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Three Different M_1 RNA-Containing Viruslike Particle Types in *Saccharomyces cerevisiae*: In Vitro M_1 Double-Stranded RNA Synthesis

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Killer strains of *Saccharomyces cerevisiae* bear at least two different double-stranded RNAs (dsRNAs) encapsidated in 39-nm viruslike particles (VLPs) of which the major coat protein is coded by the larger RNA (L-A dsRNA). The smaller dsRNA (M_1 or M_2) encodes an extracellular protein toxin (K_1 or K_2 toxin). Based on their densities on CsCl gradients, L-A- and M_1-containing particles can be separated. Using this method, we detected a new type of M_1 dsRNA-containing VLP (M_1-H VLP, for heavy) that has a higher density than those previously reported (M_1-L VLP, for light). M_1-H and M_1-L VLPs are present together in the same strains and in all those we tested. M_1-H, M_1-L, and L-A VLPs all have the same types of proteins in the same approximate proportions, but whereas L-A VLPs and M_1-L VLPs have one dsRNA molecule per particle, M_1-H VLPs contain two M_1 dsRNA molecules per particle. Their RNA polymerase produces mainly plus single strands that are all extruded in the case of M_1-H particles but are partially retained inside the M_1-L particles to be used later for dsRNA synthesis. We show that M_1-H VLPs are formed in vitro from the M_1-L VLPs. We also show that the peak of M_1 dsRNA synthesis is in fractions lighter than M_1-L VLPs, presumably those carrying only a single plus M_1 strand. We suggest that VLPs carrying two M_1 dsRNAs (each 1.8 kilobases) can exist because the particle is designed to carry one L-A dsRNA (4.5 kilobases).

Killer strains of the yeast *Saccharomyces cerevisiae* secrete a protein toxin (killer toxin) lethal to other strains. The toxin is encoded by a double-stranded RNA (dsRNA) called M, the size of which varies around 1.5 (M_2) or around 1.8 (M_1) kilobases (kb), depending on the killer determinant, and that is present in intracellular viruslike particles (VLPs). (For recent reviews, see references 12, 32, 40, and 40a.) M_1 maintenance and replication requires another dsRNA, called L-A (4.5 kb) (25, 29, 30), and the products of at least 30 chromosomal genes, including *MAK1* through *MAK28* (39, 41; for a review, see reference 40), *PET18* (4, 20), and *SPE2* (13). Most yeast strains also have three other dsRNAs, called L-BC (4.5 kb) (29, 30, 42), T (2.7 kb), and W (2.25 kb) (38). L-A encodes the major protein of the VLPs in which M_1 and L-A itself are encapsidated (1, 17–19, 30). Like L-A and M_1, L-BC is also present in intracellular VLPs, but it uses a coat protein different from that encapsidating L-A and M_1 (30). Each L-A-containing VLP is reported to have only one dsRNA molecule (7). T and W dsRNAs were not found in VLPs (38).

VLPs carrying M_1, L-A, or L-BC dsRNA have an RNA polymerase activity which synthesizes single-stranded RNA (ssRNA), and this RNA product has been shown to be the message strand (or plus strand) in the case of M_1 and L-A (5, 6, 8, 18, 30, 31, 35, 37). L VLPs (presumably L-A VLPs) from logarithmic-phase cells have also been reported, preliminarily, to have an RNA polymerase activity which produces dsRNA (3). Replication of both L-A and M_1 is apparently conservative as judged by density transfer experiments (23, 26), and L-A replicates by a sequential synthesis of the plus strand, followed by the minus strand (A. M. Newman and C. S. McLaughlin, Program Abstr. 12th Int. Congr. Genet., p. 80, 1984). The single-stranded plus strands, not the dsRNA, presumably serve as template for minus-strand synthesis.

We report here further investigation of VLPs containing M_1, M_2, L-A, or L-BC. We detected particles containing partial molecules of L-A and M_1. We also report that M_1 dsRNA-containing particles are separable by density into two varieties, one of which (M_1-H) extrudes all of its product as ssRNA into the reaction medium, whereas the other variety (M_1-L) retains some of its product within the particle, part as dsRNA and part as ssRNA. A third kind of particle, lighter than M_1-L, has little dsRNA, has a density predicted for particles containing only a single strand of M_1, and synthesizes mainly M_1 dsRNA. We suggest a replication cycle involving all of the types of particles observed and propose a "head-full" hypothesis to explain the existence of M_1-H VLPs.

MATERIALS AND METHODS

Strains and media. Strains used are listed in Table 1. Media were as previously described (39).

Cytoduction. A cytoplasmic genome can be transferred from one haploid strain to another without diploidization or other change of nuclear genotype by transient heterokaryon formation (cytoplasmic mixing, cytoduction) by using the *kar1* mutant, defective in nuclear fusion (14). The procedure was the same as described by Ridley et al. (25), with K^- strains as recipients and K^+ strains as donors.

Assay of killing activity. Colonies to be tested for killing ability were replica plated onto MB medium which had just been seeded with a lawn of the sensitive strain 5X47 (0.5 ml of a suspension with an optical density at 650 nm of 1 per ml, spread on each plate and allowed to dry). These MB plates were incubated at 20°C for 2 to 3 days. Killing was indicated by a clear zone surrounding the killer strain surrounded in turn by growth of the lawn of sensitive cells.

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TABLE 1. Strains of *S. cerevisiae* used

Designation	Genotype[a]	dsRNAs present	Source or reference
S7	α [KIL-o]	L-A-HN, L-BC	24
1101[b]	α *his4 kar1-1* [KIL-k_1] [B-o]	L-A-HN, L-BC, M_1	25
1368[b]	α *his4 kar1-1* [KIL-k_1] [B]	L-A-HN, L-BC, M_1	34
2507	a *arg1-1 ski2-2* [KIL-o]	L-BC, T, W	This work
RE23[b]	a *arg1-1 ski2-2* [KIL-k_1] [B-o]	L-A-HN, L-BC, M_1, T, W	Cytoductant from 1101 into 2507
RE24[b]	a *arg1-1 ski2-2* [KIL-k_1] [B]	L-A-HN, L-BC, M_1, T, W	Cytoductant from 1368 into 2507
1074[b]	a *leu1 kar1-1* [KIL-k_1] [B-o]	L-A-HN, M_1	25

[a] [KIL-k_1], wild-type K_1 killer cytoplasmic genome (its molecular determinant is the 1.8-kb M_1 linear dsRNA) carrying killing and resistance functions. [B] was initially described as [KIL-b] by Toh-e and Wickner (34). It is a cytoplasmic factor tnat bypasses the requirement of some MAK gene products for M_1 dsRNA maintenance. In addition, [B] gives strains a "superkiller" phenotype.

