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James E. Hopper
Brandeis University

Et al.

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Translation of the L-Species dsRNA Genome of the Killer-associated Virus-like Particles of *Saccharomyces cerevisiae**

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JAMES E. HOPPER,‡ KEITH A. BOSTIAN,§ LUCY B. ROWE,‡ AND DONALD J. TIPPER§

From the ‡Rosenstiel Basic Medical Research Center, Brandeis University, Waltham, Massachusetts 02154, and §The Department of Microbiology, University of Massachusetts Medical School, Worcester, Massachusetts 01605

Virus-like particles containing the L (P1)-species of double-stranded RNA (dsRNA) were isolated from *Saccharomyces cerevisiae*, and the translational activity of the virus-like particle-derived dsRNA was analyzed in the wheat germ cell-free system. Denaturation of the dsRNA immediately prior to *in vitro* translation resulted in the synthesis of one major and at least three minor polypeptides, whereas undenatured dsRNA, as expected, did not stimulate [³⁵S]methionine incorporation into polypeptides, but actually slightly inhibited endogenous activity.

The major *in vitro* translation product of the denatured L-dsRNA was shown to be identical with the major L-dsRNA containing virus-like particle capsid polypeptide on the basis of three criteria: co-electrophoresis on sodium dodecyl sulfate polyacrylamide gels, immunoprecipitation, and tryptic peptide analysis. We have therefore established that the L-dsRNA genome encodes the major virus-like particle capsid polypeptide. This result adds considerable support to the hypothesis that the L-dsRNA genome acts as a helper genome to the smaller (1.6×10^6 dalton) M-dsRNA genome in killer strains of yeast by providing the M-dsRNA containing virus-like particles with their major coat protein.

The killer system in the yeast, *Saccharomyces cerevisiae*, is an excellent system for investigating the molecular basis of functional relationships among viral-like genomes and between viral-like genomes and the host (nuclear) genomes in fungi (see Ref. 1 and 2 for recent reviews). Killer strains of yeast secrete a toxin which kills sensitive strains (3-5). Killer strains and strains designated neutral are normally immune to toxin. Both toxin production and immunity to toxin exhibit non-Mendelian inheritance (6-8) and both aspects of the killer phenotype are correlated with the presence of a single species of double-stranded RNA (M or P2, 1.6×10^6 daltons)¹ (10-17) which resides in the cytoplasm as an encapsulated viral-like

particle (VLP) (14, 18-20). The presence of this dsRNA² species is not, however, sufficient to confer a stable killer phenotype to the cell and its descendants: wild type alleles must be present within at least three classes of nuclear genes comprising approximately 20 distinct loci (6, 16, 17, 20-23). Mutations in any one of several Mak (maintenance of killer) genes results in the eventual loss of the M-species dsRNA (6, 12, 16, 17) and the killer phenotype. Mutations at the Kex (killer expression) and Rex (resistance to killing) loci result in a loss of killing ability and a loss of resistance, respectively. Neither class of mutation affects the presence of the M-species dsRNA (17, 21, 23).

Besides the dependence of M-species dsRNA and expression of the killer phenotype on nuclear genes, both are apparently dependent on the presence of another cytoplasmically inherited species of dsRNA, the L (or P1) species dsRNA (3.0×10^6 daltons). The L-dsRNA species, like M-dsRNA, is encapsulated in the form of an isometric virus-like particle (14, 18-20, 24). Nearly all *S. cerevisiae* strains studied in various laboratories harbor L-dsRNA, and many contain L-dsRNA without M-dsRNA, and are sensitive non-killers. Rare non-killer-sensitive strains exist which contain neither M-dsRNA nor L-dsRNA, but no strain has yet been reported which contains M-dsRNA without L-dsRNA. Recently, hybridization evidence has been presented suggesting that several copies of L-containing sequences are integrated in the yeast nuclear genome (25).

The M- and L-dsRNA species are probably separately encapsulated (14, 24), and although the full polypeptide complement of the VLPs has not been rigorously determined, a major polypeptide of 75,000 daltons and two minor species of 53,000 and 37,000 daltons have been reported to exist in the L-dsRNA-containing particle (26).³ Although it is not known whether the M-dsRNA-containing particles have the same polypeptide complement, reports of cross-antigenicity of L-dsRNA- and M-dsRNA-containing particles make cross-identity for at least one polypeptide likely.⁴

We have recently undertaken experiments aimed at understanding the relationship between the two dsRNA species

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¹ Electron microscope measurements yield a value closer to 1.1×10^6 daltons (9, and H. Fried, personal communication).

² The abbreviations used are: dsRNA, double-stranded RNA; VLP, viral-like particle; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, Sodium dodecyl sulfate.

³ G. R. Fink, personal communication.

⁴ G. R. Fink and A. J. Herring, personal communication.

and the role each plays in encapsulation and in toxin production and immunity. In the experiments reported here we have utilized a direct *in vitro* translational assay to determine the coding capacity of the L-dsRNA genome.

For the sake of clarity in discussion, we have called polypeptides derived from dsRNA-containing VLPs ScV-P_n (*S. cerevisiae* virus polypeptides) where ScV-P1 is the major L-dsRNA-containing VLP capsid polypeptide. Polypeptides derived from *in vitro* translation of denatured L-dsRNA are designated L-P_n. L-P1 is the major polypeptide product and it co-migrates with ScV-P1. The data presented here show that these two polypeptides share all major tryptic peptides, and that the L-dsRNA genome codes for at least its own major capsid polypeptide. The implications concerning the basis of the dependence of M-dsRNA on L-dsRNA are discussed.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media—A8209BNK2 (NK2), a non-killer which contains no M-dsRNA, was obtained from Dr. Gerald R. Fink (Cornell University), who derived it from a killer strain by curing with cycloheximide. Such strains characteristically overproduce L-dsRNA (8). Strain 108-3c was from Dr. Howard Douglas⁵ (University of Washington) stock collection. 3/A1, a non-killer, was received from Dr. Alan J. Herring (Queen Mary College, London) and originated from Dr. E. A. Bevan's (Queen Mary College, London) collection. All strains were haploids. YEP media contained 10 g of Bacto-yeast extract and 20 g of Bacto-peptone/liter. YEPD and YEP-galactose were prepared by addition of dextrose or galactose to YEP to yield a final 2% (w/v) solution of the sugar. YNB + YEP/5 contained, per liter of final solution: 2 g of yeast extract, 4 g of Bacto-peptone, 6.7 g of yeast nitrogen base without amino acids (Difco, Nutley, N.Y.), 25 mg of uracil, 25 mg of adenine, and 0.1 g each of histidine, isoleucine, leucine, lysine, tryptophan, threonine, tyrosine, phenylalanine, and arginine. Low phosphate YEPD, as used for [³²P]phosphate-labeling studies, was prepared as described elsewhere (27).

