PRIMER-MEDIATED INHIBITION OF THE HYDROLYSIS OF TEMPLATE DNA BY T5-INDUCED DNA POLYMERASE

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Summary — Bacteriophage T5-induced DNA polymerase has an associated 3'→5' exonuclease activity for which both single-stranded and duplex DNA serve as substrate (1). In this report, we demonstrate that hydrolysis of single-stranded DNA homopolymers (template) is inhibited in the presence of complementary (Watson-Crick sense) oligonucleotides (primer). Almost complete inhibition is observed at a primer/template ratio of $\simeq 0.1$. Formation of "H-bonded" primer-template complex seems to be necessary for the inhibition of template hydrolysis because (a) similar amounts of noncomplementary oligonucleotides have no detectable effect on the rate of template hydrolysis, and (b) complementary oligonucleotides lose their inhibitory potential at temperatures where the H-bonded primer-template complex is expected to be unstable. From our data, it appears that the inhibition of template hydrolysis in the presence of primer molecules is due to the preferential binding of the enzyme at the 3'-OH terminus of the primer in the primer-template complex.

Bacteriophage T5-induced DNA polymerase has an associated 3'→5' exonuclease activity for which both denatured and duplex DNA act as substrate (1). However, in the presence of the appropriate dNTPs needed for synthesis, the exonuclease activity is differently affected depending upon the type of primer template used. In the case of denatured DNA, during the first few minutes after the reaction is started by the addition of the enzyme, there is a limited hydrolysis of primer-template when dNTPs are present. When nicked DNA is used as primer-template, there is no detectable hydrolysis. Similar observations and suggestions have also been made by Englund and by Hershfield & Nossal for the T4 DNA polymerase (2,3).

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In this communication we demonstrate that the hydrolysis of single-stranded DNA by T5-induced DNA polymerase (hereforth referred to as template) is markedly inhibited in the presence of oligonucleotide primer molecules.

MATERIALS AND METHODS

$T5^+$-induced polymerase was purified by the method of Fujimura and Roop (4). Preparations thus obtained showed a single band on both native and sodium dodecyl sulfate polyacrylamide gels, and had no detectable endonuclease or 5'→3' exonuclease activity.

Poly(dA), oligo(dT_{12-18}), oligo(dC_{12-18}), oligo(dA_{12-18}), and TTP were obtained from PL Biochemicals. Poly(dA) had a number-average chain length of ~280 as determined by end-labeling with polynucleotide kinase (5). [^{3}H]poly(dA) was obtained from Miles and had a number-average chain length of ~150 as determined by alkaline sucrose density gradient velocity sedimentation. Oligo(dT_{12-18})·($dT_{3.0}$) [^{3}H] was synthesized by the method of Bollum et al. (6). [^{3}H]dTTP was from Schwarz-Mann. Polythyleneimine (PEI)-cellulose strips were from Brinkmann Instruments Ltd. (MN Polygram Cel 300-PEI).

Conditions for nuclease and polymerase assay were the same as described earlier (1), except that TTP, instead of 4dNTPs, was used for polymerase assay. Amounts of DNA used and time of incubation have been indicated in the figure legends. After an appropriate time of incubation, aliquots of 20–30 µl were spotted on DE81 strips (4 cm X 2 cm), which were then dried. The dry strips were washed with 1.5 ml of 0.3 M NH_4-formate for exonuclease assay (7). The amount of hydrolysis was quantified by counting the eluate in ACS (aqueous counting scintillant) scintillation fluid obtained from Amersham Searle. Washing of strips for polymerase assay was carried out the same way except that 50 ml of 0.3 M NH_4-formate was used three times for three washes. Washings were discarded and the strips were then washed with ethanol, dried, and counted in a toluene-based scintillator as described previously (1).

All incubations were carried out at 23°C unless otherwise noted.

