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A DNA–Protein Complex Involved in Bacteriophage φX174 Particle Formation

(intermediates/assembly/gene *D*-protein)

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ABSTRACT **A φX-specific DNA–protein complex has been isolated from φX-infected cells. This complex contains infective circular single-stranded DNA, the proteins of the genes *F*, *G*, *H*, and *J* in the same proportions as in the phage particle, and, in addition, the gene *D*-protein. The *D*-protein makes up 12–22% of the protein part of the complex. The sedimentation value of the complex is about 140 S. *In vitro*, the complex can be converted to the normal 114S phage particle (with a concomitant loss of its D-protein) or to an uninfective 70S particle and a small amount of free single-stranded DNA. The fast sedimenting particle is not associated with membranes.**

The assembly of the mature φX174 phage particle seems to be closely linked to the synthesis of the viral single-stranded DNA (1). No condition is known where single-stranded DNA is formed without concomitant formation of phage particles. Under some conditions, a small amount of free single-stranded DNA has been found in infected cells (2), but always in combination with the synthesis of large amounts of phage.

The products of five of the nine known genes, *B*, *C*, *D*, *F*, and *G*, are required for the synthesis of single-stranded progeny DNA. The absence of single-stranded DNA synthesis upon infection with mutants in the coat protein genes *F* and *G* may indicate that if the growing single strands are not immediately and properly packed in a coat, they become degraded by host nucleases. The functions of the products of genes *B*, *C*, and *D* are unknown; probably they are involved in initiation of single-strand synthesis, protection of the nascent single-stranded chains from duplication [as with the gene 5 protein of M13 (3)], and virion assembly. Because of its abundant presence in the infected cell (4), the gene *D*-protein is an attractive candidate for the protection function.

In this paper we describe the isolation and properties of a possible intermediate in the φX particle formation, containing large amounts of the gene *D*-protein.

MATERIALS AND METHODS

Media. TPG (5) and TPA (6), media for phage growth and titration (7) and spheroplast infection (8) have been described. Washing buffer contains 0.05 M borate–0.05 M Tris·HCl 0.05 M ethylenediaminetetraacetate (EDTA), pH 8.0. L-[^{3}H]leucine (2 Ci/mmol), [*1*-^{14}C]leucine (316 Ci/mol), and [*methyl*-^{3}H]thymidine (10 Ci/mmol) were purchased from Schwarz/Mann.

Bacterial and Viral Strains. Escherichia coli HF4714 is an amber-suppressing host (9). *E. coli* HF4704 is a nonpermissive, thymine-requiring host (5). φX *am*3 is a lysis-defective mutant (10).

Isolation of the DNA–Protein Complex. HF4704 in 30 ml of TPA + 1 μg/ml of thymine was grown at 36° to a cell concentration of 2 × 10^8/ml. φX *am*3 phage was added with a multiplicity of infection (MOI) of 5. Twenty-five minutes after infection 1 mCi [^{3}H]dThd was added. At 80 min after infection the culture was cooled, pelleted, and washed five times with washing buffer. To the final pellet was added 0.5 ml of lysozyme (10 mg/ml in 0.25 M Tris·HCl–0.25 M EDTA, pH 7.2) and 4.5 ml of 0.25 M Tris·HCl–saturated disodium EDTA, pH 7.2. This suspension was incubated for 20 min at 36° and then frozen and thawed three times. Cell debris was removed by centrifugation for 10 min at 12,000 × *g*. The supernatant was layered on a 5–20% sucrose gradient in 0.5 M Tris·HCl–0.5 M EDTA, pH 7.2, and centrifuged in the Spinco SW25.2 rotor for 5 hr at 25,000 rpm. Fractions were collected dropwise. Those fractions containing label were appropriately pooled, dialyzed against 0.25 M Tris·HCl–saturated EDTA, pH 7.2, and stored at 4°.

