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Plasmid Models for Bacteriophage T4 DNA Replication: Requirements for Fork Proteins

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Bacteriophage T4 DNA replication initiates from origins at early times of infection and from recombinational intermediates as the infection progresses. Plasmids containing cloned T4 origins replicate during T4 infection, providing a model system for studying origin-dependent replication. In addition, recombination-dependent replication can be analyzed by using cloned nonorigin fragments of T4 DNA, which direct plasmid replication that requires phage-encoded recombination proteins. We have tested in vivo requirements for both plasmid replication model systems by infecting plasmid-containing cells with mutant phage. Replication of origin and nonorigin plasmids strictly required components of the T4 DNA polymerase holoenzyme complex. Recombination-dependent plasmid replication also strictly required the T4 single-stranded DNA-binding protein (gene product 32 [gp32]), and replication of origin-containing plasmids was greatly reduced by *32* amber mutations. gp32 is therefore important in both modes of replication. An amber mutation in gene *41*, which encodes the replicative helicase of T4, reduced but did not eliminate both recombination- and origin-dependent plasmid replication. Therefore, gp41 may normally be utilized for replication of both plasmids but is apparently not required for either. An amber mutation in gene *61*, which encodes the T4 RNA primase, did not eliminate either recombination- or origin-dependent plasmid replication. However, plasmid replication was severely delayed by the *61* amber mutation, suggesting that the protein may normally play an important, though nonessential, role in replication. We deleted gene *61* from the T4 genome to test whether the observed replication was due to residual gp61 in the amber mutant infection. The replication phenotype of the deletion mutant was identical to that of the amber mutant. Therefore, gp61 is not required for in vivo T4 replication. Furthermore, the deletion mutant is viable, demonstrating that the gp61 primase is not an essential T4 protein.

Bacteriophage T4 uses multiple interdependent pathways of replication and recombination during its infection cycle. T4 mutants deficient in replication and/or recombination pathways were isolated beginning in the early 1960s with the classic study of Epstein et al. (20). In particular, mutations in genes *32*, *41*, *43*, *44*, *45*, and *62* were classified as DNA negative because they exhibited no DNA synthesis, while gene *61* mutations caused a DNA delay phenotype (18, 20). Mutations in several other genes (e.g., *46*, *47*, *59*, *uvsX*, and *uvsY*) result in a DNA arrest phenotype, with normal levels of early replication but an abrupt cessation of replication when late gene expression is activated (13, 18, 20). Mutations in many of the above-mentioned genes also reduce or alter phage recombination processes (for a review, see reference 45).

The products of many replication/recombination genes have been purified and studied extensively in vitro (for reviews, see references 46 and 50). Replication forks have been reconstituted on artificially primed templates with the DNA polymerase (gene product 43 [gp43]), DNA polymerase accessory proteins (gp45 and gp44/62), single-stranded DNA-binding protein (gp32), and helicase-primase complex (gp41/61). In the absence of other proteins, the T4 DNA polymerase catalyzes nucleotide polymerization with a very low processivity on primed single-stranded DNA templates (9, 19, 49). The polymerase accessory proteins greatly stimulate the processivity of the T4 DNA polymerase and have therefore been referred to as a sliding clamp (1, 53). The T4 gene *32* protein plays a central role in phage DNA replication, recombination, and repair (20, 59, 64). gp32 protects single-stranded DNA from nucleases, participates in DNA denaturation and renaturation, and interacts directly with many phage-encoded proteins (for a review, see reference 16). gp32 is required for replication of nicked-duplex templates by the DNA polymerase holoenzyme (37, 51) unless the template has a special structure that allows loading of the T4 replication helicase (gp41) (7). Either in the presence or absence of gp32, gp41 stimulates DNA synthesis by opening the parental helix ahead of the replication fork (60). To complete the fork, lagging-strand synthesis depends on the pentaribonucleotide primers synthesized by the T4 RNA primase (gp61) in conjunction with gp41 (6, 8, 26).

T4 DNA replication initiates by two different strategies as the infection proceeds (31, 38). At early times, replication begins at any of several origins, three of which have been analyzed at the level of nucleotide sequence (31, 39, 40, 42; also see references 23, 24, 28, and 66). Origin-dependent replication becomes repressed at about the time when late gene expression is activated (38; also see reference 11). At late times of infection, replication initiates by a recombination-dependent strategy that does not use specific origin sequences (38). The T4 in vitro replication system described above does not initiate replication from T4 origins; however, the addition of T4-encoded recombination proteins allows in vitro recombination-dependent DNA synthesis (21, 43).

Two T4 origins [*ori*(*uvsY*) and *ori*(*34*)] have been shown to direct autonomous replication during phage infection when cloned in a pBR322 vector (31–33). This in vivo plasmid replication allows a direct analysis of the proteins and DNA sequences necessary for the function of an individual T4 origin (33, 35, 42). The replication of plasmids containing

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ori(uvsY) or *ori(34)* is independent of T4 recombination proteins (e.g., UvsY) and does not require homology with the phage genome (33). Deletion analyses defined minimal origin sequences of less than 100 bp that include two elements (42). The first is a T4 middle-mode promoter sequence, which implicates *Escherichia coli* RNA polymerase in origin initiation. The second is the region just downstream of the promoter, which is required for optimal replication but not for transcription. This downstream region probably acts as a DNA-unwinding element, because a functional origin was maintained when the native downstream region was replaced with a pBR322 DNA-unwinding element (54a; also see reference 29). The precise roles of the middle-mode promoter and downstream region in the initiation of replication have not been elucidated.

