*THE RECONSTITUTION OF INFECTIVE BACTERIOPHAGE R17**

By JEFFREY W. ROBERTS† AND JOAN E. ARGETSINGER STEITZ†

THE BIOLOGICAL LABORATORIES, HARVARD UNIVERSITY, CAMBRIDGE, MASSACHUSETTS

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Bacteriophage R17 contains two major components: a single RNA molecule of molecular weight 1.1×10^6,[1] and approximately 180 identical coat protein subunits of molecular weight 14,000.[2] This apparently simple structure invites attempts to reassemble the phage from isolated RNA and coat protein in analogy with the classic reconstitution of tobacco mosaic virus.[3] Such reconstitution experiments have been performed with the closely related phages MS2[4] and fr,[5] yielding particles which appeared similar to normal bacteriophage in electron micrographs. However, the MS2 particles had no significant infectivity, and the fr assembly produced only a slight increase in infectivity over the isolated components. In addition, the assembled particles in both cases sedimented at 70*S* in sucrose density gradients, whereas normal phage sediments at 80*S*.

The failure to achieve significant infectivity in these reconstitution experiments was not surprising in view of various genetic and physiological evidence which has long suggested that another structural component might be present in mature RNA phage particles. First, studies of RNA phage amber mutants have revealed that uninfective progeny particles are produced by phages whose genomes contain a mutation in the A cistron,[6–8] which can be distinguished genetically from the cistrons encoding coat protein and the phage-specific RNA synthetase.[9, 10] Second, the use of polyacrylamide gel electrophoresis has both detected the protein encoded by the A cistron within phage-infected cells[11, 12] and provided hints that the A cistron product itself is present in small amounts in the virus particle.[12] Aided by the fact that the A cistron protein contains histidine, whereas the coat protein does not, one of us has shown recently[13] that it is present in wild-type R17 and absent from the defective particles produced by A cistron amber mutants. These experiments, and the fact that both the defective particles[14] and the reassembled particles of MS2 and fr sediment at 70*S*, suggested that the uninfective reconstituted particles might differ from normal bacteriophage by lacking the A protein.[4, 5]

Subsequent work has now led to the purification of the A protein.[13] It has a molecular weight of approximately 35,000 and is present probably in only one copy per phage particle. It is highly insoluble in aqueous buffers and can be obtained at high concentrations only in denaturing solvents. Possession of the A protein provides the opportunity to perform reconstitution experiments with all the known components of an RNA bacteriophage present in the assembly mixture. Here we report such experiments in which a significant synthesis of infective particles occurs only when isolated A protein is added to the assembly mixture.

Materials and Methods.—Phage and bacterial strains: Bacteriophage R17, and S26, the Hfr bacteria on which it was grown, were described previously.[10] Infectivity assays were performed on S26 or on S26RIE, a strain isogenic with S26 but containing the amber suppressor Su_I.[15] The coat protein amber mutant *am*B_2 was isolated by G. Gussin.[16, 10]

Purification of bacteriophage: R17 was purified according to the method of Gesteland and Boedtker,[1] with the addition of a second CsCl banding step.

Buffers and enzymes: The basic buffer, TSE, contains 0.02 *M* Tris HCl, 0.15 *M* NaCl, and 0.001 *M* ethylenediaminetetraacetate.[5] The dialysis fluid used for reconstitution was TSE, pH 9.0, supplemented with 1 per cent β-mercaptoethanol and 0.01 *M* $MgCl_2$. RNase was Sigma, 5 × recrystallized.

RNA: R17 RNA was purified from a suspension of phage at a concentration of 3.4 mg/ml in TSE, pH 7.0, by three phenol extractions followed by three ethanol precipitations. It was finally dissolved in TSE, pH 7.0, at a concentration of 1.0 mg/ml. Bentonite was not used. The mutant $am B_2$ RNA was a gift of M. Capecchi.

Coat protein: R17 coat protein was prepared by the procedure of Sugiyama *et al.*[4] One volume of R17 at a concentration of 17 mg/ml in 0.15 *M* NaCl, 0.015 *M* sodium citrate, pH 7.0, was added gradually to two volumes of glacial acetic acid at 0°C, the precipitated RNA was removed by centrifugation, and the supernate was dialyzed against three changes of 10^{-3} *M* acetic acid. This procedure yielded a stable protein solution at a concentration of 1.1 mg/ml at pH 4.2.

