A unique loop in the DNA-binding crevice of bacteriophage T7 DNA polymerase influences primer utilization

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The three-dimensional structure of bacteriophage T7 DNA polymerase reveals the presence of a loop of 4 aa (residues 401–404) within the DNA-binding groove; this loop is not present in other members of the DNA polymerase I family. A genetically altered T7 DNA polymerase, T7 polΔ401–404, lacking these residues, has been characterized biochemically. The polymerase activity of T7 polΔ401–404 on primed M13 single-stranded DNA template is one-third of the wild-type enzyme and has a 3′-to-5′ exonuclease activity indistinguishable from that of wild-type T7 DNA polymerase. T7 polΔ401–404 polymerizes nucleotides processively on a primed M13 single-stranded DNA template. T7 DNA polymerase cannot initiate *de novo* DNA synthesis; it requires tetraribonucleotides synthesized by the primase activity of the T7 gene 4 protein to serve as primers. T7 primase-dependent DNA synthesis on single-stranded DNA is 3- to 6-fold less with T7 polΔ401–404 compared with the wild-type enzyme. Furthermore, the altered polymerase is defective (10-fold) in its ability to use preformed tetraribonucleotides to initiate DNA synthesis in the presence of gene 4 protein. The location of the loop places it in precisely the position to interact with the tetraribonucleotide primer and, presumably, with the T7 gene 4 primase. Gene 4 protein also provides helicase activity for the replication of duplex DNA. T7 polΔ401–404 and T7 gene 4 protein catalyze strand-displacement DNA synthesis at nearly the same rate as does wild-type polymerase and T7 gene 4 protein, suggesting that the coupling of helicase and polymerase activities is unaffected.

Specific protein–protein interactions are essential for the coordination of leading and lagging strand-DNA synthesis at a replication fork. The relatively few proteins required for coordinated DNA synthesis in the bacteriophage T7 replication system make it an attractive model for studying these interactions (1). Only four proteins are required for the basic steps at the replication fork: T7 gene 5 DNA polymerase, gene 4 helicase/primase, gene 2.5 single-stranded DNA (ssDNA)-binding protein, and *Escherichia coli* thioredoxin (1, 2). T7 gene 5 DNA polymerase by itself has low processivity of DNA synthesis and requires *E. coli* thioredoxin in a 1:1 complex to achieve high processivity (3–7). In this communication we will refer to this functional 1:1 complex of T7 gene 5 protein and thioredoxin as T7 DNA polymerase. T7 gene 2.5 ssDNA-binding protein interacts with T7 gene 4 helicase/primase (8–10) and also with T7 DNA polymerase through its acidic carboxyl-terminal domain (10, 11).

T7 gene 4 protein exists in two forms with molecular weights of 56,000 and 63,000. The 56-kDa species is translated from an internal initiation codon, in-frame with the 63-kDa species (12–15). Both forms of gene 4 protein bind to ssDNA as a hexamer (16–18) in the presence of nucleoside 5′-triphosphates, preferentially dTTP, and translocate 5′ to 3′ by using the energy of nucleotide hydrolysis (13, 19–21). Upon encountering duplex DNA with six to seven unpaired nucleotides at the 3′ end, the gene 4 protein unwinds the DNA strands processively (20, 22–24). The acidic carboxyl terminus of T7 gene 4 protein interacts with T7 DNA polymerase (25).

The 63-kDa gene 4 protein contains a Cys_4 zinc-binding motif (26, 27) within the additional 63 aa at its N terminus not present in the 56-kDa species. In addition to having helicase activity, the 63-kDa gene 4 protein acts as a primase to catalyze the synthesis of template-directed oligoribonucleotides that serve as primers for T7 DNA polymerase (9, 19, 26, 28, 29). Gene 4 primase synthesizes functional tetraribonucleotide primers 5′-ppACCC-3′, 5′-pppACCA-3′, and 5′-pppACAC-3′ at primase recognition sequences 5′-GGGTC-3′, 5′-TGGTC-3′, and 5′-GTGTC-3′, respectively (30–33). All of these primase recognition sequences contain the core recognition sequence 5′-GTC-3′. Interestingly, the 3′-cytidine in the primase recognition sequence is required but not copied into the primer. The utilization of the tetraribonucleotides as primers by T7 DNA polymerase requires the presence of T7 gene 4 protein; other primases will not function in this reaction (29, 32). One role of the gene 4 protein in this reaction is to stabilize the tetraribonucleotide onto the template until it can be extended by the polymerase (32). In addition, the specific requirement for gene 4 helicase/primase may reflect an interaction between the primase domain of the gene 4 protein and T7 DNA polymerase for the primer to be transferred to the active site of the polymerase. In addition to the postulated interactions of the primase with the polymerase, it is likely that the helicase domain of the gene 4 protein also interacts with the polymerase. A carboxyl-terminal deletion mutant of gene 4 protein that retains a normal level of helicase and primase activities fails to support strand displacement DNA synthesis by T7 DNA polymerase (25). Furthermore, an N-terminal fragment of T7 gene 4 protein retaining only primase activity is unable to prime DNA synthesis catalyzed by T7 DNA polymerase (34), indicating the importance of the helicase domain in the proper utilization of the tetraribonucleotide primer by T7 DNA polymerase.

