Interaction of a DNA-Binding Protein, the Product of Gene D5 of Bacteriophage T5, with Double-Stranded DNA: Effects on T5 DNA Polymerase Functions In Vitro

ROBERT K. FUJIMURA* AND BARBARA C. ROOP

Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830

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The gene D5 product (gpD5) of bacteriophage T5 is a DNA-binding protein that binds preferentially to double-stranded DNA and is essential for T5 DNA replication, yet it inhibits DNA synthesis in vitro. Mechanisms of inhibition were studied by using nicked DNA and primed single-stranded DNA as a primer-template. Inhibition of T5 DNA polymerase activity by gpD5 occurred when double-stranded regions of DNA were saturated with gpD5. The 3' → 5' exonuclease associated with T5 DNA polymerase was not very active with nicked DNA, but inhibition of hydrolysis of substrates at 3'-hydroxyl termini by gpD5 could be observed. T5 DNA polymerase appears to be capable of binding to the 3' termini even when double-stranded regions are saturated with gpD5. The interaction of gpD5 with the polymerases at the primer terminus is apparently the primary cause of inhibition of polymerization.

Several proteins that bind preferentially to double-stranded DNA are known. Some, as in nucleosomes, are involved in maintaining DNA in a condensed state (13). Others, such as λ repressor and cro protein (22), bind to specific sites on DNA controlling transcription. Some, such as the T antigen of simian virus 40 (18) and the O protein of bacteriophage λ (27), bind to specific sites controlling initiation of DNA replication. There are many others with no obvious roles and no obvious enzymatic activities. Generally, they stimulate transcription and inhibit DNA synthesis in vitro (16).

The product of gene D5 (gpD5) of bacteriophage T5 has been observed to be involved in some of these functions. It is an asymmetric protein with an Mr of 28,000 and is the most abundant protein synthesized by phage T5-infected cells (23). It binds preferentially and cooperatively to duplex DNA; at saturation, one protein covers 40 base pairs (bp) (11, 23). Our calculation based on the size and the number per cell of gpD5 suggests that it is possible for duplex DNA to be in complex with a chain of gpD5 molecules from one end to the other. Analysis of DNA isolated from a crude extract of the infected cells suggests that DNA is complexed to saturation with proteins at the late phase of infection (unpublished observation). Work with amber mutants has shown that gpD5 is essential for DNA replication, for shutoff of some early transcription, and for the expression of late genes (2, 20, 23). However, both DNA polymerase and RNA polymerase are inhibited by gpD5 in vitro (8; this report). The molecular basis of the functions of gpD5 is not clear. This report concentrates on the effects of gpD5 on the functions of T5 DNA polymerase in vitro.

MATERIALS AND METHODS

DNA preparations. The T5 DNA was extracted from T5HA23, a mutant with no genetically determined single-stranded breaks in DNA (24). Unless otherwise specified, both labeled and unlabeled T5 DNA were prepared from purified bacteriophage as described previously (12). The intact nature of T5 DNA was tested by alkaline sucrose gradient centrifugation (9). PM2 DNA was prepared as described previously (9).

DNA was nicked with DNase I (Worthington Diagnostics) as described previously (9). DNA was denatured by boiling for 2 min.

Restriction enzyme digests of DNAs were prepared under conditions given by the commercial sources, but the optimum amount was determined for each lot of enzymes by agarose gel electrophoresis of products. SmaI was from Boehringer Mannheim Corp., and HindIII was from Miles Laboratories, Inc.

DNA labeled with 32P to a high specific activity was prepared by nick translation in vitro. T5 DNA was treated with 1.3 ng of DNase I (9) per ml and nick translated with Escherichia coli polymerase I with [32P]dTTP (specific activity, 8 × 10⁶ cpm/pmol) essentially as described previously (19). The product was extracted with an equal volume of phenol and purified by passing through a Sephadex G100 column (10 by 0.7 cm). The column was equilibrated with 20 mM Tris-hydrochloride (pH 8.1) containing 0.1 M NaCl, and the sample was loaded and eluted with the same buffer.

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The pooled peak fraction was dialyzed overnight against 200 times the sample volume of 10 mM Tris-hydrochloride (pH 7.6) containing 1 mM EDTA.

