The Acidic Carboxyl Terminus of the Bacteriophage T7 Gene 4 Helicase/Primase Interacts with T7 DNA Polymerase

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The gene 4 proteins of bacteriophage T7 provide both primase and helicase activities at the replication fork. Efficient DNA replication requires that the functions of the gene 4 protein be coordinated with the movement of the T7 DNA polymerase. We show that a carboxyl-terminal domain of the gene 4 protein is required for interaction with T7 DNA polymerase during leading strand DNA synthesis. The carboxyl terminus of the gene 4 protein is highly acidic: of the 17 carboxyl-terminal amino acids 7 are negatively charged. Deletion of the coding region for these 17 residues results in a gene 4 protein that cannot support the growth of T7 phage. The purified mutated gene 4 protein has wild-type levels of both helicase and primase activities; however, DNA synthesis catalyzed by T7 DNA polymerase on a duplex DNA substrate is stimulated by this mutant protein to only about 5% of the level of synthesis obtained with wild-type protein. The mutant gene 4 protein can form hexamers and bind single-stranded DNA, but as determined by native PAGE analysis, the protein cannot form a stable complex with the DNA polymerase. The mutant gene 4 protein can prime DNA synthesis normally, indicating that for lagging strand synthesis a different set of helicase/primase-DNA polymerase interactions are involved. These findings have implications for the mechanisms coupling leading and lagging strand DNA synthesis at the T7 replication fork.

The economy of proteins involved in the replication of the linear double-stranded DNA chromosome of bacteriophage T7 has made it an attractive model for dissecting the protein-protein interactions that are essential for coordination of the multiple reactions that occur at a replication fork (1). The four proteins that account for the basic reactions at the T7 replication fork are T7 gene 5 DNA polymerase, the host Escherichia coli thioredoxin, T7 gene 4 helicase/primase, and the T7 gene 2.5 single-stranded DNA (ssDNA)1 binding protein. The specific interactions that occur among these relatively few proteins are essential for T7 DNA replication and, hence, phage growth (1–3). For example, the binding of E. coli thioredoxin and T7 gene 5 protein forms a stable one-to-one complex with the ability to catalyze the template-directed polymerization of nucleotides in a highly processive manner (4–11). This dramatic effect of a specific protein-protein interaction has led to the identification of a unique 76-residue domain in the T7 gene 5 protein that is responsible for this interaction with its processivity factor (4–7).

In addition to the interaction of the gene 5 protein with thioredoxin, each of the three phage-encoded replication proteins interacts with one another. The gene 5 protein interacts with the hexameric gene 4 protein that provides both helicase and primase activities at the replication fork (12). In turn, the essential gene 2.5 ssDNA binding protein interacts with both the polymerase and the gene 4 protein, interactions that enhance both polymerase and primase activities (12–14).

The interactions of the T7 DNA polymerase with the T7 gene 4 protein warrant specific attention because these interactions are thought to coordinate leading and lagging strand DNA synthesis at the replication fork (3). In considering the interactions between these two proteins, it is important to consider the multiple reactions catalyzed by the gene 4 protein. Gene 4 encodes two co-linear polypeptides of 56 and 63 kDa. The 56-kDa form is translated from an internal initiation codon that is in-frame with the coding sequence for the 63-kDa protein (15). Both forms of the protein have helicase activity, bind ssDNA in the presence of a nucleoside triphosphate, and translocate 5′ to 3′ along the DNA strand using the energy of nucleoside 5′-triphosphate hydrolysis (15–18). Upon encountering duplex DNA, the gene 4 protein separates the strands processively, provided that there are 6 to 7 unpaired nucleotides on the 3′ strand (19). The T7 DNA polymerase-thioredoxin complex by itself is unable to synthesize DNA if it encounters double-stranded regions of DNA and so requires the strand separation ability of the helicase to replicate a duplex template (20, 21).

The 63-kDa gene 4 protein has 63 amino acids at its amino terminus that are not found on the 56-kDa protein, and this domain contains a Cys5 zinc binding motif (22, 23). The 63-kDa gene 4 protein catalyzes the template-directed synthesis of oligoribonucleotides at specific recognition sites on ssDNA, a reaction that is dependent on the presence of the zinc binding motif (18, 22–26). T7 DNA polymerase then uses these oligoribonucleotides as primers to initiate synthesis on ssDNA templates. Inasmuch as the 63-kDa gene 4 protein has both helicase and primase activities, it alone is sufficient to support T7 DNA replication and phage growth (27, 28).

The quaternary structure of the active form of the gene 4 protein, with regard to both helicase and primase activities, is a hexamer (29–31). In vivo the hexamer is most likely composed of both small and large forms of the gene 4 protein (32). Bound ssDNA appears, in electron micrographs, to pass through the center of the ring-shaped gene 4 hexamer (33). It would not be an oversimplification to conclude that this observation most likely explains the requirement for gene 4 protein.

