Direct role for the RNA polymerase domain of T7 primase in primer delivery

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Gene 4 protein (gp4) encoded by bacteriophage T7 contains a C-terminal helicase and an N-terminal primase domain. After synthesis of tetraribonucleotides, gp4 must transfer them to the polymerase for use as primers to initiate DNA synthesis. In vivo gp4 exists in two molecular weight forms, a 56-kDa form and the full-length 63-kDa form. The 56-kDa gp4 lacks the N-terminal Cys$_4$ zinc-binding motif important in the recognition of primase sites in DNA. The 56-kDa gp4 is defective in primer synthesis but delivers a wider range of primers to initiate DNA synthesis compared to the 63-kDa gp4. Suppressors exist that enable the 56-kDa gp4 to support the growth of T7 phage lacking gene 4 (T7Δ4). We have identified 56-kDa DNA primases defective in primer delivery by screening for their ability to support growth of T7Δ4 phage in the presence of this suppressor. Trp69 is critical for primer delivery. Replacement of Trp69 with lysine in either the 56- or 63-kDa gp4 results in defective primer delivery with other functions unaffected. DNA primase harboring lysine at position 69 fails to stabilize the primer on DNA. Thus, a primase subdomain not directly involved in primer synthesis is involved in primer delivery. The stabilization of the primer by DNA primase is necessary for DNA polymerase to initiate synthesis.

T7 bacteriophage | primer handoff

DNA polymerases cannot initiate synthesis of DNA de novo, requiring a 3′-hydroxyl terminated primer positioned at the catalytic site. During DNA replication, primers are synthesized by a class of enzymes designated DNA primases (1). DNA primases catalyze the synthesis of short oligoribonucleotides that can then be used as primers by DNA polymerase. DNA primases play roles in the loading of DNA helicase (2), temporal regulation of replication (3–5), and handoff of the primer to the DNA polymerase (6–8).

Based on their sequences and structures, DNA primases can be grouped into two families—the prokaryotic and the eukaryotic/archaeal primases (1). Prokaryotic primases (see Fig. 1A for T7 DNA primase) contain six conserved sequence motifs (9). An N-terminal zinc-binding domain (ZBD) formed by motif I is a determinant for recognition of a specific sequence in DNA (10–13). Motifs II–VI are located in the C-terminal RNA polymerase domain (RPD) that consists of two subdomains (Fig. 1A). The C-terminal topoisomerase-primase (TOPRIM) fold (14) contains the active site where the metal-mediated condensation of nucleotides occurs. The N-terminal subdomain of the RPD is less conserved (6), and its role remains elusive. A recent study suggests that this subdomain contains a ssDNA binding groove through which the template can track (15).

Gene 4 of bacteriophage T7 encodes two colinear proteins. The full-length product is a 63-kDa protein (63-kDa gp4) in which the C-terminal half encodes the helicase and the N-terminal half the primase (Fig. 1B). The coexistence of both primase and helicase in a single polypeptide is unique in that the two activities are physically associated in other systems but are encoded by separate genes (1). A short form of gene 4 protein is produced in equal amount from an internal start codon and ribosome binding site located within the coding sequence for the full-length protein (16). This 56-kDa gp4 has full helicase activity but lacks the ZBD (Fig. 1B). The 56-kDa gp4 cannot
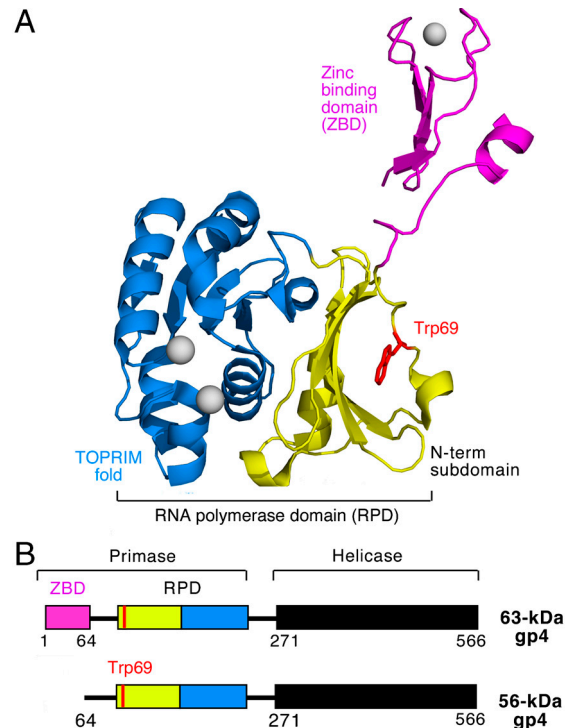

Fig. 1. Gene 4 proteins of bacteriophage T7. (A) Structure of T7 DNA primase [PDB ID code 1NUI, (6)]. The primase of T7 gp4 contains three major parts: the ZBD (magenta), the TOPRIM subdomain (blue), and the N-terminal subdomain of RPD (yellow). The latter two are collectively designated the RPD. Metal atoms are presented as silver dots, and tryptophan 69 is highlighted in red. (B) Schematic representation of the 56- and 63 kDa gp4. The primase is located in the N-terminal half of gp4, and the helicase is in the C-terminal half.

support the growth of T7 phage lacking gene 4 (T7Δ4) (17) although DNA synthesis is reduced in its absence (17).

