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STUDIES ON THE T4 BACTERIOPHAGE DNA REPLICATION SYSTEM

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ABSTRACT The study of DNA replication has been greatly facilitated by the development of in vitro systems that render the process much more amenable to study than it is within the living cell. The bacteriophage T4 in vitro system has proven particularly valuable for understanding the detailed events that take place at the moving replication fork. The current model of the T4 replication complex envisions a sophisticated assemblage of proteins, in which leading and lagging strand DNA syntheses are coupled by means of a true "replication machine". In this article, we address the following questions with regard to this replication machine: What determines the length of the Okazaki fragments and what are the template sequences required to begin their synthesis? Which protein is the actual RNA primase that is required for Okazaki fragment synthesis? Does the helix-destabilizing protein - like the primosome and the two DNA polymerase molecules - recycle within the replication complex? What is the architecture of the DNA polymerase holoenzyme on a primer-template junction and why is this enzyme so complicated? Finally, what additional proteins may be required to reconstitute the origin-specific initiation of T4 DNA replication forks in vitro?

INTRODUCTION

Approximately twenty genes have been shown by genetic analysis to be involved in T4 DNA replication (1). Two laboratories have been using this genetic analysis as a tool for reconstructing the biochemistry of the T4 DNA replication

process in vitro (2). Eleven gene products that are believed to be directly involved in the formation and propagation of DNA replication forks have thus far been isolated and purified to near homogeneity in our laboratory and are listed in Table 1 (3-7). The products of genes 43, 44/62, and 45 comprise the T4 DNA polymerase holoenzyme. By itself, the T4 gene 43 protein (DNA polymerase) is capable of elongating pre-existing primers on single-stranded DNA templates (8), while its 3'→5' exonuclease activity provides a proofreading function (9). The products of genes 44/62 and 45 comprise the polymerase accessory proteins and display a DNA-dependent ATPase activity (10,11). On a primed single-stranded DNA template, the polymerase accessory proteins interact with the DNA polymerase in a reaction that requires ATP hydrolysis by the 44/62 complex. This interaction can result in a dramatic increase in both the rate and processivity of DNA synthesis by the polymerase molecule (12-15). The activity of the accessory proteins is most consistent with their formation of a complex with the polymerase, which acts as a "sliding clamp" that keeps each polymerase molecule at the 3' end of a growing DNA chain for many cycles of synthesis (15,16).

The gene 32 protein is a helix destabilizing protein (or single-stranded DNA binding protein), and it is required to allow the polymerase holoenzyme to synthesize DNA on a double-stranded DNA template (17,18). This protein binds

Table 1. Properties of Bacteriophage T4 Replication Proteins

Type of protein	T4 gene	Molecular weight ($\times 10^{-3}$)	Current purity (%)	Activities[†]
DNA polymerase	*43*	103.5	99	5′→3′ polymerase; 3′→5′ exonuclease
Polymerase accessory proteins	*44/62;45*	35.7/21.4;24.7	99;99	SS DNA-termini-dependent ATPase, dATPase
Helix-destabilizing protein	*32*	34.5	99	cooperative binding to SS DNA
RNA-priming proteins	*41*	53.8	99	long SS DNA-dependent GTPase, ATPase
	61	39.5	99	binds DNA
Type-II topoisomerase	*39/52/60*	57.2/50.6/17	99	DS DNA strand passage; DS DNA-dependent ATPase
DNA helicase	*dda*	47	95	SS DNA-dependent ATPase; DNA unwinding

[†]SS indicates single-stranded; DS indicates double-stranded.

cooperatively to the single-stranded regions of the DNA template, eliminating short stretches of secondary structure, while leaving the bases accessible to the polymerase (19,20). The 32 protein also stimulates synthesis by the polymerase on a primed single-stranded template, and it seems to exert part of its effect through a direct interaction with the T4 DNA polymerase (21,22).

The addition of gene 41 protein to the above five-protein "core replication system" makes possible in vivo rates of fork movement at physiological concentrations of 32 protein (16). The 41 protein utilizes the energy of GTP or ATP hydrolysis to unwind the DNA helix ahead of the advancing replication complex (16,23); by itself, it has been shown to function as a DNA helicase whose direction of movement along the DNA is consistent with its association with the template on the lagging strand (24). In addition to its helicase activity, the 41 protein interacts with the gene 61 protein to form the T4 "primosome". Together, these two proteins synthesize the RNA pentamers that prime Okazaki fragment synthesis on the lagging strand (25-27). While the 61 protein alone can act as an RNA primase in vitro (28,29), our data shows that the 41/61 complex is required to produce the RNA primers that are utilized within the cell (see below).

The mixture of the seven T4 replication proteins just described is sufficient to move a DNA replication fork through a naked double-stranded DNA template in a manner that - with regard to protein requirements, fidelity, rate of fork migration, size of Okazaki fragments and processivity - closely approximates the fork movement process in the cell (2,30). However, the DNA template in vivo is not a naked DNA molecule, but rather one that is covered with a variety of other DNA binding proteins - including RNA polymerase. In vitro DNA replication on such templates requires an eighth T4 protein, the product of the dda gene (31,32). The dda protein, like the 41 protein, is a DNA helicase that utilizes the energy of nucleotide hydrolysis to unwind the helix in front of the growing fork (33,34). Unlike the 41 protein, however, the dda protein possesses the ability to push the fork past bound RNA polymerase molecules, whether the replication complex is colliding head-on with or traveling in the same direction as the transcription apparatus (32). This "snowplow" effect of the dda helicase seems to be a general one, since it has recently been shown to be required for the rapid passage of the replication complex through a lac operator DNA sequence bound by the lac repressor protein (J. Barry, unpublished observations).

The products of genes 39, 52, and 60 encode the three subunits of the T4 type II DNA topoisomerase (6,35). Mutations in these genes seem to affect the initiation of DNA replication rather than the rate of fork movement (36). However, the precise role of this topoisomerase in replication is as yet unclear.

RESULTS

Template Restrictions on RNA-Primed DNA Chain Starts.

Based upon our previous results, we proposed a model for replication fork movement in which lagging strand DNA synthesis is coupled to that on the leading strand (30). The mechanism permits efficient replication of the lagging strand by preventing the dissociation of its polymerase molecule from the replication complex, which allows this lagging strand DNA polymerase to be recycled after the completion of each successive Okazaki fragment. Implicit in this "trombone model" of DNA synthesis is the notion that the selection of successive RNA primer sites for Okazaki fragment synthesis is triggered by the completion of the synthesis of the previous Okazaki fragment on an individual lagging strand. However, as suggested by the sequence analysis of the RNA primers themselves (25-27), primer site selection will also be constrained by the template DNA sequence. In order to determine the full extent of this constraint, we have performed an analysis of the template sequence requirements for primer synthesis on a simple single-stranded DNA template, using an approach similar to that previously employed in the T7 bacteriophage system (37). In Figure 1 we present a representative selection from a compilation of thirty-two different RNA primer sites that have been mapped on an M13 single-stranded template. In these experiments, both the 41/61 primosome and the DNA polymerase holoenzyme were present, and the "primers" shown are all oligoribonucleotides that were found to prime DNA synthesis (29).

The earlier direct sequencing of the pentaribonucleotides synthesized by the 41/61 primosome in vitro revealed primers with the sequences pppApCpNpNpN, in addition to a set of sequences starting with pppG that were less well characterized (25-27). Figure 1 shows that template DNA sequences that are complementary to the first two residues of pppApC- and pppGpC-start primers are recognized by the primosome, as expected. In addition, the data reveals that there is a

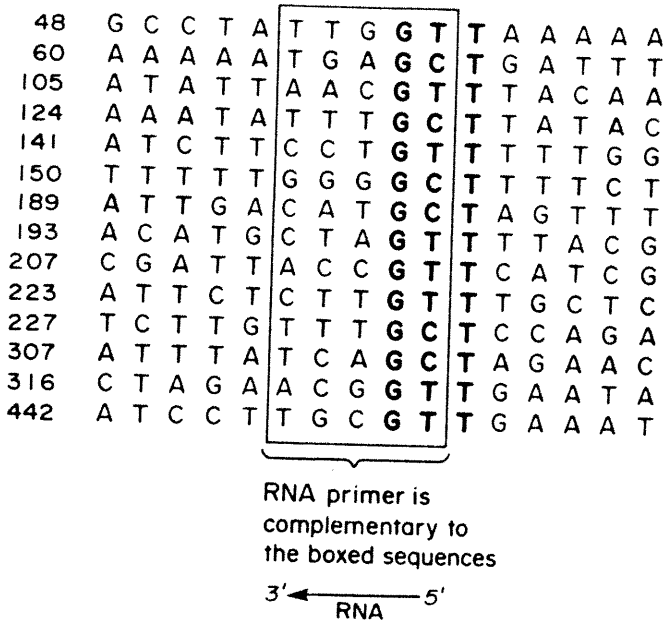


FIGURE 1. The template sequence requirements for RNA primer synthesis. DNA sequences corresponding to several different RNA primer sites which have been mapped on a single-stranded M13 DNA template are presented. The numbers refer to the distance from the primer start site to the unique AvaI site on the M13 bacteriophage chromosome.

conserved T residue at the -1 position, analogous to the conserved C residue at the -1 position of T7 bacteriophage primer start sites (37). A more detailed analysis shows that the trinucleotide sequences GTT or GCT are both necessary and sufficient to specify the efficient synthesis of RNA primers by the 41/61 primosome complex (29).