Structure–function studies of T7 DNA polymerase have been facilitated by the solution of its crystal structure (35). One approach to identify domains of T7 DNA polymerase that interact with other T7 replication proteins is to identify regions in the proteins that are unique among other members of the DNA polymerase I (pol I) family. This approach revealed the presence of a loop of four residues (Glu-Gly-Asp-Lys) spanning residues 401–404. Such a loop is absent in the other members of the pol I family whose structures are known (35–38). Interestingly, this loop is 4 bases away from 3′-OH terminus of the primer. Because T7 primase stabilizes the tetraribonucleotide primer (32), this location of the loop likely places it in close proximity to the primase domain of gene 4 protein.

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Abbreviations: pol I, DNA polymerase I; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA.

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To assess the role of the loop in interactions of T7 DNA polymerase with gene 4 helicase/primase and with DNA, we have constructed a plasmid encoding for T7 gene 5 lacking the four-residue loop. We have purified and characterized the altered T7 DNA polymerase lacking the loop (T7 polΔ401–404). Our results indicate that this four-residue loop indeed is important for interacting with the T7 gene 4 primase–tetraribonucleotide primer complex.

Materials and Methods

Mutagenesis of T7 Gene 5. *In vitro* mutagenesis of bacteriophage T7 gene 5 to delete the region encoding amino acids 401–404 was accomplished by standard PCR techniques by using two synthetic primers that encode the deletion and the plasmid pGP5–3 (7) as a DNA template. After amplification, the resulting amplified DNA fragment was digested with restriction enzymes and inserted into pGP5–3 to generate the plasmid pGP5–3Δ401–404. The DNA sequence of gene 5 in pGP5–3Δ401–404 was sequenced to confirm that the 12-bp deletion was the only mutation in the gene.

Enzymes. T7 wild-type gene 5 protein and polΔ401–404 proteins were purified either complexed to thioredoxin or in the absence of thioredoxin. When the polymerases were purified complexed to thioredoxin, T7 wild-type gene 5 protein and polΔ401–404 proteins were overproduced in *E. coli* BL21 (DE3)/pLysS (Novagen) carrying the plasmid pGP5–3 and pGP5–3Δ401–404, respectively, and the one-to-one functional complex of polymerase and thioredoxin was purified to homogeneity as described (7, 35). When the polymerases were purified in the absence of thioredoxin, both T7 wild-type gene 5 protein and polΔ401–404 proteins were overproduced in *E. coli* A179 *trxA*$^-$/pGP1–3/pGP5–3 and in *E. coli* A179 *trxA*$^-$/pGP1–3/pGP5–3Δ401–404, respectively, lacking the thioredoxin gene as described (7). Before each assay, the polymerase was mixed with an excess of thioredoxin to reconstitute a functional complex. T7 63-kDa gene 4 protein was purified as described (17).

DNA Polymerase Assay. DNA polymerase activity was measured as described previously (7). The primer–template used in the assay was circular M13 ssDNA annealed to a 25-nt complementary oligomer. The reaction mixture (50 μl) contained 50 mM Tris·HCl (pH 7.5), 50 mM NaCl, 1 mM DTT, 10 mM $MgCl_2$, 0.8 nM M13 primer–template, and 300 mM each of dATP, dCTP, dGTP, and [^{3}H]dTTP. The reaction was initiated by the addition of T7 DNA polymerase, incubated at 37°C for 10 min, and terminated by the addition of 10 μl of 0.5 M EDTA. [^{3}H]DNA product was adsorbed on DE-81 filters and washed three times with 0.3 M ammonium formate (pH 8.0), and the radioactivity was measured by liquid scintillation counting.

Exonuclease Assay. The 3′-to-5′ double-stranded DNA (dsDNA) exonuclease activity of T7 DNA polymerase was assayed essentially as described (39). The ^{32}P-labeled M13 dsDNA was prepared by extending a 25-mer primer annealed to M13 ssDNA in the presence of 300 mM each of dATP, dTTP, [α-^{32}P]dCTP, and [α-^{32}P]dGTP, 10 mM $MgCl_2$, 1 mM DTT, 50 mM Tris·HCl (pH 7.5), and 50 mM NaCl by incubation with 5 nM T7 DNA polymerase for 3 min. The reaction was terminated by heat-inactivating the enzyme at 95°C for 5 min. The labeled DNA was purified by phenol-chloroform extraction followed by purifying through Biospin columns with Bio-gel P-6 (Bio-Rad) to remove the free nucleotides.

