JOURNAL OF BACTERIOLOGY, July 1980, p. 463-470
0021-9193/80/01-0463/08$02.00/0

Vol. 143, No. 1

Encapsidation of Yeast Killer Double-Stranded Ribonucleic Acids: Dependence of M on L

KEITH A. BOSTIAN,[1]* JOYCE A. STURGEON,[2] AND DONALD J. TIPPER[2]

Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, Massachusetts 02254,[1] and Department of Microbiology, University of Massachusetts Medical School, Worcester, Massachusetts 01605[2]

Virus-like particles containing either L or M double-stranded ribonucleic acid (dsRNA) were isolated from a killer toxin-producing strain of *Saccharomyces cerevisiae* (K^+ R^+). At least 95% of M- and 87% of L-dsRNA were recovered in virus-like particle-containing fractions. The major capsid polypeptides (ScV-P1) of both L and M virus-like particles were shown to be identical, and 95% of the cellular ScV-P1 was found in the virus-like particle-containing fractions. Since L-dsRNA encodes ScV-P1, provision of this protein for encapsidation of M-dsRNA defines at least one functional relationship between these dsRNA genomes and associates the L-dsRNA with the killer character. If encapsidation of M-dsRNA is essential for its replication or expression, then L-dsRNA plays an essential role in maintenance or expression of the killer phenotype. The relationship between the L- and M-dsRNA genomes would be analogous to that between a helper and a defective virus. The presence of only minor quantities of uncomplexed dsRNA and ScV-P1 suggests that their production is stringently coupled.

Cytoplasmic virus-like particles (VLPs) containing multiple species of separately encapsidated double-stranded RNA (dsRNA) are prevalent in fungi (22, 23, 41). In *Saccharomyces cerevisiae* at least two distinct dsRNA species exist: L-dsRNA (3×10^6 daltons) and M-dsRNA (1.6×10^6 daltons) (1, 3, 10, 18, 32, 33). The killer phenotype of yeast (E. A. Bevan and M. Makower, Abstr. Proc. XI Int. Congr. Genet. 1:202, 1963) is associated with the presence of M-dsRNA (4, 25, 33) and is dependent upon a number of host nuclear genes for maintenance and replication (25, 30, 34, 36, 38). This phenotype includes killing capacity (K^+ phenotype) due to production of an exocellular polypeptide toxin of about 11,500 daltons (27), and resistance to this toxin (R^+ phenotype). Most strains of yeast which are sensitive to this toxin also fail to produce it and have the (K^- R^-) phenotype. The facility with which the killer phenotype can be tested and the relative ease with which the dsRNA and nuclear genomes can be isolated, both physically and genetically, make this system particularly suitable for the study of nuclear-cytoplasmic relationships (see reference 37 for a recent review).

Physical investigations have failed to detect M-dsRNA in any strain lacking L-dsRNA. These observations suggest a dependency of M-dsRNA on L-dsRNA for its maintenance or replication. However, no functional relationship between these separately encapsidated dsRNA's has been demonstrated, nor do most of the nuclear genes necessary for M-dsRNA maintenance affect L-dsRNA, although *mak-3* causes a reduction in L-dsRNA content (40). In addition, L-dsRNA is almost always present in laboratory strains of *S. cerevisiae* and is not subject to loss under a variety of stressful growth conditions (high temperature, presence of subinhibitory concentrations of cycloheximide or 5-fluorouracil) which result in loss of M-dsRNA and the K^+ phenotype (14, 24, 35). It has therefore been suggested that unlike the better studied segmented *Reoviridae* genomes (21, 29) the L-dsRNA and M-dsRNA of the yeast VLP system may be unrelated and the L-dsRNA may be an autonomous plasmid unrelated to the killer character associated with the M-dsRNA genome (7, 39). Fungal cells are known to be capable of harboring more than one distinct, segmented, dsRNA-containing particle system that do not undergo mutual phenotypic or genotypic recombination (8, 9, 11).

In earlier publications (6, 20) we established, by direct in vitro translation, that ScV-P1 (88,000 daltons), the capsid polypeptide of L-VLPs, is encoded by L-dsRNA. We also demonstrated that translation of M-dsRNA in vitro results in the synthesis of a 32,000-dalton polypeptide (M-P1) containing the polypeptide sequence of toxin (ScV-P4). Bearing in mind that VLPs containing M- and L-dsRNA are serologically related (16; A. J. Herring, personal com-

munication) and that ScV-P1, by itself, exceeds the coding capacity of M-dsRNA, we proposed (6, 20) that L-dsRNA acts like a helper virus in providing some necessary function or functions (i.e., capsid polypeptide, formation or control of replicase or transcriptase activity, etc.) essential for the maintenance of the M-dsRNA. Previously, L- and M-dsRNA-containing VLPs have not been critically compared for particle-associated polypeptides, mainly because of the difficulty in obtaining homogeneous preparations. Similar problems have hampered attempts at identifying L or M particle-associated replicase or transcriptase activities (M. Leibowitz and J. Bruenn, personal communications).

We have developed a purification method that generates homogeneous L- or M-dsRNA-containing VLPs. We have shown the major capsid polypeptide of the L- and M-dsRNA-containing particles to be identical by peptide mapping. In addition, we have demonstrated that at least 85% of the L- and M-dsRNA species found in late log- to stationary-phase cultures are encapsidated and that there is little uncomplexed ScV-P1 in the cell. From these results we conclude that, if encapsidation is essential for replication or expression of M-dsRNA, then L-dsRNA plays an essential role in maintenance or expression of the killer phenotype as a helper genome encoding the major capsid polypeptide for both L- and M-dsRNA particles.

