structure of the filamentous bacteriophage *fl*

**LOCATION OF THE A, C, AND D MINOR COAT PROTEINS**

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(Received for publication, August 15, 1980)

The location within the virion of the A, C, and D minor coat proteins of the filamentous bacteriophage *fl* has been analyzed. The A protein is present in ~5 copies/particle and is located at the tip of normal length phage, miniphage, and *fl/pBR322* chimeric phage, a longer than normal length phage. The mole ratios of the A, C, and D proteins are the same for each type of particle, consistent with a model of phage organization in which the minor coat proteins are clustered near or at the ends of the phage. Normal length phage were fragmented by passing them through a French press, and those fragments that contained the A protein were separated from those that did not by treating the mixture with anti-A protein antibody. Analysis of the protein compositions of the two populations of fragments showed that the A and D proteins were found together in one population of fragments and that most, if not all, of the C protein was found in the other. These results show that the D protein is located near or at the A protein end of the phage and that the C protein is located in a region near or at the opposite end. Treatment of the virion with proteases which lowered the infectivity of the phage resulted in particles in which only the A protein was cleaved to any detectable extent. These particles remained resistant to the action of micrococcal nuclease.

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Studies in many laboratories on the structure of the filamentous bacteriophage *fl*, *fd*, and *M13* have established that the bulk of the phage particle consists of a circular, single-stranded DNA molecule coated with ~2710 molecules of the major coat protein (product of phage gene VIII, B protein) (1). The virion also contains minor amounts of three other phage-encoded proteins which have been shown to be the products of gene III (A protein), gene IX (C protein), and gene VI (D protein) (2-5). The A, C, and D proteins are present in ~5, 10, and 5 copies/phage particle, respectively (5). The A protein is necessary for adsorption of these virions to their host (6) and has been located at one end of the phage particle (2, 3). Neither the function nor the location within the virion of the C and D proteins has been established as yet.

The filamentous bacteriophage are unique among bacteriophage in that the size of the DNA which can be packaged into a particle is highly variable. Miniphage ranging in length from 0.2 to 0.8 times the normal particle length have been isolated (7-9). These particles contain no intact genes and require the presence of helper phage for infection (10). In addition, the ability to clone long stretches of foreign DNA into the filamentous phage genome has led to the production of phage particles which are longer than normal (maxiphage) (For example see Refs. 11-14). The exact length of such particles is directly proportional to the size of the DNA insert. Although both miniphage and maxiphage have a filamentous phage appearance by electron microscopy, little is known about the amounts or location of the minor A, C, and D proteins in these particles.

In this paper, we show that the A and D minor coat proteins are located near or at one end of the normal length phage particle and that the C protein is located near at the opposite end. In addition, we present evidence that the protein organization of miniphage and maxiphage is probably the same as that for normal length bacteriophage.

**EXPERIMENTAL PROCEDURES**

**Materials—** Carrier-free [32P]orthophosphoric acid, I-[35S]cystine (>300 Ci/mmol), and I-[3H]leucine (47.7 Ci/mmol) were purchased from New England Nuclear. Sepharose 4B was from Pharmacia. Goat anti-rabbit IgG was obtained from Miles Laboratories, and ferritin, electron microscopic grade was from Polysciences. Bovine serum albumin (A grade) was from Cabiochem. Protein A, thrombin (pro tease Type X), and subtilisin BPN' (pro tease Type VII) were purchased from Sigma. a-Chymotrypsin (5X crystallized) and trypsin were from Worthington. Sources of all other materials used in this study have been given by Lin et al. (6).

**Growth and Purification of Bacteriophage—** *Escherichia coli* strains K38 (15) and JM101 (A[lac, pro]), F lac obtained from J. Messing through D. Bastia of Duke University) were used for the growth of wild type *fl*, miniphage, and R208, a *fl/pBR322* chimeric phage (16) obtained from J. Boeke (The Rockefeller University). Wild type (normal length) *fl* was grown on K38 in supplemented MTPA media, labeled with [35S]cystine or [3H]leucine, and purified as previously described (5). Phage containing radioactive DNA were prepared in the same manner except that 1 mCi of [32P]orthophosphate was added 15 min after infection in place of cystine. Miniphage were grown on K38, labeled with [35S]cystine or [3H]leucine, precipitated with polyethylene glycol, washed with 0.1% sarcosyl, and precipitated again with polyethylene glycol as described (5). The miniphage were then isolated by two successive sucrose gradient centrifugations and were further purified by cesium chloride gradient centrifugation (5).

