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The Linker Region between the Helicase and Primase Domains of the Bacteriophage T7 Gene 4 Protein Is Critical for Hexamer Formation

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The gene 4 protein of bacteriophage T7, a functional hexamer, comprises DNA helicase and primase activities. Both activities depend on the unidirectional movement of the protein along single-stranded DNA in a reaction coupled to the hydrolysis of dTTP. We have characterized dTTPase activity and hexamer formation for the full-length gene 4 protein (gp4) as well as for three carboxyl-terminal fragments starting at residues 219 (gp4-C219), 241 (gp4-C241), and 272 (gp4-C272). The region between residues 242 and 271, residing between the primase and helicase domains, is critical for oligomerization of the gene 4 protein. A functional TPase active site is dependent on oligomerization. During native gel electrophoresis, gp4, gp4-C219, and gp4-C241 migrate as oligomers, whereas gp4-C272 is monomeric. The steady-state \( k_{\text{cat}} \) for dTTPase activity of gp4-C272 increases sharply with protein concentration, indicating that it forms oligomers only at high concentrations. gp4-C219 and gp4-C241 both form a stable complex with gp4, whereas gp4-C272 interacts only weakly with gp4. Measurements of surface plasmon resonance indicate that a monomer of T7 DNA polymerase binds to a dimer of gp4, gp4-C219, or gp4-C241 but to a monomer of gp4-C272. Like the homologous RecA and F, ATPase proteins, the oligomerization domain of the gene 4 protein is adjacent to the amino terminus of the NTP-binding domain.

The efficient and economical replication system of bacteriophage T7 provides an in vitro model for studying essential protein-protein interactions at a replication fork (1). Synthesis of the leading and lagging strands is the result of the coordinated action of T7 DNA polymerase (gene 5 protein), its processivity factor Escherichia coli thioredoxin, T7 gene 4 helicase-primase, and T7 gene 2.5 single-stranded DNA (ssDNA)3'-5' binding protein. A 2.2-Å crystal structure has been determined for the T7 DNA polymerase-thioredoxin complex bound to a primer-template and with a nucleoside triphosphate at the active site (2). Leading and lagging strand synthesis are coupled, and the lagging strand DNA polymerase recycles from a completed Okazaki fragment to a new primer (3–5). Physical and functional interactions have been demonstrated between gene 2.5 protein and both the DNA polymerase (6) and gene 4 protein (6–8). The gene 4 protein and DNA polymerase also interact (9–11). Gene 4 of T7 encodes two co-linear polypeptides of 63 and 56 kDa, the latter arising from an in-frame translation start codon (12–15). Both polypeptides function as DNA helicases (13, 16), and both form hexamers that encircle ssDNA (17). Helicase activity results from the hydrolysis of dTTP that drives 5' to 3'-unidirectional translocation along ssDNA (18–20).

The gene 4 proteins contain two domains responsible for the primase (amino-terminal 245 residues) and helicase (carboxyl-terminal 295 residues) activities (21) (Fig. 1). The amino-terminal 63-residue region is unique to the 63-kDa gene 4 protein and contains a Cys\(^4\) zinc finger essential for recognition of the template sequences that signal the primase domain to synthesize a tetraribonucleotide that can subsequently be used to prime DNA synthesis (22). Amino acid residues of the primase domain have been aligned with those of other primases from bacteria and phage, leading to the identification of six highly conserved blocks of residues (Fig. 1) (21). A gene encoding the 271 amino-terminal residues of the 63-kDa gene 4 protein has been overexpressed in E. coli, and the purified product has been shown to have the same level of oligoribonucleotide synthesis activity as that of the 63-kDa gene 4 protein in the absence of dTTP, conditions under which there is no helicase activity (23).

Residues that compose the helicase domain have also been aligned with those of helicases from bacteria and phage (21), leading to the identification of five conserved blocks of residues (Fig. 1). Considerations of secondary structure suggest that the helicase domain is structurally homologous to the ATP-binding domain of the F, ATPase (24). Bird et al. (25) constructed carboxyl-terminal gene 4 protein fragments starting at residues 219 and 241 and showed that they form hexamers, have dTTPase activity, and function as helicases. This observation suggests that the protein interface responsible for oligomerization resides after residue 241, either in the helicase domain or in the linker region joining the primase and helicase domains (Fig. 1). In order to define the role of the linker region in oligomerization, we have constructed three truncated polypeptides that start either immediately before or after this region; we have purified the proteins and have examined their ability to form a functional hexamer.

