DNA Helicase Requirements for DNA Replication during Bacteriophage T4 Infection

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The lytic bacteriophage T4 uses multiple mechanisms to initiate the replication of its DNA. Initiation occurs predominantly at replication origins at early times of infection, but there is a switch to genetic recombination-dependent initiation at late times of infection. The T4 insertion-substitution system was used to create a deletion in the T4 dda gene, which encodes a 5'-3' DNA helicase that stimulates both DNA replication and recombination reactions in vitro. The deletion caused a delay in T4 DNA synthesis at early times of infection, suggesting that the Dda protein is involved in the initiation of origin-dependent DNA synthesis. However, DNA synthesis eventually reached nearly wild-type levels, and the final number of phages produced per bacterium was similar to that of the wild type. When the dda mutant phage also contained a mutation in T4 gene 59 (a gene normally required only for recombination-dependent DNA replication), essentially no DNA was synthesized. Recent in vitro studies have shown that the gene 59 protein loads a component of the primosome, the T4 gene 41 DNA helicase, onto DNA. A molecular model for replication initiation is presented that is based on our genetic data.

One of the key events in the initiation of bacteriophage T4 DNA replication is likely to be loading of the T4 gene 41-encoded DNA helicase onto the DNA template. Protein 41 is essential for DNA synthesis during T4 infection (11). In vitro, the gene 41 DNA helicase processively unwinds DNA in the 5'-to-3' direction (i.e., moving along the lagging strand at the replication fork) (25, 31) and greatly stimulates the rate of DNA strand displacement DNA synthesis at a replication fork. It also interacts with the T4 gene 61 protein (the DNA primase that makes the RNA primers for Okazaki fragment synthesis) to form the T4 primosome (25, 29).

The molecular events that lead to the loading of the gene 41 protein at replication origins during early times of infection are not known, but the phenotype of T4 dda helicase mutants suggests that the Dda protein plays a role. Little (24) found that infections with phage carrying deletions that remove a significant fraction of the DNA between genes 39 and 56 (which delete the dda gene) show a substantial delay in DNA synthesis at early times of infection, but—because nearly normal amounts of DNA are eventually produced—phage burst size is reduced only slightly. We have previously shown that the dda gene is essential only when the phage also carries a mutation in T4 gene 59 (10, 13; this gene is discussed below). Unfortunately, the dda mutant and dda 59 double mutant phage strains used in those studies either carried extensive deletions that also removed genes flanking the dda gene or contained mutations in additional genes. As a result, it was uncertain whether the phenotype of these phage was due only to a dda deficiency.

Although the physiological role of the Dda helicase is not clear, a great deal has been learned about its biochemical properties from in vitro studies. The Dda protein was originally isolated as a DNA-dependent ATPase by Ebisuzaki and co-workers (2, 7). Like the gene 41 helicase, the Dda protein unwinds DNA in the 5'-to-3' direction, stimulates the rate of DNA strand displacement DNA synthesis at an in vitro replication fork, and removes DNA-binding proteins that block replication fork movement (1a, 16). Since no increase in the rate of replication fork movement is observed when the Dda protein is added to in vitro reactions that have been stimulated by the gene 41 protein, the two DNA helicases do not appear to act synergistically at the fork (16). The Dda protein differs from the gene 41 protein by (i) acting distributively (continuously dissociating and reassociating with the DNA molecule being unwound) rather than processively and (ii) not forming a primosome with the gene 61 protein (16).

Although no UV sensitivity or genetic recombination deficiencies have been detected during growth of dda mutant phage, involvement of the Dda protein in DNA recombination has been suggested by results of in vitro studies. The Dda protein stimulates the rate of UvsX protein-catalyzed DNA branch migration fourfold and binds to the UvsX protein (a T4-encoded RecA analog) (12, 15, 18).

Recent studies suggest that during late times of infection, which are dominated by recombination-mediated initiation of DNA replication, the gene 41 protein is loaded onto the DNA by the gene 59 protein. Gene 59 mutant phage are unable to synthesize DNA at late times of infection and therefore display a DNA arrest phenotype (5, 11, 34). Biochemical characterization of the gene 59 protein by Jack Barry and Bruce Alberts has shown that it loads the gene 41 helicase onto single-stranded DNA in vitro (1). The role of the gene 59 protein in the earlier origin-dependent replication is not clear. Removal of gene 59 does not affect the replication of plasmids containing a T4 replication origin (22), but replication intermediates in gene 59 mutant infections are abnormal (5, 33).