Recombination-dependent replication initiation requires several T4-encoded proteins, including a synaptase (UvsX), a synaptase accessory protein (UvsY), an exonuclease (gp46/47), a type II DNA topoisomerase (gp39/52/60), and the product of gene *59* (for reviews, see references 44 and 45). This strategy of initiation is believed to involve the conversion of recombinational intermediates into replication forks (38). A plasmid model system for the analysis of T4 recombination-dependent replication has been established: any non-origin-containing fragment of T4 DNA allows plasmid replication following phage infection, but this replication is blocked when the phage is deficient in any of the recombination proteins listed above (35). Furthermore, no T4 sequence is required on the plasmid as long as the plasmid and phage genomes contain homologous sequences (19a). The recombination-dependent replication of nonorigin plasmids should be useful in determining the mechanism by which recombination triggers DNA synthesis.

In this study, we further analyze the in vivo requirements for both origin-dependent and recombination-dependent replication. Both plasmid model systems are shown to require components of the T4 replication machinery, providing further evidence that these model systems mimic T4 strategies of replication initiation. In addition, we describe a novel T4 strain with a nearly complete deletion of gene *61*. The viability of the gene *61* deletion mutant demonstrates that the primase is not essential for T4 growth and allows analysis of gp61-independent replication.

MATERIALS AND METHODS

Materials. Restriction enzymes, oligonucleotides, T4 DNA ligase, a random-primed DNA labelling kit, and [α-^{32}P]dATP were purchased from commercial sources. T4 DNA polymerase was the generous gift of B. M. Alberts (University of California, San Francisco). Plasmids pGJB1, pKK061, and pKK467 are described elsewhere (34, 35). L broth contains NaCl (10 g/liter), Bacto Tryptone (10 g/liter), and yeast extract (5 g/liter) and was supplemented with ampicillin (25 mg/liter in liquid or 40 mg/liter in solid media) for selection of pBR322-derived plasmids.

Strains. *E. coli* host strains B_E (nonsuppressing [*sup*^{0}]) and CR63 (carrying a *supD* amber suppressor) were described by Edgar et al. (17). MCS1 (*supD* and transformation competent) and AB1 (nonsuppressing and transformation competent) were described by Kreuzer et al. (33). EST2764 (C1412 *ssb*$^+$) and EST2765 [C1412 *ssb-1*(Ts)] were a generous gift from I. Tessman (Purdue University). DG76 (*leuB6 thyA47 deoC3 rpsL153*) and PC3 [*leuB6 thyA47 deoC3 rpsL153 dnaG3*(Ts)] were from the *E. coli* Genetic Stock Center (Yale University).

T4 strain K10, which is considered the wild-type control in this study, has the following mutations: *am*B262 (gene *38*), *am*S29 (gene *51*), *nd*28 (*denA*), and *r*IIPT8 (*r*II-*denB* deletion) (55). The absence of *r*II proteins has no apparent effect on origin- or recombination-dependent plasmid replication or on phage genomic replication, as judged by experiments with a K10-*r*II$^+$ derivative (4a). Strain K10-*uvsY*Δ is isogenic except for the presence of a 0.12-kb deletion that removes *ori(uvsY)* and renders the phage *uvsY* mutant (33); K10-*uvsY*Δ has also been referred to as K10-608 by Derr and Kreuzer (11). Phage strains with the following mutations were from the collection of B. M. Alberts: *am*A453 (gene *32*), *am*N81 (gene *41*), *am*E4332 (gene *43*), *am*N82 (gene *44*), *am*E10 (gene *45*), *am*HL627 (gene *61*), and *am*E1140 (gene *62*). The T4 K10 (and K10-*uvsY*Δ) derivatives of each amber mutant were generated by two successive crosses with an unequal parental input (1 *am*:10 K10) (see reference 33 for analogous constructions). The presence of the *uvsY* mutation in the K10-*uvsY*Δ derivatives was tested by complementation of the small-plaque phenotype by a plasmid that produces UvsY protein.

Construction of gene *61* deletion mutant phage. The gene *61* deletion mutation was generated by in vitro construction of plasmid pKB16 and then deposited in the T4 genome by using the insertion/substitution system (55). Plasmid pKB16 incorporates an upstream and a downstream fragment cloned from the gene *61* region, without an intervening 969-bp region that contains most of the gene *61* coding sequence. The upstream fragment consisted of a 400-bp *Taq*I-*Sty*I fragment with *Kpn*I linkers attached at the *Sty*I site. This fragment was ligated to the T4 insertion/substitution vector pBSPL0- (55), which had been treated with *Cla*I and *Kpn*I and then with alkaline phosphatase, to generate plasmid pKB615. The downstream fragment consisted of a 304-bp *Bst*NI-*Nde*I fragment with *Kpn*I linkers attached at the *Bst*NI site. This fragment was ligated to plasmid pKB615, which had been treated with *Kpn*I and *Nde*I and then with alkaline phosphatase, to generate plasmid pKB16. A phage with integrated pKB16 was obtained by selecting for homologous recombination between the plasmid and the phage K10 genome. Because T4 K10 contains *38* amber and *51* amber mutations, only phage with integrated *supF*-containing plasmid were able to grow on a nonsuppressing host (55). Such integrants were then grown in suppressing cells, allowing propagation of plasmid-free segregant phage that resulted from a second homologous recombination event. Segregant phage containing the *61*Δ mutation were identified by finding segregants that were unable to form *61*$^+$ recombinants when crossed with a *61* amber mutant. The presence of the *61*Δ mutation was confirmed by extracting DNA from a plaque-purified lysate and digesting it with *Pac*I, which cleaves T4-modified DNA. The K10-*61*Δ mutant lacks a 5-kb *Pac*I fragment and displays a novel 4-kb band, as predicted by the size and location of the deletion mutation.