In order to determine the minimum length required for a functional primer, the primer activities of truncated oligoribonucleotides - resulting from the omission of each one of the four rNTPs from the reaction mix - were determined. It was thereby found that trinucleotides are completely inactive, while tetranucleotides can prime synthesis as efficiently as the full-length pentaribonucleotides (29).

Under conditions designed to mimic in vivo replication as closely as possible, there is an absolute requirement for both 41 and 61 proteins to observe primer synthesis in vitro. However, when the concentration of 61 protein is increased

from 1.3 µg/ml to 37 µg/ml, RNA-primed DNA chain starts can be observed at specific sites in the absence of 41 protein. This is in agreement with the findings of Hinton and Nossal that 61 protein alone is capable of synthesizing short oligoribonucleotides in vitro when the concentration of the protein is raised to high levels (ref. 28; see also this Symposium). However, a template primer site analysis, similar to that performed for the 41/61 primosome complex, reveals that 61 protein alone utilizes only the GCT subset of primer sites. The specific rNTP requirements for 61 protein-primed DNA synthesis confirm this finding (29).

Our previous results demonstrate that the GC-start primers are not synthesized in vitro by the 41/61 primosome when single-stranded T4 DNA is used as the template. We interpret those results as showing that the hydroxymethylation of the cytosine residues in natural T4 DNA interferes with the recognition of the GCT sites by the primosome (27) and conclude that the only primer sites that are expected to be utilized within the T4-infected cell are those which 61 protein alone cannot recognize - the GTT sites. Hence, while the 61 primase alone is capable of synthesizing some RNA primers in vitro, only the 41/61 primosome complex is presumed to function in the normal T4-infected cell.

The Initiation of Okazaki Fragment Synthesis is Determined by a Timing Mechanism.

Because the average size of an Okazaki fragment is about 1200 nucleotides, both in vivo and in vitro, only a minority of the potential primer sites in the T4 chromosome can be utilized in any one pass of a replication fork. For example, in the sequenced region of the T4 chromosome depicted in Figure 2, potential GTT primer sites are randomly distributed with an average spacing of 50 to 60 nucleotides. Thus, on average only about one in twenty-five of these potential sites are utilized. How is this subset of sites selected? One possibility is that a primer is synthesized each time the replication fork passes a GTT site, but that some other mechanism is responsible for selecting a subset of these primers for the actual start of a DNA chain. However, this hypothesis is inconsistent with our earlier finding that more than 90% of all of the primers synthesized in the in vitro system are actually used (26). It is more reasonable to suppose that there is either a timing or a measuring mechanism for determining when enough single-stranded DNA has been

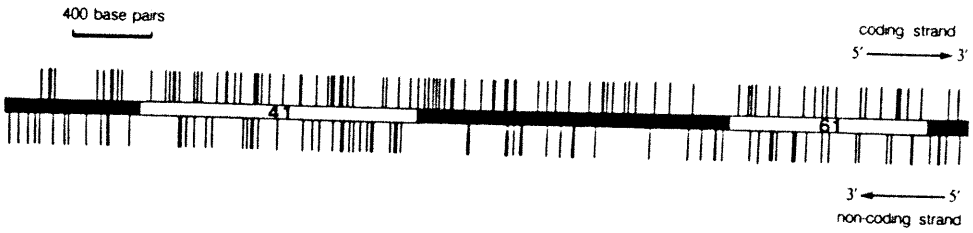


FIGURE 2. The distribution of RNA primer start sites within a sequenced 5 kb region of the T4 genome. The positions of the sequence GTT in the vicinity of genes 41 and 61 on the T4 chromosome are presented. These sites correspond to all of the potential primer sites and do not necessarily reflect those sites that are actually utilized in vivo.

exposed on the lagging strand to trigger RNA primer synthesis (2,30). In any trombone type of model, the lagging strand DNA polymerase cannot initiate synthesis of a new Okazaki fragment until it completes the synthesis of the previous one. Moreover, the very efficient utilization of RNA primers which we have observed suggests that the release of the lagging strand template by its polymerase is the signal that activates the primosome to synthesize a new primer at the next available GTT site. Using this primer, the lagging strand polymerase then restarts another cycle of Okazaki fragment synthesis.

If the rates of polymerization on the leading and lagging strands were identical, the length of each successive Okazaki fragment on any particular template molecule would be set equal to the length of the previously-synthesized fragment (30). In this view, the polydisperse size range of Okazaki fragments observed (see Figure 3B, below) would reflect a corresponding difference in the size of the first Okazaki fragment synthesized on each template DNA molecule in vitro. An attractive aspect of this proposal is that the total amount of single-stranded DNA on the lagging strand would remain constant, so that it would be possible for the many molecules of 32 protein at the fork to be recycled within the replication complex, as observed for the lagging strand DNA polymerase molecule (30).

We have tested whether such a templating mechanism determines Okazaki fragment size in the experiment schematized in Figure 3A. By severely limiting the rNTPs required for lagging strand primer synthesis in the in vitro system,

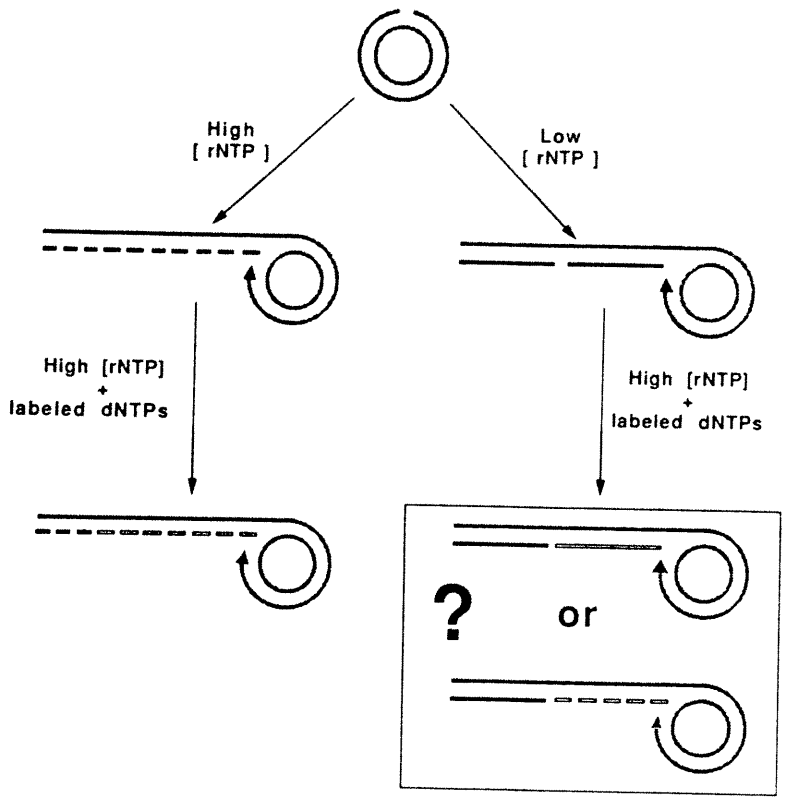


FIGURE 3A. Is Okazaki fragment length templated at a replication fork? Two possible outcomes are presented of an experiment designed to determine whether the size of an Okazaki fragment is dependent upon the size of the Okazaki fragment that was previously synthesized at a fork. Synthesis on a nicked double-stranded DNA template in the presence of non-limiting ("high") concentrations of each of the ribonucleoside triphosphates (rNTPs) yields Okazaki fragments with an average length of 1.2 kb, whereas synthesis in the presence of limiting ("low") rNTPs produces abnormally long Okazaki fragments. In this experiment, once the long pattern of Okazaki fragment synthesis was established, the concentration of rNTPs was restored to the normal "high" levels, and labeled dNTPs were added to allow the size of the newly-synthesized fragments to be measured by alkaline agarose gel electrophoresis followed by autoradiography. The continued synthesis of the long fragments would be predicted by a templating mechanism, while a rapid conversion to short fragments would be suggestive of some other mechanism for determining Okazaki fragment size.

