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Recombination-Dependent DNA Replication Stimulated by Double-Strand Breaks in Bacteriophage T4

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We analyzed the mechanism of recombination-dependent DNA replication in bacteriophage T4-infected *Escherichia coli* using plasmids that have sequence homology to the infecting phage chromosome. Consistent with prior studies, a pBR322 plasmid, initially resident in the infected host cell, does not replicate following infection by T4. However, the resident plasmid can be induced to replicate when an integrated copy of pBR322 vector is present in the phage chromosome. As expected for recombination-dependent DNA replication, the induced replication of pBR322 required the phage-encoded UvsY protein. Therefore, recombination-dependent plasmid replication requires homology between the plasmid and phage genomes but does not depend on the presence of any particular T4 DNA sequence on the test plasmid. We next asked whether T4 recombination-dependent DNA replication can be triggered by a double-strand break (dsb). For these experiments, we generated a novel phage strain that cleaves its own genome within the nonessential *frd* gene by means of the I-*Tev*I endonuclease (encoded within the intron of the wild-type *td* gene). The dsb within the phage chromosome substantially increased the replication of plasmids that carry T4 inserts homologous to the region of the dsb (the plasmids are not themselves cleaved by the endonuclease). The dsb stimulated replication when the plasmid was homologous to either or both sides of the break but did not stimulate the replication of plasmids with homology to distant regions of the phage chromosome. As expected for recombination-dependent replication, plasmid replication triggered by dsbs was dependent on T4-encoded recombination proteins. These results confirm two important predictions of the model for T4 recombination-dependent DNA replication proposed by Gisela Mosig (p. 120–130, *in* C. K. Mathews, E. M. Kutter, G. Mosig, and P. B. Berget (ed.), *Bacteriophage T4*, 1983). In addition, replication stimulated by dsbs provides a site-specific version of the process, which should be very useful for mechanistic studies.

The processes of DNA replication and homologous recombination are often interconnected. For example, localized DNA replication is invoked in most current models for homologous recombination (40, 42, 44) and origins of DNA replication create recombination hotspots (7, 13, 46). In addition, bulk chromosomal DNA replication in some organisms can be triggered by recombinational processes. For example, two distinct modes of *Escherichia coli* replication initiation depend on the strand-exchange activity of the RecA protein (see reference 3 and Discussion). While these two modes of *E. coli* replication initiation are repressed under normal growth conditions, the major mode of bacteriophage T4 replication initiation under normal growth conditions involves a recombinational process (for a review, see reference 23).

The involvement of recombination in phage T4 DNA replication was first inferred from the pleiotropic phenotypes caused by the mutational inactivation of phage genes *46*, *47*, *59*, *uvsX*, and *uvsY*. In each case, the total amount of phage recombination was reduced by about 3- to 10-fold (for a review, see reference 33). In addition, each of the mutants displayed the DNA-arrest phenotype, in which phage DNA replication ceases prematurely after several minutes of infection. Genetic studies of the segregation of genome-terminal markers argued that the ends of the T4 genome are recombinogenic and that recombination intermediates generated from the termini are used in the initiation of DNA replication (for a review, see reference 32). These and other data led Gisela Mosig to formulate the model shown in Fig. 1. In this model, replication initiates from one of several internal replication origins in the infecting phage DNA and the resulting two forks traverse the genome. Upon reaching the termini, however, each parental 3′ end cannot be completed because of the polarity of DNA polymerase and the inability of DNA polymerase to initiate chains de novo (9, 43). The parental single-stranded 3′ ends then invade homologous sequences on the same or another DNA molecule, forming a Y structure with a D-loop at the point of invasion. The key step in this model occurs when the T4 replication machinery is somehow loaded within the D-loop, and the invading 3′ end is then used as a primer for leading-strand synthesis in the rightward direction. Subsequent replication results in T4 DNA that is longer than the unit-length genome and also generates another unreplicated 3′ terminus when the fork reaches another genome end. Thus, the process of T4 recombination-dependent replication should be self perpetuating and repeated cycles would result in very long concatemers of T4 DNA.

T4 recombination-dependent DNA replication is closely related to the process of recombinational repair (for a review, see reference 20). Recombinational repair of DNA damage was first discovered in T4 (27), and subsequent studies of numerous systems indicate that it is a nearly universal process (or more likely, a collection of related processes). The five recombination genes listed above (*46*, *47*, *59*, *uvsX*, and *uvsY*) are also required for T4 recombinational repair, arguing that the mechanisms of recombination-dependent replication and

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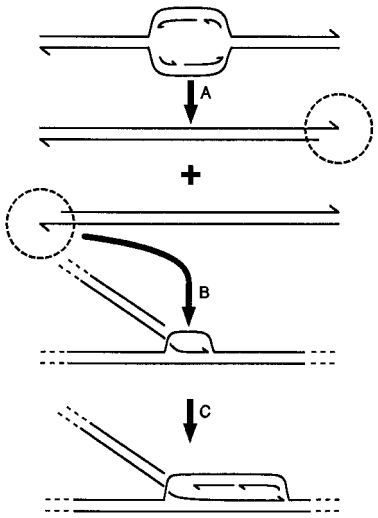

FIG. 1. Model for T4 recombination-dependent DNA replication. This model was adapted from Mosig (33). In step A, origin-dependent replication of the infecting genomic DNA leads to unreplicated 3′ parental DNA ends. Strand invasion (step B) can occur into the other end of the terminally redundant phage genome or into the middle of a coinfecting DNA (because T4 DNA is circularly permuted). The assembly of the replication complex (step C) then leads to net genomic replication, with free 3′ ends again being generated in the products.

recombinational repair are similar. While nearly all organisms use recombinational repair sparingly (only when necessary to repair breaks or other damage), T4 has apparently coopted the repair pathway into a major mechanism of DNA replication, making it much more amenable to detailed study.

The molecular mechanism of T4 recombination-dependent DNA replication has been illuminated by the development of a system that reproduces the process in vitro (for a review, see reference 23). Formosa and Alberts (15) found that extensive DNA replication could occur when T4 recombination and replication proteins were added to a circular duplex DNA template and a homologous single-stranded DNA fragment. The single-stranded DNA fragment was added to mimic the single-stranded 3′ genomic DNA end that is proposed to be involved in recombination-dependent replication in vivo. In this early study, lagging-strand replication was not achieved and single-stranded (leading-strand) product was displaced from the duplex template as replication proceeded. However, subsequent experiments succeeded in reconstituting lagging-strand replication, thereby generating normal (semiconservative) duplex DNA products (5a). Biochemical analyses have clarified the roles of individual proteins in T4 recombination-dependent replication (see reference 23). The T4-encoded UvsX protein (the RecA analog), aided by UvsY (the accessory factor for UvsX) and gp32 (the single-stranded DNA-binding protein), can promote the initial strand synapsis reaction. gp59 appears to play a key role in loading the replication fork helicase-primase complex (gp41/61) onto the synapsed recombination intermediate (5, 31). The roles of DNA polymerase and the polymerase accessory proteins at the replication fork are quite well understood from over two decades of detailed investigations (for a review, see reference 34).

While the in vitro system has clarified some of the individual steps in recombination-dependent replication, many important issues remain unresolved. First, what is the precise nature of the recombination intermediate that triggers the process in vivo? The in vitro reaction mimics the strand invasion in the first step of the Mosig model by providing a free single-stranded fragment, but there is no compelling evidence supporting the nature of the first step in vivo. For example, exonucleolytic degradation rather than (or in addition to) DNA replication could be responsible for generating 3′ single-stranded parental DNA ends. Second, why is gp46/47 required for in vivo recombination-dependent replication but not for the in vitro reaction? Third, it is not known whether recombination-dependent replication in vivo establishes bidirectional or only unidirectional replication. Fourth, the in vitro system appears capable of template strand switching (30a), which could be a critical step in recombinational repair of DNA damage, but no evidence for in vivo strand switching is yet available. Fifth, the strand-exchange protein UvsX is strictly required in the in vitro system, but may not be required for all recombination-dependent replication in vivo. Sixth, as with any in vitro system, there is always some uncertainty about how faithfully the reaction mimics the process that actually occurs in vivo. With these and other issues to be resolved, further analyses of in vivo recombination-dependent DNA replication are warranted.

