Integration of Plasmids Into the Bacteriophage T4 Genome

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ABSTRACT

We have analyzed the integration of plasmids into the bacteriophage T4 genome via homologous recombination. As judged by genetic selection for a plasmid-borne marker, a mutation in phage gene uvsX or uvsY essentially blocked the integration of a plasmid with homology to the T4 genome but no phage replication origin (non-origin plasmid). The strict requirement for these two proteins suggests that plasmid integration can proceed via a strand-invasion reaction similar to that catalyzed in vitro by the T4-encoded strand-exchange protein (UvsX) in concert with UvsY and gp32. In contrast to the results with the non-origin plasmid, a mutation in uvsX or uvsY reduced the integration of a T4 replication origin-containing plasmid by only 3-10-fold. These results suggest that the origin-containing plasmid integrates by both the UvsXY-dependent pathway used by the non-origin plasmid and by a UvsXY-independent pathway. The origin-containing plasmid integrated into the phage genome during a uvsX- or uvsY-mutant infection of a recA-mutant host, and therefore origin-dependent integration can occur in the absence of both phage- and host-encoded strand-exchange proteins (UvsX and RecA, respectively).

GENETIC recombination plays a prominent role in the life cycle of bacteriophage T4 (for review, see MOSIG 1987, 1994). Independent segregation occurs between genetic markers separated by as little as 10 kilobase pairs (kb), demonstrating that every 169-kb progeny phage genome participated in multiple recombination events in the course of a single infection. Mutations that reduce recombination also impair phage growth, blocking DNA synthesis at late times of infection when the majority of phage replication normally occurs. In addition, recombination events between phage DNA molecules are thought to be required to produce the concatemeric DNA necessary for packaging. Finally, the repair of damaged T4 DNA by post-replication recombination mechanisms is also dependent upon phage recombination proteins.

Studies with multiply mutant phage have implied that T4 uses two or three recombination pathways that utilize different subsets of T4 recombination proteins (HAMELT and BERGER 1975; CUNNINGHAM and BERGER 1977; also see MOSIG 1994 for recent review). Table 1 summarizes gene products that have been implicated in T4 recombination. Mutations that inactivate each protein have previously been shown to influence recombination between coinfected phage DNAs, and biochemical activities have been uncovered for each. UvsX is a strand-exchange protein with many similarities to the RecA protein of Escherichia coli (FUSISAWA et al. 1985; GRIFFITH and FORMOSA 1985; YONESAKI and MINAGAWA 1985; YONESAKI et al. 1985; FORMOSA and ALBERTS 1986; HINTON and NOSSL 1986). UvsY is an accessory protein which greatly stimulates binding of UvsX to a single-stranded DNA-(ssDNA)-gp32 complex (HARRIS and GRIFFITH 1989; YONESAKI and MINAGAWA 1989; KODADEK et al. 1989). Mutations in uvsX and/or uvsY reduce recombination between rII markers on coinfected phage chromosomes by only two- to fourfold (HAMELT and BERGER 1975; CUNNINGHAM and BERGER 1977). The product of phage gene 32 is the archetypal ssDNA binding protein (ALBERTS and FREY 1970). Gp32 is important in UvsX-promoted strand invasion and, by itself, can facilitate renaturation of homologous single-stranded DNA molecules (ALBERTS and FREY 1970). Amber mutations in gene 32 are lethal to the phage and block recombination as judged by electron microscopic and density-shift analyses of intracellular phage DNA (BRESCHKIN and MOSIG 1977; DANNEBERG and MOSIG 1981). Under semi-permissive conditions, a gene 32 amber mutation reduces recombination between rII markers by about threefold (BERGER et al. 1969). The products of genes 46 and 47 reportedly form a membrane-associated exonuclease (MICKELSON and WIEBER 1981; also see ALBRIGHT and GIUDICHEK 1983), and mutations that inactivate gp46 and/or gp47 reduce recombination between phage rII markers by approximately six- to eightfold (SHAH and BERGER 1971; HOSODA et al. 1971; CUNNINGHAM and BERGER 1977; WAKEM and EBISUZAKI 1981). The product of gene 50 has recently been shown to assemble the T4 helicase/primase complex onto ssDNA in vitro (J. BARRY and B. M. ALBERTS, personal communication) and is thereby thought to play an important role in recombination-dependent T4 DNA replication (see...
We decided to approach T4 recombination mechanisms by studying the integration of a plasmid into the T4 chromosome, a recombination event that can be easily measured when the plasmid carries a selectable marker appropriate for phage T4 (Selick et al. 1988). Plasmid integration can occur, at least in principle, by a simple reciprocal recombination event (see Mattson et al. 1983b; Selick et al. 1988). It is possible that plasmid integration can also occur by a more complex series of steps. For example, Mattson et al. (1983b) demonstrated that plasmid-phage recombination often generates non-viable phage that carry multiple copies of inserted plasmid, and an intramolecular excision event could presumably convert a fraction of these non-viable phage into viable integrants with only a single plasmid insert.

