong termination sites appear no more than once for most combinations. Two observations appear noteworthy, however. The combination -A-G-dCTP, where A and G are newly incorporated bases and dCTP is the incoming substrate, is a strong termination site 6 of the 6 times that it occurs on the φX region analyzed. In addition, the combination -A-Pur-X, where Pur is a pyrimidine and X is an incoming substrate other than dATP, is a strong termination site 9 of the 20 times it occurs (tabulation not shown).

These correlations and the analysis of Table I suggest that sequence is important for termination, but cannot solely explain this phenomenon, since termination sites appear several times for each incoming dNTP and for three of the possible 3'-nucleotides. This implies that either longer range interactions between enzyme and template are important for termination, or that a more complex relationship between sequence and termination is involved.

Secondary Structure Adds Termination Sites—We found that new strong termination sites could be created when Pol I if was forced to carry out synthesis through a region of double-stranded DNA. Fig. 2 represents an experiment where both Primer-A and Primer-B were annealed to the φX template and the elongation products of Primer-A were displayed. Under these conditions, the polymerase carried out strand displacement synthesis into the double-stranded region, but three new strong termination sites were observed at positions 601–629, where the polymerase must disrupt a run of four G-C base pairs to extend the primer. DNA synthesis becomes moderately higher strong termination frequency when dGMP is the 3' base, but most of these termination sites occur when the incoming substrate is a pyrimidine. Compressing the data to purine/pyrimidine combinations, Table I shows that the highest frequency of strong termination sites occurs with a pyrimidine as newly inserted nucleotide and a pyrimidine as the incoming substrate. We find that there is no significant correlation of strong termination site frequency with the penultimate nucleotide (inserted just prior to the 3'-nucleotide) (data not shown).

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Parameters That Alter the Termination Pattern—We found that the termination pattern shown in Fig. 1 was not significantly changed despite alterations of several different parameters: Increasing salt (Tris or NaCl) will decrease the average product length, as will pH changes outside an optimal range of 7.0–8.0, but the basic pattern of strong sites does not change with salt concentration, pH from 4.8 to 10.0, change of metal
from Mg$^{2+}$ to Mn$^{2+}$, the presence of 10 mM dNMPs (which inhibit Pol I 3'→5' exonuclease activity) (11), or preincubation of enzyme with template-primer (data not shown).

We did find that changes in the dNTP substrate could alter the termination pattern. Specifically, as shown in Fig. 3, reducing concentrations of individual dNTPs to 1 μM or replacing individual substrates with the corresponding α-thiol-substituted dNTP changed the termination pattern. When these altered patterns are analyzed in Table II, one observes a high frequency of strong termination sites at positions where the altered substrate was the incoming nucleotide. In several cases, the effect of the altered substrate is so great that bands which are strong termination sites under the baseline conditions (15 μM normal dNTPs) become relatively less in intensity and are no longer scored as strong sites.

Nonetheless, not all strong sites observed could be explained by the altered reaction conditions: Some sites still were observed that are independent of the incoming altered substrate. The most complicated pattern is seen when dTTP is changed: At 1 μM dTTP, 4 of 6 positions where dTTP is the incoming substrate are strong termination sites, but 4 more strong sites occur with other incoming substrates. Similarly, with dTTPαS, 5 of 6 positions for this incoming substrate are strong termination sites, but 8 other strong sites are also seen. Thus, it appears that factors in addition to substrate level and α-phosphate reactivity may also be important in influencing termination of DNA synthesis. We also
**TABLE I**

**Analysis of termination site sequence dependence**

The strong termination sites indicated in Fig. 1 were analyzed for frequency of occurrence at combinations of incoming dNTP substrate and 3' base (newly inserted nucleotide). For each combination, the number of occurrences in the φX DNA sequence from position 644 to 556 is displayed in the denominator, and number of occurrences at strong termination sites is displayed in the numerator. The data in the table are summarized for purine/pyrimidine combinations. For each combination, the symbol to the left of the arrow represents the 3' nucleotide, and the symbol to the right represents the incoming substrate. Pur, purine; Pyr, pyrimidine.