Materials—Carrier-free H₃³²PO₄ was obtained from New England Nuclear. L-[³⁵S]Methionine preparations at 695 Ci/mmol and 400 Ci/mmol were obtained from Amersham/Searle and New England Nuclear, respectively. Pancreatic ribonuclease (A) and pancreatic deoxyribonuclease (I) were purchased from Worthington Biochemical Corp. RNase III was a gift from Dr. Fred Hagen. The sodium salts of ATP and GTP were obtained from Boehringer Mannheim, and phosphocreatine from GIBCO. Phosphocreatine kinase (EC 2.7.3.2), and spermidine-free base were from Sigma Chemical Co. Proteinase K was from E.M. Laboratories. Commercial raw wheat germ was a gift from Dr. Ray Erikson and came originally from the Peavey Co., Denver, Col. Goat antiserum prepared against rabbit IgG was obtained from Miles Laboratory. Sephadex G25 was purchased from Pharmacia Fine Chemicals. DEAE-cellulose (DE52), CM-cellulose, and CF11 cellulose were all Whatman products. Acrylamide and *N,N'*-methylenebisacrylamide (electrophoresis grade) were purchased from Bio-Rad Laboratories; BH specially pure sodium dodecyl sulfate was from Gallard Schlesinger; ribonuclease-free ultrapure sucrose and enzyme grade ammonium sulfate were from Schwarz/Mann Research Corp. All inorganic salts were of ACS analytical grade.

Preparation, Purification, and Fractionation of Virus-like Particles—Cells for VLP production were grown to late stationary phase (5 to 7 days) at 28° in YEPD (adjusted to pH 4.7), with shaking for the first 2 days. For *in vivo* labeling with H₃³²PO₄, low phosphate YEPD was used. Label was added at a cell density of 10⁷ cells/ml to a final concentration of 2.5 mCi/liter. Labeling with [³⁵S]methionine was accomplished in YNB + 1/5 YEP plus 2% (w/v) glucose, pH 4.7. Label was added at a cell density of 4 × 10⁶ cells/ml to a final concentration of 6.25 mCi/liter.

Cells were harvested by centrifugation, washed once with cold-distilled H₂O, and finally resuspended in 1.5 volumes of PKE buffer (30 mM Na₂HPO₄/NaH₂PO₄, 150 mM KCl, 10 mM EDTA, pH 7.6). Cells were disrupted by shaking with glass beads for 15 min on an orbital shaker at 350 rpm at a ratio of 3 g of 0.45 μm glass beads/ml of cell suspension. Breakage was estimated to be 90 to 95% complete. The resulting slurry was passed through a glass fiber plug and the beads washed with PKE buffer. This yielded a combined filtrate at a 4- to 5-fold dilution of the original suspension. The pH was

adjusted to 5.0 and left to precipitate overnight at 4° with stirring. The precipitate was collected by centrifugation at 10,000 × *g* for 30 min and resuspended in the same volume of PKE by gentle homogenization with a Teflon-coated glass homogenizer.

The crude VLP preparation was then purified essentially as described by A. J. Herring⁵ by two to three cycles of hi-low speed centrifugation followed by sucrose density gradient centrifugations, as follows: the crude VLP suspension was adjusted to pH 7.6 by addition of 1 M KOH, and centrifuged at 10,000 × *g* for 30 min. The resultant supernatant was subjected to a high speed spin for 90 min at 30,000 rpm in the Spinco type 30 rotor, or for larger volumes at 19,000 rpm for 5.5 h in a Spinco type 19 rotor. The pellet was gently resuspended in PKE buffer and one to two additional cycles of centrifugation performed. The final pellet was resuspended in PK buffer (30 mM Na₂HPO₄/NaH₂PO₄, 600 mM KCl, pH 7.6) and loaded onto 10 to 40% (w/v) sucrose gradients made in PK buffer. Gradients were spun for 3 h at 24,000 rpm in a Spinco SW 27 rotor. Fractions from these gradients containing VLPs were pooled and loaded onto 30 to 60% (w/w) sucrose gradients from which the upper fourth had been removed to accommodate the large volume of pooled fractions. These gradients were then spun for 18 to 20 h at 24,000 rpm in a Spinco SW 27 rotor, and the fractions containing purified VLPs were pooled and stored at 4°.

Purified VLPs were prepared for SDS-polyacrylamide gel electrophoresis by dialyzing against 30 mM NaH₂PO₄, pH 7.6, 20% (v/v) glycerol, for 6 to 8 h. The dialyzed VLP solution was then made 3% (w/v) in SDS, 1% (v/v) mercaptoethanol, and 0.01% in bromophenol blue, and heat-denatured for 3 min in a boiling water bath. Slab gel electrophoresis and autoradiography of gel-fractionated proteins were according to the method of Studier (28).

Purification of the major VLP polypeptide, ScV-P1, was accomplished by preparative SDS-polyacrylamide gel electrophoresis. Purified VLPs, dialyzed, and dissociated as described above, were fractionated on cylindrical 1.4-cm diameter by 9 cm long 7.5% gels in Tris/glycine buffer (29) containing 0.5% (w/v) SDS. The protein band was excised from the unfixed gel after location by Coomassie blue staining of a thin longitudinal slice. The protein was then eluted for 24 h at 30° in 0.01 M NH₄HCO₃ containing 0.05% (w/v) SDS.

dsRNA was fractionated from purified VLPs by dilution 10-fold into 150 mM NaCl/50 EDTA/0.05% (w/v) SDS. To this mixture was added 0.1 volume of a 10 mg/ml solution of proteinase K, which had been self-hydrolyzed for 45 min at 24° in 50 mM Tris/HCl, 2 mM CaCl₂, pH 7.5. After a 1-h digestion at 30°, additional SDS was added to give a final concentration of 2% (w/v). Three standard phenol extractions were carried out using redistilled, water-saturated phenol. The nucleic acids in the aqueous phase were precipitated with ethanol at -20°. The precipitated material was pelleted at 20,000 × *g* for 10 min, the pellet dissolved in 200 mM potassium acetate, 10 mM Hepes, pH 7.5, and the ethanol precipitation repeated. The final pellet was resuspended in distilled H₂O, adjusted in 200 mM potassium acetate, 10 mM Hepes, pH 7.5, to a final concentration of about 5 mg/ml, and stored in small aliquots at -20°. Spectral analysis of the nucleic acid preparations gave A₂₈₀/A₂₆₀ ratios between 1.9 and 2.1.

