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Multiple Origins of Replication Contribute to a Discontinuous Pattern of DNA Synthesis Across the T4 Genome During Infection

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Chromosomes provide a template for a number of DNA transactions, including replication and transcription, but the dynamic interplay between these activities is poorly understood at the genomic level. The bacteriophage T4 has long served as a model for the study of DNA replication, transcription, and recombination, and should be an excellent model organism in which to integrate *in vitro* biochemistry into a chromosomal context. As a first step in characterizing the dynamics of chromosomal transactions during T4 infection, we have employed a unique set of macro array strategies to identify the origins of viral DNA synthesis and monitor the actual accumulation of nascent DNA across the genome in real time. We show that T4 DNA synthesis originates from at least five discrete loci within a single population of infected cells, near *oriA*, *oriC*, *oriE*, *oriF*, and *oriG*, the first direct evidence of multiple, active origins within a single population of infected cells. Although early T4 DNA replication is initiated at defined origins, continued synthesis requires viral recombination. The relationship between these two modes of replication during infection has not been well understood, but we observe that the switch between origin and recombination-mediated replication is dependent on the number of infecting viruses. Finally, we demonstrate that the nascent DNAs produced from origin loci are regulated spatially and temporally, leading to the accumulation of multiple, short DNAs near the origins, which are presumably used to prime subsequent recombination-mediated replication. These results provide the foundation for the future characterization of the molecular dynamics that contribute to T4 genome function and evolution and may provide insights into the replication of other multi origin chromosomes.

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Keywords: bacteriophage; T4; origins; DNA; replication

Introduction

All organisms share a common need to replicate their genomes. This process is required to distribute genetic material to progeny during reproduction and to create daughter cells during development of multi-cellular organisms. Often replication occurs concurrently with transcription and other DNA transactions on a genomic template cluttered with bound proteins, presumably creating an obstacle course of molecular machines, performing the various tasks involved in the maintenance of chromosomes. Such a scenario would imply that DNA replication, transcription, and other genomic events are orchestrated to prevent one transaction from impeding another, creating a complex molecular system, which provides a context for chromosome utility and arbitrates genome evolution.

The dynamics of DNA replication and other chromosomal transactions remain poorly understood on a genomic level and, even in the well-defined *Escherichia coli* system, where the mechanics of replication initiation and termination have been determined,[1,2] there is still some controversy surrounding the progression of DNA synthesis across

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Abbreviations used: wt, wild-type; MOI, multiplicity of infection.

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the genome.[3,4] The situation in eukaryotic systems is even less clear, as many origins contribute to chromosomal replication, raising a number of questions regarding the regulation of origin activity and the resolution of the multiple nascent DNAs arising from the various origins into a single, genomic polymer. Several groups have employed DNA micro arrays to address these questions,[5,6] and these studies have helped to identify the origins and timing of replication across entire genomes.

It is not a simple task to map the dynamic accumulation of DNA synthesis products in most systems. Typically, the chromosome is replicated only once, highlighting background and noise issues, and it can be difficult to maintain synchrony within a population of replicating cells throughout the entire cycle. We sought to avoid these problems by using the bacteriophage T4 as a model system in which to study the mechanics and dynamics of chromosomal replication. Like the chromosomes of eukaryotic cells, the linear, 172 kb, T4 chromosome contains multiple origins of replication, yet, in contrast, T4 is replicated several hundred times during a single, 30–60 min infection. This rapid genome synthesis is accomplished by a viral replisome that polymerizes DNA at a rate of 30–45 kb per minute,[7] a spectacular feat given that transcription is concurrent with replication during infection.

The T4 chromosome is terminally redundant, and about 3 kb of DNA sequence is repeated randomly at the ends of the chromosome, creating a circularly permutated genetic map. The lack of defined chromosomal telomeres and the rapid production of progeny virions presumably contribute to a unique T4 replication strategy that includes both origin-dependent and recombination-dependent replication. Initial T4 DNA synthesis is thought to originate *de novo* from one of several loci, and it has been assumed that nascent DNAs initiated at these origins are extended bi-directionally until the resultant duplex polymer reaches the ends of the parental genome.[8] Although this origin-mediated replication is sufficient to produce some full-length, infectious virus, the vast majority of T4 DNA synthesis is dependent on the viral recombination machinery.[8] The transition from origin-mediated to recombination-mediated replication is not well understood but, once the parental viral genome has been copied, there should be two possible ways in which homologous recombination can be initiated: Nascent daughter chromosomes could recombine with the homologous parental sequences, or the terminally redundant, circularly permutated viral chromosome could recombine with itself, in either case producing a primer for continued DNA synthesis.

Both origin-mediated and recombination-mediated DNA replication use an overlapping set of T4 gene products, which include the gene *43* DNA polymerase, *45* polymerase clamp, and *44/62* clamp loader, as well as the *41* helicase and the *59* helicase loader.[8–10] The 59 protein binds several DNA structures that mimic recombination intermediates used to prime recombination-mediated replication and targets 41 helicase loading to these substrates, allowing formation of a processive DNA replisome.[11] The role of 59 protein during origin-mediated replication is less clear. It stimulates replication on R-loop substrates designed to mimic RNA primers presumed to initiate origin-mediated replication *in vitro*,[12,13] but is not absolutely required for viral replication during infection.[8,14,15]

Like other T4 recombination genes, including the *recA* homologue *uvsX*, mutation of the helicase loader gene *59* causes viral DNA synthesis to halt prematurely, giving rise to the so-called DNA arrest phenotype.[8,15,16] Although the timing of this arrest is thought to mark the transition from origin-mediated to recombination-mediated replication, little is known about the switch between the two modes of replication, and the null recombination-deficient phenotype is poorly defined. Many of the T4 recombination genes contribute to multiple biological activities, making it difficult to ascribe their replication phenotypes to a particular defect, and the actual amount of origin-mediated DNA synthesis occurring in either mutant or normal infection remains unclear.

Historically, T4 DNA synthesis *in vivo* has been measured by supplementing infected cultures with radioactive thymidine and measuring the incorporation of this pulsed nucleotide into growing DNA polymers. Unfortunately, using this methodology, the actual amount of DNA synthesized is not readily apparent, as there is no way to correlate the incorporation of label with the number of genomes synthesized. Rather, genome replication can be estimated only by infectious virion production, a method that may not account for all DNA synthesis. Moreover, early during T4 infection, exogenous label is not incorporated efficiently into nascent viral DNA, as nucleotides are scavenged from the degraded host chromosome,[17] so pulsed nucleotides may not provide an accurate measurement of initial DNA synthesis.

The experimental difficulties presented by the bipartite T4 replication strategy are not limited to shared protein requirements, as is aptly demonstrated by earlier attempts to identify the viral origins of replication.[18] In one of the most exhaustive studies, Kreuzer and Alberts restricted and shotgun-cloned the T4 genome and simply asked if any of the resultant clones could be maintained *in trans* and packaged as concatemers during an otherwise wild-type (wt) infection. After several rounds of selection, a number of putative origins were recovered. Several of these mapped to regions of the T4 genome previously shown to be hotspots for recombination,[18] and at least one putative origin supports recombination-mediated replication,[19] implying that origin-mediated and recombination-mediated replication are biochemically and physically linked to the same regions of the T4 genome.

