Double-Strand Break Repair in Tandem Repeats During Bacteriophage T4 Infection

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ABSTRACT
Recombinational repair of double-strand breaks in tandemly repeated sequences often results in the loss of one or more copies of the repeat. The single-strand annealing (SSA) model for repair has been proposed to account for this nonconservative recombination. In this study we present a plasmid-based physical assay that measures SSA during bacteriophage T4 infection and apply this assay to the genetic analysis of break repair. SSA occurs readily in broken plasmid DNA and is independent of the strand exchange protein UvsX and its accessory factor UvsY. We use the unique features of T4 DNA metabolism to examine the link between SSA repair and DNA replication and demonstrate directly that the DNA polymerase and the major replicative helicase of the phage are not required for SSA repair. We also show that the Escherichia coli RecBCD enzyme can mediate the degradation of broken DNA during early, but not late, times of infection. Finally, we consider the status of broken ends during the course of the infection and propose a model for SSA during T4 infections.

THE efficient repair of double-strand DNA breaks (DSBs) is of vital importance in virtually every organism. Multiple mechanisms of repair have been described and grouped into pathways that differ in key features, such as the nature of the DNA substrates, the types of proteins that catalyze repair, and the conformation of product DNA. Except for pathways that depend on nonhomologous end joining, double-strand break repair (DSBR) requires the interaction of homologous DNA sequences. Repair mechanisms that require homology are closely associated with recombination and typically use a second copy of the damaged sequence to restore information lost at the site of the break.

Homologous recombination is often dependent on strand exchange proteins (e.g., RecA in Escherichia coli) that catalyze the invasion of a single-stranded DNA into a homologous duplex. However, a major pathway of homologous DSBR in a variety of organisms is independent of known strand exchange proteins. This pathway, called single-strand annealing (SSA), has been proposed to operate in bacteria, phage, yeast, higher plants, Xenopus oocytes, and mammalian cells (Lin et al. 1984; Maryon and Carroll 1991; Puchta and Hohn 1991; de Groot et al. 1992; Luisi-DeLuca and Kolodner 1992; Daly and Minton 1996; Ivanov et al. 1996; Stahl et al. 1997; Lai and Masker 1998; Paques and Haber 1999). Generally, SSA was proposed when the DSB was positioned between tandem (direct) repeats or when complementary sequences could be exposed at two broken ends. Transformation of linear dimer plasmids into E. coli can result in monomer circular recombinants by a RecA-independent pathway, which may involve SSA (Luisi-DeLuca and Kolodner 1992). Intermolecular SSA between two broken phage λ chromosomes was catalyzed by the Red recombination system when replication was blocked and the host RecA protein was absent (Stahl et al. 1997). In the yeast system, SSA was independent of Rad51, a known homologue of the bacterial RecA protein (Ivanov et al. 1996).

The defining feature of SSA repair is the annealing of two single-stranded homologous of opposite polarity; this single-stranded DNA could potentially be generated from double-strand breaks through the action of nucleases or helicases. The complementary strands can arise from opposite sides of a break, in the case of tandemly repeated DNA sequences, or from separate molecules that have terminal homology. An important consequence of SSA repair is the loss of at least one copy of the homologous DNA, along with any intervening sequence that flanks the break site. Thus, SSA is inherently nonconservative, a feature that distinguishes it from many other homologous DSBR mechanisms.

Several features of T4 biology suggest that SSA repair plays an important role in the phage life cycle. Almost 30 years ago, Broker and Lehman observed structures under the electron microscope that were suggestive of annealed single strands (Broker and Lehman 1971; Broker 1973). They analyzed infections in which DNA replication was severely impaired; consequently, the role of the annealed structures in a normal infection or in the context of break repair was not established. Furthermore, mutations in the bacteriophage T4 UvsX protein,
a RecA homologue, reduce phage recombination by only a fewfold, even in a recA mutant host (Hamett and Berger 1975; Cunningham and Berger 1977; Yonesaki et al. 1984; Kreuzer and Kreuzer 1994). This robust recombination in the absence of a known strand exchange protein, coupled with the ability to study mutations in essential genes, makes T4 an excellent model system in which to investigate SSA. Indeed, Mosig (1988) and others (Kozinski and Felgenhauer 1967; Broker 1973) have suggested that SSA contributes to phage chromosomal recombination during infections.

Plasmid model systems have been used for the physical characterization of DSBR in several systems. Haber and colleagues developed plasmid-based assays for repair in Saccharomyces cerevisiae and used them to describe SSA in that organism (Haber 1995; Paques and Haber 1999). Using similar methodology, George and Kreuzer (1996) used an inverted-repeat plasmid to measure DSBR during T4 infections. In both cases, plasmid DNA was monitored by Southern blotting after a DSBR was introduced in a controlled fashion. This type of physical analysis has two significant advantages over traditional genetic approaches. First, the repair reaction can be tracked in various stages by assaying the status of the digested and undigested DNA, and thereby in the repair of plasmids that are not repaired (or incompletely so) can be identified and characterized. Second, a wide variety of mutants, including conditional lethals, can be studied, since the approach does not demand the production of viable progeny. This is particularly appealing in the T4 system because virtually every substitution protocol (demand the production of viable progeny. This is particular-}

