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Double-Stranded Ribonucleic Acid Killer Systems in Yeasts

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* Corresponding author.
INTRODUCTION

Killer yeasts secrete polypeptide toxins which kill sensitive cells of their own species and frequently those of other species and genera of yeast (Fig. 1). This phenomenon has been observed in many of the yeast genera investigated, but its prevalence varies widely among natural isolates (111, 116, 130, 170). Although a majority of natural isolates apparently lack both toxin production and the associated specific toxin immunity, this must be interpreted cautiously, since assays for both toxin production and immunity require choice of an appropriate sensitive strain and determination of optimal conditions for toxin activity. Nevertheless, it seems that, whereas toxin production is probably important in yeast ecology, the ecological advantage conferred by toxin production in most of their determinants are unstable, or such production is associated with deleterious effects. Although this uncertainty reflects a lack of data on the persistence of killers in the wild, no deleterious effects are known, and their genetic determinants are stable in the laboratory. Low prevalence may indicate that yeast killers are inefficient assassins. Their determinants might often be maintained as parasitic passengers rather than as selected, offensive machinery essential to survival in intergeneric warfare.

In the few instances where killer determinants have been identified, they are cytoplasmically inherited (7, 127), double-stranded (ds) RNA (4, 6, 95, 124) or DNA (53) plasmids which encode toxin precursors (11, 51) and which are dependent on a similar plasmid for maintenance. The basis of this dependency is known only for the Saccharomyces cerevisiae killer plasmid M-dsRNAs. These dsRNAs are found only in cells also containing an additional dsRNA species called L. Both types of dsRNA exist in cytoplasmic virus-like particles (VLPs) (22, 58), and both dsRNAs require a protein encoded by the L-dsRNA (62) for encapsidation (13, 55). Killers are a minority among natural S. cerevisiae isolates, although most strains contain L-dsRNAs. Such strains are sensitive nonkillers. The helper L-dsRNA plasmids, unlike the killer determinants, have no known dependence on other plasmids, confer no known phenotype on the host cell apart from their effects on M-dsRNA maintenance, and, when alone, appear to exist as truly parasitic entities.

Clear parallels exist between the secretion of yeast killer toxins and the production of bacteriocins. Production of both is associated with specific immunity, and toxicity is restricted to similar cell types. Killer yeast strains might, therefore, more properly be called mycocinogenic strains and their toxins, mycocins. However, the original nomenclature is now well established and, as pointed out by Bussey (23), has helped to bring an esoteric aspect of yeast molecular biology to the attention of a broad range of scientists.

In this review we will focus on the molecular biology of the most intensively investigated killer system, the M₁-dsRNA-determined K₁ killer system of S. cerevisiae. We hope to demonstrate the relevance of this system to many of the vital issues in yeast and general eucaryotic molecular biology.

It should be emphasized that cytoplasmic dsRNA species have been shown to determine the killer phenotype only in Saccharomyces sp. and Ustilago (73) sp. strains. Only in one other species, Kluyveromyces lactis (51), has cytoplasmic inheritance of the phenotype and the nature of its determinant (one of two linear dsDNA species [53]) been clearly demonstrated. Brief summaries of recent information on the K. lactis and Ustilago maydis systems are included, because of their comparative value and intrinsic interest.

KILLER dsRNAs, MYCOVIRUSES OR ENCAPSIDATED PLASMIDS?

The existence of cytoplasmic VLPs containing dsRNAs was well established in the mycoviruses of filamentous fungi (85, 86) well before the discovery in 1963 (M. Makower and E.A. van, Proc. Int. Congr. Genet. XI 1:202, 1963) of the killer phenotype in laboratory strains of S. cerevisiae. These mycovirus VLPs were found to contain a limited number of capsid proteins and an associated dsRNA transcriptase activity (21) and to consist of a population of individually encapsidated, apparently interdependent dsRNA species. These properties led to their designation as fungal "virus-like particles" (VLPs).

In considering interactions between the multiple dsRNA components of mycoviruses, it should be remembered that, whereas the Reoviridae and other dsRNA viruses of higher eucaryotic cell types (reviewed in reference 36) contain a single copy of each of their component dsRNA segments in each of their virus particles, ensuring equimolar representation, mycovirus dsRNAs are under no such numerical restraint, so that differential demand for gene products can be reflected in the relative copy numbers of the relevant dsRNAs.

When the yeast killer phenotype was shown to be associated with particular dsRNA species (M₁, etc.) (6, 95, 131, 142), which were later shown to be present in cytoplasmic VLPs (22, 58), it became clear that the killer phenotype was an attribute of a yeast mycovirus which, following the established nomenclature, was called the S. cerevisiae "Virus," ScV (62). The VLPs containing various M₁ and L-dsRNA species can, therefore, be designated ScV-M₁, ScV-L₁α, etc., since reciprocal interdependence between these species apparently does not exist (see below, "Role of L-dsRNA in VLP Capsid Production and M₁-dsRNA Maintenance").

No mycovirus, including the yeast killer system, has been shown to be capable of extracellular transmission. One recent report of transmission of the dsRNAs of Helminthosporium victoria would be an exception, if confirmed (see below, "Effects of dsRNA Mycoviruses in Pathogenic Fungi") (48). On the contrary, mycoviruses and the killer dsRNAs are stably maintained, at relatively constant copy number, by vertical transmission. Transmission occurs only by cytoplasmic mixing during budding, mating, or other natural or artificial forms of cell fusion. The S. cerevisiae dsRNA killer determinants may thus be regarded as virus-like plasmids. They clearly possess properties relevant to both the truly infectious dsRNA viruses (36) and to plasmids of eucaryotic cells. Their genetic analysis resembles that of multicopy cytoplasmic plasmids, and for the best-studied species (M₁-, M₂-, and L₁α-dsRNAs), their dependence on multiple nuclear genetic loci clearly demonstrates their lack...
of autonomy. The relatively stable copy number of these dsRNAs implies some degree of coupling of VLP replication to the yeast cell cycle. Partition of VLPs during mitotic and meiotic cell division may also be controlled (5).

The S. cerevisiae killer system has become the best understood of the mycoviruses, primarily because of the facility of genetic and biochemical analyses in its host and the simplicity of scoring for killer toxin production and toxin immunity. Genetic analysis of this system has been extensive (reviewed in references 152, 156, 158, 160) and illustrates the complexity of nuclear-cytoplasmic and plasmid-plasmid interactions involved in maintenance and expression of the killer M-dsRNAs. Relationships observed between different ScVs or dsRNA species include dependency, indifferece, and incompatibility or exclusion. The killer system is now a prime model for the investigation of such phenomena in eucaryotic microorganisms. Genetic analysis of this system continues to reveal levels of complexity that outstrip biochemical analysis of their implications, although recent advances have clarified many previously obscure issues. Description of these findings is the major rationale for writing a new review of this subject.

Maturation of preprotoxin, the product of the M₁-dsRNA toxin gene, is a useful model for the complex protein secretion pathway in yeast (see "Structure of the M₁-dsRNA Preprotoxin Gene and Steps in Toxin Maturation"). At the same time, recent data on the mode of action of the toxin suggest that toxin and immunity determinants will be useful
probes of yeast cytoplasmic membrane function and models for transmembrane ionic pore formation. It also seems likely that analysis of MAK and SKI gene functions (see "Nuclear Mutations Affecting M-dsRNA Maintenance") will yield valuable insights into the molecular biology of gene expression in yeasts.

Several excellent reviews of this field already exist, and the reader is especially directed to recent reviews by Wickner (160) and Bussey (23) for more extensive coverage of information in their areas of expertise, respectively, the genetics and physiology of the S. cerevisiae killer system. Older reviews by Wickner (156) and Bruenn (17, 112) are also valuable summaries of genetic and molecular aspects of the field.

**VARIETIES AND PREVALENCE OF YEAST KILLERS**

The killer phenotype was first described in laboratory strains of S. cerevisiae by Makower and Bevan (M. Makower and E. A. Bevan, Proc. Int. Congr. Genet. XI 1:202, 1963). Killing was found to be caused by an exocellular, heat- and protease-sensitive toxin (169) that was only active and reasonably stable in the pH range of 4.2 to 4.7. This is now known to be close to its pI of 4.5 (107). Several more recent surveys of yeast strain collections and natural isolates have demonstrated the presence of similar phenomena in many genera (29, 65, 90, 92, 111, 116, 130, 170).

Most surveys have used rich agar media containing methylene blue in the pH range of 4.2 to 4.7, seeded or thinly surrounding the latter, bounded by blue-stained dead yeast cells, indicates killer toxin secretion. Figure 1 shows two natural K1 killer strains and an artificial killer strain containing no killer dsRNA but containing, instead, a phosphate-repressible cDNA copy of such a dsRNA (see "In Vivo Expression of Preprotoxin cDNAs").

An S. cerevisiae strain sensitive to the type K1 S. cerevisiae killers has often been used for initial screening, followed by cross-testing of killers to define immunity patterns. Such cross-testing matrices (116, 130, 170) usually demonstrate that, within a given genus, each killer strain is immune to its own toxin, but sensitive to toxins of other immune classes. This simple pattern is complicated by variation in the sensitivity of nonproducing strains, or of strains which all produce a single toxin type, to other toxins. As pointed out by Young and Yagiu (170), such strain variation in sensitivity is probably a result of nuclear mutations to resistance.

It is useful to distinguish semantically between killer-specific, plasmid-determined immunity and less specific resistance related to nuclear genotype. For example, whereas intergeneric killing does occur, many yeast strains are intrinsically resistant to killer toxins produced by members of other genera. Known S. cerevisiae nuclear mutations (KREL1, 2, 3) leading to resistance to M1-dsRNA-determined K1 toxin also confer resistance to a broad range of other toxin types (1, 26). However, neutral strains carrying a mutant M1-dsRNA which fails to cause toxin production (7, 24) retain immunity to the K1 toxin only.

The most thorough survey of killer types was performed by Young and Yagiu (170), using 20 killer strains found earlier in the British National Collection of Yeast Cultures (NCYC) (111). Species in 7 of 28 genera produced toxin active against an S. cerevisiae strain. Thirteen negative genera were represented by only one or two species, and since prevalence of killers among positive genera varied from 2 of 51 (Candida) to 12 of 29 (Hansenula), it seems likely that broader screening would turn up killers in several other genera. Exocellular toxins produced by the killers could be distinguished on the basis of pH optima, temperature stability, and relative susceptibility to proteases, although all were protease sensitive and optimally active below pH 5. Ten toxin types (K1 to K10) were distinguished by killer toxin and immunity specificities, including three (K1, K2, and K3) in Saccharomyces sp. strains. K1 is the type originally recognized in laboratory strains of S. cerevisiae (6, 7; Makower and Bevan, Proc. Int. Congr. Genet. XI 1:202, 1963). K2 killers were first recognized in Russian wine yeast and were shown to be capable of killing K1 killers (97, 98). Similar strains were found as contaminants of a two-stage continuous beer fermentation (88) and were later shown to belong to the same K2 killer group (116). K3 is currently defined by strain 761 of S. capensis (170). K1, K2, and K3 killers were found to carry physically distinct species of M-dsRNA (1.9, 1.7, and 1.5 kilobases [kb], respectively) (170). Minor differences were also reported in the mobilities of their associated L-dsRNAs (170). The plasmid nature of the M1-dsRNA determinant of the K1 killer strains had been previously indicated by demonstrating that this could be expressed by growth at high temperature (151) or in the presence of subinhibitory concentrations of cycloheximide (46) or 5-fluorouracil (95, 99) with concomitant loss of the killer character. This was also shown to be true for the K2 and K3 killer types (170). No dsRNA species were detected in any member of other killer classes, and none of these killers were curable under these conditions.

A homothallic wine yeast (S. cerevisiae) has recently been described (43) that kills both K1 and K2 S. cerevisiae killers. After nitrosoguanidine mutagenesis, strains killing both, called "K3," and strains bearing the K2 killer phenotype were found. The original strain contained two M-dsRNA species of about 2.0 and 1.5 kb. The K2 derivatives contained the 1.5-kb species, presumably closely related to 1.7-kb M2-dsRNA, whereas the K3 derivatives contained the 2.0-kb species. This is apparently a new killer plasmid, compatible with M2-dsRNA. It is considerably larger than the S. capensis M1-dsRNA of Young and Yagiu (170), so it is probably a fourth type of M-dsRNA, though this has not been tested directly. If the unusual killer toxin produced by S. cerevisiae strain 28 (110) is associated with a unique dsRNA, this will presumably be a fifth type. This strain produces an unusually stable toxin with a pH optimum of 5, higher than for any other known killer. Its genetic determinant is unknown (110).

Killing of S. cerevisiae by Torulopsis glabrata strain ATCC 15126 was demonstrated in 1975 (29, 30). Its toxin, dubbed pool efflux-stimulating toxin (PEST), acts very much like the other yeast killer toxins (27, 77, 89) except that its action is apparently independent of energy consumption by the victim (124). The toxin specificity of this strain differs from all of those tested by Young and Yagiu (170), including their K4 strain, T. glabrata 388. It was called "Tox 3" by Rogers and Bevan (116), but has been appended by Wickner (156) to the Young and Yagiu classification (170) as K11 specificity.

Extensive investigations of killing in natural yeast isolates by Middelbeek et al. (89-94, 130) have almost certainly uncovered other unique killer types. However, no complete comparison of these strains with the K1 to K11 types (156) has been reported, nor has the nature of the genetic determinants or their mode of transmission been reported for any of these isolates. Among 157 natural isolates, 17%, representing seven separate genera, were killers of S. cerevisiae (130).
Among yeast genera commonly found as opportunistic human pathogens, K1 killers were first observed in *Candida* spp. by Mitchell et al. (Abstr. IV Int. Congr. Yeast Genet. Mol. Biol., p. 24, 1975), and K11 was seen in *Torulopsis* sp. by Bussey and Skipper (29). An extensive survey by Middelbeek et al. (91), using a wide variety of killer strains, showed sensitivity among 116 of 142 strains of *Candida* and *Torulopsis* spp., especially to toxins produced by strains of *Hansenula* sp. (130) and *Pichia kluyveri* (90). Sensitivity is now being used epidemiologically for typing of fungal pathogens (113). A much lower prevalence of sensitivity was found in a similar survey by Kandel and Stern (69). However, the latter study used only *S. cerevisiae* and *Torulopsis* sp. strains as sources of toxin. *Cryptococcus* spp. strains were found to be sensitive only to toxins produced by other cryptococci and by certain *Torulopsis* sp. strains (92).

MODE OF ACTION OF KILLER TOXIN

The mode of action of killer toxins has been studied extensively in the *S. cerevisiae* K1 system, using strain K12-1, a normal laboratory strain killer, or derivatives of strain T158c. This strain contains a mutant “superkiller” form of M1-dsRNA encoding normal quantities of a more stable toxin (107) (see below, subsection “dsRNA Determinants”). The toxin of strain K12-1 consists of two polypeptide components (10), barely resolvable on reducing gels into slower (α) and faster (β) migrating species of about 9.5 and 9.0 kilodaltons (kd) (Fig. 2). These components are disulfide linked and run as a 17-kd dimer on nonreducing gels. It is assumed, but not proven, that this is an αβ dimer. Strain M5c contains the T158c superkiller M1-dsRNA (143) and its α component migrates more slowly than that of K12-1 (Fig. 2), although its β component migrates identically. Sequence analysis of cDNAs (10, 125; see “In Vivo Expression of Preprotoxin cDNAs”) confirms that the only amino acid variations occur in the α component. In strain M5c, secreted toxin comprises about 5% of the total secreted protein (Fig. 2) (107).

Type K1 toxin is irreversibly inactivated at pH values over 6.5, is most stable at pH values near its pl (4.5), and is inactivated with a half-life of about 60 min in culture media of normal strains (31). Degradation is inhibited by phenylmethylsulfonyl fluoride and a mutation (called ski2) results in loss of this phenylmethylsulfonyl fluoride-inhibitable exocellular protease and markedly enhanced toxin accumulation, equivalent to that seen in the presence of phenylmethylsulfonyl fluoride in normal strains (31). A considerable fraction of toxin, partially purified according to size, is inactive, 1,6β-D-Glucan affinity (64) and controlled-pore glycated glass (107) columns separate active toxin (10 to 30% of the total) from inactive toxin, with essentially complete retention of activity. However, the two fractions are indistinguishable on gels (25) or by interaction with polyvalent toxin-specific antisera (11).

The α and β components of toxin have a high content of hydrophobic and charged amino acids (10), with a combined pl of 4.5 (107), and are stabilized by 15% glycerol at this pH (107). Toxin action involves an initial, rapid, energy-independent binding of toxin to a cell wall receptor (1, 26) that has now been identified as a 1,6β-D-glucan (64). Both sensitive and immune (M1-dsRNA containing) cells have a large number of such receptors, whereas mutations in either of two nuclear loci (*kre*1 and *kre*2) (1) drastically reduce cell wall binding and modify 1,6β-D-glucan content. *kre* mutants are resistant to a wide range of killer toxins, suggesting a common initial binding step in their action (1, 26, 64).

After binding to the wall, an energy-dependent process (124) transfers toxin to its site of action, the cytoplasmic membrane. As few as 3 × 10⁶ of the 10⁷ cell wall-binding sites need to be occupied for this step and lethality to occur (23). *kre*1 and *kre*2 mutant cells have toxin-sensitive protoplasts, whereas the protoplasts of immune, toxin-producing cells retain their immunity (28). Mutants in a third nuclear gene, *kre*3 (1), are resistant in spite of normal wall binding and their protoplasts remain resistant (H. Bussey, personal communication).