In total, at least seven putative origins have been identified by a number of groups, *oriA*, *oriB*, *oriC*, *oriD*, *oriE*, *oriF*, and *oriG* (see Figure 1).[8,20,21] Several of these loci were identified as restriction

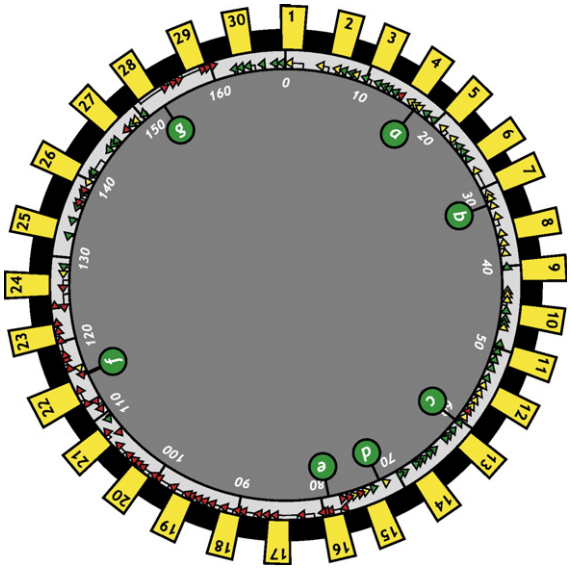

Figure 1. The T4 genomic macro array. Although the terminally redundant, 172 kb T4 chromosome is linear, the ends are circularly permutated, and there is no fixed telomere. This results in a circular genetic map as depicted here. The location and position of PCR fragments included in the genomic array are indicated with yellow tabs with identifying numbers that correspond to the PCR loci (LP numbers) given in Table 1. Putative T4 origins of DNA replication have been placed on the map as reported,[8,21] and are indicated with green lollipops and identifiers (*a*, *b*, etc.). Major open reading frames (>100 amino acids) are indicated with arrows. These were placed on the genetic map using pDRAW32 and were color-coded to indicate the timing of transcription: green, early; yellow, middle; and red, late transcripts.[44] Additionally, the position of the two smaller T4 late genes *soc* and *rI.-1* are indicated with red arrows near *oriA* and *oriC*, respectively.

fragments enriched for nascent T4 DNA early during infection, including *oriA*,[22] *oriB*,[23] *oriC*,[23] *oriE*,[22,24,25] and *oriF*.[22] Some, including *oriD*,[26] were observed using electron microscopy, while others, *oriF* and *oriG*, were recovered by Kreuzer and Alberts as autonomously replicating sequences.[18,27] With the exception of *oriF* and *oriG*, most of these putative origins remain uncharacterized, and neither the *cis* elements necessary for origin function nor the actual start sites of DNA synthesis have been defined.

Despite previous efforts, it has been unclear to what extent any of the putative origins are used during the course of a normal T4 infection, as incomplete representation of the T4 genome in the restriction fragment libraries used to map nascent DNA, disparate experimental conditions between studies, and incongruent experimental results have prevented a consensus. Without such basic information, it is impossible to determine exactly how DNA synthesis proceeds across the T4 genome, not to mention how the interplay between origin-mediated and recombination-mediated replication and other DNA transactions contribute to the dynamics of replication and the fruition of full-length genomes. Hence, in this study, we have mapped the origins of replication used during T4 infections and monitored the DNA synthesis originating from these loci, in real time, across the viral chromosome.

Results

The switch between origin-mediated and recombination-mediated replication during infection

To begin our characterization of T4 DNA synthesis during infection, we empirically defined the switch between origin-mediated and recombination-mediated synthesis during infection using viral recombination mutants. Needing an accurate way to measure viral DNA synthesis early during infection, we developed a simple dot blot assay, wherein DNA was harvested at various lengths of time post infection, blotted to nylon, and hybridized to a random-primed, ^{32}P-labeled probe derived from full-length virus. The amount of T4 DNA associated with each time-point was then measured with a phosphorimager and accumulation over time was calculated as the fold-increase in viral DNA over that present at 2 min post infection, before the onset of replication. This allowed us to estimate the average number of T4 genome equivalents in each infected cell at various lengths of time post infection.

We used a straightforward infection protocol, in which *Escherichia coli* was grown in LB at 37 °C and infected directly with T4 at a multiplicity of infection (MOI) of five viruses to each cell with no additional manipulation of cultures. One advantage of the T4 system is that a population of viruses can be effectively synchronized during infection and, under these conditions, host cells absorb more than 90% of the virus within 4 min of infection (data not shown). To control for experimental variance, multiple, independent infections were repeated on several different days for all viruses, and two replicates of each independent infection were blotted, probed, and averaged.

We chose to monitor the onset of recombination-mediated DNA synthesis using a virus harboring a mutation in *uvsX*, a recombinase gene. Many of the previously characterized *uvsX* mutants contain *amber* stop codons, which can be leaky, though the small amount of full-length protein expressed seems to have little affect on the much-reduced virus production.[28] In our hands, the *uvsX* mutant used in this study produced an average of 15 infectious virions per cell after infection for 1 h, compared to 260 for wt (data not shown), essentially the same amount of virus produced by verified *uvsX* null mutants.[28]

As expected, the *uvsX* mutation caused an arrest in DNA synthesis, and mutant DNA accumulation was much reduced compared to wt (Figure 2(a)). However, this reduction began much earlier than anticipated and was noticeable as early as 7 min post infection, as is evident in the expanded graph

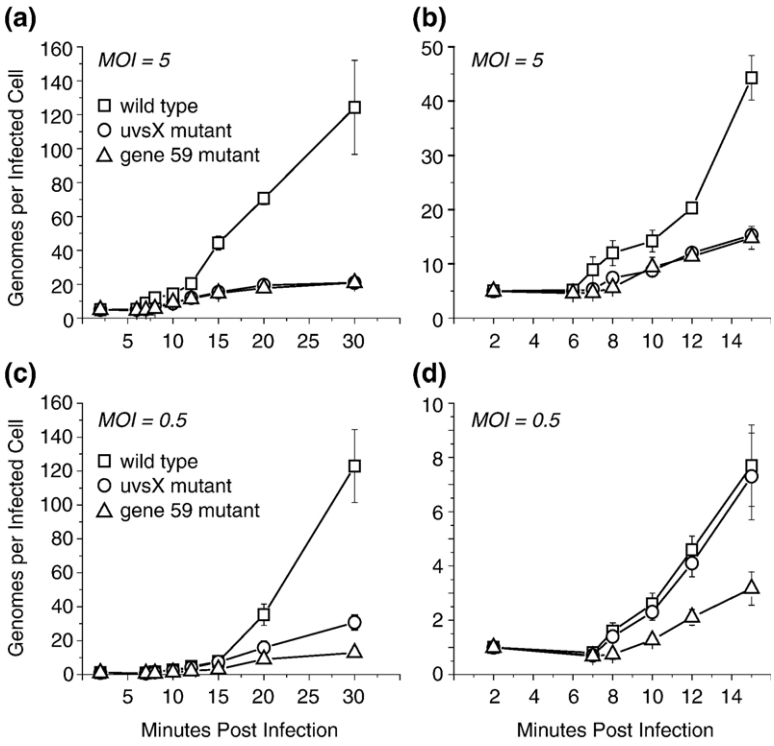

Figure 2. T4 DNA synthesis during wild-type (wt) and recombination-deficient infections. The increase in T4 genomes over the course of infection was monitored by hybridization to viral T4 DNA as described in Materials and Methods. (a) *E. coli* BL21(DE3) cells were infected at a multiplicity of five viruses per cell with either wt T4D ($n=6$), *uvsX* mutant (*recA* homologue, $n=2$), or gene*59* mutant (helicase loader, $n=5$), where n is the number of independent experiments. (b) The first 15 min of the infection in (a) are plotted on an expanded scale. (c) *E. coli* BL21 (DE3) cells were infected at a multiplicity of 0.5 virus per cell with either wt T4 ($n=6$), *uvsX* mutant ($n=5$), or gene*59* mutant ($n=3$). Thus, on average, most infected cells contain a single virus. (d) The first 15 minutes of the infection in (c) are plotted. The symbols are as follows: wt, open squares; *uvsX* mutant, open circles; gene*59* mutant , open triangles. Error bars indicate standard error.

