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K_{28}, A Unique Double-Stranded RNA Killer Virus of *Saccharomyces cerevisiae*

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The double-stranded RNA (dsRNA) viruses of *Saccharomyces cerevisiae* consist of 4.5-kilobase-pair (kb) L species and 1.7- to 2.1-kb M species, both found in cytoplasmic viruslike particles (VLPs). The L species encode their own capsid protein, and one (L_A) has been shown to encode a putative capsid-polymerase fusion protein (cap-pol) that presumably provides VLPs with their transcriptase and replicase functions. The M_1 and M_2 dsRNAs encode the K_1 and K_2 toxins and specific immunity mechanisms. Maintenance of M_1 and M_2 is dependent on the presence of L_A, which provides capsid and cap-pol for M dsRNA maintenance. Although a number of different *S. cerevisiae* killers have been described, only K_1 and K_2 have been studied in any detail. Their secreted polypeptide toxins disrupt cytoplasmic membrane functions in sensitive yeast cells. K_{28}, named for the wine *S. cerevisiae* strain 28, appears to be unique; its toxin is unusually stable and disrupts DNA synthesis in sensitive cells. We have now demonstrated that 4.5-kb L_{28} and 2.1-kb M_{28} dsRNAs can be isolated from strain 28 in typical VLPs, that these VLPs are sufficient to confer K_{28} toxin and immunity phenotypes on transfected spheroplasts, and that the immunity of the transfectants is distinct from that of either M_1 or M_2. In vitro transcripts from the M_{28} VLPs show no cross-hybridization to denatured M_1 or M_2 dsRNAs, while L_{28} is an L_A species competent for maintenance of M_1. K_{28}, encoded by M_{28}, is thus the third unique killer system in *S. cerevisiae* to be clearly defined. It is now amenable to genetic analysis in standard laboratory strains.

Double-stranded RNA (dsRNA) viruses are the only viruses known to infect cells of all organisms, including fungi. The dsRNA viruses of the yeast *Saccharomyces cerevisiae*, called ScVs, are typical of fungal dsRNA viruses in that each dsRNA species is separately encapsidated in a cytoplasmic viruslike particle (VLP). ScVs are also typical in that their replication is not normally lethal and confers a phenotype only indirectly on their host cell. dsRNA viruses in several plant-pathogenic fungi, for example, are important modulators of pathogenicity, while ScVs are associated with killer phenomena (reviewed in references 30 and 36). Killer phenomena are widespread in fungi, although only those in *S. cerevisiae* and *Ustilago maydis* are known to have dsRNA determinants (30, 36, 37). In most instances investigated, these phenomena are associated with the secretion of polypeptide toxins which disrupt cytoplasmic membrane functions of sensitive cells of the same or related genera.

Two related families of 4.5-kilobase-pair (kb) dsRNAs, L_A and L_{BC}, coexist in many laboratory strains of *S. cerevisiae* (1, 27) and encode the capsid proteins that exclusively encapsidate their own dsRNA genomes (8, 16, 28). L_B and L_C show 50% cross-hybridization and have been distinguished in only a few strains (27). L_{BC} refers to cross-hybridizing species detected in other strains. By sequence analysis of cDNAs of L_A, it has recently been demonstrated (6, 17) that a frameshift near the C terminus of the capsid gene would also allow this dsRNA to encode a putative capsid-polymerase fusion protein (cap-pol). This RNA-dependent RNA polymerase is presumed to be responsible for the transcriptase and replicase functions found to be associated with ScV VLPs in vitro (32–34, 36). L_{BC} presumably encodes similar autonomous functions.

At least two different *S. cerevisiae* killer classes (K_1 and K_2) are recognized and are defined by lack of cross-immunity. A third, K_3, is less well characterized and is not clearly distinct from K_2 in immunity (14, 20, 30, 36, 37). The corresponding killer toxins and the poorly defined resistance functions that render killer cells immune to their own toxin are encoded by 1.7- to 2.0-kb dsRNAs (M_1, M_2, and M_3 [2, 3, 13, 18]) also present in intracellular VLPs. M_1 and M_2 differ in molecular weight and show little, if any, sequence homology. The M dsRNA-containing VLPs (ScV M_1, etc.) are defective particles that depend on ScV L_A for maintenance and replication (4, 26, 30, 36). The functions of L_A and M have been fully defined only for the K_1 killer system. M_1 dsRNA (1.8 kb) encodes a precursor of the secreted toxin that is also sufficient to confer immunity (2, 13, 18, 25). L_A encodes the capsids of both L_A and M_1 VLPs (4), so that M_1 is a satellite of L_A. Thus, K_1 killers always contain both M_1 and L_A, while cells containing only L_A are sensitive nonkillers. The virion-associated polymerase synthesizes, in vitro, full-length plus-strand copies (l_A and m_1) of L_A and M_1. These transcripts are released from the VLPs and serve as messengers for translation of K_1 preprotoxin (3), capsid protein (16), and presumably the cap-pol fusion protein. They also serve as templates for dsRNA synthesis (9, 29, 32–34, 36). Capsid protein and cap-pol presumably compose the determinants of the help of killer (HOK) phenotype associated with most L_A isolates (27). The L_A species found in natural isolates of K_1 and K_2 killer strains have been called L_{1A} and L_{2A} (8). Although considerable sequence divergence between L_{1A} and L_{2A} isolates is indicated by the limited extent of their cross-hybridization and distinct differences in their oligonucleotide and associated capsid peptide maps (8, 27), no functional differences showing specificity for maintenance of individual M dsRNAs have been demon-

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TABLE 1. Strains of *S. cerevisiae* used

Strain[a]	Genotype or description	Killer phenotype[b]	dsRNAs[c]
28	Wild type	K_{28}	L_{28}, M_{28}
28c	28 cured with cycloheximide	Sensitive[d]	L_{28}
2820	α *leu2 ura3-52 ski2-2*	K_1	L_A, L_{BC}, M_1, "S_1"[e]
2820c	2820 cured at 38°C	Sensitive	L_A, L_{BC}
2267	**a** *leu2 ura3 mkt1 ski2-11*	K_2	L_A, M_2
2267c	2267 cured with cycloheximide	Sensitive	L-0[f]
1385	α *lys1 ura1*	K_2	L_A, L_B, M_2
K7	**a** *arg9*	K_1	L_A, M_1
K12	**a** *ade2 ade5*	K_1	L_{1A}[g], M_1
K7-S1	**a** *arg9*	Sensitive	L_{1A}
GC100-14D	α *ura3-52 his3 trp1 pho3 pho5*	Sensitive	L_{1A}, L_{BC}
1938	**a** *leu2 arg9*	Sensitive	L-0
1686	α *arg1 thr1*	Sensitive	L_C
381	Wild type	Sensitive	U[h]
1406	α *ade1 ski2-2*	K_1	L_{1AE}, M_1

