Virus-Like Particles in an Extranuclear Mutant of *Neurospora crassa*

(mitochondria/RNA virus/lipoprotein/glycoprotein)

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ABSTRACT A particulate fraction not present in wild-type cells has been isolated from the cytoplasm and from lysed mitochondria of the respiratory-deficient extranuclear mutant "abnormal-1" of *Neurospora crassa*. The particles have a density between 1.13 and 1.2 g/ml. They appear in thin sections after OsO_4 fixation as virus-like polymorphic vesicles containing an electron-dense nucleoid of 120-170 nm in diameter. The central core is surrounded by one or two "unit membrane" envelopes of 100 Å thickness. The particles contain a single-stranded 33S RNA that is converted to 7-9S RNA by heat treatment in the presence of sodium dodecyl sulfate and that differs in its base composition from mitochondrial and cytoplasmic ribosomal RNA. They contain 7% phospholipids rich in phosphatidylethanolamine and two major proteins, a lipoprotein of molecular weight 15,000 and a glycoprotein of molecular weight 95,000. It is suggested that these virus-like particles originate within mitochondria.

It is generally believed that the respiratory-deficient genotype of maternally inherited mutants of *Neurospora crassa* (1) is transmitted by the mitochondrial genome (2). One of these extranuclear mutants, the slow growth variant "abnormal-1" (3) is of special interest because the "abnormal" character can be transmitted to normal cells by hyphal injection of "abnormal" mitochondria, and "abnormal" mitochondria are dominant over normal mitochondria in heteroplasmons (4).

This dominant or suppressive effect can be explained either by a replicative advantage of "abnormal" mitochondria or by an extramitochondrial exchange of mitochondrial gene products (4). The recent discovery of intracellular virus-like particles† in "abnormal-1" and "poky" mutants of *N. crassa* (5) raises the interesting possibility that these particles are responsible for the dominant character of "abnormal" genotype in heteroplasmons by infecting wild-type mitochondria. This view is strengthened by our finding that a large fraction of virus-like particles is located in, or at least associated with, the mitochondrial preparation isolated in the same way as the fraction that has been used for micro injection (4).

In this communication, we describe the morphological appearance and chemical composition of these particles. They appear to be unique among all other known fungal virus-like particles that contain either a double-stranded RNA (6-10) or DNA (11).

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† The term "virus-like particles" is used merely to describe the morphological and structural appearance of a cellular fraction and does not imply a virus function like infectivity.

METHODS

Growth Conditions. The extranuclear mutant "abnormal-1" of *N. crassa* (inos 37401, FGSC Nr. 1448) was propagated by hyphal transfer on slants containing potato-dextrose agar (Oxoid CM 139) supplemented with 5 µg/ml of inositol. The growth medium contained per liter: 4 g of potato extract (Oxoid L 101), 5 g of yeast extract (Difco), 20 g of glucose, and 5 mg of inositol. 250-ml Erlenmeyer flasks containing 100 ml of medium were inoculated with a small piece of hyphal mat scraped from the slant culture and were shaken for 5 days at 30° in a rotary shaker. The contents were then transferred to two or three 1-liter erlenmeyer flasks containing 250 ml of medium and shaken for another 4 days.

Isolation of Virus-Like Particles. The hyphae were collected by filtration and homogenized by grinding with a double wet weight of sea sand in a mortar. The mixture was extracted with standard buffer (0.1 M glycine-0.01 M MgCl_2 , pH 7.1) (5) containing 0.44 M sucrose, and centrifuged for 5 min at 3000 rpm. The supernatant was centrifuged for 15 min at 11,000 rpm in a Servall centrifuge at 0° to remove mitochondria. The postmitochondrial supernatant was layered on discontinuous sucrose gradients containing the following sucrose concentrations (w/v) in standard buffer: 60% (5 ml), 49% (4 ml), 37% (4 ml), 25% (20 ml). The gradients were centrifuged in a Spinco SW 25.2 rotor for 45 min at 24,000 rpm and 2°. The greenish band in the interphase between 49 and 37% sucrose was recovered, diluted twice with standard buffer, and centrifuged for 20 min at 150,000 × *g*. The pellet was suspended in standard buffer, and the suspension was layered on a second sucrose gradient containing the following sucrose layers: 50%, 40%, 30% (2 ml each), 20%, and 10% (1.5 ml each). The gradients were centrifuged in a Spinco SW 40 rotor for 30 min at 30,000 rpm. The band between 40 and 30% was recovered, diluted, pelleted, and suspended as described above. The crude mitochondrial pellet was suspended in standard buffer containing 2% Triton X-100 to lyse mitochondria. The lysate was treated exactly as described for the postmitochondrial supernatant.

