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DNA replication *in vitro* starting with an intact φX174 phage

(membranes/phage infection)

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ABSTRACT **Conversion of the single-stranded DNA in the intact φX174 phage particle to the duplex replicative form (RF) has been demonstrated in lysates from phage-sensitive cells. The conversion is resistant to rifampicin and requires participation of both a "membrane" fraction of the lysate and a multienzyme replicative system. The lipopolysaccharide phage receptor, while essential, does not replace the membrane fraction. Clear, nonsedimentable extract fractions prepared with a certain nonionic detergent can replace the membrane fraction. Purification of the activity in these extracts by adsorption to polypropylene film yields a fraction with a 5-fold increase in activity relative to lipopolysaccharide and 50-fold increase relative to protein. The low buoyant density (1.03 g/cm^3) suggests a high phospholipid or detergent content in this fraction.**

φX174 is an icosahedral coliphage with spikes at the 12 vertices and contains a single-stranded (SS), circular DNA (1). Infection proceeds through stages of phage binding, eclipse, and DNA penetration (2) dependent on the presence of the correct lipopolysaccharide (LPS) phage receptor at the cell surface (3). The initial event in viral DNA replication, synthesis of the double-stranded, circular, parental-replicative form (RF), is tightly coupled to DNA penetration (4), and requires no new protein synthesis (1). The parental gene H spike protein, the phage adsorption protein (5), is in some way involved in the conversion of the DNA of intact φX174 phage particles to RF (φX → RF) *in vivo* (6).

Progress in the dissection and understanding of the host enzymes directing the conversion of naked φX174 SS to RF *in vitro* (7) has led us to inquire about the host and viral requirements for uncoating and replicating the DNA of intact φX174 phage particles. To this extent we have sought to devise an *in vitro* system for studying RF formation starting with intact phage particles (φX → RF). We have found and we report on two such systems and their properties.

φX → RF conversion in cell extracts prepared with nonionic detergents requires a "membrane" fraction and soluble, replicative enzymes. One of the membrane components required is the LPS phage receptor. Adsorption to polypropylene film from crude cell extracts leads to a partial purification of the φX → RF activity.

MATERIALS AND METHODS

Pluronic P103 was a gift from BASF Wyandotte (Wyandotte, Mich.); Celgard 2400W polypropylene film was a gift from Celanese Plastics Co. (Greer, S.C.). Bacterial and phage strains and sources, as well as preparation of radioactively labeled phage, have been described (3, 8). ^{32}P-labeled φX174 (*am3*) preparations contained less than 6% eclipsed particles.

Extracts and Fractionation. *Escherichia coli* cells were grown in M medium (3) to 0.5 A_{595} at 37° on a shaker. Cells were sedimented for 10 min at 20° and resuspended in 0.01 volume of Buffer A (10% sucrose–50 mM Tris·HCl, pH 7.5). The suspension was frozen in liquid N_2 and thawed at 0° in 250-μl aliquots. Cells were converted to spheroplasts by incubation with lysozyme (200 μg/ml) and 0.1 M NaCl for 30 min at 0°. Triton X-100 (10%) was added to a concentration of 0.5% and the spheroplasts were incubated for 70 sec at 30°. Over 95% of the cells were lysed by this treatment, as determined in a phase-contrast microscope. The suspension (lysate) was kept at 0° or sedimented at 30,000 × *g* for 30 min (0°) and the pellet ("membranes") and supernatant kept at 0°. Pluronic P103 replaced Triton X-100 in some instances. Extracts were fractionated by adsorption to polypropylene film.

Strips (7 × 4 mm) were added (15 strips per ml of Pluronic extract) and the extract was kept at 0° for 12 hr. The extract was then withdrawn (supernatant) and the strips were eluted with an equal volume of 1% Pluronic P103 in Buffer A for 8 hr at 0° (eluate). Replicative fractions I and II were prepared from *E. coli* H560 (9).

