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A Complex of the Bacteriophage T7 Primase-Helicase and DNA Polymerase Directs Primer Utilization*

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The lagging strand of the replication fork is initially copied as short Okazaki fragments produced by the coupled activities of two template-dependent enzymes, a primase that synthesizes RNA primers and a DNA polymerase that elongates them. *Gene 4* of bacteriophage T7 encodes a bifunctional primase-helicase that assembles into a ring-shaped hexamer with both DNA unwinding and primer synthesis activities. The primase is also required for the utilization of RNA primers by T7 DNA polymerase. It is not known how many subunits of the primase-helicase hexamer participate directly in the priming of DNA synthesis. In order to determine the minimal requirements for RNA primer utilization by T7 DNA polymerase, we created an altered gene 4 protein that does not form functional hexamers and consequently lacks detectable DNA unwinding activity. Remarkably, this monomeric primase readily primes DNA synthesis by T7 DNA polymerase on single-stranded templates. The monomeric gene 4 protein forms a specific and stable complex with T7 DNA polymerase and thereby delivers the RNA primer to the polymerase for the onset of DNA synthesis. These results show that a single subunit of the primase-helicase hexamer contains all of the residues required for primer synthesis and for utilization of primers by T7 DNA polymerase.

DNA replication is mediated by a complex of proteins that assembles at the replication fork and directs the coordinated synthesis of two DNA strands. Four proteins account for the major reactions occurring at the replication fork of bacteriophage T7 as follows: the gene 5 DNA polymerase and its processivity factor, *Escherichia coli* thioredoxin, the gene 2.5 single-stranded DNA-binding protein, and the gene 4 primase-helicase (1). The activities of these replication proteins are coordinated by their physical interactions during replication, which serve to couple the synthesis of the leading strand with that of the lagging strand (2). The primase-helicase is a fixture of the T7 replisome that directly contacts both the DNA polymerase and the single-stranded DNA-binding protein (2–6). A

C-terminal acidic segment of the primase-helicase is required for its interaction with T7 DNA polymerase (4). This stable protein-protein interaction could correspond to an interaction between the helicase bound to the lagging strand of the replication fork and the polymerase on the leading strand.

As the replication fork moves along a DNA duplex, DNA primase periodically deposits short RNA primers at specific priming sequences on the lagging strand, triggering the synthesis of Okazaki fragments that are subsequently processed to form a continuous DNA strand (7, 8). In most DNA replication systems, a separate primase protein transiently interacts with the DNA helicase to initiate primer synthesis on the lagging strand. In *E. coli*, the strength of this interaction affects the frequency of priming and thereby sets the average length of Okazaki fragments (9). The primase and helicase activities of bacteriophage T7 are fused in a single polypeptide that assembles into a ring-shaped hexamer (10–12). The bifunctional primase-helicase unwinds DNA ahead of the replication fork, and it primes the discontinuous synthesis of the lagging strand of the replication fork. The short tetranucleotides synthesized by the primase domain of the primase-helicase are not extended by T7 DNA polymerase alone (13–15); they are elongated by the polymerase only if the primase-helicase is also present during primer extension. It is not known how many subunits of the hexameric primase-helicase directly participate in the priming of DNA synthesis, nor is it known how the primase-helicase stimulates primer utilization by T7 DNA polymerase. The primase-helicase protein consists of an N-terminal primase domain and C-terminal helicase domain (12, 16, 17) that will separately catalyze tetraribonucleotide synthesis and DNA unwinding, respectively (16, 18, 19). However, the primase domain alone does not support the extension of primers by T7 DNA polymerase (18). The dual requirement for the primase-helicase and the T7 DNA polymerase during RNA-primed synthesis of DNA suggests that these proteins associate in a complex that initiates the elongation of RNA primers synthesized by the primase (20).

The ring-shaped T7 primase-helicase catalyzes DNA unwinding by encircling one strand of DNA (12, 21) and moving in a 5′ to 3′ direction along one DNA strand while displacing the complementary strand (22–26). The vectorial movement of the protein on DNA is coupled to the hydrolysis of 2′-deoxythymidine triphosphate (dTTP) (25, 27, 28), and thus, the helicase is a type of molecular motor. Crystal structures of the helicase domain of the primase-helicase (29, 30) revealed that the nucleotide-binding sites are located at the interfaces between subunits of the hexamer, where changes in the relative orientations of the subunits could influence the catalytic activities of the six potential active sites within the helicase. Three different relative orientations of adjacent subunits are observed in

the hexameric helicase that was crystallized, and these different orientations affect the nucleotide binding properties of individual subunits (30). This conformational flexibility, together with previous biochemical and genetic data revealing the identities of functionally important residues and the cooperative behaviors of nucleotide binding and hydrolysis by the hexameric helicase, are the basis for several proposed mechanisms of DNA unwinding (30, 31). In these models, nucleotide hydrolysis is coupled to changes in protein conformation and DNA binding affinity that allow the protein to step along DNA, not unlike other motor proteins that travel along protein filaments in response to nucleotide hydrolysis (32, 33). Some aspects of the proposed mechanisms of DNA unwinding resemble those of the bind-change mechanism of rotary catalysis proposed for the mitochondrial F_1-ATPase (34, 35).

There are currently few high resolution structures of primases, and none with substrates bound (36–38). The DNA binding and catalytic properties of the T7 primase are well characterized, making it an attractive candidate for structural analysis. Like the intact primase-helicase, a primase fragment (residues 1–271) of the T7 primase-helicase catalyzes the template-dependent synthesis of RNA oligomers at specific priming sites as follows: 5′-(G/T)(G/T)GTC-3′ (18, 39, 40), making predominantly pppAC, pppACC(C/A), and pppACAC. The conserved 3′-C of the priming sites is required for primer synthesis, but it is not copied into the RNA products. The tetraribonucleotides synthesized by an isolated primase fragment are *bona fide* primers that can be extended by T7 DNA polymerase, provided the intact primase-helicase protein is added during primer extension (18). The primase domain fragment of the T7 primase-helicase is monomeric even at very high protein concentrations, and its failure to support primer utilization by T7 DNA polymerase suggested that several subunits of the hexameric primase-helicase might cooperate to deliver tetranucleotide primers to the polymerase. Such cooperation could occur either by recruiting the polymerase through protein-protein interactions or by preventing dissociation of the RNA primer from the DNA template by sequestering the primer in a stable protein-DNA complex. A long lived complex of the primase-helicase and M13 single-stranded DNA forms in the presence of ribonucleotide substrates for primer synthesis (13). This primase-helicase-DNA complex most likely contains the ribonucleotide product of the primase annealed to DNA and ready for elongation by T7 DNA polymerase (14).

We wished to determine if a single subunit of the primase-helicase could provide all of the necessary DNA binding and protein contacts for priming synthesis of DNA by T7 DNA polymerase. In order to define the minimal requirements for primer utilization by T7 DNA polymerase, we have genetically engineered an altered primase-helicase that does not assemble into functional hexamers and therefore lacks DNA unwinding activity. We show that the monomeric primase physically associates with T7 DNA polymerase in a protein-DNA complex that initiates RNA-primed synthesis of DNA.

EXPERIMENTAL PROCEDURES

Materials—The oligonucleotides used in these studies were synthesized on an Applied Biosystems model 394 DNA synthesizer and purified by high performance liquid chromatography using an HQ20 anion exchange column (Applied Biosystems) or by gel electrophoresis using 20% acrylamide, 7 M urea gels. The T7 primase-helicase and T7 DNA polymerase (a 1:1 complex of T7 gene 5 protein and *E. coli* thioredoxin) were purified by published procedures (41, 42). A variant of T7 DNA polymerase with two amino acid substitutions in the active site of the 3′–5′-exonuclease (Asp-5 → Ala and Glu-7 → Ala; constructed by Stanley Tabor) was used for the assembly of stable protein-DNA complexes. This altered DNA polymerase has a wild-type level of DNA polymerase activity, but the exonuclease activity of the altered polymerase is reduced by a factor of 10^6 (43). We therefore refer to this

modified polymerase as exo⁻ T7 DNA polymerase. The primase fragment (residues 1–271) of the T7 primase-helicase was purified as described previously (18). *E. coli* DH5α was obtained from Life Technologies, Inc., and *E. coli* BL21(DE3) was obtained from Novagen. M13 single-stranded DNA (ssDNA)[1] and restriction enzymes were obtained from New England Biolabs. dNTPs were purchased from Promega Corp. ATP, CTP, and all radiolabeled materials were from Amersham Pharmacia Biotech. The diribonucleotide ApC was obtained from Sigmao. The ribonucleoside triphosphate pppApC was prepared as described under "Exonuclease III Protection Assay" below.

Mutagenesis of the T7 Primase-Helicase—The mutant T7 gene 4 protein, gp4ΔD2D3, has the sequence SASASG substituted for residues 368–382 of the primase-helicase, and consequently, it is nine residues shorter than the wild-type protein (Fig. 1). This amino acid substitution eliminates two α-helices (helices D2 and D3) located at the subunit interface of the helicase domain of the primase-helicase (29, 30) and thus should prevent the formation of the hexamer. An expression plasmid for gp4ΔD2D3 was constructed by cloning a *Bsa*I-*Afl*II fragment containing the desired modifications from the plasmid m4D1 (encoding a C-terminal fragment of the gene 4 protein, provided by Leo Guo, Harvard Medical School) into an expression plasmid for the full-length primase-helicase, pETgp4A′-A (obtained from David Frick, Harvard Medical School). The resulting plasmid, pGP4ΔD2D3, is based on the pET24a vector (Novagen Inc.), and it places the gene encoding the modified primase-helicase under control of a T7 promoter with a binding site for the lac repressor, which in turn suppresses background expression of protein prior to induction. Codon 64 of the gp4ΔD2D3 coding sequence has been changed from methionine to a glycine codon in order to prevent internal initiation of translation at codon 64 (44).