**EXPERIMENTAL PROCEDURES**

**Materials—**Oligonucleotides were from Integrated DNA Technologies. Restriction endonucleases NdeI and BamHI and T4 DNA ligase were from New England Biolabs. Expression vectors pET17b and pET19b as well as the expression host E. coli cells BL21(DE3)pLysS were available on line at http://www.jbc.org.

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‡ The abbreviations used are: ssDNA, single-stranded DNA; PCR, polymerase chain reaction; His-tag, histidine tag; gp4, gene 4 protein; RU, resonance units; DTT, dithiothreitol; NTA, nitrilotriacetic acid.
were from Novagen. Ni²⁺-NTA superflo resin was from Qiagen. PO-
ROS® 20 HQ media was from Perspective Biosystems, Inc. Centriprep®
centrifugal concentrators were from Amicon®. Tris glycine Ready Gels and the dye reagent concentrate for protein assay were from Bio-Rad.
The high molecular weight electrophoresis calibration kit, dTTP, ATP, and
T4 DNA polymerase were from Amersham Pharmacia Biotech. Sensor chips CM5 (certified grade) were from BiACore. Polyethylene-
mine-cellulose thin layer chromatography plates were from J. T. Baker
Chemical Co.

Construction of Clones for the Overproduction of Carboxyl-terminal
Fragments of Gene 4 Protein—Clones overproducing the carboxyl-termi-
nal fragments with their histidine tag at their amino end were con-
structed using the polymerase chain reaction (PCR). For all the
constructs, the wild-type gene 4 DNA was used as the template, and the
downstream primer was 5′ GCC GAC TGC GGA TCC TCA GAA GTC
AGT GTC 3′. The upstream primers used to generate the PCR
fragments for expressing the genes for gp4-C219, gp4-C241, and gp4-C272
were 5′ GGG GAC TGC CAT ATG GCT GCC CAG GCT ATG CTA CC 3′,
5′ GGG GAC TGC CAT ATG TCT CAC CTA AAT GGT C 3′, and 5′ GGG
GAC TGC CAT ATG GCT GGA CAA ATC GGT C 3′, respectively.

Amplification, each PCR product was purified and then digested with
NdeI and BamHI. The resulting fragments were ligated between the
Ndel and BamHI sites of pET-19b. For the construction of clones that
overproduce the carboxyl-terminal fragments of gene 4 protein without
a histidine tag at their amino end, the NdeI and BamHI-digested PCR
fragments were ligated into pET-17b that had been digested with
NdeI and BamHI.

Protein Overproduction and Purification—The full-length gene 4 pro-
tein was overproduced and purified as described (26). Native T7 DNA
polymerase (one-to-one complex of wild-type T7 gene 5 protein and E.
coli thioredoxin) was overproduced and purified as described (27).

Overproduction of gp4-C219, gp4-C241, and gp4-C272 was carried out
by transforming the cells BL21(DE3)/pLysS with the appropriate
plasmids. Cells were grown in 2 liters of LB media containing 100
µg/ml ampicillin and 35 µg/ml chloramphenicol at 37 °C. At an A600 of 0.6,
expression was induced by the addition of isopropyl-1-thio-
β-galactopyranoside to a final concentration of 1.0 mM. After induction for 2 h
at 37 °C, the cells were harvested by centrifugation, and the cell pellets
were stored at −80 °C.

gp4-C219, gp4-C241, and gp4-C272 were purified by affinity chromato-
graphy specific for the histidine tags at their amino termini. All steps
were carried out at 4 °C. The frozen cell pellet from 1 liter of culture was
resuspended in 20 ml of Buffer A (50 mM potassium phosphate, pH 8.0,
500 mM NaCl) containing 10 mM imidazole, and the resuspended cells
were sonicated on ice for 15 min. The cells were sonicated and then centrifuged at 10,000 × g for 30 min. The supernatant was collected as
fraction I (20 ml). A column (0.7 × 5 cm) containing 2 ml of Ni²⁺-NTA
resin was equilibrated with 20 mU of Buffer A containing 10 mM
imidazole. Fraction I (20 ml) was applied to the column at 1 ml/min at 4 °C.

A flow rate of 5 ml/min. A solution of native

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initially at a constant current of 20 mA for 30 min, followed by 40 mA
for 2 h. At the end of electrophoresis, the temperature of the running
buffer was less than 27 °C.

Native electrophoresis in 4–20% linear gradient polyacrylamide
Ready Gels was as described above except that EDTA in the running
buffer was replaced by 2 mM ATP and 4 mM MgCl₂. Gels were stained
with Coomassie Brilliant Blue.

dTTPase Activity Assay—Assays for the dTTPase activity were car-
ried out in a mixture (10 µl) containing 30 mM Tris-HCl, pH 7.5, 10 mM
MgCl₂, 10 mM dTT, 5 mM dTTP, and 5 µCi of [α-32P]dTTP (0.1 mg/ml
bovine serum albumin, and dilutions of the different gene 4 proteins.