DNA with phosphorylated 3' termini (3'-P DNA) was prepared by treatment of T5 DNA with micrococcal nuclease in 50 mM Tris-hydrochloride-2 mM CaCl₂-50 mM K₃PO₄ (pH 8.5) at 37°C for 15 min. The reaction was stopped with 5 mM ethylene glycol bis(β-aminooethyl ether)-N,N'-tetraacetic acid and heated at 75°C for 5 min. The optimal amount of micrococcal nuclease was determined by preparation of T5 DNA treated with various amounts of the nuclease. These preparations were mixed with equal amounts of DNA optimally treated with DNase I and used for synthesis with T5 DNA polymerase. The one that maximally inhibited the synthesis was used as the 3'-P DNA. For our micrococcal nuclease stock, the optimum was about 0.01 U per 15 µg of DNA in 80 µl.

Calf thymus DNA (PL Biochemical) was made 0.5 mg/ml in 75 mM Tris-hydrochloride (pH 7.6) containing 20 mM NaCl and denatured by boiling for 5 min. Polyoxymethylene imidacylic acid and deoxyoxymethylic acid were from PL Biochemicals. The chain length of polyoxymethylene imidacylic acid was estimated to be about 4000 from the "zero time intercept" of the kinetics of synthesis with T5 DNA polymerase (5).

Polymerases. T5 DNA polymerase was prepared as described previously (9).

E. coli RNA polymerase, purified to apparent homogeneity, was a gift from D. M. DeMarini (Biological Division, Oak Ridge National Laboratory). Gene D5 product. The purification procedure is given in detail elsewhere (24a). Briefly, E. coli lysate was prepared as for T5 DNA polymerase (9). DNA was precipitated with polyethylene glycol 6000 and protein purified by passage through three columns (nicked DNA-cellulose, DEAE-Sephadex, and denatured DNA-cellulose) in sequence. When about 35 µg of the protein was analyzed by polyacrylamide gel electrophoresis, only one band was detectable with an Mₐ of about 28,000. Radioactively labeled gpD5 was prepared by the same method with cells labeled by the following procedure. Concentrated E. coli F was prepared essentially as described previously (15) and infected with T5" phage. After 15 min of adsorption, the infected cells were diluted into a fresh growth medium (0 min). Starting at 7 min at 2-min intervals, 100 µCi of [3H]deoxyguanine (50.4 Ci/mmol) was added to the total of 1 mCi per 250 ml of the culture. At 27 min, 0.2 µg of unlabeled leucine per ml was added, and at 37 min, 0.1 µg of chloramphenicol per ml was added; the culture was then cooled with ice, harvested, frozen, and stored in liquid N₂.

Enzyme assays. Each reaction mixture described below was prepared at 0°C and transferred to a water bath to commence reaction. Specific amounts and conditions are given under the figure legends.

(i) DNA synthesis. The standard reaction mixture (30 µl) consisted of 67 mM Tris-hydrochloride (pH 7.6) containing 10 mM MgCl₂, 10 mM dithiothreitol, and 50 µM each of the four dinucleotide triphosphates (dNTPs) with the dTTP labeled to a specific activity of 300 cpm/pmol with [methyl-3H]dTTP unless otherwise specified. Usually DNA was added first, and DNA polymerase was added last, just before incubation.

(ii) DNA hydrolysis. The standard reaction mixture for the polymerase-associating 3' → 5' exonuclease was as above without the four dNTPs. T5 [32P]dNTP was the usual substrate. After the reaction, a sample was placed on three layers of 1.5-cm² DE81 paper, processed, and counted (1, 3). Washing of the filters by the buffer (0.3 M NH₄ formate-0.01 M NaPP, pH 7.8) was done in 1.5 ml twice, and each wash was counted by using 15 ml of ACS scintillation fluids (Amerham). For 3P-labeled substrates, the sum of the radioactivity on the filter and that in the wash solution was equal to the input. For 3H-labeled substrates, the filter quenched the activities even after washing with ethanol and ether; therefore, only the value from the wash was used. For exonuclease III (Miles Laboratories, Inc.), the reaction mixture prescribed by Miles was used and assayed with DE81 papers as above.