In this communication, we investigate the requirements for plasmid integration into the T4 genome. The results suggest that integration can occur via two distinct pathways, one dependent upon the phage UvsX and UvsY gene products, and the other independent of UvsXY but dependent on the presence of a T4 replication origin on the plasmid.

### MATERIALS AND METHODS

**Materials:** Restriction enzymes and DNA ligase were purchased from commercial sources. L broth contained NaCl (10 g/liter), Bacto-Tryptone (10 g/liter), and yeast extract (5 g/liter); it was supplemented with MgSO$_4$ (0.1% w/v) and L-tryptophan (20 mg/liter) for T4 adsorption and/or with ampicillin (25 mg/liter in liquid and 40 mg/liter in solid media) for selection of pBR322-derived plasmids.

**Strains:** *E. coli* strain CR65 (supD) was originally from the collection of B. M. Alberts (University of California, San Francisco). *E. coli* strain AB1 (nonsuppressing) and MCS1 (supD) are isogenic except that MCS1 contains the supD marker (which was transduced from CR63) and a mutation of uvsX, amBl4 (gene 38), and am HL628 (gene 51), am14 (gene 46), and amS29 (gene 51); T4 K10-uvsY is a K10 derivative which contains a 0.12-kb deletion of the gene uvsY promoter and initiation codon (also removes replication origin ori(uvsY); for a complete description, see Kreuzer et al. 1988a). The construction of T4 K10 derivatives with the following additional mutations has also been described (Kreuzer et al. 1988b): am11 (uvsX), amB14 (gene 46), amHI628 (gene 59) and amN116 (gene 39); the T4 K10-amE727 (gene 49) mutant was generated by the same procedure.

**Generation of T4 HE11 and T4 HE11-uvsY11:** T4 HE11 [24a1 nd28 (denA) riHT8 (denB-III deletion)] was generated by crossing T4 HE10 [24a1 nd28 (denA) riHT8 (denB-III deletion)] with amB262 (gene 38) and amS29 (gene 51); ENGMAN and Kreuzer 1993a to a 38* 51* derivative of T4 K10; this cross eliminated the amber mutations in genes 38 and 51. The presence of the 24a1 mutation was monitored by the failure to grow in the absence of a gp24-providing plasmid (pKK032 or pKK208) and confirmed by Southern hybridization, the denB-III deletion mutation (riHT8) by testing for exclusion.

### TABLE 1

<table>
<thead>
<tr>
<th>Protein</th>
<th>Reported function</th>
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<tr>
<td>UvsX</td>
<td>DNA strand-exchange protein</td>
</tr>
<tr>
<td>UvsY</td>
<td>UvsX accessory protein</td>
</tr>
<tr>
<td>gp92</td>
<td>Single-stranded DNA binding protein</td>
</tr>
<tr>
<td>gp46/47</td>
<td>Putative exonuclease</td>
</tr>
<tr>
<td>gp59</td>
<td>Assembly of helicase/primase</td>
</tr>
<tr>
<td>gp39/52/60</td>
<td>T4 DNA topoisomerase</td>
</tr>
<tr>
<td>gp49</td>
<td>Endonuclease VII; cleaves branched structures</td>
</tr>
</tbody>
</table>

*See text for references.*
on a λ lysogen (Benzer 1955), and the denA mutation (nd28) by hydroxyurea sensitivity (Warner et al. 1970). T4 strain HE11-uvsYΔ1 (24Δ1 nd28 [denA] rIIPT8 [denB-rII deletion] uvsYΔ1) was generated by crossing T4 HE11 to T4 K10-uvsYΔ1 at a ratio of 1 T4 K10-uvsYΔ1 to 10 T4 HE11 to favor the T4 HE11 genetic background. Among the segregants, the 24Δ1 mutation was monitored as described above and the uvsYΔ1 mutation was tentatively identified by the minute-plaque phenotype and the failure to generate large plaques in a cross-streak with a known uvsY mutant. The presence of the uvsYΔ1 mutation in T4 HE11-uvsYΔ1 was confirmed by Southern hybridization analysis, as described (see Kreuzer et al. 1988a). Stocks of all T4 strains carrying the gene 24 deletion were propagated on E. coli MCS1 containing the gp24-providing plasmid pKK032 (Kreuzer and Alberts 1986; Engman and Kreuzer 1993; also see below).