Reactions were carried out for 15 min at 30 °C and stopped by the
addition of 5 µl of 0.5 M EDTA. Aliquots of 2 µl were spotted onto a
polyethylene-cellulose thin layer chromatography plate and dTTPase activity was measured from dTTP by thin layer chromatography (19). The amount of dTTP hydrolyzed
to dTDP was measured using a PhosphorImager (Fuji).

The inhibition of ssDNA-dependent dTTPase activity of the full-
length gene 4 protein by either gp4-C219 or gp4-C272 was examined by
incubating the two proteins together for 10 min at 4 °C, and then
 aliquots were added to the reaction mixture described above that also
 contained 0.5 µM ssDNA.

Analysis of the Interaction of Gene 4 Proteins with T7 DNA Poly-
merase by Surface Plasmon Resonance—Surface plasmon resonance (28)
was carried out using a BiACore instrument. A stable gp4-T7 DNA
polymerase complex has been observed during the slow dissociation
phase (11), and in this study we examined the stoichiometry of this
stable protein complex. T7 DNA polymerase-thioredoxin complex was
used as the chip (29), and upon binding to the chip the two proteins
antibodies were covalently bound to a CM5 chip via its amine groups
using procedures described by the manufacturer, using a 7-min pulse of
the antibody at a concentration of 50 µg/ml in 10 mM sodium acetate,
pH 5. Flow Buffer contained 10 mM Heps, pH 7.5, 250 mM potassium
glutamate, 10 mM MgCl₂, 1% glycerol, 0.05% Tween 20, and 5 mM
DTT. The flow rate used in all experiments was 5 µl/min. A solution of native
T7 DNA polymerase (a one-to-one complex of gene 5 protein and thi-
oredoxin) (27) was passed over the chip at a concentration of 20
µg/ml for 10 min. Nonspecifically bound T7 DNA polymerase/thioredoxin
was removed by washing with 30 µl of 1 M NaCl in Flow Buffer. Each gene
4 protein sample was injected at least twice at a concentration of 20
µg/ml (25 µl each time) to ensure that binding to T7 DNA polymerase
was saturated. Between the injections of different gene 4 protein
samples, the chip was regenerated by flushing with 25 µl of 1 M NaCl in
Flow Buffer washed away any weakly retained gene 4 proteins. Because only the stable protein complex is focused on
this study, the stoichiometry of the two bound proteins refers to the
protein complex retained on the chip surface during the slow dissocia-
tion phase after Flow Buffer washed away any weakly retained gene 4
proteins.

The Effect of gp4-C272 on the Growth of Wild-type Bacteriophage T7
Cell C600 (22) was transformed with the plasmid that produced
the gene 4 protein fragment gp4-C272 (see above) or the full-length
gene 4 protein as a control. Cells were grown in LB media containing
100 µg/ml ampicillin at 37 °C. When the A600 reached 0.5, phase T7
were added at a multiplicity of infection of 1 and incubated at 37 °C
without shaking. After 10 min, 1 ml of cells were centrifuged for 30 s,
and the cells were washed with 1 ml of LB media to remove the
unabsorbed phage. The cells were washed a second time, then taken up
in 1 ml of LB, and incubated at 37 °C for 60 min to allow lysis to occur.
Dilutions of the lysate were titrated on E. coli C600.

RESULTS

Overproduction and Purification of Carboxyl-terminal Frag-
ments of Gene 4 Protein—In order to investigate the function of
the region linking the primase and helicase domains of T7 gene
4 protein (Fig. 1), we constructed truncations of the gene that
express three carboxyl-terminal fragments of the gene 4 protein.
Two fragments start just before this region (initiating at residues
219 and 241) and are referred to here as gp4-C219 and
gp4-C241, whereas the third starts just after this region (gp4-
C272). Proteins gp4-C219 and gp4-C241 correspond to previ-
ously identified proteolysis fragments, and gp4-C272 is three
residues longer than another proteolysis fragment that begins at
residue 275 (25, 30). In order to facilitate purification of
these overproduced fragments, and to ensure that the amino

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FIG. 1. Organization of the gene 4 protein. Boundaries for the primase and helicase domains are defined according to Ilyina et al. (21). The hatched boxes indicate conserved motifs within homologous primases (left half) and helicases (right half). The sequences in the bottom three rows correspond to the amino termini of the three carboxy-terminal fragments described in this paper; the starting residue of each is shown on the left. Each of the three fragments has a His-tag fused to its amino terminus, having the sequence Gly-His-His-His-His-His-His-His-Asp-Ser-Glu-His-Ile-Asp-Asp-Asp-Asp-Asp-Asp-Lys-His-Met. The dark region indicates the residues linking the primase and helicase domains.

