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Bacteriophage T4 Baseplate Components

III. Location and Properties of the Bacteriophage Structural Thymidylate Synthetase

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Two T4D thymidylate synthetase (*td*) temperature-sensitive mutants have been isolated and characterized. Both mutants produce heat-labile phage particles. This observation supports the view that this viral-induced protein is a phage structural component. Further, antiserum to *td* has been shown to block a specific step in tail plate morphogenesis. The results indicated that the *td* protein is largely covered by the T4D tail plate gene 11 protein. Since the phage-induced dihydrofolate reductase (*dfr*) also is partially covered by the gene 11 protein, it appears that *td* was adjacent to the tail plate *dfr*. This location has been confirmed by constructing a T4D mutant which is $dfr^{ts}td^{ts}$ and showing that these two tail plate constituents interact and give altered physical properties to the phage particles produced. A structural relationship for the tail plate folate, *dfr*, and *td* has been reported.

In 1973 Capco and Mathews (2) presented evidence that the T4 phage-induced thymidylate synthetase (*td*) was a phage capsid component. They found that antiserum prepared against purified T4D *td* enzyme inactivated T4 phage. Further, when the T6 *td* gene was genetically transferred to T4 phage particles the physical properties of the T4D td^{T6}, especially its sensitivity to heat, were quite different from either parent. However, there was also the report that two *td* amber mutants, originally isolated by D. H. Hall and studied extensively by Krauss et al. (17), could produce viable phage particles in the apparent absence of the complete *td* polypeptide after infection of the nonpermissive host. It should be noted that the *td* gene is a neighbor of the dihydrofolate reductase (*dfr*) gene in the phage genome and that both gene products previously were thought to be solely early enzymes and to be "non-essential." Mutations that destroyed enzymatic activity, whether missense or nonsense, decreased phage yield by about 50%, but they did not prevent phage production (8).

While searching for temperature-sensitive mutants of T4D, we isolated two strains of T4D td^{ts} (L. M. Kozloff and L. K. Crosby, Abstr. Annu. Meet. Am. Soc. Microbiol., 1974, V69, p. 212). It was shown that these two strains produced heat-labile phage particles and that the *td* protein appeared to be a tail plate constituent. This paper gives the detailed properties of these two mutants and presents additional evidence that the *td* gene product is a tail plate component adjacent to the *dfr* in the tail plate. A tentative scheme for the structural relationships of the tail plate folate (14), *dfr* (12), and *td* is proposed.

MATERIALS AND METHODS

Preparation and purification of bacteriophage stocks and substructures. The procedures described in the accompanying papers (12, 14) were also used in this work. In addition, T4D *td*6 (T4D *wh*6), a mutation in the *td* gene (7), was furnished by D. H. Hall. On *Escherichia coli* B3, a strain of *E. coli* B lacking *td* activity, also furnished by D. H. Hall, the *td*6 mutant gives very small or no plaques in the presence of limiting thymidine (7). Two other *td* mutants, *td* N43 and *td* N54, both amber mutants in the *td* gene, were also furnished by D. H. Hall.

Enzyme assays. The assay procedure for *dfr* was that described earlier (16). *td* activity was measured by a modification of the procedure of Lomac and Greenberg (19). The reaction mixture contained 0.04 M Tris, pH 7.4, 0.1 M 2-mercaptoethanol, 0.001 M EDTA, 0.025 M $MgCl_2$, 0.0002 M tetrahydrofolic acid, 100 μl of enzyme extract, and 0.015 M formaldehyde and was initiated by the addition of [^{3}H]dUMP, which had a final molarity in the reaction of 5×10^{-5} M. The reaction was carried out at room temperature in a total volume of 200 μl. Prior to use in the assay ^{3}H-labeled dUMP (10^{-4} M) was diluted with 20 volumes of distilled water and lyophilized and resuspended in 20 volumes of unlabeled dUMP (5×10^{-4} M). The reaction was stopped by removing 20-μl aliquots to a 200-μl suspension of Norite (50 mg/ml) in 0.4 M perchloric acid. The samples were centrifuged at room temperature, and

100 μl of the supernatant was dissolved in Aquasol and counted in a liquid scintillation counter. The supernatant fluid contains the ^3H released from dUMP when the methyl group is added.

The tetrahydrofolic acid and unlabeled dUMP were obtained from Sigma, and both the labeled dUMP and Aquasol were from Amersham/Searle. The FdUMP was a gift from S. S. Cohen, and the showdomycin, (3-β-D-ribofuranosylmaleimide) was purchased from Calbiochem.

Isolation of wh^{ts} mutants. T4D at 1.1×10^{12}/ml was mutagenized with 0.4 M NH_2OH in the presence of 0.1 M sodium phosphate buffer, pH 6.0, and 0.001 M EDTA, pH 6.0 (5). The reaction was stopped after 36 h by a 1:100 dilution into cold broth containing 1.0 M NaCl and 0.001 M EDTA. A total of 8,800 plaques were examined for the formation of "white halos" (*wh*) on the plating bacteria OK305 when incubated at 42 C. The *wh* characteristic reflects a mutation in either the *td* or *dfr* gene. Two hundred and fifty-seven *wh* plaques were selected and replated for the *wh* characteristic at 30 C. Forty-one stocks were obtained which were *wh*$^-$ at 42 C and *wh*$^+$ at 30 C. Of these 41, two stocks formed heat-sensitive virus particles. These two stocks were backcrossed against excess wild-type parental T4D (10 parental T4D/1 *wh*) a total of four times. This resulted in the preparation of two stocks, mutant 408 and mutant 604, each of which possessed only the *wh*^{ts} mutation. The characterization of these two mutants as T4D *td*^{ts} is given later.

