Published in final edited form as:

J Mol Biol. 2008 April 11; 377(5): 1304–1313.

Origin activation requires both replicative and accessory helicases during T4 infection

J. Rodney Brister

Laboratory of Molecular and Cellular Biology, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Department of Health and Human Services, Bethesda, Maryland, 20892-1770

Abstract

Summary—The bacteriophage T4 has served as an in vitro model for the study of DNA replication for several decades, yet less is known about this process during infection. Recent work has shown that viral DNA synthesis is initiated from at least five origins of replication distributed across the 172 kb chromosome, but continued synthesis is dependent on recombination. Two proteins are predicted to facilitate loading of the hexameric 41 helicase at the origins, the Dda accessory helicase and the 59 loading protein. Using a real time, genome-wide assay to monitor replication during infections, it is shown here that *dda* mutant viruses no longer preferentially initiate synthesis near the origins, implying that the Dda accessory helicase has a fundamental role in origin selection and activation. In contrast, at least two origins function efficiently without 59 loading protein, indicating that other factors load 41 helicase at these loci. Hence, normal T4 replication includes two mechanistically distinct classes of origins, one requiring 59 helicase loader, and a second that does not. Since both mechanisms require an additional factor, *repEB*, for sustained activation, normal T4 origin function appears to include at least three common elements, origin selection and initial activation, replisome loading, and persistence.

Keywords

DNA; replication; origins; helicase; T4

Introduction

Helicases are motile enzymes, central to the molecular biology of all living organisms, which cycle through several conformations in response to the binding and hydrolysis of ribo- or deoxyribo-nucleotides. This conformational switching is harnessed as a mechanical force, which helicases use to move directionally along an individual strand, unwinding duplex DNA, and in some cases, displacing proteins. These activities in turn facilitate a number of DNA transactions, including replication, transcription and recombination, making helicases critical to the function of chromosomes as hereditary units.

During chromosomal duplication, replicative helicases must be loaded onto the DNA template and assembled into the active form, often a hexamer, or a functionally equivalent, ring ^{1; 2}. Typically this helicase loading is directed to specific loci along the chromosome, the so called

Corresponding author: J. Rodney Brister, Laboratory of Molecular and Cellular Biology, NIDDK, National Institutes of Health, Building 8, Room 2A-22, Bethesda, MD 20892-0830, USA, PHONE 301-594-3193, FAX 301-402-0053, jamesbr@niddk.nih.gov.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

origins of replication. Though few universal elements have been identified, most origins include an AT rich downstream unwinding element (DUE), which comes unraveled during origin activation, serving as a target for replisome assembly^{3; 4}. Once integrated into the processive DNA synthesis complex, the helicase is thought to be the leading edge of the polymerase containing replisome, plowing through duplex DNA and spooling out single stranded templates for leading and lagging strand duplication.

The bacterial virus T4 initiates DNA synthesis from at least five origins of replication, including *oriA*, *oriC*, *oriE*, *oriF* and *oriG*, each producing several nascent DNAs during the course of infection⁵. Studies of *oriF* and *oriG* undertaken by the Kreuzer laboratory indicate that DNA synthesis at these origins is primed by a transcript produced from an upstream promoter^{6; 7; 8}. Less is known about *oriA*, *oriC*, and *oriE*, which, together, account for the bulk of replication⁵. One of these, *oriE*, includes a sequence element conserved among other T4-like viruses⁹ and predicted to contribute to origin activation¹⁰, so it is possible that different mechanisms are used to initiate DNA synthesis at the T4 origins.

Whatever the mechanism of activation, each origin is thought to facilitate formation of a processive replisome that includes the viral encoded 43 DNA polymerase, 45 sliding clamp, 61 primase and 41 helicase. Though little is known of replisome assembly during infection, *in vitro* loading of the hexameric 41 helicase onto origin substrates and subsequent replisome mediated replication is greatly stimulated by 59 loading protein¹¹. This helicase loader is a structure specific DNA binding protein and has a strong affinity for single strands and DNA forks formed when one strand of a duplex is displaced by an RNA or DNA polymer annealed to the second strand. Although these R- and D-loops are probably bound by T4 encoded 32 single-stranded DNA binding protein during infection¹², this should not interfere with 41 loading because 59 protein also has a strong affinity for 32 protein and facilitates 41 helicase loading on both naked and 32 coated DNA¹³.

The structural, rather than sequence, specificity of helicase loading reflects the bipartite T4 replication strategy, which involves both origin-initiated DNA synthesis and subsequent replication that requires viral recombination proteins (reviewed in⁶). During this latter process, homologous pairing between newly replicated strands and the chromosomal template creates D-loops used to prime the majority of viral DNA synthesis. This recombination-mediated replication occurs throughout the genome with little apparent specificity⁶, and, as might be expected, 59 protein is an absolute requirement¹⁴, presumably directing 41 helicase to the sites of homologous strand invasion.