Preparation of gp4ΔD2D3—A 5-ml overnight culture of *E. coli* BL21(DE3) cells transformed with the pGP4ΔD2D3 expression plasmid was inoculated into 1 liter of Luria-Bertani (LB) medium containing 60 μg/ml kanamycin and was shaken at 37 °C for about 6 h until the culture reached an A_{600} of ~2.0. The culture flasks were then chilled on ice for 15 min; isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM, and the induced cultures were incubated with shaking at 10 °C for an additional 18 h. The cells did not grow during incubation at 10 °C, yet the gp4ΔD2D3 protein was expressed as a mixture of soluble and insoluble protein. The soluble protein accounted for ~10% of the total gp4ΔD2D3 produced. Protein expression at 22 or 37 °C resulted in higher levels of gp4ΔD2D3 expression, but all of the protein was insoluble in native buffers. After overnight induction at 10 °C, the cells (25 g wet weight) were collected by centrifugation and resuspended in 120 ml of lysis buffer (100 mM Tris-HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride). The suspended cells were lysed by sonication. The cell lysate was centrifuged at 49,000 × g for 40 min at 4 °C, and the supernatant (fraction I) was collected for purification of gp4ΔD2D3. About 90% of the gp4ΔD2D3 precipitated in the cell lysate, and no attempt was made to resuspend this insoluble material. 7.7 g of ammonium sulfate was added to 145 ml of fraction I (10% saturation), and the solution was centrifuged at 40,000 × g for 40 min at 4 °C. To the supernatant, ammonium sulfate (24.5 g, 40% saturation) was again added with stirring, and the solution was centrifuged as before. The resulting pellet containing the gp4ΔD2D3 protein (fraction II) was resuspended in 100 ml of Buffer A (20 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, and 0.5 mM DTT) and again centrifuged at 49,000 × g for 20 min at 4 °C to remove insoluble material. The clarified fraction II was loaded on a DEAE-Sepharose column (4.9 cm² × 15 cm) and washed with 100 ml of Buffer A. The DEAE column was then eluted with a 400-ml gradient of NaCl (0–400 mM) in Buffer A. The fractions containing gp4ΔD2D3 were identified by SDS-PAGE and Coomassie Blue G-250 staining and then pooled (72 ml, fraction III). Fraction III was diluted to 180 ml with Buffer A to decrease the salt concentration, and then it was applied in 2 equal aliquots to a Mono Q column (0.79 cm² × 10 cm, Amersham Pharmacia Biotech). The Mono Q column was washed with 20 ml of Buffer A and then eluted with a 120-ml gradient of NaCl (0–400 mM NaCl) in Buffer A. The fractions containing gp4ΔD2D3 were combined from both Mono Q separations (50 ml, fraction IV), diluted to 200 ml with Buffer A, and loaded onto two 5-ml Hi-Trap heparin-Sepharose columns (2 cm² × 2.5 cm; Amersham Pharmacia Biotech) connected in series. After washing the heparin-Sepharose with 50 ml of Buffer A, the

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[1] The abbreviations used are: ssDNA, single-stranded DNA; DTT, dithiothreitol; AMP-PCP, adenosine 5′-(β,γ-methylene triphosphate); PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; ddTMP, 2′,3′-dideoxythymidine.
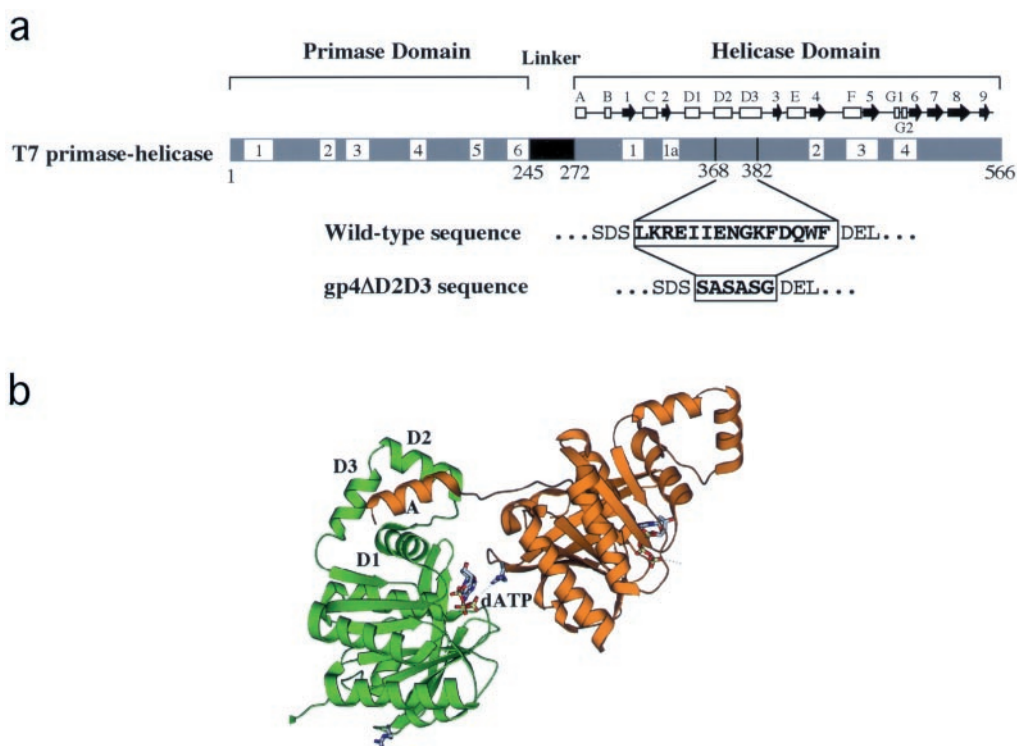
FIG. 1. **Domain organization of T7 gene 4 protein.** *a,* the modified region of the gp4ΔD2D3 primase is shown (*boxed region*) along with the corresponding sequence of the primase-helicase. A linker between the primase domain and the helicase domain (residues 245–272) is susceptible to proteolysis (19). The secondary structure of the helicase domain (29, 30) is shown *above* the conserved sequence motifs, with *boxes* denoting α-helices and *arrows* for β-strands. Helices D2 and D3 (residues 368–382) within the helicase domain are replaced in gp4ΔD2D3 with the polar segment SASASG. The boundaries and conserved motifs of the primase and helicase domains are shown, as defined by Ilyina *et al.* (55). *b,* two subunits of the hexamer are shown, colored *green* and *orange,* respectively. The interface between subunits of the primase-helicase includes the active site of the helicase (with bound dATP) and the swapped helix A that packs against helices D1–D3 of the neighboring subunit (29, 30).
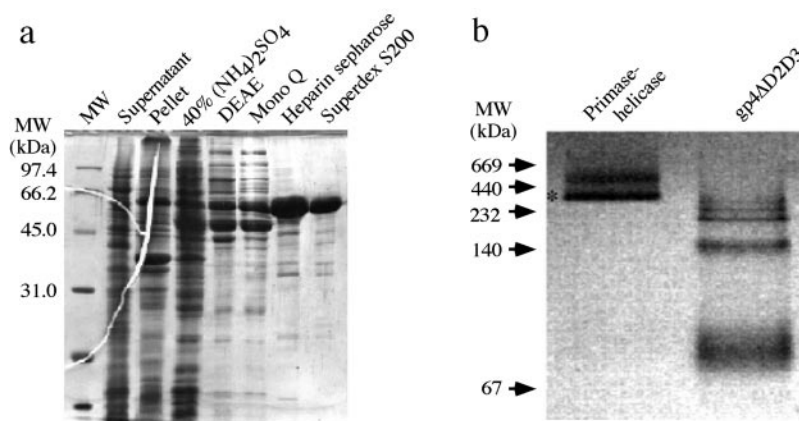

FIG. 2. **Purification of the gp4ΔD2D3 primase.** *a,* the purification scheme of the soluble gp4ΔD2D3 is described in detail under "Experimental Procedures" and is summarized by the gel analysis of the column fractions shown here. *Lanes MW,* molecular weight markers showing their molecular mass to the *left* of the gel; *Supernatant,* the supernatant of the cell lysate; *Pellet,* the precipitate of the cell lysate; *40% (NH₄)₂SO₄,* the pellet of 40% ammonium sulfate precipitation; *DEAE,* the pool of fractions from the DEAE column; *Mono Q,* the pooled fractions after the Mono Q column; *Heparin-Sepharose,* the pool of fractions of the Hi-trap Heparin column; *Superdex S200,* the collected fractions from the gel filtration column. *b,* T7 primase-helicase and gp4ΔD2D3 were analyzed on a 15% native gel. The sample buffer contained 5 mM β,γ-methylene ATP, and the electrophoresis buffer contained 1 mM ATP to stabilize oligomers of the proteins (10, 11). The positions of molecular weight markers are indicated. During electrophoresis, the wild-type primase-helicase (10 μM) migrates with an apparent mass of 380 kDa (marked with an *asterisk*), consistent with the formation of a protein hexamer. A slowly migrating species is also evident, and it might consist of two hexamers stacked together. gp4ΔD2D3 (10 μM) predominantly migrates as a monomer (≅70 kDa). Although several gp4ΔD2D3 oligomers are also present in the gel, no hexamers are detected.

proteins were eluted with a 120-ml gradient of NaCl (0–400 mM) in Buffer A. The fractions containing gp4ΔD2D3 were combined, and the protein was precipitated by adding ammonium sulfate to 60% saturation and then resuspended in 2 ml of Buffer A (fraction V). Fraction V was loaded onto a Superdex 200 gel filtration column (5.3 cm² × 60 cm; Amersham Pharmacia Biotech) that had been equilibrated with Buffer A containing 100 mM NaCl. gp4ΔD2D3 eluted from the gel filtration column at the position of a 66-kDa protein standard, consistent with the monomeric protein. The purified fractions containing gp4ΔD2D3 were combined (45 ml, fraction VI) and concentrated by ultrafiltration (Centriprep; Amicon Inc.) to a protein concentration of ~20 mg/ml in Buffer A plus 100 mM NaCl. gp4ΔD2D3 purified from the soluble fraction of the cell lysate shows no signs of aggregation. The concentrated protein was used immediately or diluted 2-fold with glycerol (50% v/v final concentration) and stored at −20 °C. gp4ΔD2D3 prepared by this procedure is typically more than 95% pure, as judged by Coomassie Blue G-250 staining of the protein sample after SDS-PAGE. The purification procedure typically results in a yield of 2 mg of pure gp4ΔD2D3 per liter of culture.
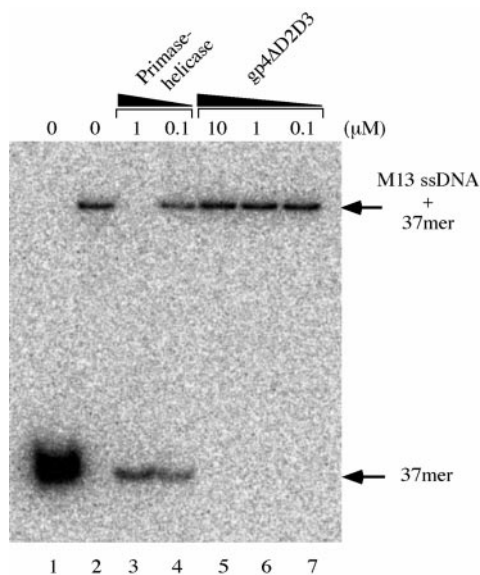
FIG. 3. **gp4ΔD2D3 lacks DNA unwinding activity.** The DNA unwinding activity of the primase-helicase was measured by the displacement of a radiolabeled 37-mer oligonucleotide annealed to M13 ssDNA. After incubation with gene 4 proteins, the substrates were separated by electrophoresis through a native gel (15%) and visualized by autoradiography. *Lane 1* shows the 5′-^{32}P-labeled oligonucleotide alone, and *lane 2* shows the starting substrate with the oligonucleotide annealed to M13 ssDNA. The primase-helicase has significant DNA unwinding activity, assayed at several different protein concentrations in the presence of dTTP (*lanes 3* and *4*; see "Experimental Procedures" for details). gp4ΔD2D3 lacks detectable DNA unwinding activity even at 10-fold higher protein concentration (*lanes 5–7*).