Two distinct mechanisms of T4 replication initiation, origin-dependent replication and recombination-dependent replication, have been analyzed by plasmid model systems. Plasmid pBR322 derivatives that contain phage replication origins replicate autonomously during a phage infection in a manner that is independent of T4-encoded recombination proteins (21, 29). In contrast, pBR322 derivatives with nonorigin fragments of the T4 genome replicate only in recombination-proficient infections (25). This replication of nonorigin plasmids very likely occurs by a mechanism closely related to phage genomic recombination-dependent replication. The strongest evidence for this point is that nonorigin plasmid replication depends on the same phage-encoded proteins used in phage genomic recombination-dependent replication (recombination proteins UvsX, UvsY, gp32, gp46, and gp47 and replication proteins gp39, gp41, gp43, gp44, gp45, gp52, gp59, gp61, and gp62 [8, 25]). If nonorigin plasmid replication follows the Mosig model described above, then each particular nonorigin plasmid may be replicated only in those infected cells which contain a phage chromosome with one end that is homologous to the inserted sequence in the chimeric plasmid.

In this report, we further investigate the mechanism of T4 recombination-dependent DNA replication using the in vivo plasmid model system. The requirement for homology is tested by generating a phage that carries the pBR322 DNA sequence and asking whether this phage can replicate a pBR322 plasmid that originates in the infected cell. In addition, the requirement for an invasive DNA end is tested by generating a T4 phage that cleaves its own genome and by asking whether the site-specific DNA break in the phage DNA stimulates the replication of a plasmid that carries a DNA insert homologous to the broken region.

MATERIALS AND METHODS

Materials. Restriction enzymes, T4 DNA ligase, alkaline phosphatase, and DNA polymerases were purchased from commercial sources. A random primed DNA labeling kit was obtained from Boehringer-Mannheim Biochemicals, and radiolabeled nucleotides were obtained from Amersham. Nitrocellulose and Nytran membranes were obtained from Schleicher & Schuell. Oligonucleotides were synthesized by National Biosciences and by the Duke University Botany Department Oligonucleotide Synthesis Facility. The topoisomerase inhibitor *m*-AMSA (4′-[9-acridinylamino]-methanesulfon-*m*-anisidide) was provided by the Drug Synthesis and Chemistry Branch of the National Cancer Institute. L broth for liquid growth of the bacterial strains contained NaCl (10 g/liter), Bacto-Tryptone (10 g/liter), yeast extract (5 g/liter), and when cells carried a pBR322 derivative, ampicillin (25 mg/liter). The EHA plates used for titering T4 phage 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).

Bacterial and phage strains. *E. coli* MCS [*supD araD139* Δ(*ara-leu*)*7697* Δ*lacX74 galU galK hsdR rpsL pro* (uncharacterized proline auxotrophy)] (21) and CR63 (*supD*) were used as permissive hosts for T4 amber mutants, whereas *E. coli* AB1 [*araD139* Δ(*ara-leu*)*7697* Δ*lacX74 galU galK hsdR rpsL*] (21) and B^E were used as nonpermissive hosts. A particular subline of B^E, called B^E-BS, was used to select plasmid-phage cointegrants in the T4 insertion/substitution (I/S) system (24).

T4 strain K10 has the following mutations: *am*B262 (gene *38*), *am*S29 (gene *51*), *nd*28 (*denA*), and *rII*PT8 (a *denB-rII* deletion) (37). K10-*uvsY* is isogenic except for the presence of a 0.12-kb deletion (*uvsY*Δ1) that renders the phage *uvsY* mutant (the strain previously referred to as K10-608 [12]; see reference 21 for the generation of the *uvsY*Δ1 mutation). K10 derivatives with *am*B14 (gene *46*), *am*HL628 (gene *59*), and *am*11 (gene *uvsX*) were generated by genetic crosses (see reference 21 for analogous constructions). The T4 strain HE10s carries the following mutations: *am*B262 (gene *38*), *am*S29 (gene *51*), *nd28* (*denA*), *rII*PT8 (a *denB-rII* deletion), and *24*Δ1 (a deletion of the 2.443-kb gene *24*-containing *Eco*RI fragment, located at kb 106.987 to 109.430 on the T4 genomic map; all T4 genome coordinates in this report are based on the recent map of Kutter et al. [26]). The HE10s strain is identical to previously described T4 HE10 (14), except that HE10s was generated from T4 I/S, whereas HE10 was generated from T4 K10 (the "s" designates the I/S background; the only known difference between T4 I/S and K10 is the presence of an unmapped temperature-sensitive mutation in T4 I/S [37]). T4 strain HE10s-*uvsY*Δ1 was generated by crossing the *uvsY*Δ1 mutation into the HE10s background.

Plasmids. pBSE0f+ and pBSPLO+ carry a *supF* gene fused to a T4 promoter and are used in the I/S system to substitute in vitro-generated mutations into the T4 genome (24, 37). pBSE0f+ also carries a portion of the T4 *frd* gene (T4 genome coordinates, 145.324 to 146.018 kb) with a 20-bp *Xba*I-*Eco*RV linker insertion which disrupts the native *Sal*I site (T4 genome coordinate, 145.644 kb) (37); pBSPLO+ contains a polylinker region (24).

The following plasmids are pBR322 derivatives which contain the indicated portions of the T4 *frd* gene: pMSH1 contains the 839-bp *Eco*RI fragment from pMS636 (a T4 *frd* DNA fragment with the homing site insert; see the construction of pMS636 below) inserted at the vector *Eco*RI site; pMSF11 contains the 742-bp *Eco*RI fragment from pBSE0f+ (a T4 *frd* DNA fragment with no homing site insert; see above) inserted at the vector *Eco*RI site; pMSF12 is nearly identical to pMSF11 except that it does not contain a 28-bp inverted repeat of pBR322 sequence (*Eco*RI-*Hin*dIII) generated in the construction of pMSF11 (no differences in the replication behavior of pMSF11 and pMSF12 have been detected); pMS744 contains the 385-bp *Xba*I-*Hin*dIII fragment from pBSE0f+ (including the upstream region and a portion of the T4 *frd* gene; T4 genome coordinates, 145.644 to 146.018 kb) cloned between the vector *Eco*RI and HindIII sites (an intermediate cloning step introduced an additional 27-bp *Xba*I-*Eco*RI region from the polylinker of pGEM4Z); and pMS747 contains the 331-bp *Eco*RI-*Xba*I fragment from pBSE0f+ (T4 genome coordinates, 145.324 to 145.644 kb) inserted at the vector *Eco*RI site (an intermediate cloning step introduced a 27-bp *Xba*I-*Eco*RI region from the polylinker of pGEM4Z). The constructions of plasmids pMS636 and pMS692 are described below.

The following plasmids are pBR322 derivatives with the indicated T4 DNA inserts from regions other than those of *frd*: pGJB1 contains a *Bgl*II-*Pst*I fragment (T4 genome coordinates, 115.300 to 115.417 kb) with the T4 replication origin *ori*(*uvsY*) (22); pKK467 contains a *Hin*dIII-*Sac*I nonorigin fragment of the T4 genome (114.420 to 114.977 kb [25]); pKK464 contains a *Hin*dIII-*Bgl*II nonorigin fragment of the T4 genome (114.420 to 115.300 kb [25]); and pKK032 contains the T4 gene *24*-containing *Eco*RI fragment (106.987 to 109.430 kb [19]).