Despite the importance of replicative ring helicases like 41 protein, they make up only a portion of known helicases, many of which appear to have distinctly non-replicative biological functions. T4 encodes at least two accessory helicases, UvsW and Dda, both of which apparently contribute to genome replication. UvsW is a 3' to 5' helicase that both unwinds DNA/DNA and DNA/RNA duplexes and stimulates annealing of unpaired strands¹⁵. UvsW is thought to unwind the R-loops used to prime replication, clearing them from origins, and 59 protein is thought to stabilize these primers, preventing their removal by UvsW¹⁶. Dda is a monomeric helicase that is able to displace bound proteins as it moves along DNA templates, 5' to 3', using an "inch worm" mechanism^{17; 18}. This displacing activity is of keen interest as replication occurs concurrently with transcription and other DNA transactions, and one expects the replisome to encounter a number of bound proteins as it travels along the chromosome¹⁹.

The current model of T4 helicase action at viral origins of replication was framed by Barry and Alberts and Gauss et al. over a decade ago. These two groups separately proposed that 59 and Dda act synergistically at the T4 origins of replication and that 41 helicase loading requires

either of the two proteins, but not both^{13; 20}. They predicted that Dda unwinds origin sequences, clearing bound proteins, and creating a single stranded helicase landing zone. Under normal conditions 59 protein would target 41 helicase loading to these unwound regions, and, in the absence of 59 protein, 41 helicase would self assemble on the cleared single-stranded DNA. However, the validity of this model remains unclear, and there has been no definitive evidence that either Dda or 59 protein is directly involved in origin-mediated replication.

Here a genome-wide assay is used to test the predicted roles of 41 replicative helicase, 59 helicase loader, and Dda accessory helicase on viral DNA replication over the course of infection. As expected, there is very little DNA synthesis, either at the origins or elsewhere, in the absence of 41 helicase. In contrast, without Dda, there is some reduced replication across the T4 genome, but peaks in viral replication are no longer visible at the origins. This unanticipated result indicates that Dda is required at a basic level at all of the T4 origins. Unlike Dda, deletion of gene 59 sharply reduces DNA at most, but not all, origins, evoking a new model wherein there are two types of helicase loading, one which is dependent on 59 protein and one where other factors direct loading of 41 protein. Both of these mechanisms require an additional viral factor, *repEB*, for persistent DNA synthesis, suggesting that origin activation is a multi step process involving a number of factors.

Results

To establish the impact of the T4 helicase systems on replication, the kinetics of viral DNA synthesis was measured using a previously developed dot blot assay⁵. Briefly, *E. coli* cells were infected with wt or mutant viruses, each harboring independent disruptions to the accessory helicase, *dda*, the replicative helicase, *gene 41*, or the helicase loader, *gene 59*. Viral DNA was harvested 2 minutes after infection, prior to the onset of replication and at several later time points, blotted onto nylon membranes, and detected with random probes derived from full length T4 chromosomes. The amount of DNA present at several time points during the infection was then compared to that present at 2 minutes, and the fold increase in DNA accumulation was calculated as the increase in viral genomes over time (Figure 1A and B).

DNA replication plateaus early during 59 deficient infections (Figure 1B). This replication arrest is a common attribute among the characterized T4 recombination mutants, and presumably marks the transition from origin- to recombination-mediated replication²¹. During this transition, priming of DNA synthesis becomes dependent on UvsX recombinase, which catalyzes homologous pairing between DNA strands, forming D-loops that are recognized by the 59 helicase loading protein^{22; 23}. This sequence of events predicts that replication kinetics should be similar in *gene 59* and *UvsX* mutant infections. Although this is the case when cells are infected with an average of five viruses, this is not seen in singly infected cells. Rather, when *gene 59* is deleted in singly infected cells, as here, viral replication stops sooner and less DNA is produced, compared to *UvsX* mutants⁵, portending additional roles for 59 protein.

The kinetics of DNA accumulation are markedly different in *dda* and *gene 41* helicase mutants. When *dda* is deleted from the viral genome, DNA synthesis is initially reduced (Figure 1A). Yet, as the infection precedes, the rate of replication increases, and ultimately, near normal levels of DNA synthesis are observed (Figure 1B). This suggests that Dda functions during early, origin-mediated replication but is not absolutely required for later, presumably recombination-mediated replication. In contrast to *dda* mutants, DNA replication is barely detectable throughout *gene 41* mutant infections (Figure 1A and B).

Patterns of DNA synthesis during infection

The predicted roles of Dda, 41 helicase and 59 loading protein at the viral origins were directly tested using a genome wide assay, which allows the accumulation of nascent DNA to be

monitored across the 172 kb T4 chromosome over time⁵. In this case, DNA was harvested from infections and blotted to nylon as before, but this time it was probed with an array of radioactive PCR fragments, amplified from 30 discrete loci across the T4 genome. The amount of T4 DNA at a particular chromosomal locus was then calculated as the fold increase over that present prior to the onset of replication. This approach allows one to follow the initiation and progression of replication across the viral chromosomes over the course of infection, in real time, without timing scores or other algorithms, yielding a qualitative, as well as, quantitative picture of DNA synthesis (Figure 2A–D).

In normal infections the pattern of T4 DNA synthesis is punctate early during infection, with significant accumulations near 5 loci, including *oriA*, *oriC*, *oriE*, *oriF*, and *oriG*, but little replication across intervening regions (Figure 2D). Similar synthesis patterns were seen in *UvsX* mutant infections, deficient in recombination, so origin-mediated, not recombination-mediated synthesis produces the normal replication pattern⁵. The normal, discontinuous pattern arises from the synthesis of small DNAs, less than 27 kb, early during infection⁵, leaving open the possibility that initial replication does not require a fully processive replisome, one including the 41 helicase, capable of traversing the entire genome. Yet, little DNA synthesis was observed in *gene 41* mutants (Figure 1), and there are no discernible peaks in DNA accumulation near *oriE* or any of the other T4 origins over the course of infection (Figure 2A). This demonstrates that 41 helicase is fundamental to DNA synthesis from all the viral origins of replication and that other helicases, such as Dda, can not substitute for 41 helicase.