DNA replication assays. *E. coli* MCS1 or AB1 containing the indicated plasmid was incubated with vigorous shaking at 37°C in L broth to a density of 4×10^8 cells per ml and then infected with the indicated T4 strain at a multiplicity of 3 PFU per cell. After a 3-min incubation without shaking to allow phage adsorption, the infected cultures were incubated for the indicated times with vigorous shaking (the 3-min adsorption period is not included in the stated times of infection). Total DNA was then prepared as previously described (33). Briefly, the infected cells and any released phage particles were collected by centrifugation and pooled, treated with sodium dodecyl sulfate-proteinase K, extracted

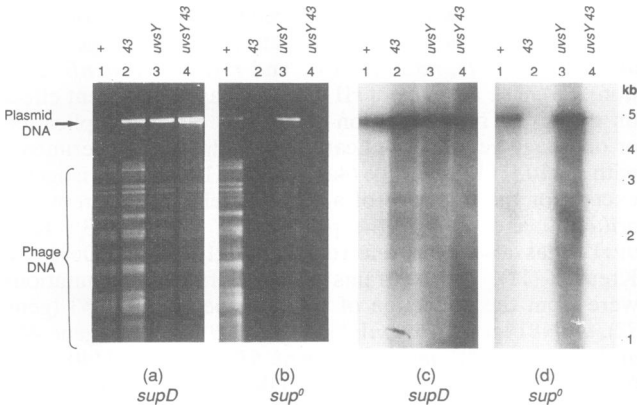

FIG. 1. Replication of origin-containing plasmid by DNA polymerase mutant. *E. coli* MCS1 (*supD*; a and c) and AB1 (*sup*^{0}; b and d) with the origin-containing plasmid pGJB1 were infected with T4 K10 (lanes 1), K10-*43* (lanes 2), K10-*uvsY*Δ (lanes 3), or K10-*43 uvsY*Δ (lanes 4). Samples were harvested at 60 min postinfection, and total DNA was cleaved with *Ssp*I and *Hae*III. The resulting fragments were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide (a and b). The same gel was subjected to Southern hybridization, using a radioactively labelled pBR322 probe to reveal more clearly the extent of plasmid replication (c and d). Phage DNA is indicated by a brace, and plasmid DNA is indicated by an arrow. The size scale was generated from the migration of *Xba*I fragments of unmodified T4 DNA (36).

sequentially with phenol, phenol-chloroform-isoamyl alcohol, and chloroform-isoamyl alcohol, and finally dialyzed against 10 mM Tris [pH 7.8]–0.5 mM Na_3EDTA. The DNA samples were digested with *Ssp*I and *Hae*III, and the resulting DNA fragments were resolved by electrophoresis through 0.8% agarose gels and visualized by ethidium bromide staining. Quantitative estimates of fold reduction in plasmid replication were determined by performing serial dilutions of restriction digests and matching the band intensities between the two relevant samples. Southern hybridization was performed as described by Maniatis et al. (41). The probe for the Southern blots was *Eco*RI-linearized pBR322, labelled by the random-primed method (Boehringer Mannheim kit).

RESULTS

Replication of plasmid models requires T4 DNA polymerase and accessory proteins. Plasmid model systems for T4 replication strategies are valid only if the plasmid replication depends on the T4 DNA polymerase (gp43), which is required for all phage genomic replication (12, 20, 61). We therefore tested whether replication of an origin-containing plasmid (pGJB1) requires the T4 DNA polymerase. Replicated DNA was examined following infection of plasmid-bearing cells by the control T4 strain K10 (Fig. 1, lanes 1) or by a K10 derivative with the gene *43* amber mutation *am*E4332 (lanes 2). To verify the importance of the amber allele, each infection was carried out in a suppressing host (MCS1; Fig. 1a and c) and in a nonsuppressing host (AB1; Fig. 1b and d). The DNA samples were digested with *Ssp*I, which cleaves T4 DNA into a series of fragments that are approximately 3 kb and smaller while cleaving replicated plasmid DNA into an approximately 4.5-kb fragment. *Hae*III was included in the digests to increase the sensitivity of the replication assay. This enzyme cleaves unreplicated (unmodified) plasmid DNA into a series of much smaller fragments but does not cleave T4-replicated (T4-modified) plasmid DNA. The resulting DNA fragments were separated by electrophoresis and visualized by ethidium bromide staining (Fig. 1a and b) and by Southern hybridization with a pBR322 probe (Fig. 1c and d). Mutational inactivation of the phage DNA polymerase completely eliminated replication of both the phage genomic and origin-containing plasmid DNA (Fig. 1b and d, lanes 2). As expected, suppression of the *43* amber mutation restored both phage and plasmid DNA replication (Fig. 1a and c, lanes 2). Surprisingly, the suppressed amber mutant consistently demonstrated increased replication of both phage and plasmid DNA compared with the *43*$^+$ control (compare lanes 1 and 2 in Fig. 1a and c). It appears that suppression of the *43* amber mutation by *supD* creates a missense protein with properties different from those of the wild-type polymerase.

The origin on plasmid pGJB1 [*ori*(*uvsY*)] has approximately 120-bp homology to the K10 phage genome, which could allow recombination-dependent replication of the plasmid (35). We limited the T4-induced replication of this plasmid to origin-dependent initiation by infection with T4 strain K10-*uvsY*Δ. This phage carries a precise deletion of the T4 restriction fragment present in pGJB1 and therefore presents no homology to the plasmid. In addition, the deletion eliminates the production of UvsY protein, which is required for recombination-dependent initiation of plasmid and phage genomic DNA synthesis (33, 35). As expected from the results with the *uvsY*$^+$ strains, the double mutant K10-*43 uvsY*Δ induced no genomic or plasmid DNA replication (Fig. 1b and d, lanes 4). The single *uvsY*Δ mutant showed reduced genomic replication and enhanced plasmid replication (Fig. 1b and d, lanes 3) compared with the K10 control (Fig. 1b and d, lanes 1). The reduction in genomic replication is due to the involvement of UvsY in phage recombination-dependent replication (33, 35). The enhanced replication of origin-containing plasmids by *uvsY* mutants is not understood but could arise either from an increased initiation frequency or from an enhanced transition to rolling-circle forms that produce many copies of product per initiation event (see Discussion and reference 33). The main conclusion from the results shown in Fig. 1, however, is that a plasmid model system for origin-dependent replication requires the T4 DNA polymerase.