Velocity Sedimentation. Samples were applied to 5–20% linear sucrose gradients, either neutral (10 mM Tris·HCl, pH 8–1 mM EDTA–1 M NaCl) or alkaline (0.2 M NaOH–1 mM EDTA–0.8 M NaCl). Gradients were formed over a 6.5 M CsCl shelf. Neutral gradients were centrifuged for 16 hr at 24,000 rpm (5°) in a Spinco SW27 rotor. Alkaline gradients were centrifuged for 12 hr at 36,000 rpm (5°) in a Spinco SW41 rotor.

Other Methods. LPS receptor from *E. coli* HF4704 was prepared as described (3). Protein was determined according to Lowry *et al.* (10). LPS was determined according to Janda and Work (11) with *E. coli* HF4704 LPS as standard.

RESULTS

DNA of intact φX174 phage particles is converted to RF in cell-free extracts

Cells (*E. coli* HF4704, sensitive to φX174) were converted to spheroplasts and lysed with the nonionic detergent Triton X-100. Such lysates, fortified with Mg^{2+} and ribo- and deoxynucleoside triphosphates, converted the DNA of intact φX174 phage particles to RF (Table 1). Neither the sedimentable ("membrane") fraction of the lysate by itself nor the soluble replicative enzyme system in the supernatant which converts SS to RF (12) was capable of carrying out this conversion (Table 1). When the "membrane" fraction was recombined with the soluble replicative enzymes, full activity was restored (Table 1, Exp. 1). The soluble enzymes could be supplied as the supernatant fraction of the deter-

Abbreviations: SS, (phage) single-stranded DNA; RF, (phage) double-stranded circular replicative form; RF I, (phage) covalently closed RF; RF II, (phage) RF with a discontinuity in at least one strand; φX → RF, conversion of DNA of intact φX174 phage particles to RF; LPS, lipopolysaccharide; Buffer A, 10% sucrose–50 mM Tris·HCl (pH 7.5); Buffer B, 10% sucrose–50 mM Tris·HCl (pH 7.5)–50 mM NaCl–50 mM dithiothreitol–0.4 mg/ml of bovine serum albumin; PFU, plaque forming units.

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Table 1. $\phi X \rightarrow$ RF conversion in Triton X-100 lysates

Exp.	Lysate	Membranes	Enzyme fraction	Phage	RF (% of total)
1	+	—	—	+	57
	—	+	—	+	<1
	—	—	Supernatant	+	<1
	—	+	Supernatant	+	46
2	+	—	—	+	41
	—	+	I	+	42
	—	+	II	+	67
3	+	—	—	+	47
	—	—	I	+	<2
	—	—	I	LPS complex	<1
	+	—	—	LPS complex	<10
4	+	—	—	+	28
	+[a]	—	—	+	36
5	+	—	—	+	36
	+	—	—	+[b]	22
	+[c]	—	—	+	<1
	—	+[d]	II	+	37

Triton-lysate and the membrane and supernatant fractions from this lysate were prepared from *E. coli* HF4704 cells as described in *Materials and Methods*. Membranes were resuspended either in Buffer A (for incubation without Fraction I or Fraction II), in Fraction I, in supernatant, or in Buffer B (10% sucrose-50 mM Tris·HCl, pH 7.5–50 mM NaCl–50 mM dithiothreitol–0.4 mg/ml of bovine serum albumin) for incubation with Fraction II. Phage-*E. coli* HF4704 LPS complex was prepared by incubating ^{32}P-labeled ϕX174 *am3* (1.5×10^{11} PFU) with 100 μg of LPS in 300 μl of 10 mM Tris·HCl, pH 7.5–1 mM EDTA–3 mM $CaCl_2$ for 15 min at 37°. To the lysate, supernatant, or membranes were added: 25 μl of 100 mM $MgCl_2$; 50 μl of a mixture of rNTPs (10 mM ATP and 1 mM each of CTP, GTP, and UTP); 50 μl of a mixture of dNTPs (1 mM each dATP, dCTP, dGTP, and dTTP); 50 μl of Fraction II (where indicated); and 100 μl of ^{32}P-labeled ϕX174 *am3* phage or phage-LPS complex. Phage was added at 5 PFU per cell equivalent and phage-LPS complex was added at 4 PFU per cell equivalent. The final volume of the mixture was 520 μl. Incubation was performed for 20 min at 30°. The tubes were placed on ice, and 0.5 M EDTA and 10% sodium dodecyl sulfate were added to a concentration of 20 mM and 1%, respectively. Incubation was then continued for 15 min at 37°. The samples were chilled to 0°; [^{3}H]thymine-labeled M13 phage was added as marker; samples were applied to neutral sucrose gradients. Gradients were fractionated directly into vials in 6-drop fractions, and radioactivity was measured in Triton-toluene scintillation fluid. Results are expressed as % RF in the gradients based on the recovered ^{32}P radioactivity (81–97% of the ^{32}P label applied to gradients).