Mutations in gene 59, and other genes involved in DNA recombination, are suppressed by a deficiency in the T4-encoded UvsW protein (5, 33–35). The suppression is not due to restoration of recombination-dependent DNA replication but rather to the mutant allowing some other mode of DNA replication (possibly origin-independent replication) to occur (9). The UvsW protein is thought to govern the switch from origin-dependent to recombination-dependent initiation of DNA replication by inhibiting initiation at replication origins.
Although the UvsW protein has not been characterized biochemically, in vivo experiments have led some researchers to suggest that it has RNase H or RNA-DNA helicase activity (9, 23).

To further examine the role of the dda and 59 genes during T4 DNA replication, the T4 insertion-substitution system (30) was used to construct a deletion that removes only DNA sequences within the dda gene, and DNA synthesis was monitored during infection by the mutant. Phage strains carrying mutations in both the 59 and dda genes were also constructed and analyzed. In addition, 59 dda double mutant pseudorevertants were isolated and their sensitivity to production of the UvsW protein was examined.

MATERIALS AND METHODS

Bacterial and phage strains. Escherichia coli CR63 (supD), NapIV (supD), and NapIV (supD) optA1 stocks are maintained in our laboratories and have already been described (14). E. coli MH1, which can be made competent for high-frequency transformation, was obtained from Michael Hall, University of Basel, Basel, Switzerland. T4 phage strains L148 (dexA and dda point mutations), sud1 (dexA and dda deletions), amHL628 (59 mutant), and amB22 (43 mutant) have been previously described (13, 14). T4 I/S (38 51 den4 den8) (30) was from stocks in the laboratory of Bruce Alberts. Construction of some phage strains required genetic crosses and screening of progeny for the recombinant phenotype (see below).

Chemicals. [methyl-3H]Thymidine was purchased from New England Nuclear, and thymidine and deoxyadenosine were from Sigma Biochemicals. Whatman GFC glass fiber filters were from VWR Scientific. Agarose was purchased from FMC Bioproducts, nitrocellulose filters were from Schleicher & Schuell, and [γ-32P]ATP was from Amersham. Oligonucleotides were synthesized by the Biomolecular Resource Center at the University of California, San Francisco.

Enzymes. All restriction enzymes and DNA-modifying enzymes were purchased from New England BioLabs unless otherwise noted. T4 polynucleotide kinase was obtained from Pharmacia LKB Biotechnology Inc., and the T4 DNA polymerase was purified in the laboratory of Bruce Alberts in accordance with published procedures (27).

Plasmids. Plasmid pKHddda contains the dda gene downstream of the λ leftward promoter, psL, controlled by the cI857 repressor encoded by the plasmid; this plasmid has been previously described (15). Plasmid pBPSPLO+ is a pBR322 derivative containing a fusion of the T4 gene 23 promoter to the supF gene (30). Plasmid pLD-del6, which expresses the uvsW gene, and plasmid pLD-del4, which does not express the uvsW gene, were the generous gifts of Leslie Derr and Ken Kreuzer.

Growth of phage and bacteria. The procedures and media used for genetic crosses have been previously described (11). Phage and bacteria were grown in M9 medium (14) for measurement of DNA synthesis. Equal inputs of parental phage were used for crosses during strain constructions (10).

Measurement of DNA synthesis. DNA synthesis was measured as incorporation of [methyl-3H]thymidine into trichloroacetic acid-insoluble material as described by Gauss et al. (14). pH 7.8; 10 mM magnesium acetate; 0.5 mM dihydrothreitol; 100 μg of human serum albumin per ml) for 1 min at 37°C. The DNA polymerase was inactivated by incubation at 65°C for 5 min. The DNA was further digested with BamHI and Sall, and the DNA fragments were electrophoresed on a 2% low-melting-point agarose gel. The 103-bp BamHI-KpnI and 1,041-bp XmnI-Sall fragments were recovered by melting the agarose at 65°C, followed by phenol extraction and ethanol precipitation. The above-described dda gene fragments (minus the KpnI-XmnI fragment, which contains nucleotides 104 to 284 of the dda gene (15)) were ligated into plasmid pBSPLIO+. This created a dda gene with an internal 181-bp deletion which removes the Walker consensus sequence for ATP binding (32). After transformation into bacterial strain MH1 by standard methods (26), the plasmid construct called pKHDdadel1 was verified by restriction enzyme mapping.