Dda facilitates origin activation

Unlike *41* mutants, DNA synthesis is reduced early during *dda* mutant infections (Figure 1A) but later rebounds to nearly normal levels (Figure 1B). This delay raised the possibility that Dda contributed to origin activity²⁰, and the replication patterns observed in *dda* mutants indicate that this is indeed the case (see Figure 2B and 2D). There is essentially no DNA synthesis anywhere along the chromosome in the first 8 minutes of *dda* mutant infections, implying that Dda serves a fundamental role in origin activation (Figure 2B). Later a relatively flat replication pattern develops across the genome, with no well defined peaks in DNA synthesis near any of the origins, including *oriE*, further supporting this conclusion. It is worth noting that nearly normal amounts of replication are seen near *oriG* at 10, 12 and 15 minutes, so some origins may recover from the *dda* mutant defect later during infection. Indeed, it is not clear what portion of the synthesis seen without Dda is initiated at the identified origins, and what portion is initiated at other loci, and for that matter, if the reduced synthesis is initiated by normal origin-mediated means, or by other means.

59 helicase loader is not required at some T4 origins

One might expect DNA synthesis in the absence of 59 protein to be similar to that observed in *gene 41* mutants. After all, this protein stimulates loading of 41 helicase onto primed DNA substrates *in vitro*, and deletion of *gene 59* reduces DNA synthesis during infection (Figure 1 and 5; 14; 20). However, as can be seen in Figure 2C, there is a unique replication pattern without 59 protein, and peaks in DNA synthesis are no longer observed near *oriA*, *oriC*, *oriF*, and *oriG*. Instead, a delayed replication is observed near *oriE*, and a second region between *oriF* and *oriG*. Though the error bars are too large to pin point the exact position of this second site of synthesis during the first 8 minutes of infection, a clear peak in replication is observed between genome positions 120 kb and 140 kb at later times.

Synthesis is also observed in the region between *oriF* and *oriG* during normal infections (see Figure 2D), particularly at early times, implying that at least one, yet uncharacterized origin is located in this region. Apparently, this normally low efficiency origin(s) can be activated under certain molecular conditions, like the absence of 59 loader, demonstrating plasticity of T4

origin usage. Since 41 helicase is required for significant DNA synthesis during infection (Figure 1 and Figure 2A), it appears that two functionally distinct mechanisms can be used to load 41 helicase at the T4 origins, one at *oriA*, *oriC*, *oriF*, and *oriG* that requires 59 loading protein, and a second mechanism used at *oriE* and the uncharacterized origin(s) between 120 and 140 kb that does not.

A low level of background replication is observed at other loci over time during *gene 59* mutant infections, and it is not clear if this synthesis is initiated. These results are somewhat different from a recent study where normal amounts of DNA synthesis were observed near *oriF* and *oriG* during *gene 59* mutant infections. The replication forks at these origins were abnormal, and synthesis proceeded normally in one direction from the origins but not the opposite direction²⁴. The reduced origin synthesis seen in Figure 2C could reflect this defect, but it could also reflect differences between the multiply infected cells used in the previous study compared to the singly infected cells used here. The number of infecting viruses alters the kinetics of viral replication, and deletion of *gene 59* has a greater effect on early replication in singly infected cells, than it does on multiply infected cells⁵.

***repEB* is required for efficient origin activation**

Unlike the other origins, the region near *oriE* includes a set of five to eight evenly spaced, sequence repeats, which together have been termed iterons^{9; 10}. Six copies of the conserved iteron sequence, 5'-AT(T/C)(T/A)CC(A/T)T(T/C)AC-3', are also located outside the *oriE* region, at 91,788 bp, 129,021 bp, 130,158 bp, 138,642 bp, 148,011 bp, and 160,580 bp (data not shown), near the second peak of DNA synthesis seen in the absence of 59 loading protein, and, to a lesser extent, in normal infections (Figure 2C and D). It is thought that a viral encoded peptide termed RepEB binds to iteron sequences, facilitating helicase loading at *oriE* and stimulating synthesis¹⁰. If this idea is correct it could explain why replication persists at iteron directed origins, despite the absence of 59 loading protein.

When *repEB* is mutated, initial viral replication is reduced (Figure 3A), but DNA synthesis later approaches normal levels (Figure 3B). This mostly early and ultimately modest reduction in synthesis may explain why a previous study detected a replication defect only when *repEB* was mutated in concert with the MotA transcription factor, a situation that would presumably disrupt transcription primed DNA synthesis from *oriA*, *oriF* and *oriG* in addition to *repEB* activities¹⁰. In any event, the delayed synthesis in *repEB* infections is similar to that observed in *dda* mutant infections and is consistent with a defect in origin function, which is later rescued by recombination-mediated replication.