Generation of T4 HE11 derivatives with additional mutations: The T4 HE11 derivatives used in this study were generated from genetic crosses between T4 HE11 and T4 K10 derivatives carrying the indicated mutation. For example, T4 HE11 was crossed with T4 K10-amB14 (gene 46) at a ratio of ten T4 HE11 to one T4 K10-amB14 to favor the T4 HE11 genetic background. Among the segregants, the gene 46, 38, and 51 mutations were tested by cross-streaking with the corresponding single mutants to check for recombination. The gene 24 deletion was monitored through the requirement for a gp24-providing plasmid, the denA mutation by sensitivity to hydroxyurea (Warner et al. 1970), and the denB-rII deletion by exclusion on a λ lysogen (Benzer 1955).

To generate the T4 HE11-uvsYΔ1 derivatives carrying additional mutations, T4 HE11-uvsYΔ1 was crossed with the appropriate T4 HE11 derivative at a ratio of 1:1. The desired amber mutations, the gene 24 deletion, and the denA and denB mutations were tested as described above. The presence of the uvsYΔ1 deletion was monitored by testing for the failure to generate large plaques in cross-streaks with known uvsY mutants, and/or by testing for complementation of the uvsYΔ1 minute-plaque phenotype by a UvsY-providing plasmid.

Plasmids: All plasmids used in this study are derivatives of pBR322. Plasmid pKK032 is pBR322 with the T4 EcoRI fragment 106,984–109,432 kb inserted at the vector EcoRI site (Kreuzer and Alberts 1986). Plasmid pKK1208 is identical to pKK032 except that pKK1208 also contains a 247-bp (EcoRI-HindIII) fragment (T4 genome coordinates 152.881–153.127 kb) with the T4 replication origin ori(34) inserted between the EcoRI and HindIII sites of the vector (see Menkens and Kreuzer 1988). Plasmid pHE1105 was constructed by linearizing pKK032 with EcoRI and then ligating the linear DNA to a 730-bp EcoRI fragment containing part of the T4 frd gene; the frd DNA fragment was purified from an EcoRI digest of pBSEof+ (Selick et al. 1988), and originally consisted of an EcoRI-HindIII fragment of the T4 genome (map coordinates 145,321–146,020 kb). Plasmid pHE111 was constructed by linearizing pHE1105 with EcoRI and ligating to an approximately 250-bp EcoRI fragment containing ori(34) (fragment identical to origin fragment in pKK1208 except that an EcoRI linker is present at the HindIII site; see Menkens and Kreuzer 1988). The gene 24 integration assay: The T4 insertion/substitution (I/S) system was designed as a means of introducing in vitro-generated mutations into the T4 chromosome, but can also be used to conveniently measure the integration of plasmids into the phage genome (Selick et al. 1988). Homologous recombination between the I/S phage chromosome and a plasmid bearing a T4 DNA insert results in integrant phage that can be selected by virtue of a plasmid-borne suppressor tRNA gene (supF). Integration of the I/S plasmid is dependent upon the presence of at least 50 base pairs (bp) of sequence homology between the plasmid and the infecting phage chromosome, and the integration

or(34) and allows for optimal growth of gene 24 deletion phage (Engman and Kreuzer 1993). The total pfu/ml values shown have been corrected for the dilution caused by addition of phage lysate. Integrants were measured by plating on E. coli MCS1 or CR63. In several parallel experiments, the number of phage that fail to attach to cells during the adsorption period was found to be between about 2 × 10⁵ and 4 × 10⁷ per ml. This small number of unattached phage was not subtracted from the pfu/ml values shown in the tables.

RESULTS

The gene 24 integration assay: The T4 insertion/substitution (I/S) system was designed as a means of introducing in vitro-generated mutations into the T4 chromosome, but can also be used to conveniently measure the integration of plasmids into the phage genome (Selick et al. 1988). Homologous recombination between the I/S phage chromosome and a plasmid bearing a T4 DNA insert results in integrant phage that can be selected by virtue of a plasmid-borne suppressor tRNA gene (supF). Integration of the I/S plasmid is dependent upon the presence of at least 50 base pairs (bp) of sequence homology between the plasmid and the infecting phage chromosome, and the integration
frequency increases with length of homology until a plateau is reached at approximately 500 bp (also see Goldberg; 1966; Bautz and Bautz; 1967; Drake; 1967; Singer et al. 1982). The I/S assay is not ideal for analyzing the proteins necessary for plasmid-phage recombination, because most available T4 mutants carry amber mutations that could be suppressed by the supF gene of the plasmid. We therefore developed an integration assay based on a different selectable marker, the essential gene 24 (Figure 1A). Gene 24 encodes the vertex protein of the phage head and plays no known role in phage recombination or replication (Eiseling 1983). Gene 24 was deleted from the T4 chromosome, yielding a 24A1 phage that is normally grown in the presence of a gp24-providing (supply) plasmid (Engman and Kreuzer 1993). The gp24 supply plasmid (pKK032) has no homology with the genome of the 24A1 phage, and therefore the supply plasmid rarely integrates into the phage genome. Phage which integrate a gene 24-containing selection plasmid that does have homology to the phage genome are capable of growth on a plasmid-free host and thus can be easily quantitated. To conduct the assay, cells containing the gene 24 selection plasmid are infected with the phage of interest. The resulting lysate is titered on a host that carries a gp24 supply plasmid to measure total plaque-forming units (pfu) and on a plasmid-free host to measure integrants (ints). Integration frequency is then expressed as ints/pfu.