For the separation of 5'-dAMP and 5'-dTMP, ascending thin layer chromatography was performed PEI-cellulose strips (20 cm X 3 cm). The solvent used was 0.25 M HCOOH (8). After the run, the strips were cut into 1-cm pieces along the length. PEI-cellulose was scraped into scintillation vials and the nucleotides were eluted with 1.5 ml of 0.5 M NaCl in 1 N HCL (9). Counting was done in ACS scintillation fluid.

RESULTS

Hydrolysis of single-stranded homopolymer DNA by T5 DNA polymerase — The kinetics of poly(dA) hydrolysis by T5 polymerase is shown in Fig. 1A. Previously we have demonstrated that the enzyme acts as exonuclease on both denatured and duplex DNA from the 3'-OH end, and that >95% of the product is 5'-dNMP (1). In the case of poly(dA) virtually all the product is 5'-dAMP, as judged by thin layer chromatography on PEI-cellulose plates with 1.0 M LiCl as solvent (data not shown). From the initial rate, the calculated turnover number under the conditions of incubation is ~3000/min.

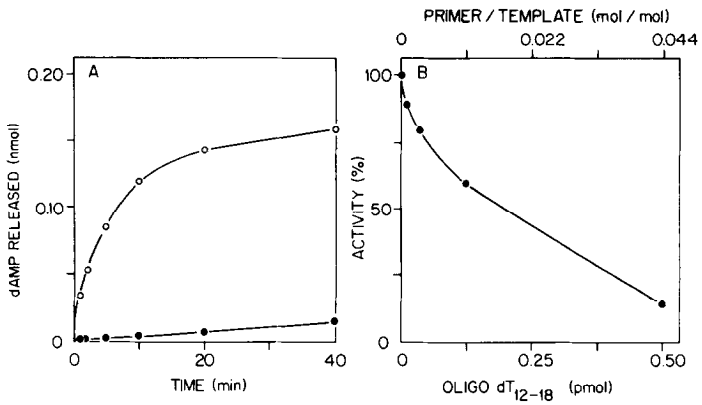
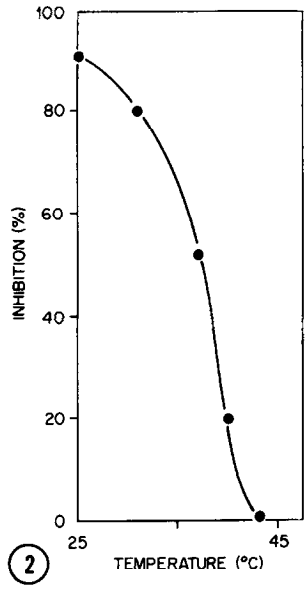


Figure 1. (A) Inhibition of template hydrolysis by oligo(dT_{12-18}). In a reaction volume of 300 µl, 130 pmol of [^{3}H]poly(dA) (5'-end basis) and 0.4 pmol of enzyme was used. Specific activity of poly(dA) was 3300 cpm/nmol of nucleotide P. At indicated times, 20-µl aliquots were spotted on DE81 paper strips and processed as described in Materials and Methods. Control (O); + oligo(dT_{12-18}) (●). Primer/template ratio was 0.3 (mol/mol). (B) Inhibition of template hydrolysis with various amounts of primer. Primer/template ratios have been indicated at the top. 0.24 pmol of enzyme was used for each incubation. Time of incubation was 1 min.

Inhibition of hydrolysis of template DNA by oligonucleotide primer — When the oligo(dT_{12-18}) and [^{3}H]poly(dA) (1:3 mol/mol) mixture is incubated with the enzyme, there is very little hydrolysis of template DNA. This is shown in Fig. 1A. To investigate whether the inhibition was dependent on primer concentration, we varied the amount of primer for a fixed amount of template. As shown in Fig. 1B, with increasing amounts of primer, greater inhibition was obtained. The rate of template hydrolysis was reduced to 50% at a primer/template ratio of 0.02. At this point, primer/enzyme ratio was approximately 0.7. Greater than 80% inhibition was obtained at a primer/template ratio of 0.044.