**Materials and Methods**

**Materials**: T4 DNA ligase, E. coli exonuclease III, and restriction enzymes were purchased from New England Biolabs (Beverly, MA), Nytren nylon transfer membranes from Schleicher and Schuell (Keene, NH), sequencing kits from National Diagnostics (Atlanta, GA), random-primed labeling kits from Boehringer-Mannheim Biochemicals (Indianapolis, IN), [α-32P]dATP from Amersham (Piscataway, NJ) and DuPont-NEN (Wilmington, DE), oligonucleotides from National Biosciences Inc. (Plymouth, MN) and the Duke University Oligo-nucleotide Synthesis Facility, and frozen electrocompetent cells (ElectromAX DH10B) from Gibco-BRL (Gathersburg, MD). Growth media were formulated as follows: L-broth: 10 g/liter sodium chloride, 10 g/liter Bacto Tryptone, and 5 g/liter yeast extract; EHA top agar: 8 g/liter sodium chloride, 13 g/liter Bacto Tryptone, 2 g/liter sodium citrate, 1.3 g/liter glucose, and 6.5 g/liter agar.

**Strains**: E. coli strain JG995 (recA1 recA spot1 thi-1 deoB38) has been previously described (George and Kreuzer 1996). Strains KL185 (galK35 λ− pyrD34 trpC45 his-68 rpsL118I(Str); malT1(λh8) xylA7 mtEB2) and KL186 (galK35 λ− pyrD34 trpC45 his-68 rpsL118I(Str); malT1(λh8) xylA7 mtEB2) were obtained from the E. coli Genetic Stock Center at Yale University. T4 strains are described in Table 1. PhenacreA and denB mutations prevent breakdown of host chromosomal and plasmid DNA, and the amber mutations in genes 38 and 51 prevent phage assembly when not suppressed, but do not affect DNA metabolism (Karmam 1994).

**Plasmids**: Plasmid pSTL55, a pBR322 derivative with a duplication of part of the tet gene, was a generous gift from Dr. Susan Lovett (Brandeis University; Lovett et al. 1993). A duplex oligonucleotide encoding the I-Tev recognition sequence was generated by annealing the following single-strand oligonucleotides: 5′-CTAGAGAAATTCACGGCTCAGTAGAT GTTTTCTGGGTCTACCTTTATATTGGCTGCAATTCT-3′ and 5′-CTAGAGAAATTCTACGGCAATATTAACCGGT AGACCCGAAAAATACCTACTGAAGCTTGGATT-3′. This duplex was ligated into pSTL55 that had been partially digested with NheI. A clone containing the oligonucleotide insert at the NheI site near the junction of the repeats was selected by restriction analysis, and the identity of the insert was confirmed by sequencing with the Sequenase kit.

**Construction of the ITM strain**: A T4 strain with a consensus middle-module promoter in place of the native late promoter of the I-Tev gene was constructed using the T4 insertion/substitution protocol (Selick et al. 1988; Kreuzer and Selick 1994). A duplex oligonucleotide was generated by annealing single-stranded oligonucleotides: 5′-GGCCGCAATTCTAACCGGGAACCTCTCTAGTAGACCGTTATGTTTCTGGGTCTACCTTTATATTGGCTGCAATTCT-3′ and 5′-GGCCGCTCAGATTATTATTTACCTATTGCAATACCGGCTTCTCTGTTCAATAATACCTCTTTTAATTTAGAGATTTAAAAATTTTATCATAGTTTTGAGCATTATTGATAAGG-3′ and extending them with Taq DNA polymerase. This duplex was digested with EcoRI and XhoI and cloned into the polylinker of plasmid pBSPL0+. Identity of the insert in the plasmid was confirmed by automated sequencing at the Duke University Sequencing Facility. The phage substitution mutant (T4 ITM) was obtained via homologous recombination using the insertion/substitution system, resulting in a phage with the desired promoter substitution and no plasmid sequences.

**Sample collection and Southern blots**: Aliquots of frozen log-phase JG995, KL185, or KL186 cells harboring the appropriate plasmid were diluted 1:200 in fresh L-broth and grown with vigorous shaking at 37° C to a density of 4 × 10^8 cells/ml. Phage were added at a multiplicity of 3 pfu/cell and incubated at 37° C for 4 min to allow phage adsorption. Cultures were returned to vigorous shaking at 37° C and aliquots were removed at the times indicated. The infected cells were collected by brief centrifugation and frozen in a dry ice/ethanol slurry, and total nucleic acids were then prepared as previously described (George and Kreuzer 1996). Aliquots of prepared nucleic acids were digested for 4–6 h with the indicated restriction enzymes, and the resulting fragments were separated by elec-trophoresis in a 1% agarose gel (13 × 25 cm or 20 × 25 cm gel) in 0.5× TBE buffer (44.5 mm Tris base, 44.5 mm boric acid, 1 mm EDTA) at 3 V/cm for 16 hr. Gels were prepared for Southern blotting using the rapid alkaline protocol of
TABLE 1