Nondenaturing Gel Electrophoresis—Native PAGE was performed in a 15% acrylamide Tris-glycine gel (Bio-Rad) with a Mini-PROTEAN II electrophoresis system (Bio-Rad). The electrophoresis buffer was 25 mM Tris-HCl, 190 mM glycine, 5 mM MgCl$_2$, with 1 mM ATP added to stabilize the hexameric form of the primase-helicase (10, 19). Protein samples (10 μM monomer) in 20 mM Tris-HCl (pH 7.5), 10 mM DTT, 5 mM MgCl$_2$, 5 mM β, γ-methylene ATP (AMP-PCP, to stabilize the hexamer), and 40% glycerol were incubated on ice for 30 min before loading on the gel and electrophoresing the samples for 4 h at 150 V at an ambient temperature of 4 °C. The proteins were visualized after electrophoresis by staining the gel with Coomassie Blue G-250.

dTTP Hydrolysis—The dTTPase activity of the helicase domain of the primase-helicase is a sensitive indicator of hexamer formation (19) because the active site of the helicase is located at the interface between subunits of the hexamer (29). Hydrolysis of dTTP was monitored using thin layer chromatography as described previously (11). The 10-μl reaction mixture contained 30 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, 10 mM DTT, 5 mM dTTP (including 5 μCi of [^{3}H] dTTP), and the indicated concentrations of the wild-type primase-helicase or of gp4ΔD2D3. The hydrolysis reactions were incubated for 15 min at 30 °C and stopped by adding 5 μl of 500 mM EDTA. One microliter of the reaction was spotted on a cellulose polyethyleneimine plate (J. T. Baker Inc.), which was subsequently developed with 1 M formic acid and 0.8 M LiCl for 1 h. After drying the gel, the separated substrate and products were visualized by autoradiography and quantitated by densitometry.

Helicase Activity Assays—The helicase activity of the T7 primase-helicase was measured with two different assays, either by monitoring the enzymatic separation of two DNA strands (27) or by its ability to support DNA replication in a rolling circle DNA replication reaction (2). DNA strand separation activity was monitored as the dissociation of a 5′-^{32}P-labeled 37-mer oligonucleotide (5′-TCACGACGTTGTAAAAC-GACGGCCAGTTTTTTTTTTTT-3′) annealed to M13 ssDNA (11). The oligo(dT) sequence at the 3′ end of the oligonucleotide is not complementary to M13 ssDNA, which allows the primase-helicase to load onto the M13 DNA at the junction between single-stranded and double-stranded regions. The helicase catalyzed strand separation in a 5′–3′ direction along the circular M13 DNA template. The 10-μl reaction mixture contained 40 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, 10 mM DTT, 50 μg/ml BSA, 5 mM dTTP, 20 ng/μl DNA substrate (described above), and the indicated concentrations of the primase-helicase. The mixture was incubated for 15 min at 23 °C, and then the reaction was stopped by
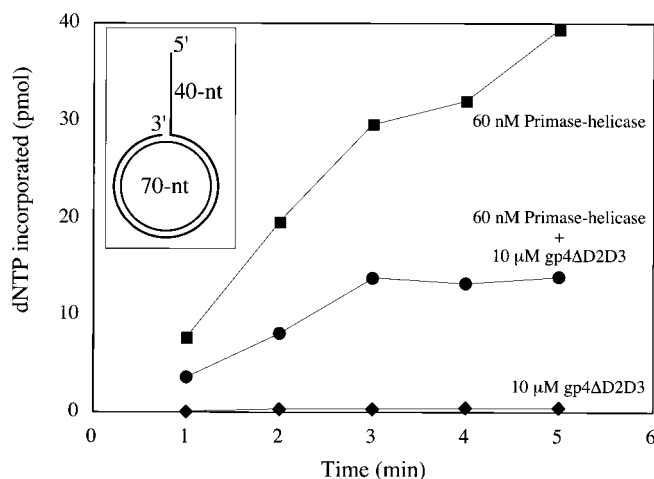

FIG. 4. **gp4ΔD2D3 does not support synthesis of DNA catalyzed by T7 DNA polymerase on duplex templates.** A rolling circle DNA replication reaction (2) was used to determine if gp4ΔD2D3 supports the replication of double-stranded DNA. The reaction contained 0.6 mM each of dATP, dCTP, dGTP, and dTTP with 333 mCi/mmol [α-^{32}P]dGTP, 100 nM DNA template (see *inset*), 80 nM T7 DNA polymerase, and the indicated concentrations of the primase-helicase or gp4ΔD2D3. The incorporation of [α-^{32}P] dGMP into DNA was monitored (see "Experimental Procedures" for the details). The primase-helicase functions in the efficient replication of a 70-nucleotide (*70-nt*) circular DNA, whereas gp4ΔD2D3 does not, apparently because it lacks DNA unwinding activity even within the context of the replication protein complex.

adding 2 μl of 500 mM EDTA. The samples were loaded on the 15% nondenaturing polyacrylamide gel with TBE buffer (90 mM Tris-HCl, 90 mM borate, 2 mM EDTA (pH 8.0)) and electrophoresed at 300 V for 2 h at an ambient temperature of 4 °C. The amounts of radiolabeled oligonucleotide remaining annealed to the substrate or in the dissociated product strand were visualized by autoradiography.

We also examined the ability of the primase-helicase to stimulate DNA synthesis by T7 DNA polymerase on a duplex DNA template in a reaction that requires DNA strand separation by the helicase (2). The DNA template for this assay was a circular 70-nucleotide DNA template annealed to an oligonucleotide with a 3′-hydroxyl annealed to the template and an unpaired 5′ end that facilitates the loading of the primase-helicase onto DNA (Fig. 4, *inset*). The sequence of this substrate was designed to identify and quantitate specifically the leading strand synthesis that is linked to the DNA unwinding by measuring the incorporation of [α-^{32}P]dGMP into DNA. The DNA replication reaction (25 μl) contained 40 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, 10 mM DTT, 100 μg/ml BSA, 50 mM potassium glutamate, 0.6 mM each of dATP, dCTP, dGTP, and dTTP, with 333 mCi/mmol [α-^{32}P]dGTP, 100 nM DNA template, 80 nM T7 DNA polymerase, and the indicated concentrations of the T7 primase-helicase or gp4ΔD2D3. The reaction mixture was incubated at 30 °C, and 4-μl aliquots were removed at 1-min intervals and quenched immediately by the addition of EDTA. The amounts of DNA synthesized were measured by spotting the reaction aliquots onto DE81 filters, washing the filters 3 times with 0.3 M ammonium formate (pH 8.0), and measuring the bound radioactivity by scintillation counting.

Oligoribonucleotide Synthesis—Oligoribonucleotides synthesized by the primase-helicase on a synthetic oligonucleotide (5′-CAGT-GACGG<u>GTC</u>GTTTATCGTCGGC-3′; template 3 shown in Table IV) were analyzed by gel electrophoresis. The reaction mixture (10 μl) contained 40 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, 10 mM DTT, 100 μg/ml BSA, 50 mM potassium glutamate, 0.1 mM 25-mer template, 1 mM each of [γ-^{32}P]ATP and CTP, and 100 nM T7 primase-helicase or gp4ΔD2D3. The reaction mixtures were incubated at 25 °C for 30 min and stopped by the addition of 2 μl of 98% formamide, 20 mM EDTA, 0.1% bromphenol blue, 0.1% xylene cyanol. The products were separated by electrophoresis in a 25% acrylamide gel containing 3 M urea and visualized by autoradiography.