When the proteins were to be labeled, the cells were grown in TPG medium + 10 μg/ml of thymine + 10 μg/ml of adenine, exposed to ultraviolet irradiation for 5 min at 19 ergs/mm^2 per sec, aerated for 10 min and then mixed with phage plus 250 μCi of [^{3}H]leucine or 125 μCi of [^{14}C]leucine.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. The gels used consisted of a 15% gel and a 3% stacking gel. The 15% gels are described by Benbow (9). The stacking gel has the following composition: 1 ml of 30% acrylamide–0.8% bisacrylamide, 1.25 ml of 0.47 M Tris–0.26 M H_3PO_4 solution, pH 6.9, 2.65 ml of distilled water, 0.1 ml of 10% sodium dodecyl sulfate, 5 μl N,N′,N′,N-tetramethylethylenediamine (TEMED) and 5 ml of 0.4% ammonium persulfate. Electrophoresis was performed in Pyrex glass tubes with an inner diameter of 0.7 cm, with 9 cm of 15% gel and 1.5 cm of stacking gel. The running buffer was a solution of 0.12 M glycine–0.025 M Tris–0.1% sodium dodecyl sulfate, pH 8.3.

Samples of 0.3 ml (or less) could be run on these gels. Each protein sample contained 2% sodium dodecyl sulfate, 3.3% mercaptoethanol, and 10% glycerol and was heated in boiling water for 2 min before use. Bromophenol blue was used as a migration marker. The gels were sliced with a Mickle gel-slicer and the radioactivity of the slices was measured in 5 ml of scintillation fluid [858 ml of toluene, 90 ml of NCS (Amersham/Searle), 42 ml of Liquifluor and 10 ml of distilled water].

Abbreviation: EDTA, disodium ethylenediaminetetraacetate.

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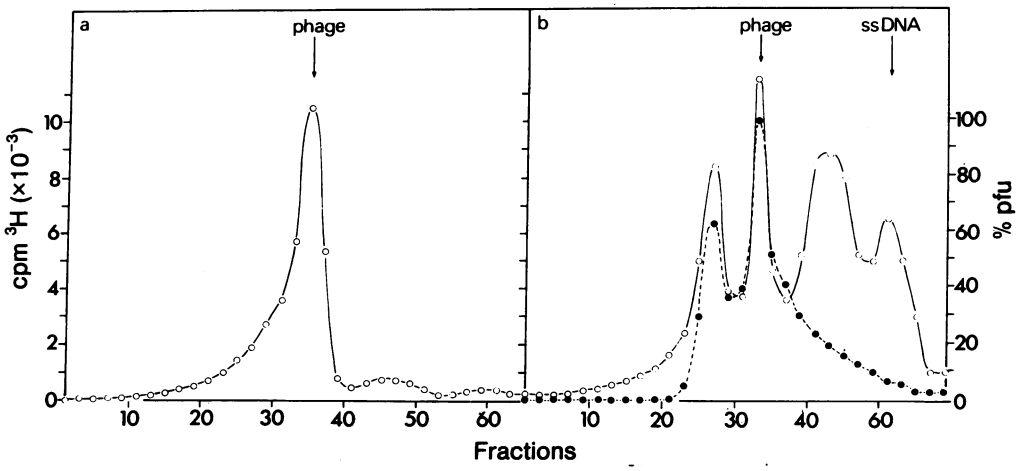

FIG. 1. Sedimentation of [^{3}H]dThd-labeled lysates of *am*3-infected HF4704 cells in 5–20% sucrose gradients containing 0.5 M Tris·HCl–0.5 M EDTA, pH 7.2. The lysates were centrifuged at 25,000 rpm for 5 hr in a Spinco SW25.2 rotor at 5°. (*a*) After lysis in 1 M NaCl–0.025 M Tris·HCl–0.025 M EDTA, pH 7.0. (*b*) After lysis in 0.25 M Tris-saturated EDTA, pH 7.2. The *arrows* indicate the position of ^{32}P-labeled phage or single-stranded (ss) DNA markers. O, [^{3}H]dThd; ●, infectivity, in plaque-forming units, pfu. Sedimentation is from right to left.

Membrane Labeling. HF4704 was grown at 37° in TPG + 10 μg/ml of thymine + 10 μg/ml of adenine. When the culture reached a concentration of 5 × 10^7 cells per ml, [*2*-^{3}H]glycerol was added to a final concentration of 3.2 μCi/ml. When the cell density reached 2 × 10^8 cells per ml, the cells were exposed to ultraviolet irradiation for 5 min at 19 ergs/mm^2 per sec, aerated for 10 min and then infected with *am*3 phage (multiplicity of infection = 5). [^{14}C]Leucine was added immediately after infection to a concentration of 1.6 μCi/ml. The infected culture was aerated for 80 min, and the cell contents were analyzed as described above.