Electron Microscopy. Pellets of purified virus-like particles were fixed with 3% glutaraldehyde in isotonic phosphate buffer, after postfixation with 1% osmium tetroxide (21), dehydration with ethanol, epon embedding, and polymerization at 60° for 48 hr. Sections were taken on a 400 mesh copper grid and stained with 1% uranyl acetate. Photographs were taken in a JEM 100-B electron microscope at 80 kV.

RNA Analysis. "Abnormal" cells were grown for 4–5 days in the presence of 8 $\mu\text{Ci/ml}$ of sodium [^{32}P]phosphate. Purified virus-like particles were suspended in standard buffer (about 3 mg/ml of protein) and lysed with 1% sodium dodecyl sulfate. After addition of a few drops of diethylpyrocarbonate (13), they were extracted twice with buffer-saturated phenol. The aqueous phase was mixed with 0.1 volume of 20% potassium acetate (pH 5), and RNA was precipitated with 2.5 volumes of ethanol, dissolved in standard buffer, and precipitated again.

Mitochondrial and cytoplasmic ribosomes of "abnormal-1" strain were isolated from the supernatant fraction of the first discontinuous sucrose gradients used to isolate virus-like particles from the postmitochondrial supernatant and from the mitochondrial lysate (see above), and purified by centrifugation in exponential sucrose gradients (14). *Escherichia coli* ribosomes were isolated from cells grown overnight in the presence of 0.1 $\mu\text{Ci/ml}$ of [^3H]adenosine (15). RNA was extracted from ribosomes as described for virus-like particles. ^3H -Labeled tRNA was a gift of Dr. von der Haar, Göttingen.

The RNA was analyzed by isokinetic sucrose gradient centrifugation (24) in the presence of 10 mM NaCl–10 mM Tris·HCl (pH 7.5). The mixing chamber contained 12.3 ml of 5% (w/w) sucrose; 13 ml of 32.9% (w/w) sucrose was added from a burette. The gradients were centrifuged for 14 hr at 32,000 rpm at 4° in a Spinco SW 40 rotor. Drop fractions were precipitated with 5% trichloroacetic acid after the addition of 0.1 mg/ml of bovine-serum albumin, collected on Millipore filters, and counted in a liquid scintillation counter. Base analysis was performed with a nucleoside analyzer (16, 17).

Protein Analysis. Protein of virus-like particles was analyzed either with sodium dodecyl sulfate–polyacrylamide electrophoresis (18) or by Sephadex filtration in the presence of 0.5% sodium dodecyl sulfate (19). In the Sephadex procedure, virus-like particles were dissolved in standard buffer containing 2.5% sodium dodecyl sulfate (3 mg/ml of protein), heated for 1 min at 100°, and passed through a Sephadex G-

FIG. 1. Electron micrograph of a thin section of purified virus-like particles from "abnormal-1" after OsO_4 fixation and staining with uranyl acetate. The bar represents 100 nm.

FIG. 2. Analysis of RNA extracted from virus-like particles by isokinetic sucrose gradient centrifugation. ^{32}P -Labeled RNA from virus-like particles (●—●) was mixed with ^3H -labeled rRNA and tRNA (B) from *E. coli* (○—○) and analyzed either directly (A) or after heating at 80° for 2 min in the presence of 0.2% sodium dodecyl sulfate (B).

100 or G-200 column (2×50 cm) equilibrated with 50 mM Tris·HCl (pH 7.4)–1 mM dithiothreitol–0.5% sodium dodecyl sulfate. 1-ml Fractions were dissolved in 10 ml of Aquasol and counted in a Packard liquid scintillation counter.

Lipid Analysis. Total lipids were extracted from lyophilized virus-like particles with chloroform–methanol 2:1. Phospholipids were separated by one- and two-dimensional thin-layer chromatography and determined by fluorescence measurements (20).

RESULTS

The virus-like particles from "abnormal-1" represent a well-defined fraction not found in wild-type cells (less than 1% of the virus-like particles in "abnormal" would have been detectable). The density of the virus-like particles (1.13–1.2 g/ml) is somewhat lower than that of mitochondria (1.20–1.25 g/ml). About 40% of the virus-like particles are separated from mitochondria by differential centrifugation. Another 20% can be washed off from mitochondria with buffer containing 10 mM EDTA; the residual 40% are liberated by lysis of mitochondria with Triton X-100, which obviously does not affect the morphological and structural integrity of virus-like particles. No structural differences could be found between virus-like particles isolated from the postmitochondrial supernatant and those liberated from mitochondria with Triton X-100.

