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THE CONSTRUCTION OF THE HYBRID PLASMID CONTAINING GENES OF THE CENTRAL PART OF PHAGE T4 BASEPLATE AND GENE 29 PRODUCT SEPARATION

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A fragment of *E. coli* bacteriophage T4 genome including the four genes (genes 51, 27, 28, 29) coding for the central plug proteins was cloned into plasmid pMCC17. The genes present on this fragment were expressed in *E. coli* in the absence of phage infection producing hub proteins, which could be identified on polyacrylamide gels. By applying affinity chromatography protein 29 was purified from extracts of *E. coli* transformed with this hybrid plasmid. The isolated protein had the ability to complement T4 29 amber mutants. The molecular weight of the purified protein was estimated as 75000 to 85000 depending on the composition of SDS-polyacrylamide gel used for the assay.

During bacteriophage T4 morphogenesis many substructures are assembled independently. The most complicated substructure of this phage is a baseplate which is assembled by joining six wedge-like structures around the central hub. Genes which specify proteins for these two substructures are grouped in two separate clusters. Gene 29 belongs to the cluster of genes coding for proteins of the central hub of the baseplate. According to Kikuchi & King [1] the presence of gene 29 protein is necessary for the formation of 14S complex. This complex consists of the following proteins: 51, 26, 28, 29. Together with 12S complex (proteins 5 and 27) 22S complex is created which is considered the main substructure of the hub. During infection, between attachment of long and short tail fibres, the baseplate undergoes conformational changes from a thick compact hexagon to a thin extended star. During these conformational changes the sheath is detached from the upper surface of baseplate and relieves the tail tube. At that time the tail tube is delivered through the cell wall to the cytoplasmatic

membrane, this leading in some way to the release of DNA from the phage head. In this process some of the hub proteins are undoubtedly involved. Such interaction was predicted but not proved by Goldberg [2]. These proteins were identified in Eiserling's laboratory [3] as products of genes 29, 48 and probably 54. The affinity of protein 29 to 48 or 54 is quite unexpected as it has never been reported before. Proteins 48 and 54 do not participate in the main assembly process of the hub, but are added during polymerization of the wedges (15S complex) around the 22S complex. These proteins can be considered as supplementary proteins, allowing for the attachment of the tube and polymerization of the sheath. The organization of these proteins (some of them penetrate the tube) is unknown and difficult to determine in isolated tubes. According to Duda *et al.* [3] the potential candidate for the penetration of the tail tube could be protein 29 but this hypothesis does not agree with the role of this protein during self-assembly of the hub and its property as an enzyme involved in the synthesis of folylpolyglutamic acid [4].

At present our knowledge about the assembly of this structure is fragmentary and controversial. The contribution of hub proteins to the total mass of baseplate is low (a few percent). During *in vitro* complementation experiments, Kikuchi & King [1] were not able to identify hub proteins on polyacrylamide gels from isolated complexes with the exception of protein 29. The "chasing" experiments of Kozloff & Zorzopulos [5] in which phages had labelled hub and unlabelled wedges do not explain the contribution of particular proteins to formation of 22S complex.

We think that the final answer to the above questions should be obtained by examination *in vitro* of complex formation from purified proteins. Identification of hub proteins can be achieved by cloning the respective genes onto bacterial plasmid allowing the synthesis of higher amounts of hub proteins.

MATERIAL AND METHODS

Bacteria, bacteriophages and plasmids. E. coli CR63 (supD) a permissive host for T4 amber mutants and *E. coli* Bb a non-permissive host for amber mutants were used for phage growth and plating. The following T4 amber mutants were used: 29 (B7), 14/15 (LO N 13), 28 (A452, 27 (P21), 51 (S29), 26 (P68), 48 (N85). All mutants were from the collection of the Kozloff laboratory originally from Edgar & Wood [6]. Plasmid pMG835 (containing *Hind*III fragment with genes 51, 27, 28, 29) was from M. Gruidl (Vanderbilt University, U.S.A.). Plasmid pMCC17 was kindly provided by A. Podhajska (Madison, Wisconsin, U.S.A.). Cloning of *Hind*III fragment

into plasmid pMCC17 was performed by standard procedures [7] and the resulting hybrid designated pBS835. For induction 0.1 mM IPTG (isopropyl β-D-thiogalactopyranoside) was used.

