



Available online at www.sciencedirect.com



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, MD 20892-1770, USA

Received 15 September 2007;
received in revised form
28 January 2008;
accepted 1 February 2008
Available online
9 February 2008

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 the 59 loading protein, indicating that other factors load the 41 helicase at these loci. Hence, normal T4 replication includes two mechanistically distinct classes of origins, one requiring the 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.

© 2008 Elsevier Ltd. All rights reserved.

Edited by J. Karn

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 deoxyribonucleotides. 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 origins of replication. Though few universal elements have been identified, most origins include an A+T-rich downstream unwinding element (DUE) that 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 infection.⁵ 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.^{6–8} Less is known about *oriA*, *oriC*, and *oriE*, which, together, account for the bulk of replication.⁵ One origin, *oriE*, includes a unique sequence element that is conserved within the genomes of other T4-like viruses.⁹ This element is

E-mail address: jamesbr@niddk.nih.gov.

† This paper is dedicated to the living memory of Nancy G. Nossal, my mentor and friend, who's voice still drifts among the science.

Abbreviations used: wt, wild type; HDHB, human DNA helicase B; MDHB, mouse orthologue of HDHB.

predicted to contribute to origin activation at *oriE* but is not found near the other origins,¹⁰ so it is possible that different mechanisms are used to initiate DNA synthesis at the various 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 the 59 loading protein.¹¹ This helicase loader is a structure-specific DNA-binding protein and has a strong affinity for 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 infection,¹² this should not interfere with 41 loading because the 59 protein facilitates loading on both naked and 32-coated DNA.¹³

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.⁶ 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 specificity,⁶ and, as might be expected, the 59 protein is an absolute requirement,¹⁴ presumably directing the 41 helicase to the sites of homologous strand invasion.

Despite the importance of replicative ring helicases like the 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 unwinds both DNA/DNA and DNA/RNA duplexes and stimulates annealing of unpaired strands.¹⁵ UvsW is thought to unwind the R-loops used to prime replication, clearing them from origins, and the 59 protein is thought to stabilize these primers, preventing their removal by UvsW.¹⁶ 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 chromosome.¹⁹

The current model of T4 helicase action at viral origins of replication was framed by Barry and Alberts and by 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 involved directly 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 that 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 assay.⁵ Briefly, *Escherichia coli* cells were infected with wild type (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 min after infection, before 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 min, and the fold increase in DNA accumulation was calculated as the increase in viral genomes over time (Fig. 1a and b).

DNA replication plateaus early during 59-deficient infections (Fig. 1b). This replication arrest is a common attribute among the characterized T4 recombination mutants, and presumably marks the transition from origin- to recombination-mediated replication.²¹ 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

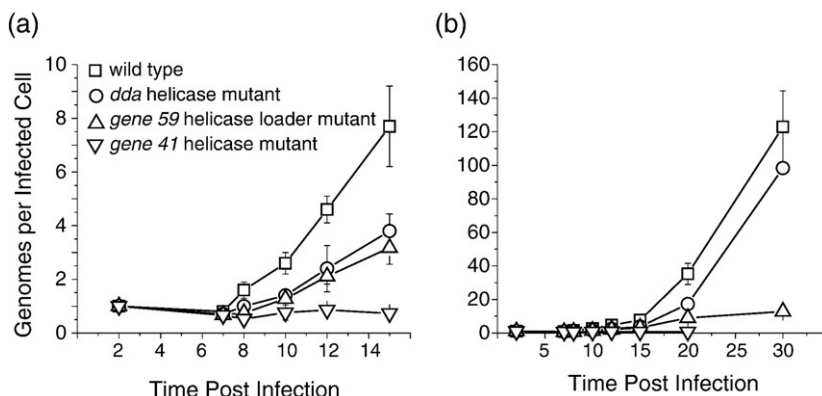


Fig. 1. T4 DNA synthesis during wt and recombination-deficient infections. The increase in T4 genomes over the course of infection was monitored by hybridization to viral T4 DNA as described in Materials and Methods. (a) *E. coli* BL21(DE3) cells were infected at a multiplicity of 0.5 virus/cell with either wt T4 ($n=6$), *gene 41* helicase mutant ($n=3$), *gene 59* loader mutant ($n=3$), or *dda* mutant ($n=4$), where n is the number of independent trials. Thus, on average, most

infected cells contain a single virus. (b) The first 15 min 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 have been published,⁵ and are presented here for comparison. The wt experiments were done in parallel with the others presented here.

