Two Types of Recombination Hotspots in Bacteriophage T4: One Requires DNA Damage and a Replication Origin and the Other Does Not

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ABSTRACT

Recombination hotspots have previously been discovered in bacteriophage T4 by two different approaches, marker rescue recombination from heavily damaged phage genomes and recombination during co-infection by two undamaged phage genomes. The phage replication origin *ori(34)* is located in a region that has a hotspot in both assays. To determine the relationship between the origin and the two kinds of hotspots, we generated phage carrying point mutations that should inactivate *ori(34)* but not affect the gene *34* reading frame (within which *ori(34)* is located). The mutations eliminated the function of the origin, as judged by both autonomous replication of plasmids during T4 infection and two-dimensional gel analysis of phage genomic replication intermediates. As expected from past studies, the *ori(34)* mutations also eliminated the hotspot for marker rescue recombination from UV-irradiated genomes. However, the origin mutations had no effect on the recombination hotspot that is observed with co-infecting undamaged phage genomes, demonstrating that some DNA sequence other than the origin is responsible for inflated recombination between undamaged genomes. The hotspots for marker rescue recombination may result from a replication fork restart process that acts upon origin-initiated replication forks that become blocked at nearby DNA damage. The two-dimensional gel analysis also revealed phage T4 replication intermediates not previously detected by this method, including origin theta forms.

RECOMBINATION hotspots provide useful windows into the detailed mechanisms of recombination. Study of meiotic recombination hotspots in yeast led to the identification of the causative double-strand breaks that are induced by SPO11 and processed by RAD51 and other yeast recombination proteins (reviewed by Stahl 1996; Pâques and Haber 1999). Analyses of χ-sites in *Escherichia coli* have likewise been instrumental in understanding the major mechanism of conjugal recombination in bacteria (Kowalczykowski *et al.* 1994; Myers and Stahl 1994) and have recently led to insight concerning the fate of broken replication forks (Kuzminov *et al.* 1994; Kuzminov 1999; Kowalczykowski 2000). Although much remains to be learned about the mechanisms of these and other pathways of recombination, the hotspots themselves facilitate progress by focusing homologous recombination into a more or less site-specific event.

Bacteriophage T4 provides one of the best-developed systems for studying recombination and particularly the relationships between recombination and DNA replication (Mosig 1998; Kreuzer 2000). Recombination hotspots were first recognized in phage T4 during studies of the process of marker rescue, which involves the recovery of genetic alleles from heavily irradiated genomes into unirradiated co-infecting genomes (Womack 1965). The frequency of marker rescue was found to vary across the genome, with three strong peaks where marker rescue was several-fold higher than elsewhere. Enhanced marker rescue has been detected from phage DNA damaged by UV, X rays, or ^{32}P decay (Womack 1965; Campbell 1969; Levy 1975). The peak of each hotspot maps very near a well-characterized phage replication origin, *ori(uvsY)*, *ori(34)*, or *oriE* (Kreuzer and Alberts 1985; Kreuzer and Morrical 1994; Mosig 1994). Furthermore, deletion of *ori(uvsY)* eliminated the hotspot normally found in its vicinity, and insertion of *ori(uvsY)* or *ori(34)* transformed a coldspot into a hotspot (Yap and Kreuzer 1991). Thus, the replication origin or an element within the origin must be responsible for generating the marker rescue recombination hotspot.

At least one recombination hotspot has also been detected during genetic crosses involving two undamaged phage chromosomes (Mosig 1966, 1968; Beckendorf and Wilson 1972; Levy and Goldberg 1980). The gene *34-35* region has a higher frequency of recombination per unit length DNA than other regions, and this hotspot overlaps with the hotspot for marker rescue recombination at *ori(34)* [*ori(34)* is located within gene *34*]. Although it is tempting to assume that the hotspot with undamaged DNA is related to the hotspot for marker rescue, the other two origin regions did not appear to exhibit enhanced recombination when tested in simple phage crosses with undamaged DNA. Therefore, either the *ori(34)* region has additional sequence

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element(s) necessary to activate a hotspot with undamaged DNA or the two kinds of hotspots are mechanistically unrelated.

T4 replication origins were first localized by hybridization analyses of early replicating DNA, although technical difficulties and other complexities led to imprecision and a certain amount of controversy over origin locations (for reviews, see Kozinski 1983; Mosig 1983; Kreuzer and Morrical 1994). Two of the regions implicated as origins by this approach later were found to contain sequences capable of driving autonomous replication, *ori(uvsY)* and *ori(34)* (Kreuzer and Alberts 1985). By analyzing replication of plasmids containing these elements and various mutant derivatives, both *ori(uvsY)* and *ori(34)* were found to consist of two elements that together are sufficient for replication (Menkens and Kreuzer 1988). The first is a middle-mode promoter containing a binding site for the MotA transcriptional activator in the −30 region (the mot box) and a consensus −10 sequence. The second element is a region of ~50 bp downstream of the promoter. The downstream region behaves like a DNA-unwinding element *in vitro*, in that single-strand specific nucleases can cleave this region within supercoiled DNA (Carles-Kinch and Kreuzer 1997). Furthermore, *in vivo* replication was maintained when the downstream region was replaced with a heterologous DNA-unwinding element. An understanding of the relationship between the promoter and the DNA-unwinding element emerged when *in vivo* chemical probing strongly suggested that a persistent RNA-DNA hybrid (R-loop) forms in the downstream region (Carles-Kinch and Kreuzer 1997). The simplest model is that the RNA within the R-loop serves as the primer for leading-strand replication, and the displaced DNA strand serves as the loading site for the replicative helicase. Consistent with this model, putative RNA-DNA copolymers have been reported in the region downstream of the *ori(uvsY)* promoter (Mosig *et al.* 1995; Vaiskunaite *et al.* 1999).

The mechanism of replication initiation at *ori(uvsY)* was recently probed by identifying replicative intermediates using two-dimensional (neutral-neutral) gels (Bell and Byers 1983; Brewer and Fangman 1987; Friedman and Brewer 1995). In this method, restriction enzyme-digested DNA is subjected to first-dimension electrophoresis that separates DNA only on the basis of total mass and then second-dimension electrophoresis that separates on the basis of both mass and shape. Replicative intermediates, containing one or more branches, form unique arcs depending on their structure. A series of simple Y molecules was found in the *ori(uvsY)* region of the T4 genome, with the branch points mapping to the region that encodes the *ori(uvsY)* transcript (Belanger and Kreuzer 1998). On the basis of the appearance of the arc containing these Y molecules, it was dubbed a "comet." Several results argued strongly that the Y molecules within the comet were intermediates in which unidirectional replication had progressed from the origin region out of the restriction fragment, but in which the second direction of replication had not yet begun. These experiments also provided additional evidence that the RNA within the R-loop serves as a primer for leading-strand replication (Belanger and Kreuzer 1998).

