A Recombinant DNA Plasmid which Inhibits Bacteriophage T7 Reproduction in *Escherichia coli*

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**SUMMARY**

*Escherichia coli* plasmids carrying a DNA restriction fragment corresponding to the extreme right end of the T7 bacteriophage genome render cells incompetent to support reproduction of wild-type T7. Processing of intracellular concatemeric phage DNA and cell lysis are perturbed by one such plasmid, pRS148. The results are discussed with reference to the possibility that the right end of the T7 genome encodes a lysis-related function.

**INTRODUCTION**

Recombinant DNA plasmids carrying phage genes in *Escherichia coli* have proved to be valuable tools for probing the control of replication, gene regulation, and for precise alignment of genetic and physical maps (Mattson et al., 1977; Campbell et al., 1978; Young et al., 1980; Studier & Rosenberg, 1981; Miller et al., 1981; Smith & Miller, 1981, 1982). Recombinant DNA plasmids that have been shown to complement and/or recombine with various phage mutations have put strict physical limits on genetic elements that previously were defined only by genetic means. In addition, these plasmids have been invaluable aids in DNA sequence analysis. Furthermore, these plasmids may ultimately prove valuable in the physical characterization of phage gene products.

Some phage genes cannot be cloned intact, and in some cases DNA segments can only be recovered in one orientation relative to adjacent plasmid promoters (Studier & Rosenberg, 1981). This is not unexpected since some phage gene products are expected to be lethal to the host cells. However, an unexpected type of plasmid, one which inhibits the reproduction of the parental phage type, has also been described. Bacteriophage T7 is unable to reproduce in cells which harbour a plasmid carrying an insert of the DNA corresponding to the extreme right end of the T7 genome. Specifically, insertion of the *HpaI* fragment into pMB9 (Campbell et al., 1978) or pBR322 (this study) produces a plasmid which renders host cells incompetent to support phage reproduction. Studies presented here indicate that many events normal after infection by T7 are unaffected by one such plasmid. However, very few progeny phage are produced.

From published genetic and molecular maps (Studier & Rosenberg, 1981) it can be concluded that the *HpaI* fragment includes sequences from the extreme right end of the genome which presumably are important in concatemer formation and maturation. The fragment contains the C-terminal coding region of gene 19, which directs the synthesis of a concatemer maturation protein (Kerr & Sadowski, 1974). The *HpaI* fragment might be expected to contain gene 20, since a mutant used to define this gene was mapped by genetic analysis to the right of gene 19 (Pao & Speyer, 1975). However, J. J. Dunn & F. W. Studier (personal communication) state that a proposed gene 20 mutant is in fact in gene 5-7. This conclusion is based on plasmid–phage recombination experiments. Gene 20 mutants of T7 are analogous to rII mutants in bacteriophage T4 in that they do not reproduce in a *rex*⁺ *λ* lysogen (Pao & Speyer, 1975). DNA sequence
analysis (J. Dunn & F. W. Studier, personal communication) has shown that the HpaI J fragment contains two potential coding sequences, called genes 19-2 and 19-3, which overlap gene 19 in a different reading frame. Finally, an adjacent potential coding sequence to the right of gene 19 has been labelled 19-5.

We document here the construction and characterization of a recombinant plasmid, pRS148, which contains part or possibly all of the HpaI J fragment inserted into pBR322. To gain some insight into the properties of pRS148, we have compared phage-directed macromolecular synthesis in pRS148- and pBR322-containing T7-infected cells. Sucrose gradient analysis of [3H]thymidine-labelled intracellular phage DNA has shown that pRS148-containing cells fail to convert concatemeric DNA into the monomeric form. Studies of cell lysis revealed that T7-infected pRS148-containing cells lyse prematurely.

METHODS

Bacterial and phage strains

Bacterial strains. E. coli HMS174 (rK12 mK12 recA rpoB1(r)) (Campbell et al., 1978) and B23 were used as non-permissive hosts for amber mutants. E. coli C600 (rK12 mK12 supE44) (Bachman, 1972) was used as the permissive host for amber mutants and Shigella sonnei strain D2 371-48 was used as the non-permissive host for ss+ phage (Hausmann et al., 1968). E. coli 594 (Campbell, 1965) was used for packaging experiments in vitro.

