Replication and Recombination in Ligase-Deficient rII Bacteriophage T4D

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Deoxyribonucleic acid replication and genetic recombination were investigated after infection of Escherichia coli with ligase-deficient rII bacteriophage T4D. The major observations are: (i) deoxyribonucleic acid synthesis is discontinuous, (ii) the discontinuities are more slowly repaired than in wild-type infection, (iii) host ligase is required for viability, and (iv) genetic recombination is increased.

Karam (5) and Berger and Kozinski (1) have observed that amber mutations in the structural gene for polynucleotide ligase (gene 30) of phage T4D are no longer lethal when an rII mutation is present in the same phage. Physicochemical analysis of 32P-labeled parental phage deoxyribonucleic acid (DNA) indicated that the rII mutation prevents the extensive endonucleolytic degradation of phage DNA which is normally observed with ligase-deficient rII+ phage (1). These results suggest that an endonuclease is responsible for the lethality associated with ligase-defective phage and that the rII mutation restores viability by directly or indirectly decreasing endonucleolytic action. This interpretation of the mechanism of suppression is consistent with the observation that ligase-deficient phage viability is restored when chloramphenicol is added 5 min postinfection and then removed 25 min later (1, 6, 8). Presumably, chloramphenicol during this phase of infection partially blocks expression of functions which allow endonucleolytic degradation of parental DNA. Limited DNA synthesis can nevertheless occur under these conditions, utilizing those enzymes synthesized before chloramphenicol addition (8).

In this communication, we report on the replication and recombination which occur after infection with a phage which is defective in both ligase and rII function.

MATERIALS AND METHODS

Phage stocks. The amber mutants of phage T4D, amX39 (gene 30), amN69 (gene 12), and amB255 (gene 10), were kindly supplied by R. S. Edgar. The rIIA mutant, r59, was obtained from A. H. Doermann. The mutant tsN2 was isolated as a spontaneous pseudorevertant of amX39 at 42 C in Escherichia coli B. The double mutant amX39-tsN2K2 does not grow at 30 C in E. coli B. Since T4D does not grow on bacterial strain TAU bar or TAU bar TS7, studies with these strains utilized a T4D derivative designated T4Dγ. This derivative was isolated as a spontaneous mutant of T4D+ which had a high efficiency of plating and a nearly normal plaque morphology on these hosts. Phage stocks containing combinations of these markers were constructed by recombination.

Bacterial strains. E. coli B was used as the nonpermissive host for in vivo and in vitro experiments with amber mutants. E. coli CR63 was used to prepare stocks and as the permissive host for phage containing amber mutations. The nonpermissive host for rII mutants was CR63(α). For studies on the role of host ligase in the restoration of viability to ligase-deficient phage, bacterial strains TAU bar and a ligase temperature-sensitive mutant TAU bar TS7 (12, 13), kindly provided by E. C. Pauling, were used.

Phage crosses. Crosses of amX39-r59-amN69 by amX39-r59-amB255 were carried out in E. coli B by the methods of Chase and Doermann (2). In each experiment, a minimum of 250 total progeny and 250 late function am+ recombinants were counted. Control crosses, r59-amB255 by r59-amN69, were done simultaneously. For studies on the role of chloramphenicol on recombination, crosses were carried out as above, but at the indicated times after infection the media were supplemented with 100 μg of chloramphenicol per ml (Parke, Davis & Co.). Chloramphenicol was removed to allow maturation by diluting 5,000-fold into unsupplemented media.

Cross lysates were plated on CR63 to determine total progeny and on E. coli W3110AB24 Su A (obtained from C. Yanofsky), which is restrictive for the late-function amber mutations used, to determine the frequency of wild-type late-function recombinants.

Physicochemical characterization of phage DNA. Methods were identical to those previously employed by Shah and Berger (J. Mol. Biol., in press).