Like other prokaryotic primase homologs, the primase domain of the 63-kDa gp4 recognizes a trinucleotide sequence (1). The recognition sequence for T7 DNA primase is 5′-d(GTC)-3′ at which it catalyzes the template-directed synthesis of r(AC); the 3′-cytosine is essential for recognition although this "cryptic" nucleotide is not copied into the product (18). The dinucleotide is then extended by T7 primase, provided the proper nucleotides, T and G, are present in the template. Consequently, T7 primase predominantly recognizes 5′-d(GGGTC)-3′, 5′-d(TGGTC)-3′,

and 5′-d(GTGTC)-3′ to catalyze the synthesis of functional tetraribonucleotides, r(ACCC), r(ACCA), and r(ACAC) that the lagging strand DNA polymerase can use to initiate the synthesis of Okazaki fragments (19, 20). The 56-kDa gp4 cannot catalyze template-dependent tetraribonucleotide synthesis because it lacks the ZBD.

The 63-kDa gp4 also delivers the primer to DNA polymerase. T7 DNA polymerase alone cannot extend a tetraribonucleotide. However, the 63-kDa gp4 enables T7 DNA polymerase to extend preformed tetranucleotides such as r(ACCA) or r(ACCC) provided the primase recognition sequence is present in the template (21). The ZBD stimulates the use of the preformed primers (6, 7).

We have examined primer delivery by the 56-kDa and 63-kDa gp4 using a minicircle DNA (22, 23). In this system four proteins—gp4 helicase-primase, gp5 DNA polymerase, gp2.5 ssDNA-binding protein, and the processivity factor *Escherichia coli* thioredoxin—constitute the replisome. Both leading and lagging strand synthesis proceed at the same rate, and Okazaki fragments are found within a transient replication loop (22, 23). Surprisingly, 56-kDa gp4 delivers a wider range of preformed primers more efficiently than does the 63-kDa gp4. We have used this property of the 56-kDa gp4 to isolate suppressors that allow the 56-kDa gp4 to support the growth of T7Δ4 phage and, in turn, to identify Trp69 as a residue critical for primer delivery.

Results

Primase Functions of Two Versions of T7 Gp4. Extracts of phage T7-infected cells contain equal amounts of a 63-kDa and 56-kDa gp4 (16). The 56-kDa protein lacks 63 N-terminal residues (11) containing the Cys_4 zinc-binding motif required for primase activity (17). The DNA coding the two forms has been cloned separately and the two proteins purified (11). Both forms contain a fully functional helicase domain (24–26).

The ZBD of gp4 is critical for the recognition of primase recognition sites in DNA (27). The 63-kDa gp4 catalyzes the DNA-dependent synthesis of di-, tri-, and tetranucleotides, whereas the 56-kDa gp4 does not (Fig. S1*A*). The oligoribonucleotides synthesized by 63-kDa gp4 are then used as primers by T7 DNA polymerase. T7 DNA polymerase alone does not initiate DNA synthesis on M13 ssDNA templates, whereas the addition of 63-kDa gp4 provides for synthesis (Fig. S1*B*). Therefore it is not surprising that the 56 kDa-gp4 fails to support the growth of T7 phage lacking gene 4 (Fig. S1*C*).

One of the primers synthesized at primase recognition sites is r(ACCA). In the absence of gp4, a preformed r(ACCA) cannot be used by T7 DNA polymerase to initiate DNA synthesis (21). However, in the presence of the 63-kDa gp4, this preformed oligonucleotide can prime DNA synthesis (Fig. S1*D*). The primase recognition sequence 5′-d(TGGTC)-3′ must also be present in the DNA template (21). Surprisingly, we find that the 56-kDa gp4 can likewise enable T7 DNA polymerase to use this oligoribonucleotide as a primer albeit less efficiently (Fig. S1*D*).

Efficient and Adaptable Primer Delivery by 56-kDa Gp4. During DNA replication, primers are synthesized at primase recognition sites on the lagging strand extruded through the central core of the helicase. To mimic this process we have used a minicircular DNA (22, 23). The minicircle consists of a dsDNA circle (70 bp) bearing a 5′-ssDNA tail (40 nt). One strand of the circle is devoid of cytosine residues except for two residues located in the two primase recognition sites, 5′-d(TGGTC)-3′. Consequently, the complementary strand has only two guanine residues. Leading and lagging strand synthesis can be measured by monitoring the incorporation of dGMP and dCMP, respectively (22, 23). A replisome consisting of T7 DNA polymerase, *E. coli* thioredoxin, gp4, and gp2.5 ssDNA-binding protein mediates extensive (greater than 10-kb product) coordinated DNA synthesis (22). The leading strand is synthesized continuously, whereas the lagging strand consists of

Okazaki fragments whose length centers around 3 kb. Coordinated DNA synthesis gives rise to equal rates of leading- and lagging-strand DNA synthesis. Consequently, the ratio of the rate of lagging-strand synthesis to the rate of leading-strand synthesis provides a measure of coordination.