[a] Strains 2820, 2267, 1385, 1938, 1686, and 1406 were provided by R. Wickner.
[b] The killer phenotype listed refers to the specific immunity class determined by the M dsRNA species present. All killer strains also produce the corresponding toxin.
[c] The L_A species listed are all derived from K_1 killers.
[d] Sensitive strains lack immunity and M dsRNAs.
[e] Strain 2820 contains a 0.6-kb deleted form of M_1 resembling S_1 (see text).
[f] L-0 strains lack detectable L dsRNA.
[g] The L_As from strains GG100-14D, K12, and K7-S1 share the capsid peptide pattern representative of L_{1A}s (8).
[h] The dsRNA phenotype of strain 381, which was used to assay K_{28} toxin, is undetermined (U).

strated and variation may only reflect evolutionary drift. We continue to use this terminology only to distinguish between L_As from natural isolates of divergent killer types encoding capsids with distinctive peptide maps. L_{BC} species are present in most killer and nonkiller strains; they lack HOK activity, are incapable of maintaining M_1, and show no appreciable sequence homology to L_A (1, 27, 28).

The K_{28} killer phenotype of *S. cerevisiae* 28 is quite different from that of K_1, K_2, or K_3 strains, although its toxin shows some physical similarities to toxins of the K_2 type (20). The secreted K_{28} killer toxin is an unusually stable 16-kilodalton (kDa) glycoprotein whose optimal pH (5.8) is also unusually high (20). Unlike the K_1 and K_2 toxins, which bind to β-1,6-linked glucans in the cell walls of sensitive yeast, K_{28} toxin binds to a cell wall mannoprotein (23). Also, whereas the primary target of K_1 and K_2 toxins is the cytoplasmic membrane, K_{28} toxin appears to inhibit DNA synthesis (22).

Strain 28 was previously shown to contain small amounts of two dsRNAs of 4.5 and 2.1 kb, called L_{28} and M_{28}, respectively (38). Simultaneous loss of killer phenotype and M_{28} during curing (38) implicated M_{28} as the determinant of the K_{28} killer phenotype. By analogy with M_1, therefore, it seemed likely that maintenance of M_{28} was dependent on capsid and replicase encoded by L_{28} and that both L_{28} and M_{28} would be found in VLPs. To test this hypothesis and to confirm the role of M_{28} as the determinant of the K_{28} phenotype, we have isolated these VLPs and used them to transfer the K_{28} phenotype to standard laboratory strains of *S. cerevisiae*. Because strain 28 is a sterile, nonsporulating wine yeast that is probably polyploid or aneuploid, it is not amenable to standard genetic analysis. The isolation of stable K_{28} derivatives of normal laboratory strains will enable us to test the effects of nuclear mutations which affect the maintenance or expression of M_1 and M_2 dsRNAs (30, 36) in cells with the K_{28} phenotype. *SKI2* is representative of a class of nuclear loci which appear to negatively control M_1 and M_2 copy number by unknown mechanisms, so that *ski2* mutants are superkillers that overproduce M dsRNA (21). We have demonstrated that K_{28} derivatives of *ski2* mutants are also superkillers that overproduce K_{28} toxin and M_{28} VLPs. In vitro transcripts have been isolated from VLPs of strain 28 and several K_{28}-infected clones. These transcripts were used as probes to characterize homologies between L and M dsRNAs from K_1, K_2, and K_{28} killers. We also analyzed VLP capsid homologies by peptide mapping and demonstrated that although L_{28} and L_{1A} share some sequence homology, they are encapsidated in distinctly different capsid proteins.

MATERIALS AND METHODS

Yeast strains and media. The genotypes and dsRNA contents of all strains of *S. cerevisiae* used in this work as are listed in Table 1. YEPD, methylene blue agar (MBA), and minimal media were described previously (16, 24). Yeast strains were cured of their killer phenotype by cultivation in YEPD medium either at elevated temperature (38°C [10, 35]) or in the presence of cycloheximide (up to 1 μg/ml [19]).

Preparation and purification of VLPs. For VLP production, the appropriate yeast strain was grown in 500 ml of YEPD medium for 3 days at 30°C. The cells were harvested by centrifugation, washed with SEKS buffer (1 M sorbitol, 0.1 M EDTA, 0.1 M Na_2SO_3, 0.8 M KCl [pH 7.5]), and resuspended in 10 ml of the same buffer. The cells were treated with either 0.1% (vol/vol) Glusulase (Endo Laboratories) or Zymolyase 100T (Seikagaku Kogyo; 50 μg/g of wet cells) and incubated at 30°C for 1.5 h essentially as described by El-Sherbeini and Bostian (7). The resultant spheroplasts were washed with SEKS buffer, suspended in 15 ml of PKE buffer (30 mM Na_2HPO_4 [pH 7.6], 150 mM KCl, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) containing 1.5 mg of bentonite per ml (bentonite was prepared as described elsewhere [11]), and disrupted at 4°C by vortexing with sterile glass beads (0.45 mm in diameter) six times for 1 min each time. The resulting cell extract was cleared of cellular debris by centrifugation (10,000 × *g*, 30 min, 4°C), and the VLP-containing supernatant was fractionated on 10 to 40%

(wt/vol) sucrose gradients as previously described (4). After centrifugation in an SW28 rotor at 28,000 rpm for 2.5 h, the gradients were fractionated by collecting 2-ml fractions from the bottom of the tube, and samples of each fraction were analyzed for dsRNA content by gel electrophoresis on 1% (wt/vol) agarose gels. VLP-containing fractions were combined and centrifuged in an SW28 rotor at 19,000 rpm for 15 h. The pelleted VLPs were resuspended in PKE buffer and either used immediately for infection experiments or stored at −70°C. Virus preparations were tested for purity by analysis of capsid content, as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and concentrations were determined as micrograms of total capsid protein per ml by the micro BCA method (Pierce Chemical Co., Rockford, Ill.).