The non-saturability of immunity argues against direct interaction of toxin and immunity determinants. This and the existence of the *kre*3 and *rex* mutants (in which killer plasmid-carrying strains kill themselves; 150) suggest the existence of a receptor in the membrane for the second stage.
of toxin action. At present it is not known whether both α and β toxin components interact with this putative receptor, or whether one toxin component is required only for initial binding to the wall, serving as a concentrated source of the lethal component for delivery to the membrane (see “Sequence of the M2-P1 Preprotoxin Gene,” subsection “Functional Analysis of the Toxin Subunit Structure and γ”).

The events leading to cell death also require expenditure of metabolic energy (27). Killing is only apparent after a lag period which is dependent on medium composition and growth phase. The effects of toxin are reversible during this lag, and stationary-phase cells are relatively resistant to toxin. In growing cells, inhibition of net proton efflux (39) occurs rapidly, coincident with inhibition of K+ influx and coupled amino acid-proton transport activity (38). All of these effects are apparently due to a rapidly induced increase in the permeability of the cytoplasmic membrane to protons (38). The proton extruding ATPase is probably not directly affected, but its effects are overwhelmed, leading, over a period of 40 to 90 min, to a marked drop in intracellular pH, metabolic inhibition, potassium ion efflux, and cell death associated with the leakage of metabolic pools such as ATP (27, 38). Macromolecules do not leak.

Very similar observations have been made with the toxin from a sake strain of S. cerevisiae (65, 77) which shares K1 immunity (23). It was also observed with this strain that Ca2+ ions prevented toxin-induced membrane damage (77).

Recent and extensive investigations of the mode of action of the purified toxin from P. kluveri 1002 have extended these observations. This yeast strain kills S. cerevisiae K1 killers (90), although it shares most of their killing spectrum (130). Its relationship to the current killer classification and the nature of its killer determinant are unknown. Like the K12-1 toxin, the P. kluveri toxin is most active and stable at pH values near its pI of 4.3. It has an apparent molecular weight of 19,000 and may be a glycoprotein (90). Binding to a cell wall receptor is energy independent, and when added to cells pretreated with metabolic inhibitors such as dinitrophenol or antimony A, cells can be rescued simply by raising the pH to 7, inactivating cell wall-bound toxin (94). After energy-dependent insertion into the membrane, killing can still be reversed for some time, but now requires removal of toxin and adjusting medium pH and K+ concentrations to physiological levels (pH 6.5, 50 mM KCl; 89). Cells are still killed if continuously exposed to toxin under these conditions for 60 min (89, 94) since membrane-associated toxin apparently remains active at pH 6.5, so that cumulative damage still occurs. It has recently been shown (68) that this toxin forms relatively nonselective, ion-permeable channels in artificial phospholipid bilayer membranes. Similar observations have been made with the T158c type K1 toxin (Bussey, personal communication). These observations further exemplify the resemblance between the yeast killer toxins and the E1 functional class of colicins (119).

As pointed out by Kagan (68), the conductance of killer toxin channels is approximately 100 times that of colicin E1 channels, consistent with the observation that toxin action on yeast cells, whose volume is about 1,000 times that of an Escherichia coli cell, like colicin action on E. coli cells, can cause an immediate leak large enough to inhibit net proton pumping and to dissipate preexisting membrane potential. This leak is also large enough to allow the cumulative lethal effects of leakage to occur within a cell’s generation time in both E. coli and yeasts.

### SUMMARY OF PHENOTYPES, dsRNA DETERMINANTS, AND PLASMID GENOTYPES OF S. CEREVISIAE KILLERS

#### Nomenclature

The nomenclature used for killer phenotypes is summarized in Table 1. S. cerevisiae killers of types K1, K2, and K3 have immunity to homologous toxin (R1+ R2+, and R3+ phenotypes, respectively) and secrete toxins of appropriate specificity (K1+, K2+, and K3+, respectively). Standard genetic tests (see below) distinguish the cytoplasmically inherited killer phenotypes determined by multi-copy plasmid genomes from those phenotypes affecting killer maintenance and expression which are determined by nuclear genes subject to Mendelian segregation (MAK, SKI, etc.; see “Nuclear Mutations Affecting M-dsRNA Maintenance”). (The dominant alleles of yeast nuclear loci are capitalized [e.g., SKI5].)

<table>
<thead>
<tr>
<th>Phenotype(s)</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Killer</td>
<td>Secretion of a polypeptide toxin that kills sensitive yeast cells</td>
</tr>
<tr>
<td>Immune</td>
<td>Plasmid-determined resistance to homologous killer toxin</td>
</tr>
<tr>
<td>Resistance</td>
<td>Resistance to a broad range of toxins, dependent on growth conditions or nuclear genotype (e.g., lack of receptor)</td>
</tr>
<tr>
<td>K1, K2, or K3</td>
<td>Strain having the type 1, 2, or 3 killer phenotype</td>
</tr>
<tr>
<td>R-</td>
<td>Lack of immunity to known killer toxins</td>
</tr>
<tr>
<td>R1+ R2+ R3+</td>
<td>Immunity to K1, K2, or K3 toxin, respectively</td>
</tr>
<tr>
<td>R1- R2+ R3+</td>
<td>Weak immunity to K1 toxin</td>
</tr>
<tr>
<td>K1+</td>
<td>Production of K1 toxin; ability to kill R-, R2-, or R3- strains</td>
</tr>
<tr>
<td>K2+</td>
<td>Production of K2 toxin: ability to kill R-, R1-, or R3- strains</td>
</tr>
<tr>
<td>K3+</td>
<td>Production of K3 toxin: ability to kill R-, R1-, or R3- strains</td>
</tr>
<tr>
<td>K1++</td>
<td>Superkiller producing more active or more stable K1 toxin</td>
</tr>
<tr>
<td>(K1+ R1-)</td>
<td>Normal phenotypes of K1 and K2 killer strains, respectively</td>
</tr>
<tr>
<td>(K1+ R1+)</td>
<td>Neutral phenotype; strain carries a mutant K1 plasmid</td>
</tr>
<tr>
<td>(K1+ R1- w)</td>
<td>Suicidal phenotype; strain carries a mutant K1 plasmid</td>
</tr>
<tr>
<td>Exclusion</td>
<td>Interference with replication of a killer plasmid leading to its loss by dilution; can be a consequence of plasmid or nuclear genotype</td>
</tr>
<tr>
<td>Suppressive</td>
<td>Plasmid causing exclusion</td>
</tr>
<tr>
<td>Helper</td>
<td>Plasmid preventing exclusion or enabling replication</td>
</tr>
<tr>
<td>Satellite</td>
<td>Replication-defective plasmid dependent on another; e.g., ScV-L1A is a satellite of ScV-L1A</td>
</tr>
<tr>
<td>VLP</td>
<td>Virus-like particle</td>
</tr>
<tr>
<td>ScV-L1A</td>
<td>S. cerevisiae virus containing L1A-dsRNA in VLPs</td>
</tr>
</tbody>
</table>
TABLE 2. dsRNAs in *S. cerevisiae* killers

<table>
<thead>
<tr>
<th>dsRNA(s)</th>
<th>Definition/comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>1.9-kb dsRNA; determinant of the K1+ R1+ phenotype</td>
</tr>
<tr>
<td>M2</td>
<td>1.7-kb dsRNA; determinant of the K2+ R2+ phenotype</td>
</tr>
<tr>
<td>M3</td>
<td>1.5-kb dsRNA; determinant of the K3+ R3+ phenotype</td>
</tr>
<tr>
<td>S3</td>
<td>0.73-kb dsRNA derived from M1 by an internal deletion</td>
</tr>
<tr>
<td>LA</td>
<td>4.7-kb dsRNA competent and required for maintenance of M1 or M2-dsRNAs; requirements for M3 maintenance not determined</td>
</tr>
<tr>
<td>L1A</td>
<td>4.7-kb class L-A dsRNA found in natural K1 killers; encodes an 88-kd polypeptide that encapsidates itself and M1</td>
</tr>
<tr>
<td>L2A</td>
<td>4.7-kb class L-A dsRNA found in naturally isolated type K2 killers; only partly homologous to L1A but probably has similar functional capabilities; encodes the 82-kd capsid of its own VLPs</td>
</tr>
<tr>
<td>LB, Lc, Lbc</td>
<td>4.7-kb dsRNAs found in most K1 and K2 killers; related to each other but not to L1A or L2A; not required for, nor probably capable of, maintenance of M1 or M2; encode the 82-kd capsid of their own VLPs</td>
</tr>
<tr>
<td>T, W</td>
<td>Minor 2.7- and 2.25-kb dsRNA species of unknown function present in each of the three S. cerevisiae strains; unrelated to each other or to other known dsRNA species</td>
</tr>
<tr>
<td>XL</td>
<td>Minor dsRNA slightly larger than L1A, occasionally reported in K1 killers; identity unknown</td>
</tr>
</tbody>
</table>

**dsRNA Determinants**

The dsRNAs associated with these plasmid phenotypes are listed in Table 2. The *S. cerevisiae* system is comprised of essentially two segments of dsRNA distinguished by size, L and M, which may exist in multiple subspecies. In addition, minor species designated T, W, and XL have been observed in strains. The products of these dsRNAs (after denaturation) and their presumed or proven in vivo counterparts are shown in Table 3.

Most isolates of *S. cerevisiae* contain at least one species of 4.7-kb L-dsRNA. Maintenance of an M-dsRNA is dependent on a subspecies, called L4, in which considerable variation is found. Variants associated with naturally isolated type K1 and K2 killers are called L1A and L2A, respectively (Table 2; "Role of L-dsRNA Species in VLP Capsid Production and M-dsRNA Maintenance"). A second subspecies, Lbc, is often present as a minor additional component. It cannot, by itself, support M-dsRNA maintenance (Table 2).

**Functional Model of M1-dsRNA**

A model of M1-dsRNA is shown in Fig. 3. The relevant data for its derivation are described elsewhere in this review. In summary, M1-dsRNA consists of approximately 1.0- and 0.6-kb segments separated by a highly adenine-uracil (AU)-rich denaturation "bubble" sequence, probably of variable size (here shown as 200 base pairs [bp]). The longer segment encodes the toxin precursor, preprotoxin. Protoxin, the primary in vivo translation product of M1-dsRNA transcripts, is believed to be identical to M1-P1, the product of in vitro translation of denatured M1-dsRNA (Table 3). The two disulfide-linked toxin polypeptides, a and β, are about 9.5 and 9.0 kd, respectively (Table 3) (10). They are separated in preprotoxin by a segment called γ, predicted to be the immunity determinant, and are preceded by an N-terminal segment called δ. The site of action of nuclear gene products involved in expression (maturation of toxin from preprotoxin: SEC, KEX) and maintenance (MAK, SKI) of M-dsRNA are also shown.

**Plasmid Genotypes of *S. cerevisiae* Killers**

The plasmid genotypes of the M-dsRNA variants in *S. cerevisiae* killers are listed in Table 4. These are designated [KIL-k1], [KIL-k2], and [KIL-k3], respectively, for M1-, M2-, and M3-dsRNAs. Thus, a normal K1 killer strain contains M1-dsRNA in ScV-M1 VLPs and has a [KIL-k1] genotype and a (K1+ R1+) killer phenotype. Strains carrying no M-dsRNA are sensitive (K- R-) with a [KIL-o] genotype usually containing an L-dsRNA.

Because the M-dsRNAs are stably maintained and segregated, mating of a sensitive, MAK- [KIL-o] strain with a K1 killer ([KIL-k1] genotype) produces a K1+ R1+ diploid, which, on sporulation, gives rise to 4:0 segregation of [KIL-k1] killers (7, 127). Strains carrying a recessive mak nuclear

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**TABLE 3. dsRNA gene products**

<table>
<thead>
<tr>
<th>dsRNA</th>
<th>Product name</th>
<th>In vitro translation</th>
<th>In vivo products</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>New</td>
<td>Old</td>
</tr>
<tr>
<td>L1A-P1</td>
<td>L1A-P1</td>
<td>L-P1</td>
<td>88</td>
</tr>
<tr>
<td>L2A-P1</td>
<td>L2A-P1</td>
<td>L-P5</td>
<td>84</td>
</tr>
<tr>
<td>L3-P1</td>
<td>L3-P1</td>
<td>L-P2</td>
<td>82</td>
</tr>
<tr>
<td>M1-P1</td>
<td>M1-P1</td>
<td>L-P3</td>
<td>78</td>
</tr>
<tr>
<td>M2-P1</td>
<td>M2-P1</td>
<td>34.8 (preprotoxin)</td>
<td>42</td>
</tr>
<tr>
<td>M3-P1</td>
<td>M3-P1</td>
<td>42 (preprotoxin)</td>
<td>42</td>
</tr>
<tr>
<td>T, W</td>
<td>T, W</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>S3-P1</td>
<td>S3-P1</td>
<td>8 (contains N terminus of preprotoxin)</td>
<td>42</td>
</tr>
</tbody>
</table>

\* ND, Not determined.
FIG. 3. Schematic structure of M₁-dsRNA. Segments encoding the β, γ, and δ components of the preprotoxin gene, from bases 14 to 964 of the plus (upper) strand, are shown to scale. Cleavage sites in the cDNA covering most of this gene (heavy line, bases 111 to 1,140) are shown. The AU-rich denaturation bubble is the site of termination of the m₉ partial-length transcript. ER, Endoplasmic reticulum. For other details, see text.
TABLE 4. Plasmid genotypes in S. cerevisiae killers

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Description</th>
<th>M-dsRNA</th>
<th>Killer and immunity phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>[KIL-o]</td>
<td>Lack known killer plasmids</td>
<td>None</td>
<td>$K^{-} R^{+}$</td>
</tr>
<tr>
<td>[KIL-k1]</td>
<td>Normal K1 killer plasmid</td>
<td>M1, M2</td>
<td>$K^{-} R_{j}^{+}$, $K_{j}^{+} R^{-}$</td>
</tr>
<tr>
<td>[KIL-k2]</td>
<td>Normal K2 killer plasmid</td>
<td>M2</td>
<td>$K_{j}^{+} R_{j}^{+}$</td>
</tr>
<tr>
<td>[KIL-k3]</td>
<td>Normal K3 killer plasmid</td>
<td>M3</td>
<td>$K_{j}^{+} R_{j}^{+}$</td>
</tr>
</tbody>
</table>

Mutants of K1 killer plasmid

| [KIL-n1] | Neutral | $M_{n1}$ | $K^{-} R_{j}^{+}$ |
| [KIL-n2] | Immunodeficient (suicidal) | $M_{n2}$ | $K_{j}^{+} R^{-}$ |
| [KIL-s1] | Dominant suppressive (e.g., $S^{-} R^{+}$) | $M_{s1}$ | $K_{j}^{+} R_{j}^{+}$ |
| [KIL-sk1] | Superkiller | M1 mutant | $K_{j}^{+} R_{j}^{+}$ |
| [KIL-b1] | Bypasses need for many MAK gene products | M1 mutant | $K_{j}^{+} R_{j}^{+}$ |
| [KIL-d1] | Dependent on a diploid host | M1 mutant | $K_{j}^{+} R_{j}^{+}$ |

Few natural mutants of ScV-M1 have been identified (7, 24, 96, 99, 115, 126, 141, 143), possibly because of the high copy number of its dsRNA genome. Vertical transmission of this plasmid, by cytoplasmic exchange, is presumably accompanied by transfer of several copies. As a consequence, segregation appears to be slow. Moreover, selective pressure is constantly applied for sequences that are replicated and expressed at an optimal level: too low a rate will result in plasmid loss, whereas too high a rate would probably be growth inhibitory or lethal. M1-dsRNA plasmid mutations have been defined which affect the expression of toxin activity (neutral, [KIL-n1]), expression of immunity (suicidal, [KIL-i1]), and M1-dsRNA replication (mak bypass, [KIL-b1], and diploid dependent, [KIL-d1]) (Table 4). Their presumed locations are shown in Fig. 3. The [KIL-b1] mutant is discussed in “Nuclear Mutations Affecting M-dsRNA Maintenance,” subsection “Superkiller (ski) Mutants and the [KIL-b1] Plasmid Mutant.”

Neutral and suicidal mutants ([KIL-n1], [KIL-i1]). Neutral mutants of M1-dsRNA (genotype [KIL-n1]) conferring a $K^{-} R_{j}^{+}$ phenotype (Table 1) were observed early among natural isolates (7). They have a defect in the toxin precursor gene but not in the component responsible for immunity. Some neutral strains, but by no means all, secrete an inactive toxin, a cross-reactive protein (CRM) of apparently normal size that is precipitated by antitoxin (see “Effect of Plasmid Mutants on Toxin Maturation”) (24). This presumably arises from a missense mutation in the toxin structural gene (Fig. 3).

A superkiller mutant strain (T158c) with a $K_{j}^{+} R_{j}^{+}$ phenotype (Table 1) was isolated (143) and later shown to produce presumably normal quantities of a more stable toxin (107) with a modified $\alpha$ component (Fig. 2).

Plasmid mutants have been isolated recently by growth of a strain carrying the M1-dsRNA derived from T158c at 37°C, causing gradual curing of the M1-dsRNA plasmid (24).