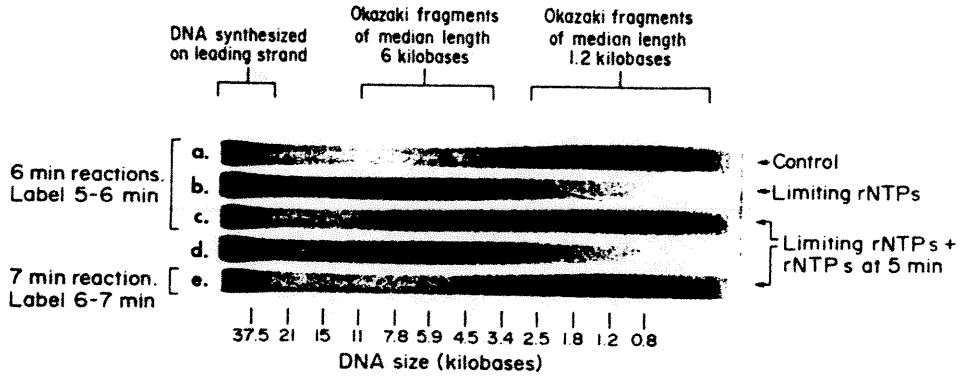


FIGURE 3B. The size of newly synthesized Okazaki fragments is not fixed by the size of the previously synthesized fragment. The results of the type of experiment described in Figure 3A are presented. DNA Synthesis was carried out on a high molecular weight cytosine-containing T4 DNA template in the presence of the seven T4 replication proteins as described previously (30) -- except that replication was initiated in the absence of added rCTP and in the presence of 20 μM rUTP, 300 μM rGTP, and 500 μM rATP. Under these conditions the average size of the Okazaki fragments is 6 kb (lanes b and d), while with non-limiting concentrations of rNTPs the average Okazaki fragment length is approximately 1.2 kb (lane a). A low level of rCTP contamination in the other rNTPs may permit the low level of primer synthesis required to produce the 6 kb Okazaki fragments. The high molecular weight DNA characteristic of leading strand synthesis is present in all of the lanes. Lanes a and b represent 6 min synthesis reactions without and with limiting rNTPs, in which the newly synthesized Okazaki fragments have been labeled during the final minute of synthesis (from 5 to 6 min). Lane c shows the DNA products when synthesis is initiated with limiting rNTPs and the normal concentration of rNTPs is restored coincident with the addition of label at 5 min (labelling from 5 to 6 min). Lane d is simply a replica of the lane b control. Lane e depicts a reaction similar to that in lane c, except that synthesis was allowed to proceed at high rNTP concentrations for 1 min prior to the addition of label from 6-7 min.

As is evident from a comparison of the distribution of fragment lengths in lane e with the other lanes, conversion of 6 kb to 1.2 kb Okazaki fragment synthesis is essentially complete within 1 min of restoration of the non-limiting concentrations of rNTPs; the lane c results show the type of intermediate fragment lengths predicted from Figure 6, below.

fewer primers are synthesized and unusually long Okazaki fragments are produced. After first establishing such a pattern of long Okazaki fragment synthesis, normal concentrations of rNTPs can be restored along with labeled dNTPs, and the size of the subsequently synthesized Okazaki fragments can be assessed by alkaline agarose gel electrophoresis. A continued synthesis of long fragments would be predicted by a simple length templating mechanism. As can be seen in the data of Figure 3B, however, the size of the subsequent Okazaki fragments does not appear to be influenced by the size of the previously synthesized fragment. In fact, the bottom lane shows that conversion to the normal 1.2 kb length Okazaki fragments is complete within 1 min of restoration of the normal concentrations of rNTPs.

The results in Figure 3 suggest that the original model of coupled leading and lagging strand DNA synthesis should be modified as depicted in Figure 4. In this view, because the DNA helix has already been opened to expose the single-stranded lagging strand template, the lagging strand DNA polymerase is free to move at a rate that is faster than that of the leading strand polymerase molecule (or rather, to pull its template past it more rapidly) - even though these two polymerase molecules are physically linked together. However, once it reaches the previously synthesized primer, the lagging strand polymerase is forced to pause before releasing its DNA template. During this pause, the leading strand polymerase continues its translocation, displacing an additional amount of single-stranded template for the next round of lagging strand synthesis. Finally, the release of the DNA by the lagging strand polymerase signals the associated primosome to synthesize an RNA primer at the next available primer site (providing that the requisite rNTPs are present), thereby restarting the next cycle of Okazaki fragment synthesis.

There are two predictions of the model in Figure 4 that are readily tested. First, because the leading and lagging strand polymerases move at different rates, the total amount of single-stranded DNA on the lagging strand will change throughout each cycle of Okazaki fragment synthesis, ruling out the possibility that all of the 32 protein recycles. In order to test for such recycling, we have used a synthetic RNA molecule that binds free 32 protein tightly (polyribo I; ref. 38) as a trap for free 32 protein during a DNA synthesis reaction. The addition of polyribo I was found to arrest the progression of replication forks nearly immediately during a DNA synthesis reaction that requires 32 protein, whereas DNA

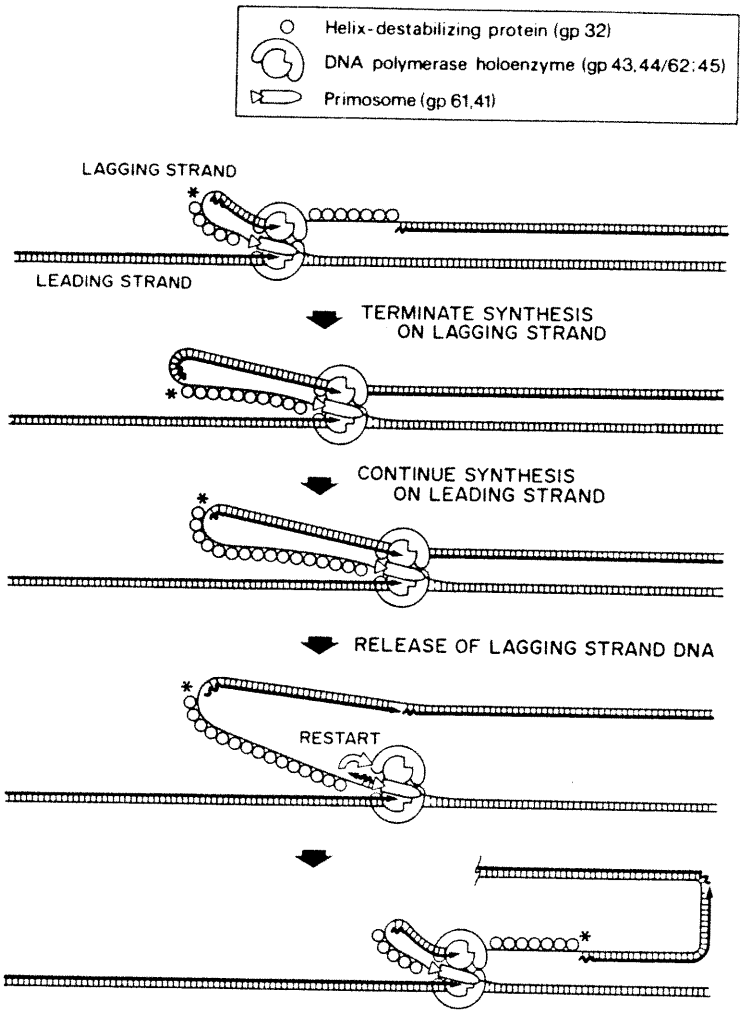


FIGURE 4. A model for DNA synthesis at a replication fork. This model of coupled leading and lagging strand DNA synthesis differs from our previous one (2,30) in assuming that the rate of lagging strand DNA synthesis is faster than the rate of leading strand DNA synthesis. This refinement incorporates a prolonged pause by the lagging strand DNA polymerase molecule before it releases its DNA template strand, and it demands that the total amount of single-stranded DNA exposed on the lagging strand template changes during each cycle of Okazaki fragment synthesis, being greatest when the fragment is initiated and least at the moment when the fragment is completed.

synthesis was not inhibited in other reactions that require the DNA polymerase holoenzyme alone (J. Barry, unpublished results). This result suggests that 32 protein is continuously undergoing dissociation and reassociation in the replication complex at the growing fork, rather than being recycled. This is presumably permissible in vivo because the concentration of free 32 protein within the cell is relatively high (estimated at about 70 µg/ml; ref. 39).

A second prediction of the model in Figure 4 stems from the rapid movement of the lagging strand polymerase molecule, which should greatly reduce the frequency of the gapped replication intermediates depicted schematically in the top panel of Figure 5. In contrast, these are expected to be the

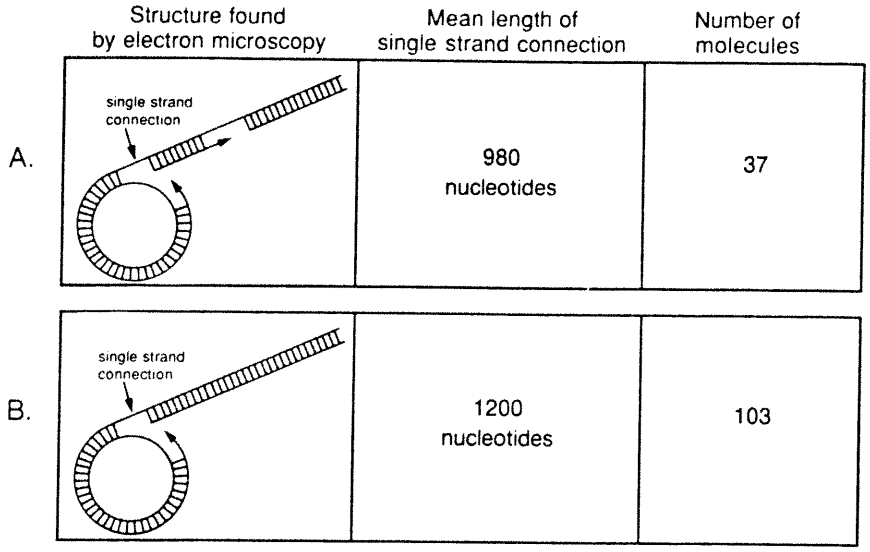


FIGURE 5. Distribution of the replication intermediates visualized in rapidly-quenched samples. DNA was synthesized in a standard reaction mixture (17) using 2 µg/ml pJMC110 plasmid DNA template, specifically nicked by the fd gene II protein to provide a unique 3'OH to prime DNA synthesis. After 4 min of synthesis, the reaction was rapidly stopped by the addition of an excess of cold EDTA and the DNA spread for electron microscopy (a variety of more sophisticated instant quenching protocols gave indistinguishable results). DNA replication intermediates were selected at random and the nature and length of their individual single-strand connections were determined. The types of structures that were observed were classified as either A or B. The type A