[a] Rifampicin was present at 10 μg/ml.

[b] Fifty plaque forming units (PFU) per cell equivalent added instead of 5.

[c] Prepared from a ϕX174-resistant strain (*E. coli* 5274).

[d] Membranes were washed in Buffer A at 0°.

gent-lysate, or another particle-free extract (Fraction I), or an ammonium sulfate fraction of the extract (Fraction II) (Exp. 2). The activity present in the "membrane" fraction was not removed by washing with buffered solutions (Exp. 5). This ϕX174 conversion to RF was also unaffected by rifampicin (Exp. 4), a specific inhibitor of RNA polymerase, just as is the *in vitro* conversion of naked ϕX174 SS (12). Lysates with similar properties were obtained when Brij-58 replaced Triton X-100 for spheroplast lysis (data not shown).

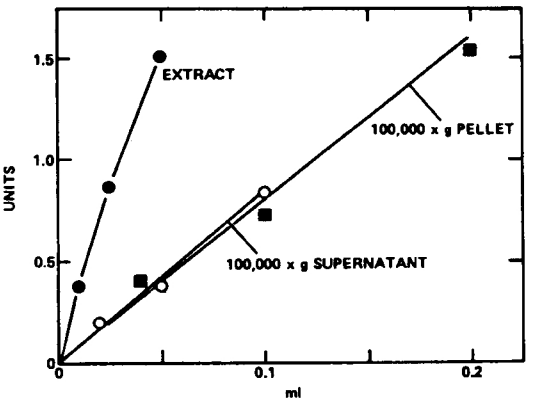

FIG. 1. $\phi X \rightarrow$ RF conversion in Pluronic extracts. Pluronic extract was prepared from *E. coli* HF4704 cells as described in *Materials and Methods*. The extract was then centrifuged for 4 hr at 40,000 rpm (0°) in a Spinco type 40 rotor in a polypropylene tube. The supernatant and the pellet, resuspended in an equal volume of Buffer A, were kept on ice. Activity was determined by incubating a sample of the fraction with: 25 μl of $MgCl_2$; 50 μl of rNTPs; 50 μl of dNTPs; 10 μl of Fraction II (a saturating amount); and 80 μl of ^{32}P-labeled ϕX174 *am3* phage (3.7×10^{11} PFU/ml), as in Table 1 made up to 475 μl with Buffer B (added first), for 20 min at 30°. The samples were then treated and analyzed by velocity sedimentation in neutral gradients as described in Table 1. % RF formed was based on the recovered radioactivity (86–102% of that applied to gradients).

It is unlikely that this conversion can be attributed to intact cells that survived lysis. *In vivo*, 20 to 30 phage particles per cell are uncoated and their DNA converted to RF (13). Inasmuch as at least 95% of the cells were lysed by the Triton treatment, a conversion of 22% of a phage input of 50 per cell equivalent (Table 1, Exp. 5) would have required that each intact cell remaining convert over 200 phage particles. This conclusion is further supported by the fact that the sedimentable fraction of the lysate was inert in $\phi X \rightarrow$ RF conversion.

Lysates prepared from cells lacking the receptor for ϕX174 (*E. coli* 5274) (3), were not capable of converting ϕX174 to RF (Table 1, Exp. 5). More than the LPS receptor component of the "membrane" fraction appears to be involved, since a complex of phage with purified LPS receptor was not converted to RF by the soluble replicative enzymes (Exp. 3). However, such a complex was converted poorly, if at all, by the lysate, suggesting that exogenous LPS inhibits conversion.

