Roles of bacteriophage T7 gene 4 proteins in providing primase and helicase functions in vivo

(DNA replication/leading- and lagging-strand DNA synthesis/site-directed mutagenesis)

LYNN V. MENDELMAN, STEPHEN M. NOTARNICOLA, AND CHARLES C. RICHARDSON

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115

Contributed by Charles C. Richardson, July 30, 1992

ABSTRACT  The helicase and primase activities of bacteriophage T7 are distributed between the 56- and 63-kDa gene 4 proteins. The 56-kDa gene 4 protein lacks 63 amino acids found at the N terminus of the colinear 63-kDa protein and catalyzes helicase activity. The 63-kDa gene 4 protein catalyzes both primase and helicase activities. A bacteriophage deleted for gene 4, T7 Δ4-1, has been tested for growth by complementation on Escherichia coli strains that contain plasmids expressing either one or both of the gene 4 proteins. T7 Δ4-1 cannot grow (efficiency of plating, 10−4) on E. coli cells that express only 56-kDa gene 4 protein. In contrast, T7 Δ4-1 has an efficiency of plating of 0.1 on an E. coli strain that expresses only 63-kDa gene 4 protein in which glycine is substituted for methionine at position 64. A bacteriophage, T7 4B−, in which methionine at residue 64 is replaced by glycine, expresses only 63-kDa gene 4 protein. The burst sizes, latency periods, and Okazaki fragment sizes of T7 4B− are similar in the presence and absence of the 56-kDa gene 4 protein; however, T7 4B− has a reduced rate of DNA synthesis when compared with a phage that synthesizes both gene 4 proteins.

Leading- and lagging-strand DNA synthesis can be reconstituted in vitro with only four proteins; the bacteriophage T7 gene 5 protein, the two gene 4 proteins, and one Escherichia coli protein, thioredoxin (1). Gene 5 protein is a nonprocessive DNA polymerase (2). E. coli thioredoxin forms a tight complex with gene 5 protein, the consequence of which is to increase the affinity of the gene 5 protein for a primer template and to markedly increase the processivity of DNA synthesis (2, 3).

The two gene 4 proteins catalyze helicase (4–6) and primase activities (7, 8). Helicase activity is required for the T7 DNA polymerase/thioredoxin complex to catalyze strand displacement synthesis (5, 9). Primase activity is required for synthesis of tetrmeric oligoribonucleotides (pppACC/C and pppACAC) at specific template recognition sequences (3′-CTGGG/T-5′ and 3′-CTGTG-5′) for use as primers by T7 DNA polymerase during lagging-strand DNA synthesis (7, 8, 10–14). Both gene 4 proteins preferentially hydrolyze TTP in the presence of single-stranded DNA; the hydrolysis of TTP supports unidirectional 5′ to 3′ translocation on a DNA strand (15–17).

The gene 4 coding sequence is translated into two colinear proteins: the 56-kDa gene 4 protein is translated from an internal ribosome-binding site and start codon located 189 base pairs from the beginning of the gene encoding the 63-kDa gene 4 protein (18). Gene 4 coding sequences have been engineered to allow for expression, overproduction, and characterization of the individual 56- and 63-kDa gene 4 proteins (19, 20). The 56-kDa protein catalyzes single-strand DNA-dependent dNTP hydrolysis and helicase activities (19, 21). The 63-kDa gene 4 protein catalyzes primer synthesis in addition to catalyzing helicase and TTP hydrolysis activities (20, 22). High specific primase activity, however, requires the presence of both 56- and 63-kDa gene 4 proteins (14, 20, 22).

