MORPHOGENESIS OF BACTERIOPHAGE T4 IN EXTRACTS OF MUTANT-INFECTED CELLS*

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The complex structure of bacteriophage T4 includes a variety of proteins[1] which become assembled into mature particles during intracellular development of the virus. Some insight into the genetic control of this process has been provided by physiological studies with conditional lethal mutants, which show that over 40 phage genes are involved in T4 morphogenesis[2] (Fig. 1). However, the mechanisms by which components are assembled have remained obscure, due in part to the lack of a suitable system for their study. In the experiments reported below, conditional lethal mutants of strain T4D have been exploited to develop an *in vitro* system in which several of the steps in phage morphogenesis can be demonstrated.

Methods and Materials.—Incubations and growth of liquid cultures were carried out at 30°C unless otherwise indicated. Previously described procedures were employed for preparation and assay of phage stocks, and for complementation tests between amber (*am*) mutants.[2]

Phage strains: All strains are derivatives of T4D unless otherwise indicated. Wild type, the *r*I mutant *r*48, and most of the *am* mutants employed have been described previously.[2] *Am* mutants of phage T2L were isolated and characterized by R. L. Russell. Mutations carried by the T2 *am* strains were assigned to homologous T4 genes on the basis of T2 *am* × T4 *am* complementation tests. Additional information on the phenotypic defects of the various mutants is given in Figure 1 and the tables.

Escherichia coli host strains were employed as follows: CR 63 (permissive for *am* mutants) for all phage assays and preparation of stocks; B/5 (nonpermissive) for preparation of infected cell extracts; S/6 (nonpermissive) as a selective plating indicator for am^+ phage; B/2, S/4 (resistant to adsorption of T2 and T4, respectively) for adsorption experiments (see Table 1).

Media and reagents: H broth, used for growth of bacteria, and EHA top and bottom agar, used for plating assays, were prepared as described previously.[3] Buffer contained Na_2HPO_4 (0.039 *M*), KH_2PO_4 (0.022 *M*), NaCl (0.07 *M*), and $MgSO_4$ (0.01 *M*) at pH 7.4. Crystalline bovine pancreatic DNase was obtained from Sigma Chemical Co.

Tail-fiberless particles were prepared using a multiple *am* mutant (X4E) defective in the tail-fiber genes 34, 35, 37, and 38 (*am* mutations: B25, A455, B252, N52, B280, and B262). A culture of *coli* B/5 was grown to 4×10^8 cells/ml, infected with X4E phage at a multiplicity (m.o.i.) of 4, aerated for 3 hr, and then treated with $CHCl_3$ to lyse the infected cells. The defective particles were purified by two cycles of low- and high-speed centrifugation and suspended in buffer. The particle concentration was estimated from the optical density of the suspension at 265 mμ, assuming $OD_{265} = 1.0$ for a suspension of 1.2×10^{11} particles/ml.[4] Fewer than 0.01% of the particles in such preparations formed plaques when plated on CR 63 indicator bacteria.

Infected-cell extracts: A culture of B/5 was grown in H broth at 37°C to 4×10^8 cells/ml, cooled to 30°C, infected (0 min) at m.o.i ~4 with phage of the desired genotype, and aerated vigorously at 30°C. Assays of surviving bacteria were generally made to verify that most of the cells were infected. At 30 min the culture was rapidly chilled by pouring into large iced Erlenmeyer flasks. (Following infection with wild-type T4D at 30°C, intracellular phage first appear at 23 min and spontaneous cell lysis does not begin until about 40 min.) The chilled culture was concentrated about 200 times by centrifugation at 5000 × *g* for 8 min and resuspension of the viscous pellet in buffer containing DNase (10 μg/ml). Microscope counts indicated 10–20% recovery of intact cells. (The low recovery is probably due to the fragility of the cells at this stage of the latent period.) The resuspended pellet was frozen at −70°C in a dry ice–ethanol

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bath, thawed at 30°C, and either used immediately or refrozen at −70°C and stored at −20°C. (Extracts retain considerable activity upon storage of up to 1 month.) As determined by microscope count, this procedure disrupted 99% of the cells. Protein content of the extracts, estimated colorimetrically,[5] varied between 20 and 30 mg/ml. Infectious phage were present at levels of 10^8 to 3×10^{10} per ml, due to unadsorbed phage and the low but finite transmission of *am* mutants.