| 3'-Nucleotide | Incoming substrate | dATP | dCTP | dGTP | dTTP | Totals |
|---------------|--------------------|------|------|------|------|--------|
| dA            | 0/14               | 1/6  | 3/10 | 2/6  | 6/36 | 16     |
| dC            | 1/10               | 1/4  | 0/4  | 2/3  | 4/21 | 19     |
| dG            | 1/4                | 3/8  | 1/5  | 3/3  | 8/20 | 40     |
| dT            | 0/7                | 0/3  | 0/1  | 0    | 0/11 | 0      |
| Totals        | 2/35               | 5/21 | 4/20 | 7/12 | 18/88| 20     |

Summary

| Summary       | lPur  | lPur | lPyr  | lPyr | lPur  | lPyr | lPyr  |
|---------------|-------|------|-------|------|-------|------|-------|
| Pur → Pur     | 0/33  | 15%  |
| Pur → Pyr     | 9/23  | 39%  |
| Pyr → Pur     | 1/22  | 5%   |
| Pyr → Pyr     | 3/10  | 30%  |

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**FIG. 2.** Termination induction by a region of double-stranded DNA. Reaction conditions and product analysis were as described in Fig. 1, with only Primer-A labeled, and these additional conditions: lane 1, Primer-A hybridized, 3-min incubation; lane 2, Primer-A hybridized, 10-min incubation; lane 3, both primers hybridized, 3-min incubation; lane 4, both primers hybridized, 10-min incubation. The markers correspond to positions on the φX174 DNA sequence shown in Fig. 1. Position 634 is one nucleotide before the 5' terminus of Primer-B.

 cannot rule out the possibility that altering one dNTP might have indirect effects on termination probability when another dNTP is the incoming substrate.

**Study of Termination Mechanism Using a Defined Template-Primer**

Termination at Multiple Steps of the DNA Synthesis Reaction—With the observation that dNTP concentration and thiol-substituted substrates can alter the Pol I If termination pattern on the φX template, we decided to study termination at a single site on a synthetic template-primer by manipulating substrate level and other parameters. Scheme 1 is a hypothetical kinetic model showing several steps in the DNA synthesis pathway at which a DNA polymerase could terminate synthesis. From the scheme, termination could occur following incorporation but before release of pyrophosphate (Step 1), after pyrophosphate release and before or after translocation to the next 3'-OH group (Step 2), after binding dNTP but before base pairing (Step 3), or polymerase may dissociate from the template during the base-pairing step but before incorporation is completed (Step 4). The experiments to be described below are consistent with and support this model. We will show that termination of DNA synthesis is reduced by increasing dNTP concentration and this is not counteracted by pyrophosphate; that termination is increased with a dNTPαS substrate, which undergoes slower nucleophilic attack on the α-phosphate atom; and that interfering with base pairing can increase termination.

**Modulation of Termination by Concentration of dCTP and dCTPαS—**The defined template-primer used to examine ter-
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Analysis of changed termination site patterns

The strong termination sites indicated in Fig. 3 were analyzed for correlation with incoming dNTP; this is compared to the frequency of occurrence of all incoming substrates in the φX DNA sequence over each region examined.

| Substrate variation | Tabulation to sequence position | Incoming substrate, occurrence over sequence region analyzed | Incoming substrate at strong termination sites with substrate variation |
|---------------------|--------------------------------|------------------------------------------------------------|---------------------------------------------------------------------|
|                     |                                | dATP        | dCTP        | dGTP        | dTTP        | dATP        | dCTP        | dGTP        | dTTP        |
| 1 μM dATP           | 615                            | 18          | 7           | 8           | 6           | 6 (0)       | 2 (3)       | 1 (1)       | 1 (2)       |
| dATP*S              | 606                            | 22          | 9           | 11          | 6           | 10 (1)      | 2 (3)       | 1 (1)       | 1 (2)       |
| 1 μM dCTP           | 618                            | 17          | 6           | 8           | 5           | 0 (0)       | 3 (3)       | 1 (1)       | 0 (1)       |
| dCTP*S              | 618                            | 17          | 6           | 8           | 5           | 0 (0)       | 4 (3)       | 1 (1)       | 1 (1)       |
| 1 μM dGTP           | 626                            | 12          | 4           | 8           | 4           | 0 (0)       | 1 (1)       | 5 (1)       | 0 (1)       |
| dGTP*S              | 626                            | 12          | 4           | 8           | 4           | 0 (0)       | 0 (1)       | 4 (1)       | 1 (1)       |
| 1 μM dTTP           | 617                            | 17          | 6           | 8           | 6           | 0 (0)       | 2 (3)       | 2 (1)       | 4 (2)       |
| dTTP*S              | 617                            | 17          | 6           | 8           | 6           | 3 (0)       | 3 (2)       | 2 (1)       | 5 (2)       |