The T7 replisome containing the 63-kDa gp4, dNTPs, ATP, and CTP mediates coordinated DNA synthesis; the ratio of lagging-strand synthesis to leading-strand synthesis is close to 1. When the 63-kDa gp4 is replaced with the 56-kDa gp4, leading-strand synthesis occurs at a slower rate but essentially no lagging-strand DNA synthesis is observed (Fig. 2*A*). This result is not surprising due to the inability of the 56-kDa gp4 to synthesize oligoribonucleotides (Fig. S1*A*). When a manufactured tetranucleotide, r(ACCA), complementary to the two primase
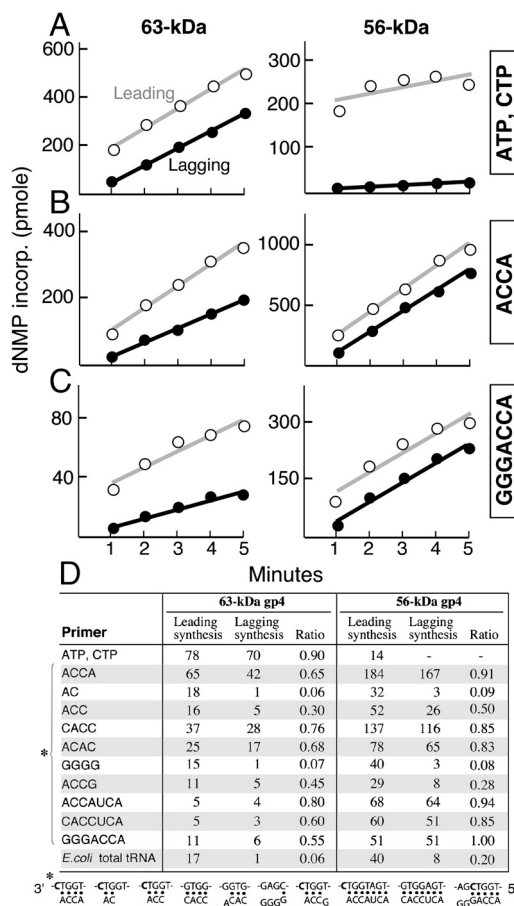

Fig. 2. Delivery of oligonucleotides by gp4 to DNA polymerase during coordinated DNA synthesis. Coordinated DNA synthesis was carried out as described previously (22, 23). The reaction mixture contained 40 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, 10 mM DTT, 100 mg/mL bovine serum albumin, 50 mM potassium glutamate, 600 μM each dATP, dCTP, dGTP, and dTTP, 100 nM minicircle, 80 nM T7 DNA polymerase, 60 nM gp4 (63-kDa gp4 or 56-kDa gp4 as indicated) monomer (10 nM gp4 hexamer), and 4 μM gp2.5. Incorporation of [³H] dGMP into the leading strand (open symbols) or [³H] dCMP into the lagging strand (solid symbols) was measured and plotted against time. (*A*) Coordinated DNA synthesis in the presence of 300 μM each ATP and CTP. (*B*) 150 μM oligoribonucleotide r(ACCA) replaced ATP and CTP in the reaction in *A*. (*C*) 150 μM oligoribonucleotide r(GGGACCA) replaced ATP and CTP in the reaction in *A*. (*D*) Coordinated DNA synthesis supported by T7 63-kDa or 56-kDa gene 4 protein in the presence of various primers. The rates of leading- and lagging-strand synthesis and the ratio of lagging-strand synthesis to leading-strand synthesis are listed. The oligonucleotides and their complementarity to the lagging strand template (some of them to the primase recognition sequence 5′-TG**GTC**-3′) are presented. Final concentration of all the performed primers is 150 μM and *E. coli* total tRNA is 6 μM in the assay. Data were obtained from duplicate experiments.

recognition sites, is substituted for ATP and CTP, leading- and lagging-strand synthesis are observed with both forms of gp4 (Fig. 2B). Interestingly, now coordination of DNA synthesis does occur with the 56-kDa gp4 as seen by the almost identical rates of leading- and lagging-strand synthesis. In Fig. 2D the rates of leading- and lagging-strand synthesis are listed along with the ratios of the rates of synthesis of the two strands. The ratio for the 63-kDa gp4 and the 56-kDa gp4 are 0.6 and 0.9, respectively, at 150 uM r (ACCA). The 56-kDa gp4 can also deliver r(CACC) to the polymerase as a primer for DNA synthesis. This oligonucleotide is complementary to a sequence in the template but not to the sequence of the primase recognition site. Although the 63-kDa protein can also use this primer, coordination is slightly better with the 56-kDa form. The ratio of lagging- to leading-strand synthesis by the 63- and 56-kDa gp4 are 0.76 and 0.85, respectively. When r(GGGACCA), a longer primer with a 5′-tail (Fig. 2C) is present, the 63-kDa gp4 has difficulty in delivering it to the polymerase whereas the 56-kDa gp4 does not.