MATERIALS AND METHODS

Yeast strains and media. The yeast strains used in this work are listed in Table 1. Strains 4/4D and 3/A1 were obtained from E. A. Bevan and D. Mitchell (Queen Mary College, London). Strain S3 was obtained from G. Fink and H. Fried (Cornell University, Ithaca, N.Y.). Strain 2-1 was obtained in our laboratory by subcloning a stable K^+ R^+ clone from strain 4/4D (J.A. Sturgeon, K.A. Bostian, K.E. Kerr, and D.J. Tipper, Abstr. IX Int. Congr. Yeast Genet. Mol. Biol. 1978, p. 102). YEPD, pH 5.5, and low-phosphate YEPD and SMD media, pH 4.7, were prepared as described previously (30; K.A. Bostian, J.M. Lemire, L.E. Cannon, and H.O. Halvorson, Proc. Natl. Acad. Sci. U.S.A., in press).

Materials. Carrier-free $^{32}P_i$ and 5,6-[^{3}H]uracil (50 Ci/mmol) were obtained from New England Nuclear. L-[^{35}S]methionine (1,200 Ci/mmol) was purchased from Amersham Searle. Pancreatic RNase A and pancreatic DNase (I) were purchased from Worthington Biochemical Corp. *Staphylococcus aureus* V8 protease was obtained from Miles Laboratories. Electrophoresis grade agarose (Seakem) was from Marine Colloids, Inc. All other reagents were as previously described (6, 20; Bostian et al., in press).

TABLE 1. *S. cerevisiae strains*

Strain	Killer phenotype	dsRNA's	Killer genotype
3/A1	Sensitive, K^- R^-	L	*KIL-o*
4/4D	Killer, K^+ R^+	L, M	*KIL-k*
2-1	Killer, K^+ R^+	L, M	*KIL-k*
S3	Suppressive, K^- R^-	L, S	*KIL-s*

Preparation and purification of ^{32}P-labeled dsRNA. Preparation of ^{32}P-labeled dsRNA was made from cells grown with vigorous aeration for 1 to 2 days in 1 to 20 liters of low-phosphate YEPD. Label was added at a cell density of 10^7 cells per ml to a final concentration of 2.5 mCi/ml. After cell disruption and preliminary RNA fractionation (6, 20), individual dsRNA species were resolved and additionally purified from rRNA and DNA by preparative agrose slab gel electrophoresis on 1% agarose gels in TA buffer (40 mM Tris [pH 8.0], 20 mM NaOAc, 2 mM EDTA) containing 1 μg of ethidium bromide per ml. The dsRNA bands were localized by UV irradiation and excised, and after freeze-thawing, the dsRNA was extracted from the gel by centrifugation (16,000 × *g* for 1 h). The extraction procedure was repeated twice with equal volumes of 5 mM EDTA. Supernatant fluids were pooled, and the dsRNA was precipitated by addition of 0.1 vol of 5 M NaCl and 2 volumes of −20°C absolute ethanol.

DNase and RNase hydrolysis. RNA preparations were treated with DNase or RNase or both by incubation at 37°C for 30 min in 20 mM K_2HPO_4 (pH 7.6)–4 mM $MgCl_2$, with or without 0.6 M NaCl. Pancreatic RNase (heated at 70°C for 1 h to destroy contaminating DNase) and pancreatic DNase were used at a final concentration of 1 and 10 μg/ml, respectively. Samples were treated with proteinase K at a concentration of 1 mg/ml for 20 min at 30°C, followed by standard phenol extraction and ethanol precipitation (20), and analyzed by agarose gel electrophoresis.

Preparation and partial purification of VLPs. Cells for VLP production were grown to late stationary phase (5 to 7 days) in 5 to 10 liters of YEPD with shaking on an orbital shaker at 160 rpm for the first 2 days and 40 rpm for the remainder. Cells were harvested by centrifugation, washed once with cold distilled water, and finally resuspended in 1.5 ml of PKE buffer (30 mM Na_2HPO_4/NaH_2PO_4–150 mM KCl–10 mM EDTA, pH 7.6) per ml of packed cells. Cells were disrupted at 4°C by shaking with glass beads for 15 to 18 min in an Erlenmeyer flask on an orbital shaker at 350 rpm at a ratio of 3 g of 0.45-mm glass beads per ml of cell suspension. Breakage was monitored by phase microscopy until 90 to 95% of the cells were broken. After passing the slurry through a glass fiber mat, the beads were washed with enough PKE buffer to yield a combined filtrate at a four- to fivefold dilution of the original suspension. The filtrate was cleared of cellular debris by centrifugation at 10,000 × *g* for 30 min. The resulting precipitate is referred to as the low-speed precipitate (Table 2). The low-speed supernatant fluid (Table 2) was then centrifuged to collect particles with an S value equal to 150 in either a Spinco type 30 rotor (90 min at 30,000 rpm) or in a Spinco type 19 rotor (5.5 h at 19,000 rpm). The high-speed precipitate was gently resuspended in PKE buffer with a Teflon-coated glass homogenizer. This crude VLP preparation was then enriched for VLPs by sucrose gradient centrifugation on 10 to 40% (wt/vol) sucrose gradients in

a Spinco type SW27 rotor (24,000 rpm for 3 h). Fractions of 2 ml were collected from the bottom of the gradient at a rate of 1.6 ml/min. A small portion of each fraction (50 to 100 μl) was extracted with water-saturated phenol in microfuge tubes. After centrifugation of the emulsion, the aqueous phase was removed and adjusted to 30 mM EDTA. Samples were directly and rapidly analyzed by electrophoresis on 0.5% agarose gels run at 8 mA/cm^2 for 2 h.

