Physical Mapping and Cloning of Bacteriophage T4 Anti-Restriction Endonuclease Gene

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We have proposed that the ability of T4 to produce non-glucosylated progeny after a single cycle of growth on a galU rglA rglB+ mutant of Escherichia coli is due to the inhibition of the rglB+ function by a phage-coded, anti-restriction endonuclease protein. Based on this hypothesis, we screened T4 deletion mutants for failure to give a burst in this host. The absence of an arn gene in phage mutants lacking the 55.5- to 58.4-kilobase region is verified by their inability to protect secondary infecting non-glucosylated phage from rglB-controlled cleavage. A functional arn gene was cloned on plasmid pBR325, and the 0.8-kilobase insert DNA was shown to be homologous to the DNA missing in the arn deletion phage.

Non-glucosylated (Glu−) T-even phage are restricted by wild-type Escherichia coli, unlike wild-type glucosylated (Glu+) phage. This restriction is specific for hydroxymethylcytosine (HMC) DNA (cytosine residues of T4 DNA are replaced by HMC) and is controlled by two distinct E. coli genes, rglA and rglB (formerly r6 and r2,4 [20–22]). The rglA-coded activity appears to be located in the membrane (9) and recognizes sites in all Glu− T-even phage (20). In contrast, the rglB-coded activity is located in the cytoplasm (9, 12) and recognizes sites in T2 and T4 Glu− DNA but not in T6 Glu− DNA (20). These two activities seem to be endonucleolytic (9, 12) and to cleave a small number of sites (9) in the Glu− T4 genomes.

T-even phage grown in galU strains of E. coli are non-glucosylated since the glucose donor, uridine diphosphoglucose, is not synthesized in these mutants (13, 23, 27). It was reported earlier (13, 23) that although parental Glu− DNA is restricted in galU mutants, progeny Glu− DNA (from a Glu+ phage infection) is not. This paradox was explained by the observation (10) that, early after infection, T4 synthesizes an anti-restriction endonuclease (arn) activity directed against rglB activity, permitting unrestricted Glu− progeny phage DNA replication in galU mutants.

In this study, the inability of T4 arn mutants to produce single-cycle progeny in a galU host was used to screen T4 deletion phage for the absence of arn activity. When coupled with the imm mutation (to permit normal injection by a superinfecting phage), the arn deletion phage failed to protect a secondary infecting Glu− phage from rglB restriction. A functional arn gene was cloned in pBR325 and shown to be homologous to the genetic region missing in the Arn− deletion phage. The arn gene is located in the nrdC-I region (30) between 55.5 and 58.4 kilobases (kb).

MATERIALS AND METHODS

Bacteria, bacteriophages, and plasmids. Derivatives of Escherichia coli K-12: HR112 (F′ rglA rglB λ*), formerly K-12 r6− r2,4− or K rgl− (21–23), was the permissive host for Glu− T4; U95 (galU rglA rglB+) (23) and its derivative HR141 (galU rglA rglB), formerly U95 r6− r2,4− (21), was the isogenic pair of galU strains used to screen T4 deletion mutants for the absence of arn function; DO100 [Δ(lac-pro) rglA rglB+], the restrictive host for Glu− T4 used to clone and detect Arn+ plasmids, was from D. Oliver; JCT729 (F′ rglA+ rglB+ recB21 sbeB15 his-327 trpE9829 leu lan rpsL32 thi), a restrictive host lacking exonuclease V activity used in sucrose gradient analysis of Arn+ and Arn− T4, was from A. J. Clark. Escherichia coli B strain B634 (hsdS/ met galU56), used to grow T4 alc7 to produce cytosine-containing T4 DNA, was from L. Snyder. T4 bacteriophage strains: T4D was the wild-type phage: Δ(39-56)12, Δ(63-32)11, Δ(63-32)7, and Δ(63-32)9 were from Homyk and Weil (14); Δ2 and Δ5 were from G. G. Wilson (Ph.D. thesis, Sussex University, Sussex, England, 1976); Δkl2 and Δarp13 were from D. Hall (5); imm2 was from Vallée (28); and alc7, a multiple mutant of T4 (amE51 [gene 56], amC87 [gene 42], NBS060 [genes denB-rHl], alc7 [gene alc]) used for making cytosine-containing T4 phage, was from Wilson et al. (29). Plasmid pBR325 (5.4 kb) confers ampicillin, tetracycline, and chloramphenicol resistance to the host (2) and was received from John Watson.