Oligonucleotides shorter than a tetramer, such as r(AC) and r(ACC), with a noncomplementary 3′-end r(ACCG), or that are almost noncomplementary r(GGGG) are not delivered by either form of gp4. Longer oligonucleotides such as tRNA and 5S RNA cannot be delivered well at low concentration. Interestingly, the tetramers r(CACC) and r(ACAC) with more than three nucleotides complementary to the template at their 3′ ends but not complementary to the primase recognition site, and heptamers r(ACCAUCA), r(CACCUCA), and r(GGGACCA) with more than four nucleotides complementary to the template at their 3′ end can be delivered by 56-kDa gp4 but not the 63-kDa gp4 (Fig. 2D).

Tryptophan at Position 69 Is Crucial for Primer Delivery. We have taken advantage of the ability of the 56-kDa gp4 to deliver a variety of oligonucleotides to identify residues in gp4 important in primer delivery. In separate studies we isolated a suppressor mutation in T7 (T7Δ4 sup) that enables the 56-kDa gp4 to complement T7 phage lacking gene 4 for phage growth. Preliminary evidence suggests that the suppressor mutation results in the reduced expression of phage gene 5.5. Gene 5.5 protein binds to the E. coli H-NS protein and thus has the potential to regulate transcription (28). The modified transcription could produce RNA transcripts that are transferred by the 56-kDa gp4 for use as primers by T7 DNA polymerase. Any modification that eliminates the function of 56-kDa gp4 leads to a loss of complementation. In a study where incremental portions of the N terminus of the 56 kDa-gp4 were deleted, we found that proteins lacking more than the first five residues (Thr65 through Trp69 of the 63-kDa gp4) lose the ability to support T7Δ4-sup growth.

A subsequent alanine screen showed that only the substitution for Trp69 significantly affected growth of the suppressor phage (Table 1). Consequently, we examined the ability of amino acids other than alanine to replace Trp69. Replacement of Trp69 with tyrosine has the least effect, whereas replacement with aspartic acid has the most dramatic effect on complementation. Substitution of alanine or lysine results in a reduced ability to support T7Δ4-sup growth. Tryptophan at position 69 plays an essential role in the 63-kDa gp4 as well (Table S1). The ability of 63-kDa gp4 with amino substitutions for Trp69 to complement for growth of T7Δ4 is very similar to that observed with 56-kDa gp4 harboring the same replacements.

Effects of Alteration of Trp69 on Activities of 63-kDa Gp4. To examine the effects of amino acid substitutions for Trp69 in the 63-kDa gp4, Trp69 was replaced with other amino acids to yield 63-kDa gp4-W69A, -W69K, -W69D, and -W69Y. After purification of the altered proteins, we found that the DNA-dependent hydrolysis of dTTP by the helicase was not dramatically affected

Table 1. Screening of primer-delivery mutants of 56-kDa gp4 using T7Δ4 phage suppressor (T7Δ4-sup)

	e.o.p.* of T7Δ4-sup	Plaque size
56-kDa gp4	1.0	Large
56-kDa gp4-T65A	1.1	Large
56-kDa gp4-Y66A	1.3	Large
56-kDa gp4-N67A	0.89	Large
56-kDa gp4-V68A	1.0	Large
56-kDa gp4-W69A	0.13	Small
56-kDa gp4-W69K	0.05	Small
56-kDa gp4-W69D	$<10^{-5}$	—
56-kDa gp4-W69Y	0.26	Large

The ability of the 56-kDa gp4 variants to support the growth of T7Δ4 suppressor phage (T7Δ4-sup) was tested as described in *Materials and Methods*. A diluted phage solution was mixed with the *E. coli* culture expressing various 56-kDa gp4s and plated. After incubation, the number and size of plaques were determined.
*Efficiency of plating, number of plaques relative to wild-type 56-kDa gp4.

(Fig. 3A); activities of 63-kDa gp4-W69A and -W69D were reduced by 25%.

On the other hand, the primase activity is affected by the substitutions. 63-kDa gp4-W69D is unable to catalyze the synthesis of oligonucleotides and 63-kDa gp4-W69A has only 10% of the activity seen with wild-type gp4 (Fig. 3B). Neither of these proteins supports DNA synthesis catalyzed by T7 DNA polymerase on M13ssDNA (Fig. 3C). 63-kDa gp4-W69Y catalyzes the synthesis of oligonucleotides as well as wild-type primase, and these oligonucleotides are effective primers for T7 DNA polymerase (Fig. 3 B and C). The altered proteins, except for 63-kDa
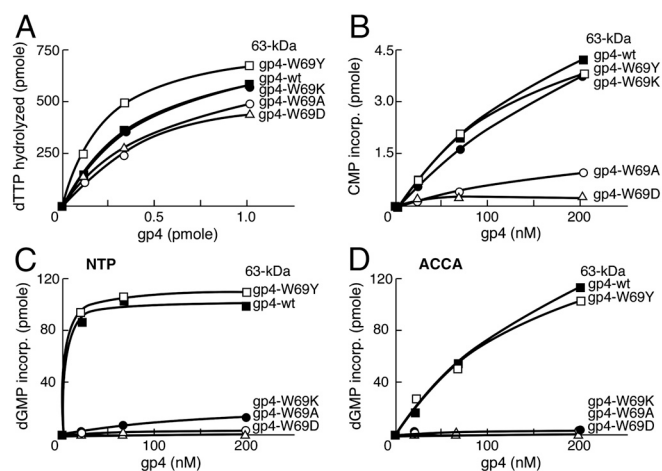