If the 45 amino acid RepEB peptide is required for iteron mediated replication as predicted¹⁰, then one would expect that mutation of *repEB* would cause a reduction in DNA synthesis from *oriE*, and perhaps region between 120 kb and 140 kb, but not the other origins. Yet, this is not the pattern of viral replication during *repEB* mutant infections. Initially, there are small, distinct peaks of DNA synthesis near *oriE*, *oriF*, and *oriG* during the first 8 minutes, but this early pattern gives way to a generally flat and reduced replication pattern after 10 minutes (compare Figure 4 to 2D), with no DNA synthesis peaks near any of the origins. Taken together these observations indicate that RepEB is not necessary for origin selection and initial nascent synthesis, at least at *oriE*, *oriF*, and *oriG*, but is required for efficient, sustained activation of all origins, not just *oriE*.

Discussion

Helicases are enzymes that melt duplex DNA polymers and translocate directionally along the displaced single strands. These activities foster the replication, repair, and transcription of DNA, and, as a result, helicases play a decidedly important role during the life cycles of all

organisms. The T4 genome encodes at least three helicases, including the 41 replicative helicase and the Dda accessory helicase, but it is not clear how these contribute to viral development. Here patterns of DNA synthesis were monitored across the T4 genome during normal and helicase deficient infections, and it was determined that both the replicative 41 helicase and the accessory Dda helicase are necessary for normal origin activation. Moreover, mutant replication patterns indicate that there are at least two mechanisms used to load 41 helicase onto the various viral origins, one dependent on 59 loading protein and another at *oriE* that is not. Although RepEB was predicted to facilitate iteron mediated helicase loading at *oriE*, this now seems unlikely because disruption of *repEB* affects replication at all origins, not just those with iterons. This mechanistic diversity is somewhat surprising because origin-mediated replication is at least partially dispensable during infection.

Origin activation requires replicative and accessory helicases

Like replicative helicases in other organisms, the 41 helicase from T4 is necessary for processive DNA synthesis *in vitro*¹³, and it was anticipated, as was seen here, that 41 helicase would be crucial to replication from the viral origins. However, it was not clear what role the accessory helicase Dda would play during viral replication. Previous models predicted that Dda served in conjunction with 59 protein to load 41 helicase at the origins and that Dda was not absolutely required for origin function^{13; 20}. Yet, the enhanced DNA synthesis normally seen near the origins is eliminated during *dda* mutant infections (Figure 2B), indicating that Dda is vital to origin activation and, in turn, raising a number of questions regarding the function of this and other homologous, accessory helicases.

Dda shares significant sequence homology with a small subset of accessory helicases, including Human DNA Helicase B (HDHB)²⁵. Although HDHB is more than twice the size of Dda, 1087 amino acids compared to 439, the region of amino acid sequence overlap is 26% identical and 47% similar between the two helicases²⁵. HDHB and its mouse orthologue, MDHB, share a number of biochemical activities thought to be involved in chromosomal replication. Both helicases physically interact with DNA polymerase α -primase (pol-prim) and stimulate synthesis of short RNA primers on DNA templates coated with the mammalian single-stranded binding protein, replication protein A (RP-A)^{25; 26; 27}. MDHB supports *in vitro* DNA synthesis catalyzed by pol-prim, RP-A and DNA gyrase²⁷, and microinjection of mutant HDHB inhibits cellular progression from G1 to S phase, blocking the onset of chromosomal duplication²⁵.

At present, the roles of MDHB and HDHB during replication remain ill defined, but given their sequence homology to Dda, it is possible that these three helicases also share functional homologies that contribute to origin activation. Indeed, all of these helicases appear to be active at the onset of chromosomal replication (25; 28, Figure 2B), perhaps suggesting a role during the initial events of origin activation. Similar to its mammalian homologues, Dda stimulates short-range DNA synthesis on double-stranded substrates *in vitro*^{13; 29; 30}, yet this activity does not appear to be important to origin activation in T4, as there is no appreciable DNA synthesis at the origins without 41 helicase (Figure 2A). Although small nascent polymers may escape detection by the genomic array used in this study, the search for these hypothetical DNAs continues with other methods, but as of yet, none have been found.

How do the T4 origins of replication work?

Once activated, the T4 origins of replication fire multiple times⁵, making it an ideal system in which to study the molecular events involved in origin operation. Unfortunately, the mechanism of origin activation remains poorly defined in T4, as well as many other multi origin systems, and it is not known what factors facilitate origin usage. Without obvious shared sequence elements at the various T4 origins, other than an upstream promoter and a DUE⁶;

³³, it is not clear how particular loci are selected as origins of replication, or how the replisome is assembled at these sequences. Nor is it clear how these sites maintain their activated state over time, allowing repetitive initiation events and the production of multiple nascent DNA polymers. This study sets the foundation for understanding these processes by delineating three molecular steps necessary for efficient origin function, selection, activation and persistence, and identifying viral factors necessary for each step.

Preferential DNA synthesis at the T4 origins requires Dda (see Figure 2B), implying that this protein is involved in the initial events of origin selection and activation. Since Dda interacts directly with 32 single-stranded binding protein ³⁰, it seems plausible that Dda targets 32 coated regions of the T4 chromosome and facilitates formation of a pre-replicative complexes. Given the well characterized protein displacement activity of Dda ¹⁸, once placed on the chromosome, this helicase could unwind duplex DNA, creating an extended single-stranded region, devoid of bound proteins, upon which the T4 replisome can assemble ^{13; 20}. Such a pre-replicative activity may also be relevant to origin activation in other systems. For example, the *Saccharomyces cerevisiae* origin recognition complex binds single-stranded DNA in a length dependent manner, stimulating endogenous ATPase activity and suggesting a functional significance during replisome assembly ³².