Fractionation of L- and M-dsRNA-containing VLPs. Fractions from the 10 to 40% (wt/vol) sucrose gradients, which were shown by dsRNA analysis to be enriched for encapsidated L- or M-dsRNA, were pooled and immediately loaded onto 35 to 55% (wt/vol) sucrose gradients. After centrifugation in a Spinco SW27 rotor (24,000 rpm for 20 h), 0.5-ml fractions were collected. A portion of each fraction was treated at 60°C for 3 min to disrupt particles and analyzed for dsRNA content by agarose gel electrophoresis on 1% agarose gels. The protein composition of these fractions was analyzed by treating samples at 100°C for 3 min in Studier buffer (31) and fractionating the denatured proteins by electrophoresis on 7.5% polyacrylamide-sodium dodecyl sulfate (SDS) slab gels. If necessary, L- and M-dsRNA peaks were separately pooled and centrifuged again on 35 to 55% (wt/vol) sucrose gradients.

dsRNA assay. dsRNA was quantitated in different subcellular fractions after cell disruption and partial purification of the dsRNA. Exogenous ^{32}P-labeled S-dsRNA (a discrete species of 0.73 × 10^6 daltons) was added just after cell disruption as an internal indicator of recovery during subsequent fractionation. This S-dsRNA was obtained from strain S3 by purification as described above. Small cultures (150 ml) of strain 2-1 were grown in low-phosphate YEPD medium with [^{32}P]orthophosphate (1.5 mCi/liter) to early stationary phase, and cells were harvested, washed, and broken in 4 ml of PKE buffer by blending in a Vortex mixer three times for 15 s each with 2/3 vol of 0.45-mm glass beads. The bead slurry mixture was washed five times with 4 ml of PKE buffer, and to the pooled supernatant material 15 μg of S-dsRNA (3.2 × 10^4 cpm/μg) was added. Portions of this total cell extract were fractionated by low-speed or high-speed centrifugation (as described for VLP preparation) and from the supernatants or redissolved precipitates the nucleic acids were extracted with phenol and isolated by ethanol precipitation. The dsRNA was purified from contaminating single-stranded RNA and DNA by LiCl precipitation as described by Fried (15) and by hydrolysis with DNase and RNase at high-salt concentration as described above. The amount of L-, M-, and S-dsRNA in each sample was determined after electrophoretic fractionation on agarose gels by densitometry of the gel autoradiograms on a Joyce-Loebl densitometer.

Preparation of immune sera and immunoglobulin G fractions. Antisera to VLPs and ScV-P1 were prepared by immunization of New Zealand white rabbits with VLPs isolated from strain 3/A1, or with polyacrylamide preparative gel electrophoretically purified ScV-P1, as previously described (20). The immunoglobulin G fractions of the immune sera were purified (20) and used for immunoprecipitation assay.

Immunoprecipitation assay of ScV-P1. The subcellular distribution of ScV-P1 polypeptide was determined by immunoprecipitation assay after cell disruption and fractionation. Cultures (30 ml) of strain 2-1 grown in SMD were labeled by addition of [^{35}S]methionine at a cell density of 10^7 cells per ml to a final concentration of 15 μCi/ml. Cells were grown to early stationary phase, and extracts were prepared essentially as described above for dsRNA assay. Portions of the extract were fractionated by low-speed or high-speed centrifugation, and supernatants and redissolved precipitates were assayed by immunoprecipitation. Solid-state immunoprecipitation reactions were performed as previously described (Bostian et al., in press). Each reaction contained 40 μg of immune or preimmune rabbit immunoglobulin, 25 μl of sample, and 1.5 mg of protein A-Sepharose 4BCL. Immunoprecipitates were analyzed after dissociation in 2× Studier buffer for 4 to 5 min at 100°C by electrophoresis on 10% polyacrylamide-SDS slab gels. The amount of radiolabeled protein in the ScV-P1 band was determined by densitometry after autoradiography of the gel by the method of Bonner and Laskey (5).

Peptide mapping. Pooled fractions of highly purified VLPs containing L- or M-dsRNA were dialyzed extensively against 0.05 M Na_2HPO_4/NaH_2PO_4 (pH 7.6), lyophilized, and dissolved in a small volume of water. These were digested in situ with *S. aureus* V8 protease as described by Cleveland et al. (13). Reactions were performed at 37°C in 0.125 M Tris-hydrochloride (pH 6.8), 0.1% (wt/vol) SDS, and 1 mM EDTA in a total volume of 350 μl. Protein concentrations were approximately 2 mg/ml at either 25 or 125 μg of protease per ml. After different incubation intervals, samples were taken and made 2% (wt/vol) SDS-1% (vol/vol) β-mercaptoethanol, and heated at 100°C for 3 min. The p ›ptides generated by these procedures were then analyzed and compared by electrophoresis on SDS-polyacrylamide slab gels.