To assess the relative role of each of the two gene 4 proteins, we have examined the growth characteristics of bacteriophage T7 in the presence and absence of each of the gene 4 proteins. The results show that the 56-kDa gene 4 protein alone is not sufficient for T7 growth. The 63-kDa gene 4 protein is essential and sufficient for T7 growth, but phage DNA replication is more efficient in the presence of the 56-kDa gene 4 protein.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. E. coli HMS174 (23), E. coli C600 (24), and E. coli 71.18 (25) have been described. Plasmids pGP1-2 (26) and pGP4-5wt and pGP4-6wt (20) have been described. The protocol for construction of plasmid pGP4-6L64 is essentially as described for pGP4-6wt (20), using the primer 5′-GTAAGTCCCGGGCTTTCCACCG-3′ and 5′-CTGGT-3′ for site-directed mutagenesis at the underlined bases of the T7 sequences 11730–11763 (18). Plasmid pGP4-6L64BstI was constructed by deletion of a 1.4-kilobase (kb) Bsm I restriction fragment that includes sequences encoding the C terminus of gene 4 protein from pGP4-6L64. Plasmids pGP4-5 and pGP4-6wt were constructed by Stan Tabor (Harvard Medical School) (27). Plasmid pGP4-5 is similar to pGP4-6wt but has the 5′ end of gene 4 deleted as a Sal I/SnaBI restriction fragment and the gene for β-lactamase is oriented in the same direction as the gene 4 coding sequences. On pGP4-5, T7 gene 4 coding sequences begin at nucleotide 11673 (18). All vectors place gene 4 coding sequences under transcriptional control of the T7 710 promoter.

Bacteriophages. Bacteriophage T7 Δ4-1 (27) (provided by Stan Tabor) is deleted for a 2.1-kb Pvu II restriction fragment encompassing T7 sequences 11481–13621 (18). A second phage, bacteriophage T7 4B−, was constructed by homologous recombination (23). E. coli C600/pGP4-6L64BstI (Su+) was infected with T7 containing an amber mutation in gene 4, T7 4-245 (28). The lysate was plated on E. coli HMS174 (Su+) and individual plaques were selected. Phage DNA with mutations from pGP4-6L64BstI have a specific site for cleavage by Ava I at position 11751 (18). Aliquots of phage DNA and two primers, 5′-GGGACATTTAGCACAATTCGCA-CG-3′ and 5′-GGCGCCCGCTAGGACAAATGTGTGCGCG-CTCGT-3′, were used to amplify T7 DNA sequences between nucleotides 11581 and 11924 (18) using a standard PCR protocol (29) for 30 cycles and a temperature profile of 55°C-94°C, 2 min; 94°C, 45 sec; 94°C-55°C, 1 min; 55°C, 1 min. DNA samples from PCRs that were cleavable by Ava I were sequenced by using the first oligonucleotide listed above as a sequencing primer. Two phases, T7 4B−, which contains the mutations, and T7 4-245R, which does not, were plaque purified three times before making phage stocks (30).

10638
Measurement of Burst Size. Burst sizes were calculated by a modification of the published procedure (31). *E. coli* HMS174 was grown to a cell density of $2 \times 10^8$ (A$_{600}$ = 0.7) in LB medium (32) at 37°C. A 0.1-ml aliquot of *E. coli* HMS174 was mixed with 0.1 ml of phage stock ($2 \times 10^8$ plaque-forming units/ml). Phages were adsorbed for 5 min at 37°C. The cells were collected by centrifugation, resuspended in 0.1 ml of LB medium, and diluted in series to $\approx$6 cells in 5 ml. The bacterial suspension was distributed in 0.2-ml aliquots to 24 tubes at 37°C. After 60 min, top agar (2.5 ml) and 0.1 ml of a saturated culture of *E. coli* HMS174 was added to each tube and the suspension was mixed and plated. Burst sizes were determined by using the Poisson distribution (33).