Electron Microscopy—Preparations of purified VLPs (from 30 to 60% (w/w) sucrose gradients) were dialyzed for 5 h against 150 mM ammonium acetate, pH 7.0, and negatively stained with 1% (w/v) uranyl acetate, pH 4.5, on Formvar and carbon-coated 200-mesh copper grids pretreated with 0.01% bovine serum albumin. Specimens were examined in a Phillips 300 microscope at 80 kV with nominal magnifications of 50,000 to 90,000.

Amino Acid Analysis—Samples of ScV-P1 from preparative gel electrophoresis were exhaustively dialyzed against distilled, deionized H₂O, and were lyophilized and dried *in vacuo* over P₂O₅. Approximately 1-mg samples were weighed and hydrolyzed with 1 ml of constant boiling HCl *in vacuo* for 24 h at 110°. The hydrolysates were then taken to dryness under vacuum over NaOH and P₂O₅. Hydrolyses were performed in duplicate. Samples were then taken up in lithium citrate buffer (30) and analyzed on a Durrum D-500 amino acid analyzer (31). Half-cystine and methionine were determined after performate oxidation (32) as cysteic acid and methionine sulfone.

Preparation of Total Cellular and Polysomal RNA—The preparation of polysomal RNA from mid-log phase cells (2 × 10⁷ cells/ml) of strain 108-3c, grown in either YEPD or YEP-galactose, was carried

⁵ A. J. Herring, personal communication.

out as previously described (33) except for slight modification. When small amounts of cells were used (2 to 4 g wet weight), cell breakage was accomplished by cell/buffer/glass bead mixture blending on a Vortex mixer in a 30-ml Corex tube for four 15-s bursts at 4°. Total cellular RNA was isolated from whole cell homogenates and extracted with phenol, as described for the extraction of dsRNA from VLPs (see above).

Separation of dsRNA and ssRNA—dsRNA was isolated from total cellular nucleic acids by two different methods. First, nucleic acids extracted from strain NK2 with phenol were hydrolyzed with pancreatic DNase followed by pancreatic RNase in the presence of 150 mM NaCl (16). The products were then fractionated on a Sepharose 4B column (112 × 1.5 cm) equilibrated in 150 mM NaCl, 10 mM Hepes, 2 mM EDTA, pH 7.2. A single peak of RNA eluted in the void volume. This material was subsequently characterized as dsRNA. Secondly, CF11 chromatography was used to separate dsRNA from ssRNA and DNA in preparations of total cellular nucleic acids from strains NK2 and 3/A1, and also for RNA extracted from purified VLPs. The procedure of Franklin (34) was followed. We routinely chromatographed the dsRNA fraction obtained from a primary CF11 column on a second column, to ensure a minimum of ssRNA or DNA contamination of the dsRNA fractions.

Preparation of Immune Serum and γ -Globulin Fraction—Sucrose gradient (30 to 60% (w/w)) fractions containing VLPs isolated from strain 3/A1 were pooled and dialyzed against phosphate-buffered saline buffer (7.5 mM Na_2HPO_4 , 2.5 mM NaH_2PO_4 , 150 mM NaCl, pH 7.2) at 4° for 4 h. The dialyzed VLPs were added to an equal volume of complete Freund's adjuvant and emulsified by sonication. Aliquots of this emulsion containing a total of 140 μg of protein were injected intradermally into four places on the back of a New Zealand White rabbit. Injections were repeated 4 weeks later, and 10 days after this second injection the rabbit was bled from the ear vein.

A crude γ -globulin fraction was prepared from the serum by three consecutive ammonium sulfate precipitations (35). The final ammonium sulfate precipitate was dialyzed against 10 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, 15 mM NaCl, pH 7.2, and the γ -globulin fraction further purified by CM-cellulose and DEAE-cellulose column chromatography as described by Palacios *et al.* (36).

Preparation of Wheat Germ Cell-free Protein Synthesis System—Wheat germ extracts were prepared by modifications of several procedures (33, 37–39). Seven grams of raw wheat germ were ground moderately hard with 7 g of 120- μm acid-washed glass beads in a cold mortar for 1 min and then extracted with 25 ml of lysis buffer consisting of 40 mM KAc, 2.0 mM magnesium acetate, 20 mM Hepes, 2 mM CaCl_2 , 1 mM dithiothreitol, pH 7.2. The lysate was centrifuged at 23,000 × *g* for 10 min, and 0.01 volume of 1 M Hepes, pH 7.2, and 0.01 volume of 100 mM magnesium acetate were added while mixing. After centrifugation at 23,000 × *g* for 20 min the supernatant was immediately applied to a column (2.6 × 30 cm) (175-ml bed volume) of Sephadex G-25 coarse equilibrated with 20 mM Hepes, pH 7.2, containing 40 mM KAc, 2 mM magnesium acetate, 1 mM dithiothreitol. The void volume of the column was approximately 70 ml, and the flow rate was maintained at approximately 1.5 ml/min. The fractions in the void volume having the highest A_{260} were pooled and immediately quick-frozen in small aliquots for storage at -70°.

Cell-free Protein Synthesis—Total polysomal RNA and purified dsRNA (natured and denatured), were routinely translated in 50- μl reaction volumes containing 15 μl of wheat germ lysate (A_{260} of 50 to 70). Conditions used for RNA derived from dsRNA and polysome preparations differed only with respect to the concentrations of KAc and RNA employed. Optima for these were 100 mM potassium acetate and 40 $\mu\text{g}/\text{ml}$ of RNA for denatured dsRNA and 160 mM potassium acetate and 100 $\mu\text{g}/\text{ml}$ of RNA for total polysome-derived RNA. The concentrations of all other components used were: 1.6 mM magnesium acetate, 0.16 mM spermidine-free base, 40 $\mu\text{g}/\text{ml}$ of creatine phosphokinase, 3 mg/ml of creatine phosphate, 0.6 mg/ml of ATP, 0.1 mg/ml of GTP, 1.4 mM dithiothreitol, 0.44 mM concentration of each of 19 nonradioactive amino acids, 1 μM nonradioactive methionine, and [^{35}S]methionine to a final specific radioactivity of 4.5×10^5 cpm/pmol. Reaction mixtures were incubated at 22° for 3 h. Samples for the determination of radioactivity in trichloroacetic acid-precipitable material and for SDS-polyacrylamide slab gel electrophoresis were processed as described before (40).