The transition from a pre-replicative to an activated state presumably facilitates formation of a primer used to initiate nascent DNA synthesis and fosters replisome assembly onto the primed origin. *In vitro*, 59 loader is required for efficient 41 helicase loading ²² and replication of R-loop substrates designed to mimic a primed *oriF* ¹¹. Yet, there are at least two mechanisms used to load 41 helicase during infection, as neither *oriE* nor the origin(s) between 120 kb and 140 kb require 59 loading protein (see Figure 2D). The basis for this mechanistic diversity is not clear, but it could arise if a yet unidentified protein interacts specifically at these loci, perhaps mediated through iteron sequences, promoting helicase loading. However, this in itself would not account for the increased DNA synthesis from the 120 kb to 140 kb region observed in 59 mutant infections. Rather, there is some plasticity to T4 origin activation, and it appears that activity from the other origins somehow suppresses replication from 120 kb to 140 kb region under normal conditions.

Both Dda and RepEB are expressed at the onset of T4 infection, prior to 59 protein and 41 helicase ³¹, but these peptides appear to have different roles during origin activation. In contrast to Dda, a small amount of origin specific DNA synthesis is observed without RepEB, at least at *oriE*, *oriF*, and *oriG* (see Figure 4). This implies that RepEB is active after the initial selection of these origins and functions once origins have been remodeled by Dda. However, the origin activity in *repEB* mutant infections is not persistent, indicating that this peptide helps to maintain origins in an operative state. RepEB is thought to bind single-stranded DNA with some preference for iteron sequences ¹⁰, so this protein could target origins unwound by Dda. Once bound to origin sequences, RepEB could then facilitate origin activity through at least two, non-exclusive mechanisms: First, RepEB could serve an architectural role, holding origin sequences in an open conformation, passively contributing to primer formation or other origin activities. Second, RepEB could actively recruit 41 helicase, or other replisome factors, to the T4 origins.

Dda is expressed early during T4 infection, prior to 59 protein and 41 helicase ³¹, implying that it is active during the initial steps of origin replication. Since Dda interacts directly with 32 single-stranded binding protein ³⁰, it seems plausible that Dda targets 32 coated regions of the T4 chromosome and facilitates formation of a pre-replicative complexes. Given the well characterized protein displacement activity of Dda ¹⁸, once placed on the chromosome, this helicase could unwind duplex DNA, creating an extended single-stranded region, devoid of bound proteins, upon which the T4 replisome can assemble ^{13; 20}. Such a pre-replicative

activity may be relevant to origin activation in other systems. For example, the *Saccharomyces cerevisiae* origin recognition complex binds single-stranded DNA in a length dependent manner, stimulating endogenous ATPase activity and suggesting a functional significance during replisome assembly³².

Origin-mediated replication is dispensable during infection

The overwhelming majority of T4 replication is initiated by recombination, and origin-mediated replication is at least partially dispensable during infection. So it is not clear why the virus would evolve and maintain discrete origins of replication, and, for that matter, multiple mechanisms for origin activation. One idea is that origins help to regulate DNA synthesis. This makes sense in the context of cellular organisms, because the cell needs to react to environmental cues, time genome replication with cellular division, and prevent origins from firing inappropriately. However, unlike cellular chromosomes that replicate once per cycle, two to three hundred copies of the T4 chromosome are made during a single infection, suggesting that origins have been maintained for different reasons.

Early during infection, each T4 origin produces several short DNAs, which are later elongated in a recombination-dependent process^{5; 34}. So, in a way, the origins provide primers for continued synthesis, and, given the delayed kinetics of *dda* mutants, these origin primers enhance the rate of viral replication. Certainly this would be advantageous to the virus in some environments, like the rapidly dividing cells infected under laboratory conditions, but the discontinuous synthesis produced by this replication strategy may also influence gene expression, providing multiple transcriptional templates near the origins. This idea makes some sense because *oriE*, *oriF* and *oriG* are near clusters of genes necessary for viral maturation and packaging, so enhanced transcription from these regions may be important during T4 development. Perhaps the easily manipulated T4 system can serve as a model wherein this and other questions regarding genome mechanics and evolution can be experimentally addressed.

Materials and Methods

Strains

E. coli BL21(DE3) was obtained from Stratagene. *E. coli* B and CR63, as well as wt T4D, have been maintained in this laboratory. Construction of the gene 59 mutant was described previously⁵, as was the *repEB* mutant¹⁰. The T4 *dda* helicase deletion mutant was a gift from Kenneth N. Kreuzer.

Growth of bacteria and phages

All bacteria were grown in LB broth at 37°C. All T4 infections were done at 37°C. T4 isolates were plaque purified from stock and expanded in either *E. coli* CR63 or BL21(DE3) cells. Phage stocks were stored at 4°C in 10mM Tris (pH7.4), 150mM NaCl, 0.03% gelatin.