The reaction mixture (50 μl) to measure 3′-to-5′ dsDNA exonuclease activity contained 0.5 nM M13 primer–template, 50 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, and 5 nM T7 DNA polymerase. The reactions were initiated by the addition of the enzyme and incubated at 37°C for the indicated time periods. The reaction was terminated by the addition of 25 μl of 0.5 mM EDTA. The [^{32}P]DNA remaining was adsorbed onto DE81 filter paper and washed three times with 0.3 M ammonium formate (pH 8.0), and the radioactivity was measured by liquid scintillation counting to calculate the amount of DNA hydrolyzed.

T7 Gene 4-Dependent DNA Synthesis by T7 DNA Polymerase. The ability of T7 DNA polymerase to extend a tetraribonucleotide in the presence of gene 4 primase was assayed by using two different methods. In one case, T7 63-kDa gene 4 protein was allowed to synthesize the tetraribonucleotide primers at the major primase recognition sites on a circular M13 ssDNA template in the presence of ATP and CTP as described (9, 32), and the ability of T7 DNA polymerase to extend the tetraribonucleotide primers was measured. Alternatively, the process of primer synthesis was uncoupled from primer extension by directly using the oligoribonucleotide 5′-pACCA-3′, and the rate of extension of the ribonucleotide primers by T7 DNA polymerase in the presence of T7 63-kDa gene 4 protein was measured as described (30, 40).

In each case, the reaction mixture (50 μl) contained 50 mM Tris·HCl (pH 7.5), 50 mM potassium glutamate, 10 mM $MgCl_2$, 10 mM DTT, 100 μg BSA, 0.5 nM M13 ssDNA, 300 mM each of dATP, dCTP, [^{3}H]dTTP dGTP, ATP, and CTP, and 60 nM T7 63-kDa gene 4 protein. In the absence of ATP and CTP, the oligoribonucleotide primer 5′-pACCA-3′ was supplied in different amounts to the above reaction mixture. The reactions were initiated by the addition of T7 DNA polymerase, incubated at 37°C for 10 min, and terminated by the addition of 25 μl of 0.5 M EDTA. [^{3}H]DNA was adsorbed onto DE-81 filters and washed three times with 0.3 M ammonium formate (pH 8.0), and the radioactivity was measured by liquid scintillation counting.

Strand Displacement DNA Synthesis. Strand displacement DNA synthesis requires the coupling of the helicase activity of gene 4 protein with T7 DNA polymerase. It is measured by the ability of T7 gene 4 protein to stimulate DNA synthesis catalyzed by T7 DNA polymerase on a preformed replication fork. DNA used for this assay was a 70-nt circular ssDNA annealed to a 70-base partially complimentary oligonucleotide. The sequence is such that 30 bases at the 3′ end of the oligonucleotide anneal to the circular DNA, resulting in a 40-base 5′ oligo(dT) tail (1, 25). The reaction mixture (50 μl) contained 50 mM Tris·HCl (pH 7.5), 50 mM potassium glutamate, 10 mM $MgCl_2$, 5 mM DTT, 2.5 μg BSA, 300 mM each of dATP, dCTP, dTTP, and [α-^{32}P]dGTP, 200 nM of the preformed replication fork, and 60 nM gene 4 protein (either 63 or 56 kDa). The reaction was initiated by adding varying amounts of T7 DNA polymerase and terminated by adding 10 μl of 500 mM EDTA. [^{32}P]DNA was adsorbed onto DE-81 filters and washed three times with 0.3 M ammonium formate (pH 8.0), and the radioactivity was measured by liquid scintillation counting.