(iii) Effects of gpD5. To study effects on the above reactions, various amounts of gpD5 were added immediately after DNA addition in each reaction tube unless otherwise specified. Variations in the volumes of gpD5 added were normalized by the addition of the same buffer, 20 mM Tris-hydrochloride (pH 8.2) containing 1 mM mercaptoethanol, 1 mM EDTA, and 50% glycerol.

Agarose gel electrophoresis. All gel electrophoresis was carried out in a 22-cm horizontal gel electrophoresis apparatus (Aquebogue Shop, Long Island, N.Y.). Gel electrophoresis of native DNA was usually carried out in 0.6% (wt/vol) agarose (SeaKem) in the Tris- acetate system of Hayward and Smith (14). Runs were made at 30 mA constant current for about 16 h.

Gel electrophoresis of denatured DNA was carried out with the system of McDonell et al. (21). Agarose (0.8%) was made in 30 mM NaCl-2 mM EDTA. The solvent system was 30 mM NaOH containing 2 mM EDTA. Runs were made at 22 V (constant) for about 18 h.

RESULTS

Effect of gpD5 on the functions of T5 DNA polymerase with nicked DNA. The effectiveness of nicked DNA as a primer-template for DNA synthesis with T5 DNA polymerase depends on the degree of nicking. On both sides of the optimum, the amount of nucleotides incorporated decreases. As shown in Fig. 1A, the usual optimal concentration of DNase I under our conditions is about 1.3 ng/ml. A rough estimate of the number of cuts per molecule, as determined by agarose gel electrophoresis, was about 30.

T5 DNA, nicked to the optimal degree for synthesis, was complexed with increasing amounts of gpD5. Such complexes were used as primer-template for T5 DNA polymerase. As shown in Fig. 1B, the synthesis was inhibited by gpD5, and the inhibition was linearly dependent on gpD5 concentration on a semilog plot. At the 50% inhibition point, the molar ratios of the essential macromolecules were as follows: [DNA(bp)]/[gpD5]/[DNA polymerase] = 36:1:0:006. The number of nicks was calculated to be about the same as the number of DNA
polymerase molecules, and under these conditions the initial rate of synthesis was dependent on both DNA polymerase and nicks. The ratio of [DNA]/[gpD5] of 36 was about the same as the saturation value for binding of gpD5 to DNA (11, 23).

With nicked T5 DNA more extensively nicked, there was less synthesis and no inhibition of this synthesis by gpD5 (Fig. 1B. 13 ng of DNase per ml). This extensively nicked DNA is a very good substrate for the 3' → 5' exonuclease associated with the polymerase and suggests that this preparation of nicked DNA contains many single-stranded 3'-hydroxyl (3'-OH) termini where the enzyme binds preferentially.

Similar studies were carried out with PM2 DNA, with almost identical results (data not shown).

The effect of gpD5 on hydrolysis was studied by using constant amounts of DNA (1 μg/30 μl) and DNA polymerase (10 nM). The DNA was nicked to various degrees by use of different amounts of DNase I, leaving the rest of the variables constant for the reactions. When DNA was treated with 1.3 ng of DNase per ml (which produced optimal number of nicks for synthesis), the hydrolysis by the 3' → 5' exonuclease was barely detectable (data not shown), and we could not study the effect of gpD5. When DNA was treated with a larger amount of DNase I (13 ng of DNase per ml), about 60% of the DNA was hydrolyzed in 20 min, and there was no effect of gpD5 on hydrolysis. Judging from the properties of the 3' → 5' exonuclease of the DNA polymerase and gpD5, their binding sites on DNA differ: gpD5 binds to double-stranded regions, whereas 3' → 5' exonuclease of the DNA polymerase acts preferentially at the single-stranded 3'-OH ends of the DNA, which are produced in increased quantity by the increased DNase I treatment. The hydrolysis of a heat-denatured sample of DNase-treated DNA was only slightly higher, suggesting that the 3'-OH ends were already in abundance before denaturation; under these conditions, there was no effect of gpD5.