RESULTS

Purification and fractionation of VLPs. When crude VLP preparations from the killer strain 2-1 were centrifuged on 10 to 40% (wt/vol) sucrose gradients, analysis of the dsRNA content of fractions (Fig. 1) demonstrated a substantial enrichment for VLPs in the fractions pooled (9–14). This approach enables a rapid assessment of particle location in overloaded gradients, thus avoiding loss in yield by more extensive preliminary purification. The VLPs in the pooled fractions were then directly applied to a second 35 to 55% (wt/vol) sucrose gradient to avoid additional time-consuming manipulations that lead to particle disruption. The separation of VLPs containing L- or M-dsRNA thus achieved is demonstrated by the dsRNA and protein profiles shown in Fig. 2. These indicate that the pooled outer shoulders of the L and M VLP peaks should yield preparations with less than 5% cross-contamination. Electrophoretic analysis of the VLP proteins on polyacrylamide-SDS

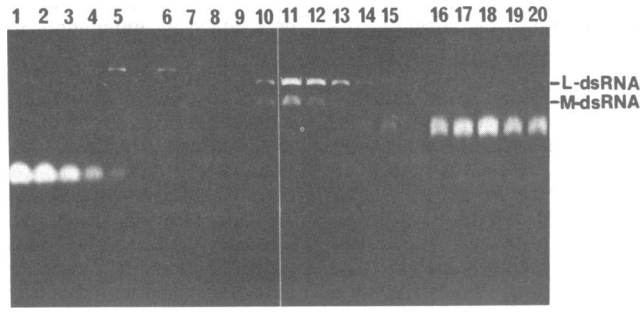

FIG. 1. *Preparation and partial purification of VLPs. Crude VLP preparations of strain 2-1 were made as described in the text and enriched for VLPs by centrifugation on 10 to 40% (wt/vol) sucrose gradients. Fractions containing VLPs were determined by assaying for dsRNA. Portions of each fraction were taken and analyzed by ethidium bromide-stained 1% agarose slab gel electrophoresis as shown. Fractions 1 to 20 are from top to bottom of the gradient, respectively.*

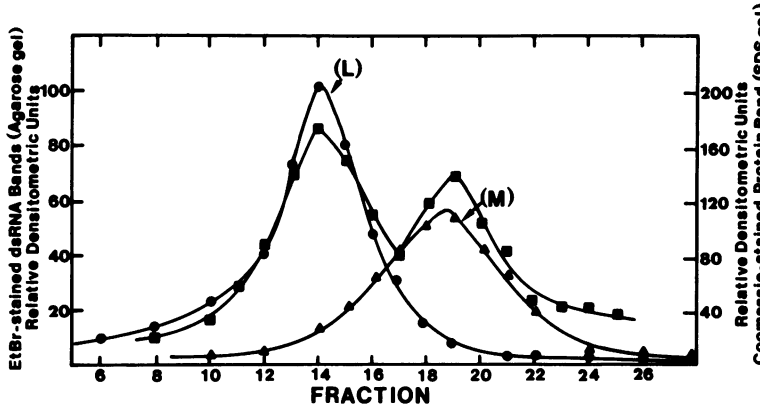

FIG. 2. *Separation of VLPs containing L- or M-dsRNA. The enriched VLP preparation of strain 2-1 generated by the 10 to 40% (wt/vol) sucrose gradient centrifugation of Fig. 1 was fractionated by a 35 to 55% (wt/vol) sucrose gradient centrifugation and the gradient fractions analyzed for dsRNA and protein content as described in the text. The quantity of L-dsRNA (●) and M-dsRNA (▲) were determined by densitometry of their EtBr-stained gel bands after electrophoresis on a 1% agarose slab gel. The quantity of capsid polypeptide (■) was determined by densitometry of its Coomassie blue-stained gel band after electrophoresis on a 10% SDS-polyacrylamide slab gel.*

gels reveals a single major polypeptide in both L and M particles (Fig. 3). For L-dsRNA VLPs, this protein has already been identified as ScV-P1, previously estimated at 88,000 daltons (20). Also observed in both L and M particles are two minor polypeptides migrating at the same rate as the species ScV-P2 and ScV-P3 previously observed in particle preparations from strain 3/A1, a nonkiller lacking M-dsRNA (20). No other particle-associated polypeptides were consistently detected by electrophoretic analysis of these gradients.

ScV-P1 polypeptide encapsidates both L- and M-dsRNA. L and M particles from the distal shoulders of the 35 to 55% (wt/vol) sucrose gradient fractionation were essentially free of cross-contamination. The major polypeptides in both preparations were shown to co-migrate on SDS-polyacrylamide gels (Fig. 3) and to be specifically precipitated by anti-ScV-P1 antibody (data not shown). These polypeptides were further compared with each other and to ScV-P1 obtained from the VLPs of strain 3/A1 by peptide mapping. Digestions were performed in parallel for various times with a constant amount of *S. aureus* V8 protease. The partial hydrolysis fragments were then resolved by electrophoresis on a 10 to 15% polyacrylamide-SDS slab gel as shown in Fig. 4. As seen, all three polypeptides (ScV-P1 from strain 3/A1 and the major polypeptide of L- and M-VLPs from strain 2-1) give similar patterns. The proteins from VLPs of strain 2-1 had been lyophilized and were consequently less sensitive to protease, accounting for the higher concentrations of enzyme used for its hydrolysis (Fig. 4B and C). The enzyme accounts

for the two bands at about 38,000 daltons which are prominent in Fig. 4B and C. The major products at 30,000 to 32,000 daltons and at about 45,000 daltons in Fig. 4A are seen to be partial hydrolysis products, since they are transiently present in the more complete hydrolysis series shown in Fig. 4B and C. The coincidence of both partial and limit hydrolysis products confirms the identity of the capsid proteins in the three samples.

