The gene 41 protein is the DNA helicase associated with the bacteriophage T4 DNA replication fork. This protein is a major component of the primosome, being essential for coordinated leading and lagging strand DNA synthesis. Models suggest that such DNA helicases are loaded only onto DNA at origins of replication, and that they remain with the ensuing replication fork until replication is terminated. To test this idea, we have measured the extent of processivity of the 41 protein in the context of an in vitro DNA replication system composed of eight purified proteins (the gene 43, 44/62, 45, 32, 41, 59, and 61 proteins). After starting DNA replication in the presence of these proteins, we diluted the 41 helicase enough to prevent any association of new helicase molecules and analyzed the replication products. We measured an association half-life of 11 min, revealing that the 41 protein is processive enough to finish replicating the entire 169-kilobase T4 genome at the observed replication rate of ~400 nucleotides/s. This processivity of the 41 protein does not require the 59 protein, the protein that catalyzes 41 protein assembly onto 32 protein-covered single-stranded DNA. The stability we measure for the 41 protein as part of the replication fork is greater than estimated for it alone on single-stranded DNA. We suggest that the 41 protein interacts with the polymerase holoenzyme at the fork, both stabilizing the other protein components and being stabilized thereby.

DNA is replicated during bacteriophage T4 infection by a protein complex that is composed entirely of viral proteins. This “protein machine” serves as a universal model for the apparatus that catalyzes DNA replication (1–3), and it is composed of several proteins that have distinct activities. The gene 43, 44, 62, and 45 proteins combine to form the DNA polymerase holoenzyme. Two molecules of this holoenzyme are required during replication, one on the leading strand and one on the lagging strand. The gene 43 protein is the DNA polymerase while the other three proteins are “accessory” proteins, forming an ATP-dependent sliding clamp that greatly increases the processivity of the polymerase (4, 5). The gene 32 protein binds in cooperative clusters to single-stranded DNA and is required for any replication of double-stranded DNA in the absence of a helicase (6, 7).

The DNA helicase associated with the T4 DNA replication fork is the gene 41 protein (7). A ring-shaped hexamer (8), the 41 protein unwinds the double helix ahead of the DNA polymerase on the leading strand, translocating in the 5′→3′ direction along the opposite strand (the lagging strand template). The 41 protein promotes DNA synthesis on the leading strand at a rate of ~400 nucleotides/s in vitro (9), close to the rate observed in vivo (10). The newest addition to the T4 in vitro DNA replication system, the gene 59 protein, greatly facilitates the loading of the 41 helicase onto 32 protein-covered single-stranded regions of the double-stranded DNA template (9, 11–13). The T4 gene 41 and 61 proteins together form the primosome. Although the 41 protein is required for the gene 61 DNA primase to recognize its priming sites efficiently (14), it is the primase alone that synthesizes the RNA primer (15–17).

Gene 41 mutations strongly reduce the amount of DNA replication in the phage-infected cell (18) and eliminate synthesis on the lagging strand (19). DNA helicases that are part of the primosome, including this one, have been shown to stay with a single replication fork for a substantial period before dissociating (20). However, in this report, we present the first quantitation of the processivity of a DNA helicase that is associated with a DNA replication fork, demonstrating that the 41 protein remains with the replication fork long enough to replicate the entire 169-kb T4 chromosome without dissociating.

**EXPERIMENTAL PROCEDURES**

Reagents, Enzymes, and Substrate DNA—Nucleoside triphosphates were obtained from Pharmacia Biotech Inc., human serum albumin from Worthington, and [α-32P]dCTP from Amersham Corp. The bacteriophage T4 gene 32, 41, 43, 44/62, 45, 59, and 61 proteins were purified to homogeneity as described elsewhere (9, 21). All proteins were free of detectable nuclease activity. The 44/62 proteins were prepared from T4-infected cells, whereas the other proteins were the products of donors T4 genes expressed on plasmids in Escherichia coli. The purified template DNA used for replication was circular double-stranded DNA, isolated after growing plasmid pMC110 in E. coli (22).