Replication of plasmids that incorporate nonorigin fragments of T4 DNA (nonorigin plasmids; e.g., pKK467) requires T4 recombination proteins, including UvsY, UvsX, and gp46 (35). To determine whether the T4 DNA polymerase is also required for recombination-dependent replication of a nonorigin plasmid, pKK467-bearing host cells were infected by K10 (Fig. 2, lanes 1) or by K10-*43* (lanes 2). The suppressed *43* amber mutant again produced elevated levels of replicated plasmid and phage genomic DNA (Fig. 2a and c, lanes 2), and the nonsuppressed *43* amber mutant failed to replicate either the plasmid or the phage DNA (Fig. 2b and d, lanes 2). As expected, the nonorigin plasmid did not replicate in a *uvsY*Δ infection, regardless of the presence of the *43* mutation and amber suppressor (data not shown; also see reference 35). These results demonstrate that the plasmid model system for T4 recombination-dependent initiation of replication requires the T4 DNA polymerase.

Mutations in T4 genes *45*, *44*, and *62* also eliminate phage genomic replication (18, 20); the corresponding gene products constitute the T4 DNA polymerase accessory proteins (for a review, see reference 50). Replication of both plasmid model systems was found to depend on the polymerase

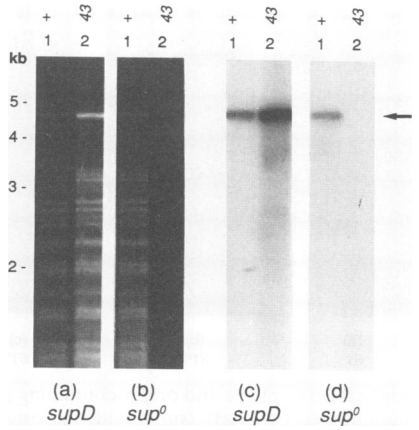

FIG. 2. Replication of nonorigin plasmid by T4 DNA polymerase mutant. *E. coli* MCS1 (*supD*; a and c) and AB1 (*sup*^{0}; b and d) with the nonorigin plasmid pKK467 were infected with K10 (lanes 1) or K10-*43* (lanes 2). Samples were harvested at 60 min postinfection, and total DNA was isolated and cleaved with *Ssp*I and *Hae*III. The resulting fragments were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide (a and b). The same gel was subjected to Southern hybridization, using a radioactively labelled pBR322 probe (c and d). Plasmid DNA is indicated by an arrow on the right, and the size scale on the left was generated as for Fig. 1.

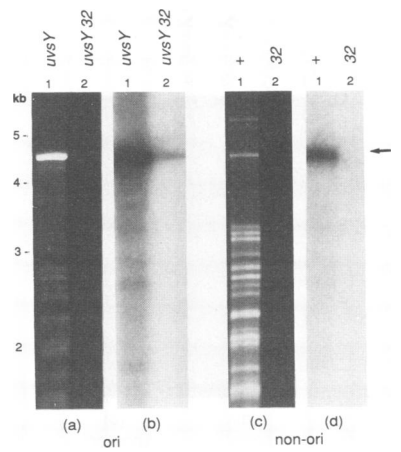

FIG. 3. Replication of origin-containing and nonorigin plasmids by the gene *32* mutant. *E. coli* AB1 (*sup*^{0}) with the origin-containing plasmid pGJB1 (a and b) or the nonorigin plasmid pKK467 (c and d) was infected with K10-*uvsY*Δ (a and b, lanes 1), K10-*32 uvsY*Δ (a and b, lanes 2), K10 (c and d, lanes 1), or K10-*32* (c and d, lanes 2). Samples were harvested at 60 min postinfection, and total DNA was isolated and cleaved with *Ssp*I and *Hae*III. The resulting fragments were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide (a and c). The same gel was subjected to Southern hybridization, using a radioactively labelled pBR322 probe (b and d). Plasmid DNA is indicated by an arrow on the right, and the size scale on the left was generated as for Fig. 1.

accessory proteins. The *45* amber and *44* amber mutations eliminated replication of both the origin-containing and nonorigin plasmids (data not shown). The *62* amber mutation greatly reduced but did not eliminate plasmid replication; the very low level of replication observed could be due to ribosomal suppression of the *am*E1140 allele (65).

Replication of plasmid models by a gene *32* amber mutant. Gene *32* mutants are deficient in phage DNA replication, recombination, and repair (20, 59, 64). However, a small amount of phage DNA synthesis has previously been detected in gene *32* mutant infections, suggesting that one or more pathways of T4 replication can operate, albeit inefficiently, in the absence of gp32 (5, 48). We therefore tested the involvement of gp32 in the replication of origin and nonorigin plasmid models systems. A very low level of replication of the *ori*(*uvsY*)-containing plasmid (pGJB1) was detected in the *uvsY*Δ *32* (*am*A453) double-mutant infection of nonsuppressing cells (Fig. 3a and b, lanes 2). Comparison of serial dilutions of the *uvsY*Δ digest with the *uvsY*Δ *32* digest revealed that the *32* amber mutation reduced replication of the origin-containing plasmid by about 60-fold. A different gene *32* amber mutation (*am*E315) gave similar results, and a plasmid incorporating *ori*(*34*) instead of *ori*(*uvsY*) replicated to a similar level (data not shown). In addition, the same low level of plasmid replication was observed with a *uvsY*$^+$ *32* infecting phage (data not shown); apparently, the enhancement of origin-containing plasmid replication by a *uvsY* mutation does not occur in the absence of gp32.