In this article, we investigate the relationship between the hotspots for recombination with damaged *vs.* undamaged DNA by constructing phage with point mutations that inactivate *ori(34)*. We use both plasmid replication assays and two-dimensional gel analysis of phage replicative intermediates to confirm the replication defect of the origin mutant and in the process uncover θ replication intermediates in the wild-type *ori(34)* region.

MATERIALS AND METHODS

Materials: Oligonucleotides were synthesized by the Duke University Botany Department Oligonucleotide Facility. Restriction enzymes, random-primed labeling kit, and radiolabeled nucleotides were purchased from commercial sources. L broth contained NaCl (10 g/liter), Bacto Tryptone (10 g/liter), and yeast extract (5 g/liter) and was supplemented with ampicillin (25 mg/liter) for plasmid-bearing strains. Hershey agar plates contained Bacto Tryptone (13 g/liter), agar (10 g/liter), NaCl (8 g/liter), sodium citrate (2 g/liter), and glucose (1.3 g/liter).

Strains: *E. coli* strains CR63 (*supD*) and B_E (nonsuppressing) were the permissive and restrictive hosts for amber mutant phage, respectively. *E. coli* MCS1 (*supD*) and AB1 (nonsuppressing) are transformation-competent strains described by Kreuzer *et al.* (1988a). The restrictive host for selecting rII^+ phage was a λ^+ lysogen of strain MCS1 (which also carried plasmid pKK467, which is irrelevant for this experiment).

$T4^+D$ (wild type) was originally from the collection of B. M. Alberts (University of California, San Francisco). T4 strain K10-*uvsY* (Kreuzer *et al.* 1988a) carries the following mutations: *uvsY*Δ1 (*uvsY* deletion), *amB262* (gene *38*), *amS29* (gene *51*), *nd28* (*denA*), and *rIIPT8* (*rII-denB* deletion). T4 strains carrying a mutation in *rIIA* (Δ*250*) and in *rIIB* (*UV232*) were obtained from Carolina Biological Supply Co. (Burlington, NC). T4 23^{am}, which carries the *amB17* mutation in gene *23*, was originally from the collection of W. B. Wood (University of Colorado, Boulder). T4 35^{am}, which carries the *amB252* mutation in gene *35*, was obtained from B. Kutter (Evergreen State College, Olympia, WA).

Sequences of T4 mutations: The nucleotide sequences of *amB252* in gene *35*, *rIIA* Δ*250*, and *rIIB UV232* were determined by automated sequencing at the Duke University Comprehensive Cancer Center Facility, using appropriate PCR fragments from the T4 genome as templates. The *amB252* mutation was found to be a C to T transition at position 155,398 in the T4 genome (all genome coordinates in this article are from the 10/98 release), which alters codon 162 (CAG, encoding Q) to the amber codon TAG. The *rIIA* Δ250 mutation was found to be a 39-bp deletion that removes nucleotides 1289 to 1327 of the T4 genome (creating the new sequence 5′-TGCTT^GAGTG-3′ with ^ indicating the location of the deletion). The *rIIB UV232* mutation, which was previously known to be a frameshift, was found to be an insertion of an additional T in a run of two T's that are located at 168,468 and 168,469 in the genome (creating the new sequence 5′-AAGCTTTCCACC-3′).

Plasmids: Plasmid pKK025 is a pBR322 derivative with a 1.05-kb T4 DNA fragment (map coordinates 152,883–153,935 bp) cloned at the vector *Eco*RI site (Kreuzer and Alberts

1985, 1986). This *Eco*RI fragment of the T4 genome is an internal fragment of gene *34* and contains the intact *ori(34)*.

Plasmid pPLD2, which carries the *ori(34)* and gene *34* amber mutations, was constructed as follows: pKK025 was first cleaved with *Dra*III and *Bam*HI to excise part of the T4 insert [including *ori(34)*] and adjacent vector sequence. The large linear fragment was ligated to a duplex oligonucleotide that recapitulated the native T4 sequence except for the indicated mutations (see Figure 1; the mutations also introduced diagnostic restriction sites). The duplex oligonucleotide (ends compatible with the *Dra*III and *Bam*HI sticky ends) had the following sequence: 5′-GTGTACCACGTTGTGTCTCATTTGCCTCGA GAATATTTAACGTGTAATGGTCCCAGAGAGTTCCTGATT CAACTAATCCAGATAGAGCAACAACCTAG-3′/5′-GATCCT AGGTTGTTGCTCTATCTGGATTAGTTGAATCAGGAACTC TCTGGGACCATTACACGTTAAATATTCTCGAGGCAAATG AGACACAACGTGGTACACTTC-3′. The insert in the resulting plasmid, pPLD1, was verified by DNA sequencing. A second segment of homology to the T4 genome was inserted beyond the origin and amber mutations to allow efficient transfer of the mutations into the T4 genome (see below). In this step, pPLD1 was cleaved with *Avr*II (cleaves near the end of the insert from the above step) and *Sal*I (cleaves in the vector), and the large linear fragment was ligated to the following duplex oligonucleotide: 5′-CTAGGTACGATCAGTACTATT AAATCTGGTTTTAATTTTTAATGGTGTAGAGATACGAGT ATCG-3′/5′-TCGACGATACTCGTATCTCTACACCATTAAA AATTAAAACCAGATTTAATAGTACTGATCGTAC-3′. The insert in the resulting plasmid, pPLD2, was verified by DNA sequencing.

Construction of new T4 strains: A T4 strain carrying the *ori(34)* mutations and the nearby 34^{am} mutation (*S721am*, altering serine-721 of gp34 to amber) was generated by marker rescue from plasmid pPLD2. Cells carrying pPLD2 were infected with $T4^{+}D$, and plaque-purified progeny were screened for amber mutants. Amber mutants were then tested for the presence of the diagnostic restriction sites after PCR amplification of the gene *34* region, using PCR primers 5′-GCGGCC AATGAACCACCA-3′ and 5′-CCAACACAGCAAGGTGCAG-3′ (amplifies T4 map coordinates 152,616–153,607 bp). We found several progeny that carried all three restriction sites (T4 34^{am} $ori(34^{-})$] and one that carried only the *Avr*II site [T4 34^{am} (wild type for *ori(34)*); resulting from crossover between the origin and amber mutations].

A phage strain carrying the *ori(34)* mutations without the amber mutation was generated by a second round of plasmid marker rescue. In this case, T4 34^{am} $ori(34)^{-}$ was used to infect cells carrying a plasmid with wild-type gene *34* sequence, and progeny without the amber mutation were selected by plating on nonsuppressing cells. PCR analysis identified a progeny phage that had lost the *Avr*II restriction site (diagnostic of the amber mutation) but maintained the two sites diagnostic of the origin mutations [T4 $ori(34)^{-}$(gene 34^{+})].