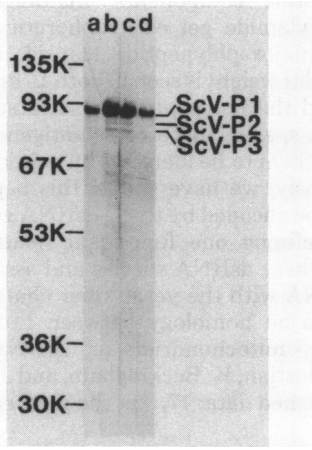

FIG. 3. *Protein profiles of fractionated VLPs. VLP fractions containing L- or M-dsRNA from the 35 to 55% (wt/vol) sucrose gradients of Fig. 2 were analyzed for their protein content by electrophoresis on a 10% SDS-polyacrylamide slab gel. Lanes are (a) fraction 11 from distal shoulder of L-VLPs; (b) fraction 14 from peak of L-VLPs; (c) fraction 19 from peak of M-VLPs; and (d) fraction 22 from distal shoulder of M-VLPs.*

Association of VLP components. The encapsidation of M-dsRNA by ScV-P1, a protein coded by L-dsRNA (20), suggests that encapsidation is important for M-dsRNA maintenance. We therefore wished to investigate the extent of encapsidation of both M- and L-dsRNA in vivo and also to obtain an estimate of the fraction of ScV-P1 in particulate form. The susceptibility of particles to breakage during cell disruption and fractionation limits the recovery of both protein and dsRNA in VLPs employing the experimental procedures described. We overcame these problems by assaying for dsRNA or ScV-P1 in crude extracts fractionated by differential centrifugation. Reconstitution experiments with exogenous radiolabeled ScV-P1 or S-dsRNA show little nonspecific association of these soluble components with membrane fractions obtained from low-speed centrifugation or with the particle fraction obtained by ultracentrifugation of the clarified extract. The relatively high percentage of dsRNA recovered in encapsidated form (Table 2) shows that VLPs are apparently stable during cell breakage with glass beads. Although 100% of the exogenous S-dsRNA is found in the high-speed supernatant, 95% of M-dsRNA and 87% of L-dsRNA are found in the membrane or particle fraction, of which the majority is found in particles. Immunoprecipitation of these fractions with anti-ScV-P1 immunoglobulin G reveals that the majority of the poly-

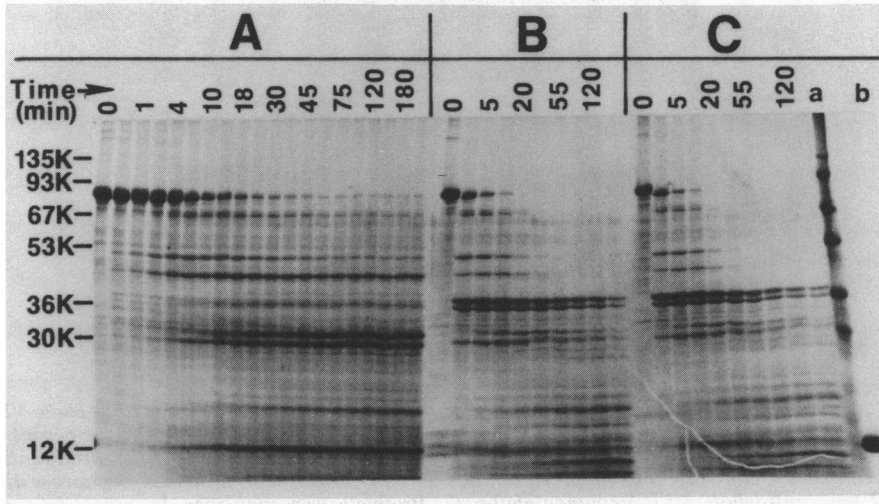

FIG. 4. *Peptide maps of capsid polypeptide from VLPs containing L- or M-dsRNA. The capsid polypeptides derived from (A) L-VLPs of strain 3/A1 (K^- R^-), or from (B) L-VLPs, or (C) M-VLPs of strain 2-1 (K^+ R^+) were compared by limited S. aureus V8 protease hydrolysis. Digestions were performed in parallel for various times, and the resulting peptides were analyzed by electrophoretic fractionation on a 10 to 15% SDS-polyacrylamide slab gel. Lanes (a) and (b) are marker proteins of known molecular weight (20).*

peptide is associated with dsRNA in the high-speed precipitate, presumably as intact capsid, with a significant amount (17%) membrane associated. No additional particle-associated polypeptides were found after immunoprecipitation with anti-whole VLP immunoglobulin G (Fig. 5). From these results it is clear that most of the dsRNA and ScV-P1 found in these cells exist in particles with little of either free in the cytoplasm.

