JOURNAL OF BACTERIOLOGY, June 1977, p. 1303-1309

Vol. 130, No. 3
Printed in U.S.A.

Biochemical and Physiological Studies of the Yeast Virus-Like Particle

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Received for publication 24 February 1977

A study was made of the virus-like particle (VLP) of *Saccharomyces cerevisiae* S7. This strain contains elevated amounts of P1 double-stranded ribonucleic acid (dsRNA) but no P2 dsRNA. The amount of dsRNA contained in cells grown on a fermentable carbon source (glucose) was compared with that in cells grown on a nonfermentable carbon source (ethanol). It was found that ethanol-grown cells contain higher levels of dsRNA than glucose-grown cells. In the former, the amount of dsRNA increased during the logarithmic phase of growth, whereas in the latter it increased during the transition from the logarithmic to the stationary phase. A method was devised to isolate VLPs from these cells by using CsCl gradients, and the yield was assessed by monitoring the recovery of dsRNA. Three proteins were found to be tightly associated with these particles. They have molecular weights of 75,000, 53,000, and 37,000. Together they account for almost all of the coding capacity of the P1 dsRNA that the VLP contains.

The discovery of a double-stranded ribonucleic acid (dsRNA)-containing virus-like particle (VLP) in the yeast *Saccharomyces cerevisiae* (7) seemed to offer an excellent tool for the study of the transcription and translation of specific genes in this simple eucaryote. Moreover, the finding that the VLP is cytoplasmically located (16; C. Reardon and C. N. Gordon, personal communication; S. G. Oliver, unpublished data) indicated that a comparison of the transcription of viral and nuclear genes might provide a novel approach to the study of the role of the nucleus in the control of gene expression.

Before the yeast VLP can be used as such an experimental tool, some basic information must first be obtained. (i) A strain must be selected that has a high yield of VLPs. We chose to work with the nonkiller strain, S7 (a gift from P. Lhoas), which contains elevated amounts of P1 dsRNA but no P2 dsRNA. P1 (molecular weight, 2.5×10^6) is probably the viral genome, whereas a second dsRNA molecule (P2; molecular weight, 1.4×10^6) is associated with the yeast killer character (2, 15). (ii) Since an infectious cycle has yet to be established for the yeast VLP, it is necessary to determine whether viral replication is always coordinate with cell growth or whether there is a period of preferential viral replication that the experimenter may exploit. We show that a burst of accumulation of dsRNA occurs on the transition from the logarithmic to the stationary phase in glucose-grown cells. (iii) An efficient method of isolation of VLPs is required. We developed a method that gives an estimated 25% yield of VLPs as determined by the recovery of dsRNA. (iv) It is necessary to know the protein composition of the VLP. We demonstrate that the VLP contains three proteins, which, if they were all encoded by the VLP genome, would account for most of its information content. These findings should form the basis for future studies of the molecular biology of the yeast VLP.

(A preliminary account of these results was given at the Pacific Slope Biochemical Conference, June 1975.)

MATERIALS AND METHODS

Yeast strains. S7, a prototrophic, nonkiller haploid, containing P1 but no P2 dsRNA, was obtained from P. Lhoas, Imperial College, London, United Kingdom. S7EBρ is a petite mutant derived from S7 by ethidium bromide mutagenesis (13).

Medium. YM-1 medium was described previously (6). Either 2% (wt/vol) glucose or 3% (vol/vol) ethanol was used as a carbon source.

Solutions. Virus phosphate buffer (VPB) contained 0.15 M NaCl in 0.03 M phosphate buffer (pH 7.5). TNS crushing cocktail contained 2% (wt/vol) triisopropyl naphthalene sulfonate (Eastman-Kodak)

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and 0.1 M NaCl in 50 mM tris(hydroxymethyl)-aminomethane-hydrochloride (pH 7.5).

Cell counts. Cell counts were made either with a Coulter particle counter or with an Improved Neubauer hemocytometer. Ultrasonic treatment was used to separate clumps and unseparated but divided pairs.