* Sequences were tabulated in the region from φX position 652 to the last strong termination stop that could be clearly identified, which varied for each lane in Fig. 3.

** These four columns represent the occurrence of all incoming substrates over the φX DNA region analyzed for a particular substrate variation.

 Termination at a single site is shown at the top of Fig. 4. The template is a synthetic 40-mer, with 16 nucleotides complementary to Primer-A, and the template region consisting of 23 dT residues and 1 inosine at position 30. It was thus possible to study termination at position 30 by modulating the concentration of dCTP as the complementary substrate. A typical experiment varying dCTP concentration is shown in Fig. 4. Termination at position 30 would result in a product 29 nucleotides in length, while incorporation at position 30 would lead to longer products. One can see by inspection that increasing dCTP concentration decreases termination: As substrate concentration is raised, the number of product molecules at position 29 decreases relative to longer products. One also finds that, as pyrophosphate concentration increases, the distribution of synthesized molecules shifts toward smaller products (data not shown).

** Additional conditions were as follows: lane 1, no dCTP present; lane 2, 0.1 μM dCTP; lane 3, 1 μM dCTP; lane 4, 30 μM dCTP.

The template-primer system is depicted at the top of the figure, with an inosine residue at position 30. Reaction conditions and product analysis were as described under "Experimental Procedures." The products of synthesis are displayed for position 29 and above, with markers indicated for positions 29 and 40. Additional conditions were as follows: lane 1, no dCTP present; lane 2, 0.1 μM dCTP; lane 3, 1 μM dCTP; lane 4, 30 μM dCTP.

** Hill Plot Analysis—The observations with the inosine-containing template were extended by examining the molecular order of reaction by data using Hill plot analysis. Fig. 6 shows analysis for an experiment with this template and either termination probability at position 30. Reaction conditions and product analysis were as described under "Experimental Procedures." The products of synthesis are displayed for position 29 and above, with markers indicated for positions 29 and 40. Additional conditions were as follows: lane 1, no dCTP present; lane 2, 0.1 μM dCTP; lane 3, 1 μM dCTP; lane 4, 30 μM dCTP.

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FIG. 5. Plot of termination probability versus dCTP concentration on a log-log scale. Reaction conditions were as described in Fig. 4, with the additional dCTP (closed circles) or dCTPaS (open circles) concentration shown. Termination probability at position 30 is plotted and is described as the ratio of product molecules at position 29, to product molecules at position 29 plus all product molecules at greater lengths. Results are also shown for 2 mM PPI added to reaction mixtures with dCTP, and 1 mM PPI added to reactions with dCTPaS.

FIG. 6. Hill plot of termination probability versus dCTP concentration. Reaction conditions were as described in Figs. 4 and 5, with dCTP (closed circles) or dCTPaS (open circles). Incorporation and termination probabilities, \( P_i \) and \( P_o \), respectively, are defined under "Experimental Procedures," as is the parameter \( z \), which equals \( P_i/P_o \). Best fit lines are shown, with the data point at 150 pM dCTP omitted.

dCTP or dCTPaS as substrate. As described under "Experimental Procedures," the data can be analyzed by the equation

\[
\log z = n \log[dCTP] + k,
\]

where \( z \) is \( P_i/P_o \) and \( n \) is the Hill coefficient derived from the slope. Both plots were linear (Fig. 6). The best fit line through the experimental data for dCTPaS gives 0.41 for \( n \) and -0.34 for \( k \). For dCTP, omitting the data point at 150 \( \mu \)M gives a best fit line with 0.34 for \( n \) and 0.24 for \( k \); if the last data point is included, the best fit gives 0.30 for \( n \) and 0.26 for \( k \) (data not shown). Thus, for both substrates the Hill coefficient is much less than 1, indicating negative cooperativity.