In contrast, gp4-C272 migrates as a monomer (data not shown). Whereas the gp4-C272 fragment (like gp4-C219 and gp4-C241) has a 23-residue tail containing the His-tag at its amino terminus (Fig. 1), this appendage has no effect on its ability to oligomerize; we have overproduced and purified the untagged form of gp4-C272, and this protein also migrates as a monomer (data not shown).

Oligomerization Is Essential for dTTPase Activity—gp4 has a dTTPase that provides the energy for translocation and helicase activities on ssDNA molecules (19). gp4 also has an inherent dTTPase activity that can be stimulated 50–100-fold by ssDNA (32). As one measure of the functional integrity of the helicase domains in gp4-C219, gp4-C241, and gp4-C272, we compared their specific dTTPase activities to that of gp4 in the absence of DNA effectors. Because the different peptides have markedly different abilities to form oligomers, and because previous results suggested that oligomerization might be necessary for dTTPase activity and helicase activity (31, 33), we determined the steady-state $k_{\text{cat}}$ values for dTTPase activity of each protein at varying protein concentrations.

The $k_{\text{cat}}$ value of dTTPase activity for gp4 was moderately sensitive to protein concentration; varying the concentration of monomers from 0.6 to 6 $\mu$M increased the $k_{\text{cat}}$ by 10-fold (Fig. 3). This result suggests that the $K_d$ for oligomerization of gp4 is at least 2 to 3 $\mu$M. In contrast, the $k_{\text{cat}}$ values for gp4-C219 and gp4-C241 were affected less than 2-fold upon variation of the protein concentrations over the same range, suggesting the $K_d$ for oligomerization to be less than 1 $\mu$M. The $K_m$ (dTTP) for gp4, gp4-C241, and gp4-C241 was approximately 100 $\mu$M.

The $k_{\text{cat}}$ value of dTTPase activity for gp4-C272 is affected dramatically by protein concentration; a 10-fold increase in protein concentration (from 6 to 60 $\mu$M monomers) increased $k_{\text{cat}}$ by more than 100-fold (Fig. 3). We estimate the $K_d$ for oligomerization to be between 30 and 40 $\mu$M. Interestingly, at 100 $\mu$M gp4-C272, the $k_{\text{cat}}$ value was comparable to that for gp4 assayed at 0.6 $\mu$M. At 50 $\mu$M gp4-C272, the $K_m$ (dTTP) was approximately 1 mM.

Based on the data presented in Figs. 2 and 3, we conclude that dTTPase activity requires oligomerization, even in the absence of DNA. Furthermore, they indicate that gp4-C272 does indeed form oligomers, but only at very high concentrations, much higher than the concentrations used for the native gel electrophoresis (Fig. 2D). Finally, the data with gp4-C219 and gp4-C241 suggest that these two proteins form oligomers that are considerably more stable than are those formed by gp4. Thus the linker region (residues 241 to 272) between the helicase and primase domains has a major effect on the ability of the protein to oligomerize, and hence on its dTTPase activity.

Mixed Oligomers of gp4 and Either gp4-C219 or gp4-C242 Have Reduced dTTPase Activity—Since both gp4-C219 and gp4-C242 can each form oligomers and catalyze the hydrolysis of dTTP, it was of interest to determine whether they can form mixed oligomers with gp4, and whether such mixtures would also have dTTPase activity. We addressed this question in two ways. First, we examined the ssDNA-dependent dTTPase activity of a mixture of gp4 and either gp4-C219 or gp4-C242. Second, we carried out native polyacrylamide gel electrophoresis to screen for the presence of mixed oligomer species.

The dTTP hydrolysis catalyzed by gp4 is stimulated 50–100-fold by the presence of M13 ssDNA (32). In contrast, the dTTPase activity catalyzed by either gp4-C219 or gp4-C242 is...

![Native gel electrophoretic analysis of gene 4 polypeptides.](http://www.jbc.org/content/30305/36/32763/FIG-2)
unaffected by the presence of M13 ssDNA. When either gp4-C219 or gp4-C272 is mixed with gp4, they each dramatically inhibit the dTTPase activity of gp4 on M13 ssDNA (Fig. 4). A 5-fold reduction in the dTTPase activity is observed in the presence of a one-to-one molar ratio of gp4 to gp4-C272 and in the presence of a one-to-five molar ratio of gp4 to gp4-C219. As a control, the addition of a 40-fold excess of a T7 primase fragment (23) with a His-tag at its amino terminus has no effect on the dTTPase activity of the full-length gene 4 protein, ruling out the possibility that the histidine tag might be causing this inhibition. These results show that both gp4-C219 and gp4-C272 form hetero-oligomers that have greatly reduced ssDNA-dependent dTTPase activity compared with gp4 alone.

