Encapsidation of the La France Disease-Specific Double-Stranded RNAs in 36-nm Isometric Viruslike Particles

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ABSTRACT

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We investigated the relationship between the conserved electrophoretic pattern of nine double-stranded RNAs (dsRNAs) and the viruslike particles (VLPs) associated with La France disease of the button mushroom, *Agaricus bisporus*. Using a purification procedure involving chloroform extraction, PEG-NaCl precipitation, differential centrifugation, and equilibrium centrifugation in cesium-sulphate gradients, we have obtained preparations from diseased sporophores that were highly enriched in a 36-nm isometric VLP and contained minor amounts of both a 25-nm isometric VLP and the 19- × 50-nm single-stranded RNA bacilliform virus. Cesium-sulphate gradient fractions that contained these particles (average buoyant density = 1.25 g/cc) also contained the nine disease-specific dsRNAs of 3.8–0.8 kb and three disease-associated polypeptides with molecular weights of 63, 66, and 129 K. Neither the VLPs, dsRNAs, nor the polypeptides were present in healthy sporophores analyzed under identical conditions. Our data suggest that the nine dsRNAs implicated in the etiology of La France disease constitute the genome of a 36-nm isometric virus.

Additional keywords: mushroom die-back, mycovirus.

More than 40 years ago, Sinden and Hauser (20) described an infectious malady of the button mushroom *Agaricus bisporus* (Lange) Imbach as it occurred at a commercial farm in southeastern Pennsylvania. Today this disease, referred to as La France disease, has been described throughout the world and is considered an important limiting factor in the commercial cultivation of mushrooms (24). Symptoms of the disease range from an insidious infection involving an almost imperceptible yield loss to acute outbreaks associated with slow mycelial growth, malformed sporophores, and total crop failure (17,24,25).

In 1962, Hollings (11) first proposed a viral etiology for La France disease based on the observation of several types of virus-like particles (VLPs) in symptomatic sporophores. Although a variety of VLPs have been detected in diseased tissues, the most common are isometric particles measuring 25 nm and 35 nm in diameter and a 19- × 50-nm bacilliform particle (15,24). Recently, double-stranded RNA (dsRNA) analysis demonstrated a close association between a conserved set of nine dsRNAs and the disease in mycelial cultures (12) and sporophores (17,25).

Considering that mycoviruses typically have dsRNA genomes (3), it seems reasonable to suggest that the dsRNAs represent the genome of a virus(es) that is the causal agent of the disease. However, the possible relationship between the dsRNAs and the VLPS remains obscure. In this paper, as well as in a preliminary report (7), we show that all nine disease-related dsRNAs are encapsidated in 36-nm isometric VLPs. This finding together with previous evidence (17,22,23,25) implicates a viral complex involving a dsRNA isometric virus and a single-stranded RNA (ssRNA) bacilliform virus (mushroom bacilliform virus, MBV) in the etiology of La France disease.

MATERIALS AND METHODS

Source of tissue. A total of six healthy and nine diseased sporophore isolates of hybrid off-white mushroom varieties were used in this study. All sporophores were collected at commercial sites located in Chester Co., PA, with the exception of two healthy and two diseased sporophore isolates that were obtained from farms in England. Sporophores were trimmed of the basal portion of the stipe, washed with tap water, rinsed briefly with deionized water, and stored at −80 C.

Purification of virus. All steps were done at 4 C or on ice. Frozen sporophores (100 g) were homogenized in 300 ml of cold 50 mM Tris-HCl, 1 mM ethylene diaminetetraacetate (EDTA), and 0.1% 2-mercaptoethanol, pH 8.0, for 2 min in a Waring blender on a high-speed setting. The homogenate was clarified by centrifugation at 6,000 *g* for 20 min (low-speed centrifugation), and the resultant supernatant was recovered and shaken briefly with 0.1 vol of chloroform. The aqueous phase was resolved by