Infection of yeast spheroplasts with VLPs. Host strains for infection studies were grown to late logarithmic phase (10^7 cells per ml) in 50 ml of YEPD medium, harvested, and washed twice with 1 M sorbitol. The washed cells were suspended in 5 ml of sorbitol (1 M) that contained 42 μl of 2-mercaptoethanol, incubated at 30°C for 15 min, and again washed twice with sorbitol. Spheroplasts were prepared from cells suspended in 5 ml of sorbitol as described above. The spheroplasts were washed and resuspended in 5 ml of sorbitol (1 M) and kept on ice. The spheroplasts (10^8 cells) were transfected with 20 μg of purified K_{28} killer virus in the presence of 2 μg of an appropriate plasmid (YEp 351 [*LEU2*] or YEp 352 [*URA3*] [15]) to allow selection of transformants (7) in the presence of 10 μg of calf thymus DNA. Transfection mixtures were incubated on ice for 10 min and then at 30°C for 20 min. After dilution with 10 volumes of buffered polyethylene glycol (20% polyethylene glycol 4000, 10 mM Tris hydrochloride [pH 7.5], 10 mM $CaCl_2$) and incubation at 30°C for at least 10 min, the spheroplasts were harvested, suspended in 150 μl of rinse buffer (1 M sorbitol, 33% YEPD medium, 6.5 mM $CaCl_2$, 6 μg of uracil per ml), and mixed with 10 ml of minimal regeneration agar. Selection was made for Ura^+ or Leu^+ transformants, depending on the genotype of the host strain and plasmid employed, by plating onto uracil- or leucine-deficient minimal medium (SC-Ura or SC-Leu, respectively). After 3 to 5 days at 30°C, prototrophic transformants were picked, cloned, and screened for viral uptake by testing for their K_{28} killer phenotype.

dsRNA preparation. dsRNAs were prepared from yeast cells grown to late logarithmic phase in YEPD medium (3). A rapid procedure (1, 31) was used for initial strain characterization. This procedure isolates total RNA. rRNA and other single-stranded RNAs were subsequently removed by precipitation with 4 M LiCl (28) before gel electrophoresis. For purification of dsRNAs to allow more complete analysis, cells were harvested, washed with extraction buffer (10 mM Tris hydrochloride [pH 7.4], 10 mM EDTA) and disrupted at 4°C by vortexing with glass beads (0.45 mm in diameter) for 6 min. After addition of SDS to a final concentration of 0.5% (wt/vol), extraction with phenol, and precipitation with ethanol, dsRNAs were further purified from contaminating single-stranded RNA, rRNA, and DNA by treatment with 4 M LiCl followed by chromatography on Whatman CF 11 cellulose (11). Purified dsRNAs were subsequently analyzed by electrophoresis on 1% (wt/vol) agarose gels.

RNA polymerase assay. Virion-associated RNA polymerase activity was measured by a modification (9) of the method of Welsh et al. (34). Purified VLPs (32 μg of capsid protein) were incubated in an assay mixture (50 μl) containing 50 mM Tris hydrochloride (pH 7.4); 5 mM $MgCl_2$; 0.1 mM EDTA; 20 mM NaCl; 5 mM KCl; 10 mM 2-mercaptoethanol; 0.5 mM each ATP, GTP, and UTP; 16 μM CTP; 4 μM [^{32}P]CTP (specific activity, 400 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.), and 1.5 mg of bentonite per ml. After incubation at 30°C for 2 h, the reaction products were precipitated by the addition of 0.9 mg of *Torula* RNA (0.3 ml of 0.3% RNA in 10 mM sodium pyrophosphate) as carrier, followed by the addition of 0.5 ml of trichloroacetic acid (10%, wt/vol). The precipitate was incubated on ice for 10 min, centrifuged, and dried under a vacuum. The dried pellet was dissolved in 50 μl of 8× SSC (1× SSC is 15 mM sodium citrate at pH 7.5 plus 0.15 M NaCl) and used for Northern (RNA) blot analysis.

Northern blot hybridization. RNA-RNA hybridizations were performed essentially as described by Thiele and Leibowitz (29). Equal quantities of denatured dsRNAs were fractionated by electrophoresis through 1.1% agarose–formaldehyde gels, blotted to nylon membranes, and probed with ^{32}P-labeled in vitro transcripts of the appropriate K_{28} VLPs. Autoradiographic exposures were made with X-ray film (Kodak XAR-5; Eastman Kodak Co., Rochester, N.Y.) at −70°C with an intensifying screen (Du Pont Co., Wilmington, Del.).

Capsid proteins and peptide mapping. The major capsid proteins were extracted from purified VLPs by precipitation with 10% trichloroacetic acid essentially as described by Esteban and Wickner (9). The purified capsid proteins were redissolved in gel-loading buffer and analyzed by SDS-PAGE on 10% polyacrylamide gels with Coomassie brilliant blue for detection. Peptide mapping of the capsid proteins was performed as described previously (4). About 100 μg of the purified protein was dissolved in 70 μl of 0.125 M Tris hydrochloride buffer (containing 0.1% SDS and 1 mM EDTA [pH 6.8]) and digested with *Staphylococcus aureus* V8 protease (100 μg/ml) as described by Cleveland et al. (5). After various intervals, aliquots (10 μl) were analyzed for the generated peptide patterns by SDS-PAGE.

Assay of killing activity. Yeast strains that had been infected with the K_{28} killer virus were tested for their killer phenotype by being streaked onto MBA plates (pH 5.8) seeded with 10^5 cells of the sensitive strain 381. A zone of growth inhibition surrounding the streak indicates toxin production. To quantify toxin activity in culture supernatants, samples (0.1 ml) were pipetted into wells (10 mm in diameter) cut into the agar, and the plates were incubated for 72 h at 23°C. The diameter of the growth-free zone around the wells is proportional to the logarithm of the killer toxin activity. Toxin activities are expressed in arbitrary units; 1 U of K_{28} toxin activity corresponds to about 0.1 ng of purified killer protein (24). All values given in this paper are means of three independent assays.

Western blots. Secreted K_{28} toxin was detected by Western blot (immunoblot) analysis using polyclonal antibodies generated to purified toxin in goats (M. Schmitt and P. Pfeiffer, unpublished data). Culture supernatants of the appropriate killer clones were concentrated 30-fold by precipitation with ethanol (70% [vol/vol] final concentration) and dialysis against citrate-phosphate buffer (10 mM, pH 3.5) containing 15% glycerol. Samples equivalent to equal numbers of cells were fractionated by SDS-PAGE with a 7.5 to 17.5% polyacrylamide gel and blotted electrophoretically onto a nitrocellulose membrane. The membrane was incubated with the polyclonal antitoxin antibody, followed by incubation with a rabbit anti-goat immunoglobulin G secondary antibody (Sigma Chemical Co., St. Louis, Mo.). The antigen-antibody conjugate was detected by ^{125}I-labeled protein A binding and autoradiography.