Hydrogen-bonded primer-template complex is necessary for inhibition — The results of the following experiments demonstrate that H-bonded primer-template complex formation is essential for the inhibition of hydrolysis of template DNA.

(a) As indicated above, the hydrolysis of poly(dA) was inhibited quite effectively by oligo(dT_{12-18}) under our assay conditions. However, noncomplementary oligonucleotides,



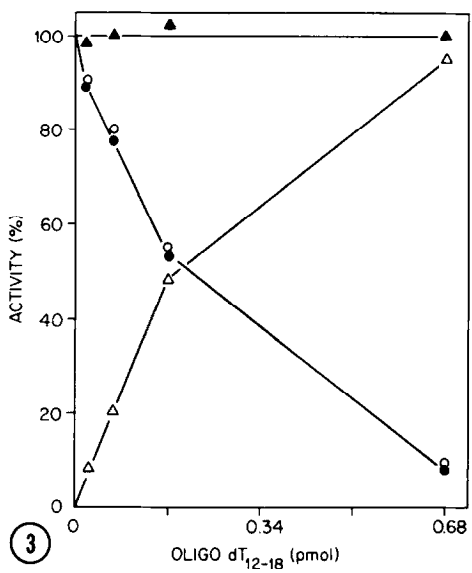


Figure 2. Reversal of inhibition of template hydrolysis at elevated temperatures. Primer/template ratio was 0.05. 1.45 pmol of primer and 0.4 pmol of enzyme were used for each time point. For calculation of percent inhibition at a given temperature, both incubations (i.e., with and without primer) were done at the same temperature. Time of incubation was 30 sec.

Figure 3. Correlation between the amount of inhibition of template hydrolysis and fraction of enzyme molecules bound at the primer site. Conditions were essentially as in Fig. 1B. Control (O); + dTTP 200 μM (●); Fraction of enzyme molecules functioning as polymerase (△). Conditions for polymerase assay as in Ref. 1. Sum of enzyme functioning as exonuclease and polymerase (▲).

such as (dC_{12-18}) and (dA_{12-18}), did not cause any inhibition even when larger amounts were used (data not shown).

(b) When an oligo(dT_{12-18}) and poly(dA) mixture was incubated with enzyme at various temperatures, the degree of inhibition diminished with increasing temperature at a given primer/template ratio (Fig. 2). It should be mentioned here that, at 43°C, the control activity (i.e., hydrolysis of the template in the absence of primer) was 50% of the corresponding activity at 25°C.

Inhibition of template hydrolysis is due to the preferential binding at the 3'-OH primer site — The large amount of inhibitions obtained at relatively low primer/template

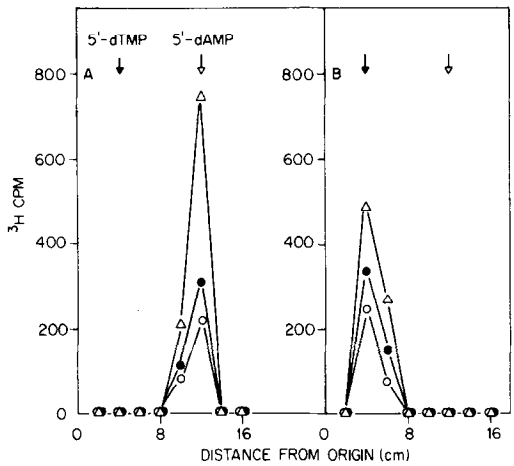


Figure 4. Hydrolysis of primer during the inhibition of template hydrolysis. [^{3}H]poly(dA) and oligo(dT_{12-18}) · $dT_{3,0}[^3H]$ were used. After spotting on DE81 paper, mononucleotides were eluted with 0.3 M NH_4-formate, and part of it was applied to PEI-cellulose strips for chromatography. (A) Control; (B) + oligo(dT_{12-18}) · $dT_{3,0}[^3H]$. Primer/template ≈ 0.1. 1 min(O); 5 min (●); 20 min (△). 4.0 pmol primer and 0.24 pmol enzyme were used for each time point. The amount of ^{3}H label on the primer was 1100 cpm/pmol primer (5'-end). Specific activity of [^{3}H]poly(dA) was ~400 cpm/nmol nucleotide P.