If the above interpretation is correct, hydrolysis of DNA by E. coli exonuclease III, which specifically hydrolyzes from the 3'-OH ends of duplex DNA (1), should be inhibited by gpD5. The result with DNA treated with 2.9 ng of DNase I per ml (Fig. 2) shows that exonuclease III is indeed inhibited; at 50% inhibition, the ratio of DNA to gpD5 was 48 bp per molecule. Therefore, the inhibition was occurring near the saturation point of the binding of gpD5, as was the synthesis with DNA polymerase and nicked DNA.

On the basis of these observations, we conclude that the inhibition of DNA polymerase by gpD5 occurs when double-stranded DNA is sat-
urated with gpD5. Judging from the results with E. coli exonuclease III, the polymerase associated 3' → 5' exonuclease will also be inhibited if the substrate is double stranded. The inhibition occurs when DNA is saturated because either the 3'-OH ends of primers are covered with gpD5 so that the polymerase cannot bind, or the polymerase does bind to the primer end but is inhibited. To clarify these possibilities the following experiments were carried out.

In a separate communication we characterized the gpD5-DNA complex by band sedimentation in a metrizamide gradient to apparent equilibrium positions for both free DNA and the complex (buoyant density of 1.12 and 1.17 g/cm³, respectively) (11). A similar technique was used to show that DNA polymerase does bind to nicked DNA saturated with gpD5 (Fig. 3). The gpD5 was labeled with ³H, and DNA polymerase was identified by assay with denatured calf thymus DNA as a primer-template. In the absence of DNA, DNA polymerase and gpD5 sediment separately; gpD5 remains at the meniscus, and the DNA polymerase peak sediments to fraction no. 17 (Fig. 3A). When nicked PM2 DNA was saturated with gpD5 and then mixed with T5 polymerase, the polymerase binds to the complex and cosediments (Fig. 3B). The polymerase peak was identified with denatured calf thymus DNA in 60 mM (NH₄)₂SO₄. Under this condition, freed polymerase preferentially uses denatured DNA as a primer-template. The free DNA sediments to fraction no. 15 under these conditions (11). The complex of polymerase and gpD5-DNA is slightly active in DNA synthesis, and synthesis is detectable only with precursors of high specific radioactivity. This suggests that at least some polymerases are bound at the primer termini.

In another experiment, DNA was labeled

FIG. 2. Effects of gpD5 on hydrolysis by exonuclease III. PM2 [³H]DNA was nicked with 2.9 ng of DNase per ml, and 22 μM (bp) was used per reaction mixture containing 1.6 U of exonuclease III. Incubation was at 36°C for 35 min. The rest of the procedures are described in the text.

FIG. 3. Analysis of the complex between gpD5 and PM2 DNA. (A) A 1.2-pmol sample of DNA polymerase was mixed with 64 pmol of ³H-labeled gpD5 in the standard reaction mixture for DNA synthesis, but without the four dNTPs, and incubated at 37°C for 20 min. It was layered on a metrizamide gradient (16% to 40%, wt/vol) as described elsewhere (11) and centrifuged for 16 h at 4°C in an SW50.1 rotor at 45,000 rpm. Fractions were collected from the bottom and assayed for [³H]gpD5 in 50 μl of each fraction by using Amersham ACS scintillant (O). DNA polymerase was assayed in 11 μl of each fraction with denatured calf thymus DNA (5 μg) in 60 mM (NH₄)₂SO₄ and 300 cpm of [³²P]dTTP per pmol in the standard reaction mixture at 37°C for 20 min (O). (B) A 1.3-nmol (bp) sample of PM2 DNA nicked with 1.3 ng of DNase per ml was mixed with 64 pmol of [³H]gpD5 and then 1.2 pmol of DNA polymerase in the same reaction mixture as above. Fractions after the centrifugation were assayed as above. In addition, PM2 DNA-DNA polymerase complex was assayed using 11 μl of each fraction with [³²P]dTTP at specific activity of 2 × 10⁴ cpm/pmol, otherwise in the standard reaction mixture at 37°C for 20 min (O).
proximal to 3'-OH termini with [32P]dTTP of high specific activity by nick translation with *E. coli* DNA polymerase I to increase the detectability of the 3' → 5' exonuclease activity (Fig. 4). Such DNA was complexed with T5 DNA polymerase and 3'-P DNA was added in excess, and hydrolysis by 3' → 5' exonuclease of the polymerase was started by addition of Mg2+. Under these conditions, the polymerases that are not in complex with 3'-OH DNAs are complexed to 3'-P DNA and inhibited from further action. If during the hydrolysis, the four dNTPs are added, the enzyme molecules in the process of hydrolysis, and only these, will act to incorporate dNMPs in the synthesis process (6). Thus, hydrolysis and synthesis can be studied on the same substrate.

