Virus-like Particles Associated with the Double-stranded RNA Species Found in Killer and Sensitive Strains of the Yeast *Saccharomyces cerevisiae*

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**SUMMARY**

Two high mol. wt. double-stranded RNA species have been discovered in yeast; some strains possess both species (killers) whilst other strains possess only one. Strains of the latter type have been isolated which possess relatively large amounts of dsRNA. Cell fractionation experiments utilizing these strains have shown that the dsRNA is associated with isometric virus-like particles similar to those found in other fungi. Similar particles, containing both species of dsRNA, have been isolated from a killer strain. The two species of dsRNA appear to be separately encapsidated.

**INTRODUCTION**

The discovery of high mol. wt. double-stranded RNA (dsRNA) in several strains of yeast was made as a result of the search for the genetic determinant of the cytoplasmically inherited killer character (Berry & Bevan, 1972). Further investigation of this dsRNA has revealed that killer strains possess two species of mol. wt. \(2.5 \times 10^8\) and \(1.4 \times 10^6\), referred to as P1 and P2, respectively, while sensitive strains possess only the larger P1 dsRNA molecule or have no detectable dsRNA at all (Bevan, Herring & Mitchell, 1973). Genetic analyses of cells segregating the killer character have shown that the inheritance of dsRNA follows a cytoplasmic pattern (Mitchell, Bevan & Herring, 1973). However, sensitive segregants receiving the nuclear maintenance allele \(m\) (Somers & Bevan, 1968) lose P2 dsRNA or, in one particular cross, both dsRNA species (Mitchell *et al.* 1973). Additionally, exposure of the cells to the base analogue 5-fluorouracil induces a large proportion of the cells to mutate to a stable sensitive phenotype and this change is accompanied by the loss of P2 dsRNA (Mitchell *et al.* 1973). These results have led to the conclusion that P1 and P2 dsRNAs together determine the killer character in yeast (Bevan *et al.* 1973).

The initial finding of dsRNA in killer strains led to a search for virus-like structures in those strains but no such structures were seen in ultrathin sections of killer cells (Berry & Bevan, 1972). Earlier cell fractionation experiments also failed to reveal virus-like particles (VLPs) but in these experiments a freeze pressing method was used to disrupt the cell wall and it has since been shown that isolated dsRNA containing virus from *Penicillium chrysogenum* is sensitive to one cycle of freezing and thawing (Nash *et al.* 1973). The ultimate discovery of VLPs, reported below, was made by first disrupting cells with a French pressure cell and examining those cell fractions found to contain dsRNA. This approach was greatly assisted by the isolation of sensitive strains which yielded much higher amounts of P1.
dsRNA than any killer strains investigated to date. These high yielding strains were discovered during the analysis of haploid segregants from a sensitive by sensitive cross (S3 wtz by S9 ad21a) all the spore cultures yielding elevated levels of P1 dsRNA. It has since been frequently observed that a large increase in the level of P1 dsRNA maintained by a strain may accompany a change in genetic background.

METHODS

Strains and cell growth. Four yeast strains were used for this study, three of which were derived from genetic crosses performed in this laboratory. These three strains are designated 3/A1 (sensitive, haploid), LD/1 (sensitive, diploid) and 4/4D (killer, haploid). The other strain used was a. ad21M (haploid, sensitive). Cells were grown for 2 to 4 days in yeast complete medium consisting of 2% (w/v) yeast extract, 1% bacto-peptone and 2% (w/v) glucose at 28 °C. The cells were harvested by centrifuging and were washed once with distilled water before resuspension in buffer for disruption.

Cell disruption was achieved using an Aminco French pressure cell model 4-3398A at a pressure of 1200 lb/sq inch and a temperature of 4 °C.

Preparation of total nucleic acid. Cells were disrupted in 0.05 M-tris HCl buffer, pH 7.6, containing 0.01 M-KCl, 0.005 M-MgCH3COO, 0.01 M-mercaptoethanol and 0.005 M-spermidine HCl (YTBM buffer, Marcus & Halvorson, 1967), unbroken cells were removed from the suspension by centrifuging for 5 min at 1100 g, the pellet was washed once and the supernatant fluids pooled. The suspension was then diluted to twice its vol. with 0.05 M-tris HCl buffer, pH 7.5, containing 0.1 M-NaCl and 2% (w/v) Na-tri-iso-propylphthalenesulphonate (Eastman-Kodak) (TNS buffer) and nucleic acid prepared from it by two cycles of extraction with the phenol-cresol mixture described by Parish & Kirby (1966) followed by precipitation with two vol. of cold ethanol. The precipitate was then taken up in 0.15 M-NaCH3COO buffer, pH 6.9, containing 0.5% SDS and reprecipitated with ethanol to remove traces of phenol.