Immunoprecipitation Assay—The double antibody method (indirect method) employing goat anti-rabbit γ -globulin was used. The equivalent point for the reaction of goat anti-rabbit γ -globulin against rabbit γ -globulin was determined routinely for each prepa-

ration. In a typical immunoprecipitation assay, 30 μl of 0.5% (w/v) SDS, followed by 0.25 ml of BSM buffer (100 mM H_3BO_3 , 135 mM NaCl, 100 mM methionine, pH 8.0), was added to 20 μl of an *in vitro* translation reaction mixture in a glass tube. To the tubes incubated at 35° were added 5 μl of a mixture of purified γ -globulin from immunized and nonimmunized rabbits (0.2 mg/ml immune – 1.88 mg/ml non immune). The reaction mixtures were allowed to precipitate for 60 min. Subsequently, 10 μl of goat anti-rabbit γ -globulin (26 mg/ml) was added, the reaction tubes incubated at 35° for 30 min, and finally left at 4° overnight. The resulting immunoprecipitates were washed three times with BSM buffer using a clinical centrifuge at 4°, and the final pellet was prepared for SDS-polyacrylamide gel electrophoresis by dissociating in SDS-gel electrophoresis sample buffer for 3 min in a boiling water bath.

Tryptic Peptide Analysis—The ScV-P1 band from SDS-gels of [^{35}S]methionine-labeled VLP preparations and the major L-P1 band from SDS gels of [^{35}S]methionine labeled *in vitro* translation products of the L-dsRNA were excised, hydrolyzed with trypsin, and fractionated in parallel by paper ionophoresis as described by Morrison and Lodish (41), with slight modification. One and two-tenths milligrams of [^{35}S]methionine-labeled VLP protein or a 1300- μl aliquot of the *in vitro* translation reaction mixture supplemented with 0.9 mg of unlabeled VLP protein were fractionated on 3 mm thick SDS-polyacrylamide slab gels, and the excised protein bands from the stained and dried gels were cut into small pieces and digested for 14 to 16 h at 37° with stirring, in 8 ml of 1% (w/v) NH_4HCO_3 containing 0.1 mg/ml of trypsin. The incubation of gel slices was then repeated with 5 ml of fresh NH_4HCO_3 containing 0.05 mg/ml of trypsin. After 12 h at 37° the supernatant from this second digest solution was combined with the first and the total volume was lyophilized three times and dissolved in 100 μl of solvent for paper ionophoresis. Ionophoresis was carried out for 2.5 h at 45 V/cm in pyridine acetate buffer, pH 3.5 (41). Following ionophoresis the paper was cut at 5-mm intervals from the origin and the radioactivity in each segment was determined by counting in toluene-based scintillation fluid.

RESULTS

Purification of VLPs—When crude VLP preparations from strain 3/A1 were centrifuged on 10 to 40% (w/v) sucrose gradients, and the pooled VLP fractions subsequently centrifuged on 30 to 60% (w/w) sucrose gradients, two bands were observed upon fractionation (Fig. 1). Electron microscopic examination of the major, faster sedimenting band reveals isometric virus-like particles measuring approximately 40 nm in diameter (Fig. 2), as previously reported (14). Phenol extractions of pooled fractions of this major band yield L-

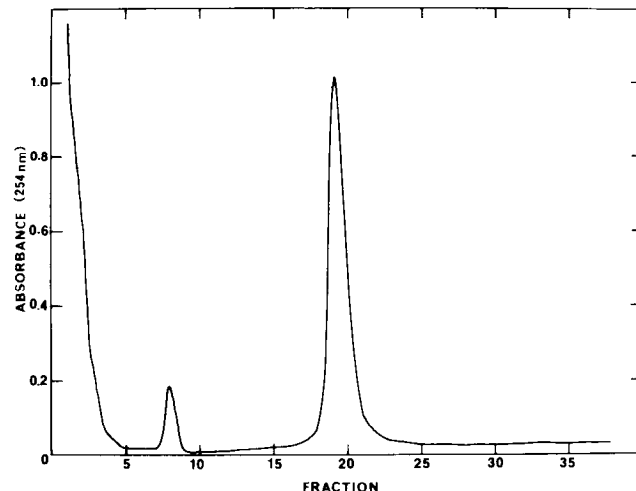


FIG. 1. Fractionation of VLPs from *Saccharomyces cerevisiae* strain 3/A1 on a 30 to 60% (w/w) sucrose gradient. After centrifugation, fractions (20 drops) were collected and scanned at 254 nm in a Beckman model 25 spectrophotometer equipped with a flow cell. Fraction 38 represents the bottom of the gradient. The major peak at Fraction 20 contained L-dsRNA.