Ethylmethanesulfonate mutagenesis was applied at a stage when a maximal fraction of cells contained a single M1-dsRNA molecule. Cells regenerated a normal copy number when returned to 30°C and were screened for mutant killer phenotypes. A diploid strain was used to avoid detection of recessive nuclear mutations, and cytoplasmic inheritance of the phenotype was confirmed by standard tests (7). A variety of neutral (NLP) and “suicidal” (SLP) mutants were isolated.

The suicidal mutants ([KIL-i1] genotype) produce wild-type quantities of normal toxin, but have a much reduced immunity. These are thought to be defective in the immunity determinant encoded by M1-dsRNA (Fig. 3). They still survive, even when grown at pH 4.7 (the optimal pH for toxin stability and activity), but unlike normal killers, which survive high toxin concentrations, SLP mutants were killed by concentrations only about 100-fold greater than those required to kill sensitives derived by complete heat curing of the killer plasmid from the parent cell line (24).

Suppressive mutants ([KIL-s1]). Suppressive deletion mutants of M1-dsRNA (genotype [KIL-s1]), called S-dsRNAs, were reported in 1973 (126) and shown, by heteroduplex analysis, to result from internal deletions (47, 70). Such deleted plasmids confer neither toxin production nor immunity, but on mating of a strain carrying a [KIL-s1] plasmid to a [KIL-k1] strain, an unstable ($K_{j}^{+} R_{j}^{+}$) diploid is produced. It slowly segregates $K^{-} R^{+}$ [KIL-s1] suppressives. If the unstable diploid is immediately sporulated, killer haploids produced also segregate suppressives, 4:0. The mutant plasmid excludes the normal M1-dsRNA plasmid, giving suppressive strains a dominant ($K^{-} R^{+}$) phenotype, much as defective-interfering virus mutants interfere with replication of wild-type viruses (63). However, unlike viral defective-interfering particles, S-dsRNAs are not dependent on the parent M1-dsRNA; rather, both M1- and S-dsRNAs are defective satellites dependent on Ldio-dsRNA for encapsidation, so that M1 can be completely eliminated and still allow maintenance of an S-dsRNA. Since L-dsRNAs produce capsid proteins essential for their own maintenance (see “Role of L-dsRNA Species in VLP Capsid Production and M1-dsRNA Maintenance”), one might expect to find L deletions which are the equivalent of classical viral defective-interfering particles, but which are not suppressives. Such L deletions have not, however, been observed.

In a recent study (115), it was shown that suppression is not a consequence of growth advantage of cells containing the S-dsRNA, nor of a higher copy number of S- than M1-dsRNA. Rather, as anticipated, it seems to be due to preferential replication of the S-dsRNA, possibly because of competition for a limiting supply of some essential cellular component. Three S-dsRNAs derived from the same M1-dsRNA were found to fail to coexist stably. The most suppressive was actually the largest of the three, so that reduced genome length is not the cause of suppression. Neutral mutants of unaltered size found coexisting with the parental M1-dsRNA in one unstable K1 killer strain (13) were themselves suppressive (J. Sturgeon, K. A. Bostian, and D. J. Tipper, Abstr. IX Int. Congr. Yeast Genet. Mol. Biol., p. 102, 1978). This suggests that relatively subtle mutations can lead to altered dsRNA replication efficiency. This may explain the origin of natural isolates containing a single mutant type of M-dsRNA, in spite of a presumed initial excess of normal M1-dsRNA.

The best-characterized S-dsRNA is S3, derived from the mutant M1-dsRNA of superkiller strain T158c by an internal 1.2-kb deletion; 500- and 230-bp terminal fragments are left.
Sequence analysis (D. J. Thiele, E. M. Hannig, and M. J. Leibowitz, Virology, in press) has recently located these fragments in M₁-dsRNA (Fig. 3). Two modified forms of S₃ segregated during replication: a dimeric molecule (S1) and a form (S₄) derived from S₁ by a second small deletion (47). Similar duplication and size variation have been seen in other suppressives (115) and presumably arise by some copy choice mechanism during transcription within the VLPs.

Diploid-dependent mutant ([KIL-d]). A [KIL-d] mutant has been isolated in which the M₁-dsRNA is only maintained in a/o diploid cells (155). Maintenance of this dsRNA has apparently become subject to MAT locus controls.

STRUCTURAL COMPARISONS OF KILLER dsRNAs

In spite of early reports to the contrary (reviewed in reference 156), and in keeping with all of the genetic evidence, recent hybridization experiments have failed to find any homology between any killer dsRNA species and any DNA genome in S. cerevisiae (8, 9, 57, 129). Probes used in Southern blots of genomic DNA have included denatured and fragmented dsRNAs, labeled with [γ-32P]ATP and T4 polynucleotide kinase, representing all of the L₁₄A-, L₁₄BC-, and M₁-dsRNAs (9) and nick-translated cDNA complementary to 50% of M₁-dsRNA (9). Use of these probes and nick-translated cDNAs complementary to segments at one terminus of L₁₄A-dsRNA (8) also demonstrate the lack of detectable homology between M₁ and any of the L-dsRNAs and between L₄ and L₁₄BC.

Except for their single unpaired 3' bases, each dsRNA is a perfect duplex. M₁-dsRNA has an internal, asymmetrically located, highly AU-rich bubble sequence of about 200 bp, visible as a denatured region in electron micrographs (47) and removed by treatment with RNase S₁, leaving terminal fragments of about 1,000 and 600 bp (Fig. 3) (145). No similar region has been detected among L-dsRNAs. This difference is believed to account for the fact that, whereas L-dsRNA size is stable, M₁-dsRNA size varies by up to 12% in subclones of K₁ killer strains (S. S. Sommer and R. Wickner, personal communication). The transcriptase may slip or skip while transcribing oligouridyllic acid regions on the negative strand in the bubble, leading to variation in bubble size and insertion or deletion of oligoadenyllic acid [oligo(A)] sequences in the plus strand.

Each dsRNA has a relatively AU-rich terminus, the source of a large RNase T₁ oligonucleotide, and a relatively guanine-cytosine-rich terminus (15). The AU-rich 5'-terminal sequences reported for L₁₄A, L₁₄₂₂A, M₁, M₂, and L₁₄BC-dsRNAs are shown in Table 5.

Sequencing of dsRNAs from their 3' termini has provided data for 100 to 250 bp of both ends of M₁- and L₁₄A-dsRNAs (133–135; Thiele et al., in press). Earlier reports of sequence heterogeneity in L-dsRNA (19) were, at least in part, due to the presence of a mixture of L₁₄A and L₁₄BC and probably also to the presence in dsRNA preparations of multiple small RNA species of about 100 bp (L. A. Weinstein and M. J. Leibowitz, personal communication). These appear to be specifically associated with the dsRNAs, but are not related to the partial-length "pause products" synthesized by VLP transcriptase (14) (see "Transcription and Replication of Killer dsRNAs"). Some heterogeneity has been found in the sequences of overlapping cDNAs, representing fragments of a single L-dsRNA preparation (8; J. Bruenn; personal communication), suggesting rapid sequence divergence. In contrast, very little divergence has been seen in comparing sequences coding for toxin precursors in M₁-dsRNAs from normal and superkiller strains (see "Sequence of the M₁-P1 Preprotoxin Gene" and "In Vivo Expression of Preprotoxin cDNAs").

CELL CYCLE STUDIES OF dsRNA REPLICATION IN S. CEREVISIAE

Replication of the killer dsRNAs probably occurs in two distinct steps, as in reoviruses (122): a plus-strand transcript, defined as encoding the known dsRNA gene products (VLP capsid and toxin precursor), is produced in vitro by a VLP-associated transcriptase (see "Transcription and Replication of Killer dsRNAs") (14, 18, 59, 144). It must initiate at the AU-rich dsRNA terminus (see Fig. 3). It presumably acts as a template for a replicase which regenerates dsRNA by synthesis of the minus strand. Encapsulation of the dsRNA product would regenerate a VLP.

Both 5' ends of all L- and M-dsRNAs are ppG (20), presumably the acceptor for polymerization of each strand. All 3' ends are -CA-OH, with C pairing with ppG and the A being unpaired (Fig. 3; Table 5) (19), with the exception of L₁₄BC, in which both -CA-OH and -CG-OH 3' termini have been found (44). This minor L-dsRNA species resembles the Ustilago sp. dsRNAs in this respect (see "dsRNA Killer System of U. maydis").

<table>
<thead>
<tr>
<th>dsRNA</th>
<th>&quot;Plus&quot; genomic strand</th>
</tr>
</thead>
<tbody>
<tr>
<td>L₁₄A</td>
<td>ppGGGAUUUUUUAAUCAAUAACUCCCAUGCUAAAGA...UAGGGAAAACCAUGCA(OH)</td>
</tr>
<tr>
<td>L₁₄₂₂A</td>
<td>ppp GAUAAUUGAUAAUC... . . . GCUUACCAUAUGCA(OH)</td>
</tr>
<tr>
<td>L₁₄BC</td>
<td>ppp GAUAAUUGAUAAUC... . . . GCUUACCAUAUGCA(OH)</td>
</tr>
<tr>
<td>M₁</td>
<td>ppp GAUAAUUGAUAAUC... . . . GCUUACCAUAUGCA(OH)</td>
</tr>
<tr>
<td>M₂</td>
<td>ppp GAUAAUUGAUAAUC... . . . GCUUACCAUAUGCA(OH)</td>
</tr>
</tbody>
</table>

*In each case sequence is shown for the plus genomic strand. The complementary sequence of the minus genomic strand is shown only for M₁, to illustrate the 3'-unpaired A(OH) residues. The AUGs followed by open reading frames are underlined in the M₁ and L₁₄A sequences.
Several experiments demonstrate that ScV replication occurs continuously throughout the yeast cell cycle (100), although Zakian et al. report a pause in ScV-L replication during the S phase (171). Our own unpublished data indicate continuous VLP capsid, ScV-L, and ScV-M1, synthesis in pulse-labeled cells, in balanced growth, fractionated on sucrose gradients. The stability of ScV maintenance and the relative stability but wide strain variation in ScV copy number remain unexplained. In any given strain, nutrient conditions can cause ScV-L copy number to vary as much as threefold; higher numbers are found in ethanol-grown cells than in glucose-grown cells (105), and starvation for nitrogen source causes extensive degradation of preexisting L-dsRNA (33). The return of ScV-L1A and ScV-M1 to a normal copy number at 25 to 30°C, after partial heat curing by growth at 37 to 39°C (24), suggests feedback regulation of dsRNA copy number or recovery of some temperature-sensitive, rate-limiting step in VLP replication.

It has recently been shown (R. A. Sclafani and W. Fangman, personal communication) that full-length and partial transcripts of the killer L- and M-dsRNAs (see "Transcription and Replication of Killer dsRNAs") accumulate 50- to 100-fold in cells blocked at the G1/S cell cycle boundary by the use of α-factor and cdc7 blocks. Entry into S after relief of the block leads to reduction in l, possibly due to replication, but further increase in m. Replication of dsRNA thus appears to require some cellular components involved in the initiation of DNA synthesis. These must be constitutively present to allow dsRNA synthesis in G1. They are either inactivated or selectively occupied by DNA synthesis on entry into S phase.

TRANSCRIPTION AND REPLICATION OF KILLER dsRNAs

Isolated intact VLPs containing both L- and M-dsRNAs also contain a transcriptase activity which, in the presence of nucleotide triphosphates, produces full-length, non-complementary, single-stranded transcripts having messenger activity for the known dsRNA gene products (14, 15, 18, 59, 144, 146). The mechanism of transcription appears to be conservative (10; Sclafani and Fangman, Mol. Cell. Biol., in press), with extrusion of the newly synthesized plus strand. However, this is difficult to establish with certainty.

The 4.7-kb in vitro, single-stranded I transcript from ScV-L1A encodes L1A-P1 capsid (Table 3), whereas the 1.9-kb m transcript from ScV-M1 encodes M1-P1, the preprotoxin. Both 5′ and 3′ sequencing of the transcript from VLPs containing M1-dsRNA have been performed (54). The transcript is a faithful copy of the genomic dsRNA, initiating and terminating at the ends, without polyadenylation. Addition of an extra 3′-terminal purine occurs in vitro, 53% A and 38% G, although only A has been identified in vivo (19). The strands of M1- and L1A-dsRNAs have been separated, and the minus strands have been identified by hybridization with the in vitro plus-strand transcripts (133, 134). Determination of their 3′ sequences confirms the 5′ sequences of the in vitro transcripts, which initiate at the AU-rich terminus of both L1A- and M1-dsRNA (Table 5). This is also the AU-rich terminus present in the larger (1 kb) S1 nuclease fragment of M1 (145), demonstrating that the M1-P1 gene in this fragment is transcribed toward the bubble (Fig. 3).

Full-length, plus-stranded transcripts of the killer dsRNAs are also found in vivo (9), together with unique partial-length transcripts. These partial-length transcripts, l1 (2.3 kb) derived from L1A and m1 (1.2 kb) derived from M1, share the mRNA activities of their full-length counterparts (9). Hybridization to the separated plus and minus strands of M1-dsRNA confirms the positive polarity of both full-length and partial transcripts (54). It is not known whether any of these transcripts are capped. The ratios of these dsRNAs and their transcripts in killer strain K12-1 are shown in Table 6. Transcripts of the LBC species, present as a minor L component in strain K12-1, were not investigated (9).

The existence of both full-length and partial-length plus-strand transcripts for both dsRNA species investigated suggests differential function for these two species. It seems plausible that the full-length transcripts are intermediates in a reovirus-like replication mechanism (122), whereas the partial-length transcripts, which are just long enough to encode the preprotoxin and capsid gene products, are responsible for expression of the dsRNA genes in vivo. Perhaps secondary structure or blocking of capsid or other proteins masks the mRNA activities of m and l in vivo, avoiding the potential collision of ribosomes and replication machinery converging from opposite ends of a single plus strand. Alternatively, the partial-length mRNAs may not express all of the dsRNA genes: if secondary structure or bound proteins mask the known initiator AUG in the full-length transcripts, they might serve as messengers for different (unidentified) gene products derived from downstream initiation or ribosome binding sites, absent or inaccessible in the partial-length transcripts. The VLP-associated transcriptase and the immunity determinant are candidates for such unidentified products. However, immunity probably forms part of the preprotoxin (see "In Vivo Expression of Preprotoxin cDNAs").

The AU-rich 3′ termini of L1A-, L2A-, M1-, and M2-dsRNAs (Table 5) share a common eight-base sequence, AUU(U/A)UUC-A-OH, postulated to be the transcriptase recognition site (15). The sequence in LBC-AAAAAUC(A/G)-OH, differs considerably (Table 5). Since the M1-P1 gene initiates only 6 bp upstream of this site (see below), the 1.2-kb m transcript must initiate at the same terminus as the full-length transcript. It probably terminates within the AU-rich bubble sequence of M1, just beyond the end of the M1-P1 gene (Fig. 3). It is not known whether m is a product of premature transcription termination or of cleavage of m. Cleavage, as in the processing of some mitochondrial transcripts, might be dependent on translation initiation.

Both m and mb bind tightly to polyuridylic acid [poly(U)]-Sepharose, suggesting polyadenylation (9). However, it has recently been shown (54) that the separated plus strand of M1-dsRNA and the in vitro synthesized m plus strand, neither of which bears a 3′-terminal polyadenylic acid

<table>
<thead>
<tr>
<th>Species</th>
<th>kb</th>
<th>Relative concn</th>
<th>Molecules per cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1A-dsRNA</td>
<td>4.7</td>
<td>100</td>
<td>30</td>
</tr>
<tr>
<td>Lbc-dsRNA</td>
<td>4.7</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>l transcript of L1A</td>
<td>4.7</td>
<td>2</td>
<td>0.6</td>
</tr>
<tr>
<td>l transcript of L1A</td>
<td>2.3</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>M1-dsRNA</td>
<td>1.9</td>
<td>69 (100)</td>
<td>21</td>
</tr>
<tr>
<td>m transcript of M1</td>
<td>1.9</td>
<td>9 (14)</td>
<td>3</td>
</tr>
<tr>
<td>m transcript of M1</td>
<td>1.2</td>
<td>11 (17)</td>
<td>3</td>
</tr>
</tbody>
</table>

* L1A-dsRNA represents 0.03% of the total RNA in these cells, giving a copy number of about 30. The Lbc relative concentration is estimated from stained gels. All other relative concentrations are derived from Northern blots (9).
[poly(A)] sequence, both bind to oligodeoxynucleosydric acid (oligo(dt))-cellulose and have precisely the same thermal elution properties on poly(U)-Sepharose as the m and m5 in vivo transcripts (9). In contrast, the minus M1 genomic strand and both strands of S3 dsRNA (deleted for the bubble; Fig. 3) fail to bind to oligo(dt)-cellulose. Binding is, therefore, due to the presence of oligo(A) regions in the M1 plus strand, almost certainly within the AU-rich bubble sequence. Thus, the in vivo m5 transcript probably terminates after this A-rich bubble sequence, which may perform the functions usually provided by the post-transcriptionally added 3'-oligo(A) regions of yeast nuclear mRNA transcripts. The genomic RNAs of the single-stranded polioviruses and alphaviruses both possess 3'-terminal poly(A) tracts (118).

The site of the L1A-P1 capsid gene in L1A is not known. However, an open reading frame does start with AUG at bases 30 to 32, continuing through the known 113-bp sequence (133). Since translation from yeast transcripts normally initiates at the first AUG, this may be the start of the capsid gene unless its context (79) is preceded by CCC, not a favored sequence) makes it considerably less favorable than the next downstream AUG (F. Sherman, personal communication). No other AUG exists within the 113-bp sequence and, in common with M1- and reovirus dsRNAs (36), it seems likely that translation initiates close to the terminal transcription initiation site, probably at this first AUG. If so, then L1A, which is just long enough to encode L1A-P1, probably resembles m5 in being a 5' fragment of the full-length transcript (9). It should terminate halfway through L1A. The function of the 3'-terminal 2.5 kb of L-dsRNA is unknown. The stability of L-dsRNA size suggests an essential structural or genetic role.