[b] These strains were examined for the presence of M_1-H and M_1-L VLPs, and all were found to have both types (see Results). We observed, however, that the *ski2-2* strains RE23 and RE24 had, in general, a higher proportion of M_1-H VLPs than the *SKI*$^+$ strains.

VLP preparation. Cells were cultured in YPE broth (1% yeast extract, 2% peptone, 4% ethanol, 0.4 g of adenine sulfate per liter) at 30°C for 3 to 4 days, harvested, and washed once with distilled water. Washed cells were treated with 2 ml of 0.2 M Tris sulfate (pH 9.3)–10 mM EDTA–1:20 (vol/vol) 2-mercaptoethanol per g wet weight for 30 min at room temperature. After centrifugation for 5 min at 5,000 rpm in an SS34 rotor, cells were suspended in 1 ml of 1 M sorbitol–20 mM potassium phosphate (pH 6.8)–Zymolyase 60000 (0.5 mg/ml; Kirin Brewing, Tokyo, Japan) per g original wet weight and incubated at 30°C for 1 h. The resultant spheroplasts were collected, suspended in buffer A (50 mM Tris hydrochloride [pH 7.6], 10 mM EDTA, 0.15 M NaCl, 1 mM dithiothreitol) that contained 1 mg of bentonite per ml, and lysed by passing through a French pressure cell at 14,000 lb/in^2. Cell debris was removed by low-speed centrifugation (9,600 × *g*) for 30 min. VLPs were collected by high-speed centrifugation (100,000 × *g*) for 40 min, suspended in buffer A, and centrifuged again at low speed. The density of the final supernatant, that contained VLPs, was adjusted to 1.35 by addition of CsCl, and the suspension was centrifuged (130,000 × *g*) for 20 h at 4°C. Fractions were collected and dialyzed overnight against buffer A containing 20% glycerol; the dsRNA and protein content of each fraction were examined by agarose gel and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, respectively. Small aliquots were used for RNA polymerase assay.

RNA polymerase assay. RNA polymerase activity was measured by the method described by Welsh et al. (37), with slight modifications. VLPs were incubated in an assay mixture containing 50 mM Tris hydrochloride (pH 7.6), 5 mM $MgCl_2$, 0.1 mM EDTA, 20 mM NaCl, 5 mM KCl, 10 mM 2-mercaptoethanol, 0.5 mM each of ATP, GTP, and CTP, 20 μM [α-^{32}P]UTP (1 mCi/μmol; New England Nuclear Corp., Boston, Mass.), and 1.5 mg of bentonite per ml. After incubation at 30°C for 1 to 2 h, the reaction products were precipitated by the addition of 0.3 ml of 0.3% *Torula* RNA (Sigma Chemical Co., St. Louis, Mo.) as carrier and 10 mM sodium pyrophosphate, followed by 10% trichloroacetic acid (TCA). After incubation on ice for 10 min, the precipitate was collected on a Whatman GF/B glass fiber filter, and its radioactivity was measured. Bentonite was prepared as previously described (15). All the experiments were done under conditions in which the activity was linear with time and with the amount of VLPs.

dsRNA. Different dsRNAs (L-A, L-BC, T, W, M_1, and M_2) were purified by cellulose chromatography as previously described (33). To analyze dsRNA in cells, the rapid method for extraction described by Fried and Fink (16) was used, and the extracted dsRNAs were analyzed on 1.5% agarose gels.

M13 DNA. Plasmid pKC2 (27) carries a 1-kb cDNA of M_1, including most of the preprotoxin-coding region. M13 clones carrying the insert of pKC2 in each orientation (mp10 KC2 and mp11 KC2) were kindly provided by N. Skipper. Viral DNA was purified as previously described (22).

Hybridization. The hybridization procedure was essentially the same as the method described by Welsh and Leibowitz (35). RNA polymerase reaction products, labeled with ^{32}P as described above, were purified by phenol extraction in the presence of 0.2% sodium dodecyl sulfate and 7 mM EDTA, precipitated with 70% ethanol, and washed once with 70% ethanol. The precipitate was dried and dissolved in 8 × SSC containing 0.2% sodium dodecyl sulfate and 200 μg of Torula RNA per ml as carrier. (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate.) A 2-μg amount of purified, cold ssDNA from the two different KC2 clones, with plus and minus strands as inserts (or H_2O alone, as control), was

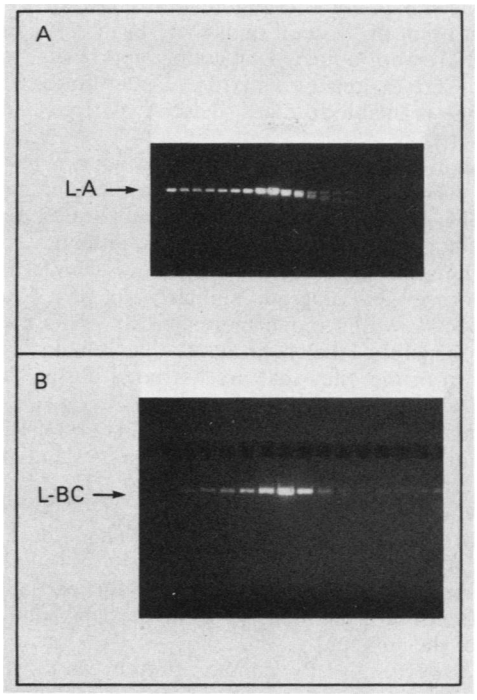

FIG. 1. Agarose gel electrophoresis of L-A (A) and L-BC (B) dsRNAs from VLPs purified by CsCl gradient equilibrium sedimentation. *S. cerevisiae* strains used were S7 (A) and 2507 (B) (Table 1). Portions of each fraction of the CsCl gradient were diluted 10-fold with buffer B (50 mM Tris hydrochloride, 1 mM EDTA, 0.2% sodium dodecyl sulfate) before extraction with 1 volume of phenol. The samples used for agarose gel electrophoresis were taken directly from the aqueous phase without ethanol precipitation. In the figure, the left corresponds to the bottom of the gradient (the denser fractions), and the right corresponds to the lighter fractions.

denatured by heating at 83°C for 3 min in the presence of 90% formamide (final volume, 10 μl); then the same volume of ^{32}P-labeled RNA polymerase reaction products, dissolved in 8 × SSC, was added. After another 1-min incubation at 83°C, annealing was begun at 46°C. After a 30-min incubation at 46°C, annealing was stopped by the addition of 50 volumes of 2 × SSC alone or 2 × SSC containing RNase A (40 μg/ml) and RNase T_1 (160 U/ml). Unhybridized ssRNA was digested for 15 min at 37°C. RNA-DNA hybrids were precipitated by 10% TCA in the presence of 1 mg of Torula RNA and 10 mM sodium pyrophosphate, collected on Whatman GF/B glass fiber filters, and their radioactivity was measured.