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1 The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; RU, resonance units; bp, base pair.
form a hexamer to bind ssDNA (29). Studies using a nucleotide binding site mutant have shown that translocation of the gene 4 protein on ssDNA is inhibited when the hexamer consists of a mixture of wild-type monomers and monomers of the nucleotide binding site mutant gene 4 protein (32). These latter studies show that the subunits within a hexamer must interact in a coordinated manner to translocate on ssDNA and to function as a helicase and a primase.

The potential interactions between the gene 4 protein and T7 DNA polymerase are numerous, as evidenced by the above considerations, and suggest that the gene 4 protein may play a pivotal role in coordinating leading and lagging strand DNA synthesis since it functions in both. First, an interaction of the two proteins would appear essential to coordinate the movement of the polymerase with that of the helicase as it unwinds the duplex during leading strand synthesis. Second, on the lagging strand the primase must stabilize the newly synthesized tetra-ribonucleotides until they are extended by the polymerase (26, 34, 35). The ability of the polymerase to use the oligoribonucleotides as primers is dependent on the presence of the gene 4 protein, a requirement that dictates an interaction between the two proteins. Finally, the coupling of leading and lagging strand DNA synthesis at the T7 replication fork (3) most likely relies on the ability of the gene 4 hexamer to interact with both the leading and lagging strand DNA polymerases.

Earlier studies demonstrated an interaction between T7 DNA polymerase and gene 4 protein in the presence and absence of M13 ssDNA (12, 36), and numerous studies have provided indirect evidence of physical interactions based on the activities of the two proteins (3, 20, 21, 26, 34, 35). In this report we show a direct interaction of the T7 DNA polymerase and the gene 4 protein and identify the acidic carboxyl terminus of the gene 4 protein as an essential domain for this interaction. Furthermore, we find that an altered gene 4 protein that cannot interact with T7 DNA polymerase is, nevertheless, capable of catalyzing the unwinding of duplex DNA. At a replication fork the helicase activity of the mutant protein is uncoupled from the polymerization reaction resulting in a cessation of DNA synthesis.

**EXPERIMENTAL PROCEDURES**

**Materials**

*Bacterial Strains and Bacteriophage*—*E. coli* HMS174(DE3) was used for protein production (37), and *E. coli* DH5α (Life Technologies, Inc.) was used for subcloning and complementation analysis. Bacteriophage T7 wild-type (38) and T7 Δ4-1 lacking gene 4 have been described (39).

*DNA and Enzymes*—The mutant T7 gene 4 carboxyl-terminal deletion protein was purified following the procedure described previously for the wild-type gene 4 protein (29). The gene II endonuclease of bacteriophage Φ1 was purified from *E. coli* strain DH5α/pM13/pDG117IIa-G79A (provided by K. Horiiuchi, National Institute of Genetics, Japan) as described (40). T7 DNA polymerase (gene 5 protein-thioredoxin complex), native and Δ28, has been described (41). The plasmid used as a substrate for the gene II protein, pET24a(+), containing a bacteriophage Φ1 origin of replication sequence, was purchased from Novagen. M13mp6 ssDNA was prepared as described (42). Oligoribonucleotides were synthesized by the core facility of the Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School.

**Methods**

*Mutagenesis and Complementation Analysis*—*In vitro* mutagenesis of bacteriophage T7 gene 4 to delete the region encoding the last 17 amino acids of the protein was accomplished using T7 DNA as the template in the polymerase chain reaction. The following oligoribonucleotides were used: (CTD) 5′-AATCCACAGGTGGTTAACCCGTAACCTGAGGTATCCGCGCC-3′ is complementary to T7 nucleotides 13186–13211 with a new termination codon (bold type) and an introduced PfMI site (underlined), and (SN101 5′-CTGGGGTGGTGGCCTGCTAC-3′, which is complementary to T7 bases 12931–12948. Amplification of the DNA was performed using “Ultrim” DNA polymerase from Perkin-Elmer, and the resulting 301-bp DNA fragment was purified, cleaved with PfMI and AflI, and inserted into pP4A4. Each of the 20 nucleotide mutants (32) that were made with the same primer pairs described above was cloned in a new region which was confirmed to determine that no undesired changes were present. The complementation analysis was performed as described previously (29).

**Nucleotide Hydrolysis Assay**—The hydrolysis of dTTP by gene 4 protein in the presence of ssDNA was measured as described previously (32). Each 20-μl reaction contained 200 nM gene 4 protein, 50 μM M13mp6 ssDNA (nucleotide equivalents), and the indicated concentration of [α32P]dTTP in dTTPase buffer (40 mM Tris–HCl, pH 7.5, 50 mM NaCl, 10 mM MgCl2, and 10 mM DTT). The reaction mixtures were incubated at 30 °C for 20 min, and the amount of dTTP converted to dTDP was determined by polyethyleneimine-cellulose thin layer chromatography and Phosphor-Image analysis (Molecular Dynamics).