(Figure 2(b)). This observation was confirmed with a second recombination mutant, containing a nearly complete deletion within gene *59*, constructed as described in Materials and Methods. At an MOI of 5, gene *59* mutant infections showed amounts of DNA synthesis similar to that of *uvsX* mutant infections. The accumulation of viral DNA was clearly reduced compared to wt, and this reduction in DNA synthesis was apparent from at least 7 min post infection (Figure 2(a) and (b).

To determine whether our experimental conditions contributed to the early onset of DNA arrest observed with the recombination mutants, we repeated the experiment with the MOI reduced to 0.5 virus per cell, so that each infected cell contained, on average, a single virus. As can be seen in Figure 2(c) and (d), this change in the MOI had a profound effect on viral DNA synthesis. Under otherwise identical conditions, at an MOI of 0.5, the *uvsX* mutation has little affect on synthesis, compared to wt, until 15 min post infection. Thus, the number of infecting viruses appears to influence the timing at which recombination begins to contribute to T4 DNA synthesis. Notably, replication of the gene *59* mutant at the lower MOI of 0.5 arrested earlier than the *uvsX* mutant, implying that 59 protein is required before the onset of recombination-mediated replication.

Mapping early nascent T4 DNA synthesis

Having defined the time-course of origin-dependent replication under our conditions, we next wanted to map the nascent DNA produced during T4 infection at an MOI of 0.5. The hybridization strategy used in Figure 1 proved to be quite useful in quantifying the amount of nascent DNA made over time, and we wanted to apply a similar strategy to the mapping of these synthesis products to discrete regions within the T4 genome. Hence, we created an array of PCR products that provided equal representation of the entire T4 genome and could be used for mapping studies. This array is comprised of discrete 2.5–3.0 kb PCR products amplified from 30 loci, spaced evenly across the phage genome, and includes products positioned near seven putative origins of DNA replication, *oriA*, *oriB*, *oriC*, *oriD*, *oriE*, *oriF*, and *oriG*, identified in previous studies (see Figure 1).

Although the T4 replisome progresses at a rapid rate of 30–45 kb per minute,[7] at the onset of replication in a population of infected cells, there should be some number of origin-initiated, leading strands less than 10 kb, but longer than the 2 kb average Okazaki fragments.[29] To map these nascent DNAs, we harvested DNA from infections and fragmented contaminating *E. coli* chromosomal DNA with the restriction enzyme HaeIII, resolved the undigested T4 DNA on alkaline agarose gels. HaeIII cleavage is blocked by methylation of the internal cytosine bases within the recognition sequence 5′-GGCC-3′,[30] and it does not appear to cut the hydroxymethylated cytosine-containing T4 DNA produced early during our infections (data not shown).

The shorter, nascent viral DNAs were then excised from the gels, purified, random primed labeled, and used to probe the T4 genomic macro array. The enrichment of these size selected DNAs at specific loci, and hence their origin, was determined using

a relative abundance score. This score allows the abundance of DNA at a locus during infection to be compared to the abundance of DNA at that same locus in the packaged virus, before infection and subsequent replication, and is calculated as the ratio between the percentage signal at a given locus compared to all loci on the array and the same percentage obtained with a control probe derived from packaged viral DNA. Thus, a relative abundance score at a particular locus greater than 1 indicates that this locus is enriched in nascent DNA as compared to the packaged, full-length T4 genome.

A large enrichment at all three time-points was observed in the nascent 3–6 kb wt DNA at a PCR locus near the putative T4 origin, *oriE* (Figure 3(a)).[22,24,25] A similar enrichment pattern was seen in the 6–10 kb size selected wt DNA, with the greatest relative abundance observed near *oriE* (Figure 3(b)). There was also a less pronounced enrichment at another locus, which overlaps a second previously identified T4 origin, *oriF*.[18,31] Although the sequence elements required for *oriE* activity have not been delineated, *oriE* and *oriF* are thought to be distinct mechanistically.[32] Thus, it appears that two functional classes of origins are active within a single population ofT4-infected cells.

To determine if the synthesis observed in Figure 3(a) and (b) was true origin-dependent rather than recombination-dependent synthesis, size-selected nascent DNA from *uvsX* mutant infections was prepared and hybridized to the array. Similar to wt, the greatest relative abundance in both size-selected populations of mutant DNA was observed near *oriE* and *oriF*, indicating that DNA synthesis originating from these loci during the time monitored is not dependent on uvsX protein (Figure 3(c) and (d)). Thus, it appears that there are at least two origins of *de novo* synthesis active in a population of infected cells under our conditions. Of these, *oriE* appears to be the most active.

Analysis of T4 replication dynamics

Though the previous analysis gives some indication as to the origins of phage DNA synthesis, our size-selection strategies preferentially enriched for nascent DNA derived from origins at which 3–10 kb nascent DNA polymers accumulate. This could be problematic if, as one would expect, once initiated, nascent DNA polymers continue to grow at 30–45 kb per minute, until the entire genome has been replicated. By size-selecting nascent DNAs, most of these predicted, growing polymers would be excluded from analysis, and with them, the ability to follow T4 DNA synthesis across the genome and illuminate the dynamics replication.

A second mapping strategy was employed to address these issues. Herein, a time-course of total