Isolation of T4-pBR322 cointegrant phage. A T4 strain with integrated pBR322 sequences was isolated as a pseudorevertant of the gene *24*-deletion phage T4 HE10s (14). This phage carries *denA* and *denB* mutations, which prevent the breakdown of host chromosomal and plasmid DNA, along with the gene *24*Δ1 mutation (see above). Because gene *24* is an essential late gene, T4 HE10s grows only in the presence of a gp24-providing plasmid such as pKK032. This plasmid is a pBR322 derivative with the same *Eco*RI fragment that was deleted from the phage; therefore, the pKK032 and T4 HE10s genomes have no homology. Pseudorevertants of T4 HE10s which are capable of growth in the absence of a gp24-providing plasmid arise at a low frequency; some of these result from integration of the plasmid into the phage genome via nonhomologous recombination (14, 45). One pseudorevertant, named T4 HE13s, was analyzed in detail and found to contain integrated pKK032. Southern hybridization of an *Nde*I digest of (cytosine-modified) DNA from T4 HE13s demonstrated the presence of pBR322 sequences in the phage genome. To confirm the plasmid integration and locate the integration site, T4 HE13s was crossed with another gene *24* deletion phage, T4 CD17 (14). The genetic background of T4 CD17 allows the production of unmodified T4 DNA that can be cleaved by the vast majority of restriction enzymes. The integrated plasmid was moved into this background by selecting for gp24 independence among the progeny of the cross. Several restriction digests of unmodified DNA from the progeny phage confirmed the presence of an integrated pKK032 plasmid and mapped the insertion site to a 1,206-bp *Xba*I fragment on the T4 genome (coordinates, 41.878 to 43.084 kb), a nonessential region between genes *55* and *nrdD* (data not shown). A derivative of T4 HE13s carrying the *uvsY*Δ1 mutation (21) was generated by crossing T4 HE13s with T4 HE10s-*uvsY*Δ1.

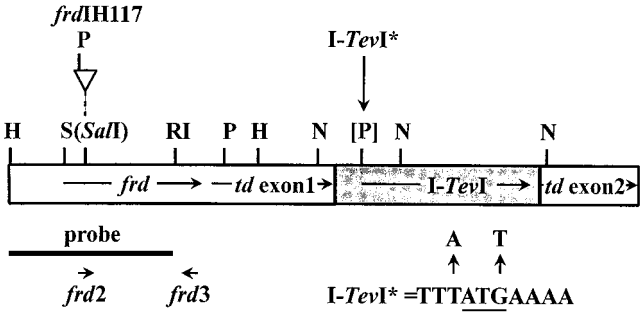

FIG. 2. Map of the *frd-td* region of T4. The T4 genomic map from 146.018 (left) to 143.233 kb (right) is shown with relevant restriction sites (H, *Hin*dIII; S, *Ssp*I; RI, *Eco*RI; P, *Pac*I; N, *Nde*I). The *Sal*I site, which is destroyed upon the insertion of *frd*IH117, is shown in parentheses, and the *Pac*I site introduced with this insertion is indicated. The *Pac*I site introduced with the I-*Tev*I* mutation is shown in brackets, and the sequence of the mutation is shown at the bottom (with the I-*Tev*I initiation codon underlined). The open reading frames of *frd*, *td*, and I-*Tev*I are indicated by horizontal arrows, with the *td* intron being represented by the shaded box. The region labeled "probe" was used as probe in the experiments reflected in Fig. 4 and 5. The *frd*2 and *frd*3 primers, indicated by horizontal arrows, were used in a PCR to generate the probe for Fig. 7.

Isolation of T4 strain KT with I-*Tev*I endonuclease mutation. The following oligonucleotide, with homology to the N terminus of the I-*Tev*I-encoded endonuclease gene, was designed to introduce a mutation (called I-*Tev*I*) that would abolish the initiation codon of the endonuclease: 5′-*GCATCG*ATACTTTATT GTTTAAAGTATTTTTAATCTGATAAATTCCGCTTTTAATTAATACCT CTTTAAATATAGAAGTATTTATTAAAGGGCAAGTC*GACTTGCCC*-3′. The nucleotides in boldface type indicate substitutions from the wild-type sequence, the underlined nucleotides comprise a *Pac*I site that was introduced as a result of the mutations, and the italicized nucleotides are additional bases used in the cloning step but not present in the T4 genomic sequence. The oligonucleotide was synthesized as single-stranded DNA, annealed at the self-complementary 3′ ends to form dimers, and made duplex by extension with Klenow polymerase (35). The resulting DNA was digested with *Cla*I and *Sal*I and cloned into the phage T4 I/S vector pBSPLO+ (24, 37) to form pMS692. After verifying the insert by DNA sequencing, the I-*Tev*I* mutation was introduced into the T4 genome by the T4 I/S system (37). *E. coli* MCS1 cells containing pMS692 were infected with T4 K10, and plasmid integrants and later segregants were selected by the methods outlined by Kreuzer and Selick (24). The segregants were screened for the presence of the I-*Tev*I* mutation by the digestion of purified phage DNA with *Pac*I; the mutations change a 2.420-kb *Pac*I fragment into two smaller fragments (Fig. 2). The T4 strain with the I-*Tev*I* mutation is designated T4 KT.

Isolation of T4 double mutant, KTH, with I-*Tev*I* mutation and I-*Tev*I homing site insert. An oligonucleotide was designed to introduce the homing site for the I-*Tev*I endonuclease into the nonessential *frd* gene of T4 KT. The sequence is based on the I-*Tev*I binding-cleavage site within an intronless version of the T4 *td* gene (6, 11). The oligonucleotide was synthesized as single-stranded DNA, annealed at the self-complementary 3′ ends, and made duplex by extension with Klenow polymerase (35). The duplex products were digested with *Xba*I (which cleaves in the two flanking regions) and cloned into the *Xba*I site of pBSE0f+ to form plasmid pMS636. The insertion is located between the following two sequence blocks of *frd* DNA: AAAGCCGGTC.....GACCGTTTTA. The sequence of the insertion (including the remnant of the *Xba*I-*Eco*RV linker) is as follows: 5′-GAAGATA*TCTAGA*GTTCTATCAGTTTAATGTGCGTAATGG CTATTTGGATTTGCAGTGGTATCAA**CG**CTCAGTAGATGTTTTCTTGG GTCTACCGTTTAATTAA*TCTAGA*TATCTTC-3′ (the I-*Tev*I cleavage site is between the two bases in boldface type, a novel *Pac*I site is underlined, and the *Xba*I sites within the flanking linker regions are italicized). After verifying the homing-site insert by DNA sequencing, the insert was introduced into the genome of T4 KT by the T4 I/S system (Fig. 2). Segregants were screened for the presence of the homing-site insert (called *frd*IH117) by digestion with *Pac*I, which yields a unique 2.25-kb band with phage DNA containing the insert. The double mutant phage is designated T4 KTH.

Isolation of T4 KTH derivatives with recombination-deficient mutations. Triple mutants with the I-*Tev*I* mutation, the *frd*IH117 insert, and either a *46* (*am*B14), *59* (*am*HL628), *uvsX* (*am*11), or *uvsY* (*uvsY*Δ1) mutation were constructed by genetic crosses. T4 K10 strains carrying the single recombination-deficient mutations (21) were first crossed with T4 KT to introduce the I-*Tev*I* mutation, and the resulting double mutants were then crossed with T4 KTH to introduce the homing site. The progeny of the two crosses were screened for the desired mutation as follows: (i) the *46*, *59*, and *uvsX* mutations were detected by cross-streaking (which detects both complementation and recombination) using appropriate tester phage on nonsuppressing host cells (18); (ii) the *uvsY* muta-

tion was detected by spotting candidate phage on a lawn of *E. coli* CR63 on plates containing *m*-AMSA (25 μg/ml), which blocks the growth of *uvsY* mutants (33a); (iii) the I-*Tev*I* mutation and homing-site insert were detected either by restriction digests of phage DNA or by PCR analysis. For PCR analysis, primers *td*987 (5′-GAACAATTGCTCTGTTCGGATC-3′) and *td*2036 (5′-GGGTTTCTG GATTCCAACG-3′) were used to amplify a 1,049-bp DNA fragment that is cleaved into 607- and 442-bp fragments with *Pac*I when the I-*Tev*I* mutation is present, and primers *frd*2 (5′-CCAAGTAAGCATTCG-3′) and *frd*3 (5′-CAAC CATTTCACGCTTGC-3′) were used to amplify a DNA fragment that increased from 564 to 681 bp with the *frd*IH117 insertion.