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* mutants,⁵ portending additional roles for the 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 (Fig. 1a). Yet, as the infection precedes, the rate of replication increases, and ultimately, near-normal levels of DNA synthesis are observed (Fig. 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 (Fig. 1a and b).

Patterns of DNA synthesis during infection

The predicted roles of Dda, 41 helicase and the 59 loading protein at the viral origins were tested directly using a genome-wide assay, which allows the accumulation of nascent DNA to be monitored across the 172 kb T4 chromosome over time.⁵ 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 before 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 (Fig. 2a–d).

In normal infections the pattern of T4 DNA synthesis is punctate early during infection, with

significant accumulations near five loci, including *oriA*, *oriC*, *oriE*, *oriF*, and *oriG*, but little replication across intervening regions (Fig. 2d). Similar synthesis was seen in *uvsX* mutant infections, deficient in recombination, so origin-mediated, not recombination-mediated replication produces the punctate pattern.⁵ This discontinuous replication arises from the synthesis of small DNAs, less than 27 kb, early during infection,⁵ 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 (Fig. 1), and there are no discernible peaks in DNA accumulation near *oriE* or any of the other T4 origins over the course of infection (Fig. 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, cannot substitute for the 41 helicase.

Dda facilitates origin activation

Unlike *41* mutants, DNA synthesis is reduced early during *dda* mutant infections (Fig. 1a) but later rebounds to nearly normal levels (Fig. 1b). This delay raised the possibility that Dda contributed to origin activity,²⁰ and the replication patterns observed in *dda* mutants indicate that this is indeed the case (see Fig. 2b and d). There is essentially no DNA synthesis anywhere along the chromosome in the first 8 min of *dda* mutant infections, implying that Dda serves a fundamental role in origin activation (Fig. 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 min, 12 min and 15 min, 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