Cell breakage. Cells were broken by passage of a suspension of 1 volume of packed cells in 1 volume of buffer through a French pressure cell (Aminco) at ca. 24,000 lb/in^2. The average cell breakage obtained by this method was ca. 90%.

Preparation and estimation of RNA. Nucleic acid was extracted from cells broken (as described above) in TNS crushing cocktail or from cell fractions essentially as described by others (2). The method is based on that of Kirby (8) and involves two extractions with phenol-cresol and two precipitations with ethanol at −20°C. Polyacrylamide gel electrophoresis was carried out in 2.6% gels at 3.3 mA/gel for 7 h at room temperature as described by Loening (9). The amount of nucleic acid applied to the gels was ca. 100 μg of whole-cell extract for the ratio gels and ca. 50 μg of LiCl supernatant material, together with exactly 100 μg of pure yeast ribosomal RNA (rRNA), for the determination of absolute amounts (see below). The estimation of RNA in gels was made by ultraviolet light scanning at 260 nm with a Gilford gel scanner. Areas under the peaks were determined by cutting them out and weighing the paper; high chart speeds were used to reduce weighing errors.

The amount of P1 dsRNA in a growing culture was determined both as an absolute amount per milliliter of culture and as a proportion of the rRNA in that culture. The following procedure, in which all steps were quantitated, was used. A known volume of culture was taken and a cell count was made. The cells were harvested, suspended in a known volume of TNS crushing cocktail, and passed through a French pressure cell. Both the percent breakage and any losses in the cell were determined. Nucleic acid was extracted into a known volume, and the extract was divided in two. One portion was used to determine the relative proportion of dsRNA and rRNA, and the other was used for the determination of the absolute amount of dsRNA in the extract. This latter portion was freed from rRNA by LiCl precipitation (1), and the extract, together with a known amount of pure yeast rRNA, was subjected to electrophoresis. The amount of P1 dsRNA in the gel was then calculated from the relative areas under the dsRNA and rRNA peaks, appropriate correction being made for their differences in hyperchromicity (3).

Electrophoresis and estimation of protein. The amount of protein in a sample was determined by a modification of the Lowry et al. procedure (10), and the sample was concentrated to ca. 1 mg/ml by rotary evaporation. A 100-μl volume of this concentrated material was prepared for electrophoresis by adding 37 μl of loading mixture (2.7% sodium dodecyl sulfate, 2.7% 2-mercaptoethanol, 27% sucrose, and 0.014% bromophenol blue in 27 mM phosphate buffer, pH 7.0) and boiling for 1 to 2 min. Neutral sodium dodecyl sulfate-polyacrylamide gels were prepared as described by Maizel (11) and were run for 16 to 18 h at 2.5 mA/gel. After electrophoresis, gels were fixed in trichloroacetic acid (5%, wt/vol), stained in Coomassie brilliant blue (0.5% [wt/vol] in 50% methanol), and destained in 7% acetic acid. The gels were scanned at 590 nm with a Gilford gel scanner, and areas under peaks were determined by planimetry. Molecular weights were determined by the method of Weber and Osborn (18), using appropriate protein standards for calibration. Gels were stained for glycoproteins by the periodic acid-Schiff procedure of Fairbanks et al. (4).

Electron microscopy. Samples were prepared for electron microscopy by a modification of the negative-staining procedure of Gordon (5). A drop of VLP suspension was placed on a carbon-coated grid and allowed to absorb for 1 min; the grid was then washed three or four times with double (glass)-distilled water. The preparation was stained by dipping the grid into a 0.3% solution of uranyl acetate and then wetting with 0.5 μl of 0.015% octadecanol in hexane. After drying, grids were examined in a Siemens 1A electron microscope.