It has been noted (82) that the m plus strand has a plausible ribosome binding site just upstream of the M1-P1 initiation AUG codon (Fig. 3) capable of significant base pairing to yeast 16S and 5.8S rRNAs. This is believed to have little if any significance for eucaryotic nuclear transcripts where interactions with the cap and cap binding protein may be of primary importance in translation initiation. It might be significant for the cytoplasmic dsRNA transcripts, if they are uncapped; however, this is only speculation at this point. Moreover, the L1A sequence lacks an obvious ribosome binding site (133). Although this might explain why denatured M1-dsRNA is a far better in vitro messenger than denatured L1A-dsRNA (the dsRNAs are uncapped), this may, alternatively, reflect the different contexts of the initiator AUG sequences. Both the short 5' sequence in M preceding the initiation AUG and that in the L1A plus strand (133) are A rich and G poor, characteristics of many yeast mRNAs (60).

The full-length in vivo transcript l, but not l5, binds to poly(U)-Sepharose (9). No AU-rich sequence is known to exist in L1A, and Hannig et al. (54) found no binding of the L1A in vitro transcript to oligo(dT)-cellulose, so that l, the in vivo equivalent, may be polyadenylated. However, an oligo(dT)-primed, 4.3-kb cDNA has been made from denatured L-dsRNA (16). Also, Haylock and Bevan (56) failed to find binding of the full-length l in vivo transcript to oligo(dT)-cellulose. Possibly differential binding to poly(U)-Sepharose, which requires shorter oligo(A) stretches than binding to oligo(dT)-cellulose, indicates the presence of relatively short oligo(A) stretches within the L1A plus strand, perhaps terminating l5 and the L1A-P1 gene. It was suggested (9) that post-transcriptional polyadenylation may be common to the presumptive in vivo replication intermediates m and l and that subsequent removal of poly(A) might leave the terminal unpaired -A-OH on the dsRNAs. However, there is no precedent for such a mechanism. Poly(A) addition now seems unlikely, and the existence of terminal -CG-OH on a major fraction of L1C molecules (44) argues against a poly(A) origin for terminal -CA-OH. Perhaps post-transcriptional addition of a single 3'-terminal A or G occurs as in E. coli SPB RNA phage (2).

Yeast cells appear to lack significant pools of dsRNA or ScV-P1 capsid except in VLPs (13). Production of either may, therefore, limit the rate of VLP replication. Strain K12-1 contains only about 30 molecules of L- and 20 of M1-dsRNA per cell (Table 6). The copy numbers of full-length transcripts, averaging about 0.6 of l and 3 of m per cell in this strain (Table 6), should depend on ScV full-length transcript copy number, transcription rate, transcript stability, and the rate of initiation of dsRNA replication on these transcripts. The existence of these small pools of l and m suggests that these transcripts are sequestered in polysomes or that initiation of negative-strand synthesis is rate limiting for dsRNA replication.

Replication of dsRNAs on their plus-strand transcripts initiates at their C-rich 3' termini. There is little sequence homology among dsRNAs at this site (Table 5), and Brennan et al. (15) have suggested that a short 3' stem-and-loop structure, present just before a terminal AUG-CGA-OH sequence, may be the replicase recognition site. In plant viral genomic RNAs, such as alfalfa mosaic virus, such a structure serves to bind capsid as a necessary part of a replication complex (78). If this is also true of the Scv-dsRNAs, then, as suggested by Brennan et al. (15), capsid production, potentially controlled by l5 concentration, could control initiation of dsRNA replication as well as encapsidation. It seems likely that capsid binding to nascent dsRNA and encapsidation would at least accompany replication, protecting dsRNA from RNase activity and regenerating a VLP. A dsRNA-specific nuclease has recently been identified in yeasts (D. J. Mead, and S. G. Oliver, Proc. XI Int. Conf. Yeast Genet. Mol. Biol., p. 46, 1982). Thus, the rate of production of the l5 partial transcript, and the efficiency of its interaction with ribosomes, may ultimately control the Scv copy number. For ScV-L1A, the copy number varies from 30 to 50, in strains such as K12-1, to several thousand in the atypical sensitive strain S7 (17). As suggested by Field et al. (44), competition for a limited capsid supply among different dsRNA species might account for the reduction in copy numbers seen in mixtures of compatible species such as L1A and m5 (S. G. Ball, C. Tirtiaux, and R. B. Wickner, Genetics, in press) as well as exclusion among apparently incompatible species such as M1 and its suppressive derivatives (see “Summary of Phenotypes . . . ,” subsection “Plasmid Mutations in M1-dsRNA”) and M1 and M2 (see [HOK], [EXL], and [NEX] . . . ,” subsection “Exclusion of M2- by M1-dsRNA”). No in vitro model of second-strand synthesis currently exists for testing the role of capsid in this process.

ROLE OF L-dsRNA SPECIES IN VLP CAPSID PRODUCTION AND M1-dsRNA MAINTENANCE

All K1, K2, or K3 killers contain at least one species of L-dsRNA (about 4.7 kb; Table 2) as well as the M-dsRNA killer determinant. This is also as true of cells maintaining the various mutant forms of M1-dsRNA. In fact, almost all S. cerevisiae isolates contain a form of ScV-L, even though a majority are sensitive lacking M-dsRNA.

The first successful attempt at using in vitro translation of denatured dsRNA to determine gene function demonstrated
that the (major) L-dsRNA of sensitive strain 3/Al codes for the capsid protein, ScV-P1 (88 kd), which comprises at least 95% of the VLP protein in this strain (62). Antiseras raised against ScV-L were shown to precipitate both ScV-L and ScV-M1 (55), and it was later shown, in the K1 killer strain 2-1, that ScV-P1 capsid comprised at least 95% of the protein in both separated ScV-L and ScV-M1 VLPs (13). Minor proteins of unknown function, called ScV-P2 (82 kd) and ScV-P3 (78 kd), were consistently found in VLPs of strain 3/ Al (62) and in both ScV-L and ScV-M1 in strain 2-1 (13). It was proposed that encapsidation is essential for dsRNA maintenance and that M1 is a satellite “mycovirus” dependent on L as a source of VLP capsid (62). Reciprocal dependence of L on M1 clearly does not exist, so that M1 and L are not parts of a single mycovirus system.

**Nomenclature and Function of the Various L-dsRNA Species**

It has now been shown that most S. cerevisiae strains contain at least two unrelated L-dsRNA species, as first suggested by Sommer and Wickner (128). The M-dsRNA killer plasmids are independent satellites of one L-dsRNA class, whereas the second L-dsRNA class is unrelated to the first or to M. A third class of dsRNA molecules, called T and W, has recently been described (see “T-, W-, and XL-dsRNAs”) and others may exist. Laboratory yeast strains are known which have most combinations of these three potentially independent ScV types, including absence of any detectable dsRNA. Their role, if any, in cell metabolism is, therefore, obscure. This is discussed further below (“T-, W-, and XL-dsRNAs”), following descriptions of the structural relatedness, gene contents, dependence on nuclear genes, and relationship to M-dsRNA maintenance of the different L-dsRNA species.

The L-dsRNAs found in K1 killers and related strains were called L1 and La by Field et al. (44), L2, and Lc by Sommer and Wickner (129), and “L2” and “L1” by El Sherbeini et al. (41) (L1 = La = “L1”; Lc = L2 or Lc = “L1”). “L1” in native form migrates slightly more slowly than “L2” on agarose gels (41). Whereas this difference has been seen reproducibly, it is only obvious after application of a rapid isolation procedure (41) and might reflect a modification specific to “L1” (e.g., bound protein) rather than true size difference. In general, all recognized L-dsRNAs are of approximately the same size (estimated at 4.5 to 5.0 kb). Field et al. (44) called the major L species in K2 killers L1, since they found it to differ from L1 in terminal sequence (15) (Table 5), and noted that their K2 killers also contained L2.

Nomenclature in the L-dsRNAs needs clarification. Because numbers have already been adopted to designate the K1, K2, etc., killer phenotypes, any numbers associated with L-dsRNAs should reflect a relationship to the M1-, M2- (etc.)-dsRNA killer determinants. The L1, L2, Lc nomenclature of Sommer and Wickner (129), adopted here, seems the most useful primary distinguishing system. L1 and L2 have been distinguished in four strains (129) and appear to be closely related. The similar species found in other strains will be called L1c. Variants of L1 are all competent, to a variable extent, for the maintenance of M1- and M2-dsRNAs, whereas variants of L1c are not. Evidence is accumulating to indicate that this reflects the ability of capsid encoded by Lc to encapsidate M-dsRNA in VLPs. Capsid encoded by L1c is known only to encapsidate itself (see below).

Although the known variation in L1c properties is small, a broad spectrum of L1 phenotypes is already known. The variants found in naturally isolated K1 killer strains share only partial sequence homology with those of K2 strains studied and encode distinctly different capsid proteins. We shall call the species isolated from killer strains L1A and L2A to denote the associated killer type, after Bruenn (44), although it appears probable that they are quite similar in their abilities to maintain M1- and M2-dsRNAs. L1A subspecies vary in their ability to maintain M1, and L2A dsRNAs in different nuclear and cytoplasmic genetic backgrounds (SKI" or ski", etc.; see next section, subsection “Superkiller (ski) Mutants and the [KIL-b] Plasmid Mutant”). The associated [HOK], [NEX], and [EXL] phenotypes (see below) have been denoted L1A-HN, L1A-E, etc. (129). We shall use this nomenclature where natural association with M-dsRNAs is not known, as for L1A-E from the sensitive strain AN33 (129). The L1A type found in all naturally isolated K1 killers analyzed is L1A-HN, which we shall call L1A-HN.

The potentially independent VLP systems which include these different L-dsRNAs are called ScV-LA (including ScV-L1A and ScV-L2A) and ScV-LBC (Table 3). ScV-LBC is apparently found in both K1 and K2 killers (44; our unpublished data) (Table 2), so it must be compatible with L1A plus M1 or L2A plus M2-dsRNAs.

**Comparison of Sequences of L-dsRNAs**

Apart from the apparent slight difference in size between LBC and Lc detected on agarose gels (41), L-dsRNAs appear to be remarkably constant in size even when isolated from S. cerevisiae strains of diverse origin. It has recently been shown (133) that the separated, single strands of L1A are easily resolved from those of L2B or L2C on native 5% acrylamide gels, presumably as a consequence of different secondary structures. The strands of L2B, however, were not clearly resolved from those of L2C by this procedure.

In most K1 killers studied, L1A comprises 90 to 95% of the L-dsRNA and L1B detected on agarose gels (41). L-dsRNAs appear to be remarkably constant in size even when isolated from S. cerevisiae strains of diverse origin. It has recently been shown (133) that the separated, single strands of L1A are easily resolved from those of L1B or L1C on native 5% acrylamide gels, presumably as a consequence of different secondary structures. The strands of L2B, however, were not clearly resolved from those of L2C by this procedure.

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It is calculated that as little as 12% random sequence modification is required to give totally different T1 fingerprints (129). "Northern" blot analyses confirm these findings. Portions of L1A (200 to 400 bp) have been cloned as cDNAs, using short in vitro transcription products derived from the 5' terminus as template (8). A cDNA probe representing 325 bp close to the AU-rich terminus of L1A and a second probe derived from a region near this terminus, but separated from it by at least 585 bp, failed to react with LBC (8). Use of labeled fragments representing all of L1A and LBC as probes also confirms the lack of detectable homology between L1A and Lb or Lc, and between LBC and L1A or L2A, and confirms the marked similarity of Lb and Lc (137; our unpublished data). The L1A probe cross-hybridized about 20 to 30% with L2A (our unpublished data). The terminal cDNA probe derived from L1A also reacted moderately strongly with L2A, whereas the internal cDNA probe failed to react (8). Only limited regions of L1A and L2A may, therefore, be sufficiently homologous in sequence to cross-hybridize. L1A and L2A are distinctly less related than Lb is to Lc.

Capsid Proteins Encoded by the Various L-dsRNAs

The gene products presumed or proven to be encoded by various L-dsRNAs are listed in Table 3. Translation of denatured L1A produces L1A-P1, the previously identified 88-kd major Scv-P1 capsid in K1 killers (62), which is capable of encapsidating M1-dsRNA (13). This is now called VL1A-P1 (Table 3).

Translation of L2A produces L2A-P1 (84 kd; Table 3), identical to the VL2A-P1 major capsid protein in strain 482 killer VLPs. L2A-P1 is quite distinct from L1A-P1 in its peptide map (34; M. E. Sherbeini et al., submitted for publication).

Translation of denatured Lb, Lc, or Lbc produces Lbc-P1, an 82-kd polypeptide that has been shown, by peptide mapping (34), to be identical to the single capsid protein in VLPs containing Lbc (VLbc-P1, Table 3) (El Sherbeini et al., submitted for publication). VLP capsid proteins from strains containing only Lbc were previously reported (129) to comprise a mixture of components smaller than Lbc-P1. However, Lbc-P1 is extremely sensitive to proteolysis and the lower-molecular-weight species may be degradation products. Peptide maps fail to distinguish between the 82-kd capsids from VLPs isolated from strains containing only Lb, only Lc, or Lbc-dsRNAs. However, these capsids are clearly different from those of VLPs containing L1A- and L2A-dsRNAs (El Sherbeini et al., submitted for publication).

Lbc-P1 was previously called L-P2, a major translation product of denatured L-dsRNA from strains such as K12-1 which contain both L1A and Lbc. Even though Lbc is only 5 to 10% of the dsRNA in these strains, it is apparently a much more efficient in vitro messenger and L-P2 (Lbc-P1) is often the predominant translation product. VLbc-P1 comigrates with Scv-P2, first identified as a minor VLP component in strain 3/A1, a strain containing Lbc (62; our unpublished data). However, Scv-P2 is also seen in strain 2-1, which contains only L1A (our unpublished data), where its source is unknown. The source of Scv-P3, seen in VLPs of both strains 3/A1 (62) and 2-1 (13), is also unknown. It comigrates with Lbc-P1 (Table 3), a translation product of Lbc which is distinctly different in peptide map from Lbc-P1, but which has no identified in vivo equivalent.

VLPs from strain K23, a K1 strain having relatively high Lbc levels, can be separated into a lighter Scv-M1 peak and a heavier Scv-L peak in which Scv-L1A and Scv-Lbc are partially resolved. The Scv-M1 peak contained only VL1A-P1, confirming the role of this capsid in M1 maintenance (13) and consistent with the inability of VLbc-P1 to perform this function. The VL1A-P1 and VLbc-P1 peaks in the Scv-L peak coincided with the L1A- and Lbc-dsRNA peaks, respectively, suggesting that each L-dsRNA is encapsidated only by its homologous capsid protein (E. Sherbeini et al., submitted for publication). This has been clearly demonstrated by Thiele et al. (133), who fractionated the VLPs of strain NK3 (KIL-3). Heavy VLPs contained L1A- and S3-dsRNAs (not fractionated) and VL1A-P1. A very light VLP peak contained Lbc and was found to contain three smaller proteins, essentially as previously reported for Lbc VLPs by Sommer and Wickner (129). It seems likely that the low density of the VLPs containing Lbc-dsRNA, which were found near the top of the gradient, was due to damage, for which the protease sensitivity of Lbc-P1 may have been responsible.

In a similar experiment, it was shown that the dsRNA transcriptase activity in the light VLP peak from a K1 killer was much more heat labile than in the heavier peak (87). On the assumption that this peak contained Scv-M1, this result was postulated to explain the heat curability of Scv-M1. However, these VLPs were almost certainly damaged forms of Scv-Lbc.

NUCLEAR MUTATIONS AFFECTING M-dsRNA MAINTENANCE

Maintenance of Killer (mak) Genes: the KRBI Mutation

By screening K1 killer strains for (K^- R^-) segregants, easily scored by replicating onto a lawn of sensitive cells (Fig. 1), mutants in 30 nuclear loci (spe2, spe10, pet18, mak1, mak3-28) that cause loss of M1-dsRNA have been identified (96, 139a, 150, 155, 162, 165). This screen will also include kex mutants (see "Structure of the M1-dsRNA Preprotoxin Gene and Steps in Toxin Maturation"), but unlike mak mutants, kex mutants retain immunity, also easily scored as in Fig. 1. Many mak mutants are marked by a single allele, and it is estimated (156) that 100 such loci may exist, a surprisingly high number. They are not clustered and have been mapped to 15 of the 16 known yeast chromosomes (165).

Because the presence of LA or Lbc confers no obvious phenotype on a sensitive S. cerevisiae strain lacking M- dsRNA, their loss cannot be so easily monitored. However, the dependence of M on L-dsRNA allows detection, in killers, of mutants required by both M and LA or whose primary defect is in LA-dsRNA maintenance. Three of the killer maintenance mutants (pet18, mak3, and mak10) have recently been found to fall into this category (see next section). None of the MAK genes is required for Lbc or W (see "T-, W-, and XL-dsRNAs").