low-speed centrifugation, collected, adjusted slowly to 10% polyethylene glycol (mol wt 8,000, Sigma, St. Louis, MO) and 0.6 M NaCl, and stirred for 2.5 h. The precipitate that formed was collected by low-speed centrifugation and resuspended in 200 ml of 50 mM Tris-HCl and 1 mM EDTA, pH 8.0 (TCE). The suspension was clarified by low-speed centrifugation and then centrifuged at 102,000 *g* for 2 h. The resulting high-speed pellets were resuspended overnight in a total of 40 ml of TCE. The suspension was clarified by centrifugation at 10,000 *g* for 20 min, overlaid on 10 ml of TCE containing 35% (w/v) sucrose in 30-ml tubes, and centrifuged at 102,000 *g* for 1 h. The resulting pellets were resuspended overnight in a total volume of 1 ml of TCE (sucrose pad pellet). One milliliter of the sucrose pad pellet suspension was layered onto preformed 10–50% (w/w) cesium-sulphate gradients prepared in TCE and centrifuged in a Beckman SW-28 swinging bucket rotor at 112,000 *g* for 24 h

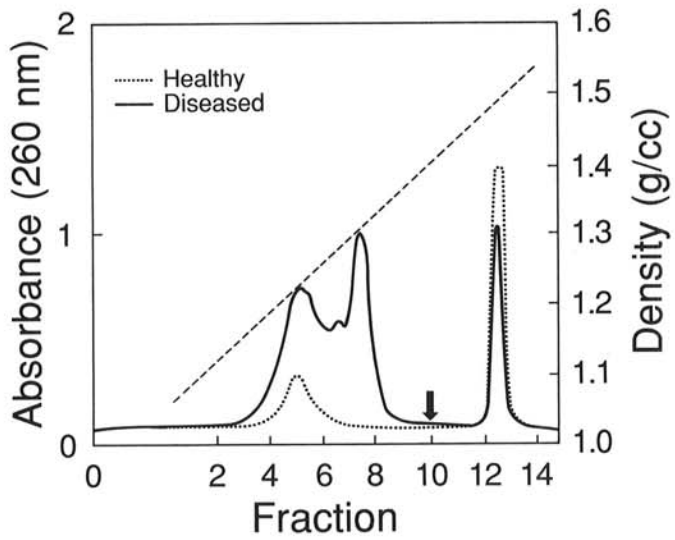

Fig. 1. Absorbance profiles (260 nm) of 10–50% cesium-sulphate gradients containing sucrose pad pellets from healthy and diseased sporophores after equilibrium centrifugation. Centrifugation was at 112,000 *g* for 24 h in a Beckman SW-28 swinging bucket rotor. Volumes of the fractions are: 6 ml (fraction 1), 3 ml (fraction 2), 2 ml each (fractions 3–14). Buoyant density is indicated by the hatched line. The arrow shows the position in the gradient at which the phenol-extracted and cellulose-column chromatography-purified dsRNAs banded.

(Beckman Instruments, Fullerton, CA). Gradients were prepared in 38-ml centrifuge tubes and consisted of 8 ml each of 10, 20, 30 and 40%, and 6 ml of 50% cesium-sulphate solutions. Gradients were fractionated with an ISCO density-gradient fractionator (ISCO, Lincoln, NE) and monitored at 260 nm with a Beckman Model 3600 spectrophotometer. Fractions were dialyzed for 24–36 h against four 800-ml changes of TCE, centrifuged at 102,000 *g* for 1 h, and the resulting pellets were resuspended overnight in 100 μl of TCE. Samples of each gradient fraction were analyzed for dsRNA, protein, and VLPs.

Analysis of dsRNA. The dsRNA was isolated from sporophores by phenol extraction and cellulose column chromatography after the method of Morris and Dodds (14) as modified by Wach et al (25).

To analyze cesium-sulphate gradient fractions for dsRNA, 1 vol of the fraction was vortexed briefly with 4 vol of 100 mM Tris-HCl, 0.2 M NaCl, 2 mM EDTA, and 1% sodium dodecyl sulphate (SDS), pH 7.0, and 5 vol of phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v). The resultant emulsion was centrifuged at 14,000 *g* for 2 min, the upper aqueous phase was recovered, and the nucleic acids were precipitated with 2.5 vol of cold 95% ethanol at −20 C for a minimum of 1 h. The precipitate that formed was collected by centrifugation at 14,000 *g* for 10 min, dried in vacuo, and resuspended in 30 μl of 10 mM Tris-HCl and 1 mM EDTA, pH 8.0 (TE).