VLP isolation. The VLP isolation procedure is outlined in Table 1, where the recovery of P1 dsRNA, relative to the amount found in the whole-cell lysate, is also given. Briefly, the procedure was as follows. Five- to seven-day cultures of strain S7 in YM-1 medium plus 2% glucose were harvested by centrifugation and washed twice in VPB. The washed cell pellet was resuspended in an equal volume of VPB and lysed by passage through a French pressure cell. (The cell suspension was kept on ice, the French pressure cell was precooled, and the delivery tube and collection flask were kept on ice during the breakage procedure.) Unbroken cells and cell debris were removed by centrifugation at 12,000

TABLE 1. *VLP isolation procedure*

Procedure	Recovery (%) of dsRNA
Harvest cells and wash twice in VPB	
↓	
Resuspend in VPB and lyse in French press at 24,000 lb/in^2	100
↓	
Centrifuge lysate at 12,000 × *g* for 30 min	
↓	
Centrifuge supernatant at 100,000 × *g* for 90 min (Beckman 50 or Ti75)	73.6
→ Discard supernatant	8
↓	
Pellet	38.4
↓	
Suspend pellet in CsCl (1.3 g/cm^3) and layer over 1.4-g/cm^3 and 1.7-g/cm^3 CsCl step gradient; centrifuge at 29,000 rpm in an SW41 Ti for 18 h	
↓	
Collect VLP band; 86% of the dsRNA in gradient is in this band	24.3

× g for 30 min. The supernatant fluid was then spun at 100,000 × g in either an Angle 50 or a Ti75 rotor in an L2-65B centrifuge (Beckman) for 90 min. The pellet was resuspended in VPB by mild sonic oscillation (1.2 A; Branson Power Sonifier) for 1 to 3 s with constant cooling. This suspension was brought to 1.3 g/cm³ with CsCl, layered over 1.4- and 1.7-g/cm³ steps of CsCl in VPB, and centrifuged at 29,000 rpm at 15°C for 18 h in either an SW40 or an SW41 Ti (Beckman) rotor. This gradient was then fractionated, or the bands were removed, and diluted into VPB before pelleting at 100,000 × g for 90 min to removed CsCl. These sediments could then be suspended and layered onto gradients of 10 to 45% sucrose in VPB and centrifuged at 35,000 rpm in an SW40 or SW41 Ti (Beckman) rotor for 2 h. These gradients were used for the determination of S values (12) and for the preparation of protein samples.

RESULTS

Synthesis of P1 dsRNA in yeast batch culture. We first established that strain S7 contained P1 dsRNA by using the criteria established by others (2). The material referred to here as P1 dsRNA is alkali soluble, is ribonuclease sensitive at low, but not at high, salt concentrations, and runs to the position characteristic of P1 on polyacrylamide gels (see Fig. 4). The band in polyacrylamide stains pink with toluidine blue dye and shows relatively greater ultraviolet light-induced fluorescence than rRNA when stained with ethidium bromide.

We measured both the absolute amount of P1 dsRNA per milliliter of culture and its relative amount as a proportion of the rRNA in the culture. This was done for both glucose- and ethanol-grown cultures of strain S7 as they progressed through the growth curve to the stationary phase. The stationary-phase cell densities were 2.7 × 10^8 cells per ml for glucose-grown cultures and 1.2 × 10^8 cells per ml for ethanol-grown cultures.

Figure 1 shows the data from a typical experiment. In the glucose culture, both the absolute amount and the relative amount of dsRNA rose as the culture made the transition from the logarithmic to the stationary phase of growth. In contrast, the amount of P1 dsRNA in ethanol-grown cells rose during the mid-logarithmic phase. dsRNA reached higher levels in the ethanol-grown cells than in the glucose-grown cells. When calculated on a per cell basis, these data indicate that there is a maximum of ca. 24,000 molecules of P1 dsRNA per ethanol-grown cell and ca. half that number per glucose-grown cell. Weber and Lindner (17) obtained similar figures for acetate-grown cells by counting the number of VLPs observable in serial thin-section under an electron microscope. These numbers are in excess of the estimates of Vodkin et al. (16) for killer strains and, therefore, confirm previous observations (7) that P1 dsRNA levels are elevated in non-killer strains, which possess only the P1 molecule but not the P2 molecule.

