Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 3345–3349, May 1985
Genetics

A defective phage system reveals bacteriophage T4 replication origins that coincide with recombination hot spots

(initiation of DNA synthesis/plasmid transduction/site-specific recombination/modified DNA)

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Contributed by Bruce M. Alberts, December 26, 1984

ABSTRACT **Plasmid transduction mediated by bacteriophage T4 has been used to study putative T4 DNA replication origins cloned as inserts in the *Escherichia coli* plasmid pBR322. Two particular inserts from the T4 genome allow high-frequency plasmid transduction, suggesting that each insert might contain a T4 replication origin. T4 infection of these plasmid-containing cells produces large numbers of defective phage particles that contain long linear concatamers of the plasmid DNA. During a second cycle of infection, these defective phage genomes can be replicated better than normal phage chromosomes present in the same infected cell; consequently, the T4 DNA inserts must be functioning as replication origins. Both of these origins appear to utilize a previously unrecognized mode of T4 replication initiation. Moreover, each origin coincides with a major recombination hot spot in the phage genome, and therefore this mode of replication initiation seems to involve a local stimulation of homologous genetic recombination. From a purely practical standpoint, additional DNA fragments can be cloned in an origin-containing plasmid, allowing isolation of large amounts of any DNA sequence with the glucosylated hydroxymethylcytosine modifications of T4 DNA.**

Bacteriophage T4 provides one of the best-developed systems for studying the mechanisms involved in DNA replication. A mixture of seven highly-purified T4 proteins catalyzes efficient *in vitro* DNA synthesis with characteristics very similar to those observed *in vivo* (1). The one glaring deficiency of this *in vitro* system is its inability to initiate replication forks at natural origins of T4 replication; this problem is exacerbated by the apparent complexity of T4 fork-initiation *in vivo*.

One major obstacle to studying T4 initiation stems from the extensive DNA modifications of the phage genome (2). Every deoxycytidine residue in the phage genome is modified at the 5 position with hydroxymethyl groups, and virtually all are glucosylated as well. Most restriction nucleases do not cleave T4-modified DNA, and this complicates the localization of T4 replication origins. However, several apparent origins have been mapped by isolating pulse-labeled DNA at various times after infection and then hybridizing this labeled DNA to restriction enzyme digests of unmodified T4 DNA (reviewed in refs. 3 and 4). The unmodified DNAs used in these studies have been either cloned fragments of the T4 genome or T4 DNA prepared from a special multiple-mutant phage (5, 6). Unfortunately, these methods are unable to define the minimum nucleotide sequence necessary for a functional origin, and there is still controversy over origin locations.

In view of these complexities, we decided to develop a procedure that allows both genetic selection and functional tests for cloned T4 replication origins. The procedure will be described in detail elsewhere. Its most important feature is that cloned replication origins can be manipulated *in vitro* in the form of simple plasmids and then tested for *in vivo* origin activity as linear plasmid concatamers that closely mimic the structure of the natural phage genome. This approach should in principle allow an exact determination of the DNA sequence elements necessary for a T4 replication origin.

The selection and subsequent analysis of putative DNA replication origins depends on the T4-induced transduction of plasmid pBR322 derivatives, which has been studied in other laboratories (7–9). When a T4 mutant deficient in host-DNA breakdown infects a host carrying pBR322, the resulting phage lysate can transduce the plasmid to a new host, albeit at a fairly low frequency. The transducing particles contain fully-modified linear concatamers of pBR322, and the plasmid can be reestablished in naive cells by *recA*-dependent recombination (G. Wilson and W. Konigsberg, personal communication; refs. 8 and 9). By using variations on this procedure, we were able to select from a library of cloned T4 DNA sequences two plasmids (pKK405 and pKK025) carrying inserts that increase the frequency of T4-induced plasmid transduction by about 100-fold (unpublished results). In this communication, we demonstrate that these two plasmids contain T4 DNA inserts that function as replication origins in the T4-infected cell.