A globulin fraction from antiserum against homogeneous T2L *td* was generously provided by Frank Maley (6). This serum is known to cross-react with both the T4 and T6 *td*'s. The serum preparation contained 30 mg of globulin per ml.

RESULTS

Identification of T4D wh^{ts} mutants. The two temperature-sensitive mutants, 408 and 604, which gave "white halo" plaques at 42 C and normal plaques at 30 C, were characterized after they were genetically purified by backcrossing four times against parental T4D. Since plaques with a white halo can be produced by either a mutation in the *dfr* or the *td* gene (8), both plating tests and the heat sensitivity of the enzyme activities were compared to parental T4D and to nonsense (or missense) mutations in both genes. When plated on *E. coli* B3, a *thy*$^-$ host, in the presence of 4 μg of thymidine per ml which is limiting, both 408 and 604 formed no plaques and behaved like the known *td* mutant at the nonpermissive temperature, but both formed normal plaques like wild type (*td*$^+$ or *dfr* mutant) at the permissive temperature (Table 1). When plated on *E. coli* B in the presence of trimethoprim (0.55 μg/ml), an inhibitor of *E. coli dfr* activity, these two mutants formed plaques like wild type and thus were different from the known *dfr* mutant. These plating properties strongly suggested that both mutant 408 and mutant 604 were *td*^{ts} mutants.

TABLE 1. *Plating properties of T4Dwh^{ts} mutants*[a]

Phage type	Plaque size (mm) on *E. coli* B-3 (broth and thymidine)		Plaque size (mm) on *E. coli* B (broth and trimethoprim)	
	30 C	43 C	32 C	42 C
T4D *dfr*$^+$*td*$^+$	1.2–2.0	0.3	1.0	2.5
T4D *dfr*$^-$*td*+	1.2–2.5	0.2	0.1	0.1
T4D *dfr*$^+$*td*$^-$	0	0	1.0	1.5
T4D *wh*^{ts}-408	1.0–2.0	0	1.0	1.5
T4D *wh*^{ts}-604	1.8–2.2	0	1.0	1.5

[a] *E. coli* B-3 was grown with 4 μg of thymidine per ml in Burroughs-Wellcome broth. *E. coli* B was grown in tryptone broth (Difco), and the plates contained 0.55 μg of trimethoprim per ml. T4D *dfr*$^-$*td*$^+$ was the *wh*1 mutant of Hall (7), and T4D *dfr*$^+$*td*$^-$ was the *td*6 mutant of Hall (7).

Extracts were prepared in the usual manner to measure the heat sensitivity of the induced *td* and *dfr* activities. The heat sensitivity of the *td* activities induced in *E. coli* B3 by wild-type T4D and the two mutants is shown in Fig. 1. Both mutants induced a *td* activity which was considerably more heat labile than that induced by the parental T4D. On the other hand, the heat sensitivities of *dfr* activities induced by 408 and 604 were identical to that induced by the parental phage (Fig. 2). These enzymological properties confirm the conclusion from the plating properties that 408 and 604 are both *td*^{ts} mutants.

Properties of T4D td^{ts} mutant particles. The heat lability of preparations of *td*^{ts} mutants 408 and 604 as compared to parental T4D are shown in Fig. 3 and 4. Both stocks were grown at low and high temperatures and then heated at 60 C in the standard way. Eight separate preparations of mutant 408 and four separate preparations of mutant 604 were grown. All phage stocks prepared at either temperature were always more heat labile then parental T4D. Further, when assembled at the high temperature the phage particles have significantly different heat labilities than when assembled at a lower temperature. These results, which are both qualitatively and quantitatively quite similar to those with *dfs*^{ts} *far* mutants, indicate that the temperature-sensitive *td* gene product must be a viral structural component. In view of the fact that the tail plate is the most heat-labile phage structural component (16), these observations suggest that the *td* protein is a tail plate constituent.