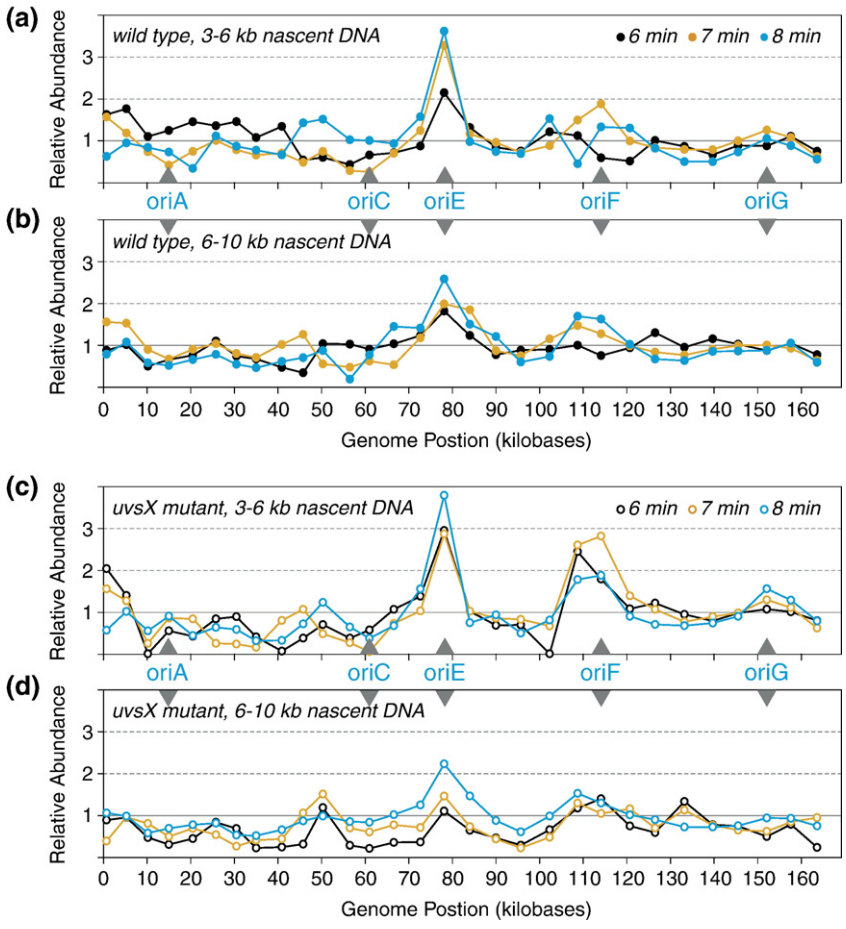

Figure 3. Relative abundance of size-fractionated nascent T4 DNA synthesized early during infection. Wild-type (wt) T4D and the recombination *uvsX* mutant were used to infect *E. coli* BL21(DE3) cells at a multiplicity of 0.5 virus per cell. Total viral DNA harvested from infections at 6, 7, and 8 min post infection was size-fractionated on alkaline agarose gels and used to generate random primed probes as described in Materials and Methods. These labeled DNAs were then used to probe the T4 genomic macro array, immobilized on nylon membranes, and the relative abundance of viral DNA at each locus along the array was calculated as described in Materials and Methods. (a) Wt nascent T4D DNA, 3–6 kb. (b) Wt nascent T4D DNA, 6–10 kb. Filled circles used in (a) and (b) are 6 min, black: 7 min, orange: 8 min, blue. (c) Mutant *uvsX* nascent DNA, 3–6 kb. (d) Mutant *uvsX* nascent DNA, 6–10 kb. The open circles used in (c) and (d) are 6 min, black: 7 min, orange: 8 min, blue. The position along the 168 kb T4 genome is identified (in kb) along the *x*-axis.

T4 DNA harvested early during infection was blotted onto nylon strips, and these strips were probed independently with each of the PCR loci from the genomic array. This allowed the amount of DNA synthesized at a particular locus, at a particular time-point to be calculated as the fold increase over the amount at the same locus present at 2 min post infection, before significant replication of the parental genome. An advantage of this methodology is that the fold increase in DNA at a given locus should reflect the number of times that locus is replicated, providing a direct measurement of DNA accumulation. Hence, this approach allows one to plot the synthesis of DNA across the genome in real time and to visualize the dynamics of viral replication.

Similar to our total viral DNA analysis, multiple, independent infections were repeated on several different days, and the results from each were averaged. During the first 8 min of wt infections a two- to threefold increase in the amount of DNA was observed near *oriE* (Figure 4(a)), an expected result, given that the greatest enrichment of size-selected nascent DNAs was observed at the same PCR locus. As the infection progressed, the DNA accumulation near *oriE* continued, and by 12 min a nearly tenfold increase was observed at this locus (Figure 4(b)), implying that, on average, replication was initiated from this region nearly ten times per infecting virus during this time.

DNA also accumulated at several other loci with the T4 genome during the first 8 min of infection, including those near *oriA*, *oriC*, *oriF*, and *oriG*, as well as a discrete locus located between the latter two origins (Figure 4(a)). Moreover, the amount of DNA continues to increase near *oriA*, *oriC*, *oriF*, and *oriG*, and by 12 min post infection an approximately fivefold increase in accumulation is observed near all of these loci (Figure 4(b)). Thus, multiple T4 origins are active within a single population of infected cells, and these origins continue to fire, producing multiple nascent DNAs, as the infection proceeds.

Replication dynamics in the *uvsX* recombination mutant

Our data indicate that at an MOI of 0.5 both wt and *uvsX* mutant viruses produce essentially the same amount of DNA during the first 15 min of infection (Figure 2(c) and (d)). After that point, DNA synthesis in the *uvsX* mutant reaches a plateau, and the virus exhibits the DNA arrest phenotype associated with T4 recombination mutants. The obvious conclusion is that T4 recombination-mediated DNA synthesis does not contribute significantly to phage DNA replication at the lower MOI until

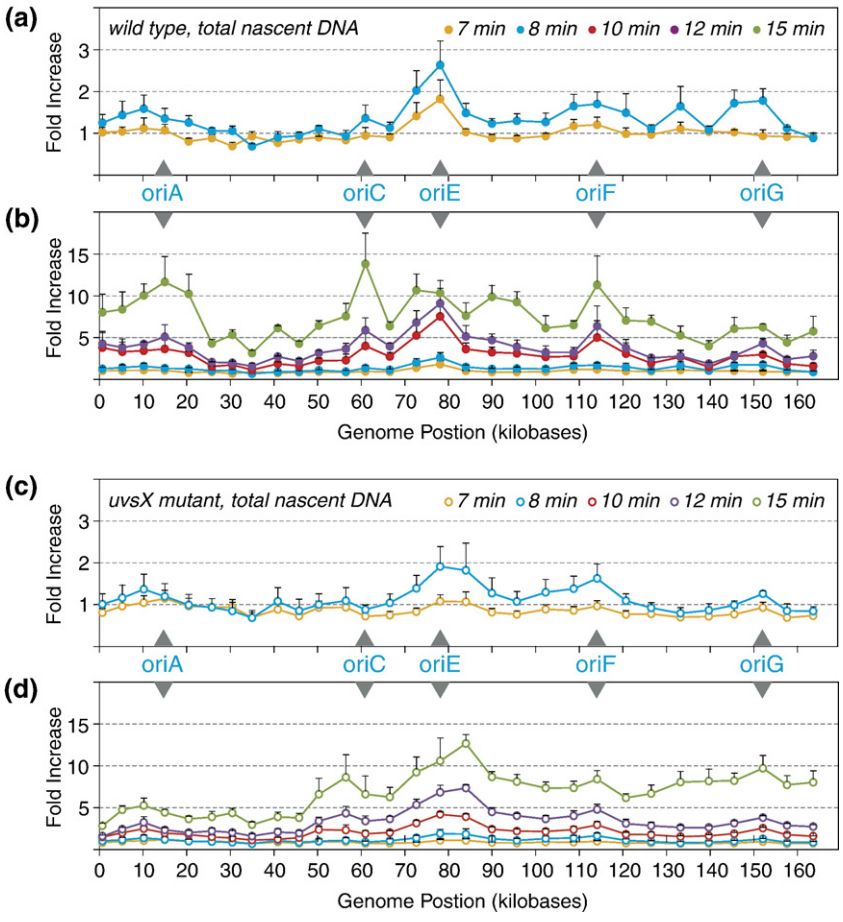

Figure 4. T4 DNA replication dynamics in wild-type (wt) and recombination-deficient infections. DNA synthesis was monitored across the viral genome using labeled PCR fragments from the T4 macro array to probe blotted DNA from infections. The amount of viral DNA present at a given time-point at a given 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 wt T4D ($n=3$) at a multiplicity of 0.5 virus per cell, where n is the number of independent experiments. (b) The same wt T4D infections at later time-points. Filled circles in (a) and (b) are 7 min, orange: 8 min, blue: 10 min, red; 12 min, purple; 15 min, green. (c) *E. coli* BL21 (DE3) cells were infected with mutant *uvsX* ($n=4$) phage at a multiplicity of 0.5 virus per cell. (d) The same *uvsX* infections at later time-points. Open circles in (c) and (d) are 7 min, orange: 8 min, blue: 10 min, red; 12 min, purple; 15 min, green. The position along the 168 kb T4 genome is identified (in kb) 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.