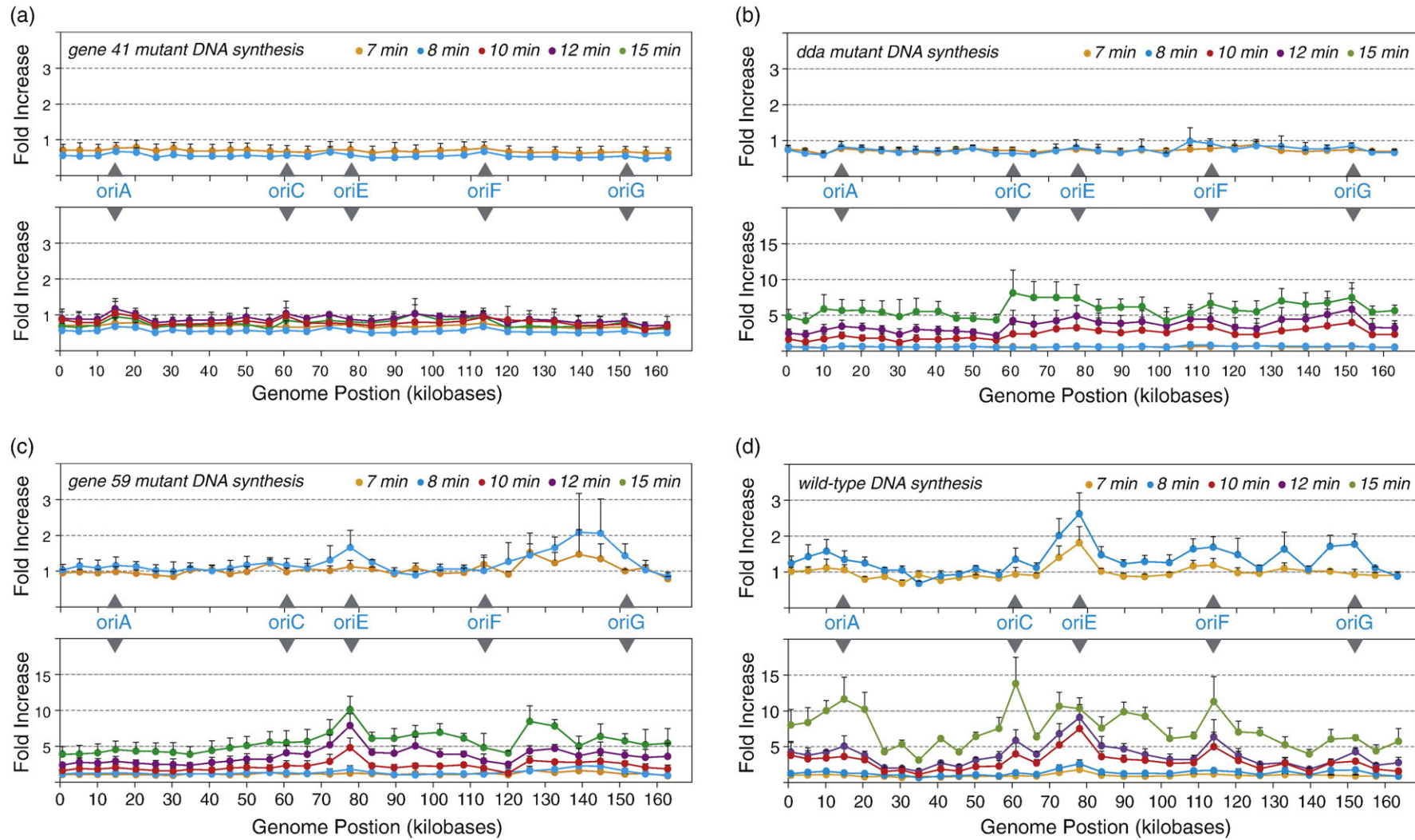


Fig. 2. T4 DNA replication dynamics in wt and recombination-deficient infections. DNA synthesis was monitored across the viral genome using labeled PCR fragments from the T4 macroarray to probe blotted DNA from infections. The amount of viral DNA present at a given time-point at a given locus was plotted as a fold increase over the DNA in the same infection at the same locus at 2 min 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 virus/cell, where n is 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 virus/cell. (c) *E. coli* BL21(DE3) cells were infected with mutant *gene 59* ($n=3$) phage at a multiplicity of 0.5 virus/cell. (d) *E. coli* BL21(DE3) cells were infected with wt T4D ($n=3$) at a multiplicity of 0.5 virus/cell. The wt T4D data have been published,⁵ and are presented here for comparison. The filled circles used in all panels are 7 min, orange; 8 min, blue; 10 min, red; 12 min, purple; 15 min, 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 datum point is indicated with error bars.

other loci and, for that matter, if the reduced synthesis is initiated by normal origin-mediated means, or by other means.

The 59 helicase loader is not required at some T4 origins

One might expect DNA synthesis in the absence of the 59 protein to be similar to that observed in gene 41 mutants. After all, this protein stimulates loading of the 41 helicase onto primed DNA substrates *in vitro*, and deletion of gene 59 reduces DNA synthesis during infection (Fig. 1).^{5,14,20} However, as can be seen in Fig. 2c, there is a unique replication pattern without the 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 pinpoint the exact position of this second site of synthesis during the first 8 min of infection, a clear peak in replication is observed between genome positions 120 kb and 140 kb at later times.

Synthesis is observed also in the region between *oriF* and *oriG* during normal infections (see Fig. 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 the 59 loader, demonstrating plasticity of T4 origin usage. Since the 41 helicase is required for significant DNA synthesis during infection (Figs. 1 and 2a), it appears that two functionally distinct mechanisms can be used to load the 41 helicase at the T4 origins, one at *oriA*, *oriC*, *oriF*, and *oriG* that requires the 59 loading protein, and a second mechanism used at *oriE* and the uncharacterized origin(s) between 120 kb 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 where this synthesis is initiated. These results are somewhat different from a recent study in which 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 direction.²⁴ The reduced origin synthesis seen in Fig. 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 cells.⁵

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 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 the 59 loading protein and, to a lesser extent, in normal infections (Fig. 2c and d). It is thought that a viral-encoded peptide termed RepEB binds to iteron sequences, facilitating helicase loading at *oriE* and stimulating synthesis.¹⁰ If this idea is correct, it could explain why replication persists at iteron-directed origins, despite the absence of the 59 loading protein.