Preparation and analysis of total DNA from phage infections. Unless otherwise indicated, *E. coli* MCS1 (suppressing) or AB1 (nonsuppressing) with the indicated plasmid was grown at 37°C in L broth to a cell density of 4×10^8/ml and then infected with T4 at a multiplicity of 3 PFU per cell. After a 3-min incubation without shaking (for phage adsorption), the infected cultures were incubated for the indicated period of time (see figure legends) with vigorous shaking at 37°C. Total DNA was then prepared from the infected cells by pelleting the cells in 1.5-ml microfuge tubes for 2 min at 4°C. The pellets were frozen in a dry ice-ethanol bath, and any released phage particles in the supernatant were pelleted by a 1-h centrifugation at 4°C and then frozen in the dry ice-ethanol bath. The two pellets were thawed and resuspended together in 300 μl of lysis buffer (100 mM NaCl, 50 mM Tris-HCl [pH 7.4], 0.2% sodium dodecyl sulfate [SDS], 10 mM EDTA, 0.3 mg of proteinase K per ml) and incubated for 1 h at 65°C. The lysates were then extracted with equal volumes of phenol, phenol: chloroform:isoamyl alcohol (25:24:1, vol/vol/vol), and then chloroform:isoamyl alcohol (24:1, vol/vol) and dialyzed against Tris-EDTA at 4°C overnight.

Quantitation of plasmid replication. Total DNA (5 μl) was digested with *Nde*I and subjected to electrophoresis in a 0.8% agarose gel in TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) at 3 V/cm for 4 h. The gels were transferred to Nytran membranes in alkaline solution, as specified by the manufacturer. The membranes were neutralized in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 15 min at room temperature and then prehybridized (in 50% formamide–6× SSC–0.5% SDS–1× Denhardt's solution–100 μg of denatured herring sperm DNA per ml) at 42°C for 6 h. The membranes were hybridized overnight at 42°C with ^{32}P-labeled DNA from the *frd* region of T4 (a 694-bp *Eco*RI-*Hin*dIII fragment or a 564-bp PCR product generated with primers *frd*2 and *frd*3 labeled by the random primed method). The probe hybridizes to both the *Nde*I-linearized plasmid (pMSH1 or pMSF11) DNA and a 3.537-kb T4 genomic *frd* fragment, allowing quantitation of both plasmid and phage DNA on the same blot. The membranes were washed in 1× SSC–0.5% SDS at 65°C for 30 min and washed twice in 0.1× SSC–0.5% SDS at 65°C for 30 min. The amount of hybridization was measured directly from the membranes by a Scanalytics AMBIS 100 radioisotope imaging system.

RESULTS

Plasmid replication triggered by homology to the T4 genome. Any one of 25 different nonorigin fragments from the T4 genome, when cloned into plasmid pBR322, allowed T4 recombination-dependent plasmid replication (25). This result suggested that recombination-dependent DNA replication initiates at random locations and does not require any specific elements of T4 DNA sequence, although it did not eliminate the possibility that initiation depends on a T4 DNA sequence element that is repeated throughout the genome (and present on each of the 25 inserts). If replication occurs at random locations and does not require any T4 DNA sequence elements, then a phage carrying an insert of pBR322 DNA would direct the replication of a pBR322 plasmid originally resident in the infected cell (even though that plasmid contains no phage DNA insert). To test this prediction, we isolated a T4 phage strain, HE13s, with pBR322 integrated stably in the phage genome. As described in more detail in Materials and Methods, T4 HE13s is an illegitimate plasmid-phage recombinant that was derived from the gene *24*-deletion phage, T4 HE10s (also, see Table 1 for T4 strain genotypes). The integrated plasmid in T4 HE13s consists of pBR322 with the same gene *24* fragment that is deleted from T4 HE10s. Thus, T4 HE13s contains an intact gene *24*, albeit at an unusual location.

E. coli host cells containing pBR322 were infected with T4 HE13s or with either of two control phage strains, the parent T4 HE10s and the grandparent T4 I/S (T4 I/S is the gene 24^+ parent of T4 HE10s; see Materials and Methods and reference 14). After a 1-h infection, DNA was prepared from the infected cells and digested with restriction enzymes *Hae*III and *Ssp*I. T4-modified DNA, with glucosylated hydroxymethylcytosine residues, is resistant to most restriction enzymes, including *Hae*III. Resistance to *Hae*III thereby provides a convenient tool for specifically examining T4-replicated (i.e., modified) plasmid DNA, because *Hae*III cleaves unreplicated plasmid into many small fragments. T4-modified DNA is sensitive to *Ssp*I, which cleaves the phage chromosome into a series of fragments of less than 3.5 kb and linearizes T4-replicated pBR322 DNA (4.3 kb).

TABLE 1. Genetic background of bacteriophage T4 strains[a]

Strain	Genotype
HE10s	*denA* (*denB-rII*)$^\Delta$ *38*^{am} *51*^{am} *24*$^\Delta$
HE13s	*denA* (*denB-rII*)$^\Delta$ *38*^{am} *51*^{am} *24*$^\Delta$ (with integrated plasmid pKK032)
K10	*denA* (*denB-rII*)$^\Delta$ *38*^{am} *51*^{am}
KT	*denA* (*denB-rII*)$^\Delta$ *38*^{am} *51*^{am} I-*Tev*I*
KTH	*denA* (*denB-rII*)$^\Delta$ *38*^{am} *51*^{am} I-*Tev*I* *frd*IH117

[a] Allele designations and information on the origin of each strain are presented in Materials and Methods. Derivatives of these strains with one additional mutation are referred to by appending the mutant gene number on the parental strain name (e.g., KT-*46* is a *46*^{am} derivative of KT).

The control phages T4 HE10s and T4 I/S failed to replicate the pBR322 originally resident in the infected host cells. Both infections yielded only phage genomic DNA fragments, which are 3.5 kb and smaller (Fig. 3A, lanes 1 and 2). Digestion of the DNA from the HE13s-infected cells, however, yielded two fragments larger than 3.5 kb (Fig. 3A, lane 3). The 6.2-kb fragment is the integrated plasmid DNA that originates from the genome of HE13s and is also seen with phage genomic DNA that has been grown on plasmid-free host cells (data not shown). The novel 4.3-kb fragment is the size expected for replicated pBR322 DNA; the identity of this fragment was

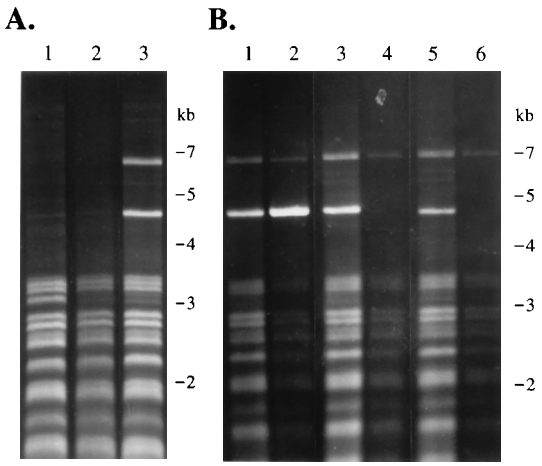

FIG. 3. Replication of plasmids by T4-pBR322 cointegrant phage. (A) *E. coli* MCS1 bearing plasmid pBR322 was infected with T4 I/S (lane 1), T4 HE10s (lane 2), or T4 HE13s (lane 3). (B) *E. coli* MCS1 bearing plasmid pGJB1 (lanes 1 and 2), pKK467 (lanes 3 and 4), or pBR322 (lanes 5 and 6) were infected with either T4 HE13s (lanes 1, 3, and 5) or its *uvsY*-mutant derivative (lanes 2, 4, and 6). In all cases, the infected cells were incubated for 1 h at 37°C. DNA was then prepared from the infected cultures and digested with *Ssp*I and *Hae*III. The resulting fragments were separated by agarose gel electrophoresis and stained with ethidium bromide. The molecular size scale was generated from the migration of *Xba*I fragments of T4 cytosine-containing DNA. Due to the presence of the glucosyl residues, the T4-replicated plasmid and phage DNA restriction fragments migrate slightly slower than expected on the basis of the molecular size scale, which was generated with nonglucosylated DNA.

confirmed by Southern hybridization with a pBR322 probe (data not shown). Therefore, a pBR322 plasmid, initially resident in the infected host cell, can replicate following infection by T4 HE13s. We conclude that the presence of homology between the T4 HE13s chromosome and pBR322 permits plasmid replication by T4 because the closely related T4 HE10s without the plasmid insert was unable to replicate pBR322. Also, the replication of pBR322 by T4 HE13s demonstrates that recombination-dependent plasmid replication does not require any T4 DNA sequences on the plasmid.