Polyacrylamide gel electrophoresis was performed as described by Loening (1967). Running buffer was 0.036 M-tris, 0.03 M-Na dihydrogen phosphate, pH 7.8, containing 0.001 M-EDTA and 0.2% SDS (BDH, specially pure grade). Gel concentration was 2.6% acrylamide and electrophoresis was carried out at 3.7 mA per gel for 6 to 8 h. This relatively low current was used as it has been found to increase the separation between P1 dsRNA and DNA (Fisher & Dingman, 1971). To reveal P2 dsRNA as in Fig. 1 it was necessary to remove single-stranded RNA from the nucleic acid loaded onto the gel. This was done by taking up the nucleic acid in 1 x SSC (0.15 M-NaCl, 0.015 M-Na citrate, pH 7.0) containing 15% (w/v) sucrose and 1 µg/ml ribonuclease A (Sigma, crystalline grade) and leaving for 30 min at room temperature prior to loading on to the gel. The gels shown in Fig. 1 were loaded with 100 µl of this solution containing 500 to 1000 µg of total nucleic acid. RNA prepared from sucrose gradient fractions (see below) was loaded in half strength running buffer containing 15% (w/v) sucrose and 2% (w/v) SDS. Gels were scanned with a Joyce Leol ‘Polyfrac’ u.v. gel scanner and were then washed by gentle agitation in distilled water for at least 3 h to remove traces of SDS, stained with aqueous 0.01% toluidine blue for 12 h and destained by further washing.

Identification and quantification of dsRNA. Double-stranded RNA was identified by its mobility on the gel, the characteristic sharpness of the dsRNA band and its property of staining pink with toluidine blue (single-stranded nucleic acids stain blue, Berry & Bevan, 1972). However, as the mobility of P1 dsRNA and DNA are quite similar a further check
was made by digesting with 50 µg/ml ribonuclease A in 1 x SSC, 15% sucrose for 30 min prior to loading the gel; this treatment removes the dsRNA bands. For the purpose of comparison the amount of dsRNA or DNA in the bands was estimated by measuring the areas under the relevant peaks in the u.v. gel scans. Measuring peak areas of scans of gels loaded with a known amount of dsRNA has shown that this method is only accurate when the band contains several µg of dsRNA. At the levels of dsRNA being estimated in the result shown in Fig. 2, this method provides only a rough estimate of dsRNA per band.

**Cell fractionation** was performed essentially by the method described by Marcus & Halvorson (1967) for the preparation of yeast polysomes. Cells from 200 ml of culture were disrupted in YTBM buffer (see above) and the homogenate spun at 10000 g for 20 min to remove cell wall, nuclei and mitochondria. The supernatant fluid was removed carefully avoiding the fatty pellicle which forms during this centrifuging, diluted to have an E_{260} of between 50 and 100, and 0.5 ml samples of this suspension were loaded on to 21 ml 10 to 30% (w/v) sucrose gradients in YTBM buffer and spun for 180 min at 60000 g (25000 rev/min) in the 3 x 23 ml swing-out rotor of a MSE superspeed 65 centrifuge at 4 °C. The gradients were analysed by displacement from below with heavy sucrose solution through a LKB ‘Uvicord’ u.v. monitor, and 0.75 to 1.0 ml fractions were collected. RNA was prepared from the fractions by diluting them with one vol. of TNS buffer followed by phenol extraction. Yeast tRNA was added to each fraction as carrier before extraction. The RNA was analysed by gel electrophoresis without prior ribonuclease digestion. At least two identical gradients were run at one time, one being used for RNA analysis, and the contents of the fractions of the sister gradient were collected by pelleting at 20000 g for 150 min and examined in the electron microscope.