Isolation of VLPs. Our VLP isolation procedure has been described in Materials and Methods and is outlined in Table 1. In the absence of an infectivity assay, we used the recovery of dsRNA as an indication of the yield at the various stages of the isolation procedure. This method has limitations; for instance, the major loss of dsRNA is seen (Table 1) to be the formation of the 100,000 × g pellet; however, we have no way of knowing whether this represents handling losses for the VLP or the separation of encapsulated from unencapsulated molecules.

The CsCl gradient afforded a separation of

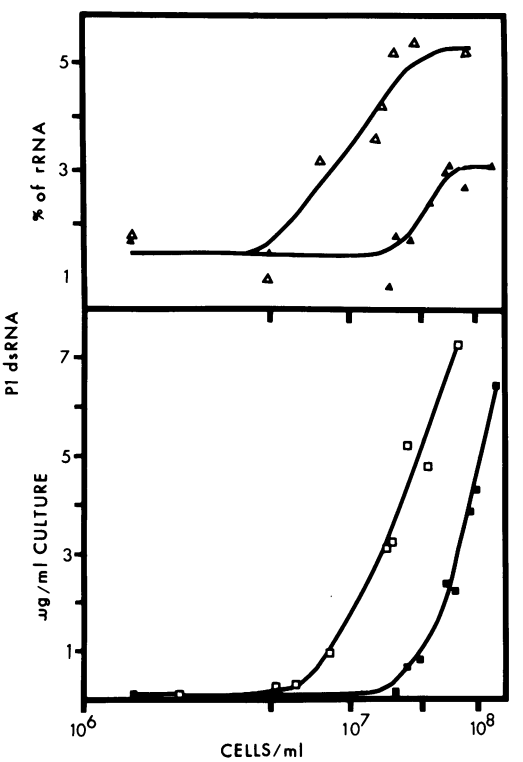

FIG. 1. *Determination of the absolute and relative amounts of P1 dsRNA in batch cultures of strain S7 grown in YM-1 medium plus glucose (▲, ■) and YM-1 medium plus ethanol (△, □). Samples were taken at the indicated cell densities, and the amount of P1 that they contained was determined as described in the text. Symbols: ■ and □, absolute amount (micrograms) of P1 per milliliter of culture; ▲ and △, amount of P1 relative to rRNA in the same two cultures.*

"full" (dsRNA-containing) VLPs, which banded at 1.42 g/cm^3, from "empty" capsids, which banded at 1.32 g/cm^3. The electron micrograph of negatively stained material from the "full" VLP band of CsCl gradient (Fig. 2) shows the preparation to be free from other subcellular particles. Almost 90% of the dsRNA in the gradient was found in the "full" VLP band (Fig. 3 and Table 1). The "empty" VLP band contained particles that were identifiable by electron microscopy and that had the normal protein complement. However, no nucleic acid was detectable in extracts from this band. The dense ribosomes formed a tightly packed pellet at the bottom of the tube in these gradients. The VLP is therefore free from contamination with rRNA (Fig. 4).

Figure 5 shows a 260-nm absorption profile from a sucrose density gradient sedimentation of "full" VLPs prepared from CsCl. Some loss of dsRNA from the particles occurred, since both a "full" and an "empty" band can be seen. Direct measurement of the band position yielded approximate *S* values of 160*S* for the "full" and 110*S* for the "empty" band by the formula of Martin and Ames (12). The small peak at about 40*S* contains dsRNA and presumably represents degraded VLPs since free dsRNA would not have such a high *S* value.