**DNA Synthesis**—A specific DNA 3′ end, produced by treating plasmid DNA with bacteriophage fd gene 2 protein, serves as the primer to start strand-displacement DNA replication on a double-stranded circular DNA template (9, 22). Reactions contained assay buffer (33 mM Tris acetate, pH 7.8, 66 mM potassium acetate, and 10 mM magnesium acetate), 1 mM dithiothreitol, 0.1 mg/ml human serum albumin, deoxyribonucleoside triphosphates (0.3 mM each of dATP, dGTP, and dtTP, plus 0.08 mM [α-32P]dCTP at a specific activity of 1200 Ci/mol), and ribonucleoside triphosphates (1.25 mM ATP and 0.5 mM GTP). The DNA concentration before dilution was 0.8 μg/ml (0.26 nM plasmid). Unless otherwise noted, the 32 protein concentration was 40 μg/ml and the concentrations of the DNA polymerase holoenzyme components were: 3 μM 43 protein, 16 μM 44/62 proteins, and 10 μM 45 protein. The gene 41, 59, and 61 proteins were used at the concentrations specified in each experiment. In reactions containing the 61 protein, 0.25 mM UTP and CTP were added.
Alkaline Agarose Gel Electrophoresis—DNA synthesis was stopped by the addition of alkaline electrophoresis loading buffer to produce a final concentration of 8 mM NaOH, 20 mM Na3EDTA, 10% w/v sucrose, and 0.04% w/v bromocresol green. The radioactive DNA products were analyzed by electrophoresis through alkaline agarose gels, as described elsewhere (23). Following electrophoresis, gels were soaked in two changes of 7% trichloroacetic acid for 30 min each to remove residual \([\alpha\text{-}^{32}\text{P}]\text{dCTP}\). The gels were dried under vacuum onto Whatman DE81 DEAE-cellulose paper and autoradiographed at \(-80^\circ\text{C}\) using an intensifying screen. In addition, the dried gels were analyzed using a PhosphorImager SF with ImageQuant software, IQ 3.3j C15 (Molecular Dynamics, Sunnyvale, CA). The sizes of the radioactively labeled DNA strands were determined by comparison with 5'-end-labeled \([32\text{P}]\) standards (T7 DNA at 40 kb and restriction fragments of \(\alpha\text{-}^{32}\text{P}]\)dCTP. Thispulselabeling screen. In addition, the dried gels were analyzed using a PhosphorImager SF with ImageQuant software, IQ 3.3j C15 (Molecular Dynamics, Sunnyvale, CA). The sizes of the radioactively labeled DNA strands were determined by comparison with 5'-end-labeled \([32\text{P}]\) standards (T7 DNA at 40 kb and restriction fragments of DNA of known size).

RESULTS

Our experimental approach to assess the processivity of the 41 protein relies on establishing a moving replication fork in vitro and then diluting the mixture enough to prevent all further 41 protein associations with the DNA. Double-stranded DNA, nicked at a unique sequence by the bacteriophage \(\phi\) gene 2 protein (22), served as both the primer and template for this DNA replication reaction. Synthesis begins at the 3'-OH side of the nick. Initially, a double-stranded rolling circle with a 5' single-stranded tail is produced by the polymerase holoenzyme in a relatively slow strand-displacement reaction requiring the 32 protein. The 5' single-stranded tail then provides a "loading zone" onto which the 59 protein assembles the 41 helicase (9). After a brief period of DNA synthesis in the presence of all of these proteins, the samples are diluted 10-fold into solutions that maintain the original protein and reagent concentrations, except for DNA and the 41 and 59 proteins. Before dilution, the 59 protein very rapidly loads the 41 helicase onto the DNA template, whereas after dilution no more loading occurs. Therefore, by observing the course of DNA synthesis after dilution, we could determine the degree of 41 protein processivity at the replication fork (how long it stays on the DNA without dissociating).

The Gene 41 DNA Helicase at the Replication Fork Is Highly Processive—In our initial experiments, rolling circle DNA replication was performed in the absence of primase (61 protein), so that only leading strand DNA synthesis occurred (Fig. 1A). The template was first incubated with the DNA polymerase holoenzyme and 32 protein for 1 min, and the slowly elongating leading strand was labeled with \([\alpha\text{-}^{32}\text{P}]\text{dCTP}\). This pulse labeling of DNA was terminated by the addition of excess dCTP at the same time that the 41 and 59 proteins were added to begin rapid leading strand synthesis. The rate of replication fork movement was then determined by measuring the increase in the length of the pulse-labeled DNA products with time, using a denaturing agarose gel.