If the homology-driven replication of pBR322 by T4 HE13s occurs by T4 recombination-dependent DNA replication, then it should also require phage-encoded recombination proteins such as the product of gene *uvsY*. We previously showed that UvsY is required for the T4-directed replication of pBR322 derivatives carrying T4 DNA inserts but is not required for replication of pBR322 derivatives containing cloned T4 replication origins (21, 25). A *uvsY* deletion mutation was introduced into T4 HE13s by a genetic cross. T4 HE13s and its *uvsY* mutant derivative were then used to infect host cells containing each of three different plasmids: (i) pGJB1, a pBR322 derivative with a cloned *ori*(*uvsY*) DNA fragment; (ii) pKK467, a pBR322 derivative containing a 558-bp insert of T4 DNA with no origin that was previously used to analyze T4 recombination-dependent DNA replication (25); and (iii) pBR322 with no insert. As in the above-described experiment, DNA was prepared from the infected cells and digested with *Hae*III and *Ssp*I.

The total amount of phage DNA recovered from each *uvsY* mutant infection was significantly reduced compared with that from the *uvsY*$^+$ infection, reflecting the dependence of the majority of T4 DNA synthesis on phage recombination proteins (Fig. 3B, compare even- and odd-numbered lanes). As expected from previous studies (21), the replication of the origin-containing plasmid pGJB1 was not dependent on UvsY protein but rather was increased by the *uvsY* mutation (Fig. 3B, lanes 1 and 2). Also as expected, the replication of the T4 insert-containing plasmid pKK467 was blocked by the *uvsY* mutation (Fig. 3B, lanes 3 and 4). Importantly, the replication of pBR322 by T4 HE13s was also blocked by the *uvsY* mutation (Fig. 3B, lanes 5 and 6). This result is consistent with the requirement for homology between phage and plasmid and strongly supports the conclusion that T4 HE13s replicates pBR322 via the recombination-dependent initiation mode of T4. The conclusion is further supported by the finding that most of the replicated pBR322 DNA is in the form of plasmid concatemers (data not shown), as was previously observed for recombination-dependent plasmid replication (25).

Construction of T4 phage strains that cleave their own genome. According to the model of Mosig (32), the recombination-dependent replication of T4 DNA is initiated when an unreplicated 3′ genomic end invades the homologous region of the same or another T4 chromosome (see the introduction). The recombination-dependent replication of plasmids with homology to the phage genome might likewise depend on the generation of an unreplicated end of the T4 genome. Because the ends of T4 DNA are random, any plasmid with homology to the phage genome would be homologous to the ends of a subset of genomic phage DNA molecules. We wished to directly test whether the ends of genomic T4 DNA trigger recombination-dependent plasmid DNA replication by introducing a site-specific double-strand break (dsb) at a defined location of the phage genome and then measuring replication of a plasmid with homology to this genomic end. In this section we describe phage strains that cleave their own genome, and in the next section we use these phage strains to demonstrate that genomic ends can indeed trigger plasmid replication.

The construction of phage strains that cleave their own genome was based on analyses of intron mobility in the T-even phages (for a review, see references 6 and 11). Wild-type T4 contains an intron within the *td* gene, and the intron itself encodes a site-specific double-stranded DNA endonuclease, I-*Tev*I. Wild-type T4 does not contain recognition sites for I-*Tev*I. However, relatives or variants of T4 that are missing the *td* intron contain such a recognition site, called the homing site, at the junction between the two exon sequences in their genomic DNA. In a mixed infection, the intronless phage DNA is cleaved by the I-*Tev*I produced from the intron-containing phage. Repair of the dsb then occurs, with the intron-containing phage genome as the template. The net result is a very efficient gene conversion event in which the intronless phage genome gains a copy of the intron (6, 11).

We first attempted to introduce a synthetic homing site into the nonessential *frd* gene of T4 using the I/S system for T4 genome substitution (24, 37). In the I/S system, a plasmid containing an insert with homology to T4 DNA integrates into the phage genome via homologous recombination, creating a duplication of the cloned insert. Subsequent excision via a second act of homologous recombination eliminates the plasmid vector and one copy of the sequence duplication. Depending on the sites of crossovers, a constructed mutation within the cloned plasmid insert can be left behind in the phage genome during the excision event, resulting in the desired mutant phage (which no longer carries plasmid vector sequences). We cloned a synthetic homing site near the middle of a 0.7-kb *frd* DNA fragment within the I/S plasmid vector and attempted the genome substitution. However, we were unable to isolate a phage carrying the homing site in the *frd* gene, even though another insertion at this same location was previously generated by the same technique (37). Presumably, either the desired phage is inviable because it cleaves its genome too frequently or the homing site in the plasmid is cleaved very efficiently and lost upon plasmid integration.

In an attempt to circumvent this problem, we next mutated I-*Tev*I, the coding sequence for the intron endonuclease. The overall strategy was to eventually generate a double mutant that was I-*Tev*I deficient and carried the homing site insert in *frd* and then coinfect cells with both the double mutant and wild-type T4 to generate cleaved T4 chromosomes. A synthetic oligonucleotide was designed to mimic the translational initiation region of the I-*Tev*I gene, except that the initiator ATG was changed to ATT. We expected that this mutation (I-*Tev*I*) would abolish the translation of I-*Tev*I. As described in Materials and Methods, the genome substitution was successful and the resultant phage strain is called T4 KT (see Fig. 2 for a map). To analyze the possible endonuclease activity of this phage strain, we also generated a gene *46* mutant derivative called T4 KT-*46*. Albright and Geiduschek (1) had previously shown that plasmid DNA with a dsb is greatly stabilized in vivo by a mutation that inactivates gp46/47, presumably because this protein is, or controls the activity of, an exonuclease involved in linear DNA degradation.

The I-*Tev*I activity induced by KT, KT-*46*, and the I-*Tev*I$^+$ control strains K10 and K10-*46* were analyzed by infecting cells that carry a pBR322 derivative, pMSH1, with a cloned homing site within a T4 *frd* DNA fragment. DNA was prepared at various times after infection, cleaved with *Pst*I (which linearizes unreplicated plasmid), and analyzed by Southern hybridization with a plasmid probe. Several major plasmid DNA forms were detected from the T4 K10 control infection (Fig. 4A). The bulk of the plasmid DNA at the earliest time was

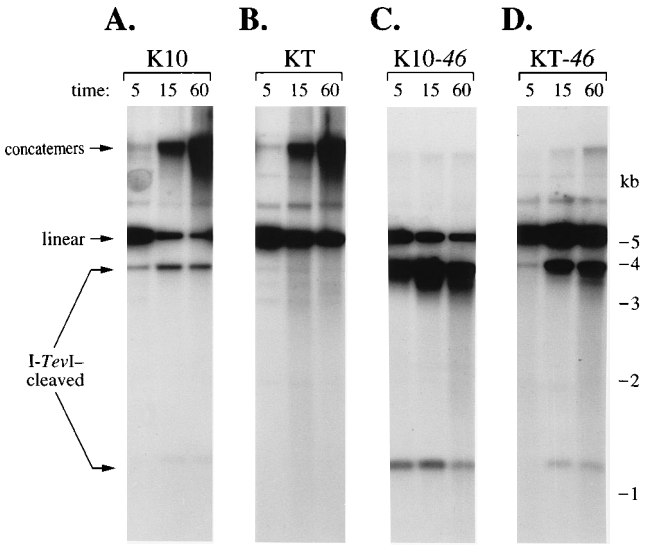

FIG. 4. Cleavage of plasmid with *td* homing site by T4 phage with and without the I-*Tev*I* mutation. *E. coli* AB1 containing plasmid pMSH1 was infected with either T4 K10 (I-*Tev*I$^+$ *46*$^+$) (A), T4 KT (I-*Tev*I* *46*$^+$) (B), T4 K10-*46* (I-*Tev*I$^+$ *46* mutant) (C), or T4 KT-*46* (I-*Tev*I* *46* mutant) (D) and incubated for the indicated times at 37°C. Total DNA was then prepared from the infected cultures and digested with *Pst*I, subjected to electrophoresis through a 1% agarose gel, and transferred to nitrocellulose. The nitrocellulose was probed with radiolabeled pBR322 DNA. The positions of T4-replicated plasmid concatemers (resistant to *Pst*I cleavage), *Pst*I-linearized plasmid, and I-*Tev*I-cleaved plasmid DNA are indicated. The molecular size scale was generated from the migration of *Xba*I fragments of T4 cytosine-containing DNA.