**Helicase and Primase Assays**—The DNA used as a substrate in the helicase assay consisted of circular M13mp6 ssDNA annealed to a 36-base radiolabeled oligonucleotide, 5′-[α32P]-GGATCGGGAATTCG-GAACCTAAGGCCTAAGCG-3′. The 20-5′-bases of the oligonucleotide are complementary to M13mp6 ssDNA, and the 16-3′-bases do not base pair, so that after annealing the oligonucleotide has a 16-base 3′-single stranded tail. T7 gene 4 helicase requires a 3′-tail of at least 8 nucleotides to initiate strand separation (19). The 36-mer was 5′-end labeled using [γ32P]-ATP and T4 polynucleotide kinase. The substrate was assembled by incubating the M13 DNA with the radiolabeled oligonucleotide in a slight molar excess, at 65 °C for 5 min, and cooling to 30 °C over 20 min. The substrate was used in the helicase reaction without further purification.

The helicase reaction mixture (60 μl) consisted of 20 mM helicase substrate, 10 nM gene 4 protein, 2 mM dTTP, and dTTPase buffer. The reaction mixture was incubated at 30 °C, samples were removed at the indicated time, and the reaction was stopped by adding EDTA to 20 mM. Helicase activity was measured by separation of the DNA in the reaction in a nondenaturing agarose gel followed by Phosphor-Image analysis to quantify the amount of radiolabeled DNA in each band. Helicase activity was reported as the percentage of oligonucleotide displaced from the M13 DNA.

The primase activity of the gene 4 proteins was measured as described previously (29). The reaction mixtures (20 μl) contained dTTPase buffer, 50 mM potassium glutamate, 40 μM M13mp6 ssDNA (nucleotide equivalents), 5 nM T7 DNA polymerase, 300 μM each nucleotide (dTTP, dCTP, dGTP, and dTTP), 0.3 mM each of ATP, [α32P]CTP, 10 nM M13 ssDNA, and 60 nM gene 4 protein. After incubation at 30 °C for 5 or 10 min, the reaction was stopped by the addition of EDTA to 20 mM. The products of the reaction were examined by electrophoresis in a 25% polyacrylamide gel containing 2 M urea.

**Strand Displacement DNA Synthesis**—DNA synthesis catalyzed by T7 DNA polymerase and gene 4 protein through regions of dsDNA was examined using two different DNA substrates. One assay used a double-stranded plasmid containing a single site-specific nick, and the second assay used a 70-nucleotide circular dsDNA molecule containing a preferred replication fork.

In the first assay the DNA substrate containing a site-specific nick was constructed by incubating plasmid DNA containing the cloned Φ1 origin of replication with the bacteriophage Φ1 gene II endonuclease. The gene II endonuclease introduces a single phosphoester bond cleavage at a specific site within the Φ1 origin of replication (40). The nicking reaction (25 μl containing 10 pmol of [α32P]-labeled DNA, 20 nM Tris–HCl, pH 8.0, 80 mM KCl, 5 mM MgCl2, 5 mM DTT, and 10 nM of gene II protein were incubated at 30 °C for 30 min. Control reactions indicated that these conditions were sufficient to nick all of the plasmid DNA as determined by agarose gel electrophoresis and ethidium bromide staining. The nicked plasmid was dispensed directly into the DNA synthesis reactions.

**Oligoribonucleotide Synthesis**—The assay measuring the synthesis of oligoribonucleotides by the T7 gene 4 protein has been described (23, 24). Briefly, the reaction mixture (10 μl) contained 40 nM Tris–HCl, pH 7.5, 10 mM MgCl2, 10 mM DTT, 100 μg/ml bovine serum albumin, 50 mM potassium glutamate, pH 7.5, 0.6 mM each of dATP, dCTP, dGTP, and dTTP, and 0.3 mM each of ATP, [α32P]CTP, 10 nM M13 ssDNA, and 60 nM gene 4 protein. After incubation at 30 °C for 5 or 10 min, the reaction was stopped by the addition of EDTA to 20 mM. The products of the reaction were examined by electrophoresis in a 25% polyacrylamide gel containing 2 M urea.
DNA synthesis was measured in reactions containing 2 mM \([\alpha-\text{32P}]\text{dTTP}, 300\ \mu\text{M} \text{dGTP}, \text{dATP}, \text{and dCTP}, 40\ \text{nM} \text{T7 DNA polymerase} \Delta28\), and the indicated concentration of gene 4 protein in \text{dTTPase buffer}. The 20-\mu\text{l} reaction mixtures were incubated at 30 °C for 10 min and then terminated by the addition of 4 \mu\text{M} of EDTA, 0.6% SDS, 10% glycerol, and 0.01% bromphenol blue. The products of the reaction were separated by electrophoresis in a 0.8% agarose gel, dried, and analyzed by PhosphorImaging.