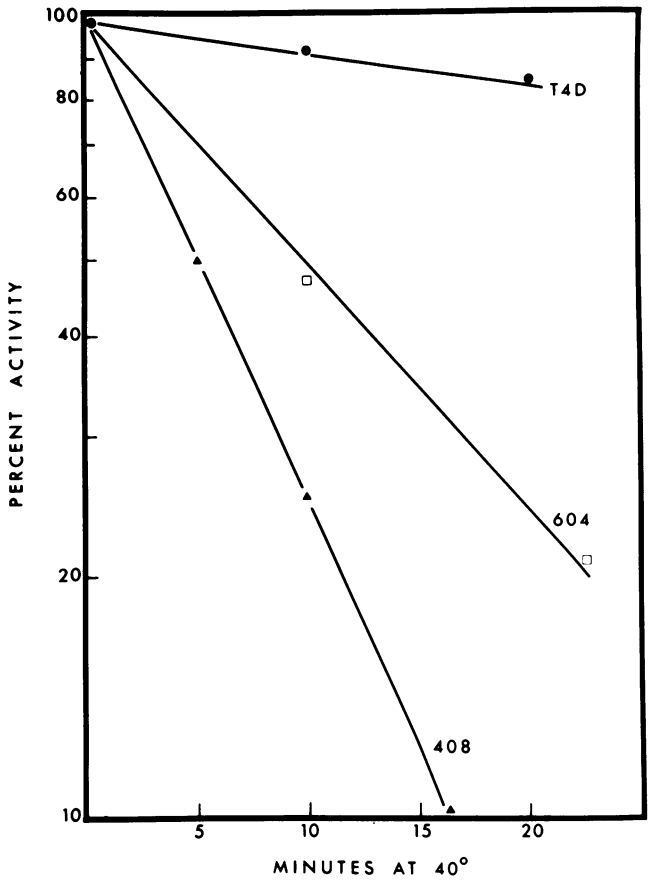

FIG. 1. *Heat inactivation of td induced by T4D mutants 408 and 604 and wild-type T4D. The extracts were prepared after infection at 30 C as described earlier for the measurement of dfr activity (12).*

The different heat labilities of 408 (and 604) when assembled at the high temperature as compared to phage formed at a lower temperature suggest that *td* can be used as a structural component with some variations in its tertiary or quaternary structure. Presumably at one temperature a segment of the *td* polypeptide assumes a heat-labile configuration, and later, upon heating at 60 C, it is denatured more rapidly than when synthesized and used for phage assembly at the different temperature.

Preparation and properties of a T4d dfr^{ts}td^{ts} mutant. A phage double mutant was obtained by infecting *E. coli* B simultaneously with the T4D *td*^{ts} 408 mutant as the majority parent (multiplicity of infection of 10) and with the T4D *far* mutant, P1, which is *dfr*^{ts} (9), as the minority parent (multiplicity of infection of 1). The progeny of this cross were plated on *E. coli* B on plates containing pyrimethamine, and plaques were picked at random. The picked plaques were then tested for their plating properties, and one recombinant was found which had the properties shown in Table 2. This phage stock was both resistant to pyrimethamine and did not give plaques at the nonpermissive temperature on *E. coli* B3. These plating tests and the enzymological properties described in Table 3 indicate that this stock is T4D *td*^{td}*dfr*^{ts}.

The heat sensitivity of this double mutant was compared to the majority parent 408. Figure 5 shows typical results obtained in several experiments. When assembled at 42 C the double mutant, as expected, was more heat labile than 408 asembled at 42 C, but when assembled at 30 C the double mutant was less heat labile than the 408 mutant assembled at this temperature. These properties indicate that heat labilities of these two viral components are not necessarily additive. The heat labilities of these two components appear to be dependent upon the quaternary or tertiary structure of each other.

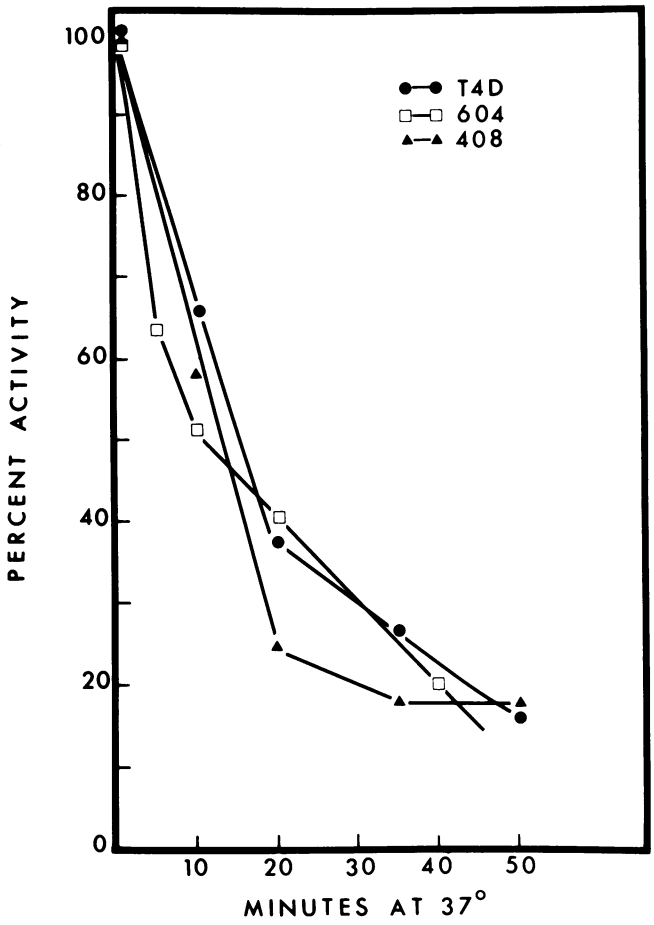

FIG. 2. *Heat inactivation of dfr induced by T4D mutants 408 and 604 and by wild-type T4D. The extracts were prepared after infection at 30 C as described earlier (12).*

The properties of this double mutant, which is $td^{ts}dfr^{ts}$, suggest that these two components interact physically and are probably adjacent to each other in the baseplate.