MATERIALS AND METHODS

Materials. Restriction enzymes were purchased from New England Biolabs; T4 DNA ligase, from Boehringer Mannheim; proteinase K, from EM Laboratories, Elmsford, NY; human serum albumin, from Worthington; and rifampicin, from Sigma. L broth contains NaCl (10 g/liter), Bacto-Tryptone (10 g/liter), and yeast extract (5 g/liter). Medium M9C contains K_2HPO_4 (7 g/liter), KH_2PO_4 (3 g/liter), NH_4Cl (1 g/liter), NaCl (500 mg/liter), $MgSO_4$ (120 mg/liter), $CaCl_2$ (11 mg/liter), $FeCl_3 \cdot 6H_2O$ (270 μg/liter), $MnCl_2 \cdot 4H_2O$ (20 μg/liter), glucose (10 g/liter), Casamino acids (3 g/liter), tryptophan (20 mg/liter), and thiamine (1 mg/liter).

Strains. *Escherichia coli* B_E (nonsuppressing) and CR63 (*supD*) have been maintained in this laboratory, and the construction of strain MCS-1 (*supD* and transformation-competent) will be described elsewhere. The plasmids pKK024, pKK025, and pKK405 are derivatives of pBR322 with the indicated *Eco*RI fragments of T4 DNA inserted at the *Eco*RI site of the vector. T4 [*33*$^-$(*am*N134) *55*$^-$(*am*BL292) *46*$^-$(*am*N130) *47*$^-$(*am*A456)] was provided by G. Mosig (Vanderbilt University) and T4 [*42*$^-$(*am*C87) *56*$^-$(*am*E51) *denB*(ΔNB5060) *alc*(W7)] was obtained from E. Kutter (Evergreen College). The T4 multiple-mutant strain KK172 was constructed by infecting *E. coli* CR63 (*supD*) with a mixture of T4 (*33*$^-$ *55*$^-$ *46*$^-$ *47*$^-$) and T4 (*42*$^-$ *56*$^-$ *denB alc*), each at a

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Abbreviation: kb, kilobase pair(s).

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multiplicity of infection of 5. Single plaques from the resulting lysate were isolated on CR63, and complementation tests were performed to determine the genotype of each recombinant. The T4 mutant KK172 is *42*$^+$ *56*$^+$ *33*$^-$ *55*$^-$ *46*$^-$ (the genotype of *47* was not determined due to the lack of a suitable tester strain); in addition, it is competent for T4-mediated transduction, which probably requires both the *denB* and *alc* alleles (7, 9).

Preparation of Phage Lysates Containing Concatameric Plasmid DNA. *E. coli* MCS-1 (*supD*) containing the indicated pBR322-derived recombinant plasmid was grown to 2.5 × 10^8 cells per ml at 37°C with vigorous shaking in L broth supplemented with 0.1% (wt/vol) $MgSO_4$ and tryptophan at 20 μg/ml. The cells were infected with T4 strain KK172 (see above) at a multiplicity of 3. After a 3-min period without shaking for phage adsorption, the infected culture was incubated with shaking for 2 hr at 37°C. Lysis was completed by the addition of chloroform, and cellular debris was removed by centrifugation at 7700 × *g* for 10 min.

***In Vivo* DNA Replication Measurements.** *E. coli* B_E was grown at 30°C with vigorous shaking in M9C medium to 2 × 10^8 cells per ml. The cells were infected with the indicated T4 lysate at a multiplicity of 5, and phage adsorption was allowed for 2 min without shaking (not included in subsequent time measurements). The infected culture was then incubated with shaking at 30°C and intracellular DNA was prepared as a function of time. In the experiment shown in Fig. 3*A*, an additional control was added to better duplicate the growth conditions of Luder and Mosig (10)—the cells were preconcentrated to 10^9 per ml by gentle centrifugation prior to infection; this had no significant effect on the results obtained (data not shown).