Without the 41 and 59 proteins, replication forks produced DNA on the leading strand at a rate of about 10 nucleotides/s (Fig. 1B, lanes A–C). When the 41 and 59 proteins were present at 5 and 0.3 \(\mu\text{g/ml}\), respectively, the rate of the DNA replication fork increased 40-fold to 380 nucleotides/s (Fig. 1B, lanes D–F). This rapid rate of replication was not decreased by a dilution of the 41 and 59 proteins; it even increased slightly to 430 nucleo-

32 protein concentration was maintained at 40 \(\mu\text{g/ml}\). Aliquots were taken at 25 s intervals after dilution, and the products were run on a denaturing 0.5% agarose gel. Autoradiographs of the dried gel show the pre- and post-dilution concentrations of the 41 and 59 proteins as indicated. C, quantitation of data in panel B. The percentage of replication forks that contained a helicase was determined by dividing the radioactivity in the fast-moving band (representing forks with helicase) by the total radioactivity in the lane, as determined with a PhosphorImager. Data from lanes D–I are shown; in lanes A–C and J–L no forks with helicase were detected.
The percentage of replication forks that contained a helicase could be determined by comparing the amount of radioactivity in the fast-moving band (representing forks with helicase) with the total amount of radioactivity in each lane. As shown in Fig. 1C, the percentage of replication forks that contained a helicase after dilution of the 41 and 59 proteins remained constant throughout the 75-s incubation. This result was expected, inasmuch as controls show that none of the slow-moving replication forks can acquire a DNA helicase at the diluted concentration of the 41 and 59 proteins (0.5 and 0.03 μg/ml, respectively; Fig. 1B, lanes J–L). We conclude that, once loaded, a 41 protein molecule can function for at least 75 s at a single replication fork without dissociating.

Half-life of the 41 Protein at the DNA Replication Fork—To determine the half-life of the gene 41 helicase at a complete replication fork, we performed a rolling circle replication reaction in which the primase (61 protein) was included, resulting in both leading and lagging strand DNA synthesis. In addition, we needed a way to measure 41 protein association for much longer times after dilution. Since dilution of the 41 and 59 proteins does not decrease the rate of movement of replication forks with a helicase (see Fig. 1), any change in the rate of dCTP incorporation into the leading strand after dilution of these two proteins can be attributed to a slowing of forks due to helicase dissociation. An experiment like that in Fig. 1 was therefore performed, except that after the 10-fold dilution, the dCTP incorporation was measured between 2 and 16 min of incubation. When products were analyzed by electrophoresis through a denaturing agarose gel, the very long leading strand products (>40 kb) migrated through the gel as a sharp band and were easily separated from any lagging strand products (data not shown). The total amount of [α-32P]dCTP incorporated into the leading strand at each time point was quantitated and used to determine the effect of 41 and 59 protein dilution.

As shown in Fig. 2A, without dilution of these proteins (●), the rate of dCTP incorporation was linear for 16 min, revealing that there was no measurable decrease in the number of replication forks with helicase. However, after a 10-fold 41 and 59 protein dilution (Fig. 2, panels A and B; □) there was a small but detectable decrease in the rate of dCTP incorporation throughout the 16 min time course. This indicated a very slow decrease in the number of functional replication forks due to a loss of helicase. The rate of fork loss obeyed the first-order rate law:

\[
\frac{d[F]}{dt} = -k[F] \quad \text{(Eq. 1)}
\]

and

\[
[F] = [F]_0 e^{-kt} \quad \text{(Eq. 2)}
\]

where \(k\) = rate constant, \(t\) = time after dilution, \(F\) = number of forks at a given time, \([F]_0\) = initial number of forks at \(t = 0\).

If \(y\) = total dCTP incorporated into DNA, then

\[
y = \int_0^t [F]_0 R \cdot dt \quad \text{(Eq. 3)}
\]

where \(R\) = rate of fork. After substituting \([F]_0 e^{-kt}\) for \([F]\), integrating, and assuming that at \(t = 0\), \(y = 0\), we find that

\[
y = \frac{[F]_0 R}{k} (1 - e^{-kt}) \quad \text{(Eq. 4)}
\]

The equation of the best fit line shown in Fig. 2B fit the following equation:

\[
y = 3.09(1 - e^{-0.063t}) \quad \text{(Eq. 5)}
\]

As time \(t\) becomes very large, \(y\) approaches a maximum incorporation of 3.09 pmol. To find the half-life, we solved the equation for \(t\) when \(y = \) one half of 3.09 pmol, revealing a half-time for 41 protein association of 11 min at 37°C.