Generation of T4 KH1 phage. T4 I/S phage containing the deletion in the dda gene were generated by substituting the modified dda gene from plasmid pKHddadel1 for the wild-type dda gene in the T4 I/S phage by using the T4 insertion-substitution protocol as described by Selick et al. (30). To screen for T4 I/S phage containing the deletion in the dda gene, phage were plated on host CR63 on NZCYM media plates (26), transferred to nitrocellulose filters, and after prehybridization, hybridized with 1 ng of the 5′-end-labeled oligonucleotide 5′-CATATCGAGGAAGAGCCAGG-3′ (which is complementary to the deleted region of the dda gene) per ml. Hybridization was carried out in 6× SSC [0.9 M sodium chloride and 90 mM sodium citrate, pH 7]–1× Denhardt’s reagent (8–100 μg of Saccharomyces cerevisiae tRNA per ml)–0.05% sodium PP, for 15 h at 35°C. The filters were washed four times for 5 min each time with 6× SSC–0.05% sodium PP, at 25°C and once for 30 min at 45°C and autoradiographed (26). Plaques that did not hybridize with the oligonucleotide were isolated, added to 5 ml of CR63 in Luria-Bertani medium, and incubated for 2 h at 37°C. Cells were removed by centrifugation, and phage DNA was isolated and digested with EcoRV and with both EcoRV and SFal as described by Kreuzer and Alberts (21). Southern blotting with the above-described 32P-labeled oligonucleotide as a probe was used to confirm the identity of the phage called T4 I/S KH1, whose DNA did not hybridize with this oligonucleotide.

Phage T4 I/S KH1 carries the new deletion mutation in the dda gene, as well as the mutations in genes riI, 37, and 51 of the original I/S phage (30). To remove these mutations, a cross was performed with T4 phage sudI, which contains a large deletion in the gene 39 to 56 interval that encompasses the dda and dexA genes. The progeny of the cross were plated on bacterial host strain LG1900 (NapIV optA1), which restricts the growth of dexA, 37, or 51 mutant phage. Only phage carrying the constructed dda mutation form plaques. The progeny phage were then screened for the presence of an riI mutation by being plated on host strain CR63 (λ) (riI mutants are restricted on a host with a λ lysogen). One of the nonrestricted phages, designated T4 KH1, was crossed to amHL628 (5, 11, 33) to construct the dda 59 double mutants described in this report.

RESULTS

Deletion of the dda gene results in a delay in early DNA synthesis during T4 infection. To determine whether the previously reported delay in DNA synthesis during infection by dda mutant phage containing multiple mutations was due solely to a dda gene mutation, the T4 insertion-substitution system was used to construct mutant KH1, which is identical to the wild type except for deletion of DNA sequences within the dda gene (15, 30). To ensure that KH1 phage had not picked
up a *dda* mutation found in many previously characterized *dda* mutant phage strains, the phage were plated on an opt*4* mutant host, LG1900. Because, as shown in Table 1, no restriction of KH1 phage growth was observed, the KH1 phage is *dda*/*dde* (14).

DNA synthesis during infection by wild-type and various *dda* mutant phages is shown in Fig. 1. Like the *dda* mutants previously characterized (sud1 and L148), the KH1 *dda* mutant phage shows a DNA delay phenotype. Recovery by the sud1 and L148 mutants from the delay appears to be more rapid than recovery by KH1. Since sud1 and L148 are known to carry additional mutations in other genes, it is possible that those mutations partially enhance this recovery period.

**Either the 59 or the Dda protein is necessary to obtain a significant amount of DNA synthesis during infection.** A gene 59 amber mutant, amHL628, was crossed with the KH1 *dda* phage to generate a *dda* 59 double mutant. This was done to analyze the phenotype of a *dda* 59 double mutant in the absence of other deficiencies that complicated the previous analyses (10). DNA synthesis during infection by the resulting *dda* 59 phage, PG20, is shown in Fig. 2A. Like previously studied *dda* 59 strains (Fig. 2B), the PG20 mutant displays a “DNA zero” phenotype (Fig. 2A). No DNA synthesis at early or late times of infection was observed and no phage were produced (Table 1). The defect in early DNA synthesis is therefore more severe in the *dda* 59 double mutant than in either of the single mutants (Fig. 2A and B).

