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NEW DEVELOPMENTS IN FUNGAL VIROLOGY

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I. Introduction

Although viruses are widely distributed in fungi, their biological significance to their hosts is still poorly understood. A large number of fungal viruses are associated with latent infections of their hosts. With the exception of the killer-immune character in the yeasts and smuts and hypovirulence in the chestnut blight fungus, fungal proper-
ties that can specifically be related to virus infection are not well defined. Typically, fungal viruses are isometric particles 25–50 nm in diameter, and possess dsRNA genomes. Depending on whether the genome is undivided or segmented, the dsRNA viruses are classified into two families, Totiviridae and Partitiviridae (Buck and Ghabrial, 1991a,b).

Mycoviruses are not known to have natural vectors; they are transmitted in nature intracellularly by hyphal anastomosis and heterokaryosis, and are disseminated via spores. Because fungi have a potential for plasmogamy and cytoplasmic exchange during extended periods of their life cycles, and because they produce many types of propagules (sexual and asexual spores), often in great profusion, mycoviruses have accessible to them highly efficient means for transmission and spread. It is no surprise, therefore, that fungal viruses are not known to have an extracellular phase to their life cycles.

Although extracellular transmission of a few fungal viruses has been demonstrated using fungal protoplasts, the lack of conventional methods for experimental transmission of these viruses has been, and remains, an obstacle to understanding their biology (for reviews see Ghabrial, 1980; Buck, 1986; Nuss and Koltin, 1990). The recent application of molecular biological approaches to the study of mycoviral dsRNAs, and the improvements in DNA-mediated fungal transformation systems, have allowed a clearer understanding of the molecular biology of mycoviruses to emerge. Considerable progress has been made in elucidating the genome organization and expression strategies of the yeast L-A virus and the unencapsidated RNA virus associated with hypovirulence in the chestnut blight fungus. These recent advances in biochemical and molecular characterization of the genomes of fungal viruses and associated satellite dsRNAs as they relate to the biological properties of these viruses and to their interactions with their hosts are the focus of this chapter.

II. ISOMETRIC dsRNA MYCOVIRUSES WITH MONOPARTITE GENOMES: THE GENUS Totivirus

The discovery in the early 1960s of the killer phenomenon in the yeast Saccharomyces cerevisiae (Bevan and Makower, 1963), and in the late 1960s, of a similar phenomenon in the smut fungus Ustilago maydis (Puhalla, 1968), eventually led to the discovery of the isometric dsRNA mycoviruses with undivided genomes (Bevan et al., 1973; Wood and Bozarth, 1973; Adler et al., 1976; Buck and Ghabrial, 1991a). Yeast
or smut killer strains secrete a protein toxin to which they are immune or resistant, but which is lethal to sensitive cells (reviewed by Bruenn, 1980; Bussey, 1981; Tipper and Bostian, 1984; Koltin, 1986; Wickner, 1986). Both toxin production and immunity to it are cytoplasmically inherited traits in the yeast killer system. The killer toxins are encoded by satellite dsRNAs, denoted M dsRNAs, which are dependent on helper viruses with monopartite dsRNA genomes for encapsidation (Koltin et al., 1978, 1980; Bostian et al., 1980a,b; Peery et al., 1982; Dalton et al., 1985).

The helper viruses, which belong to the genus Totivirus (family Totiviridae) (Buck and Ghabrial, 1991a), are autonomously replicating viruses that do not require M dsRNA for replication. Moreover, they also encode the RNA polymerase protein required for the replication of both the M dsRNA and their own genomic dsRNA. The helper virus dsRNA and M dsRNA are separately packaged in capsids coded for by the helper virus. From the virological point of view, the killer system in each of the yeast and smut fungi is comprised of a totivirus with associated satellite dsRNAs. The monopartite nature of the genome of the helper virus, particularly in the case of the yeast killer system, is well documented, and its structure, organization, and expression strategy have been elucidated in detail. Therefore, the use of the terms "satellite viruses" or "killer viruses with segmented genomes" to refer to the killer system or to the viruses associated with the killer strains of the yeast and smut is contrary to the accepted terminology, and leads to confusion as to the nature of the genome. In satellite viruses the RNA codes for its own coat protein, whereas in the satellite RNAs the RNA becomes packaged in capsid coded for by the helper virus. Thus, in killer strains, infections with totiviruses are associated with satellite dsRNAs, not satellite viruses. Because the satellite RNAs that encode the killer toxins are not required for the replication of the helper totiviruses, they do not comprise parts of the essential genomes. Therefore, it is inappropriate from the virological point of view to refer to the viruses associated with killer fungi as having segmented or multipartite genomes. For example, we do not consider the cucumber mosaic virus (CMV) satellite RNA, which elicits lethal necrosis in tomato plants coinoculated with CMV, as part of the CMV genome (Simon, 1988).