Agarose gel electrophoresis of dsRNA. The dsRNA samples were adjusted to 0.01% bromophenol blue and 3.5% sucrose and separated on 10-cm long 1% agarose horizontal minigels (Mini the Gel-cicle, Hoeffer, San Francisco, CA). Electrophoresis was carried out at a constant 75 V for 1.5 h. Electrophoresis buffer was 80 mM Tris-phosphate and 8 mM EDTA, pH 7.8, containing ethidium bromide (50 ng/ml). Nucleic acids were visualized by transillumination with UV light and photographed with type 55 Polaroid film. The sizes of the dsRNAs were estimated from their electrophoretic mobilities relative to the *Bst*II restriction endonuclease fragments of lambda DNA ranging from 8.4 to 0.17 kb (New England Bio Labs, Inc., Beverly, MA).

Confirmation of dsRNA. Nucleic acid was isolated from 10 μl of the cesium-sulphate gradient-purified VLPs by phenol extraction and ethanol precipitation as before. The nucleic acid pellet was resuspended in 20 μl of TE and adjusted to a final concentration of either 0.3 M NaCl or 0.03 M NaCl and 1 ng of RNase A (DNase-free RNase A, Boehringer-Mannheim, Indianapolis, IN) per microliter (final volume of 100 μl). The nucleic acids were incubated at 37 C for 1.5 h, extracted twice with an

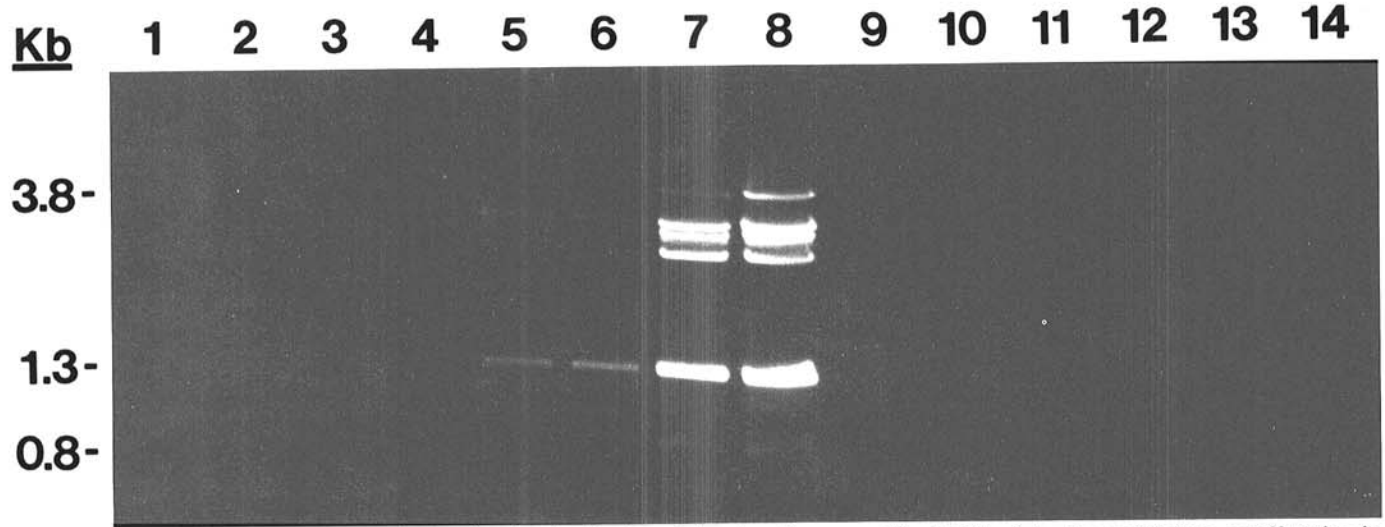

Fig. 2. Agarose gel electrophoretic analysis showing the distribution of the nine disease-specific dsRNAs following equilibrium centrifugation in cesium sulphate. Conditions for centrifugation and fractionation of the gradient (fractions 1–14) were as described in Figure 1. Electrophoresis was carried out in 1% agarose gel at 75 V for 1.5 h. Size (kb) of the dsRNAs relative to *Bst*II-digested lambda DNA fragments (data not shown) is indicated at left. It should be noted that the 2.8-kb dsRNA is only partially resolved from the 3.0-kb dsRNA, and that the minor 1.7-, 0.9-, and 0.8-kb dsRNAs are faintly visible.