The second assay used a preformed replication fork, consisting of a 70-bp circular DNA molecule with a 5′-single-stranded tail of 40 nucleotides, to examine strand displacement synthesis. This mini-replication fork was synthesized by converting a 70-base oligonucleotide (AE06, 5′-GATCCGACGCTCTATGTCAGTTGGAGAGATGAATCC-3′) into a 70-base single-stranded circular using a 20-base oligonucleotide to splint the ends together. The 70-mer, AE06, was first phosphorylated with T4 polynucleotide kinase in a standard reaction and then hybridized with a 20-mer (AE07 5′-GGTGCATGCTCTCATCAATC′-3′). The 20-base oligonucleotide is complementary to the 5′-10 bases and 3′-10 bases of the 70-mer so that, when the two oligonucleotides are annealed, the ends of the 70-mer are brought together and can be covalently joined into a circle by T4 DNA ligase. The single-stranded 70-nucleotide circle was purified by separating the products of the ligation reaction on a 6.5% urea, 10% polyacrylamide gel and eluting the circle from the corresponding region of the gel. The circular molecule was then annealed to a partially complementary concentration of 180 mM in flow buffer. Proteins were removed from the polymerase reaction mixture (50 mM Tris, pH 7.5, 1 mM MgCl2, 10 mM DTT, and 1.8 mM each of \([\alpha-\text{32P}]\text{dTTP}, \text{dATP}, \text{dCTP}, \text{and dGTP}]) with a sequence such that the 5′-prime 40 bases form a single-stranded tail. The annealing reaction contained the 70-nucleotide circle in a slight molar excess (1.08) over the 110-base oligonucleotide. Since the excess circular DNA cannot contribute to the DNA synthesis reaction, the substrate was used without further purification.

DNA synthesis using the mini-fork DNA substrate was assayed in a reaction mixture (50 \mu\text{l}) containing, 40 mM Tris, pH 7.5, 10 mM MgCl2, 10 mM DTT, 100 mg/ml bovine serum albumin, 50 mM potassium glutamate, pH 7.5, and 600 \mu\text{M} each of \([\alpha-\text{32P}]\text{dTTP}, \text{dATP}, \text{dCTP}, \text{and dGTP}]) with 100 nM DNA substrate. The DNA polymerase and gene 4 protein were mixed and preincubated for 5 min at 4 °C at a concentration of 180 mM in flow buffer. Proteins were removed from the polymerase was examined using a non-denaturing PAGE gel-shift assay. The analysis was performed as described previously (29), with the polymerase examined using a non-denaturing PAGE gel-shift analysis.
to dissociate the DNA strands of the substrate is essentially the same as that of wild-type protein.

This assay also demonstrates the ability of the gene 4 proteins to translocate 5’ to 3’ on ssDNA. Since the gene 4 protein binds ssDNA randomly (43), it must translocate along the M13 ssDNA to displace the bound oligonucleotide. The average distance the gene 4 protein must translocate to reach the oligonucleotide from its initial binding location is half the number of nucleotides in M13 ssDNA or about 3,600 nucleotides. Consequently, the time course of strand separation on this hybrid DNA substrate indicates that both the wild-type and gene 4A-ΔCt protein translocate on ssDNA at approximately the same rate.

Primase Activity—The primase activity of the gene 4A protein is required for the initiation of lagging strand DNA synthesis and is essential for phage viability (27, 28). In the presence of ribonucleoside 5’-triphosphates, the T7 gene 4A protein catalyzes the synthesis of oligoribonucleotides at specific primase recognition sites on ssDNA (22, 44). These oligoribonucleotides are then used as primers by T7 DNA polymerase to initiate DNA synthesis. To assess the primase activity of the gene 4A-ΔCt protein, we measured both its ability to prime DNA synthesis catalyzed by T7 DNA polymerase on M13 ssDNA and its ability to synthesize the template-dependent oligoribonucleotides used as primers. The results of these assays demonstrate that the gene 4A-ΔCt protein can perform both of these related functions as well as the wild-type protein (Fig. 3, B and C). The ability of the mutant gene 4 protein to synthesize and present primers in a manner that they can be used by the DNA polymerase is shown by the comparable levels of DNA synthesis observed in the coupled assay (Fig. 3B). We also demonstrate directly that the mutant gene 4 protein synthesizes levels of tetramic oligoribonucleotides equivalent to that of the wild-type protein (Fig. 3C). Thus, the gene 4A-ΔCt protein is not defective in either its ability to catalyze the synthesis of oligoribonucleotides or to prime lagging strand synthesis.