**Mutations in the *usw* gene partially suppress the DNA synthesis defect in *dda* double mutant infections.** When growing the *dda* 59 double mutant phage, a significant number of apparent revertants capable of making plaques on nonsuppressing bacterial strains were observed. In previously reported studies, pseudorevertants of gene 59 mutants have been found to map to the T4-encoded *usw* gene (5, 33, 35). To determine whether the suppressing mutation in our *dda* 59 double mutant was a deficiency in the *usw* gene, hosts containing a plasmid that expresses the *usw* gene, pLD-del6, were infected with our revertants (9). If a deficiency in the *usw* gene allows our *dda* 59 mutant to grow, then expression of the wild-type *usw* gene from a plasmid should stop phage growth. A plasmid identical to pLD-del6 except for deletion of the promoter and Shine-Dalgarno sequences necessary for *usw* gene expression was used as a control (9).

*dda* 59 pseudorevertant PG21, like many other similar revertant mutants that we tested, did not produce plaques on hosts harboring a plasmid expressing the *usw* gene (Table 2). This result is not simply due to inappropriate expression of the *usw* gene, since wild-type phage growth was reduced only twofold in this host (Table 2). When hosts containing the expression vector alone were infected by *dda* 59 *usw* pseudorevertant PG21, there was a delay in DNA synthesis compared with wild-type phage, like that seen with *dda* mutant phage (Fig. 3). These results suggest that the gene 59 deficiency, but not the *dda* deficiency, is suppressed in this pseudorevertant and that the suppression is due to a deficiency in the *usw* gene.

**DISCUSSION**

Although *dda* mutant phage show a DNA delay phenotype, *dda* is a nonessential gene because the amount of DNA synthesized eventually reaches nearly wild-type levels during late times of infection and the phage burst size is only slightly reduced (if at all). However, when a phage carries both *dda* and 59 mutations, little or no DNA synthesis is observed and no phage are produced. A deficiency in gene 59 alone results in arrest of DNA synthesis at late times of infection, but because DNA synthesis is normal at early times (33), some phage are produced (5, 11, 33). The defect in early DNA synthesis for the double mutant is more severe than that observed in either a *dda* or a 59 mutant alone, suggesting that the two gene products interact synergistically during origin-dependent replication. In summary, either the 59 or the *dda* gene, but not both, is necessary for phage growth.

A molecular model to explain the genetic results is presented in Fig. 4. This model is based on the properties of the 59 and Dda proteins in vitro: the gene 59 protein loads the gene 41 DNA helicase onto single-stranded DNA (1), whereas the Dda protein is a DNA helicase that, unlike the 41 DNA helicase, loads easily onto single-stranded DNA by itself (17). Thus, we propose that the Dda protein facilitates loading of the gene 59-41 protein complex at replication origins by increasing the length of single-stranded DNA available for binding of the complex. In *dda* mutant phage, less DNA is unwound at the origin and binding of the gene 59-41 protein complex is inefficient, although the gene 41 protein alone is eventually loaded onto the template to form a primosome at the replication fork. In 59 mutant phage, the Dda protein helps the gene 41 protein load onto the template by generating a long region of single-stranded DNA at the origin to serve as a loading zone for the gene 41 protein (1). In *dda* 59 double mutant phage, the length of DNA unwound at the origin may
be too short for binding by gene 41 protein alone, so few, if any, normal replication forks are formed. We have proposed in the model in Fig. 4 that the first steps in both origin unwinding (early) and genetic recombination (late) are normal in the dda 59 double mutant. These initial steps could involve some DNA synthesis, which escapes detection when [3H]thymidine incorporation is measured.

The uvsW mutation suppresses the gene 59 protein deficiency but not the Dda protein deficiency. In the scheme diagrammed in Fig. 4, an additional mutation in the uvsW gene would allow the 59 dda double mutant to produce phage by enabling DNA synthesis to occur during late times of infection—possibly by allowing an alternate form of replication initiation (9, 28) that does not require either Dda or the gene 59 product.

The model in Fig. 4 suggests that there is a synergistic interaction of the Dda and gene 59 proteins during origin-dependent DNA replication. However, the gene 59 protein may not normally play a role in loading of the gene 41 protein onto the DNA at early times after infection with wild-type phage, being needed only when the phage is deficient in the Dda protein. This view remains viable, inasmuch as gene 59 mutants alone display few (if any) defects in DNA synthesis at early times after infection (Fig. 2 and references 5 and 33) or in the replication of plasmids containing a T4 origin (22).