(FIGURE 5. cont'd.) molecules, representative of those in which the lagging strand polymerase was captured in the process of translocation, comprise only about 25% of the total. The remaining 75% have type B structures, which represent molecules in which the lagging strand polymerase has paused after completing the synthesis of an Okazaki fragment.

predominant intermediate if the speeds of the leading and lagging strand polymerase molecules are the same. When the lagging strand polymerase has completed synthesis of its current Okazaki fragment and is paused adjacent to the previously synthesized primer, molecules like those in the bottom panel of Figure 5 are produced. The fraction of molecules of this type should approximate the fraction of the cycle time that the lagging strand polymerase spends waiting. Our results indicate that the lagging strand polymerase spends only about 25% of its time moving and 75% of its time pausing between successive rounds of Okazaki fragment synthesis (Figure 5). Thus, the lagging strand molecule appears to move along its template at more than twice the rate of its leading strand counterpart. Consistent with this rate difference on the two sides of the fork is the observation that the measured rates of polymerase translocation are faster on single-stranded than on double-stranded DNA templates in vitro (15,16).

Since the selection of RNA primer sites is independent of the length of the previously-synthesized Okazaki fragment, some other mechanism must be involved in primer site selection. According to the model in Figure 4, the primosome becomes "activated" as a consequence of the release of the DNA by the lagging strand polymerase molecule. Since the potential GTT primer sites will be encountered with a frequency of about once in every 60 nucleotides, primer site selection by such an activated primosome should be a very fast event in the synthesis reaction. If we therefore ignore the brief time required to find a primer site, the major parameters that will influence the frequency of primed DNA starts and hence Okazaki fragment size will be the rates of movement of the leading and lagging strand polymerases (R1 and R2, respectively) and the length of time, T, that the lagging strand polymerase pauses before releasing the lagging strand template. For the synthesis of any particular pair of successive Okazaki fragments, these parameters are related to the lengths of the two fragments by the following equation:

$$\left[\frac{L_{(n)}}{R_2} + T\right] R_1 = L_{(n+1)} \qquad \text{(eq.1)}$$

where $L_{(n)}$ = length of current Okazaki fragment (nucleotides)
$L_{(n+1)}$ = length of next Okazaki fragment (nucleotides)
T = duration of lagging strand polymerase pause (sec)
R_1 = rate of leading strand polymerase translocation (nucleotides/sec)
R_2 = rate of lagging strand polymerase translocation (nucleotides/sec)

Here we have assumed that T is a constant, whereas in reality a distribution of pause times is expected, reflecting a first order rate constant for the dissociation of the polymerase from its template. Thus, a broad distribution of Okazaki lengths is expected, as observed (see Figure 3B, above).

From the above equation, L(n) is related to the length of the first Okazaki fragment, L(o), by the equation:

$$L_{(n)} = \left(\frac{R_1}{R_2}\right)^n L_{(o)} + T(R_1)\left[\frac{\left(\frac{R_1}{R_2}\right)^n - 1}{\left(\frac{R_1}{R_2}\right) - 1}\right] \qquad \text{(eq.2)}$$

where $L_{(o)}$ = length of the first Okazaki fragment (nucleotides)
$L_{(n)}$ = length of the nth consecutive Okazaki fragment (nucleotides)

If we assume that R1 and R2 do not change with time, that R1 < R2, and that T is a constant, then the sizes of successively synthesized Okazaki fragments will rapidly converge to a uniform length, L(n), that is independent of the length of the initial fragment, L(o):

$$\text{as } n \to \infty \text{ , } L_{(n)} \approx \frac{R_1 R_2}{R_2 - R_1}(T) \qquad \text{(eq.3)}$$

Thus, the ultimate size or "equilibrium length" of the Okazaki fragments to be synthesized on a particular template will be independent of the size of the very first fragment synthesized. An example is presented in Figure 6, where we have assumed leading and lagging strand polymerase translocation rates of 300 and 600 basepairs per sec, respectively, and a pause time, T, of 2 sec; here an equilibrium length of approximately 1200 basepairs per Okazaki fragment is attained

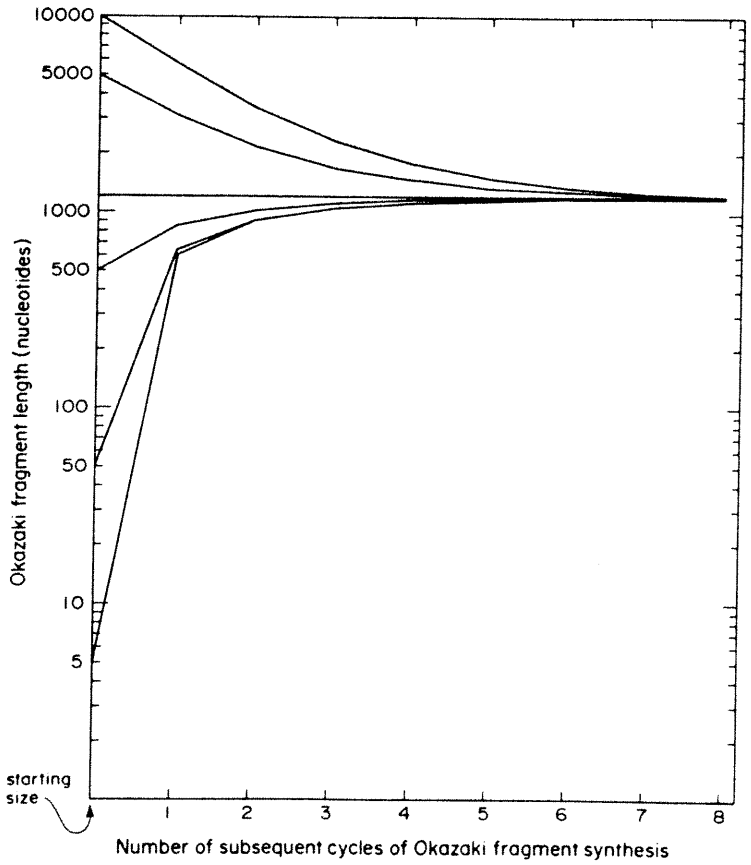


FIGURE 6. According to the Figure 4 model, the sizes of successively synthesized Okazaki fragments converge to a uniform length that is independent of the size of the first Okazaki fragment synthesized on a particular template. This plot is calculated from equation 2 in the text, using R1 = 300 nucleotides/sec, R2 = 600 nucleotides/sec and T = 2 sec. Separate curves are given for L(o) = 5, 50, 500, 1200, 5000, and 10,000 nucleotides.

within eight cycles of Okazaki fragment synthesis, regardless of whether the first fragment was 5 or 10,000 nucleotides long. Therefore, by manipulating the parameters within reasonable limits, one predicts that an equilibrium length of Okazaki fragments can be reached quite rapidly, as observed in the experiment depicted in Figure 3B and as might be expected following the initiation of replication of the T4 chromosome in vivo.

Essential to the mechanism in Figure 4 is some timing mechanism that meters the duration of the lagging strand polymerase pause, signaling the release of this polymerase over a range of set times following the completion of an Okazaki fragment. The exact nature of such a timing mechanism or "clock" remains to be determined; however, a likely possibility can be inferred from the following set of experiments designed to study the association of the holoenzyme complex with a primer-template junction.

Maintenance of a Stable Holoenzyme Complex Requires Continuous Polymerase Translocation.

Although the term "multienzyme complex" is used to describe the T4 replication machine, a stable complex has never been isolated. Presumably, the associations of the individual components are either weak or else dependent upon stabilizing factors that are lost during the isolation procedures. In order to better understand the architecture of the replication complex, the interactions on a primer-template junction between DNA polymerase, its accessory proteins and the helix destabilizing protein have been probed using DNA footprinting techniques. The primer-template molecule illustrated in Figure 7 was chosen for these studies for the following reasons: 1) While its double-stranded and single-stranded regions are large enough to accomodate the replication complex, the entire molecule is small enough for an accurate footprinting analysis on standard sequencing gels. 2) Because the primer and template strands are covalently linked, the footprint analysis of both strands can be conducted in a single experiment. 3) In the absence of dATP, the polymerase stalls on the substrate, remaining in one place while it catalyzes successive rounds of excision and incorporation of the terminal nucleotides. 4) The molecule can be constructed with its 3' terminus in two different places, enabling sequence-specific and structure-specific interactions to be distinguished.