Fig. 1 shows a thin section of purified virus-like particles after OsO_4 fixation. The particles appear as polymorphic vesicles of 250–400 nm in diameter containing an electron-dense "nucleoid" of 120–170 nm in diameter. This core region

FIG. 3. Densitogram of sodium dodecyl sulfate-polyacrylamide gels stained with Coomassie Blue after electrophoresis of dissociated virus-like particles alone (A) or together with subunits α , β , and β' of RNA polymerase (core enzyme) from *E. coli* (B). Before electrophoresis, samples were heated for 2 min at 100° in the presence of 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol.

is surrounded by one or, in some cases, by two or more envelopes that have the trilamellar "unit membrane" appearance typical for most biological and artificial membranes in thin sections (22, 23). The membranes have a rather constant thickness of 10 nm, which is significantly higher than the thickness of mitochondrial membranes in sections treated in the same way (23).

Chemical analysis of virus-like particles suggests that they are composed of 8–10% RNA, 83–85% protein, and 7% phospholipids. Fig. 2A shows the sedimentation profile of ^{32}P -labeled RNA extracted from virus-like particles with phenol-sodium dodecyl sulfate after centrifugation in an isokinetic sucrose gradient (24) that contained ^3H -labeled rRNA and tRNA from *E. coli* as internal marker. Most of the trichloroacetic acid-insoluble RNA sediments at 33 S, with some material sedimenting in the 4–9S region. After heat treatment in the presence of 1% sodium dodecyl sulfate, the 33S peak is converted into a heterodisperse RNA with two main peaks sedimenting at 9 S and 7 S (Fig. 2B). The S-value of the 33S species has been verified by cosedimentation with ^3H -labeled ribosomal 30S subunit from *E. coli* in a separate experiment.

Table 1 shows the base composition of RNA from virus-like particles in comparison to that of rRNA from mitochondria and cytoplasm. It is obvious that RNA from virus-like particles differs significantly from both rRNA species, having an intermediate G+C content. The base composition also suggests a noncomplementary structure of RNA from virus-like particles; this suggestion is supported by our finding that more than 90% of the RNA is RNase-sensitive at high-salt concentration, indicating a single-stranded structure (25). In some cases ^{32}P -labeled RNA from virus-like particles was con-

taminated by RNase-resistant trichloroacetic acid-precipitable radioactivity that could be removed by additional purification steps (26). No DNase-sensitive material could be detected in virus-like particles.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (18) reveals a rather simple protein composition of virus-like particles. Fig. 3 shows the densitogram of sodium dodecyl sulfate gels stained with Coomassie Blue containing proteins of virus-like particles together with subunits of *E. coli* RNA polymerase (core enzyme) as internal markers (27). The pattern of virus-like particles exhibits two major bands of estimated molecular weights of 15,000 (1) and 95,000 (2), with some diffuse stain in the intermediate region. Similar molecular weights can be estimated from the elution pattern from Sephadex G-200 shown in Fig. 4. In this case, cytochrome *c* from horse heart (molecular weight 13,000) (28) and bovine-serum albumin (molecular weight 69,000) (29) have been used as marker proteins. The elution profile of proteins of virus-like particles labeled *in vivo* with both [^{14}C]leucine and [^3H]glucosamine (Fig. 4A) indicates that the heavy component (protein 2) is a glycoprotein, whereas only traces of [^3H]glucosamine have been incorporated into the small component 1.

FIG. 4. Gel filtration of proteins from virus-like particles in the presence of sodium dodecyl sulfate. Virus-like particles were isolated from 4- to 5-day-old cultures of "abnormal-1" cells labeled for 24 hr with 1 $\mu\text{Ci}/\text{ml}$ of [^3H]glucosamine and 0.1 $\mu\text{Ci}/\text{ml}$ of [^{14}C]leucine, or with 1 $\mu\text{Ci}/\text{ml}$ of [^3H]leucine and 5 $\mu\text{Ci}/\text{ml}$ of [^{32}P]sodium phosphate. The samples of virus-like particles were heated with 1% sodium dodecyl sulfate and passed through Sephadex G-200 as described in *Methods*. The column was standardized with bovine-serum albumin and cytochrome *c* from horse heart, which gave elution peaks at fractions 38 and 57, respectively. (A) Proteins of virus-like particles labeled with [^{14}C]leucine (●—●) and [^3H]glucosamine (O—O). (B) Methanol-soluble proteins of virus-like particles labeled with [^3H]leucine (●—●) and [^{32}P]sodium phosphate (O—O).

About 40% of the radioactivity of virus-like particles labeled with [³H]leucine is extractable with 90% methanol. Sephadex filtration reveals that protein 1 is the lipophilic component. If ³²P-labeled virus-like particles are extracted under the same conditions, about 10% of the radioactivity is solubilized and elutes together with protein 1 (Fig. 4B). We conclude that the small component is a lipoprotein.