Protein composition of the VLP. Figure 6 shows a 590-nm absorption scan of a sodium dodecyl sulfate-gel separation of proteins from VLPs isolated by the CsCl gradient procedure followed by sedimentation in sucrose. Proteins V, B, and D were consistently present in such preparations and occurred in a ratio of approximately 10V:1B:1D. The amounts of protein A and of another protein, C (intermediate in

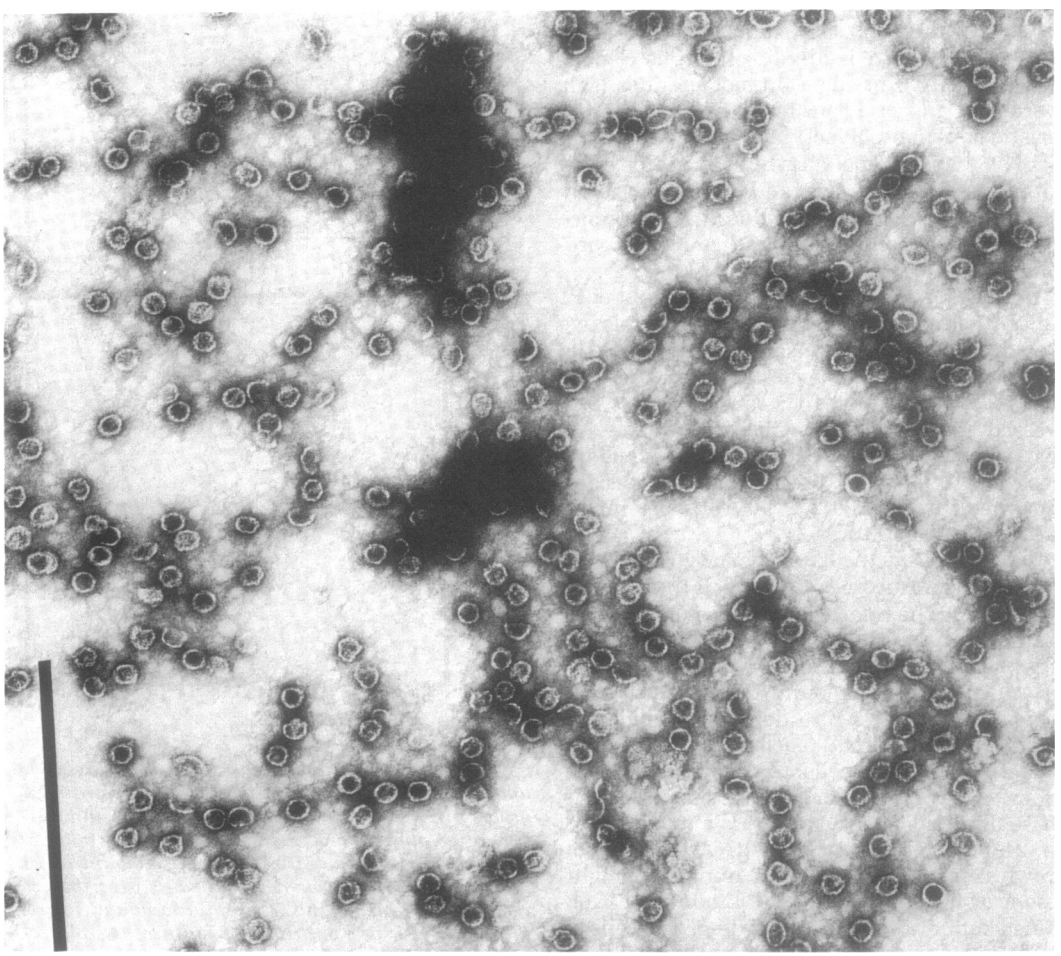

FIG. 2. *Electron micrograph of material from the VLP band of a CsCl gradient. The material was negatively stained with uranyl acetate by a modification of the Gordon procedure (5). Bar represents 500 nm.*