TABLE 2. *Amount of free and particle-associated dsRNA and ScV-P1 in the killer strain 2-1*

Cellular fraction	% total dsRNA			% Total immunoprecipitable ScV-P1
	L	M	S	
Low-speed precipitate	16.4	10	0	17.3
Low-speed supernatant	83.6	90.0	100	82.7
High-speed precipitate	70.6	84.4	0	79.1
High-speed supernatant	13.0	5.6	100	3.5

DISCUSSION

Having obtained adequate amounts of homogeneous VLPs containing L- or M-dsRNA, we have for the first time been able to assess their polypeptide components unambiguously. By polyacrylamide gel electrophoretic analysis, a single major polypeptide (capsid) of identical molecular weight is seen in both L- and M-VLPs. We find the polypeptides associated with each dsRNA species to be cross-antigenic and have shown them to be identical by peptide mapping. Previously, we have shown this peptide (ScV-P1) to be encoded by the L-dsRNA genome (20), thus defining one functional relationship between these dsRNA species and associating the L-dsRNA with the yeast killer character. Since there is no homology between L-dsRNA and nuclear, mitochondrial, or 2μDNA genomes (K.A. Bostian, K. Beckingham, and J.E. Hopper, unpublished data; 17, 39), the gene encoding the

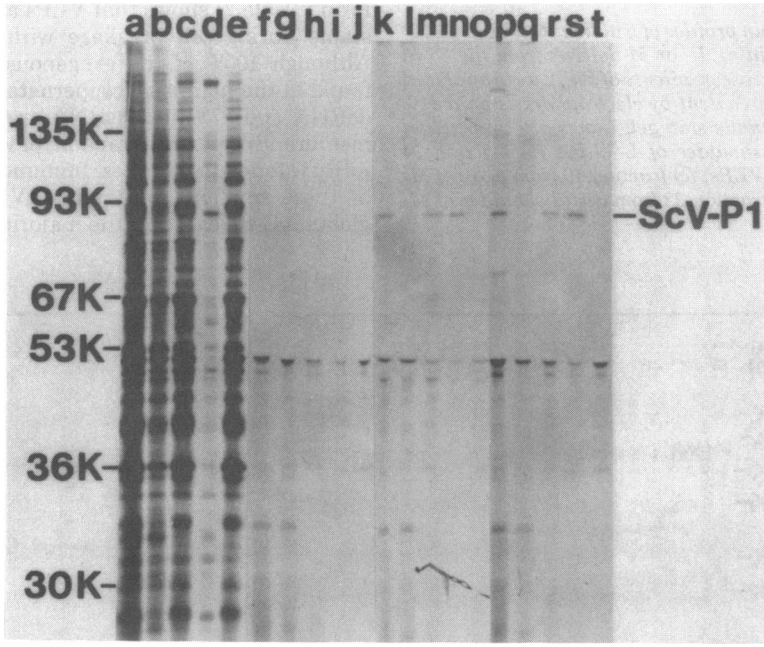

FIG. 5. *Immunoprecipitation of various subcellular fractions with anti-ScV-P1 or anti-whole VLP immunoglobulin G. Cells of strain 2-1 were grown in SMD containing 15 μCi of [^{35}S]methionine per ml, and cells were harvested in early stationary phase. Cells were disrupted and fractionated by differential centrifugation as described above. Solid-state immunoprecipitations were performed with protein A-Sepharose 4B CL with anti-ScV-P1 or anti-whole VLP immunoglobulin G. Products were analyzed by autoradiography after electrophoresis on a 10% SDS-polyacrylamide gel. Lanes (a to e) are total [^{35}S]methionine-labeled proteins present before immunoprecipitation in: (a) total cell extract; (b) low-speed precipitate; (c) low-speed supernatant; (d) high-speed precipitate; (e) high-speed supernatant. Lanes (f to j) are the fractions of (a to e), respectively, precipitated with pre-immune immunoglobulin G, and similarly lanes (k to o) precipitated with anti-ScV-P1 immunoglobulin G, and lanes (p to t) precipitated with anti-whole VLP IgG.*

M-dsRNA particle capsid polypeptide is solely resident in the L-dsRNA genome, supporting our hypothesis (6, 20) that encapsidation of M-dsRNA by ScV-P1 may be one basis for the apparent dependency of the M-dsRNA genome on the L-species dsRNA (1, 3, 6, 20, 25, 32). Because L-dsRNA maintenance is independent of any M-dsRNA-encoded function, since L-dsRNA can exist autonomously, the association between M- and L-dsRNA resembles that between a defective and a helper virus rather than that of components of an interdependent segmented mycovirus genome.

If M-dsRNA maintenance or replication is dependent upon encapsidation, then this finding is sufficient to account for the apparent dependency of M-dsRNA on L-dsRNA. Moreover, since the nonoverlapping single-stranded coding capacity of L-dsRNA is about 160,000 daltons of protein, in addition to ScV-P1, L-dsRNA could encode other polypeptides utilized by both dsRNA species. A number of less abundant polypeptide translation products other than ScV-P1 have been observed when L-dsRNA is translated in a reticulocyte cell-free system (K.A. Bostian and R.L. Lee, unpublished data), and these may represent such functions.

We have also demonstrated that at least 85% of the total L-dsRNA and M-dsRNA is encapsidated in virus-like particles. Furthermore, 95% of the capsid polypeptide was found in particulate form and not free in the cytoplasm. This indicates a stringent control of VLP replication, involving control of dsRNA maintenance or ScV-P1 production or both. This control could be exerted over both dsRNA and capsid protein synthesis, or VLP production could be controlled by dsRNA replication alone, with a rapid turnover of capsid protein not incorporated into VLPs. Conversely, control over capsid production alone could control VLP production if unencapsidated dsRNA were unstable or if dsRNA were only synthesized from fully encapsidated single-stranded primary transcript. Any of these patterns would be consistent with the observed paucity of free dsRNA and capsid protein. The latter model, however, is supported by the findings of Herring and Bevan that VLPs from growing cells synthesize both dsRNA as well as complete single-stranded RNA transcripts of the dsRNA genome (2, 19). The yeast system may therefore undergo a replicative cycle similar to reovirus (28), involving an intermediate mRNA transcript which becomes a substrate for synthesis of a complementary strand only when complexed with virion polypeptide. In this way translation of the mRNA strand is autoregulated. By analogy, it is probably that although L- and M-dsRNA genomes are separately encapsidated, their replicative cycles are not entirely autonomous, but rather both are dependent upon ScV-P1 derived from L-dsRNA for antisense strand replication. It is not known, however, whether transcription or replication of the M- or L-dsRNA genomes requires intact particles. The availability of homogeneous particle preparations, gently and rapidly isolated, may aid in investigating their properties.