equal volume of phenol/chloroform/isoamyl alcohol, and precipitated with ethanol. The final nucleic acid pellet was resuspended in 20 μl of TE and analyzed by agarose gel electrophoresis. Nucleic acid that hydrolyzed in low, but not high salt, was regarded as dsRNA.

Analysis of protein. Polypeptides were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the discontinuous buffer system described by Laemmli (13). Before electrophoresis, proteins were denatured for 5 min at 95 C in 2 vol of 125 mM Tris-HCl, pH 6.8, containing 20% glycerol, 4% SDS, and 10% 2-mercaptoethanol. Denatured proteins were separated in 0.75-mm thick polyacrylamide gels (MiniProtean II cell, Bio-Rad, Richmond, CA) with a 4% stacking gel and a 7.5% separating gel. Electrophoresis was carried out at room temperature with a constant voltage of 100 V until the dye reached the bottom of the separating gel (about 1.5 h). Molecular weight standards included myosin (205 K), β-galactosidase (116 K), phosphorylase B (94 K), albumin (67 K), ovalbumin (43 K), carbonic anhydrase (30 K), and trypsin inhibitor (20 K) (Sigma, and Pharmacia, Piscataway, NJ). Gels were stained for at least 30 min at room temperature with 0.1% Coomassie Blue R 250 (Gibco-BRL, Gaithersburg, MD) in a 10% acetic acid and 40% methanol solution. Gels were destained in a 10% acetic acid and 40% methanol solution until sufficiently clear and then stored in either a 2% glycerol or 7.5% acetic acid solution.

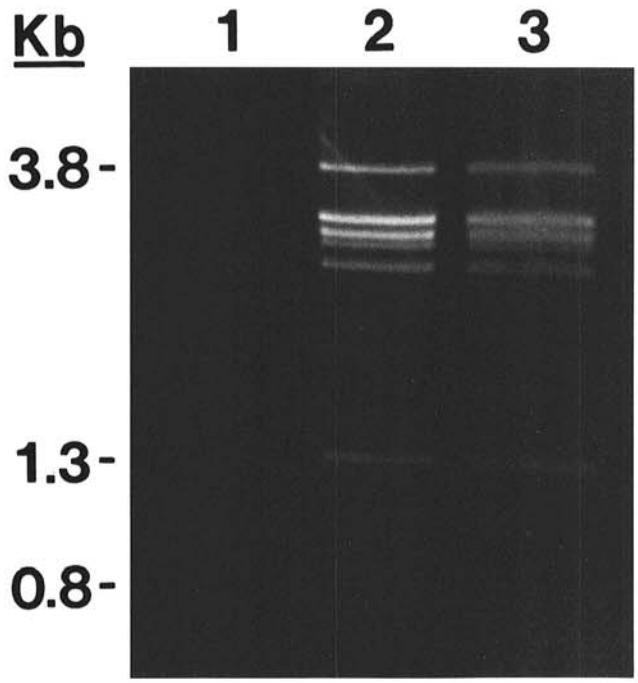

Fig. 3. Ionic strength-dependent sensitivity of the cesium-sulphate gradient purified disease-specific nucleic acids to hydrolysis by RNase A. Nucleic acids were isolated by phenol extraction and ethanol precipitation and then incubated with RNase A (1 μg/ml) in 0.03 M NaCl (lane 1) or 0.3 M NaCl (lane 2) for 30 min at 37 C. Lane 3 contains dsRNA isolated from diseased sporophores by phenol extraction and cellulose column chromatography and incubated in TE (10 mM Tris-HCl and 1 mM EDTA) for 30 min at 37 C. Size (kb) of the dsRNAs relative to *Bst*II-digested lambda DNA fragments (data not shown) is indicated at left.