To grow R208 (maxiphage), strain JM101 was grown in supplemented MTPA medium to a density of 2 x 10^8 bacteria/ml and infected with R208 at a multiplicity of infection of 10. After 46 min, the bacteria were plated on tryptone plates containing 50 µg/ml of ampicillin. An ampicillin-resistant colony was then picked into supplemented MTPA media lacking cystine or leucine but containing 50 µg/ml of ampicillin. An ampicillin-resistant colony was then picked into supplemented MTPA media lacking cystine or leucine but containing 50 µg/ml of ampicillin and grown to a density of 2 x 10^8 bacteria/ml. [35S]Cystine or [3H]leucine was added, and the phage were grown, harvested, and purified as described (5).

The homogeneity of the radioactive phage preparations used in

* This work was supported by Public Health Service Grants GM18305 (to R. E. W.) and GM12607 (to W. K.) from the National Institute of General Medical Sciences and a National Science Foundation Grant PCM7924132 (to R. E. W.). The costs of publication of this article were defrayed in part by the payment of page charges.

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this study was determined by agarose gel electrophoresis of whole phage (9, 10) and by electron microscopy. 

Normal length phage preparations contained 90 to 95% normal length phage with small amounts of contaminating diploid, triploid, and miniphage particles. Miniphage preparations contained less than 10% normal length phage. Maxiphage preparations were greater than 90% homogeneous, the remaining species consisting of what appeared to be to be highly fragmented. All normal length phage preparations were subject to electron microscopy and analyzed by densitometry on a drop of sample for 30 s, washed 3 to 4 times by floating on H2O or 0.1 m ammonium acetate, and stained for 15 to 40 s with 2% uranyl acetate in H2O. Samples were viewed at 80 kV in a JEM 100C electron microscope and photographed at a magnification of 20,000 to 35,000. Actual magnifications were determined by reference to a grating replica. Particle lengths for normal length phage, miniphage, and maxiphage were determined by tracing micrographs that were enlarged 3 times.

RESULTS

Proteins A, C, and D are Present in Specific Regions of Normal Length Phage, Miniphage, and Maxiphage—We have shown previously that the average length phage particle contains 5 molecules of the A protein (2, 3, 5). This was done by determining the percentage of various radioactive amino acids in the A and B proteins using this approach due to the present lack of antibodies directed against these proteins. Since in all three phage types the same amount of A protein is located only at one end of the virion, comparison of the amounts of C and D proteins relative to the A protein should give some information as to the relative location of these proteins in the phage particle. If the C and/or D proteins are located in specific regions, such as the ends of the phage particles, then their amounts relative to the A protein should remain the same in each type of phage. If the C and/or D proteins are distributed along the lengths of the particles, then their amounts relative to the A protein would be expected to vary with the length of the phage particle. The predicted mole ratios for both possibilities are given in Table I. Normal length phage, miniphage, and maxiphage labeled with [35S]cysteine were grown and isolated

1 The abbreviation used is: SDS, sodium dodecyl sulfate.

2 D. F. Hill and G. B. Petersen, personal communication.
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TABLE I

| Phage       | Phage length | A Protein molecules/phage | Predicted mole ratio (A:C:D) | Observed mole ratio (A:C:D) |
|-------------|--------------|---------------------------|-----------------------------|-----------------------------|
|             |              |                           | C and D at ends | C and D along length | A | C | D | A | C | D | A | C | D |
| Normal length | 843 (20)     | 4.1 (0.1)                 | 1 | 2 | 1 | 1 | 2 | 1 | 1 | 2.4 (0.3) | 1.1 (0.1) |
| Miniphage   | 380 (29)     | 5.3                       | 1 | 2 | 1 | 1 | 0.9 | 0.5 | 1 | 2.3 | 1.0 |
| Maxiphage   | 1449 (29)    | 5.0 (0.4)                 | 1 | 2 | 1 | 1 | 3.4 | 1.7 | 1 | 2.3 (0.2) | 1.1 (0.1) |

* The mean lengths of 40 normal length phage, 48 miniphage, and 36 maxiphage are listed. Values in parentheses are standard deviations.

