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Unique Double-Stranded RNAs Responsible for the Anti-*Candida* Activity of the Yeast *Hanseniaspora uvarum*

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Killer strains of the yeast *Hanseniaspora uvarum* contain cytoplasmic double-stranded RNAs (dsRNAs) of 4.7-kbp L and 1.0-kbp M species, which were shown to be separately packaged into icosahedral virus-like particles exhibiting RNA-dependent RNA polymerase activity. The L genome of the *H. uvarum* L-dsRNA virion HuV-L was shown to encode a 77-kDa major capsid protein. Peptide maps of the purified HuV coat protein and the 81-kDa major capsid protein from K1 killer viruses of *Saccharomyces cerevisiae* revealed distinctly different peptide patterns, suggesting significant sequence divergence at the level of the capsid-coding L-dsRNAs. In vitro transcripts from purified HuV-L particles showed no cross-hybridization to denatured L_A, L_B, or L_C, indicating that L from *H. uvarum* represents a unique L-dsRNA species. Weak, but clearly detectable cross-hybridization of the 1.0-kb dsRNA of HuV-M, encoding the secreted 18-kDa anti-*Candida* toxin, to the toxin-coding M genomes of *S. cerevisiae* K1, K2, and K28 killers indicated partial sequence homology among all of the M-dsRNAs tested.

Double-stranded RNA (dsRNA) viruses in yeast belong to the constantly growing class of mycoviruses which are widely distributed among fungi (3). While the persistence of most of these noninfectious viruses (which are therefore referred to as virus-like particles [VLPs]) is symptomless in the appropriate host cell and only rarely culminates in pathology, some VLPs are known to be associated with certain adverse phenotypic effects on the fungus: La France (die-back) disease of the white-button mushroom *Agaricus bisporus*, plaque formation in *Penicillium* spp., and hypovirulence in *Endothia* spp. (reviewed in reference 3). In *Saccharomyces cerevisiae*, *Ustilago maydis*, and *Zygosaccharomyces bailii*, dsRNA VLPs have been identified which confer a killer phenotype on the corresponding host cell which is based on the secretion of a virally encoded killer toxin that kills sensitive strains of the same or closely related species in a receptor-mediated two-step process (4, 10, 16).

The well-characterized dsRNA viruses in *S. cerevisiae*, ScV-L_A and ScV-M, are members of the unsegmented *Totiviridae* family which (per particle) contain (i) one dsRNA molecule, (ii) 120 copies of the major capsid protein (Cap), and (iii) 2 copies of the Cap-Pol protein (1, 5, 15). The 4.6-kb L_A genome of ScV-L_A virions encodes the major capsid protein (Cap) and the RNA-dependent RNA polymerase (Pol), which is expressed as a Cap-Pol fusion protein by a ribosomal frameshift event (6, 17). In addition to L_A, which itself does not confer a phenotype on the infected host cell, toxin-secreting killer strains contain one of several known satellite dsRNAs (M_1, M_2, M_{28} . . .) which all depend on the coexistence of ScV-L_A to be stably maintained and replicated within the yeast cell (16, 18). Each M-dsRNA virion contains the genetic information for a killer toxin and a specific and as yet unidentified immunity component which renders killer cells immune to their own toxin (4, 16). A similar dsRNA-based killer system has recently been identified in the yeasts *Z. bailii* and *Hanseniaspora uvarum* (13, 19). Their dsRNA viruses (ZbV and HuV, respectively) were successfully transferred to *S. cerevisiae* nonkiller strains by VLP transfection (in the case of ZbV) or intergeneric protoplast fusion (in the case of HuV), resulting in yeast transfectants and/or heterokaryons that stably expressed the corresponding killer phenotype (13, 19). In both yeasts, the smaller M-dsRNA genomes (which are 1.8 kb [M_{Zb}] and 1.0 kb [M_{Hu}] in size) encode secreted killer proteins which (compared to the killer toxins of *S. cerevisiae*) have a broader killing spectrum, being lethal to different yeast genera, including pathogenic and nonpathogenic strains of *Candida albicans*, *C. glabrata*, and *C. krusei* (9, 11, 13). The secreted anti-*Candida* toxin of *H. uvarum* is a monomeric, nonglycosylated 18-kDa protein (11) which is encoded by a 1.0-kb M-dsRNA genome. Because of the lethality of this toxin for pathogenic yeasts and its antifungal drug potential, it was the purpose of this study to characterize the dsRNAs in *H. uvarum* in more detail.