Effect of antiserum to TD on tail plate assembly. Capco and Mathews reported that high concentrations of antiserum to purified but not homogeneous T4D *td* inactivated T4D (2). Recently Galivan et al. (6) prepared antiserum to homogeneous *td* isolated from bacteria infected with T2L. This antiserum was reported to cross-react with *td* induced by T4 or T6 phages. It was found that the antiserum to the T2-induced *td* did not inactivate either T2, T4, or T6 phage particles even at a dilution of 1:3 when incubated for as long as 18 h.

Using procedures identical to those in the accompanying papers (12, 14), this serum was incubated with either incomplete T4D particles lacking both the 11 and 12 proteins, T4D 11^-, or with T4D particles lacking just 12P, T4D 12^-. Later, the extract containing 11P and 12P was added to treated particles. It can be seen in the typical experiment shown in Fig. 6a and b that antiserum to T2 *td* inhibits the step in morphogenesis involving addition of the 11 protein to the baseplate, but that this antiserum had no effect on the addition of the 12 protein to 12^- particles. These observations confirm that *td* is a T4D baseplate component lying largely under the 11 protein in close proximity to the baseplate *dfr* (12) and folate (14).

Effect of cofactors or inhibitors of *td* activity on addition of 11 protein to 11^- phage particles. Since cofactors or inhibitors of *dfr* react with both whole phage particles and even more readily with incomplete particles lacking the 11 (and 12) protein (12, 20), the effect of analogous compounds known to bind to *td* was examined. Intact T4D, T2L, and T2H were incu-

bated with either dUMP, FdUMP, showdomycin, or tetrahydrofolate plus formaldehyde. dUMP and the mixture of tetrahydrofolate plus formaldehyde are normal substrates for *td*, whereas FdUMP (18, 21) and showdomycin (10) are known to react stoichiometrically and possibly covalently with the enzyme and to cause irreversible inhibition. None of the four compounds in any combination affected phage viability or the rate of morphogenesis involving 11 (and 12) protein addition to 11^- (and 12^-) particles. It seems likely that the folate-binding site on the tail plate *td* is occupied by the phage dihydropteroyl hexaglutamate. Further, it appears that the binding site for dUMP (and showdomycin) is buried within the complex tail plate structure and is not free to react with these low-molecular-weight compounds.

Properties of T4D having an amber mutation in its *td* gene. Two T4D mutants, N43 and N54, derived from T4D C1, having an amber mutation in their *td* gene were originally isolated by D. H. Hall and partially characterized by Krauss et al. (17). In view of the evidence by Capco and Mathews (2) that *td* is a phage component and that td^{ts} mutants form heat-labile particles and other evidence presented earlier in this report, the properties of particles produced by these two *td* amber mutants were examined. Phage stocks were grown at 28 or 42 C on both the nonpermissive bacterial host (*E. coli* B) and the permissive bacterial host (CR63).

The heat sensitivities of these four phage preparations are shown in Fig. 7. It is apparent that the heat sensitivity of the phage particles is dependent on the *td* mutation, since the sensitivity reflects both the temperature of assembly and the completion or noncompletion of the *td* polypeptide. When grown at 28 C all the stocks were considerably more heat sensitive than when grown at 42 C. Further, N54 when grown at 42 C on *E. coli* B is much more sensitive than when it was grown at 42 C on CR63

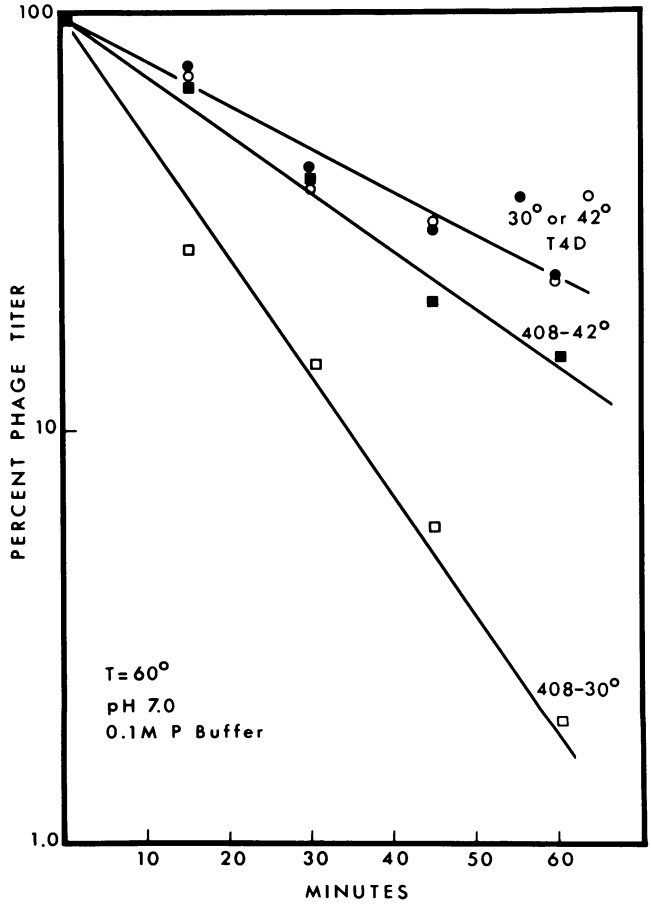

FIG. 3. *Heat inactivation at 60 C of T4D and T4D mutant 408 assembled either at 30 or 42 C.*