T4 35^{am} $ori(34)^{-}$ was constructed by a genetic cross, using an equal multiplicity of T4 $ori(34)^{-}$ and T4 35^{am}. Individual plaques were first screened for amber mutants, and then a progeny phage with the origin mutations was identified using PCR and the two restriction sites diagnostic for $ori(34)^{-}$. T4 34^{am} *rIIA* and T4 34^{am} *rIIA* $ori(34)^{-}$ were generated by genetic crosses between T4 Δ*250* (*rIIA*) and either T4 34^{am} or T4 34^{am} $ori(34)^{-}$, respectively. The *rIIA* mutation was recognized by failure to grow on a λ-lysogen, and the amber and origin mutations were verified by the diagnostic restriction sites in the gene *34* PCR fragment. Similarly, T4 35^{am} *rIIB* and T4 35^{am} *rIIB* $ori(34)^{-}$ were generated by genetic crosses between T4 *UV232* (*rIIB*) and either T4 35^{am} or T4 35^{am} $ori(34)^{-}$.

Plasmid replication assay: MCS1 cells carrying either plasmid pPLD2 or pKK025 were grown at 37° in L broth to an A_{560} of 0.5 ($\sim 4 \times 10^{8}$ cells/ml) and then infected with T4 K10-*uvsY* at a multiplicity of 3 pfu per cell. After a 3-min attachment period without shaking, the infected cells were incubated with shaking at 37° for 1 hr. Total nucleic acids were prepared as described by Kreuzer *et al.* (1988a). The purified DNA was digested with *Hae*III and *Ssp*I, and the resulting fragments were separated on a 0.8% agarose gel and visualized by ethidium bromide staining.

Two-dimensional agarose gel analysis of phage chromosomal origin activity: Replication intermediates were visualized on two-dimensional gels, essentially as described by Belanger and Kreuzer (1998). Host AB1 was grown to an A_{560} of 0.4 and infected with phage T4 34^{am} or T4 34^{am} $ori(34)^{-}$ at a multiplicity of 6 pfu per cell. At the indicated time after infection, DNA was purified and cleaved with *Pac*I, generating a 6203-bp *ori(34)*-containing fragment (T4 map coordinates 147,877–154,079 bp; see Figure 1). The fragments were resolved in a first-dimension 0.4% agarose gel run with 1× TBE (89 mm Tris base, 89 mm boric acid, 2 mm Na_3EDTA) at 15 V for 29 hr. DNA lanes were cut out from the first-dimension gel and cast across the top of a second-dimension 1% agarose gel containing ethidium bromide (0.3 μg/ml), which was run with 0.5× TBE at 150 V for 15 hr. Replication intermediates of the *Pac*I restriction fragment were visualized by Southern blotting using a radiolabeled probe prepared from the 1.05-kb *Eco*RI fragment of pKK025 (T4 map coordinates 152,883–153,935 bp).

UV marker rescue: The procedure for marker rescue from UV-irradiated phage was essentially as described by Yap and Kreuzer (1991). Briefly, donor phage with or without the *ori(34)* mutations were irradiated with enough UV light to cause ~50 lethal hits. *E. coli* CR63 (*supD*) was grown in L broth at 37° to an A_{560} of 0.125 ($\sim 10^{8}$ cells/ml) and then infected with donor phage at a multiplicity of 2.4 pfu per cell and amber mutant helper phage (either 34^{am} or 23^{am}) at a multiplicity of 5 pfu per cell. The infection and subsequent steps were conducted under yellow light to prevent photoreactivation. After a 10-min attachment, the infected cells were diluted 25-fold with L broth, shaken for 70 min at 37°, chilled, lysed with chloroform, and subjected to centrifugation to remove cell debris. The total phage titer was measured by plating on CR63 and the recombinant phage titer (34^{+} or 23^{+}) by plating on B_E, in each case using the average of duplicate dilution series. The starting lysates of amber mutant phage contained revertants at frequencies of 10^{-5} or less, at least 1000-fold lower than the measured marker rescue recombination frequencies.

Recombination frequencies in crosses between undamaged phage: *E. coli* CR63 was grown in L broth at 37° to an A_{560} of 0.5 ($\sim 4 \times 10^{8}$ cells/ml) and then infected with the two indicated phage strains, each at a multiplicity of 3 pfu per cell. In parallel experiments with related phage strains, the efficiency of adsorption was >0.95. The infected cells were incubated for 70 min at 37° and then chilled and lysed by the addition of chloroform. After centrifugation to remove cell debris, the total phage titer was measured by plating on CR63, am^{+} (34^{+} 35^{+}) recombinant phage titer by plating on B_E, and rII^{+} recombinant phage titer by plating on MCS1 pKK467 λ^{+} (averages of duplicate dilution series). In all cases, the frequency of revertants (am^{+} or rII^{+}) in the starting lysates was at least 1000-fold lower than the measured recombination frequencies from the crosses.

RESULTS

Construction of T4 with mutations in *ori(34)*: The T4 replication origin *ori(34)* lies within the coding region of gene *34*, which encodes an essential tail fiber protein (Figure 1A). To inactivate *ori(34)* without destroying

