Bacteriophage-Host Interaction and Restriction of Nonglucosylated T6

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Nonglucosylated T6 phage (T6agt\(-\)) were found to have two structural anomalies when compared with wild-type T6. The DNA of T6agt\(-\) phage contains single-strand interruptions. These can be seen both during infection, in the pool of replicating DNA, and in DNA extracted from purified phage. In addition, the sodium dodecyl sulfate-polyacrylamide gel pattern of T6agt\(-\) phage structural proteins reveals a protein band not found in T6. The altered protein has a mobility slightly faster than that of the major head protein, and it is not removed by osmotic shock. The restriction activity of Escherichia coli B directed against T6agt\(-\) phage is abolished by preinfection of the cells for 4 min with T4 imm2. The shut-off of restriction is observed either by the rescue of superinfecting T6agt\(-\) or by the failure to detect degradation of incoming T6agt\(-\) DNA. This effect is resistant to rifampin and chloramphenicol.

Host-specific degradation of nonglucosylated T-even bacteriophage DNA (i.e., restriction) occurs after infection of wild-type Escherichia coli. The exact mechanism responsible for this nucleolytic attack on the incoming phage DNA is not known. The initial observation (14) and characterization (10, 23-25) of this phenomenon established that restriction depends upon the state of glucosylation of the infecting phage genome. T-even phage DNA contains specific glucosyl moieties attached via carbon one to the hydroxymethyl group of 5-hydroxymethyl cytosine. When these glucosyl residues are absent, either due to a lack of the phage-speciﬁc glucosyl transferase or to infection of a host cell deﬁcient in uridine diphosphoglucose, infection of wild-type E. coli is abortive and the phage DNA is partially converted to acid-soluble fragments (for a review, see reference 21). Phages which do not contain 5-hydroxymethyl cytosine (e.g., lambda or P1) are not restricted by this system. Host mutants have been isolated which do not restrict nonglucosylated phage, and, for both E. coli B and K-12, these mutations are found in two genetic loci (19).

No enzyme or enzyme system which is specific for nonglucosylated 5-hydroxymethyl cytosine-containing DNA has been detected. An analysis of the known E. coli nuclease has revealed no speciﬁcity for nonglucosylated phage DNA (6, 22). Further, when host mutants lacking restriction activity were compared with wild-type cells, no difference was found either in their complement of nucleases or in the ability of cytoplasmic extracts and membrane preparations to degrade nonglucosylated phage DNA (21; M. J. Hewlett, Ph.D. thesis, University of Arizona, Tucson, 1973). It may thus be necessary to consider alternative explanations for the mechanism of this speciﬁc host function.

The present study demonstrates that T6 phage containing a double amber mutation in the \(\alpha\)-glucosyl transferase gene have two altered structural properties: (i) the nonglucosylated phage DNA contains single-strand breaks, and (ii) the capsid of nonglucosylated T6 contains an altered structural protein. In addition, we will show that the restriction function of the host cell is abolished by prior phage infection. These data suggest that restriction of nonglucosylated phage may not initially involve a specific nucleolytic activity, but rather may be a function of a defective phage-host interaction.

(The work described in this paper was taken from a dissertation submitted by the senior author to the Faculty of the University of Arizona in partial fulfillment of the requirements for the degree of Doctor of Philosophy.)

MATERIALS AND METHODS

Cells and bacteriophage strains. Cell and phage strains used in this study, together with their relevant properties and sources, are listed in Table 1.

Media. Minimal medium was M9 (2), containing 2 to 4 mg of glucose per ml and supplemented (when necessary) with thiamine at 1 \(\mu\)g/ml. Broth medium was 3 g of nutrient broth (Difco) and 5 g of NaCl per liter, supplemented with thiamine as indicated above.

Cell and phage growth. Cells were grown to 10\(^8\)
cells per ml for preparation of phage stocks or to 3 x 10⁸ cells per ml for individual experiments. Non-glucosylated phage were maintained by growth in E. coli K-12 -r2.4- Derivative of ER22, permissive for growth of T6, requires -pL- for growth.