RNA-primed DNA Synthesis—The synthesis of tetraribonucleotides by the primase-helicase and their elongation by T7 DNA polymerase were measured as described previously (4). The reaction mixture (10 μl) contained 40 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, 10 mM DTT, 100 mg/ml BSA, 50 mM potassium glutamate, 25 ng/μl M13 ssDNA, 1 mM each of ATP and CTP, 0.3 mM each of dATP, dCTP, and dGTP, 0.3 mM
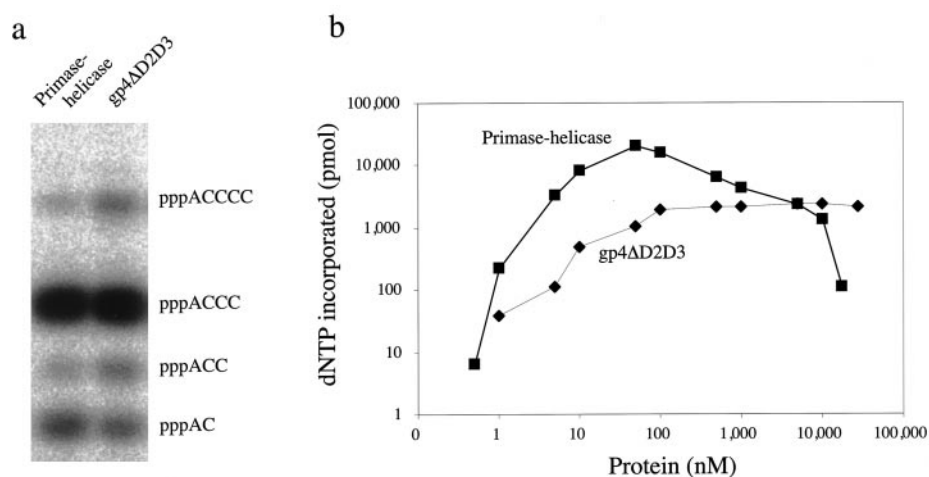
FIG. 5. **Synthesis of RNA primers and utilization for DNA synthesis.** *a*, the primase activities of the primase-helicase and gp4ΔD2D3 mainly synthesize a tetraribonucleotide that can be utilized by T7 DNA polymerase and lesser amounts of a pppAC dinucleotide that cannot be elongated by the polymerase (15). The incorporation of [γ-^{32}P]ATP into oligoribonucleotides by T7 primase was analyzed in reactions containing 0.1 mM 25-mer template (template 3 in Table IV), 1 mM each of [γ-^{32}P]ATP and CTP, and 100 nM T7 primase-helicase or gp4ΔD2D3. The products of this reaction were separated by electrophoresis in a 25% polyacrylamide gel containing 3 M urea and visualized by autoradiography. Both primases produce similar amounts of the RNA products shown. *b*, the synthesis of tetraribonucleotides by the primase-helicase and their elongation by T7 DNA polymerase were carried in the reactions containing 25 ng/μl M13 ssDNA, 1 mM each of ATP and CTP, 0.3 mM each of dATP, dCTP, and dGTP, 0.3 mM [^{3}H]dTTP, 100 nM of exo^{-} T7 DNA polymerase, and the indicated concentrations of the primase-helicase or gp4ΔD2D3. After incubation at 37 °C for 10 min, incorporation of [^{3}H]dTTP into DNA was measured by scintillation counting. Like the wild-type primase-helicase, gp4ΔD2D3 catalyzes the RNA-primed synthesis of DNA on a M13 ssDNA template. The gp4ΔD2D3 protein, which is predominantly monomeric and lacks the dTTPase activity associated with the primase-helicase, does not inhibit DNA synthesis at high concentrations, and it supports DNA synthesis at a rate that is about 1/10th the maximal rate of the wild-type primase-helicase in this assay.

[^{3}H]dTTP, 100 nM of exo^{-} T7 DNA polymerase, and the indicated concentrations of the primase-helicase or gp4ΔD2D3. The mixtures were incubated for 30 min at 37 °C, and then DNA synthesis was quantitated as the amount of radioactive dTMP incorporated into DNA using the DE81 filter binding described above.

Formation of a Priming Complex—We examined the stability of a protein-DNA complex consisting of gp4ΔD2D3 and T7 DNA polymerase bound to a primed DNA template using an exonuclease protection assay (20, 45). DNA complexed to the primase-helicase and the DNA polymerase is protected from degradation by exonuclease III, whereas the DNA that dissociates from the complex is rapidly degraded. The assay consists of a reaction in which a radiolabeled primer is synthesized by the T7 primase and elongated by T7 DNA polymerase, followed by dilution of the reaction and the re-addition of one or both T7 protein(s) prior to the addition of a large molar excess of exonuclease III (refer to Fig. 6*a*). The conditions for synthesis of the radiolabeled primer are similar to those described above for RNA-primed synthesis of DNA. The 25-mer DNA template for this reaction, 5′-CAGTGAC<u>GGGTC</u>GTT-TATCGTCGGC-3′ (template 3 in Table IV), includes a primase recognition site (underlined) for synthesis of the tetraribonucleotide pppACCC by the primase. The template sequence for synthesis of the tetraribonucleotide by the primase is followed by a sequence that allows primer extension by T7 DNA polymerase to be terminated site-specifically, after the incorporation of 1–4 deoxynucleotides. For example, a 6-mer primer strand (pppACCCdGddT) was synthesized in a 10-μl reaction containing 40 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, 10 mM DTT, 100 μg/ml BSA, 50 mM potassium glutamate, 0.5 mM template DNA, 1 mM each of [γ-^{32}P]ATP and CTP, 0.3 mM each of dGTP and ddTTP, and 100 nM each of gp4ΔD2D3 and exo^{-} T7 DNA polymerase. The primer synthesis reaction was incubated at 25 °C for 30 min, and then it was diluted 100-fold before adding back one or both of the replication proteins (10 μM) and challenging with exonuclease III. By diluting the initial reaction mixture to 1 nM protein, we could then add back 10 μM gp4ΔD2D3 and/or 10 μM T7 DNA polymerase to determine if both proteins are required for the formation of a stable priming complex. A high concentration (10 mM) of the next 2′-deoxynucleotide matching the template was added to stabilize the 3′ end of the primer in the polymerase active site (20). The final reaction for exonuclease digestion (30 μl) consisted of 40 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, 10 mM DTT, 100 μg/ml BSA, 50 mM potassium glutamate, 10 mM dCTP, 10 μM each gp4ΔD2D3 and/or exo^{-} T7 DNA polymerase, and a 100-fold dilution of the initial primer synthesis reaction (final concentration of the template DNA was 5 μM). A 4-μl aliquot was taken before the addition of exonuclease III, and the sample was quenched by addition of 3 μl of formamide with bromphenol blue. This sample is regarded as the 0 min time

point of exonuclease III digestion. The nuclease digestion was initiated by adding exonuclease III to the reaction (4 units/μl; New England Biolabs). The reaction was incubated at room temperature, and aliquots (4 μl) were removed at the indicated times and quenched with formamide/dye solution (3 μl). The radiolabeled products were separated by electrophoresis in a 25% acrylamide gel containing 3 M urea and then visualized by autoradiography. Exonuclease III efficiently removes deoxynucleotides from the 3′ end of the radiolabeled primer strand of the unprotected DNA, leaving a tetraribonucleotide (pppACCC) that is resistant to further degradation. The time course of DNA dissociation from the priming complex was determined from the intensity ratio of the fully extended primer strand at each time point to the intensity at 0 min. Under these conditions the amount of exonuclease III was not completely saturating. The addition of 3-fold more exonuclease decreased the apparent lifetime of the priming complex by about 10–20%. The continued synthesis of tetraribonucleotides during exonuclease digestion presents another complication (described below) in determining the half-life of the priming complex. We have therefore operationally defined the apparent half-lives of the various priming complexes (Tables I–V) as the time of exonuclease digestion required to decrease the amount of extended primer strand to one-half of the amount present at 0 min.

To determine the effect of primer length on the stability of the priming complex, the compositions of 2′-deoxynucleotide(s) and 2′,3′-dideoxynucleotide were adjusted in the primer synthesis reaction to create RNA-DNA products of the desired lengths. The appropriate dNTP (10 mM) complementary to the template nucleotide adjacent to the primer 3′ end was then added to each protein-DNA complex prior to challenge with exonuclease III. For example, the complex with a 5-mer (pppACCCddG) primer strand was prepared with ATP, CTP, and ddGTP for the primer synthesis reaction, followed by the addition of dTTP for the assembly of the stable priming complex. DNA templates of different lengths (Table IV) were examined using similar methods.

To determine the contribution of the 5′-triphosphate moiety of the primer to the stability of the priming complex, primer synthesis was initiated with a preformed diribonucleotide triphosphate (pppAC), or an unphosphorylated diribonucleotide (AC), in a reaction that included [α-^{32}P]dGTP to radiolabel the primer strand. After primer synthesis, exonuclease challenge was carried out as described above. pppAC was prepared by enzymatic synthesis using the primase fragment (residues 1–271) of the primase-helicase (18) and a short DNA template (5′-<u>GTCAA</u>-3′). The reaction (2 ml) contained 40 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, 10 mM DTT, 100 μg/ml BSA, 50 mM potassium glutamate, 2 μM template DNA, 2 mM each ATP and CTP, and 1 μM T7 primase fragment. The reaction was incubated for 1 h at 37 °C, and the products were purified by reverse phase high performance chromatography