The product of $\phi X \rightarrow$ RF conversion was characterized as RF II on the basis of its sedimentation in neutral sucrose gradients, alkaline sucrose gradients, and buoyant density in neutral CsCl gradients (data not shown). Comparable analyses performed on the product obtained with another form of lysate are presented below.

"Soluble" extract for $\phi X \rightarrow$ RF conversion

The Triton-lysate described in Table 1 is viscous and difficult to manipulate. Another lysis procedure was therefore sought. When spheroplasts were treated with the nonionic detergent Pluronic P103, the cell DNA and the bulk of the cell membranes could be sedimented. The clear, nonviscous supernatant (Pluronic extract), fortified with soluble replicative enzymes, was capable of converting the DNA of intact ϕX174 phage particles to RF (Fig. 1). $\phi X \rightarrow$ RF conversion was dependent on Pluronic extract and soluble replicative enzymes (Fraction II); conversion was not detected when a

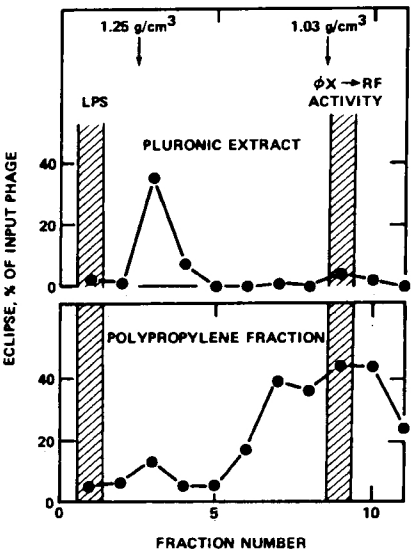

FIG. 2. Equilibrium sedimentation of $\phi X \rightarrow$ RF activity. Pluronic extract and polypropylene eluate were prepared from *E. coli* HF4704 cells (*Materials and Methods*) and 0.25 and 1.5 ml, respectively, applied to gradients composed of the following layers: 6.5 M CsCl, 70, 60, 50, and 25% sucrose (w/v) in 50 mM Tris-HCl (pH 7.5). The samples were overlayered with the same buffer. Gradients were centrifuged for 44 hr at 54,000 rpm (5°) in a Spinco SW56 rotor. Gradients were fractionated in 8-drop fractions. Phage-eclipsing activity was determined in 5- and 30-μl aliquots in the gradients in the top and bottom panels, respectively. $\phi X \rightarrow$ RF activity was determined as in the legend of Fig. 1 on the fractions indicated by the shaded zone. Another zone indicates the position at which purified *E. coli* HF4704 LPS banded in a parallel gradient.

Pluronic extract from ϕX-resistant cells (*E. coli* 5274) was used or when phage were incubated with purified LPS from *E. coli* HF4704 together with the detergent, Fraction II, Mg^{2+}, and ribo- and deoxynucleotides (data not shown).

Upon high-speed centrifugation, 21% of the activity was recovered in the pellet, and 23% was assayed in the supernatant (Fig. 1). When the supernatant and pellet fraction were combined, the activities were only additive (data not shown). As will be seen, this loss of activity was due largely to adsorption to the polypropylene centrifuge tube.

The activity of the extracts in eleven successive preparations was 38, 26, 45, 150, 12, 32, 22, 15, 20, 25, and 33 units/ml. A unit is defined as the conversion of 10^{10} particles ($\phi X \rightarrow$ RF) in 20 min at 30°. The activities of these Pluronic extracts ranged from the conversion of 2 to 30 phage particles per cell equivalent.

Fractionation and characterization of Pluronic extract

The loss of $\phi X \rightarrow$ RF conversion activity upon centrifugation of Pluronic extracts in polypropylene tubes (Fig. 1) suggested adsorption to polypropylene film as a possible fractionation procedure. Strips of polypropylene film (Celgard 2400 W) adsorbed nearly half of the $\phi X \rightarrow$ RF stimulating activity from extracts, and most of this was eluted with 1% Pluronic P103 (Table 2). In six successive preparations, the percent of the activity adsorbed and eluted from polypropylene film was 15, 29, 39, 40, 26, and 30. Exposure of the activity initially unadsorbed to fresh polypropylene strips resulted in improved recoveries.