T4 strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>K10</td>
<td>amB262 (gene 38)</td>
<td>Selick et al. (1988)</td>
</tr>
<tr>
<td></td>
<td>amS29 (gene 51)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>nd28 (dnaA)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rllPT8 (del8-rll)</td>
<td>Kreuzer et al. (1988a,b)</td>
</tr>
<tr>
<td>K10-uvSX</td>
<td>K10, am11 (gene uvsX)</td>
<td>Derr and Kreuzer (1990)</td>
</tr>
<tr>
<td>K10-uvSY</td>
<td>K10, uvsYΔ1</td>
<td>Benson and Kreuzer (1992)</td>
</tr>
<tr>
<td>K10-32</td>
<td>K10, amA453 (gene 32)</td>
<td>Kreuzer et al. (1988a,b)</td>
</tr>
<tr>
<td>K10-46</td>
<td>K10, amB14 (gene 46)</td>
<td>This work</td>
</tr>
<tr>
<td>ITM</td>
<td>K10, ITM (I-Tev1 promoter)</td>
<td>This work</td>
</tr>
<tr>
<td>ITM-32</td>
<td>ITM, amA453 (gene 32)</td>
<td>This work</td>
</tr>
<tr>
<td>ITM-46</td>
<td>ITM, amB14 (gene 46)</td>
<td>This work</td>
</tr>
<tr>
<td>ITM-41</td>
<td>ITM, amN81 (gene 41)</td>
<td>This work; Benson and Kreuzer (1992)</td>
</tr>
<tr>
<td>ITM-43</td>
<td>ITM, amE4332 (gene 43)</td>
<td>This work; Benson and Kreuzer (1992)</td>
</tr>
<tr>
<td>ITM-59</td>
<td>ITM, amHL628 (gene 59)</td>
<td>This work; Kreuzer et al. (1992)</td>
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RESULTS

DSBR in tandem repeats during bacteriophage T4 infection produces two distinct products: We have developed a plasmid assay to monitor the repair of DSBs during phage infection, primarily through direct physical analysis of plasmid DNA isolated from infected cells. The relevant plasmids are maintained in E. coli, and the analysis is initiated by infecting with T4. Plasmid pTD001 is a modified version of pSTL55 (Lovett et al. 1993), which was originally derived from pBR322. pTD001 contains a recognition sequence for the phage-encoded endonuclease I-Tev1, which creates a DSB at a defined site in the plasmid during the phage infection (Figure 1). The DNA sequences adjacent to the I-Tev1 cleavage site in pTD001 are arranged as tandem (direct) repeats (787 bp long), with the cleavage site centered between the repeats. This repeated DNA sequence disrupts the coding region of the tetracycline resistance gene of the plasmid; therefore, pTD001 does not confer tetracycline resistance. A DSBR event that deletes one of the two tandem repeats will regenerate pBR322, a smaller plasmid (4361 bp) that confers tetracycline resistance. pTD001 is maintained in E. coli host cells, which are infected with phage to initiate the analysis.

The interaction of the repair and replication systems in T4-infected cells increases the complexity of our anal-

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Figure 1.—Schematic of plasmids used in the DSBR assay. pTD001 contains a 787-bp duplication in the tetracycline resistance gene and an I-Tev1 cleavage site near the junction of the repeats. Cleavage generates a staggered DSB flanked by 53 and 51 bp of nonhomology on the left and right sides of the break, respectively. Nonconservative but precise repair of the break eliminates the duplicated sequence and regenerates pBR322.

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ysis. Bacteriophage T4 is known to initiate DNA replication from DSBs, and break-directed plasmid DNA replication has been shown to accompany DSBR in an inverted-repeat plasmid (George and Kreuzer 1996). Replication by phage enzymes incorporates hydroxymethylcytosine (hmC) into the DNA chain in place of cytosine; the replicated DNA is further modified by the addition of glucose residues to the hmC-DNA. Consequently, DNA that has been replicated by the phage becomes resistant to most restriction enzymes (including A\textsc{f}III and H\textsc{ae}III). Phage-replicated plasmid DNA is typically recovered as long concatamers that have a low mobility in agarose gels (Mattson et al. 1983; Kreuzer and Alberts 1986; Kreuzer et al. 1988a). We therefore anticipated that some repaired DNA might be in the form of replicated concatamers that would not be cut by A\textsc{f}III. The starting pTD001 plasmid, along with any cleaved or repaired DNA that has not undergone extensive replication, should be cut normally by A\textsc{f}III.

The generation and repair of DSBs were detected in the plasmid DNA soon after the T4 infection began. In vivo cleavage by I-TevI, followed by in vitro restriction with A\textsc{f}III, generated the expected plasmid DNA fragments of \(\sim 2295\) and 2913 bp. These cleaved bands were evident 10 min into the infection but quickly faded thereafter (Figure 2A, d bands). Concomitant with the appearance of these cleavage bands, another band appeared at the position expected for the deletion repair product, in which one copy of the tandem repeat has been precisely removed (Figure 2A, band c). The expected position of the I-TevI cleavage in the d bands and the expected nature of the deletion in band c have both been confirmed by extensive restriction mapping (data not shown). A short time after the appearance of the cleaved DNA and the deletion product, a band containing high-molecular-weight plasmid DNA begins to accumulate (Figure 2A, band a). This band is presumably composed of replicated plasmid concatamers that are resistant to A\textsc{f}III digestion.

A control plasmid, pSTL55, that does not contain an I-TevI cleavage site but is otherwise identical to pTD001, was analyzed to test the importance of the introduced DSB. This plasmid produced no cleaved DNA, no deletion products, and no replicated concatamers at 10 or 40 min (Figure 2A, lane 8; data not shown). We conclude that the cleavage and deletion formation we observe in plasmid pTD001 are specifically induced by I-TevI cleavage and that the deletion represents a bona fide DSBR event.