Stability of T7 Gene 4 Primase/Polymerase/DNA Complex. The stability of the primase–polymerase–primer/template complex was measured by using a 22-mer DNA template, 5′-CAGTCACGGGTCGTTTATCGTC-3′. The primase recognition sequence 5′-GGGTC-3′ (underlined) allows T7 gene 4 primase to synthesize a tetraribonucleotide 5′-pppACCC-3′, which is then extended by T7 DNA polymerase. The extension stops at the fourth base from the oligoribonucleotide 3′ end because of the incorporation of [α-^{32}P]ddAMP. The complex of primer–template, primase, and polymerase remains stable in the presence of the next incoming nucleotide, dCTP (S.T., unpublished results). The primase–polymerase complex can

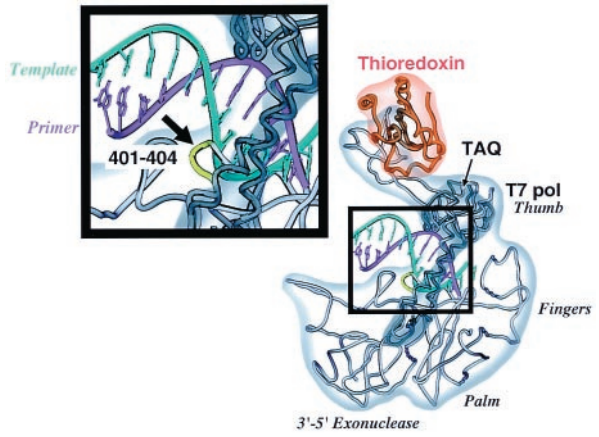

Fig. 1. Structural alignment of T7 and *Taq* DNA polymerases. Atomic coordinates of the crystal structures of *Taq* DNA polymerase (38) (PDB entry 2KTQ) and T7 DNA polymerase (35) (PDB entry 1T7P) were superimposed by using the program INSIGHT II (Biosym Technologies, San Diego). The superimposed structures were visualized by using the program SETOR (41). T7 DNA polymerase (light grey) bound to the thioredoxin molecule (orange), primer (purple), and the template (cyan) are shown. The different domains of the T7 DNA polymerase are indicated. Only the I and I1 helices of *Taq* DNA polymerase (dark grey) are shown here, superimposed on the corresponding helices of T7 DNA polymerase. The loop unique to T7 DNA polymerase, comprising residues Glu-Gly-Asp-Lys spanning from residue 401 through 404, is shown in green.

protect the labeled primer from degradation by exonuclease III, and the degradation of the labeled primer is monitored with time.

A 25-μl reaction contained 50 mM Tris·HCl (pH 7.5), 50 mM potassium glutamate, 1 mM DTT, 160 nM T7 gene 4 protein (63 kDa), 200 nM T7 DNA polymerase, 1 μM DNA template, and 500 mM each of dATP, dGTP, dCTP, [α-^{32}P]ddATP (Amersham Pharmacia), ATP, and CTP. The reaction mixture was incubated at 20°C for 5 min to allow the primase to synthesize the oligoribonucleotide primer and polymerase to extend it. The reaction mixture was incubated with 40 units of Exonuclease III (New England Biolabs) at 20°C. Aliquots of 6 μl were withdrawn at 1, 5, 10, and 60 min and added to 6 μl of stop solution containing formamide and EDTA. The reaction products were analyzed by electrophoresis through a 20% polyacrylamide-urea gel.

Results

Superimposition of the structure of T7 DNA polymerase onto that of other members of the DNA polymerase I family, whose structures are known (35–38), revealed the presence of a unique loop of a 4-aa residue located between helices I and I1 at the base of the thumb region. The structure of I and I1 helices of *Taq* DNA polymerase was chosen as an example and superimposed to that of T7 DNA polymerase (Fig. 1). We have generated an altered polymerase, T7 polΔ401–404, lacking the loop, and characterized it to understand the role of this loop in the polymerization reaction.

Effect of the Deletion of the 401–404 Loop on Phage Growth. To determine the effect of the deletion of the 401–404 loop *in vivo*, plating efficiencies of T7 phage lacking gene 5 were compared on *E. coli* strain containing expression vectors for wild-type T7 DNA polymerase or T7 polΔ401–404. T7 phage lacking gene 5 cannot grow on *E. coli* in the absence of exogenous gene 5. The plaque sizes of T7 phage lacking gene 5 were similar when wild-type T7 DNA polymerase or T7 polΔ401–404 was produced from a plasmid, pGP5–3 or pGP5–3Δ401–404, respectively.

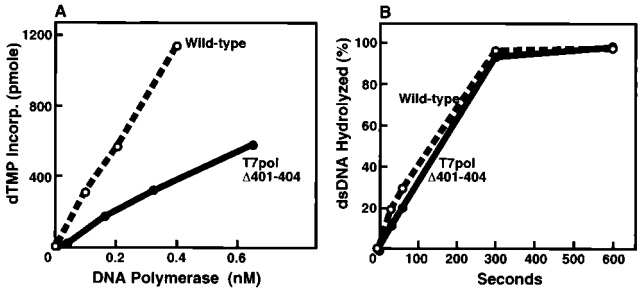

Fig. 2. Effect of deletion of residues 401–404 on the polymerase and exonuclease activity of T7 DNA polymerase. (*A*) The DNA polymerase activity of wild-type T7 DNA polymerase (○) or T7 polΔ401–404 (●) was assayed by using a circular M13 ssDNA template annealed to a 25-nt DNA primer. The rate of incorporation of [^{3}H]dTMP was plotted against different enzyme concentrations. (*B*) The 3′-to-5′ dsDNA exonuclease activity of wild-type (○) or T7 polΔ401–404 (●) was assayed by using a circular M13 ssDNA template annealed to a radiolabeled oligonucleotide as described in *Materials and Methods*. The rate of degradation of the primer was measured with time. One hundred percent primer–template corresponds to 25 fmol M13 DNA.