Enzymes and chemicals. Restriction endonuclease EcoRI, T4 DNA ligase, and E. coli RNA polymerase holoenzyme were from New England Biolabs. DNA

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polymerase was from Miles Laboratories, Inc. [α-32P]ATP, 111 TBq/nmol, and phosphorus-32 (carrier free) were from New England Nuclear Corp. Ribonucleoside triphosphates and deoxyribonucleoside triphosphates were from Boehringer Mannheim Corp. Other chemicals and reagents were of the highest purity available.

**Bacterial, phage growth, and other techniques.** Bacterial and phage techniques were described by Adams (1). Preparation of 32P-labeled HMC T4 DNA and sucrose gradient analysis of intracellular phase DNA were described earlier (9). T4 Δ5 imm double mutants were constructed by crossing T4 Δ5 and T4 immys mutants at a ratio of 20:1. Since T4 imm mutants make larger plaques than do Δ5 or wild-type T4, 10 large plaques were picked, purified, and used to make stocks. The imm phenotype was confirmed by the absence of superinfection breakdown of 32P-labeled wild-type T4 DNA (11). The Δ5 imm phenotype was verified by the inability to give a burst on a galU rglB+ host cell. We presume that the particular recombinant we used in this study is actually a T4 Δ5 immys triple mutant, since this recombinant is Imm+ at 30°C. T4 imm single mutants are deficient in immunity only at 37°C, unlike T4 immys (28).

**Nick translation and DNA-DNA hybridization.** Plasmid pDH346 DNA carrying the T4 arn gene insert was nick translated with [α-32P]ATP as described by Davis et al. (7) to produce a labeled probe. DNA hybridization was carried out as described by Denhardt (8). In brief, 1 μg of the unlabeled denatured T4 DNA bound to nitrocellulose filters was annealed with 0.025 μg (1.1 × 106 cpm/μg) of denatured 32P-nick-translation plasmid DNA fragments. Phage DNA for hybridization experiments was isolated by phenol extraction from cesium chloride gradient-purified phage particles.

**Isolation of phage and plasmid DNA.** T4 cytosine-containing DNA was prepared from the T4 multiple mutant of alc7 as described by Wilson et al. (29). Plasmid DNA was isolated from cultures grown in M9 (7) minimal medium (supplemented with 0.4% glucose and 0.2% Casamino Acids) by a modification of the method of Katz et al. (15) which gives a high yield of plasmid DNA with very little contamination of chromosomal DNA. Cultures were grown to 5 × 109 cells per ml, and plasmid DNA was amplified by growing the cultures in the presence of spectinomycin (300 μg/ml) for 16 h. Cells from 500-ml cultures were concentrated and washed and incubated in TE buffer (10 mM Tris-hydrochloride [pH 8.0]–1 mM EDTA). Washed cells were suspended in 9 ml of cold 25% sucrose buffer with 0.05 M Tris-hydrochloride (pH 8.0). A 1.8-ml amount of lysozyme (5 mg/ml in 0.25 M Tris-hydrochloride [pH 8.0]) was added and incubated on ice for 10 min with occasional shaking. At this point, 3.6 ml of 0.25 M EDTA was added, and the incubation on ice was continued for 10 min. A 14.4-ml amount of Brid lysis mix (1.0% Brid 58; 0.4% sodium dodecyl sulfate; 0.0625 M EDTA; 0.05 M Tris-hydrochloride, pH 8.0) was added, and the cells were incubated for 60 min on ice with gentle shaking. The lysate was cleared by centrifugation at 20,000 rpm for 60 min at 4°C in a 60 Ti rotor (Beckman Instruments, Inc.). The supernatant fluid was decanted carefully, and the volume was adjusted to 30 ml with TE buffer. For most of the experiments, a portion of the cleared lysate was treated with RNase (10 μg/ml) and then deproteinized with phenol. Phenol was removed by chloroform-isooamyl-alcohol (99:1) extraction, and the DNA, precipitated with ethanol and redissolved in TE buffer, was used without further purification. In some cases, covalently closed DNA was purified by equilibrium centrifugation after adding 31.5 g of CsCl (density, 1.64) and 5 ml of 7.5-mg/ml ethidium bromide to 30 ml of cleared lysate. The plasmid DNA was collected, freed of ethidium bromide by extraction with isopropanol, dialyzed against TE buffer, and concentrated by ethanol precipitation.