RESULTS

Isolation and in vivo characterization of an rII temperature-sensitive mutant. To characterize the time course of the lethality of rII function in the absence of gene 30 product, a temperature-sensitive rII mutant was isolated. This mutant was
selected as a spontaneous temperature-sensitive pseudorevertant of amX39. The doubly mutant strain has been designated amX39-tsNK2. It grows well on E. coli B at 42°C but does not grow at 30°C. Table 1 shows the burst size of the revertant and the parental amX39 mutant in E. coli B, E. coli CR63, and E. coli CR63(λh) at 30 and 42°C. Since the double mutant grows equally well at high and low temperature on E. coli CR63, it is unlikely that the reversion event is due to a change of the amber codon in amX39 to a codon which confers cold sensitivity to the ligase function. The growth response in CR63 also indicates that the temperature-sensitive mutation responsible for suppression does not reduce phage yields when there is normal ligase function. The ability of the revertant to grow nearly normally in E. coli CR63 (λh) at high temperature shows that this suppressor mutation does not have typical rII phenotype. Gene 30 suppressors of this type which are within the rII region have been described (1).

Confirmation of the rII site of tsNK2 mutation was found by mapping it with respect to known rII mutations in both two- and three-factor crosses. The site of tsNK2 was very close to the site of the marker r59 (rIIA). Table 1 also shows that tsNK2, as with other previously described rII mutations, was recessive to the wild-type allele.

Since tsNK2 suppresses gene 30 mutants only at high temperature, we have performed temperature-shift experiments to define the time at which rII+ function is lethal in the absence of phage ligase. Phage production after a temperature shift from 30 to 41°C at various times after infection of E. coli B with amX39-tsNK2 indicates that incubation at 41°C must be initiated within 15 min after infection for the production of viable progeny (Fig. 1). This result is in good agreement with a similar experiment of J. D. Karam (personal communication).

**DNA synthesis with amX39-tsNK2.** The observation that gene 30 function is required for T4 viability has been interpreted to mean that phage-induced polynucleotide ligase is normally required for replication and cannot be replaced by bacterial ligase. It is clear, however, that the requirement for phage ligase is removed by the rII mutation, presumably by creating intracellular conditions which permit DNA synthesis by using only preexisting bacterial ligase. It has been previously shown that suppression of amX39 by rII mutations prevents endonucleolytic degradation of parental DNA (1). This endonucleolytic attack of the input template molecule, before extensive replication has occurred, is probably the early lethal event which was demonstrated in the temperature-shift experiment (Fig. 1).

The DNA synthesized after infection with amX39-tsNK2 has been examined on neutral and alkaline sucrose gradients. The results of these sedimentation studies of DNA labeled with 3H-thymidine for long times after infection with either amX39-tsNK2 or amX39 at 42°C are shown in Fig. 2. The DNA synthesized by the double mutant (rII-ligase-deficient) was mature-sized even when denatured; the gene 30 single mutant (rII+-ligase-deficient), however, synthesized only very short segments of DNA which failed to be covalently joined into mature molecules. Figure 3A shows results from an infection at 31°C with amX39-tsNK2; at this temperature, the double mutant behaved like the single mutant. The results obtained at 42°C were similar to those in Fig. 2C. Figure 3B shows the results of a mixed infection of amX39-tsNK2 and amX39 at 42°C; as expected, the rII mutation was recessive to the wild-type allele and only low-molecular-weight DNA was synthesized.

To characterize more completely the DNA synthesis under ligase-deficient conditions, the replicative intermediates were examined in pulse-labeling experiments. In accord with previous findings of Okazaki et al. (11) in wild-type infec-
that the erally of less infection, amX39-tsNK2 was handled and DNA; marker used to multiplicity times of fragments in infection DNA were produced, ample, after alkaline 4A). (Fig.

4.51)

Fig. 4. Temperature-shift experiment. E. coli B cells were infected at 30°C with amX39-tsNK2 at a multiplicity of 5 PFU per bacterium, and at the indicated times samples were diluted 20-fold into fresh medium at 41°C. Chloroform was added 90 min after infection to lyse the cells. Burst sizes are given as a percentage of those observed with T4D-infected cells handled identically.

tion, DNA labeled with a short pulse sedimented in alkaline sucrose in a peak of approximately 8 to 10S; with longer pulse time, the bulk of the label was converted into mature-size molecules (Fig. 4A). In contrast (Fig. 4B), after infection with amX39-tsNK2 at 42°C, short fragments were produced, but the rate of conversion of short fragments of newly synthesized DNA into mature-size molecules was markedly reduced. For example, after a 150-sec pulse more than 90% of the DNA from the wild type sedimated rapidly with marker DNA; under the same conditions in an amX39-tsNK2 infection, the newly synthesized DNA was much more heterogeneous and generally of less than mature size. It should be noted that the amount of label incorporated during the pulse differed less than twofold between wild-type and amX39-tsNK2-infected cells.

