Morphogenesis of Bacteriophage $\phi 80$: Identification of the Cistron 13 Product

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Received for publication 1 September 1971

An *in vitro* complementation reaction leading to the assembly of bacteriophage $\phi 80$ tails from component proteins is described. Tail assembly occurs when a lysate of any mutant in cistron 13 is mixed with a second lysate of a mutant in any of the other cistrons involved in tail formation. Lysates of mutants that are blocked in tail formation contain phage heads that can unite with free tails to form infective particles. The rate of the complementation reaction shows little dependence upon temperature, suggesting that the assembly depends largely upon the kinetic encounter of the interacting components. The tail component missing in cistron 13 mutant lysates was purified approximately 55-fold and shown to be, at least in part, a protein having a molecular weight of approximately 22,000. This protein was also released from highly purified infective $\phi 80$ particles after osmotic shock followed by heat-treatment, suggesting that it most probably is an integral structural protein of the phage tail. Lysates of mutants of bacteriophage λ that are defective in tail formation were shown to contain a tail component identical with or similar to the $\phi 80$ cistron 13 product.

The morphogenesis of the related bacteriophages $\phi 80$ and λ is controlled by two gene clusters located on the left arm of the chromosome of each phage (3, 4, 10). The proximal gene cluster is involved in tail formation, whereas the distal one specifies head formation (4, 10, 13). Furthermore, *in vitro* complementation experiments coupled with observations with the electron microscope (7, 8) have shown that phage mutants defective in head formation synthesize normal tails and that tail mutants produce normal heads.

Weigle (14) showed that the union of λ heads and tails to form infective particles depends solely on the probability of encounter of the two components and that no other factor is necessary for the reaction.

In vitro cross-complementation studies between $\phi 80$ and λ have shown that $\phi 80$ tails can combine with λ heads as efficiently as with $\phi 80$ heads to form infective particles, whereas λ tails combine with $\phi 80$ heads relatively very poorly (4, 6).

The present study was initiated with the aim of investigating reactions leading to the assembly of λ and $\phi 80$ heads and tails from protein and nucleic acid components. This report describes one such reaction which leads to the *in vitro*

assembly $\phi 80$ tails. One of the components in this reaction, the cistron 13 product, has been identified as a relatively heat stable, low-molecular-weight protein. A similar protein was shown to exist in lysates of some of the mutants in bacteriophage λ that are blocked in tail formation.

MATERIALS AND METHODS

Chemicals and media. Mitomycin C (crystalline) and bovine serum albumin (fraction V) were purchased from the Sigma Chemical Co. Egg white lysozyme, α -chymotrypsinogen, ovalbumin, bovine pancreas deoxyribonuclease I (crystalline), ribonuclease (crystalline), trypsin (2X crystallized), and soybean trypsin inhibitor were products of Worthington Biochemical Corp.

Sephadex G-100 (particle size, 40 to 120 μ m) was purchased from Pharmacia Fine Chemicals Inc.

Media used for growth of bacteria, preparation of phage lysates, and for plaque assays have been described (4).

Bacterial and phage strains. Bacterial strains and various mutants of bacteriophage $\phi 80$ have been described previously (4). The suppressor sensitive (*sus*) mutants of bacteriophage λ used in this study were kindly provided by A. Campbell (3) and J. Weigle (13).

Electron microscopy. The specimen supports used were carbonized Formvar-coated 400-mesh copper grids. A specimen was prepared by applying a drop of suspension on the grids, waiting 30 sec, and removing the drop with a filter paper strip. This was followed

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by negatively staining the material by adding one drop of 2% potassium phosphotungstate, pH 7.3 (2); waiting for 30 sec; and withdrawing the solution with a filter paper strip. The grids were allowed to air-dry before examination. Specimens were examined with an RCA EM-4 electron microscope.

Preparation of wild-type and mutant phage lysates. Wild-type $\phi 80$ and λ phages were prepared and purified to a high degree by a procedure described previously (5).

Lysates of various *sus* mutants of λ and $\phi 80$ were prepared as follows. A culture of *Escherichia coli* K-12 strain 594 or W3350 lysogenic for a *sus* mutant was grown at 37°C to a cell concentration of approximately 5×10^8 per ml in Penassay Broth (Difco). The culture was then induced by adding mitomycin C (1 $\mu\text{g}/\text{ml}$) or, in the case of λ clts mutants, by heating at 44°C for 25 min. Lysis in the case of $\phi 80$ starts at approximately 150 min after induction and is complete 30 min later. In the case of λ , lysis occurred at about 110 min after induction. At the end of lysis, chloroform was added to kill surviving bacteria and the cell debris was removed by centrifugation at approximately $8,000 \times g$ for 20 min.