We used the unique characteristics of T4-replicated DNA to further analyze the products of DSBR in this system. The restriction enzyme A\textsc{s}I is able to cut glucosylated hmC-DNA efficiently. After A\textsc{s}I digestion, product DNA was liberated from the concatemeric forms that were evident in the A\textsc{f}III-digested sample (compare Figure 2B, lane 1, and Figure 2A, lane 6). Significantly, this liberated material is in the form of deletion product, which migrates more quickly than the starting pTD001 plasmid. Its position in the gel just above the unreplicated deletion product is caused by a small reduction in mobility imparted by the glucosyl modifications. The relatively low intensity of the replicated concatemer band (A\textsc{f}III digests) vs. the replicated monomer band (A\textsc{s}I digests) is presumably caused by poor transfer of large molecules during the Southern blot procedure.

Restriction enzyme H\textsc{ae}III cleaves unmodified plasmid DNA to further analyze the products of DSBR in this system. The restriction enzyme A\textsc{s}I is able to cut glucosylated hmC-DNA efficiently. After A\textsc{s}I digestion, product DNA was liberated from the concatemeric forms that were evident in the A\textsc{f}III-digested sample (compare Figure 2B, lane 1, and Figure 2A, lane 6). Significantly, this liberated material is in the form of deletion product, which migrates more quickly than the starting pTD001 plasmid. Its position in the gel just above the unreplicated deletion product is caused by a small reduction in mobility imparted by the glucosyl modifications. The relatively low intensity of the replicated concatemer band (A\textsc{f}III digests) vs. the replicated monomer band (A\textsc{s}I digests) is presumably caused by poor transfer of large molecules during the Southern blot procedure.

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mid DNA into very small fragments but cannot cleave modified DNA. Adding HaeII to the AsI digest effectively removes any unreplicated DNA, since the small HaeII-cleaved fragments run off the bottom of the gel. As expected, only the replicated deletion product remains intact after HaeII digestion (Figure 2B, lanes 3 and 4). These results imply that replication of this product is complete, with no intact DNA remaining in the entire plasmid. These results also demonstrate that replication only occurs in tight association with DSBR, since the control plasmid lacking the I-Tev site produced neither replicated material nor repair products.

In this study, we will focus mainly on the repair pathway that results in unreplicated deletion product. This pathway potentially involves an SSA mechanism of DSBR, which was not detected in a similar system using inverted repeats (George and Kreuzer 1996).

**DSBR in tandem repeats occurs in the absence of strand exchange proteins:** T4 recombination proteins are well characterized in vitro, and many have homologues in both bacterial and eukaryotic systems (for reviews see Kreuzer and Morrical 1994; Mosig 1994). To test the possible roles of these proteins in DSBR, E. coli cells harboring plasmid pTD001 were infected with mutant phage, and DNA from infected cells was digested with AflIII to reveal the repaired but unreplicated DNA.

In each case, samples were collected at 10 and 40 min after infection, to reveal both the initial and final accumulated levels of repair and cleavage products.

Of particular interest in the context of SSA are the phage protein UvsX, a DNA strand exchange protein (a functional and structural homologue of RecA in E. coli and Rad51 in both yeast and mammalian cells; Bianco et al. 1998), and its accessory factor UvsY (a functional analogue of Rad52; Benson et al. 1998). Eliminating these activities by mutation had no impact on the level of unreplicated deletion product (Figure 3, lanes 4–7, band c; E. coli strain JG99S, the host for these experiments, is recA−). Interestingly, production of the replicated concatemer product is greatly reduced, but not abolished, by these mutations (see discussion).

Other phage mutations significantly affected the production of the unreplicated deletion product. An amber mutation in gene 32, which encodes the single-strand DNA binding protein of the phage, completely abolished the reaction (Figure 3, lanes 8 and 9). Phage gene 46 encodes one subunit of a putative exonuclease that is thought to be directly involved in recombination and recombinational repair (Mickelson and Wiberg 1981). Mutations in this gene also had a dramatic effect on the fate of the DSB. I-Tev cleaved DNA accumulated to very high levels during the 46-mutant infection (Figure 3, lanes 10 and 11, d bands), arguing that gp46 is normally involved in processing the DNA ends. Furthermore, accumulation of the unreplicated deletion product was reduced and delayed (Figure 3, lanes 10 and 11, band c). We conclude that gp32 and gp46 are necessary for the normal production of the unreplicated deletion product.

**Construction of a mutant phage with altered I-Tev expression:** The link between DSBR and DNA replication was of particular interest to us, but the programmed nature of T4 gene expression initially prevented us from applying the DSBR assay to phage replication mutants. The endonuclease I-Tev, used to generate DSBs in pTD001, is normally expressed during the late phase of T4 gene expression (Clyman et al. 1994). However, T4 late gene expression requires simultaneous DNA replication (Williams et al. 1994), and thus mutations that abolish phage DNA replication also abolish expression of I-Tev (data not shown). To avoid this problem and to allow a direct investigation of T4 replication mutants, we created a phage mutant that expresses I-Tev even in the absence of DNA replication.