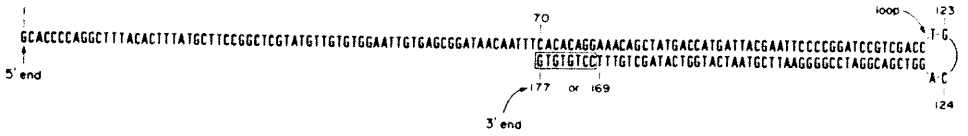


FIGURE 7. The sequence and structure of the 177/169 nucleotide-long DNA primer-template molecule used for the DNA footprinting analyses. The construction of this molecule, derived from a longer M13 mp7 HaeIII fragment, is described in ref. 40. The molecule has been constructed with the two different 3' termini shown, in order to distinguish sequence-specific from structure-specific protein-DNA interactions.

The positions of the T4 replication proteins on the primer-template molecule were assessed by determining the extent of protection they afford to cleavage by several different cleaving agents (40). The results can be briefly summarized as follows:

(1) Polymerase alone binds the DNA substrate adjacent to the 3' terminus, protecting a segment of the duplex region from both neocarzinostatin and DNase I cleavage. The presence of 32 protein on the single-stranded region lowers the Kd of the polymerase for this DNA site by a factor of thirty, presumably as a consequence of the direct interaction observed between these two proteins (21,41).

(2) The addition of 45 protein extends the region of DNase I protection into the duplex several basepairs beyond that conferred by 43 alone. In the absence of polymerase however, the 45 protein does not protect any region of the primer/template molecule, with or without 32 protein present.

(3) The polymerase accessory protein complex, in the presence of the nonhydrolyzable ATP analog, ATPγS, protects 20 basepairs of duplex and 8 nucleotides of single-stranded template DNA abutting a primer-template junction from DNase I cleavage. Addition of 32 protein reduces the concentrations of 44/62 and 45 proteins that are required to achieve this protection by a factor of 100. The binding of the non-hydrolyzable ATP analogue is required for the formation of this DNA-protein complex; no footprint can be detected in the presence of ATP, indicating that the hydrolysis of ATP greatly weakens the complex.

In order to carry out the footprint analysis on the entire holoenzyme complex, footprinting experiments were performed using a mixture of the accessory proteins, 32

protein, and DNA polymerase with ATP added, since ATP hydrolysis is required for the effects of the accessory proteins on polymerase activity (13,14,42). Surprisingly, the protection conferred by this combination of proteins is no different from that provided by the same components in the absence of the 44/62 protein. Moreover, no decrease is observed in the Kd of the polymerase for the primer-template junction in the presence of the accessory proteins and ATP, even though a major decrease would be expected from the pronounced effects of the accessory proteins on both DNA synthesis (14) and polymerase exonuclease activities (42).

In the presence of ATPγS and 32 protein, the polymerase appears to compete with the ATPγS-bound accessory protein complex for binding to the primer-template junction. Instead of an additivity of the protection patterns of the individual components, the observed protections appear to result from a competition between two non-interactive assemblies. Furthermore, the accessory proteins under these conditions inhibit rather than stimulate polymerase activity (40).

Our inability to detect the complete holoenzyme complex in the presence of either ATP or ATPγS suggests that active polymerase translocation is an essential requirement for stable complex formation; such translocation is of course not occurring in any of the footprinting experiments. Kinetic studies show that the accessory proteins are a stably-bound component of a moving replication complex, with only one molecule of the 44/62 protein required per primer-template end (42,43). In contrast, on the stalled replication forks used in these experiments, concentrations of accessory proteins that are thousands of times higher than the concentration required to saturate a moving replication complex are insufficient to produce a footprint in the presence of ATP.

We propose that the replication complex is designed to dissociate from the template in the absence of a moving polymerase, and that the timing of the steps leading to this dissociation is governed by an energy-driven "clock". Our rendering of the moving holoenzyme complex, presented in Figure 8, is a composite of the results of all of our footprinting analyses as well as similar studies of the Klenow fragment of E. coli DNA polymerase I (44). We suggest that the assembly of the holoenzyme complex begins when the primer-template junction binds to an ATP-bound accessory protein complex, activating its ATP hydrolysis (32). The polymerase quickly interacts with this complex, positioning itself at the primer-template junction and displacing the accessory proteins to an adjacent position on the duplex.

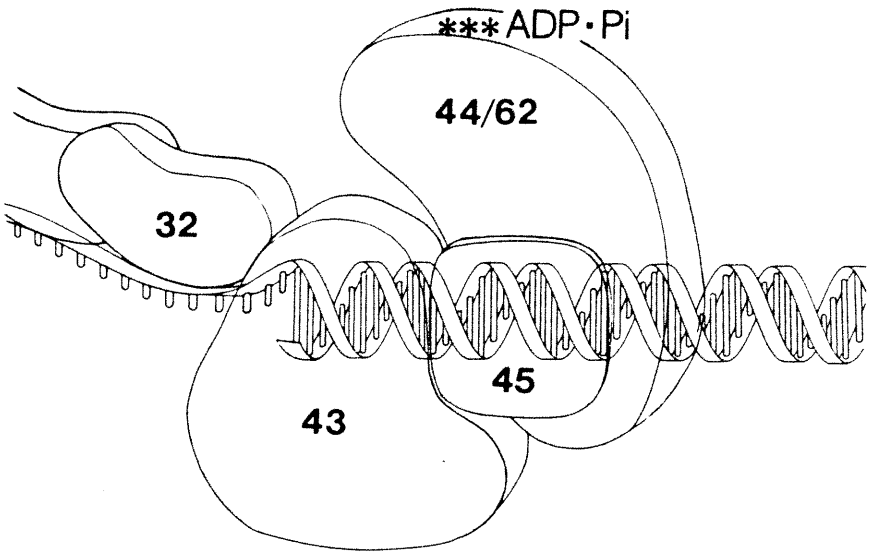


FIGURE 8. A model of the moving T4 DNA polymerase holoenzyme complex on a primer-template junction. This very low resolution view of the holoenzyme/DNA template/32 protein complex has been deduced from our DNA footprint analyses, as well as from a comparison with similar studies of the Klenow fragment of E. coli DNA polymerase I (44). Essential to the stability of this presumed structure is the active translocation of the holoenzyme complex, which is postulated to be required to maintain the polymerase accessory proteins in their "energized" (***ADP·Pi-bound) active clamp conformation.

The resulting accessory protein complex, which we propose remains bound to ADP + Pi, represents a high energy state and constitutes the active polymerase clamp. In the absence of a rapidly translocating polymerase, the decay of this complex to release ADP + Pi leads to its dissociation from the DNA molecule, explaining our inability to detect it in our footprint analyses.

A simple diagram depicting the coupling of polymerase translocation to the maintenance of a tightly-bound holoenzyme complex is depicted in Figure 9. The accessory protein component of the holoenzyme complex is represented in several energy states, with E***, E**, and E* representing several activated conformations of the accessory protein complex, each of which forms a stable clamp for the DNA

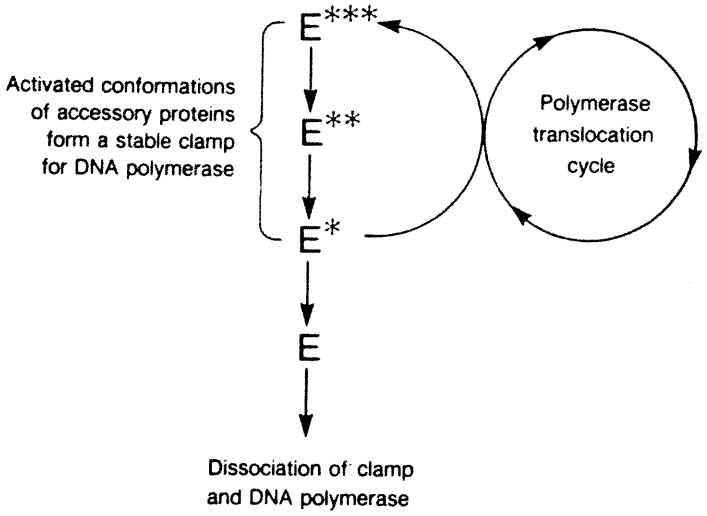


FIGURE 9. Diagram of an energy-driven clock to measure polymerase pausing. In this simple model, the activated holoenzyme complexes denoted E***, E**, and E* are maintained by their coupling to the polymerase translocation cycle. In the absence of polymerase translocation, the accessory protein complex decays through a series of intermediate conformations, each of which is characterized by a particular half-life. Decay to the lowest energy state, E, allows the dissociation of the accessory protein clamp and the release of the DNA polymerase.

polymerase. E represents the lowest energy state of the accessory protein complex which, having released its bound ADP and Pi, has lost its ability to clamp the polymerase to the template. In this view, active polymerase translocation causes the periodic reactivation of the accessory protein complex, thereby keeping it "pumped up" to the high energy states that are required to keep the polymerase tightly bound. As soon as the polymerase pauses, however, the activated accessory protein complex begins to decay through a series of intermediate energy states, each characterized by a particular half-life. As long as the energy state of the polymerase accessory protein complex remains at or above E* (that is, so long as the pause time is less than the sum of the half-lives for each decay step), the accessory protein complex can be recycled to its activated conformation E*** via the coupling of the reaction E* → E*** to polymerase translocation. For longer pauses, however -- such as when

the polymerase complex encounters a region of DNA damage over which it cannot replicate, when it is stalled on the primer template junction by the omission of a nucleoside triphosphate (as in our footprinting experiments), or when it encounters the 5' end of the previously synthesized Okazaki fragment during lagging strand DNA synthesis -- the polymerase accessory protein complex dissociates, allowing the DNA polymerase to leave the template.