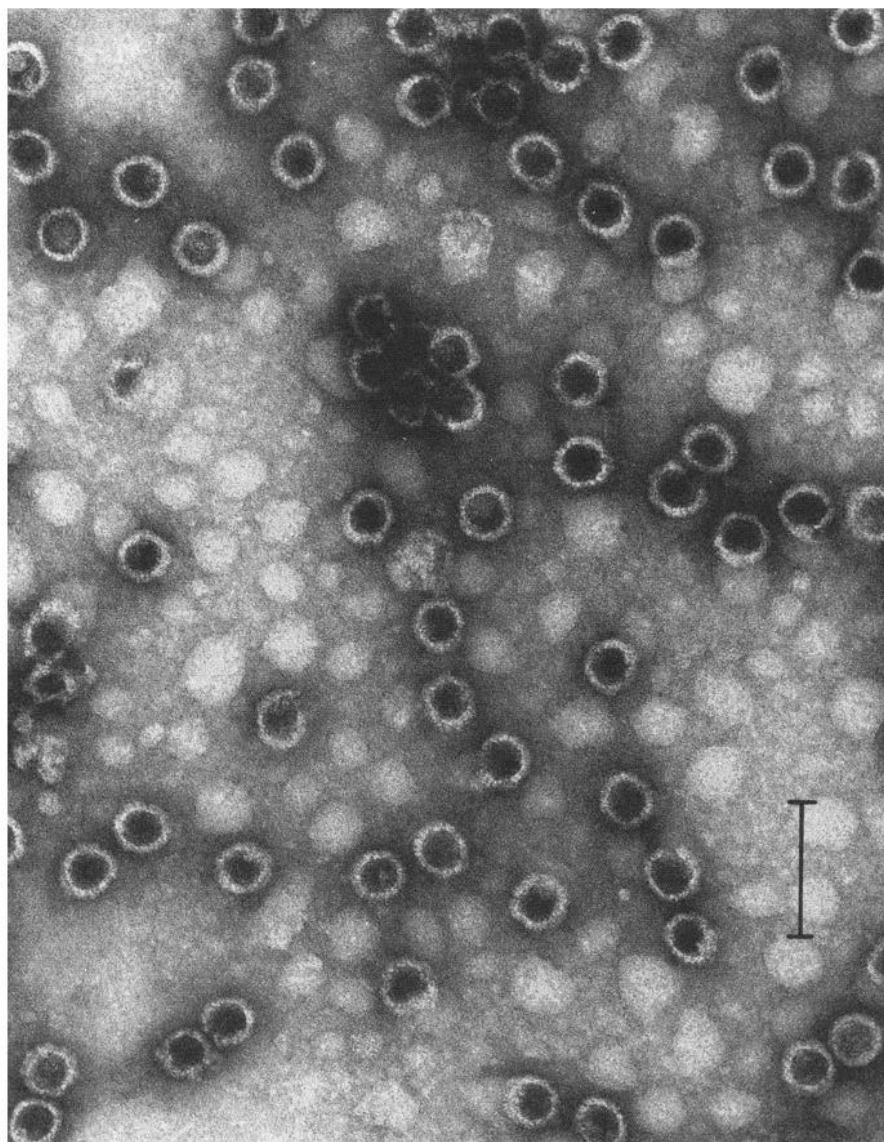


FIG. 2. Electron micrograph of a representative field of a negative stained VLP preparation from the major peak of the sucrose gradient fractionation shown in Fig. 1. The bar represents 100 nm.

dsRNA. The slower sedimenting band, representing about 5% of the A_{260} material, contains no dsRNA, but consist of empty particles as judged by electron microscopy.

Analysis of VLP proteins on SDS-polyacrylamide gels (7.5 or 10%) consistently gave a single major polypeptide (Fig. 3) migrating just below phosphorylase A (92,000 daltons). This major VLP protein has previously been estimated to have a size of 75,000 daltons (14), but the calculated molecular weight from these gels is about 88,000. At least three other minor bands (at approximately 140,000, 82,000, and 78,000 daltons) are always seen regardless of the conditions of VLP preparation and dissociation. The prominent bands at 53,000 and 37,000 daltons, reported by Oliver *et al.* (26), have not been reproducibly detected in our preparations. The amino acid content of purified ScV-P1 polypeptide is shown in Table I.

Isolation of L-dsRNA – L-dsRNA was isolated for *in vitro* translation by three different methods. Initially, total cellular nucleic acids obtained from strain NK2 by phenol extraction were hydrolyzed with pancreatic DNase and pancreatic RNase, and the products fractionated on Sepharose 4B columns. The RNA was characterized as dsRNA on the basis of

insensitivity to pancreatic RNase in 150 mM NaCl sensitivity to pancreatic RNase in 15 mM NaCl and sensitivity to RNase III in 150 mM NaCl (11, 12). Polyacrylamide gel electrophoretic molecular weight determination and staining with ethidium bromide, which is specific for ds nucleic acid, confirmed the double strandedness of the RNA, as described by Vodkin *et al.* (13). The yield of dsRNA from strain NK2 by this method was 0.33% of the total nucleic acid fraction. This value agrees well with previous values for this strain.³

Alternatively, dsRNA from strains 3/A1 and NK2 was separated from the total nucleic acid fraction by Whatman CF11 chromatography (34), as previously described (21). Yields of dsRNA by this procedure were 0.4% of the total nucleic acid for both strains. Finally, L-dsRNA was extracted from purified VLPs by phenol extraction after hydrolysis with proteinase K. The RNA products derived from the VLPs were then fractionated by Whatman CF11 chromatography. The “dsRNA” fractions from CF11 chromatography were determined to be dsRNA on the basis of insensitivity to pancreatic RNase in high salt, sensitivity to pancreatic RNase in low salt, insensitivity to pancreatic DNase. They were character-

ized as *L*-species by electrophoretic migration behavior and ethidium bromide staining following standard procedures (13, 21).

In Vitro Translation—*In vitro* translation of these dsRNA preparations was carried out using the wheat cell-free translational system. Incorporation of L-[³⁵S]methionine into products was detected by SDS-polyacrylamide slab gel electrophoresis and autoradiography. Undenatured L-dsRNA does not direct the *in vitro* synthesis of any [³⁵S]methionine-containing polypeptides detectable by long autoradiographic exposures (Fig. 4). Slight inhibition of translation programmed by en-

dogenous (wheat embryo) mRNAs did occur, as revealed by comparison with the control (no added RNA).

In contrast to the lack of stimulation by dsRNA, prior denaturation of L-dsRNA by a 1- or 2-min incubation at 100° in the presence of EDTA, followed by quick cooling, results in the *in vitro* synthesis of a single major and three or four minor molecular weight species of [³⁵S]methionine-containing polypeptides which do not co-migrate in SDS-gels with major endogenous polypeptides (Fig. 4), and are consequently judged to be encoded by L-dsRNA. The major band synthesized *in vitro*, L-P1, has a migration rate corresponding to a polypeptide of about 88,000 daltons and co-migrates on SDS-gels with ScV-P1 (Fig. 4). The most prominent minor bands migrate faster, although a small amount of a component migrating more slowly than L-P1 has been observed after more prolonged incubation. The pattern of L-dsRNA specified polypeptides does not change if the dsRNA is exposed to pancreatic RNase in high salt and then deproteinized prior to denaturation and *in vitro* translation (data not shown).

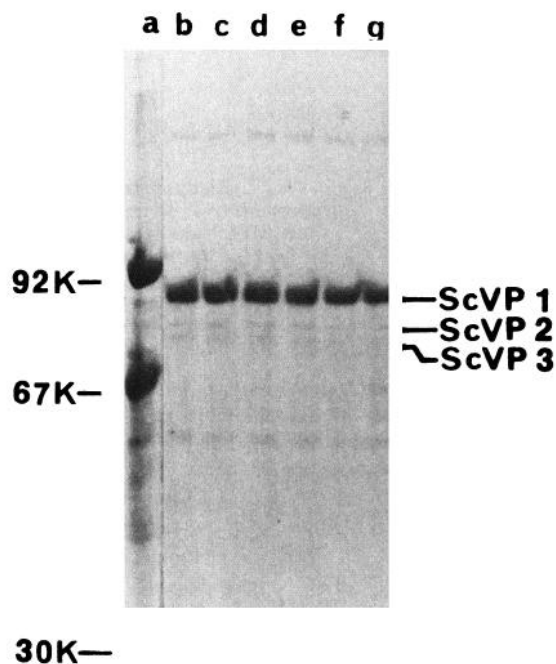


FIG. 3. 7.5% SDS-polyacrylamide gel electrophoresis of the major peak of VLPs from the sucrose gradient fractionation shown in Fig. 1. The gel was stained with Coomassie blue. The markers (slot *a*) are phosphorylase A (92,000), bovine serum albumin (67,000), and carbonic anhydrase (30,000). Times of heating at 100° in SDS sample buffer prior to application to the gel were 0 min (*g*), 0.5 min (*f*), 1 min (*e*), 2 min (*b*, *d*), and 5 min (*c*). All were dialyzed against phosphate/glycerol buffer prior to denaturation. For one sample (*b*), this was performed in the presence of 1.2 mM phenylmethylsulfonyl fluoride.