We have shown previously with denatured DNA, that the kinetics of nucleotide incorporation under the conditions where [polymerase] << [primer-template] are biphasic (5). The initial phase of synthesis is very fast due to processive incorporation of nucleotide as the polymerase translocates along the template. The second phase is slower because transfer of the polymerase from the end of one template to a new primer-template is a slower process and is rate limiting. When nicked DNA is used as a primer-template, and where [polymerase] < [primer], the nucleotide incorporation follows the similar biphasic curve (Fig. 5A, sample 1). Presumably the same explanation can be given. We have used the amount of T5 DNA polymerase that gave slightly less than the maximal initial rate of synthesis and assumed that the amount of the polymerase used was slightly less than the number of effective primer ends. If the second phase of the time course in the reaction is slower due to transfer of polymerases to new primer-templates, then the process can be inhibited by DNA that is nonfunctional as primer-templates, such as 3'-P DNA (Fig. 4). Thus, 3'-P DNA was added in 10-fold excess to a reaction similar to sample 1.

The second phase of the synthesis is inhibited as expected due to dissociated polymerase complexing with 3'-P DNA (Fig. 5A, sample 2). As a control for the above experiment when 3'-OH DNA and 3'-P DNA were mixed together before the addition of the polymerase, even the first phase was inhibited because 3'-P DNA was in excess and the enzymes were preferentially bound to it (Fig. 5A, sample 3).

We have also shown previously that in the presence of Mg2+ without dNTPs, the polymerase bound to a primer-template acts as 3' → 5' exonuclease, acting processively. Thereafter, when the four dNTPs are added, the same enzyme acts as a polymerase (6). Sample 4 was hydrolyzed by this technique in the presence of 3'-P DNA, and the four dNTPs were added 2 min later (0 min in Fig. 5A) to initiate synthesis. Since a separate experiment showed that, under similar conditions, hydrolysis for the initial 2 min was not affected by the presence of 3'-P DNA, we have assumed that most of the polymerases are still in complex with the initial substrates at the end of this period. Thus, when the four dNTPs were added after 2 min of hydrolysis, the synthesis occurred on the same primer-templates. The result (sample 4) is consistent with this explanation as judged from an initial rate equal to that of the control (sample 1). The fractions of the template hydrolyzed during the synthesis in samples 1 and 3 are presumably on the single-stranded 3'-OH ends.