Stimulation of T7 DNA Polymerase Activity on dsDNA—The experiments presented so far demonstrate that the abilities of the gene 4A-ΔCt protein to hydrolyze nucleotides, translocate on ssDNA, unwind duplex DNA, and prime lagging strand DNA synthesis are unaffected by the carboxyl-terminal deletion. Nevertheless, this mutant protein cannot support phage replication in vivo. It seemed likely that the carboxyl-terminal deletion affects an essential protein-protein interaction required for DNA replication.

The helicase activity of the gene 4 protein is required for T7 DNA polymerase to catalyze synthesis on duplex DNA (21). In this reaction there is a specific interaction between the T7 proteins, as demonstrated by the fact that other DNA polymerases cannot substitute for T7 DNA polymerase (21). Thus, we used plasmid dsDNA containing a single site-specific nick to examine the ability of the gene 4 protein to interact with T7 DNA polymerase and stimulate its activity on a duplex template. The phage f1 gene II protein was used to introduce a single nick at the f1 origin of replication contained in plasmid pET24a(+) (refer to inset Fig. 4A). The gene 4 protein requires a 5’-single-stranded tail to which it can bind and continue strand separation. Thus T7 DNA polymerase Δ28 lacking 3’ to 5’ exonuclease activity was used as this enzyme will initiate DNA synthesis at a nick and catalyze strand displacement synthesis of approximately a hundred nucleotides (21). The gene 4 protein then enters the reaction by binding to regions of ssDNA created by the DNA polymerase.

In the results shown in Fig. 4A there is essentially no DNA
Helicase and primase activities of the wild-type and gene 4A-ΔCt proteins. The experiments were performed as described under “Experimental Procedures.” A, helicase activity was measured as the ability of the gene 4A proteins (labeled G4p in inset) to separate an annealed 36-base radiolabeled oligonucleotide from circular M13 ssDNA (refer to inset for a diagram of the DNA substrate). Samples were removed at the indicated time, and the reaction was stopped. The percent of oligonucleotide displaced was determined by non-denaturing-PAGE and PhosphorImager analysis. B, primase activity was measured as the ability of the gene 4A proteins to prime DNA synthesis catalyzed by T7 DNA polymerase on M13 ssDNA. The concentrations of gene 4 proteins used and the amount of dNMP incorporated by the polymerase are shown. C, the oligoribonucleotide synthesis reaction mixtures contained 60 nM gene 4 protein and 10 nM M13 ssDNA. The synthesized oligoribonucleotides were labeled by the incorporation of [α-32P]CTP during 5-or 10-min incubations at 30 °C. The reaction products, identified at the right of the panel, were separated by PAGE and visualized by autoradiography. The curves in A and B, and the lanes in C are labeled WT, wild-type gene 4A protein; and ΔCt, gene 4A-ΔCt protein.

synthesis on the nicked duplex template in the absence of gene 4 protein. The addition of wild-type gene 4 protein to the reaction leads to a marked increase in DNA synthesis, but addition of the carboxyl-terminal deleted protein results in less than 10% the stimulation level of the wild-type protein. Examination of the products of these reactions by agarose gel electrophoresis shows that the DNA molecules synthesized in the presence of the gene 4A-ΔCt protein are never more than a few hundred nucleotides long, whereas the wild-type helicase permits the continuous addition of well over 50,000 nucleotides (Fig. 4B). Increases in the concentration of wild-type gene 4A protein produce an increase in the amount of DNA synthesized, not in the length of DNA synthesized.

We also used a preformed replication fork to compare the abilities of the wild-type and gene 4A-ΔCt proteins to stimulate T7 DNA polymerase activity (21). Beside using a different DNA substrate to confirm our findings, this assay allowed us to determine if the inability of the mutant gene 4 protein to stimulate DNA synthesis is due, in whole or part, to the mutation in T7 DNA polymerase Δ28. The preformed replication fork used in this experiment consists of a 70-bp circular duplex DNA molecule with a 5'-single-stranded tail of 40 nucleotides (diagrammed in the inset of Fig. 5A). The presence of the 5'-tail allows the use of wild-type T7 DNA polymerase rather than the Δ28 deletion form lacking exonuclease activity.

The data presented in Fig. 5 confirm that the gene 4A-ΔCt protein is defective in its ability to support synthesis by the DNA polymerase through duplex DNA. The time course reveals that the amount of DNA synthesized is lower and the length of the product molecules is shorter in reactions with the mutant gene 4A protein relative to that obtained with the wild-type protein. Even at the longest reaction time (5 min) the gene 4A-ΔCt protein cannot support the synthesis of DNA molecules as long as those synthesized with the wild-type gene 4 protein in 1 min (Fig. 5B).