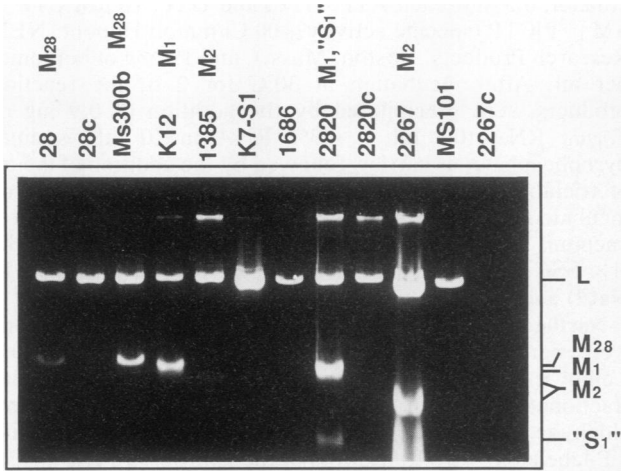

FIG. 1. dsRNA patterns of killer and cured yeast strains. dsRNAs were isolated from cultures of the indicated strains (Table 1) by the rapid procedure described in Materials and Methods. The M dsRNA species present, if any, are also indicated. Samples corresponding to approximately equal quantities of dsRNA were fractionated by agarose gel electrophoresis and stained with ethidium bromide. Strains MS300b, 2820, and 2820c carry *ski2-2* alleles. Strains 2267 (overloaded) and 2267c carry *ski2-11* alleles. Strain MS300b is a K_{28} transfectant of strain 2820c (Table 2). Strain MS101 was obtained from strain MS100, a K_{28} transfectant of strain 1938 (Table 2), by curing of M_{28} (see text).

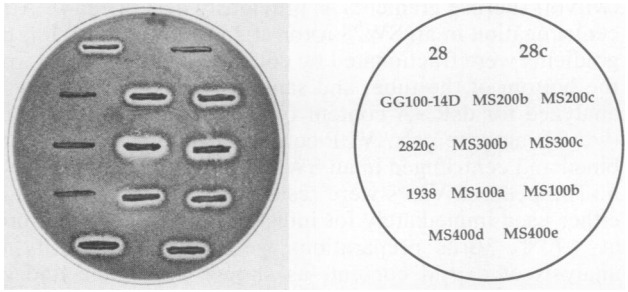

FIG. 2. Killing-zone assays of clones infected with K_{28} killer virus. The indicated transfected clones of the host strains GG100-14D, 2820c, 2267c, and 1938 were tested for their killer phenotype on MBA plates (pH 5.8) seeded with an overlay of the sensitive strain 381.

RESULTS

dsRNAs of strain 28, representative K_1 and K_2 killer strains, and cured derivatives. The genotypes of strains used in these experiments are listed in Table 1, which also lists their known dsRNA contents. All L_A species listed derive from K_1 killers. Some of the dsRNA patterns are illustrated in Fig. 1, which shows a gel with approximately equal amounts of dsRNA loaded in each lane. Relative dsRNA contents were estimated from separate gels loaded with RNA from equal numbers of cells (data not shown). All of the L dsRNAs had similar mobilities. Strain 28 (Fig. 1) contained low levels of 4.5-kb L_{28} and much lower levels of M_{28} (2.1 kb). After being cured (see below), it retained only L_{28} (strain 28c). Strain K7-S1 contained only L_A. Strain 1686 contained only L_C. Strain K12, a standard K_1 killer, contained L_A, L_{BC}, and M_1. Strain 1385, a K_2 killer, contained L_A, L_B, and M_2. M_2 in such K_2 killers is typically present at levels significantly lower than the M_1 in K_1 killers. The dsRNAs of the K_2 *ski2-11* strain 2267 were overloaded, showing a streak of residual rRNA at the bottom. Nevertheless, it can be seen that this strain, which contained a high level of L_A, also had a high ratio of M_2 to L_A compared with that of strain 1385. The difference in mobility of the M_2 isolates from these two strains illustrates previously observed variation (36). The dsRNA isolate shown from the K_1 *ski2-2* strain 2820 had a high content of L_A, L_B, and M_1. However, the recovery of M_1 from this strain was variable and usually low, even lower than that from strain K12; this strain is a stable but weak K_1 killer. This is probably because strain 2820 also contains a 0.6-kb dsRNA species labeled "S_1" in Fig. 1. "S_1" is apparently a deletion form of M_1, since it reacts strongly with an m_1 probe in Northern blots (data not shown); it is very similar in size to the suppressive deletion form of M_1 called S_1 (30), although such deletion derivatives normally suppress M_1 maintenance completely. The stable maintenance of M_1 and K_1 phenotypes in the presence of a putative suppressive derivative is unusual and may reflect an aspect of the *ski2* phenotype or the phenotype "S_1" itself.

To test the effects of the different *ski2* alleles in strains 2820 and 2267 on maintenance levels of M_{28}, it was first necessary to eliminate the resident M dsRNAs of these strains. Two procedures have frequently been used to cure killer strains of *S. cerevisiae*, causing the loss of the killer phenotype and the corresponding M dsRNA. The first is growth at elevated temperatures, which is effective in causing the loss of both M_1 and L_A (but not M_2 [10, 35]), and the second is growth in the presence of subinhibitory concentrations of cycloheximide or 5-fluorouracil (19), which are effective in curing both M_1 and M_2 dsRNAs. Use of the latter procedure resulted in the loss of both M_2 and L_A from the K_2 *ski2-11* strain 2267, producing the dsRNA-free nonkiller strain 2267c (Table 1; Fig. 1). Growth at 38°C resulted in curing of both M_1 and the S_1-like species from the K_1 *ski2-2* strain 2820, producing the nonkiller strain 2820c (Table 1; Fig. 1). This cured *ski2-2* strain retained an elevated L_A content. Use of the cycloheximide curing procedure also resulted in curing of strain 28, producing the nonkiller strain 28c, which retained only L_{28}, though at increased levels (Fig. 1). The killer phenotypes of strains 28 and 28c are illustrated in Fig. 2, and their relative dsRNA contents are more clearly illustrated in the purified samples shown in Fig. 3 (lanes 1 and 2).