FIG. 6. **Formation of a long lived priming complex.** *a,* a stable complex of T7 DNA polymerase and gp4ΔD2D3 bound to a primed DNA template can be assembled under conditions that block extension of the primer strand by the polymerase. The primase synthesizes a tetraribonucleotide (*red*) that can be extended by T7 DNA polymerase. DNA synthesis is terminated by the incorporation of a 2′,3′-dideoxynucleotide. The primer synthesis reaction is then diluted 100-fold so that the priming complex can be assembled on the primed template using one or both T7 replication proteins. The protein-DNA complex protects the radiolabeled primer strand from degradation by exonuclease III. The addition of dCTP, which binds to the polymerase active site, secures the polymerase to the 3′ end of the primer strand (45). dCTP is not present during primer synthesis, so the polymerase and primase readily dissociate from the primed DNA template (*step 1*), prior to assembly of the stable priming complex in *step 2*. The stability of this primer elongation complex is measured by challenging the protein-DNA complex with exonuclease III and monitoring the degradation of the radiolabeled primer strand as the complex dissociates. Exonuclease III removes the deoxynucleotides from the 3′ end of the primer, leaving a tetraribonucleotide (pppACCC) that is resistant to further degradation. *b,* an autoradiograph showing the radiolabeled products of the primer extension reaction described in *a*, after challenge with exonuclease III. The exonuclease challenge assays were carried out as described under "Experimental Procedures," and the degradation of the radiolabeled product (pppACCCdGddT) at the indicated times was analyzed by electrophoresis in a 25% native gel and visualized by autoradiography. The lengths of the radiolabeled products are shown at the *left side* of the figure, including the triribonucleotide (pppACC; labeled *3*) and the tetraribonucleotide (pppACCC; labeled *4*) synthesized by the primase. The primer extension products resulting from the incorporation of deoxynucleotides by T7 DNA polymerase include a 5-mer (pppACCCdG) and a specifically terminated 6-mer product (pppACCCdGddT). In *reaction 1*, the addition of both T7 replication proteins (10 μM each) and a bound nucleotide substrate (dCTP) results in a very stable protein-DNA complex with a half-life of more than 60 min. Omitting any of these components (*reactions 2–5*) from the binding reaction results in the loss of protection of the radiolabeled strand. *c,* the intensities of the 6-mer primer extension products of reactions 1–5 shown in *b* are plotted as a fraction of the starting material at 0 min for each reaction. The continued synthesis of new primer strands with residual nucleotide substrate is a likely explanation for the initial increase in product abundance (*reaction 1*) during exposure to exonuclease III.
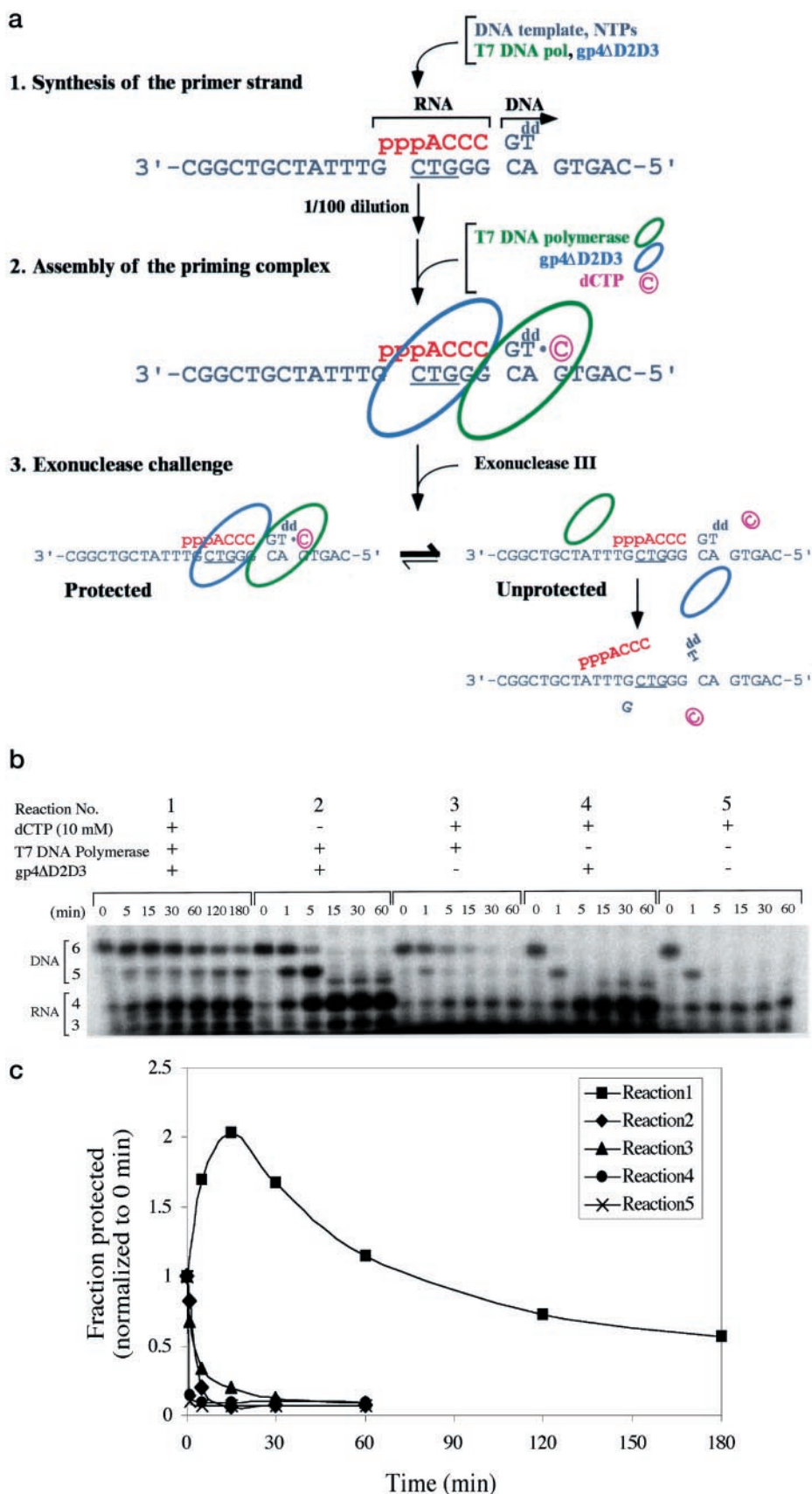
Isolation of a Protein Complex That Mediates Primer Utilization—

The protein-DNA complex containing the primase-helicase and T7 DNA polymerase was isolated by affinity capture on an immobilized DNA template (template 3 in Table IV) containing a 3′-biotin group bound to avidin-agarose beads. To form the immobilized complex, a 6-mer primer strand was enzymatically synthesized using the reaction conditions

TABLE I

The apparent lifetime of the priming complex depends upon the concentration of the bound nucleotide substrate

The intensity of the fully extended primer strand at each time point (Figs. 6*b* and 7) was quantitated by densitometry and normalized to the intensity at 0 min (Fig. 6*c*). The half-life of the protein-DNA complex is approximated by the time at which the normalized intensity was one-half the starting value at 0 min.

Concentration of dCTP	Apparent half-life
mM	*min*
0	<5
0.2	~10
1	~20
5	~60
10	>60[a]

[a] This value is estimated from Fig. 6*c*. The others are from Fig. 7.

described above, and then the reaction was diluted 10-fold into a binding solution (10 μl) consisting of 40 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, 10 mM dCTP, 10 mM DTT, 50 mM potassium glutamate, 0.2% Tween 20, 3.5 μl of NeutrAvidin-agarose resin (Pierce), and 10 μM each of gp4ΔD2D3 and exo$^-$ T7 DNA polymerase (final concentration of the template DNA was 10 μM in the binding reaction). The binding mixture was incubated on ice for 30 min, and the avidin resin was washed three times with 200 μl of wash buffer at 4 °C using a centrifugal filtration device (Nanosep MF; Pall Filtron). The wash buffer consisted of 40 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, 10 mM DTT, 50 mM potassium glutamate, 0.2% Tween 20, 200 mM NaCl, and 5% glycerol. The Tween 20 detergent was added to prevent nonspecific binding of proteins to the filtration membrane. 10 mM dCTP was added to the wash solution of complexes that were initially prepared in the presence of dCTP. The washed avidin beads were resuspended in 20 μl of SDS-PAGE sample buffer and centrifuged to elute the bound proteins. The eluted proteins were separated by SDS-PAGE and visualized by staining with Coomassie Blue G-250.

RESULTS

Construction of a Monomeric Primase-Helicase—In order to study the minimal requirements for primer utilization by T7 DNA polymerase, we constructed a monomeric variant of the T7 primase-helicase. Crystal structures of the helicase domain of the primase-helicase (29, 30) have revealed an interlocking arrangement of neighboring subunits within the hexamer. The N-terminal α-helix of the helicase domain (helix A; residues 272–281; Fig. 1*b*) extends from each subunit to pack against three helices of the adjacent subunit (helices D1–D3; residues 345–388) in a "helix swapping" arrangement (46). The hexamer is further stabilized by additional residues immediately N-terminal to helix A (19, 30) and several loops surrounding the nucleotide-binding site. Because the isolated primase domain is monomeric and shows no evidence of self-association even at high protein concentrations (19), it seemed likely that the main interactions stabilizing the primase-helicase hexamer were those observed in crystal structures of the helicase domain.

Our strategy to prevent oligomerization of the primase-helicase was to eliminate two helices located at the subunit interface (helices D2 and D3; residues 368–382), and to replace this segment with a short polar sequence (NH$_2$-SASASG-COOH; Fig. 1*a*) that would be unlikely to stabilize the hexameric packing seen in the crystal structure (29, 30). The resulting 61-kDa protein, which we have named gp4ΔD2D3 to indicate the deletion of helices D2 and D3, lacks half the residues contributing to the helix swapping interaction that links adjacent subunits of the primase-helicase. Although gp4ΔD2D3 is insoluble when expressed in *E. coli* grown at 37 °C, a significant amount of soluble protein can be obtained by inducing protein expression at 10 °C for 18 h (Fig. 2*a*; see "Experimental Procedures"). The gp4ΔD2D3 purified from the soluble fraction is well behaved and completely soluble at 25 mg/ml in buffer containing 100 mM NaCl. About 18 mg of soluble protein can be obtained from a 9-liter culture.

The oligomeric state of gp4ΔD2D3 was examined by native gel electrophoresis (Fig. 2*b*). 5 mM β,γ-methylene ATP and 1 mM ATP was included in the protein sample and in the gel running buffer, respectively, in order to favor the formation of oligomers (11, 47). Nucleotides bind at the interfaces between subunits of the primase-helicase (29, 30) and stabilize the hexameric form of the protein (10). The T7 primase-helicase has an apparent mass of ~400 kDa, indicating that the hexamer forms under these conditions (Fig. 2*b*). Variable amounts of a second, slowly migrating species are observed in current and previous experiments (11, 48). The apparent mass of this slowly migrating species is consistent with two hexamers associated in the low ionic strength buffer used for native PAGE. In contrast to the wild-type primase-helicase, about 85% of the gp4ΔD2D3 is monomeric ($\cong$70 kDa) in the gel, and there is no detectable hexamer. However, trace amounts of several higher order oligomers of gp4ΔD2D3 are evident in the native gel, indicating some self-association into dimers and trimers.

gp4ΔD2D3 Lacks dTTPase and DNA Unwinding Activities—Although the gp4ΔD2D3 is predominantly monomeric in solution, it was important to determine whether the modified protein can assemble into functional hexamers on DNA. We therefore assayed gp4ΔD2D3 for its ability to hydrolyze dTTP and to unwind DNA, activities that depend upon the oligomerization of gp4. The hydrolysis of dTTP fuels the translocation of the primase-helicase along DNA in a 5′ to 3′ direction, separating the strands of duplex DNA (22–26). The active site of the helicase consists of residues from two adjacent subunits, and oligomerization is required for nucleotide hydrolysis (19). The addition of single-stranded DNA to the primase-helicase stimulates its rate of dTTP hydrolysis about 20-fold and promotes hexamer formation (11, 47). In contrast, gp4ΔD2D3 lacks detectable dTTPase activity in the presence or absence of M13 ssDNA (not shown), even at protein concentrations as high as 50 μM using conditions that support a high level of nucleotide hydrolysis activity by the wild-type primase-helicase. The results indicate that gp4ΔD2D3 does not form functional hexamers, even at protein concentrations that are much higher than those required to assemble a hexamer of the wild-type primase-helicase (Fig. 2*b*).