Fig. 3. Effect of alteration of tryptophan 69 on functions of 63-kDa gp4. (A) DNA-dependent dTTP hydrolysis activity of 63-kDa gp4. The indicated amounts of wild-type gp4 and gp4 with amino acid substitutions for Trp69 were incubated with 0.25 mM [α-^{32}P] dTTP (0.1 μCi) in the presence of 8 nM M13 ssDNA at 37 °C for 20 min. Reaction products were separated by TLC, and dTTP hydrolysis was determined by measuring the amount of dTDP present in the reaction. (B) Template-directed primer synthesis of 63-kDa gp4. Reaction mixtures containing 10 μM template DNA, 5′-d(GGGTCA$_{10}$), 0.1 mM each of ATP and [α-^{32}P] CTP (0.1 μCi), and various amounts of the indicated gp4 were incubated at 37 °C for 30 min, and the reaction products were analyzed on denaturing polyacrylamide gels. The amount of CMP incorporated into tetraribonucleotide r(ACCC) was measured. (C and D) RNA-primed DNA synthesis in the presence of NTPs (C) or preformed primer r(ACCA) (D). Reaction mixtures containing 10 nM M13 ssDNA, 0.1 mM all four NTPs or 25 μM r(ACCA) as indicated, 0.3 mM all four dNTPs, 0.1 μCi of [α-^{32}P] dGTP, 100 nM T7 DNA polymerase (a 1:1 complex of T7 gene 5 protein and *E. coli* thioredoxin), and various amounts of the indicated gp4 were incubated at 37 °C for 10 min. Amounts of dGMP incorporated into DNA were measured and plotted against the concentration of gp4 used. Data were obtained from triplicate experiments.

gp4-W69Y, are unable to deliver a preformed oligonucleotide to the polymerase (Fig. 3D).

The most interesting protein is the 63-kDa gp4-W69K in which lysine replaces tryptophan. Although this altered protein synthesizes oligonucleotides as efficiently as does the wild-type 63-kDa gp4 (Fig. 4B), the oligonucleotides are not used effectively by T7 DNA polymerase (Fig. 3C). In addition 63-kDa gp4-W69K is unable to deliver a preformed oligonucleotide to the polymerase (Fig. 3D).

Substitution of Lysine for Tryptophan at Position 69 Selectively Abolishes Primer Delivery. The single amino acid alteration in gp4-W69K does not affect the helicase activity of either the 56- or 63-kDa gp4 (Fig. 4A). 63-kDa gp4-W69K catalyzes the synthesis of oligoribonucleotides as well as does wild-type gp4 (Fig. 4 B and C). In these experiments we examined oligonucleotide synthesis on M13 DNA where all three major primase recognition sites are present as well as minor recognition sites (19). As with wild-type gp4 the altered primase synthesizes tetraribonucleotides as well as the precursors, di- and trinucleotides (Fig. 4B). 63-kDa gp4-W69K recognizes the same trinucleotide sequence, d(GTC), in the template as wild-type gp4; a template containing a modified recognition site, d(GGGTG) or d(TGGTG), where the cryptic cytosine is changed to guanosine does not support synthesis. In view of its inability to transfer oligonucleotides to the polymerase, 63-kDa gp4-W69K cannot support coordinated DNA synthesis using ATP and CTP. Furthermore, 56-kDa gp4-W69K cannot support coordinated DNA synthesis using the preformed olgionucleotide r(ACCA) (Fig. 4D).
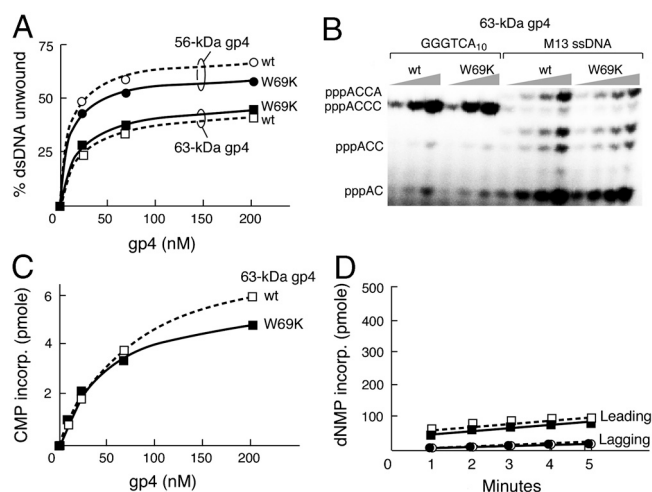
T7 Primase-W69K Fails to Stabilize the Primer/template Complex as Determined by Surface Plasmon Resonance. Despite weak binding affinity, binding of the T7 primase fragment to the primase recognition sequence 5′-d(TGGTC) can be measured by surface plasmon resonance (29). The presence of ATP and CTP for primer synthesis or the preformed primer r(ACCA) stimulates this binding (Fig. 5). The W69K primase fragment binds slightly less tightly than does wild-type primase in the presence of NTP (Fig. 5B), consistent with the result that it behaves similar to wild-type primase in oligoribonucleotide synthesis. However, binding of the altered protein is significantly lower than that of the wild-type protein in the presence of a preformed primer (Fig. 5C), indicating that the primase-W69K cannot form a stable complex with the primer/template.