Extracts are referred to in the text by the number of the defective gene of the *am* mutant used to infect the cells.

Results.—Activation of tail-fiberless particles: Since gene 23 controls the synthesis of the major structural component of the head membrane,[6] a 23-extract

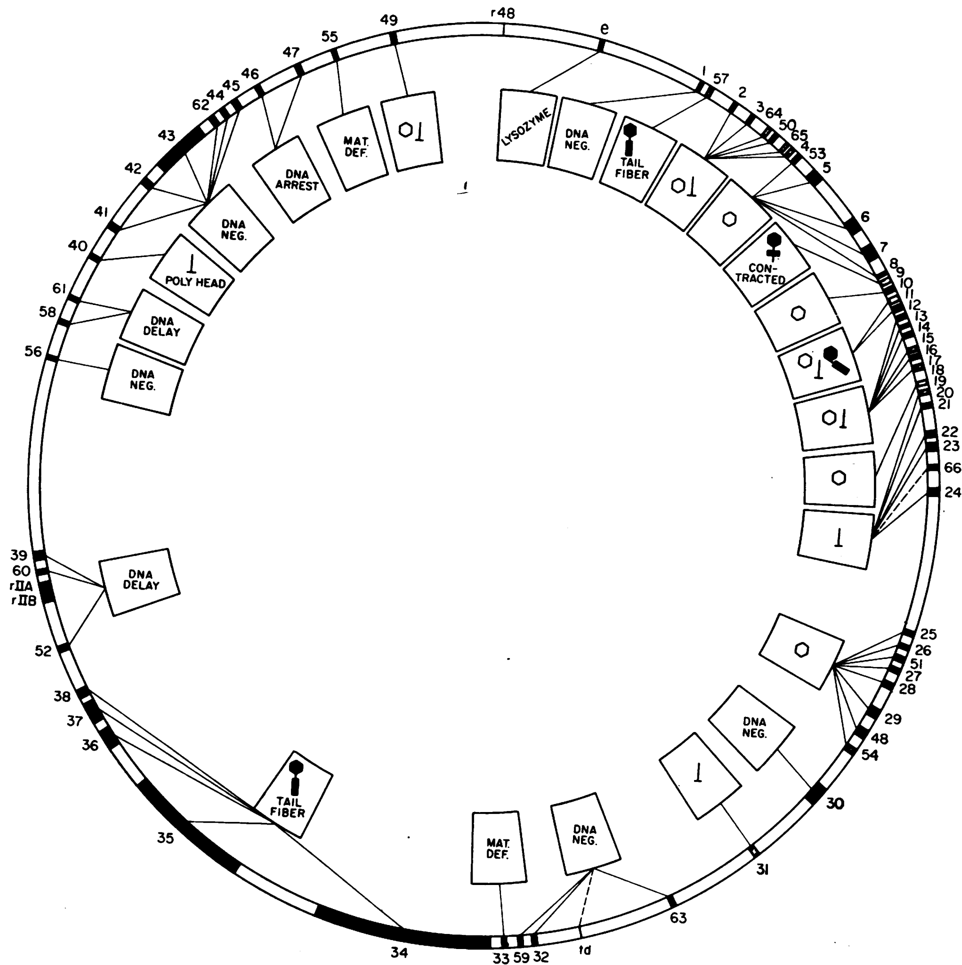

FIG. 1.—Defective phenotypes of conditional lethal mutants of T4D under restrictive conditions. Characterized genes are represented by shaded areas illustrating relative locations and, if known, approximate map lengths.