Isolation of VLPs from strain 28 and transfection of the K_{28} killer phenotype. Use of procedures previously described for the isolation of VLPs from K_1 strains (4) resulted in the isolation of VLPs with very similar properties from strain 28. Analysis of sucrose gradient fractions for dsRNA content showed the presence of both L_{28} and M_{28} (data not shown). The sedimentation velocities of VLPs from strains 28 and K12 were identical. Yields from strain 28 were lower by a factor of 5, proportional to the relative dsRNA contents of the whole cells (Fig. 1). As described below, analysis of the protein contents of the VLP fractions from strain 28 by SDS-PAGE showed the presence of a single major protein of 80 kDa in amounts, relative to the dsRNA content, expected for a VLP capsid protein. This protein typically constituted >80% of the total protein in these preparations, and the total protein content was used to estimate VLP concentrations. We attempted to fractionate L_{28} and M_{28} VLPs on CsCl gradients (9), without success. Transfection experiments, therefore, employed the mixture of L_{28} and M_{28} VLPs. The

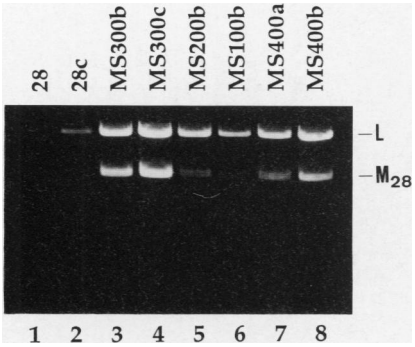

FIG. 3. Agarose gel electrophoresis of dsRNAs from strain 28 and VLP_{28}-infected host strains. dsRNAs were extracted and purified as described in Materials and Methods. Samples corresponding to equal numbers of cells of the indicated strains were fractionated by electrophoresis on 1% agarose gels and stained with ethidium bromide.

donors, recipients, and resultant transfectant clones are listed in Table 2.

The sensitive strain 1938 was free of detectable dsRNAs (Table 1). As illustrated in Table 3, spheroplasts of this strain, selected by cotransformation with the *LEU2* plasmid YEp351, could be transfected by VLPs from strain 28 with 66% efficiency by using minor modifications of the procedure described by El-Sherbeini and Bostian (7). This relatively high efficiency is similar to that observed for transfection of the same strain by VLPs from the K_1 strain K12 (data not shown). The representative transfected strains MS100a and MS100b showed characteristic K_{28} killing zones on sensitive strain 381 (Fig. 2). These zones were detectably larger than the rather weak zones produced by strain 28 itself (Fig. 2). Infected clones that had been selected for further analysis were cultivated in YEPD medium for 50 generations and showed a stable K_{28} killer phenotype, indicating stable inheritance of the K_{28} killer virus. By using the agar diffusion assay (Table 4) for a more quantitative comparison of the killer toxin activities, both MS100 clones were shown to produce about four times more toxin than strain 28 did. As shown in Fig. 3 (lane 6), strain MS100b contains both L_{28} and M_{28} in amounts four- to fivefold higher than those in strain 28. The role of L_{28} and M_{28} in determining the K_{28} killer phenotype is therefore confirmed.

The sensitive strain GG100-14D, which contains both L_A and L_{BC} (Table 1), has frequently been employed both for transfection with M_1 VLPs and for transformation by M_1 cDNAs (7, 13). It was transfected by strain 28 VLPs with low efficiency (Table 3), but the three transfectant clones studied, MS200a, MS200b, and MS200c, behaved indistinguishably from MS100a and MS100b: they produced essentially identical K_{28} killing zones (Fig. 2) and quantities of K_{28} toxin (Table 4) and contained very similar quantities of M_{28} dsRNA (Fig. 3, lane 5). These transfectants were also completely stable on prolonged cultivation. As shown below, they apparently retain low and variable levels of L_{28}.

TABLE 3. Efficiency of VLP transfections[a]

VLP donor strain	No. of killer transformants obtained with host strain[a]:			
	GG100-14D	2820c	2267c	1938
28	3	1	ND	33
MS200a	ND	ND	50	50
MS200b	ND	17	50	50
MS200c	ND	35	50	50

[a] M_{28} VLP infections were performed by cotransformation with YEp351 (*LEU2*) or YEp352 (*URA3*) (15), depending on the genotype of the host strain (Table 1). In each experiment, 50 plasmid transformants were tested. The resultant transfectant clones are listed in Table 2.
[b] ND, Not determined.

***HOK* function of L_{28}.** The HOK function of L_A dsRNAs is required for maintenance of both M_1 and M_2 dsRNAs in Ski^+ hosts (21). Maintenance of M_{28} by L_A in MS200 strains and by L_{28} in MS100 strains implied that L_{28} may also have HOK function. This can be determined by a cross to the *ski2* K_1 killer strain 1406 (Table 1). This strain contains L_{AE}, a variant of L_A which is defective in HOK function; maintenance of M_1 depends on the recessive *ski2* phenotype. A diploid resulting from a cross to a Ski^+ strain will lose M_1 unless HOK is introduced from this Ski^+ strain (26). Strain MS100a was cured of M_{28} by growth on cycloheximide, producing strain MS101, which retained only L_{28} (Fig. 1). The diploid produced by crossing this strain with strain 1406 was a stable K_1 killer, demonstrating that L_{28} has HOK function.

Transfection of *ski2* host cells. Spheroplasts of the dsRNA-free *ski2-11* strain 2267c were transfected with 100% efficiency by fresh VLPs from strain MS200a, MS200b, or MS200c (Table 3), producing strains MS400a, MS400b, and MS400c (Table 2). Strain 2820c (*ski2-2*), which contains L_A and L_{BC}, was transfected by MS200 VLPs with lower efficiency (average, 52%; Table 3), producing strains MS300b and MS300c (Table 2). These *ski2-2* transfectants produced unusually large killing zones on lawns of strain 381 (Fig. 2), while the *ski2-11* transfectants produced zones of intermediate size (Fig. 2). Transfection of strain 2820c by strain 28 VLPs was very inefficient, producing the single strain MS300a (Tables 2 and 3). Nevertheless, this strain, like all of the other transfectants, was completely stable on prolonged cultivation. Like the other *ski2-2* strains, it showed unusually large killing zones on lawns of strain 381

TABLE 2. dsRNA contents of VLP transfectant strains[a]

Donor strain	dsRNAs	Recipient strain	dsRNAs	Transfectant strain(s)	dsRNAs
28	L_{28}, M_{28}	1938	None	MS100a, MS100b	L_{28}, M_{28}
28	L_{28}, M_{28}	GG100-14D	L_A, L_{BC}	MS200a, MS200b, MS200c	L_A, L_{BC}, $(L_{28})^b$, M_{28}
28	L_{28}, M_{28}	2820c	L_A, L_{BC}	MS300a	L_A, L_{BC}, (L_{28}), M_{28}
MS200c	L_{28}, M_{28}, L_A, L_{BC}	2820c	L_A, L_{BC}	MS300b, MS300c	L_A, L_{BC}, (L_{28}), M_{28}
MS200c	L_{28}, M_{28}, L_A, L_{BC}	2267c	None	MS400a, MS400b, MS400c	L_A, L_{BC}, (L_{28}), M_{28}

[a] Spheroplasts of the indicated recipient strains were transfected with VLPs from the indicated donor strains by cotransformation with the appropriate plasmid (see Materials and Methods).
[b] L_{28} could be detected clearly only in the MS100 transfectants (see text). L_{28} may have been present in other transfectants [indicated as (L_{28})], although capsid protein patterns suggested that quantities were low (see text).