Although conditional mutants were not deliberately sought, several mak mutants result in slow growth (spe2, pet18, mak13, 15, 17, 20, 22, 27), whereas mak1, mak16, mak30, and pet18 alleles are temperature sensitive for growth and mak6 is cold sensitive (156, 165). MAK6 has recently been identified with a previously known locus, LTS5, causing growth to be cold sensitive at 8°C (114). A few conditional (temperature-sensitive) mak mutants were found in a deliberate search (50). Thus, M1-dsRNA maintenance is critically dependent on the function of a number of nuclear
genes whose products are important or essential for cell growth.

*PET18* is required for *M*1-dsRNA maintenance and also for maintenance of mitochondrial DNA (84); other mutants tested (a large number are now known) do not lead to loss of *M*-dsRNA.

Genes of known function required for *M*1 maintenance include *SPE2*, which encodes adenosylmethionine decarboxylase, required for spermine and spermidine biosynthesis (35), and *SPE10*, which encodes ornithine decarboxylase, required for synthesis of putrescine (139a). Cells grow slowly (but fail to undergo meiosis or to sporulate) in complete absence of *SPE2* function. Both *spe2* and *spe10* mutants lose *M*1-dsRNA. No other dsRNAs are affected, except for the loss of *L3-E*, an unusual *L*-variant (see “[HOK], [EXL], and [NEX] ... subsection “[EXL], *L3-E*, and *L3-A*-VE”) in *spe10* mutants (139a). This variant, unlike all other L-dsRNAs, also requires *MAK27* (159). The physical characteristics of the different *S. cerevisiae* dsRNAs and their differential chromosomal gene requirements are listed in Table 7.

*MAK8* is identical to *TCM1* (166), the gene for trichodermin resistance, encoding ribosomal protein L3. Some function of this protein is apparently more critical for *M*1-dsRNA maintenance than for growth. This function could involve translation, as part of the ribosome, or might, for example, involve direct interaction of protein L3 with *M*1-dsRNA or its transcripts, just as the *E. coli* translational factors Tu, Ts, and S1 are involved in replication of Q6 RNA phage (2).

*MAK1* has recently been identified with the gene for yeast topoisomerase-1 (136). Screening of extracts of temperature-sensitive mutants identified a mutant with a topoisomerase-1 (temperature-sensitive) defect. This was found to be allelic with *mak1-1*, which has less than 1% wild-type topoisomerase-1 activity by this in vitro assay, a surprising result.

Loss of functional mitochondrial DNA bypasses the requirement for *MAK10* for maintenance of *M*1-dsRNA; antibiotic-induced respiratory deficiency does not (154). A dominant nuclear mutation in the *KRBI* locus, isolated as a suppressor of *mak7*, also bypasses *pet18* (164) (Table 8), but not several other *mak* mutations. Although loss of *M*1-dsRNA is prevented in *pet18 KRBI* strains, mitochondrial DNA loss is not prevented. The interplay between these nuclear loci, mitochondrial DNA, and *M*1-dsRNA maintenance is not understood. *KRBI* is centromere linked, but is not located on any of the 16 known yeast chromosomes. It thus defines a new chromosome, XVII (161).

### Table 7. Summary of biochemical properties and known genetic requirements of *S. cerevisiae* dsRNA types

<table>
<thead>
<tr>
<th>dsRNA species</th>
<th>[HOK], etc.</th>
<th>Size (kb)</th>
<th>Hybrids</th>
<th>Enodes</th>
<th><em>PET18</em></th>
<th><em>MAK1</em></th>
<th><em>SPE2</em></th>
<th><em>SPE10</em></th>
<th><em>MAK27</em></th>
<th><em>SKI</em></th>
<th><em>CLI</em></th>
<th>Temp</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L</em>1-A</td>
<td><em>L</em>1-A-HN</td>
<td>4.7</td>
<td><em>L</em>1-A(+)</td>
<td>Capsid</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>T1</td>
</tr>
<tr>
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<td><em>L</em>1-H</td>
<td>4.7</td>
<td><em>L</em>1-A(+)</td>
<td>Capsid</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>T1</td>
</tr>
<tr>
<td><em>L</em>1-A</td>
<td><em>L</em>1-A-E</td>
<td>4.7</td>
<td>NT</td>
<td>Capsid</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>T1</td>
</tr>
<tr>
<td><em>L</em>1-B</td>
<td><em>L</em>1-B</td>
<td>4.7</td>
<td>Capsid</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>?</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>M1</td>
<td>1.9</td>
<td>M1</td>
<td>Preprotoxin</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>T1</td>
</tr>
</tbody>
</table>

* Characteristics listed include associated [HOK], [NEX], and [EXL] phenotypes (see text, “[HOK], [EXL], and [NEX] ... variations in ability of *L*- to maintain *M*-dsRNAs”), indicated by *H* and *E* suffixes, and potential hybridization interactions. Only those probes listed have been tested, each against all species listed, indicating also the nonomorphism between *M*1, *L1-A*, *L1-B*, T, and W. Nuclear genes required for maintenance in *SKI*1 hosts are indicated by + (described in text). “Nuclear Mutations affecting M-dsRNA Maintenance”; “Effects of *mak* and *ski* Mutations on L-dsRNA Maintenance”; “TS-, W-, and XL-ds-RNAs”. “Temp” indicates temperature sensitivity of maintenance: ts, loss (curing) at high temperature; TI, enhancement at high temperature. NT, Not tested.

**Superkiller (ski) Mutants and the [KIL-b] Plasmid Mutant**

The same plate test for killer zone size (Fig. 1) which allows detection of (K"- R") *mak* mutants derived from (K"+ R") killers also allows identification of "superkiller*" (K"+ R") strains in which the zone of killing is larger than normal. Such strains fall into several categories, as follows:

(i) loss of toxin cell wall receptor (*krel2* mutants; see “Mode of Action of Killer Toxin”) resulting in escape of a larger fraction of toxin produced into the medium (32);

(ii) a *ski* mutation resulting in loss of a toxin-degrading exocellular protease and increased toxin accumulation (see “Mode of Action of Killer Toxin”; 31);

(iii) a [KIL-sk1] mutation in *M*1-dsRNA, as in strain T158c, resulting in production of a more stable toxin, presumably less sensitive to the *ski2* gene product;

(iv) a [KIL-b] plasmid mutation, presumed but not proven to reside in *M*1-dsRNA, which results in an increased *M*1-dsRNA copy number (139; R. B. Wickner, personal communication) and is presumably responsible for increased toxin production (common in laboratory yeast strains);

(v) mutations in nuclear *ski2*2,3,4,6,7,8 loci which also increase the *M*1-dsRNA copy number (and also that of L-dsRNAs; see next section) (114, 139); and

(vi) a *ski1* mutation, which increases toxin production without an effect on *M*1-dsRNA copy number. Unlike other *ski* mutations, *ski1* fails to suppress *mkt* (“[HOK], [EXL], and [NEX] ...” subsection “[NEX], *L1-HN*, and *mkt1.2*”,)

The *ski1*,2,3,4,6,7,8 mutations were firstly identified by this superkiller phenotype and later found to suppress certain *mak* mutations (139). The *ski5*,6,7,8 mutants were later discovered by a second phenotype, suppression of *mkt* (see section cited in (iv) above). Their effects on *mak* mutations have not yet been described. The [KIL-b] plasmid mutation also "bypasses" a requirement for certain intact *MAK* genes

**Table 8. Classification of *MAK* and *ski* mutations**

<table>
<thead>
<tr>
<th>mak group</th>
<th>Genes</th>
<th>Suppressed by:</th>
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</thead>
<tbody>
<tr>
<td>MI</td>
<td><em>mak16</em></td>
<td><em>ski1,2,3,4</em></td>
</tr>
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<td>MI1</td>
<td><em>mak3,10 pet18</em></td>
<td><em>ski1,2,3,4</em></td>
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<td><em>mak12,21,26</em></td>
<td><em>ski1,2,3,4</em></td>
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<tr>
<td>MIV</td>
<td><em>mak1,4,6,7,11 spe2</em></td>
<td><em>ski1,2,3,4</em></td>
</tr>
<tr>
<td>MIV</td>
<td><em>mak3,3 pet18</em></td>
<td><em>ski1,2,3,4</em></td>
</tr>
</tbody>
</table>

* *ski6,7,8* (see text) have not yet been fitted into this scheme.
phenotype three genes the classification ski5), ski, only variant, LA-E, function, speculation in now plus [KIL-bl], [HOK], (see between premature. However, LA to tcml, dsRNAs of dsRNA, with maintenance of genetic interaction 3). Second-strand "T-, "Structure toxin ski5) than tion 3) seconds-strand "M-dsRNA). It is through the yeast acid phosphatase genes (137). A likely focus of this regulation is initiation of dsRNA replication by second-strand synthesis on a primary plus-strand transcript (Fig. 3). Interactions with different dsRNAs are discussed in "T-, W-, and XL-dsRNAs."

Significance of mak and ski (etc.) Mutations

The mutations listed in Table 7 are of direct value in studies of the killer system since they can be used to manipulate cellular dsRNA contents. Mutations affecting toxin secretion, such as krel and -2, ski5, and kexl and -2 (see "Structure of the M1-dsRNA Preprotoxin Gene and Steps in Toxin Maturation"), are of considerable interest to individuals attempting to analyze and optimize protein secretion in S. cerevisiae. It seems likely that the SKI loci (other than ski5) are involved in relatively general, negative regulation of dsRNA replication (see "[HOK], [EXL], and [NEX] . . . .", subsection "Conclusion"). The value of other mutations may derive principally from analysis of their effects on basic cell metabolism, as illustrated by the spe, tcm, and topoisomerase 1 mutations.

It appears that identification of loci affecting killer phenotype allows isolation of mutations apparently partially defective in a wide variety of fundamentally important nuclear gene products. This is valuable only after identification of the primary defect, a haphazard process so far, involving the demonstration of allelism between mutants isolated for apparently independent phenotypes. However, it seems possible that any mak or other killer mutation resulting in retarded or temperature-sensitive growth may be worthy of biochemical analysis if clues to the primary defect can be elucidated.

Wickner (156) has pointed out that retarded growth is not, by itself, responsible for M1-dsRNA loss in mak mutants. For example, a spe2 ski2 mutant still grows slowly, but retains M1. Similarly, a pet18 ski1 strain remains petite and temperature sensitive for growth although competent for M1 maintenance.

**EFFECT OF mak AND ski MUTATIONS ON L-dsRNA MAINTENANCE**

Until recently, curing of M1-dsRNA or loss due to introduction of a mak mutation was not thought to cause loss of L-dsRNA, although a reduction by about threefold was observed on introduction of mak3 into a K1 killer (165). One killer strain, K7, was known to be an exception since introduction of mak10 led to loss of both M1 and L-dsRNAs (96). It is now known that this reflects the dependence of L-dsRNA maintenance on MAK3, MAK10, and PET18 (41, 129) and that this is the only L species in strain K7; the residual L-dsRNA in other strains is LBC, which is independent of these genes (Table 7). The loss of all L-dsRNA from strain K7 and the loss of a major fraction of the total L-dsRNA in other K1 killers during heat curing of the killer phenotype (151) is similarly due to the temperature sensitivity of LA but not of LBC maintenance (Table 7). M1 is heat cured more readily than LA (128) and may have a more stringent requirement than LA for an unidentified temperature-labile cell component, or it may simply require a high LA copy number for its maintenance.

In these experiments (128), it was shown that, after heat curing of a K1 killer to the point that LA was eliminated from 90% and M1 from 99% of the progeny, the few remaining killer clones (retaining M1) all retained LA. This is the most stringent demonstration of the dependence of M1 on LA.

Maintenance of M2-dsRNA in strains containing L2A is known to require MAK10 (97). By analogy with the K1 system, this suggests that L2A shares with LA at least the requirement for MAK10. M2 also requires MAK8 and MAK16. Other mak loci have not been tested (160). M2 is heat curable and resembles M1 in all characteristics tested, except in its requirement for MTK (see next section, subsection "[NEX], LAH2, and mkel.2")

Several variants of L4-dsRNA have been identified by Wickner and co-workers according to their ability to maintain or exclude M2- and M1-dsRNAs in different genetic backgrounds (128, 129, 157, 159, 167). These are listed in Table 9 together with their associated plasmid phenotypes, [HOK], [NEX], and [EXL], and some of their critical properties. These cytoplasmically inherited phenotypes were initially defined as unidentified plasmids but are now known to be phenotypic variants of the L4-dsRNA plasmid. L1A, the dsRNA normally found in K1 killers, carries [HOK] and [NEX] phenotypes and so is called LAH2. L2A, on the other hand, has only weak [HOK] activity (159) and lacks [NEX] function. It can mutate to LAH2, with strong [HOK] and (EXL8) activity (159) (see below). LAHE and LAHE are L-dsRNA species with unique properties which were found in particular sensitive strains (AN33 and 200, respectively; 157, 159) with no known descent from K1 or K2 killers. These forms of LAH2 seem to be part of a continuous spectrum in their ability to maintain M1- and M2-dsRNAs (Table 9). All seem less competent for M2- than for M1-dsRNA maintenance.
TABLE 9. Competence of Lₐ-dsRNA variants for M-dsRNA maintenance*  

<table>
<thead>
<tr>
<th>Lₐ variant†</th>
<th>Genotype</th>
<th>M₁-dsRNA</th>
<th>M₂-dsRNA (MKT⁺ strains)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SKI⁺ (Lₐ-E)</td>
<td>SKI⁺ (Lₐ-H)</td>
</tr>
<tr>
<td>Lₐ-E [EXL]</td>
<td>−</td>
<td>± (low)</td>
<td>−</td>
</tr>
<tr>
<td>Lₐ-H [HOK] [EXL]</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lₐ₂ [HOK]</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Lₐ₃ [HOK]</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>Lₐ₂-H [EXL] [HOK]</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Lₐ₃-H [HOK] [NEX]</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* The copy number of M-dsRNA maintained ranges from zero (−) through low (±), moderate (+), to high (++). ND, Not determined.
† Lₐ-E = Incompetent for maintenance of M₁- or M₂-dsRNA in a SKI⁺ host; excludes M₁-dsRNA from combination with Lₐ-E and maintains M₁-dsRNA in a ski− host at low copy number, a cold-resistant combination; Lₐ-H = Competent for maintenance of M₁-d, but not M₂-dsRNA in a SKI⁺ host; excludes M₂-dsRNA from combination with Lₐ₂, Lₐ₂ = Competent for maintenance of M₁ and M₂-dsRNA in a SKI⁺ host at low copy number, but maintenance of M₁ is recessive to Lₐ₂-E weak (HOK*) Lₐ₂-H = (EXL²) mutant of Lₐ₂ now dominant to Lₐ-E for maintenance of both M₁ and M₂-dsRNA; strong (HOK), Lₐ₃-H (Lₐ₃) = Competent for maintenance of M₁ and M₂-dsRNAs at moderate copy numbers in SKI⁺ hosts (the high levels of M₁-dsRNA supported in ski− hosts leads to cold sensitivity); prevents exclusion of M₁ by Lₐ₃-E, but excludes M₁ in a SKI⁺ mkt− host.

[HOK] and Lₐ-H

The form of Lₐ-dsRNA that is competent for the maintenance of M₁- or M₂-dsRNA in a SKI⁺ nuclear background (Table 9) is called Lₐ-H, where the H subscript stands for [HOK], a cytoplasmically inherited genotype originally called Help Of Killer (157), because of its ability to maintain a purpurated M₁-dsRNA mutant called [KIL-sd1] for ski dependent (167) (see below).

[EXL], Lₐ-E, and Lₐ-H

A sensitive yeast strain, AN33, was found to harbor a form of Lₐ-dsRNA that, on mating with a K2 killer (carrying M₂- and L₂ₐ-dsRNAs) resulted in exclusion of the M₂-dsRNA, producing a sensitive diploid (157). The AN33 plasmid was called [EXL] and later found to be a form of Lₐ-dsRNA now called Lₐ-E (129, 157, 159).

It is now known that Lₐ-E is incompetent for the maintenance of either M₁- or M₂-dsRNA in a SKI⁺ background (Table 9). In a ski− background, it is able to maintain M₁-dsRNA. [KIL-sd1] is now known to be a combination of normal M₁ with Lₐ-E-dsRNA and a ski− mutation. Thus, the test for [HOK] is introduction into a SKI⁺ strain of Lₐ-E and M₁ from a ski host. Maintenance of the K₁− R₁− phenotype in the diploid indicates the presence of Lₐ-E in the SKI⁺ strain. Since SKO⁺ is dominant, since exclusion of Lₐ-H renders the M₁-dsRNA in such strains independent of the ski mutation, Lₐ-H is either replicated with or excludes Lₐ-E. Conversely, the ability of Lₐ-E to exclude M₂ on mating to a K2 killer means that Lₐ-E excludes L₂ₐ or is dominant in its effect on M₂-dsRNA maintenance. It is now known (159; Ball and Wickner, in press; Wickner, personal communication) that L₂ₐ has weak [HOK] activity, being able to maintain both itself and either M₁- or M₂-dsRNA at relatively low copy numbers in a SKI⁺ host. A SKI⁺ diploid produced by mating of an Lₐ-E strain with a strain in which M₁ was maintained by Lₐ-E is a stable, weak killer retaining low M₁ content (Wickner, personal communication). Presumably, it also retained low L₂ₐ content. Possibly a reduction in L₂ₐ on introduction of Lₐ-E is sufficient to cause complete loss of M₂ but only reduction in M₁-dsRNA.