cleaved into a linear unit-length fragment by the restriction enzyme, indicating that it was neither replicated (which would make it resistant to *Pst*I cleavage) nor cleaved by I-*Tev*I. As the infection progressed, a major portion of plasmid DNA migrated near the top of the gel, at the position of limiting migration of long linear DNA fragments. This band represents concatemers of T4-replicated plasmid DNA, which were generated by recombination-dependent replication of the plasmid because of homology with the phage genome (see the introduction). Similar replication was detected in a control plasmid without the I-*Tev*I cleavage site (data not shown), indicating that this plasmid replication is not dependent on the presence of the cleavage site in the plasmid. The most important bands for the purpose of this experiment were the two with sizes of 4.1 and 1.1 kb, which were generated in small amounts in the wild-type K10 infection (Fig. 4A). These two bands result from in vivo cleavage of the plasmid at the homing site followed by in vitro cleavage with *Pst*I. The site of cleavage was mapped to the inserted homing site by analysis with three different restriction enzymes, and these two bands were not detected with a control plasmid lacking the homing site (data not shown).

Dramatically different amounts of the plasmid DNA forms were detected from the K10-*46* infection (Fig. 4C). First, the slowly migrating T4-replicated plasmid DNA was virtually absent, as expected on the basis of the requirement of gp46/47 in plasmid recombination-dependent DNA replication (25). Second, large amounts of I-*Tev*I-cleaved plasmid DNA were detected, even at the earliest time point. Indeed, the bulk of the plasmid DNA had been cleaved by I-*Tev*I within the first 8 min of infection (a 3-min adsorption period plus a 5-min incubation). This result confirms the conclusion that inactivation of gp46/47 greatly stabilizes cleaved plasmid DNA during a T4 infection (1). Presumably, the same amount of I-*Tev*I cleavage also occurred in the K10 (*46*$^+$) infection, but most of the cleaved plasmid DNA was either degraded or repaired.

The effect of the I-*Tev*I* mutation on homing site cleavage can now be considered. In the KT (I-*Tev*I* *46*$^+$) infection, only a trace amount of I-*Tev*I-cleaved plasmid DNA was detected (Fig. 4B). However, when cleaved DNA processing was prevented by the *46* mutation, substantial amounts of I-*Tev*I-cleaved plasmid DNA were evident in spite of the I-*Tev*I* mutation (Fig. 4D). The extent of cleavage was reduced compared with the K10-*46* (I-*Tev*I$^+$) infection, and the time course of cleavage appeared to be somewhat delayed. Nonetheless, the I-*Tev*I* mutation clearly does not abolish I-*Tev*I-directed DNA cleavage. Presumably, the mutated initiation codon (ATT) functions at a reduced level to initiate translation or an alternative start codon is functional.

Because the I-*Tev*I* mutation reduces but does not abolish I-*Tev*I-directed DNA cleavage, it seemed possible that the homing site could now be successfully introduced into the mutant phage chromosome to create a T4 strain that cleaves its own genome. We therefore repeated the I/S procedure described above using the I/S plasmid with a T4 *frd* DNA insert containing the cloned homing site (the insertion mutation is called *frd*IH117, for insertion of homing site 117 bp; see Fig. 2 for a map). The substitution was indeed successful, generating the new phage strain KTH (see Materials and Methods).

To determine whether T4 KTH actually cleaves its own genome, we infected plasmid-free cells with this phage strain, with a *46* mutant derivative of this strain, or with either of two control strains that do not contain the homing site insert (KT and KT-*46*). Total DNA was digested with *Nde*I (which cleaves T4-modified DNA) and analyzed by Southern hybridization with a T4 *frd* probe. The T4 genomic *frd* DNA fragment was cleaved at the expected location, and cleavage occurred only in the two phage strains that contain the homing site insert (Fig. 5, lanes 2 and 4). The amount of detectably cleaved DNA was increased substantially by the gene *46* mutation, just as with cleaved plasmid DNA in the previous experiment. We conclude that T4 KTH cleaves its own genome at the inserted homing site and that gp46/47 participates in the repair and/or degradation of T4 genomic DNA that has sustained a dsb.

Plasmid replication triggered by a dsb in the phage genome. With the availability of a T4 phage strain that cleaves its own genome, we could return to the original question: does an artificially introduced DNA end in the phage chromosome stimulate the recombination-dependent DNA replication of a plasmid that has homology to the broken region? Host cells carrying pMSF11, a pBR322 derivative with a T4 *frd* fragment (but no homing site), were infected with T4 KTH, which cleaves its genome in the *frd* region, or with the control strain KT, which does not. Total intracellular DNA was prepared after 60 min of infection and cleaved with *Ssp*I and *Hae*III, and replicated plasmid DNA was visualized by hybridization with a plasmid DNA probe. A modest amount of plasmid pMSF11 DNA replication was detected in the control infection with T4 KT, the phage that does not carry the homing site (Fig. 6, lane 1). This represents the background level of recombination-dependent plasmid replication that we had previously detected with plasmids that have homology to the phage genome (25). However, the presence of the homing site in the genome of T4 KTH increased the replication of the *frd*-containing plasmid approximately twofold (Fig. 6, lane 2). The stimulation of plasmid DNA replication is limited to plasmids that have homology near or at the break site, because control plasmids with T4 DNA inserts from other regions of the phage chromosome were replicated to similar levels by T4 KT and T4 KTH (Fig. 6, lanes 7 and 8; also, data not shown). We conclude that a dsb in

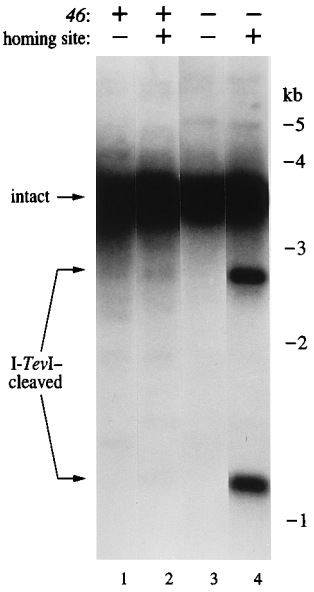

FIG. 5. Self cleavage of genomic DNA by phage with the I-*Tev*I* mutation and homing-site insert. *E. coli* B^E (with no plasmid) was infected with either T4 KT (lane 1), KTH (lane 2), KT-*46* (lane 3), or KTH-*46* (lane 4) at a multiplicity of infection of 8 PFU per cell (the genotype for gene *46* and the presence or absence of the homing site insertion are indicated above the lanes). The infected cells were incubated for 30 min at 37°C, and total DNA was then prepared and digested with *Nde*I (which cleaves T4-modified DNA). The DNA fragments were subjected to electrophoresis through a 0.8% agarose gel and transferred to nitrocellulose. The probe for the Southern blot consisted of the 0.694-kb *Eco*RI-*Hin*dIII fragment of the T4 *frd* gene, labeled by the random primed method. The positions of the intact 3.537-kb *Nde*I genomic fragment and the two products resulting from I-*Tev*I cleavage are indicated. The molecular size scale was generated from the migration of *Xba*I fragments of T4 cytosine-containing DNA.