TABLE 4. K_{28} toxin activities[a]

S. cerevisiae strain	Inhibition zone diam (mm)	K_{28} toxin activity (10^3 U/ml)[b]	Relative K_{28} toxin activity
28	9	1.3	1
GG100-14D	0	0	0
MS200b	12	5.6	4
MS200c	12	5.6	4
2820c	0	0	0
MS300b	16	45	35
MS300c	16	45	35
2267c	0	0	0
MS400d	14	16	12
MS400e	14	16	12
1938	0	0	0
MS100a	12	5.6	4
MS100b	12	5.6	4
MS101	0	0	0

[a] K_{28} toxin activities were determined in supernatants of cultures of killer strain 28 and of the M_{28} VLP-containing transfectant strains indicated.
[b] 10^4 U of K_{28} toxin is equivalent to approximately 1 μg of protein.

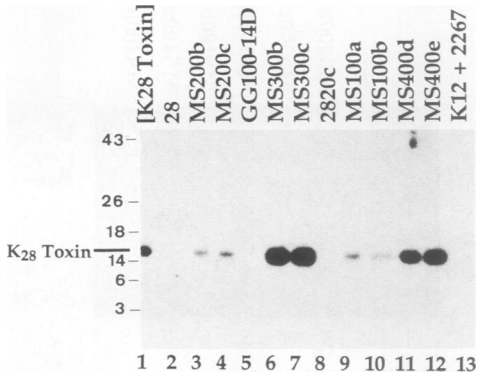

FIG. 4. Western blot analysis of K_{28} toxin in concentrated supernatants obtained from cultures of VLP_{28}-infected clones. Culture supernatants of the indicated strains were concentrated, fractionated by SDS-PAGE, blotted onto a nitrocellulose membrane, and detected with antibody to K_{28} toxin, as described in Materials and Methods. An autoradiograph with bound ^{125}I-protein A is shown. Lane 1 contains 100 ng of purified K_{28} toxin. Molecular mass markers are indicated on the left in kilodaltons.

(data not shown). To demonstrate the dependency of the high toxin production by the MS300 strains on the *ski2-2* mutation, strain MS300a was crossed with the Ski$^+$ strain 1938. The diploid produced toxin at the moderate level shown by the MS100 transfectants of strain 1938. On sporulation, each tetrad tested showed 2:2 segregation of this wild-type toxin level from the high level seen in the MS300 parent (data not shown). The MS300 strains produced about 36 times more toxin and the MS400 strains produced about 12 times more toxin than strain 28 (Table 4). As for M_1 dsRNA, the *ski2*-induced superkiller phenotypes of MS400 and MS300 strains correlated with the presence of comparable increases in the contents of both L and M_{28} dsRNAs (Fig. 1, lane MS300b; Fig. 3, lanes 3, 4, 7, and 8).

Western blot analysis of secreted K_{28} toxin. Culture supernatants of different VLP_{28}-infected clones were concentrated 30-fold and analyzed by SDS-PAGE and Western blotting with polyclonal antibodies to K_{28} toxin. The secreted K_{28} toxin was barely detectable in supernatants from cultures of the wild-type killer strain 28, whereas supernatants from cultures of all M_{28} VLP-infected clones gave considerably stronger signals (Fig. 4). The infected clones from the L-0 host 1938 (lanes 9 and 10) and from GG100-14D (lanes 3 and 4) clearly secreted significantly higher amounts of toxin than the wild-type strain did. Two clones of the infected *ski2-2* host 2820c (lanes 6 and 7) secreted approximately 35 to 40 times more killer toxin than the original VLP donor did. The infected *ski2-11* host 2267c produced an intermediate level of toxin (lanes 11 and 12). Thus, signals on this Western blot correlated well with assays of toxin activity (Table 4) and M_{28} content (Fig. 3). Lane 13 of the Western blot (Fig. 4) contained a mixture of K_1 and K_2 killer toxins from strains K12 and 2267, respectively. Neither demonstrated any cross-reactivity with the anti-K_{28} toxin antibody.

Killer phenotypes of strain 28 and K_{28} transfectants. Analysis of the killer phenotype of strain 28 is difficult because of its weak toxin activity. Nevertheless, it was previously shown (20) that strain 28 yielded killing zones when plated on both K_1 and K_2 killer strains. Killing of strain 28, however, could be clearly demonstrated only with K_1 killers. The established toxin sensitivities of the transfection recipients and higher toxin production by the transfectant clones allowed their K_{28} killer phenotypes to be more readily observed. All killed both K_1 and K_2 strains, and killing of all K_{28} strains by both K_1 and K_2 toxins was demonstrable (Table 5). Thus, these transfectants portray the characteristic K_{28} killer phenotype, whose distinctive immunity pattern is clarified.

Characterization of L_{28} and M_{28} dsRNAs by RNA blot hybridization. To further characterize the L_{28} dsRNA from the wild type killer strain 28, we used purified L_{28} VLPs from the cured strain 28c as a source of RNA transcriptase activity to synthesize l_{28} single-stranded RNA transcripts in vitro (32–34). This labeled l_{28} transcript was used as a probe for hybridization with denatured L_A, L_B, L_C, and L_{28} dsRNAs. The resulting Northern blot (Fig. 5) showed that the l_{28} transcript hybridized strongly with denatured L_{28} (lane 4), weakly with L_A (lane 1), and weakly with a mixture of L_A and L_B (lane 2) but not at all with L_C (lane 3). This

TABLE 5. Killer and sensitivity phenotypes[a]

Toxin source	Killer or sensitivity phenotype[b] of strain:						
	381	S6	GG100-14D	28 (K_{28})	MS100a (K_{28})	K12 (K_1)	1385 (K_2)
28 (K_{28})	+	−	−	−	−	+	+
MS100a (K_{28})	+	−	−	−	−	++	++
K12 (K_1)	+	+	+	++	++	−	++
1385 (K_2)	+	+	+	(+)	+	+	−

[a] The strain to be tested for killing (toxin source) was streaked onto a lawn of the strain to be tested for sensitivity on MBA plates at pH 5.8 (K_{28} toxin), 4.7 (for K_1 toxin), or 4.3 (for K_2 toxin). All plates were incubated at 23°C, and zones of inhibition were recorded after 2 to 3 days. The killer phenotypes of strains tested and used as toxin sources are indicated in parentheses.
[b] Phenotype designations: +, lawn was killed; −, lawn was resistant; ++, large killing zone; (+), killing could not be clearly determined.