Interaction of DNA Polymerase and Gene 4 Protein—Considering that the gene 4 protein and DNA polymerase have been shown to interact (12) and that the gene 4A-ΔCt protein is equally as active as the wild-type protein in translocation and unwinding dsDNA (Fig. 3), it seems likely that the decreased stimulation of T7 DNA polymerase activity by the carboxyl-terminal deleted gene 4A protein could be caused a defect in its ability to interact properly with the polymerase at a replication fork. The interaction of the gene 4 protein and DNA polymerase was examined directly by a gel-shift assay and surface plasmon resonance.

In the presence of the non-hydrolyzable nucleotide analog β,γ-methylene dTTP the wild-type gene 4 hexamer binds a single-stranded radiolabeled oligonucleotide resulting in decreased mobility of the labeled oligonucleotide (Fig. 6, lane 1, solid arrowhead). The gene 4A-ΔCt protein binds to single-stranded oligonucleotide equally as well as does the wild-type protein (Fig. 6, lane 5). When DNA polymerase is included in the reaction there is a further decrease in the mobility of the wild-type gene 4A protein-oligonucleotide complex indicating an increase in the size of the complex due to the interaction of
the polymerase with the gene 4 protein (Fig. 6, lane 2, open arrowhead). In contrast, T7 DNA polymerase has little effect on the mobility of the gene 4A-Ct protein-oligonucleotide complex, suggesting that the polymerase cannot stably interact with the mutant gene 4 protein (Fig. 6, lane 4). Under the conditions used in this assay the T7 DNA polymerase does not bind ssDNA (Fig. 6, lane 3) demonstrating that the decrease in migration of the polymerase-wild-type gene 4 protein-DNA complex (Fig. 6, lane 2) is due to protein-protein interactions.

The interaction between the gene 4 proteins and DNA polymerase was also examined using a biosensor and surface plasmon resonance measurements (BIAcore, Pharmacia Biosensor). This analysis provides a second, direct and independent method of assessing the effect of the carboxyl-terminal deletion of the gene 4 protein on its ability to interact with DNA polymerase. Plasmon resonance changes in direct proportion to the mass of the molecules adsorbed on the surface of the biosensor chip, providing a real-time measurement of protein-protein interactions under native conditions (45). In these experiments a biosensor chip with covalently attached avidin was used to bind a 3′-biotinylated 33-base oligonucleotide. Under the buffer and flow rate conditions used neither T7 DNA polymerase A28 nor gene 4 protein binds the avidin biosensor chip before ssDNA is applied (data not shown). After ssDNA is bound to the chip the gene 4 proteins will bind only in the presence of β,γ-methylene dTPP as described under “Experimental Procedures.” The results presented in this report confirm this observation shown in Fig. 7 equivalent amounts of wild-type and mutant gene 4 proteins, in the presence of β,γ-methylene dTPP, were bound to the ssDNA on the chip (9512 RU ± 390). DNA polymerase was then injected over the gene 4 proteins-ssDNA complex bound to the biosensor chip. As shown in Fig. 7A T7 DNA polymerase binds to the wild-type gene 4 protein-ssDNA complex as evidenced by the increased resonance response (810 RU) above the DNA polymerase base line. In contrast, no binding (less than 30 RU) of T7 DNA polymerase to the mutant gene 4 protein-ssDNA complex on the chip was observed (Fig. 7B). We conclude that the primary defect caused by the deletion of the carboxyl terminus of the gene 4 protein is a loss of the ability to form a stable complex with T7 DNA polymerase.

DISCUSSION

The gene 4 protein of bacteriophage T7 provides both helicase and primase functions at the replication fork (16, 21, 25, 34, 44). In this capacity the gene 4 protein interacts with T7 DNA polymerase on both the leading and lagging strands to unwind the duplex and synthesize primers, respectively. Through these interactions with DNA polymerases on each strand, the gene 4 protein coordinates leading and lagging strand synthesis at the replication fork (3). The dramatic and specific effects of the helicase and primase activities of the gene 4 protein on polymerase activity imply a direct interaction of the two proteins, and such an interaction has been demonstrated (12). The results presented in this report confirm this interaction and lead to the identification of the carboxyl terminus of the gene 4 protein as the domain responsible for its interaction with the T7 DNA polymerase during leading strand synthesis.

Interestingly, the carboxyl-terminal domain of the T7 gene 4 protein is not essential for any of its multiple enzymatic activ-
ties, but is absolutely required for its function in vivo. The DNA dependent nucleotide hydrolysis, helicase, and primase activities of the gene 4A-ΔCt protein are essentially identical to those of the wild-type protein. Only when we examined the coupling of polymerase activity on duplex DNA to helicase activity of the gene 4 protein did we detect a defect. In these assays, where DNA synthesis is dependent on the coordinated activities of both proteins, the carboxyl-terminal truncated gene 4 protein is at least 10-fold less effective in stimulating DNA synthesis catalyzed by T7 DNA polymerase than is the wild-type gene 4 protein.