We replaced the native late promoter of I-Tev with an artificial sequence that contained consensus elements for a T4 middle-mode promoter (for review see Stitt and Hinton 1994). Middle-mode gene expression requires the host RNA polymerase in conjunction with several T4 proteins, but does not require concurrent phage DNA replication. We moved the artificial promoter sequence into the phage genome by using the T4 insertion/substitution system (Selick et al. 1988) and identified positive clones by PCR analysis. We then

![Figure 3.—Cleavage and repair of plasmid DNA in T4 recombination mutant infections. Samples were prepared from infected cells carrying plasmid pTD001. Plasmid DNA was digested with AflIII and analyzed by Southern blotting using a pBR322 probe. The uninfected control (UI) is in lane 1, and all other lanes are loaded in pairs, representing 10-min (even-numbered lanes) and 40-min (odd-numbered lanes) infections. The infecting phages were K10 (WT), K10-uvsX− (uvsX), K10-uvsY− (uvsY), K10-32− (32), and K10-46− (46).](image-url)
tested the efficacy of the middle-mode promoter substitution by crossing in additional mutations in phage genes 33 and 55 that block late gene expression. T4 33−55 mutants with the native I-TevI late promoter did not produce I-TevI, as judged by in vivo cleavage of pTD001, but the middle-mode promoter substitution mutant produced copious amounts of cleaved plasmid (data not shown). We named the promoter substitution mutant T4 ITM (I-TevI Middle; strain lacks the 33−55 mutations) and used it as a basis for additional experiments.

Our initial attempts to measure DSBR during ITM infections revealed a profound difference in the nature of the cleavage and repair reactions. Although a small amount of I-TevI-cleaved DNA was evident, almost no deletion product was observed after 30 min of infection (Figure 4A, lane 2). We then crossed the 46− mutation into the ITM background, but this double mutant showed only a slight increase in cleaved DNA and no measurable deletion product formation above background (Figure 4A, lane 3; compare to Figure 3, lane 11). Thus, the accumulation of cleaved plasmid DNA was greatly diminished in the ITM background, even when gene 46 was mutated, a condition that normally favors such accumulation.

We reasoned that a host nuclease might be degrading the cleaved plasmid DNA, which was being produced earlier than normal in the phage infection. E. coli exonuclease V (RecBCD) is known to be highly active on double-stranded ends and is thought to be active during the early part of T4 infections (Lipska et al. 1989; Appasani et al. 1999). We moved plasmid pTD001 into a host strain that carried the recB21 mutation, which inactivates exonuclease V, and repeated the assay with phage ITM. Under these conditions, cleaved plasmid DNA accumulated to high levels and both types of deletion product were evident (Figure 4A, lane 5). Infection with the double mutant ITM-46− resulted in a large accumulation of cleaved plasmid DNA, as well as the production of a significant amount of unreplicated deletion product at late times (Figure 4A, lane 6). For both the 46− and 46− phage, the amount of cleaved plasmid DNA was increased relative to comparable infections in the recB+ HindII and a supporting nuclease activity is sufficient to repair the break (Appasani et al. 1999). Alternative models suggest that the combination of annealing and a supporting nuclease activity is sufficient to repair the break (Maryon and Carroll 1991; Dornfeld and Livingston 1992). The behavior of phage DNA replication mutants should be particularly informative with regard to the mechanism of single-strand annealing, and so we moved appropriate mutations into the ITM background. Host cells carrying plasmid pTD001 were infected with ITM phage deficient in one of several important DNA replication or recombination activities, and DNA was collected and analyzed as above.

Although the amounts of unreplicated deletion product were lower in the ITM background, significant effects of the replication and recombination mutations...
mutation in gene 41 totally eliminated the replicated concatamer product. However, mutations in either 41 or 59 had little or no effect on the unreplicated deletion product (Figure 5, lanes 10–13, band c). A small amount of replicated deletion product was still produced during the 59\textsuperscript{−} infection, similar to the result seen in the uvsX\textsuperscript{−} and uvsY\textsuperscript{−} infections above (see discussion).

**Plasmid transformation measures DSBR in pTD001:**
The reduced intensity of the unreplicated deletion product in ITM infections made accurate quantitation difficult. We therefore developed a companion assay that provides an alternative estimate of break repair. Precise deletion of one copy of the tandem repeat in pTD001 restores the tetracycline resistance gene of the plasmid (Lovett et al. 1993). By transforming DNA isolated from infected cells into competent E. coli, we measured the number of plasmids that had acquired an intact \texttt{tet} gene. This transformable signal is almost entirely sensitive (>90%) to H\texttt{adII} digestion (data not shown), demonstrating that replicated plasmid concatemers do not contribute appreciably and that the assay measures only unreplicated deletion product. Unlike the physical assay, which is insensitive to small sequence alterations or subtle repair defects, the transformation assay demands perfect restoration of the original \texttt{tet} reading frame.

We transformed DH 10B cells with DNA from infected cells and plated the transformed cells on carbenicillin alone (all plasmids support growth) and on carbenicillin plus tetracycline (only repaired deletion plasmids support growth). The proportion of recombinant plasmids in each sample was then estimated by comparing the two numbers. A low frequency of recombinants was observed prior to phage infection, and a wild-type phage infection stimulated recombination \textasciitilde 11-fold (Figure 6). Mutations in genes 32 and 46 significantly inhibited break repair, with the former mutation having the most pronounced effect. Gene 41 and gene 43 mutants showed an intermediate level of break repair in this assay, reducing the amount of transformable signal roughly 2-fold. Since gp41 and gp43 are not strictly required for repair, phage-directed DNA replication is presumably not obligatory in this pathway. The mutation in gene 59 did not affect the reaction, which is surprising because this protein is thought to interact with gp41 during replication initiation (see discussion).