[^35S]Metionine Labeling of T7 Proteins. *E. coli* HMS174 was grown to an A$_{600}$ of 0.75 at 37°C. Ten milliliters of culture was transferred to a Petri dish and irradiated with UV light from a germicidal lamp at a distance of 20 cm for 2 min. The cells were transferred to 37°C for 15 min to clear *E. coli* RNA. T7 phages were added to 2.5 ml of UV-irradiated cells at a multiplicity of infection of 10, and the mixture was incubated at 37°C for 8 or 10 min. At each time point, 1 ml of cell mixture was added to 150 $\mu$Ci of[^35S]methionine (1 Ci = 37 GBq) in an Eppendorf tube and incubated at 37°C for 2 min. The cells were collected by centrifugation and the supernatant was removed. The cells were resuspended in 50 $\mu$l of 50 mM Tris-HCl (pH 8.0) and then 50 $\mu$l of 50 mM Tris-HCl, pH 8.0/4% SDS was added; the mixture was boiled for 10 min and chilled on ice. A 40-$\mu$l vol of 50 mM Tris-HCl, pH 8.0/1% Triton X-100 was added and the mixture was sonicated for 10 bursts with a Branson Sonifier 450 at a 40% setting on an output of 1; then it was centrifuged at 15,000 $\times$ g for 10 min at 4°C. The supernatant was collected and stored at -80°C.

Other Methods. The protocols for immunoblot analysis of gene 4 proteins (20) and [^3H]thymidine uptake into DNA after phage infection (34) have been described. The size of Okazaki fragments synthesized at 25°C was determined by alkaline sucrose gradients (35, 36) with the following modifications. Four-drop fractions were collected into 96-well microtiteration plates containing 50 $\mu$g of calf thymus DNA per well. Fractions were collected on 0.7-cm diameter GF/C filters in a Milliflip I filtration manifold from Schleicher & Schuell and washed three times with 200 $\mu$l of ice-cold 1 M HCl and two times with 95% ethanol.

**RESULTS**

Bacteriophage T7 Δ4-1 and Gene 4 Protein Expression Vectors. A T7 phage with a deletion of gene 4 was constructed by excision of a 2.1-kb Pvu II restriction fragment from wild-type bacteriophage T7. The deleted fragment includes coding sequences for T7 genes 3.8, 4, 4.1, 4.2, 4.3, and 4.5. T7 Δ4-1 is not viable on *E. coli* C600, but it grows in *E. coli* C600/pPG4-6WT. The latter strain harbors a plasmid that expresses both 56- and 63-kDa gene 4 proteins (20).

Two gene 4 expression vectors were constructed to permit synthesis of the 63-kDa gene 4 protein, yet decrease or eliminate synthesis of the 56-kDa gene 4 protein. In these plasmids (Fig. 1), the sequences near the start codon of the 56-kDa protein were mutated to either decrease consensus in the ribosome-binding site of the 56-kDa protein (pGP4-6RBS) or decrease consensus in the ribosome-binding site and substitute the amino acid glycine for the methionine start codon of the 56-kDa gene 4 protein (pGP4-6GSP). A third plasmid, pGP4-5, has the ribosome-binding site and start codon of the 63-kDa gene 4 protein deleted from gene 4 coding sequences and only expresses the 56-kDa gene 4 protein (described in Materials and Methods).

Gene 4 protein expression vectors were tested for their ability to synthesize the 56- and 63-kDa gene 4 proteins. Each plasmid was transformed into *E. coli* 71.18/pGP1-2 (26). In this strain, an increase in temperature is used to induce the synthesis of T7 RNA polymerase, which, in turn, will transcribe cloned genes from a T7 φ10 promoter. The relative level of 56- and 63-kDa gene 4 proteins produced from each plasmid was determined by immunoblot analysis with a rabbit polyclonal antibody raised against the 56-kDa gene 4 protein (Fig. 2). pGP4-6WT directs synthesis of both 56- and 63-kDa gene 4 proteins, whereas pGP4-6GSP, expresses only the 56-kDa gene 4 protein. Small but detectable levels of 56-kDa gene 4 protein are synthesized by pGP4-6RBS. In the strain containing pGP4-5, only the 56-kDa gene 4 protein is synthesized. The lower limit of detection for gene 4 protein by immunoblot analysis is $\approx$0.5 ng and the lane marked pGP4-5 contains $\approx$50 ng of 56-kDa protein.