Lₐ-H is the L-dsRNA found in strain 200 (159). It excludes M₂ on mating with a K2 killer ([EXL] phenotype), but is capable of maintaining M₁-dsRNA at moderate copy number in a SKI⁺ background ([HOK] phenotype). It will maintain both in a ski− background.

L₂ₐ and L₂ₐ-H ([EXL]²)

The L₂ₐ found in natural K2 killers has weak [HOK] activity. Introduction of Lₐ-E causes loss of M₂. After mutagenesis of such a K2 strain, ([EXL]²) variants resistant to M₂ exclusion by Lₐ-E were obtained (159). They were found to have acquired a strong [HOK] phenotype in their L-dsRNA, which has tentatively been called L₂ₐ-H (Table 9).

[NEX], Lₐ-HN, and mkt1,2

Lₐ₁, the Lₐ-dsRNA normally found in K1 killers, has strong [HOK] activity. Also, when associated with M₂-dsRNA, it prevents exclusion by [EXL] (Lₐ₁-E). This phenotype is called [NEX] and this Lₐ type is called Lₐ₁-HN (163). Lₐ₁ is, therefore, Lₐ₁-HN. Lₐ₁-HN is also the Lₐ species found in sensitive strains S288c and S7, which have been much used in killer studies. It is the only L-dsRNA found in strain S7 and killer strains K7, K12, and K23.

Lₐ-HN itself excludes M₂ in crosses with K2 killers containing L₂ₐ if the haploid segregant contains a defect in either of two nuclear loci called mkt₁ and mkt₂. Exclusion occurs at 30°C, but not at 20°C (157, 159). L₂ₐ-HN variant form of Lₐ₁ has the ([EXL]²) part of the [NEX] phenotype, but fails to cause exclusion of M₂ in an mkt− strain.

Exclusion of M₂ by Lₐ₁-HN in an mkt− strain is prevented by a ski−, -3, or -4 mutation, but not by ski. Selection for mkt− suppressors of M₂ exclusion has led to the discovery of three further ski− loci called skiₙ, -7, and -8 (114). Mutations in any of these six ski loci lead to a marked increase in Lₐ₁, Lₐ₁C, and M₁-dsRNA copy number (Ball et al., in press). An Lₐ₁-HN M₁ ski− combination causes cold sensitivity which is lost if M₁ is removed. This suggests that M₁ replication requires some cell component critical for growth at low temperature, such as the LTS5 gene product, which is titrated by a high M₁ copy number (114). If Lₐ₁-E replaces Lₐ₁-HN in a ski− strain, M₁ copy number is low and the strain is cold resistant. It has also been observed that the presence of M₁-dsRNA causes a marked reduction in Lₐ₁ copy number (Ball et al., in press).

Mutations in three further loci, called mks₁, mks₂, and MKS50, were found to suppress [NEX] exclusion of M₂ in mkt strains. These mutants, however, did not portray other aspects of the ski phenotype (114).

Exclusion of M₂ by Lₐ₁-dsRNA

Besides exclusion of M₂-dsRNA by [EXL], or by [NEX] in an mkt− background, M₂− is excluded by M₁-dsRNA (98). This is unrelated to the L₁₁₁₁₉₁₉₁-dsRNA also present in K₁ killers, since cells cured of M₁-dsRNA, but retaining L₁₁₁₁₉₁₉₁-dsRNA, no longer exclude M₂ (159).

Conclusion: Mechanisms of Exclusion and Models for Control of dsRNA replication

At present, no coherent explanation exists for the interactions of Lₐ-dsRNAs with themselves, with M₁- and M₂-dsRNAs, and with the products of SKI, MAK, and MKT loci. The differential effects of mutations in these loci on maintenance of different dsRNAs (Table 7) indicate significant differences in mechanisms of replication or its control.
At the same time, the increase in copy number of M1, M2, L-A+, and LBC-dsRNAs caused by ski mutations suggests convergence on a pathway negatively controlled by SKI gene products (Ball et al., in press). Data (our unpublished observations) suggesting mutual down-regulation of L-A and LBC similarly suggest competition in replication. It would be surprising if the dsRNAs failed to share common chromosomally encoded replication components. Nevertheless, a chromosomal defect (clo−) in LBC replication has only recently been recognized (149), and no mutant causing loss of W- or T-dsRNAs is known (see next section). The known maintenance mutants can be organized into a converging hierarchy (Fig. 4), as suggested by Wickner (160). The bias towards recognition of mutations affecting M- or L-A- dsRNAs clearly reflects the ease with which their loss can be detected. One suspects that most of the genetic loci indicated only modulate the efficiency of the actual replication event and that this scheme should not be regarded as a biosynthetic pathway. It is not known whether MAK3,10 and PET18 are required directly only by L-A, or also by M1.

The dependence of M1 on L-A (Fig. 4, positive arrow) seems clearly related to the provision of capsid peptide. Ball et al. (in press) suggest, alternatively, that L-A helps M1 maintenance by antagonizing the action of SKI gene products. The reduction of L-A-HN in the presence of high M1 in a ski− strain (114) (Fig. 4, negative arrow) may reflect competition for a limited capsid supply but might equally reflect competition for an unidentified limiting cellular component, such as the MAK3,10 or PET18 gene product. Investigation of the effects of mixing various combinations of L-A, L2A, L-A-HN, and LBC on their respective copy numbers, in identical nuclear genetic backgrounds, would clarify some of these issues. The permutations of presence of M-dsRNAs, ski, mak, and mkt mutations are obviously large. However, specific issues should soon be resolved. For example, is L2A excluded by L-A and is L-A, itself excluded by L-A-HN?

If recognition by capsid is involved in initiation or elongation events in dsRNA synthesis (14), then exclusion phenomena could arise from competition for capsid between different transcripts, or from abortive binding of nonhomologous capsid. This implies that [HOK], [NEX], and [EXL] phenotypes are aspects of capsid structure. This may become clear upon comparison of capsids and capsid genes in VLPs containing L-A-E, L-A-HN, and other L-A species. The quite distinct peptide maps for L-A-P1 and L2A-P1, however (see above, "Role of L-dsRNA Species in Capsid Production and M1-dsRNA Maintenance"), suggest that results may be difficult to interpret, since both have, or can apparently acquire, M1-specific replication functions implied by the [HOK] genotype. It seems equally likely that exclusion phenomena are due to selective competition between dsRNA species for nuclear gene products required for dsRNA maintenance. For example, L-A-HN and M2 may both require MKT gene products, although these are not required (directly) by M1. Similarly, M1 and M2 may require capsid or an unidentified gene product, for which M1 competes most effectively. This type of competition may also explain the phenotype of suppressive-deletion mutants of M1-dsRNAs ("Summary of Phenotypes ...," subsection "Plasmid Mutations in M1-dsRNA").

The variation in properties of L-A found in different strains may reflect either discrete types or the products of rapid divergent evolution of the dsRNA genome (see "Evolution of the Killer System"). Thus, L-A-E may have evolved for self-maintenance in the absence of a requirement for M1 maintenance, whereas L1A (L-A-HN) has been selected for M1 maintenance and L2A has been selected for M2 maintenance. The production of quantities of K2 toxin sufficient for competition with other yeast strains may require only a relatively low M2 copy number.

T-, W-, and XL-dsRNAs clo− and the Discovery of T and W

Discovery of a complex nuclear chromosomal defect called clo−, which results in failure to maintain LBC at 25°C (maintenance persists at 30°C or higher temperatures), has allowed study of two low-copy-number dsRNA species, unrelated to L and M-dsRNAs, called T and W (149).

Vodkin (141) unexpectedly obtained strains lacking all detectable dsRNA (including L, M, T, and W) by heavy ethyl methanesulfonate mutagenesis of killer strain FM11 [PSI+], selection for [PSI+] phenotype (cytoplasmically inherited enhancement of nonsense suppression), and screening for nonkillers. One such strain, JM6 (141), has recently been found (149) to give a second unexpected result: 2 to 2 segregation of progeny retaining or losing L-dsRNA when crossed with a pet18 strain presumably containing LBC. Strain 1815, a segregant of this cross lacking L-dsRNA (L- phenotype), contains the clo− ("chromosomal L-o") defect which results in frequent segregation of L-o meiotic progeny in crosses to certain strains containing L or but not in crosses to strains carrying L2 or Lc. Because clo− depends on at least two unlinked chromosomal defects, a karl clo− L-o strain was constructed and used to test the dependence of various dsRNAs on clo+. L9, L15, and L28 but not L1A, M1, or M2 were found to require clo+ by this test. The lack of consistency with the meiotic data on Lc is not understood (149).

Electrophoretic analysis of the dsRNAs isolated from clo− L-o strains (149) showed the persistence or enhancement of two unique bands of 2.7 and 2.25 kb, migrating between L- and M-dsRNAs, whose presence has frequently been noted but whose independence of M and L was not previously apparent. They have been called T (2.7 kb) and W (2.25 kb). They are inherited cytoplasmically and strains have been found carrying neither, both, or only W. Thus, T may be
dependent on W, but neither is dependent on any L-dsRNA. Maintenance of W is independent of 25 MAK genes tested, and neither T nor W shows cross-hybridization to each other or to any of the known L- and M-dsRNAs. The copy numbers of both are increased about 10-fold by growth at 37°C, except in strains carrying an unidentified cytoplasmic gene. Encapsulation has not been demonstrated; however, a sucrose gradient peak of T-dsRNA was observed in a crude extract of cells of a strain containing both T and W, migrating between ScV-L and free T-dsRNA (149).

**Independent dsRNA Families and Their Roles in S. cerevisiae**

At least three apparently independent dsRNA families are now recognized in S. cerevisiae: L₁₄-dsRNAs and their M- dsRNA satellites, L₁₄C-dsRNAs, and T and W. Other dsRNA species may exist, and minor bands in addition to T and W are frequently seen on electrophoresis of dsRNA preparations, migrating between L and M. XL, another minor species, is sometimes observed migrating slightly slower than L. The relationship of these species to other dsRNAs is unknown.

None of the three dsRNA families correlates with mitochondrial DNA, the 2μm plasmid, [URE3], [PSI], 20S RNA, or other cytoplasmically inherited phenotypes or genomes (149, 160), and none of them is apparently required for cell maintenance in the laboratory. Their role in the wild is also unknown and their prevalence has not been studied. It is possible that wild-type homothallic yeasts may segregate strains containing unique dsRNA species which have only become mixed in the laboratory. The evidence on "wild-type" Saccharomyces sp. strains is fragmentary, but suggests no pattern. Thus, most wild-type strains seem to contain L₁₄C, W, and frequently T and L₁₄. Exceptions are S3, a mak10 L-o strain (T and W content unknown; 127), and the S. carlsbergensis strains Y3795D andNCYC CB1 which lack 2-μm circular DNA and detectable dsRNA. If these are competent in the wild, then the various dsRNA species may be merely parasitic, tolerated entities lacking survival value. The origin of the satellite killer M-dsRNAs is a separate mystery (see "Evolution of the Killer System").

**STRUCTURE OF THE M₁₄-dsRNA PREPROTOXIN GENE AND STEPS IN TOXIN MATURATION**

**Toxin Is Derived from a Glycosylated Intracellular Proteolysing**

The only radiolabeled species specifically immunoprecipitated from extracts of pulse-labeled cells of K1 killers by antitoxin immunoglobulin G is a 42- to 44-kd species (12). This species is chased into extracellular toxin with a half-life of about 25 min at 30°C (24, 25) and so is a protoxin. This protoxin is converted to a 34- to 35-kd species by endoglycosidase H (12). Since this enzyme cleaves the N-acetyl chitobiose units that link the oligomannosyl side chains in eucaryotic glycoproteins to asparaginyl residues (132), protoxin is a glycoprotein of this type. This is confirmed by the effects of tunicamycin, an inhibitor of the synthesis of the glycosyl donor for this type of protein modification, on protoxin synthesis. Preincubation of cells with tunicamycin (to exhaust donor) and pulse-labeling reveal a modified protoxin of about 34 to 35 kd. This is unstable, but chases only poorly into extracellular toxin (25). Inhibition of glycosylation apparently prevents efficient toxin secretion and lability of the protoxin to intracellular degradation.

Neither α nor β toxin component is glycosylated (107), so that the glycosylated portion of protoxin must be removed during maturation. A model of toxin maturation (see below) is shown in Fig. 5.

**Toxin Is Encoded by M₁₄-dsRNA Which Also Determines Immunity**

Genetic evidence clearly demonstrates M₁₄-dsRNA to be the determinant of both toxin production and immunity. First, both phenotypes are lost concomitantly with loss of M₁₄- dsRNA by curing (heat, cycloheximide, etc.) (46, 95, 151). Second, induced plasmid mutants include both neutral and suicidal types (24). Like naturally isolated killers and neutral mutants, these phenotypes correlate with M₁₄-dsRNA presence in curing and transmission experiments (7, 24).

The identity of M₁₄-dsRNA and the killer determinant was confirmed by demonstrating that M₁₄-P1, the 34-kd single polypeptide comprising more than 90% of the proteins produced from denatured M₁₄-dsRNA by a rabbit reticulo- cyte translation system, contains all of the 20 to 23 Staphylococcus aureus V8 protease-generated peptide components of secreted toxin (11). The cDNA sequence predicts 24 V8 peptides in mature toxin (see below). M₁₄-P1 is very similar in size to deglycosylated protoxin and is specifically immunoprecipitated by antiserum raised against purified toxin (13). M₁₄-P1 is also the product of in vitro translation of both the m and m₁₄ single-stranded in vivo transcripts of M₁₄-dsRNA (9).

M₁₄-P1 was predicted to be identical to the primary in vivo translation product of M₁₄-dsRNA, preprotoxin (11). Since M₁₄-P1 (34 kd) exceeded the size of toxin (19 kd) and its in vitro assayed leader peptide (estimated at 1.6 kd; see below) by about 13 kd, and since toxin was known to lack glycosylation, it was postulated (11, 12) that a glycosylated fragment of protoxin is the immunity determinant and that M₁₄-dsRNA is monocistronic.

**Protoxin Maturation Involves the Normal Protein Secretion (sec) Pathway**

The normal pathway for protein secretion in yeasts resembles that in higher eucaryotic cells and is defined by the series of temperature-sensitive sec mutants isolated by Nivick et al. (102–104). A group including sec18 accumulates secreted proteins in the endoplasmic reticulum (ER) at 37°C and is blocked in transfer to the Golgi. A single mutant, sec7, accumulates protein in Golgi structures and has a leaky block in transfer to secretion vesicles. A third group, including sec1, is blocked in post-Golgi transfer from secretion vesicles to the exterior (Fig. 5) (102). Use of these mutants to study the maturation of invertase demonstrates that core glycosylation occurs in the ER and elongation occurs in the Golgi (42). K1 killer strains of sec18, sec7, and sec1 mutants all produce protoxin of apparently normal size at 37°C (25), indicating that most protoxin modifications occur during cotranslational entry into the ER, with none of the massive elongation of the carbohydrate chains in the Golgi common to secreted proteins such as invertase and acid phosphatase (42), even though protoxin accumulation in sec7 at 37°C indicates Golgi involvement in toxin secretion (Table 10).

The effects of the presence of dog pancreas membrane vesicles, a model of ER function (121), on translation of denatured M₁₄-dsRNA or of the m and m₁₄ in vivo transcripts are consistent with these in vivo results. M₁₄-P1 (34 kd) is converted to a mixture of M₁₄-P1a (about 1.6 kd smaller) and M₁₄-P1b, the size of protoxin (43 kd) (12). Both species become protected against added protease (25), so are presumably located inside the vesicles. Purified M₁₄-P1b is converted to M₁₄-P1a by endoglycosidase H (12). It is probable, therefore, that transport into these vesicles is accompa-
FIG. 5. Proposed maturation of preprotoxin via the yeast secretion pathway. A partial-length plus-strand transcript of M₁-dsRNA is extruded from ScV-M₁ VLPs, associates with ribosomes very near its N terminus, and associates with the ER secretory mechanism by virtue of the hydrophobic leader sequence in the N-terminal δ component of preprotoxin. This component may subsequently anchor the preprotoxin to the membranes of the secretory pathway. Glycosylation of the γ segment occurs in the ER. No known further modifications occur during passage through the Golgi apparatus (defined by the effects of sec18 and sec7 mutations), although KEX1 and KEX2 gene products may function at this stage. Fragmentation of preprotoxin, involving a TPCK-inhibitable protease, occurs in secretory vesicles, before release of the disulfide-bonded α- and β-toxin components by fusion with the cytoplasmic membrane. The γ component is hypothesized to interact with an unidentified cytoplasmic membrane toxin receptor, rendering the cell immune. The fate of δ is unknown. Toxin attacks sensitive cells by interacting with a 1,6β-d-glucan cell wall receptor, possibly via its β component, labilizing linkage to the α component which interacts with the membrane receptor to create the lethal cation-permeable pores. Full-length transcripts are involved in replication, involving encapsidation of nascent dsRNA as it is formed.
nied by removal of a 1.6-kd N-terminal secretion leader or signal peptide by leader peptidase and by asparagine N-glycosylation. Consistent with this model (see Fig. 5), both protoxin and mRNA for M1-P1 were found to be membrane associated in vivo (9).