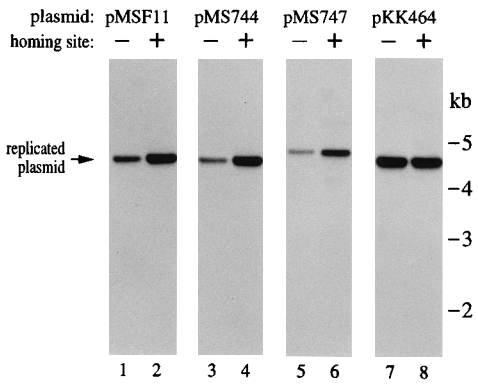

FIG. 6. Test for dsb-directed replication of plasmids with homology to the T4 genome. *E. coli* MCS1 cells containing the indicated plasmids were infected with either T4 KT (which has no homing site; −) or T4 KTH (with the homing site insert; +) and incubated for 1 h at 37°C. Total DNA was then prepared from the infected cultures, digested with *Ssp*I and *Hae*III, subjected to electrophoresis through a 0.8% agarose gel, and transferred to Nytran membranes. The membranes were probed with a radiolabeled *Hin*dIII-*Sal*I fragment of pBR322. The autoradiogram shows T4-replicated (*Hae*III-resistant) plasmid DNA. Each of the plasmids consists of pBR322 with a T4 DNA insert. pMSF11, which contains the *Hin*dIII-*Eco*RI fragment of *frd* that spans the region where *frd*IH117 was inserted, is digested by *Ssp*I into 4.47- and 0.64-kb fragments. pMS744, which contains the upstream portion of *frd* (to the left of the *frd*IH117 insertion in Fig. 2), is cleaved into 4.41- and 0.33-kb fragments. Only the larger fragments of pMSF11 and pMS744 hybridize to the probe. Plasmid pMS747, which includes the region of *frd* to the right of the *frd*IH117 insertion, is linearized into a 4.72-kb fragment by *Ssp*I. pKK464, which contains a region of T4 DNA (114.420 to 115.300 kb) remote from the introduced dsb site, is cleaved by *Ssp*I into 4.45- and 0.77-kb fragments (the larger of which hybridizes to the probe). The molecular size scale was generated from the migration of *Bst*EII fragments of λ DNA.

the phage chromosome can trigger the replication of a plasmid with homology to the broken region, even though the plasmid itself contains no break site. Thus, recombination-dependent DNA replication can be triggered by a double-stranded DNA end during T4 infections.

The experiment just described used a plasmid (pMSF11) with homology to both sides of the dsb in the phage chromosome. It seemed possible that many of the phage chromosomal dsbs in that experiment were repaired using a plasmid DNA template, with no net replication of plasmid DNA. If so, this conventional dsb repair might compete with dsb-directed plasmid replication and thereby reduce the level of plasmid replication. To test this proposition, the experiment was repeated with two plasmids (pMS744 and pMS747) that each contained only one side of the homology present in the larger pMSF11 plasmid. In each case, plasmid replication was again stimulated approximately twofold by cleavage of the phage chromosome (Fig. 6, lanes 3 to 6; quantitation of parallel DNA samples was conducted with a direct radioisotope imaging system). The presence of homology on both sides of the dsb does not appear to inhibit dsb-directed plasmid replication.

The products of phage genes *uvsX*, *uvsY*, *46*/*47*, and *59* are required for maximal levels of recombination-dependent replication of the phage genome or of plasmids with homology to the phage chromosome (see the introduction). We therefore tested whether each of these gene products is also necessary for dsb-directed plasmid DNA replication by crossing mutations into the T4 KTH genetic background and then repeating the dsb-directed plasmid replication experiment described above. The levels of replicated plasmid and phage DNA were quantitated in four repetitions of the experiment, which gave consistent results (the Southern blot for one experiment is shown in Fig. 7, and the quantitated data from this blot is presented in Table 2).

The mutations in *uvsX* and *uvsY* essentially abolished recombination-dependent plasmid replication, whether or not the infecting phage chromosome contained the engineered dsbs (Fig. 7; Table 2). In contrast, the gene *46* and *59* mutations reduced, but did not abolish, dsb-directed plasmid replication. Several points should be considered in judging the extent of the deficiency caused by these two mutations. First, the total amount of replicated plasmid DNA in infections with phage carrying the engineered dsbs was strongly reduced by the *46* and *59* mutations (12- and 14-fold, respectively). Second, the ratio of replicated plasmid DNA to phage DNA was also reduced by the *46* and *59* mutations (2.8- and 1.65-fold, respectively) but not nearly as much as was the total amount of plasmid DNA. This ratio might be considered a more realistic estimate of dsb-directed plasmid replication: the amount of one of the participating DNA molecules (the phage DNA) was significantly reduced in these two mutant infections, which might reduce plasmid replication by an indirect mechanism. Third, the presence of the dsb in the phage chromosome clearly did stimulate additional plasmid replication in either the gene *46*- or *59*-mutant infection. Considering these three points, we think that gp46 and gp59 are probably involved in dsb-directed plasmid replication. The residual replication detected in the two mutant infections implies that either the amber mutations are leaky or that these two proteins are not strictly required for dsb-directed plasmid replication. It is also important to note that mutations in gene *46* and *59* are expected to reduce late gene expression (which requires concurrent phage DNA replication) and therefore could reduce the

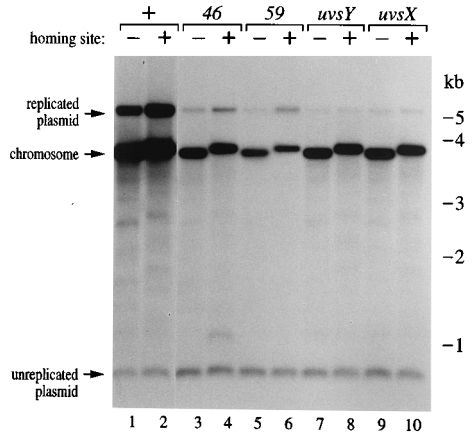

FIG. 7. Dsb-directed plasmid replication in wild-type and mutant T4 phage. *E. coli* AB1 containing plasmid pMSF12 was infected with the following T4 strains: KT (lane 1); KTH (lane 2); KT-*46* (lane 3); KTH-*46* (lane 4); KT-*59* (lane 5); KTH-*59* (lane 6); KT-*uvsY* (lane 7); KTH-*uvsY* (lane 8); KT-*uvsX* (lane 9); and KTH-*uvsX* (lane 10). Following a 1-h incubation at 37°C, total DNA was prepared and then digested with *Nde*I and *Hae*III. The DNAs were subjected to electrophoresis through a 0.8% agarose gel and transferred to a Nytran membrane. The probe for the Southern blot was prepared by amplifying the *frd* region by PCR with the *frd*2 and *frd*3 primers (see Materials and Methods). The T4-replicated plasmid DNA is linearized by *Nde*I and resistant to *Hae*III, giving a 5.05-kb band (indicated by an arrow). The probe also hybridizes to a 3.53- or 3.65-kb chromosomal *Nde*I fragment from the *frd* region (the size of the fragment is increased by the *frd*IH117 insertion). One fragment of the unreplicated plasmid is detected as a 0.6-kb *Hae*III fragment. A quantitation of the replicated plasmid and phage chromosomal DNA fragments from this experiment is presented in Table 2. The molecular size scale was generated from the migration of *Bst*EII fragments of λ DNA.

expression of I-*Tev*I from its late promoter. Thus, we cannot eliminate the possibility that the reduction in dsb-directed plasmid replication in the *46*- and *59*-mutant infections is an indirect consequence of reduced I-*Tev*I expression. Further work is necessary to explore the roles of these two important proteins in dsb-directed DNA replication. Nevertheless, it is clear that maximal levels of dsb-directed plasmid replication require the same recombination proteins that are involved in recombination-dependent replication of the phage genome or of plasmids with homology to the phage genome (in the absence of engineered dsbs). Furthermore, as we had previously observed for plasmid recombination-dependent DNA replication without engineered breaks (25), a mutation in *uvsX* or *uvsY* is much more debilitating to the process than is a mutation in gene *46* or *59*.