TABLE I
Amino acid analysis of ScV-P1

Amino acid	Integral residues/75,000 g protein
Aspartic acid	51
Threonine	42
Serine	47
Glutamic acid	58
Glycine	65
Alanine	66
Valine	42
Isoleucine	38
Leucine	57
Tyrosine	33
Phenylalanine	44
Lysine	20
Histidine	12
Arginine	18
Proline	2
Half-cystine	8
Methionine	7

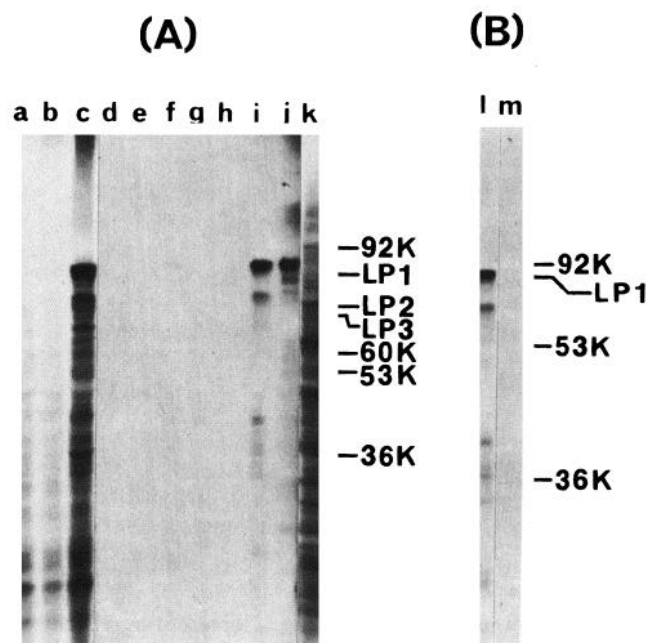


FIG. 4. Radioautograms of 10% SDS-polyacrylamide slab gels demonstrating co-migration and immunological cross-reactivity of ScV-P1 and the major polypeptide (L-P1) synthesized in the wheat germ cell-free system in response to denatured L-dsRNA. The *in vitro* translation reactions were run under optimum conditions as described in text. The indirect method of immunoprecipitation was carried out as detailed under "Experimental Procedures." **A**, (*a*) endogenous mRNA activity in the wheat embryo system; (*b*) undenatured L-dsRNA added; (*c*) denatured L-dsRNA added; (*d*, *e*, and *f*) reaction of preimmune serum γ -globulin with translation reactions programmed with (*d*) no RNA; (*e*) undenatured L-dsRNA; and (*f*) denatured L-dsRNA, respectively; (*g*, *h*, and *i*) reaction of γ -globulin prepared against purified VLPs with translation reactions programmed with (*g*) no RNA, (*h*) undenatured L-dsRNA, and (*i*) denatured L-dsRNA; (*j*) [³⁵S]methionine-labeled polypeptides from purified VLPs; ScV-P2 and ScV-P3 are not readily visible in this preparation; (*k*) [³⁵S]methionine-labeled polypeptides synthesized in the wheat embryo system programmed by yeast polysomal RNA. **B**, competition experiment confirming the specificity of the immunoprecipitation reaction, (*l*) reaction of anti-VLP γ -globulin with translation products synthesized in response to denatured L-dsRNA (same as *i*); (*m*) identical assay as in *l* except that 1 μ g of unlabeled VLPs were added to the translation reaction prior to addition to the anti-VLP γ -globulin.

Optimization for Denatured dsRNA-directed Cell-free Polypeptide Synthesis of L-P1—Several parameters of the wheat embryo cell-free system were varied to determine optimum conditions for denatured dsRNA-directed polypeptide synthesis. Optima were assessed not on the basis of total [³⁵S]methionine incorporation into trichloroacetic acid-precipitable material but rather on the basis of incorporation into L-P1, the major *in vitro* synthesized polypeptide. Optima for Mg²⁺ and for spermidine were found to be 1.6 mM and 0.16 mM, respectively. These are very close to the optima routinely observed for yeast polysomal RNA-directed polypeptide synthesis. The K⁺ optimum was consistently very broad, ranging from 80 to 110 mM, thus somewhat lower than the 120 to 160 mM K⁺ optimum observed for yeast polysomal RNA.⁶

The optimum range of denatured L-dsRNA concentration in the cell-free system was found to be between 0.02 and 0.04 mg/ml (Fig. 5). This optimum is 3- to 4-fold lower than that observed for polysomal RNA directed polypeptide synthesis⁶ and possibly reflects the formation of double-stranded RNA in the cell-free reaction mixture. Preliminary experiments conducted to determine the extent of dsRNA formation in the cell-free reaction mixture under conditions of translation do reveal that as much as 30% of the input denatured dsRNA becomes immediately insensitive to pancreatic RNase, and that this insensitive fraction may increase to as much as 65% during the first 25 to 45 min at 22° (data not shown). These preliminary experiments do not, however, distinguish insensitivity due to dsRNA formation from that due to some other cause. The nature of the pancreatic RNase-insensitive form is under further study.

Denatured L-dsRNA Directs Synthesis of Major VLP Polypeptide—L-P1, labeled *in vitro* with [³⁵S]methionine, was compared with the major ScV-P1 polypeptide, obtained from purified L-dsRNA containing VLPs labeled *in vivo* with [³⁵S]methionine. Both polypeptides were purified by gel electrophoresis and hydrolyzed with trypsin under identical conditions. The tryptic peptides, fractionated by paper ionophoresis, gave nearly identical patterns (Fig. 6), although differences in minor slow migrating peptides were seen.