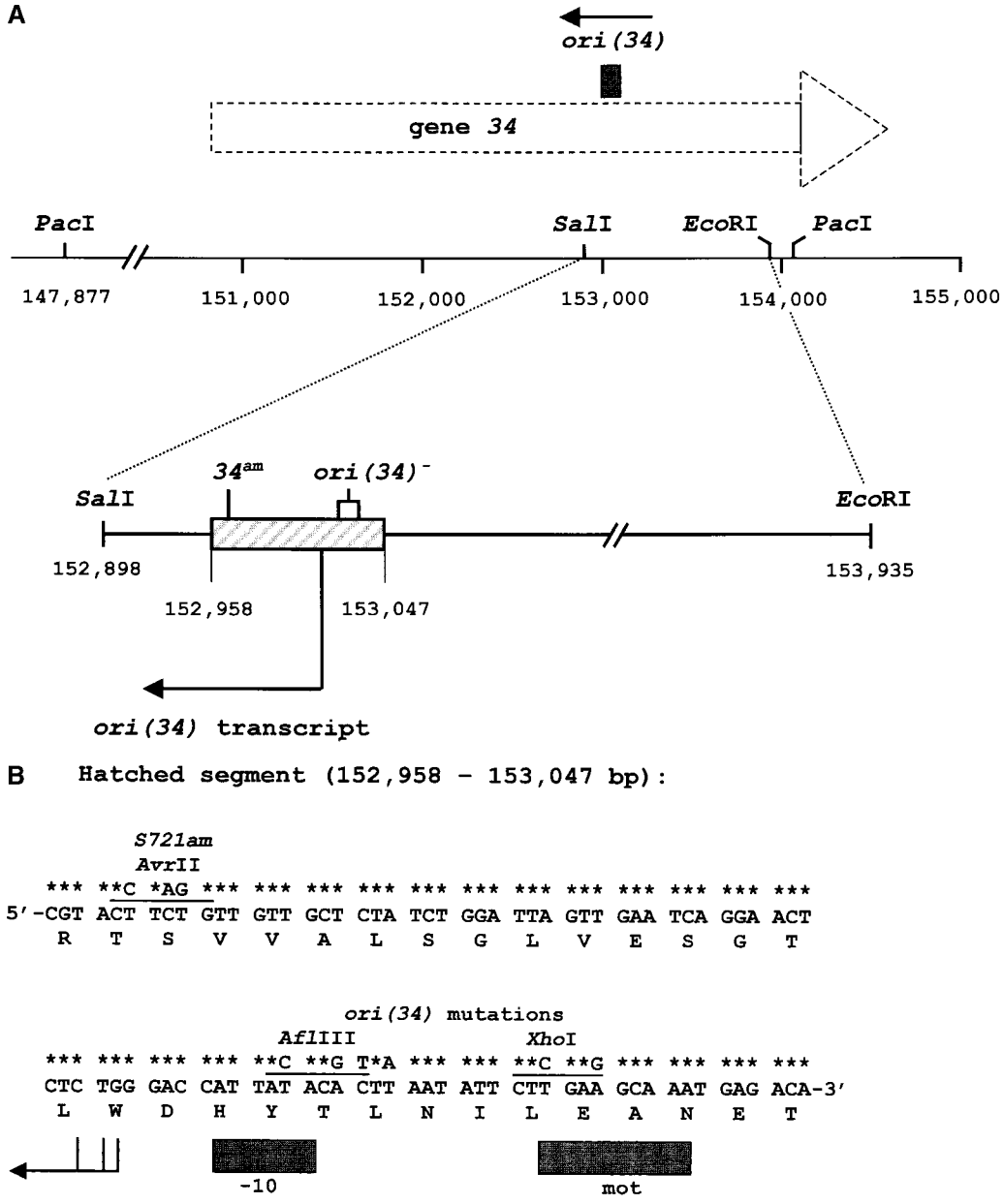

FIGURE 1.—Diagram of the *ori(34)* region and the origin mutations. The gene *34* region of phage T4 is diagrammed in A. All coordinates are in base pairs with respect to the T4 genomic sequence (10/98 release). The gene *34* reading frame extends from 150,804 to 154,673 bp in the rightward direction (clockwise on the circular T4 map), while the *ori(34)* middle-mode promoter is oriented in the opposite direction and lies within the gene *34* reading frame. The two *Pac*I sites used in two-dimensional gel analyses below are indicated on the map, along with a *Sal*I site and an *Eco*RI site that are important in this study (the map does not show other *Eco*RI sites, located outside the *Sal*I-*Eco*RI fragment). The *Sal*I-*Eco*RI fragment present in plasmid pPLD2 is shown in the blow-up at the bottom of A. The blow-up indicates the positions of the engineered 34^{am} and *ori(34)* mutations, originally within plasmid pPLD2 and subsequently transferred into the T4 genome. B represents the DNA and amino acid sequence of the segment of gene *34* that is within the hatched box in A. The six base substitutions that constitute the *ori(34)* mutations are indicated above the sequence (asterisks indicate unchanged sequence). The mot box and −10 sequences of the *ori(34)* promoter are indicated, with the three MotA-dependent 5′ RNA ends (GUILD *et al.* 1988) indicated by the three adjacent leftward facing arrows. The six mutations at *ori(34)* do not alter the gene *34* reading frame but do create diagnostic cleavage sites for *Afl*III and *Xho*I. The newly created amber mutation (serine-721 to amber; *S721am*) in gene *34* is shown above the main sequence; this triple mutation creates a diagnostic *Avr*II site in gene *34*.

the function of gp34, we introduced base substitution mutations within the *ori(34)* promoter that did not alter the amino acid sequence of gp34. A total of six base substitutions were introduced within and between the consensus −10 and mot box elements of the *ori(34)* promoter, and these were designed so that they also introduced diagnostic *Afl*III and *Xho*I restriction sites (Figure 1B). These mutations would very likely abolish origin function, on the basis of previous deletion and base-substitution analyses of origin function (MENKENS and KREUZER, 1988; A. MENKENS and K. KREUZER, unpublished results).

In addition to the origin mutations, we also introduced a triple mutation ~50 bp downstream of the origin promoter, changing the S721 codon of gene *34* into an amber codon and introducing a diagnostic *Avr*II site in the DNA (Figure 1B). This amber mutation was important for introducing the origin mutations into the phage genome and was also useful as a marker in recombination experiments. All nine mutations were generated within synthetic oligonucleotides and cloned into a plasmid (see MATERIALS AND METHODS).

The final plasmid, pPLD2, carries the origin and amber mutations within a region of just over 1 kb of

T4 sequence (*Sal*I-*Eco*RI insert diagrammed in Figure 1A). Phage T4 undergoes recombination with plasmids carrying such homologous segments, generating both plasmid-phage co-integrants and phage that have undergone a gene conversion event without acquiring the plasmid. For plasmid pPLD2, the co-integrants would be lethal due to interruption of gene *34*. Gene convertants carrying the engineered amber mutation were obtained at a frequency of ~1%. By screening PCR-amplified DNA from these amber mutant phage with the diagnostic restriction enzymes, strains with and without the *ori(34)* mutations were obtained [T4 34^{am} $ori(34)^{-}$ and T4 34^{am}]. A phage strain carrying the *ori(34)* mutations without the amber mutation [T4 $ori(34)^{-}$] was also generated by a second round of marker rescue from a plasmid with the wild-type sequence.

Replication is abolished by the origin mutations: We began to assess the impact of the origin mutations on *ori(34)* function by measuring plasmid replication. Phage T4 shuts off the replication of pBR322-based plasmids from the ColE1 origin shortly after infection (Mattson *et al.* 1983). However, plasmids that contain a cloned *ori(34)* replicate extensively after infection, using the T4 replication machinery (Kreuzer and Alberts 1985). For this experiment, we used a *uvsY*-mutant phage so that the plasmid could not replicate by the recombination-dependent mechanism that operates with any plasmid that has homology to the phage genome (Kreuzer *et al.* 1988b). DNA purified from the infections was cleaved with restriction enzymes *Ssp*I and *Hae*III, which permits visualization of T4-replicated plasmid DNA without any unreplicated plasmid. This assay takes advantage of the fact that T4 incorporates modified 5-hydroxymethyl dCMP residues during DNA replication (these residues are further modified with glucosyl groups after replication). T4-replicated plasmid DNA containing these modified dCMP residues is resistant to *Hae*III, which cleaves unreplicated plasmid DNA into many small fragments that run off the agarose gel. *Ssp*I is one of the few enzymes that cuts T4-modified DNA, cleaving the plasmids used in this experiment into two fragments, the larger of which is easily separated from all T4 *Ssp*I restriction fragments. As expected, the control plasmid with intact *ori(34)* generated large amounts of T4-replicated plasmid DNA (Figure 2, lane 1). However, plasmid pPLD2, with the origin point mutations, was completely inactive (Figure 2, lane 2). We conclude that the origin point mutations abolish replication from *ori(34)*, at least in the context of the plasmid.