Discussion

Whereas the synthesis of oligoribonucleotides by the primase is fairly well delineated, relatively little is known about the stabilization of the oligonucleotide and its transfer to the polymerase. Earlier studies suggested that the ZBD is responsible for primer delivery (6, 7). However, the 56-kDa gp4, lacking the ZBD, is also capable of primer delivery, necessitating the involvement of other elements. In fact, comparison of 63-kDa and 56-kDa gp4 for their efficiency in primer delivery (Fig. 2D) showed that the ZBD restricts primer handoff to those oligonucleotides complementary to the primase recognition site only. Thus, primer delivery by ZBD alone observed in previous studies (6, 7) could be explained by some unknown protein-DNA/RNA interaction in the presence of the high concentration of ZBD. The DNA primase of gp4 has three major parts: the ZBD, a C-terminal TOPRIM fold located in the RPD, and an N-terminal subdomain of the RPD (Fig. 6A). Compared with the ZBD and TOPRIM subdomains, both of which contribute to oligoribonucleotide synthesis, the role of the N-terminal RPD subdomain is less clear. Recent studies on *E. coli* DnaG primase, in which the N-terminal subdomain of the RPD was cross-linked to ssDNA, revealed two primase-template complexes in which a slight translocation of ssDNA relative to the primase can be observed. In that case, the N-terminal RPD subdomain was proposed to function as a template-tracking
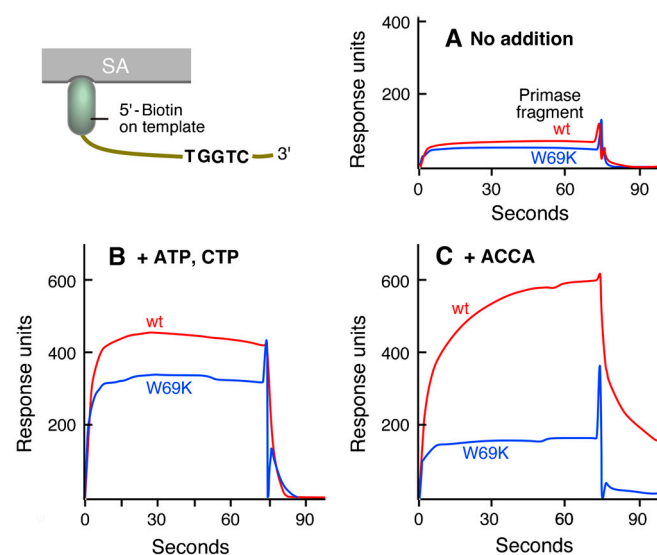

Fig. 4. Substitution of lysine for tryptophan 69 of gp4. (A) DNA unwinding activity was determined by measuring the amount of radiolabeled ssDNA displaced from a minireplication fork (500 fmol) by the indicated gp4. (B) Template-directed oligoribonucleotide synthesis catalyzed by 63-kDa gp4. Reaction mixtures containing 20 μM 15mer DNA 5′-d(GGGTCA₁₀) or 10 μM M13 ssDNA, 0.1 mM each of [α-³²P] CTP (0.1 μCi) and ATP (replaced with 0.1 mM each of four NTPs plus 0.5 mM dTTP when M13 ssDNA was used as template), and 22, 67, or 200 nM gp4 were incubated at 37 °C for 30 min, and the reaction products were analyzed on denaturing polyacrylamide gels. (C) The amount of CMP incorporated into tetraribonucleotide as shown in B with M13 ssDNA template was measured. (D) Coordinated DNA synthesis supported by 63-kDa gp4-W69K or 56-kDa gp4-W69K. Reactions were carried out as described for Fig. 3. The reaction mixture contained 40 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 10 mM DTT, 100 mg/mL bovine serum albumin, 50 mM potassium glutamate, 600 μM each dATP, dCTP, dGTP, and dTTP, 100 nM minicircle, 80 nM T7 DNA polymerase, 60 nM gp4 monomer (10 nM gp4 hexamer), 4 μM gp2.5, and 300 μM each ATP and CTP for 63-kDa gp4-W69K or 150 μM r(ACCA) for 56-kDa gp4-W69K. Incorporation of [³H] dGMP into the leading strand (■ for 63-kDa gp4-W69K and □ for 56-kDa gp4-W69K) or [³H] dCMP into the lagging strand (● for 63-kDa gp4-W69K and ○ for 56-kDa gp4-W69K) was measured and plotted against time. Data were obtained from at least duplicate experiments.