Figure 1 compares dsRNA patterns and associated (killer) phenotypes of *H. uvarum* killer strain 470 and one of its nonkiller derivatives (strain 469c; obtained after curing with 5-fluorouracil) with the dsRNA patterns of representative *S. cerevisiae* K1, K2, and K28 killer strains. All bands represented dsRNA since the material was resistant to DNase I and to RNase A under high-salt conditions (0.6 M NaCl) but RNase A sensitive under low-salt conditions (Fig. 1). While both *H. uvarum* strains contained a 4.6-kb dsRNA species not distinguishable in size from L_A of *S. cerevisiae*, all toxin-coding M-dsRNA genomes were clearly different from each other, ranging in size from 1.0 to 1.9 kb. By using procedures that have been previously described for the isolation of dsRNA VLPs from *S. cerevisiae* and *Z. bailii* (13), we identified dsRNA virions with similar properties in the yeast *H. uvarum*. As shown in Fig. 2A, dsRNA analysis of fractionated sucrose gradients revealed the presence of L- and M-dsRNAs in *H. uvarum* with sedimentation rates similar to those of the representative *S. cerevisiae* K1 and K28 killer strains (13). Subsequent analysis of combined dsRNA-containing sucrose gradient fractions 7 to 11 by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (Fig. 2B) identified only one single protein with an apparent molecular mass of 77 kDa which most likely represents the major coat protein in which both dsRNAs

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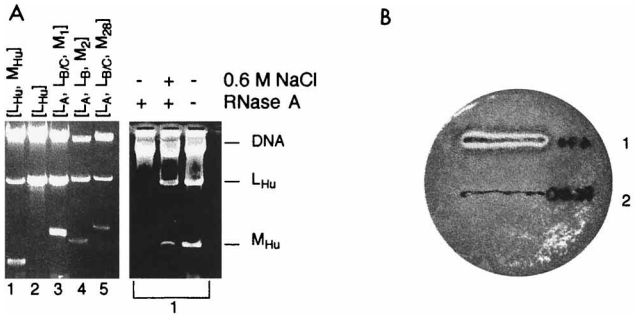

FIG. 1. dsRNA patterns (A) and killer phenotype (B) of killer and cured nonkiller yeast strains. dsRNAs were isolated from *H. uvarum* 470 and 469c (lanes 1 and 2), and *S. cerevisiae* killer strains 466 (lane 3), 1385 (lane 4), and MS300 (lane 5) as previously described (14). The presence and positions of L- and M-dsRNAs are indicated. The RNase A resistance of the *H. uvarum* dsRNAs under high-salt conditions is also indicated. L_{Hu} and M_{Hu} represent natural dsRNA species present in killer strains of *H. uvarum*. The killer phenotype was tested for by streaking *H. uvarum* killer strain 470 and its cured nonkiller derivative (strain 469c) on methylene blue agar plates (pH 4.5) that had been seeded with a lawn (10^5 cells) of the sensitive tester strain *C. glabrata* 453.

TABLE 1. Characteristic features of the HuV-associated RNA polymerase of L- and M-VLPs from *H. uvarum*

Reaction condition[a]	RNA polymerase activity (%)[b]
Control	100
Without $MgCl_2$	18
Without ATP	7
Without CTP	5
Without GTP	9
Without ATP, CTP, or GTP	0
With rifampin (80 μg/ml)	85
With actinomycin D (80 μg/ml)	94
With α-amanitin (80 μg/ml)	98
With ethidium bromide (100 μg/ml)	6

[a] Purified VLPs from *H. uvarum* 470 were assayed for virion-associated RNA polymerase activity by incubating HuV at a concentration of 250 μg/ml (determined as micrograms of total capsid protein per milliliter by the bicinchoninic acid assay method [Sigma]) in an assay mixture of 50 μl as previously described (12).