Radioactive labeling of phage DNA was carried out by infection of appropriate hosts in M9 medium containing 2 µg of uracil per ml and either [2-¹⁴C]uracil (1.25 µCi/ml) or [5-³H]uracil (5 µCi/ml). Labeling of phage capsid proteins was similarly performed by infection in M9 medium containing 10 µg of L-leucine per ml and either L-[¹-¹⁴C]leucine (0.5 µCi/ml) or L-[4,5-³H]leucine (2.5 µCi/ml). All radiolabeled compounds were obtained from New England Nuclear.

Extraction of phage DNA. DNA was obtained from purified phage by a low-shear phenol extraction as described by Thomas and Abelson (27). The resulting DNA was dialyzed against and stored in 0.1 x 0.15 M NaCl plus 0.015 M sodium citrate (pH 7.0). For analysis on alkaline sucrose gradients, DNA was released from purified phage by treatment with 0.5 N KOH and analyzed directly.

When it was necessary to examine the intracellular DNA of phage-infected cells, such cells were lysed as described previously (18). The resulting lysates could be analyzed directly on sucrose gradients.

Sucrose gradient analysis. Neutral or alkaline 5% to 20% (wt/vol) sucrose gradients were prepared and run as previously described (18).

Measurement of DNA degradation after infection. Appropriate hosts were grown to 3 x 10⁸ cells/ml and infected with phage containing labeled DNA. At various times after infection, 0.5 ml of the culture was added to 0.5 ml of ice cold 10% trichloroacetic acid. After 15 min in ice, the samples were centrifuged at 1,000 x g for 15 min. Portions (0.5 ml) of the resulting supernatant were counted in 10 ml of Aquasol (New England Nuclear).

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of phage proteins. Analysis of virion proteins of labeled T6 and T6agt phage or phage ghosts was carried out essentially as described by Laemmli (13). Phage ghosts were prepared by osmotic shock as described by J. Cornett (Ph.D. thesis, University of Arizona, Tucson, 1973). Gels (6 mm in diameter, 10% acrylamide) were run and cut into slices approximately 1 mm thick. The gel fractions were treated with 0.5 ml of Protosol (New England Nuclear):water (9:1, vol/vol) overnight at 55 C and counted in 10 ml of Omnifluor (New England Nuclear).

RESULTS

Intactness of T6agt DNA. Figure 1 shows the sedimentation pattern on neutral sucrose gradients of intracellular phage DNA from E. coli B₄⁺B₄- cells infected for 25 min with either T6 or T6agt. In both cases, the DNA cosediments with added marker T6 DNA. However,
when the same intracellular DNA is analyzed on alkaline sucrose gradients, the material derived from T6-infected cells sediments heterogeneously with a front cosedimenting with added marker, as observed previously (16), whereas DNA from T6agt- infected cells sediments heterogeneously and slower than added marker (Fig. 2). This suggests that T6agt- phage DNA made during infection of a permissive host contains single-strand breaks.

When the DNA of purified T6agt- phage is analyzed on an alkaline sucrose gradient (Fig. 3), it can be seen that a significant proportion of the DNA molecules contain single-strand breaks. We have found that T6 and T6agt- DNA cosediment on neutral sucrose gradients (data not shown).

Since T6agt- phage contain structurally abnormal DNA, we wondered if, in addition, the structural proteins of these phage were altered in any way. To ascertain this, [14C]leucine- or [3H]leucine-labeled phage were disaggregated in SDS, and the proteins were separated on SDS-polyacrylamide gels (13). Figure 4 represents a control electrophoretic pattern obtained with proteins from T6 phage ([14C] versus [3H]). This demonstrates that the choice of isotopic precursor does not affect the observed labeling pattern. When T6 and T6agt- phage proteins are compared (Fig. 5), a band of protein appears (around slice 49) in the case of the mutant, migrating slightly ahead of the major head protein band. This altered band (or bands) is still in evidence when the labeling regime is reversed (Fig. 6). When T6 and T6agt- phage ghosts are prepared by osmotic shock, and the resulting protein patterns are observed, the altered T6agt- band remains in evidence (Fig. 7). The low viability of phage ghosts prepared in this study (less than 2% relative plating efficiency) indicates that the altered protein is not an internal (or osmotically sensitive) component of the phage.