When *repEB* is mutated, initial viral replication is reduced (Fig. 3a), but DNA synthesis later approaches normal levels (Fig. 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* activities.¹⁰ 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 that is later rescued by recombination-mediated replication.

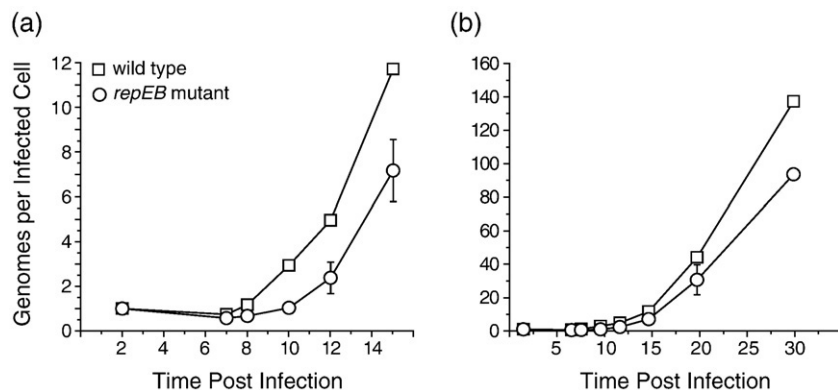


Fig. 3. T4 DNA synthesis during RepEB deficient infections. The increase in T4 genomes over the course of infection was monitored by hybridization to viral T4 DNA as described in Materials and Methods. (a) *E. coli* BL21(DE3) cells were infected at a multiplicity of 0.5 virus/cell with either wt T4 ($n=1$) or *repEB* mutant ($n=3$, except for the 30 min time-point where $n=1$), where n is the number of independent trials. Thus, on average, most infected cells contain a single virus.

(b) The first 15 min 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.

If the 45 amino acid RepEB peptide is required for iteron-mediated replication as predicted,¹⁰ then one would expect that mutation of *repEB* would cause a reduction in DNA synthesis from *oriE*, and perhaps the 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 min, but this early pattern gives way to a generally flat and reduced replication pattern after 10 min (compare Figs. 4 and 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 the 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 the 41 helicase would be crucial to replication from the viral origins. However, it was not clear what role the accessory helicase Dda would have during viral replication. Previous models predicted that Dda served in conjunction with the 59 protein to load the 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 (Fig. 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 helicases.²⁵ HDHB and its mouse orthologue, MDHB, share a number of biochemical acti-