Total intracellular DNA was prepared by centrifuging the cells for 2 min in an Eppendorf microcentrifuge, freezing the cell pellets in a dry ice/ethanol bath, and resuspending in 50 mM Tris Cl, pH 7.8/100 mM NaCl/10 mM NaEDTA, pH 8/0.2% $NaDodSO_4$ containing proteinase K at 500 μg/ml. After 2 hr at 65°C, the lysate was extracted with buffer-saturated, neutralized phenol; the aqueous phase was treated then with a mixture containing equal volumes of phenol and chloroform/isoamyl alcohol (24:1) and finally with chloroform/isoamyl alcohol alone. The DNA samples were then dialyzed extensively against 10 mM Tris Cl, pH 7.8/0.5 mM NaEDTA, pH 8, and stored at 4°C.

Restriction digests with *Eco*RV (1000 units/ml) were incubated at 37°C for 2 hr in 5% (wt/vol) glycerol/50 mM Tris Cl, pH 9/10 mM $MgCl_2$/10 mM dithiothreitol/human serum albumin (100 μg/ml). Restriction digests with *Aha* III (200 units/ml) were incubated at 50°C for 4 hr in 66 mM potassium acetate/33 mM Tris acetate, pH 7.8/10 mM magnesium acetate/0.5 mM dithiothreitol/albumin (100 μg/ml). These are conditions optimized for cleavage of T4-modified DNA by the two enzymes. The restriction fragments were separated by electrophoresis in 0.6% (Fig. 1) or 0.5% (Fig. 3) agarose gels in TBE buffer (90 mM Tris/90 mM boric acid/2.5 mM NaEDTA, pH 8) and visualized by ethidium bromide staining.

Deletion Analysis of the Cloned Origin from Gene *34*. The deletions of plasmids pKK024 and pKK025 described in Fig. 4 were all generated by cleaving the plasmids at two restriction sites, followed by resealing with T4 DNA ligase. pKK061 and pKK075 result from *Hin*dIII cleavage of plasmids pKK024 and pKK025, respectively. This removes the indicated segments of the T4 DNA insert (see Fig. 4) and a small region of pBR322 vector sequences (from the *Eco*RI to the *Hin*dIII site of the original vector). pKK062 and pKK076 were constructed from pKK024 and pKK025, respectively, in a similar fashion and lack the segment of DNA between the *Sal* I site of the insert and the *Sal* I site of the vector pBR322. *E. coli* MCS-1 cultures separately carrying each plasmid were infected with T4 (*42*$^-$ *56*$^-$ *denB alc*) and the titers of plaque-forming units and plasmid transducing particles were determined, as will be described elsewhere.

RESULTS

Replication of Multiply Mutant T4 Bacteriophages. Our tests of the plasmids pKK405 and pKK025 for T4 replication origins were based on the earlier results of Luder and Mosig (10), which strongly suggested that T4 uses at least two modes of initiation of DNA synthesis. The so-called "primary initiation" mechanism requires a direct involvement of host RNA polymerase and its recognition of some early T4 promoter(s), whereas "secondary initiation" reportedly requires the phage-induced recombination exonuclease encoded by genes *46* and *47* (10). In part, this model was derived from an analysis of a *46*$^-$ *47*$^-$ *33*$^-$ *55*$^-$ quadruple-mutant phage. Because of the gene *46/47* defect, the quadruple mutant should be blocked in secondary initiation (see *Discussion*). Moreover, primary initiation continues indefinitely because RNA polymerase remains able to recognize early promoters (due to the gene *33/55* defect) (10, 11). As measured by [^{3}H]thymidine labeling, DNA synthesis in this mutant was sensitive to rifampicin, a specific inhibitor of the host RNA polymerase (10). This is in marked contrast to the rifampicin-resistance of late DNA synthesis in a wild-type phage infection.