In order to examine physically whether gp4 can form a complex with gp4-C219, gp4-C241, or gp4-C272, we compared the electrophoretic mobility of each species on native polyacrylamide gels, either alone or in combination (Fig. 5). The samples contained Mg²⁺ and ATP, which have been shown to promote the oligomerization of full-length gene 4 protein (34).

In the presence of Mg²⁺ and ATP, gp4, gp4-C219, and gp4-C241 predominantly form hexamers (Fig. 5, lanes 1, 5, and 6), whereas gp4-C272 forms exclusively monomers (Fig. 5, lane 7). When either gp4-C219 or gp4-C241 is mixed with gp4, the mixtures migrated as a smear between the hexameric gp4 and hexameric gp4-C219 (Fig. 5, lane 2) or gp4-C241 (Fig. 5, lane 3), indicating that hetero-oligomers had formed. In contrast, the presence of the monomeric gp4-C272 had little or no effect on the mobility of gp4 (Fig. 5, lane 4), indicating that the interaction between these two proteins is too weak to allow the complex to remain intact during gel electrophoresis.

**Stable Complexes of Gene 4 Protein Fragments and T7 DNA Polymerase—**Specific interactions between gp4 and T7 DNA polymerase are essential for both leading and lagging strand synthesis. During leading strand synthesis, the helicase activity of gp4 promotes strand displacement DNA synthesis. During lagging strand synthesis, the primase activity of gp4 catalyzes the synthesis of a tetraribonucleotide that is used to initiate the synthesis of Okazaki fragments. Specific interactions between the helicase domain of the gp4 and T7 DNA polymerase have been demonstrated both functionally (9) and physically (10, 11). Notarnicola et al. (11) showed that the acidic carboxyl-terminal tail of gp4 is essential for interaction with T7 DNA polymerase. Since the helicase domain, including the acidic carboxyl-terminal tail, is intact in the three carboxyl-terminal fragments, we examined their ability to bind to T7 DNA polymerase.

The interactions between each of the gene 4 protein fragments and T7 DNA polymerase were analyzed by surface plasmon resonance (Fig. 6). This technique was used previously to analyze the interactions of a domain of T7 DNA polymerase with its processivity factor, thioredoxin (29); T7 DNA polymerase reactions were carried out as described under “Experimental Procedures.” Each mixture contained 0.01 μM M13 ssDNA molecules, 0.3 μM gp4 (where indicated), and the indicated amounts of either gp4-C219 or gp4-C272 (expressed as monomers). Prior to each reaction, gp4 was mixed with gp4-C219 or gp4-C272 in 30 mM Tris-HCl, pH 7.5, 0.1 mg/ml bovine serum albumin, and 10 mM DTT for 10 min at 25 °C. The proteins were then added to the dTTPase reaction mixture, and the reaction was allowed to proceed for 15 min at 30 °C. The amount of dTTP hydrolyzed to dTDP was measured by thin layer chromatography. The dTTPase activity of the full-length gene 4 protein alone is represented as 100%. 0.3 μM gp4 plus indicated concentrations of gp4-C219. □, gp4-C219 alone. ■, 0.3 μM gp4 plus indicated concentrations of gp4-C219. Fragment gp4-C272 alone had no detectable dTTPase activity at the protein concentrations tested (not shown).

**Fig. 3.** $k_{\text{cat}}$ value of dTTPase activity as a function of gene 4 protein concentration. dTTPase activity was measured in the presence of 5 mM dTTP as described under “Experimental Procedures.” The $k_{\text{cat}}$ values (nanomoles of dTDP formed per s/nmol of monomers of polypeptides) were plotted as a function of the concentration of either gp4, gp4-C219, gp4-C241, or gp4-C272.

**Fig. 4.** Inhibition of the ssDNA-dependent dTTPase activity of the full-length gene 4 protein (gp4) by gp4-C241 and gp4-C272. dTTPase reactions were carried out as described under “Experimental Procedures.” Each mixture contained 0.01 μM M13 ssDNA molecules, 0.3 μM gp4 (where indicated), and the indicated amounts of either gp4-C219 or gp4-C272 (expressed as monomers). Prior to each reaction, gp4 was mixed with gp4-C219 or gp4-C272 in 30 mM Tris-HCl, pH 7.5, 0.1 mg/ml bovine serum albumin, and 10 mM DTT for 10 min at 25 °C. The proteins were then added to the dTTPase reaction mixture, and the reaction was allowed to proceed for 15 min at 30 °C. The amount of dTTP hydrolyzed to dTDP was measured by thin layer chromatography. The dTTPase activity of the full-length gene 4 protein alone is represented as 100%. 0.3 μM gp4 plus indicated concentrations of gp4-C219. □, gp4-C219 alone. ■, 0.3 μM gp4 plus indicated concentrations of gp4-C219. Fragment gp4-C272 alone had no detectable dTTPase activity at the protein concentrations tested (not shown).