Similar pulse-labeling experiments have been carried out with the double mutant amX39-r59 used to infect E. coli B at 30°C. The r59 marker is a missense mutation within the rIIA cistron which has normal rII phenotype and is strongly restricted on λ lysogens. Results obtained in these experiments (not shown) were similar to those obtained with amX39-tsNK2 infection at 42°C. Iwatsuki and Okazaki have obtained comparable results in unpublished experiments [cited in Iwatsuki and Okazaki, (4)] on the replicative intermediates after infection with ligase-deficient rII phage.

The above results indicate that after infection with ligase-deficient rII phage (i) DNA synthesis is discontinuous and (ii) the short fragments of DNA produced by discontinuous synthesis are slowly covalently linked to produce mature-size molecules. A likely explanation for these findings is that in the absence of rII function bacterial ligase joins the newly synthesized Okazaki fragments.

Role of bacterial ligase. To determine the role of bacterial ligase in the growth of ligase-deficient rII phage, we have used a bacterial strain, TAU bar TS7, which is temperature-sensitive and has low levels of temperature-sensitive polynucleotide ligase activity (12, 13). At 42°C, the mutant bacteria have less than 2 to 4% of wild-type levels of ligase (3). To get adequate phage growth on this bacterial strain or its parental strain, TAU bar, it was first necessary to isolate a mutant (T4Dγ). The inability of wild-type T4D to grow in TAU bar is not understood; however, it is possible that TAU bar restricts T4D+ and that the phage mutant is less sensitive to restriction. AmX39, amX39-r59, and amX39-tsNK2 containing the γ marker were constructed by recombination and tested for the ability to produce phage in the absence of host ligase. In the control experiments, TAU bar TS7 was infected with T4Dγ and produced phage progeny. As would be expected, amX39-γ, amX39-tsNK2-γ, and amX39-r59-γ were equally nonviable at 42°C in the absence of host ligase (Table 2). In contrast to this, the parental strain TAU bar, which produces normal bacterial ligase, did produce progeny after infection at 42°C with amX39-tsNK2-γ or amX39-r59-γ but not with amX39-γ. Thus, it can be concluded that for ligase-deficient phage to replicate two criteria must be satisfied: (i) normal levels of host ligase must be present and (ii) the endonucleolytic attack of the DNA early in the infection must be prevented by inactivation of rII function.

Since these experiments have been carried out, a new ligase-deficient bacterial strain has been isolated (3). This strain, which has reduced levels of ligase activity in enzyme assays, differs from the strain used here in several aspects. In
vivo it shows no deficiency in joining of newly synthesized Okazaki fragments to form chromosomal DNA. Unlike TAU bar TS7, this strain does not show aberrant growth characteristics under nonpermissive conditions. In spite of these profound differences in phenotype from the TS7 mutant, this strain, when used as a host for ligase-deficient rII phage, is also unable to produce
Fig. 3. Integrity of newly synthesized DNA: alkaline sucrose gradients. E. coli B cells were infected in TCG medium supplemented with 200 μg of 2'-deoxyadenosine per ml with either amX39-tsNK2 (multiplicity of infection 15) at 31 C (A) or amX39-tsNK2 and amX39 (multiplicity of infection 7.5 each) at 42 C (B). At 7.5 min after infection, 12.5 μCi of 3H-thymidine (7 Ci/m mole) per ml was added. Infected cells were labeled for 8 min and then lysed in the presence of 32P-labeled phage by the lysozyme-Sarkosyl-low temperature method. Samples were centrifuged for 100 min at 189,000 × g at 5 C through 5 to 20% alkaline sucrose gradients. Symbols: ■, 3H-labeled DNA; ○, 32P-labeled T4 marker DNA.

