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

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Virus-like particles containing the L (P1)-species of double-stranded RNA (dsRNA) were isolated from Saccharomyces cerevisiae, and the transnational 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 vitrotranslation 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 species of homologous dsRNA genome in killer strains of yeast by providing the M-dsRNA as a helper genome to the smaller (1.6 × 10^6 daltons) M-dsRNA-containing 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 the 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 L-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 daltons have been reported to exist in the L-dsRNA-containing particle (26). 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.

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

1 The abbreviations used are: dsRNA, double-stranded RNA; VLP, virus-like particle; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, Sodium dodecyl sulfate.
2 G. R. Fink, personal communication.
3 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 polyssopptides derived from dsRNA-containing VLPs ScV-P1, ScV-P2, ScV-P3, ScV-P4, and ScV-P5. The major polyssopptide and product it co-migrated with ScV-P1. The data presented here show that these two polyssopptides share all major tryptic peptides, and that the L-dsRNA genome codes for at least its major capsid polyssopptide. 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 chloramphenicol. Such strains characteristically overproduce L-dsRNA (8). Strain 108-3c was from Dr. Howard Douglas (University of Washington) stock collection. 3/4A1, 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. The 25 mm CuCl2 solution was prepared as described above (3). 2% yeast extract, 4 g Bacto-peptone, 6.7 g yeast nitrogen base without amino acids (Difco, Nutley, N.J.), 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 tRNA and tRNA-labeling studies, was prepared as described elsewhere (9, 10).

**Materials**—Carrier-free H32PO4 was obtained from New England Nuclear. L-[35S]Methionine preparations at 695 Ci/mmol and 400 Ci/mmol were obtained from Amer sham/Atlas and New England Nuclear, respectively. Pancreatic ribonuclease (I) and pancreatic deoxyribonuclease (I) were purchased from Worthington Biochemical Corp. RNase III was a gift from Dr. Fred Hagen. The sodium dister; ribonuclease-free ultrapure sucrose and enzyme grade ammophoresis grade) were purchased from Bio-Rad Laboratories; BH salts were of ACS analytical grade. For in vivo labeling with H32PO4, low phosphate YEPD was used. Label was added at a cell density of about 5 mg/ml, and stored in small aliquots at -20°C. Spectral analysis of the nucleic acid preparations gave A260/A280 ratios of 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. Ultrathin sections were prepared by conventional methods. The grids were then washed in 100 mM sodium citrate buffer (pH 7.0) and negatively stained with a 1% solution of uranyl acetate. The protein was then visible as a dense homogeneous precipitate. The globular particles were about 100 nm in diameter and appeared to be homogenous.

**Preparation, Purification, and Fractionation of Virus-like Particles**—Cells for VLP production were grown to late stationary phase (5 to 7 days of growth) in YEPD (adjusted to pH 4.7), with shaking for the first 2 days. For in vivo labelling with H32PO4, low phosphate YEPD was used. Label was added at a cell density of 106 cells/ml to a final concentration of 2.5 mcg/mg. 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 x 106 cells/ml to a final concentration of 0.6 mcg/mg.

Cells were harvested by centrifugation, washed once with cold distilled H2O, and finally resuspended in 1.5 volumes of PKE buffer (30 mm NaHPO4/Na2HPO4, 150 mm KCI, 10 mm EDTA, pH 7.0). Cells were disrupted by shaking with glass beads for 15 min on an orbital shaker at 37°C at a rate of 250 rpm, at a ratio of 3.45 mm 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 3- to 5-fold dilution of the original suspension. The pH was adjusted to 6.0 and to precipitate overnight at 4°C with stirring. The precipitate was collected by centrifugation at 10,000 x g for 30 min, resuspended in the same volume of PKE and gently homogenized with a Teflon-coated glass homogenizer.

The crude VLP preparation was then purified essentially as described by A. J. Herring (28) by two to three cycles of low speed centrifugation followed by sucrose density gradient centrifugations, as described below. The crude VLP suspension was adjusted to pH 7.6 by addition of 1 M KOH, and centrifuged at 10,000 x g for 30 min. The resultant supernatant was subjected to a high speed spin for 90 min at 30,000 rpm in the Spincino type 30 rotor, or for larger volumes at 19,000 rpm for 5.5 h in a Spincino 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 NaHPO4/Na2HPO4, 600 mM KCl, pH 7.6) and loaded onto 10 to 40% (w/v) sucrose gradients made in PK buffer. Gradients were spun in 3 h at 24,000 rpm in a Spincino SW 27 rotor. Fractions from these fractions containing VLPs were pooled and loaded onto 30 to 60% (w/v) sucrose gradients from which the upper trough 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 Spincino SW 27 rotor, and the fractions containing purified VLPs were pooled and stored at 4°C.

Purified VLPs were prepared for SDS-polyacrylamide gel electrophoresis by dialyzing against 30 mM NaHPO4, pH 7.6, 20% (v/v) glycerol, for 6 to 8 h. The dialyzed VLP solution was then made 3% (w/v) SDS. The dialyzed VLPs were then added to 5% (v/v) meraepropanol, and 0.1% in bromphenol blue, and heat-denatured for 3 min in a boiling water bath. Gel electrophoresis and autoradiography of gel-fractionated proteins were according to the method of Studier (28).