Analysis of RNA polymerase reaction products. RNA polymerase reaction products were analyzed by agarose gel electrophoresis. The entire 20-μl reaction mixture was loaded onto 1 to 1.5% agarose gels after the reaction was stopped by addition of 10 mM EDTA. ^{32}P-labeled products, extruded from the particles, could be detected by staining directly with ethidium bromide or by autoradiography. In addition, the products that were not extruded from the VLPs were detected by autoradiography, with XAR-5 X-ray film, with overnight exposures. Labeled RNA products inside the particles were purified from the ones extruded by electroelution of the VLP bands in the agarose gel. After extraction with phenol, the nature of the products was examined by RNase treatment in the presence of different salt concentrations.

RNase treatments. RNase treatment of RNA polymerase reaction products was carried out in low or high salt concentrations at 37°C for 1 h. Pancreatic RNase A was used at a concentration of 5 μg/ml or higher. High salt concentrations (0.8 M) were obtained by adding appropriate volumes of 5 M NaCl to the reaction mixture. Under these conditions the enzyme is unable to digest dsRNA, whereas ssRNA is fully sensitive.

Separation of M_1-H and M_1-L VLPs by agarose gel electrophoresis. Based upon their different densities and molecular weights, M_1-H and M_1-L VLPs can be separated (or distinguished) by agarose gel electrophoresis, although they both contain the same M_1 dsRNA molecule. M_1-H VLPs on 1 to 1.5% agarose gels migrate similarly to naked dsRNA, whereas M_1-L VLPs move more quickly. This method is useful to separate labeled products that remain inside the particles from the ones that are extruded during the RNA polymerase reaction.

Determination of VLP molecular weights and dsRNA content. Relative molecular weights of whole L-A and M_1 VLPs were determined by the following equation (21): $M_r = Nfs/(1 - \bar{\nu}\rho)$, where N = Avogadro's number, f = the frictional coefficient (for spherical particles, $f = 6\pi r\eta$, where r is the radius of the particle and η is the viscosity of the solution), s = the sedimentation coefficient, $\bar{\nu}$ = the partial specific volume (inverse of the density) of the particles, and ρ = the density of the solvent.

Saccharomyces VLPs contain protein and dsRNA in different ratios, depending on the type of particle; the RNA fraction can be estimated by the following equation (21): $F = (Sn/Sv)\,[(Sv - Sp)/(Sn - Sp)]$, where Sn = the density of RNA in CsCl = 1.9 g/ml (28), Sp = the density in CsCl of VLP protein (empty capsid) = 1.31 g/ml (1, 24), and Sv = the density in CsCl of the VLPs.

RESULTS

Large-scale VLP purification (see Materials and Methods). VLPs can be separated from a majority of ribosomes by

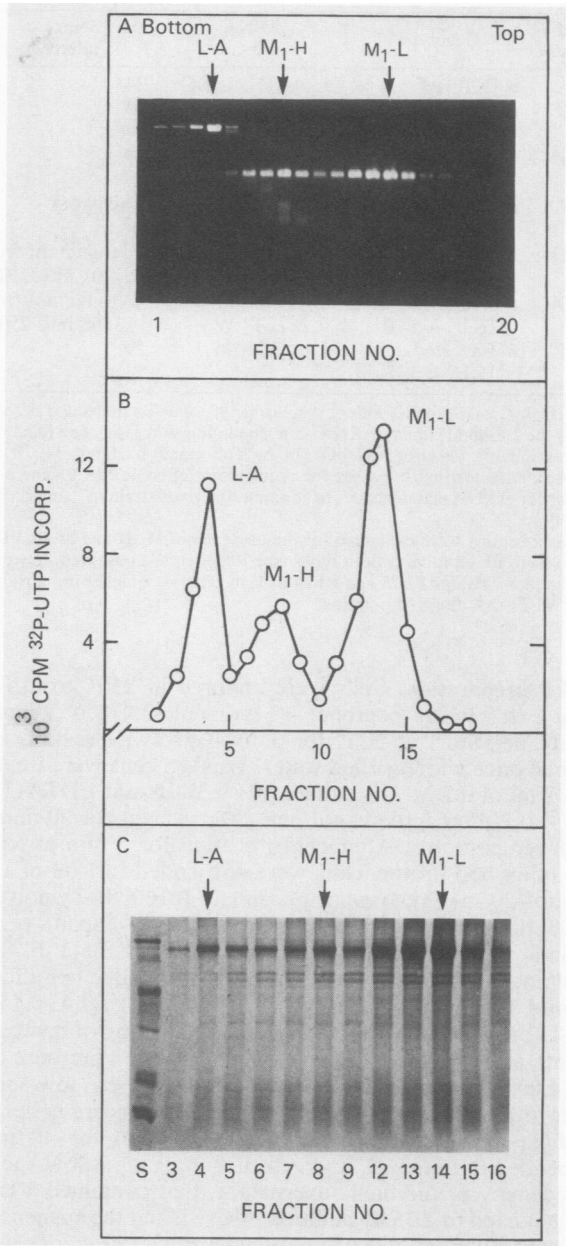

FIG. 2. (A) Agarose gel electrophoresis of L-A and M_1 dsRNAs from VLPs purified by CsCl gradient equilibrium sedimentation. Strain 1368 (Table 1) was used as a source of particles. Samples were prepared as explained in the legend to Fig. 1. (B) RNA polymerase activity in the same fractions measured as indicated in Materials and Methods. (C) Polyacrylamide gel electrophoresis of proteins from the same VLPs. To one portion of each fraction (10 to 20 μl), 0.5 ml of 10% TCA was added. After a 10-min incubation in ice, samples were spun down, and pellets were washed with 1 ml of 0.1% TCA. The precipitates were dissolved in electrophoresis loading buffer that contained 0.1% sodium dodecyl sulfate and β-MET and were boiled for 5 min. Electrophoresis was carried out in 10% acrylamide gel with 0.1% sodium dodecyl sulfate for 3 h, and the gel was stained with Coomassie brilliant blue. Molecular-weight standards included phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400). The locations of L-A, M_1-H, and M_1-L VLPs in the gradient are indicated by arrows (peak fractions). The major band is the major capsid protein.