Two Methylated Bases Produce Nearly Absolute Blocks to Processive Synthesis—Fig. 7 shows the effects of termination when methylated bases are present in the defined template. Under low enzyme:template ratios, even after 10-min incubation time, these altered bases represent very strong blocks to elongation by Pol I If. Strong stops remain with these bases even with all dNTPs present at 250 \( \mu \)M. We have found that synthesis to the end of the template can be produced with a 10-fold higher molar ratio of enzyme:template, although one still sees a strong band at position 29 even under those conditions. These data suggest that Pol I If experiences difficulty in base pairing with the methylated derivatives, causing the polymerase to "stall" at this site, and consequently to have a greater likelihood of dissociating from template before elongation. That additional enzyme can synthesize past the methylated site suggests that the methyl base may provide a block to reinitiation that can be partially overcome with a larger number of polymerase molecules.

**DISCUSSION**

We have extended earlier work on termination of DNA synthesis on homopolymers (1, 2) by examining termination on a natural DNA template and on other defined template primers. We find that on a \( \phi X 174 \) DNA template lacking secondary structure, Pol I If terminates in a nonrandom

\footnote{J. Abbots, G. Zon, and S. H. Wilson, unpublished experiments.}
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fashion; certain sites are favored for termination (Fig. 1). Use of the natural DNA template allowed an investigation of the effects of sequence composition on termination, which could not be examined with homopolymers. We identified conditions including substrate variation that could be used to modulate termination on the φX template. With this knowledge, observations were extended by examining termination at a single site on a defined template-primer system. We note that Fairfield et al. (29) reported that T4 DNA polymerase also terminates processive synthesis in a nonrandom manner on an M13 DNA template, but these workers did not identify individual termination sites.

Scheme 1 displays an ordered reaction mechanism for DNA synthesis. In this scheme, termination of synthesis can occur at any of several steps, and the same form of DNA polymerase that binds to template can also dissociate: The enzyme at a particular reaction step need not convert to a different form in order to terminate. Our results with Pol I If are consistent with such an ordered reaction model.

Analysis of strong termination sites over the φX DNA template (Table I) indicated a preference for termination when the incoming substrate was dTTP. We also found that decreasing dNTP concentration and use of dNTPoS substrates will increase termination (Fig. 3 and Table II). In addition, the introduction of secondary structure causes termination during strand displacement synthesis (Fig. 2). On a defined synthetic template-primer system, termination probability at a site where dCTP was the incoming nucleotide showed a distinct relationship with dCTP concentration (Figs. 4 and 5). A similar relationship was seen with dCTPoS, but termination with the thiol derivative was always higher at the conditions examined (Fig. 5). Pyrophosphate also decreased processivity, but its effect appeared to be general, producing no preferential termination at a particular site (Fig. 5). Lastly, we observed that two particular methylated bases produce very strong blocks to processive DNA synthesis (Fig. 7).

Benkovic and colleagues (12) have advanced a model in which processive synthesis is treated as a simple competition between continued incorporation of nucleotides and dissociation of enzyme from the template-primer under steady-state conditions. Consistent with this straightforward model, one would expect that phenomena which impede continued nucleotide incorporation would increase the frequency of termination of DNA synthesis. We have identified several factors above which modulate termination.

By kinetic studies with DNA Pol I on a poly(dA)-oligo(dT) template-primer, Bryant et al. (12) found two rate-determining steps, one following dTTP binding and preceding the chemical bond-forming step and a second step following chemical bond formation. These workers hypothesized that the first rate-determining step was a change in the enzyme-template-dTTP complex to a form "poised" for correct base pairing and/or catalysis (13). They suggested that the second rate-determining step could be the translocation of the polymerase following phosphate bond formation in preparation for binding of the following dNTP substrate (12). The model of these workers can be related to Scheme 1 in the following manner: Step C, binding of dNTP to the enzyme-template complex is a rapid equilibrium step. The first rate-determining step is analogous to Step D, to produce a ternary complex in which base pairing occurs, or a complex prepared for base pairing. By the latter scheme, Step D could represent two separate steps producing different ternary complexes. Following formation of the poised complex, Step F represents formation of the chemical bond. Step A represents subsequent release of pyrophosphate. Step B is translocation of the polymerase to the 3'-hydroxyl of the newly incorporated nucleotide and represents the second rate-determining step. Termination of synthesis can occur at any of Steps 1 through 4 in Scheme 1.