RESULTS

Isolation of the Fast Sedimenting Component. Fig. 1*a* and *b* shows the sedimentation patterns of an *am*3-infected HF4704 culture after lysis in 1 M NaCl–0.025 M Tris·HCl–0.025 M EDTA, pH 7.0, and 0.25 M Tris·HCl–saturated EDTA, pH 7.2, respectively. The phage DNA had been labeled, late in infection, with [^{3}H]dThd. In Fig. 1*a* the only labeled component found is the normal, infective 114S particle. This is the usual result, although occasionally a minor amount (about 10%) of free single-stranded DNA can also be observed.

When the lysis is performed in 0.25 M Tris·HCl–saturated EDTA, pH 7.2 (Fig. 1*b*), however, the sedimentation pattern becomes more complex. In addition to the 114S particle we find 140S, 70S, and free single-stranded DNA components. Sometimes a peak at 100 S is also found (not shown). The relative amounts of each peak vary somewhat in different experiments.

When the different fractions are diluted in 1 M NaCl–0.025 M Tris·HCl–0.025 M EDTA, pH 7.0, and tested for their plaque-forming ability, only the 140S peak and the 114S peak are infective. If the dilution is performed in 0.1 M NaCl–0.025 M Tris·HCl–0.025 M EDTA, pH 7.2, only 1/20 as much infectivity is recovered from the 140S peak. The DNA of the 140S component hss been analyzed in neutral and alkaline sucrose gradients and by CsCl equilibrium centrifugation and is a circular single-stranded molecule of the viral (+) type.

The broad peak around 70 S has been described previously (11–13) and consists of particles with DNA extruded out from the phage coat or of particles with only a small amount of DNA. Because of its lack of infectivity and its instability (it is converted into 70S particles) the 100S peak is thought also to be a damaged phage particle.

To test whether the 140S component might be only the product of an aspecific interaction between the 114S particle and a host component, we added purified mutant 114S phage particles to the infected cells before lysis. After lysis (in 0.25 M Tris·HCl–saturated EDTA, pH 7.2), the lysate was analyzed by sucrose gradient centrifugation and it was found that the added phages had retained their original sedimentation value of 114 S.

Stability and Conversion of the 140S Component. When the 140S component in 0.25 M Tris·HCl–saturated EDTA was stored for 2 weeks at 4°, about 50% was converted into a mixture of 114S and 70S particles. This instability was studied by dialysis for 24 hr of pure [^{3}H]dThd-labeled 140S particles against different solutions (Fig. 2). In 1 M NaCl the majority of the 140S particles are converted into 114S phage with a small amount of 70S material (Fig. 2*a*). After dialysis against 0.4 M NaCl (Fig. 2*b*), a larger 70S peak is observed and a peak of free single-stranded DNA appears. In 0.1 M NaCl (Fig. 2*c*) the 140S peak has completely disappeared; also the 114S peak has nearly disappeared and almost all the label is found in the 70S and the free single-stranded DNA peaks. After dialysis against 1 M NaCl–0.1 M $MgCl_2$–0.025 M Tris·HCl, pH 7.2, the only labeled particle found is the 114S phage (Fig. 2*d*). Thus, the addition of Mg^{++} is an efficient way to convert the 140S particle into phage, whereas the presence of EDTA is always required for the conservation of the 140S particle.

Similar experiments were performed with [^{14}C]leucine-labeled 140S particles; the results were the same, although of course the free single-stranded DNA peak was not detected. These experiments clearly indicate that the 140S particle can be, *in vitro*, a precursor of the normal phage particle.