Similar substrates were used to study the effect of gpD5 (Fig. 5B). Sample 6 is the control and is similar to sample 4 of Fig. 5A. Sample 7 shows the effect of gpD5 added in saturating amount at 0 min along with the four dNTPs after 2 min of the hydrolysis by 3' → 5' exonuclease of the polymerase to create single-stranded regions. It shows that the synthesis was inhibited from 0 min. For sample 5, gpD5 was added before hydrolysis, and it inhibited even the 3' → 5' exonuclease activity to the level of sample 8, where the hydrolysis occurred only concomitantly with the synthesis. The concomitant hydrolysis occurred presumably with single-stranded 3'-OH ends that were present even before the initiation of the reactions. During the prior hydrolysis all types of 3'-OH ends were probably being hydrolyzed, and part of them were inhibited by gpD5. Our results are consistent with the explanation that the enzymes bound to 3'-OH ends that are properly hydrogen
FIG. 5. Effects of gpD5 on nick-translated DNA. T5 DNA was nicked, and regions proximal to 3′OH termini were labeled with [32P]dTTP by nick translation as described in the text (see the legend to Fig. 4). Such DNA had [32P]-labeled regions with specific activity of 42 to 57 cpm/fmol at the time of experiments. Resynthesized regions were labeled with [3H]dTTP at specific activity of 200 cpm/fmol. Samples of 30 µl were taken out at each time point, acid precipitated, and counted. About 350 fmol/30 µl corresponds to 100% hydrolysis of [32P]-labeled regions. Synthesis and hydrolysis are shown with solid and broken lines, respectively. (A) Effects of 3′P DNA on synthesis and hydrolysis with nick-translated DNA complexed with DNA polymerase. Sample 1 (□), In 200 µl of the standard reaction mixture, but without MgCl₂ and the four dNTPs, 0.34 µg of [32P]DNA (about 10⁵ cpm) and 2.7 pmol of DNA polymerase were mixed, and then at 0 min the standard amounts of MgCl₂ and the four dNTPs were added to start the reaction at 37°C. Sample 2 (●), The reaction mixture was the same as sample 1, except that after the mixing of [32P]DNA and DNA polymerase, 3.3 µg of 3′-P DNA was added. Sample 3 (○), Same as sample 1, except that [32P]DNA and 3′-P DNA were mixed before the addition of the polymerase. Sample 4 (■), Same as sample 2, except that MgCl₂ was added at -2 min to start the hydrolysis, and the four dNTPs were added at 0 min to start the synthesis. (B) Effect of gpD5 on the synthesis and hydrolysis. Sample 5 (▲), The reaction mixture consisted of the same amount of [32P]DNA and DNA polymerase as sample 1, and then 240 pmol gpD5 was added followed by addition of 3.3 µg of 3′-P DNA. At -2 min, MgCl₂ was added, and then the four dNTPs were added at 0 min. Sample 6 (■), The repeat of sample 4. Sample 7 (■), Same as sample 5, except that gpD5 was not added until the 0 min. Sample 8 (○), Same as sample 5, except MgCl₂ was not added till 0 min, that is, no prior hydrolysis.

FIG. 6 shows another set of experiments indicating that the 3′ → 5′ exonuclease can be inhibited by gpD5 added in the saturating amount. It also shows that the amount of synthesis is not affected by the prior hydrolysis, which presumably creates single-stranded regions along the template over which the synthesis occurs. In these experiments (Fig. 6), the prior hydrolysis was carried out for various times, and the synthesis was carried out for 4 min. (Note that the amount of synthesis was much greater than the amount of prior hydrolysis.) The concentrations of the substrate and the enzyme were the same as those in Fig. 5A and B. Sample 1 (Fig. 6) is the control that shows the time course of the prior hydrolysis when no inhibitors were present. The amount synthesized was not affected by the degree of the prior hydrolysis.

Sample 2 (Fig. 6) shows the effect of adding excess 3′-P DNA to the complex of DNA polymerase and 3′-OH DNA. The time course of the prior hydrolysis was not affected for at least 2 min, but then the hydrolysis was inhibited. The hydrolysis of labeled regions of the initial substrate was completed in about 3 min. However, the amount synthesized from each time point of the prior hydrolysis was about the same. This suggests that the enzyme is still bound to the initial substrate even after 10 min of the prior hydrolysis, and such a complex is capable of bonding and that can serve as primer-templates are preferentially inhibited by gpD5.

The R1 elements are selectively degraded when hybridized to gpD5. This suggests that gpD5 may be involved in the degradation of R1 elements.
acting as primer-template for synthesis when the four dNTPs are added. Sample 3 (Fig. 6) is the same as sample 1, except gpD5 was added in saturating amount before the initiation of hydrolysis. The prior hydrolysis was obviously inhibited, but it was still going on at a much reduced rate. The synthesis from each time point of the prior hydrolysis was almost completely inhibited, and the degree of inhibition of the synthesis was independent of time of the prior hydrolysis or the length of the single-stranded regions. A similar experiment in which gpD5 was added before the polymerase showed that it does not matter which is a added first. Sample 4 (Fig. 6) is similar to sample 3, except that 3'-P DNA was present in excess to complex preferentially with any enzyme dissociated from the initial complex. The effect on the prior hydrolysis was about the same as for sample 3, and the synthesis was slightly less inhibited. This is consistent with our interpretation for sample 3 that most of the enzymes were still bound to the initial substrate.