**Fig. 5.** Formation of mixed oligomers between full-length gene 4 protein (gp4) and gp4-C219, gp4-C241, or gp4-C272. Lane 1, gp4 alone. Lane 2, gp4 plus gp4-C219. Lane 3, gp4 plus gp4-C241. Lane 4, gp4 plus gp4-C272. Lane 5, gp4-C219 alone. Lane 6, gp4-C241 alone. Lane 7, gp4-C272 alone. Each lane contains 20 μg of each protein. Protein samples were incubated in 20 mM Tris-HCl, pH 7.5, 2 mM ATP, 4 mM MgCl₂, and 10 mM DTT for 15 min at 23 °C prior to gel electrophoresis. Molecular mass markers are indicated on the left.

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*S. Guo and C. C. Richardson, unpublished observations.
*S. Guo and C. C. Richardson, unpublished data.

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**FIG. 3.** $k_{\text{cat}}$ value of dTTPase activity as a function of gene 4 protein concentration. dTTPase activity was measured in the presence of 5 mM dTTP as described under “Experimental Procedures.” The $k_{\text{cat}}$ values (nanomoles of dTDP formed per s/nmol of monomers of polypeptides) were plotted as a function of the concentration of either gp4, gp4-C219, gp4-C241, or gp4-C272.
Hexamer Domain of T7 Helicase

T7 helicase is a single polypeptide, capable of performing both helicase and primase activities. Sequence alignment (21), electron microscopy (17), and limited proteolysis (24, 25) provide evidence for separate domains in the full-length protein responsible for each of the two activities. Expression of truncated genes encoding either the amino- or carboxyl-half of the protein results in peptides that retain primase (23) or helicase (25) activity, respectively. The tandem placement of the two domains on the same polypeptide may facilitate the regulation of these two activities that play such important roles in leading and lagging strand DNA synthesis at a replication fork.

Oligomerization of the gene 4 protein plays a critical role in both helicase and primase functions of the protein. Analysis of the native molecular weight of the primase fragment, using a series of gels of different percentages of polyacrylamide, indicates that it migrates on the native gel as a monomer. This observation contradicts an earlier report by Frick et al. (23) that suggested that the primase fragment, although not forming a hexamer, was a dimer. We believe that this discrepancy is due to the use of only one polyacrylamide gel concentration (10%) in the earlier report and the large variability that can be observed in the apparent molecular weights of proteins determined by this method depending on the percentage of the polyacrylamide gel used (39, 40). We find that mobility of the primase fragment on a single 18% gel corresponds to that of a monomer. Whereas the primase fragment alone catalyzes the synthesis of oligoribonucleotides, it binds DNA less tightly than does gp4 and is thus dependent on its association with the helicase domain to transport it along ssDNA to a primase recognition site (23).

Native gel electrophoresis (32, 41) and electron microscopy (17, 32) provide evidence that the gene 4 protein is primarily a hexamer in both the presence and absence of DNA. Although nucleotides are not required for hexamer formation, they stabilize the complex (31, 32, 34), even in the absence of Mg

Dissociation constant K0.2 of 5 nM (35). We coupled a monoclonal antibody to thioredoxin to the solid support on the sensor chip, and we tested the chip to ensure that gp4 and its fragments are not retained nonspecifically (data not shown). Then a one-to-one complex of T7 DNA polymerase and thioredoxin was bound to the chip via the antibody (A). The following four gene 4 proteins were then passed over this complex in separate experiments: gp4 (B), gp4-C219 (C), gp4-C241 (D), and gp4-C272 (E). In each case each of the four proteins was injected at least twice to ensure that the binding sites on T7 DNA polymerase were saturated. The molar ratios between bound proteins was injected at least twice to ensure that the binding sites on T7 DNA polymerase and gene 4 proteins are obtained by dividing their RU ratio with their molecular weight ratio and are summarized in Table I. We coupled a monoclonal antibody to thioredoxin to the chip via the antibody (A). The following four gene 4 proteins were then passed over this complex in separate experiments: gp4 (B), gp4-C219 (C), gp4-C241 (D), and gp4-C272 (E). In each case each of the four proteins was injected at least twice to ensure that the binding sites on T7 DNA polymerase were saturated. The molar ratios between bound proteins was injected at least twice to ensure that the binding sites on T7 DNA polymerase and gene 4 proteins are obtained by dividing their RU ratio with their molecular weight ratio and are summarized in F. gp4 binds stably to T7 DNA polymerase (Fig. 6B). Based on the change in RU values, a molar ratio of two monomers of gp4 were bound for each T7 DNA polymerase, suggesting that a dimer of gp4 binds a monomer of T7 DNA polymerase. Likewise, both gp4-C219 and gp4-C241 also each interacted with T7 DNA polymerase stably in a two-to-one ratio (Fig. 6C and D), suggesting that they also bind the polymerase as dimers. In contrast, whereas gp4-C272 interacts tightly with the T7 DNA polymerase (Fig. 6E), the stoichiometry of its binding is one monomer of gp4-C272 per T7 DNA polymerase. These data further reveal the monomeric nature of gp4-C272, in contrast to the propensity of the other gene 4 protein fragments to form oligomers.