Protein Composition. The different components of Fig. 1*b* were prepared with label in their proteins and isolated. The proteins were then analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Purified *am*3 phage

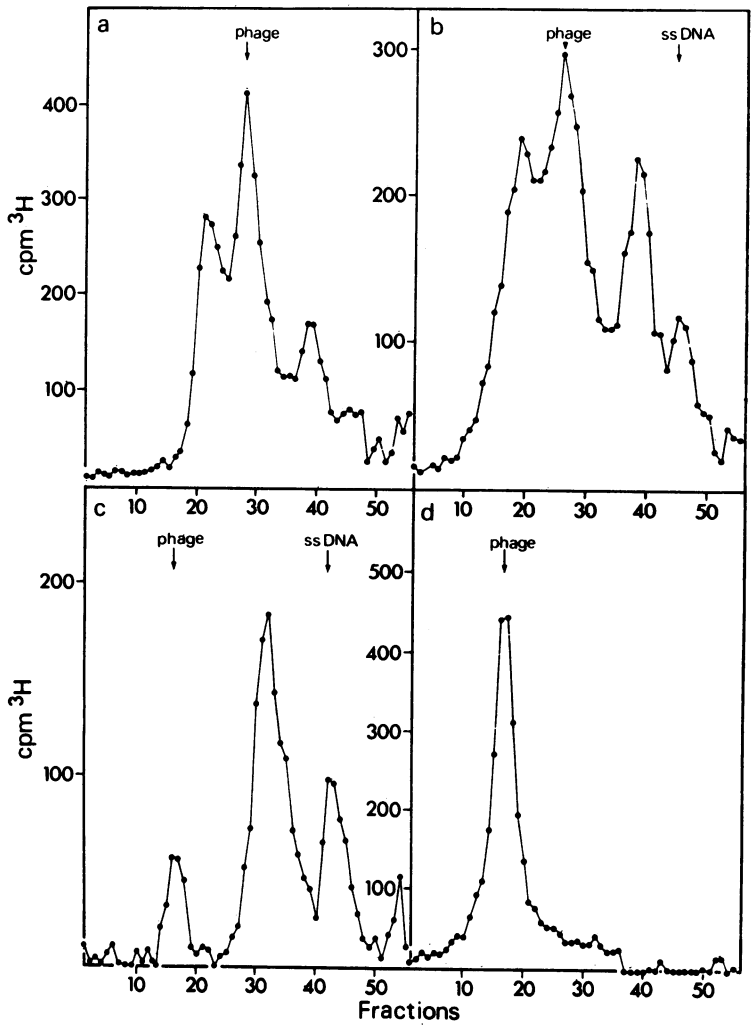

FIG. 2. Sedimentation in 5–20% sucrose gradients containing 0.5 M Tris·HCl–0.5 M EDTA, pH 7.2, of the [³H]dThd-labeled 140S component after dialysis against (*a*) 1 M NaCl–0.025 M Tris·HCl, pH 7.2; (*b*) 0.4 M NaCl–0.025 M Tris·HCl, pH 7.2; (*c*) 0.1 M NaCl–0.025 M Tris·HCl, pH 7.2; and (*d*) 1 M NaCl–0.1 M $MgCl_2$–0.025 M Tris·HCl, pH 7.2. Centrifugation was performed in a Spinco SW65 rotor for 35 min at 55,000 rpm. ³²P-labeled phage and single-stranded DNA markers were used. Sedimentation is from right to left.

particles or lysates of ultraviolet-irradiated cells infected with wild-type phage (14) were used as controls.

Phage particles are known to contain four major proteins, of which three are well defined as the products of the genes *F*, *G*, and *H* and the fourth has been attributed to gene *J* (9). The major φX-proteins of normal lysates are F, H, G, J, and D.

Fig. 3 shows the migration patterns derived from the various phage components of Fig. 1*b*. The proteins of the 114S particle (Fig. 3*b*) are found in four peaks that co-migrate with the peaks of the F, H, G, and J proteins of the purified phage; so also in this respect the 114S particle is indistinguishable from the normal phage. The proteins of the 140S component (Fig. 3*a*) are found in five peaks; in addition to the peaks of the F, H, G, and J proteins, a large protein peak is found at the position of the gene *D*-protein. The gene *D*-protein is also present in the 100S component (Fig. 3*c*) and to a much lesser amount also in the 70S particle (Fig. 3*d*).