Two selection plasmids, pHE1105 and pHE111, were constructed for these assays (Figure 1B). Both are pBR322 derivatives containing a 2.4-kb EcoRI fragment of T4 DNA that includes the promoter and entire coding region of gene 24. As with the supply plasmid, the chromosomes of 24A1 phage strains are not homologous to the gene 24 insert fragment. Plasmid pHE111 contains a T4 replication origin (ori(34)) and is referred to as an origin plasmid, while pHE1105 contains no known T4 origin and is referred to as a non-origin plasmid. The T4 replication origin in pHE111 maps within an essential gene (34) in the T4 genome, and therefore plasmid integration into this gene would not yield viable progeny. To provide a target for homologous recombination between the infecting 24A1 phage and the gene 24 plasmids, both plasmids contain a 700-bp fragment of the nonessential phage frd (dihydrofolate reductase) gene. The plasmid integration described below is dependent on the frd DNA insert; plasmids that lack the frd insert but are otherwise identical to pHE111 and pHE1105 resulted in integration frequencies of about $10^{-2}$ ints/pfu (data not shown).

**Non-origin plasmid integration:** To determine the phage protein requirements for plasmid integration, amber or deletion mutations in genes of interest were crossed into T4 strain HE11 (genotype: 24A1 denA denB+II deletion) and the resulting derivatives were tested for plasmid integration frequencies. The results of integration assays with host cells containing the non-origin plasmid (pHE1105) are presented in the left half of Table 2. A mutation in gene uvsX or uvsY reduced non-origin plasmid integration by about 100-fold. The large reductions caused by the uvsX and uvsY mutations contrast with the very modest decreases (e.g., two- to fourfold) in phage-phage recombination caused by mutations in these genes (Hamlett and Berger 1975; Cunningham and Berger 1977; our unpublished data). These data therefore suggest that integration of a non-origin plasmid occurs via only a subset of T4 recombination pathways, and that phage-phage recombination uses at least one additional pathway (i.e., a UvsXY-independent pathway).

A mutation in gene 46 also decreased the integration frequency of the non-origin plasmid, but only by a factor of about 12, and the gene 59 and 49 mutations caused four- and sixfold reductions, respectively. The products of genes 46, 59, and 49 apparently play stimulatory though nonessential roles in the formation of integrants with the non-origin plasmid. Interestingly, a mutation in gene 39 caused an approximately twofold stimulation of plasmid integration. A mutation in gene 32 was lethal under these conditions, and therefore could not be analyzed for plasmid integration.

### TABLE 2

<table>
<thead>
<tr>
<th>Phage mutant</th>
<th>Non-origin plasmid</th>
<th>Origin plasmid</th>
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<tbody>
<tr>
<td></td>
<td>Ints/pfu (rel)</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>$4.3 \times 10^{-3}$ (1)</td>
<td>$3.2 \times 10^{-3}$ (1)</td>
</tr>
<tr>
<td>uvsX</td>
<td>$2.8 \times 10^{-5}$ (0.007)</td>
<td>$9.6 \times 10^{-4}$ (0.3)</td>
</tr>
<tr>
<td>uvsY</td>
<td>$4.0 \times 10^{-5}$ (0.011)</td>
<td>$1.2 \times 10^{-3}$ (0.38)</td>
</tr>
<tr>
<td>46</td>
<td>$3.5 \times 10^{-4}$ (0.08)</td>
<td>$6.8 \times 10^{-4}$ (0.21)</td>
</tr>
<tr>
<td>59</td>
<td>$1.2 \times 10^{-3}$ (0.28)</td>
<td>$2.9 \times 10^{-3}$ (0.91)</td>
</tr>
<tr>
<td>49</td>
<td>$7.0 \times 10^{-4}$ (0.16)</td>
<td>$6.7 \times 10^{-4}$ (0.21)</td>
</tr>
<tr>
<td>39</td>
<td>$9.7 \times 10^{-3}$ (2.5)</td>
<td>$1.0 \times 10^{-2}$ (3.1)</td>
</tr>
</tbody>
</table>

* Additional mutations present in T4 HE11 (24Δ1 denA denB+II deletion).
* Non origin plasmid (pHE1105) and origin plasmid (pHE111) were each resident in host strain AB1; see Figure 1 for plasmid maps.
* Numerical average of the int/pfu values measured in each experiment; relative values (rel) compared to wild-type control are in parentheses.
* Numerical average of the pfu/ml values measured in each experiment.