The decay from E*** through intermediate states to E* introduces a lag-time before the accessory proteins begin the final decay process to E and dissociation. This time delay represents a clock that enables the accessory proteins to measure the time of polymerase stalling in a manner that prevents the polymerase from dissociating until a certain minimum stalling time is exceeded. Such a timing mechanism is biologically useful, because it prevents the unnecessary dissociation of a moving DNA polymerase that has only transiently paused, thereby allowing the leading strand DNA polymerase molecule to move processively for tens of minutes or more. As pointed out by Hopfield (45), these types of clock mechanisms require an input of energy. Here, the energy required initially comes from the ATP hydrolysis that establishes E*** from E, and it is subsequently provided by the energetically favorable polymerization and excision cycles of the DNA polymerase. We believe that the usefulness of such a clock, which enables the holoenzyme complex to distinguish between significant and insignificant barriers to its continued translocation, explains why nature evolved the polymerase accessory proteins rather than merely settling for a more tightly-binding type of DNA polymerase.

A T4 I/S Vector System Can be Used to Identify New Replication Genes.

While the present in vitro system is contributing to an increasingly detailed picture of the events taking place at the moving replication fork, no one has yet been able to reconstitute the initiation of replication in vitro in the manner presumed to occur within the T4 infected cell. T4 possesses at least three different modes of initiation. Primary initiation, discussed by Mosig and her co-workers in this volume, appears to be dependent upon host RNA polymerase and independent of genetic recombination (46). Secondary initiation, as proposed by Luder and Mosig and partially reconstituted in vitro by Tim Formosa in our laboratory (47),

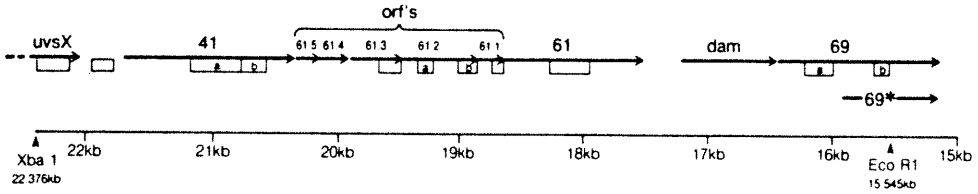
is resistant to rifampicin but dependent upon the recombination functions of T4 genes 46 and 47 (46). Tertiary initiation, as discussed in this volume by Kreuzer, is both rifampicin-resistant and recombination-independent (48).

Replication from primary origins proceeds bidirectionally and from one or several specific ori sequences within the T4 chromosome (49), analogous to the initiation of replication for E. coli and bacteriophage lambda (this volume). Part of our work has focused on the identification of the additional proteins that are suspected to be involved in this mode of initiation. The placement of the primosome complex onto the DNA has always been the rate-limiting step in our in vitro reactions, and it seems likely that this reflects the non-biological manner in which we are carrying out this step. We presume that our system is lacking one or more proteins, analogous to the bacteriophage lambda O and P or the E. coli dnaA proteins. As demonstrated by the McMacken and Kornberg laboratories (this volume), these proteins are responsible for loading the primosome complexes onto the DNA at their respective replication origins.

One approach that we are taking to find the suspected missing T4 initiation proteins is via the identification of new replication genes. As previously shown by Epstein and his co-workers, genes that encode related functions tend to be clustered on the T4 chromosome (50). Thus, for example, the genes that encode the three components of the polymerase accessory protein complex are next to one another, as are the genes for the two primosome components, the 41 and 61 proteins. In order to identify open reading frames (orfs) which might encode primosome-loading proteins, a DNA sequence analysis of the region of the T4 chromosome encompassing genes 41 and 61 was performed. The result of this study, including some sequence analysis of the flanking regions from the labs of Minagawa (51) and Mosig (52), is presented in Figure 10. Between genes 41 and 61, we have identified five orfs, the largest of which would encode a 23 Kd protein and the smallest of which would encode a 6 Kd protein, as indicated in the Figure.

In order to determine which of these orfs might be essential for T4 growth, we have constructed an insertion/substitution vector (I/S) system that allows us to target mutations to specific orf sequences in the T4 chromosome and to then assess their effects. The I/S system, based upon the πVX system of Brian Seed (53) and constructed in collaboration with K.N. Kreuzer, is used to shuttle mutations into the T4 chromosome as depicted in Figure 11.

While the I/S system can be used as an effective mechanism for targeting stable mutations to the T4 chromosome by a cycle of integration and segregation, we have also used it to examine the effect of gene-disruptions performed on several of the orfs, as well as on several control genes. To insure gene disruption upon homologous recombination of the plasmid into the phage chromosome, cloned gene fragments that were completely internal to the predicted coding sequences in Figure 10 were used as the plasmid-borne T4 target sequences.



Predicted sizes of the orf gene products	
61.1	54 amino acids (5940 daltons)
61.2	208 amino acids (22880 daltons)
61.3	97 amino acids (10670 daltons)
61.4	85 amino acids (9350 daltons)
61.5	60 amino acids (6600 daltons)

FIGURE 10. A map of a region of the T4 chromosome suspected to contain additional replication genes. The genetic map of T4 extending from the middle of the uvsX gene through gene 69 is presented. Gene 69* is presumed to be transcribed from within the coding sequence of gene 69 (52). The precise map positions within this region are included, along with the locations of the XbaI site at 22.376 kb and the EcoRI site at 15.545 kb. Our DNA sequence analysis between genes 41 and 61 has revealed five open reading frames, or orfs, which can be translated into the predicted gene products indicated (approximate molecular weights were calculated by assuming 110 daltons/ amino acid). The various open boxes that are distributed along the length of this map denote the target DNA fragments that were used for the gene disruption analyses discussed in the text and presented in Table 2.

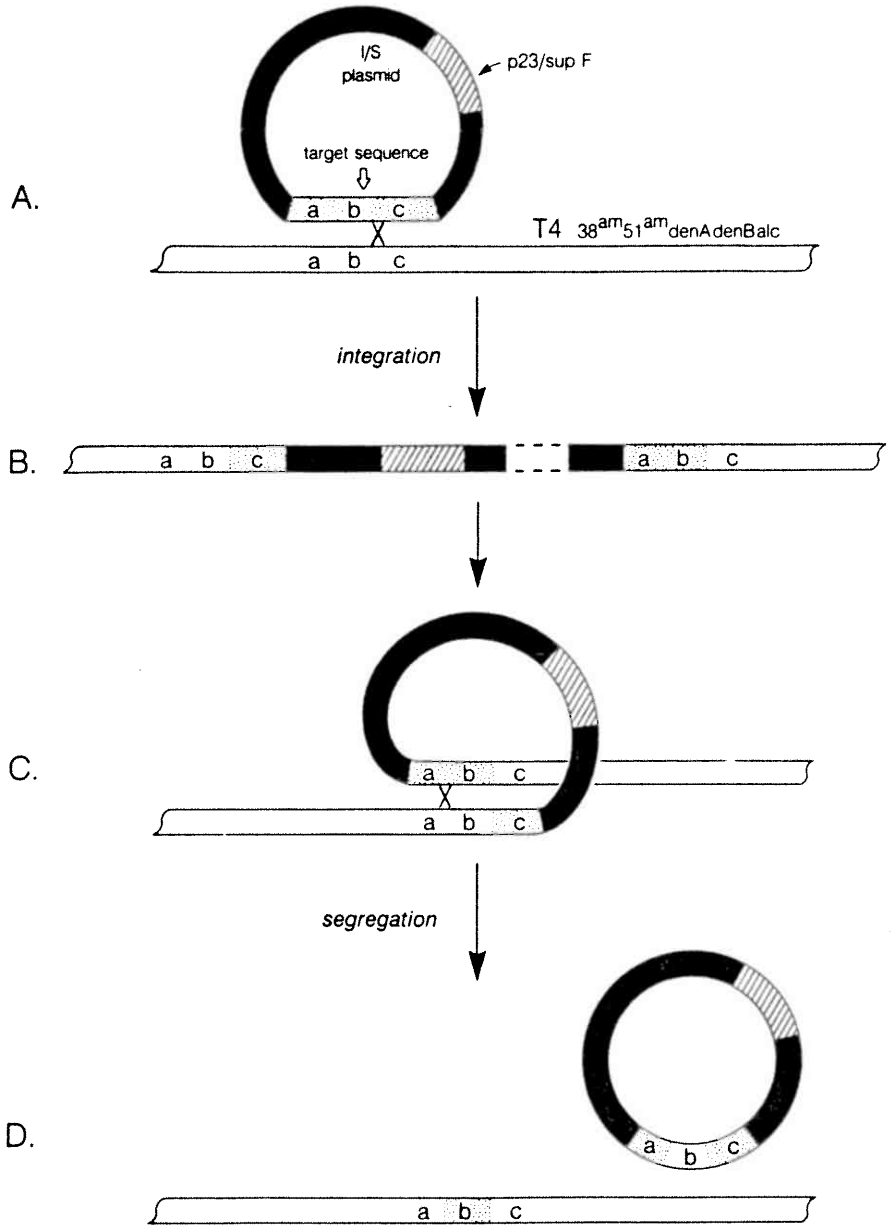


FIGURE 11. An insertion/substitution vector system that can be used to substitute plasmid-borne T4 "target sequences" for their chromosomal counterparts.