**Two-dimensional gel analysis of cleaved DNA reveals degradation of both strands:**
The bands corresponding to I-Tev\textsuperscript{I}-cleaved DNA were visibly smeared in most samples analyzed above. In a yeast model system, cleaved DNA was shown to be progressively resected on one strand, presumably as a key intermediate in SSA repair (Haber 1995). We therefore analyzed the smeared DNA bands generated after I-Tev\textsuperscript{I} cleavage to determine if they contained resected DNA. Samples were run on agarose gels under conditions comparable to those used.
Figure 6.—Plasmid transformation measures DSBR. DNA from infected cells was transformed into recA^-E. coli cells. Cells were plated on carbenicillin alone (all plasmids support growth; Carb^R) or on carbenicillin plus tetracycline (only deletion products support growth; Tet^R Carb^R). Frequency of repair was calculated by dividing the number of colonies on carbenicillin plus tetracycline by the number on carbenicillin alone. The data represent a typical transformation of the DNA samples in Figure 5. Samples were collected from recB^- host cells, uninfected (UI) or infected for 10 or 30 min with T4 ITM (WT), ITM-32^- (32), ITM-46^- (46), ITM-43^- (43), ITM-41^- (41), or ITM-59^- (59).

Figure 7.—Southern blot of cleaved DNA analyzed by twodimensional neutral/alkaline gel electrophoresis. The firstdimension gel was a neutral gel run in the presence of ethidium bromide. Each gel lane was cut out and DNA was denatured by soaking in NaOH. The slice was then cast and run in the second-dimension gel in the presence of NaOH, and the DNA was visualized by Southern blotting with a pBR322 probe. A control with a linear plasmid DNA fragment (2713 bp), resected from one end by E. coli exonuclease III in vitro, is shown in A. B shows a sample collected from recB^- host cells, carrying plasmid pTD001, infected with T4 K10 phage for 10 min, and digested with A^III prior to electrophoresis. The heavy spot in the top left is A^III-linearized full-length pTD001. Other spots along the diagonal are repaired, deleted pTD001 and the 2913- and 2295-bp I-Tev-cleaved fragments of pTD001 (spots from upper left to lower right, respectively).

In Figures 2–5, except that ethidium bromide was added to accentuate the difference in mobility between fully duplex and partially single-stranded molecules. Lanes from these first-dimension gels were cut out and soaked in NaOH to denature DNA and then run on a seconddimension gel in the presence of NaOH along the perpendicular axis.

In this type of two-dimensional gel, linear fragments with resection on one strand produce a characteristic “caret” pattern (Maryon and Carroll 1991). This caret pattern develops because, upon denaturation between dimensions, resected molecules release one full-length strand and one strand of variable, shorter length. All full-length strands comigrate during the second dimension, producing the horizontal arm of the caret, while the ever-shorter resected strands migrate faster and thus produce the sloping arm. This assay would not detect molecules in which resection has proceeded past the A^III site (used to linearize the plasmids prior to analysis) or molecules that have been rendered fully single stranded, but it should readily detect plasmids with limited resection at one or both ends. As a control, linear plasmid DNA with one end resected in vitro by E. coli exonuclease III produced a prominent caret pattern (Figure 7A). In contrast, every in vivo sample we analyzed produced a contrasting pattern—a diagonal smear with no hint of a caret (Figure 7B). This diagonal smear represents uniform degradation of both strands, since the mobility of the unpaired strands remains constant after denaturation. We obtained this result with DNA from the wild-type infection (Figure 7B), as well as DNA from a variety of mutant infections (K10, K10-32^-, K10-46^-, ITM, ITM-41^-, ITM-43^-, and ITM-46^-) collected from several E. coli backgrounds (data not shown). Numerous time points were analyzed, both early and late in the infection, including a series from a low-temperature infection in which the reaction is much slower (presumably extending the life span of any intermediates). In each case, no resection was detected and the pattern was consistent with double-stranded degradation (data...
not shown). We conclude that the smeared DNA is generated by double-strand degradation after I-TevI cleavage.

**DISCUSSION**

We have found that double-strand breaks between tandem repeats are repaired efficiently during bacteriophage T4 infection. Three distinct repair pathways were uncovered, differing in their genetic requirements, their time of occurrence during the infection, and the nature of the repaired products that are produced. The predominant repair products late in the infection are long, replicated concatemers that have lost one copy of the tandem repeat. Production of this material is largely dependent on the UvsX and UvsY proteins of the phage and as such probably requires one or more strand invasion events. We propose that this product is generated by an extensive chromosomal replication (ECR) mechanism (George and Kreuzer 1996) in which an initial strand invasion establishes one or more replication forks that subsequently generate multiple copies of the repaired plasmid. Although this is the predominant repair product, this pathway need not be the predominant mechanism for repairing the DSB; the extensive replication of the repaired molecules leads to overrepresentation of these products. A small amount of replicated deletion product is produced during infections by uvsX, uvsY, or 59 mutants, suggesting one or more additional pathways for replication-coupled repair.