We found that dNTP concentration can influence termination, both in the φX natural DNA template system and in the defined template-primer system. A linear relationship on a log-log plot was observed between termination probability at a specific site and the concentration of the incoming dNTP for that site (Fig. 5). When data for termination at a specific site were analyzed by Hill plot, linear relationships were found for dCTP and dCTPoS substrates (Fig. 6). The slopes of the lines, or Hill coefficients, in Fig. 6 were 0.34 for dCTP and 0.41 for dCTPoS. For negative cooperativity, a Hill coefficient of <1 is expected, as observed here. This is consistent with termination from two or more forms of the enzyme that are reversibly connected in a sequence. Such an interpretation is represented in the ordered, equilibrium model in Scheme 1, where there are multiple steps or enzyme forms, at which dNTP concentration can modulate termination, including the rate-determining steps identified by Benkovic and co-workers (12, 13). Since the enzyme forms are in equilibrium, increasing dNTP concentrations could act by "pulling" the polymerase through the translocation step or could facilitate formation of the enzyme-template-dNTP complex poised for catalysis.

The fact that termination on the φX template appears to at least partially depend on the incoming dNTP substrate may also be related to the models of Benkovic and co-workers; dNTPs may differ in their abilities to facilitate translocation or formation of the poised complex. In that regard, recent NMR studies by Ferrin and Mildvan (14) are noteworthy. These workers found a single Pol I If binding site for dATP and dTTP, in agreement with equilibrium dialysis experiments that identified a single dNTP binding site for Pol I (15). Ferrin and Mildvan (14) also found that dATP and dTTP assume unique conformations when bound by either Pol I or its large fragment. Since bound dNTPs can assume different conformations, it seems plausible that dNTPs could require different times to assume the poised complex postulated by Benkovic and colleagues, which in turn could produce a dependence on termination for incoming substrate.

The fact that the same termination sites on the φX template are seen with either of two primers suggest that position effects (turns along the DNA helix) are less important than sequence effects. The use of these two primers also reduces the possibility that some Pol I If "memory" effect at initiation is important in determining termination sites; the first three nucleotides incorporated onto Primer-A are purines, and the first three onto Primer-B are pyrimidines. The statistical samples of Tables I and II are limited to a small region of DNA, and even then the overall correlation of strong termination sites is complex: Each incoming dNTP is represented several times as strong sites, and 3 of 4 bases are represented several times as the 3'-nucleotide (Table I). In addition, while strong termination sites correlate with the incoming dNTP when substrate is reduced in concentration or replaced by its α-thiol derivative, some strong sites remain which appear independent of these changes in incoming substrate. Within the limits of sample size, we conclude that, although there is a preference for termination with dTTP incoming onto dG, there must be additional complex phenomena or action at longer range that influence termination on natural DNA templates. We note that Kuchta et al. (30) recently found that both binding and dissociation of duplex DNA oligomers from Pol I If were sequence dependent.

Introducing a double-stranded region to the φX template
induced termination (Fig. 2). This observation could have implications for the identification of strong termination sites on this template. The region examined is presumed to be lacking in secondary structure, based on the absence of sites that “arrest” synthesis by DNA polymerase α, and an absence of hairpin structures of 11 or more bases predicted by computer analysis (10). However, one cannot exclude the possibility that the 5′ position in solution could assume transient secondary structures which conceivably could be important in producing the termination sites seen in Fig. 1. In this regard, an advantage of short synthetic templates is the ability to study termination at specific sites where possible secondary structure has been eliminated.