A more accurate estimate of the size of the peptide removed by these vesicles is derived from translation of S3-dsRNA. The deletion in M1-P1 which produces S3 initiates after 230 bases, within the α toxin peptide region of the M1-P1 gene (Fig. 3; see below). In vitro translation of S3, S3-P1, is an 8-kd species that is precipitated by antitoxin immunoglobulin (G12). It is predicted to consist of the first 72 amino acids of M1-P1, including 28 amino acids of α, and a short C-terminal region derived from sequence at the other side of the deletion, toward the 3′ end of the M1-dsRNA plus strand (Fig. 3). In the presence of membrane vesicles, S3-P1 is converted to S3-P1a (6.4 kd), which does not become glycosylated. It has the normal initiation and leader sequence but lacks the normal glycosylation sites of M1-P1. It is undetectable in vivo (12).

These results demonstrate that toxin maturation involves the normal pathway for protein secretion. Secreted yeast glycoproteins such as invertase gain a core (GlcNAc)2-Manα2(Glu)3 glycosyl subunit in the ER. The estimated size of protoxin indicates that it carries three of these 2.5-kd core subunits.

If this glycosylation event and leader peptidase action were the only modifications to M1-P1 in the in vivo synthesis of protoxin, then endoglycosidase H should convert protoxin to an approximately 32-kd species of the size of the in vitro M1-P1a product. Since the deglycosylated protoxin and the product formed in vivo in the presence of tunicamycin are the same size as M1-P1 preprotoxin, about 2 kd larger than expected (12, 25), either additional modifications occur or the leader peptide removal seen in vitro does not occur in vivo. Other possible modifications might be O-glycosylation or palmitoylation, as found for mammalian secreted proteins (120). Evidence is accumulating that the leader peptide remains intact and may be responsible for the binding of protoxin to membranes in vivo (Fig. 5). It has recently been reported (67), on the basis of parallel data, that secretion into the ER of the yeast α mating factor precursor, encoded by the MFαl gene, also occurs without removal of the N-terminal leader. It is possible that maturation events unique to precursors of secreted proteins, such as protoxin and pro-α factor, may require the N-terminal attachment of these precursors to the membranes of the secretion pathway. The KEX2 function (see below) is a candidate for such an event.

### Conversion of Protoxin to Toxin in Secretion Vesicles Involves a TPCK-Sensitive Protease

Protoxin is stable in a sec18 mutant at 37°C, but has a half-life of only 15 min in a sec1 strain at 37°C, where it is chased into intracellular species of about toxin size. Stability in the leaky mutant sec7 is intermediate (Table 10). This suggests that cleavage of protoxin to toxin normally occurs in secretion vesicles (25). This was substantiated by the observation that tolylsulfonfonyl phenylalanyl chloromethyl ketone (TPCK), an inhibitor of chymotrypsin-like proteases, causes accumulation of protoxin in normal, sec7, or sec1 mutant strains at any temperature (Table 10) (25). The maturation of protoxin in secretion vesicles apparently depends on a TPCK-sensitive protease (Fig. 5). Tolylsulfonfonyl lysyl chloromethyl ketone, the lysyl analog of TPCK and an inhibitor of trypsin-like enzymes, failed to stabilize protoxin in vivo (25).

### Effects of kex1, kex2, and sec Mutations

Nonconditional mutations in two nuclear loci, KEX1 and KEX2, were found to prevent expression of killing while allowing normal expression of immunity in a strain carrying a normal [KIL-k1] plasmid (163). Strains carrying either kex1 or kex2 mutations grow slowly, and the kex2 mutation has a pleiotropic effect on all exocellular proteins and glycoproteins: all are larger and more basic than usual (117). kex2 strains also fail to mate or sporulate (83). They fail to secrete active α factor (66), secreting instead a massively overglycosylated pro-α factor (67). No such effects are seen in a kex1 strain, but both mutations may affect some step common to the maturation of unglycosylated secreted proteins. Neither type of mutant strain secretes an active toxin or antigenically cross-reactive material. Protoxin is stable in a kex1 strain but unstable in a kex2 strain (Table 10). A kex1 kex2 double mutant behaves like a kex1 strain, so that the kex1-determined step probably precedes that determined by kex2. The protoxin is not stabilized by TPCK in a kex2 strain (Table 10), suggesting that the KEX2 step precedes exposure to TPCK-sensitive protease (transfer to vesicles) and that a kex2 mutation renders protoxin sensitive to a TPCK-insensitive protease system. It is suggested (25; Fig. 5) that KEX1 and KEX2 events occur in the Golgi and that protoxin is shunted toward the vacuolar degradative machinery if normal processing is inhibited by a kex2 mutation. Action of the KEX2 product may protect protoxin from this shunt and presumably precedes the sec7 event. Investigation of the biochemical events controlled by these nonessential genes may illuminate aspects of the yeast secretion pathway peculiar to proteins such as protoxin.

### Table 10. Nuclear genes affecting expression of K1 toxin and effects of TPCK

<table>
<thead>
<tr>
<th>Gene</th>
<th>Conditional</th>
<th>K1 killer phenotype</th>
<th>Protoxin half-life (min)*</th>
<th>Comments</th>
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<td>~100 (30)</td>
<td>~100</td>
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<td>~100</td>
</tr>
<tr>
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<td></td>
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<td>~100</td>
</tr>
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<td>ts (37°C)</td>
<td></td>
<td>15 (37)</td>
<td>~100</td>
</tr>
</tbody>
</table>

* Data on half-lives are approximate (25).
* ts, Temperature sensitive.
[KIL-k_{1}] strains carrying the rexl mutation (150) are suicidal and kill themselves at pH 4.7. This mutation has not been studied extensively. It might result in premature toxin activation, degradation of immunity factor, or modification of the hypothetical membrane receptor for toxin so that it is not protected by the immunity determinant.

SEQUENCE OF THE M_{1}-P_{1} PREPROTOXIN GENE

Localization Within the dsRNA of a cDNA Covering Most of the Gene

The in vivo transcripts of M_{1}-dsRNA were fractionated from the bulk of yeast strain K12-1 polyadenylated RNAs by virtue of their tight binding to poly(U)-Sepharose and were used for oligo(T)-primed cDNA synthesis (K. A. Bostian, L. Villa-Komaroff, and D. J. Tipper, unpublished data; 10). After guanine-cytosine tailing and insertion into the PstI site of pBR322, a 900-bp insert was cloned. This plasmid hybridized specifically with denatured M_{1}-dsRNA and its transcripts in Northern blots (9). Sequence analysis of the cDNA (10) and comparison with the available data on the terminal sequences of T158C M_{1}-dsRNA (134, 135) (Fig. 6) demonstrated that one end of the cDNA started with a PstI site encoded in the dsRNA at bases 111 to 116, counting the pppG at the 5' end of the plus strand of M_{1}-dsRNA as the first base. The cDNA sequence corresponding to the N-terminal 20 amino acids of the α toxin component commences at base 146. Sequence corresponding to the N-terminal 20 amino acids of the β toxin component commences at base 713, in the same reading frame as the α component (10). A single TAG stop codon was found in this frame at bases 962 to 964, and if the β component comprises the entire C-terminal fragment (Fig. 6), its predicted size (83 amino acids: 9.5 kd) corresponds well to the estimated 9-kd size derived from gel electrophoresis (Fig. 2). In vivo C-terminal processing of the β component cannot be excluded on the basis of present data. Both alternate reading frames have termination codons scattered throughout the sequence.

Only two ATG triplets, at bases 14 to 16 and 99 to 101, exist within the dsRNA and cDNA sequence preceding the α N terminus. Only the former is in frame with the α and β toxin sequences, and labeling of the N terminus of M_{1}-P_{1} with leucine and valine (20 and 30 amino acids sequenced, respectively) confirmed that in vitro synthesis initiates at the former (10). The length of M_{1}-P_{1} is predicted to be 316 amino acids, with a molecular weight of 34,787, in good agreement with gel estimates.

Domain Structure of the Preprotoxin Processing Sites

A domain structure for M_{1}-P_{1} illustrating its potential processing sites is shown in Fig. 7. The cleavages producing the N termini of α and β are trypsin-like (Arg-Glu, Lys and Arg-Tyr, respectively) and are identified as P2 and P4. No Lys or Arg residues exist between amino acid residues 109 and 134, covering the possible size limits for the C-terminal sequence (134 to 135), a sequence which is cleaved by no known protease type. However, several potential chymotrypsin-type cleavage sites are clustered in the 120 to 132 region where the C terminus of α probably exists. The Trp-Gly bond at 130 to 131 (Fig. 6) is the prime candidate for cleavage by the TPCK-inhibitable enzyme that cleaves proprotoxin in vivo. This site is located in a generally hydrophilic area of this rather hydrophobic protein and is likely to be exposed on the surface of M_{1}-P_{1}. To rationalize the stabilizing effect of TPCK on proprotoxin, and the lack of such an effect for tolylsulfonyl lysyl chloromethyl ketone, we postulate that the C terminus of α is produced by a chymotrypsin-like endoproteolysis (labeled P3, Fig. 7) and that this precedes, and is required for, access of a trypsin-like enzyme to the P2 and P4 sites.

The remaining preprotoxin processing site, P1 (Fig. 7), is that which sequence analysis predicts will be hydrolyzed by leader peptidase during cotranslational transport of M_{1}-P_{1} into the lumen of the ER. The in vitro data using dog pancreas membranes, indicating a loss of about 14 amino acids, make it unlikely that the entire 44-amino acid sequence preceding α is removed in this first step. Recent analyses of leader peptidase sites (e.g., reference 109) indicate that all are preceded by a stretch of 8 to 12 primarily hydrophobic amino acids which may favor a β structure in aqueous and an α-helical structure in a membrane environment (32). This stretch is usually preceded by a positively charged amino acid, which may anchor the preceding region on the cytoplasmic side of the membrane (109), allowing the helical hydrophobic region to span the membrane exposing the leader peptidase site to the luminal surface (32). This site consists most commonly of a β-structure-breaking residue followed, one to three residues later, by an ala-X-ala sequence and a β turn, with cleavage occurring after the second ala residue. Val-X-Ala is the most frequent alternate cleavage site (109). This pattern is followed in the M_{1}-P_{1} N terminus by Arg (residue 10), the hydrophobic stretch Val-Leu (residues 12 to 21) terminated by His followed by Val-Ala-Leu (residues 24 to 27). A cotranslational P1 processing site is predicted at the Ala-Leu (residues 26 to 27) bond (Fig. 7). A further 18 amino acids would have to be removed to expose the α N terminus. The discrepancy of the predicted size of the leader peptide (2.9 kd) with that observed in vitro (12) is large, and both estimates of the size of unglycosylated proprotoxin and experiments on in vivo expression of the cDNA (see "In Vivo Expression of Preprotoxin cDNAs") indicate that, in spite of predictions based on sequence (109), this leader peptide is not cotranslationally removed in yeasts.

All of the Asn-X-Thr/Ser glycosylation sites in M_{1}-P_{1} occur in the region between α and β that is excised during processing. This region (γ, predicted to be 103 amino acids: Fig. 6 and 7) is now the candidate for the immunity determinant (Fig. 5). Both α and β contain three Cys residues. No other Cys residues are found in M_{1}-P_{1}, so a maximum of three disulfide bonds could join α and β. Those shown in Fig. 7 are chosen at random.

Analogies to Insulin and α-Factor Maturation

The maturation of toxin resembles that of insulin (32). Both are processed by removal of N-terminal and internal peptides, leaving two disulfide-linked peptides. Terminal cleavages in both cases occurs within secretion vesicles. A trypsin-like endopeptidase cleaving at Arg, Lys-X is involved in both cases. However, the fragment removed in M_{1}-P_{1} is much larger than the insulin C peptides (25 to 35 residues). The insulin C peptide is highly variable in sequence and, it has been suggested (32), mainly serves to ensure that proinsulin exceeds a critical, minimal size for the mammalian secretion pathway. In larger secreted mammalian proteins such as insulin-like growth factor, the C segment is much shorter. A specific function for γ is suggested by its large size, although it may simply be needed to carry sites for glycosylation. Glycosylation may be a prerequisite for channeling of a protein through the secretion pathway in yeasts. Several additional roles for γ may be envisaged: first, after excision, γ may be the immunity determinant; second, as a
FIG. 6. Sequence of M₁-dsRNA. Sequence from plus-strand bases 1 to 110 is derived from RNA sequencing of the T158c variant (135). The rest of the sequence derives from the cDNA from wild-type M₁-dsRNA of strain K12-1 (10). The start of preprotoxin at “6” and the N termini of the α- and β-toxin components are shown. The start of the γ segment is hypothetical. The cysteine residues potentially involved in binding α and β subunits and the Asn-X-thr/ser glycosylation sites are boxed.

part of protoxin, it may mask the toxin-active site, protecting the producer cell internal membranes. Third, γ may serve to direct the folding and disulfide bond formation of α- and β-toxin subunits. The membrane association of protoxin is unusual and perhaps unique among precursors of secreted yeast proteins, other than pro-α factor.

Secretion of toxin is strikingly similar to secretion of α factor in yeasts. This factor is a 13-amino acid peptide processed from a precursor which, according to its gene sequence, is a 165-amino acid protein with an N-terminal secretion leader, a 60-amino acid central region containing three glycosylation sites, and four tandemly repeated C-terminal α-factor sequences separated by 6- to 8-amino acid spacer regions (80). Release of the proximal α-factor precursor occurs by cleavage at Lys,Arg-Glu bonds and is followed by action of a membrane-associated aminopeptidase (66). It has been recently shown (67) that the three sites do become glycosylated in the ER and fail to become elongated.
Cleavage may initiate in the Golgi and occurs mostly in secretion vesicles. The leader peptide is not removed in the ER. The similarities in organization and maturation of preprotoxin and pre-α factor are obvious, though clear differences in processing exist. There is no evidence for a role for aminodipeptidase in protoxin maturation, and kexl does not affect α-mating-type expression. Since overglycosylation of pre-α factor occurs in a kex2 mutant (67) and normal glycosylation chain extension in invertase apparently occurs in the Golgi (42), this substantiates our prediction of the site of kex2 action as part of traffic control in the Golgi (25) (Fig. 5). Data on both the MFA1 and protoxin systems suggest that a nonglycosylated secreted protein in yeasts must have a glycosylated precursor and that maturation of these particular precursors may require retention of the N-terminal leader sequence as a membrane “anchor.”

Functional Analysis of the Toxin Subunit Structure and γ

Both α- and β-toxin subunits have a relatively high content of both charged and hydrophobic amino acids. All of the excess charge on toxin responsible for its pi of 4.5 resides in the β subunit. This subunit might, therefore, be supposed to provide the proton binding sites for the cation channels produced by toxin in target cell membranes. However, analysis of the preprotoxin sequence for hydrophobicity, averaged over seven amino acids, shows no long stretches of adjacent amino acids in β with high average hydrophobicity which could correspond to the membrane spanning regions of an integral membrane protein (81). In contrast, the α subunit has two such regions of very high hydrophobicity in its C-terminal two-thirds (preprotoxin residues 72 to 91 and 112 to 127) separated by a highly hydrophilic region which contains four acidic amino acid residues and all three of the cysteine residues of α. It seems likely that spontaneous insertion of this region of α into a membrane might be accompanied by a large conformational shift, with topological eversion of this region, so that previously buried hydrophobic regions interact at their surface with the lipid alkane chains and the hydrophilic region forms a central hydrated core, producing the cation channel. Disulfide binding of β to this hydrophilic region in intact toxin could prevent such a shift, protecting the producer cell membranes against attack.

FIG. 7. Schematic domain structure of preprotoxin. Within the N-terminal domain “δ,” the hypothetical leader peptidase site (P1) (109) is shown between residues 26 and 27. Processing sites P2 and P4 release the N termini of the α- and β-toxin components. Disulfide bonds drawn between these two subunits are arbitrarily chosen. The subunits are only separable (in sodium dodecyl sulfate) after reduction (25). The chymotrypsin-like site between α and γ is also hypothetical. Glycosylation (G) sites in γ are indicated. Symbols: □, hydrophobic amino acids; △, acidic amino acids; ▲, basic amino acids; ◇, other.
The killer toxin may resemble the abrin and ricin class of toxins in which receptor binding and toxic domains reside on separate, disulfide-bonded polypeptide chains (106). If so, then β should have affinity for the 1.69-g-d-glucan wall receptor, interaction with which may abrogate its linkage to α, potentially releasing it for interaction with the membrane (Fig. 5).

Since the toxin is predicted to attack the cytoplasmic membrane via a secondary receptor, then immunity factor might interact with this receptor rather than with toxin. After processing of proprotease, fusion of secretion vesicles with the cytoplasmic membrane would expose the hydrophilic, glycosylated portion of M1-P1 (now predicted to be the γ peptide) on the exterior of this membrane where the receptor for toxin presumably exists (Fig. 5). Analysis of hydrophobicity (81) has shown that, like β-toxin component, the γ peptide has no long hydrophobic region that would correspond to a membrane-spanning segment. Thus, if membrane association occurs, it is presumed to involve binding to a membrane protein. No means exists for identifying the immunity factor at present, but coupling of appropriate parts of the M1-cDNA to an expression vector (experiments in progress) should yield a fusion protein which could be used to develop antisera to search for the γ-peptide sequence in vivo. Experiments on in vivo expression (see below) seem to rule out the possibility that immunity factor is derived from a separate reading frame in M1-dsRNA or from the δ portion of M1-P1.

Hydrophobicity analysis (81) emphasizes the capacity of the region of δ from residues 10 to 29 to form a membrane-spanning region, consistent with secretion leader functions and also consistent with a role for δ in anchoring proprotease to the membranes of the secretory pathway (Fig. 5). Its ultimate fate is unknown. Fragmentation of M1-dsRNA at the bubble leads (after denaturation) to in vitro production of a minor 19-kd translation product, derived from the shorter 600-bp terminal fragment (145). This is not produced from intact, denatured M1-dsRNA. Although it appears, therefore, to be an in vitro artifact, it does demonstrate the presence of a long open reading frame in this region of M1-dsRNA that might have significance.