Preparation of bacteriophage T4 tube-baseplate substructures. The phage tail substructure, consisting of tail plate attached to tail core, were isolated from lysates of the T4D mutants defective in two genes 14/15 by a series of differential and zonal centrifugation (Kozloff *et al.* [8]). The fractions containing the tube-baseplate tail structure were identified by electron microscopy and SDS-PAGE electrophoresis.

Electroelution of protein from the SDS-PAGE gels. Purified baseplates were analysed on SDS-PAGE gels (9% with low bis-acrylamide concentration), and then about 30 preparative gels were made to isolate the region corresponding to the 29 product. The bands were visualized by immersing the gel in 1.5 M KCl for 5 - 10 min, then the bands were excized with a razor edge and stored at −20°C. The bands were subjected to electrophoretic elution as described by the manufacturer of the elution apparatus (IBI). The combined eluate was precipitated by cold acetone. The precipitate was then suspended in a small volume of 0.1 M phosphate buffer, pH 7.0. This preparation was checked again by SDS-PAGE and injected to rabbits to raise antiserum.

Antisera. Antiserum was prepared by injecting rabbits with the antigen in complete Freund adjuvant intradermally and then boosting them subcutaneously 3 weeks after the first injection. The resulting antiserum was preadsorbed with extract of *E. coli* Bb to remove any antibody against *E. coli* antigens which could be present in the rabbit serum.

Cyanogen bromide activation of Sepharose CL-4B and covalent binding of immunoglobulin fraction. The coupling of immunoglobulin to Sepharose was carried out by a modified version of the procedure developed by Cuatrecasas *et al.* [9]. Sepharose CL-4B (25 ml) was washed 4 times with distilled water and suspended in 50 ml of 0.5 M phosphate buffer, pH 11.5. A solution of 5 g cyanogen bromide dissolved in 8 ml of ice-cold dimethylformamide was added dropwise to the gel gently stirred with a magnetic stirrer; the pH of the suspension was kept at 11.5 by adding 4 M NaOH. After 20 min of mixing, the suspension was filtered with suction and the gel was washed first with cold distilled water (50 ml) and then with 0.1 M sodium bicarbonate, pH 9.5 (500 ml). IgG fraction was added to the gel (about 10 mg protein per 1 ml of the gel in 0.2 M sodium carbonate buffer, pH 9.0, containing 0.5 M NaCl) and the suspension was stirred mechanically for 24 h at 4°C. The gel was washed with 0.2 M sodium carbonate, pH 9.0, plus 0.5 M NaCl, and the remaining active sites on Sepharose were saturated with glycine (100 ml of 0.2 M glycine in 0.2 M sodium carbonate buffer, pH 9.0, plus 0.5 M NaCl with

overnight mechanical stirring). Finally, the gel was washed successively with 0.2 M sodium carbonate, pH 9.5, 0.5 M NaCl, then with 0.1 M acetate buffer, pH 4.0, and finally with 0.2 M carbonate, pH 9.5, 0.5 M NaCl.

Extract preparation and purification of gene 29 product. The bacteria *E. coli* Bb harbouring the plasmid pBS835 were grown in tryptone broth (50 ml) in the presence of ampicillin (50 μg/ml) to $A_{575} = 0.4$ (4×10^8 cells/ml) and then IPTG was added (final concentration 0.1 mM). Usually induction was carried out for 1 h (by this time the cultur reached $A_{575} = 0.8$). The culture was then spun down and resuspended in equal volume of buffer A (0.002 M KH_2PO_4, 0.007 M NaCl, 0.004 M K_2HPO_4 and 0.002 M $MgSO_4$), pH 7.4, plus lysozyme (25 μg/ml). After 15 min of incubation on ice the resulting spheroplasts were spun down at 4000 rev/min for 15 min (if higher speed is used they tend to lyse) and resuspended in 2 ml of 50 mM Tris/HCl, pH 7.5, 0.5 M NaCl, DNAase (50 μg/ml) and PMSF (phenylmethylsulphonyl fluoride) 50 μg/ml were added and the mixture was frozen and thawed 3 times, then sonicated for 30 s. After sonication Triton X-100 was added to a concentration of 0.5%. Lysates were centrifuged at 40000 rev/min in Beckman rotor Ti50 for 0.5 - 1 h. The supernatant was applied to a Sepharose column (25 ml) in which the globulin fraction of the anti-29 serum was convalently bound to the gel. After extensive washing with 50 mM Tris/HCl, pH 7.5, 0.5 M NaCl, the enzyme was eluted with 3 M KSCN in 50 Tris/HCl, pH 7.5; 5 ml fractions of the eluate were subjected to fast dialysis against 50 mM Tris/HCl, pH 7.5. One millilitre of each fraction was then precipitated with 15% TCA for 0.5 h and subjected to SDS-PAGE electrophoresis. The remaining volumes of the eluate were concentrated in glycerol to 2 ml (6 × concentration) and then dialysed against buffer A. Half of that volume was used for complementation of phage T4 29 extracts. To concentrate the eluate ammonium sulphate (0.85 saturation) was also used. The precipitate was then dissolved in 2 ml of 50 mM Tris/HCl, dialysed against buffer A, and used for the complementation experiments.