For purification of phage components (heads, tails, and cistron 13 product), more concentrated defective lysates were prepared by collecting the induced cells at 150 min after induction (at the onset of lysis) by centrifugation at $5,000 \times g$ for 15 min at 4°C. The pelleted cells were frozen and thawed once and allowed to lyse in a volume of 0.02 M potassium phosphate buffer (pH 7.2) equal to 5% of the original cell suspension. The lysate was treated with deoxyribonuclease I (2 $\mu\text{g}/\text{ml}$) and ribonuclease (3 $\mu\text{g}/\text{ml}$) at 37°C for 20 min. Chloroform was then added, and the suspension was centrifuged at $8,000 \times g$ for 20 min. The supernatant fluid was used as a source of phage components.

In vitro complementation leading to tail assembly. Tests for in vitro complementation between any two mutant lysates defective in tail formation were performed by incubating 0.3 ml of each at 37°C for 2 hr, after which the reaction was essentially complete. Since the above lysates already contain heads that can unite with tails to form infective units, tail assembly was therefore estimated by measuring the increase in the number of infective units per milliliter of incubation mixture.

Assay for cistron 13 product. The assay mixture for cistron 13 protein in the in vitro tail assembly reaction contained, in a total volume of 0.6 ml, 0.3 ml of *sus* 31 lysate (7.0 mg/ml of protein) and a dilution of cistron 13 protein that resulted in the assembly of between 2×10^6 and 3×10^6 plaque-forming units in a 2-hr incubation period. A unit of activity is equal to the formation of one infective unit of the bacteriophage. All lysates and protein fractions were in 0.02 M potassium phosphate buffer (pH 7.2).

Sephadex-gel filtration. Sephadex G-100 (particle size, 40 to 120 μm) was suspended in 0.02 M potassium phosphate buffer (pH 7.2) and allowed to swell for 2 to 3 days at 4°C. Fine particles were removed by decantation. Gel suspensions were deaerated under reduced pressure and packed into columns. The gel

bed was equilibrated with 0.02 M potassium phosphate buffer (pH 7.2) by maintaining a flow of the buffer through the column for 2 days at a rate of approximately 20 ml/hr. All gel filtration experiments were performed at 4°C. Other details of the procedure are found in the legends to Fig. 5 and 6.

The proteins used as standards for molecular weight determination were dissolved in the equilibration buffer at a concentration of 2.0 mg/ml. A volume of 2.0 ml of the protein solution was applied to the column. The proteins in the effluent were estimated by measuring the absorbance at 280 nm. Symmetrical peaks with sharp maxima were obtained in all cases. The molecular weights of the standard proteins used were: α -chymotrypsinogen, 25,000 (15), ovalbumin, 45,000 (12); bovine serum albumin (fraction V), 67,000 (9); lysozyme, 14,400 (11).

RESULTS

In vitro complementation among mutants blocked in tail formation. In a previous publication (4), in vitro complementation among *sus* mutants allowed mapping of various cistrons involved in tail and head formation on prophage $\phi 80$. A recent and more complete prophage map is shown in Fig. 1. Three additional cistrons involved in tail formation were discovered as a result of isolating more mutants.

In vitro complementation tests involving all possible combinations of two *sus* mutants blocked in tail formation (head donors) were performed to investigate the possibility of assembly of complete phage tails from various components in the mutant lysates. It was observed that lysates of mutants belonging to cistron 13, which were shown earlier to complement quite efficiently with tail-donor mutants, could also form infective units when mixed with any other head-donor mutant lysate. This complementation behavior, which is unique to cistron 13 mutants, is shown in Table 1. Complementation of cistron 13 mutants with tail-donor mutants is at least 10 times as efficient as with other head-donor mutants. Extensive examination of lysates of cistron 13 mutants by electron microscopy revealed the presence of intact heads and the absence of either attached or free tails. An electron micrograph of a lysate of *sus* 31 is shown in Fig. 2. In addition to complete heads, cistron 13 lysates contained short, electron-transparent rods which could possibly be tail fragments. This possibility is under investigation. These results show that in vitro assembly of functional tails takes place when two lysates (one of which is of a cistron 13 mutant) containing complete heads but no tails are combined. The assembled tails then unite with heads to form infective units.