FIGURE 11 (cont'd).

A) The T4 target sequence is cloned into a pBR322-derived plasmid vector, whose most striking feature is the presence of a supF tRNA gene to which has been fused the promoter of the T4 late gene 23. Cells containing this plasmid are infected with the indicator phage, defective in host/plasmid DNA breakdown ($denA^-denB^-alc^-$) and carrying two amber mutations in the late genes 38 and 51. The target sequence recombines with its homologous site on the T4 chromosome, which results in the integration of the entire plasmid with a duplication of the target sequence.

B) Because the amber mutations in the indicator phage are suppressible by the p23-supF following infection by T4, the integrant resulting from (A) can be readily selected by its ability to grow on a non-suppressing E. coli host cell. While target sequences that disrupt an essential gene will yield no viable phage at this point, target sequences that integrate into a non-essential region or that reconstruct an essential gene by duplication will produce normal-size plaques.

C,D) The vector and one copy of the target sequences will often be segregated out of the phage chromosome by intramolecular homologous recombination during growth on a non-selective, suppressing host. In this way, portions of the plasmid-borne target sequence can be readily substituted for their corresponding sequence in the phage chromosome.

The results of an analysis extending from the uvsX gene through the newly discovered replication genes, 69 and 69* (52), are presented in Table 2. As expected, insertional-inactivation of genes 41 and 61 is lethal. Disruption of the recombination protein gene, uvsX, which we find to be an essential component of a recombination-mediated replication machine (47), is also lethal. The carboxy-terminus of genes 69 and 69* appears to be non-essential, but disruption of the amino-terminus of gene 69 is lethal. Among the orfs tested, only the disruption of orf 61.1 appears to have an effect, as indicated by its growth-limited phenotype.

Table 2. Results of a Gene Disruption Analysis of the uvsX-69 Region of T4

Gene or orf	Target fragment, length	Plaque size
uvsX	Xba I(22.376)-Alu I(22.159), 217 bp	—
uvsX-41 intergenic	Cla I(22.001)-Aha III(21.708), 293 bp	normal
41a	Eco RI(21.155)-Sal I(20.757), 398 bp	—
41b	Sal I(20.757)-Hind III(20.609), 148 bp	—
orf 61.3	Xmn I(19.695)-Mbo I(19.592), 103 bp	normal
orf 61.2a	Rsa I(19.372)-Hpa II(19.227), 145 bp	normal
orf 61.2b	Hinf I(19.016)-Mbo I(18.885), 131 bp	normal
orf 61.1	Aha III(18.767)-Xmn I(18.662), 105 bp	minute
61	Ava I(18.273)-Hind III(17.979), 294 bp	—
69a	Taq I(16.400)-Taq I(16.058), 342 bp	—
69b/69*	Xba I(15.727)-Eco RI(15.545), 182 bp	normal
frd (control)	Hind III(143.945)-Eco RI(143.250), 695 bp	normal

A gene disruption analysis of the region of the T4 chromosome spanning genes uvsX to 69* was performed using the indicated target sequences, and the corresponding effects on plaque size are noted. The precise target fragments have been indentified by the locations of their termini within the T4 chromosome (0-166.488 kb). Disruption of an essential gene or orf results in the production of no plaques while disruption of a non-essential region yields normal plaques. Minute plaques are indicative of a growth-limited phenotype. The disruption of the non-essential dihydrofolate reductase gene, frd, was included in each experiment as a control.

As depicted in Figure 12, the in vivo rate of DNA synthesis of the orf 61.1 disruption mutant is much lower than that of the wild type phage. A stable orf 61.1 mutant, created by using the I/S system to place an 8 basepair

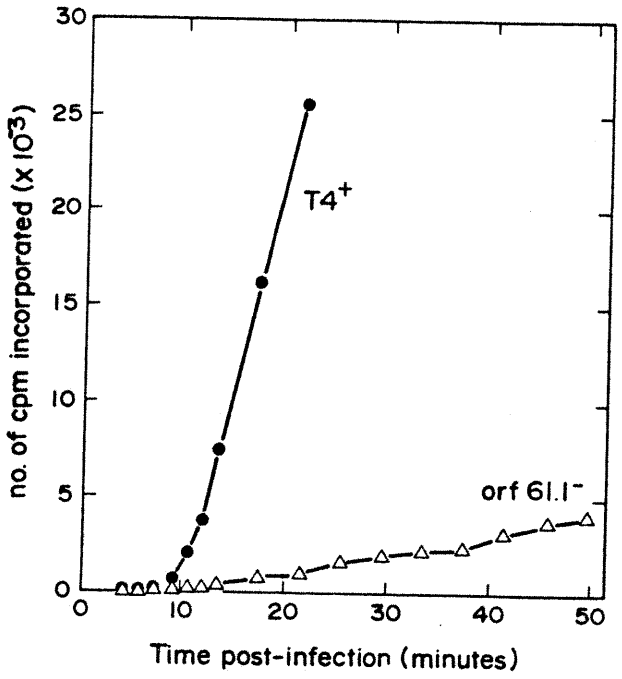


FIGURE 12. Intracellular DNA synthesis observed after infection of E. coli with an orf 61.1- mutant. Using a non-suppressing strain of E. coli, ^{14}C-thymidine was added to cultures 3 min after infection at 37°C and acid precipitable counts measured at subsequent times as indicated. Under these conditions, the indicator phage itself displays a profile of ^{14}C-thymidine uptake that is identical to that observed for the wild-type T4 - shown here as a control.

insertion into the middle of orf 61.1 (Figure 11), is similarly growth-limited. Orfs 61.4 and 61.5 remain to be tested.

What might be the function of orf 61.1? A comparison of the sequence of this 54 amino acid protein with the consensus DNA binding domain of several DNA-binding regulatory proteins (54) reveals a perfect homology between the amino-terminus of orf 61.1 and the most stringently conserved residues of the DNA binding domain. In addition, individual amino acid matches can be found at virtually every position of orf 61.1 with corresponding sites in the consensus domains of the known DNA binding proteins, spanning 13 amino acid residues. Could the orf 61.1 protein, whose coding sequence we have recently engineered for overproduction, be the T4 analog of the lambda O and the E. coli dnaA proteins?

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REFERENCES

1. Wood WB, Revel HR (1976). The genome of bacteriophage T4. Bacteriol Rev 40:847.
2. Nossal NG, Alberts BM (1983). Mechanism of DNA replication catalyzed by purified bacteriophage T4 replication proteins. In Mathews C, Kutter E, Mosig G, Berget P (eds): "Bacteriophage T4", Washington, DC: Amer Soc Microbiol, p 71.
3. Bittner M, Burke RL, Alberts BM (1979). Purification of the T4 gene 32 protein free from detectable deoxyribonuclease activities. J Biol Chem 254:9565.
4. Morris CF, Hama-Inaba H, Mace D, Sinha NK, Alberts B (1979). Purification of the gene 43, 44, 45 and 62 proteins of the bacteriophage T4 DNA replication apparatus. J Biol Chem 254:6787.
5. Morris CF, Moran LA, Alberts BM (1979). Purification of gene 41 protein of bacteriophage T4. J Biol Chem 254:6797.
6. Liu LF, Liu C-C, Alberts BM (1979). T4 DNA topoisomerase: a new ATP-dependent enzyme essential for initiation of T4 bacteriophage DNA replication. Nature 281:456.
7. Jongeneel CV, Formosa T, Alberts BM (1984). Purification and characterization of the bacteriophage T4 dda protein. A DNA helicase that associates with the viral helix-destabilizing protein. J Biol Chem 259:12925.
8. Goulian M, Lucas ZJ, Kornberg A (1968). Enzymatic synthesis of deoxyribonucleic acid XXV: Purification and properties of deoxyribonucleic acid polymerase induced by infection with phage T4. J Biol Chem 243:627.
9. Brutlag D, Kornberg A (1972). Enzymatic synthesis of deoxyribonucleic acid. J Biol Chem 247:241.
10. Mace DC, Alberts BM (1984). The complex of T4 bacteriophage gene 44 and 62 replication proteins forms an ATPase that is stimulated by DNA and by T4 gene 45 protein. J Mol Biol 177:279.