Isolation of T4 DNA from Infected Cells

Infections were conducted as described previously⁵. *E. coli* BL21(DE3) host cells were grown to a density of 3×10^8 cells per ml at 37°C, and infected in parallel with pre-warmed wt or mutant phage at a multiplicity of 0.5 viruses per cell. After addition of phage to bacterial cultures at 37°C and thorough mixing for 1 min, phages were allowed to absorb for an additional 45 seconds without mixing. Cultures were then mixed again for 15 seconds, mixing was stopped and two minute samples were withdrawn. Mixing was again started and maintained through the course of infection, except for brief stops to withdraw samples. Aliquots of infected cultures were withdrawn and immediately submersed in ½ volume phenol and ½ volume chloroform. Tubes were inverted 5–7 times to completely lyse the cells. After all aliquots had

been withdrawn, all phenol/chloroform extractions were inverted another 10–14 times and centrifuged to separate aqueous and organic phases. The recovered aqueous phase was extracted with 1 volume of chloroform and stored at 4°C overnight.

Quantification of *in vivo* T4 DNA synthesis

T4 DNA synthesis *in vivo* was monitored by a previously described dot blot assay⁵. Recovered aqueous phase aliquots from the chloroform extractions detailed above were digested with 40 units/ml RNase If (New England Biolabs) at 37°C for 30 minutes. Digested samples (100 µL) were denatured by the addition of 1 volume of 0.5M NaOH, 1.5M NaCl at 65°C for 10 minutes and cooled to room temperature. Denatured aliquots were applied to Hybond-XL membranes (Amersham Biosciences) using a Minifold-1 dot blot system (Schleicher and Schuell) in accordance with manufacturer's instructions. Blots were dried at room temperature for 15–30 minutes, neutralized in 6× SSPE (3M NaCl, 0.2 M NaH₂PO₄, 0.05 M EDTA, pH 7.4), and dried completely.

In preparation for hybridization, blots of viral DNA samples were pretreated in 6× SSPE (pH 7.4), 1% SDS at 62°C for 4 to 6 hours. This buffer was then replaced with fresh hybridization buffer, 6× SSPE (pH 7.4), 1% SDS, 10% dextran. Blots were hybridized to probes generated from full length T4 DNA isolated from purified virions or from PCR fragments amplified from the T4 genome. In either case, probes were radiolabeled using the Prime-It II random primer labeling kit (Stratagene) and cleaned using ProbeQuant G50 spin columns (GE Healthcare). Approximately 5× 10⁵ cpm of probe per ml of hybridization solution was denatured at 95°C for 10–15 minutes and hybridized with blots at 62°C for 12 to 16 hours.

After hybridization, blots were washed 3 times in 2× SSPE (pH 7.4), 0.1% SDS at room temperature for 30 minutes each, and once in 2× SSPE (pH 7.4), 0.1% SDS at 62°C for 30 minutes. These hybridization and wash conditions were empirically determined to prevent cross-hybridization with *E. coli* DNA as judged by dot blotting. Washed blots were exposed to a phosphorimager screen, which was scanned by a FUJIFILM FLA-3000 phosphorimager. Data was analyzed using FUJIFILM Image Gauge V3.12 software, and the amount of signal present at a given time point was divided by the amount of signal from the same infection at 2 minutes, prior to the onset of viral replication.

Measuring T4 DNA replication dynamics with the genomic macro array

DNA synthesis *in vivo* was measured along the T4 chromosome using a genomic macroarray⁵. Details of the experimental process have been described before⁵, and are essentially identical to those in the previous section. However, once DNA aliquots recovered from infections were processed and blotted to nylon membranes, they were then hybridized with 30 random primed probes generated from each of the PCR fragments from the T4 genomic macroarray. Once washed, the amount of DNA present at a given locus at a given time point was divided by the amount at the same locus, in the same infection, at the 2 minute time point, and the fold increase at each of these loci was determined.

Acknowledgments

This paper is dedicated to the living memory of Nancy G. Nossal, my mentor and friend, who's voice still drifts among the science. I would like to thank Ken Kreuzer for kindly providing the *dda* mutant. I would like to thank India Hook-Barnard, Rick Bonocora, Charlie Jones and Debbie Hinton for helpful discussions.

