

In Vitro Reassembly of Shell-Like Particles from Disrupted Polyoma Virus

(hemagglutination/infectivity/electron microscopy/density-gradient centrifugation)

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ABSTRACT When purified polyoma virus is exposed to 0.01 M dithiothreitol in the presence of 0.2 M Na₂CO₃-NaHCO₃ (pH 10.6) at 0-4°C, the capsids are rapidly disrupted to protein subunits of capsomere size, as judged by density gradient centrifugation, sedimentation equilibrium centrifugation, and electron microscopy. Hemagglutination activity and infectivity of disrupted virus are reduced to below detectable amounts. Removal of the disruption reagents by dialysis at 4°C against 0.05 M Tris-0.14 M NaCl-1 mM EDTA and 0.1 mM 2-mercaptoethanol (pH 8.0) results in a time-dependent reappearance of up to 17% of the starting hemagglutination titer, under optimum conditions of ionic strength, pH, temperature, and virus protein concentration. The recovered hemagglutination activity is found in glycerol gradients associated with a 100S DNA-protein complex consisting mostly of linear aggregates of capsomeres. When the linear complex is treated with pancreatic DNase, the complex is converted into spherical particles, of approximately virus size, that sediment at 140 S (with aggregates at 180 S), as well as on the cushion of half-saturated CsCl at the bottom of the gradients. All reassembled particles are not infectious and have markedly reduced DNA to protein ratios.

The detailed mechanisms of maturation and assembly of the DNA tumor viruses, polyoma and simian virus 40, as well as other icosahedral viruses, are not completely understood. The successful *in vitro* reassembly of various RNA phages (1-4) and plant viruses (5-7) suggest that assembly of these viruses involve, at least in part, self-assembly mechanisms (8). To date, no reassembly systems have been reported for any DNA or mammalian viruses.

Polyoma is one of the simplest of the mammalian DNA viruses. Each virion consists of a closed circular double-stranded DNA of molecular weight 3×10^6 (9), enclosed in an icosahedral capsid containing five or six minor proteins, in addition to the major capsid protein (10). The simplicity of the virus suggested studies of the mechanism of virus assembly; this report describes attempts to define the conditions under which purified polyoma virus can be disrupted and reassembled *in vitro*.

MATERIALS AND METHODS

Large-plaque polyoma, LP 32, was used throughout this study. All reagents were analytical grade, when available. Dithiothreitol (DTT) was purchased from Calbiochem and electrophoretically purified pancreatic DNase was obtained from Worthington Co. Bovine-serum albumin (Fraction V) was from Pentex. All radioisotopes were purchased from Schwarz Bioresearch Co.

Abbreviations: DTT, dithiothreitol; HA, hemagglutination activity.

Virus Purification. Virus was made from infected primary baby-mouse kidney cells and purified (10); more recently, purification has been by precipitation with polyethylene glycol (11).

Hemagglutination. Hemagglutination assays were performed as described (11).

Virus Disruption and Reassembly. Purified virus, at protein concentrations of 0.01-1.4 mg/ml, was treated with 0.2 M Na₂CO₃-NaHCO₃, 0.01 M DTT (pH 10.6; disruption buffer) for 3-4 hr at 0°C. For reassembly, 0.1-0.2 ml of disrupted virus was dialyzed at 4°C against 250 ml of 0.05 M Tris-0.15M NaCl-1mM EDTA-0.1 mM 2-mercaptoethanol (pH 8.0) overnight without stirring. After dialysis against 0.05M Tris (pH 8.0) for 2 hr, the final reassembled samples were stored at 4°C, where they were stable for several weeks.

Ultracentrifugation. Density-gradient band sedimentation was performed in 10-30% glycerol or 5-20% sucrose, either in disruption buffer or in 0.05 M Tris-1 mM EDTA-0.05% bovine-serum albumin (pH 8.0).

Determination of DNA conformation under alkaline conditions (12) was made by layering 0.1-ml samples onto 3.5 ml of CsCl solution (1.5 g/ml in 0.05 M NaOH, pH > 12.4) in polyallomer tubes, followed by centrifugation in an SW 50 L rotor for 90 min at 35,000 rpm. Samples were collected directly onto Whatman glass-fiber filters, washed with cold 5% Cl₂CCOOH, and counted in a scintillation counter.