11. Piperno JR, Kallen RG, Alberts BM (1978). Analysis of a T4 DNA replication protein complex: studies of the DNA recognition site for T4 gene 44/62 and 45 protein-catalyzed ATP hydrolysis. J Biol Chem 253:5180.
12. Alberts B, Morris CF, Mace D, Sinha N, Bittner M, Moran L. (1975). Reconstruction of the T4 bacteriophage DNA replication apparatus from purified components. ICN-UCLA Symp Molec Cell Biol 3:241.
13. Piperno JR, Alberts BM (1978). An ATP stimulation of T4 DNA polymerase mediated via T4 gene 44/62 and 45 proteins: The requirement for ATP hydrolysis. J Biol Chem 253:5174.
14. Huang C-C, Hearst JE, Alberts BM (1981). Two types of replication proteins increase the rate at which T4 DNA polymerase traverses the helical regions in a single-stranded DNA template. J Biol Chem 256:4087.
15. Mace DC, Alberts BM (1984). Characterization of the stimulatory effect of T4 gene 45 protein and the gene 44/62 protein complex on DNA synthesis by T4 DNA polymerase. J Mol Biol 177:313.
16. Alberts BM, Barry J, Bedinger P, Burke RL, Hibner U, Liu C-C, Sheridan R (1980). Studies of replication mechanisms with the T4 bacteriophage in vitro system. ICN-UCLA Symp Molec Cell Biol 19:449.
17. Liu C-C, Burke RL, Hibner U, Barry J, Alberts BM (1979). Probing DNA replication mechanisms with the T4 bacteriophage in vitro system. Cold Spring Harbor Symp Quant Biol 43:469.
18. Nossal NG, Peterlin BM (1979). DNA replication by bacteriophage T4 proteins: the T4 43, 32, 44-62, and 45 proteins are required for strand displacement synthesis at nicks in duplex DNA. J Biol Chem 254:6032.
19. Alberts BM, Amodio FJ, Jenkins M, Guttman ED, Ferris FL (1969). Studies with DNA-cellulose chromatography. I. DNA-binding proteins from Escherichia coli. Cold Spring Harbor Symp Quant Biol 33:289.
20. Alberts BM, Frey L (1970). T4 bacteriophage gene 32: a structural protein in the replication and recombination of DNA. Nature 227:1313.
21. Huberman JS, Kornberg A, Alberts BM (1971). Stimulation of T4 bacteriophage DNA polymerase by the protein product of T4 gene 32. J Mol Biol 62:39.
22. Burke RL, Alberts BM, Hosoda J (1980). Proteolytic removal of the COOH terminus of the T4 gene 32 helix-destabilizing protein alters the T4 in vitro replication complex. J Biol Chem 255:11484.

23. Liu C-C, Alberts BM (1981). Characterization of the DNA-dependent GTPase activity of T4 gene 41 protein, an essential component of the T4 bacteriophage DNA replication apparatus. J Biol Chem 256:2813.
24. Venkatesan M, Silver LL, Nossal NG (1982). Bacteriophage T4 gene 41 protein, required for the synthesis of RNA primers, is also a DNA helicase. J Biol Chem 257:12426.
25. Nossal NG (1980). RNA priming of DNA replication by bacteriophage T4 proteins. J Biol Chem 255:2176.
26. Liu C-C, Alberts BM (1980). Pentaribonucleotides of mixed sequence are synthesized and efficiently prime de novo DNA chain starts in the T4 bacteriophage DNA replication system. Proc Natl Acad Sci USA 77:5698.
27. Liu C-C, Alberts BM (1981). Characterization of RNA primer synthesis in the T4 bacteriophage in vitro DNA replication system. J Biol Chem 256:2821.
28. Hinton DM, Nossal NG (1985) Bacteriophage T4 DNA replication protein 61. Cloning of the gene and purification of the expressed protein. J Biol Chem 260:12858.
29. Cha T-A, Alberts BM (1986). Studies of the DNA helicase-RNA primase unit from bacteriophage T4: a trinucleotide sequence on the DNA template starts RNA primer synthesis. J Biol Chem 261:7001.
30. Alberts BM, Barry J, Bedinger P, Formosa T, Jongeneel CV, Kreuzer KN (1983). Studies on DNA replication in the T4 bacteriophage in vitro system. Cold Spring Harbor Symp Quant Biol 47:655.
31. Bedinger P, Hochstrasser M, Jongeneel CV, Alberts BM (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.
32. Jongeneel CV, Formosa T, Munn M, Alberts BM (1984). Enzymological studies on the T4 bacteriophage DNA replication proteins. In Hubscher U, Spadari S (eds): "Proteins Involved in DNA Replication", Advances in Experimental Medicine and Biology, Vol. 179, New York: Plenum Publishing Corp, p 17.
33. Krell H, Durwald H, Hoffman-Berling H (1979). A DNA-unwinding enzyme induced in bacteriophage T4-infected Escherichia coli cells. Eur J Biochem 93:387.
34. Jongeneel CV, Bedinger P, Alberts BM (1984). Effects of the bacteriophage T4 dda protein on DNA synthesis catalyzed by purified T4 replication proteins. J Biol Chem 259:12933.

35. Stetler GL, King GJ, Huang WM (1979). T4 DNA-delay proteins, required for specific DNA replication, form a complex that has ATP-dependent DNA topoisomerase activity. Proc Natl Acad Sci USA 76:3737.
36. McCarthy D, Minner C, Bernstein H, Bernstein C (1976). DNA elongation rates and growing point distributions of wild-type phage T4 and a DNA-delay amber mutant. J Mol Biol 106:963.
37. Tabor S, Richardson CC (1981). Template recognition sequence for RNA primer synthesis by gene 4 protein of bacteriophage T7. Proc Natl Acad Sci USA 78:205.
38. Newport JW, Kowalczykowski SC, Lonberg N, Paul LS, von Hippel PH (1980). Molecular aspects of the interactions of T4-encoded gene 32-protein and DNA polymerase (gene 43-protein) with nucleic acids. ICN-UCLA Symp Molec Cell Biol 19:485.
39. von Hippel PH, Kowalczykowski SC, Lonberg N, Newport JW, Paul LS, Stormo GD, 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.
40. Munn M (1986). Analysis of the bacteriophage T4 DNA replication complex. PhD Thesis, University of California, San Francisco.
41. Formosa T, Burke RL, Alberts BM (1983). Affinity purification of bacteriophage T4 proteins essential for DNA replication and genetic recombination. Proc Natl Acad Sci USA 80:2442.
42. Bedinger P, Alberts BM (1983). The 3'-5' proofreading exonuclease of bacteriophage T4 DNA polymerase is stimulated by other T4 DNA replication proteins. J Biol Chem 258:9649.
43. Newport J (1980). A study of the proteins involved in bacteriophage T4 replication. PhD thesis, University of Oregon, Eugene, Oregon.
44. Joyce CM, Ollis DL, Rush J, Steitz TA, Konigsberg WH, Grindley NDF (1985). Relating structure to function for DNA polymerase I of E. coli. ICN-UCLA Symp Molec Cell Biol, in press.
45. Hopfield JJ (1974). Kinetic proofreading: a new mechanism for reducing errors in biosynthetic processes requiring high specificity. Proc Natl Acad Sci USA 71:4135.

46. Luder A, Mosig G (1982). Two alternative mechanisms for initiation of DNA replication forks in bacteriophage T4: priming by RNA polymerase and by recombination. Proc Natl Acad Sci USA 79:1101.
47. Formosa, T. (1985). Recombination-dependent initiation of DNA synthesis by bacteriophage T4 proteins in vitro. PhD thesis, University of California, San Francisco, CA.
48. Kreuzer KN, Alberts BM (1985). A defective phage system reveals bacteriophage T4 replication origins that coincide with recombination hot spots. Proc Natl Acad Sci USA82:3345.
49. Delius H, Howe C, Kozinski AW (1971). Structure of the replicating DNA from bacteriophage T4. Proc Natl Acad Sci USA 68:3049.
50. Epstein RH, Bolle A, Steinberg CM, Kellenberger E, Boy De La Tour E, Chevalley R, Edgar RS, Susman M, Denhardt GH, Lielausis A (1964). Physiological studies of conditional lethal mutants of bacteriophage T4D. Cold Spring Harbor Symp Quant Biol 28:375.
51. Fujisawa H, Yonesaki T, Minagawa T (1985). Sequence of the T4 recombination gene, uvsX, and its comparison with that of the recA gene of Escherichia coli. Nucl Acids Res 13:7473.
52. MacDonald PM, Mosig G (1984). Regulation of a new bacteriophage T4 gene, 69, that spans an origin of DNA replication. EMBO J 3:2863.
53. Seed B (1983). Purification of genomic sequences from bacteriophage libraries by recombination and selection in vivo. Nucl Acids Res 11:2427.
54. Pabo CO and Sauer RT (1984). Protein-DNA recognition. Ann Rev Biochem 53:293.