Kinetics of the complementation reaction. The

Deletion 6

Deletion 25

Deletion 13

Deletion 10

Deletion 16

Deletion 9

Deletion 8

Deletion 6

Deletion 17

Deletion 15

FIG. 1. Head- and tail-specifying cistrons of $\phi 80$. Only that portion of the prophage specifying head and tail formation is shown. Numbers above the line representing the prophage correspond to various *sus* mutants, whereas those below the prophage represent cistrons to which the mutants belong. Dotted lines represent bacterial chromosome material. The solid lines below the prophage map represent prophage segments present in deletion lysogens used in mapping the $\phi 80$ *sus* mutants. The sequence of cistrons 15 and 16 is unknown.

TABLE 1. *In vitro* complementation between lysates of $\phi 80$ cistron 13 mutants and those of other cistrons specifying tail formation

Combination of <i>sus</i> mutant lysates	Complementation ^a
$\phi 80$ <i>sus</i> 31 (13) ^b + $\phi 80$ <i>sus</i> 9 (3) ^c	1,036
$\phi 80$ <i>sus</i> 31 (13) + $\phi 80$ <i>sus</i> 57 (7).....	32
$\phi 80$ <i>sus</i> 31 (13) + $\phi 80$ <i>sus</i> 58 (8).....	27
$\phi 80$ <i>sus</i> 31 (13) + $\phi 80$ <i>sus</i> 24 (9).....	18
$\phi 80$ <i>sus</i> 31 (13) + $\phi 80$ <i>sus</i> 107 (10).....	68
$\phi 80$ <i>sus</i> 31 (13) + $\phi 80$ <i>sus</i> 21 (11).....	36
$\phi 80$ <i>sus</i> 31 (13) + $\phi 80$ <i>sus</i> 109 (12).....	72
$\phi 80$ <i>sus</i> 31 (13) + $\phi 80$ <i>sus</i> 23 (13).....	1.2
$\phi 80$ <i>sus</i> 31 (13) + $\phi 80$ <i>sus</i> 108 (14).....	53
$\phi 80$ <i>sus</i> 31 (13) + $\phi 80$ <i>sus</i> 46 (15).....	64
$\phi 80$ <i>sus</i> 31 (13) + $\phi 80$ <i>sus</i> 42 (16).....	142
$\phi 80$ <i>sus</i> 54 (13) + $\phi 80$ <i>sus</i> 107 (10).....	72
$\phi 80$ <i>sus</i> 107 (10) + $\phi 80$ <i>sus</i> 42 (16).....	0.92
$\phi 80$ <i>sus</i> 109 (12) + $\phi 80$ <i>sus</i> 46 (15).....	1.1
$\phi 80$ <i>sus</i> 31 (13) + heat-dissociated $\phi 80$..	97

^a Values represent ratios of the number of infective particles in mixtures of two lysates (0.3 ml of each) after an incubation period of 2 hr to that in individual uncombined lysates.

^b Numbers in parentheses represent the cistrons to which the various mutants belong.

^c $\phi 80$ *sus* 9 is a mutant defective in head formation; all other mutants shown are defective in tail formation.

kinetics of complementation between lysates of $\phi 80$ *sus* 31 (cistron 13) and *sus* 107 (cistron 10) leading to tail assembly and subsequent formation of phage-infective units are shown in Fig. 3. The production of phage was measured by diluting and plating as a function of time. The background of active phage (leakers and revertants) in the lysates varies between 4×10^4 and 2×10^5 per ml. The production of infective phage is very rapid and is almost complete after 30 min. A decrease by a factor of about 2.5 in the rate was observed when the temperature of the reaction was lowered to 5 C, which is approximately equivalent to the increase in the viscosity of the reaction mixture. This suggests that the assembly is mainly dependent upon the probability of encounter of the interacting components.

Purification of the cistron 13 product. The product of cistron 13, which is found in lysates of mutants in other tail-specifying cistrons, was purified approximately 55-fold with a yield of 58%. This was accomplished by a heat-treatment step followed by filtration on Sephadex G-100 gel. The cistron 13 product was purified from *sus* 107 (cistron 10) lysates. The dependence of phage assembly on the concentration of *sus* 107 lysate in the complementation

FIG. 2. Electron micrograph of a lysate of $\phi 80$ sus 31 stained with sodium phosphotungstate. Magnification, 95,000 $\times$. The inset is an electron micrograph of wild-type infective $\phi 80$. Magnification, 180,000 $\times$.

FIG. 3. Kinetics of complementation between $\phi 80$ *sus* 31 and *sus* 107 lysates. The incubation mixtures contained 1 ml of each of lysates of *sus* 31 and 107 (prepared as described under Materials and Methods). Samples (0.1 ml) were withdrawn at various intervals, diluted in Penassay Broth, and plated to determine the number of infective units. Symbols: ○, at 37°C; ●, at 5°C.

mixture is shown in Fig. 4. A linear relationship was observed up to a rate of assembly of approximately 3×10^6 infective units in a 2-hr incubation period.