<table>
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<tr>
<th>Phage mutant</th>
<th>Non-origin plasmid</th>
<th>Origin plasmid</th>
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<tbody>
<tr>
<td></td>
<td>Av pfu/ml</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>$8.2 \times 10^{5}$</td>
<td>$4.1 \times 10^{10}$</td>
</tr>
<tr>
<td>uvsX</td>
<td>$1.3 \times 10^{6}$</td>
<td>$2.4 \times 10^{9}$</td>
</tr>
<tr>
<td>uvsY</td>
<td>$1.8 \times 10^{5}$</td>
<td>$1.6 \times 10^{6}$</td>
</tr>
<tr>
<td>46</td>
<td>$2.2 \times 10^{5}$</td>
<td>$3.8 \times 10^{6}$</td>
</tr>
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<td>59</td>
<td>$3.3 \times 10^{5}$</td>
<td>$6.0 \times 10^{6}$</td>
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<tr>
<td>49</td>
<td>$5.0 \times 10^{5}$</td>
<td>$1.4 \times 10^{6}$</td>
</tr>
<tr>
<td>39</td>
<td>$9.7 \times 10^{5}$</td>
<td>$4.6 \times 10^{6}$</td>
</tr>
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</table>
Origin-dependent plasmid integration: T4 replication origins map to recombination hotspots in the phage genome and are necessary and probably sufficient for activating the recombination hotspots (KREUZER and ALBERTS 1985; YAP and KREUZER 1991). We therefore tested the effect of including a 0.25-kb fragment containing a T4 replication origin (ori(?4)) in the integration plasmid. As described above, the T4 origin is from an essential phage gene and therefore plasmid integration into this gene would not yield viable progeny.

The presence of the origin fragment in the frd-containing plasmid had little noticeable effect on plasmid integration in the wild-type infection, but stimulated integration about 30-fold in the uvsX- and uvsY-mutant infections (Table 2). This marked stimulation suggests the existence of a recombination pathway that is independent of UvsX and UvsY proteins but dependent on the presence of a T4 replication origin in the test plasmid. Although the presence of the origin fragment stimulated plasmid integration during infections by the uvsX and uvsY mutants, the total amount of origin plasmid integration in either of these mutants was about threefold less than in the wild-type infection. The simplest interpretation is that the origin plasmid can integrate by either of the two pathways suggested above: the UvsXY-dependent pathway (accounting for about ⅓ of plasmid integration in wild-type infections) and the origin-dependent but UvsXY-independent pathway (accounting for about ⅔ of plasmid integration in wild-type infections). According to this view, the mutation in uvsX or uvsY essentially eliminates the only pathway available to the non-origin plasmid and eliminates one of two pathways available to the origin plasmid.

While the origin fragment markedly stimulated plasmid integration in the uvsX- or uvsY-mutant infection, little or no stimulation was detected in the other mutant infections. However, the small or nonexistent stimulation in these infections is difficult to interpret, primarily because each of these mutations allows much more integration of the non-origin plasmid than do the uvsX or uvsY mutations. To further explore the requirements for origin-dependent integration, we tested a series of double mutants each containing the uvsYΔ1 mutation. According to the above interpretation, the presence of the uvsY mutation in the double mutants should eliminate the UvsXY-dependent integration pathway so that the effect of the second mutation on origin-dependent plasmid integration could be easily assessed. We attempted to introduce amber mutations in each of the following genes into the uvsYΔ1 genetic background: uvsX, 46, 59, 39, and 49. The uvsYΔ1 46 double mutant produced no detectable burst in the non-amber-suppressing host, indicating that the combination of these two mutations is lethal (also see WAKEM and EBISUZAKI 1981). The lethality of this double mutant combination unfortunately prevented a test of plasmid integration frequencies. We also were unable to measure integration into a uvsYΔ1 39 double mutant, which we failed to isolate for unknown reasons (perhaps involving lethality). We tested each of the three viable double mutants in integration assays with both the non-origin and origin plasmids (Table 3).

As expected from the single mutant analyses above, origin-dependent integration in the uvsYΔ1 background was essentially unaffected by addition of a uvsX mutation. This result provides evidence that the same integration pathway is operative in uvsX and uvsY single-mutant infections and in double-mutant infections. The gene 39 mutation also had no inhibitory effect on origin-dependent plasmid integration, and the mutation in gene 49 apparently reduced the frequency of integrants by a small factor. These results therefore indicate that origin-dependent integration can occur in the absence of UvsX, UvsY, gp59, or gp49.