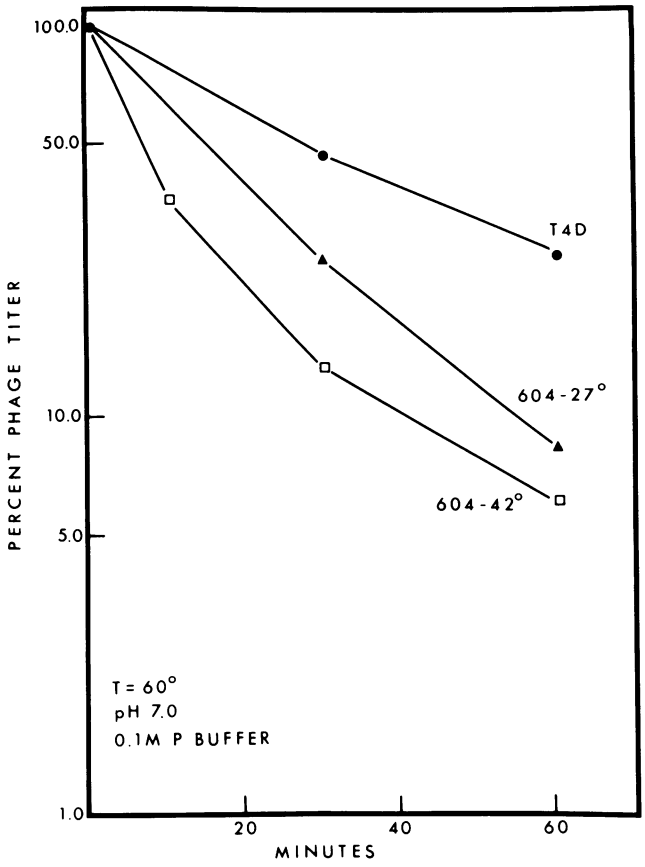

FIG. 4. *Heat inactivation at 60 C of T4D and T4D mutant 604 assembled either at 27 or 42 C.*

TABLE 2. *Plating properties of various T4D mutants*

T4D phage type[a]	Plaque size (mm) on *E. coli* B-3		Pyrimethamine sensitivity
	30 C	43 C	
td^+far^+	1.6	0.5	Sensitive
408-(td^{ts})	1.5	0	Sensitive
P1-far^-(dfr^{ts})	1.4	0.4	Resistant
408 × P1-$dfr^{ts}td^{ts}$	1.4	0	Resistant

[a] far = folate analogue resistant = *dfr* resistant. The plating conditions are those described for Table 1.

TABLE 3. *Properties of enzymes induced by infection with various T4D mutants*[a]

Phage	*td* activity after 10 min at 40 C (%)	*dfr* activity in presence of 4 × 10^{-6} pyrimethamine (%)
T4D	62	7
T4D td^{ts} (408)	18	6
T4D dfr^{ts} (P1)	82	47
Recombinant T4D $td^{ts}dfr^{ts}$	30	64

[a] The activities were measured as described in Materials and Methods.

and was more sensitive than N43 grown at 42 C on either host.

A similar small, but reproducible, influence of the host cell and temperature of assembly in phage particles produced is seen in Fig. 8, describing the interaction of these four T4D *td* amber mutant stocks with anti-*dfr* serum. The ability of the serum globulin to react with the phage tail plate *dfr* was influenced by the nature of the *td* formed. For three of the stocks, N43 grown on CR63 and N54 grown on either host, the phage assembled at 42 C has its *dfr* more exposed to the antiserum than phage produced at 28 C.

It should be emphasized again that parental T4D particles have identical properties with regard to both heat sensitivity and inactivation rates with anti-*dfr* serum irrespective of their host or temperature of assembly. It can be concluded that the *td* gene product is a constituent of the virion, in agreement with the data on td^{ts} mutants, and is most likely a tail plate component adjacent to the *dfr*. These properties support the view that the partial peptide products of the *td* gene in N43- and N54-infected cells are large enough to serve this structural role.

DISCUSSION

A proposal for the structural relationship between baseplate folate (14), *dfr* (12), and *td* is shown in Fig. 9. All three components are shown partially or completely buried within the tail plate. They are tentatively placed close to the center of the tail plate near the center "plug" and the tail core. One reason for this location is that the structure consisting of the tail core plus plate contains folic acid but free tail plates do not contain the folate. During morphogenesis the addition of the tail core to the plate must stabilize the structure and help bury the folate within the tail plate.