The enclosed symbols indicate defective phenotypes as follows: *DNA NEG.*, no DNA synthesis; *DNA ARREST*, DNA synthesis arrested after a short time; *DNA DELAY*, DNA synthesis commences after some delay; *MAT DEF.*, maturation defective, DNA synthesis is normal but late functions are not expressed; a hexagon indicates that free heads are produced, an inverted T, that free tails are produced; *TAIL FIBER*, fiberless particles produced; gene 9 mutants produce inactive particles with contracted sheaths; gene 11 and 12 mutants produce fragile particles which dissociate to free heads and free tails. Based on previously published[2] and unpublished experiments.[3]

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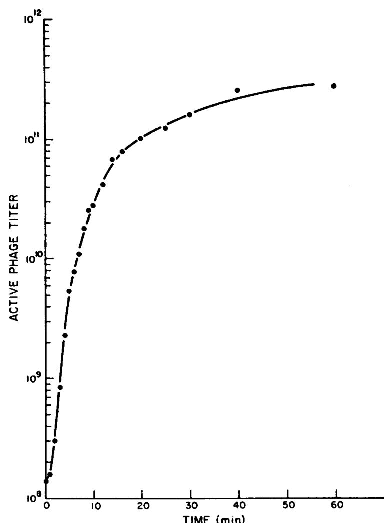

FIG. 2.—Kinetics of activation of fiberless particles in the presence of 23-extract. Buffer containing 8 × 10^{11} purified fiberless particles/ml was mixed with an equal volume of 23-extract and incubated at 30°C. At the times indicated, samples were removed for plaque assay on CR 63 indicator bacteria. Titers shown represent active phage/ml of reaction mixture.

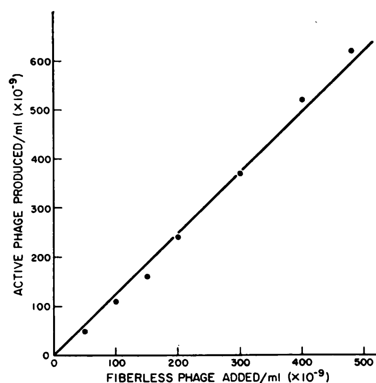

FIG. 3.—Proportionality between fiberless particles added and final yield of active phage in the presence of 23-extract. Buffer, fiberless particles, and 50 μl of 23-extract were mixed as indicated in total volumes of 100 μl. After 200 min of incubation, aliquots of each reaction mixture were assayed for plaque-forming phage. The 20% excess of active phage recovered over fiberless particles added probably reflects the uncertainty in determining titer of the particle suspension by optical density (see *Methods and Materials*).

(prepared from nonpermissive cells infected with *am* B17, a T4D mutant defective in gene 23) should contain all of the components required for tail-fiber assembly but no phage heads or complete particles. When purified tail-fiberless particles (see *Methods and Materials*) are incubated with 23-extract, the titer of active phage in the mixture rapidly increases over 3 orders of magnitude to a level approaching that of the fiberless particles initially added (Fig. 2). The kinetics of active phage production are not linear. No increase in active titer is observed upon incubation of either the extract or the fiberless particles alone.

As shown in Figure 3, the number of active phage produced is proportional to the number of fiberless particles added to the mixture. Within the error of measure-

TABLE 1

ADSORPTION AND SEROLOGICAL SPECIFICITIES OF ACTIVATED PHAGE

Phages	Fraction Unadsorbed to: B/5	B/2	S/4	Fraction surviving anti-T4 serum
T4*r*48	0.002	0.01	0.8	0.01
T2L	0.001	0.9	0.00	0.43
X4E-4	0.004	0.003	1.00	0.01
X4E-4P	0.06	0.1	0.7	0.01
X4E-2	0.001	0.9	0.2	0.13
X4E-2P	0.1	0.2	1.2	0.01

Phages: T4*r*48: T4 control; T2L: T2 control; X4E-4: fiberless T4 particles activated by 23-extract; X4E-4P: progeny of X4E-4; X4E-2: fiberless T4 particles activated by T2 *am* 108 (gene 20) extract; X4E-2P: progeny of X4E-2.