**Plating Efficiencies of T7 Δ4-1.** T7 Δ4-1 was titered on *E. coli* C600 containing each of the gene 4 expression vectors to examine the requirements for individual gene 4 proteins during T7 growth (Table 1). *E. coli* C600/pGP4-5, which only expresses 56-kDa gene 4 protein, does not support the growth of T7 Δ4-1. On *E. coli* C600/pPG4-6GSP, the strain without...
Fig. 2. Immunoblot analysis of gene 4 proteins. E. coli 71.18/pGP1-2 (26) was transformed with the gene 4 expression plasmid indicated above each lane. Individual strains were grown at 30°C to an A600 = 0.6. The cells were shaken at 42°C for 20 min and then at 37°C for an additional 100 min. One milliliter of each cell culture was pelleted in an Eppendorf tube. The cell pellet was resuspended in 0.1 ml of loading buffer [60 mM Tris-HCl, pH 6.8/1%/2-mercaptoethanol/3%/SDS/10% (vol/vol) glycerol/0.01% bromphenol blue] and heated to 95°C for 5 min; 10 μl of each sample was loaded onto a SDS/10% polyacrylamide gel (7.5 × 8 cm). The procedures for transfer of proteins to nitrocellulose membrane and detection of gene 4 proteins by immunoblot analysis have been described (20).

detectable 56-kDa gene 4 protein, T7 Δ4-1 has a plating efficiency of 0.1. The strain that can synthesize reduced levels of the 56-kDa gene 4 protein, E. coli C600/pGP4-6RS, also supports the growth of T7 Δ4-1, with a 5-fold lower efficiency of plating than on E. coli C600/pGP4-6WT.

Rates of DNA Synthesis During T7 Δ4-1 Infection. As discussed in the Introduction, loss of the 56-kDa gene 4 protein helicase activity could affect the rate of leading-strand DNA synthesis or the synthesis of primers on the lagging strand. The rate of DNA synthesis of T7 Δ4-1 was compared in E. coli C600 strains that harbor pGP4-6WT or pGP4-6RS. Time courses of the rate of DNA synthesis did not differ significantly in either strain after infection with T7 Δ4-1 at a multiplicity of infection of 1 (Fig. 3).

Bacteriophage T7 4B-. In the process of characterizing the growth of T7 Δ4-1, we found that when the multiplicity of infection was ≥1, the burst size was diminished markedly and the rate of DNA synthesis was delayed and reduced. Thus, we questioned the results shown in Fig. 3 because, in order to fully characterize T7 growth in the presence of the 63-kDa gene 4 protein alone, it is necessary to use a high multiplicity of infection to ensure that nearly all the bacteria are infected with at least one phage. To circumvent the problems associated with the complementation assay, we constructed a mutantophage that expresses only the 63-kDa gene 4 protein. Mutant gene 4 sequences from vector pGP4-6RS were crossed into the genome of bacteriophage T7 4-245, a phage containing an amber mutation in the gene 4 coding sequences (see Materials and Methods). Two phages were purified: T7 4B-, whose genome contains point mutations in gene 4 from pGP4-6RS (Fig. 1) and, for comparison, T7 4-245R, a revertant of the gene 4 amber mutation. The genotypes of these phages were confirmed by restriction enzyme and DNA sequence analyses.

Table 1. Plating efficiencies of T7 Δ4-1 on various E. coli C600 strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plaque-forming units/ml</th>
<th>EOP</th>
</tr>
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<tbody>
<tr>
<td>C600/pGP4-6WT</td>
<td>5 × 10^9</td>
<td>1</td>
</tr>
<tr>
<td>C600/pGP4-6RS</td>
<td>4 × 10^9</td>
<td>0.1</td>
</tr>
<tr>
<td>C600/pGP4-6RS</td>
<td>1 × 10^9</td>
<td>0.2</td>
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<tr>
<td>C600/pGP4-5</td>
<td>1 × 10^9</td>
<td>2 × 10^-7</td>
</tr>
<tr>
<td>C600</td>
<td>&lt;5</td>
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Efficiency of plating (EOP) is calculated by dividing the number of plaque-forming units on a given strain by the plaque-forming units on E. coli C600/pGP4-6WT.