Unlike the totiviruses associated with the yeast and smut killer systems, member viruses in the family Totiviridae that infect filamentous fungi are not known to be associated with killer phenotypes. However, purified preparations of these viruses are often associated with dsRNA species of unknown origin and could be satellite or defective dsRNAs in nature (Buck, 1986).
A. Taxonomic Considerations

In addition to the genus *Totivirus* (The monopartite dsRNA mycovirus group), the family Totiviridae (the monopartite dsRNA viruses) includes two genera of viruses that infect protozoal hosts: *Giardiaivirus* and *Leishmaniaivirus*. The yeast *S. cerevisiae* virus ScV-L-A (synonymous with ScV-L1) is the type species of the family (Buck and Ghabrial, 1991a). Virus members in the three genera are listed in Table I. A comparison of the properties of the three genera is shown in Table II.

Because the yeast L-A and L1 viruses, which have been the subject of comprehensive studies in two laboratories, are very closely related variants of the same virus (12-base substitution in 4579 bp), no attempt is made in this review to distinguish between them. The designation "yeast L-A virus" is used throughout this article to refer to both ScV-L-A and ScV-L1 viruses.

In addition to the family Totiviridae that comprises viruses with monopartite dsRNA genomes, the International Committee on Taxonomy of Viruses (ICTV) has approved a second family of isometric dsRNA mycoviruses with bipartite genomes, "Partitiviridae" (Buck and Ghabrial, 1991b). This family includes mycoviruses with genomes of two monocistronic dsRNA components, which vary in size among virus members from 1.4 to 2.2 kbp (the dsRNA segments of the individual partitivirus are usually of similar size). One dsRNA segment

| Genus       | Virus                                                                 |
|-------------|----------------------------------------------------------------------|
| *Totivirus* | *Saccharomyces cerevisiae* virus ScV-L-A (synonym ScV-L1)-type species |
|             | *Ustilago maydis* virus UmV-H (P6)                                    |
|             | *Helminthosporium victoriae* 190 S (Hv190S) virus                     |
| Possible members | *Aspergillus foetidus* virus S (AfV-S)                            |
|             | *Aspergillus niger* virus S (AnV-S)                                  |
|             | *Gaeumannomyces graminis* virus 87-1-H (GgV-87-1-H)                  |
|             | *Mycogone perniciosa* virus (MpV)                                   |
|             | *S. cerevisiae* virus L-BC (ScV-L-BC; synonym ScV-La)                |
|             | *U. maydis* virus UmV H (P1 and P4)                                  |
|             | *Yarrowia lipolytica* virus (YIV)                                    |
| *Giardiaivirus* | *Giardia lamblia* virus (GLV)-type species                        |
|             | *Trichomonas vaginalis* virus (TVV)                                  |
| *Leishmaniaivirus* | *Leishmania braziliensis* virus 1-1 (LRV1-1)-type species          |
TABLE II

COMPARATIVE PROPERTIES OF THE GENERA IN THE FAMILY Totiviridae

| Property                  | Giardiaivirus | Leishmaniavirus | Totivirus |
|---------------------------|---------------|-----------------|-----------|
| Particle diameter         | 33⁺           | 30⁺             | 40–43     |
| Density in CsCl           | 1.40–1.42     | ND              | 1.40–1.43 |
| dsRNA size (kbp)          | 7.0–7.5       | 5.0             | 4.6–6.7   |
| Capsid protein size (kDa) | 100           | 80              | 73–88     |