We have reexamined the DNA replication that occurs after infection with the quadruple-mutant phage by measuring increases in intracellular T4 DNA directly (see *Materials and Methods*). As shown in Fig. 1*A*, DNA synthesis continues at late times of infection, with the total amount of phage DNA increasing about 40-fold from 10 min (lane 2) to 90 min (lane 3) post-infection. Therefore, as in the studies of Luder

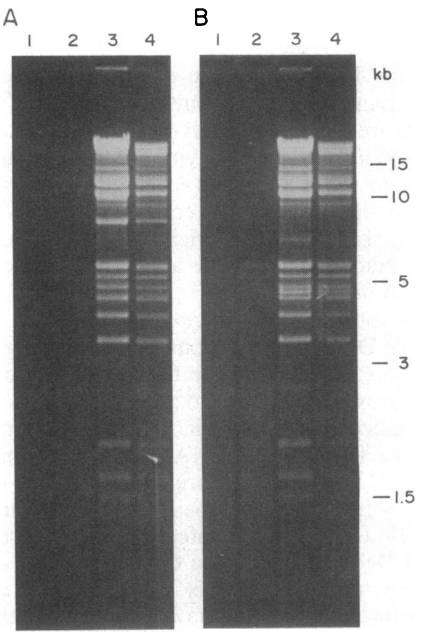

FIG. 1. Effect of rifampicin on the intracellular DNA replication of a multiply mutant T4 bacteriophage. *E. coli* B_E was infected with T4 (*33*$^-$ *55*$^-$ *46*$^-$ *47*$^-$) (*A*) or with T4 strain KK172 (*B*). Intracellular DNA was isolated at 0 min (lanes 1), 10 min (lanes 2), and 90 min (lanes 3) postinfection or at 90 min postinfection after rifampicin was added at 10 min postinfection (lanes 4). The isolated DNAs were treated with *Eco*RV, which cleaves fully modified DNA, and then separated by agarose gel electrophoresis and visualized by staining with ethidium bromide. The restriction patterns of the two phage strains differ slightly due to the NB5060 deletion in T4 KK172. The scale on the right was generated from the migration of *Xba* I restriction fragments of unmodified T4 DNA (12).

and Mosig (10), the *33⁻ 55⁻* defect suppresses the DNA replication deficiency that would otherwise be caused by the *46⁻ 47⁻* mutations. However, rifampicin addition (at 10 min of infection) failed to block all replication in the quadruple mutant. Judging from the relative increase in the amount of phage DNA, approximately four successive rounds of replication occurred in the presence of rifampicin, whereas only slightly more than five occurred in its absence. This raises the possibility that there is a third significant mode of T4 replication initiation, one that is independent of both RNA polymerase and the gene *46/47* exonuclease. In following the nomenclature of Mosig (4), this new mode will be tentatively referred to as "tertiary initiation."

A new multiply mutant T4 strain (KK172), containing the *33*, *55*, and *46* mutations from the quadruple-mutant phage along with mutations that make it competent for plasmid transduction, showed the same response to rifampicin in this experiment (Fig. 1*B*). This new phage strain was used for the plasmid studies to be described below, since, unlike the *33⁻ 55⁻ 46⁻ 47⁻* mutant, it allows the formation of plasmid concatamers.

Replication of Plasmid Concatamers in the T4-Infected Cell. The test for replication origins contained on the recombinant plasmids was carried out with plasmid concatamers that closely resemble the natural substrate for T4 replication initiation, since they are fully modified linear DNA molecules. In an initial infection by T4 strain KK172, a plasmid containing a T4 DNA insert was converted into plasmid concatamers that are packaged into defective phage particles. This first infection (Fig. 2*A*) was performed in a *supD* host to suppress the amber mutations of T4 KK172. The test for concatamer replication was then performed by using this lysate at a high multiplicity in a second infection, so that each cell infected with a defective phage was also infected with several intact phage particles (Fig. 2*B*). Because the amber mutations of T4 KK172 are not suppressed in the second infection, secondary replication initiation should be blocked, whereas primary and the putative tertiary initiation should continue. The extent of DNA replication was judged by