The values given were determined as described under "Experimental Procedures" and are average values based on 4, 1, and 4 determinations from single preparations of [3H]leucine-labeled normal length phage, miniphage, and maxiphage, respectively. Values in parentheses are standard deviations. The values given are based on the presence of 19 leucines/A protein and 2 leucines/B protein, as determined from the fl DNA sequence. The number of B protein molecules/particle was assumed to be 2710, 1222, and 4658 for normal length phage, miniphage, and maxiphage, respectively. The predicted mole ratios are based on the approximate A:C:D ratio of 1:2:1 previously determined by Lin et al. (5) for normal length phage.

The observed mole ratios were determined as described under "Experimental Procedures" and are average values based on determinations from 7, 1, and 2 independent preparations of [35S]cysteine-labeled normal length phage, miniphage, and maxiphage, respectively. Values in parentheses are standard deviations. The mole ratios are based on the presence of 8 cysteines/A protein and 1 cysteine/C or D protein, as determined from the fl DNA sequence. The B protein does not contain cysteine.

![Fig. 1. Electron micrographs of normal length phage, miniphage, and maxiphage reacted with ferritin-conjugated antibodies. Normal length phage (A), miniphage (B), and maxiphage (C) were reacted with anti-A protein IgG and ferritin-conjugated goat anti-rabbit IgG as previously described (3) and prepared for electron microscopy as described under “Experimental Procedures.” Bar equals 0.1 μm.](image1)

and their lengths were determined by electron microscopy (Table I). The amounts of the A, C, and D proteins were determined by the combined agarose gel filtration and SDS-polyacrylamide gel electrophoresis procedure described by Lin et al. (5). The phage were solubilized in SDS, and the DNA and proteins were partially separated on a Bio-Gel A-5m column. All three types of phage particles gave an elution profile identical with that shown in Fig. 5B. The amount of A, C, and D proteins in each fraction of the A-5m column was determined by polyacrylamide gel electrophoresis and the mole ratios were calculated. The observed mole ratios shown in Table I for normal length phage, miniphage, and maxiphage are virtually indistinguishable from one another and are in excellent agreement with those ratios predicted by a model in which all of the minor phage coat proteins are located in specific regions, possibly near or at the ends of the phage.

**Preparation and Separation of the Ends of the Phage**—It was necessary to isolate each end of the phage to determine the location of the C and D proteins. Anti-A protein IgG was used to separate the two ends of fragmented phage using the procedure outlined in Fig. 2. Normal length phage were fragmented by passing them through a French press. The random fragments generated by such treatment were incubated with anti-A protein IgG as described under "Experimental Procedures." Phage fragments that originated from the end of the phage containing the A protein formed rosette-
like aggregates due to the cross-linking effect of anti-A protein IgG under these conditions. These rosette-like structures were then separated from fragments derived from the opposite end and from regions of the particle initially located between the ends of the phage on a sucrose velocity gradient layered above a dense shelf of metrizamide. The aggregates composed of fragments derived from the A protein end of the phage collected on the metrizamide shelf (Peak X, Fig. 2) while the remaining fragments migrated more slowly in the sucrose gradient (Peak Y, Fig. 2).