The amount of *D*-protein associated with the 140S particle is rather variable; in different experiments the label in the *D*-protein comprised between 12 and 22% of the total of the combined peaks. This observation and the loss of *D*-protein when the 140S particles are converted into 114S phage suggest a loose attachment of *D*-protein to the 140S particle, such that a portion of the *D*-protein may, variably, be lost during isolation.

If we assume that the other proteins are present in the same molar amounts as found in the 114S phage, we can calculate from Fig. 3*b* that the number of molecules of *D*-protein (molecular weight 14,000) present per 140S particle is about 80.

Membrane Association. When phage DNA molecules are associated with cell membranes their sedimentation rate is increased (16). Although the difference in sedimentation rates found here is rather small, as compared to the rates of membrane-associated DNA, there was a possibility that part of the increase in $s_{20,w}$ might be caused by an association of the 140S component with some host membrane. This possibility was tested by labeling of the host membranes with ³[H]glycerol, infection of the cells with phage, and isolation of the 140S component. The result of such an experiment is shown in Fig. 4. The label derived from glycerol is observed in a component at about 40 S and is also found distributed through the entire

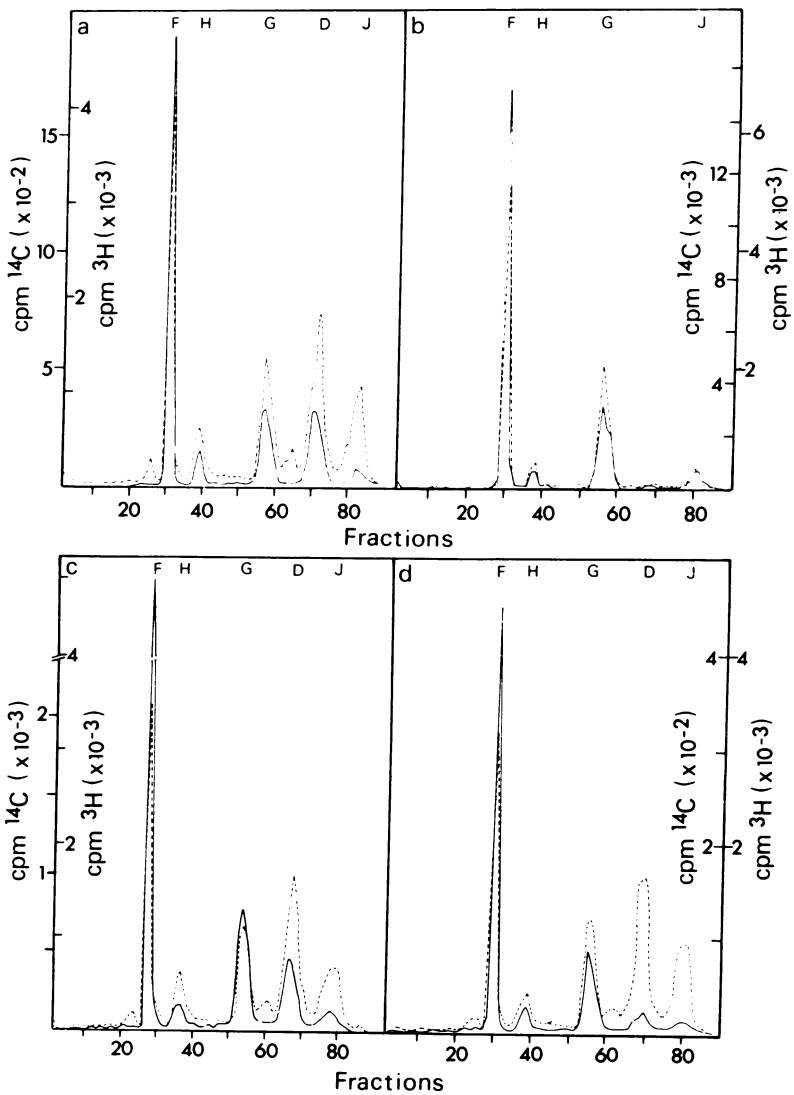

FIG. 3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of (*a*) 140S, (*b*) 114S, (*c*) 100S, and (*d*) 70S particles. All samples are [^{14}C]leucine-labeled. To (*a*), (*c*), and (*d*) was added [^{3}H]leucine-labeled lysate from cells infected by wild-type phage; to (*b*), purified [^{3}H]leucine-labeled phage. Migration is from left to right. *Solid line*, ^{14}C; *broken line*, ^{3}H.

gradient. There are, however, no peaks of label seen at the positions of the different φX particles. Although this result does not exclude the presence of a minor amount of membrane, it seems reasonable to assume that no membranes are present in the 140S particle.