Infectivity. Native virus and reassembled particles were assayed at 33°C for infectivity with secondary mouse-embryo cells (13).

Molecular-Weight-Determinations. The molecular weight of disrupted viral protein was estimated by the equilibrium-sedimentation method (14), using a model E Spinco ultracentrifuge equipped with Rayleigh interference optics. Centrifugation was for 24 hr at 6110 rpm, and all manipulations were performed at 0.4°C. A value of 0.72 was assumed for \bar{v} .

Molecular weights of disrupted viral proteins were also estimated from their sedimentation rates in 5-20% sucrose in disruption buffer, by the method of Martin and Ames (15). An internal marker of *S*-carboxymethylated polyoma protein, with a molecular weight of 45,000 (10), was used. If the axial ratio of capsomeres is assumed to be about six times (16) that of an average globular protein (3.3; ref. 17) and we assume that there are no significant differences in solvation or partial specific volume between monomeric subunits and

TABLE 1. Properties of purified native polyoma and of reassembled particles

Particle	Sedimentation coefficient(s)*	Molecular weight	Infectivity, plaque-forming units per mg of protein	Hemagglutination units per mg of protein	DNA to protein ratio†
Native virus	240		3.3×10^9	1.6×10^2	1.00
Disrupted virus			<10	<0.2	
Major protein component	7	221,000‡ 272,000§			
Minor protein components	10				
	1.5-2.65				
Reassembled particle	100		<10	4.8	0.94
Reassembled (DNase) (unfractionated)			<10	19.2	
Reassembled (DNase)	140		<10	38	0.33
Reassembled (DNase)	180		<10	27	0.34
Reassembled (DNase)	240		<10	7	0.36
Reassembled (DNase)	CsCl cushion		<10	45	0.54

* Measured in sucrose and glycerol gradients; not corrected for temperature and solvent.

† Defined as 1.00 for virions.

‡ Sedimentation equilibrium (17).

§ Sedimentation in sucrose gradients (18).

capsomeres, the frictional coefficient of an oblate ellipsoid capsomere can be shown to be 1.7 times greater than that for an average globular protein of equal volume by the Perrin equation (18):

$$\frac{f}{f_0} = \frac{(a^2/b^2 - 1)^{1/2}}{(a/b^{3/2} \tan^{-1}(a^2/b^2 - 1)^{1/2})}$$

Since apparent molecular weight is inversely related to the frictional coefficient, observed values for molecular weight have been increased by a factor of 1.7.

Electron Microscopy. An Hitachi HU11B electron microscope was used at an accelerating voltage of 75 kV. For negative staining with 1% uranyl acetate (pH 4.6) or 2% phosphotungstate (pH 7.2; ref. 19), samples were applied to nitrocellulose-carbon-backed grids for 5 min, removed by blotting with filter paper, covered with stain for an additional 5 min, and air-dried before examination. For chromium shadowing, grids coated with 3% parlodion film were used. Samples were applied for 5 min, removed by blotting with filter paper, and air-dried. Chromium shadowing was performed at an angle of 25°.

RESULTS

Disruption

When purified virus was exposed to disruption conditions at 0–4°C, hemagglutination activity (HA) was rapidly lost, and both HA and infectivity were below detectable amounts within 30 min (Table 1). Neither pH 10.6 nor 0.01 M DTT alone at 0°C resulted in the loss of HA. Addition of DTT at alkaline pH causes disruption of polyoma virions (20, 21).

Characterization of the disrupted virus indicated that more than 80% of the protein was in the form of a 7S particle, while smaller amounts were found at 10 S and at 1.5–2.6 S. (Fig. 1). No detectable virus-sized (240 S) material remained. The molecular weight estimates for the major component of disrupted virus, summarized in Table 1, are 221,000 by the equilibrium sedimentation method and 272,000 by sedimentation in sucrose gradients in the presence of alkylated

viral-protein markers. Since the major polyoma capsid protein has a molecular weight of 42,000–48,000 (10, 22), pentamers and hexamers (23, 24) would be expected to have molecular weights of about 225,000 and 270,000, respectively. These molecular weight estimates suggested that the protein of disrupted virions was largely in the form of capsomeres, and electron microscopy confirmed the presence of many capsomere-like particles. Negatively-stained (Plate 1a) samples of native virus showed the spherical, 48-nm particles previously described for polyoma (16). After disruption, only free, doughnut-shaped capsomeres were seen (Plate 1b).