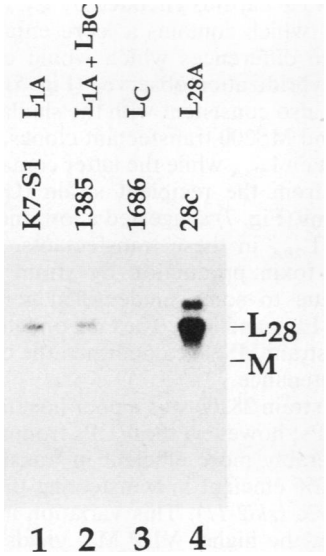

FIG. 5. Northern blot analysis of L dsRNAs. Denatured dsRNAs from the indicated strains were subjected to electrophoresis on a 1.1% agarose–formaldehyde gel, transferred to a nylon membrane, and hybridized with a ^{32}P-labeled in vitro transcript of L_{28} VLPs from strain 28c. The L dsRNAs present in each strain are also indicated.

indicates that strain 28 lacks L_{BC} and that L_{28} dsRNA shares partial homology with L_A, consistent with its HOK function. It is, therefore, analogous to L_{1A} and L_{2A} in natural isolates of K_1 and K_2 killers and will henceforth be designated L_{28A}.

To characterize M_{28} dsRNA, we used in vitro transcripts from VLPs of the infected clone MS300c as a probe for Northern blot analysis of denatured M_1 and M_2 dsRNAs (Fig. 6). By using this probe, which should contain transcripts of L_A, L_{BC}, M_{28}, and possibly also L_{28A} (Table 2), a

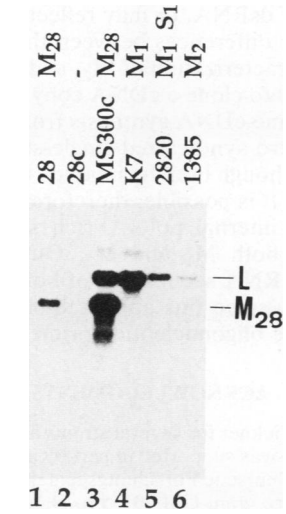

FIG. 6. Northern blot analysis of M dsRNAs. Denatured total dsRNAs from the indicated strains were fractionated as described in the legend to Fig. 5 and detected with the ^{32}P-labeled in vitro transcripts obtained from VLPs of the VLP M_{28}-transfected clone MS300c. The M dsRNAs present in each strain are indicated. The probe apparently contained transcripts of L_A, L_{BC}, and M_{28} (see text).

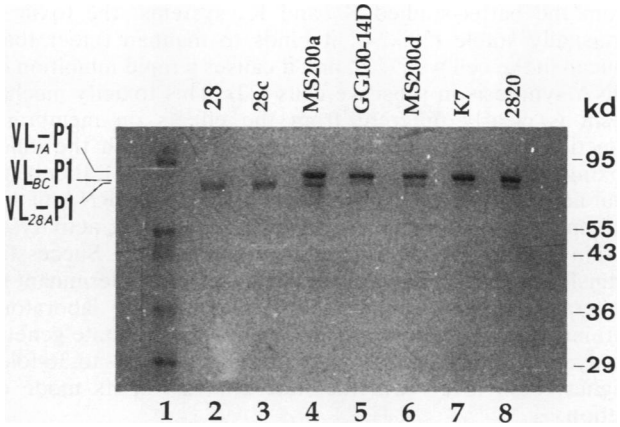

FIG. 7. SDS-PAGE of purified capsid proteins. Capsid proteins of purified VLPs isolated from the indicated strains were fractionated by SDS-PAGE and detected by staining with Coomassie brilliant blue. Lane 1 contains marker proteins of the sizes indicated on the right in kilodaltons (kd).

very strong signal was obtained with the L and M species of transfected clone MS300c (lane 3) and with the M_{28} species of strain 28, but no signal could be detected with either M_1 (1.9 kb; lanes 4 and 5), M_2 (1.7 kb; lane 6), or the S_1-like species (0.6 kb; lane 5). This probe also clearly detected L_A and L_{BC} (lanes 3, 4, and 5) but not L_{28A} (lanes 1 and 2). Presumably L_{28A} hybridizes too weakly to L_A to be detectable with this mixed probe. Strain MS300c, therefore, apparently failed to retain L_{28A}. This again illustrates the competence of L_A for maintenance of M_{28} and shows that M_{28} in the absence of detectable L_{28A} is sufficient for expression of the K_{28} phenotype.

Encapsidation of M_{28} and L_{28A} dsRNAs. Electrophoretic analysis (SDS-PAGE) of the capsid protein from L_{28A} VLPs (isolated from strain 28c) or a mixture of L_{28A} and M_{28} VLPs (isolated from strain 28) revealed a single 80-kDa polypeptide in both particles (Fig. 7, lanes 2 and 3) that could clearly be differentiated from the capsid proteins of L_{1A} (VL_{1A}-P1, 88 kDa; lane 7) and less clearly from the weaker band of VL_{BC}-P1 (82 kDa; the minor component in lanes 5 and 8), which is derived from L_{BC} (8). On the basis of the revised nomenclature for VLP capsids (8), we designated this new 80-kDa capsid protein of the K_{28} killer virus VL_{28A}-P1. It was visible as a minor component in VLPs from transfected clone MS200a (lane 4), but may be absent in the sister clone MS200d (lane 6). Digestion of VL_{28A}-P1 with *S. aureus* V8 protease, followed by SDS-PAGE of the products of partial hydrolysis, revealed a pattern of peptides distinctly different from that obtained after protease digestion of the 88-kDa VL_{1A}-P1 or the 82-kDa VL_{BC}-P1 (data not shown). Peptide mapping of VL_{28A}-P1 from L_{28A} VLPs and from fractions highly enriched for M_{28} VLPs from strain MS100a resulted in identical patterns, indicating that both dsRNAs are encapsidated in the same 80-kDa capsid protein (data not shown). Proof that this new capsid protein is encoded by L_{28A} dsRNA will require analysis of the in vitro translation products of denatured L_{28A}; such analysis is planned for future experiments.