The lack of a VLP infectious cycle and host cell lysis (2) and the observation that the VLP content of a given strain is relatively independent of growth phase (A. J. Herring, personal communication; 26, 33) suggest that VLPs replicate in step with the yeast cell. The above results do not rule out a transient production of naked dsRNA during a brief replicative phase of a prolonged cell cycle. Density shift experiments indicate that L-dsRNA synthesis occurs in all phases of the cell cycle except in S (W. L. Fangman, personal communication), so that coupling to DNA replication must be indirect. Clearly, clarification of the replicative cycle and morphogenic development of the killer VLPs will require analysis within the yeast cell cycle, which should be aided by the dsRNA, particle, and protein assays utilized above.

Because heteroduplex analysis of the yeast dsRNA's and extensive genetic analysis of the yeast killer system indicate that the L- and M-dsRNA genomes are unrelated, and therefore did not evolve from some common sequence, the evolution of the present dependence of M-dsRNA on L-dsRNA is not immediately obvious. Presumably, the interdependent segmented genomes of mycoviruses have evolved from a linked genetic precursor, and perhaps, L- and M-dsRNA evolved from a single genome by fragmentation into segments essential for toxin production and replication. However, the autonomy of L-dsRNA and its ubiquitous existence in most yeast strains suggest that the yeast killer system may have developed fortuitously from autonomous and independent replicons. A suggested mechanism for this comes from work by Clare and Oliver (12). They have shown that during adverse cellular growth conditions, such as nitrogen starvation, L-dsRNA is degraded. Presumably, the degraded nucleotides enable the host cell to complete its cell division. However, not all of the L-dsRNA is lost during starvation, and the authors suggest that the encapsidated dsRNAs are the ones preserved. Perhaps unencapsidated cytoplasmic dsRNAs could persist in progenitors of contemporary yeast cells, but ScV-P1 may have helped maintain the M-dsRNA genome under stress conditions.

ACKNOWLEDGMENTS

This research was supported in part by Public Health Service grants GM 20755 (D. J. T.) and GM 10610 awarded to Harlyn O. Halvorson and by an American Cancer Society Postdoctoral Fellowship 1438 (K.A.B.).