DNA Polymerase and Exonuclease Activities. T7 DNA polymerase, like most prokaryotic DNA polymerases, also has an inherent 3′-to-5′ exonuclease activity. DNA polymerase activity was assayed by using an M13 ssDNA template, to which a 25-nt DNA primer had been annealed. As shown in Fig.2*A*, the polymerase activity of the T7 polΔ401–404, measured as the initial rate of DNA synthesis, is approximately one-third that of the wild-type T7 DNA polymerase. We have tested the ability of T7 polΔ401–404 to form a stable ternary complex on a 26-mer DNA template annealed to 21-mer DNA primer as described previously (35). T7 polΔ401–404 could form a stable ternary complex with equal efficiency as the wild-type enzyme. The lower polymerase activity of the T7 polΔ401–404 could be due to a weaker interaction with the primer or a lower processivity.

We therefore measured the processivity of DNA synthesis by using an M13 primer–template as described (42). A 22-nt primer ^{32}P-labeled at the 5′ end was annealed to circular M13 ssDNA. DNA synthesis was carried out by using a 1:50 and 1:250 molar ratio of primer–template to enzyme. Reactions were stopped after 60, 90, and 120 sec, and the reaction products were analyzed by 6% polyacrylamide-urea gel. The processivity of DNA synthesis of T7 polΔ401–404 was found to be greater than 300 nt per binding event, similar to that of wild-type T7 DNA polymerase (data not shown).

The 3′-to-5′ dsDNA exonuclease activity of the wild type and the mutant enzyme were assayed by using internally labeled M13 dsDNA as described in *Materials and Methods*. The fraction of dsDNA degraded by the exonuclease activity of T7 DNA polymerase was measured against time. The dsDNA exonuclease activity of the mutant protein was indistinguishable from that of the wild-type enzyme (Fig. 2*B*).

Gene 4 Primase-Dependent DNA Synthesis. The primase domain of T7 gene 4 protein catalyzes the synthesis of tetraribonucleotides from primase recognition sites in the presence of ATP and CTP (30). The gene 4 protein then stabilizes the newly synthesized oligoribonucleotides on the DNA template until T7 DNA polymerase uses them as primers (32, 40). In the absence of gene 4 protein, T7 DNA polymerase cannot extend the previously synthesized primer (30). The requirement for gene 4 protein is specific. The DNA polymerase from bacteriophage T4 cannot extend tetraribonucleotide primers generated by the T7 gene 4 protein (40). A defective interaction of the T7 DNA polymerase with the T7 gene 4 primase/primer complex could result in a

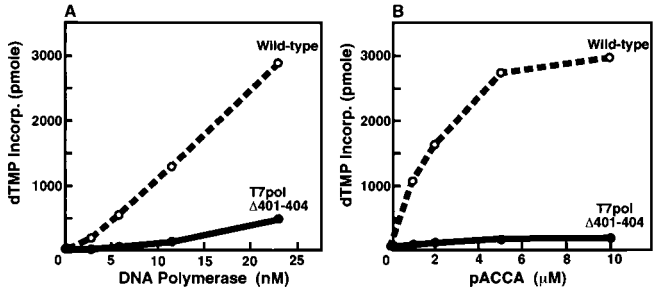

Fig. 3. T7 gene 4 primase-dependent DNA synthesis. (*A*) The extension of RNA primers synthesized by T7 63-kDa gene 4 protein in the presence of ATP and CTP by wild-type T7 DNA polymerase (○) or T7 polΔ401–404 (●) was determined as described in *Materials and Methods*. The amount of incorporation of [^{3}H]dTMP is plotted against different polymerase concentrations. (*B*) The ability of the wild-type T7 DNA polymerase (○) or T7 polΔ401–404 (●) to extend synthetic tetraribonucleotide primers in a reaction containing T7 63-kDa gene 4 protein was determined as described in *Materials and Methods*. The amount of incorporation of [^{3}H]dTMP was plotted against different concentrations of the tetraribonucleotide 5′-pACCA-3′.