The small amount of residual replication in the *32* amber mutant infections could be due to substitution by the host SSB protein. Therefore, infections were carried out in isogenic, nonsuppressing *ssb*$^+$ and *ssb-1*(Ts) host strains (58). The same level of plasmid replication occurred during *32* amber mutant infections of the *ssb*$^+$ or *ssb-1* host cells at high temperature (data not shown), indicating that the host SSB protein is not responsible for the low level of replication. The residual replication of the origin-containing plasmids may depend on a low level of read-through of the gene *32* amber mutations or on some residual activity of the amber peptides, which are believed to retain the DNA-binding domain of gp32 (22, 57). Alternatively, gp32 may not be strictly required for origin-dependent plasmid replication.

In contrast, replication of the nonorigin plasmid in a nonsuppressing host was abolished by the *32* amber mutation (Fig. 3c and d; compare lanes 1 and 2). Thus, T4 single-stranded DNA-binding protein is strictly required for recombination-dependent replication of a plasmid, indicating that recombination-dependent initiation is more critically dependent on gp32 than is origin-directed initiation.

Replication of plasmid models by a T4 helicase mutant. Amber mutants deficient in the T4 helicase (gp41) were originally classified as DNA negative (20). However, *41* amber mutants produce a low level of newly replicated DNA, some of which consists of single-stranded fragments (52, 62). As in the case of gp32 deficiency, one or more pathways of T4 replication may have some function in the absence of gp41. An amber mutation in gene *41* was therefore assayed for its effect on the replication of the phage genome and plasmid model systems. The *41* amber mutation substantially reduced, but did not eliminate, T4 genomic replication in either the *uvsY*$^+$ (Fig. 4a and b, lanes 2) or *uvsY*Δ (Fig. 4a, lane 4) genetic background. The *41* amber mutation reduced the replication of the origin-containing plasmid by less than 2-fold in the *uvsY*$^+$ (Fig. 4a, lanes 1 and 2) background and by about 10-fold in the *uvsY*Δ (Fig. 4a, lanes 3 and 4) background. The relatively large reduction in the *uvsY*Δ background could be explained, at least in part, by proposing that gp41 is necessary for the enhancement of plasmid replication in the absence of UvsY (see Discussion). This explanation is supported by the similar levels of plasmid replication in the *41* and *41 uvsY*Δ infections (Fig. 4a, lanes

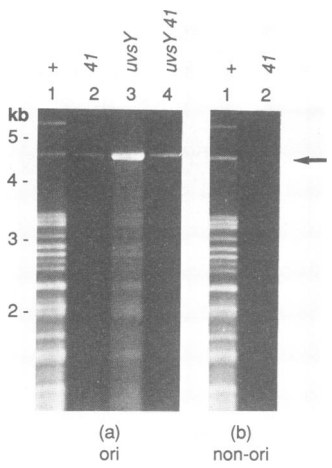

FIG. 4. Replication of origin-containing and nonorigin plasmids by a gene *41* mutant. *E. coli* AB1 (*sup*^{0}) with the origin-containing plasmid pGJB1 (a) or the nonorigin plasmid pKK467 (b) was infected with K10 (lanes 1), K10-*41* (lanes 2), K10-*uvsY*Δ (lane 3), or K10-*41 uvsY*Δ (lane 4). Samples were harvested at 60 min postinfection, and total DNA was isolated and cleaved with *Ssp*I and *Hae*III. The resulting fragments were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide. Plasmid DNA is indicated by an arrow on the right, and the size scale on the left was generated as for Fig. 1.

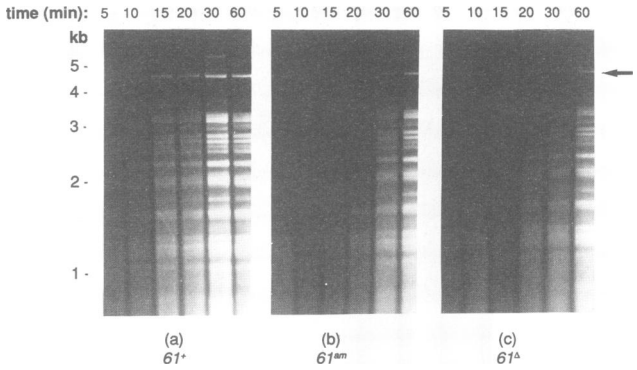

FIG. 5. Replication of phage and origin-containing plasmid DNA by gene *61* mutants. *E. coli* AB1 (*sup*^{0}) with the origin-containing plasmid pGJB1 was infected with K10 (a), K10-*61* (b), or K10-*61*Δ (c). Samples were harvested at the indicated times postinfection, and total DNA was isolated and cleaved with *Ssp*I and *Hae*III. The resulting fragments were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide. Plasmid DNA is indicated by an arrow on the right, and the molecular size scale on the left was generated as for Fig. 1.

2 and 4). With respect to recombination-dependent replication, the single *41* amber mutation reduced the replication of the nonorigin plasmid by approximately sixfold (Fig. 4b). These results indicate that replication of both plasmids normally utilizes gp41 when it is available but that significant amounts of plasmid replication can occur in the amber mutant infections (see Discussion).