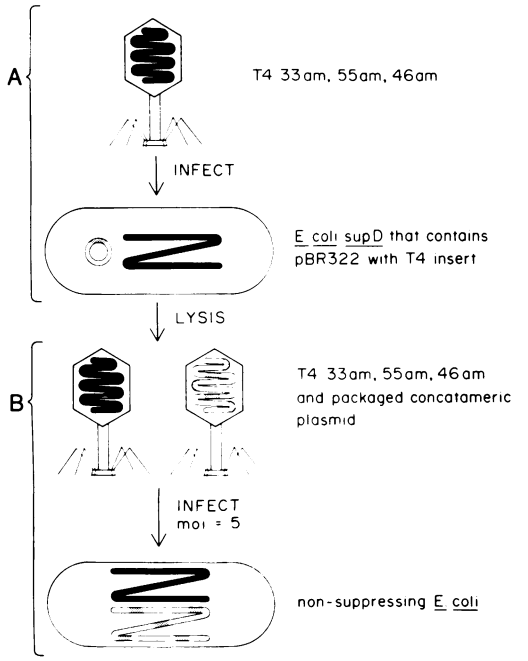

FIG. 2. Test for cloned T4 replication origins. T4 genomic DNA is represented by the filled lines, pBR322 vector DNA by the open lines, and T4 DNA inserted in pBR322 by the stippled lines. See text for details. moi, Multiplicity of infection; am, amber (nonsense) mutation.

treating the purified intracellular DNA with restriction enzyme *Aha* III (which cleaves fully modified T4 DNA) and analyzing the resultant restriction fragments. In an identical experiment, concatamers of pBR322 with no insert were not replicated, as judged by Southern blotting (data not shown).

The plasmids pKK405 and pKK025 both showed striking *in vivo* replication activity in this assay (Fig. 3 *A* and *B*). Both types of plasmid concatamer were replicated significantly in the absence of rifampicin (lane 4) but not more so than the phage genomic DNA. When rifampicin was present from 10 min to 90 min after infection (lane 5), both concatameric plasmids were more efficiently replicated than the phage genome. This is most readily seen from a comparison of the relative band intensities at 90 min (lane 5) with those in a concentrated aliquot of the starting phage DNA sample (lane 2).

Since the concatamers are capable of being replicated better than the phage genomes, they must contain sites able to function as replication origins. The extent of replication eliminates the alternative possibility that these plasmid concatamers are replicated only by virtue of homologous recombination events that allow their integration into the phage genome, as found for other T4 inserts (9). (Some of the concatameric plasmid DNA is nonetheless integrated into phage genomic DNA, as will be described elsewhere.) A second important conclusion is that the function of these two origins is not dependent on a direct involvement of either the host RNA polymerase or the phage gene *46/47* exonuclease. This supports the idea that T4 utilizes three separate modes of replication initiation, and it suggests that these two cloned replication origins operate in the tertiary mode (see *Discus-*