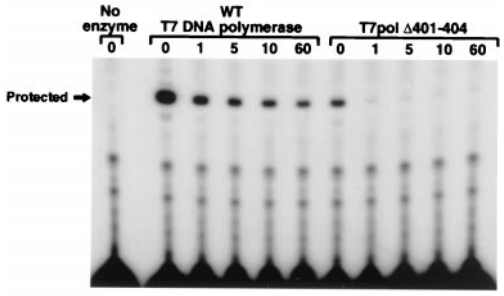

Fig. 4. Stability of gene 4 primase and polymerase complex. T7 gene 4 primase was allowed to synthesize a tetraribonucleotide primer, 5′-pppACCC-3′, on a synthetic oligonucleotide template, 5′-CAGTCACGGGTCGTTTATCGTC-3′, containing the primase recognition sequence 5′-GGGTC-3′ (underlined). T7 DNA polymerase extends this oligoribonucleotide to the fourth nucleotide, at which point the extension terminates because of the incorporation of ddAMP at the fourth nucleotide. Using this strategy, a complex of gene 4 helicase/primase, ^{32}P-labeled primer–template, and DNA polymerase was formed as described in *Materials and Methods*. The protection of the labeled primer by the helicase/primase–polymerase complex from exonuclease III digestion was measured at different time points as indicated. The products of exonuclease III digestion were analyzed by 20% polyacrylamide-urea gel electrophoresis. An autoradiogram of the gel is shown above.

lower rate of gene 4 primase-dependent DNA synthesis on ssDNA templates.

To examine the role of the 401–404 loop in the proper utilization of tetraribonucleotide primers generated by the gene 4 primase/helicase, we assayed gene 4 primase-dependent DNA synthesis on a circular M13 ssDNA template. T7 polΔ401–404 has a 6-fold lower rate of DNA synthesis as compared with the wild-type enzyme in the gene 4 primase-dependent DNA polymerase assay (Fig. 3*A*). This result shows that T7 polΔ401–404 is defective in its ability to extend the tetraribonucleotide primers synthesized by the gene 4 primase.

To obtain additional evidence for the interaction of the 401–404 loop with T7 primase/primer complex, we examined the ability of T7 DNA polymerase to extend a synthetic tetraribonucleotide pACCA on M13 circular ssDNA in the presence of the T7 63-kDa gene 4 primase. As discussed above, T7 DNA polymerase requires the specific presence of T7 gene 4 primase to extend such an oligoribonucleotide. In the presence of gene 4 primase, T7 DNA polymerase will use a preformed tetraribonucleotide as a primer but only at primase sites (5′-GTC-3′) in the template containing the cryptic cytosine (29, 32, 40). T7 polΔ401–404 is unable to extend efficiently the preformed tetraribonucleotide in the presence of the T7 63-kDa gene 4 primase. T7 polΔ401–404 had less than 10% of the activity observed with wild-type gene 4 protein (Fig. 3*B*). This result supports a model in which the four-residue loop of T7 DNA polymerase is important for interaction with the primase domain of gene 4 protein. Alternatively, the loop could assist in the stabilization of the short primer in a reaction in which the primase domain of gene 4 protein contacts only the stabilized primer, an interaction that remains essential for primer use.

Stability of T7 Gene 4 Protein–T7 DNA Polymerase Complex. A stable complex of T7 DNA polymerase and gene 4 protein is formed when DNA polymerase incorporates a chain-terminating nucleotide during primer extension in the presence of the next incoming nucleotide (S.T., unpublished results). The complex of primase and polymerase will protect the 3′ end of the extended primer from cleavage by exonuclease III. An analogous strategy has been used to characterize T7 DNA polymerase and rat β polymerase complexed to a primer–template and a nucleoside triphosphate (35, 43).

Gene 4 protein will catalyze the synthesis of the tetraribonucleotide pppACCC on the oligonucleotide template 5′-CAGTCACGGGTCGTTTATCGTC-3′ containing the primase recognition sequence 5′-GGGTC-3′ (underlined). If [α-^{32}P]ddATP is present along with dCTP, dGTP, and dTTP, the primer will be extended by T7 DNA polymerase to 4 nt. Incorporation of [α-^{32}P]ddAMP at the fourth base from the primer 3′-OH end will prevent further extension. In the presence of dCTP the next nucleotide that should be incorporated, a stable complex of polymerase/gene 4 protein/primer–template, is formed. This stable complex protects the newly synthesized DNA from hydrolysis by exonuclease III. The stability of the complex is determined by following the fate of 3′ end-labeled primer using urea-PAGE.