A T4 primase deletion mutant is viable and displays a DNA delay phenotype. Mutations in gene *61* cause a DNA delay phenotype, with most phage genomic replication occurring at an unusually late time of infection (18, 62, 67). Unlike the T4 amber mutations analyzed above, unsuppressed gene *61* amber mutations do not normally cause lethality although they do reduce the burst and plaque sizes (20, 67). The relatively modest growth defect of a *61* amber mutant is difficult to understand, because gp61 is the only known RNA primase that functions with the T4 replication machinery (6, 26; for a review, see reference 50). To further investigate the phenotype of a *61* amber mutant, we conducted replication experiments of the type described above. The *61* amber mutant was found to replicate both origin-containing and nonorigin plasmids, with the same delayed kinetics as for the mutant's genomic DNA synthesis (46; see below).

These results imply that either the gp61 primase is not strictly required for T4 replication or the amber mutant produces a low level of functioning protein (by read-through of the stop codon, a partially functional amber peptide, or an internal restart codon following the amber codon). To ensure the complete absence of gp61 and to ascertain whether the primase is strictly required for T4 replication, a novel phage strain was created by deleting nearly the entire gene *61*. This was accomplished by constructing a recombinant plasmid with the deletion mutation and then substituting the deletion into the T4 genome by using the T4 insertion/substitution system (see Materials and Methods) (55). The *61*Δ mutation removes 969 bp of the 1,029-bp gene *61* coding region and causes a small-plaque phenotype resembling that of a *61* amber mutant. Because the deletion mutant is viable, gp61 is not an essential phage protein.

The replication phenotype of the *61*Δ mutant was compared with that of the *61* amber mutant and the *61*$^+$ control (K10) by isolating DNA samples at various times after infection of host cells that carried an origin-containing plasmid (pGJB1; Fig. 5). T4 *61*$^+$ genomic replication was detected by 10 min postinfection and continued until at least the 30-min time point (Fig. 5a). In contrast, most of the genomic replication by the *61* amber or *61*Δ mutant occurred in the period between 30 and 60 min postinfection (Fig. 5b and c, respectively). By 60 min, each mutant had replicated nearly as much DNA as had the *61*$^+$ control phage, clearly demonstrating that T4 DNA replication does not require the gp61 primase. Nonetheless, the T4 replication machinery presumably utilizes gp61 when it is available, as indicated by the severe delay in replication when the protein was absent. The phage DNA replication observed in the *61*Δ mutant infection apparently does not require the host DnaG primase, because a *dnaG*(Ts) mutation did not affect *61*Δ genomic replication (data not shown; also see reference 47).

As in the case of the phage genome, the origin-containing plasmid replicated at relatively early times in the *61*$^+$ control infection (Fig. 5a) but at very late times in the *61* mutant infections (Fig. 5b and c). gp61 is therefore not required for plasmid replication directed by *ori*(*uvsY*), although the primase is apparently utilized when it is available, and replication is severely delayed when it is not.

The genomic replication of the *61*Δ mutant is unusual because it does not utilize the gp61 primase and because it occurs mostly at very late times. Extensive genomic replication begins after the onset of late protein synthesis, which is itself delayed by the *61*Δ mutation (data not shown). Why is the *61*Δ mutant replication delayed, and what are the requirements for genomic replication in the absence of the gp61 primase? Previously, genomic replication by a *61* amber mutant was shown to require gp46, suggesting that it is a form of recombination-dependent replication (25). We therefore tested the requirement for gp46 in a *61*Δ mutant

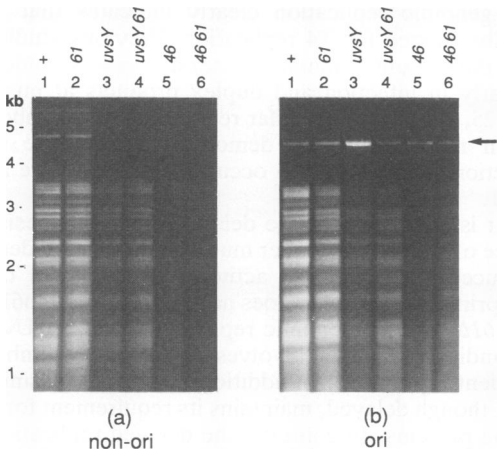

FIG. 6. Evidence that *61Δ* phage and nonorigin plasmid DNA replication requires UvsY and gp46. *E. coli* AB1 (*sup*^{0}) with the nonorigin plasmid pKK467 (a) or the origin-containing plasmid pGJB1 (b) was infected with K10 (lanes 1), K10-*61Δ* (lanes 2), K10-*uvsYΔ* (lanes 3), K10-*61Δ uvsYΔ* (lanes 4), K10-*46* (lanes 5), or K10-*61Δ 46* (lanes 6). Samples were harvested at 60 min postinfection, and total DNA was isolated and cleaved with *Ssp*I and *Hae*III. The resulting fragments were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide. Plasmid DNA is indicated by an arrow on the right, and the size scale on the left was generated as for Fig. 1.