Restriction is abolished by prior phage infection. To determine the effect of phage infection upon the process of restriction, we used mutants of T4 phage which fail to establish immunity to superinfection, T4imm2 (28). We examined the yield of progeny T6agt phage after infection of E. coli ER22 or ER22R-B1- by T4imm2am42 and superinfection by T6agt-. The total progeny (determined on ER22R-B1- to allow T6agt but not T4imm2am42 growth) increased with time after the initial infection (Table 2). When the cells were superinfected at 8 min after the initial infection, the yield of progeny from both wild-type and permissive cells was identical. This implies that the mechanism of restriction is no longer active after the infection.

We wondered if this shut-off of restriction also applied to the degradation of T6agt DNA in nonpermissive cells. In this set of experiments, we used the mutant T4imm2s. The addition of the “spackle” (s) mutation insures
that immunity to superinfection does not develop at a slow rate with time (J. B. Cornett, Ph.D. thesis, University of Arizona, Tucson, 1973). Figure 8 shows the effect of prior infection (for 4 min) on the degradation of superinfecting T6agt DNA in E. coli B. The initial infection completely inhibits the host degradation of nonglucosylated DNA. This phenomenon is not dependent on the expression of phage functions, since neither rifampin (100 μg/ml) nor chloramphenicol (100 μg/ml) could prevent the effect (Fig. 9). Rifampin at 100 μg/ml
Fig. 6. Effect of reversing the labeling regime on the SDS gel electrophoretic pattern of proteins from T6 and T6agt-. Experimental protocol is the same as that of Fig. 4, except that the proteins are from [3H]leucine-labeled T6 and [14C]leucine-labeled T6agt- phage.

Fig. 7. Comparison of proteins from T6 ghosts and T6agt- ghosts by SDS-polyacrylamide gel electrophoresis. Experimental protocol is the same as that of Fig. 4, except that the proteins are from [14C]leucine-labeled T6 ghosts and [3H]leucine-labeled T6agt- ghosts.

reduces phage RNA synthesis to less than 1% of the normal level in our hands (data not shown). Thus, the abolition of restriction by prior infection appears to be a function of the initial interaction between the phage and host cell.

In interpreting the experiments which demonstrate shut-off of the restriction activity, one must consider the possibility of a trivial explanation—namely, that incoming T6agt- DNA becomes glucosylated by glucosyl transferase of the preinfecting imm mutant, and hence protected from degradation. This glucosyl transferase could either have entered the cell with the primary phage (if it is a virion protein; see
The following table summarizes the results of the experiment described above.

### Table 2. T6agt- total progeny after superinfection of T4imm2am42-infected cells

<table>
<thead>
<tr>
<th>Host</th>
<th>Total progeny $\times 10^4$ (PFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0'</td>
</tr>
<tr>
<td>ER22</td>
<td>7.7</td>
</tr>
<tr>
<td>ER22+s-n,-</td>
<td>23.5</td>
</tr>
</tbody>
</table>

*Cells grown in nutrient broth at 37°C to 3 x 10$^8$ cells per ml were infected with T4imm2am42 at a multiplicity of infection of 5. At the indicated times, 1.0 ml of each infected culture was transferred to an iced culture tube containing T6agt- in 4.0 ml of nutrient broth at a superinfection multiplicity of infection of 5. After an additional 4 min for adsorption, each tube was transferred to a 37°C bath, aerated for 50 min, and lysed with chloroform. Total T6 progeny was determined by plating on E. coli ER22+s-n,- which is nonpermissive for growth of T4imm2am42.

*Unadsorbed phage titer, 5.0 x 10$^4$ plaque-forming units (PFU)/ml.

*Time (minutes) of superinfection.

*Total progeny with T6agt- alone, 5.0 x 10$^4$ PFU/ml.

*Total progeny with T6agt- alone, 33.5 x 10$^4$ PFU/ml.

below) or been synthesized in the interval between primary and secondary infection. The following facts militate against this interpretation. (i) As stated above, the resistance to degradation of nonglucosylated DNA develops normally in the presence of inhibitors which would block de novo enzyme synthesis (Fig. 9). (ii) As shown by Kornberg et al. (11), DNA glucosyl transferase activity is not detectable in infected cells until at least 6 min after infection. The assay procedure is extremely sensitive, and it seems likely that the presence of even a few enzyme molecules per cell would have been detected. We have obtained results similar to those of Kornberg et al. (data not shown). (iii) The onset of resistance to degradation is extremely rapid. Considerable protection of the nonglucosylated DNA is evident even when the interval between primary and secondary infection is reduced to zero (Fig. 10). It seems unlikely that significant glucosylation could have occurred in this time interval.