Origin-dependent plasmid integration occurs in a recA-mutant host: Origin-dependent plasmid integration is independent of the T4 strand-exchange protein UvsX and its accessory protein UvsY. We therefore tested whether the host RecA protein participates in origin-dependent integration. The origin and non-origin test plasmids were introduced into isogenic recA+ and

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**TABLE 3**

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<th>Phage mutant</th>
<th>Non-origin plasmid</th>
<th>Origin plasmid</th>
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<tbody>
<tr>
<td></td>
<td>Ints/pfu (rel)</td>
<td>Av pfu/ml</td>
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<td>av</td>
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* Additional mutations present in T4 HE11 (24Δ1 denA denB+II deletion).
* As in Table 2.
* Numerical average of the int/pfu values measured in each experiment; relative values (rel) compared to uvsY single mutant are in parentheses.
* Standard deviation of the int/pfu values measured in each experiment.
* Numerical average of the pfu/ml values measured in each experiment.
recA56-mutant strains, and plasmid integration frequencies were measured for the wild-type 24Δ1 phage and its uvsX amber mutant and uvsYΔ1 derivatives. All the measured integration frequencies were about 2-fold lower in the recA-mutant host than in the recA+ host (Table 4). We believe that this small reduction is due to some secondary effect related to the altered physiology of recA mutants, because: (1) the reduction was detected with either plasmid; (2) the reduction was detected with all three phage genotypes; and (3) all burst sizes were also reduced in the recA-mutant host.

As in the experiments above (which used a different bacterial host), the mutation in either uvsX or uvsY reduced the integration of the non-origin plasmid by about 100-fold in either the recA+ or recA56 host (Table 4). These results thereby support the conclusion that nearly all integration of the non-origin plasmid requires the phage-encoded strand-exchange protein UvsX and its accessory protein UvsY. The main purpose of this experiment was to analyze origin-dependent plasmid integration. In the recA+ host, the presence of the T4 replication origin just as much in the uvsX- and uvsY-mutant infections, although not as dramatically as in the previous recA+ bacterial host (approximately 8-fold vs. 30-fold; compare Tables 4 and 2). We do not understand why the extent of stimulation was different in these two bacterial hosts. Nonetheless, the important conclusion is that plasmid integration was stimulated by the origin just as much in the uvsX- and uvsY-mutant infections of the recA56 host as in the recA+ host (Table 4). Thus, origin-dependent plasmid integration occurs in the absence of both the phage- and host-encoded DNA strand-exchange proteins.

**DISCUSSION**

**The gene 24 integration assay:** The gene 24 integration assay appears useful for studying recombination mechanisms during phage T4 infection. Mutations in genes uvsX or uvsY reduce non-origin plasmid integration by about 100-fold (Tables 2 and 4), but reduce phage-phage recombination by only a few fold (HAMLETT and BERGER 1975; CUNNINGHAM and BERGER 1977). Thus, non-origin plasmids apparently integrate into the phage chromosome only by a uvsXY-dependent pathway, while phage-phage recombination occurs by both uvsXY-dependent and -independent pathways. The effects of particular DNA segments on recombination can be readily tested using plasmid integration approaches. In this way, the addition of a fragment containing a T4 replication origin was found to activate plasmid integration in the absence of UvsX, UvsY or both (Tables 2–4).

Several limitations of this plasmid-based recombination assay must be considered. First, plasmids may exist in a topological form and/or subcellular localization that differs from that of the phage chromosome. At least at the very start of infection, plasmid but not phage genomic DNA would be in a negative superhelical form, which could affect recombination capacities (see SINDEN and PETTJOHN 1982; ALBRIGHT and GEIDUSCHEK 1986). Second, unreplicated plasmid DNA is not modified with the glucosyl and hydroxymethyl groups characteristic of native T4 DNA, and this might affect participation of plasmid DNA in phage-promoted recombination. For example, the origin-dependent pathway of plasmid integration could require a plasmid-borne origin only to generate modified plasmid DNA that acts as a substrate in a recombination pathway that requires modified DNA. Third, as already mentioned in the Introduction, it is not certain that plasmid integration occurs only by a simple reciprocal recombination event. Fourth, the assay is compromised by unexpected plasmid replication caused by the gene 24 DNA insert. The promoter region of gene 24 induces a small amount of plasmid replication that is independent of T4 recombination proteins (ENGMAN and KREUZER 1993), and therefore the "non-origin" plasmid used in this study replicates weakly.
This low level of replication may contribute to the background level of non-origin plasmid integration in the \textit{supF} (and presumably \textit{uvsX}) mutant infections. This inference is based on an independent measure of plasmid integration using the T4 I/S system, which relies on a plasmid containing the \textit{supF} selectable marker instead of gene 24. Integration of the non-origin \textit{supF}-containing plasmid was reduced nearly 10-fold by the \textit{uvsY} mutation (unpublished data), rather than the 100-fold reduction detected with the non-origin gene 24-containing plasmid. Nevertheless, the small amount of replication of the gene 24-containing plasmid does not prevent detection of a strong stimulation of integration by the origin-containing fragment.