Complementation experiments. These experiments were performed using: 1, crude extract preparation, 2, purified enzyme. Lysozyme was added to the suspension of cells to facilitate the lysis. Phage extract (0.5 ml) was mixed with an equal volume of an extract of bacterial cells harbouring T4 plasmids or with 0.5 ml of purified protein 29. As a control 0.5 ml of phage extract with 0.5 ml of buffer A was used. Complementation was completed after 2 - 6 h. Other details were as in the procedure of complementation described by Kikuchi & King [10].

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis. This was carried out according to Laemmli [11] using 12.5% or 10% acrylamide concentration in gels.

RESULTS AND DISCUSSION

Construction of hybrid plasmid and properties of E. coli Bb strain containing the T4-pMCC17 hybrid (pBS835)

The DNA of *E. coli* bacteriophage T4 contains 5-hydroxymethyl-cytosine in place of cytosine. In normal wild type phage this base is glucosylated and this makes the T4 DNA resistant to most restriction nucleases [12]. Because of this resistance DNA from a mutant of T4 phage containing non-modified cytosine was used for cloning.

As we mentioned before, genes responsible for the synthesis of the hub proteins are clustered together except for gene 5, which is located at the beginning of the first cluster coding for proteins of wedges substructure. Some of the restriction fragments from this cluster have already been identified [13]. Because of our interest in protein 29 we cloned *Hind*III fragment (identified by M. Gruidl, Vanderbilt University, U.S.A.), 3.5 kb long, to bacterial plasmid pMCC17 (a derivative of pKK223-3 plasmid constructed by D. Cox, Madison University, U.S.A., which contains the strong *trp-lac* promoter). The resulting hybrid was designated pBS835. According

Table 1

Plating efficiency of T4 amber mutants on various E. coli strains

Genes 26 and 48 are not located on the cloning fragment. On the genetic map of phage T4 gene 26 is neighbouring to gene 51 and 48 to gene 29.

Amber mutant	*E. coli* CR 63	*E. coli* Bb	*E. coli* Bb transformed with plasmid pBS835
Wild type T4D	1×10^{12}	8×10^{11}	1×10^{12}
Amber 26	1×10^{13}	2×10^{7}	2×10^{7}
Amber 51	1×10^{12}	3×10^{9}	1×10^{12}
Amber 27	1×10^{11}	9×10^{6}	7×10^{9}
Amber 28	1×10^{11}	6×10^{9}	3×10^{10}
Amber 29	3×10^{11}	1×10^{7}	2×10^{7}
Amber 48	4×10^{12}	1×10^{7}	1×10^{7}

to marker rescue experiments this fragment contains genes: 51, 27, 28, 29 (M. Gruidl. personal communication).