As shown in Fig. 4, the complex of wild-type T7 DNA polymerase with gene 4 primase protects the 3′ end-labeled primer from exonuclease III digestion for more than 60 min. In striking contrast, T7 polΔ401–404 poorly extends the tetraribonucleotide primer, and the complex of T7 polΔ401–404 with gene 4 primase fails to protect the extended primer from degradation by exonuclease III. This result demonstrates a defective interaction within the complex consisting of T7 polΔ401–404, gene 4 protein, and primer–template.

Strand Displacement DNA Synthesis. Inasmuch as the 63-kDa gene 4 protein has helicase as well as primase activity, we examined the ability of the gene 4 helicase to interact functionally with T7 polΔ401–404. T7 DNA polymerase by itself is unable to synthesize DNA once it encounters a duplex DNA region in the template and requires the T7 gene 4 helicase for further synthesis (44, 45). The helicase domain of T7 gene 4 protein, moving from a 5′-to-3′ direction on the strand to be displaced, unwinds the duplex DNA to provide a ssDNA template for the polymerase (24). The resulting strand displacement DNA synthesis requires a coupling of polymerase and helicase activity through direct interaction of the T7 helicase and DNA polymerase (25, 45). To examine the interaction of T7 polΔ401–404 with T7 gene 4 protein, strand displacement DNA synthesis was performed by using both the 56- and 64-kDa forms of the gene 4 protein. The template was a 70-nt circular ssDNA annealed to a 70-base oligonucleotide, in which only the 30 3′ bases of the linear oligonucleotide anneal to the circular DNA whereas the 40 5′ bases 5′ form an

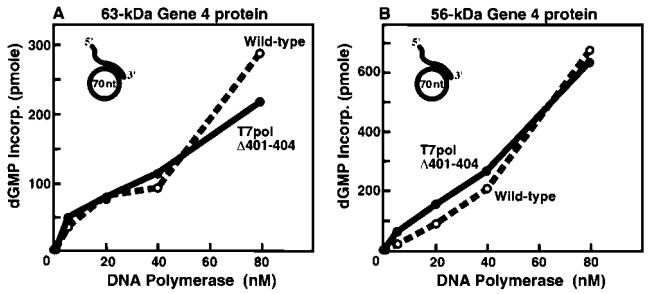

Fig. 5. Strand displacement DNA synthesis. Strand displacement DNA synthesis was measured by using a 70-nt circular dsDNA template with a 40-nt 5′-oligo(dT) DNA tail (as shown in *Insets*). The 5′ ssDNA tail allowed the gene 4 protein to assemble on the ssDNA. The hexameric gene 4 protein translocates 5′ to 3′ along the ssDNA direction until it encounters the 70-nt circular duplex DNA region. The helicase activity of gene 4 protein unwinds the duplex region and allows T7 DNA polymerase to carry out DNA synthesis on the circular ssDNA template. The reaction was performed as described in *Materials and Methods*. The amount of [α-^{32}P]dGMP incorporated into the DNA by the wild-type T7 DNA polymerase (○) or T7 polΔ401–404 (●) is plotted vs. increasing polymerase concentrations either in the presence of the 63-kDa gene 4 protein (*A*) or the 56-kDa gene 4 protein (*B*).

oligo(dT) tail. In both cases, the rate of strand displacement synthesis by T7 polΔ401–404 was similar to that observed with wild-type DNA polymerase (Fig. 5). The results show that the deletion of the four-residue loop of T7 DNA polymerase did not affect the ability of T7 gene 4 protein to provide helicase activity for strand displacement synthesis catalyzed by T7 DNA polymerase, a reaction in which the acidic C terminus of the gene 4 protein interacts with the polymerase.

Discussion

Using *in vitro* replication systems, it has been shown previously that leading and lagging strand DNA synthesis at the T7 replication fork are coordinated in that both proceed at the same rate (1, 46) even though lagging strand synthesis inherently requires more reactions than does leading strand synthesis. It seems likely that specific protein–protein interactions are essential to coordinate the multiple reactions that are occurring on the two strands. For example, the helicase translocating on the lagging strand and unwinding the duplex must be coordinated with the polymerase on the leading strand. Likewise, DNA synthesis catalyzed by the polymerase on the lagging strand must be coordinated with synthesis on the leading strand. Such a coordination could be mediated by interactions between the polymerases themselves or through interactions with the gene 4 protein. In the bacteriophage T7 replication system a number of such interactions have been demonstrated with the purified proteins (25, 29, 30, 32, 46, 47).