Analysis of VLPs. Mica sheets (Electron Microscopy Supplies, Fort Washington, PA) were coated with carbon film using an Edwards vacuum coater (Sussex, England). The carbon films were layered onto 400-mesh copper grids on which samples of the cesium-sulphate gradient fractions were incubated for 5 min and negatively stained with 2% aqueous uranyl acetate (pH 4.5) for 1 min. Grids were examined with a Phillips model 300 transmission electron microscope (Phillips, Mahwah, NJ) and photographed with Kodak 4489 EM film (Eastman Kodak, Rochester, NY).

RESULTS

Equilibrium centrifugation. Following equilibrium centrifugation of sucrose pad pellets from diseased tissue, the 260-nm absorbance profile of the cesium-sulphate gradients showed three major peaks corresponding to fractions 5, 7, and 12 (Fig. 1). These regions of the gradient were also associated with an intense light-scattering material. For some diseased isolates, the peaks in fractions 5 and 7 could not be resolved, but appeared as one broad zone. By comparison, absorbance profiles of gradients containing sucrose pad pellets from healthy sporophores revealed only the two peaks in fractions 5 and 12.

Analysis of cesium-sulphate gradients for dsRNA. Agarose gel electrophoretic analysis of cesium-sulphate gradients containing sucrose pad pellets from diseased sporophores revealed that the six major dsRNAs of 3.8, 3.1, 3.0, 2.8, 2.6, and 1.3 kb and the three minor dsRNAs of 1.7, 0.9, and 0.8 kb banded together in fractions 5–8 with an average buoyant density of 1.25 g/cc (Fig. 2). Typical of dsRNA, the nine nucleic acid molecules showed an ionic strength-dependent sensitivity to hydrolysis by RNase A (Fig. 3). The dsRNA was not found in other regions of the gradient, including fraction 12 that was associated with a UV-absorbing material, nor was dsRNA detected in gradients containing sucrose pad pellets from healthy sporophores. In a replicated experiment, we observed that the disease-specific dsRNAs isolated from sporophores by phenol extraction banded at a higher buoyant density (1.38 g/cc) than those associated with the sucrose pad pellets (Fig. 1, arrow).