Purified γ -globulin fractions prepared from sera raised in rabbits against purified L-dsRNA containing VLPs precipitated both ScV-P1 and L-P1 (Fig. 4). The lower molecular weight species produced *in vitro* were also precipitated. Specificity of the immune precipitation was determined by competition experiments. The addition of unlabeled VLPs, but not the addition of any one of several other purified proteins, competed with the immunoprecipitation of *in vitro* synthesized, [³⁵S]methionine-labeled L-P1 and minor polypeptides (Fig. 4).

DISCUSSION

One of the major *in vitro* translation products of denatured *L*-species dsRNA has been shown to correspond to the major L-dsRNA-containing VLP polypeptide, ScV-P1, by three criteria: (a) co-migration during SDS-gel electrophoresis; (b) immunological cross-reactivity, and (c) identical tryptic peptide patterns. dsRNA obtained either from isolated VLPs or by CF11 chromatography of total cellular nucleic acids requires denaturation in order to direct the cell-free synthesis of [³⁵S]methionine polypeptides, indicating that we have translated the sense strand of the VLP genomic L-dsRNA rather than a single-stranded RNA molecule either carried within VLPs or co-purifying with VLPs and VLP-derived

⁶ J. E. Hopper and L. B. Rowe, unpublished results.

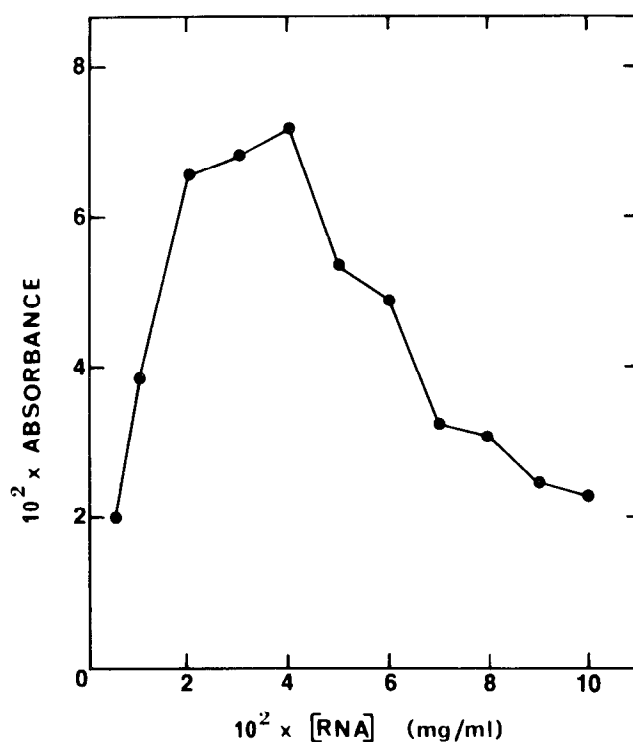


FIG. 5. *In vitro* [³⁵S]methionine incorporation into L-P1 as a function of denatured L-dsRNA concentration. Data are expressed in relative densitometric units obtained from scans of a radioautogram of a 10% SDS-polyacrylamide slab gel. The wheat embryo system translational reaction was carried out at 22° for 3 h as described under "Experimental Procedures."

dsRNA. Translation of ScV-P1 *in vitro* is not programmed by a single-stranded region (tail) of a transcriptional or replicative intermediate, since we observe no decrease in synthesis of dsRNA-specified polypeptides following exposure of the dsRNA to pancreatic RNase at high ionic strength prior to denaturation and translation.

The translational activity of denatured L-dsRNA in wheat embryo extracts was relatively low. This could be due to the presence of dsRNA caused by renaturation, or to the known lack of 5'-terminal caps (m⁷GpppGp or m⁷GpppAp) in the L-dsRNA (42), or both. The low RNA concentration optimum and the rapid formation of pancreatic RNase-insensitive structures following denaturation support the notion that at least part of the reason for low activity may be due to renatured dsRNA, or snap-back regions, or both. Messenger RNAs lacking 5'-terminal caps can be capped by methylating enzymes present in wheat embryo extracts, and the addition of *S*-adenosylmethionine enhances this activity (43, 44). The attainment of high levels of translational activity for at least some eukaryotic mRNAs is dependent on the presence of 5'-terminal caps (45-47). Since it is conceivable that the mRNAs derived *in vitro* from L-dsRNA are capped, the addition of *S*-adenosylmethionine to our wheat embryo extracts may enhance polypeptide synthesis directed by genomic L-dsRNA *in vitro*.

In addition to L-P1, the denatured L-dsRNA directs the synthesis of two other slightly smaller, moderately abundant polypeptides, and sometimes of small amounts of a larger species at approximately 120,000 to 160,000 daltons (not shown). It is unlikely that these four polypeptides represent entirely non-overlapping sequences within the L-dsRNA unless both strands are translated, because the single strand