Phage strains. Amber mutants used, with their allele designations, were as follows: am1-342, am3-29, am4-208, am5-28 and am6-147. The am4 am5 and am3 am5 am6 double and triple mutants were kindly provided by P. Sadowski, as was the ss− strain. The am4 am5 double mutant fails to synthesize DNA primase and DNA polymerase in a non-suppressing host.

Media. Bacterial strains were grown routinely in LB containing 10 μg thiamine/ml and 10 μg thymidine/ml. When cells contained plasmids, 10 μg tetracycline/ml was included.

Labelling of intracellular DNA. For labelling of intracellular phage DNA, cells were grown in TCG phosphate medium (Benbasat et al., 1978). At 7 min post-infection [3H]thymidine (approx. 6.7 Ci/mmol) was added to 10 μCi/ml final concentration. Labelled cells were harvested as described below.

Construction and identification of pRS148. The construction of plasmids carrying segments of T7+ DNA has been described (Smith & Miller, 1981). In brief, HpaI-digested T7 DNA was inserted into the PstI site of pBR322 (Bolivar et al., 1977) by means of poly(dA)-poly(dT) tails (Lobban & Kaiser, 1973). Plasmids carrying various segments of the T7 genome were identified after transformation into E. coli by hybridization with 32P-labelled T7 DNA and by virtue of their ability to produce wild-type recombinants with one or more amber mutants in a spot test (Smith & Miller, 1981). pRS148 was identified as a plasmid that annealed with T7 DNA but would not recombine with any amber mutant, nor support the growth of control, T7+, phage.

Phyiscal characterization of pRS148. pRS148 was purified in CsCl-ethidium bromide gradients as described by Smith & Miller (1981). Purified plasmid was linearized by digestion with EcoRI, and the molecular weight of the plasmid was determined by electrophoresis in agarose (McDonnell et al., 1977).

For hybridization analysis, 1 μg of purified pRS148 DNA was 32P-labelled by nick translation (Maniatis et al., 1977). The labelled plasmid was used to probe nitrocellulose blots of FnuC- and HpaI-digested T7 DNA which had been electrophoresed in 0.5% agarose gels (Southern, 1975).

Isolation of DNA. DNA was isolated from concentrated phage suspensions by phenol extraction (Miller et al., 1976), followed by dialysis.

To isolate [3H]thymidine-labelled intracellular phage DNA for sucrose gradient analysis, infected, labelled cells were ice-chilled at 15 or 20 min post-infection and concentrated by centrifugation. Cells were resuspended in 0-01 M-Tris-HCl pH 7-4, 0-1 M-NaCl and 0-005 M-EDTA (TNE buffer) and lysed by addition of SDS to a final concentration of 0.5% (w/v). After incubation at 37°C for 10 min, pre-digested Pronase was added to a final concentration of 1 mg/ml. The lysates were incubated at 37°C overnight, phenol-extracted, ether-washed, and loaded on sucrose gradients with wide-bore pipettes.

To co-extract ss− concatemeric and T7+ monomeric DNA, E. coli B23 was infected with ss− phage at a m.o.i. of 5, and chloramphenicol was added to a final concentration of 100 μg/ml at 12 min post-infection. At 15 min after infection, cells were chilled on ice and pelleted by centrifugation. After suspension in TNE, aliquots of the cell suspension were mixed on ice with various amounts of concentrated T7+ phage. DNA was extracted as described above for [3H]thymidine-labelled intracellular DNA. Then aliquots of each DNA sample were packaged in a control in vitro packaging extract, and the proportions of packaged ss− and T7+ DNA were determined by plating on D2 and B23. A mixture of ss− concatemeric and T7+ monomeric DNA that resulted in the packaging of approximately equal numbers of ss− and T7+ was selected for the experiment described in Table 2.

In a separate experiment it was shown that chloramphenicol treatment at 12 min post-infection resulted in the accumulation of concatemeric molecules, as evidenced by the appearance of the MboI B−C fusion fragment diagnostic of the head-to-tail arrangement of phage genomes (Langman et al., 1978).