15 min post infection. This conclusion is supported by the fact that short nascent DNA recovered early during *uvsX* infections maps to the same loci as wt (Figure 3). So, it is not surprising that the pattern of total DNA accumulation in the *uvsX* mutant is also similar to that of wt virus, at least during the first 12 min of infection, with most synthesis mapping near *oriE*, and less mapping near *oriA*, *oriF* and *oriG* (Figure 4(c) and (d)). Yet, as the time-course progresses to 15 min post infection, some distinct differences are observed between the two viruses, most notably that DNA accumulation near *oriA* is reduced during *uvsX* mutant infections, as compared to wt. This could indicate that the T4 genome contains at least two functionally distinct classes of origins, one more dependent on recombination proteins for activity under our experimental conditions.

Short nascent DNAs accumulate early during infection

If nascent DNA polymers grew continuously from their origin to the ends of the genome, one would expect to see the amount of DNA increase sequentially across the genome over time. So, as a single replication fork moved from an origin to adjacent loci, the amount of DNA should increase by a factor of 2, initially at the origin and subsequently at adjacent loci. This dynamic should be measured easily by our assay and, given a physiological replisome rate of 30–45 kb per minute,[7] one would expect the twofold increase in DNA associated with each nascent replication fork to travel across the T4 genomic array at the rate of four to six adjacent loci per minute.

In contrast to the expected DNA synthesis dynamic, the fold increases observed initially at the origin loci do not move evenly across the genome with time (Figure 4). Instead, the pattern of T4 DNA synthesis early during infection is one of peaks and valleys, wherein five- to tenfold increases in the amount of DNA are observed near the origins during the first 12 min of infection, but much smaller increases are observed in the regions adjacent to these loci. So, it appears that nascent replication forks are moving slower than anticipated through some regions of the T4 genome and, as a result, short nascent DNAs are accumulating near the origins.

To determine the size distribution of nascent DNAs directly, viral DNA harvested early during infection was size-fractionated on alkaline agarose gels, blotted to nylon membranes and hybridized to random-primed probes derived from full-length T4 DNA and a PCR product near *oriE* (Figure 5). To simplify the analysis, the fraction of DNA less than 27 kb was graphed for each time-point and, as expected from the replication dynamics in Figure 3, most of the DNA detected with either full-length T4 (Figure 5(b)) or *oriE* probes (Figure 5(c)) was less than 27 kb, until 12 min post infection. After this time, most of the DNA detected with both probes was greater than 27 kb.

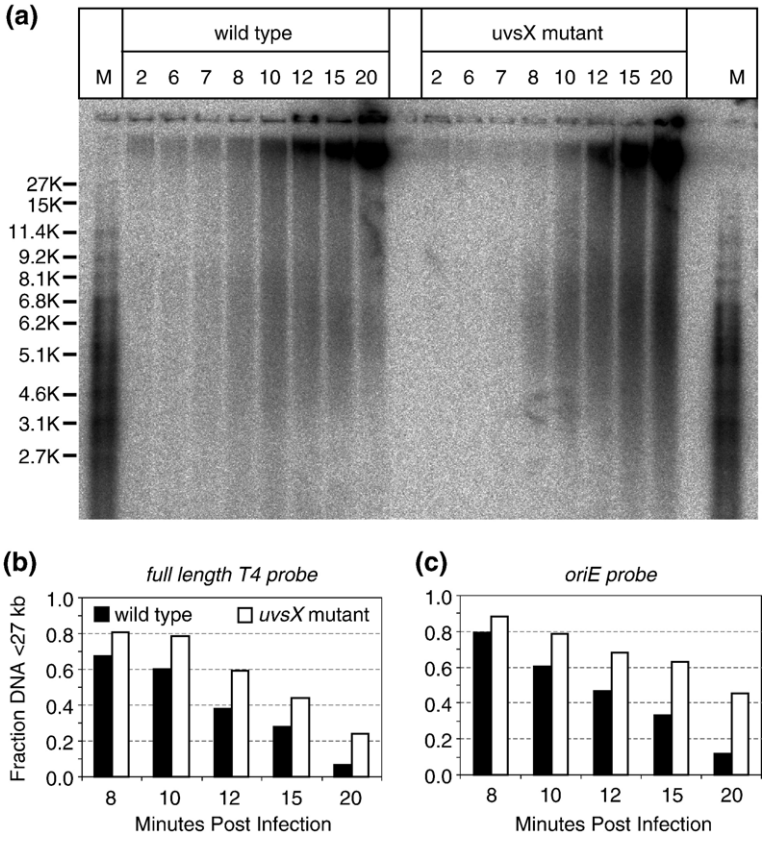

Figure 5. Size distribution of nascent T4 DNA synthesized early during infection. (a) Wild-type (wt) T4D and the recombination mutant *uvsX* were used to infect *E. coli* BL21 (DE3) cells at a multiplicity of 0.5 virus per cell. Total viral DNA, harvested from wt and *uvsX* infections at 2 min, 6 min, 8 min, 10 min, 12 min, 15 min, and 20 min post infection, was size-fractionated on an alkaline agarose gel, transferred to nylon and probed with full-length T4 DNA as described in Materials and Methods. Numbers above lanes indicate time (min) post infection and M identifies the PacI-digested viral DNA molecular mass standard. (b) The blot in (a) was exposed to a phosphorimager screen and scanned. To determine the fraction of DNA shorter than 27 kb, the PSL (detected radiation) in a given lane associated with 2.7–27 kb DNA was divided by the PSL present in the total DNA in the same lane, from 2.7 kb to the top of the well, as described in Materials and Methods. Results from wt infections are shown with filled bars and *uvsX* infections with open bars. (c) The blot in (a) was stripped and reprobed with a PCR fragment that overlaps the putative position of *oriE*, LP16 from Table 1. The fraction of DNA less than 27 kb was then calculated as in (b).

It is worth noting that the smaller DNAs observed early during the wt infection do not simply disappear, as would be expected if a certain number of nascent polymers were made initially, then elongated over time. Rather, a certain abundance of short DNAs is apparent throughout the first 20 min of wt infection, suggesting that they are produced at a more or less constant rate during this time. This does not occur in *uvsX* mutant infections, where the abundance of short DNAs increases throughout the time studied (Figure 5(a)), as if short polymers were still being made, but not elongated efficiently, culminating in a larger percentage of polymers less than 27 kb, compared to wt. This observation is consistent with the result of a previous study,[33] and indicates that *uvsX* influences the normal size distribution of nascent DNA.

Discussion

The chromosome is the fundamental unit of heredity, a dynamic macromolecular structure that serves as a template for transcription of genes, replication of genetic material, and the various DNA transactions required for genome maintenance. As a first step in characterizing the interplay between these activities, we have developed a unique set of experimental strategies that allowed us to measure the actual accumulation of nascent DNA, across the T4 genome, over the course of infection. Our data indicate that early T4 DNA synthesis is discontinuous, with several origins contributing to a punctate replication pattern, comprised of multiple, short, origin-derived polymers, an observation that raises a number of questions regarding the regulation of T4 DNA replication.