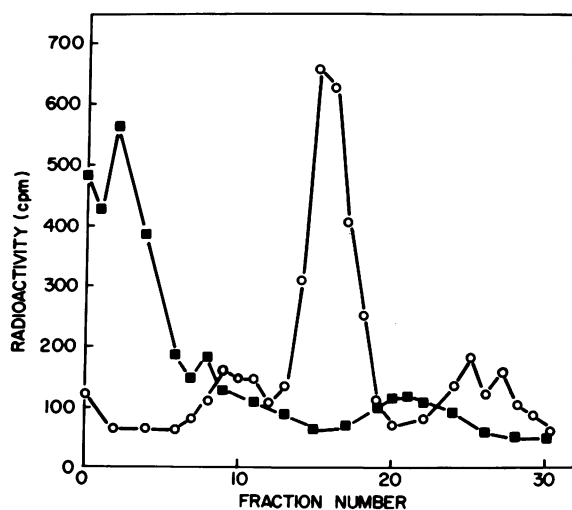


FIG. 1. Sedimentation of disrupted polyoma in a 5–20% sucrose gradient. 10 μ l of double-labeled polyoma virus was treated with 150 μ l of 0.2 M NaHCO_3 – Na_2CO_3 (pH 10.6) and 0.01 M DTT for 4 hr. The solution was layered onto a linear 5–20% sucrose gradient in the same buffer with DTT, and centrifuged for 9 hr at 46,000 rpm in a SW 50L rotor at 4°C. 0.14-ml aliquots were collected and the acid-insoluble [^3H]DNA (■—■) and [^{14}C]protein (○—○) counted.

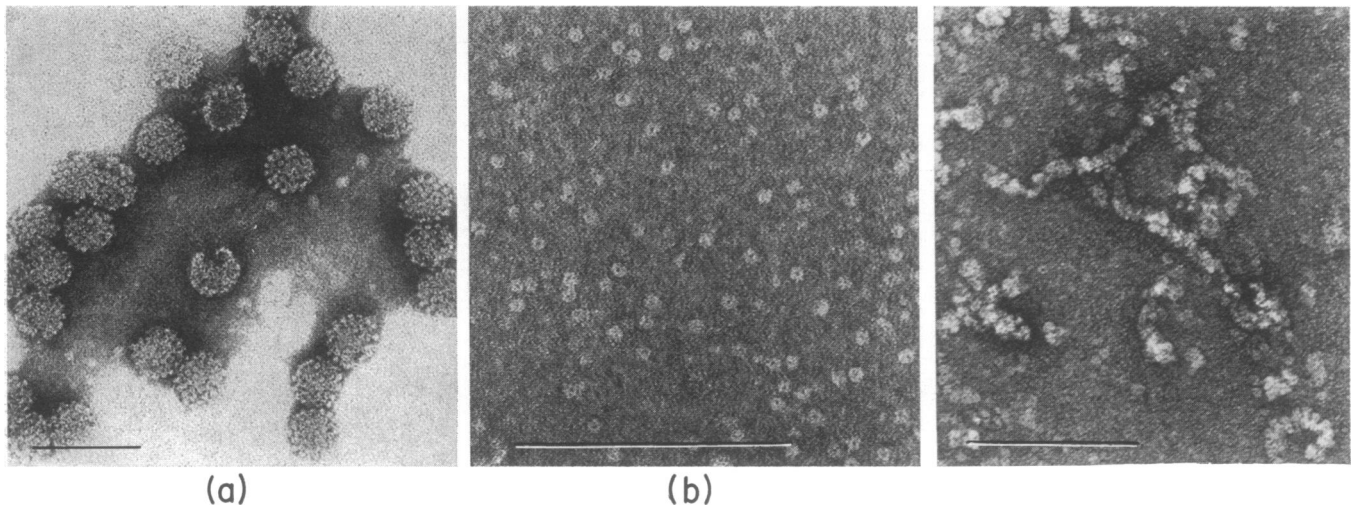


PLATE 1. (a left) Native purified polyoma stained with 2% phosphotungstate. (b, center) Native purified polyoma treated with 0.2 M Na_2CO_3 - NaHCO_3 -0.01 M DTT (pH 10.6) for 2 hr at 4°C at a virus protein concentration of 200 $\mu\text{g}/\text{ml}$, and stained with 2% phosphotungstate. No virus particles are seen, and only the doughnut-shaped particles resembling capsomeres are evident.