Two-dimensional agarose gel analysis with Southern blotting was recently used to visualize replication intermediates formed within phage chromosomal DNA at the T4 origin *ori(uvsY)* (Belanger and Kreuzer 1998). As described above (see Introduction), a comet was attributed to intermediates in which unidirectional replication had progressed from the origin region out of the restriction fragment, but in which the second direction of replication had not yet begun. We applied this assay to the *ori(34)* region to determine whether the origin mutations abolish origin function within the context of the phage chromosome.

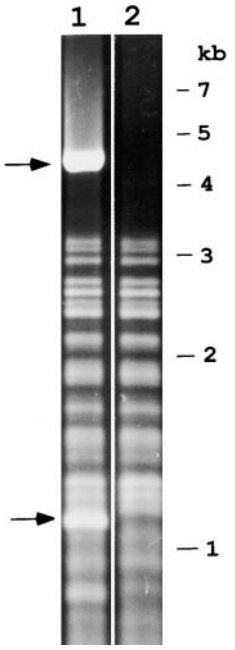

Figure 2.—The *ori(34)* mutations abolish origin-directed plasmid replication in T4-infected cells. Cells carrying plasmid pKK025 [wild-type *ori(34)*; lane 1] or pPLD2 [mutated *ori(34)*; lane 2] were infected with T4 strain K10-*uvsY*. After a 1-hr infection, total nucleic acids were purified from each infection. DNA was digested with *Ssp*I and *Hae*III, subjected to electrophoresis through a 0.8% agarose gel, and visualized by ethidium bromide staining. The replicated plasmid pKK025 restriction fragments (4.31 and 1.10 kb) are indicated by arrows, and the other bands are phage genomic restriction fragments. Plasmid pPLD2 lacks a small (15-bp) segment of T4 DNA contained within pKK025 (T4 genome coordinates 152,883–153,935 bp for pKK025 and 152,898–153,935 bp for pPLD2), but the minimal *ori(34)* sequence lies completely within the pPLD2 fragment (Menkens and Kreuzer 1988). As a result of the construction steps, plasmid pPLD2 is also missing the vector sequence from *Eco*RI to *Sal*I and generates *Ssp*I fragments of 3.65 and 1.10 kb (deletion of this segment of vector was previously shown to have no effect on T4 origin-directed replication; see Menkens and Kreuzer 1988). The size scale was generated from the migration of *Xba*I fragments of T4 dC DNA.

As with *ori(uvsY)*, the wild-type *ori(34)* generated a prominent spot along the Y arc, analogous to the previously defined comet nucleus (Figure 3). The comet nucleus at *ori(34)* was produced at similar times of infection as the one previously detected at *ori(uvsY)*, consistent with the time period during which T4 origins are active. The position of the comet nucleus with *ori(34)* is different than that with *ori(uvsY)* because the origin is in a different location with respect to the restriction sites used to cleave the DNA (see Figure 3 legend). The size of the intermediates within the comet nucleus can be estimated from their migration in the first dimension

(albeit with relatively low precision). As in the case of *ori(uvsY)*, the molecules within the *ori(34)* comet nucleus have a size consistent with Y-branched DNA with the branch point located at the 5′ end of the origin transcript (see Figure 4). The comet detected at *ori(uvsY)* also contained a tail, consistent with molecules that have branch points downstream of the 5′ end of the origin transcript (Belanger and Kreuzer 1998). However, a tail was not obvious in the comet at *ori(34)* in these blots (Figure 3; see discussion).

The *ori(34)* region also generated two arcs that were not evident in the previous study of *ori(uvsY)*, a bubble arc and an X arc (Figure 3). A bubble arc is diagnostic of origin-containing restriction fragments, being composed of bubbles generated by either uni- or bidirectional replication from an internally located replication origin (Brewer and Fangman 1987; Friedman and Brewer 1995; Kuzminov *et al.* 1997). The X arc detected at *ori(34)* seems to merge with the comet nucleus (Figure 3), suggesting that the X-arc molecules are related to the comet molecules (Figure 4). The origin mutations abolished the comet nucleus, the bubble arc, and the X arc (Figure 3), providing very strong evidence that the mutations obliterate origin function in the phage chromosome. The nature of the bubble and X arcs and possible reasons why the two origins differ in these forms are discussed below (see discussion).

The *ori(34)* mutations eliminate the hotspot for marker rescue recombination: As described in the Introduction, deletion of *ori(uvsY)* eliminated a hotspot for marker rescue recombination, and insertion of either *ori(uvsY)* or *ori(34)* into a coldspot created a new hotspot for this type of recombination (Yap and Kreuzer 1991). We therefore had a strong expectation that the *ori(34)* mutations would eliminate the hotspot for marker rescue recombination in the gene *34* region.

Gene 34^+ phage strains with and without the origin mutations were irradiated with UV at a dose that causes ~50 lethal hits per phage particle. These heavily irradiated donor phage were then co-infected with nonirradiated helper phage that carry an amber mutation in either gene *23* (coldspot control) or gene *34* (tested hotspot), and marker rescue was measured as the frequency of 23^+ or 34^+ recombinants in the progeny.

As expected from the original Womack (1965) study, the frequency of marker rescue recombination from the $ori(34)^+$ irradiated phage for the gene *34* amber mutation was about fourfold higher than that for the gene *23* mutation (Figure 5A). This inflation in recombination was abolished by the origin mutations (Figure 5A), consistent with the previous conclusion that T4 origins create hotspots for marker rescue recombination (Yap and Kreuzer 1991).