To test the efficacy of the procedure, normal length phage containing $^{32}$P-labeled DNA were fragmented, incubated with nonimmune or anti-A protein IgG, and subjected to sucrose velocity centrifugation as outlined above. As a control, unfragmented phage were subjected to the same procedure. Inspection of Fig. 3A clearly shows that passage of the phage through the French press produced fragments smaller than the intact phage. (The small amount of material on the metrizamide shelf may result from side-by-side clustering of phage which was observed by electron microscopy.) Treatment of intact phage with anti-A protein IgG resulted in the aggregation of all particles, which upon centrifugation migrated to the metrizamide shelf (Fractions 4 to 7, Fig. 3B). This showed that the amount of antibody used would interact with enough A protein to aggregate all of the phage present in the solution. However, treatment of fragmented phage under the same conditions (Fig. 3B) resulted in only one-third of the total $^{32}$P radioactivity sedimenting to the metrizamide shelf (Fractions 4 to 7, Peak X material), while the remaining two-thirds of the radioactivity (fractions 9 to 22, Peak Y material) migrated to the same position in the gradients as the fragments treated with nonimmune IgG (Fig. 3A). These results suggested that the fragments were approximately one-third of the original phage length. This was confirmed by an examination of electron micrographs of the rosettes in Peak X material and the fragments in Peak Y material where the length of the fragments ranged in size from 1/5 to 1/2 of the original phage length (Fig. 4).

The same experiment was repeated using phage labeled with $^{35}$S-cysteine (Fig. 3, C and D). In this case, most of the radioactivity should be at the A protein end of the phage since the A protein contains 8 cysteines and the C and D proteins only 1 cysteine each. The major coat, or B protein, does not contain cysteine.) Fig. 3D shows that $\sim$80% of the radioactivity sedimented to the metrizamide shelf when fragmented phage containing this label were treated with anti-A protein IgG. These data show that the material in Peak X contains those fragments derived from the A protein end of the phage and that the material in Peak Y is made up of the middle and opposite end of the phage particle.

The Protein Compositions of the Separated Fragments—Large amounts of Peak X and Peak Y material were fragmented, incubated with anti-A protein IgG, and run on a sucrose gradient as described under “Experimental Procedures.” Samples were taken from fractions corresponding to Fraction 5 (Peak X) and Fraction 16 (Peak Y) of the gradient shown in Fig. 2B and were prepared for electron microscopy. A, Peak X material; B, Peak Y material. Bar equals 0.1 µm.

**Fig. 3.** Sucrose velocity sedimentation of fragmented and unfragmented phage incubated with immune or nonimmune IgG. Normal length phage labeled with $[^{32}]$P DNA or $[^{35}]$S-cysteine were fragmented or left intact, incubated with anti-A protein IgG or nonimmune IgG, and centrifuged on sucrose velocity gradients that were layered above a metrizamide shelf as described under “Experimental Procedures.” The metrizamide shelf extends from the bottom of the centrifuge tube to about fraction 4. Phage in A and C were incubated with nonimmune IgG. Phage in B and D were incubated with anti-A protein IgG. ○—○ unfragmented phage; ○—○ fragmented phage.

**Fig. 4.** Electron micrographs of material taken from Peaks X and Y. Normal length phage were fragmented, incubated with anti-A protein IgG, and run on a sucrose gradient as described under “Experimental Procedures.” Samples were taken from fractions corresponding to Fraction 5 (Peak X) and Fraction 16 (Peak Y) of the gradient shown in Fig. 2B and were prepared for electron microscopy. A, Peak X material; B, Peak Y material. Bar equals 0.1 µm.
fractions indicated in Fig. 2 were subjected to gel filtration on a Bio-Gel A-5m column, and the gels were pooled and concentrated as described under "Experimental Procedures." 

A portion of each fraction was analyzed by SDS-polyacrylamide gel electrophoresis, only the A, C, and D proteins given in Table II were recovered from the gels. 

Approximately 95% of the [35S] cysteine counts initially incubated with anti-A protein IgG were recovered from the gels.

The mole ratios are based on the cysteine content of the A, C, and D proteins given in Table II.

The counts per minute of each protein refer to the sum of the radioactivity in each protein from all fractions of the gel filtration columns in Fig. 5.

The mole ratios are based on the cysteine content of the A, C, and D proteins. Quanti- tation of the amount of A, C, and D proteins as described above showed that the mole ratios of these proteins in fragmented or unfragmented phage that were not treated with antibody were identical with each other and to the preparations of the Peak X and Peak Y samples.