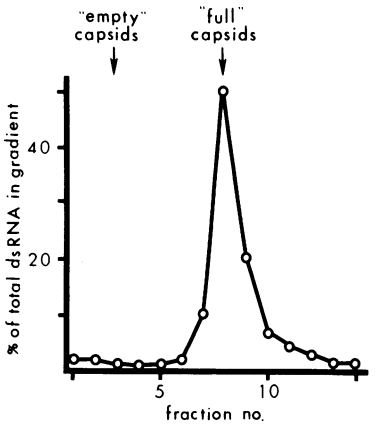

FIG. 3. *Distribution of dsRNA in a CsCl gradient. After centrifugation, fractions (1 ml) were collected from the CsCl gradient by upward displacement with dibromoethane. The fractions were diluted in an excess of TNS crushing cocktail to reduce the salt concentration prior to extracting nucleic acid. Gels were run to confirm the identity of the dsRNA as the P1 species and to permit quantitation as described in the text.*

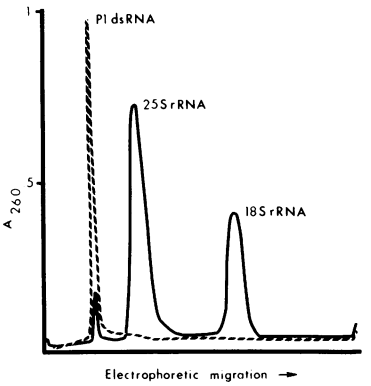

FIG. 4. *Absorption scans at 260 nm (A_{260}) of 2.6% polyacrylamide gels containing nucleic acid extracted from early-log-phase glucose-grown cells (——) and from the "full" VLP band from a CsCl gradient (-----).*

molecular weight between B and D), were found to vary widely from preparation to preparation. (For instance, in the preparation shown in Fig. 6, protein C is undetectable.) Moreover, an analysis of fractions from a sucrose density gradient showed that A and C did not peak together with the "full" VLP band. We conclude, therefore, that proteins A and C are either cellular contaminants or are only loosely associated with the VLP.

Figure 7 shows the molecular weights of V, B, and D calibrated against a standard curve produced by the co-electrophoresis of bovine se-

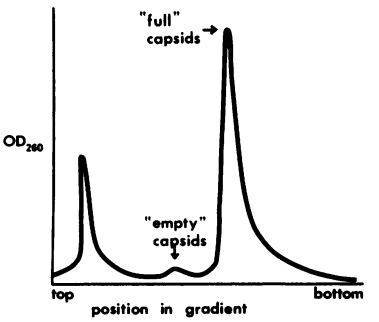

FIG. 5. *Absorption scan of a VLP-sucrose gradient. "Full" VLPs were isolated from a CsCl gradient, pelleted at 100,000 × g for 90 min, and suspended in VPB. The suspension was applied to a gradient of 10 to 45% sucrose in VPB and centrifuged at 35,000 rpm in an SW41 Ti rotor for 2 h. The scan was obtained by pumping the gradient through the flow cell of a recording spectrophotometer set to monitor optical density at 260 nm (OD_{260}).*

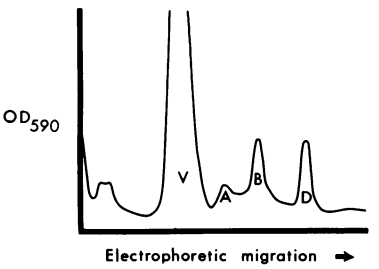

FIG. 6. *Neutral sodium dodecyl sulfate-gel electrophoresis of VLP proteins. VLPs isolated after both CsCl and sucrose gradient centrifugations were disrupted for electrophoresis as described in the text. After electrophoresis at 2.5 mA/gel for 17 h, the gels were fixed and then stained with Coomassie brilliant blue. The gels were then scanned for optical density at 590 nm (OD_{590}).*