This complex structure illustrates the extremely tight binding of the folate to the phage tail protein, as shown by the fact that folate cannot be removed from whole phage particles or even 11^- particles by stirring with a suspension of activated charcoal for 18 h. The ability of two pteridine-binding proteins (the antifolate serum and milk protein [14]) to greatly inhibit the rate of completion of morphogenesis of 11^- particles, but to only decrease somewhat the amount of final phage formed, suggests that the pteridine is buried and that, whereas the pteridine binds reversibly with these two proteins, the addition of the 11 protein to this

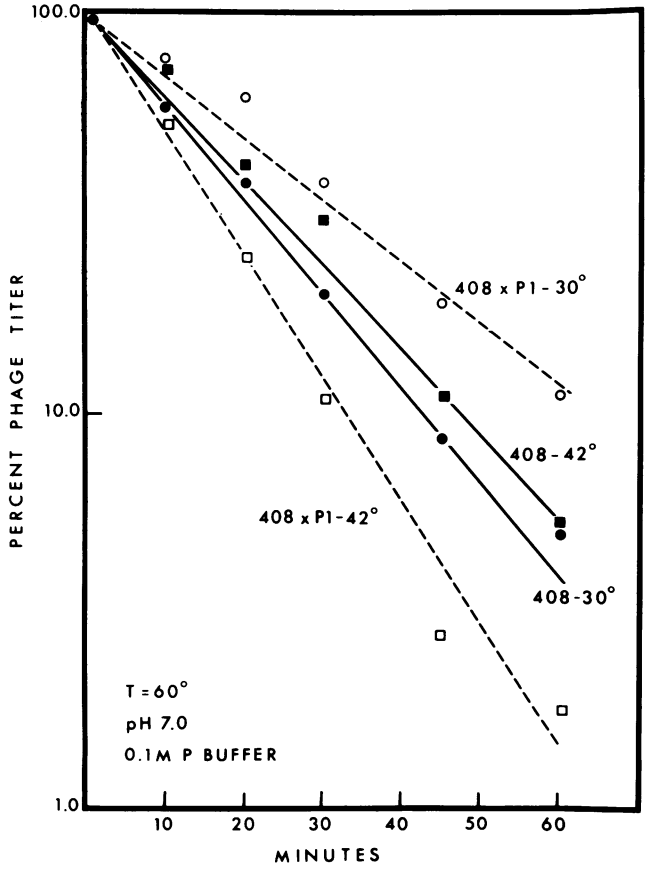

FIG. 5. *Heat sensitivity of recombinant T4D phage stock prepared from a cross of 408 × P1 compared to parental T4D 408 mutant. P1 is* dfr^{ts} *and 408 is* td^{ts}*, so the recombinant is T4D* dfr^{ts} td^{ts} *(see Tables 2 and 3). The procedure for preparing this stock is given in the text.*

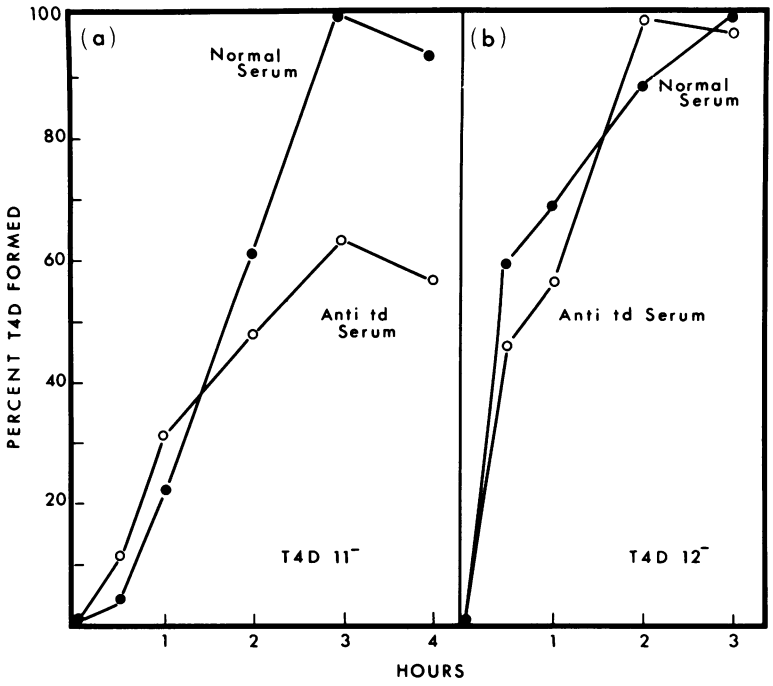

FIG. 6. *(a) Effect of antiserum to td on the addition of P11 (and P12) to T4D 11⁻ particles. The antiserum preparation was incubated with T4D 11⁻ particles overnight in the cold at a final globulin concentration of 12 mg/ml. The extract containing the P11 (and P12) was added later, as described earlier (14). The control titer was 4 × 10⁶, and after 3 h at 30 C the titer was 4.4 × 10⁹ (= 100%). (b) Effect of antiserum to td on the addition of P12 to T4D 12⁻ particles. The antiserum was incubated as in (a), and the reaction was started by addition of the same extract as in (a). The initial titer was 1.2 × 10⁸, and after 3 h the T4D titer was 9.8 × 10⁹ (= 100%).*