A protein: The procedure used to purify A protein will be published in detail elsewhere.[13] Basically the procedure consists of solubilizing purified phage in 6 *M* guanidinium HCl, dialyzing into 8 *M* urea, and chromatographing the mixture on cellulose phosphate. The A protein used for reconstitution was eluted from the phosphocellulose column with a linear NaCl gradient and was dissolved in 8 *M* urea, 0.05 *M* Tris HCl, pH 8.5, and 0.2 *M* NaCl. The concentration of A protein was estimated by assuming an extinction coefficient of 1.0/mg/ml at 280 mμ.

Dialysis membrane: Dialysis tubing (Union Carbide, 23/32) was prepared by boiling successively in 2 per cent Na_2CO_3, distilled water, 0.002 *M* EDTA, and distilled water again.

Sucrose gradient analysis: Five-ml, 5 to 20 per cent sucrose density gradients in TSE, pH 7.0, were centrifuged one hour at 50,000 rpm in the Spinco SW50 rotor. Approximately 20 three-drop fractions were collected.

Results.—Our basic reconstitution experiment consists of combining the three phage components in the presence of 5.7 *M* urea, dialyzing the reaction mixture for at least two hours against two changes of buffer, and then assaying directly for infectivity. A complete experiment is summarized in Table 1; for controls, we

TABLE 1

DEPENDENCE OF THE RECONSTITUTION OF INFECTIVE R17 UPON ADDED A PROTEIN

	Combined before Dialysis				
	RNA	Coat protein	A protein	Other	Resulting plaque-forming units/ml
1*A*	+	+	+	−	2.7×10^7
1*B*	+	+	+	−	2.7×10^7
2*A*	+	+	−	−	4.3×10^4
2*B*	+	+	−	−	1.2×10^5
3*A*	+	−	+	−	1.2×10^3
3*B*	+	−	+	−	9×10^3
4*A*	−	+	+	−	$<10^2$
4*B*	−	+	+	−	$<10^2$
5	+	+	+	5 μg/ml RNase	$<10^2$

Components were mixed at 0°C, placed in dialysis sacs, and dialyzed overnight at 4°C against two changes of TSE, pH 9, containing 1% β-mercaptoethanol and 0.01 *M* $MgCl_2$. Each reaction mixture was then assayed for infectivity on strain S26. The following quantities of the three components were used: 50 μg RNA from a 1.0 mg/ml solution; 80 μg coat protein from a 1.1 mg/ml solution; and 2 μg A protein from a 0.10 mg/ml solution. A buffer containing 8 *M* urea in 0.05 *M* Tris, pH 8.5, was added to yield a final urea concentration of 5.7 *M*. When any constituent was omitted, an equivalent volume of its solvent was added to the assembly mixture. *A* and *B* refer to duplicate experiments.

mixed and dialyzed all three combinations of two components taken together. The addition of A protein increases the yield of infective phage by several hundred-fold over the assembly mixture from which A protein is omitted, a result which has been obtained repeatedly with different preparations of all three components. The yield is not increased further by adding more than the indicated amount of A protein, which represents one A protein molecule per RNA molecule. In other experiments we have obtained up to 1.3×10^8 infective phage per milliliter, representing an efficiency of conversion of RNA strands to infective particles of 2×10^{-6}.

Although the inclusion of all three components in the reaction mixture is necessary for a full yield of infective particles, more infectivity is obtained when either protein is omitted than when RNA is omitted; this is consistent with the fact that neither protein is completely free of the other, as has been determined by polyacrylamide gel electrophoresis. The A protein, which is taken directly after elution from the phosphocellulose column, may contain up to 50 per cent coat protein. The coat protein purification does not specifically remove the A protein, although much of it is lost during the dialysis steps of the purification procedure. Any of the components plated alone gives less than one hundred plaques per milliliter of reaction mixture.