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Sucrose gradient analysis of intracellular DNA. [3H]Thymidine-labelled intracellular phage DNA was sedimented in linear 5 to 20% neutral sucrose gradients containing 0.15 m-NaCl, 0.01 m-Tris-HCl pH 7.4, 1 mM-EDTA. Centrifugation was for 3 h at 30,000 rev/min in a SW50.1 rotor. Approximately 35 ten-drop fractions were collected from the bottom of each gradient onto filter paper discs, and the radioactivity in each fraction was then determined by liquid scintillation counting.

In vitro packaging of T7 DNA. This was done as described by Roeder & Sadowski (1979).

RESULTS

Characterization of pRS148

As indicated in Methods, pRS148 was identified by spot test as not permitting T7+ growth. In order to identify the region of the genome carried by pRS148, hybridization analysis was performed according to Southern (1975). Wild-type T7 DNA was digested with the restriction enzyme FnuC, electrophoresed and blotted, and the blot was probed with 32P-labelled pRS148. The FnuC C fragment was homologous to pRS148 showing the insert to be derived from the right end of the genome. In order to refine our localization, a similar experiment was performed with a HpaI digest of phage DNA. The results demonstrated that pRS148 hybridizes to a fragment with the electrophoretic mobility of fragment: HpaI 1 or HpaI J. Since only fragment HpaI J is within the FnuC C fragment we conclude that pRS148 contains DNA homologous to the HpaI J fragment. From the molecular weight of linearized pRS148, as determined by agarose electrophoresis, we estimate that the size of the insert including homopolymer tails corresponds to a length of 2.0 kilobases (kb). Since homopolymer tails in these and related experiments were approximately 40 to 50 base pairs (bp), we conclude that pRS148 contains most or all of the 2.1 kb HpaI J fragment.

The efficiency of plating of T7+ on HMS174/pRS148 was found to be 2 to 3% relative to HMS174/pBR322 or HMS174 with no plasmid. The plaques observed on HMS174/pRS148 were very small and irregular in shape. Single-step growth experiments showed that pRS148-containing cells produce 1 or 2 phage/cell on average. Other strains of E. coli containing pRS148 similarly do not support T7+ reproduction.

Macromolecular synthesis

In an attempt to identify the event in T7+ infection which was being affected by pRS148, the patterns of macromolecular synthesis were examined in T7+-infected, pRS148-containing cells. The results were compared to those obtained with pBR322-containing cells infected in parallel. DNA synthesis was measured by [3H]thymidine incorporation according to Studier (1969), RNA synthesis was measured by [3H]uracil incorporation according to Young et al. (1980) and protein synthesis was monitored by incorporation of 14C-labelled amino acids and polyacrylamide gel electrophoretic analysis of the proteins according to Russel (1973). The results of these analyses showed that the rate and extent of DNA, RNA and protein syntheses was decreased by at most 50% in T7+-infected, pRS148-containing cells compared to T7+-infected, pBR322-containing cells. No obvious differences in the amounts of any T7+ proteins were observed by polyacrylamide gel electrophoresis either.

Fate of intracellular phage DNA

When the fate of progeny phage DNA was examined by sedimentation of intracellular DNA in neutral sucrose gradients, a significant difference was observed between infected pRS148- and pBR322-containing cells. At 15 min after infection of pBR322-containing cells, much of the progeny phage DNA consisted of molecules that sedimented faster than monomeric T7 DNA (Fig. 1a). By 20 min post-infection a considerable fraction of this DNA had been processed to monomeric form (Fig. 1c). The sucrose gradient profile obtained with pRS148-containing cells at 15 min post-infection is similar to that obtained with the control cells at the same time (Fig. 1b). However, at 20 min after infection pRS148-containing cells did not contain significant amounts of monomeric T7 DNA (Fig. 1d). Therefore, processing of concatemers is either retarded or defective in pRS148-containing cells.
Fig. 1. Sucrose gradient sedimentation analysis of intracellular T7 DNA. *E. coli* containing either pBR322 (a, c) or pRS148 (b, d) were grown to $3 \times 10^8$ cells/ml in TCG medium and were infected with T7+ phage. At 7 min post-infection, the cells were labelled with [3H]thymidine. At 15 min (a, b) or 20 min (c, d) post-infection, the DNA was extracted, mixed with $^{32}$P-labelled T7+ DNA, and analysed by sucrose gradient sedimentation. The arrow in each panel represents the position of the $^{32}$P-labelled T7+ reference DNA. The graphs show the percentage of $^3$H-labelled, T7+ DNA recovered in each fraction of the gradient. Sedimentation is from right to left (Langman *et al.*, 1978).