The *ori(34)* mutations do not affect recombination between undamaged phage genomes: The frequency of homologous recombination between undamaged phage genomes was measured during co-infections of two phage that differ in both the gene *34-35* region and the *rII* region, *i.e.*, T4 34^{am} *rIIA* × T4 35^{am} *rIIB*. The frequency of recombination between the *34* and *35* amber

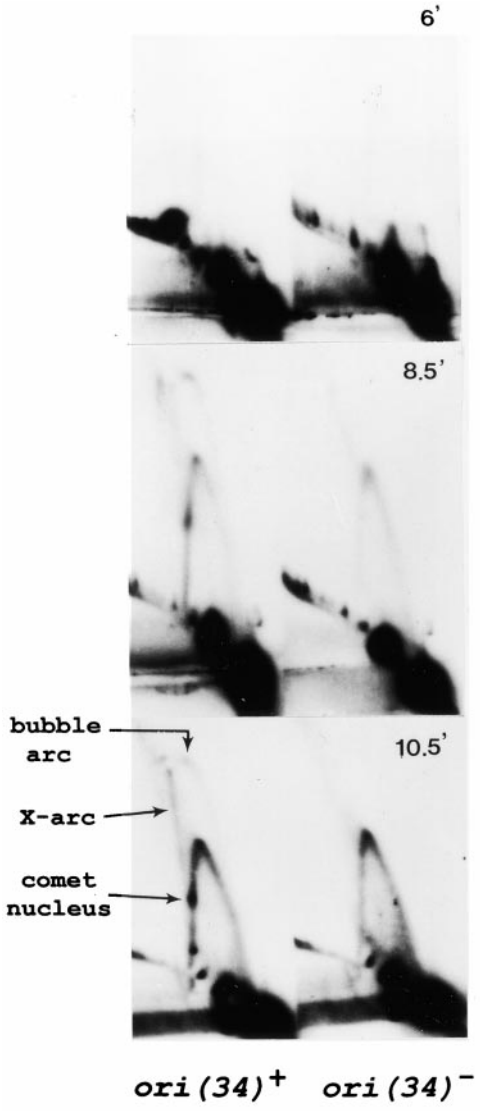

Figure 3.—The *ori(34)* mutations abolish phage genomic replication intermediates. DNA was purified after 6.0, 8.5, and 10.5 min of infection with either T4-34^{am} [$ori(34)^+$] (left) or T4-34^{am} $ori(34)^-$ (right). The DNA was digested with *Pad*I, which cleaves at genome coordinates 147,877 and 154,079 bp (see map in Figure 1A). The resulting fragments were separated by two-dimensional gel electrophoresis as described by Belanger and Kreuzer (1998) and visualized by Southern blotting with an *ori(34)* probe (T4 genome coordinates 152,883–153,935 bp). The positions of the comet nucleus, bubble arc, and X arc are indicated in the bottom left. Note that the position of the comet is much farther along the Y arc (more than half replicated) than in most of the gels of Belanger and Kreuzer (1998) due to the location of restriction sites relative to the origin. The arrangement of restriction sites and origin is most similar to the analysis of *ori(uvsY)* in Figure 5D of the aforementioned study [although that restriction fragment was ~1.6 kb larger than the *ori(34)* restriction fragment analyzed here].

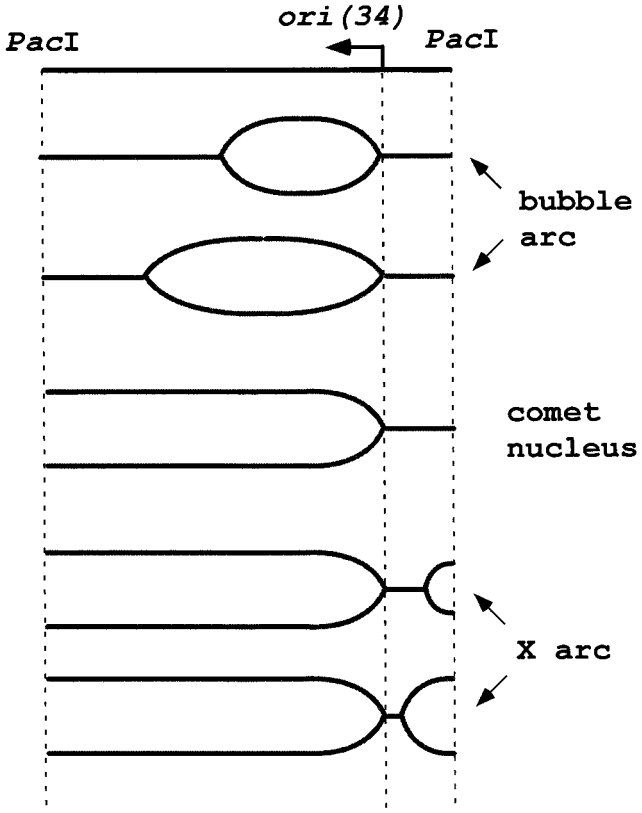

FIGURE 4.—Interpretation of branched molecules at *ori(34)*. The map at the top indicates the *Pac*I sites used in the digest and the relative location and direction of *ori(34)* within the region (also see Figure 1A). The proposed structures of branched DNA within the bubble arc, comet nucleus, and X arc are indicated below the map. The simplest model is that the bubble arc contains the early intermediates of unidirectional replication from the origin, the comet nucleus contains molecules in which this unidirectional fork has exited the restriction fragment, and the X arc contains molecules in which another replication fork has entered the fragment from the right flank.