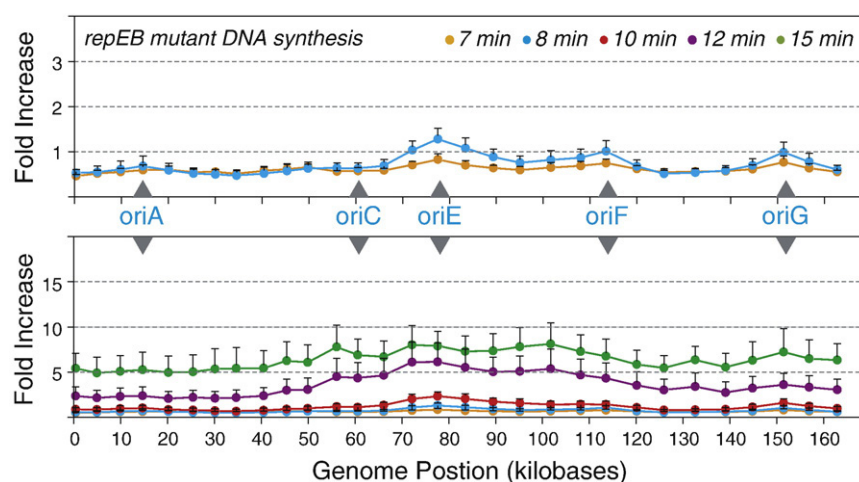


Fig. 4. T4 DNA replication dynamics in RepEB-deficient infections. DNA synthesis was monitored across the viral genome using labeled PCR fragments from the T4 macroarray to probe blotted DNA from infections. The amount of viral DNA present at a given time-point at a given locus was plotted as a fold increase over the DNA in the same infection at the same locus at 2 min 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 virus/cell, where n is the number of independent trials. The filled circles used in all panels are: orange, 7 min; blue, 8 min; red, 10 min; purple, 12 min; and green, 15 min. 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 datum point is indicated with error bars.

vities 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)^{25–27}. MDHB supports *in vitro* DNA synthesis catalyzed by pol-prim, RP-A and DNA gyrase,²⁷ and microinjection of mutant HDHB inhibits cellular progression from G1 to S phase, blocking the onset of chromosomal duplication.²⁵

At present, the roles of MDHB and HDHB during replication are ill defined, but given their sequence homology to Dda, it is possible that these three helicases share functional homologies that contribute to origin activation. Indeed, all of these helicases appear to be active at the onset of chromosomal replication (Fig. 2b),^{25,28} 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 the 41 helicase (Fig. 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 has been found.

How do the T4 origins of replication work?

Once activated, the T4 origins of replication fire multiple times,⁵ making it an ideal system in which to study the molecular events involved in origin operation. Unfortunately, the mechanism of origin activation is 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 downstream unwinding element,^{6,33} 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 Fig. 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 *S. 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*, the 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 the 59 loading protein (see Fig. 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, before the 59 protein and the 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 Fig. 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 facilitate origin activity through at least two, non-exclusive mechanisms: First, RepEB could serve an architectural role, holding origin sequences in an open conformation, contributing passively to primer formation or other origin activities. Second, RepEB could actively recruit 41 helicase, or other replisome factors, to the T4 origins.

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 or 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, 200 to 300 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. This would certainly 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 addressed experimentally.

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 as described,⁵ as was the *repEB* mutant.¹⁰ 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 10 mM Tris (pH 7.4), 150 mM NaCl, 0.03% (w/v) gelatin.

Isolation of T4 DNA from infected cells

Infections were conducted as described.⁵ *E. coli* BL21 (DE3) host cells were grown to a density of 3×10^8 cells/ml at 37 °C, and infected in parallel with pre-warmed wt or mutant phage at a multiplicity of 0.5 virus/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 s without mixing. Cultures were then mixed again for 15 s, mixing was stopped and samples were withdrawn at 2 min. Mixing was started again and maintained through the course of infection, except for brief stops to withdraw samples. Aliquots of infected cultures were withdrawn and submersed imme-

diately in 0.5 volume of phenol and 0.5 volume of chloroform. Tubes were inverted 5–7 times to lyse the cells completely. After all aliquots had been withdrawn, all phenol/chloroform extractions were inverted another 10–14 times and centrifuged to separate the aqueous and organic phases. The recovered aqueous phase was extracted with one volume of chloroform and kept at 4 °C overnight.

Quantification of *in vivo* T4 DNA synthesis

T4 DNA synthesis *in vivo* was monitored by a dot blot assay as described.⁵ Recovered aqueous phase aliquots from the chloroform extractions described above were digested with 40 units/ml RNase If (New England Biolabs) at 37 °C for 30 min. Digested samples (100 µl) were denatured by the addition of one volume of 0.5 M NaOH, 1.5 M NaCl at 65 °C for 10 min 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 min, neutralized in 6×SSPE (3 M 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% (w/v) SDS at 62 °C for 4–6 h. This buffer was replaced with fresh hybridization buffer, 6×SSPE (pH 7.4), 1% SDS, 10% (w/v) 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 5×10^5 cpm of probe per 1 ml of hybridization solution was denatured at 95 °C for 10–15 min and hybridized with blots at 62 °C for 12–16 h.