[b] 100% RNA polymerase activity corresponds to 0.12 nmol of [α-^{32}P]UTP incorporated per hour per microgram of capsid protein.

are separately encapsidated. Since the same 77-kDa polypeptide was seen in VLP preparations of the cured nonkiller derivative strain *H. uvarum* 469c (see Fig. 3), it can be assumed that the toxin-coding M-dsRNA genome of *H. uvarum* killer strains is encapsidated by the same protein, which therefore is likely to be provided by HuV-L. On the basis of the revised nomenclature of VLP capsids (7), we designated this new 77-kDa HuV capsid protein VL_{Hu}-P1. Incorporation of [α-^{32}P]UTP in all dsRNA-containing fractions indicated a particle-associated RNA polymerase activity (Fig. 2C) sharing characteristic properties with RNA-dependent RNA polymerases: the HuV-associated RNA polymerase activity was dependent on the presence of all of the ribonucleoside triphosphates, drastically decreased in the presence of 5 mM ethidium bromide (which interacts mainly with double-stranded nucleic acids), but unaffected by either rifampin or α-amanitin, which both are known to inhibit DNA-dependent RNA polymerases (Table 1). All of these data confirm that the HuV-associated RNA polymerase resembles an RNA-dependent RNA polymerase acting as transcriptase on the dsRNA template (2, 12).

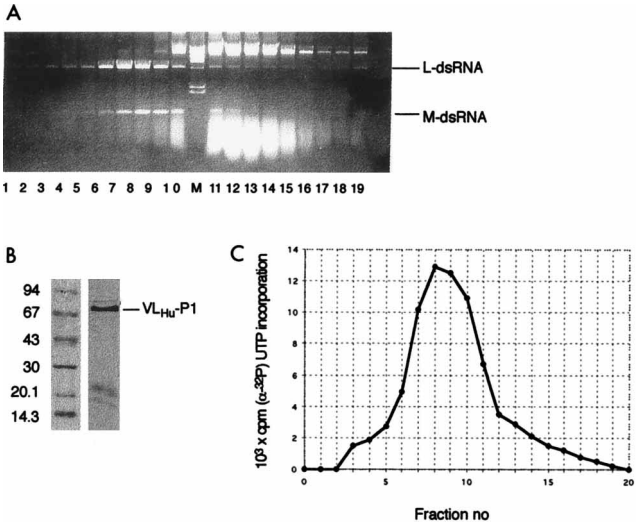

FIG. 2. Agarose gel electrophoresis of virion-associated dsRNAs (A), SDS-PAGE of the major capsid protein (B), and RNA-dependent RNA polymerase activity (C) of HuV-L and HuV-M particles from the killer yeast *H. uvarum* 470 purified by sucrose gradient equilibrium sedimentation. (A) Portions (20 μl) of each fraction were extracted with 1 volume of phenol-chloroform, directly taken from the aqueous phase, and used for agarose gel electrophoresis (M represents an *Hin*dIII-digested λ DNA marker). (B) Viral capsid proteins in a 20-μl aliquot of combined fractions 7 to 11 were precipitated by addition of 0.5 ml of trichloroacetic acid (10% [wt/vol]), washed with 1 ml of 0.1% trichloroacetic acid, dissolved in gel loading buffer (containing 2.5% SDS and 5% β-mercaptoethanol), and used for SDS-PAGE (5 to 22.5% gradient gel). (C) The virion-associated RNA polymerase activity in each fraction was measured by determining the incorporation of [α-^{32}P]UTP (400 Ci/mmol; New England Nuclear) into the trichloroacetic acid-precipitable material as previously described (12). The values to the left of panel B are molecular sizes in kilodaltons.