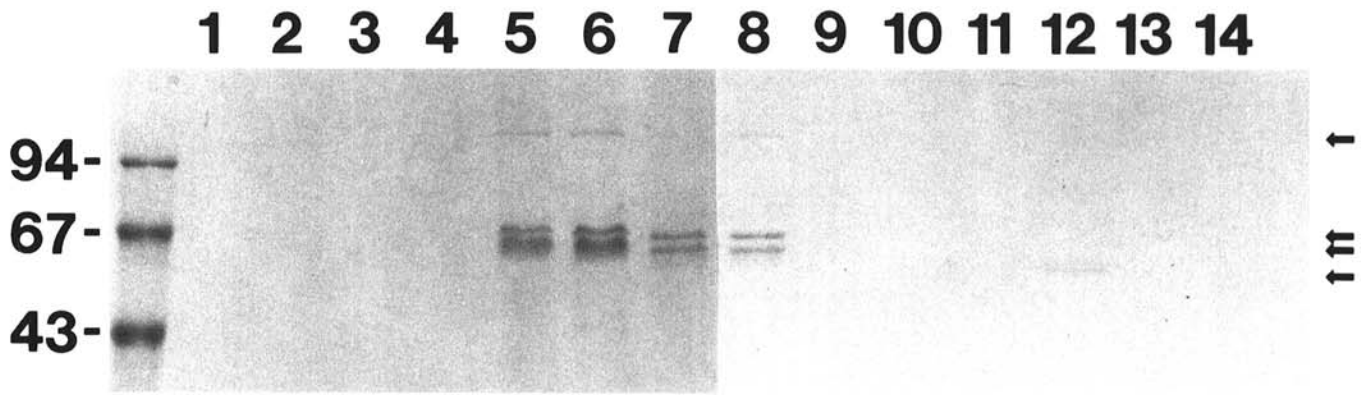

Fig. 4. SDS-PAGE showing the distribution of polypeptides following equilibrium centrifugation in cesium sulphate. Conditions for centrifugation and fractionation of the gradient (fractions 1–14) were as described in Figure 1. Arrows at right indicate the positions of the 129-, 66-, and 63-K polypeptides associated with the dsRNA-containing fractions (5–8) and the approximately 61-K polypeptides in fraction 12. Electrophoresis was carried out in a 4% stacking gel and 7.5% separating gel at 100 V for 1.5 h. The molecular weight (K) of the protein markers phosphorylase B, bovine serum albumin, and ovalbumin is shown at left.

Analysis of cesium-sulphate gradients for protein. Two major polypeptides of 63 and 66 K and a minor 129-K polypeptide were detected by SDS-PAGE in the dsRNA-containing fractions of cesium gradients (Fig. 4). The 63- and 66-K polypeptides were always found in equimolar amounts among the different diseased sporophore isolates. These three polypeptides were disease-specific as evidenced by their absence in gradients containing sucrose pad pellets of healthy sporophores (Fig. 5). In addition, two poorly resolved minor polypeptides of about 61 K were associated with the dsRNA fractions. Fraction 12, which showed UV absorbance not attributed to nucleic acid, also contained two approximately 61-K polypeptides that were present in both healthy and diseased tissues.

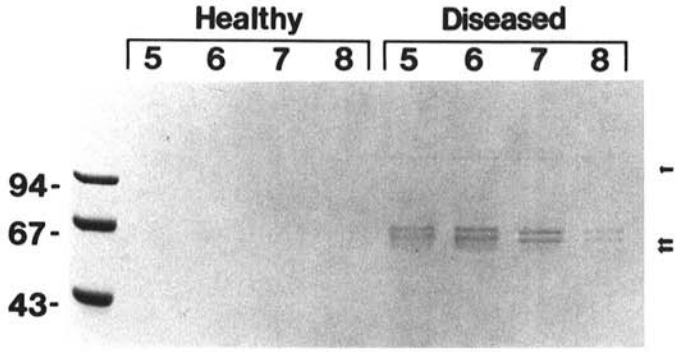

Fig. 5. Demonstration of the disease-specific nature of the polypeptides associated with the dsRNA-containing fractions (5–8) of cesium-sulphate gradients following equilibrium centrifugation. Polypeptides present in fractions 5–8 of gradients containing sucrose pad pellets from either healthy or diseased sporophores were separated by SDS-PAGE as described in the legend to Figure 4. Arrows at right indicate the positions of the 129-, 66- and 63-K polypeptides. Molecular weight (K) of the protein markers phosphorylase B, bovine serum albumin, and ovalbumin is shown at left.

Analysis of cesium-sulphate gradients for VLPs. Isometric VLPs with a mean diameter of 36 nm (SD = ±2.8, $N = 300$) were observed in gradient fractions that contained the disease-specific dsRNAs and polypeptides (Fig. 6A). These particles were neither observed in other regions of the gradient devoid of the dsRNAs and polypeptides, nor in gradients containing extracts of healthy sporophores. Cesium-sulphate gradient-purified preparations of the 36-nm isometric VLP generally contained low levels of the 19- × 50-nm bacilliform virions of MBV and an occasional 25-nm isometric VLP (Fig. 6B). A dot blot hybridization test, using a cloned complementary DNA specific for MBV RNA as a probe (18), confirmed the presence of MBV RNA sequences in purified VLP preparations (data not shown).

DISCUSSION

Evidence for the viral nature of La France disease of *A. bisporus* is based principally on the disclosed association with VLPs (11) and a highly conserved electrophoretic pattern of nine dsRNAs (9,12,17,25). A viral etiology was advanced following the induction of disease symptoms in healthy sporophores by hyphal anastomosis with mycelium and spores from diseased cultures (19) and by mechanical inoculation with cell-free preparations (4).