Transition from origin-mediated to recombination-mediated replication

The maintenance of genome integrity during multiple replication cycles is a key priority in the evolution of chromosomes. Lacking defined telomeres, the linear T4 chromosome has evolved a unique replication strategy that includes both origin-mediated and recombination-mediated DNA synthesis. This bipartite strategy ensures the production of full-length genomes and allows the virus to rapidly synthesize several hundred progeny genomes during the short time-course of a single infection. Although the individual components of this replication strategy have been studied *in vitro*, little is known about the interplay between these two modes of replication and the dynamics of resultant DNA synthesis during actual infection.

The switch between these two modes of replication was thought to depend on the synthesis of proteins made at later times.[15,16] However, we found that the interval before the onset of recombination-mediated T4 replication is not a fixed duration. Rather, it varies with the number of viruses per infected cell. In singly infected cells, the *recA* homologue *uvsX* does not contribute to the total amount of viral DNA produced during the first 15 min of infection. In contrast, when multiple viruses infect the same cell, under otherwise identical conditions, the transition to recombination-mediated replication occurs much earlier, and both *uvsX* and gene59 are required for viral DNA synthesis as early as 7 min post infection.

It is not clear how increasing numbers of infecting viruses influence the T4 replication strategy such that recombination is favored, but a scarcity of required host factors such as RNA polymerase or altered expression of specific viral genes could cause an imbalance in protein levels, creating an environment wherein origin activation proteins are limited or that recombination proteins are overly abundant. Of course, increasing the number of viral copies may also increase the substrates available for recombination and, thus, may in itself be a factor in the switch to recombination-mediated replication.

It is also not clear why replication slows earlier in the gene *59* mutant compared to the *uvsX* mutant in singly infected cells. Taken at face value, this observation implies that gene *59* is required before the onset of recombination-mediated replication. However, it is not likely that the 59 protein is absolutely required during the initial steps of origin- mediated replication, since one would expect no DNA synthesis in gene*59* mutants if this was the case. Rather, our observations are consistent with secondary role for protein 59 during origin-mediated replication, as asserted in a previous study, where mutation of gene *59* had no effect on initial leading strand synthesis from *oriF* but disrupted subsequent retrograde origin-mediated synthesis.[14] Such a role at the T4 origins would presumably cause DNA synthesis to arrest earlier in gene *59* mutants than in *uvsX* mutants.

Multiple origins of T4 DNA replication

There are clearly multiple origins of T4 DNA replication, including a major origin near *oriE* and at least four secondary origins located near *oriA*, *oriC*, *oriF*, and *oriG*. Unlike previous studies,[22,24,25] we observed synthesis near these origins concomitantly, within a single population of infected cells, raising the possibility that some or all of these origins are active simultaneously within a single viral genome. However, this issue is still unresolved, and it is entirely possible that micro-environmental differences between infected cells influence origin activity such that particular origins are used discretely within individual infected cells.

There is some reason to suspect that environmental factors may influence origin usage, as the characterized T4 origins do not appear to be mechanistically similar. Of the five, the best characterized is *oriF*. This origin, which was recovered initially by Kreuzer and Alberts, is able to support replication of episomal plasmids during T4 infection.[18,27] Normal origin activity from this locus is dependent on several *cis* elements, including a T4

middle mode promoter,[34,35] a downstream unwinding element or DUE,[31] and a sequence element thought to form a hairpin structure.[35] The current model predicts that the T4 middle mode *uvsY* promoter upstream from *oriF* produces an RNA, which is then used as primer for DNA synthesis by the T4 replisome. Both *oriA* and *oriG* are located near middle mode promoters, and each is thought to operate in a manner similar to that of *oriF*.[21]

The region near *oriE* appears to be quite different from that near *oriF*. There is no middle mode promoter in the region, which led Gisela Mosig's group to suggest that DNA replication is primed by an RNA produced from an early promoter downstream from six copies of a directly repeated sequence element:[32]

$$5'-AT(T/C)(T/A)CC(A/T)T(T/C)(A/G)AC-3'$$

These so called iterons are each separated by 12 bp of non-repeated sequence and are maintained in the anti-sense direction of a large open reading frame encoding the gene *5* base-plate lysozyme, a protein necessary for viral entry into host cells. Similar repeats are found in the syntenic gene *5* region regions of other T4-like bacteriophages,[36] but it is not clear if these iterons are linked functionally either to gene *5* or to origin activity from *oriE*.

Thus, T4 may have mechanistically distinct classes of origins, and the difference between these classes could contribute to their functional usage during infection. This would make sense on at least two levels. First, as genomes evolve, one would expect them to encounter multiple selective pressures over time, which may lead to the maintenance of several types of origins, allowing the organism to reproduce under a variety of environmental conditions and, hence, increasing fitness. Second, the individual origins may fulfil specific functions during the replication of a single viral chromosome. Yet, it remains entirely unclear how T4 origin activity is regulated, and how several origin loci, with distinct genetic elements, function during the course of infection.

Punctate patterns of T4 DNA synthesis

Early during infection, short, nascent DNAs accumulate near the T4 origins, suggesting that these loci function as amplicons, producing a number of subgenomic length DNAs that can be used subsequently during recombination-mediated synthesis (Figures 3 and 4). However, the physical state of these origin-derived DNAs is not clear. Perhaps, initially, each nascent DNA is displaced from the origin and later recombines with the parental template where it is used to prime subsequent DNA synthesis. This might explain why the proportion of short nascent DNA remained high in the absence of the RecA homologue uvsX, a protein involved in both recombination and the restart of stalled replication forks.[37] Alternatively, the nascent polymers may remain associated with the template, creating an "onion skin" structure wherein the multiple nascent strands are held in place inside the parental duplex. These structures have been seen before in electron micrographs of replicating T4 DNA,[38] though not the five to ten layers implied by the data presented here.

It is not clear why replication forks do not progress efficiently into the regions adjacent to the T4 origins early during infection. Fork progression is regulated by both active and passive mechanisms in other systems. Terminator sequences impede *E. coli* DNA synthesis in a defined polarity through a mechanism mediated by *tus* proteins,[1,39] but there is reason to suspect that other mechanisms contribute to discontinuous DNA synthesis observed throughout the *E coli* genome.[3,4] Both replication pause sites and initiation rates influence DNA synthesis patterns in Epstein-Barr virus.[40] In eukaryotic cells, DNA synthesis rates differ from one genomic region to another,[41] and several factors, including local and global transcription patterns, appear to influence replication dynamics.[5,42]

No replication terminator has been identified in T4 and, although it has been suggested that transcription may impede T4 DNA synthesis,[43] the relationship between viral transcription and replication is not established. There has been a genomic study of T4 transcription during infection using a micro array but, unfortunately, the conditions used in this study were different than ours, preventing any direct comparisons of replication and transcription timing.[44] Yet, it is worth noting that all of the origins of replication identified in Figure 4 are near T4 genes expressed during late transcription (see Figure 1), suggesting that further investigation into the interplay between T4 transcription and replication is warranted.