**Cloning the arn gene.** DNA cleaved with restriction endonucleases by the supplier's instructions was ligated with T4 DNA ligase at 12°C for 16 h in a buffer containing 0.05 M Tris-hydrochloride (pH 7.5), 10 mM MgCl2, 20 mM dithiothreitol, and 1 mM ATP. An EcoRI partial digest of T4 cytosine-containing DNA was ligated to EcoRI-cleaved plasmid pBR325 DNA and used for transformation calcium-shocked (7) DO100 cells. Since the insertion of foreign DNA at the EcoRI site of pBR325 inactivates the chloramphenicol transacylase gene, we screened the Amp' Cam' transformants for the arn+ property which renders the rglB+ restricting cells permissive for Glu T4 phage. The purified transformants were grown in wells (fraction collection tray model no. 26107; Gilson Medical Electronics, Inc.) overnight at 37°C. A duplicating device with 25 prongs was used to transfer droplets to a set of three plates that had been seeded with either no phage (master plate) or 106 T4 Glu- or T4 Glu+ phage particles in the soft agar. The transformants which failed to grow on both T4 Glu- and T4 Glu+ plates were picked from the master plates and tested for efficiency of plating of T4 Glu+ phage.

**RESULTS**

Strategy for mapping the T4 arn gene. T-even phage-coded glycosyltransferases transfer the glucose moiety from uridine diphosphoglucose to HMC residues of the phage DNA (6). Since galU mutants of E. coli fail to synthesize uridine diphosphoglucose, progeny phage grown in these mutants lack the glucosyl modification of HMC residues in their DNA (13, 22). The growth properties of T4 on two galU host strains are shown in Table 1. The burst sizes of T4 in galU rglA rglB+ and galU rglA rglB- host cells were similar when the progeny phage were assayed on a nonrestricting host. T4 forms plaques, however, on only galU rglA rglB+ and not on galU rglA rglB+ cells (13, 21, 23, 27). To explain why the replicating HMC DNA is immune to restriction, it has been suggested that both rglA- and rglB-restricting DNase activities are localized in the host membrane (29). However, Dharmalingam and Goldberg (9) have shown that only rglA may be membrane bound; the rglB activity has a cytoplasmic location (12). An additional observation which explains the lack of restriction of HMC DNA in galU mutants is that, early after infection, T4 makes an anti-restriction endonuclease protein (Arn) which inhibits rglB activity (10). Therefore, in such
cells, progeny HMC DNA can replicate since the rglA product (located in the membrane) is also inactive against the replicating HMC DNA.

If we presume that the arn gene is nonessential for glucosylated T4 phage growth, we could screen for T4 arn mutants since such mutants would liberate only two or less phage per infected cell in galU-restricting strains, whereas in galU rglA rglB mutants, a normal burst would be obtained. Therefore, we screened available T4 mutants which have deletions in different parts of the genome to locate the arn gene.

Physical mapping of the arn gene. The burst sizes of various T4 mutants deleted in nonessential regions of the genome are shown in Table 2. The ratio of burst sizes on galU rglA rglB+ and galU rglA rglB host cells was close to 1.0 for T4 wild type and the deletions Δ(39-56)12, Δ(63-32)1, Δ(63-32)7, and Δ(63-32)9. In contrast, ΔfarP13, which covers the region extending from 48.4 to 65 kb clockwise from the rIIA-rIIB junction (Fig. 1), gave a burst size ratio of 0.01. This result implies that the region of DNA missing in the ΔfarP13 mutant codes for an activity which overcomes rglB restriction. To further localize the presumptive arn gene, we tested shorter deletions in the same region. Deletion Δk2 (49.5 to 60 kb) gave a burst size ratio of 0.03 and must also lack the arn gene. However, Δ2 (deleted from 49.4 to 55.5 kb) gave a ratio of 1.3. These results show that at least part of the arn gene is located between the rIII-distal ends of Δ2 (55.5 kb) and Δk2 (60 kb). The absence of arn gene expression in Δ5 (deleted from 53 to 58.4 kb; ratio, 0.028) indicates that at least a segment of the arn gene is in the 2.9-kb region extending from 55.5 to 58.4 kb, the region between the rIII-distal ends of Δ2 and Δ5.