It was observed that cistron 13 product was relatively stable at high temperatures. This was taken advantage of in purifying this product. The first step in the purification involved incubating the *sus* 107 lysate (see above for preparation) at 92°C for 3 min. The precipitated proteins were removed by centrifugation at $2,000 \times g$ for 10 min. This resulted in a purification of approximately $4.3 \times$ with a yield of 87%. The supernatant fluid from this step was then subjected to gel filtration on a Sephadex G-100 column. The pattern of fractionation on Sephadex G-100 is shown in Fig. 5. Assay of fractions for ability to effect tail assembly when added to a lysate of *sus* 31 (cistron 13) showed that this activity is probably associated with a single component that eluted just after the bulk of proteins. The three fractions with highest activity were pooled and used for characterization

studies. The specific activity of the pooled fractions was 3.1×10^7 . Other details of gel filtration on Sephadex G-100 are given above and in the legend to Fig. 5. A summary of the purification procedure is given in Table 2.

Nature and properties of the cistron 13 product. Treatment of the partially purified preparation with trypsin (0.2 mg/ml at 37°C for 120 min) followed by addition of soybean trypsin inhibitor (0.4 mg/ml) resulted in 87% loss of activity in the complementation reaction. Incubation with either a mixture of trypsin (0.2 mg/ml) and trypsin inhibitor (0.4 mg/ml) or just trypsin inhibitor (0.4 mg/ml) had no effect. This suggests that the cistron 13 product is at least in part protein in nature. Furthermore, this product was insensitive to treatment with pancreatic deoxyribonuclease (50 μ g/ml) and ribonuclease (50 μ g/ml).

Estimation of the molecular weight of cistron 13 product was made by the gel filtration method with Sephadex G-100 (1). A plot of $\log_{10}$ molecular weight versus elution volume is shown in Fig. 6. The cistron 13 product has a molecular weight of approximately 22,000. Details of gel

FIG. 4. Dependence of complementation on the concentration of *sus* 107 lysate. The reaction mixture contained 0.3 ml of *sus* 31 lysate (7.0 mg of protein per ml) and varying amounts of *sus* 107 lysate in a total volume of 0.6 ml. All lysates were in 0.02 M potassium phosphate buffer (pH 7.2). Mixtures were assayed for infective units after a 2-hr incubation period.

filtration are given above and in the legend to Fig. 6.

Release of the cistron 13 product from complete phage particles. The question asked here was whether the cistron 13 product is one of the tail structural components or does it merely direct the assembly of the tail proteins. One way of getting information pertaining to this is by examining purified complete phage particles for the presence of this product.

Highly purified wild-type $\phi 80$ was prepared by ammonium sulfate fractionation, differential centrifugation, and banding twice in CsCl gradients according to a procedure described earlier (5). A stock suspension of $\phi 80$ (5×10^{12} plaque-forming units/ml) in 44% (w/w) CsCl solution was diluted 100-fold into 0.02 M potassium phosphate buffer (pH 7.0) and subsequently incubated at 92 C for 3 min. The phage preparation, which had lost its plaque-forming ability as a result of this treatment, was then

FIG. 5. Gel filtration of the heat-treated *sus* 107 lysate on a Sephadex G-100 column (22 by 1.7 cm). The column was equilibrated with 0.02 M potassium phosphate buffer (pH 7.2). The flow rate was maintained at approximately 32 ml/hr. A 6.8-ml amount of the heat-treated *sus* 107 lysate (1.7 mg of protein per ml) was applied on the column. Symbols: O, absorbance at 280 nm; ●, activity.

assayed for content of the cistron 13 product by the in vitro complementation reaction. The result given at the bottom of Table 1 shows that this osmotically shocked and heat-inactivated $\phi 80$ preparation does indeed contain the missing component in *sus* 31 lysates. This observation strongly suggests that the cistron 13 product is one of the protein tail components.

In vitro complementation between $\phi 80$ cistron 13 mutants and λ mutants defective in tail formation. These complementation tests were performed to determine whether the tail component missing in cistron 13 mutants can be supplied by mutants of bacteriophage λ that are also defective in tail formation. The results presented in Table 3 show that lysates of λ mutants

FIG. 6. Plot of elution volume against log molecular weight for some standard proteins along with the partially purified cistron 13 product on a Sephadex G-100 column (30 by 2.5 cm). A 2-ml amount of the protein solutions (2.0 mg/ml) was applied on the column and eluted separately. The column was equilibrated with 0.02 M potassium phosphate buffer (pH 7.2). Fractions (3.0 ml) were collected at a flow rate of 42 ml/hr.