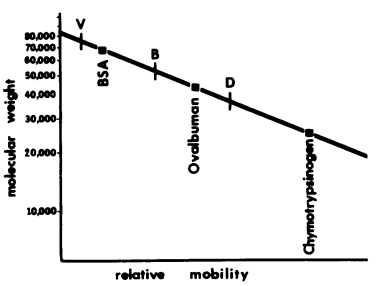

FIG. 7. *Molecular weight of the three VLP-associated proteins. Three molecular weight standards (bovine serum albumin, 67,000; ovalbumin, 45,000; chymotrypsinogen, 25,000) were subjected to co-electrophoresis with the VLP proteins. To determine the molecular weight of the unknowns, the log molecular weight is plotted against relative mobility (18).*

rum albumin (molecular weight, 67,000), ovalbumin (molecular weight, 45,000), and chymotrypsinogen (molecular weight, 25,000). The molecular weights from V, B, and D are 75,000, 53,000, and 37,000, respectively. We applied the periodic acid-Schiff test for glycoproteins (4) and concluded that, if any of the VLP proteins is glycosylated, then it is to a lesser extent than human immunoglobulin G (3% sugar by weight [13]), which was readily detectable by our procedure.

It cannot be formally ruled out that the two minor proteins, B and D, are breakdown products of the major protein, V. However, this seems unlikely since the sum of the molecular weights of B and D is greater than the molecular weight of V. Furthermore, the inclusion of protease inhibitors such as phenylmethyl sulfonyl fluoride at all stages of the isolation procedure did not alter the results. If V, B, and D are indeed the only three VLP proteins, then together they account for a total of 165,000 daltons of the estimated 167,000 daltons of the protein-coding capacity of P1 dsRNA (the putative viral genome).

DISCUSSION

The continuing study of the "killer" phenomenon in yeast by Bevan and his co-workers (7, 15) has opened up an interesting area of study—that of the yeast VLP and its associated dsRNA's. Two species of dsRNA, P1 (molecular weight, 2.5×10^6) and P2, are found in killer strains of yeast (2, 16). P2 is directly associated with the killer phenomenon since sensitive and neutral stains either lack this species entirely or possess a P2 of altered molecular weight (16). P1, on the other hand, appears to be almost universally present in laboratory strains of *S. cerevisiae*. In an unpublished survey, we found that none of the *S. cerevisiae* strains in our collection lacks P1, with the exception of a commercial bakers' yeast of uncertain genetic origins.

Since our principal interest in the yeast VLP is as a tool for yeast molecular biology, we concentrated our efforts on a nonkiller strain, S7, which has elevated amounts of P1 but no P2. We showed that cells grown on a nonfermentable substrate (ethanol) carry higher levels of P1 than do those grown on a fermentable medium (glucose). Moreover, the increase in the level of dsRNA in glucose-grown cells towards the end of the log phase occurs at a time when the transition from fermentative to oxidative metabolism is expected to occur. These data imply a subtle (that is to say, non-obligatory) relationship between the VLP and mitochondrial function.

The method of VLP isolation that we developed gives a reasonable yield, as determined by the recovery of dsRNA. It has enabled us to determine the protein composition of the VLP. We have concluded that there are three VLP proteins, which we have designated V (molecular weight, 75,000), B (molecular weight, 53,000), and D (molecular weight, 37,000). Protein V is present in approximately 10-fold excess over B and D and probably represents the major capsid protein. We have tried a number of different denaturing procedures without reducing the apparent molecular weight of V. We conclude, therefore, that there are indeed only three proteins that are tightly VLP associated. Their total molecular weight accounts for 165,000 daltons of the estimated 167,000 daltons of protein for which the putative viral genome P1 dsRNA can code.

ACKNOWLEDGMENTS

This work was supported by a Science Research Council Postdoctoral Fellowship to Stephen G. Oliver, by Public Health Service grant CA 10628 to Calvin S. McLaughlin from the National Cancer Institute, by the Cancer Research Co-ordinating Committee of the University of California to Calvin S. McLaughlin, and by the Burns Family Foundation to Calvin S. McLaughlin.

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