As a further demonstration that each component of the reaction mixture contributes to the infective assembled particles, we performed an assembly with RNA prepared from an R17 amber mutant, *am*B_2,[16, 10] which grows only in a bacterial strain containing an amber suppressor. The mutant phage stock from which the RNA was isolated contained about one-tenth per cent wild-type revertants, which grow equally well in suppressing and nonsuppressing bacteria. For the assembly we used coat protein and A protein prepared from wild-type phage. Table 2 reveals that the reconstituted infective particles have the genotype of the input RNA; only about one-tenth per cent of the assembled particles which plate on the suppressing bacteria also plate on the nonsuppressing bacteria.

Part of a reaction mixture which included A protein was analyzed on a sucrose gradient in order to determine the sedimentation constant of the infective particles relative to normal R17 and to the bulk of the particles made. The result is shown in Fig. 1*A*. The optical density profile reveals that most of the assembled particles sediment at 70*S*, indicating that they are probably identical to the particles of MS2 and fr which were assembled without added A protein. This is not surprising since a relatively small fraction of the particles is infective even with added A protein. A second peak, at about 30*S*, represents both intact RNA molecules and a complex of RNA and a few coat protein molecules.[16, 4] In contrast to the optical

TABLE 2

RECONSTITUTION WITH *am*B_2 RNA

Combined before Dialysis			Resulting Plaque-Forming Units/ml	
amB_2 RNA	Coat protein	A protein	S26RIE (Su^+)	S26 (Su^-)
+	+	+	1.1×10^6	1×10^3
+	+	−	3×10^3	$<10^2$

The procedure was as in Table 1, except that the reaction mixtures were assayed for infectivity on both S26 (Su^-) and S26RIE (Su^+), a strain which contains the amber suppressor Su_I. The following quantities were used: 50 μg *am*B_2 RNA from a 1.0 mg/ml solution; 80 μg coat protein from a 1.1 mg/ml solution; and 1 μg A protein from a 0.11 mg/ml solution. Sufficient 8 *M* urea in 0.05 *M* Tris, pH 8.5, was added to yield a final urea concentration of 5.7 *M*. The *am*B_2 RNA was purified in the presence of Macaloid (see text), which probably accounts for the lower titers than those found in Table 1.

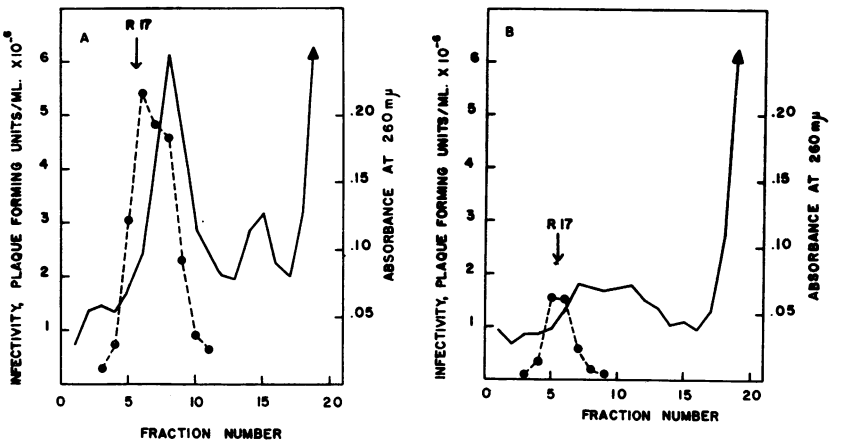

FIG. 1.—Sucrose gradient analysis of a reconstitution reaction mixture. (*A*) A 200-μl portion of a reaction mixture which was prepared as in Table 1 and yielded 4×10^7 plaque-forming units per milliliter was centrifuged on a sucrose density gradient as described in *Materials and Methods*. A 50-μl portion was taken from each fraction for infectivity assays on S26; the remainder of each fraction was diluted with 0.16 ml TSE, pH 7.0, and its A_{260} determined. The position of radioactive R17 run in a parallel gradient is indicated by the arrow. —— A_{260}; - - -●- - - infectivity. (*B*) A 200-μl portion of the same reaction mixture was analyzed in *A* was treated with 5 μg/ml of RNase for 5 min at room temperature before it was layered on a parallel gradient.

density peak, the main infectivity peak is coincident with an R17 marker run in a parallel gradient, although it has a shoulder which coincides with the peak of assembled particles. Thus the majority of infective particles made are identical to normal R17 by this criterion.