Very little protein was adsorbed to polypropylene film (Table 2). Only 8% of the LPS was adsorbed (Table 2); for two other preparations, the values were 4 and 7%. Nevertheless, these fractions were, respectively, 60, 21, and 50% as active as the Pluronic extract in promoting eclipse of phage particles (determined by nuclease sensitivity after Sarkosyl treatment) (3). (The total phage-eclipsing activity recovered in the polypropylene supernatant and eluate fractions was 120–150% that of the Pluronic extract.) These data indicate that the LPS is heterogeneous in terms of its capacity to support phage eclipse. Whether this is due to physical heterogeneity, such as the state of aggregation, or to association with other cell components, is not clear. The fraction of LPS that is more efficient in promoting eclipse may be the same as that which participates in $\phi X \rightarrow$ RF conversion.

Table 2. Polypropylene fractionation of Pluronic extract

	Activity (units)	Protein (mg)	LPS (mg)	Specific activity: Units/mg of protein	Specific activity: Units/mg of LPS
Pluronic extract	45	3.4	0.12	13	375
Polypropylene:					
Supernatant	20	3.2	0.12	6	170
Eluate	17	0.025	0.01	680	1700

Pluronic extract, and polypropylene supernatant and eluate were prepared from *E. coli* HF4704 cells as described in *Materials and Methods*. Activity in the fractions was determined as in the legend of Fig. 1.

The Pluronic extract and the polypropylene eluate were centrifuged to equilibrium in sucrose gradients and assayed for phage-eclipsing activity. The bulk of the eclipsing activity of the Pluronic extract banded at the density of outer cell membrane (Fig. 2) (3, 14), whereas that of the polypropylene eluate banded near the top of the gradient at a much lower density. A fraction from the less dense band (see shaded zone in Fig. 2) contained the $\phi X \rightarrow$ RF conversion activity, at 5 units/ml. This represents a recovery of 74% of the activity applied to the gradient, assuming the $\phi X \rightarrow$ RF activity to be proportional to eclipsing activity in this band. There was no $\phi X \rightarrow$ RF activity associated with the small amount of eclipse activity in the band at the density of outer membrane. Similarly, $\phi X \rightarrow$ RF activity was found only near the top of the gradient of the Pluronic extract, corresponding to the small peak of eclipse activity at that position. The low buoyant density (approximately 1.03 g/cm^3) suggests that the material has either a high phospholipid or detergent content. The activity is not homogeneous in terms of buoyant density (Fig. 2).

When viewed in the electron microscope, the polypropylene eluate contained membrane fragments of 500–2000 Å in size (data not shown); no such particles were seen in the reagents themselves.

Product of $\phi X \rightarrow$ RF conversion

The products of $\phi X \rightarrow$ RF conversion by the Pluronic extract and polypropylene eluate were analyzed by velocity sedimentation, with the synthetic strand labeled with [^{3}H]dTTP (Fig. 3). The Pluronic extract product contained the ^{3}H label in material sedimenting at neutral pH as RF I and RF II (Fig. 3A) and coincident with peaks of ^{32}P label representing the phage strand. The SS appearing in these gradients (Fig. 3A and B) was derived from eclipsed phage disrupted by sodium dodecyl sulfate treatment during sample preparation. When the peak fraction sedimenting as