Adsorption experiments: Phage were mixed with suspensions of the indicated bacteria (4 × 10^8/ml) in H broth containing 0.004 *M* KCN. After 15 min, incubation samples were shaken with $CHCl_3$-saturated broth and assayed to determine the fraction of input phage remaining unadsorbed.

Inactivation by antiserum: Hyperimmune anti-T4 rabbit serum was used at a dilution giving 1% survival of T4D phage in H broth after 5 min at 30°C.

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ment, activation is quantitative when the incubation period is extended to 200 min, and no further increase in active titer is observed beyond this point. Lowering the concentration of 23-extract by a factor of 2 decreases the rate of the reaction, but not the final yield of active phage. No reaction is observed at 0°C.

The following experiments provide evidence that activation reflects the attachment of tail-fiber components present in the extract to the fiberless phage particles.

(1) Complementation spot tests were used to determine the genotype of 40 of the phage particles activated in two separate experiments. All 40 were of genotype X4E, that of the fiberless particles added.

(2) An extract of cells infected with a T2L mutant (*am* 108) defective in gene 20 (required for head membrane formation) was found to activate fiberless T4D particles as described above. The progeny of particles activated in the presence of this extract and of particles activated with a T4D 23-extract were obtained by growth for one cycle on strain CR 63. Samples of the T2 extract-activated and T4D extract-activated phage and their progenies were then compared with T2L wild type and T4D *r*48 for adsorption to B, B/2, and S/4 bacteria, and for neutralization by anti-T4 serum (Table 1). Phage activated with the T2 extract showed the adsorption characteristics, and, to some extent at least, the serological properties of T2L. Their progeny, however, behaved like the T4D extract-activated phage and the T4D control samples. These results support the view that activated phage are T4D particles with either T2 or T4D tail fibers, depending upon the extract used in the activation reaction.

(3) As a more direct test for attachment of fibers, samples of phage from the reaction mixture of Figure 2 were removed after 0, 12, and 200 min of incubation and photographed in the electron microscope by Dr. M. Moody, after purification by three cycles of high- and low-speed centrifugation and negative staining with uranyl acetate. The fractions of active particles in the three samples were, respectively, 0.1, 10, and 100 per cent. Counts of the number of fibers per particle in the three samples (Fig. 4) indicate that the phage acquire tail fibers during incubation with the extract. The results suggest that more than one fiber is necessary for activity, since over 70 per cent of the particles in the 12-min sample show one or more fibers, whereas only 10 per cent are able to form plaques on CR 63.

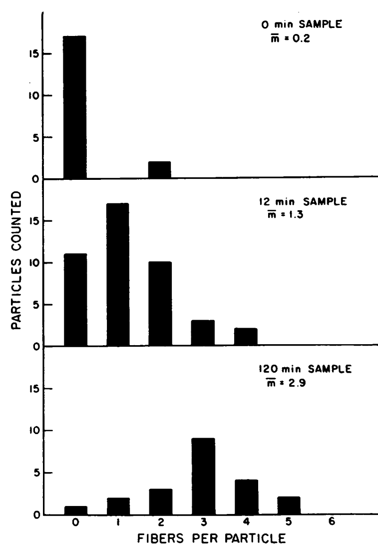

Fig. 4.—Tail-fiber counts from electron micrographs of extract-activated phage (see text). Counts were made on two or more fields from each sample, scoring only particles with clearly visible end plates on an unobstructed background. For each time point the average number of visible fibers per phage ($\bar{m}$) is given. It should be noted that six fibers per phage are seldom visible even in preparations of wild-type T4D.