The most straightforward explanation for increased termination with dNTPpS substrates is that lower nucleophilic reactivity (16) reduces the rate of phosphodiester bond formation. Kuchta et al. (30) recently reported that on heterogeneous oligomer DNA templates, incorporation by Pol I If is severalfold slower for dATPpS than for dATP. It remains possible, however, that the dNTPpS influence on termination could occur at the steps identified as rate-limiting by Benkovic and colleagues, if the thiol derivatives were less effective than dNTPs in facilitating translocation or in forming the complex poised for catalysis.

An alternative explanation for the thiol effect can be related to studies on a poly(dA)-oligo(dT) template primer (13), where the observation that dTTPpS reduced processivity for both Pol I and Pol I If was attributed to the helix-destabilizing effects of incorporated phosphorothioate substrate (17). We do not favor this explanation of helix instability for our observations on the 5′X template, since strong termination sites appear where dNTPpS is an incoming substrate, rather than following incorporation. An additional argument is that, where the dNTPpS substrate is a mixture of the A isomer, which Pol I does use, and the B isomer, which it does not incorporate (3), the effects on termination are due to extra time for the polymerase to “sample” the mixture for the proper isomer. This could not explain, however, the results with dATPpS, which consists only of the A isomer.

Pyrophosphate severely inhibits the rate of DNA synthesis, consistent with earlier reports (18), and reduces processivity. Pyrophosphate appears to have a general effect on processivity: It does not increase termination probability at a specific site, and it cannot counteract the effect of increasing dCTP or dCTPpS concentration. We cannot rule out the possibility that pyrophosphate may have its dominant effect on initiation.

The most likely explanation for the termination induced by methylated bases seems to be interference with hydrogen bonding. Previous work on synthetic templates has shown that N2′-Me-dT severely inhibits incorporation by Pol II (19), and O6-Me-dG inhibits incorporation by Pol I (20). In addition, Larson et al. (21) showed that methylation of DNA produces "pause" sites for Pol II. To our knowledge, however, this is the first demonstration that methylated bases represent essentially absolute blocks to processive DNA synthesis by Pol II. If we have previously shown that methylated bases produce pause sites with DNA polymerase β (22). These results suggest that methyl groups must be removed from damaged DNA before DNA synthesis can occur. That DNA repair polymerases stall at sites opposite a methylated base may provide the cell with a defense against misincorporation, allowing alkyl removal before the extension of synthesis. Because Pol II If does not have 5′- to 3′-exonuclease activity, it represents a simpler model enzyme for DNA polymerase processivity studies than intact Pol II. Experiments with homopolymer templates showed no significant difference in processivity results with Pol II or Pol II If (23). We cannot rule out the possibility that the generalized termination during the first few nucleotides incorporated (Fig. 1) may be due to a sub-population of enzyme. However, this same early termination phenomenon with Pol II If was seen on homopolymer templates, and we believe that the explanation of a subspecies of enzyme is unlikely for several reasons: 1) the enzyme preparation was homogeneous; 2) the phenomenon was seen with three additional enzyme preparations; and 3) the intercept replot from the substrate kinetics was linear, consistent with the presence of only one active enzyme form (22).

The physiological significance of the strong termination sites is not clear. We do note, however, that the human immunodeficiency virus reverse transcriptase conducts processive synthesis and shows a termination pattern on the 5′X template that is distinctly different from the pattern with Pol I If. We have also found that DNA polymerase β, an enzyme which is chiefly distributive in mode of synthesis, shows a pattern of strong pause sites on the 5′X template that is clearly different from the termination pattern of Pol II If (22). It thus seems plausible that the patterns of termination may reflect different inherent characteristics of DNA polymerases, possibly including differences in the active site.

Previous work in several cases has predicted a correlation of processivity with DNA replication fidelity. Hopfield's energy relay model predicted that the first nucleotide inserted should be more error-prone than subsequent insertions (23). Kunkel (24) has found that polymerases which are more processive generally show greater fidelity; this correlation is particularly striking with frameshift mutations (25), but is also seen with single-base misinsertions (26). Finally, Panico et al. (27) find evidence that the exonuclease/polymerase ratio of Pol I increases with processive synthesis. All of these models suggest that, for a processive DNA polymerase, incorporation of the initial nucleotide following a strong termination site should be error-prone. It would, therefore, be of interest to compare the termination pattern of DNA polymerases with their mutational spectra on specified templates.

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