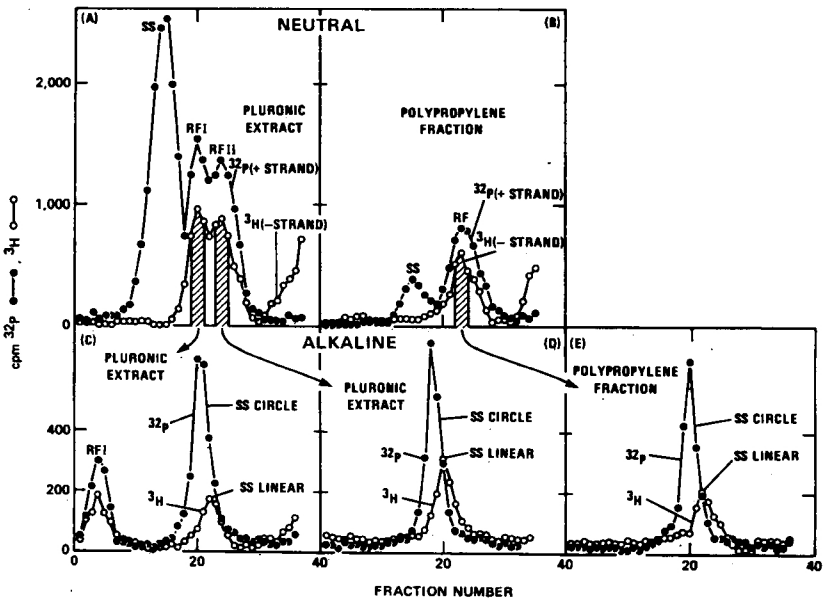

FIG. 3. Product of $\phi X \rightarrow RF$ *in vitro*. Pluronic extract and polypropylene eluate were prepared from *E. coli* HF4704 cells (*Materials and Methods*). [^{3}H]dTTP (47 Ci/mmol, 100 μl) was added to a tube and dried under nitrogen. To this were added: 100 μl of Pluronic extract or 200 μl of polypropylene eluate; 50 μl of $MgCl_2$; 100 μl of rNTPs; 100 μl of dNTPs; 25 μl of Fraction II; and 200 μl of ^{32}P-labeled ϕX174 *am3* phage (as in Fig. 1). The samples were made up to 0.96 ml with Buffer B (added first). Incubation was for 20 min at 30°. The samples were then treated and applied to neutral sucrose gradients as in Fig. 1 but without the CsCl shelf. The gradients were analyzed in 15-drop fractions. DNA was precipitated in 250-μl aliquots of the fractions and collected on Whatman GF/C filters. Radioactivity was measured in toluene scintillation fluid. To 250 μl of the RF I and RF II peak fractions in A (Pluronic extract) and the RF peak fraction in B (polypropylene eluate) were added 250 μl of 10 mM Tris-HCl (pH 7.5)–1 mM EDTA, 20 μl of 5 M NaOH and 1 μl of ^{32}P-labeled ϕX174 *am3* phage; after 10 min at 37° the samples were applied to alkaline sucrose gradients. Gradients were fractionated in 5-drop fractions; the DNA was precipitated and collected; and radioactivity was measured as above. The sedimentation is from right to left.

RF I was analyzed in an alkaline sucrose gradient, only 45% sedimented as a closed-circular supercoil (Fig. 3C). Thus approximately 20% of the product was RF I. The remaining ^{3}H-labeled material, as well as the material from the RF II peak, gave the pattern expected: full-length, linear complementary strand derived from RF II (Fig. 3C and D). We attribute the composition of the RF I peak when analyzed on alkaline gradients to overlap of the RF I material with the broader peak of RF II in neutral gradients in the experiment shown. The product of conversion by the polypropylene eluate was exclusively RF II, as judged by sedimentation in neutral (Fig. 3B) and alkaline (Fig. 3E) gradients. The ^{3}H label was incorporated into nearly full-length, linear complementary strands (Fig. 3E). In another experiment, however, approximately 14% of the product was RF I; also in other experiments variable amounts of label were incorporated into linear strands that were shorter than unit length.

The $\phi X \rightarrow RF$ results, based on the behavior of the ^{32}P-labeled viral strand, were verified by analyses of the complementary synthetic strand labeled with ^{3}H. The $\phi X \rightarrow RF$ conversion by the Pluronic extract (Fig. 3A) was 29 and 44%, as judged by ^{32}P and ^{3}H label, respectively. The corresponding values for the polypropylene eluate (Fig. 3B) were 13 and 19%, respectively. Since the number of phage particles was measured by plaque formation, a decrease in efficiency of plating (i.e., 0.66) could explain the discrepancy between the ^{32}P and ^{3}H results. Another possibility, however, is that some uninfectious particles are present in the phage preparation and can be converted to RF in this system.