TABLE 1. Summary of plasmid replication results

Mutation	Plasmid replication[a]		
	Origin dependent		Recombination dependent
	uvsY$^+$	*uvsY*	
None	+	++	+
43, *44*, *45*, or *62*	−	−	−
32	−[b]	−[b]	−
41	+/−	+	+/−
61	+[c]	+[c]	+[c]

[a] +, normal wild-type level; ++, enhanced over wild-type level; +/−, modest reduction from wild-type level; −, 50-fold or greater reduction from wild-type level.
[b] Approximately 2% of the wild-type level of replicated plasmid.
[c] Delayed plasmid replication.

infection by constructing a double *61Δ 46* mutant. Because UvsY is also involved in recombination-dependent replication, a *61Δ uvsYΔ* double mutant was also generated. The two double mutants, each single mutant, and the nonmutant control (K10) were used to infect nonsuppressing host cells containing either a nonorigin plasmid (pKK467; Fig. 6a) or an origin-containing plasmid (pGJB1; Fig. 6b). As expected, genomic replication was markedly reduced in the *uvsYΔ* or *46* amber single-mutant infections (Fig. 6, lanes 3 and 5, respectively) but not in the *61Δ* single-mutant infections (lanes 2). The extensive genomic replication by the *61Δ* mutant (lanes 2) was markedly reduced in either the *61Δ uvsYΔ* (lanes 4) or *61Δ 46* (lanes 6) infections. Therefore, much T4 genomic replication in the absence of gp61 occurs via recombination-dependent initiation.

Turning to the plasmid models, the nonorigin plasmid replicated to similar levels in the *61Δ* mutant infection and the nonmutant control infection (Fig. 6a, lanes 1 and 2). As in the case of phage genomic replication, nonorigin plasmid replication occurred at much later times in the *61Δ* than in the *61*$^+$ infection (data not shown). As previously observed (35), nonorigin plasmid replication was greatly reduced or abolished by a mutation in gene *uvsY* (lane 3) or *46* (lane 5). The delayed nonorigin plasmid replication in the absence of gp61 also required both UvsY and gp46, as indicated by the lack of replication in the *61Δ uvsYΔ* and *61Δ 46* infections (lanes 4 and 6, respectively). We conclude that recombination-dependent replication of nonorigin plasmids does not require gp61, although plasmid replication is severely delayed in its absence. The delayed nonorigin plasmid replication that occurs in the absence of gp61 still requires UvsY and gp46.

To summarize, in the absence of gp61, nonorigin plasmids and the phage genome both exhibit a delayed form of recombination-dependent replication. However, an origin-containing plasmid also exhibited delayed replication in the *61* mutant infections (Fig. 5). Is the delayed replication of the origin-containing plasmid also recombination dependent? Apparently not, because this plasmid replicated as extensively in the *61Δ uvsYΔ* and *61Δ 46* double-mutant infections as in the *61Δ* single-mutant infection (Fig. 6b, lanes 4, 6, and 2, respectively). We conclude that bona fide origin-dependent replication can occur in an infection lacking gp61. Interestingly, the enhanced replication of the origin-containing plasmid in the *uvsYΔ* mutant background did not occur in the *61Δ uvsYΔ* double mutant (Fig. 6b, lanes 3 and 4).

DISCUSSION

Plasmid model systems for T4 origin- and recombination-dependent initiation of replication are shown here to involve phage-directed replication, using all seven T4 replication fork proteins for normal levels and/or timing of DNA synthesis (Table 1). These results thus support the use of plasmid models to aid analysis of the multiple strategies of T4 replication initiation. As expected, the T4 DNA polymerase and at least two of its accessory proteins (gp45 and gp44) are required for any T4-related DNA synthesis. An amber mutation in gene *62*, which encodes the third accessory protein, greatly reduced but did not completely eliminate replication of both plasmid models. Either gp62 is not strictly required for plasmid replication or the residual replication in the *62* amber infection is dependent on a low level of gp62 produced in the amber mutant infection.

Origin- and recombination-dependent plasmid replication both showed strong requirements for the T4 single-stranded DNA-binding protein, gp32. However, while either of two *32* amber mutations eliminated replication of a nonorigin plasmid, both left approximately 2% of the normal level of replication of an origin-containing plasmid. The stricter requirement for gp32 in recombination-dependent replication is likely to reflect a required role of the protein in the generation or stability of recombinational intermediates prior to replication fork assembly, since gene *32* mutations markedly reduce genetic recombination (59). In addition, Mosig et al. (48) have previously presented evidence that gp32 is strictly required for recombination-dependent initiation of T4 genomic DNA replication.

A small amount of replication from a plasmid-borne origin apparently occurs in the absence of gp32, unless both amber mutants produce a partially functional fragment of gp32 or a low level of complete protein by read-through of the amber codons. The small amount of replication does not depend on the analogous *E. coli* SSB protein, as judged by the lack of effect of a host *ssb* mutation. Therefore, T4 origins may be

capable of directing inefficient replication in the absence of host or phage single-stranded DNA-binding protein. Recent experiments with the in vitro T4 replication system have shown that gp32 is not required for a functional replication fork, as long as a single-stranded region is provided for the loading of the gp41 helicase (8). If the replication fork can function in the absence of gp32, then why did both *32* amber mutations markedly reduce replication of the origin-containing plasmid? One possible explanation is that gp32 assists in the proper assembly of replication complexes at the origin, a process that is not required in the in vitro replication system. Another possibility, suggested by Mosig et al. (48), is that replicative intermediates and/or products are degraded by nucleases in the absence of gp32. Yet another possible explanation is that, in the case of origin-containing plasmids, gp32 is required for a transition from bidirectional to rolling-circle replication. A T4 origin [*ori*(*34*)] cloned into the *lac* operon of *E. coli* induced replication in both directions, indicating that replication is initially bidirectional (30a). However, a major product of plasmid replication is long plasmid concatemers, indicating that plasmid rolling circles can be formed (35). Because rolling circles generate many more product strands than do bidirectional forms, even a low frequency of conversion from bidirectional to rolling-circle replication could account for most of the product DNA in wild-type infections. A block in this conversion could therefore considerably reduce the level of replicated origin-containing plasmid.