After hybridization, blots were washed three times in 2×SSPE (pH 7.4), 0.1% SDS at room temperature for 30 min each, and once in 2×SSPE (pH 7.4), 0.1% SDS at 62 °C for 30 min. These hybridization and wash conditions were determined empirically 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 phosphor-imager. Data were 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 min, before 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 macroarray.⁵ Details of the experimental process have been described,⁵ and are essentially identical with those in the previous section. However, once DNA aliquots recovered from infections were processed and blotted to nylon membranes, they were 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 min time-point, and the fold increase at each of these loci was determined.

Acknowledgements

I thank Ken Kreuzer for kindly providing the *dda* mutant. I thank India Hook-Barnard, Rick Bonocora, Charlie Jones and Debbie Hinton for helpful discussions.

References

- Dillingham, M. S. (2006). Replicative helicases: a staircase with a twist. *Curr. Biol.* **16**, R844–R847.
- Davey, M. J. & O'Donnell, M. (2003). Replicative helicase loaders: ring breakers and ring makers. *Curr. Biol.* **13**, R594–R596.
- Costa, S. & Blow, J. J. (2007). The elusive determinants of replication origins. *EMBO Rep.* **8**, 332–334.
- Mott, M. L. & Berger, J. M. (2007). DNA replication initiation: mechanisms and regulation in bacteria. *Nature Rev. Microbiol.* **5**, 343–354.
- Brister, J. R. & Nossal, N. G. (2007). Multiple origins of replication contribute to a discontinuous pattern of DNA synthesis across the T4 genome during infection. *J. Mol. Biol.* **368**, 336–348.
- Miller, E. S., Kutter, E., Mosig, G., Arisaka, F., Kunisawa, T. & Ruger, W. (2003). Bacteriophage T4 genome. *Microbiol. Mol. Biol. Rev.* **67**, 86–156.
- Weigel, C. & Seitz, H. (2006). Bacteriophage replication modules. *FEMS Microbiol. Rev.* **30**, 321–381.
- Carles-Kinch, K. & Kreuzer, K. N. (1997). RNA-DNA hybrid formation at a bacteriophage T4 replication origin. *J. Mol. Biol.* **266**, 915–926.
- Petrov, V. M., Nolan, J. M., Bertrand, C., Levy, D., Desplats, C., Krisch, H. M. & Karam, J. D. (2006). Plasticity of the gene functions for DNA replication in the T4-like phages. *J. Mol. Biol.* **361**, 46–68.
- Vaiskunaite, R., Miller, A., Davenport, L. & Mosig, G. (1999). Two new early bacteriophage T4 genes, repEA and repEB, that are important for DNA replication initiated from origin *E*. *J. Bacteriol.* **181**, 7115–7125.
- Nossal, N. G., Dudas, K. C. & Kreuzer, K. N. (2001). Bacteriophage T4 proteins replicate plasmids with a preformed R loop at the T4 ori(uvsY) replication origin in vitro. *Mol. Cell.* **7**, 31–41.
- von Hippel, P. H., Kowalczykowski, S. C., Lonberg, N., Newport, J. W., Paul, L. S., Stormo, G. D. & Gold, L. (1982). 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.* **162**, 795–818.
- Barry, J. & Alberts, B. (1994). A role for two DNA helicases in the replication of T4 bacteriophage DNA. *J. Biol. Chem.* **269**, 33063–33068.
- Shah, D. B. (1975). Replication and recombination of gene 59 mutant of bacteriophage T4D. *J. Virol.* **17**, 175–182.
- Nelson, S. W. & Benkovic, S. J. (2007). The T4 phage UvsW protein contains both DNA unwinding and strand annealing activities. *J. Biol. Chem.* **282**, 407–416.
- Dudas, K. C. & Kreuzer, K. N. (2001). UvsW protein regulates bacteriophage T4 origin-dependent replication by unwinding R-loops. *Mol. Cell Biol.* **21**, 2706–2715.