TABLE 2. Physical properties of L-A, M_1-H, and M_1-L VLPs[a]

VLP	Parameter[b]				
	Density (g/ml)[c]	Sedimentation coefficient[d]	Molecular mass (whole particle), 10^6	% dsRNA	Molecular mass of dsRNA, 10^6
L-A	1.4076	160*s*	12.3	23.3	2.85
M_1-H	1.3834	149*s*	11.9	18.0	2.15
M_1-L	1.3541	138*s*	11.5	11.0	1.26

[a] Based on these data and the molecular mass of M_1 dsRNA of about 1.2 × 10^6 daltons, the most likely structure for the M_1-H VLPs is that with two M_1 dsRNA molecules per particle.

[b] Our electron microscopy observations of isolated L-A, M_1-H, and M_1-L VLPs showed no detectable differences in particle diameter. We thus used the average diameter determined by the extensive studies of others (1, 7, 11, 19, 37). All three VLPs had the same protein coat composition (≅120 subunits of 8.1 × 10^4 daltons) and diameter (39 nm).

[c] The values shown are the averages from 20 independent measurements on 20 different preparations from the strains listed in Table 1. Standard deviations were 0.0022, 0.0014, and 0.0014 for L-A, M_1-H, and M_1-L VLPs, respectively.

[d] The possible error in estimating S values of M_1-H and M_1-L was ± 2.7S.

using a relatively short high-speed spin. This pellet can then be resuspended and banded in a CsCl gradient to yield particles apparently free of rRNA and tRNA. With this method, approximately 100 to 150 mg of particle protein can be prepared in 2 days from 2 liters of a stationary-phase cell culture. Total amounts depend on the strain used, on conditions of growth, and also on the types of VLPs present (L-A VLPs only, L-A and M_1, or M_2 VLPs in "killer" strains).

L-A and L-BC VLPs. When particles are prepared in this way from strain S7 carrying mostly L-A dsRNA, only a single major peak of VLPs was found (Fig. 1A) at a density of 1.4076. In combination with the known 160*s* sedimentation rate of these particles (1, 11; Newman and McLaughlin, Abstr. 12th Int. Congr. Genet. 1984), their 39-nm diameter (1, 18, 19), and the density of dsRNA (28) and empty heads (1, 24), one can calculate (see Materials and Methods) (21) that the particles have a mass of about 12.3 × 10^6 daltons and contain about 23% by weight of dsRNA or about 2.85 × 10^6 daltons of L-A dsRNA per particle. Thus, there is one L-A dsRNA molecule (3.0 × 10^6 daltons) per particle.

TABLE 3. Hybridization of RNA polymerase reaction products from M_1-H and M_1-L VLPs with plus- and minus-strand cDNA clones[a]

M13 clone added	RNase treatment[b]	M_1-H VLPs		M_1-L VLPs	
		cpm	%	cpm	%
None	−	15,800	100	12,300	100
None	+	930	5.8	1,020	8.3
mp11 KC2 (plus strand)	−	16,800	105	12,700	103
mp11 KC2 (plus strand)	+	1,160	7.3	1,370	11.1
mp10 KC2 (minus strand)	−	15,700	99	12,000	97
mp10 KC2 (minus strand)	+	11,600	73	8,800	71

[a] M_1-H and M_1-L VLPs were prepared from strain RE24 grown on glucose-containing media for 4 days. (Stationary-phase cells were collected, and VLPs were purified as described in Materials and Methods.) M13 clones were used as probes to detect plus- and minus-strand synthesis. Final concentration of M13 DNA in the hybridization reaction mixture was 0.2 mg/ml (at least 10 times higher than M_1 RNA). Since the KC2 clone only covers about 1 kb of M_1 (total length 1.8 kb), and about 200 base pairs of poly(A) present in a full-length plus strand would also be resistant to pancreatic and T1 RNases, 70% RNase resistance represents the expected result if 100% of the transcript is plus strand (last line of the table).

[b] +, Treated with RNase; −, not treated with RNase.

In addition to the main peak of VLPs, there is a series of VLP bands decreasing in density and in the molecular weight of the extracted dsRNA (Fig. 1A), with full-sized L-A in the particles of density 1.4076 at one extremity. This gives a striking "staircase" effect on gels of the phenol-extracted fractions from the CsCl gradient (Fig. 1A). Similar results have been found with several other L-A-containing strains. With strain 2507 (Table 1) that lacks L-A and carries mainly L-BC, a clear peak of VLPs at approximately the same density (1.4030) was observed, but, unlike the L-A-containing strain, there was no staircase observed, even after overexposure of the film (Fig. 1B). We conclude that the staircase effect is due to the presence in some particles of partial-length L-A molecules. The role of these partial-length L-A dsRNA-containing VLPs in L-A replication, if any, remains to be elucidated.

Both L-A- and L-BC-containing VLPs from stationary-phase cells cosedimented with an RNA-dependent RNA polymerase activity, whose products are mainly plus ssRNA (data not shown).

M_1 VLPs show two peaks in CsCl gradients. When VLPs were prepared from strains carrying L-A, L-BC, and M_1 dsRNAs, two peaks of M_1 VLPs were found on CsCl gradients (Fig. 2A). The denser peak, called M_1-H (for heavy), was found at a density ρ = 1.3834; this peak, as compared with the L-A-containing one, had the density

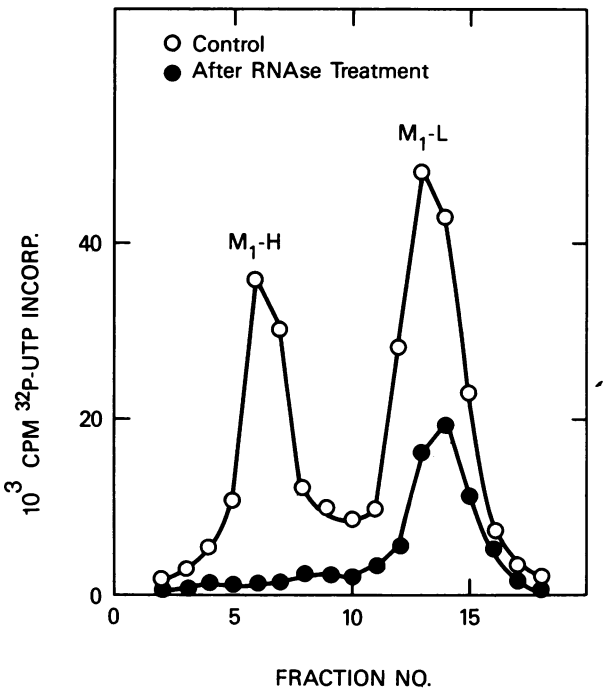

FIG. 3. Effect of RNase digestion on M_1-H and M_1-L VLP RNA polymerase reaction product. Fractions from a CsCl gradient containing M_1-H and M_1-L VLPs were incubated in RNA polymerase reactions for 2 h. The RNA polymerase reaction was stopped by the addition of 10 mM EDTA, and each fraction from the gradient was divided into two portions. To one of them, pancreatic RNase A was added to a final concentration of 5 μg/ml, and the mixture was incubated at 37°C for 1 h. TCA-precipitable radioactivity was measured. Since protein was not removed by phenol extraction, RNase resistance indicates protection by protein, not double-stranded structure. Most of the RNA polymerase product was single stranded (see text).