Fig. 5. Sequence-specific DNA binding of T7 primase. A biotinylated 25-mer ssDNA containing a primase recognition site, 5′-d(TGGTC) (1000 RU) was immobilized on the surface of a Biacore streptavidin sensor chip. T7 primase (10 μM) was flowed over the chip together with the indicated components in a buffer containing 40 mM Tris-HCl, pH 7.5, 50 mM potassium glutamate, 10 mM MgCl₂, and 1 mM DTT, and binding signals were detected. Concentrations of ATP/CTP and r(ACCA) were 50 μM. Representative data from multiple experiments are presented.
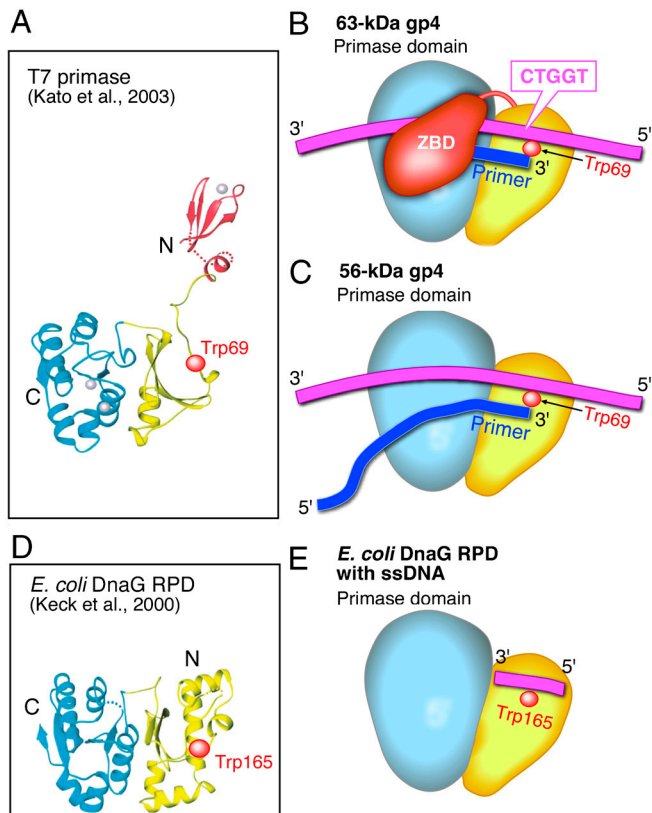
Fig. 6. Model for primer delivery by T7 primase. (*A*) Structure of T7 primase [PDB ID code 1NUI, (6)]. The ZBD is colored in red, the TOPRIM subdomain in blue, the N-terminal subdomain of RPD in yellow. Metal atoms are presented as silver dots. (*B*) Model of the predelivery complex formed by the 63-kDa gp4 (helicase not shown), DNA template (purple), and RNA primer. The 3′ end of the annealed oligonucleotide is stabilized by Trp69 in the N-terminal subdomain of the RPD while the other end of the primer/template is sandwiched by the ZBD and the TOPRIM subdomain. The 3′ end of the primer (5′ end of template) is ready to be extruded through the N-terminal subdomain of the RPD. (*C*) Model of the predelivery complex formed by the 56-kDa gp4 (helicase not shown), DNA template (purple), and RNA primer. The overall arrangement is similar as that for 63-kDa gp4. However, in the absence of the ZBD, longer oligonucleotides with noncomplementary 5′ termini can be accommodated. The 3′ end of the primer must match the template sequence and is stabilized by Trp69 as for the 63-kDa gp4. (*D*) Structure of the RPD domain of *E. coli* primase [PDB ID codes 1DD9 and 1DDE, (30)]. The TOPRIM subdomain is colored in blue and the N-terminal subdomain of RPD in yellow. (*E*) Schematic presentation of the complex (15) of *E. coli* primase with a short ssDNA from which the predelivery complex of T7 primase was derived. In both primases the tryptophan residues discussed in the text are highlighted.

site (15) (Fig. 6 *D* and *E*). Furthermore, this subdomain was suggested to play a role in primer handoff to the polymerase whereby it extrudes the primer template to expose the 3′ terminus of the primer.

Based on the similarity of the T7 and *E. coli* primases, a model for primer delivery by the two molecular weight forms of gp4 can be envisioned (Fig. 6 *B* and *C*). In this model, the N-terminal subdomain of the RPD stabilizes the primer (either synthesized de novo or from preformed RNA)-template complex and extrudes the 3′ end of the primer for extension by DNA polymerase. Most importantly, in the *E. coli* studies a crucial protein-DNA interaction was between a tryptophan (Trp165) and DNA. Trp165 is critical to maintain the interaction between the template and primase in both translocation states (15). In the current study, a tryptophan (Trp69) is also crucial for primer delivery. Although the sequences surrounding the tryptophan are less conserved, the importance and location (Fig. 6 *A* and *D*) of this

residue in gp4 and DnaG proteins strongly suggest a common mode of primer delivery among bacterial-type primases. The model can also explain the more flexible primer usage by the 56-kDa gp4. Although the 3′ end of the primer is stabilized by the N-terminal subdomain of the RPD, the 5′ end of the primer could still contact the catalytic site in the TOPRIM subdomain as with a newly synthesized primer. In the case of the 63-kDa gp4, the DNA contacts both the TOPRIM subdomain and the ZBD. Because of the spatial hindrance exerted by the ZBD and the specific interaction between the ZBD and the recognition site, the length and sequence of the primer bound to the template are restricted (Fig. 6*B*). However, in the case of the 56-kDa gp4, so long as the 3′ end of the primer is hydrogen-bonded to the template, the length and sequence of the primer are less critical (Fig. 6*C*). One ill-defined step is how the 3′ end of the newly synthesized primer moves a significant distance (>20 Å) to contact the W69 residue (6). During primer synthesis, the ZBD and TOPRIM domains are in close proximity, whereas W69 is close to the flexible linker following the ZBD. A shorter distance between W69 and the catalytic center during primer synthesis than that observed in the primase structure is likely. NMR results showing significant conformational change within both the ZBD and RPD domains upon addition of the DNA template and the preformed primer (6) support this possibility.