- Spurling, T. L., Eoff, R. L. & Raney, K. D. (2006). Dda helicase unwinds a DNA-PNA chimeric substrate: evidence for an inchworm mechanism. *Bioorg. Med. Chem. Lett.* **16**, 1816–1820.
- Byrd, A. K. & Raney, K. D. (2006). Displacement of a DNA binding protein by Dda helicase. *Nucleic Acids Res.* **34**, 3020–3029.
- Bedinger, P., Hochstrasser, M., Jongeneel, C. V. & Alberts, B. M. (1983). Properties of the T4 bacteriophage DNA replication apparatus: the T4 dda DNA helicase is required to pass a bound RNA polymerase molecule. *Cell* **34**, 115–123.
- Gauss, P., Park, K., Spencer, T. E. & Hacker, K. J. (1994). DNA helicase requirements for DNA replication during bacteriophage T4 infection. *J. Bacteriol.* **176**, 1667–1672.
- Mosig, G. & Colowick, N. (1995). DNA replication of bacteriophage T4 *in vivo*. *Methods Enzymol.* **262**, 587–604.
- Jones, C. E., Mueser, T. C. & Nossal, N. G. (2000). 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.* **275**, 27145–27154.
- Bleuit, J. S., Xu, H., Ma, Y., Wang, T., Liu, J. & Morrical, S. W. (2001). Mediator proteins orchestrate enzyme-DNA assembly during T4 recombination-dependent DNA replication and repair. *Proc. Natl Acad. Sci. USA* **98**, 8298–8305.
- Dudas, K. C. & Kreuzer, K. N. (2005). Bacteriophage T4 helicase loader protein gp59 functions as gatekeeper in origin-dependent replication *in vivo*. *J. Biol. Chem.* **280**, 15619–15621.
- Taneja, P., Gu, J., Peng, R., Carrick, R., Uchiumi, F., Ott, R. D. *et al.* (2002). A dominant-negative mutant of human DNA helicase B blocks the onset of chromosomal DNA replication. *J. Biol. Chem.* **277**, 40853–40861.
- Saitoh, A., Tada, S., Katada, T. & Enomoto, T. (1995). Stimulation of mouse DNA primase-catalyzed oligoribonucleotide synthesis by mouse DNA helicase B. *Nucleic Acids Res.* **23**, 2014–2018.
- Matsumoto, K., Seki, M., Masutani, C., Tada, S., Enomoto, T. & Ishimi, Y. (1995). Stimulation of DNA synthesis by mouse DNA helicase B in a DNA replication system containing eukaryotic replication origins. *Biochemistry* **34**, 7913–7922.
- Tada, S., Kobayashi, T., Omori, A., Kusa, Y., Okumura, N., Kodaira, H. *et al.* (2001). 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.* **29**, 3835–3840.
- Jongeneel, C. V., Bedinger, P. & Alberts, B. M. (1984). Effects of the bacteriophage T4 dda protein on DNA synthesis catalyzed by purified T4 replication proteins. *J. Biol. Chem.* **259**, 12933–12938.
- Ma, Y., Wang, T., Villemain, J. L., Giedroc, D. P. & Morrical, S. W. (2004). Dual functions of single-stranded DNA-binding protein in helicase loading at the bacteriophage T4 DNA replication fork. *J. Biol. Chem.* **279**, 19035–19045.
- Luke, K., Radek, A., Liu, X., Campbell, J., Uzan, M., Haselkorn, R. & Kogan, Y. (2002). Microarray analysis of gene expression during bacteriophage T4 infection. *Virology* **299**, 182–191.
- Lee, D. G., Makhov, A. M., Klemm, R. D., Griffith, J. D.

- & Bell, S. P. (2000). Regulation of origin recognition complex conformation and ATPase activity: differential effects of single-stranded and double-stranded DNA binding. *EMBO J.* **19**, 4774–4782.
33. Mosig, G., Colowick, N., Gruidl, M. E., Chang, A. & Harvey, A. J. (1995). Multiple initiation mechanisms adapt phage T4 DNA replication to physiological changes during T4's development. *FEMS Microbiol. Rev.* **17**, 83–98.
34. Cunningham, R. P. & Berger, H. (1977). Mutations affecting genetic recombination in bacteriophage T4D. I. Pathway analysis. *Virology* **80**, 67–82.