Production of phage specific proteins expressed by hybrid plasmid was estimated using *E. coli* Bb (which was nonpermissive for T4 amber mutants) transformed with the respective hybrid plasmid. Such a transformant should have the ability to support growth of different T4 amber mutants (containing amber mutation in genes present in our plasmid). All of the above T4 amber mutants were plated on untransformed Bb (nonpermissive) and on

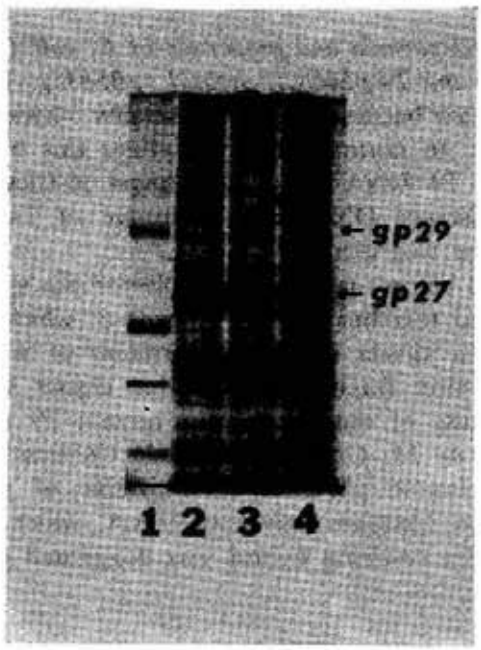

Plate 1. SDS-polyacrylamide gel electrophoresis of plasmid proteins synthesized in *E. coli* JM101. Lane 1, Sigma molecular weight standards, from top to bottom: phosphorylase *b* (*M*, 94000); bovine serum albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), soybean trypsin inhibitor (20000), lactalbumin (14000). Lane 2, non-induced *E. coli* JM101 containing plasmid pBS835. Lane 3, JM101 containing plasmid pBS835, induced with IPTG. Lane 4, *E. coli* JM101 with vector pMCC17. Tentative assignment of bands to products of genes 29 and 27 is indicated by arrows.

E. coli Cr63 permissive strains. Amber mutants defective in genes located outside of this cluster were also tested. These results are given in Table 1. The lack of appropriate protein in infected *E. coli* Bb for T4 amber mutant prevented the formation of viable phage progeny by blocking the process of morphogenesis. The phage protein provided by hybrid plasmid should compensate this defect. Titers in this test (complementation *in vivo*) for particular T4 amber mutants on *E. coli* Bb transformed with hybrid plasmid suggest that the level of biologically active protein 29 is low compared to proteins 27 or 51. As we can see from Plate 1 the level of proteins 29 and 27 in the examined cells does not reflect differences in titration for 27 and 29 T4 amber mutants (see Table 1). However, the expression of plasmid proteins shown in Plate 1 was induced by IPTG, while the expression in *E. coli* Bb was not inducible, therefore the latter can be considerably lower than in *E. coli* of JM series. Differences in complementation for particular phage with mutations in hub genes were

reported before [10]. We cannot at this time give any experimental support for this observation. The above results suggest only the necessity of purification of individual hub proteins for further examination of their properties. The next section of this paper focuses on attempted purification of protein 29 and its preliminary characterization.

Separation of gene 29 product

Purification of phage T4 proteins from a particular substructure is very difficult because of high tendency of these proteins to self-assembly. The purification of gene 29 product was undertaken before by Sadewasser & Kozloff [4] (who used Sephadex G-75 and then blue-agarose column) from extracts of *E. coli* Bb infected with the appropriate T4 amber mutants. The presence of protein 29 in eluates from the column was scored by its ability to complement T4 29 gene amber mutants. Sadewasser & Kozloff [4] did not present the electrophoretic analysis of eluate samples used for complementation. The participation of different complexes of phage proteins in those experiments cannot be therefore excluded. To avoid this risk we would rather chose the purification of phage proteins from extracts of *E. coli* cells, which were transformed with hybrid plasmid containing fragments of phage T4 DNA coding for proteins from a particular substructure. For purification of protein 29 we selected the *Hind*III fragments because *Hind*III site separates the genes 29 and 48, the products of which have a high tendency to self-assemble [3]. This fragment (3.5 kb) contained only four genes coding for hub proteins: 51, 27, 28 and 29. From the literature data [2] protein 27 could be expected to separate easily from the baseplate: the elimination of the remaining two proteins 51 and 28 is a matter of applying suitable techniques of purification. We have chosen the purification of gene 29 product by affinity chromatography on Sepharose 4B to which antibodies to this proteins were attached. The crude material used for protein 29 purification were the extracts of *E. coli* Bb transformed with plasmid pBS835. The fractions of eluates were subjected to fast dialysis against 50 mM Tris/HCl, pH 7.5 (removal of KSCN) and, after precipitation with 15% TCA, examined on 12.5% SDS polyacrylamide gels for the presence of protein 29. The electrophoretic pattern of eluate from the column is presented in Plate 2.