TABLE 2. Replicated DNA levels in wild-type and mutant phage infections[a]

Strain	Replicated plasmid DNA (cpm)	Phage chromosomal DNA (cpm)	Replicated plasmid DNA/phage chromosomal DNA[b]	Ratio for KTH/ratio for KT[c]
KT	182	1,100	0.165	
KTH	417	1,180	0.353	2.14
KT-*46*	20.3	305	0.067	
KTH-*46*	33.6	265	0.127	1.90
KT-*59*	11.0	150	0.073	
KTH-*59*	28.9	135	0.214	2.93
KT-*uvsY*	11.3	470	0.024	
KTH-*uvsY*	11.3	467	0.024	1.0
KT-*uvsX*	10.4	469	0.022	
KTH-*uvsX*	12.2	351	0.035	1.59

[a] The Southern blot shown in Fig. 7 was quantitated by an AMBIS 100 radioisotope imaging system and the results are presented here.

[b] We estimate the maximum error in this ratio to be roughly 10% on the basis of repeated quantitations of the same DNA samples.

[c] The ratio of replicated plasmid DNA to phage DNA for the KTH strain divided by the same ratio for the KT strain. This experiment was repeated four times for the wild type, *46*, *59*, and *uvsY* strains and twice for the *uvsX* strains. Quantitations of the repeats gave average ratios of 2.33 for the wild type, 1.76 for the *46* mutant, 2.23 for the *59* mutant, 1.03 for the *uvsY* mutant, and 1.26 for the *uvsX* mutant. The values for the *uvsY* and *uvsX* mutants are based on plasmid counts per minute that are just above background, and the replicated plasmid DNA/phage chromosomal DNA ratios are therefore not accurate.

DISCUSSION

In this study, we have tested two important predictions of the model that Mosig (32) proposed to explain T4 recombination-dependent DNA replication (Fig. 1). First, we tested the DNA sequence requirements for T4 recombination-dependent replication. As predicted by the model, we found that recombination-dependent replication does not require any particular T4 DNA sequences but rather requires only homology between the two participating DNA molecules. Second, the model predicts that the ends of the T4 genome, each formally equivalent to half of a dsb, are responsible for the strand invasion that leads to recombination-dependent DNA replication. We have shown that an engineered dsb can indeed trigger recombination-dependent replication in a homologous DNA molecule, supporting the importance of T4 DNA ends.

In vivo T4 recombination-dependent DNA replication has been rather difficult to study in the context of the phage genome because it can occur anywhere in the 170-kb phage genome and because origin-dependent replication also contributes to phage genomic replication. The plasmid model system for dsb-directed replication can potentially alleviate both of these problems, providing a site-specific version of a process that usually occurs throughout the phage genome. We thereby hope to identify blocked intermediates in recombination-dependent replication by analyzing DNA isolated from cells infected with one or another T4 recombination or replication mutant, providing in vivo evidence for a detailed biochemical pathway.

As described in the introduction, the biochemical activities of several critical T4 recombination proteins have been well studied, leading to a fairly detailed model for T4 recombination-dependent replication. The products of genes *uvsX*, *uvsY*, and *32* function together to catalyze a strand-exchange reaction in vitro and therefore probably catalyze this reaction in recombination-dependent replication (Fig. 1, step B). In addition, gp59 assists in the loading of the replicative helicase-primase complex onto appropriate DNA substrates in vitro (5, 31), arguing that this protein is important in assembling the replication complex within the recombination intermediate.

In contrast to these well-studied proteins, gp46/47 plays a central but poorly understood role in T4 DNA metabolism (for a review, see reference 23). In a long-standing model, gp46/47 constitutes an exonuclease involved in host DNA degradation and in T4 recombination (30). The observation that *46*/*47* mutations stabilize double-strand ends in vivo is consistent with this model and suggests that gp46/47 might catalyze 5′ to 3′ exonuclease action at double-stranded ends. This model could also explain why gp46/47 is not required for in vitro recombination-dependent DNA replication, because a single-stranded primer DNA is provided in that system (15). Importantly, *46*/*47* mutations stabilize broken DNA ends regardless of whether they are in a plasmid or in the phage genome (1; this communication). These results again indicate that gp46/47 plays a critical role both in DNA degradation (i.e., broken

plasmid DNA) and DNA recombination (i.e., broken phage DNA, presumably undergoing some form of dsb repair). If gp46/47 is a recombination nuclease, it likely has at least one additional activity to explain its role in phage recombination-dependent replication. The incomplete replication of genomic 3′ ends (initially, during origin-dependent replication) should be sufficient for the generation of the single-stranded primer DNA for recombination-dependent replication, obviating the need for a 5′ to 3′ exonuclease. Perhaps gp46/47 has additional activities involved in recombination, such as a helicase activity similar to that of the RecBCD enzyme (for a review, see references 17 and 38). Alternatively, gp46/47 may not itself be an exonuclease but may rather be indirectly required in vivo for the exonuclease activity of another protein.

Recombination-dependent DNA replication is not limited to phage T4. Following the induction of the SOS system, *E. coli* is capable of an alternative mode of replication, called iSDR (inducible stable DNA replication), which appears to be quite analogous to T4 recombination-dependent replication. iSDR depends on RecA and other *E. coli* recombination proteins and does not require various factors that are normally required for *oriC*-driven replication (DnaA protein, transcription, translation, and an intact *oriC*) (4, 28). The analogy between T4 and *E. coli* recombination-dependent replication apparently extends to the triggering mechanism. Using plasmids with engineered dsbs, Asai et al. recently showed that dsbs can indeed trigger DNA replication in SOS-induced *E. coli* (2). A recent model for iSDR in *E. coli* (2) is quite similar to the Mosig model for phage T4 recombination-dependent replication. In the model for iSDR, the induction of the SOS system somehow leads to preferential dsbs in a few specific locations of the bacterial chromosome. After exonucleolytic generation of single-stranded 3′ ends, strand invasion into an unbroken homolog leads to the assembly of bidirectional replication forks. Studies of recombination-dependent DNA replication triggered by dsbs may therefore be relevant to the duplication of both viral and cellular genomes.

Recombination-dependent DNA replication appears to be very closely linked to the process of dsb repair. Dsb repair is now implicated in many key biological processes, including DNA repair following chemical and physical damage, meiotic recombination, and generation of antibody diversity (16). In the models proposed by Resnick (36) and Szostak et al. (41), repair of a dsb or double-strand gap requires limited DNA replication within the affected region. An alternative model suggests that replication is much more extensive during certain forms of dsb repair. Specifically, recombination during the process of conjugation or generalized transduction in *E. coli* involves an invading duplex DNA with two ends, which are hypothesized to trigger the assembly of two oppositely oriented replication forks that traverse the entire bacterial genome to generate the completed recombinant (2, 39).

The mobile introns of phage T4 have provided an excellent system for analyzing the mechanism of dsb repair (for a review, see references 6 and 11). When an intron-containing and an intronless phage DNA coinfect the same cell, I-*Tev*I produced from the intron-containing phage DNA cleaves the intronless DNA at the homing site, leading to efficient dsb repair and the acquisition of the intron. Recent studies have implicated the T4 recombination proteins UvsX, UvsY, gp46/47, and gp59, along with phage-encoded replication proteins, in this mode of dsb repair (10, 6a). Thus, the requirements for dsb-directed DNA replication and dsb repair are very closely related or perhaps identical.

Phage T4 KTH undergoes repair of its self-inflicted dsbs in cells carrying the *frd* plasmid with no cleavage site (data not shown). Thus, in the same infection, the phage chromosomal dsbs can lead to either plasmid DNA replication or to repair of phage chromosomal DNA. What exactly is the relationship between dsb repair and break-stimulated DNA replication, both in the specific T4 system and in general? Is a decision made between local DNA replication and extensive genomic replication? How are the strand invasion events at the two ends of a newly broken molecule coordinated, and does lack of coordination lead to genomic replication? Answers to these and other questions are necessary for a complete understanding of the fate of dsbs in various biological systems.

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