The mole ratios of A, C, and D proteins in Peak X and Peak Y material (Fig. 2) were determined as described under "Experimental Procedures." A portion of each fraction was electrophoresed on SDS-polyacrylamide gels, and the gels were prepared for fluorography as described (5). Samples in Lanes 1 and 2 were taken from Peak X material, Fractions B and F, respectively (Fig. 5A). Samples in Lanes 3 to 8 were taken from Peak X material, Fractions A to F, respectively (Fig. 5A). The letters on the right refer to the expected positions of the phage A, B, C, and D proteins. T, top of the separating gel. BPB, position of the tracking dye bromphenol blue.

TABLE II

| A protein | C protein | D protein | Mole ratio (A:C:D)* |
|-----------|-----------|-----------|---------------------|
| Peak X**  | 127,093   | 5,651     | 18,157              | 1.04 1.1 |
| Peak Y**  | 2,617     | 34,632    | 1,075               | 105.9 3.3 |
| Peak X + Peak Y | 129,710 | 40,283    | 19,232              | 2.5 1.2 |
| Fragmented | 71,968    | 22,272    | 10,002              | 2.5 1.1 |
| Unfragmented | 78,890   | 25,114    | 10,701              | 2.5 1.1 |

*The mole ratios are based on the cysteine content of the A, C, and D proteins.

**The counts per minute of each protein refer to the sum of the radioactivity in each protein from all fractions of the gel filtration columns in Fig. 5.

To accurately determine the amount of the A, C, and D proteins in the separated phage fragments, the fractions indicated in Fig. 5A (A to F) were run on SDS-polyacrylamide gels, the radioactivity in each gel slice determined, and the amount of the A, C, and D proteins in each sample calculated as described under "Experimental Procedures." The results of this analysis are presented in Table II. Peak X material (Fig. 2) contained 94% of the total A protein, 98% of the total D protein, and 14% of the total C protein. Peak Y material contained 86% of the total C protein and only minimal amounts of the A and D proteins.

In order to be certain that passage of phage through the French press did not change the relative amounts of the A, C, and D proteins, we did control experiments using [35S]cysteine-labeled phage from the same preparation as above. Either fragmented or unfragmented phage were not treated with antibody were solubilized and subjected to gel filtration on a Bio-Gel A-5m column (Fig. 5B). Analysis of appropriate fractions (Fig. 5B, A to D) on SDS-polyacrylamide gels showed that the first major peak eluting from the column contained all the A protein, while most of the second peak was comprised of [35S]labeled C and D proteins. Quantification of the amount of A, C, and D proteins as described above showed that the mole ratios of these proteins in fragmented or unfragmented phage that were not treated with anti-A protein IgG were identical with each other and to the
with protease digestion. Cysteine-labeled phage were treated as described under "Experimental Procedures." Figure indicate the positions of the A, B, C, and D protein, and C protein, with apparent molecular weights of 69,000, 20,000, 15,000, 11,500, and 5,500, respectively. BPB, position of the tracking dye bromphenol blue.

ratio of these proteins in the combined Peak X and Peak Y samples (Table II).

Analysis of the amount of the A, C, and D proteins in Peak X and Peak Y material (Fig. 2) prepared from four separate [%35S]cysteine-labeled phage preparations gave values that were in good agreement with those presented here. It therefore appears that the D protein is located near or at the A protein end of the phage and that the C protein is located in a region near or at the opposite end.1