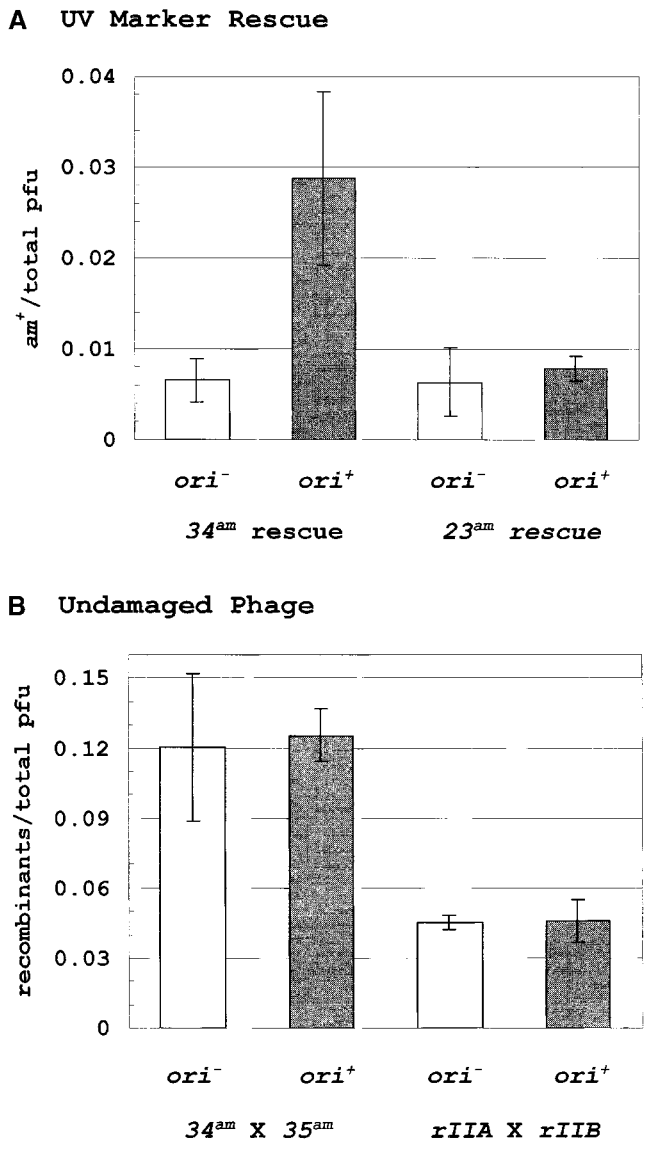

FIGURE 5.—Effect of the origin mutations on hotspot recombination. The effect of the *ori(34)* mutations on the hotspot for marker rescue recombination is shown in A. Phage with a wild-type *ori(34)* (*ori*$^+$) and the *ori(34)* mutations (*ori*$^-$) were irradiated with 50 lethal hits of UV. The irradiated donor phage were co-infected with undamaged helper phage that carry an amber mutation in gene *34* (hotspot) or gene *23* (coldspot control). The average frequencies of marker rescue from three infections (with standard deviations) are shown as *am*$^+$ (*34*$^+$ or *23*$^+$) phage over total progeny. The effect of the *ori(34)* mutations on the hotspot for recombination between undamaged phage genomes is shown in B. The two co-infecting phage genomes had the genotypes *34*^{am} *rIIA* and *35*^{am} *rIIB*; in one case both phage had the wild-type *ori(34)* (*ori*$^+$), and in the other case, both had the *ori(34)* mutations (*ori*$^-$). Recombination in the gene *34-35* hotspot was measured as the frequency of *am*$^+$ (*34*$^+$ *35*$^+$) over total progeny and that in the coldspot region as *rII*$^+$ over total progeny. The average frequencies of recombination from three infections (with standard deviations) are shown.

mutations, measured by plating on a nonsuppressing host, should reflect the recombination hotspot previously measured with undamaged phage genomes (see Introduction). The frequency of recombination between the *rII* alleles, measured by plating on a (suppressing) phage λ-lysogen, provides a control for coldspot recombination. By measuring both hotspot and coldspot recombination within the very same cross, any experimental variations that artificially alter recombination frequencies should be canceled out. The cross was performed with two pairs of phage, one wild-type for *ori(34)* and one carrying the *ori(34)* mutations.

In this case, the origin mutations caused no significant effect on the frequency of recombination in the gene *34* region (Figure 5B). It was important to establish that we were measuring inflated hotspot recombination, since we were using different alleles from those used in the previous studies of this recombination hotspot (MOSIG 1966, 1968; BECKENDORF and WILSON 1972;

Levy and Goldberg 1980). The previously unsequenced alleles were therefore sequenced, and we found that 2431 bp separates the *34*^{am} and *35*^{am} mutations, whereas 1724 bp separates the rIIA and rIIB mutations (see materials and methods). On the basis of these distances, the measured frequency of recombination per kilobase pair was 93% higher for the gene *34-35* interval than for the *rII* interval [*ori(34)*$^{+}$ phage]. This comparison argues that the hotspot for recombination between undamaged phage genomes was indeed having a significant effect on the gene *34-35* recombination that we measured. We conclude that the *ori(34)* mutations do not affect the hotspot for recombination between undamaged phage genomes.

DISCUSSION

In this study, we constructed point mutations in the promoter elements of the phage T4 origin *ori(34)* and introduced these into the phage genome. As intended, the mutations abolished all replication function. Thus, autonomous replication was eliminated in a plasmid containing the origin mutations. Also, the origin mutations eliminated three different kinds of phage genomic replication intermediates relating to origin function. Although the mutations eliminated the hotspot for marker rescue recombination from damaged genomes, they did not affect the hotspot for recombination between undamaged genomes.

Replicative intermediates at *ori(34)*: As in the past study of *ori(uvsY)* (Belanger and Kreuzer 1998), two-dimensional gel analysis of the *ori(34)* region revealed a prominent comet nucleus along the Y arc. The molecules in the comet nucleus apparently consist of intermediates in which the origin has triggered replication in only one direction, with leading-strand replication being primed from the origin transcript. The molecules are in the Y form because the replication fork has exited the restriction fragment being analyzed (see Figure 4). Several results, including a relatively short half-life of the comet, indicate that the second direction of replication from *ori(uvsY)* is induced after a short delay (Belanger and Kreuzer 1998). These results suggest that assembly of the two different replication complexes for bidirectional replication is uncoupled and mechanistically distinct.

The comets formed at *ori(uvsY)* and *ori(34)* differ somewhat in shape, with only the former displaying a prominent tail (Belanger and Kreuzer 1998; Figure 3). The molecules in this comet tail apparently result when the 5′ end of the origin transcript is processed before the branch point from the first direction of replication is established, because mutational inactivation of RNaseH activities resulted in an *ori(uvsY)* comet without a tail (Belanger and Kreuzer 1998). Therefore, the absence (or shortening) of a tail at *ori(34)* could be explained by a lack of 5′ end processing at this origin. Alternatively or in addition, the *ori(34)* transcript may be much shorter than the *ori(uvsY)* transcript, limiting all branch points to a smaller region.

Along with the comet nucleus, we also detected two kinds of *ori(34)* replication intermediates that were not observed in the previous analysis of *ori(uvsY)*. A prominent bubble arc and X arc were both detected in DNA fragments containing the wild-type *ori(34)* but not in the corresponding fragments when they contained the origin mutations. Bubble arcs are characteristic of origin-containing restriction fragments (Brewer and Fangman 1987; Friedman and Brewer 1995) and can be generated with either bidirectional or unidirectional replication. The molecules within the bubble arc had a total size less than that of the molecules in the comet nucleus, as judged by their position in the first-dimension gel, which separates molecules on the basis of size. Therefore, these molecules could be generated by unidirectional replication from the origin. One likely possibility is that these molecules are the precursors to the comet nucleus, *i.e.*, the intermediates of replication before the first replication fork exits the restriction fragment (Figure 4). Why were θ molecules not seen in the previous study of *ori(uvsY)*? One possibility is that the forks initiated at *ori(34)* initially travel slower, perhaps because replicative helicase loading is delayed at *ori(34)*. More experiments are necessary to distinguish between this and other possibilities.

The X arc detected in the *ori(34)* region is also dependent on the origin. One end of this arc appears to be very near or at the nucleus of the comet, which we infer to contain DNA molecules with a Y branch at the position of the 5′ end of the origin transcript (see above). The simplest explanation is that the X arc consists of comet-like molecules in which a second replication fork has entered the restriction fragment (from the end opposite to the one in which the initial unidirectional fork exited; see Figure 4). The X arc again appears to be unique, or at least more prominent, with *ori(34)* [compared to *ori(uvsY)*]. Perhaps another active T4 replication origin is closer, in the upstream direction, to *ori(34)* than to *ori(uvsY)*. Alternatively or in addition, the second direction of replication from *ori(34)* might be more delayed than that from *ori(uvsY)*. The presence of the X arc even raises the possibility that *ori(34)* is a strictly unidirectional origin.