Electron microscopy of negatively stained portions of L- and M-dsRNA-containing sucrose gradient fractions failed to clearly identify icosahedral virions, although some preparations showed spherical particles with an estimated diameter of 30 to 40 nm (data not shown). The failure to identify spherical VLPs in *H. uvarum* is probably due to their extreme instability and to the low levels of virions present in this yeast, which were visible only in yeast cells that had been grown in YEPG medium on the nonfermentable carbon source glycerol. The purified 77-kDa HuV capsid protein (VL_{Hu}-P1) was further compared with the major capsid protein VL_{1A}-P1 of ScV-L_A and ScV-M_1 derived form the K1 killer *S. cerevisiae* K7 (Fig. 3). Digestions were performed in parallel for various times with a constant amount (100 ng) of *Staphylococcus aureus* V8 protease, and the partial hydrolysis fragments were subsequently analyzed by SDS-PAGE on an SDS–5 to 22.5% polyacrylamide gradient gel. As shown in Fig. 3, the 77-kDa capsid protein VL_{Hu}-P1 of HuV-L revealed a pattern of peptides distinctly different from that obtained from the 81-kDa VL_{1A}-P1 capsid protein of ScV-L_A/ScV-M_1 after V8 digestion, suggesting considerable sequence divergence at the level of the Cap-coding L-dsRNA genomes. To confirm this sequence divergence, Northern blot analysis was performed in which total dsRNAs derived from *H. uvarum* 469c (L) and 470 (L and M), *S. cerevisiae* K7 (L_A and M_1), 1385 (L_A, L_B, and M_2), 1686 (L_C), and MS300c (L_A, $L_{B/C}$, and M_{28}), and *Z. bailii* killer strain 412 (L, Z, and M) were subjected to electrophoresis on a denaturing 1.1% agarose-formaldehyde gel, transferred to a positively charged nylon membrane, and hybridized with a ^{32}P-labeled in vitro transcript of purified HuV-L particles derived form cured nonkiller strain *H. uvarum* 469c. Under stringent hybridization conditions (0.2 × SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% SDS, 58°C), the labeled probe strongly

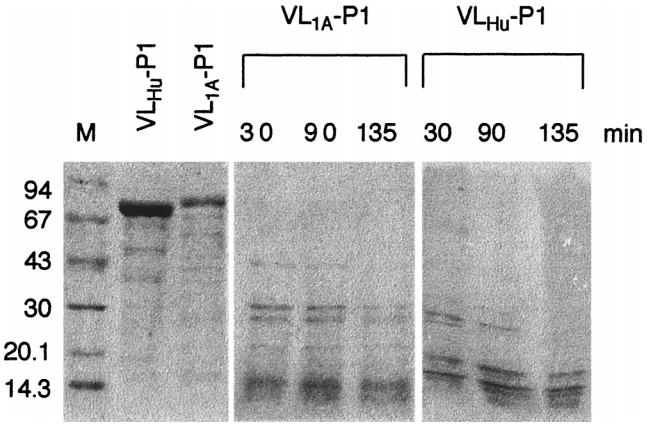

FIG. 3. Peptide maps of purified capsid proteins from HuV and ScV particles. The major capsid proteins VL_{1A}-P1 from VLPs of *S. cerevisiae* K7 and VL_{Hu}-P1 from VLPs of *H. uvarum* 469c were compared by limited *S. aureus* V8 protease hydrolysis. Digestions were performed in parallel for the indicated times, and the resulting peptides were analyzed by electrophoretic separation on an SDS–5 to 22.5% polyacrylamide gel. Sizes (in kilodaltons) of molecular mass standards (M, LMW; Pharmacia) are indicated on the left.

hybridized with denatured L-dsRNA from *H. uvarum* (Fig. 4A, lanes 6 and 7) but not at all with either L_A, L_B, or L_C of *S. cerevisiae* (lanes 1 to 4), nor with the L species from *Z. bailii* (lane 5), not even under less stringent hybridization conditions. This indicates that *H. uvarum* contains a unique L-dsRNA species that lacks detectable homology with L_A and/or $L_{B/C}$, consistent with the observed differences of the HuV-associated