In the in vitro replication system, gp41 serves as the replicative DNA helicase activity and is necessary for gp61 primase activity (6–8, 26, 27, 54, 60). Furthermore, *41* amber mutations block most T4 DNA synthesis and cause lethality (20, 62). The slight amount of phage genomic DNA replication by *41* amber mutants could be due to residual gp41 activity (e.g., due to read-through of the amber codon), or alternatively, limited genomic replication may be possible in the absence of gp41. In the latter case, an alternate helicase (30) could be involved in phage replication. In this study, a gene *41* amber mutation was found to reduce, but not eliminate, plasmid replication initiated by either an origin- or recombination-dependent mechanism. Preliminary results indicate that plasmid replication in the *41* mutant infections is delayed, as in *61* mutant infections. The reduction in replication of both plasmid models indicates that gp41 is normally utilized in both modes of replication, but the residual replication suggests that gp41 may not be strictly required for either. The residual replication could be due to leakiness of the amber mutation, as discussed above for the gene *32* mutations. A second possibility is that some other phage- or host-encoded helicase can substitute for gp41 in T4-directed plasmid replication. A final possibility is that replication can occur in the absence of a helicase. In any case, the possible gp41-independent replication of the origin-containing plasmid is of particular interest. Loading of the replication fork helicase has been shown to be one of the earliest steps in the initiation of replication at diverse origins, including those of simian virus 40, *E. coli*, and phage λ (2, 3, 10, 14, 15, 63). If the origin-dependent replication in the *41* amber mutant infection is not due to leakiness of the amber mutation, then initiation from T4 origins may be mechanistically distinct from those systems.

This report describes the generation of a deletion mutation that removes almost all of T4 gene *61*. The *61*Δ strain replicates DNA, though in a delayed fashion, and generates a productive infection. Thus, the gp61 primase is not an essential T4 protein. The severe delay in both plasmid and phage genomic replication clearly indicates that gp61 is normally utilized for T4 replication. Previous studies have shown that *61* amber mutants generate single-stranded products early in infection and duplex products at much later times (25, 47, 67). The similar replication of an amber and a deletion mutant (Fig. 5) demonstrates that the delayed production of duplex DNA occurs in the complete absence of gp61.

What is the nature of the delayed DNA synthesis in the absence of gp61? A *61* amber mutation eliminated detectable T4-induced RNA primase activity (37, 56), and the host DnaG primase apparently does not substitute for gp61. Much of the *61*Δ delayed genomic replication requires UvsY and gp46, indicating that it involves a form of recombination-dependent replication. In addition, nonorigin plasmid replication, though delayed, maintains its requirement for recombination proteins. In contrast, the delayed replication of an origin-containing plasmid in a *61*Δ infection does not require UvsY and gp46. These results demonstrate that gp61-independent replication can occur by at least two strategies, and these appear to be modifications of the wild-type recombination- and origin-dependent initiation strategies. gp61-independent replication may require a late protein that substitutes for the gp61 primase, as indicated by the delay in T4 *61*Δ replication until after the onset of late protein expression. Mosig et al. (47) have recently presented evidence for a role of a specific late protein in gp61-independent replication. A mutation in gene *49*, which is expressed mainly late in infection (4), abolished replication in a *61* amber mutant infection. This result led to a model in which gp61 is necessary for lagging-strand but not leading-strand replication from T4 origins at early times; as the infection progresses, the recombination endonuclease encoded by gene *49* provides primers for lagging-strand replication. In this model, gp49 cleaves recombinational intermediates formed by invasive single-stranded ends which are generated by origin-directed replication in the absence of lagging-strand synthesis (47). Perhaps this model can be adapted to explain the delayed replication of both origin-containing and nonorigin plasmids; long single-stranded concatemers might be generated by rolling-circle plasmid replication in the absence of gp61, and cleavage of a recombination intermediate involving the 5′ end of the concatemer might allow replication of the single-stranded product into a duplex.

The extensive replication of an origin-containing plasmid in the complete absence of gp61 demonstrates that gp61 primase is not required for the priming of leading-strand replication from the origin. Both *ori*(*uvsY*) and *ori*(*34*) contain a middle-mode promoter, which could allow priming by an RNA polymerase transcript, and multiple copies of the sequence required for priming by gp61 (42). The gp61-independent origin replication therefore strongly suggests that *E. coli* RNA polymerase can provide the primer for leading-strand synthesis from the origins. However, it is still possible that gp61 provides the primer when the primase is available; use of the RNA polymerase transcript in the absence of gp61 could be very inefficient, explaining the delay in origin-containing plasmid replication in the *61*Δ infection. Alternatively, initiation of leading-strand replication may always utilize the RNA polymerase transcript, and the delay in plasmid replication could result from a deficiency in lagging-strand replication caused by an inability to prime Okazaki fragment synthesis.

Mutations in the *uvsY* gene lead to enhanced levels of replicated origin-containing plasmid (33). As explained above, an increased production of plasmid rolling circles

could be responsible for the enhanced replication, or alternatively, the absence of UvsY may cause an increase in the frequency of origin initiation. Although we cannot now distinguish between these two models, the results presented here provide clues to the enhanced plasmid replication of *uvsY* mutants. Most importantly, mutations in genes *32*, *41*, and *61* each blocked the enhanced plasmid replication of a *uvsY* mutant. Thus, important components of the replication machinery are involved in the overreplication phenomenon. Furthermore, a suppressed *43* amber mutation also led to enhanced replication of plasmids, although perhaps by a different mechanism.

In summary, plasmid model systems for two distinct modes of T4 DNA replication utilize the seven T4 replication fork proteins for the normal amount and/or timing of DNA replication. However, mutations in genes *41* and *61* do not abolish either mode of replication, implying that the replicative helicase/primase complex of T4 is not strictly required for origin-dependent or recombination-dependent DNA replication. Alternate bypass mechanism(s) of replication may operate when the replicative helicase/primase complex is unavailable.

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