The experiment of Fig. 10 also rules out any special role of the imm mutation in generating the observed results, for coinfection with $[^3H]T6agt-$ and either T4imm2s or T4D yields essentially identical degrees of protection of the labeled DNA.

Coinfection of T6agt- and T6 results in a level of protection similar to that shown in Fig. 10 with T4 (data not shown). Thus our results evidently differ from those of Hattman (9), who observed that coinfection of T2 and T*2 did not prevent degradation of T*2 DNA.

### DISCUSSION

If we compare the restriction of nonglucosylated phage DNA with other known restriction-modification systems in *Escherichia coli* (17), we might expect that the cell contains a nuclease which is specific for nonglucosylated 5-hydroxymethyl cytosine-containing DNA. This line of reasoning has led most investigators to attempt a demonstration of such activity in preparation of wild-type cells. Since these attempts have not been successful, we sought further definition of this system. In particular, we have considered the structural properties of T6agt- and the effect of phage-host interaction on the restriction system.

T6agt- phage DNA synthesized during infection of a permissive host contains single-strand interruptions. Using the relationship derived by Abelson and Thomas (1), we estimate from the sedimentation rates of T6 and T6agt- DNA in alkali (Fig. 2) that the latter molecules contain, on the average, one or two single-strand breaks.
when isolated at 25 min postinfection. Ando et al. (3) have described a T4-induced nuclease which attacks nonglucosylated T4 (T*4) DNA more rapidly than normal T4 DNA. The pool of nascent nonglucosylated DNA within the infected cell may be subject to attack by such phage enzymes. Normal phage DNA made early during replication may be protected if these nucleases are expressed at later times during infection.

DNA obtained from a purified stock of T6agt- phage contains molecules with single-strand breaks. It has been reported that the burst size of T6agt- obtained from infection of permissive cells is one-fifth to one-tenth that observed in the case of T6 infection (19). The occurrence of nicked molecules encapsidated in a T6agt- stock suggests that the stock may contain a large population of nonviable particles. Indeed, when the viable phage titer (plaque-forming units on a permissive host) is compared with an estimate of the particle concentration (obtained from the ultraviolet absorbance of the stock) the viable particles in a purified T6agt- stock account for about 10% of the total particles (M. J. Hewlett, Ph.D. thesis, University of Arizona, Tucson, 1973).

The SDS-gel electrophoresis pattern of virion proteins reveals that T6agt- phage contain a protein of about 38,000 daltons which does not appear among the proteins of normal T6. The altered protein is not an internal component as evidenced by its osmotic stability. Therefore, the occurrence of this protein is not due to an altered pattern of DNA binding proteins resulting from nonglucosylation of the DNA.

A priori, this protein abnormality can be rationalized in at least two ways. It is possible that nonglucosylated DNA does not function properly during phage maturation and packaging. As a result, the progeny phage contain some protein which is incorrectly processed (e.g., improperly cleaved during assembly). Alternatively, the abnormality may be due to the presence of a nonfunctional phage glucosyl transferase. This may be an indirect effect, such as the participation of the glucosyl transferase in an enzyme complex required for phage maturation. The effect could also be direct if the glucosyl transferase performs both a catalytic and a structural role, as has been shown for dihydrofolate reductase (12, 15), thymidylate synthetase (4), and the 1 gene nuclease (23). In this case the apparent new protein in the T6agt- capsid may be an amber peptide of the glucosyl transferase whose presence is normally masked in the gel electrophoretic pattern by inclusion along with other proteins in one of the slower-migrating major bands.

Theoretically, we might distinguish between these two possibilities by examining the nature

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**Fig. 9.** Effect of inhibitors on protection from DNA degradation in *E. coli* B. The protocol is the same as that of Fig. 8, except that rifampin (RIF; 100 µg/ml) or chloramphenicol (CAM; 100 µg/ml) was added, where indicated, 2 min before the primary infection with T4imm2s.