The band with molecular weight of 83 000 is probably a product of gene 29. This fraction had the ability to complement T4 29 gene amber mutants (data not shown). The second band, of 50 000 molecular weight, could be the product of gene 27. Although our column can bind only baseplate proteins between 70 000 and 90 000, it is conceivable that protein 29 forms a complex with gene 27 protein and that during elution of proteins from the column with 3 M KSCN this complex dissociates to

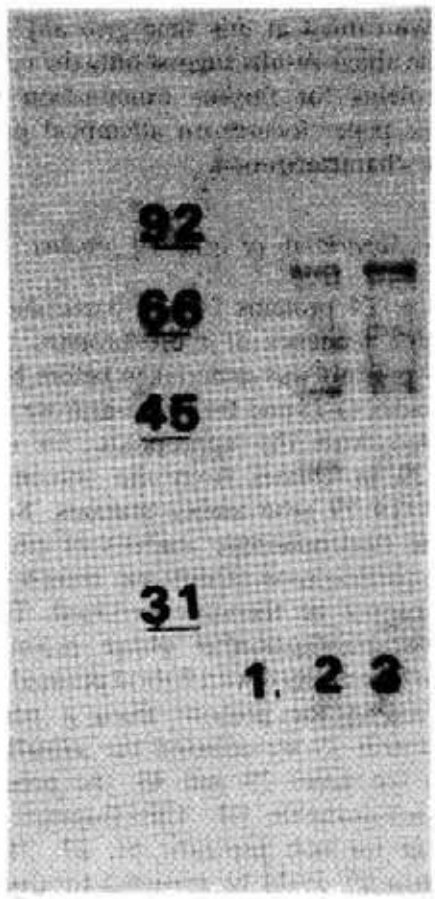

Plate 2. Electrophoretic pattern of fractions of the eluate from affinity Sepharose column (SDS - 12.5% polyacrylamide gel). Lane 1, last volume of the column wash (50 mM Tris/HCl, pH 7.5, plus 0.5 M NaCl). Lanes 2 and 3 represent proteins present in the first two fractions of the eluate (eluent 50 mM Tris/HCl, pH 7.5, and 3 M KSCN). These bands were absent in the eluates from extracts of *E. coli* Bb without pBS835 hybrid plasmid. Bio-Rad Laboratories molecular weight standards ($M_r \times 10^{-3}$), from top to bottom: phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase.

individual proteins. Our eluate, however, did not have the ability to complement gene 27 amber mutants. Therefore we rather think that the 50000 band is the degraded form of protein 29. We cannot exclude the possibility that this 50000 molecular weight band can represent bacterial protein as well. According to literature data [9] the ability to complement T4 amber mutants by crude extracts decreases quickly during storage even at −70 C. Such decreasing ability to complement can be due to the activity of proteases present in crude extracts or to the degradation of complexes during storage.

To check this possibility we prepared extracts in the presence of PMSF and purified our extracts immediately after preparation.

Electrophoretic analysis of such eluate revealed the presence of two bands; one with molecular weight of 110000 and the second corresponding to the position of protein 29 (data not shown). This eluate had the ability to complement T4 29 amber mutants in a higher degree than the previous one (see Plate 2). The band with molecular weight of 110000 can represent the complex of gene 28 and 29 products. During prolonged storage this band disappeared from our eluates. According to literature [5] the molecular weight of gene 28 product is about 30000. The affinity of protein 28 to protein 29 is in agreement with the pattern of assembly of the hub proteins proposed by Kikuchi & King [1]. There are two different hypotheses for the assembly of the hub proteins: one put forward by Kikuchi & King [1] and the other by Kozloff laboratory [14]. The self assembly pathway of the hub was established only on the basis of the complementation experiments of crude extracts of *E. coli* infected with appropriate amber T4 mutant. We rather think that only experiments with purified proteins would bring the final answer as to the assembly pathway of this substructure (main substructure of the hub contains only products of six genes: 26, 51, 27, 28, 29 and 5). The results presented above initiate such an approach which may also offer the way for the derivation of hub proteins in the state of electrophoretic purity.

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