Of the gene 4 protein, a single polypeptide, functions as both a helicase and a primase. Sequence alignment (21), electron microscopy (17), and limited proteolysis (24, 25) provide evidence for separate domains in the full-length protein responsible for each of the two activities. Expression of truncated genes encoding either the amino- or carboxyl-half of the protein results in peptides that retain primase (23) or helicase (25) activity, respectively. The tandem placement of the two domains on the same polypeptide may facilitate the regulation of these two activities that play such important roles in leading and lagging strand DNA synthesis at a replication fork.

**Discussion**

T7 gene 4 protein, a single polypeptide, functions as both a helicase and a primase. Sequence alignment (21), electron microscopy (17), and limited proteolysis (24, 25) provide evidence for separate domains in the full-length protein responsible for each of the two activities. Expression of truncated genes encoding either the amino- or carboxyl-half of the protein results in peptides that retain primase (23) or helicase (25) activity, respectively. The tandem placement of the two domains on the same polypeptide may facilitate the regulation of these two activities that play such important roles in leading and lagging strand DNA synthesis at a replication fork.

**Table I**

| Host       | pfu/cell* |
|------------|-----------|
| C600       | 150 ± 15  |
| C600/pGP4(C-272)** | <0.2     |

* Plaque-forming unit per cell was determined as described under “Experimental Procedures.” The values represent an average of three experiments.

**gp4-C272** expresses the gene encoding gp4-C272 without the His-tag (see “Experimental Procedures”).

per cell when infecting *E. coli*, the burst is <0.2 plaque-forming units per cell when infecting cells that are producing gp4-C272 from a plasmid. Thus this carboxyl-terminal fragment is dominantly lethal for growth of wild-type T7 phage in vitro, consistent with its inhibition of the dTTPase activity of gp4 in vitro.
ues 241–271) and the effect that this region has on oligomerization and dTTPase activity. The fact that the primase fragment is monomeric, and that the helicase fragment gp4-C241 retains the ability to form hexamers (25), indicates that the regions critical for oligomerization reside downstream of residue 241. In the present study we show that the linker between residues 241 and 271 is critical for stable oligomer formation, since deletion of this region results in a peptide that is a monomer during electrophoresis on a native gel and has severely diminished dTTPase activity. On the other hand, our data show that this region alone is not sufficient for oligomer formation. First, the primase fragment that terminates at residue 271 is a monomer based on native gel electrophoresis. Second, gp4-C272 inhibits the ssDNA-dependent dTTPase activity of gp4 and is dominantly lethal in vivo, observations that strongly suggest that this protein retains its ability to interact with the wild-type gp4, albeit transiently. Finally, the fact that $k_{cat}$ for dTTPase activity of gp4-C272 increases with protein concentration indicates that the dTTPase activity is dependent on its ability to oligomerize. Therefore, other regions on the helicase domain must also contribute to the hexamer formation of gene 4 protein.

Although previous data have suggested that dTTPase activity of gene 4 protein is dependent on oligomerization (13, 31), our analysis demonstrates this point definitively. The $k_{cat}$ values for dTTPase of gp4-C219 and gp4-C241 are less sensitive to protein concentration than is that of gp4, indicating that the oligomers formed by these two proteins are more stable than are those formed by gp4. In contrast, and most impressively, the $k_{cat}$ value for dTTPase of gp4-C272 varies more than 100-fold with protein concentration. This variation indicates that oligomerization of gp4-C272 is essential for dTTPase activity and that the stability of the oligomers formed is greatly diminished compared with that of the other proteins analyzed in this study.