An unreplicated deletion product is also generated during phage infections and is present early during the infection with reasonable abundance. This product is produced at wild-type levels in the absence of the phage strand invasion proteins UvsX and UvsY (even in the absence of host RecA) and appears to be stable throughout the infection. Restriction mapping has confirmed that this product has lost one copy of the tandem repeat, thus regenerating native pBR322 sequence. Furthermore, transformation of this repaired DNA into competent cells confers tetracycline resistance, indicating that the reading frame in the repaired region has been precisely restored. On the basis of its independence from strand exchange proteins and its lack of extensive DNA replication, this product is very likely generated by a single-strand annealing mechanism.

UvsX protein is homologous to strand exchange proteins from other organisms, including bacterial RecA and S. cerevisiae Rad51. Genetic data in yeast show that SSA repair is not dependent on Rad51 (Ivanov et al. 1996), and similarly, SSA between broken phage \( \lambda \) chromosomes is independent of RecA (Stahl et al. 1997). In D. radiodurans, a gram-positive bacterium, recircularization of plasmids bearing direct repeats, but not inverted repeats, was found to occur after massive radiation damage in recA mutants (Daly and Minton 1996). We see comparable results with our plasmid model system; direct repeats can be repaired by SSA in a UvsX-independent manner (this study), while inverted repeats cannot (George and Kreuzer 1996). In our studies, uvsY mutants were indistinguishable from uvsX mutants. Recent reports suggest that yeast Rad52 protein is a functional analogue of UvsY (Benson et al. 1998), and several studies show that Rad52 is not required for SSA repair of large homologies in yeast (Dornfeld and Livingston 1992; Prado and Aguilar 1995; Paques and Haber 1999). Additional evidence for SSA has been reported in several higher eukaryotes, although in general the protein requirements in those systems are not known (Lin et al. 1984; Maryon and Carroll 1991; Puchta and Hohn 1991). We infer that T4 can repair double-strand breaks by an SSA pathway that seems to be highly conserved in many diverse organisms.

Early efforts to characterize recombination during T4 infections led to a model with features similar to the SSA mechanism. Broker and Lehman (1971) proposed that the early stages of recombination involved resection of nicked DNA by an exonuclease, followed by annealing of complementary strands promoted by the phage gp32 protein. Using electron microscopy, they examined recombination during infections that lacked both DNA polymerase (gp43) and polynucleotide ligase (gp30). In their system, gp32 mutants were 10-fold less proficient at generating branched DNA structures. Our results indicate that recombination in a tandem-repeat substrate is totally dependent on gp32. The involvement of single-stranded DNA binding proteins may be general, because a mutation in the RPA protein caused a significant defect in SSA repair after induction of a double-strand break in tandem repeats in the yeast system (Umez u et al. 1998). Both T4 studies indicate that gp32 plays a key role during SSA, presumably by promoting the efficient annealing of complementary single strands, which it does in vitro (Kong et al. 1997).

Of the proteins investigated in this study, only gp46 and its accessory factor gp47 have not been purified and characterized in vitro. Several studies indicate that gp46/47 either encodes or modulates an exonuclease activity (Michelson and Wiberg 1981; Mosig 1998). Furthermore, gp47 carries a phosphoesterase motif also found in E. coli SbcD (subunit of the SbcCD nuclease) and S. cerevisiae Mre11 protein (Sharpl es and Leach 1995). Our data support the assignment of gp46/47 as a recombination nuclease, in that gp46 mutants accumulate broken DNA to much higher levels than wild-type phage. Sequence analysis of gp46 reveals several motifs common among helicases, suggesting that the gp46/47 enzyme may have helicase as well as nuclease functions (Sharpl es and Leach 1995). Our data are also consistent with an involvement of the putative helicase activity in repair of DSBs in the T4 infection, since we were unable to detect resected DNA in a wild-type infection (see below). Gene 46 mutants are significantly
impaired for SSA repair at early times of infection, although these mutants appear by Southern blot analysis to accumulate some repaired product at late times. In contrast to samples from other mutants, this accumulated material is not represented proportionately in the transformation assay. As the transformation assay demands strict restoration of the original reading frame in the repaired region, this suggests that the observed band represents incompletely or improperly repaired plasmid. We surmise that gp46, acting as either a helicase or a nuclease, is important in generating single-stranded DNA prior to strand annealing. Additionally, we propose that a substantial fraction of the unreplicated product band produced during 46\textsuperscript{-} infections is not fully repaired and may represent an intermediate or abortive side-product of repair.

The importance of DNA degradation was particularly evident in our studies of the ITM mutation, which altered the timing of the DSB and had an immense impact on the overall character of the repair reactions. A simple change in the timing of the break from late to middle times of infection (from \(\sim\)10 min to 4 min into the infection) revealed the profound influence of the host RecBCD nuclease. The RecBCD complex of E. coli is exonuclease V, which is known to play a pivotal role in a major recombination and repair pathway in the bacteria (Myers and Stahl 1994). This nuclease modulates recombination via its interaction with double-strand ends, and in the ITM infections, I-Tev\textsuperscript{-}broken DNA was greatly stabilized when RecBCD was mutated. Specific inactivation of the proposed nucleolytic subunit of the complex, RecD, was also sufficient to stabilize the cleaved DNA in our assay and to allow the generation of complete repair products (D. Tomso and K. Kreuzer, unpublished data). The apparent insensitivity of wild-type (K10; late I-Tev\textsuperscript{-} promoter) infections to RecBCD suggests that host exonuclease V activity is irrelevant by late times of infection. Indeed, the late T4 protein gp2 has been shown to protect against exonuclease V degradation and plays a key role in protecting the ends of infecting phage DNA (Lipinska et al. 1989; Appasani et al. 1999). Protection by gp2 at late but not early times could account for the differential degradation of broken plasmid DNA in K10 vs. ITM infections.