LITERATURE CITED

1. **Berry, E. A., and E. A. Bevan.** 1972. A new species of double-stranded RNA from yeast. Nature (London) New Biol. **239**:279–280.
2. **Bevan, E. A., and A. J. Herring.** 1976. The killer character in yeast: preliminary studies of virus-like particle replication. p. 153–162. *In* W. Bandlow, R. J. Schweyen, D. Y. Thomas, K. Wolf, and F. Kaudewitz (ed.), Genetics, biogenesis and bioenergetics of mitochondria. Walter deGruyter, New York.
3. **Bevan, E. A., A. J. Herring, and D. J. Mitchell.** 1973. Preliminary characterization of two species of dsRNA in yeast and their relationship to the "killer" character. Nature (London) **245**:81–86.
4. **Bevan, E. A., and J. M. Somers.** 1960. Somatic segregation of the killer (k) and neutral (n) cytoplasmic genetic determinants in yeast. Genet. Res. **14**:71–77.
5. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labeled protein and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–88.
6. **Bostian, K. A., J. E. Hopper, D. J. Rogers, and D. J. Tipper.** 1980. Translational analysis of the killer-associated virus-like particle dsRNA genome of *S. cerevisiae*: M dsRNA encodes toxin. Cell **19**:403–414.
7. **Bruenn, J., and W. Kane.** 1978. Relatedness of the double-stranded RNAs present in yeast virus-like particles. J. Virol. **26**:762–772.
8. **Buck, K. W., and R. F. Girvan.** 1977. Comparison of the biophysical and biochemical properties of *Penicillium cyaneo-fulvum* virus and *Penicillium chrysogenum* virus. J. Gen. Virol. **34**:145–154.
9. **Buck, K. W., and G. F. Kempson-Jones.** 1974. Capsid polypeptides of two viruses isolated from *Penicillium stoloniferum*. J. Gen. Virol. **22**:441–445.
10. **Buck, K. W., P. Lhoas, D. J. Border, and B. K. Street.** 1973. Virus particles in yeast. Biochem. Soc. Trans. **1**: 1141–1142.
11. **Buck, K. W., and G. Ratti.** 1975. Biophysical and biochemical properties of two viruses isolated from *Aspergillus foetidus*. J. Gen Virol. **27**:211–224.
12. **Clare, J. J., and S. G. Oliver.** 1979. The regulation of RNA synthesis in yeast IV. Synthesis of double-stranded RNA. Mol. Gen. Gent. **171**:161–166.
13. **Cleveland, D. W., S. G. Fisher, M. W. Kirschner, and U. K. Laemmli.** 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. **252**:1102–1106.
14. **Fink, G. R., and C. A. Styles.** 1972. Curing of a killer factor in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. U.S.A. **69**:2846–2849.
15. **Fried, H. M., and G. Fink.** 1978. Electron microscopic heteroduplex analysis of "killer" double-stranded RNA species from yeast. Proc. Natl. Acad. Sci. U.S.A. **75**: 4224–4228.
16. **Harris, M. S.** 1978. Virus-like particles and double-stranded RNA from killer and non-killer strains of *Saccharomyces cerevisiae*. Microbiol. **21**:161–174.
17. **Hastie, N., V. Brennan, and J. Bruenn.** 1978. No homology between double-stranded RNA and nuclear DNA of yeast. J. Virol. **28**:1002–1005.
18. **Herring, A. J., and E. A. Bevan.** 1974. Virus-like particles associated with the double-stranded RNA species found in killer and sensitive strains of the yeast *Saccharomyces cerevisiae*. J. Gen. Virol. **22**:387–394.
19. **Herring, A. J., and E. A. Bevan.** 1977. Yeast virus-like particles possess a capsid-associated single-stranded RNA polymerase. Nature (London) **268**:464–466.
20. **Hopper, J. E., K. A. Bostian, L. B. Rowe, and D. J. Tipper.** 1977. Translation of the L-species dsRNA genome of the killer-associated virus-like particles of *Saccharomyces cerevisiae*. J. Biol. Chem. **252**:9010–9017.
21. **Joklin, W. K.** 1974. Reproduction of Reoviridae, p. 231–334. *In* H. Fraenkel-Conrat and R. Wagner (ed.), Comparative virology, vol. 2. Plenum Publishing Corp., New York.
22. **Lemke, P. A.** 1976. Viruses in eucaryotic microorganisms. Annu. Rev. Microbiol. **30**:105–145.
23. **Lemke, P. A., and C. H. Nash.** 1974. Fungal viruses. Bacteriol Rev. **38**:29–56.
24. **Mitchell, D. J., E. A. Bevan, and A. J. Herring.** 1973. The correlation between dsRNA in yeast and the "killer-character." Heredity **31**:133–134.
25. **Mitchell, D. J., A. J. Herring, and E. A. Bevan.** 1976. The genetic control of dsRNA virus-like particles associated with *Saccharomyces cerevisiae* killer yeast. Heredity **37**:129–134.
26. **Oliver, S. G., S. J. McCready, C. Holm, P. A. Sutherland, C. S. McLaughlin, and B. S. Cox.** 1977. Biochemical and physiological studies of the yeast virus-like particle. J. Bacteriol. **130**:1303–1309.
27. **Palfree, R., and H. Bussey.** 1979. Yeast killer toxin: purification and characterization of the protein toxin from *Saccharomyces cerevisiae*. Eur. J. Biochem. **93**: 487–493.
28. **Schonberg, M., S. C. Silverstein, D. H. Levin, and G. Acs.** 1971. Asynchronous synthesis of the complimentary strands of the reovirus genome. Proc. Natl. Acad. Sci. U.S.A. **68**:505–508.
29. **Silverstein, S. C., J. K. Christman, and G. Acs.** 1976. The reovirus replicative cycle. Annu. Rev. Biochem. **45**:375–408.
30. **Somers, J. M., and E. A. Bevan.** 1968. The inheritance of the killer character in yeast. Genet. Res. **13**:71–83.
31. **Studier, F. W.** 1973. Analysis of bacteriophage T7 early RNAs and protein on slab gels. J. Mol. Biol. **79**:237–248.
32. **Vodkin, M. H., and G. Fink.** 1973. A nucleic acid associated with a killer strain of yeast. Proc. Natl. Acad. Sci. U.S.A. **70**:1069–1072.
33. **Vodkin, M., F. Katterman, and G. R. Fink.** 1974. Yeast killer mutants with altered double-stranded ribonucleic acid. J. Bacteriol. **117**:681–686.
34. **Wickner, R. B.** 1974. Chromosomal and nonchromosomal mutations affecting the "killer character" of *Saccharomyces cerevisiae*. Genetics **76**:423–432.
35. **Wickner, R. B.** 1974. "Killer character" of *Saccharomyces cerevisiae*: curing by growth at elevated temperatures. J. Bacteriol. **117**:1356–1357.
36. **Wickner, R. B.** 1978. Twenty-six chromosomal genes needed to maintain the killer double-stranded RNA plasmid of *Saccharomyces cerevisiae*. Genetics **88**:419–425.
37. **Wickner, R. B.** 1979. The killer double-stranded RNA plasmids of yeast. Plasmid **2**:303–322.
38. **Wickner, R. B., and M. J. Leibowitz.** 1976. Chromosomal genes essential for replication of a double-stranded RNA plasmid of *Saccharomyces cerevisiae*: the killer character of yeast. J. Mol. Biol. **105**:427–443.
39. **Wickner, R. B., and M. J. Leibowitz.** 1977. Dominant chromosomal mutation by-passing chromosomal genes needed for killer RNA plasmid replication in yeast. Genetics **87**:453–469.
40. **Wickner, R. B., and M. J. Liebowitz.** 1979. *mak* mutants of yeast: mapping and characterization. J. Bacteriol. **140**:154–160.
41. **Wood, H. A.** 1973. Viruses with double-stranded RNA genomes. J. Gen. Virol. **20**:61–85.