PLATE 2. (right) Electron micrographs of 100S reassembled particles stained with 2% phosphotungstate. There are numerous linear aggregates of capsomeres. Some free capsomeres and amorphous capsomere clumps are also present. The bar indicates 100 nm.

Reassembly

Conditions for reassembly were chosen to optimize HA recovery. Dialysis of disrupted virus against optimal condi-

tions of ionic strength, pH, and temperature gave a time-dependent reappearance of HA activity. The optimum NaCl concentration was 0.15 M; NaCl could be replaced by KCl, which was optimal at 0.3–0.5 M. The presence of both NaCl and KCl at their optima did not increase the recovery of HA, compared to that obtained with each salt separately. There was a narrow pH optimum of 7.6–8.5; HA recovery decreased markedly with increasing temperature. HA recovery upon dialysis was maximal at a virus protein concentration of about 20 $\mu\text{g}/\text{ml}$.

The presence of 0.1–100 mM Mg^{++} did not affect the recovery of HA activity, but 0.1 mM Ca^{++} (as CaCl_2) reduced HA recovery to 20% of the control. The presence of oxidizing agents, such as 0.1 mM dehydroascorbic acid or CuSO_4 did not improve HA recovery, whereas 0.1 mM 2-mercaptoethanol improved the reproducibility of HA recovery, while not markedly increasing the final titer. Under these optimum conditions, maximal HA returned within several hours.

Properties of reassembled particles

Sedimentation of reassembled particles in 10–30% glycerol gradients demonstrated a single major noninfectious DNA-protein complex, containing more than 90% of the protein and the DNA in the sample, and sedimenting at about 100 S (Fig. 2a). Sedimentation of the 100S material in an alkaline CsCl gradient showed that it contained DNA components I and II in the same proportion as in starting virions. Most of the recovered HA activity cosedimented with the complex, although a variable small amount was found on the CsCl cushion at the bottom of the gradient. After treatment with DNase, the HA titer increased 4- to 8-fold (Table 1), and the 100S complex was replaced by heterogeneously sedimenting material with HA-positive peaks at about 140 S, 180 S, and on the CsCl cushion (Fig. 2b). The DNA to protein ratio of all HA-positive regions was reduced to one-third to one-half of that found in intact virions, and even less in other similar experiments. None of the fractions contained infectious virus. Examination of reassembled particles by alkaline CsCl

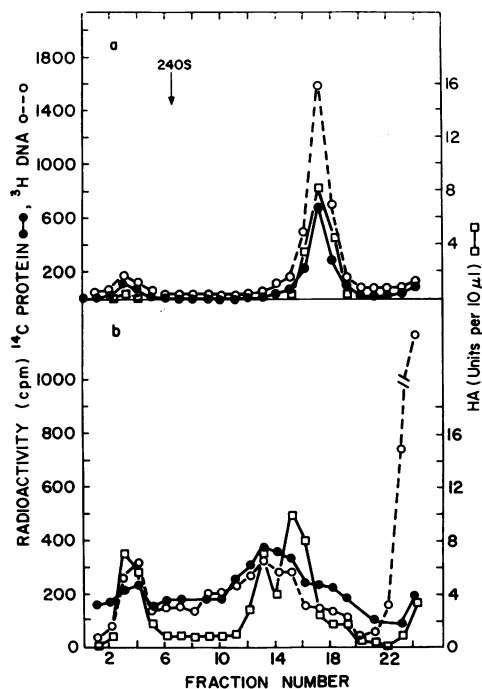


FIG. 2. Glycerol-gradient centrifugation of reassembled particles. Virus was disrupted and reassembled (640 $\mu\text{g}/\text{ml}$ of viral protein concentration); reassembled particles (a) were sedimented in 10–30% glycerol gradients in 0.05% bovine-serum albumin in an SW 50L rotor for 40 min at 45,000 rpm (10°C). The 100S peak was pooled and treated with 20 $\mu\text{g}/\text{ml}$ of pancreatic DNase (b) for 30 min in the presence of 0.01 M Mg^{++} and sedimented as in (a). 10- μl aliquots from 0.22-ml fractions were assayed for HA (\square — \square); the remainder of the samples were precipitated with cold 5% Cl_3CCOOH , washed onto glass-fiber filters, and the samples were counted by liquid scintillation.