To examine the ability of the gene 4 protein to enable T7 DNA polymerase to catalyze synthesis through ssDNA, we used two assays, each employing a distinct DNA template. One template was circular duplex plasmid DNA containing a single site-specific nick. In this instance, it was necessary to use T7 DNA polymerase Δ28, an altered form of the polymerase lacking 3’ to 5’ exonuclease activity (46). Only in the absence of exonuclease activity will the polymerase catalyze strand displacement synthesis sufficient to create a single-stranded tail, or lagging strand, to which the gene 4 protein can bind and subsequently translocate to the replication fork where it can presumably form a complex with the paused DNA polymerase (21). Coincidentally, this same form of the polymerase was required in the gel-shift assay to prevent the potent exonuclease activity of the wild-type polymerase from hydrolyzing the oligonucleotides present in the reaction mixture. The second DNA synthesis assay used a small circular duplex DNA molecule containing a replication fork (21); therefore, the wild-type T7 DNA polymerase could be used in the reaction.

Together T7 DNA polymerase and wild-type gene 4 protein polymerize nucleotides processively on a duplex template at a rate of approximately 300 nucleotides per s at 30 °C (21). Furthermore, the ability of the gene 4 protein to function in this reaction is specific for T7 DNA polymerase because other enzymes such as the phage T4 DNA polymerase cannot substitute for T7 polymerase. Our data indicate that processive DNA synthesis depends on the formation and maintenance of a stable complex at the replication fork, and this is provided by the direct interaction of the gene 4 protein and DNA polymerase. This interaction prevents the proteins from outdistancing each other and thus losing their combined effectiveness. For example if the helicase out-paced the polymerase so that a duplex region formed between the two proteins, DNA synthesis would halt since the polymerase alone cannot catalyze strand displacement synthesis through even a single base pair (21).

Alternative scenarios relying on uniform rates of movement of the enzymes along the DNA are, of course, conceivable. These situations would not rely on direct physical interactions to maintain a replication complex. For example, it is possible that the rate of nucleotide polymerization is faster than that of helicase unwinding. This would prevent a functional separation of the two proteins since a helicase moving ahead of the polymerase would be the rate-limiting step. Likewise, in a model where the polymerase is positioned in front of the helicase the inability of the polymerase to catalyze strand displacement synthesis would keep it in close proximity to the helicase. Neither model, however, can account for our results since polymerase activity would be expected to remain the same with either the wild-type or mutant gene 4 protein. Our finding that the gene 4A-ΔCt protein is severely defective in stimulating T7 DNA polymerase, in two different synthesis assays, even though it has normal helicase activity suggests strongly that coordination of the two proteins is lacking. The results, in fact, indicate that the coordination normally occurs through a protein-protein interaction to form a functional complex.

It is clear that the T7 DNA polymerase and the gene 4 protein form a complex. This interaction was shown indirectly in earlier studies (12) and is demonstrated directly in the gel-shift experiments and plasmon resonance measurements presented in this study. Furthermore, both these latter experiments demonstrate that the carboxyl-terminal truncated gene 4 protein is defective in its ability to form a complex with T7 DNA polymerase. In the gel shift assay the polymerase remains bound to the gene 4 protein through the preincubation period and subsequent gel electrophoresis, a total period of more than 2 h. In the plasmon resonance analysis the binding of DNA polymerase to the gene 4 hexamer-ssDNA complex was so tight that an off-rate could not be determined within the time course of the experiment. Formation of the gene 4 protein-DNA polymerase complex, however, is not dependent on the presence of ssDNA. Gel-shift experiments performed without oligonucleotide resulted in the formation of the same DNA polymerase-wild-type gene 4 protein complex as detected by silver staining of the gels (data not shown). This result was expected since, as shown in Fig. 6 (lane 3), T7 DNA polymerase does not bind ssDNA under the conditions used. Moreover, it is likely that the entire 26-base oligonucleotide is bound within the hexameric gene 4 protein (30, 31).

The ability of the gene 4 proteins to form hexamers and bind ssDNA was also demonstrated by the gel-shift assay. In the presence of β,γ-methylene dTTP both wild-type and the gene

![Surface plomen resonance analysis of the interactions between the gene 4 proteins and T7 DNA polymerase.](image-url)
4A-ΔCt protein bind the radiolabeled oligonucleotide (Fig. 6, lanes 1 and 5). Since the gene 4 protein must form a hexamer to bind ssDNA (29, 31), it is clear that hexamer formation was not affected by the carboxyl-terminal deletion. Furthermore, both proteins bound ssDNA with a stoichiometry of approximately 1 mol of ssDNA per mol of gene 4 hexamer, indicating that DNA binding is not affected by the deletion mutation.