Gene 4 protein expression by the phages was examined by immunoprecipitation of [35S]methionine-labeled proteins after infection of E. coli HMS174 with wild-type T7, T7 4B-, and T7 4-245R (Fig. 4). The 56-kDa gene 4 protein was undetectable in extracts from cells infected with T7 4B- (<0.1% total gene 4 protein). Wild-type T7 and T7 4-245R expressed approximately equivalent levels of 56- and 63-kDa gene 4 proteins.

Growth Characteristics of T7 4B-. Both T7 4B- and T7 4-245R have a latency period of ~25 min in E. coli HMS174. Cells that are infected with either phage strain release approximately equal numbers of phage particles: T7 4B- has a burst size of 143 ± 46 and T7 4-245R has a burst size of 119 ± 12. The sizes of nascent DNA fragments produced during phage infection were similar and ranged in size from 5 S to 15 S (data not shown).

The rate of DNA synthesis was measured in a time course during infection of E. coli HMS174 with T7 4B- and T7 4-245R. After T7 4-245R infection, the rate of DNA synthesis increases to a maximum at 12 min and then declines (Fig. 5).

Fig. 3. Rates of DNA synthesis after infection with T7 Δ4-1. The rate of DNA synthesis was measured with a 90-sec pulse of [3H]thymidine at 4-min intervals after infection of E. coli C600/pGP4-6WT (c) or E. coli C600/pGP4-6RS (b) with T7 Δ4-1 at a multiplicity of infection of 1 at 37°C. Each point represents the average and each bar represents an extreme of the data from two experiments.

Fig. 4. Autoradiogram of gene 4 protein immunoprecipitations. E. coli HMS174 was infected with wild-type T7, T7 4-245R, or T7 4B- and pulse labeled with [35S]methionine 8 or 10 min after phage infection (detailed in Materials and Methods). Cell lysates containing approximately equal numbers of phage particles: T7 4B- has a burst size of 143 ± 46 and T7 4-245R has a burst size of 119 ± 12. The sizes of nascent DNA fragments produced during phage infection were similar and ranged in size from 5 S to 15 S (data not shown).

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FIG. 5. Time course of DNA synthesis rates. Rate of DNA synthesis was measured with a 90-sec pulse of $[^{3}H]$thymidine at 4-min intervals after infection of E. coli HMS174 (1 x $10^{6}$ cells per ml) at 37°C with T7 4B− (c) or T7 4-2458R (e) at a multiplicity of infection of 10.

After infection with T7 4B−, however, the rate of DNA synthesis does not increase to the levels detected with T7 4-245R but remains constant until ~16 min postinfection, at which point the rate of DNA synthesis begins to decline.

**DISCUSSION**

The efficiency of the replication system of bacteriophage T7 is striking. With a genome size intermediate between that of the small E. coli phages such as φX174 and the larger phages such as bacteriophage T4, T7 has evolved to balance the use of a minimal number of phage-encoded proteins and the use of host-encoded proteins. For example, the phage-encoded DNA polymerase annexes a single host protein, thioredoxin, as a processivity factor (2, 3, 37). Similarly, in a compromise of coding space, the two colinear proteins of gene 4 provide T7 with both helicase and primase activities (18).

The finding that the 63-kDa gene 4 protein catalyzes both helicase and primase activities (20, 22), whereas the 56-kDa gene 4 protein catalyzes only helicase activity (19, 21), raises an intriguing question. What is the advantage of expression of the 56-kDa gene 4 protein? Part of the answer may be found in the biochemical characterizations of purified 63-kDa gene 4 protein. The 56-kDa gene 4 protein must be present to obtain optimal synthesis of tetraboronucleotide primers by the 63-kDa gene 4 protein; equal concentrations of 56- and 63-kDa gene 4 proteins increase the number of primers synthesized by the 63-kDa protein ~20-fold (20).