**Recombination-dependent DNA replication:** The majority of DNA synthesis during T4 infection occurs at late times of infection and is dependent upon the products of T4 genes \textit{uvsX}, \textit{uvsY}, 46/47, 59, and 39/52/60 (among others; for reviews, see MOSIG 1983; KREUZER and MORRICAL 1994). This late DNA replication was proposed to initiate via a unique mechanism in which recombination intermediates are converted into replication forks (LUDER and MOSIG 1982). In the first step of this model, a single-stranded 3' end (generated by the replication of linear DNA) invades an homologous duplex DNA to create a recombination intermediate. In the second step, the intermediate is converted into a replication fork, presumably utilizing the invading 3' end as a primer for leading-strand synthesis.

Plasmids bearing homology to the infecting phage chromosome, but no T4 origin sequence, are replicated during T4 infection by a process that mimics phage recombination-dependent replication (KREUZER et al. 1988b). The majority of the replicated plasmid product is in the form of long plasmid concatamers, arguing strongly that plasmid replication is an active process and not simply the result of passive replication after the integration of plasmids into the phage genome. Assuming that recombination-dependent plasmid replication involves the conversion of a phage-plasmid recombination intermediate into a replication fork, it should be possible to distinguish between phage proteins involved in the two steps outlined above. Mutations inactivating proteins required for the first step should abolish both recombination-dependent plasmid replication and plasmid-phage recombination, as do mutations in \textit{uvsX} and \textit{uvsY} (Table 2; KREUZER et al. 1988b). The products of these genes are therefore implicated in the formation of recombination intermediates during recombination-dependent replication. The assignment of these two proteins in the generation of a plasmid-phage recombination intermediate fits well with the biochemical functions of these proteins in the strand-exchange reaction (see below). A mutation in gene 46 reduces both plasmid replication (KREUZER et al. 1988b) and plasmid integration (Table 2) by about 10–20-fold, suggesting that gp46/47 also participates in formation of a plasmid-phage recombination intermediate. Mutations in genes whose products are required for the second step (the conversion of recombination intermediates into replication forks) should block plasmid replication but not plasmid-phage recombination. The gene 39 mutation greatly reduces recombination-dependent plasmid replication (KREUZER et al. 1988b) but not plasmid-phage recombination (Table 2). The T4 topoisomerase may therefore be involved in either the initiation of replication or the subsequent propagation of the fork (perhaps as a replication fork swivelase).

**Two pathways of plasmid integration:** Previous studies of T4 recombination pathways compared phage-phage recombination frequencies from singly and multiply mutant phage infections. In the most comprehensive analysis, CUNNINGHAM and BERGER (1977) concluded that UvsX and UvsY act independently of gp46/47, while all four of these gene products act independently of gp59. Based on such analyses of gene product requirements, at least two or three different pathways for homologous recombination were inferred (HAMLETT and BERGER 1975; CUNNINGHAM and BERGER 1977). Based on a consideration of the structures of recombination substrates and products, MOSIG (1994) argued that T4 uses four recombination pathways, two of which predominate in a wild-type infection. The possible relationships between the pathways proposed from gene product requirements and those proposed by MOSIG (1994) are not clear.

Our studies suggest that plasmids can integrate into the T4 chromosome by two distinct pathways; one requires the phage genes \textit{uvsX} and \textit{uvsY}, while the other is independent of these two genes but requires a T4 replication origin insert fragment on the test plasmid. Both pathways involve homologous recombination in the \textit{frd} target region because plasmids lacking the \textit{frd} insert fail to integrate (data not shown). Recombination of the non-origin plasmid with the phage genome is strongly dependent upon the products of phage genes \textit{uvsX} and \textit{uvsY} (Table 2), suggesting that a well-characterized strand-exchange reaction is involved. The invasion of single-stranded DNA into homologous duplex DNA is efficiently catalyzed by a combination of highly purified UvsX, UvsY and gp32 (YONESAKI and MiNAGAWA 1989; HARRIS and GRIFFITH 1989). A mutation in gene 46 reduced integration of the non-origin plasmid by about 12-fold, suggesting that the gp46/47 complex stimulates but is not required for integration. Based on the inferred exonuclease activity of gp46/47 (MICKELSON and WIBERC 1981), this protein may generate a single-stranded DNA substrate for the strand-exchange reaction catalyzed by UvsX, UvsY and gp32.