Table 2 shows the result of a lipid analysis. 7% of total dry weight of lyophilized virus-like particles are extractable as phospholipids. Virus-like particles show a relatively high content of cardiolipin, a phospholipid common in bacteria and found in eukaryotic cells to be located exclusively in the inner membrane of mitochondria (30). In comparison with both mitochondrial membranes, virus-like particles are low in phosphatidylcholine and unusually rich in phosphatidylethanolamine.

DISCUSSION

Several fungi like *Penicillium* or *Aspergillus* have been reported to contain polyhedral nucleoprotein particles, 25–42 nm in diameter, having a double-stranded RNA (6–10). They are generally named virus-like particles, although infectivity of purified virus-like particles has not been demonstrated. The particles isolated from the extranuclear mutant “abnormal-1” of *Neurospora crassa* have also been described as virus-like from their morphological appearance (5). It seems difficult to reconcile a possible virus function with the observation that these particles are determined by cellular genetic elements and are produced constantly during many generations like other cellular structures. However, since from their morphology and their chemical composition the particles have much more in common with enveloped viruses than with cellular structures like mitochondria, the term “virus-like” seems to be justified. It is unlikely that virus-like particles have functional properties of viruses like infectivity in its classical sense; the possible intracellular infection of mitochondria discussed below would be a novel phenomenon in virology. Virus-like particles are best described as a cellular particulate component resembling certain polymorphic viruses. Virus-like particles from “abnormal-1” share with virions of the arbo-, myxo-, paramyxo-, rhabdo-, and tumor virus groups (12) the following structural properties: they exhibit a dense nucleoid surrounded by a membrane envelope, and they contain lipo- and glycoproteins, phospholipids, and single-stranded RNA.

Most of this RNA sediments at 33 S, but is converted by heat denaturation to RNA chains of lower molecular weight (mainly 9 S and 7 S). In this respect, RNA from virus-like particles resembles 70S RNA from tumor viruses, which is converted to 30S RNA by similar treatment (31).

The protein composition of virus-like particles from “abnormal-1” is remarkably simple. Again, in analogy to RNA

TABLE 2. Phospholipid composition* of virus-like particles and mitochondrial membranes†

Phospholipids	Virus-like particles	Mitochondrial	
		Inner membrane	Outer membrane
Phosphatidylethanolamine	67.5	35	31
Cardiolipin	9.8	21	3
Phosphatidylinositol‡	1.8	2	17
Phosphatidylserine‡	9.1		
Phosphatidylcholine	11.8	41	49

* Expressed in percent of total phospholipids.

† The values are taken from ref. 30 and refer to rat-liver mitochondria. However, mitochondrial membranes from *Neurospora* have essentially the same phospholipid composition (W. Neupert, personal communication).

‡ Phosphatidylinositol and phosphatidylserine have not been separated for mitochondrial lipid analysis (30).

tumor viruses (32, 33), the heavy protein is labeled by glucosamine (Fig. 4A). Whether the glycoprotein is a component of the envelope of virus-like particles remains to be shown.

The striking similarity of the phospholipid composition of enveloped viruses and of the plasma membranes of their host cells supports the idea that these viruses acquire cell membranes by budding (34). Our finding that virus-like particles from “abnormal-1” contain cardiolipin, the marker lipid of the inner mitochondrial membrane, but only little phosphatidylcholine, a major constituent of both mitochondrial membranes, suggests that virus-like particles do not acquire mitochondrial membranes by budding, but rather use components of the inner membrane to build up a new membrane that has a simpler protein structure, a higher resistance against Triton X-100, and a somewhat different thickness.

An intramitochondrial origin of virus-like particles is also suggested by the observation that a large fraction of these particles is liberated from purified mitochondria only after lysis of mitochondrial membranes, and by recent experiments indicating that the RNA from virus-like particles is transcribed from mitochondrial DNA and that both proteins of virus-like particles are synthesized on mitochondrial ribosomes (manuscript in preparation).

Furthermore, it seems that production of virus-like particles has been triggered by a spontaneous mutation of mitochondrial DNA, the only known extranuclear genetic element in *Neurospora* (3). The dominant or suppressive character of the “abnormal” genotype in heteroplasmons has tentatively been explained as being caused by an active component of the mitochondrion or some active material present in the mitochondrial preparation (4). It now seems possible that virus-like particles are these active components that suppress normal respiration and normal growth by infecting wild-type mitochondria.

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TABLE 1. Base composition of RNA from virus-like particles and from mitochondrial and cytoplasmic ribosomes

Source of RNA	Mole percent				
	A	U	G	C	G+C
Virus-like particles	25.1	26.9	29.1	18.9	48.0
Mitochondrial ribosomes	33.5	31.7	19.5	15.3	34.8
Cytoplasmic ribosomes	25.1	24.4	28.8	21.7	50.5

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