Another possible alternative to the model presented in Fig. 4 is that early in a dda mutant phage infection, the gene 59 protein loads the gene 41 protein at D loops created by genetic recombination. However, the presence of recombination intermediates at early times is a matter of controversy. By using a density shift technique, Dannenberg and Mosig detected a change in phage DNA density that they interpreted to be a result of genetic recombination 5 to 6.5 min after phage infection (1 min after the onset of DNA replication) (6). However, others have detected genetic recombination only 15 to 20 min (4) and 11 min (19, 20) after infection. The arrest of

![Graph A](image1.png)

**FIG. 2.** DNA synthesis after infections with 59, dda, and double mutant T4 bacteriophages. Incorporation of [methyl-3H]thymidine in trichloroacetic acid-insoluble DNA was measured. (A) The dda mutant used was KH1, and the 59 dda double mutant phage was PG20. (B) Infections were carried out as described for panel A, except that different mutant phage were used: the gene 59 mutant was amHL628, and the 59 dda double mutant phage was amHL628aad1 (10, 33).

![Graph B](image2.png)

**FIG. 3.** DNA synthesis after infection with 59 dda double mutant phage carrying a suppressor mutation. All hosts contained a plasmid. As a control, hosts containing pLD-del4 (vector only) were infected with wild-type T4, PG20 (59 dda), and PG21 (59 dda pseudorevertant) phages. The same bacteria containing pLD-del6 (uvsW expression vector) were infected with PG21. Incorporation of [methyl-3H]thymidine in trichloroacetic acid-insoluble DNA was measured.

### TABLE 2. Extrachromosomal expression of T4 uvsW restricts growth of a dda 59 pseudorevertant

<table>
<thead>
<tr>
<th>Phage</th>
<th>Control plasmid pLD-del4</th>
<th>uvsW expression plasmid pLD-del6</th>
</tr>
</thead>
<tbody>
<tr>
<td>T4</td>
<td>210</td>
<td>100</td>
</tr>
<tr>
<td>PG20 (dda 59)</td>
<td>0.04</td>
<td>0.02</td>
</tr>
<tr>
<td>PG21 (dda 59 revertant)</td>
<td>31</td>
<td>0.7</td>
</tr>
</tbody>
</table>

* Host cells were grown at 30°C in M9 medium to a concentration of 2 × 10^8/ml. The cell titer was determined immediately before infection. At 90 min postinfection, the cells were lysed with chloroform and the number of phage was determined by plating the lysate on CR63 cells at 30°C. Burst size is expressed as the number of viable plaques produced per cell.
FIG. 4. Molecular model for the roles of the bacteriophage T4 Dda and gene 59 proteins during infection. (A) At early times of infection by wild-type phage, the replication origin binding proteins unwind the origin, and this single-stranded DNA is extended by the Dda helicase. The gene 59 protein efficiently loads the gene 41 DNA helicase onto the unwound region, which leads to formation of a complete DNA replication fork. At late times of infection, the gene 41 DNA helicase is loaded by the gene 59 protein at the D loop formed by strand invasion, following the synapsis step in genetic recombination. (B) During infection by dda mutant phage, the origin is unwound but the length of single-stranded DNA is not extended since the Dda protein is absent. After a period of time, the gene 59 protein loads enough molecules of the gene 41 DNA helicase to give efficient DNA synthesis, but the start of this synthesis is delayed. Recombination-dependent DNA synthesis at late times of infection is unaffected by the Dda helicase deficiency. (C) During infection by 59 mutant phage, the length of single-stranded DNA at the origin is extended by the Dda helicase and serves as a loading zone for the gene 41 protein. DNA synthesis occurs with no noticeable delay (5, 34). At late times of infection, the gene 41 protein is not loaded onto the D loop because of the gene 59 deficiency, and thus no DNA synthesis occurs.

DNA synthesis 20 to 25 min after infection with phage carrying mutations in genes involved in DNA recombination (5) may mean that DNA replication is initiated from recombinational intermediates only after several rounds of origin-dependent replication have occurred.

In summary, we have generated a T4 dda mutant phage and used this phage to construct dda 59 double mutants. The dda mutant phage have a DNA delay phenotype, which is extended to a severe block in early DNA synthesis when the phage is also a gene 59 mutant. This result suggests that both the dda and gene 59 proteins play a role in the origin-dependent DNA replication which occurs at early times of infection. A model for the roles of the Dda and gene 59 proteins based on our genetic results that is based on their in vitro biochemical properties can be derived. Verification of the suggested roles of these two proteins is likely to require reconstitution of the T4 DNA replication initiation process in a purified in vitro system containing a T4 replication origin (3, 21, 22).

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