**Purification of the major VLP polyssopptide, ScV-P1**, was accomplished by preparative SDS-polyacrylamide gel electrophoresis. Purified VLPs, dialyzed, and dissociated as described above, were fractionated on a 15% (w/v) acrylamide gel in a buffer of 50 mm Tris-HCl, 2 mm CaCl2, pH 7.5. The band corresponding to the major L-P1 was excised from the gel and subjected to staining of a thin longitudinal slice. The protein was then eluted for 24 h at 30°C in 0.1 M Na2HCO3 containing 0.05% (w/v) SDS.

dsRNA was fractionated from purified VLPs by dilution 20-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°C in 50 mm Tris HCl, 2 mm CaCl2, pH 7.5. After a 1-h digestion at 30°C, 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. To the resulting phenol phase was precipitated with ethanol at -20°C. The precipitated material was pelleted at 20,000 x g for 10 min, the pellet dissolved in 200 mM potassium acetate, 10 mm Heps, pH 7.5, and the ethanol precipitation repeated. The final pellet was resuspended in distilled H2O, adjusted to a final concentration of 5 mg/ml in a boiling water bath, and concentrated by ultrafiltration. The concentration of about 5 mg/ml, and stored in small aliquots at -20°C. Spectral analysis of the nucleic acid preparations gave A260/A280 ratios between 1.9 and 2.1.

**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 12 N HCl in a sealed vessel boiling HCl for 24 h. The hydrolysates were then taken to dryness under vacuum over NaOH and P2O5. Hydrolysates 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 performic acid oxidation (32) as cysteic acid and methionine sulfoxide.

**Preparation of Total Cellular and Polysomal RNA**—The preparation of polysomal RNA from mid-log phase cells (2 x 106 cells/ml) of strain 108-3c, grown in either YEPD or YEP-galactose, was carried out by a modification of the method of Studier (28).

*Appendix*—A. J. Herring, personal communication.
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 Sephrose 4B column (122 × 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 S/AI, and also for RNA extracted from purified VLPs. The procedure of Franklin (34) was followed.

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 Na2HPO4, 2.5 mM NaH2PO4, 150 mM NaCl, pH 7.2) at 4° for 4 h. The dialyzed VLPs were added to an equal volume of 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 Na2HPO4, NaH2PO4, 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 CaCl2, 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 0.6 M 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 A280 were pooled and immediately quick-frozen in small aliquots for storage at 70°.

Cell-free Protein Synthesis—Total polyosomal RNA and purified dsRNA (naturized and denatured) were routinely translated in 50-μl reaction volumes containing 15 μl of wheat germ lysate (A280 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 160 mM potassium acetate, 1 mg/ml of RNA for denatured dsRNA and 160 mM potassium acetate and 100 μg/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 μg/ml of creatine phosphokinase, 3 mg/ml of creatine phosphate, 0.8 μg/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 [35S]methionine to a final specific radioactivity of 4.5 × 105 cpm/pmole. Reaction mixtures were incubated at 22° for 3 h. Samples for the determination of radioactivity in trichloroacetic acid-insoluble material and for SDS-polyacrylamide 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 preparation. In a typical immunoprecipitation assay, 30 μl of 0.5% (w/v) SDS, followed by 0.25 ml of BSM buffer (100 mM HEPES, 150 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 40 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 dissolving in SDS-sample buffer for 3 min in a boiling water bath.

Tryptic Peptide Analysis—The Scv-P1 band from SDS-gels of [35S]methionine-labeled VLP preparations and the major L-P1 band from SDS gels of [35S]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 Morison and Lodish (41), with slight modification. One and two-tenths milligrams of [35S]methionine-labeled VLP protein or a 1300-μl aliquot of the in vitro translation reaction mixture supplemented with 0.3 mg of unlabeled VLP protein were fractionated on 3 mm thick polysacrylamide 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) NH4HCO3, containing 0.1 mg/ml of trypsin. The incubation of gel slices was then repeated with 5 ml of fresh NH4HCO3, 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/v) 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 (41). Phenol extractions of pooled fractions of this major band yield L-
dsRNA. The slower sedimenting band, representing about 5% of the A_{260} material, contains no dsRNA, but consists 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 characteri-
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 endogenous (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°C 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). 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).

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**TABLE I**

| Amino acid analysis of ScV-P1 | 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-cysteine                | 8                                |
| Methionine                   | 7                                |

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**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°C 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.

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**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) denatured L-dsRNA added; (d) denatured L-dsRNA; and (f) denatured L-dsRNA, respectively; (g), (h), and (i) reaction of preimmune serum γ-globulin 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, (1) 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 μg 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 Mg++ 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 concentrations 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°C (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 y-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 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 (m'GpppGp or m'GpppAp) in the L-dsRNA (L-)

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°C for 3 h as described under "Experimental Procedures."

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

6 J. E. Hopper and L. B. Rowe, unpublished results.
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). We have not observed any polypeptides derived from
our VLP preparations which consistently migrate at these
molecular weight positions. Nor have we been able to detect
the $M_r = 37,000$ or 53,000 species among those dsRNA-directed
in vitro translation products, although the pattern of polypep-
tides 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 poly-
peptide components in VLPs isolated under varying condi-
tions, and a more extensive analysis of translation products
derived from L-dsRNA is required before the relationship
between L-dsRNA encoded polypeptides and VLP poly-
peptides 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
evacculating L-dsRNA, then the results presented here sug-
gest 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-dsRNA$^{1,5}$ 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 x 10$^6$ daltons by electron
microscopic measurements (9)$^P$ 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 fur-
ther more stringent basis for the dependence of M-dsRNA

$^1$ A. J. Herring and E. A. Bevan, unpublished results cited in
Ref. 24.

$^2$ H. Fried, personal communication.
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/transferase 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-in vitro transcription for production of translatable mRNAs. 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. Preliminary results suggest that this technique also works with reovirus dsRNAs.

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