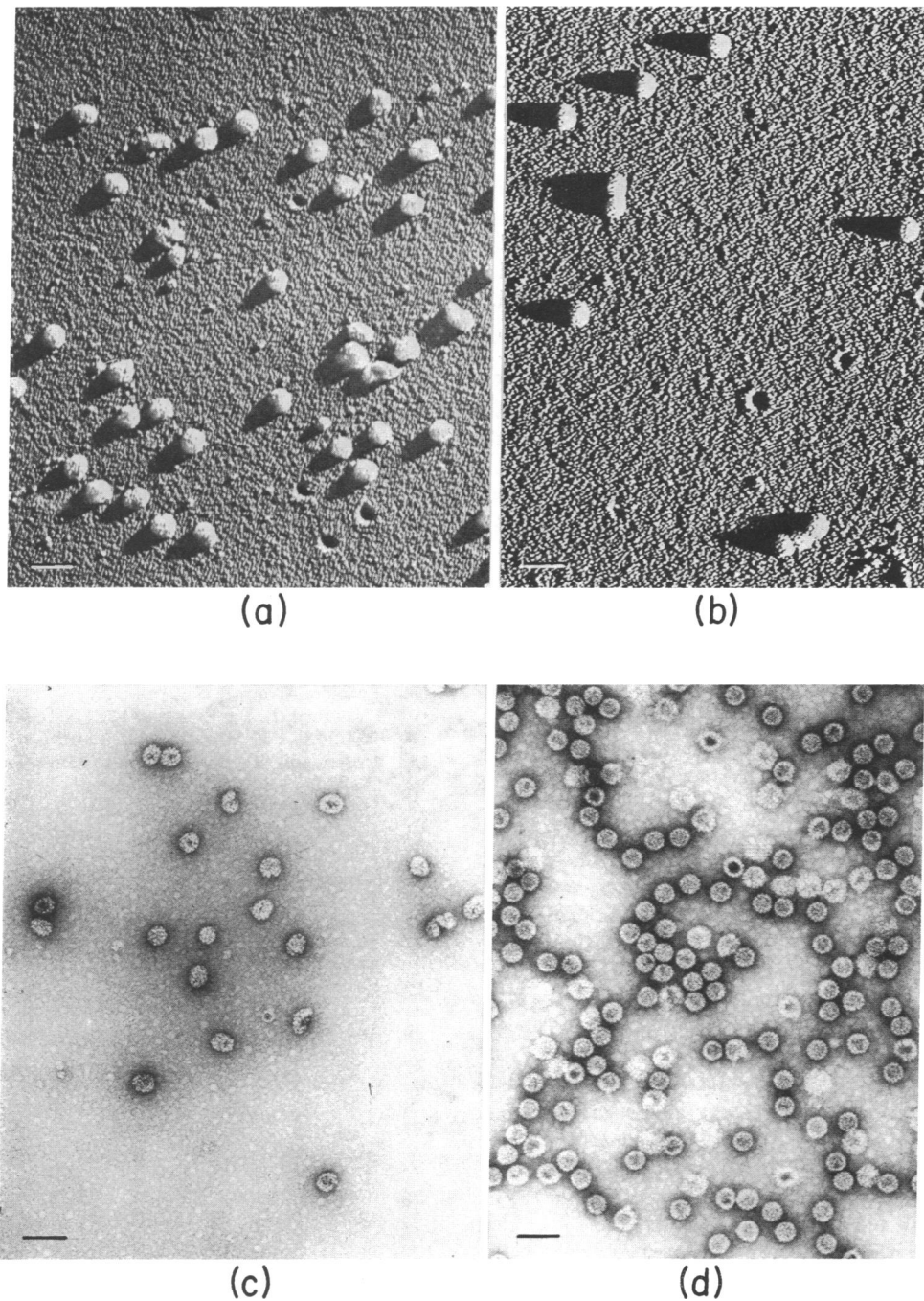


PLATE 3. (a) Reassembled 100S particles, after treatment with 20 $\mu\text{g}/\text{ml}$ of DNase, and subsequent sedimentation in a neutral glycerol gradient. Virus was disrupted and reassembled at a viral protein concentration of 640 $\mu\text{g}/\text{ml}$. After treatment with DNase, hemagglutination-positive peaks were located after sedimentation in 10-30% glycerol on 0.05 M Tris-1 mM EDTA-0.05% bovine-serum albumin (pH 8.0) in the SW 50.1 rotor for 40 min at 45,000 rpm (10°C). Samples were removed from regions of the gradient corresponding to 140 S and shadowed with chromium. (b) Native purified polyoma, shown for comparison. (c) 140S reassembled particles, stained with uranyl acetate. (d) Native virus stained with uranyl acetate. The bars indicate 100 nm.

gradients after DNase treatment has shown that the DNA does not sediment into the gradient, and is therefore extensively degraded.

The reassembled material was examined by electron microscopy after negative staining or shadowing, both before and after treatment with DNase and sedimentation in glycerol gradients. Material in the 100S peak consisted largely of linear aggregates of capsomeres and some free capsomeres

(Plate 2). After treatment with DNase and sedimentation in a glycerol gradient (Fig. 2b), the HA-positive peaks at 140 S, 180 S, and on the CsCl cushion all contained large numbers of spherical particles, which were mainly single in the 140S peak (Plate 3a), largely aggregated to duplexes in the 180S peak, and markedly aggregated in the material recovered from the CsCl cushion. Shadowed reassembled particles were somewhat similar in appearance to native

virus (Plate 3b), although some particles were flattened and irregular.

Observed by negative staining, many 140S reassembled particles were somewhat irregular and distorted (Plate 3c), and some particles appeared to be empty when compared to native virus (Plate 3d).

DISCUSSION