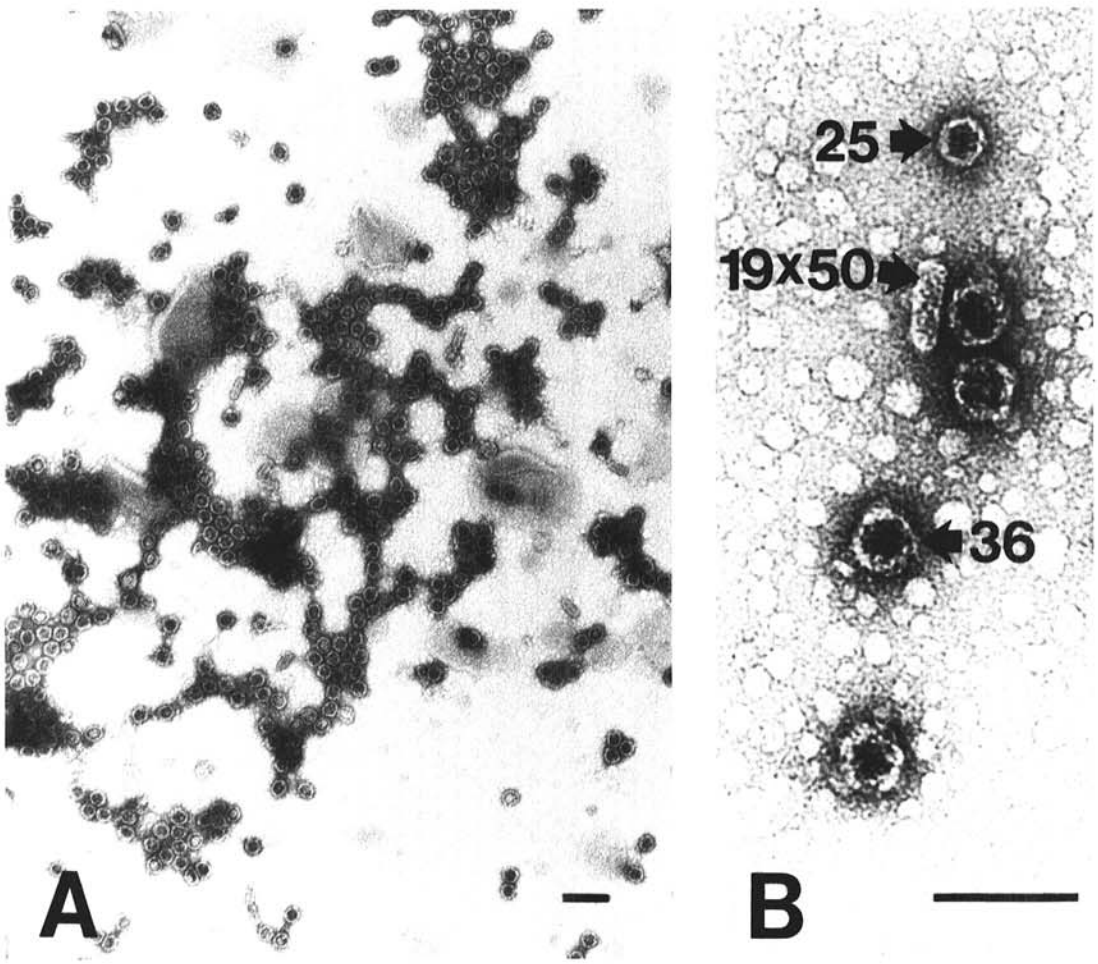

Fig. 6. A, electron micrograph of the VLPs in fractions 5–8 of cesium-sulphate gradients showing predominantly a 36-nm isometric particle. **B,** electron micrograph of the 36-nm isometric particle (36) and the 19- × 50-nm bacilliform (19 × 50 nm) and 25-nm isometric (25) particles found at low levels in purified preparations. Stained with 2% aqueous uranyl acetate, pH 4.5. The bars represent 100 nm.