We interpret these as showing that the translocation of the enzyme along the template is inhibited by gpD5. If gpD5 was forcing the enzyme off the substrate during the prior hydrolysis, then the longer time of the hydrolysis should cause more inhibition of the subsequent synthesis than occurred in the presence of 3'-P DNA. The experiments also showed that the amount of synthesis is not affected by the length of the single-stranded regions created by the prior hydrolysis. This further suggests that the strand displacement process ahead of a growing primer does not affect the synthesis either in the presence or the absence of gpD5. Therefore, the obvious possibility left is that the translocation of the polymerase is inhibited during the synthesis and the hydrolysis of DNA complexed to saturation with gpD5.

**Effect of gpD5 on T5DNA polymerase functions with primed single-stranded DNA.** If the above interpretation is correct, then the synthesis with primed single-stranded DNA, which will be free of double-stranded regions ahead of growing primer, also will be inhibited by gpD5. This was tested by use of heat-denatured *SmaI* digests of T5 DNA. This enzyme cuts T5 DNA into four pieces with average Mr values of 19 × 10⁶ (size ranges from about 8 × 10⁶ to 30 × 10⁶). Various amounts of gpD5 and DNA were used with 0.6 pmol of T5 DNA polymerase. The initial rates were determined from 4 min of synthesis, during which the time course of synthesis is about linear. The results are shown as the reciprocal of the dTMP incorporated versus the concentration of gpD5 (Fig. 7). The inhibition is greater when the primer-template concentration is less than optimal. This indicates that polymerase and gpD5 compete for primer-template. Similar results were obtained with *HindIII* digests and also with polydeoxyadenylic acid-oligo(deoxythymydyllic acid.

This was further tested by the addition of increasing amounts of native DNA to 30-μl reaction mixtures containing constant amount of denatured *SmaI* digest (0.5 μg), DNA polymerase (0.4 pmol), and gpD5 (48 pmol). The study above has shown that a concentration of about

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**Figure 6.** Effect of gpD5 with time of prior hydrolysis of nicked translated DNA under various conditions. Sample 1 (Δ), Samples (30 μl) of reaction mixtures with the same concentration of ingredients as sample 1 of Fig. 5 were hydrolyzed for various lengths of time as indicated in the figure, and then the four dNTPs were added to each sample for DNA synthesis. Synthesis time was 4 min for all samples. Reactions were stopped and analyzed as for Fig. 5. Sample 2 (○). Reaction mixtures were prepared as for sample 1, except that 3'-P DNA was added in the same concentration as sample 2 of Fig. 5 before the initiation of hydrolysis. Sample 3 (○), Reaction mixtures were prepared as sample 1, except gpD5 was added in the same concentration as for Fig. 5 (B) before the initiation of hydrolysis. Sample 4 (▲). Reaction mixtures were prepared as for sample 1, except that 3'-P DNA and gpD5 were added in sequence in the same concentrations as for samples 2 and 3, respectively.
regions of primer-templates are saturated with gpD5.

Hydrolysis of denatured DNA is not affected by gpD5 because hydrolysis occurs preferentially with single-stranded 3'-OH ends.

**Effects of gpD5 on some other nucleic acid enzymes.** RNA synthesis with E. coli RNA polymerase and native T5 DNA is also inhibited as DNA is saturated with gpD5 (data not shown). Further studies may reveal more subtle differences from the effect with DNA polymerase.

There were no apparent effects of gpD5 on the activities of restriction enzyme HindIII or on pancreatic DNase I, when examined by means of gel electrophoreses of digests.

**DISCUSSION**

T5 DNA polymerase has at least two enzymatic activities. The polymerization from 3'-OH termini of primer ends can occur with nicked DNA as a primer-template displacing a strand ahead of the growing primer strand (9). Hydrolysis in the 3' → 5' direction from nicks is not as effective (4), but can be detected easily with substrates labeled at regions proximal to 3'