TABLE 2. Purification of the cistron 13 product

Fraction	Vol (ml)	Activity ^a (units/ml)	Specific activity (units/mg of protein)	Per cent yield
Crude lysate ($8,000 \times g$ supernatant fluid)	8.0	3.9×10^6	5.6×10^5	100
Supernatant fluid after heat treatment	6.8	4.0×10^6	2.4×10^6	87
Sephadex G-100 fraction	10.2	1.8×10^6	3.1×10^7	58

^a One unit of activity is equal to the assembly of one infective unit upon mixing 0.3 ml of *sus* 31 lysate (7.0 mg of protein per ml) and an appropriate dilution of the various fractions of *sus* 107 lysate. Other details of the assay are given in Materials and Methods.

TABLE 3. *In vitro* complementation between lysates of $\phi 80$ cistron 13 mutants and those of bacteriophage λ involved in tail formation

Combination of <i>sus</i> mutant lysates	Complementation ^a
$\phi 80$ <i>sus</i> 31 (13) + lambda <i>sus</i> L.....	27
$\phi 80$ <i>sus</i> 31 (13) + lambda <i>sus</i> H12.....	38
$\phi 80$ <i>sus</i> 31 (13) + lambda <i>sus</i> K24.....	82
$\phi 80$ <i>sus</i> 31 (13) + lambda <i>sus</i> M87.....	48
$\phi 80$ <i>sus</i> 31 (13) + lambda <i>sus</i> G9.....	122
$\phi 80$ <i>sus</i> 31 (13) + lambda <i>sus</i> 145.....	0.86
$\phi 80$ <i>sus</i> 31 (13) + lambda <i>sus</i> 12.....	0.92

^a Values are comparable to those given in Table 1.

in cistrons L, H, K, M, and G contain the missing tail component in cistron 13 mutants. No complementation was observed with lysates of mutants in cistron I of λ , which could mean that cistron 13 of $\phi 80$ and cistron I of λ specify the same component that is necessary for the tail assembly.

DISCUSSION

An *in vitro* complementation reaction leading to the assembly of $\phi 80$ tails that subsequently interact with heads to form infective units was described. Complementation occurs when lysates of *sus* mutants in cistron 13, which are defective in tail formation but make complete heads, are mixed with lysates of mutants of other cistrons responsible for tail formation. This allowed the purification of the missing component in cistron 13 lysates. Since this missing component is made by all other cistrons involved in tail formation, it may at this time be assumed that it is the product of cistron 13.

Observations showing that the product of cistron 13 is inactivated by trypsin and that it can be released from intact phage particles by osmotic shock followed by heat treatment suggest that this product is an integral protein constituent of the tail structure.

Extensive examination of several cistron 13 mutant lysates by electron microscopy indicated the presence of assembled tailless heads and the absence of any assembled free tails. This suggests that the cistron 13 product is necessary for assembly of tails from small subunits and is not merely involved in linking heads to preassembled tails.

The efficiency of the complementation reaction observed upon mixing appropriate lysates is relatively low. The number of active phage assembled is between 0.002 and 0.001 that found in non-defective lysates. This probably is inherent in

the probability of effective collisions between the various interacting components. Earlier experiments on the kinetics of assembly of phage λ from heads and tails showed that only about 1 in 100 of the collisions between the two particles is effective in assembly of an infective unit (14). Since the $\phi 80$ assembly reaction described above involves both tail assembly and subsequent joining of heads to tails, the probability of effective collisions would be expected to be much lower than one in a hundred, thus possibly explaining the low efficiency of assembly observed.

Several lysates of *sus* mutants of bacteriophage λ (*sus* L, H, K, M, and G) were shown to contain a component that could replace the cistron 13 product in the *in vitro* complementation reaction. Bacteriophages λ and $\phi 80$ are related in several respects, and the above observation points to still another aspect of homology. Mutants in cistron I of λ have thus far been shown to lack the ability to form the component corresponding to that of cistron 13 in $\phi 80$, suggesting a correspondence between the two cistrons. More direct evidence for this is being sought by complementation studies among a large number of λ *sus* mutants defective in tail formation.

ACKNOWLEDGMENTS

This research was supported by a grant from the Arts and Sciences Research Committee of the American University of Beirut. The excellent technical assistance of Hermine Hajenian is greatly appreciated. The author would like to acknowledge the help of A. Afifi of the Faculty of Medical Sciences of the American University of Beirut in the use of the electron microscope.

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