DISCUSSION

The K_{28} killer phenotype of *S. cerevisiae* 28 is particularly interesting because of the characteristics that distinguish it

from the better-studied K_1 and K_2 systems: the toxin is unusually stable (20, 24), it binds to mannan rather than glucan in the cell wall (23), and it causes a rapid inhibition of DNA synthesis in sensitive cells (22). This toxicity mechanism is clearly different from the effects on membrane function seen with K_1 and K_2 toxins and with the killer toxins of many unrelated yeast genera (30, 37, 38). Biochemical and genetic analysis of the toxin and its dsRNA determinant has been hampered by the weak killing activity of strain 28 and its recalcitrance to genetic analysis. Successful transfer of the K_{28} phenotype and its dsRNA determinant to several derivatives of standard *S. cerevisiae* laboratory strains by VLP transfection will obviously facilitate genetic analysis. The production of strains producing 4- to 36-fold-higher toxin levels will facilitate analysis of its mode of action.

Extracellular transmission of the killer phenotype by VLPs was first shown by El-Sherbeini and Bostian (7), who demonstrated transfection of K_1 and K_2 killer viruses into *S. cerevisiae* cells rendered competent by spheroplasting or by treatment within lithium acetate. With strain GG100-14D, efficiency with the K_1 virus was high, while efficiency with the K_2 virus was only about 4%. Using a very similar procedure, we obtained efficient transfection of the L-0 strain 1938 by the mixture of L_{28A} and M_{28} VLPs from strain 28 but an efficiency for strain GG100-14D similar to that previously observed for K_2 virus (7). The reasons for the apparently stable variation in transfection competence of different host strains for different ScV M species are unknown. They may reflect the HOK efficiency of the resident L_A species, the efficiency with which different m single-stranded RNA transcripts become encapsidated, initial selection against toxin expression, or other aspects of the complex host-killer virus interaction. In spite of this variation, all of the K_{28} transfectants of these Ski^+ strains were stable, all produced four times more K_{28} toxin than the strain 28 VLP donor did, and all contained correspondingly higher levels of M_{28} dsRNA. The killing and immunity characteristics of these strains were more easily demonstrable than that of the original strain, 28, and these strains clearly reproduced the full K_{28} phenotype (Table 5). We were also able, for the first time, to show sensitivity of K_{28} strains to K_2 toxin, completing differentiation of the K_{28} and K_2 phenotypes (Table 5). Western blotting and analyses of dsRNA patterns confirmed that the higher K_{28} toxin activities of transfected clones resulted from higher levels of toxin secretion and correlated with higher M_{28} contents (Fig. 3 and 4). Thus, the strain 28 VLPs are sufficient to transfer the K_{28} phenotype. Since loss of this phenotype had previously been correlated with loss of M_{28} (but not of L_{28A}) during curing (38), as we have confirmed (Fig. 1, 2, and 3), it is highly probable that the K_{28} phenotype is encoded by M_{28}, just as K_1 toxin and immunity are encoded by M_1 (3, 13, 18). This is consistent with the maintenance of the K_{28} phenotype in strain MS300c, which lacks detectable L_{28A} (Fig. 6). The differences between M_{28} and M_1 or M_2 were emphasized by the lack of detectable cross-hybridization in Northern blots (Fig. 6).

The L_{28} derived from strain 28c failed to cross-hybridize with L_C from strain 1686 and hence lacks detectable L_{BC} dsRNA. Since L_{28} provides HOK activity to M_1 dsRNA, it must produce capsid protein and cap-pol capable of maintaining M_1 dsRNA and is therefore now called L_{28A}. The HOK activity of L_{28A} is consistent with the sequence similarity shown by hybridization of L_{28A} to L_A (Fig. 5), although clear differences in gel mobility (Fig. 7) and peptide maps of the VLP capsids encoded by L_{28A} and the L_A from strain K7-S1 (which contains a representative L_{1A} [8]) suggest sequence differences which would correlate with the weak RNA hybridization observed (Fig. 5). The HOK activity of L_{28A} is also consistent with the similar M_{28} contents of the MS100 and MS200 transfectant clones, since the former can only contain L_{28A} while the latter contain predominantly L_A derived from the recipient strain (GG100-14D). VLP capsid patterns (Fig. 7) suggested a low and variable level of retention of L_{28A} in these transfectants. The low dsRNA content and toxin production by strain 28 is, therefore, apparently due to some unidentified genetic determinant distinct from L_{28A} and M_{28}. The lack of detectable L_{28A} from transfectant strain MS300c confirmed the competence of L_A for M_{28} maintenance.

The *ski2-2* strain 2820c was a poor host for transfection by strain 28 VLPs; however, the VLPs from the MS200 clones were considerably more efficient in transfecting this strain and were 100% efficient in transfecting the two L-0 strains 1938 and 2267c (*ski2-11*). This variation in efficiency might merely reflect the higher VLP M_{28} yields from the MS200 clones or some advantage of L_A over L_{28A} in HOK function. No inhibitory activity was detectable in strain 28 VLP preparations when they were mixed with MS200c preparations and used to transfect strain 2820c (data not shown). The products of at least six chromosomal *SKI* genes are known to repress dsRNA replication in *S. cerevisiae* and have been referred to as the yeast antiviral system (1). The *ski* mutants show an easily detectable K_1 or K_2 superkiller phenotype, and in all except *ski-1* mutants, this is caused by an increased copy number of the corresponding M dsRNA. All infected clones of both of the *ski2* alleles transfected with K_{28} killer virus showed much higher amounts of M_{28} dsRNA than the Ski^+ MS100 and MS200 clones did, and Western blot analysis of concentrated culture supernatants probed with antitoxin antibodies indicated that the VLP_{28}-infected *ski* mutants secreted up to 40 times more killer K_{28} toxin than the original virus donor strain 28 did. The less marked superkiller phenotype of the 2267c transfectants may be characteristic of the *ski2-11* allele, which may cause less overproduction of dsRNA, or may reflect other cytoplasmic or nuclear genetic differences between these strains.

For further characterization of M_{28} and comparison to M_1 and M_2, we intend to clone a cDNA copy. $Oligo(dT)_{15}$ could not be used to prime cDNA synthesis from either denatured M_{28} or from in vitro-synthesized single-stranded transcripts from ScV M_{28}, although both techniques worked well for M_1 (data not shown). It is possible, therefore, that M_{28} does not contain the long internal poly(A)-rich sequences that are characteristic of both M_1 and M_2. Our present strategy focuses on direct RNA sequencing of both ends of M_{28} and its m_{28} transcript, since this approach should enable us to synthesize specific oligonucleotide primers.

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