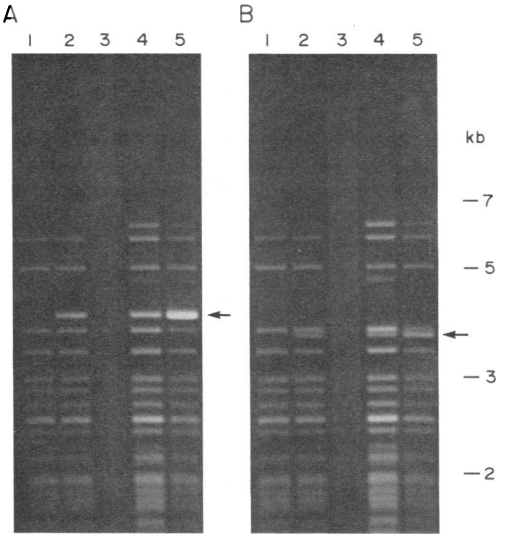

FIG. 3. Concatamers of two recombinant plasmids containing T4 inserts can be replicated autonomously. *E. coli* B_E was infected with lysates of T4 strain KK172 that had been grown on *E. coli* MCS-1 (*supD*) carrying pKK405 (*A*) or pKK025 (*B*). Intracellular DNA was isolated at 10 min (lanes 3) and 90 min postinfection (lanes 4) in the absence of rifampicin and at 90 min postinfection (lanes 5) in the presence of rifampicin (100 μg/ml, added at 10 min postinfection). DNA was also isolated from the phage lysates used for each infection (lanes 2); the concentration of phage DNA in these lanes was adjusted to approximate those in lanes 5 for ease of comparison. Thus, lanes 2 contain DNA that is concentrated 31-fold (*A*) or 40-fold (*B*), compared to lanes 3–5. T4 KK172 DNA (lanes 1) was isolated before growth on the plasmid-containing strains, to determine the positions of the phage genomic restriction bands. Arrow (*A* and *B*) indicates the respective plasmid concatamer restriction band. Each of these bands is absent from phage genomic DNA (lanes 1); their identity as the expected plasmid concatamer restriction fragments was confirmed by further restriction mapping and Southern blotting experiments (data not shown).

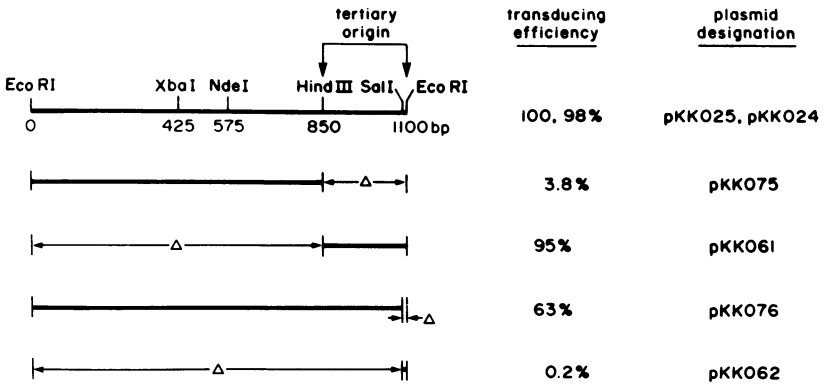

FIG. 4. Deletion analysis of the replication origin from gene *34*. The T4-mediated transducing efficiencies are expressed as relative ratios of the titer of transducing particles to plaque-forming units. The original plasmid (pKK025) allowed the production of 4.3×10^{-4} transducing particles/plaque-forming unit; this value was set at 100%. The opposite orientation of this insert in pBR322 (pKK024) gave an efficiency of 98%, and pBR322 with no insert gave an efficiency of 0.8%. The segment of the T4 DNA insert deleted in each of the derivative plasmids is indicated by the triangles under the restriction map of the insert (see *Materials and Methods*). bp, Base pairs.

sion). The plasmid concatamers were replicated better in the presence of rifampicin than in its absence (Fig. 3). This is likely to reflect a competition for some limiting component of the phage replication apparatus used commonly in all modes of T4 replication.

The Two Cloned Tertiary Origins Are Located at Recombination Hot Spots. The two T4 DNA inserts containing tertiary origins have been localized on the T4 physical map by Southern blotting experiments (results to be shown elsewhere). The insert in pKK025 is a 1.1-kilobase-pair (kb) *Eco*RI fragment from gene *34* (T4 map coordinates 150.75–151.85 kb), and that in pKK405 is a 1.4-kb *Hin*dIII fragment from the region near gene *25* (map coordinates 113.1–114.5 kb; for complete map, see ref. 12). The origin sequence in gene *34* was mapped more precisely by deletion analysis of pKK025 and pKK024 (pKK024 has the same insert as pKK025, but in the opposite orientation). As shown in Fig. 4, the high-frequency transduction of these plasmids is dependent on the 250-base-pair segment contained in plasmid pKK061. Concatamers of plasmid pKK061 were replicated better than intracellular phage DNA in the type of experiment represented in Fig. 3 (data not shown), showing that this segment also functions as a replication origin in linear, fully modified DNA.