**Isolation of VLPs** was achieved by the low pH precipitation method of Charney et al. (1961) using the methods described by Banks et al. (1969) and the buffers used by Ratti & Buck (1972) for the isolation of *Aspergillus foetidus* virus. Cells from 10 to 20 l of culture were disrupted in 0.03 M-Na phosphate buffer, pH 7.6, containing 0.15 M-KCl, spun for 30 min at 10000 g, and to the supernatant yeast RNA (BDH) was added to a concentration of 100 µg/ml. The suspension was then adjusted to pH 5.0 with 10% (v/v) acetic acid and left to precipitate overnight. The precipitate was spun down at 10000 g for 10 min, raised in 0.03 M-Na phosphate buffer, pH 7.6, containing 0.6 M-KCl, cleared by spinning at 10000 g for 10 min and the VLPs collected by pelleting (200000 g for 150 min). This crude VLP preparation was further purified by sucrose density gradient centrifuging on 10 to 30% (w/v) gradients in YTBM buffer for 150 min at 63600 g (25000 rev/min) in the SW25(1) rotor of a Spinco L2 65B centrifuge at 4 °C. Gradients were analysed as above.

**Electron microscopy.** The high speed pellets for examination were taken up in 0.1 to 0.2 ml of 0.15 M-ammonium acetate buffer, pH 7.0, and drops of this suspension were dried down on to grids coated with carbon and formvar, stained with 2% (w/v) phosphotungstic acid for 0.5 min and viewed in a Siemens-Elmiskop 1A.

**RESULTS**

**Relative levels of dsRNA**

The two scans of polyacrylamide gels shown in Fig. 1 illustrate the large difference in dsRNA levels in the nucleic acid from one of the high yielding sensitive strains, 3/A1 and a killer strain, 4/4D, which is itself high yielding relative to other killer strains investigated. The DNA:dsRNA ratios from the two strains are 1:18.9 and 1:1.34, respectively; the mean cell level of dsRNA in the sensitive strain is thus fourteen times that of the killer
strain. It should be emphasised that this is an estimation of the mean cell level of dsRNA in a given cell population; we cannot as yet discount the possibility that the observed levels of dsRNA in total nucleic acid are due to the induction of dsRNA synthesis in a small proportion of cells. However, we do observe that the level of dsRNA yielded tends to be a stable characteristic of the strain.

Initial demonstration of virus-like particles

The isolation of high yielding strains allowed the detection of P1 dsRNA by gel electrophoresis when a post-mitochondrial supernatant fluid was analysed by sucrose density gradient centrifuging. Fig. 2 shows the result of such an analysis. The $E_{260}$ profile of the gradient revealed a broad peak of 78S ribosomes as described by Marcus & Halvorson (1967). This identification was confirmed as fractions from this peak yielded the two ribosomal RNAs in roughly equimolar ratios. The P1 dsRNA which, naked, has an $S$ value of about 15 (Bevan et al. 1973), sedimented further than the ribosomes, running to a position on the gradient which suggests that it is associated with a particle of approx 150S. Observation of the pelleted contents of dsRNA containing fractions from a sister gradient in the electron microscope showed virus-like particles similar in structure to those shown in Fig. 3. This result was obtained with both strains 3/A1 and LD/1. Particles were not found at other
levels of the gradients nor were they found in fractions from the dsRNA containing level from a gradient loaded with material from the strain a \textit{ad}_{21} M, a sensitive which contains no detectable dsRNA. Analysis of gradients loaded with material from the killer strain 4/4D gave the same result as above but the lower level of dsRNA in this strain meant that P1 dsRNA was only just detectable by eye in stained gels. In the electron microscope only a few VLPs were seen. It was clear that this method would not reveal whether P2 dsRNA was associated with a fast sedimenting particle.

\textbf{Bulk isolation of virus-like particles}

Since the VLPs detected in the experiments described above clearly resembled the virus particles described in other fungi (reviewed by Wood, 1973) we thus applied the isolation technique used for \textit{Aspergillus foetidus} virus to our strains. The result may be seen in Fig. 3 which shows VLPs isolated from strain 4/4D; the preparation has been purified on a sucrose gradient and consists of pooled fractions containing VLPs. The particles are isometric, having a mean diam. of 39 nm (mean of 200 particles). Many broken particles may be seen and many of them appear to be empty. Particles of identical appearance were similarly prepared from strain 3/A1.
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Fig. 4. $E_{260}$ profile of a 10 to 30% sucrose gradient loaded with crude VLP preparation from cells of strain 4/4D. Sedimentation was from left to right. The bar indicates the fractions containing VLPs.

Fig. 5. Three $E_{260}$ scans of 2.6% polyacrylamide gels loaded with RNA prepared from fractions of the sucrose gradient shown in Fig. 4. Left hand scan; RNA from fraction 11, middle scan; RNA from fraction 13, right hand scan; RNA from fraction 15.