Our recent work (29) rendered us the possibility to examine the role of oligonucleotide stabilization by T7 primase (31). Binding of primase to a ssDNA template in the presence of the cognate primer rACCA can be detected by surface plasmon resonance. We found that the primase-W69K has a severe defect in this binding mode, consistent with its failure in primer delivery. Another binding mode of primase to the oligonucleotides was also revealed where ATP and CTP replace the preformed rACCA. This binding is highly specific (the primase recognition site including the cryptic C must be present) and a series of conformational changes during the catalysis of primer synthesis are necessary for the binding. Primase-W69K shows only a slight defect in this binding mode, suggesting that the defect of this mutant arises from neither a disturbed primase-DNA interaction nor an abnormal primer synthesis but specifically from failure in stabilization of primer/template. This altered primase also helps to further distinguish the "catalysis" and "predelivery" binding modes of primase to the DNA and primer.

Stabilization of the primer/template is likely to be a fundamental function of primase. The short oligoribonucleotides produced by T7 primase cannot form a stable DNA-RNA hybrid, and thus the primase must participate in this stabilization. Moreover, oligonucleotides less than 21 nucleotides in length are ineffective primers for T7 DNA polymerase. It seems likely that DNA primase interacts with the polymerase to secure the primer in the DNA binding cleft. Unlike in the *E. coli* system where multiple switches occur during primer handoff (8), in the concise T7 replication system it is likely that the primer is delivered directly from the primase to the polymerase. As a crucial component in the T7 replisome, the gene 2.5 ssDNA-binding protein inhibits primer delivery to the polymerase (7). Interestingly, gp2.5 also interacts with DNA polymerase through a conserved aromatic residue (3). Another component, the T7 helicase, is covalently linked to the primase so that the helicase-polymerase interaction (3) could favor primer delivery by recruiting the polymerase to the primase and primer.

Materials and Methods

Construction of Plasmids, Site-Directed Mutagenesis, Protein Overproduction, and Purification. Site-directed mutations were introduced into plasmid pET24-gp4 or pET28-gp4 following a standard procedure. Proteins were overproduced in *E. coli* BL21(DE3) and purified as described (31–33).

Assays. The ability of T7 gp4 expressed in *E. coli* DH5α to support growth of T7Δ4 phage (or T7Δ4-sup) was determined by spot or plaque assay. For the

spot assay, *E. coli* (0.1 mL) grown at log phase was mixed with soft agar and plated on LB plate. Aliquots of serially diluted phage solution were spotted on the plate and incubated at 37 °C overnight. For plaque assay, a diluted phage solution was mixed with the *E. coli* culture containing soft agar and plated. After incubation, the number and size of plaques were determined.

The biochemical assays have been described (22, 32, 33). All reactions were carried out in a buffer containing 40 mM Tris-HCl, pH 7.5, 10 mM MgCl$_2$, 10 mM DTT, and 50 mM potassium glutamate. In template-directed primer synthesis, reaction mixtures containing the indicated ssDNA template, 0.1 mM each of ATP, and [α-^{32}P] CTP (0.1 μCi), and the indicated amount of gp4 were incubated at 37 °C for 30 min. Reaction products were separated by electrophoresis through 25% denaturing polyacrylamide gel, and the incorporation of CMP into a tetraribonuclotide was determined by phosphoimager analysis. In the dTTP hydrolysis assay, the indicated amounts of gp4 were incubated with 0.25 mM [α-^{32}P] dTTP (0.1 μCi) in the presence of 8 nM M13 ssDNA at 37 °C for 20 min. Reaction products were separated by TLC, and dTTP hydrolysis was determined by measuring the amount of dTDP present in the reaction. In the DNA unwinding assay, a radiolabeled minireplication fork (500 fmol) was incubated at 37 °C for 5 min with the indicated gp4 in the presence of 1 mM dTTP. ssDNA formed as the result of unwinding was separated using a nondenaturing gel and the amount of ssDNA was determined (34). In RNA-primed DNA synthesis, reaction mixtures contain 10 nM M13 ssDNA, 0.3 mM all four dNTPs, 0.1 μCi of [α-^{32}P] dGTP, 100 nM T7 DNA polymerase (a 1:1 complex of T7 gene 5 protein and *E. coli* thioredoxin), the indicated amounts of gp4, and either 0.1 mM of all NTP or the indicated amount of oligoribonucleotide. After incubation at 37 °C for 10 min, incorporation of dGMP into the DNA product was measured. Reaction conditions for coordinated DNA synthesis assay were described previously (22, 23). In some reactions, ATP and CTP were replaced with the indicated preformed primer. Surface plasmon resonance analysis was performed as previously described (29).

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