Washington et al. (24) described the properties of a large number of randomly generated mutations in the gene 4 protein. One, threonine 257 to alanine, lies in the linker that is the focus of this study. This mutant has higher DNA-independent dTTPase activity than wild-type gene 4 protein but lower helicase activity. In addition, native gel electrophoresis carried out in this earlier study (Fig. 6A in Washington et al. (24)) showed a significantly lower amount of monomer species formed with this mutant. These results suggest that this mutationally altered protein forms more stable oligomers than does the wild-type gene 4 protein.

Both Notarnicola et al. (32) and Washington et al. (24) characterized mutants in motif 4 of the helicase domain (Fig. 1) that arginine 487 is changed to a cysteine (24). This mutant protein forms oligomers more efficiently than does wild-type protein, and its ssDNA-independent dTTPase activity is 7-fold higher than that of the wild-type protein. The three mutant proteins in motif 4 investigated by Notarnicola et al. (32) (H475A, D485G, and R487A) and the three investigated by Washington et al. (24) (R487C, G488D, and S496F) are all impaired in their ability to bind to ssDNA, in particular R487A and S496F. These data suggest that the primary role of this motif is in the binding to ssDNA; the modest effect on oligomerization observed with some of these mutants could be either an indirect one due to the interrelationship of oligomerization, DNA binding, and dTTPase activity or perhaps this region indeed makes a direct although minor contribution to the interaction of subunits.

Our results suggest that although the linker region described in this report (residues 242–271) plays a direct role in oligomerization of the gene 4 protein, as an indirect consequence it affects the dTTPase active site by influencing the conformation of the dimer interface. Immediately adjacent to this linker region toward the carboxyl terminus lies the conserved motif 1 of the helicase domain (Fig. 1), also known as the “Walker A motif” (44). This motif has been shown to make up part of the NTP hydrolysis active site in F$_1$-ATPase (45). Mutations in this motif in gene 4 protein reduce or eliminate its dTTPase activity (26, 38). The juxtaposition of these two regions critical to dTTPase is likely to have important consequences regarding the regulation of this activity.

Immediately adjacent to this linker region toward the amino terminus of the gene 4 protein is the conserved motif 6 of the primase domain (Fig. 1), a motif shared by the primases of other bacteria and bacteriophage (21). Although the exact function of motif 6 remains unknown, it is believed that this region in the E. coli primase may be involved in binding of Mg$^{2+}$ (46). Furthermore, in a proposed tertiary model of the Toprim domain in the DnaG-type primases and type II topoisomerase, motif 6 overlaps the last strand of the Toprim domain of primases (47). The juxtaposition of motif 6 of the primase domain and the region essential for oligomerization of the helicase is intriguing. As the helicase is translocating 5' to 3' on the lagging strand, it must stop when it reaches a primase recognition site to allow the primase domain to synthesize an RNA primer in the opposite direction. It is likely that the linker region described here plays a role in coordinating these two activities.

In vivo gp4 functions in a replication complex with the T7 DNA polymerase and T7 DNA-binding protein. In vitro, specific interactions between gp4 and the T7 DNA polymerase have been demonstrated (9–11), although there are presently no data regarding the stoichiometry of the two proteins in the complex. In this study we demonstrate that gp4, gp4-C219, and gp4-C241 each bind to the DNA polymerase as a stable dimer. It is interesting that a dimer rather than a hexamer of each of these species forms a stable complex with the T7 DNA polymerase. Although we cannot rule out the possibility of steric restriction being responsible for the inability of hexamer formation, we speculate that the interaction of the polymerase with one subunit of gp4 enables this gp4 subunit to in turn interact stably with one more gp4 subunit. The fact that gp4-C272 binds to T7 DNA polymerase as a monomer is consistent with its diminished ability to form oligomers with itself.

It has been suggested that the helicase domain of T7 gene 4 protein beginning at residue 306 has the same secondary structure as the known structures of RecA protein and F$_1$-ATPase (24). Both RecA protein and F$_1$-ATPase bind ATP, and the ATP-binding domains are superimposable with a root mean squared deviation of less than 2 Å (45, 48). Besides forming a helical filament on DNA, RecA also forms hexameric rings and may be a structural homologue of ring helicases (49). Analogous to gene 4 protein, both RecA protein and F$_1$-ATPase contain a region amino-terminal to the nucleotide-binding domain that plays a critical role in oligomer formation (48, 50). This similarity suggests that there may be conservation in the mechanism of oligomerization in these three proteins.

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Note Added in Proof—The crystal structure of the gene 4 protein fragment gp4-C272 has recently been determined (Sawaya, M. R., Guo, S., Tabor, S., Richardson, C. C., and Ellenberger, T. (1999) Cell 99, in press).