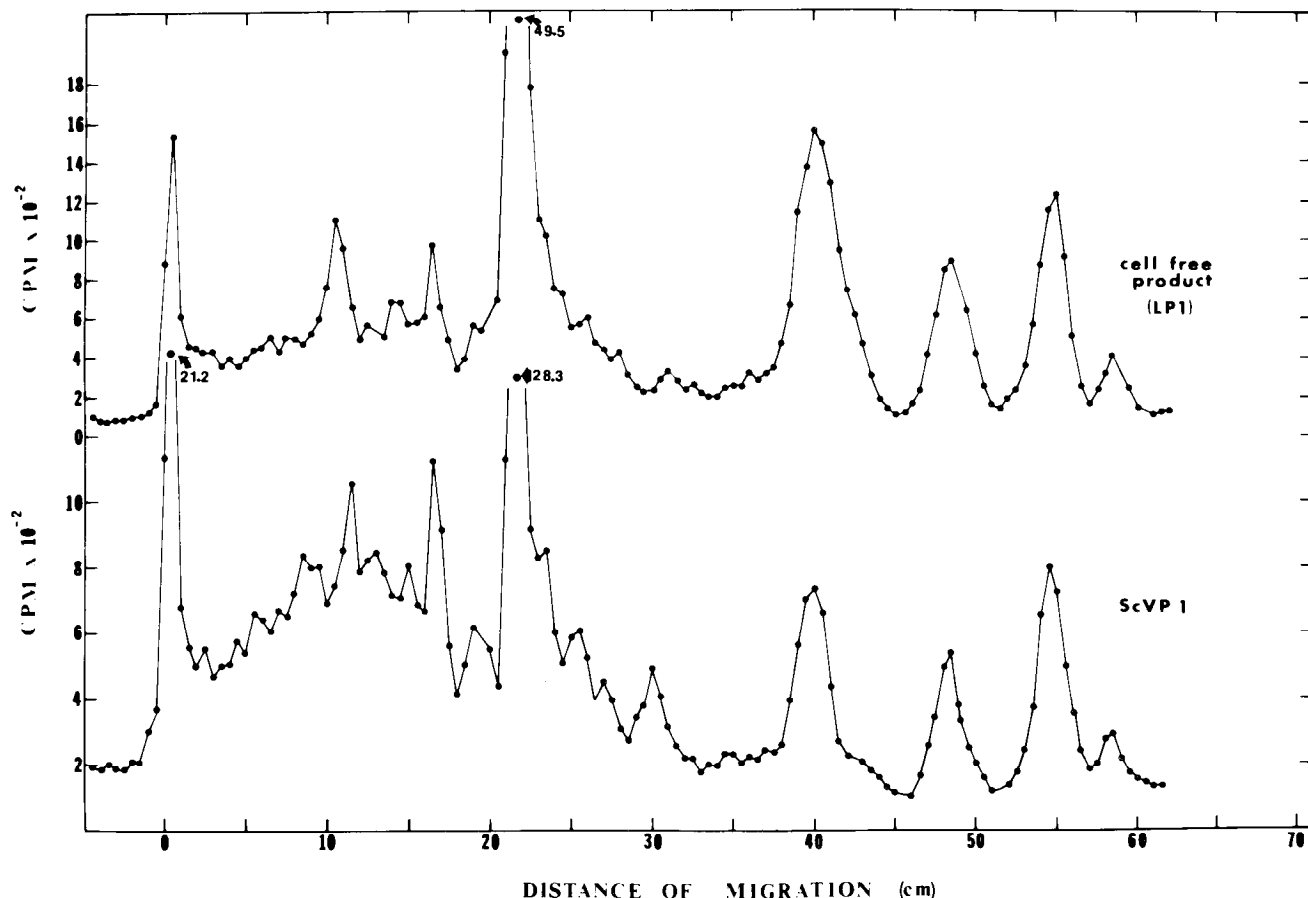


FIG. 6. Fractionation of tryptic peptides from ScV-P1 labeled *in vitro* with [³⁵S]methionine and L-P1 labeled *in vitro* with [³⁵S]methionine. After trypsin hydrolysis, peptides were fractionated by paper ionophoresis at pH 3.5. The paper was cut into strips, and counted.

theoretical maximum coding capacity of the L-species is only approximately 170,000 daltons. One or both of the smaller species may be NH₂-terminal fragments of L-P1 produced by premature termination. Premature termination is frequent in the wheat embryo cell-free system. The relationship of these and the larger species to ScV-P1 is currently being investigated by tryptic peptide analysis.

Two minor polypeptides having molecular weights of 37,000 and 53,000 have been detected in VLP preparations by others (26).³ We have not observed any polypeptides derived from our VLP preparations which consistently migrate at these molecular weight positions.⁷ Nor have we been able to detect the $M_r = 37,000$ or 53,000 species among the L-dsRNA-directed *in vitro* translation products, although the pattern of polypeptides in this region could be obscured by the products of wheat embryo endogenous mRNA translation and possibly by products of premature termination on the L-dsRNA ScV-P1 cistron. Clearly, a more rigorous analysis of the VLP polypeptide components in VLPs isolated under varying conditions, and a more extensive analysis of translation products derived from L-dsRNA is required before the relationship between L-dsRNA encoded polypeptides and VLP polypeptides is completely defined.

Although our results do not bear directly on the apparent dependency of the M-dsRNA genome on the L-dsRNA genome, they are consistent with the generally held hypothesis that L-dsRNA is a helper genome for maintenance of the M-species. This hypothesis is derived from the general observation that

although many yeast strains contain L-dsRNA without M-dsRNA, no strain has yet been reported which contains M-dsRNA without L-dsRNA. If M-dsRNA is indeed obligately encapsulated in VLPs whose proteins are shared with those encapsulating L-dsRNA, then the results presented here suggest a firm basis for M-dsRNA dependency on L-dsRNA: the L-dsRNA species encodes the major capsid polypeptide for the M-dsRNA-containing VLPs. Two types of data obtained by others support this hypothesis. Antibody raised against capsid protein of particles containing L-dsRNA precipitate VLPs containing L- or M-dsRNAs^{3,8} and SDS-polyacrylamide gel electrophoretic analysis of dissociated VLPs containing L-dsRNA or both M- and L-dsRNA reveals the same size major capsid polypeptide (24). In light of these observations and the fact that the M-dsRNA species (1.1×10^6 daltons by electron microscopic measurements (9)⁹ is not large enough to code for a 88,000-dalton polypeptide, it is highly likely that L-dsRNA, rather than a nuclear gene (*e.g.* a Mak gene), encodes the capsid polypeptide of M-dsRNA containing VLPs. We intend to isolate VLPs containing exclusively M-dsRNA in order to make a direct comparison of their polypeptide components with those of VLPs containing L-dsRNA.

The replicase and transcriptase activities required for replication and expression of the dsRNA genomes might also be functions encoded exclusively by L-dsRNA, suggesting a further more stringent basis for the dependence of M-dsRNA

⁸ A. J. Herring and E. A. Bevan, unpublished results cited in Ref. 24.

⁹ H. Fried, personal communication.

⁷ Unpublished results.

maintenance on the presence of the L-dsRNA genome. Alternatively these functions could be encoded by nuclear genes, although extensive searches have failed to produce chromosomal mutants leading to loss of both M- and L-dsRNA-containing VLPs. Thus if a putative replicase/transcriptase is encoded by nuclear genes, then these functions are also essential for cell survival.

The *in vitro* translation of VLP genomic dsRNA which we have applied to the killer system could be a generally useful approach to decoding dsRNA species in several other viral systems. If applicable, it obviates the need to first carry out *in vitro* transcription for production of translatable mRNAs. We have applied this direct translational approach to VLP-derived dsRNA from *Penicillium chrysogenum*, and have obtained *in vitro* translation of the major capsid polypeptide.¹⁰ Preliminary results suggest that this technique also works with reovirus dsRNAs.¹¹

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¹¹ L. B. Rowe and J. E. Hopper, unpublished results.

Translation of the L-species dsRNA genome of the killer-associated virus-like particles of *Saccharomyces cerevisiae*.

J E Hopper, K A Bostian, L B Rowe and D J Tipper

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