Fig. 4. Short-pulse-labeling experiment: alkaline sucrose. E. coli B was infected with T4D+ (A) or amX39-tsNK2 (B) in TCG medium supplemented with 200 μg of 2'-deoxyadenosine per ml at a multiplicity of 7.5 PFU per bacterium. After 15 min, 3H-thymidine (7 Ci/m mole) was added (50 μCi/ml for a 5-sec pulse; for longer labeling times, 12.5 μCi/ml). The pulse was terminated by lysing infected cells in the presence of 32P-labeled phage by the lysozyme-Sarkosyl-low temperature method. Samples were centrifuged for 129 min at 189,000 × g at 5 C through 5 to 20% alkaline sucrose gradients. Sedimentation is from right to left. The arrow indicates the position of 32P-labeled T4 marker DNA. Symbols: □, 5-sec pulse, 3H-labeled DNA; ○, 40-sec pulse, 3H-labeled DNA; ▲, 150-sec pulse, 3H-labeled DNA.
viable progeny. Thus, the ability to support growth of ligase-deficient rII phage appears to be a sensitive in vivo assay for host ligase levels.

**Effect of limited ligase on phage genetic recombination.** The preceding analyses of the replicating DNA after infection with ligase-deficient rII phage indicate that the newly replicated DNA exists in a highly nicked form for appreciably longer times than after infection with wild-type phage. As shown in Table 3, genetic recombination between a pair of linked genes was increased almost sevenfold under these conditions, suggesting a possible relationship between the persistence of nicks in the intracellular DNA and the degree of genetic recombination in the progeny.

An alternative explanation of the observed increases in recombination is suggested by two other manifestations of the ligase-deficient rII phenotype. As can be seen in Fig. 5, DNA synthesis lagged by approximately 10 min compared to infection with ligase-positive rII phage. Concomitant with the lag in DNA synthesis was a similar, or slightly more pronounced, delay in the appearance of mature phage (Fig. 6). Since the DNA molecules are spending a longer time in the vegetative state, this could be expected to result in some increases in recombination frequency (9). To test this possibility, delays in maturation were artificially introduced by the addition of chloramphenicol and the effect on recombination was measured. It is clear from the results of such an experiment (Table 4) that temporary chloramphenicol-induced blockage of late function expression fails to produce significant alterations in the frequency of recombinants.

In these experiments, chloramphenicol was added at a variety of times after infection. Kozinski, Kozinski, and Shannon (7) have shown that chloramphenicol addition after 9 min at 37 C has no effect on the transfer of parental label to progeny DNA molecules. Mattsson (10) has also reported that chloramphenicol-induced delays in

### Table 2. Growth in *E. coli* TAU bar and TAU bar TS7 of T4D mutants deficient in ligase

<table>
<thead>
<tr>
<th>Phage mutant</th>
<th>Host bacterium</th>
<th>Burst size at 30 C</th>
<th>Burst size at 41 C</th>
</tr>
</thead>
<tbody>
<tr>
<td>T4Dγ</td>
<td>TAU bar</td>
<td>23.2</td>
<td>18.2</td>
</tr>
<tr>
<td>amX39-γ</td>
<td>TAU bar</td>
<td>0.06</td>
<td>0.02</td>
</tr>
<tr>
<td>amX39-r59-γ</td>
<td>TAU bar</td>
<td>3.0</td>
<td>6.0</td>
</tr>
<tr>
<td>amX39-tsNK2-γ</td>
<td>TAU bar</td>
<td>0.1</td>
<td>6.9</td>
</tr>
<tr>
<td>T4Dγ</td>
<td>TAU bar TS7</td>
<td>109</td>
<td>16.5</td>
</tr>
<tr>
<td>amX39-γ</td>
<td>TAU bar TS7</td>
<td>0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>amX39-r59-γ</td>
<td>TAU bar TS7</td>
<td>5.0</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>amX39-tsNK2-γ</td>
<td>TAU bar TS7</td>
<td>0.25</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* Burst sizes were calculated as the number of progeny per infected cell.

### Table 3. Effect of limited ligase on genetic recombination

<table>
<thead>
<tr>
<th>Cross</th>
<th>Per cent recombinationa between amB255 and amX39</th>
<th>1. r59-amB255 × r59-amN69 (control)</th>
<th>3.4, 3.9</th>
</tr>
</thead>
<tbody>
<tr>
<td>2. amX39-r59-amB255 × amX39-r59-amN69 (ligase-deficient)</td>
<td>26.4, 23.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* See Materials and Methods. The percentages were calculated by multiplying by two the frequency of wild-type recombinants. Values are given for two separate crosses.

maturation have no effect on the accumulation of recombinants.