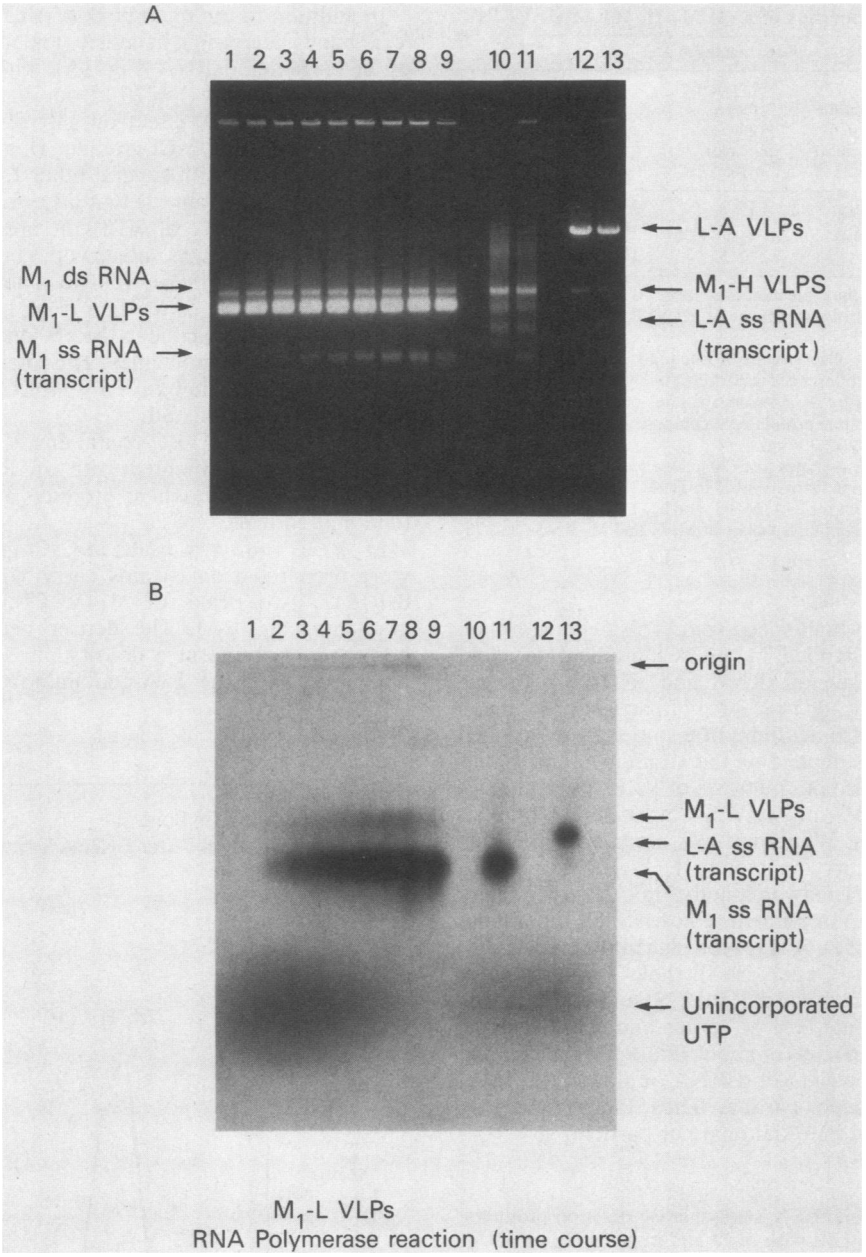

FIG. 4. Ethidium bromide-stained agarose gel (A) and autoradiogram (B) from a time course experiment of the RNA polymerase activity present in M_1-L VLPs. These particles were purified from strain 1368 by CsCl gradient equilibrium sedimentation. Samples taken at different times from the reaction mixture were loaded onto the gel without phenol extraction. Lanes 1 to 9, samples taken at 0, 10, 20, 30, 45, 60, 90, 120, and 180 min, respectively; lanes 10 and 11, M_1-H VLPs before and after 180-min incubations with the radioactive substrate ([^{32}P]UTP), respectively. Lanes 12 and 13 correspond to the main peak of L-A VLPs before and after RNA polymerase reaction (180 min), respectively. In this type of gel (1% agarose), M_1 dsRNA and M_1-H VLPs migrate together.

expected for particles in which the single L-A molecule has been replaced by two M_1 dsRNA molecules without changing the protein content in the particle. We use equation 1:

$$Sv = \frac{Mn + Mp}{\frac{Mn}{Sn} + \frac{Mp}{Sp}} \quad (1)$$

where Sv, Sn, and Sp, as defined above (see Materials and Methods), are CsCl densities of VLPs, dsRNA, and empty capsids, respectively, and Mn and Mp are molecular weights of dsRNA and empty capsids, respectively.

If, in equation 1, we let Mn be 2.4×10^6 daltons (the weight of two M_1 dsRNA molecules), then the calculated density value, Sv = 1.3868, is close to that observed for M_1-H particles.

The less-dense peak, which we called M_1-L (for light), was found at a density $\rho = 1.3541$. Its density corresponds to that expected for particles with only one M_1 dsRNA molecule ($\rho = 1.3513$). Upon measure of the protein: M_1 dsRNA ratio by