References

1. Dillingham MS. Replicative helicases: a staircase with a twist. *Curr Biol* 2006;16:R844–R847. [PubMed: 17027480]

2. Davey MJ, O'Donnell M. Replicative helicase loaders: ring breakers and ring makers. *Curr Biol* 2003;13:R594–R596. [PubMed: 12906810]
3. Costa S, Blow JJ. The elusive determinants of replication origins. *EMBO Rep* 2007;8:332–334. [PubMed: 17401406]
4. Mott ML, Berger JM. DNA replication initiation: mechanisms and regulation in bacteria. *Nat Rev Microbiol* 2007;5:343–354. [PubMed: 17435790]
5. Brister JR, Nossal NG. Multiple origins of replication contribute to a discontinuous pattern of DNA synthesis across the T4 genome during infection. *J Mol Biol* 2007;368:336–348. [PubMed: 17346743]
6. Miller ES, Kutter E, Mosig G, Arisaka F, Kunisawa T, Ruger W. Bacteriophage T4 genome. *Microbiol Mol Biol Rev* 2003;67:86–156. [PubMed: 12626685]
7. Weigel C, Seitz H. Bacteriophage replication modules. *FEMS Microbiol Rev* 2006;30:321–381. [PubMed: 16594962]
8. Carles-Kinch K, Kreuzer KN. RNA-DNA hybrid formation at a bacteriophage T4 replication origin. *J Mol Biol* 1997;266:915–926. [PubMed: 9086270]
9. Petrov VM, Nolan JM, Bertrand C, Levy D, Desplats C, Krisch HM, Karam JD. Plasticity of the gene functions for DNA replication in the T4-like phages. *J Mol Biol* 2006;361:46–68. [PubMed: 16828113]
10. Vaiskunaite R, Miller A, Davenport L, Mosig G. Two new early bacteriophage T4 genes, repEA and repEB, that are important for DNA replication initiated from origin E. *J Bacteriol* 1999;181:7115–7125. [PubMed: 10559179]
11. Nossal NG, Dudas KC, Kreuzer KN. Bacteriophage T4 proteins replicate plasmids with a preformed R loop at the T4 ori(uvsY) replication origin in vitro. *Mol Cell* 2001;7:31–41. [PubMed: 11172709]
12. von Hippel PH, Kowalczykowski SC, Lonberg N, Newport JW, Paul LS, Stormo GD, Gold L. Autoregulation of gene expression. Quantitative evaluation of the expression and function of the bacteriophage T4 gene 32 (single-stranded DNA binding) protein system. *J Mol Biol* 1982;162:795–818. [PubMed: 6984860]
13. Barry J, Alberts B. A role for two DNA helicases in the replication of T4 bacteriophage DNA. *J Biol Chem* 1994;269:33063–33068. [PubMed: 7806534]
14. Shah DB. Replication and recombination of gene 59 mutant of bacteriophage T4D. *J Virol* 1975;17:175–182. [PubMed: 1107596]
15. Nelson SW, Benkovic SJ. The T4 phage UvsW protein contains both DNA unwinding and strand annealing activities. *J Biol Chem* 2007;282:407–416. [PubMed: 17092935]
16. Dudas KC, Kreuzer KN. UvsW protein regulates bacteriophage T4 origin-dependent replication by unwinding R-loops. *Mol Cell Biol* 2001;21:2706–2715. [PubMed: 11283250]
17. Spurling TL, Eoff RL, Raney KD. Dda helicase unwinds a DNA-PNA chimeric substrate: evidence for an inchworm mechanism. *Bioorg Med Chem Lett* 2006;16:1816–1820. [PubMed: 16439125]
18. Byrd AK, Raney KD. Displacement of a DNA binding protein by Dda helicase. *Nucleic Acids Res* 2006;34:3020–3029. [PubMed: 16738140]
19. Bedinger P, Hochstrasser M, Jongeneel CV, Alberts BM. Properties of the T4 bacteriophage DNA replication apparatus: the T4 dda DNA helicase is required to pass a bound RNA polymerase molecule. *Cell* 1983;34:115–123. [PubMed: 6136341]
20. Gauss P, Park K, Spencer TE, Hacker KJ. DNA helicase requirements for DNA replication during bacteriophage T4 infection. *J Bacteriol* 1994;176:1667–1672. [PubMed: 8132462]
21. Mosig G, Colowick N. DNA replication of bacteriophage T4 in vivo. *Methods Enzymol* 1995;262:587–604. [PubMed: 8594380]
22. Jones CE, Mueser TC, Nossal NG. Interaction of the bacteriophage T4 gene 59 helicase loading protein and gene 41 helicase with each other and with fork, flap, and cruciform DNA. *J Biol Chem* 2000;275:27145–27154. [PubMed: 10871615]
23. Bleuit JS, Xu H, Ma Y, Wang T, Liu J, Morrical SW. Mediator proteins orchestrate enzyme-ssDNA assembly during T4 recombination-dependent DNA replication and repair. *Proc Natl Acad Sci U S A* 2001;98:8298–8305. [PubMed: 11459967]
24. Dudas KC, Kreuzer KN. Bacteriophage T4 helicase loader protein gp59 functions as gatekeeper in origin-dependent replication in vivo. *J Biol Chem* 2005;280:21561–21569. [PubMed: 15781450]