Electron Microscopy of Very Long DNA Products after Dilution of the 41 and 59 Proteins—We wanted to prove directly that, once loaded, the helicase-led replication fork is processive enough to replicate the length of the T4 genome (169 kb). We
performed a DNA replication reaction in which the 41 and 59 proteins were diluted 10-fold, stopped after 8 min, and the products were analyzed on a denaturing 0.5% agarose gel (see "Experimental Procedures").

FIG. 3. The 59 protein is not required for the processivity of the 41 helicase. DNA replication reactions were initiated and diluted as described in Fig. 1 and under "Experimental Procedures" with the following changes: to increase the amount 41 protein loaded onto the template, the pre-dilution loading time was 40 s (compared to 25 s); in the absence of the 59 protein, the 41 protein concentration was increased to 60 μg/ml; and the concentrations of the accessory proteins were increased to 40 μg/ml 44/62 proteins and 25 μg/ml 45 protein. The pre- and post-dilution concentrations of the 41 and 59 proteins were as indicated. Aliquots were taken at 25 s intervals after dilution, and the products were analyzed on a denaturing 0.5% agarose gel (see "Experimental Procedures"). The 59 protein is not required for the processivity of the Gene 41 Protein at the Replication Fork—Although the 59 protein has no measurable effect on the rate of replication fork movement once the 41 protein has become engaged (9), the 59 protein could remain associated with the DNA replication fork and affect its processivity. To test this possibility, we raised the 41 protein concentration in our assays to levels that allow some loading of this helicase onto DNA in the absence of the 59 protein. Under these conditions, we were able to load the 41 protein onto only ~10% of the 5′-tailed templates (Fig. 3, lanes G-I), in contrast to the >80% of templates that obtain a 41 protein-covered single-stranded DNA. Although these very long DNA products were tangled and prone to breakage, 20% of the molecules had 5′ double-stranded tails in the range of 165–197 kb (data not shown).

The Gene 59 Protein Is Not Required for the Processivity of the Gene 41 Protein at the Replication Fork—Although the 59 protein has no measurable effect on the rate of replication fork movement once the 41 protein has become engaged (9), the 59 protein could remain associated with the DNA replication fork and affect its processivity. To test this possibility, we raised the 41 protein concentration in our assays to levels that allow some loading of this helicase onto DNA in the absence of the 59 protein. Under these conditions, we were able to load the 41 protein onto only ~10% of the 5′-tailed templates (Fig. 3, lanes G-I), in contrast to the >80% of templates that obtain a 41 protein-covered single-stranded DNA. Although these very long DNA products were tangled and prone to breakage, 20% of the molecules had 5′ double-stranded tails in the range of 165–197 kb (data not shown).

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In addition to interacting with the 44/62 and 45 proteins, the 41 helicase may also interact with the DNA polymerase (43 protein). Spacciapoli and Nossal found that a mutant form of 43 protein (A737V) had an absolute requirement for the 59 protein to maintain replication forks with a helicase (13). They suggested that a domain in the polymerase interacts with the helicase; if this domain is not functional, then the helicase requires the 59 protein for continuous reloading or for stabilization. In addition, there is ample evidence that two 43 protein...
molecules are present at the replication fork (2). The simplest model of the replication fork would include a 43 protein dimer. However, sedimentation equilibrium analysis unambiguously shows that the 43 protein exists as a monomer in solution under the conditions used in our in vitro replication experiments. This suggests the 43 protein must interact with DNA and/or other components of the DNA replication machine to hold the two 43 protein molecules at the replication fork.

The interactions of the 41 protein with other replication proteins, together with the increased half-life of the helicase on DNA as part of the replication complex, point to the helicase as a central protein around which the other proteins assemble. The highly processive nature of the 41 helicase makes it a prime candidate for the cornerstone of the replication machinery, perhaps providing a physical link between the two polymerase molecules at the replication fork.

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