In spite of this in vitro observation, in the present study, we have shown that the 63-kDa gene 4 protein alone is sufficient for T7 phage growth. Surprisingly, the only observable defect in phage growth in the absence of 56-kDa gene 4 protein is a decrease in the rate of DNA synthesis. Otherwise, the size of Okazaki fragments and the burst size appear normal. We cannot, of course, rule out the possibility that limited proteolysis of the 63-kDa gene 4 protein gives rise to 56-kDa gene 4 protein. However, by immunoprecipitation, we have ruled out quantities of 56-kDa protein ~0.1% of the 63-kDa protein. The other, albeit remote, explanation for the decrease in the rate of DNA synthesis is that the substitution of glycine for methionine at position 64 in the 63-kDa protein in some way alters the properties of the 63-kDa gene 4 protein. However, a 63-kDa gene 4 protein with a leucine substitution at this position has been reported to catalyze primase, helicase, and DNA-dependent NTase activities (38).

In our earlier models for the role of the 56- and 63-kDa proteins at the replication fork, we proposed that the 56-kDa gene 4 protein functions as a helicase on the leading strand (39). The 56-kDa protein would also serve as a vehicle to transport 63-kDa gene 4 protein to a primase recognition site. One advantage of this model is that processive leading-strand DNA synthesis would not be impeded by primer synthesis since the 56-kDa protein would not recognize primase sites. Paradoxically, the ability of the 63-kDa protein to function alone and still support T7 growth may be due to the fact that it requires 56-kDa gene 4 protein for optimal primase activity. In the absence of the 56-kDa gene 4 protein, the 63-kDa gene 4 protein could function as an efficient, processive helicase since it effectively ignores most primase recognition sequences. Lagging-strand DNA synthesis might be reduced, however, due to the decreased number of initiation events.

It is clear that the 56-kDa gene 4 protein alone is not sufficient for T7 growth (this study; ref. 27). Although a requirement for primase activity for lagging-strand DNA replication is not surprising, this is not the case in bacteriophage T4, where synthesis of Okazaki fragments can be bypassed. The T4 primase and helicase are encoded by genes 61 and 41, respectively (40-43). Mutants of gene 41 show a DD phenotype; loss of T4 helicase eliminates DNA synthesis (44). Mutants of gene 61, however, show a DD phenotype; loss of primase does not eliminate but simply delays DNA synthesis (45). Luder and Mosig (46) have proposed that the requirement for RNA priming on the lagging strand is bypassed by increases in strand invasion that occur during the recombination-dependent stages of T4 replication. Double mutants in genes 61 and 49 are not viable and provide genetic evidence for this model (47). Gene 49 codes for T4 endonuclease VII, which is thought to produce the strand scissions that provide invasive 3'OH ends in the later stages of T4 replication (48).

Although no such mechanism for bypass of lagging-strand DNA synthesis has been observed in T7-infected cells, it cannot be ruled out. If such a mechanism did exist, then other essential roles must be found for the 63-kDa gene 4 protein. One such additional role for the 63-kDa gene 4 protein could be in the initiation of T7 DNA replication at the replication origin (49). It is still unclear whether initiation of T7 DNA replication in vivo is dependent on primer synthesis by the 63-kDa gene 4 protein.

Note Added in Proof. Rosenberg et al. (50) have characterized the properties of a T7 phage that produces 63-kDa gene 4 protein with a leucine substitution for methionine at residue 64.

We thank Stan Tabor for his generous contributions of phage stocks, plasmids, and advice on the construction of phage T7 4B− and plasmid pGP4-6551. We thank Hiroshi Nakai and Susannah Wurgler for critically reading this manuscript. L.V.M. was supported by Damon Runyon–Walter Winchell Cancer Fund Fellowship DRG-1044. S.M.N. was supported by Virology Training Grant T32AI07245 from the National Institutes of Health. This investigation was supported by Grant NP-1U from the American Cancer Society. Grant DE-FG0288ER60688 from the Department of Energy, and Grant AI-06045 from the United States Public Health Service.