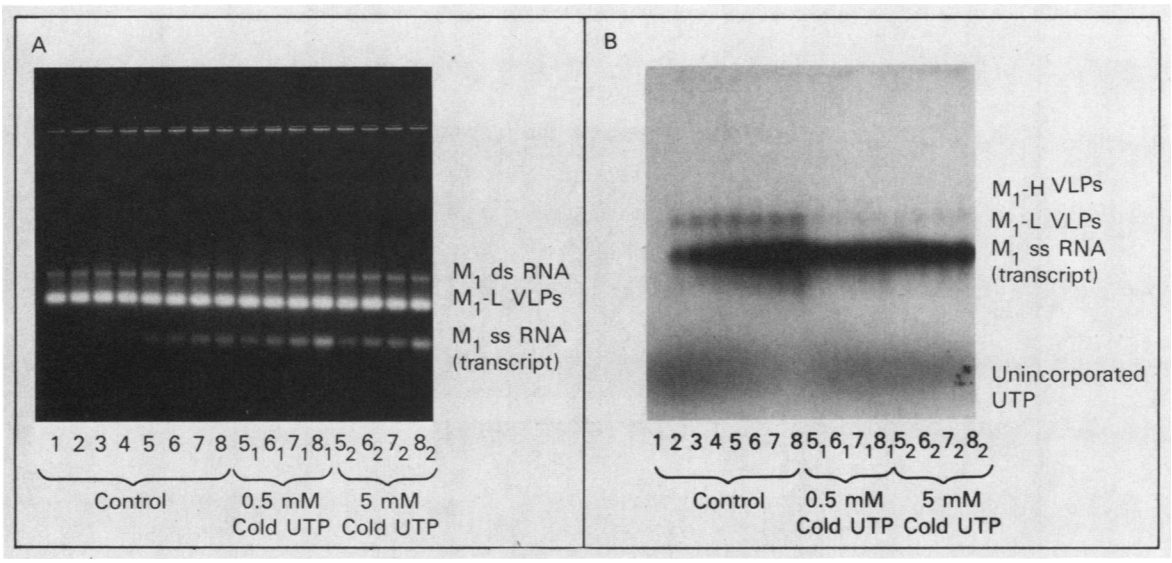

FIG. 5. Pulse-chase experiment with M_1-L VLPs from the same strain as in Fig. 4. Lanes 1 to 8, control samples taken at 0, 10, 20, 30, 45, 60, 75, and 90 min, respectively. After sample number 4 was taken, the reaction mixture was divided into three aliquots, one of which was incubated without any modification as a control to follow total incorporation. To aliquots 1 and 2, 0.5 mM and 5 mM cold UTP were added, respectively. 0.5 mM and 5 mM cold UTP represent 25- and 250-fold dilutions, respectively, from the original UTP used in the reaction. Labeled material was only partially chased out of the particles even after incubation for 1 h.

particle density, the M_1-L particles have a ratio about twice that of M_1-H particles, and indeed sodium dodecyl sulfate-polyacrylamide gels of portions from the CsCl gradient showed that the same proteins were present in the L-A VLPs, the M_1-H VLP peak, and the M_1-L VLP peak (Fig. 2C). Measuring the amount of protein by the Lowry method and M_1 dsRNA by ethidium fluorescence showed that the M_1-L peak had 2.4 times as much of the same proteins per dsRNA molecule as the M_1-H peak fractions, confirming the result obtained from particle density measurement (described above).

That the observed two varieties of M_1-containing VLPs were not artifactual was originally suggested by three lines of evidence. (i) When M_1-H and M_1-L VLPs were each rebanded on CsCl, M_1-H banded only at the M_1-H density, and M_1-L banded at the M_1-L density (data not shown). (ii) Although L-A is encapsidated in the same major coat protein as M_1, only one peak of L-A VLPs was observed, and that was at a density corresponding to one L-A dsRNA molecule per particle; that is, no L-A heavy band (two L-A dsRNA molecules per particle) was observed. (iii) The density difference between M_1-H and M_1-L particles did not vary significantly in many (>20) preparations (from logarithmic- and stationary-phase cells).

Most of our routine preparations were done with ethanol-grown cells of the *ski2-2* [B] [KIL-k_1] strain RE24 (Table 1) because it dramatically overproduces M_1 VLPs as a result of the combined effects of [B] (previously referred to as [KIL-b]) (2, 34) and the *ski2-2* mutation (25, 33). To determine whether this strain or the growth conditions were specifically producing the observed two peaks of M_1 VLPs, we examined M_1 VLPs from [B] and [B-o] strains, *ski2-2* strains, and SKI^+ strains, grown on glucose or on ethanol, for the presence of one or both types of M_1 VLPs (Table 1). We found that whereas there were significant differences from one preparation to another in the relative amounts of M_1-H and M_1-L VLPs, nevertheless both were invariably present in each preparation examined.

The difference in density between M_1-H and M_1-L VLPs is adequately explained by the difference in the protein: dsRNA ratio observed. We sought to confirm our interpretation of M_1-H and M_1-L VLPs as those having two and one M_1 dsRNA molecules per particle, respectively, by directly determining the RNA content per particle from particle size and hydrodynamic properties. By electron microscopy, both types of M_1 VLPs and L-A VLPs have about the same size. Sedimentation coefficients were estimated by sucrose gradient (10 to 40% sucrose) centrifugation after M_1-H and M_1-L VLPs were first purified by CsCl gradient equilibrium sedimentation as described above. Sucrose gradient centrifugation was carried out in an SW41 rotor at 38,000 rpm for 3 h and at 4°C. L-A VLPs were used as an internal standard; after fractionation, particles were detected by ethidium bromide-stained agarose gel electrophoresis (without extraction of the dsRNA with phenol) and also by RNA polymerase activity. M_1-H particles had an *s* value of 149, and M_1-L particles had an *s* value of 138.

Using the results summarized in Table 2 and the same type of calculations as for L-A VLPs, we estimated the molecular masses of both particles and also molecular masses of the dsRNAs that they contain. For M_1-H VLPs, the estimated particle molecular mass is 11.9×10^6 daltons, and dsRNA content is 18%, giving an RNA molecular mass of 2.15×10^6 daltons, i.e., roughly two molecules of M_1 dsRNA per particle (2.4×10^6 daltons). In the case of M_1-L VLPs, the molecular mass obtained was 11.5×10^6 daltons, with 11% of the particle mass as dsRNA, or 1.26×10^6 daltons, i.e., one molecule of M_1 dsRNA (1.2×10^6 daltons).

RNA polymerase activity of M_1-H and M_1-L VLPs. The peaks of L-A, M_1-H, and M_1-L VLPs each coincided with a peak of RNA polymerase activity (Fig. 2B). Hybridization of the labeled products of the M_1-H and M_1-L VLPs with M13 clones that contained M_1 cDNA inserts in both orientations (27) (Table 3) showed that both peaks synthesize predominantly plus-strand (i.e., message strand) RNA. The phenol-extracted RNA product is mainly single stranded as judged