Susceptibility of the A, C, and D Proteins to Protease Digestion and Sulphhydrl Reducing Agents—In an attempt to ascertain the role of the C and D proteins in the infective process, [%35S]cysteine-labeled phage were treated with various proteases as described under "Experimental Procedures." After freeing the resulting particles from the protease and phage protein fragments derived from the protease digestion, a small aliquot of the resulting particles was titered to determine the percentage of surviving phage. Trypsin showed no effect, while chymotrypsin, thermolysin, and subtilisin lowered the titer from 5.6 \( \times 10^{15} \) to 2.7 \( \times 10^{13} \), 3.0 \( \times 10^{10} \), and 3.3 \( \times 10^{9} \) phage/ml, respectively. To determine which viral components were affected by these protease treatments, we subjected the various protease-digested [%35S]cysteine-labeled phage to SDS-polyacrylamide gel electrophoresis. The only minor phage protein susceptible to cleavage, as detected by SDS-polyacrylamide gel electrophoresis, was the A protein (Fig. 7). There appears to be a good correlation between the amount of intact A protein left after digestion and the percentage of the remaining infectivity. The migration position of the B protein on SDS-polyacrylamide gels, as detected by Coomassie blue stain, appeared unchanged after treatment of phage with any of the proteases tested, and amino acid analysis of B protein from subtilisin-digested phage showed that the B protein was not susceptible to cleavage (data not shown). These results suggest that limited cleavage of the A protein is sufficient to cause the observed loss in activity. This loss in activity is probably not due to exposure of the DNA to solvent since the DNA in phage treated with thermolysin or subtilisin is resistant to the action of micrococcal nuclease.

Since the A and D proteins are located at the same end of the phage and both contain cysteine, it is possible that they might be associated via disulfide bonds. To test this, [%35S]cysteine-labeled phage were solubilized with 0.1% SDS overnight at 37°C and subjected to A-5m gel filtration in the absence of any sulfhydrl reducing agents. A pattern indistinguishable from that in Fig. 5B was obtained. When Fractions A to D (Fig. 5B) were analyzed by SDS-polyacrylamide gel electrophoresis in the absence of sulfhydrl reducing agents, the A, C, and D proteins all migrated at the expected positions (Fig. 8, part I) and were present in the expected mole ratios.

1 We believe that the C protein found in Peak X material results from contamination with Peak Y material. However, we cannot unambiguously rule out the possibility that 1 or 2 molecules of C protein reside at the same end as the A and D proteins.
This implies that any interaction between the A and D proteins or among different molecules of the same minor coat protein species is not mediated by disulfide bonds in the phage particle. However, if a fraction which contained both B and D proteins was heated in the presence of a high concentration of mercaptoethanol (1.1 M), the D protein did not enter the separating gel (Fig. 8, compare Lanes 7 of parts I and II). The D protein remained at the top of the gel only in fractions that contained large amounts of the B protein (Fig. 8, part II, compare Lanes 2 and 3 and Lanes 6 and 7). At B protein concentrations less than ~1 mg/ml, this phenomenon was not observed. Formation of these high molecular weight complexes was promoted by the presence of 1.1 M disulfide reducing agent during the process of electrophoretic fractionation, and the other domain (stem) may be essential for anchoring the A protein to the phage particle itself. It is possible that the stem might be composed of part of the A protein since the knobless phage produced by treatment with subtilisin still retain a 15,000-dalton portion of the A protein. The fact that the A and D proteins appear to be at the same end of the phage might indicate that there is some interaction between these two proteins. However, any such association cannot be due to disulfide bridges since the individual proteins can be readily dissociated from the phage and from each other in the absence of sulfhydryl reducing agents.

If any of the minor proteins recognize a specific region of the DNA, then the DNA would have to be present in a unique orientation in the phage particle with respect to these proteins since they are located near or at the ends of the phage. To date, a number of groups have addressed the question of DNA orientation within the phage and have reached somewhat conflicting conclusions (27–30). The approach presented here should allow us to determine whether a specific region of the DNA is present at the A and D protein-containing end of the phage.

Acknowledgments—We thank D. F. Hill, G. B. Peterson, and C. W. Gray for communicating results prior to publication. We also thank Ms. Lucy Hamilton and Ms. Gerda Michalsky for expert technical assistance.

Addendum—We have recently learned that J. Schoenmakers and G. Simons have results which suggest that the low molecular weight band (C protein band) obtained after subjecting disrupted M13 phage to SDS-polyacrylamide gel electrophoresis may contain some gene VII product as well as the protein coded by phage gene IX. Both of these proteins have nearly identical molecular weights, and each contains 1 residue of cysteine per molecule. Therefore, the value of 10 copies of C protein per phage particle presented in this paper would represent the sum of the number of copies of both the gene VII and gene IX products in the phage.

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