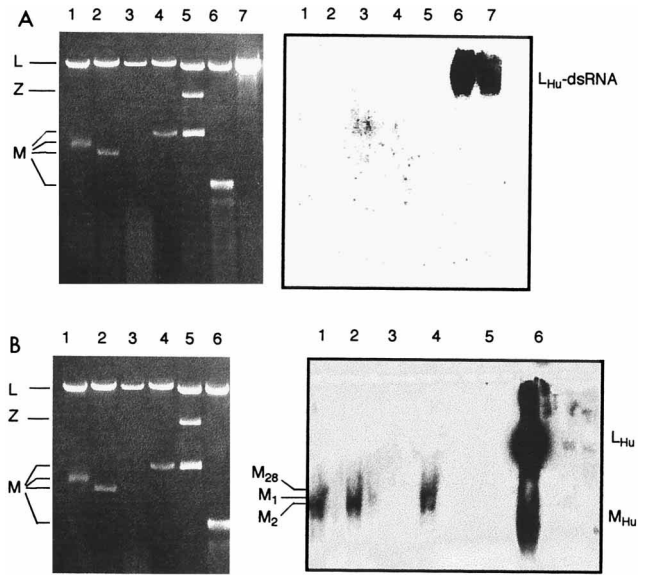

FIG. 4. Agarose gel electrophoresis and Northern blot analysis of dsRNAs from killer strains of *S. cerevisiae*, *Z. bailii*, and *H. uvarum*. Denatured dsRNAs from *S. cerevisiae* K7 (L_A and M_1, lane 1), 1385 (L_A, L_B, and M_2, lane 2), 1686 (L_C, lane 3), and MS300 (L_A, $L_{B/C}$, and M_{28}, lane 4), *Z. bailii* 412 (L_{Zb}, Z, and M_{Zb}, lane 5), and *H. uvarum* 470 (L_{Hu} and M_{Hu}) and 469c (L_{Hu}) (lanes 6 and 7, respectively) were subjected to electrophoresis on a 1.1% agarose-formaldehyde gel, transferred to a nylon membrane, and probed with a ^{32}P-labeled L single-stranded RNA in vitro transcript of purified HuV-L from *H. uvarum* 469c (A) and a mixture of ^{32}P-labeled in vitro transcripts of HuV-L and HuV-M particles derived from *H. uvarum* 470 (B). The dsRNAs present in each strain are indicated. (Note that the blot in B was overexposed to detect cross-hybridization of the HuV-M probe with denatured M-dsRNA of *S. cerevisiae* killer strains under low-stringency conditions.)

capsid protein in gel mobility and peptide maps from the L_A-encoded Cap protein of ScV-L_A (Fig. 3). Proof that this new HuV capsid protein is encoded by the L-dsRNA genome requires analysis of the in vitro translation product(s) of denatured L; such an analysis is planned for future experiments. To characterize the toxin-coding M-dsRNA of *H. uvarum*, we used a mixture of ^{32}P-labeled in vitro transcripts of HuV-L and HuV-M particles as a probe for Northern blot analysis of denatured M_1-, M_2-, and M_{28}-dsRNAs from *S. cerevisiae* killers and denatured M-dsRNA from *Z. bailii* killer strain 412 (Fig. 4B). Under moderate-stringency hybridization conditions in combination with a low-stringency wash, this probe gave a strong signal with L- and M-dsRNAs of *H. uvarum* (lane 6) and a weaker signal with the M genomes of *S. cerevisiae* (lanes 1 to 4) but no signal with the M-dsRNA of *Z. bailii* (lane 5). Although the M-dsRNAs in *S. cerevisiae* are known to lack significant sequence homology, the observed cross-hybridization of the HuV-M probe with M_1, M_2, and M_{28} might be due to the long internal poly(A)-rich tract that all *S. cerevisiae* M genomes have in common (12).

Attempts to show that HuV-L and HuV-M are sufficient to confer the *H. uvarum* killer phenotype on spheroplasts of *S. cerevisiae* (L_A) and (L-0) nonkiller strains by means of VLP transfections failed, although the same technique has been successfully used for experimental infection of *S. cerevisiae* with purified ScV and ZbV particles (13, 14). Intergeneric spheroplast fusion between *H. uvarum* killers and *S. cerevisiae* nonkillers was the only way to successfully transfer the *H. uvarum* killer phenotype (19), but even in this case, the resulting heterokaryons were unstable and lost the killer phenotype after a few generations (8). For further characterization of HuV-M and its dsRNA-encoded anti-*Candida* toxin, and for heterologous expression of the *H. uvarum* killer phenotype in *S. cerevisiae*, we intend to clone a cDNA copy of the 1.0-kb M genome.

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