30 pmol of gpD5 per 30 µl is sufficient to inhibit synthesis under these conditions; thus, the amount used was about 20 pmol in excess of that needed for maximum inhibition. The result is shown as a double-reciprocal plot and compared with the reaction without gpD5 (Fig. 8). The results were as expected. When native DNA is present in sufficient quantity to complex all the gpD5 (2 nmol of DNA, bp, or greater in this case), the rate of synthesis was the same as the maximum rate without gpD5 ($V_{\text{max}} = 14$ pmol of dTMP incorporation per min). The convergence of initial rates shows that inhibition is competitive. The abrupt change in the slope of the curve at the higher concentration of native DNA suggests preferential affinity for native DNA over primed single-stranded DNA. This is due to a positive cooperative effect (17) and suggests preferential cooperative binding to native DNA. A relationship between cooperativity and length of double-stranded regions may shed some light on the effects of gpD5 on DNA synthesis. These data are consistent with our interpretation that inhibition of DNA polymerase occurs at the 3'-OH terminus of a primer when double-stranded DNA.

![FIG. 7. Dependence of gpD5 effect on concentration of denatured restriction enzyme digests of T5 DNA. Initial rates of synthesis were determined by use of heat denatured SmaI digests in the presence of various amounts of gpD5. DNA concentrations were as follows: 0.1 (○), 0.2 (●), 0.5 (△), and 1.0 (▲) µg/30 µl; T5 DNA polymerase was 0.6 pmol. Other conditions were as described in the text.](image)

![FIG. 8. Double-reciprocal plot of effects of gpD5 on initial rates of synthesis with mixture of denatured and native SmaI digests of T5 DNA. Rates of synthesis at 37°C were determined with various concentrations of native DNA (●), with denatured DNA maintained at 0.5 µg/30 µl. The amount of gpD5 was 48 pmol, and that of T5 DNA polymerase was 0.4 pmol. The controls are without gpD5 (○) and with native DNA alone (▲).](image)
termini with isotopes of high specific radioactivity. With DNA polymerase complexed to nicked DNA, in the presence of excess 3'-P DNA as scavenger for free DNA polymerase, we have shown that DNA polymerase in complex with nicked DNA can act first as an exonuclease and then as a polymerase just as we have shown previously with polydeoxyadenylic acid-oligodeoxymethylid acid (6). The product of gene D5 binds to such duplex DNA preferentially and cooperatively (11, 23), and the present studies show that when it saturates the duplex DNA, it inhibits both hydrolysis and synthesis.

Inhibition of polymerization by gpD5 could be at 3'-OH termini or by prevention of strand displacement ahead of a growing primer. Our data indicate that the primary effect is at 3'-OH termini because syntheses with nicked, gaped, and primed single-stranded DNA are all inhibited. Although there is no evidence for direct interaction between gpD5 and DNA polymerase, gpD5 may, when complexed to DNA, hinder proper alignment of DNA polymerase at the 3'-OH end.

The above discussion suggests at least two possible mechanisms for involvement of gpD5 on DNA replication.

(i) The role of gpD5 may be indirect. A binding of gpD5 to all double-stranded regions except the origin or promoter sites (or both) induces the complex involved in initiation of DNA replication to bind to these exposed sites (10). Such a site may be at least partially single stranded and may thus have a lower affinity for gpD5. RNA synthesized in such a site may act as a primer for DNA synthesis under certain conditions (26). RNA primer complexed to DNA may also have a lower affinity for gpD5. DNA covered with gpD5 may prevent adventitious initiation of DNA replication from any 3'-OH termini.

(ii) Some fraction of gpD5 may bind specifically to certain DNA sequences or structures, such as that at the origin of DNA replication and promoter sites. About 5% of gpD5 appears to be phosphorylated (7), and such fractions may be involved in site-specific binding. However, it was not determined what was phosphorylated—certain amino acids could be phosphorylated or ADP ribosylated. There may be some other kind of modification. As mentioned above, the T antigen of simian virus 40 binds to the origin of DNA replication, and this binding is essential for DNA replication (18). According to a recent report, only a small subclass of the T antigen binds to the origin, and it corresponds to a fragment recognized by a T antigen-specific monoclonal antibody (25).

The presence of mutants of gene D5 that prevent T5 DNA synthesis in nonpermissive hosts (15, 20) indicates that the role of gpD5 cannot be just a passive one. It is probably analogous to that of the T antigen of simian virus 40 (18) and the O protein of A (27). Mechanisms of the action of these two proteins are not yet known, and the T5 system may provide another approach to understanding the role of this type of DNA-binding proteins.

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