**In vitro packaging experiments**

*In vitro* packaging experiments were performed to determine whether pRS148 was capable of directly interfering with phage assembly. Packaging extracts were prepared by infecting *E. coli* 594 (sup°) with phage of genotype am3 am5 am6 (Roeder & Sadowski, 1979). DNA purified from wild-type phage and purified pRS148 or pBR322 DNA were added to these extracts. The results of duplicate experiments were very similar and have been pooled in Table 1 (experiments 1 to 4). The production of wild-type phage was dependent on and quantitatively proportional to phage DNA added. Phage production was somewhat inhibited by added plasmid DNA, but the effects of pBR322 and pRS148 were similar. We obtained no evidence for a direct inhibitory effect of pRS148 DNA on phage assembly in cell-free extracts.

Packaging extracts also were prepared from HMS174 which contained either pRS148 or pBR322 and which were infected by am3 am5 am6 phage. Such extracts also produce wild-type phage when incubated with wild-type monomeric T7+ DNA. Because we were interested in the possibility that pRS148 might interfere with the maturation of concatemers in such extracts, we added a mixture of concatemeric and monomeric DNA. The presence of the ss- marker on the added concatemeric molecules allowed us to determine the relative efficiencies of *in vitro* packaging of these two types of molecule by each extract. The ss- marker allows T7 to plate on the *Shigella* strain D2 (Hausmann *et al.*, 1968). The results (Table 2) show that (i) extracts prepared from HMS174/pRS148 cells are about 50% as efficient as HMS174/pBR322 extracts in packaging exogenous DNA and (ii) the relative efficiency of packaging concatemers, as indicated by the percentage of total packaged phage which carried the ss- marker, is similar in the two extracts. These results show that HMS174/pRS148 extracts contain all the components necessary to package exogenous monomeric and concatemeric DNA. The reduced efficiency of HMS174/pRS148 packaging extracts may not be significant since considerable extract-to-extract variability was encountered in such experiments.
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Table 1. In vitro packaging of wild-type T7 DNA by E. coli extracts

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Extract*</th>
<th>T7+ DNA added</th>
<th>Plasmid</th>
<th>Titre on B23 (p.f.u./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>594</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>594</td>
<td>0.2 µg</td>
<td>-</td>
<td>1.04 × 10^6</td>
</tr>
<tr>
<td>3</td>
<td>594</td>
<td>0.2 µg</td>
<td>0.3 µg pBR322</td>
<td>5.6 × 10^5</td>
</tr>
<tr>
<td>4</td>
<td>594</td>
<td>0.2 µg</td>
<td>0.3 µg pRS148</td>
<td>6.7 × 10^5</td>
</tr>
</tbody>
</table>

* Cells of the indicated strain (Campbell, 1965) were infected with T7 am3 am5 am6 and used for packaging experiments as described by Roeder & Sadowski (1979).

Table 2. Efficiency of packaging T7 DNA by extracts of E. coli

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Extract*</th>
<th>T7 DNA†</th>
<th>D2 (p.f.u./ml)</th>
<th>B23 (p.f.u./ml)</th>
<th>% ss*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HMS174/pBR322</td>
<td>5 µl</td>
<td>1.1 × 10^5</td>
<td>2.9 × 10^5</td>
<td>39</td>
</tr>
<tr>
<td>2</td>
<td>HMS174/pRS148</td>
<td>5 µl</td>
<td>4.9 × 10^4</td>
<td>1.6 × 10^5</td>
<td>30</td>
</tr>
</tbody>
</table>

* Extracts were prepared from cells of the indicated strain as described by Roeder & Sadowski (1979).
† Monomeric T7+ and ss- concatemeric DNA were co-extracted as described in Methods.