DISCUSSION

The labile 140S component can be, *in vitro*, quite efficiently converted into a stable 114S phage particle. The 114S particle thus derived is identical to normal φX particles with respect to infectivity, thermal stability, buoyant density, and protein composition. The irreversibility of this process, i.e., no 114S phage is changed into 140 S when added during lysis, makes it likely that also *in vivo* there is only a precursor–product relationship between these two. We assume, therefore, that the 140S particle is an intermediate in the mature phage formation.

If so, it follows from the high proportion of phage material existing as this 140S particle that in the cell it is only slowly processed into phage (if at all—it could well be that the intact phage found normally are only produced during or after lysis of the cell). We have recently isolated a phage mutant that produces, after artificial lysis, only the 140S component (to be published); after incubation in the proper medium this component is fully converted into phage.

By means of protein labeling we have demonstrated that gene *D*-protein is present in large amount (up to 22% of the protein content) in this 140S particle. We could not establish whether this *D*-protein is attached to the DNA or to the virion coat, although the small amount of free single-stranded DNA formed after decomposition of the 140S particle, when incubated in low salt concentration (Fig. 2*d*) is not associated with phage protein. The 114S phage particles do not contain *D*-protein, so, upon conversion of the 140S particle into normal phage, all of the *D*-protein is removed.

The removal of *D*-protein, the large increase in stability when converted into 114S phage, and, alternatively, the easy conversion into 70S particles with DNA extruding out of the virion, suggest a model for the structure of the 140S particle and for the last steps in φX174 phage formation. In the 140S particle the DNA is not yet fully encapsulated by the coat and is still associated with *D*-protein. When phage is

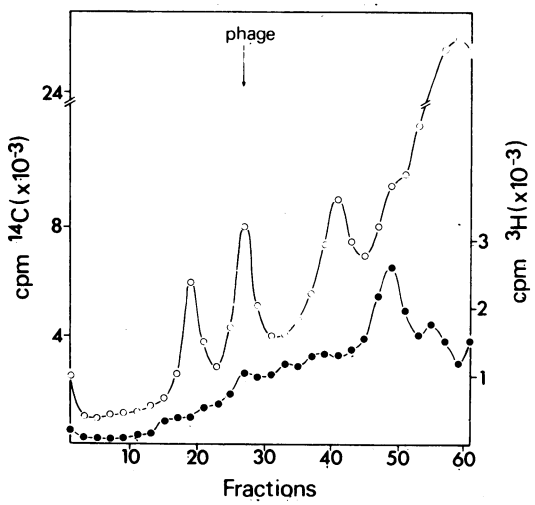

FIG. 4. Sedimentation in 5–20% sucrose gradients containing 0.5 M Tris·HCl–0.5 M EDTA, pH 7.2, of a [^{3}H]glycerol and [^{14}C]leucine-labeled lysate of *am*3-infected HF4704 cells. The lysate was centrifuged at 25,000 rpm for 5 hr in a Spinco SW25.1 rotor at 5°. ^{32}P-labeled *am*3 phage was used as sedimentation marker. ○, ^{14}C; ●, ^{3}H. Sedimentation is from right to left.

to be formed, the *D*-protein is removed and the DNA becomes completely enclosed by the coat. If the last step is not performed properly, part of the DNA is not enclosed and is then susceptible to nuclease degradation, resulting in 70S particles with small amounts of DNA.

This process resembles somewhat the final steps of phage assembly of the filamentous single-stranded DNA phages. Here a single-stranded DNA–gene 5 protein complex is found, which is converted into an intact phage with a complete stripping-off of the gene 5-protein (3, 15). Possibly the *D*-protein and the gene 5-protein have a similar function, i.e., protection of the nascent single-stranded DNA from duplication or degradation. There is, however, no direct evidence for this possibility.

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