**Separate encapsidation of P1 and P2 dsRNAs**

Polyacrylamide gel analysis of the dsRNA prepared from a crude VLP preparation derived from strain 4/4D showed that both P1 and P2 dsRNA species were present. Since P1 and P2 dsRNAs are not found in equimolar amounts in total nucleic acid (Bevan et al. 1973) it seemed likely that there would be at least two types of particles in these preparations, one type containing P1 dsRNA alone, since this species predominates, and another
either containing P2 alone or the two species jointly encapsidated. The results illustrated in Figs. 4 and 5 suggest separate encapsidation. Fig. 4 shows the $E_{205}$ profile of a sucrose gradient loaded with crude VLP preparation as described in the methods section above, the fractions containing VLPs are indicated although not all of the extinction of these fractions was due to VLPs as their contents may be further fractionated if re-run on a sucrose gradient. Fig. 5 shows the scans of three gels loaded with RNA prepared from fractions taken from different levels of the zone containing VLP. Clearly, the proportions of P1 to P2 dsRNA vary in the three fractions; P2 being the prevalent species at the top of the zone while P1 predominates at the bottom. In one such experiment the extreme top and bottom fractions containing VLPs yielded P2 dsRNA and P1 dsRNA alone, respectively. This result strongly suggests that the two killer yeast dsRNAs resemble the other fungal virus dsRNAs in that they are separately encapsidated (Wood, 1973). Comparison of the particle sizes in the top and bottom halves of the zone containing VLP failed to reveal any significant difference (Student's $t$-test) which suggests that a small difference in density may account for the slightly slower sedimentation rate of the P2 containing particles.

**DISCUSSION**

The particles which we describe above clearly may be grouped with the dsRNA viruses found in other fungi (reviewed by Wood, 1973); we have preferred the more cautious term ‘virus-like particles’ to virus or mycophage for the following reasons. First, the presence of these particles in the cell has not been shown to cause any deleterious effects on the cell whatsoever; indeed, in the case of the killer cells we believe that their presence under certain environmental conditions may confer a considerable selective advantage. Secondly, no infective cycle has yet been demonstrated for these particles although the existence of such a cycle cannot be discounted and this is an aspect which we shall investigate further. Finally, the existence of nuclear genes which have been shown to affect the maintenance of both species of dsRNA, and hence both types of virus particle, suggests that the VLP may be very closely integrated with the host cell (Bevan et al. 1973).

In one respect, however, the situation in yeast is unique; all the other dsRNA virus systems in fungi have been found to be of the type called by Wood (1973) component viruses in that they have a minimum of two separately encapsidated dsRNA components. We see this two component situation in our killer strains, and in a few sensitive strains described below, but we have also observed the loss of the P2 dsRNA component either as a spontaneous or induced genetic mutation, or as a response to a change in genetic background to give a single component with the ability to replicate alone (Bevan et al. 1973). Thus in one respect the yeast VLP represents the simplest dsRNA particle so far described. It is not the simplest, however, when judged by the criterion of size of the genome. If we leave aside the possibility of base sequence heterogeneity within the mol. wt. classes of the dsRNA components of these systems, the yeast VLP genome is larger than both the PsV-s and the PsV-f systems having a mol. wt. of $2.5 \times 10^6$ (Bevan et al. 1973) relative to a total of $1.95 \times 10^6$ and $2.11 \times 10^6$ for the two *Penicillium stoloniferum* viruses, respectively.

Our previous studies of yeast dsRNA also provide some evidence to support the theory put forward by Ratti & Buck (1972) that the multicomponent systems may arise by the survival and accumulation of defective particles. In our attempt to correlate the presence of both P1 and P2 dsRNA with the killer character we discovered three sensitive strains which had P1 dsRNA plus a second component, but in all three cases this second component proved to be lighter in mol. wt. than the P2 dsRNA found in our killer strains. One of these
strains (adIo) came from our collection but the other two (4D/13 and 4D/14) were isolated as spontaneous sensitive genetic mutants from a killer diploid with the normal dsRNA species. In the most extreme case (4D/13) there was a change in mol. wt. of the dsRNA from $1.4 \times 10^6$ to $0.66 \times 10^6$ accompanied by a loss of killer function (Bevan et al. 1973).

It is our present concern to characterize the two yeast VLPs and to investigate their mode of replication and the manner in which they interact with the yeast cell.

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REFERENCES


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