Recombination hotspot with undamaged DNA: Although the origin mutations abolished both DNA replication from the origin and the hotspot for marker rescue recombination from damaged DNA, they had no effect on the hotspot for recombination between two undamaged phage genomes. Therefore, *ori(34)* is not involved in the generation of the hotspot with undamaged DNA. This explains the long-standing mystery of why the other two hotspots for marker rescue recombination are not also hotspots for recombination between undamaged genomes. The two kinds of hotspots are

mechanistically distinct, and the fact that they both exist in the gene *34-35* region is apparently a coincidence.

What causes the gene *34-35* hotspot for recombination between undamaged genomes? One clue is that the hotspot requires the T4-directed glucosylation of DNA (Levy and Goldberg 1980). Another is that the gene *34* region of phage T2 is not normally a hotspot, but induces a hotspot when it is introduced into the homologous location in phage T4 (Beckendorf and Wilson 1972). Therefore, T2 DNA must have the necessary DNA sequence, but either the different glucosylation pattern or the lack of some other necessary component prevents it from being active in the context of a T2 infection. One reasonable model is that the hotspot with undamaged genomes results from double-strand breaks introduced by a T4-encoded site-specific endonuclease that is not also encoded by T2. Phage T4 encodes a family of site-specific endonucleases, some of which are involved in the process of intron mobility (Sharma *et al.* 1992; Clyman *et al.* 1994). Since the phage has a very efficient system of double-strand break-directed recombination, a recombination hotspot could result if there is indeed a strong cleavage site in the vicinity of gene *34-35*. Further experiments are clearly necessary to deduce the mechanism of inflated recombination between undamaged genomes.

Recombination hotspot with DNA damage: The major conclusion of this work is that *ori(34)* induces a recombination hotspot only with damaged DNA. What is the mechanism of this hotspot recombination? Yap and Kreuzer (1991) showed that marker rescue recombination was observed only when the origin was on the damaged genome, and the presence or absence of the origin on the undamaged genome was irrelevant. Similarly, we found that *ori(34)* mutations in the UV-damaged donor phage eliminated the marker rescue hotspot even though the recipient amber-mutant phage still carried an intact *ori(34)* (Figure 5). Therefore, it seems very likely that marker rescue recombination involves the encounter of an origin-generated replication fork with DNA damage. Presumably, the high level of DNA damage within the irradiated genomes prevents origin-initiated replication forks from traveling very far from the origins, leading to the localized stimulation of recombination. Similar results were obtained in studies of marker rescue recombination from UV-damaged phage T7 DNA (Burck and Miller 1978). In this case, deletion of the primary T7 replication origin shifted the peak of recombination from the location of the primary origin to the region near the secondary replication origin (at the left end of the genome). Furthermore, the T7 recombination hotspot region was preferentially replicated when cells were infected with UV-irradiated T7 phage (and no unirradiated helper phage; Burck and Miller 1978). For both the T4 and T7 systems, inflated recombination in the hotspot could depend on increased copy number of DNA near the origin and/or recombinogenic structures created when replication forks meet DNA damage.

The blockage of replication forks by bound proteins, RNA, or DNA damage has been shown to lead to homologous recombination in a variety of systems (Burck and Miller 1978; Voelkel-Meiman *et al.* 1987; Horiuchi *et al.* 1994; Horiuchi and Fujimura 1995). Therefore, a simple model for the T4 hotspots of marker rescue recombination is that the origins trigger replication forks, which are blocked soon after leaving the origin when they encounter DNA damage. The molecular pathway for activating recombination at a blocked fork is uncertain, but may well involve the induction of a double-strand break at the fork (Horiuchi and Fujimura 1995; Kuzminov 1995; Michel *et al.* 1997; Seigneur *et al.* 1998). If so, the newly generated end would presumably feed into the very active pathway of recombination-dependent DNA replication (RDR) in T4. T4 RDR can be efficiently triggered by double-strand ends, and DNA replication has been shown to be very tightly connected to the repair of double-strand breaks in this system (Kreuzer *et al.* 1995; George and Kreuzer 1996; Mueller *et al.* 1996). In those events measured genetically as marker rescue recombination, the broken end of the damaged DNA (at the blocked fork) would invade an undamaged genome (originally containing the amber mutation), presumably triggering a new replication fork on that genome by RDR.

Recent work with inhibitors of the phage T4 type II DNA topoisomerase suggest a similar mechanism for processing and repair of antitumor drug-induced protein-DNA crosslinks. A variety of antitumor agents trap the phage T4 (and mammalian) topoisomerase in the cleavage complex, a covalent protein-DNA complex in which the DNA contains an enzyme-bridged DNA break (reviewed by Kreuzer 1998). The damage caused by the antitumor agents is likely related to the cleavage complex, and a pathway of recombinational repair can mitigate the toxicity (Neece *et al.* 1996). A recent analysis of plasmid replication intermediates demonstrated that T4 replication forks are blocked when they reach covalent topoisomerase-DNA complexes *in vivo* (Hong and Kreuzer 2000). Interestingly, the fork blockage persists even after the drug-induced cleavage complexes reverse, indicating that the forks have been temporarily or permanently disabled. Although the events subsequent to fork blockage are currently unknown, a reasonable model is that the blocked forks are cleaved (*e.g.*, by a recombinational endonuclease; see Kreuzer 2000), opening up the RDR pathway in which double-strand ends trigger new DNA synthesis.

This model could also provide a simple mechanism for explaining the fascinating process of multiplicity reactivation (Luria 1947; Rayssiguier and Vigier 1977; Kreuzer and Drake 1994). In multiplicity reactivation, two or more heavily irradiated phages are able to reconstitute viable progeny by recombination during

a co-infection. Since each infecting genome has many lethal hits of damage, the molecular process that occurs must somehow actively avoid incorporating DNA damage into the progeny genomes. Perhaps the process begins much the same way as marker rescue recombination, with a replication fork being initiated at an origin but then stopping soon thereafter when it encounters damage. In this model, the blocked fork is then cleaved, and the resulting double-strand end triggers a new round of replication on another template (which is unlikely to be damaged in the same location). Repeated rounds would allow replication to traverse the entire genome, avoiding damage when necessary by jumping to a new template in the process of RDR.

These pathways are reminiscent of recent proposals for reactivating replication forks in *E. coli* after they have been blocked by damage or otherwise aborted (Kuzminov 1995, 1999; Cox *et al.* 2000; Marians 2000; Michel 2000). In this view, the major function of recombinational repair is to reconstitute broken or otherwise damaged replication forks. Evidence suggests that many, perhaps most, forks initiated at *E. coli oriC* never make it to the terminus, but instead need to be reconstituted by pathways that involve recombination. Because of their site-specific nature, the T4 hotspots for marker rescue recombination could provide a productive system for analyzing the molecular mechanism of replication fork reactivation.

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