Fig. 5*A* summarizes the gradient of genetic recombination across the T4 genome as measured by Womack (13). There is a near-perfect coincidence of the location of the two inserts containing replication origins with the location of two of the three most active recombination hot spots in the T4 genome. A more detailed map of the hot spot in the gene *34* region (Fig. 5*B*) was generated by combining the precise physical map of this region (14) with the genetic data of Womack (13). The 250-base-pair insert from plasmid pKK061 is near the peak of the recombination hot spot (Fig. 5*B*), with enhanced recombination apparent for alleles on both sides of the origin.

DISCUSSION

T4 utilizes several distinct modes of replication initiation. Primary initiation requires a direct involvement of RNA polymerase and is blocked when RNA polymerase is converted into its late form by the products of T4 genes *33* and *55* (10). The products of the first round of T4 DNA replication should have unreplicated single-stranded 3′ ends due to the 5′ → 3′ polarity of DNA polymerase, and, since T4 DNA is circularly permuted and terminally redundant, these free 3′ ends can invade homologous duplex DNA on the same or a different DNA molecule. In the model of Luder and Mosig (ref. 10; see also ref. 4), the secondary mode of T4 replication initiation occurs when this recombination intermediate is converted into a replication fork.

At present, it is not clear whether other kinds of recombination intermediates can also be converted into replication

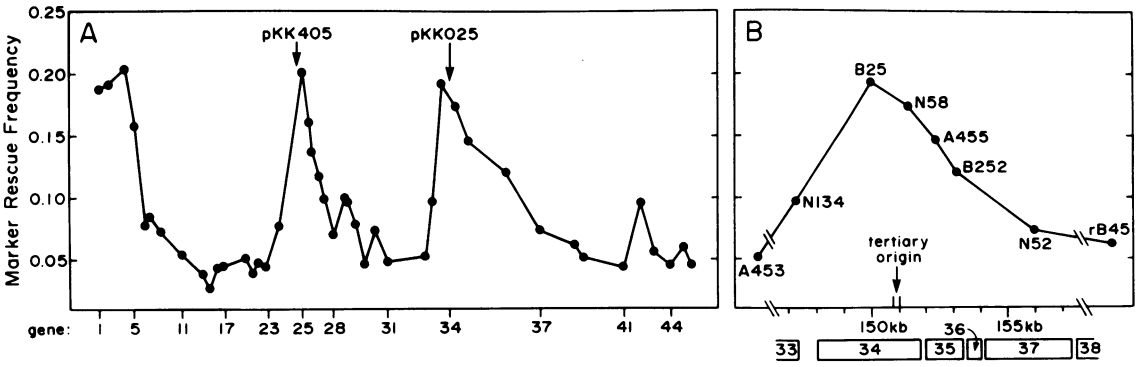

FIG. 5. Coincidence of tertiary origins to major recombination hot spots in the T4 genome. The frequency of marker-rescue for various alleles of T4 was determined by Womack (13). (*A*) Recombination frequencies are shown for all alleles tested across the T4 genome (see ref. 13 for allele designations), and the T4 gene numbers are given for some of the alleles. The location of the inserts from pKK405 and pKK025 are indicated by arrows. (*B*) The recombination data was transferred to the physical map of the gene *34* region determined by Revel (14) (also see ref. 12). Genes are represented by boxes below the map scale. The location of the 250-base-pair segment containing the tertiary origin from gene *34* (see text) is in the small interval indicated by tick marks on the physical map (T4 map coordinates 150.75–151.00 kb). The low frequencies of marker-rescue of alleles A453, N52, and rB45 are typical for regions of the genome without a recombination hot spot (see *A*).