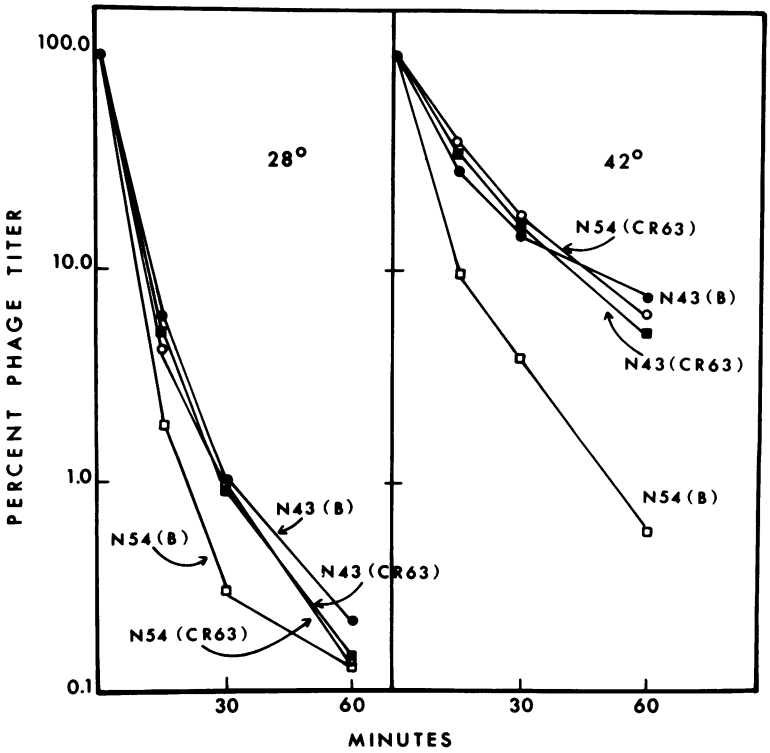

FIG. 7. *Heat sensitivities of two T4D td amber mutants, N43 and N54. The stocks were assembled at 28 or 42 C while growing in either the permissive host (CR63) or the nonpermissive host (B). Phage were diluted to an initial titer of 10⁵ in 0.1 M potassium phosphate buffer, pH 7.0, and heated in a water bath at 60 C.*

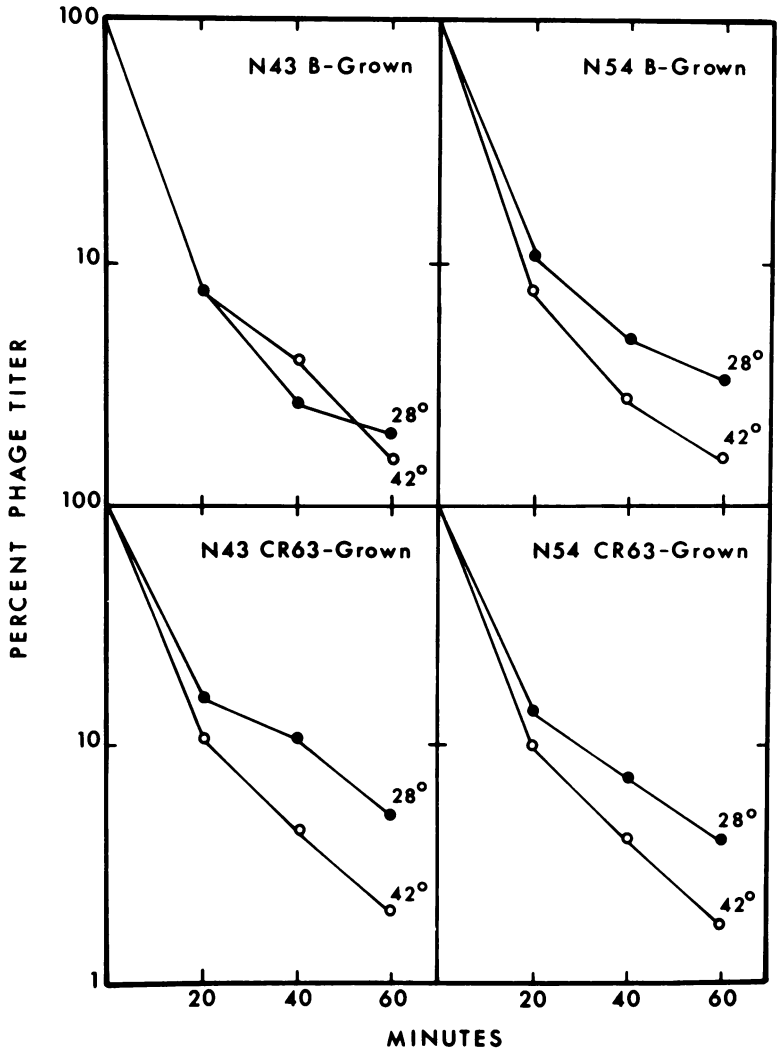

FIG. 8. *The inactivation of two T4D td amber mutants, N43 and N54, by antiserum to dfr. The stocks were assembled at 42 or 28 C while growing in either the permissive (CR63) or the nonpermissive host (B). Phage stocks and antiserum were diluted in tryptone broth adjusted to pH 5.0. The reaction mixture was incubated at 37 C. The initial phage concentration was 5 × 10⁶/ml, and the serum was used at a final dilution of 1:51.*