gp4ΔD2D3 also lacks DNA unwinding activity and thus is unable to assemble on DNA into functional hexamers (Fig. 3). The DNA unwinding activity of the wild-type primase-helicase readily displaces a radiolabeled oligonucleotide annealed to M13 ssDNA, whereas gp4ΔD2D3 is inactive, even at 100-fold higher protein concentrations (Fig. 3). Helicase activity was also assayed in a rolling circle DNA replication system in which DNA strand separation by the helicase is required for DNA synthesis (Fig. 4) (2). In the presence of the primase-helicase, T7 DNA polymerase incorporates radiolabeled nucleotides into DNA at a rate of 2–3 pmol/min. In contrast, gp4ΔD2D3 does not support DNA synthesis (incorporation of <0.1 pmol/min) in this replication system even at 10 μM gp4ΔD2D3, consistent with its lack of DNA unwinding activity. We could not exclude the possibility that gp4ΔD2D3 is inactive in this replication system because it fails to interact with the DNA or proteins of the replication fork (2, 49). We therefore examined whether or not gp4ΔD2D3 could inhibit the activity of wild-type primase-helicase in this reaction. When gp4ΔD2D3 (10 μM) was mixed with the primase-helicase (60 nM) in the replication reaction, the rate of DNA synthesis was decreased to ½–⅓ of the wild-type rate (Fig. 4). This indicates that gp4ΔD2D3 can interact with the T7 DNA replication complex and interfere with DNA synthesis, presumably because the altered protein cannot unwind the DNA duplex.

gp4ΔD2D3 Primes DNA Synthesis by T7 DNA Polymerase—The synthesis of RNA primers by the primase-helicase and by
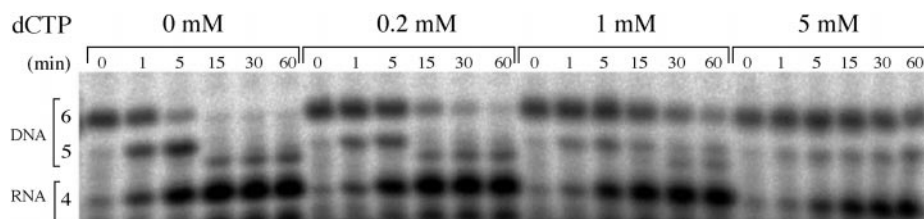
FIG. 7. **Stabilization of the priming complex by the polymerase substrate dCTP.** A high concentration of the next nucleotide (dCTP) matching the template sequence stabilizes the priming complex. The exonuclease III protection assay shown in Fig. 6 was used to monitor the formation of the priming complex in the presence of the nucleotide concentrations shown. Nucleotide concentrations higher than 5 mM (not shown) did not additionally increase the lifetime of the priming complex.
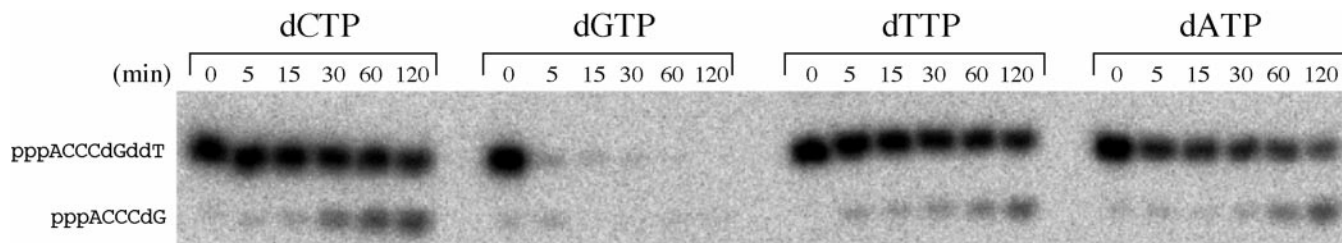

FIG. 8. **Mismatched dNTPs can stabilize the priming complex.** The stabilities of priming complexes assembled with the correct (dCTP) or mismatched nucleotide substrates were measured as described in the legend of Fig. 6, using 10 mM of each of the nucleotides listed *above* the gel. The extended primer strand was labeled by the incorporation of $[\alpha\text{-}^{32}P]$dGMP into DNA, followed by chain termination with 2′,3′-dideoxythymidine. The sequences of the extended primer strands are indicated on the *left side* of the figure.

TABLE II

The correct bound nucleotide, and some incorrect nucleotides, stabilize the priming complex

As described for the experiments shown in Table I, the intensities of the fully extended primer strands shown in Fig. 8 were quantitated by densitometry, and the half-lives were estimated for priming complexes formed in the presence of a nucleotide matching the DNA template (dCTP) or incorrect nucleotides.

Next incoming dNTP	Apparent half-life
	min
dCTP (correct)	>60
dTTP	~30
dATP	<5
dGTP	<5

gp4ΔD2D3 was examined in reactions with a 25-mer DNA template containing a primase recognition site (template 3 in Table IV). In the presence of $[\gamma\text{-}^{32}P]$ATP and CTP, gp4ΔD2D3 synthesized the same amounts of di-, tri-, and tetraribonucleotide products as the wild-type primase-helicase (Fig. 5a). The primase-helicase and gp4ΔD2D3 also synthesize a small amount of a pentaribonucleotide, presumably by misincorporating AMP or CMP opposite a cytidine at this position of the template. The major product of RNA synthesis is a tetraribonucleotide that can be elongated by T7 DNA polymerase in the presence of dNTPs (see Fig. 6b).

The tetraribonucleotides synthesized by the T7 primase-helicase can be extended by T7 DNA polymerase only in the presence of the primase-helicase (26, 28). Although a fragment spanning the N-terminal half of the primase-helicase correctly synthesizes tetraribonucleotides, the primase fragment does not support their utilization by T7 DNA polymerase (18). The primase fragment and gp4ΔD2D3 are both predominantly monomeric proteins, and primer utilization by T7 DNA polymerase might require the presence of a hexameric primase. We tested this notion by measuring the amount of DNA synthesis catalyzed by T7 DNA polymerase in reactions containing the hexameric T7 primase-helicase or the predominantly monomeric gp4ΔD2D3 protein. As expected, the wild-type primase-helicase supported the RNA-primed synthesis of DNA by T7 DNA polymerase in a reaction containing an M13 ssDNA template with ATP, CTP, and a mixture of all four dNTPs. The rate

of DNA synthesis increased with increasing concentrations of the primase-helicase protein until DNA synthesis was inhibited at very high protein concentrations (Fig. 5b). This inhibition of DNA synthesis in the presence of a large molar excess of primase-helicase probably results from the depletion of dTTP in the DNA synthesis reaction through hydrolysis by the helicase domain. Remarkably, gp4ΔD2D3 supports DNA synthesis at a rate approaching 1/10th the wild-type rate. High concentrations of gp4ΔD2D3 do not inhibit DNA synthesis, a finding consistent with its lack of dTTPase activity. The lower efficiency of gp4ΔD2D3 in promoting primer utilization and DNA synthesis might be explained by its failure to rapidly locate priming sites on the M13 ssDNA because of the defective helicase domain, which is unable to actively translocate on DNA (11).

A Priming Complex of gp4ΔD2D3 and T7 DNA Polymerase—Although it is incapable of assembling into functional hexamers, gp4ΔD2D3 primes DNA synthesis catalyzed by T7 DNA polymerase. This finding suggests that a single subunit within the hexameric primase-helicase is sufficient to prime Okazaki fragment synthesis on the lagging strand of the replication fork. The requirement for the primase during extension of RNA primers further suggests that the T7 DNA polymerase engages short, naturally occurring primers only when they are bound to the primase protein. The primase might help initiate primer elongation by securing the RNA primer to the DNA template (3) and/or by recruiting the polymerase to the priming site through protein-protein interactions. A four amino acid loop located at the base of the thumb of T7 DNA polymerase is required for lagging strand synthesis (20). The selective loss of lagging strand synthesis upon deletion of the loop, together with its location near the polymerase active site, suggests that the loop might contact the RNA primer or interact with the primase during the elongation of a primer.

The association of the hexameric T7 primase with T7 DNA polymerase during extension of a tetraribonucleotide primer was previously demonstrated using an exonuclease challenge assay (20). We investigated the minimal requirements for primer utilization by T7 DNA polymerase, using the monomeric gp4ΔD2D3 primase to synthesize and deliver tetraribonucleotide primers to the polymerase. A very stable protein-DNA complex containing gp4ΔD2D3 and T7 DNA polymerase
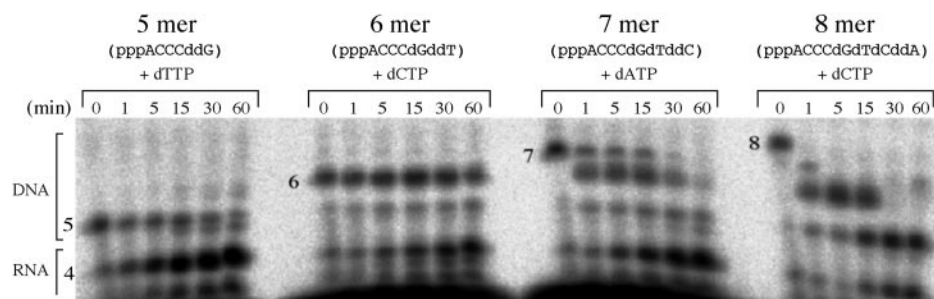
FIG. 9. **The stability of the priming complex changes during elongation of the primer strand.** The primer extension reaction (described in Fig. 6) was stopped at different positions of the DNA template by the incorporation of a chain-terminating 2′,3′-dideoxynucleotide. After the addition of gp4ΔD2D3, T7 DNA polymerase, and the appropriate dNTP, the stabilities of the resulting priming complexes were determined by exonuclease challenge. The complex containing the 6-mer primer extension product (pppACCCdGddT) is the most stable. Incorporation of fewer or more nucleotides prior to terminating synthesis resulted in faster dissociation of the protein-DNA complex (5-, 7-, and 8-mer complexes). The effect of primer strand length on the stability of the protein-DNA complex implies that specific contacts are made to the 5′ and 3′ ends of the primer strand within the complex.

TABLE III
The stability of the priming complex depends upon the length of the primer strand in the complex

The intensities of the fully extended primer strands shown in Fig. 9 were analyzed by densitometry as described in Table I.