DISCUSSION

The *in vitro* conversion of naked ϕX174 SS to RF (7) differs from *in vivo* parental RF synthesis in at least three important respects: (*a*) naked SS are not the template *in vivo*, i.e., phage DNA penetration is tightly coupled to parental RF formation (4), (*b*) the gene H protein, a component of the phage coat, is involved in parental RF synthesis *in vivo* (6), and (*c*) recovery of RF in association with the outer membrane (3) suggests that replication occurs at a membrane site (13).

In order to explore the early events of phage replication *in vivo*, an *in vitro* system capable of converting the DNA of intact ϕX174 phage particles to RF was devised. The first such system was a cell lysate prepared with the nonionic detergent Triton X-100 (Table 1). "Membranes" sedimented from the lysate were inactive, but the system could be reconstituted by adding back the soluble replicative enzymes (Table 1). The $\phi X \rightarrow RF$ conversion was resistant to rifampicin (Table 1), an inhibitor of *E. coli* RNA polymerase, just as the *in vitro* replication of naked ϕX174 SS is (12), and the product of the reaction was RF II (data not shown).

Since lysates prepared from cells resistant to ϕX174 were inactive in $\phi X \rightarrow RF$ conversion (Table 1), we conclude that the correct LPS receptor (3) is a required component of the "membrane" fraction. However, the LPS appears not to be the only factor involved, since a complex of phage with LPS receptor was not replicated by the soluble enzymes alone (Table 1).

A distinction should be made between phage complexed with the LPS receptor *in vitro* and phage released from the complex by the ionic detergent, Sarkosyl. The DNA of the phage–LPS complex is not utilized as a template by the soluble replicative enzymes; after Sarkosyl treatment, the DNA is as readily replicated by the soluble replicative enzymes as naked SS (data not shown). The phage–LPS complex formation *in vitro* leads to a release of the gene H protein (S. L.

Rowen and A. Kornberg, unpublished observations), unlike the *in vivo* transfer of this "pilot" protein (15) into the cell with the infecting DNA (6). Thus, the action of the membrane fraction should include an involvement of the gene H protein in the $\phi X \rightarrow RF$ conversion.

An inhibitory effect of antibody directed against the gene H spike protein of the phage on $\phi X \rightarrow RF$ conversion in the lysate (data not shown) suggests that the gene H protein may be involved in RF formation *in vitro*, since the antibody was added after allowing time for phage binding and eclipse. The results do not distinguish between a direct and indirect effect of antibody binding to H protein on RF formation, but they are consistent with the notion that H protein is involved in $\phi X \rightarrow RF$ conversion *in vitro*, as it is *in vivo* (6).

A Triton-lysate is viscous and unsuitable for the purification of the $\phi X \rightarrow RF$ conversion activity. A clear, "soluble" cell extract can be obtained by using the nonionic detergent Pluronic P103. Fortified with the soluble replicative enzymes (7), such extracts can carry out the $\phi X \rightarrow RF$ conversion (Fig. 1). Both the Pluronic extract and the soluble replicative enzymes are required. A full-length linear complementary DNA strand is synthesized and the product is mainly RF II, although some molecules are sealed, yielding RF I (Fig. 3).

A fractionation procedure, starting with the Pluronic extract, depends on adsorption to, and elution from, polypropylene (Table 2). A 50-fold purification of $\phi X \rightarrow RF$ activity with respect to protein was achieved (Table 2). Electron microscope analysis revealed that the polypropylene eluate contains membrane fragments (data not shown).

The polypropylene eluate retained only 4–8% of the LPS of the Pluronic extract (Table 2). Yet, this fraction was from 21 to 60% as active as the extract in promoting phage eclipse. The polypropylene eluate was enriched for phage-eclipsing activity, possessing all the $\phi X \rightarrow RF$ converting activity of the Pluronic extract (Fig. 2), and distinct from the bulk of the LPS. The low buoyant density (approximately 1.03 g/cm^3) of this fraction (Fig. 2) suggests that it has either a high phospholipid or detergent content.

Further fractionation and resolution of the activity in the Pluronic extract should improve our understanding of its components and elucidate the host function in the $\phi X \rightarrow RF$ conversion as well as the role of the gene H (pilot) protein in the process.

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