Our results also suggest a second pathway for plasmid integration when the test plasmid contains a T4 replication origin insert. The insert fragment containing
ori(34) is quite small (0.25 kb) and contains no other known sequences involved in T4 DNA metabolism. The previously defined replication origin, comprising about half of this fragment (Menkens and Kreuzer 1988), is thus likely to be responsible for stimulating integration. Based on data from both single- and double-mutant tests, integration via the origin-dependent pathway does not require the products of uvsX, uvsY, 59 and 49 (although it might be stimulated by one or more of these proteins).

Because the origin-dependent plasmid integration was found to be independent of the T4-encoded strand-exchange protein UvsX and its accessory protein UvsY, we tested for a possible requirement for the host RecA protein. Origin-dependent integration was found to occur during a uvsX- or uvsY-mutant infection of a reca- host (Table 4), and therefore origin-dependent plasmid integration is not catalyzed by either of these two strand-exchange proteins. Considering the lack of requirement for UvsX and RecA, we speculate that origin-dependent integration may proceed via a mechanism different from strand invasion. One interesting possibility is that origin-dependent integration involves single strand annealing promoted by gp32, which anneals homologous single strands in vitro (Alberts and Frey 1970). A T4 recombination pathway requiring gp32 (and gp46/47) and proceeding via a single strand annealing mechanism has previously been proposed, based upon electron microscopic studies of branched DNA structures isolated from replication-deficient phage infections (Broker and Lehman 1971). The function of the replication origin in plasmid integration could be to provide single-stranded DNA regions for gp32-promoted annealing. Nonrepli- cating plasmid DNA might not provide single-stranded regions for gp32-promoted annealing, in part because its closed circular structure precludes the putative exonuclease action of gp46/47. Single-stranded regions could be generated as displacement loops during replication initiation or as the result of the action of a helicase loaded at the origin. Alternatively, plasmid replication might generate a suitable substrate for exonuclease action by gp46/47, such as a double-strand break or the end of a rolling-circle replicative intermediate. The stimulation of plasmid integration by a phage origin is intriguing because T4 replication origins generate recombination hotspots in the phage chromosome (Yap and Kreuzer 1991). At least some of the hotspot recombination occurs by a UvsY-independent mechanism (Yap and Kreuzer 1991), and so the enhanced recombination at the hotspots could be mechanistically related to the origin-dependent plasmid integration described here.

Other homologous recombination pathways that are independent of RecA or analogous strand-exchange proteins have also been described. These include plasmid recombination by the RecE pathway of E. coli (Fishel et al. 1981; Laban and Cohen 1981; Simmons et al. 1985) and the λ Red pathway (Shulman et al. 1970; Radding 1970). Interestingly, the λ Red pathway is independent of host RecA protein only when phage DNA replication is allowed (Stahl et al. 1985; Stahl and Staehly 1986); replication could thus play a similar role in the λ Red pathway and in T4 origin-dependent plasmid integration. The RecE and Red pathways have recently been shown to utilize a second family of strand-exchange proteins, mechanistically distinct from RecA or UvsX (Hall et al. 1993; Hall and Kolodner 1994; also see Kmiec and Holloman 1981; Muniappa and Radding 1986). In vitro strand exchange by members of this second family proceeds as follows: (1) exonucleolytic degradation of the duplex to expose a 3' single-stranded end, (2) annealing of the eroded end with the complementary single-stranded molecule, and (3) strand exchange through the remaining duplex region. Therefore, as an alternative to the (gp32-dependent) single strand annealing model proposed in the paragraph above, T4 origin-dependent plasmid integration could depend on a member of this second family of strand-exchange proteins. T4 origin-dependent integration presumably does not involve the host RecE pathway, because the reca strain used in our experiments (Table 4) does not contain an sbcA mutation that is necessary for induction of this pathway. Perhaps origin-dependent integration depends on a T4-encoded protein which is an unrecognized member of this second family of strand-exchange proteins. In this context, gp46/47 seems like an excellent candidate, given its recognized role in T4 recombination and its putative exonuclease activity (see Introduction).

In closing, it is interesting to note that the gene product requirements for integration of the origin-containing plasmid closely parallel gene product requirements that have previously been deduced for phage-phage recombination (compare data in Table 2 to values quoted in the Introduction). The optimistic interpretation is that the two plasmid integration pathways that operate with the origin-containing plasmid mimic two major pathways of phage-phage recombination. Further work should test the validity of this interpretation, and explore additional mechanistic details of the two plasmid integration pathways.

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LITERATURE CITED


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