Extract complementation among tail-fiber mutants: Mutants defective in one or more of the genes 34, 35, 36, 37, 38, and 57 fail to produce active progeny under restrictive conditions *in vivo*. They do, however, produce a normal yield of noninfectious phage particles

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which lack tail fibers.[2] Antigenic studies with *am* mutants indicate that genes 34, 36, and the gene pair 37–38 control the synthesis of three antigens, designated A, B, and C, respectively, which are found in wild-type T4 lysates associated with the tail fibers of active phage.[7] Under restrictive conditions, gene 57 mutants produce low levels, and gene 35 mutants give normal levels of all three antigens. However, the inactive particles produced by all of the above tail-fiber mutants are devoid of associated antigens (J. King, unpublished).

The preceding experiments indicate that fiber attachment to fiberless particles can proceed *in vitro*. By mixing extracts made with mutants defective in different tail-fiber genes, some of the earlier steps in fiber assembly can be shown to occur as well. We shall refer to the production of active phage under these conditions as "extract complementation."

No increase in active phage titer is observed upon incubation of 34, 35, 36, 37, or 38 extracts alone or with added fiberless particles. However, when pairwise combinations of extracts are allowed to react, the fiberless particles present in the extracts become activated in some, but not all, of the mixtures (Table 2). Of particular interest are combinations such as 34 + 36 and 34 + 37. Since each of the extracts lacks one of the three antigens, the observed activity suggests that at least two steps occur in the mixture: association of the antigens and their attachment to the phage. The partial or complete inactivity of all pairwise combinations among 36, 37, and 38 indicates that not all of the reactions in fiber assembly proceed efficiently in the *in vitro* system as presently constituted. (The results of tests with

TABLE 2

EXTRACT COMPLEMENTATION AMONG PAIRS OF TAIL FIBER MUTANTS

Tail-fiber antigens present:	A, B, C	B, C	A, B, C	A, C	A	A
Defective gene no.	23*	34	35	36	37	38
38	1000	370	187	9.4	2.6	2.6
37	269	38	65	2.6	2.2	
36	268	238	70	0.4		
35	1050	55	3.3			
34	835	2.8				
23*	0.8					

Equal volumes of two extracts were mixed, incubated 90 min, and then assayed for plaque-forming phage. Results are expressed as titer (active phage/ml) $\times 10^{-9}$ in the reaction mixture. Numbers at the bottom of each column represent active titers observed after 90 min incubation of each extract alone. *Am* mutants employed for extract preparation were as follows: 38, *am* C290; 37, X2j (double mutant: *am* N52 and *am* B280); 36, *am* E1; 35, *am* B252; 34, X2d (double mutant: *am* B25 and *am* A455); 23, *am* B17. The first column heading gives the antigenic components found in defective lysates of the mutants listed in the second column heading.

* Control; defective in head formation.

TABLE 3

EXTRACT COMPLEMENTATION BETWEEN HEAD AND TAIL MUTANTS

Defective gene number	5	6	10	27	29	2	23	31
31	—	91	20	—	21	1.0	2.3	0.8
23	283	180	176	445	278	3.7	3.8	
2	189	218	137	352	132	5.3		
29	1.5	1.8	0.9	9.9	3.5			
27	1.4	1.5	0.7	0.7				
10	1.6	2.2	0.4					
6	2.6	3.4						
5	2.7							

Pairwise mixtures of extracts were allowed to react, and the results are expressed as in Table 2, except that incubation time was increased to 120 min. The *am* mutants employed are given in Table 5.