In summary, the results show that (i) exogenous pRS148 does not specifically inhibit in vitro packaging of exogenous T7 DNA and (ii) extracts prepared from cells containing pRS148 are capable of packaging exogenous monomeric and concatemeric T7 DNA. The results suggest that pRS148 causes some organizational defect in the cell, which is not preserved in cell-free extracts.

Cell lysis

To determine whether cell lysis was affected by pRS148, cultures of HMS174/pRS148 and HMS174/pBR322 were grown to 2 × 10^8 cells/ml in side-arm flasks. The cells were infected with wild-type T7 at a m.o.i. of 5 and their absorbances were monitored at 2 min intervals with a Klett colorimeter. At 4 min intervals, samples of each culture were diluted into chloroform-saturated broth and vortex-mixed vigorously. Such lysates were later diluted and plated for phage.

The results of one such experiment are shown in Fig. 2. In this experiment, cultures were maintained at 25 °C to increase the duration of infection and to accentuate the difference between pRS148- and pBR322-containing cells. However, qualitatively similar results have been obtained at 30 °C and 42 °C. The presence of pRS148 causes the infected cells to lyse 10 to 12 min prematurely relative to HMS174 containing pBR322 (Fig. 2) or relative to HMS174 with no plasmid (data not shown). Furthermore, the lysis of HMS174/pRS148 began before substantial numbers of progeny phage appeared in the HMS174/pBR322 culture. Therefore, premature lysis of infected HMS174/pRS148, or the premature alterations of membrane structure that must be supposed to precede actual lysis, provides an adequate explanation of the inability of such cells to produce phage.

Variations of the experiment described in Fig. 2 were repeated with wild-type phage six times with a consistent outcome. The results of similar experiments with mutant phage are shown in Fig. 3. The am4 am5 double mutant has a double replication block in HMS174. The HMS174/pBR322 culture lysed at the same time when infected with T7 am4 am5 as with wild-type phage. Thus, lysis is not normally dependent upon DNA synthesis. The HMS174/pRS148 culture lysed somewhat earlier than the HMS174/pBR322 control culture when infected with T7 am4 am5 phage but substantially later than when HMS174/pRS148 cells were infected with wild-type phage. When HMS174 containing either pRS148 or pBR322 was infected with am1-342-bearing phage, the absorbance of the infected cultures continued to increase slowly for 30 min, and no lysis was observed over the course of the experiment (120 min). T7 am1 phage fail to produce T7 RNA polymerase, do not express any class II or III genes, and do not lyse the host.
Fig. 2. Cell lysis after infection by T7. E. coli was grown to 2 x 10^8 cells/ml in LB at 25 °C and infected with T7+ phage. At intervals after infection, the absorbance of the cultures was measured and aliquots of the infected cells were plated for phage. ●, Absorbance of E. coli HMS174/pBR322; ○, absorbance of E. coli HMS174/pRS148; ▼, titre of T7+ phage produced from E. coli HMS174/pBR322. Less than one phage per infective centre was produced from the E. coli HMS174/pRS148 culture.

One explanation for these results is that pRS148 encodes a protein which affects stability of the cell envelope. Production of the protein at an inappropriate time or at an inappropriate place or in an inappropriate amount could lead to premature lysis of the cell. The effects of the alteration might be manifested before lysis. Therefore, the decreases in macromolecular synthesis and the defects in concatemer metabolism might arise as secondary effects of an altered cell envelope. J. J. Dunn & F. W. Studier (personal communication) have identified several possible genes in the HpaI J fragment of T7 phage. These include two possible genes...
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Fig. 3. Cell lysis after infection by T7 amber mutant phage. An experiment similar to that described in Fig. 2 was conducted with E. coli infected by various amber mutant phage. Cultures of E. coli were infected with either T7 am1 or T7 am4 am5 at 25 °C, and the absorbance of the cultures was measured at intervals after infection. The points shown are absorbances of the following: ▲, HMS174/pBR322 after infection by T7 am1 phage; △, HMS174/pRS148 after infection by T7 am1 phage; ●, HMS174/pBR322 after infection by T7 am4 am5 phage; ○, HMS174/pRS148 after infection by T7 am4 am5 phage.

(19·2 and 19·3) which have sequences that overlap gene 19 and a gene (19·5) which maps to the right of gene 19. It is quite possible that one of these genes is responsible for the observed effects of pRS148.

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