Analysis of the arn gene expression. Expression of the arn gene in T4 wild-type phage-infected cells inhibits the host rglB restriction activity so that superinfecting Glu- T4 DNA remains uncleaved (10). In cells infected with T4 imm mutants, the superinfecting Glu- T4 DNA is uncleaved (Fig. 2A), confirming the earlier observation (10). The imm mutation (11, 28) is needed to alleviate the superinfection exclusion property of the primary infecting phage. We constructed T4 Δ5 imm double mutants (as described in Materials and Methods) to study expression of the arn gene. In T4 Δ5 imm-infected cells, the secondary infecting Glu- DNA is cleaved into fragments (Fig. 2B), demonstrating that the arn gene is not expressed by T4 Δ5 imm mutants.

Molecular cloning of the arn gene. To clone the arn gene, an EcoRI partial digest of cytosine-containing T4 DNA was ligated into the single EcoRI site in plasmid pBR325. Amp+ Cam+ transformants of the restrictive host E. coli DO100 were screened for their ability to permit the growth of T4 Glu- phage. The plasmid DNA isolated from two such transformants had an insert of about 0.8 kb in length. DNA from one transformant, plasmid DNA pDH346, was further characterized (Table 3). This recombinant DNA hybridized extensively with wild-type T4 DNA as well as with Δ2 DNA but not with Δ5 or to any significant extent with ΔfarP13 DNA. These results limit the location of the arn gene to the 55.5- to 58.4-kb segment of the T4 map.

<table>
<thead>
<tr>
<th>TABLE 1. Growth properties of wild-type T4 in galU rglA rglB+ and galU rglA rglB strains</th>
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<td>Host strain</td>
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<tr>
<td>--------------</td>
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<tr>
<td>galU rglA rglB+</td>
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<td>galU rglA rglB</td>
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* Host strains used were U95 and HR141.

** The efficiency of plating is the plaque count obtained relative to the plaque count on E. coli HR112 rglA rglB.

*** In burst size experiments, log phase cells (2 x 10^8/ml) were infected with 0.1 T4 phage per cell, incubated at 37°C for 10 min, and then diluted 2,000-fold. The diluted samples were aerated for 2 h and plaque released by the addition of chloroform. Burst size is calculated from the number of plaque formations at 2 h after infection compared with the number of infective centers at 10 min after infection measured on HR112 rglA rglB.

<table>
<thead>
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<th>TABLE 2. Burst size of T4 and T4 deletion mutants</th>
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<tr>
<td>Phage strain</td>
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</tr>
<tr>
<td>T4 wild type</td>
</tr>
<tr>
<td>T4 Δ(39-56)12</td>
</tr>
<tr>
<td>T4 Δ(63-32)1</td>
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<tr>
<td>T4 Δ(63-32)9</td>
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<tr>
<td>T4 ΔfarP13</td>
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<td>T4 Δk2</td>
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* Burst sizes were determined as described in Table 1, footnote c.
VOL. 149, 1982
MAPPING T4 ANTI-RESTRICTION ENDONUCLEASE GENE 697

FIG. 1. Map location of the arn gene on the T4 genome. The distance clockwise from the rIIA-rIIB junction, the arbitrary 0 point (30), is given in kilobases at the top of the figure. Location of the arn gene (-----) was determined as described in the text. Location of the arn gene is given from points 49 and tk as well as the endpoints of Δtk2 and ΔfarP13 determined from the recombination frequencies published by Chace and Hall (5). Their recombination data was converted to map lengths in base pairs, using the four-parameter switch function of Stahl et al. (24). We used 166 kb as the total length of T4 DNA (14) instead of the value 200 kb used by Stahl et al. (24). The total map length of T4 is taken as 2,000 map units.