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TABLE 4

FUNCTIONAL PROPERTIES OF MORPHOGENETIC-MUTANT EXTRACTS

Extract		Incubated with			Mutant Phenotype	
Defective gene	*Am* mutant	Self	23-Extract (tail donor)	6-Extract (head donor)	Functional components	Components visible in EM
2	N51	5.3	3.7	218	T	HT
64	E1102	1.8	8.9	205	T	HT
50	A458	20	23	229	T	HT
65	E348	5.2	9.5	133	T	HT
4	N112	36	80	407	T	HT
53	H28	1.4	181	1.6	H	H
5	N135	2.7	283	2.6	H	H
6	N102	3.4	180	—	H	H
7	B16	1.2	263	1.6	H	H
8	N132	1.3	518	3.5	H	H
9	E17	7.8	76	458	HT	Cϕ
10	B255	0.38	176	2.2	H	H
11	N93	3.8	15	14	HT	ϕHT
12	N69	0.9	104	261	HT	ϕHT
13	E609	0.5	636	216	HT	HT
14	B20	1.1	337	426	HT	HT
15	N133	5.6	936	923	HT	HT
16	N66	1.7	1.8	149	T	HT
17	N56	0.5	0.5	96	T	HT
18	E18	0.3	447	775	HT	HT
20	N50	0.4	1.6	57	T	T
21	N90	20	8.9	165	T	T
22	B270	2.6	1.1	141	T	T
23	B17	0.83	—	180	T	T
24	N65	0.7	0.8	135	T	T
25	N67	29	608	16	H	H
26	N131	4.1	144	3.6	H	H
51	S29	1.6	67	1.8	H	H
27	N120	0.7	1670	1.5	H	H
28	A452	3.0	91	2.3	H	H
29	B7	3.5	278	1.8	H	H
48	N85	0.7	107	1.6	H	H
54	H21	0.9	137	6.4	H	H
31	N54	0.8	0.9	350	T	T
49	E727	1.3	1.4	125	T	HT

Extracts are listed in the map order of the corresponding defective genes (see Fig. 1). Incubations were carried out and the results are expressed as in Table 3. Interpretation of the results is given under the heading "Functional components." Extracts complementing 6-extract (reference head donor) but not 23-extract (reference tail donor) to produce active phage are designated as tail donors (T); those complementing 23 but not 6 as head donors (H), and those complementing both reference extracts as both head and tail donors (HT). The last column lists for comparison the previously determined defective phenotypes of the various mutants.[2] The symbols indicate the presence of free heads (H), free tails (T), complete particles (ϕ), and particles with contracted sheaths (Cϕ) in electron micrographs of defective lysates.

57-extracts, not shown, were ambiguous due to the high leakage of gene 57 mutants.)

Genotype tests of phage produced in the active combinations show that endogenous particles from *either* extract can be activated by tail-fiber attachment. Assays of the activated phage on the restrictive host S/6 revealed no am^+ recombinants, a further indication that the increase in active titer is due to an extracellular process. If the observed complementation were due to mixed infection by the two mutant phages of uninfected cells present in the extracts, then the resulting progeny would be expected to include 5–35 per cent am^+ recombinants, depending upon the map interval between the two defective genes employed.

Extract complementation among other morphogenetic mutants: The experiments described above indicate that the attachment of tail fibers to fiberless particles and at least some of the steps in fiber assembly can proceed in extracts with high efficiency. To test the *in vitro* efficiency of other steps in the maturation process, extracts were prepared from cells infected with a number of mutants defective in various

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morphogenetic genes. These were examined in pairs for extract complementation as above (Table 3). Platings on the restrictive host S/6 indicated that $< 10^{-3}$ am^{+} recombinants were generated in the many mixtures tested. In most cases the results of the test were unambiguous, either showing no increase over the controls (no complementation) or more than a tenfold increase (complementation).

A striking correlation is found between the extract complementation behavior of mutants and their defective phenotypes.[2] Mutants which produce heads but no tails as determined in the electron microscope (EM), and thus presumably are blocked in tail assembly, do not complement among themselves (genes 5, 6, 10, 27, 29) but do complement with mutants which produce tails, but no heads (genes 23, 31). Many other tests not shown in Tables 3 or 4 support this generalization. These results suggest that the attachment of heads to tails can proceed *in vitro*, and that mutant extracts can be classed as "head donors" or "tail donors," extracts from one class being active only with extracts from the other.