**DISCUSSION**

Our results indicate that replication producing mature-size phage DNA molecules can occur in the absence of the gene 30 product utilizing only the host ligase. Limitation of an endonucleolytic activity controlled by the rII genes of T4D is necessary for such replication to take place. Apparently the early endonucleolytic attack of injected DNA molecules cannot be repaired adequately by host ligase, thus accounting for the lethality of ligase-deficient rII+ phage.

It has been previously reported that chloramphenicol addition at early times after infection with *amX39* results in partial suppression of the ligase-deficient phenotype and DNA of high-molecular-weight is synthesized. In the experiments reported here and in previous experiments with ligase-deficient rII phage (1), the rII mutation has an effect very similar to chloramphenicol in suppressing the abnormal DNA synthesis phenotype of the gene 30 mutant. Preliminary experiments suggest that the mechanism of chloramphenicol rescue of gene 30 mutants is by
Fig. 5. Net DNA synthesis in phage-infected E. coli B at 30 C. 32P incorporation into acid-insoluble, alkali-resistant material. Infection was carried out in TCG medium with a multiplicity of 8 phage per bacterium. 32P (1 μCi/ml) was added 3 min after infection. Symbols: △, r59; ○, r59-amX39; □, amX39.

Table 4. Effect of chloramphenicol-induced delays in maturation on genetic recombination

<table>
<thead>
<tr>
<th>Cross</th>
<th>Chloramphenicol</th>
<th>Percent recombination between amX39 and amN69</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. r59-amB255 × r59-amN69</td>
<td>None</td>
<td>3.9</td>
</tr>
<tr>
<td>Same</td>
<td>7-37 min</td>
<td>3.7</td>
</tr>
<tr>
<td>Same</td>
<td>10-40 min</td>
<td>3.2</td>
</tr>
<tr>
<td>Same</td>
<td>13-43 min</td>
<td>3.3</td>
</tr>
<tr>
<td>Same</td>
<td>17-47 min</td>
<td>2.9</td>
</tr>
<tr>
<td>2. amX39-r59-amB255 × amX39-r59-amN69 (ligase-deficient)</td>
<td>None</td>
<td>23.0</td>
</tr>
</tbody>
</table>

* See Materials and Methods. The percentages were calculated by multiplying by two the frequency of wild-type recombinants.

its inhibition of the synthesis of the rII gene product (H. Krisch, unpublished data).

Once ligase-deficient rII replication has begun with the relatively un-nicked parental DNA as a template (1), it proceeds in a discontinuous fashion. The replicative discontinuities which are produced are only slowly repaired by rate-limiting levels of host ligase. The pattern of DNA synthesis in ligase-deficient rII infection is thus compatible with the Okazaki model of DNA synthesis. The major difference from wild-type infection is the increased lifetime of the discontinuities in the newly replicated DNA molecules.

The increases in genetic recombination observed under these conditions are probably explained by the persistence of replicative DNA intermediates which contain numerous single-stranded nicks. Such structures may facilitate break-reunion recombination. The slight delay in both DNA synthesis and the production of mature viruses after ligase-deficient rII infection may also have effects on the formation of recombi-
nants. The results of the chloramphenicol experiment indicate that maturation delays of the observed duration are in themselves inadequate to account for effects of this magnitude. However, it cannot be ruled out that the prolonged incubation of the nicked vegetative DNA molecules has some contributory role in producing increased levels of recombination. More detailed studies of this altered recombination in ligase-deficient rII infection will be published elsewhere. Preliminary experiments suggest that the increases in recombination are less pronounced when the markers used in the cross are separated by very short intervals. This result is what would be expected if the markers are so close to each other that the probability of a replicative discontinuity occurring between them is small.

It is unclear from the results reported here whether replicative discontinuities are involved in the formation of recombinants after infection with wild-type T4D. It is possible that only under conditions of limited ligase do they have a significant role in this process. Nevertheless, our results probably justify a reconsideration of the importance of replicative discontinuities in current models of recombination.

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LITERATURE CITED