from pDH346. This 0.8-kb T4 DNA fragment contains the functional arn gene since clones derived from restrictive cells containing pDH346 are nonrestricive and the plasmid DNA does not hybridize with Δ5 DNA, indicating a lack of significant homology to T4 DNA beyond the endpoints of the Δ5 deletion. This observation limits the arn gene to the 55.5- to 58.4-kb region covered by Δ5.

In an attempt to determine whether the arn gene is expressed from its own promoter or from a vector promoter, we looked for promoters on the isolated 0.8-kb insert by R-loop analysis (3, 25). pDH346 DNA was cut with EcoRI, and both fragments were transcribed with E. coli RNA polymerase holoenzyme. After 2 and 3 min of transcription, more than 95% of 450 vector DNA molecules had more than one R-loop per molecule, whereas a similar number of 0.8-kb T4 DNA fragments showed none. Incubations of up to 8 min also showed no R-loops associated with the T4 insert. This suggests to us that, in the plasmid, arn is not associated with its own promoter; however, additional work will be needed to establish this point.

The mapping data show that the arn gene is located in the 10-kb region between nrdC and rI. This was the largest segment of the T4 chromo-

FIG. 2. Restriction of superinfecting Glu^- T4 DNA by cells previously infected with phage T4 missing the arn gene. Strain JC7729, a restricting host lacking exonuclease V activity, was preinfected with (A) T4 immS or (B) T4 Δ5, immS at a multiplicity of 4.0 and superinfected at 5 min with ^3H-labeled Glu^- T4 phage at a multiplicity of 1.0. At 5 min after secondary infection, cells were lysed, and the DNA was analyzed by neutral sucrose gradients as described previously (9–11). The arrows indicate the position of ^3H-labeled marker T4 DNA. For experiments A and B, sedimentation was for different times; therefore, the graphs have been aligned at the position of the T4 ^3H marker DNA.
some (about 7% of the T4 genome) which contained no known genes. One gene, adenine methylase (dam), in the closely related phage T2, is mapped (4) in the region corresponding to this portion of T4 DNA (16). The only other gene reported to map in this region is su30 (18); however, the data for this claim has not yet been published. It is interesting that the five genes located in this region are functionally related in the sense that all genes are involved in the DNA metabolism of the phage and, more specifically, nrdC, ik, and dam are all nucleotide-modifying enzymes.

Since the original description of phage-coded activities that prevent T7 and T4 restriction (10, 26), this phenomenon has been reported for T3, T5, and bacillus phage NR2 (for review, see reference 17). More recently, the direct interaction of the T7 antirestriction protein with the host-restricting enzyme has been nicely demonstrated (19). However, the arr gene is unique in that it is a second protection mechanism apart from the glucose modification. Furthermore, the arr gene seems to be useful only for the glucosylated phage under special circumstances, i.e., in hosts where phage-mediated glucosylation is not possible. We have shown earlier that one rglB restriction site is located in the arr gene itself (11). Therefore, the arr gene function is blocked when Glu− T4 DNA enters a restricting host. Since T4 deletion mutants lacking this gene grow normally as long as the phage DNA is glucosylated, the arr gene appears to be nonessential for the development of T4. Though average burst sizes in the permissive host vary among different deletion mutants (Table 2, column 3), there is no obvious correlation between this variation in burst size and the presence or absence of the arr gene.

At this point, it is unclear of what use the arr gene might be to wild-type T4 or, for that matter, to the Glu− T4 in a restricting host. The existence of an alternative (possibly more primitive) protection mechanism which is useful only for a glucosylated phage in a host where glucosylation is not possible invites evolutionary speculation. However, this might be more useful when the regulation of arr gene expression and the interaction of the rglB enzyme with the arr protein is clearer.

ACKNOWLEDGMENTS

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LITERATURE CITED


enhances suppression of ligase mutants with rII mutations. Virology 50:291–293.