Another possible explanation for the altered processing of broken DNA in the ITM infections is that the phage may require a fairly long period of infection to generate sufficient quantities of proteins (e.g., gp32 and gp46/47) to catalyze the efficient repair of DSBs. Several important repair proteins, including gp32 and gp46/47, do not reach peak levels until at least 7–9 min into the infection (Cowan et al. 1994). According to this view, I-Tev\textsuperscript{-} cuts generated at late times would be repaired quickly, whereas those produced prematurely in the ITM infections would not be repaired efficiently and thus would become susceptible to degradation by RecBCD. Insufficient quantities of gp32 and/or gp46/47 at early times could also explain the relatively low level of repaired plasmid generated during ITM infections of the rec\textsuperscript{B} host.

The ITM mutant strain allowed us to investigate directly the role of several DNA replication proteins, including the DNA polymerase (gp43), the replicative helicase (gp41), and the helicase loading factor (gp59). Unlike gene conversion and other similar conservative repair processes, SSA is nonconservative and does not have an inherent requirement for new DNA synthesis. However, several models for SSA have suggested that synthesis may play a role in filling in gaps formed by extensive nuclelease degradation (Lin et al. 1990; Puchta and Hohn 1991; Lai and Masker 1998; Paques and Haber 1999). In our experiments, gp43- and gp41-deficient mutants both displayed measurable levels of SSA repair above background, implying that SSA can in fact operate without any phage-directed DNA synthesis. In this case, repair may depend on precise nuclelease cleavage of annealed structures to generate nicked (i.e., gap-free) intermediates or may involve gap filling by a host polymerase. Nonetheless, a significant reduction in repair compared to wild-type infections was also observed. These results suggest that a subset of SSA repair events requires some localized synthesis to restore gaps in the plasmid, although we have thus far been unable to detect any replicated patches in the repaired DNA (data not shown). Mutations in the DNA synthesis machinery of the phage may also cause a generalized disruption in the physiology of the infection, for example, by altering late gene expression (Williams et al. 1994), and thereby exert an indirect effect on repair.

In contrast to the gene 41 and gene 43 mutations, the gene 59 mutation had no effect on SSA and appeared phenotypically similar to the uvsX and uvsY mutants. Importantly, the gene 59 mutant produced a small amount of replicated concatemer, just like the uvsX and uvsY mutants. One interpretation is that UvsX, UvsY, and gp59 all operate in a single pathway to produce the bulk of the replicated concatemer product and that a small amount of concatemer product is produced by an alternative pathway in the absence of any one of these proteins. In this model, gp59 is required to load gp41 onto recombination intermediates generated by UvsX/UvsY-promoted strand invasion, but is not required to load gp41 in the alternative pathway. A different interpretation is that elimination of gp59 or UvsX/UvsY causes a quantitatively similar but mechanistically unrelated decrease in the amount of concatemer product.

In an attempt to delineate the steps of break repair, we used two-dimensional neutral/alkaline gel electrophoresis to analyze the cleaved DNA generated by I-Tev\textsuperscript{-}. Maryon and Carroll (1991) used a similar technique to track the fate of cleaved DNA injected into Xenopus oocytes and were able to document single-strand resection. Research in yeast has also revealed extensive single-strand resection following cleavage by the HO endonuclease (Haber 1995). However, we were not able to
observe similar resection in any of a number of wild-type and mutant infections. The overall reaction in T4-infected cells is much faster than that in eukaryotic systems, so it is possible that transient resected intermediates do exist but are repaired or degraded too quickly to be detected. Alternatively, repair in T4 may be initiated by substrates that have been rendered single stranded by helicases rather than nuclease. Partial denaturation at the ends of broken DNA has been proposed as an early step in recombination (Rosenberg and Hastings 1991; see also Lovett et al. 1988), but these denatured structures would be expected to reanneal during preparation and thus appear as intact duplexes in our analysis.

Figure 8 depicts two models for SSA repair during bacteriophage T4 infections. The first (Figure 8A) is an adaptation of models proposed by other investigators (Lin et al. 1984; Maryon and Caroll 1991) in which repair is nuclease driven, in that the homologous regions required for annealing are exposed by nuclease degradation. As an alternative, we propose that SSA may occur by a helicase-driven mechanism, in which the homologous regions are revealed partially or entirely by helicases, without resection (Figure 8B). At this stage we cannot distinguish between these models, although we have consistently detected homogenous degradation of both strands at broken ends, rather than single-strand resection, in spite of a sensitive assay that readily detects resection in vitro-generated samples.

In summary, phage T4 can repair broken DNA by an efficient SSA mechanism. This mechanism is independent of strand exchange proteins, but requires single-strand DNA binding protein and the putative exonuclease/helicase gp46/47. Although replication is not required for this SSA repair pathway, break repair within the tandem-repeat substrate triggers extensive DNA replication in two other pathways, one dependent and one independent of strand exchange protein UvsX. Because these repair pathways can be studied by a direct physical assay, this system has the potential to provide key insights into recombination intermediates and will potentially allow us to track the fate of intermediates as they proceed through repair and replication.

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