The recovered HA activity is associated with reassembled particles, and not with surviving native virions. Undegraded 240S virions could not be detected on neutral glycerol gradients after a short exposure to disruption conditions, and HA activity was undetectable before reassembly. Furthermore, the HA of reassembled particles differs from that of native virus, since it requires adsorption at 37°C for at least 15 min and is quickly inactivated by 0.6% glutaraldehyde at pH 8.0 in HEPES buffer (Boyle and Friedmann, unpublished results). Also, when reassembled particles labeled with [¹⁴C]protein were mixed with native virus particles labeled with [³H]protein and examined by sedimentation through a glycerol gradient in 0.05 M Tris (pH 8.0), it was not possible to detect any virions in the 100S reassembled particles, making it further unlikely that the HA activity that sediments in the 100S region could be attributed to trapped, undegraded virions.

The 100S reassembled complex, before DNA setreatment, consists mainly of linear aggregates of capsomeres presumably aligned along, and bound to, DNA. After DNase treatment, the linear aggregates disappear, to be replaced by rather uniform, spherical particles. Reassembled particles contained less DNA than native virions, and all the DNA was susceptible to cleavage with DNase, indicating defective encapsidation. All reassembled particles were noninfectious (Table 1). Lack of infectivity cannot be attributed to degradation of the DNA by DTT (25), since the distribution of viral components I and II (12, 26) [or pseudovirion component III (27)] in 100S material after sedimentation in alkaline CsCl was identical to that of native virus.

Because of the probable skew symmetry of polyoma (28), delineation of capsomere arrangement and clarification of symmetry for these reassembled particles by electron microscopy is particularly difficult. Although it is, therefore, difficult to establish that reassembled particles are structurally identical to native virions or shells, their size and ordered structure suggest that they are similar to naturally occurring 140S DNA-free shells, which are produced during lytic infection with polyoma virus (29).

The existence of linear capsomere aggregates along strands of DNA suggests that DNA participates in the assembly of a spherical virus. Nevertheless, the relevance of this capsomere reassembly and of the linear aggregates to intracellular assembly remains to be established.

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