Primer strands	Incoming dNTP	Apparent half-life
		min
5-mer (pppACCCddG)	dTTP	∼30
6-mer (pppACCCdGddT)	dCTP	>60
7-mer (pppACCCdGdTddC)	dATP	<1
8-mer (pppACCCdGdTdCddA)	dCTP	<1

is formed when DNA synthesis is halted by the incorporation of a 2′,3′-dideoxynucleotide during the initial cycles of deoxynucleotide incorporation by the polymerase (Fig. 6a). The stability of the protein-DNA complex was determined by adding a high concentration of exonuclease III and monitoring the loss of the radiolabeled primer as DNA dissociates from the complex (Fig. 6a). Both gp4ΔD2D3 and T7 DNA polymerase are required for the formation of the stable complex with the nascent primer, suggesting that both proteins remain bound to DNA (compare the extent of protection for *reaction 1* with that of *reactions 3–5* in Fig. 6b). If either protein is left out of the binding reaction, the primer is rapidly degraded to a tetraribonucleotide (labeled at the 5′ γ-phosphate position), which resists further degradation by exonuclease III. A high concentration of the substrate dCTP is also required to secure the primer in the active site of the polymerase (*reaction 2*, Fig. 6b) (20, 45). In the complete reaction, the radiolabeled primer DNA strand is very resistant to degradation by exonuclease III, and the protein-DNA complex dissociates slowly (Fig. 6c). The exposed 3′ single-stranded end of the DNA template is a poor substrate for exonuclease III (50–52), and it is degraded slowly (results not shown) in comparison to the recessed 3′ end of the primer. A determination of the lifetime of the protein-DNA complex is complicated by an observed 2-fold increase in the amount of radiolabeled primer synthesized during the first 15 min of the exonuclease digestion (compare *reaction 1* with *reactions 3–5,* Fig. 6c). This increase is the result of *de novo* primer synthesis by the primase (10 μM) present during exonuclease digestion. Because of these complications, we operationally define the apparent half-life of the priming complex as the time when the amount of extended primer decreases to one-half the amount at 0 min. The *apparent* half-lives of priming complexes measured in this way (Tables I–V) can then be compared to identify the experimental variables that affect complex stability. The priming complexes containing the gp4ΔD2D3 monomer are almost as long lived as the priming complex containing the hexameric T7 primase-helicase (20).

A different type of interaction between gp4 and T7 DNA polymerase has been described (4) that requires an acidic segment at the C terminus of the helicase domain. The analogous C-terminal region of gp4ΔD2D3 is not required for the formation of the priming complex. A truncated version of gp4ΔD2D3 lacking this acidic segment forms priming complexes with T7 DNA polymerase that are as stable as those formed by the full-length gp4ΔD2D3 protein described above (not shown). The lack of involvement of the C-terminal segment of the helicase domain further implies that the priming complex is sustained by interactions between the primase domain of the primase-helicase and the polymerase. At very high protein concentrations (100 μM primase), the primase fragment (residues 1–271 (18)) of the primase-helicase lacking the helicase domain can function in the assembly of a short lived priming complex (half-life 1–2 min determined by exonuclease challenge; not shown). The added stability provided by the helicase domain of gp4ΔD2D3 might result from tighter interactions with the DNA template (40).

Several factors strongly influence the stability of the T7 priming complex. A high concentration of the incoming dCTP substrate is required to keep the polymerase bound to the primed DNA template (Fig. 7) (45). The priming complex becomes more stable as the concentration of dCTP is increased, reaching maximal stability at 5–10 mM dCTP (Table I). Although dCTP is the correct incoming nucleotide specified by the template, the binding of several mismatched dNTP substrates in the polymerase active site measurably stabilizes the complex (Fig. 8). For these experiments, the polymerase incorporated [α-^{32}P]dGTP at the 3′ end of the tetraribonucleotide primer followed by the incorporation of ddTMP to terminate DNA synthesis. The addition of dCTP, which matches the next position of the template, produced a long lived priming complex, whereas the addition of dGTP resulted in a half-life of less than 5 min (Fig. 8 and Table II). The complex with dATP was initially degraded rapidly by the added exonuclease, but some of the radiolabeled primer was protected from degradation for more than 2 h. Surprisingly, a pairing of dTTP with the template guanosine was fairly effective at stabilizing the priming complex (Fig. 8). It is possible that the 10 mM dTTP present in the reaction permits re-synthesis of the extended primer following the exonucleolytic removal of the 3′-ddTMP.

The length of the extended primer strand has a pronounced effect on the lifetime of the complex. The long lived complex described above contains an elongated primer consisting of 6 nucleotides (5′-pppACCCdGddT-3′) that is annealed to a 25-mer template (template 3 in Table IV). Similar priming complexes were prepared by halting primer elongation after the incorporation of 1–4 deoxynucleotides by T7 DNA polymerase,

TABLE IV
DNA template requirements for the T7 priming complex

The apparent half-lives of priming complexes formed under the conditions described under "Experimental Procedures," and using the DNA templates shown below, were estimated by densitometry (data not shown). See text for a complete description of these experiments.

Template	Sequence[a]	Apparent half-life
		min
1 (19-mer)	3′-<u>CTGCTATTTG**CTG**GGCAGT</u>-5′	<5
2 (22-mer)	3′-<u>CTGCTATTTG**CTG**GGCAGT</u>GAC-5′	~20
3 (25-mer)	3′-CGG<u>CTGCTATTTG**CTG**GGCAGT</u>GAC-5′	>60
4 (28-mer)	3′-TAACGG<u>CTGCTATTTG**CTG**GGCAGT</u>GAC-5′	>60
5 (28-mer)	3′-CGG<u>CTGCTATTTG**CTG**GGCAGT</u>GACTTT-5′	~20
6 (31-mer)	3′-CGG<u>CTGCTATTTG**CTG**GGCAGT</u>GACTTTTTT-5′	~20

[a] The primase recognition sites are denoted by bold letters. The common sequences are underlined.

and the lifetimes of these complexes were determined by challenge with exonuclease III. The complex with a single dideoxynucleotide appended to the tetraribonucleotide (the 5-mer complex in Fig. 9) is less stable than the 6-mer complex described above, and it has a half-life of about 30 min (Table III). The 6-mer complex is most stable, with a half-life of more than 60 min (Figs. 6*b* and 9; Table III). Further elongation of the primer strand to create the 7- and 8-mer complexes incrementally decreases the stability of the priming complex (Fig. 9 and Table III). The destabilization of the priming complex with increasing length of the DNA product might indicate that interactions between the primase and polymerase are weakened as the polymerase moves away from the priming site during DNA synthesis. The elongating primer strand eventually becomes effectively long enough that the primase is no longer needed for DNA synthesis.

The priming complexes described above were bound to a 25-mer DNA template (template 3 in Table IV). We also examined DNA templates 19–31 nucleotides in length with the same priming sequence in complexes containing the 6-mer elongation product (pppACCCdGddT) and the bound nucleotide dCTP. All of these templates supported the synthesis of tetraribonucleotides by gp4ΔD2D3 and their elongation by T7 DNA polymerase (data not shown). However, the stabilities of the stalled priming complexes depended upon template length (Table IV). The complex with the shortest template (a 19-mer, template 1 in Table IV) was least stable and had a half-life of less than 5 min. Addition of three bases to the 3′ end of this sequence (template 2) significantly improved the stability of the priming complex (Table IV). A three-nucleotide extension to the 5′ end of template 2 to create template 3 provided optimal stability. A template with three additional nucleotides at the 3′ end (template 4) produced an equally stable priming complex. However, the addition of nucleotides to the 5′ end of the template (templates 5 and 6 in Table IV) destabilized the complex. The reason for this effect is not known.

The 5′-Triphosphate of the Primer Stabilizes the Priming Complex—The ribonucleotides synthesized by the T7 primase contain a 5′-triphosphate moiety from the ATP that initiates synthesis. The primase can also incorporate ATP analogs with chemically modified phosphate linkages at the 5′ end of ribonucleotide products (39). This relaxed specificity suggests that the primase does not interact strongly with the 5′-triphosphate moiety during RNA synthesis. The unphosphorylated dinucleotide AC is also efficiently extended by the primase to form a tetraribonucleotide (ACCC) that is utilized by T7 DNA polymerase (14). Thus, the role of the 5′-triphosphate of the naturally occurring products of T7 primase is enigmatic. We examined whether or not the 5′-triphosphate contributes to the physical stability of priming complexes formed between gp4ΔD2D3 and T7 DNA polymerase in primer extension reactions initiated with pppAC or AC dinucleotides instead of ATP. Both dinucleotides were readily extended by gp4ΔD2D3, and the resulting

tetraribonucleotide was elongated by T7 DNA polymerase in the presence dNTP and ddNTP substrates (Fig. 10). The priming complex that was initiated with pppAC was as long lived as the complex initiated with ATP. In contrast, the priming complex initiated with the unphosphorylated AC was unstable, with a half-life of less than 5 min (Fig. 10 and Table V). These results show that the 5′-triphosphate of the primer strand contributes significantly to the stability of the priming complex, and they imply that the phosphate group plays a role in primer utilization.

Purification of the Priming Complex Using an Affinity-tagged Template—The exonuclease III protection assay described above provided indirect evidence that the T7 primase and the polymerase were stably associated in the priming complex. In order to observe the complex directly, we purified the complex by a biotin-avidin affinity capture procedure. The priming complex was assembled on a 3′-biotinylated template (template 3 in Table IV) coupled to avidin-agarose beads. The beads were washed to remove proteins bound nonspecifically, and the remaining proteins complexed to the template were eluted with SDS and visualized by SDS-PAGE (Fig. 11). In the complete priming reaction (*lane 2*) both gp4ΔD2D3 and T7 DNA polymerase remained associated with the template. If either the primer-template strand or the dCTP was left out of the reaction, no detectable protein remained associated with the immobilized template (*lanes 3–5*). When the correct incoming nucleotide (dCTP) was replaced with 10 mM dTTP, dATP, or dGTP a stable complex was not isolated (data not shown), providing further evidence of the specificity of protein interactions with the immobilized DNA template. Although a mismatched dTTP provided some protection against exonuclease digestion (see above), the isolation of the priming complex requires the correct nucleotide substrate to be present during complex formation and in the wash buffer. The equal Coomassie Blue staining intensities of the gp4ΔD2D3 and T7 DNA polymerase eluted from the avidin-agarose beads (Fig. 11, *lane 2*) suggest that a 1:1 complex of primase and DNA polymerase is present in the stalled priming complex.