The synthesis and use of RNA primers on the lagging strand during coordinated DNA synthesis poses a unique set of problems. The primase must be able to efficiently recognize primase sites, synthesize oligoribonucleotides, and stabilize these short primers until they are extended by T7 DNA polymerase. In the T7 system the physical association of the helicase and primase activities within one polypeptide enables the primase to reach primase recognition sites via its association with the helicase (33, 34). In addition, the tight binding of the gene 4 protein to DNA via its helicase domain facilitates the access of primase sites by the primase domain (48). Interactions between the primase domain and the polymerase for efficient transfer and stabilization of primer/template complexes seem likely but remain to be demonstrated.

The crystal structure of T7 DNA polymerase (35) reveals the presence of a unique loop of 4 aa: Glu-Gly-Asp-Lys (residues 401–404). This loop is absent in other members of the pol I family, whose structures are known (35–38, 49). The loop is located in the DNA-binding crevice 4 nt away from the 3′-OH terminus of the primer (35). Of relevance is the fact that primers synthesized by the T7 primase are tetraribonucleotides, a length that would place the 5′-triphosphate of the terminal nucleotide within the loop region. Thus, the uniqueness and position of the loop suggest an interaction of the polymerase with the primase/primer complex at this site.

In the present study, we have investigated the role of the 401–404 loop by generating and characterizing an altered T7 DNA polymerase in which the 4-aa loop has been deleted. The deletion of the loop had no effect on plaque size and efficiency of plating. It is possible that the defects in primer utilization observed *in vitro* are not sufficiently severe to affect T7 phage growth. This finding is not surprising as mutations in T7 gene 4 that reduce the primase activity of the T7 gene 4 primase/helicase still can support the growth of T7 bacteriophage although with a lower efficiency (27, 50). It is possible that the deletion of the loop has affected the burst size of T7, which has not been determined in the present study. The deletion of the 4-aa loop had no effect on the processivity of DNA synthesis or on the 3′-to-5′ dsDNA exonuclease activity of the polymerase. The rate of DNA synthesis on M13 single-stranded template annealed to a DNA primer was 3-fold lower than that of the wild-type enzyme. There is a marked defect in the ability of T7 polΔ401–404 to use primers synthesized by gene 4 protein. T7 wild-type DNA polymerase uses tetraribonucleotide as primers, either chemically synthesized or synthesized by gene 4 protein, provided gene 4 protein is present in the reaction and a primase recognition sequence is present in the template (29, 30, 32, 40). Tetraribonucleotides alone cannot prime DNA synthesis catalyzed by T7 DNA polymerase. The altered polymerase, T7 polΔ401–404, is severely impaired (15% of that observed with wild-type DNA polymerase) in its ability to use primers synthesized by gene 4 protein on M13 DNA, on which there are multiple primase sites. An even greater impairment is observed with regard to the use of exogenously added tetraribonucleotide. In the presence of gene 4 protein, T7 polΔ401–404 uses these tetraribonucleotides less than 10% as well as the wild-type enzyme. The inability of complex of T7 polΔ401–404 and gene 4 protein to protect the primer from cleavage by exonuclease III provided additional evidence for the role of this four-residue loop in the interaction with T7 gene 4 primase/primer complex.

Inasmuch as the helicase and primase activities are inseparable in the T7 replication system, we also examined the effect of deleting the 401–404 loop on the coordination of helicase and polymerase activities. Wild-type T7 DNA polymerase and T7 helicase activities are coordinated during strand displacement synthesis of duplex DNA such that both proceed at the same rate, a coordination that requires the acidic C terminus of the gene 4 protein (25). In the present study, the T7 polΔ401–404 and the gene 4 protein functioned together to catalyze strand displacement DNA on a minicircular DNA molecule just as well as the wild-type polymerase and the gene 4 protein. This result is interesting in that some mutations in gene 4 protein that affect primer synthesis also affect translocation of gene 4 protein on ssDNA (27).

Our results show clearly that the four-residue loop in the DNA-binding crevice increases the efficiency of extension of relatively long primers or the tetraribonucleotides synthesized by the T7 gene 4 primase. In the latter case the use of such short primers requires the presence of the gene 4 primase and a primase recognition sequence. Although the absence of the loop may well destabilize the primer as a result of loss of contacts or a conformational change, we believe that the loop also plays a role in the interaction of the primase with the

polymerase. First, the structurally similar DNA polymerase I of *E. coli*, a polymerase that does not interact with a primase, is lacking a similar loop. Second, the most profound defect observed with T7 polΔ401–404 is its ability to use preformed tetraribonucleotides in a gene 4 primase-mediated reaction. Tetraribonucleotides are synthesized by the primase, and the position of the loop places it in a position to interact with the tetranucleotides and the primase domain of the gene 4 protein. Third, the inability to form a stable complex with the gene 4 protein, a primer, and T7 polΔ401–404 is compatible with such an interaction, albeit one that also involves interactions of the primer with the polymerase.

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