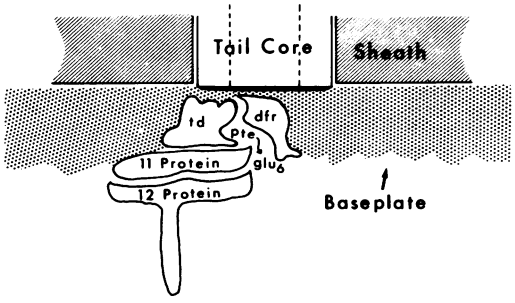

FIG. 9. *Suggested location for T4D baseplate dihydropteroyl hexaglutamate (pte-glu₆), dfr, and td.*

structure is irreversible. The tip of the folate polyglutamate portion is shown extending somewhat from the bottom of the baseplate, since it is known that hog kidney conjugate can react with this portion of the folate molecule (13). The claim by Dawes and Goldberg (3) that, for some phage strains, the inactivation by conjugase is due to conjugase binding to the polyglutamate rather than to enzymatic cleavage of the terminal glutamate residues suggests either that only a small portion extends from the tail plate or that it lies in some protected fold or crevice of the proteins on the bottom of the baseplate.

The *dfr* is shown in Fig. 9 as interacting with the pteridine polyglutamate and the *td* and to be only partially covered by the 11 protein. It is known that the *dfr* must be buried in the tail, since it required urea treatment of plates to demonstrate *dfr* activity (16). However, anti-*dfr* serum (22) does react with, and kill, whole phage particles. This serum killed 11^- particles at least fourfold more rapidly so the *dfr* can only be partially covered by the 11 protein. A portion of the *dfr* is shown exposed at the bottom of the tail, not only to react with antiserum but also to indicate a site for the interaction with reduced nicotinamide adenine dinucleotide (NADPH). NADPH does bind to and inactivate phage and, as reported earlier (16), over 10^{10} phage can be inactivated by NADPH treatment with first-order kinetics. Further, no NADPH-resistant particles have been detected. It seems that this feature of the *dfr* is essential for phage viability. It should be emphasized again that, in in vitro complementation experiments, the interaction of various nucleotides with this NADPH site before the phage is completely formed also affects the rate of morphogenesis. For the formation of the phage structure then, the *dfr* polypeptide must contain a NADPH binding site, a dihydrofolate binding site, and a *td* interacting site. The rest of the *dfr* molecule so far appears to be unnecessary, and these experiments indicate that a partial polypeptide can serve as a structural element.

The occlusion of the *td* molecule within the tail plate is suggested by the data that the presence of the 11 protein prevents inactivation by the anti-T2 *td* serum of Galivan et al. (6). However, Capco and Mathews (2) did get inactivation of T4 by high concentrations of antiserum to T4 *td*. We have been unable to demonstrate any *td* activity in phage particles, even using gentle methods to disrupt incomplete 11^- particles. This is perhaps not surprising in view of the great lability of *td* reported by Krauss et al. (17) and Capco et al. (1). Finally, with regard to the location of the *td*, the *td* binding sites for dUMP (or FdUMP or showdomycin) appear to be buried within the tail structure, possibly near the tail core since 11^- particles do not react with these reagents. The physical properties of the two T4D amber *td* mutants again indicate that, like *dfr*, partial peptides of *td* can still play a structural role. This view is supported by the recent observation by Mosher and Mathews (personal communication) that their antiserum to T4D *td* does inactivate both N43 and N54 *td* amber stocks at rates slightly higher than for T4D.

The interaction of *td* and *dfr* shown in Fig. 9 rests (i) on their common location under the 11 protein in the baseplate and (ii) on the properties of the T4D $td^{ts}dfr^{ts}$ double mutant, which suggest a physical interaction of the two proteins. In addition, both enzymes have a site which binds dihydrofolic acid. Whereas a physical interaction between these two enzymes has long been postulated, only recently has enzymological evidence been presented, by Kawai and Hillcoat (11), indicating that *dfr* and *td* from L1210 cells do form physical complexes in solution. It is of some interest that Tomich et al. (23) have recently reported that in T4-infected cells *td* forms a complex with other early enzymes and has less enzymatic activity than expected.

The observations in these three papers, especially the properties of the td^{ts} and dfr^{ts} mutants, reflect the inherent unstable nature of the tail plate. This phage structure must be flexible enough to undergo morphological changes during infection, and this would be in accord with its heat lability.

A difficult final problem is still the determination of the number of folates and *dfr* and *td* molecules in each baseplate. Perhaps the best estimate is still two or possibly six of each per tail plate. This low concentration, especially of early proteins, is perhaps the reason the late labeling of T4 capsid components, followed by disruption and polyacrylamide gel analysis of either phage or of infected cells, has not revealed these two components (4, 24). However, the failure of the current analytical procedures to detect these components does not argue against the conclusion that these phage-induced components, *dfr*, *td*, and folate, not only aid DNA synthesis but also play a critical structural role in forming the tail plate.

ACKNOWLEDGMENTS

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