DISCUSSION

As the replication fork advances, RNA primers are periodically synthesized by a primase on the lagging strand and extended by a DNA polymerase to create Okazaki fragments several hundred to several thousand nucleotides in length (8). The priming sites used by prokaryotic replication systems are short sequences that are expected to occur by chance every several hundred nucleotides. However, not every potential priming site is used, and it has been suggested that a timing mechanism regulates the frequency of primer synthesis and hence the length of Okazaki fragments produced during replication. In *E. coli*, the DnaG primase transiently associates with the replication machinery to prime Okazaki fragment synthesis, and then the primase dissociates from the replication com-
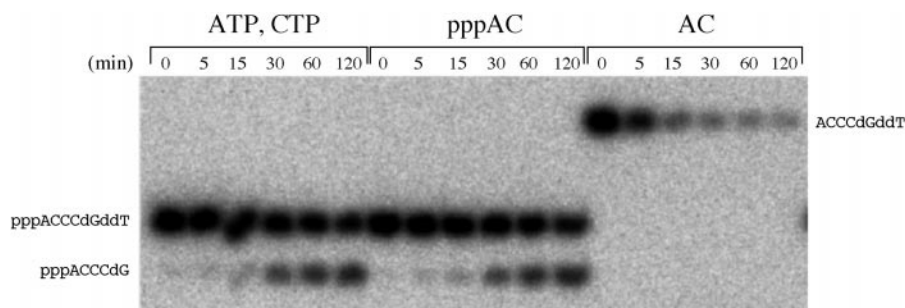
FIG. 10. **The 5′-triphosphate of the primer stabilizes the priming complex.** Priming complexes were initiated with a phosphorylated dinucleotide (pppAC) or with the unphosphorylated dinucleotide (AC) and elongated by the primase and T7 DNA polymerase in the presence of [α-³²P]dGTP to form radiolabeled products with the sequences shown in the figure. The stabilities of priming complexes initiated with either dinucleotide were compared with that of a standard complex in which primer synthesis was initiated with ATP (*left side* of figure), using the exonuclease challenge assay described in Fig. 6. The unphosphorylated primer strand migrates more slowly than the phosphorylated strands. It is evident that priming complexes containing a 5′-triphosphate group on the primer strand (initiated with pppAC or ATP) are significantly more stable than the complex with the unphosphorylated primer.

TABLE V
The 5′-triphosphate of the primer contributes to stability of the priming complex

Primers synthesized from CTP and ATP or the diribonucleotides shown below were incorporated into priming complexes, and the apparent half-lives of these complexes were determined by exonuclease challenge.

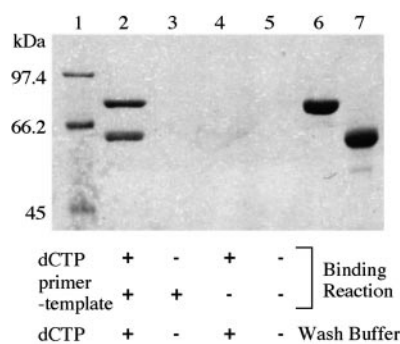| Precursor	Apparent half-life
	min
ATP, CTP	>60
pppAC, CTP	>60
AC, CTP	<5

FIG. 11. **Isolation of a stable DNA complex containing both primase and polymerase.** The priming complex described in the legend of Fig. 6 was purified using an immobilized DNA template that was attached to avidin-agarose beads through a 3′-biotin group. After washing the beads, the proteins were eluted with SDS and separated by electrophoresis on a 12% SDS-PAGE gel and then visualized by staining with Coomassie Blue. The priming complex (*lane 2*) contains both T7 DNA polymerase (the purified protein is shown in *lane 6*) and gp4ΔD2D3 (compare with pure protein in *lane 7*). The equal staining intensities of the proteins eluted from the priming complex suggest that T7 DNA polymerase and gp4ΔD2D3 are present in a 1:1 ratio. Neither protein alone stably interacts with the immobilized DNA (not shown). Immobilization of the proteins on the agarose beads requires dCTP (*lane 3*) and the DNA template (*lanes 4 and 5*), indicating that a specific complex forms on the primed DNA template.

plex. The strength of this interaction between the primase and helicase might serve as a timing mechanism that controls the frequency of priming (9). The consolidation of primase and helicase functions in the bifunctional primase-helicase of bacteriophage T7 raises questions about how many subunits of the hexamer participate in primer synthesis and utilization by T7 DNA polymerase, and how the frequency of these events is controlled during replication.

We have engineered an altered T7 primase-helicase (gp4ΔD2D3) that does not form functional hexamers, yet it primes DNA synthesis by T7 DNA polymerase (Fig. 5b). The

monomeric primase physically complexes with T7 DNA polymerase during initiation of RNA primer extension. These results suggest that the primase complexed to the DNA template delivers the newly synthesized tetraribonucleotide primer to the polymerase active site. Although gp4ΔD2D3 lacks two α-helices (D2 and D3) that account for most of the buried surface of the subunit interface of the helicase domain (Fig. 1b) (29, 30), the modified protein has some residual tendency to oligomerize (Fig. 2b). The remaining interactions between subunits might be mediated by the N-terminal primase domain or by the loops surrounding the active site of the helicase (29, 30). Additionally, the linker region (residue 241–271) between the primase and helicase domain contributes to oligomerization of the *gene 4* protein (19). gp4ΔD2D3 lacks the dTTPase and DNA unwinding activities associated with the hexameric primase-helicase, and it is predominantly monomeric, even at high protein concentrations (Fig. 2b). These results strongly suggest that a single protomer of the T7 primase-helicase can function in the utilization of tetraribonucleotide primers by T7 DNA polymerase.

gp4ΔD2D3 consists of both primase and helicase domains of the T7 *gene 4* protein. A fragment of the primase-helicase spanning only the primase domain is a monomer, and it catalyzes the template-dependent synthesis of tetraribonucleotides at specific priming sites (18), as does gp4ΔD2D3. However, the minimal primase fragment does not efficiently function in primer utilization by T7 DNA polymerase. The additional helicase domain of gp4ΔD2D3 might support primer utilization by contributing DNA interactions that secure the nascent primer on the DNA template until it is elongated by T7 DNA polymerase (40). The helicase domain might also participate in protein-protein interactions with the polymerase. The primase-helicase interacts strongly with T7 DNA polymerase in the absence of DNA (3, 4, 19, 53). However, the priming complex (Ref. 20 and this work) is different from a previously described interaction between the primase-helicase and T7 DNA polymerase that involves 17 residues at the C terminus of the helicase domain (4). This protein-protein interaction is required to couple the synthesis of the lagging strand of the replication fork with synthesis of the leading strand (2). This interaction, which can be observed with the purified proteins bound to single-stranded DNA (4), might correspond to the interaction of the helicase on the lagging strand of the replication fork with the polymerase on the leading strand. In contrast, the interaction of the T7 primase with the DNA polymerase occurs under conditions specific for primer elongation. The formation of a stable complex requires a primed template and a nucleotide substrate bound to the polymerase (Figs. 6 and 11). The removal of the acidic segment from the C terminus of gp4ΔD2D3 does not interfere with the formation of a stable priming com-

plex. These results do not exclude the possibility of additional protein-protein interactions between the primase and the polymerase when these proteins are bound to a primed DNA template.

The finding that the stability of the priming complex decreases as the primer strand is elongated (Fig. 9 and Table III) supports the notion of a direct interaction between the primase and the polymerase within the priming complex. Interactions with the 5′-triphosphate of the primer, which contribute substantially to the stability of the complex (Fig. 10 and Table V), could fix the location of the primase on the DNA template. T7 DNA polymerase engages the other (3′) end of the growing primer strand within the priming complex, as shown by the absolute requirement for a dNTP substrate to stabilize the priming complex. The length of the primer strand, and hence the distance between the primase and polymerase proteins bound to either end of the short primer strand, is a critical determinant of the stability of the priming complex (Fig. 10), implying a direct interaction between these proteins. Longer DNA primers are utilized by T7 DNA polymerase without assistance from the primase. In the crystal structure of the polymerizing complex (45), the DNA binding groove of the polymerase makes numerous contacts with DNA duplex exiting the polymerase. For the utilization of tetraribonucleotide primers, the primase protein might serve as a surrogate for these DNA contacts until the primer becomes sufficiently long to fill the DNA binding groove of the polymerase. This model suggests that the primase protein partially occupies the DNA binding groove of the polymerase, where it secures the RNA primer in the polymerase active site. A second role of the primase might be to prevent the dissociation of its short RNA product from the template by remaining bound to the nascent primer prior to its engagement by T7 DNA polymerase (3).

The formation of a stable priming complex requires a high concentration of an incoming nucleotide that can pair with the next base of the single-stranded DNA template in the active site of the polymerase (Fig. 7 and Table I). Although the nucleotide that can correctly pair with the template generates the most stable priming complex, other mismatched nucleotides can also stabilize the complex to varying extents (Fig. 8 and Table II). In particular, dTTP is able to pair with a template guanosine to produce a fairly long lived complex, although this complex cannot be isolated by the affinity capture procedure shown in Fig. 11. The addition of dGTP in combination with a guanine template base does not generate a stable complex (Fig. 8). In contrast, addition of dATP provides some protection in the exonuclease challenge assay (Fig. 8). The oversized guanine-guanine pair is apparently excluded from the polymerase active site, whereas an adenine-guanine pair might form a more favorable *syn/anti* base pairing geometry (54).

We have identified the minimal protein requirements for primer utilization by T7 DNA polymerase. A monomeric gene 4 protein lacking helicase activity is able to prime DNA synthesis by the polymerase. If DNA synthesis is halted during the first few cycles of nucleotide incorporation by the polymerase, the monomeric primase remains associated in complex with the polymerase on the primed DNA substrate. These interactions allow short RNA primers, which otherwise are not recognized by the polymerase, to be utilized and extended. The stalled primer elongation complex described here is an attractive candidate for structural analyses of interactions mediating the initial steps of DNA synthesis on the lagging strand of the T7 replication fork.

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A Complex of the Bacteriophage T7 Primase-Helicase and DNA Polymerase Directs Primer Utilization

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