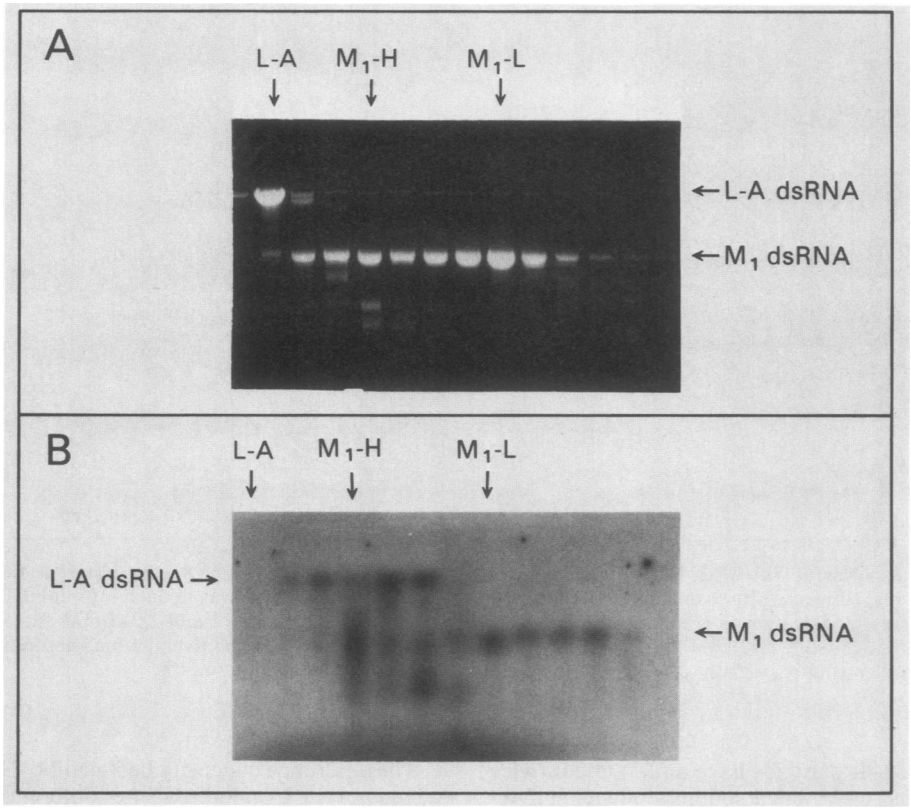

FIG. 6. (A) Effect of RNase treatment at high salt concentration (0.8 M NaCl) on the products synthesized by VLPs from a logarithmic-phase cell culture. After the RNA polymerase reaction, total RNA in the fractions of a CsCl gradient was extracted with phenol and precipitated with ethanol. After a washing with 70% ethanol, samples were dried and dissolved in a small volume of distilled water (10 to 20 μl). RNase treatment was carried out as described in Materials and Methods. Before loading on the gel, RNase was extracted with phenol, and the remaining RNA was precipitated with ethanol. Under these conditions only dsRNA is resistant to RNase treatment. (B) Autoradiogram of the gel. Arrows indicate the position of L-A, M_1-H, and M_1-L peaks of VLPs. Most of the M_1 dsRNA synthesis occurred in particles lighter than the M_1-L VLP peak and also between M_1-H and M_1-L VLPs. Some of the partial-length M_1 dsRNA-containing VLPs, banding between M_1-H and M_1-L VLPs, also had dsRNA polymerase activity.

by its sensitivity to pancreatic RNase in the presence of high salt concentrations (Table 3).

When pancreatic RNase was added to the reaction mixture after the RNA polymerase reaction was halted by adding 10 mM EDTA, the product of the M_1-H VLPs was completely degraded, implying that it had been extruded from the particles (Fig. 3). In contrast, the M_1-L VLP RNA polymerase product was 20 to 60% RNase resistant (depending on the preparation), although most of this RNase-resistant product became sensitive after phenol extraction. This suggested that part of the M_1-L VLP RNA polymerase product was inside the VLPs and thus protected from degradation by RNase. The reactions are linear for over 3 h with both M_1-L and M_1-H VLPs. Thus little VLP breakdown occurs in the reaction mixture.

To further examine the products of M_1-H and M_1-L VLPs, entire reaction mixtures were analyzed directly by agarose gel electrophoresis. The major product of both M_1-H and M_1-L VLPs was full-length single-stranded M_1 RNA which, as shown above and previously (36), was plus strand (Fig. 4). That product release from M_1-L particles was not due to breakdown of particles during the reaction is again suggested by the fact that the small proportion of free M_1 dsRNA after ethidium bromide staining (Fig. 4A) did not change during incubation of the RNA polymerase reaction of up to 3 h.

Extensive synthesis was obtained, to the point that all of the [^{32}P]UTP was converted to M_1 ssRNA product (Fig. 4B). This means that 1 full-length single-stranded M_1 RNA molecule was synthesized per 10 molecules of the template (M_1 dsRNA) in 2 h, under the UTP-limiting conditions used in this experiment.

The products of the M_1-L VLP RNA polymerase included both full-length M_1 plus ssRNA that was extruded from the particles and a substantial amount of product that remained inside the VLPs upon agarose gel electrophoresis (Fig. 4B and Fig. 5B). By CsCl gradient equilibrium sedimentation after RNA polymerase reaction with M_1-L VLPs, the extruded M_1 ssRNA product is easily separated from that which remains inside the particles, because the latter banded at the density of M_1-L VLPs (data not shown). The finding that part of the M_1-L VLP RNA polymerase product was RNase resistant in low salt concentration (before phenol extraction) (Fig. 3) showed that this RNA polymerase product must be inside the particles.

To determine whether the intraparticle RNA polymerase product was an intermediate in the synthesis of the single-stranded M_1 RNA product, a pulse-chase experiment was carried out (Fig. 5). Diluting the [^{32}P]UTP 25- or 250-fold, after 90 min rapidly chased out part of the intraparticle label (compare samples 5, 5_1, and 5_2). The remaining part was not

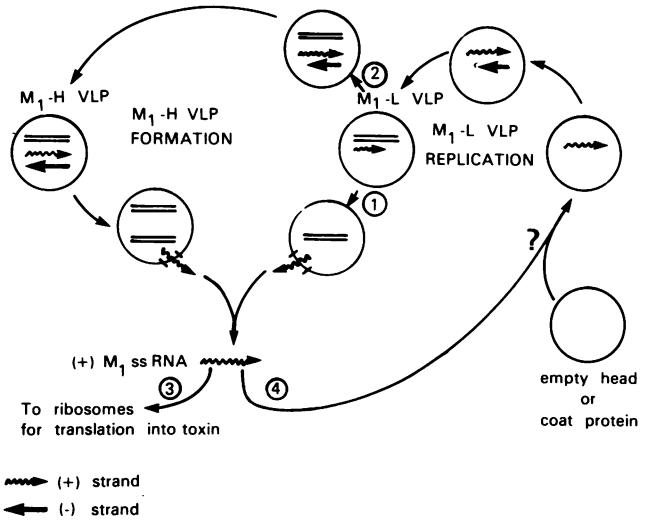

FIG. 7. A model of the VLP cycle for M_1 dsRNA transcription and replication. The various types of VLPs have the same coat proteins but differ in RNA content. M_1-L VLPs, that contain one molecule of M_1 dsRNA per particle and have RNA polymerase activity, synthesize plus M_1 ssRNA, which is used in three different ways. (i) Most of it is extruded and translated to make toxin (number 3 in the model). (ii) Some is extruded and somehow packaged in a new coat (number 4 in the model). (iii) Some of the plus M_1 ssRNA is retained within the particle (number 2 in the model), where it serves as template for minus-strand synthesis to form M_1-H VLPs. M_1-H VLPs are essentially full heads, and so their plus-strand transcript is all extruded from the particles and is used for translation and repackaging. The particles formed by association of plus strands with a new coat can then synthesize minus strands to make new M_1-L particles.

chased out, even though RNA synthesis in the unchased control continued to yield a 2.5-fold increase in extruded labeled RNA. This indicates that at least part of the intraparticle-labeled RNA is not an intermediate in the extruded plus-strand ssRNA synthesis.