Certainly, many aspects of the T4 replication dynamics observed in this study await further experimental exploration. Yet, the assays developed here should allow us to determine directly the genetic factors required for the normal pattern of DNA synthesis, and further development of these assays should provide the experimental tools necessary to define the mechanisms of origin function. The amenability of T4 to both the genomic investigations as demonstrated here and biochemical characterization demonstrated by a number of groups should provide a unique system where genomic phenomena observed *in vivo* can be correlated quickly with biochemical mechanisms defined *in vitro*. Ultimately, such studies should shed light on the dynamics and underlying mechanics of genomic transactions, and how these contribute to the evolution of chromosomes.

Materials and Methods

Strains

E. coli BL21(DE3) and SCS110 were obtained from Stratagene. *E. coli* B and CR63, as well as wt T4D, have

been maintained in this laboratory. The T4 recombination mutant T4*uvsXam11* was a gift from John W. Drake.[45]

Growth of bacteria and phages

All bacteria were grown in LB 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–HCl (pH7.4), 150 mM NaCl, 0.03% (w/v) gelatin.

Construction of T4 phage harboring a deletion of gene *59*

The T4 gene *59* deletion was made by site-directed mutagenesis of the plasmid pNN2002,[46] which contains wt gene *59* and flanking T4 DNA. The method of Kunkel *et al*.[47] was modified to include the use of T4 DNA polymerase, T4 *44/62* clamp loader, and *45* clamp to copy the ssDNA template as described.[48] Two primers, 59del1 (5′-GCATGCGGAGTTTGATCATAGTAGAAAATC-3′) and 59del2 (5′-CAATACTTGCAAGTGATCACAGTTTCA-ATG-3′), were used to introduce BclI restriction sites into pNN2002 at the termini of the gene *59* open reading frame. Mutated plasmids grown in the dam methylation-deficient host *E. coli* SCS110, were digested with BclI. The larger fragment was self-ligated to give a circular plasmid, pJRB1113, in which all but six codons of the gene *59* open reading frame had been deleted. The gene *59* deletion thus contains a very small open reading frame that codes for the amino acid sequence MITCKY.

The gene *59* deletion was introduced into the T4D viral background by homologous recombination as described.[49] Briefly, *E. coli* B (pJRB1113) was grown in LB supplemented with 50 μg/ml of carbencillin to an A_{600} of 0.28 and infected with T4D at an MOI of five viruses per cell. Infected cultures were incubated at 37 °C with vigorous shaking for 2 h, and then lysed with chloroform. Resultant phage stocks were titered and plated on *E. coli* BL21(DE3) cells harboring the gene *59* expression plasmid pNN2859.[46] Plaques were lifted onto nitrocellulose, and probed with an oligonucleotide that spans the gene *59* deletion, 59del3 (5′-TAGATTTTCTACTATGATCAC-TTG-3′), in 6× SSPE (3M NaCl, 0.2 M NaH_2PO_4, 0.05 M EDTA), 10% (w/v) dextran, 1% (w/v) SDS at 50 °C. Filters were washed twice in 2×SSPE (SSPE is 3M NaCl, 0.2 M NaH_2PO_4, 0.05 M EDTA, pH 7.4), 1% SDS at 50 °C. There was no hybridization of the gene *59* deletion probe with wt T4D plaques under these conditions.

Six plaques harboring gene *59* deleted phage were identified and isolated in this first screen. These plaques were resuspended in LB, titered, and plated again on *E. coli* BL21(DE3) cells harboring pNN2859. A secondary plaque hybridization screen was then done using the gene *59* deletion oligonucleotide as above, and several plaques were isolated. A stock designated T4gene*59*Δ was expanded from one of these plaques. The mutant phage yields pin-point plaques on *E. coli* BL21(DE3) cells and normal-sized plaques when complimented with pNN2859.

Isolation of T4 DNA from infected cells

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 and mutant phage. After addition of phage to bacterial cultures at 37 °C and through mixing for 1 min, phages were allowed to absorb for an additional 45 s without mixing. Samples were then mixed again for 15 s, then mixing was stopped and 2 min samples were withdrawn. Mixing was again started and vigorous mixing was then maintained, except for brief stops to withdraw samples. Aliquots of infected cultures were withdrawn and immediately added to phenol/chloroform (1:1, v/v). Tubes were inverted five to seven times to completely lyse the cells. After all aliquots had been withdrawn, all phenol/chloroform extractions were inverted another ten to14 times and centrifuged to separate the aqueous and organic phases. The recovered aqueous phase was extracted with one 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 dot blot assay. Recovered aqueous phase aliquots from the phenol/chloroform extractions detailed 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, 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 4–6 h. This buffer was 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 made using the Prime-It II random primer labeling kit (Stratagene), cleaned using ProbeQuant G50 spin columns (GE Healthcare), and denatured at 95 °C for 10–15 min before hybidization with blots at 62 °C for 12–16 h. Approximately 5×10^5 cpm of probe per 1 ml of hybridization solution was used.

After hybridization, blots were washed three times in 2×SSPE (pH7.4), 0.1% SDS at room temperature for 30 min each, and once in 2×SSPE (pH7.4), 0.1% SDS at 62 °C for 30 min. These hybridization and wash conditions were empirically determined to prevent cross-hybridization of T4 DNA with *E. coli* DNA as judged by dot blotting. Washed blots were exposed to a phosphorimager screen and scanned by a FUJIFILM FLA-3000 phosphorimager. Data were analyzed using FUJIFILM Image Guage V3.12 software.

The amount of DNA present at a given time-point was calculated as a fold increase over the amount in the same infection at the 2 min time-point, before the onset of viral replication. In infections done at an MOI of 0.5, each infected cell contains, on average, a single infecting virus, and the amount of DNA at 2 min post infection is equivalent to one T4 genome per infected cell. Thus, the fold increase in DNA over that at 2 min is equal to the average number of genomes per infected cell. In contrast, at an MOI of 5, on average each cell is infected with five genomes. The fold increase in DNA is not over a single genome, but five genomes, and the fold increase must be

multiplied by 5 to determine the average number of genomes per infected cell.

Amplification of the T4 macro array

The PCR fragments that comprise the T4 macro array were amplified from full-length T4 DNA isolated from purified virions using the Expand Long Template PCR kit (Roche) with buffer 2, as directed by the manufacturer, using 150 ng of T4 DNA in each 50 μl reaction. The PCR primers are listed in Table 1 and were designed such that all hybridized to the T4 genomic template at the same temperature, allowing all the reactions to be run concurrently in the same thermal cycler. The thermal cycling was done in a GeneAmp PCR system 9700 (Applied Biosystems) under the following conditions: ten cycles of 92 °C for 2 min, 55 °C for 30 s, and 68 °C for 4 min followed by 25