We have now shown that the nine dsRNA segments associated with this disease are encapsidated in 36-nm isometric VLPs. Although several different dsRNA-containing particles have been isolated from symptomatic sporophores (2,21), their possible role in the disease has never been established. The 36-nm isometric VLP described in this study is strongly implicated in the etiology of La France disease, because the presence of its encapsidated dsRNAs in sporophores (18,25) and mycelial cultures (10,12) is positively correlated with the manifestation of disease symptoms. Our conclusion is corroborated by a wealth of electron microscopic evidence that has accumulated over the last three decades identifying a 35-nm isometric particle as one of three morphologically distinct VLPs encountered in diseased tissues (5,15,24).

In recent years, it has become evident that the mere detection of dsRNA in fungal tissues does not predict an association with VLPs. In some fungi, dsRNAs occur as naked molecules residing within the mitochondrion (16) or membraneous systems (1,8). The results of the present study suggest the La France disease-related dsRNAs make up the genome of a "conventional" mycovirus in the sense they exist in vivo as nucleoprotein complexes organized as 36-nm isometric VLPs. This conclusion is supported by the co-purification of the dsRNAs, three novel polypeptides, and the VLPs by a procedure involving chloroform extraction, PEG-NaCl precipitation, differential centrifugation, and equilibrium centrifugation. Further, the absence of obvious membraneous structures in our purified VLP preparations together with an apparent resistance of the dsRNA-containing complex to chloroform as well as ether (C. P. Romaine, *unpublished results*) argues against the involvement of lipid-rich vesicles.

We consistently observed that fractions of cesium-sulphate gradients associated with the highest concentration of the dsRNAs (Fig. 2, fractions 7 and 8) did not coincide with those most highly enriched in the disease-specific polypeptides (Fig. 4, fractions 5 and 6). This phenomenon might indicate the heterogeneous encapsidation of the dsRNA segments or more probably, the presence of empty capsids of lower buoyant density. The idea that the dsRNAs might exist as naked molecules that coincidentally have the same buoyant density as the VLPs is improbable, because the dsRNAs associated with VLP preparations have a lower buoyant density than naked dsRNA molecules isolated from sporophores by phenol extraction and cellulose column chromatography. This shift to a lower buoyant density would be expected if the dsRNA molecules were complexed with protein, as for example, in the form of a VLP.

Typically, the genomes of mycoviruses consist of one to three dsRNA segments that are packaged separately in isometric particles composed of a single capsid polypeptide (3). The genome of the mycovirus described here consists of nine dsRNA segments, which are encapsidated in an undetermined arrangement by isometric particles composed of two major structural polypeptides. Although the nine dsRNAs have unique nucleotide sequences (9), three of the segments (1.7, 0.7, and 0.8 kb) occur in submolar concentrations and could conceivably be satellite RNAs (10). It is unlikely the 19- × 50-nm bacilliform and 25-nm isometric particles detected at low levels in our purified VLP preparations play a significant role in packaging the dsRNAs. Highly purified virions of MBV do not contain dsRNA, but rather a 4.4-kb ssRNA that shares no extensive sequence homology with the disease-specific dsRNAs (18). Furthermore, MBV has been shown to have a single capsid protein of 24.5 K (22). We demonstrated the presence of MBV RNA sequences in our VLP preparations by hybridization analysis, which confirmed the identity of the observed 19- × 50-nm particles as virions of MBV. On the other hand, the 25-nm isometric particle occurred at concentrations too low to accommodate the dsRNAs, although it is possible it packages the three minor dsRNAs. It is worth noting that isometric particles measuring 25 nm in diameter (24) and a 2.4-kb dsRNA are found in healthy tissues (9,10,17), but their relationship to one another or to the 25-nm particles reported here has not been explored.

Our understanding of the etiology of La France disease has now been extended to include the role of a viral complex composed of a dsRNA-containing 36-nm isometric virus, hereafter referred to as LIV (La France isometric virus) and MBV, a ssRNA 19- × 50-nm bacilliform-shaped virus. Electron microscopic studies suggest MBV only occurs in mixed infections with LIV and that it is not involved in all episodes of the disease (6,15,24). Experiments involving single and mixed infections in protoplasts should conclusively define the pathogenicity of the individual viruses and their respective causative role in the disease.

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