IN VIVO EXPRESSION OF PREPROTOXIN cDNA

The M1-cDNA sequence has been fused, in frame, to the 5′ promoter, upstream control region, and N-terminal secretion leader peptide of the yeast-repressible acid phosphatase PHOS gene (137). After leader peptidase action, a proprotease should be produced containing the last 12 amino acids of the δ region. A sensitive strain transformed with this plasmid remains sensitive in media with high phosphate content, but becomes an immune killer in low phosphate (Fig. 1; S. Hanes, V. E. Burn, D. J. Tipper, and K. A. Bostian, submitted for publication). Only a fraction of the hybrid proprotease produced in derepressed cells is converted to toxin. This fraction is processed to proprotease by leader removal and glycosylation. The residual hybrid preprotease remains unmodified and may be degraded in the cytoplasm. Inefficiency of processing in this construction may reflect problems associated with leader removal and loss of the N-terminal membrane anchor, inefficiency of insertion of the hybrid molecule into the ER membrane, or overloading of the secretion pathway.

It has recently been reported (123, 125) that a similar cDNA has been prepared from denatured strain T158c M1-dsRNA, using an oligo(dT) primer presumably initiating within the bubble (16). The cDNA sequence contains an open reading frame virtually identical to that of the K12-1 preprotoxin (Fig. 5), differing in only four base pairs. Two amino acid changes occur (Ile 103 to Ser and Thr 123 to Ala), both in the α subunit, consistent with the observed difference in α-subunit gel mobility (Fig. 2). The other two changes, within the γ sequence, are silent. The clone extends further than the K12-1 cDNA in both 3′ and 5′ directions. At the 3′ end, a 13-bp insert occurs 16 bp downstream of the TAG stop codon (Fig. 5), and an oligo(A)-rich region located 15 to 40 bp downstream of our sequence (Fig. 5) probably indicates the start of the AU-rich bubble. At the 5′ end, it contains most of the proprotease leader sequence. It has been fused, at two locations, to the N terminus and 5′ promoter of the yeast ADHI gene. Expression of the fused genes should produce preproteases with modified N termini: one differs in only two of the first three amino acids, and the other lacks the first nine. Neither modification affects the hydrophobic membrane-spanning region of δ. Plasmids carrying both fusions, transformed into a sensitive strain, produce immune killers (D. Thomas, personal communication).

The results of both promoter gene fusions demonstrate that the portion of M1-dsRNA cloned contains all of the information needed for toxin production and immunity and strengthen the hypothesis that both functions are contained within M1-P1 preprotoxin. These results seem to rule out δ as an immunity determinant.

The β killer toxin component and α factor are nonglycosylated C-terminal fragments of glycosylated precursors that carry all of the information used for routing through the yeast protein secretion pathway. Commercial interest in the genes for these precursors derives from the hope that appropriate gene fusions, at the processing sites that result in release of the C-terminal components, may result in secretion of polypeptides of interest, lacking glycosylation or an N-terminal prefix. For toxin, this target for gene fusion is the Lys, Arg, Tyr-γβ boundary.

If a DNA plasmid could be designed to give a transcript with normal M1-dsRNA termini, then expression of such a plasmid in a sensitive cell containing ScV-L1A might result in synthesis of the cRNA strand and encapsidation. This will be difficult to achieve and not all of the requirements are understood. However, in this way, stable dsRNA plasmids containing and expressing an internal sequence of choice might be synthesizable.

EFFECT OF PLASMID MUTANTS ON TOXIN MATURATION

Two naturally isolated (N1 and N2) and several ethyl methanesulfonate-induced (NPL-1 to -10) neutral variants of M1-dsRNA have been analyzed for expression of the preprotease gene (Table 11) (24). All have a normal-sized M1-dsRNA, except NLP-3, which had a doublet of M-dsRNAs on agarose gels. Strains NLP-3, -1, and -10 secrete toxin-sized material reacting with antitoxin and retaining 0.1 to 3% of normal toxin activity (Table 11). have normal-sized toxin of normal half-life, and are presumably missense mutations in the α or β toxin components (24, 25). The significance of the additional M-dsRNA band in NLP-3 is unknown. Strains N1 and N2 fail to secrete toxin activity or CRM, although they produce a toxin of normal size. This protein was unusually stable in strain N1 and presumably has a structural modification rendering it resistant to the TPCK-sensitive vesicular protease. Protoxin stability is no greater than normal in strain N2. It is either destroyed intracellularly, possibly being shunted from Golgi to vacu-
TABLE 11. Expression of preprotoxin genes in M<sub>1</sub>-dsRNA mutants (24)

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<tr>
<th>M&lt;sub&gt;1&lt;/sub&gt;-dsRNA variant</th>
<th>Killer phenotype</th>
<th>dsRNA size (kb)</th>
<th>In vitro translation product (kd)</th>
<th>In vivo toxin (kd)</th>
<th>Half-life at 30°C (min)</th>
<th>Secreted</th>
<th>Toxin activity (%)</th>
<th>Protein reacting with antidote</th>
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TABLE 12. Codon usage in the killer preprotoxin gene<sup>a</sup>

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<sup>a</sup> Data are from the preprotoxin sequence shown in Fig. 6. The codons recognized by major tRNAs and almost exclusively used by major mRNAs are underlined. The codons used by preprotoxin and only in the least abundant of eight mRNAs studied (3) are indicated by *. Those used by preprotoxin but not even in this mRNA are indicated by **.
but becomes unstable if mitochondria are reintroduced to the *S. cerevisiae* killers (52). This phenotype bears some resemblance to the effect of a *mak10* mutation on ScV-M1, and suggests incompatibility between mitochondrial DNA and the *K. lactis* plasmids in *S. cerevisiae*. No such effect is seen in the petite-negative *K. lactis* species.

Spontaneous or induced nonkiller mutants of both pGKI-1-containing *K. lactis* and *S. cerevisiae* killers fall into several classes (101, 147, 148): loss of both plasmids, loss of only pGKI-1, central or terminal deletions of pGKI-1, and point mutations in this plasmid. Some of those with central and point mutations are neutral and retain the immunity determinant. An open reading frame for 1,146 amino acids in pGKI-1 presumably codes for a preprotoxin (52), as in the *S. cerevisiae* M-dsRNA system, but no data on intracellular toxin precursors and transcripts, or on the mechanism of toxin secretion, have been published. *K. lactis* chromosomal mutations equivalent to the *S. cerevisiae* *kex* mutations have been isolated (147) and may affect toxin maturation.

The role of pGKI-2 in pGKI-1 maintenance remains conjectural. The two plasmids are not encapsidated and bear no homology either to each other or to nuclear or mitochondrial DNA.

The termini of pGKI-1 have 202-bp inverted repeats. Similar 182-bp repeats are found in pGKI-2 (51). They bear some resemblance, therefore, to the telomeres of yeast chromosomal DNA and are presumably essentially involved in replication.

The stability of these plasmids enhances their potential as cloning vectors. Restriction endonuclease maps are available (148) and sequencing is in progress. Hybrid plasmids between pGKI-1 and the *S. cerevisiae* *URA3* gene stably transform uracil-requiring *K. lactis* giving *URA4* progeny, but the reintegrated plasmids are circular rather than linear, as anticipated (40).

**dsRNA KILLER SYSTEM OF *U. MAYDIS***

*U. maydis*, the smut fungus of maize, causes callus formation in infected tissues and is one of the many plant-pathogenic fungi (37). Killers of three different immunity specificities (P1, P4, and P6) have been found among natural isolates; each type kills the other two but is immune to its own toxin (72–74, 168). The toxins do not kill *S. cerevisiae*. Each killer type is associated with cytoplasmic dsRNAs individually encapsidated in VLPs (71, 75), including at least one representative of each of three size classes: heavy (H), 3.6 to 6.2 kb; medium (M), 0.92 to 1.7 kb; and light (L), 0.35 to 0.36 kb. The distribution of dsRNAs of similar size in representative killer strains are presented in Table 13. M2(4) and M2(6) refer to the species of M2 size found in P4 and P6 killers, respectively. Since the P6 killer strain carries only H1, M2(6), and L, and variants of P1 and P4 killers are known to lack several of the H components listed (71), it appears that the minimal requirements for a killer is an H and an L species plus the appropriate M species: M1(1) for P1, M2(4) or M3 for P4, and M2(6) for P6 killers.

The *U. maydis* virus (UmV) VLPs have a single major 75-kd capsid protein (76) which is probably encoded by one or more of the H dsRNAs (S. Simon, M. Gorecki, and Y. Kolton, Genetics 97:576, 1981), since it is found both in strains containing only H1 and in related strains containing only H3 and H4. Toxin production (76) and immunity (108) segregate with the M dsRNAs (Table 13) and in vitro translation of these dsRNAs produces a 19-kd product antigenically related to the 12-kd toxin (Simon et al., Genetics 97:576, 1981). The analogy to the ScV system is obvious; however, the variety of H and M species and the presence of L species are unique.

Northern gel and heteroduplex analyses of the strains listed in Table 13 demonstrate that the L species in P1 (P1-L) is entirely derived from one terminus of M1(1), whereas P4-L is similarly homologous to one end of M2(4) or M3 (M2 and M3 were not resolved) and P6-L is homologous to one end of M2(6) (45). The significance of the existence of these small (0.36-kb) redundant dsRNAs, derived from the killer determinant dsRNAs, is unknown. L is clearly too small to encode the 19-kd toxin precursor, although it could encode a 7-kd fragment of it, possibly related to immunity.

The Northern gel analysis also demonstrated partial homology between the killer determinants M1(1) and M2(4) + M3. Neither is homologous to M2(1) and neither has homology to M2(6) (45). This is consistent with the partial overlap of immunity specificities between P1 and P4, but the lack of overlap of either with P6 (72).

Probes of H1 from P1, P4, or P6 reacted with the H1 species of all three types, showing relatedness but not identity. The same relationship exists between the H2 species of P1 and P4 but, surprisingly, H1, H2, and H3–H4 species are not related, even within the same strain (45). It seems likely, therefore, that the capsid peptides presumably encoded by these various H species will also differ, like those encoded by the ScV L1A+, L2A+, and L3C dsRNAs. A complexity at least as great as that seen in the ScV-L system is apparent in *U. maydis*.

Limited 3'-sequence analysis has been performed (45) and demonstrates that, as for the L1C–dsRNAs of *S. cerevisiae*, the UmV dsRNAs have either 3'-terminal A-OH or G-OH residues which are presumed to be unpaired and added post-translationally.

**EFFECTS OF dsRNA MYCOVIRUSES ON PATHOGENIC FUNGI**

As stated in the Introduction, killer phenomena in yeast presumably have a substantial impact on competition between related species and (less frequently) between yeast genera for their favored ecological niche. This may also apply to pathogenic species which compete for parasitization of host tissues. The effects of killer plasmids on virulence of animal pathogenic species such as *Candida* and *Cryptococcus* appear to be minimal, and their presence and toxin susceptibility are only important for epidemiological studies at present (9), (92) (113). However, effects of mycovirus infection on the virulence of plant-pathogenic fungi of major economic importance have been reported and are under investigation.

<table>
<thead>
<tr>
<th>dsRNA</th>
<th>Size (kb)</th>
<th>P1</th>
<th>P4</th>
<th>P6</th>
</tr>
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<tbody>
<tr>
<td>H1</td>
<td>6.2</td>
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<td>+</td>
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<tr>
<td>H2</td>
<td>5.0</td>
<td>+</td>
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</tr>
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<td>H3</td>
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<td>+</td>
<td>+</td>
<td>+</td>
</tr>
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<td>H4</td>
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</tr>
<tr>
<td>M1 (1)</td>
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<td>+ (K)</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>+ (K)</td>
<td></td>
</tr>
<tr>
<td>M2 (1, 4)</td>
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<td></td>
<td>+</td>
<td>+ (K)</td>
</tr>
<tr>
<td>M3</td>
<td>1.1</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>L</td>
<td>0.36</td>
<td>+</td>
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</table>

* Sizes are approximate (45). The species determining killer phenotype are indicated by (K). Numbers in parentheses indicate the killer type in which dsRNAs of similar size are found.
Whereas the killer system in \textit{U. maydis} is not known to affect virulence, cytoplasmically inherited dsRNAs found in certain strains of \textit{Endothia parasitica} profoundly affect the virulence of their host (140). \textit{Endothia parasitica} was introduced into North America early in this century, probably from the Orient, and virtually eliminated the American chestnut over the next 50 years. A similar pattern occurred in parallel in Europe; however, hypovirulent strains causing a chronic, nonlethal infection developed there. These strains carry three to five dsRNA species in the size range of 5 to 11 kb, and cytoplasmic transmission of these species correlates with hypovirulence. They have not been further characterized. Although isolated in particles, these particles appear to be membrane vesicles rich in carbohydrate and lipid, but low in protein content, rather than encapsidated VLPs (140).

\textit{Rhizoctonia solani}, a broad-range pathogen for many economically important crops, has also been found to contain dsRNAs associated with hypovirulence (140). \textit{H. victoriae}, the causal agent of Victoria blight in oats, is itself susceptible to a "disease" characterized by stunted mycelial growth, poor sporulation, generalized lysis, and distorted mycelial cell walls (48). Transmission of these characteristics, which markedly reduce virulence for plants, is associated with dsRNA-containing VLPs. Serologically unrelated 190S and 145S VLP species are found, containing one and four dsRNA species, respectively. Healthy, virulent \textit{H. victoriae} isolates contain no dsRNA or only the 190S VLPs. The 145S species is the presumed agent of the disease of the fungal mycelium and appears to be unstable (48). A low (1%) but significant rate of disease transmission was observed on mixing normal \textit{H. victoriae} protoplasts with VLP preparations containing both the 190S and 145S species (48). The 145S species may be a satellite virus of the 190S species, analogous to the relationship between the Scv-M and Scv-L\textsubscript{A} species of \textit{S. cerevisiae} VLPs.

Within the Scv system, killer dsRNA-containing VLPs have been introduced into commercial fermentation strains (160) by mating or cytoduction, to guard against the type of takeover by wild-type killers first observed in sake and beer fermentations (65, 88).

\textbf{EVOLUTION OF THE KILLER SYSTEM}

As pointed out by Holland et al. (61), RNA genomes are found only in viruses parasitic on cells with DNA chromosomes. They may represent relics of a pre-DNA biosphere or self-replicating transcripts of a DNA progenitor. They comprise most of the disease-causing viruses of eucaryotes and profoundly affect the survival and evolution of their hosts.

The error-correcting mechanisms inherent in the DNA synthetic apparatus (e.g., proof-reading exonuclease and marking of the template strand by methylation) are not believed to exist in the various RNA-dependent transcriptases, all of which have virus-encoded components, and evolution of RNA genomes by replication error is rapid, so that selection for replication efficiency is of major importance to the survival of these parasitic genomes. Thus, the frequency of spontaneous temperature-sensitive vesicular stomatitis virus mutants is about 1 to 2% and silent mutations must occur at similar frequency, $10^{-4}$ to $10^{-5}$ per nucleotide per replication (61).

The production of defective-interfering particles, dependent on parental genomes as helper viruses (63), is a graphic illustration of both the frequency and constraints on RNA virus mutation. In persistent infection by a mixture of vesicular stomatitis virus and defective-interfering particles, mutation and evolution may continue toward a state in which the virus is almost incapable of forming infectious particles. If this ability were lost, a plasmid-like state reminiscent of the mycoviruses would result. Mycoviruses might have arisen by a similar mechanism, although the absence of known infectious viruses in fungi may be related to the excellent protection provided by their cell walls.

Whatever their origin, mycoviruses such as Scv-L\textsubscript{A} now exist in apparent amicable equilibrium with their host cells, producing at least one component essential for their own survival, but dependent on many host gene products. Mutation to runaway replication has never been observed, probably because of potential lethality and the absence of transmission. Perhaps more surprising is the stability of Scv-L "infection" implied by the absence of Scv-L-free segregants, in the absence of known physiological advantage conferred by Scv-L on R- K- host cells. These dsRNA genomes seem to be peculiarly stable. The origin of the dsRNA species conferring male sterility on \textit{Vicia faba} (49) is also unknown.

The origin of the Scv-M killer plasmids, satellites totally dependent of Scv-L, is a different problem. They presumably appeared by acquisition of genes unrelated to dsRNA maintenance after the L-dsRNA systems were established. Although they negatively affect Scv-L copy number and must share some replication components, the lack of homology between L- and M-dsRNAs suggests that, if they have a common origin, their divergence is ancient. Perhaps rare nuclear RNA recombinational events (splicing errors?) lead to insertion of information from DNA genes into Scv-L transcripts. The complete killer phenotype would probably have had to evolve as a DNA system before such an event, since toxin production and immunity would presumably be inseparable. Both may have derived from a single transmembrane protein complex involved in controlling cytoplasmic ion concentrations. Perhaps further investigation of the modes of action of toxin and immunity may lead to recognition of normal cellular counterparts of their functional components.

\textbf{LITERATURE CITED}


dsRNA KILLER SYSTEM IN YEASTS


Sommer, S. S., and R. B. Wickner. 1982. Yeast L dsRNA consists of at least three distinct RNAs; evidence that the non-Mendelian genes [HOK], [NEX] and [EXL] are on one of these dsRNAs. Cell 31:429–441.