To test this generalization further, infected-cell extracts were prepared using *am* mutants defective in each of 35 genes known to be involved in morphogenetic steps other than tail-fiber assembly.[2] (Genes identified only with temperature-sensitive mutants were not tested.) Each extract was tested for activity against a reference head donor (6-extract) and a reference tail donor (23-extract). As shown in Table 4, every mutant showing the EM phenotype of heads but no tails behaves as a head donor only (14 tested), while every mutant showing tails but no heads behaves as a tail donor only (6 tested). Most of the class of mutants producing both heads and tails (unattached; see Fig. 1) also behave as tail donors only (8/12). Apparently the heads observed by EM are either incomplete or nonfunctional by-products of defective maturation which cannot be efficiently activated in extract mixtures. However, extracts made with the remaining four mutants of this class (genes 13, 14, 15, and 18) behave as both head and tail donors, suggesting that they contain functionally competent heads and tails but lack components required for their union. These preparations, in contrast to those of head donors only and tail donors only, also complement extracts made with double mutants which produce neither heads nor tails (one defective in genes 10 and 31, another in genes 27 and 23).

Mutants defective in genes 9, 11, or 12 also show extract complementation with both the reference head donor and the reference tail donor, as well as with the double mutants. Under restrictive conditions *in vivo*, 9, 11, and 12 mutants produce apparently complete but inactive phage particles characterized in the electron microscope by contracted sheaths (gene 9 mutants) or a tendency to dissociate into free

TABLE 5

GENOTYPES OF EXTRACT-ACTIVATED PHAGE; HEAD AND TAIL MUTANTS

Extract *A*		Extract *B*		Number of Phage	
Gene defect	Functional components	Gene defect	Functional components	Genotype *A*	Genotype *B*
29	H	23	T	20	0
14	HT	23	T	20	0
14	HT	6	H	11	9
6	H	2	T	20	0
14	HT	2	T	20	0
14	HT	16	T	20	0
6	H	16	T	20	0

Genotypes of the phage particles activated during 120-min incubation of the indicated extract mixtures were determined by spot testing for complementation with appropriate known mutant strains.

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heads and tails upon storage (gene 11 and 12 mutants). The extract complementation results suggest that these defective particles can be activated when a missing component is provided by another extract.

Complementation spot tests of phage produced in the active combinations of Tables 3 and 4 show that their genotype is exclusively that of the head donor (Table 5). When 14-extract, a head and tail donor, complements a tail donor extract, the phage produced are of genotype 14; when it complements a head donor extract, comparable numbers of the two genotypes are found among the active particles.

Discussion and Summary.—The results reported here show that several of the steps in the morphogenesis of bacteriophage T4D can take place in extracts of infected cells. These steps include the assembly of tail fibers and their attachment to the virus particle, as well as the union of the head and the tail. Apparently many of the larger components—fiberless particles, fibers, heads, and tails—which accumulate in mutant-infected cells under restrictive conditions are not aberrant byproducts of defective synthesis, but intermediates in the assembly process which may be utilized *in vitro* for the morphogenesis of active virus.

The limited success of our attempts at complementation in extracts indicates that many of the steps in the maturation process do not proceed efficiently under the conditions presently employed. It remains to be seen whether some of these steps, such as the formation of the head or of the tail, can be demonstrated under altered conditions, for example, at higher concentration of reactants.

As an extension of the previously reported EM characterization of defective phenotypes,[2] the extract complementation studies provide some further insight into the functions controlled by the various mutationally defined morphogenetic genes. An example is the group of 12 genes whose mutants produce unattached heads and tails recognizable in electron micrographs. While four of these are apparently involved in the union of heads and tails, the remaining eight may be required to complete or alter the head in some manner which activates it for tail attachment.

Perhaps most significantly, our results show that at least portions of the morphogenetic pathway are open to direct attack by biochemical methods. It may be hoped that purification of components and further study of individual reactions will lead to some understanding of the interactions and specificities involved in the assembly of complex supramolecular structures such as bacteriophage T4.

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