Table 1. Oligonucleotides used to construct the T4 macro array

PCR locus	Map position	Oligonucleotide sequences
LP1	168102	5′-TTACAACTCGGCTGGTTGAACTACC-3′
	1413	5′-ACCGTAACTGGCTAAGCATTCGC-3′
LP2	4033	5′-TACCACGCAATGGATAACCACCG-3′
	6512	5′-GGTGTTGATACTCTTCTGAAACGTATCGC-3′
LP3	9559	5′-GCGCTTTATGGAATGTTGATGCAGG-3′
	11721	5′-CGTTTAGAGCATACTCAGGCATCTGC-3′
LP4	14745	5′-AAGAACTTCCACATGAATCACCTGCC-3′
	16978	5′-GGGCAGTCGAATGTTTTAGAACACCAC-3′
LP5	19392	5′-CACCGACATACTCTGTGCTGCG-3′
	21773	5′-TCTCCGGAAGACCACAGCTGG-3′
LP6	24549	5′-CTCATTGACTCTATCAATGAGTTCAGCACG-3′
	27103	5′-AGATGATTCTGGACCCTTTGGATTCC-3′
LP7	29048	5′-GCTGCTAAATTTGAAGGTGAACACGC-3′
	31963	5′-CAACTGATTCCAAAGAGAATGACGGC-3′
LP8	34525	5′-GCAGGTCTTCCACAAGCTTTCTTCG-3′
	37124	5′-TGGTAAATCCACTGTAATGGAAGGTCTGG-3′
LP9	39786	5′-CACGGCTGTCATAGACATTGTGAACG-3′
	42055	5′-CCAGATGAAGTAGGTCGTTGTCCTGG-3′
LP10	44749	5′-AAGCTAATCACCTCGACCATGACC-3′
	46729	5′-TCATTCGTTCAGTCCAGTCAGTTCC-3′
LP11	49197	5′-TCGTATTATCATCAAAGGATTCAACTACGTCC-3′
	52446	5′-GGTGCTCGATTGGCTAAAGGTCTAG-3′
LP12	55230	5′-TCTCCTTGTGCATCTACCGGAGC-3′
	57809	5′-TGTAATGATGAGCGAGGCGAAACG-3′
LP13	60217	5′-GAGAAACGACTTCACAGACAGAATCGC-3′
	62007	5′-GCATCACGCCTACGGAATGTTCC-3′
LP14	65319	5′-CGATATTCAGCCATTAAGTGTTGGTCAGC-3′
	67876	5′-TGATAGAGTTGATTGGAATGGTTGTTCCG-3′
LP15	71150	5′-GAACCATCAGTCCGACGACTTACC-3′
	74155	5′-CAAGATGGATCGACTCTTGTACTTGTGC-3′
LP16	76349	5′-CTGACGAGCTTTAGGTGGAATATAGTGC-3′
	79504	5′-TTCCTTCAACTAAAGTGGTAGCATCTCC-3′
LP17	82520	5′-GATCATTCTGTCATTGGTTCATCAGCG-3′
	85510	5′-ACTTCTTGACCAGCAATTAATCGTCCC-3′
LP18	88424	5′-CCAGTTACACTAGTAGCTGCTTCCG-3′
	91406	5′-TGACCACCTCTTTGCTGGATAACG-3′
LP19	94422	5′-CCATTCCTCAACCATTTCACGAGTCC-3′
	96976	5′-CGGTCAAATTTTAGCACGCCACC-3′
LP20	100895	5′-TGCTCGTCAGAAGTTCGTTGATTGG-3′
	103871	5′-TTGTGTCAGTCAATGAACCTAATCCACG-3′
LP21	107170	5′-TCGTCAAGAGTAGCATGAGCTCCG-3′
	110021	5′-TCTGGAAAGTCATTCATTGAAATTGCTCG-3′
LP22	112743	5′-CATCGATTGTGAAGAGAGCACGTTCC-3′
	115578	5′-CTTACTGCTGACACGGTTGAACGC-3′
LP23	119325	5′-GTTGAGGATGATACTGCTGAATCTGTGG-3′
	122020	5′-GAACTGTACGTTGACTCATGGCACC-3′
LP24	125094	5′-TAAGAAGGACGTATCTACCACCTGAACG-3′
	128001	5′-TGTTACAGCATTGGGTGATTCTATTGACG-3′
LP25	131764	5′-GGCATAAAGTCTGCATCACGAGTACC-3′
	134628	5′-TAACCTGAATCCTGAACGTCGCC-3′
LP26	138347	5′-CCATCAGTACCGGATTGAAGTTGACG-3′
	141120	5′-GTTACCAACTATGCAGCTTGGCTGG-3′
LP27	144009	5′-ACTTATGGGTTTCTGGATTCCAACGC-3′
	146978	5′-GGTAGTTCATCTAGTGCTGATGACACG-3′
LP28	150898	5′-ATCAACGTAGCTTTAGCTGATCGTACC-3′
	153249	5′-AGTAGGTTCACTCTGCGCAATCC-3′
LP29	156094	5′-TGGCATTCAAGTCCCATATGCTCC-3′
	159006	5′-AATCCAGCAGGAACTGAATCACTCG-3′
LP30	162235	5′-CCACGTTCAATACTTACTTGTCCAGCG-3′
	165005	5′-CACAAACTCGCTTCTATTGCAGGTGG-3′

cycles of 92 °C, 2 min, 55 °C for 30 s, and 68 °C for 4 min plus an additional 20 s each additional cycle. Under these conditions, each primer pair produced a single band when visualized on EtBr-stained agarose gels.

The blotting of PCR products to Hybond-XL membranes was done essentially as described above. A total of 50 ng of each PCR product was blotted in 0.5 M NaOH, 1.5 M NaCl. Blots were hybridized to probes generated from T4 DNA harvested from infections as described above. A control probe generated from full-length T4 DNA isolated from purified virions was also used in duplicate. The blots were washed and scanned as described above. The relative abundance of DNA at a particular locus was then calculated.[24,25] First, the percentage abundance of a given locus in each DNA sample was determined by dividing the number of phosphorimager units (PSLs) associated with an individual PCR fragment probed with that DNA by the sum of PSLs at all PCR fragments in the array when probed with the same DNA. Second, the percentage abundance of each experimental datum point was standardized by dividing this quotient by the percentage abundance of the same PCR locus, when probed by control DNA purified from T4 virions in parallel hybridizations.

Size-selection of nascent T4 DNA

DNA recovered from infections as described above was precipitated with the addition of isopropanol. This DNA was resuspended in 10 mM Tris–HCl (pH 7.9), 10 mM $MgCl_2$, 50 mM NaCl, 1 mM EDTA and digested for 1 h at 37 °C with HaeIII, to fragment bacterial DNA, and RNase If (New England Biolabs). DNA was then precipitated in ethanol at room temperature for 15 min and loaded onto an alkaline 1% (w/v) agarose gel in 50 mM NaOH, 1 mM EDTA. A New England Biolabs 1 kb ladder was loaded as a size marker. The DNA was resolved at 25 V (160mA) for 16 h, and the gel was neutralized and stained in TBE (0.4 μg/ml of ETBr) for 1 h. DNA was visualized with UV light, excised from the gel, and purified using the Qiagen Qiaex II kit.

T4 DNA replication dynamics

T4 DNA synthesis *in vivo* at the loci along the T4 macro array was monitored by a dot blot assay. Recovered aliquots of DNA from infections were processed and blotted to nylon membranes as described above. These blots were hybridized with random primed probes generated from each of the 30 PCR fragments that comprise the T4 genomic macro array. The amount of DNA present at a given locus at a given time-point was calculated as a fold increase over the amount at the same locus in the same infection at the 2 min time-point.

Southern blots of DNA from infected cells

DNA for Southern blotting was prepared from infections as described above. HaeIII, RNase If (New England Biolabs) digested DNA samples were then resolved on an alkaline 0.7% agarose gel in 50 mM NaOH, 1 mM EDTA, at 30 V (200 mA) for 21 h. A lane containing PacI-digested T4 DNA was included as a size standard. The gel was equilibrated in 0.5 M NaOH, 1.5 M NaCl for 1 h, and blotted to Hybond-N+ (Amersham) in the same buffer overnight by capillary action. Southern blots were probed as described above and stripped according to manufacturer's instructions.

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