Not only is an interaction of the gene 4 protein and DNA polymerase required for strand displacement DNA synthesis, it is also required for priming DNA synthesis on the lagging strand of the replication fork. The gene 4A protein catalyzes the synthesis of tetra-ribonucleotides at specific sequences on ssDNA in a template-mediated reaction. These tetra-ribonucleotides are stabilized on the template by the gene 4A protein until T7 DNA polymerase can use them as primers to initiate ssDNA in a template-mediated reaction. The gene 4A protein catalyzes strand displacement DNA synthesis, it both proteins bind ssDNA with a stoichiometry of approximately 1 mol of ssDNA per mol ofgene 4 hexamer, indicating that DNA binding is not affected by the deletion mutation.

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No T7 phage suppressors of this gene 4 deletion mutation were detected in the complementation assays (Table I), a result that is most likely due to the severe nature of the deletion mutation. Site-directed mutagenesis of individual residues within this 17-amino acid carboxyl-terminal domain of the helicase/primase could be used to determine the specific residues involved in the interaction with T7 DNA polymerase. These mutant gene 4 proteins could then be used to detect suppressor mutations in the T7 DNA polymerase and thus identify the domain of the polymerase responsible for interactions with the helicase/primase. It is possible that a single residue of this region plays an essential role in the interaction with T7 DNA polymerase, rather than the region as a whole. In this regard, it is interesting to note that Rosenberg et al. (47) in a random mutagenesis of gene 4 failed to identify essential residues in this carboxyl-terminal region. They did, however, select a gene 4 mutant, Q507/Ochre, containing a termination codon positioned so that the last 60 residues of the protein were deleted. This mutant could not support the growth of a gene 4-deleted T7 phage. While this 60-amino acid deletion is more severe than the 17-residue deletion mutant studied in this report, it is likely that their mutant gene 4 protein was also defective in its ability to interact with DNA polymerase and may not have been defective in either helicase or primase activities, as suggested.

At least one other phage T7 replication protein has a negatively charged region that participates in protein-protein interactions. T7 gene 2.5 encodes a ssDNA binding protein that is required for phage replication (2). This protein has a negatively charged carboxyl terminus that functions in dimerization of the gene 2.5 protein and interactions with T7 DNA polymerase and gene 4 protein (48). It is not known if a single domain of the DNA polymerase interacts with the negatively charged carboxy-terminal domains of both the gene 2.5 protein and the gene 4 helicase/primase. However, we were not able to detect any interactions between the gene 2.5 protein and either the T7 DNA polymerase or the gene 4 helicase/primase in the native PAGE system used for the gel-shift assays studied in this investigation (data not shown). This finding indicates that the gene 4 protein-DNA polymerase interaction is considerably stronger than that of the gene 2.5 protein-DNA polymerase interaction.

Bacteriophages T3 and SP6 are closely related to phage T7; the gene 4 proteins of these phage also have negatively charged carboxyl termini, 6 of the terminal 17 residues are negatively charged in both phage. Presumably, these acidic domains also mediate interactions with the DNA polymerase during replication. The phage T7 gene 4 helicase/primase also shares regions of homology with DnaB, the replicative helicase of E. coli (49). The carboxyl terminus of DnaB is not as negatively charged as that of the T7 gene 4 helicase/primase, and this may reflect the fact that a separate protein, the 7-protein, mediates interactions between the helicase and the polymerase (50). The presence of a protein that specifically functions as a link between the DNA helicase and the DNA polymerase may be a more common replisome scheme than the direct interaction between helicase and polymerase found in T7 phage, which has evolved a minimal set of highly efficient interdependent replication enzymes.

The interaction of gene 4 protein and the DNA polymerase at the replication fork is an important aspect of the replication process. Structural studies will be required to determine where on the gene 4 protein hexamer the carboxyl-terminal domain is located and how this location orients the DNA polymerase at the replication fork. Nevertheless, we present a model of the interaction between T7 DNA polymerase and the gene 4 helicase/primase at a DNA replication fork (Fig. 8). It is not clear from the data presented whether a single monomer of the gene 4 hexamer and the DNA polymerase are in constant contact at the replication fork or if the attraction between the proteins is dispersed over a continuous region of the hexamer. If the helicase rotates around the DNA axis as it translocates and unwinds dsDNA, the interaction with the polymerase may be changing sequentially from one monomer in the hexamer to the next to relieve torsional strain. In this case the polymerase is probably not in constant contact with a single monomer of the gene 4 protein but more likely contacts a region of the hexamer composed of the carboxyl-terminal domains of each monomer.  

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2 T. Kusakabe and C. C. Richardson, unpublished observations.
If, on the other hand, the helicase moves along the DNA without any rotation relative to the DNA, a constant interaction between a single gene 4 monomer and the DNA polymerase may be all that is required to maintain a stable complex. The model presented allows either the helicase or the polymerase some rotational flexibility around the DNA as the replication fork moves through the chromosome.

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The Acidic Carboxyl Terminus of the Bacteriophage T7 Gene 4 Helicase/Primase Interacts with T7 DNA Polymerase

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