25. Taneja P, Gu J, Peng R, Carrick R, Uchiumi F, Ott RD, Gustafson E, Podust VN, Fanning E. A dominant-negative mutant of human DNA helicase B blocks the onset of chromosomal DNA replication. *J Biol Chem* 2002;277:40853–40861. [PubMed: 12181327]
26. Saitoh A, Tada S, Katada T, Enomoto T. Stimulation of mouse DNA primase-catalyzed oligoribonucleotide synthesis by mouse DNA helicase B. *Nucleic Acids Res* 1995;23:2014–2018. [PubMed: 7596831]
27. Matsumoto K, Seki M, Masutani C, Tada S, Enomoto T, Ishimi Y. Stimulation of DNA synthesis by mouse DNA helicase B in a DNA replication system containing eukaryotic replication origins. *Biochemistry* 1995;34:7913–7922. [PubMed: 7794903]
28. Tada S, Kobayashi T, Omori A, Kusa Y, Okumura N, Kodaira H, Ishimi Y, Seki M, Enomoto T. Molecular cloning of a cDNA encoding mouse DNA helicase B, which has homology to *Escherichia coli* RecD protein, and identification of a mutation in the DNA helicase B from tsFT848 temperature-sensitive DNA replication mutant cells. *Nucleic Acids Res* 2001;29:3835–3840. [PubMed: 11557815]
29. Jongeneel CV, Bedinger P, Alberts BM. Effects of the bacteriophage T4 dda protein on DNA synthesis catalyzed by purified T4 replication proteins. *J Biol Chem* 1984;259:12933–12938. [PubMed: 6092352]
30. Ma Y, Wang T, Villemain JL, Giedroc DP, Morrical SW. Dual functions of single-stranded DNA-binding protein in helicase loading at the bacteriophage T4 DNA replication fork. *J Biol Chem* 2004;279:19035–19045. [PubMed: 14871889]
31. Luke K, Radek A, Liu X, Campbell J, Uzan M, Haselkorn R, Kogan Y. Microarray analysis of gene expression during bacteriophage T4 infection. *Virology* 2002;299:182–191. [PubMed: 12202221]
32. Lee DG, Makhov AM, Klemm RD, Griffith JD, Bell SP. Regulation of origin recognition complex conformation and ATPase activity: differential effects of single-stranded and double-stranded DNA binding. *Embo J* 2000;19:4774–4782. [PubMed: 10970868]
33. Mosig G, Colowick N, Gruidl ME, Chang A, Harvey AJ. Multiple initiation mechanisms adapt phage T4 DNA replication to physiological changes during T4's development. *FEMS Microbiol Rev* 1995;17:83–98. [PubMed: 7669352]
34. Cunningham RP, Berger H. Mutations affecting genetic recombination in bacteriophage T4D. I. Pathway analysis. *Virology* 1977;80:67–82. [PubMed: 878316]

Figure 1. T4 DNA synthesis during wt and recombination deficient infections

The increase in T4 genomes over the course of infection was monitored by hybridization to viral T4 DNA as described in Materials and Methods. (A) *E. coli* BL21(DE3) cells were infected at a multiplicity of 0.5 viruses per cell with either wt T4 (n=6), *gene 41* helicase mutant (n=3), *gene 59* loader mutant (n=3), or *dda* mutant (n=4), where n equals the number of independent trials. Thus, on average, most infected cells contain a single virus. (B) The first 15 minutes of the infection in (A) are plotted on an expanded scale. The symbols used in graphs are as follows: wt, open squares; *gene 41* mutant, open upside down triangles; *gene 59* mutant, open triangles; *dda* open circles. Error bars indicate standard error. The wt and *gene 59* data was previously published⁵ and is presented here for comparison. The wt experiments were done in parallel with the others presented here.

Figure 2. T4 DNA replication dynamics in wt and recombination deficient infections

DNA synthesis was monitored across the viral genome using labeled PCR fragments from the T4 macroarray to probe blotted DNA from infections. The amount of viral DNA present at a given time point at a given locus was plotted as a fold increase over the DNA in the same infection at the same locus at 2 minutes post infection, as described in Materials and Methods. (A) *E. coli* BL21(DE3) cells were infected with mutant *gene 41* (n=3) phage at a multiplicity of 0.5 viruses per cell, where n equals the number of independent trials. (B) *E. coli* BL21(DE3) cells were infected with mutant *dda* (n=4) phage at a multiplicity of 0.5 viruses per cell. (C) *E. coli* BL21(DE3) cells were infected with mutant *gene 59* (n=3) phage at a multiplicity of 0.5 viruses per cell. (D) *E. coli* BL21(DE3) cells were infected with wt T4D (n=3) at a

multiplicity of 0.5 viruses per cell. The wt T4D data was previously published in ⁵ and is presented here for comparison. The filled circles used in all panels are 7 minutes, orange: 8 minutes, blue: 10 minutes, red; 12 minutes, purple; 15 minutes, green. The position along the T4 genome is identified in kilobases along the x-axis. To maintain graphic clarity only the upper extent of the standard error at each data point is indicated with error bars.

Figure 3. T4 DNA synthesis during RepEB deficient infections

The increase in T4 genomes over the course of infection was monitored by hybridization to viral T4 DNA as described in Materials and Methods. (A) *E. coli* BL21(DE3) cells were infected at a multiplicity of 0.5 viruses per cell with either wt T4 (n=1) or *repEB* mutant (n=3, except for the 30 minute time point where n=1), where n equals the number of independent trials. Thus, on average, most infected cells contain a single virus. (B) The first 15 minutes of the infection in (A) are plotted on an expanded scale. The symbols used in graphs are as follows: wt, open squares; *repEB* open circles. Error bars indicate standard error. Experiments were done in parallel.

Figure 4. T4 DNA replication dynamics in RepEB deficient infections

DNA synthesis was monitored across the viral genome using labeled PCR fragments from the T4 macroarray to probe blotted DNA from infections. The amount of viral DNA present at a given time point at a given locus was plotted as a fold increase over the DNA in the same infection at the same locus at 2 minutes post infection, as described in Materials and Methods. *E. coli* BL21(DE3) cells were infected with mutant *repEB* ($n=3$) phage at a multiplicity of 0.5 viruses per cell, where n equals the number of independent trials. The filled circles used in all panels are 7 minutes, orange: 8 minutes, blue: 10 minutes, red: 12 minutes, purple: 15 minutes, green. The position along the T4 genome is identified in kilobases along the x-axis. To maintain graphic clarity only the upper extent of the standard error at each data point is indicated with error bars.