forks by the secondary mode of initiation, and this question is important for deducing the replication mechanism of the two cloned origins studied here. Either of two general models can explain the coincidence of these two origins with recombination hot spots. In the first, each origin is a site that initiates genetic recombination, and DNA synthesis commences only when the resultant recombination intermediates are converted into replication forks by the secondary mode of replication initiation. In the second model, the origin is a site that initiates DNA replication, and as a result of this replication, recombination is enhanced in the vicinity of the origin. The evidence presented here favors the second alternative, since replication of the cloned origins does not require the gene *46/47* exonuclease previously implicated in secondary initiation. One could argue that the exonuclease is required for initiating replication only from certain types of recombination intermediates, but this seems unlikely. We will assume for the remainder of this discussion that the initiation of replication precedes the formation of recombination intermediates at the two cloned origins and, therefore, that a tertiary rather than a secondary mode of initiation is involved.

The data presented here suggest that tertiary initiation plays a significant role in the replication of the phage genome. First, in the absence of secondary initiation, a large amount of T4 phage DNA replication is insensitive to rifampicin (Fig. 1). Second, two distinct segments of the T4 genome operate as potent tertiary origins when contained on either circular plasmids or linear, fully modified concatamers of those plasmids. Third, both of the tertiary origins uncovered here presumably function early in infection (since these origins are active in a 33^- 55^- mutant that expresses only early functions), and both map in regions of the genome where early phage replication origins have been reported. Several groups have reported that phage genomic replication can commence in the interval between T4 map coordinates 110 and 120 kb (refs. 4, 15; C. F. Morris and M. Bittner, unpublished data quoted in ref. 3), and one of the tertiary origins is between map coordinates 113.1 and 114.5 kb. The second tertiary origin maps within the coding sequence of gene *34* at 151 kb, and Morris and Bittner (quoted in ref. 3) reported a possible origin in the region 136–150 kb. In addition, Kozinski and Ling (16) found that the strongest apparent replication origin in a T4 gene 44^- mutant was near (or within) gene *35*; this origin is thus very close to or identical with the second tertiary origin.

Both of the cloned tertiary origins allow the synthesis of long linear DNA concatamers from the circular plasmids in which they are initially contained. The simplest mechanism for this conversion is replication by a rolling-circle intermediate. The *in vitro* T4 DNA replication system is proficient at rolling-circle replication when supplied with a nicked circular plasmid, producing long plasmid concatamers (17). Therefore, the tertiary origin could contain a site that is nicked to begin DNA synthesis, either by a site-specific nuclease or by a site-specific DNA topoisomerase (as in the case of the CisA protein-dependent replication of ϕX174 DNA; refs. 18 and 19). A pivotal role for a site-specific DNA nick in the function of tertiary origins is consistent with genetic experiments suggesting that such a nick is responsible for the gene *34* recombination hot spot (20–22). However, since genomic T4 DNA is linear rather than circular, this proposed mechanism cannot account for replication of the entire T4 genome. Initiation from a nick would induce only unidirectional DNA synthesis. Additional events would be necessary to produce bidirectional replication forks or to allow the partially replicated genomes to become incorporated into the concatameric pool of intracellular T4 DNA by genetic recombination events (4).

Much additional work will be necessary to explore the various mechanisms of replication initiation used by the T4 bacteriophage. The defective phage system described here should greatly facilitate progress toward understanding these replication mechanisms and their relationship to genetic recombination events. Moreover, by introducing additional DNA fragments into the tertiary origin-containing defective phage, large amounts of fully modified DNA molecules of any sequence can now be prepared for both *in vivo* and *in vitro* studies. This DNA should be useful for molecular studies of many important processes in the T4-infected cell and could also be used to help analyze the rules of DNA sequence recognition used by any site-specific DNA binding protein.

We thank Gisela Mosig for many helpful discussions and for providing the quadruple-mutant phage strain, Geoff Wilson and Tom Mattson for informing us of their results prior to publication, and Kathleen Raneses for expert preparation of the manuscript. This work was supported by Grant GM24020 from the National Institutes of Health and by postdoctoral fellowships to K.N.K. from the American Cancer Society (California Division) and the National Institutes of Health. B.M.A. is an American Cancer Society Lifetime Research Professor.

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