**Fig. 10.** Partial protection from degradation of T6agt- DNA by coinfection with T4imm2s or T4D. The protocol is the same as that of Fig. 8, except that the primary and secondary phages were added simultaneously by addition of mixed phage suspensions.
of phage produced after T6 infection of a host cell lacking the precursor uridine diphosphoglucose. In this situation, the phage glucosyl transferase will be functional, but the DNA will be nonglucosylated. However, such experiments have been frustrated to date by an anomalous labeling pattern of phage proteins in uridine diphosphoglucose-negative cells (M. J. Hewlett, Ph.D. thesis, University of Arizona, 1973) and by the fact that such cells do not completely block the glucosylation of phage DNA. Experiments are in progress to determine whether other $agt$ mutants (amber or missense) of T6 have this abnormality.

The restriction activity of $E. coli$ B directed against T6$agt^-$ is abolished by prior infection with an immunity-negative T4 mutant (Table 2, Fig. 8). This phenomenon is detected by observing the recovery of T6$agt^-$ progeny or by the failure to see degradation of incoming phage DNA. This latter effect is not prevented by the addition of either rifampin or chloramphenicol (Fig. 9), so that the expression of phage functions is not required. This is somewhat akin to the fact that the activity of the recBC nuclease (endonuclease V) is abolished by phage infection, but this effect requires the expression of phage genes (27). On the other hand, host protein and mRNA synthesis are shut-off by phage infection, and at least one mechanism responsible for this arrest is independent of phage gene expression (5). We believe that the shut-off of restriction is due to an interaction at the cell surface during phage attachment, and thus is akin to the shut-off of macromolecular synthesis produced by infection with phage ghosts.

It should be under stood that this discussion is confined to restriction in $E. coli$ B. Restriction in K strains seems to occur by a different mechanism, as evidenced by the following. (i) Infection of $E. coli$ W3110 (a K strain) with T4$imm^-$ does not protect T6$agt^-$ DNA from degradation (M. J. Hewlett, Ph.D. thesis, University of Arizona, Tucson, 1973). Thus, phage infection evidently does not abolish restriction in this strain. (ii) As shown by Fleischman and Richardson (7), activity of the restriction system can be observed in toluenized cells of $E. coli$ K strains. The substitution of 5-hydroxymethyl dCTP for dCTP in this system abolishes observed DNA synthesis, presumably because the restriction activity degrades hydroxymethyl cytosine-containing DNA as fast as it is synthesized. This would suggest that restriction and its shut-off are not cell surface phenomena, although Fleischman and Richardson do point out that their data are consistent with the idea of a membrane site for restriction. Since pol A strains of $E. coli$ B are not available, we cannot ask whether restriction could be similarly demonstrated in toluene-treated cultures of B strains.

Our data suggest that restriction in $E. coli$ B may not be due solely to the action of a sequence-specific nuclease. It is possible that restriction results from the failure of T6$agt^-$ phage to interact efficiently with the host cell. This defect may be in a function which is required to insert the phage genome into the cytoplasm of the cell. Instead, the nonglucosylated DNA may enter a compartment of the cell (e.g., the periplasmic space) where the DNA is degraded, possibly irrespective of its state of glucosylation or hydroxymethylation. The altered protein component of T6$agt^-$ DNA may be that portion of the phage capsid involved in this event. Indeed, a phage function has recently been described, associated with the action of T4 gene 2, which is required for proper transport of the phage DNA into the host cell (J. Silverstein, P. Siegel, and E. Goldberg, Fed. Proc. 33:1487, 1974).

This view of restriction may account for several observations. It is known that wild-type cells infected with nonglucosylated phage may still express some early phage functions (20, 21). In our scheme, this might occur if partially degraded phage DNA enters the cytoplasmic membrane.

The shut-off of restriction by prior infection with T4 immunity-negative mutants may be due to alterations at the cell surface. Infection with immunity-negative mutants does not allow the development of superinfection exclusion or superinfection breakdown, both of which occur by insertion of the secondary genome into the periplasmic space. Thus, T4 imm-preinfected cells may permit the nonglucosylated genome to enter the cell cytoplasm un molested.

Finally, the existence of permissive mutants of $E. coli$ B may not be due to mutation of a specific nuclease. Instead, such cells may contain membrane alterations so that T6 or T6$agt^-$ DNA successfully enters the cytoplasm without the intervention of a phage-specific function. Preliminary evidence from SDS-polyacrylamide gel analysis of membrane proteins of $E. coli$ B$_{am}'$ indicates that these cells have altered membrane characteristics (M. J. Hewlett, Ph.D. thesis, University of Arizona, 1973).

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LITERATURE CITED