ratios indicated that the inhibition could be due to preferential binding of the enzyme at the 3'-OH end of the primer molecules, H-bonded to the template. To investigate this possibility, we measured polymerase activity at various primer/template ratios. [It should be mentioned here that only properly H-bonded oligonucleotides serve as primer for T5 DNA polymerase, and practically 100% of the enzyme molecules function as polymerase when saturating amounts of primer-template are used (Das and Fujimura, unpublished observations).] Measurement of polymerase activity at various primer/template ratios, therefore, gave us an estimate of the fraction of the enzyme molecules bound at the primer site. As shown in Fig. 3, there was an excellent correlation between the amount of inhibition of template hydrolysis and the fraction of enzyme molecules acting as polymerase. Addition of dTTP had no marked effect on the extent of inhibition obtained with various amounts of primer molecules (Fig. 3).

Another way to investigate the above-mentioned possibility is to see whether hydrolysis of primer continues under conditions where the hydrolysis of template is inhibited. If the inhibition is due to the preferential binding at the 3'-OH end of the primer molecules, then this should be the case. For this experiment we used primer molecules labeled at their 3'-OH ends. As shown in Fig. 4, primer hydrolysis was observed when there was no detectable hydrolysis of template DNA (Fig. 4).

DISCUSSION

In this communication we have presented evidence which indicates that hydrolysis of single-stranded DNA homopolymers by the 3'→5' exonuclease activity of T5 DNA polymerase can be inhibited very effectively in the presence of complementary primer molecules. For this inhibition, the presence of dNTPs is not necessary. Primer hydrolysis is, however, drastically inhibited in the presence of dNTPs (1).

The evidence which indicates that H-bonded duplex formation between the primer molecule and the template is necessary for inhibition is twofold. First, noncomplementary oligonucleotide primers have no effect on the hydrolysis of template DNA; second, complementary oligonucleotides lose their inhibitory potential at higher temperatures, where one would expect the duplex in question to be less stable.

The inhibition of poly(dA) hydrolysis in the presence of oligo(dT) is not unique to the poly(dA)-oligo(dT) pair since similar results were obtained with a poly(dT)-oligo(dA) pair (data not presented).

The inhibition of hydrolysis of template DNA is probably due to the binding of the enzyme at the primer site. The evidence that lends support to this interpretation is as follows. When oligo(dT_{12-18}) · ($dT_{3.0}$)[^{3}H]:poly(dA) [^{3}H] is used as substrate at a primer/template ratio of 0.1, there is no detectable hydrolysis of poly(dA) template. However, production of dTMP during the same period continues. This observation indicates that the affinity of the enzyme for the 3'-OH termini at the primer sites, compared with the

corresponding termini of the template molecules, is much higher.

Other evidence that supports the explanation offered in the last paragraph is that the degree of inhibition of template hydrolysis obtained at various primer/template ratios correlates very well with the fraction of polymerase activity observed at corresponding ratios when appropriate dNTPs are supplied. This indicates that the slower rate of hydrolysis of template DNA in the presence of primer molecules is due to the engagement of the enzyme molecules at the primer sites. A useful corollary of this observation is that the 3'→5' exonuclease- and polymerase-active sites of T5 DNA polymerase share a common domain. Deutscher and Kornberg have alluded to such a possibility in the case of DNA polymerase I (10). We have obtained a wide variety of evidence which indicates that this is very likely to be the case for T5 polymerase (11–13).

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