To further characterize the product remaining inside the particles, M_1-L VLPs containing labeled product were separated from the extruded ssRNA by agarose gel electrophoresis and were then electroeluted from the gel. The RNA product was then extracted from the particles with phenol. The product was approximately 30% resistant to pancreatic RNase A digestion with 0.8 M NaCl present in the incubation mixture, and this RNase A-resistant material migrated mainly with M_1 dsRNA upon agarose gel electrophoresis.

M_1 dsRNA synthesis. The presence of M_1 dsRNA synthesis in the M_1-L particle further led us to specifically examine all the fractions of a CsCl gradient of VLPs for dsRNA synthesis. The products of an RNA polymerase reaction using each fraction were phenol extracted and digested with pancreatic RNase A at 0.8 M salt concentration. An autoradiogram of agarose gel electrophoresis of the resulting material is shown in Fig. 6.

The peak of M_1 dsRNA synthesis was in fractions lighter than M_1-L VLPs, namely, at the position one would expect to find particles containing only one strand of M_1 and the same coat protein as M_1-L. M_1 dsRNA synthesis was also observed in fractions intermediate in density between M_1-H and M_1-L VLPs, at the position expected for particles having one M_1 dsRNA and one M_1 ssRNA molecule.

L dsRNA (probably L-A) synthesis is also observed in fractions lighter than L-A VLPs. This activity has been characterized in detail elsewhere (T. Fujimura, R. Esteban, and R. B. Wickner, submitted for publication).

S VLPs. In our preparations, three other minor dsRNA molecules, with sizes smaller (1.2 to 1.6 kb) than full-length (1.8 kb) M_1 dsRNA, were always observed (Fig. 2A, lanes 7 to 9). The densities of the particles containing these three dsRNAs are between those of M_1-H and M_1-L VLPs. They do not form a staircase, as do the L-A VLPs (Fig. 1A), but rather are discontinuously distributed. These molecules were separated by electroelution from agarose gels.

Hybridization of M13 pKC2 plus- and minus-strand probes to denaturing gels of these three RNA species (data not shown) showed that both plus and minus strands of each were the same size. These are thus partial-length molecules with homology to M_1, like the S (suppressive) dsRNAs previously described (16) and previously shown to be in VLPs (17).

These "S VLPs" contain RNA polymerase activity that can synthesize S dsRNAs of the same size (Fig. 6B). This activity is found in fractions lighter than those having the preformed mutant dsRNAs. The S dsRNAs seem to be defective molecules resulting from early termination of transcription or replication or from skipping some sequences in the process. They are not intermediates in M_1 dsRNA replication.

M_1-L VLPs that have been frozen and thawed several times produce, in addition to full-length plus-strand RNA, significant amounts of three partial-length ssRNAs corresponding in size to the three sizes of S dsRNAs found at denser fractions in the gradient. These partial-length transcripts, apparently from VLPs containing full-length M_1 dsRNA, may be the origin of S dsRNA.

DISCUSSION

We have used strains overproducing VLPs due to their having a mutation in the chromosomal *SKI2* gene which encodes a repressor of L-A, L-BC, and M copy number and due to their carrying the cytoplasmic element [B] which elevates M_1 copy number. The basic findings described here do not depend on those mutations, however, and have been largely confirmed by our studies of wild-type strains.

The use of CsCl gradients for VLP purification has the advantage over sucrose gradients of allowing good separation of L-containing VLPs from M-containing VLPs as well as differentiation among the several diffferent types of M_1-containing particles described here. In addition, larger amounts of VLPs can be isolated by CsCl than is practical on similar-sized sucrose velocity gradients.

Our examination of M_1 VLPs shows that particles containing one M_1 and particles containing two M_1 dsRNA molecules can be found in all strains examined. Both of these produce M_1 plus-strand ssRNA for export, presumably mostly for translation. Only the particles having one M_1 dsRNA retain part of their plus ssRNA, presumably to later serve as a template for M_1 dsRNA synthesis (Fig. 7).

Particles with only one M_1 single strand synthesize a complementary strand, forming M_1 dsRNA-containing particles. Further, we find that particles whose density suggests they may have one M_1 dsRNA and one M_1 ssRNA also synthesize detectable M_1 dsRNA. We have thus detected particles carrying out reactions apparently representing each of the steps shown in the model in Fig. 7, except for the synthesis of the particles containing only one M_1 plus ssRNA strand. One possibility for this step is that some of the plus strands that were extruded are subsequently

encapsidated by newly produced coat proteins. A second possibility is that encapsidation and plus ssRNA synthesis are coupled. That is, perhaps some of the plus ssRNA strands are extruded directly into a preformed empty head.

What is the significance of the observation that M_1-H VLPs, having two M_1 dsRNAs per particle, can exist, but that L-A VLPs all have only one L-A dsRNA per particle? We suggest that this reflects simply the inherent capacity of the particle for dsRNA—a sort of head-full hypothesis. We suggest that the L-A-encoded major coat protein forms a structure primarily adapted to hold one L-A dsRNA and that it can hold no more, not even another L-A ssRNA transcript. M_1 is less than half the length of L-A, and so two M_1 dsRNA molecules will fit comfortably inside one VLP, but the new plus ssRNA transcripts are all extruded for lack of space. Particles with only one M_1 dsRNA molecule are not crowded and so retain some of their plus ssRNA transcripts which can then be copied to make a second M_1 dsRNA molecule inside the same particle. Similarly, particles containing two M_2 dsRNAs have been observed (unpublished observations), as would be expected by this notion from the size of M_2 dsRNA (1.5 kb).

The existence of different types of viruslike particles containing one or two dsRNA molecules of the same kind per particle has also been reported in *Penicillium stoloniferum* virus PsV-S (9). In that case, replication of dsRNA occurred mainly in dsRNA-containing particles in a semiconservative way (10), and the particles with two dsRNA molecules were formed from those that contained only one dsRNA molecule.

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