It is interesting that some of the assembled infective particles sediment more slowly than normal R17. A mild RNase treatment (Fig. 1*B*) of the reaction mixture before it is layered on the gradient destroys all infectivity associated with these particles and produces a sharp peak of infectivity which coincides with the R17 marker. In addition to the slower particles, a considerable fraction of the reassembled phage which sediment at 80*S* is sensitive to RNase, whereas normal R17 is almost totally insensitive to RNase treatment. These results suggest that many of the infective reconstituted particles are not quite normal, possibly because they contain components which are partially damaged.

The yield of infective particles obtained in our reconstitution system does not depend upon the order of addition of the isolated components to the reaction mixture; in the presence of 5.7 *M* urea before dialysis all components are probably in a denatured state. Efficient reconstitution does require the inclusion of a divalent cation and the presence of a large amount of β-mercaptoethanol (1%) in the dialysis liquid. Omission of either reduces the yield of infective particles by an order of magnitude. Magnesium was used as the cation, although calcium appears to serve equally well. An attempt was made to use Macaloid (a clay similar to Bentonite) in the reaction mixture to prevent RNase action; this completely eliminates the assembly of infective particles. Possibly these materials scavenge the A protein or coat protein and thus prevent the reconstitution.

In our best experiments the efficiency of conversion of RNA strands to infective particles is about 2×10^{-6}. Sucrose gradient analysis revealed that in these experiments not all of the RNA was converted to particles, so that the fraction of particles which were infective was somewhat higher than this. It should be noted that only about 10 per cent of the particles in most purified R17 preparations are active, which may mean that a corresponding fraction of the components is defective originally.

Discussion.—These experiments show that the inclusion of A protein in a reconstitution mixture containing RNA and coat protein from bacteriophage R17 increases the yield of infective particles by more than two orders of magnitude over a similar mixture which does not contain A protein. Although Hohn[5] has reported a small increase in infectivity in the assembly of bacteriophage fr from RNA and coat protein alone, and we find some infective centers when A protein is not added to the reaction mixture, it is likely that both of these increases result from residual A protein in the coat protein preparation.

If several limitations of the assembly system were overcome, the efficiency of reconstitution of infective particles might be increased considerably. First, RNase may be present in the assembly mixture; even RNA which remains intact enough to form particles could be damaged beyond biological integrity by RNase activity. The problem could likely be eliminated by growing the phage used to prepare all the isolated components in a bacterial strain lacking RNase I;[17] such a strain was not used in these experiments because it complicates the purification of the A protein. Second, all of the phage components used in reconstitution have suffered harsh treatment during their isolation and thus may be partially inactive. This is particularly true of the A protein, which has been exposed to both guanidine and urea. The participation of slightly damaged components in reconstitution could account for the appearance of RNase-sensitive infective particles which sediment both more slowly than and at the same rate as normal phage. Finally, experiments with radioactive A protein have shown that it adheres strongly to the dialysis membrane. This interaction may either denature it or effectively remove it from the reaction mixture and could explain the disparity between the results obtained in the duplicate experiments of Table 1, since such minor variables as the total membrane surface of each dialysis sac would become important.

In contrast to the reconstitution of tobacco mosaic virus from RNA and coat protein[3] and the recently reported assembly of cowpea chlorotic mottle virus,[18] the reconstitution of infective R17 requires that a second protein take its designated position in the viral structure. We do not know the exact function of the A protein; however, it is known that defective particles made *in vivo* by phage amber mutants which produce no A protein fail to adsorb to the bacterial host.[6, 7] Thus the A protein may itself be an adsorption organelle, or it may simply be a structural component which must be present in order that the over-all shape of the particle be recognized by the attachment site of the host. The experiments reported here, in any case, offer firm proof that the A protein is a necessary constituent of infective bacteriophage.

Summary.—Infective particles have been reconstituted from the three isolated components of the RNA bacteriophage R17. This was made possible by the isolation and purification of a minor viral protein component, the A protein. When it

is added to an assembly mixture containing viral RNA and coat protein, a several hundredfold stimulation in the yield of infective particles results. The A protein is thus shown to be a necessary constituent of the infective bacteriophage.

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