Data are summarized from Wang and Wang (1991) and Buck and Ghabrial (1991). A. Most likely an underestimate, considering the size of the dsRNA packaged. B. ND, not determined.

encodes the capsid protein (CP) and the other segment encodes an unrelated protein, probably the RNA-dependent RNA polymerase (RDRP) (Buck, 1986). Another group of isometric dsRNA mycoviruses, the *Penicillium chrysogenum* virus group, comprises viruses with genomes of three or four apparently monocistronic dsRNA segments. Because it is not known how many dsRNA segments are required for replication, this group has been tentatively classified as the genus "Chrysovirus" in the family Partitiviridae. Although the physicochemical and biochemical properties of many of the viruses in the family Partitiviridae have been well characterized, it is surprising that none of these viruses have been characterized at the molecular level.

B. Virion Properties

1. Morphology and Physicochemical Properties

Virions are isometric, 40–43 nm in diameter, with no envelope. Symmetry of particles has not been determined. Negatively stained virions of *Helminthosporium victoriae* 190 (Hv190) virus are shown in Fig. 1 as an example of a totivirus.

The *Mr* of the yeast L-A virions is estimated at 12.3 × 10⁶ (Esteban and Wickner, 1986). The sedimentation coefficient *s*₂₀,ₘ (in Svedberg units) for members of the Totivirus genus are in the range of 160 S to 190 S. Particles lacking nucleic acid sediment at the rate of *s*₂₀,ₘ = 98–113 S. Buoyant density in CsCl [ρ CsCl (g/cm³)] = 1.40–1.43. Isolates of the yeast L-A virus and the smut UmV-H may have additional components, containing satellite or defective dsRNAs, with different sedimentation coefficients and buoyant densities. Purified virus preparations from killer yeast strains contain, in addition to the yeast L-A virions, two density components: ScV-M-light (ρ CsCl = 1.3513) and ScV-M-heavy (ρ CsCl = 1.3834), with one and two molecules of M.
Fig. 1. Electron micrograph of a purified preparation of the totivirus Helminthosporium victoriae (Hv190S) virus negatively stained with 1% uranyl acetate. Bar, 50 nm.

dsRNA (1.8 kbp), respectively (Esteban and Wickner, 1986). These two ScV-M components can be separated by CsCl equilibrium density gradient centrifugation. The M dsRNA encodes the killer protein. The yeast L-A virus preparations may also contain small quantities of one or more dsRNA species with sizes between 0.7 and 1.6 kbp, denoted as S (suppressible) dsRNA. All S dsRNAs are derived from M dsRNA by internal deletion, in a manner similar to the evolution of defective interfering animal virus RNAs. Suppressive sensitive strains of yeast, in which the killer functions have been lost, are known to contain S dsRNA in place of M dsRNA (Bruenn, 1986).

2. Nucleic Acid

Virions contain a single linear molecule of uncapped dsRNA 4.7–6.7 kbp in size. Some virus isolates contain additional satellite dsRNAs
which encode "killer" proteins; these satellites are encapsidated separately in capsids encoded by the helper virus. As indicated earlier, some virus isolates may contain, additionally or alternatively to the satellites, defective dsRNAs.

The complete nucleotide sequence (4579 bp) of the yeast L-A (L1) dsRNA is deposited as European Molecular Biology Laboratory accession number J04692 (X13426). The (+) strand has two large open reading frames (ORFs) that overlap by 130 bases. The first ORF encodes the viral major capsid polypeptide, with a predicted size of 76 kDa. The two reading frames together encode, via translational frame shift, the putative RDRP as a fusion protein (analogous to gag-pol fusion proteins of the retroviruses), with a predicted size of 170 kDa (Diamond et al., 1989; Icho and Wickner, 1989). Sites essential for encapsidation, transcription, and replication have been defined (see Section II,E).

3. Viral Proteins

There is a single major capsid polypeptide species, with an $M_r$ of $73-88 \times 10^3$. Protein kinase activity is associated with Hv190S virions (Ghabrial and Havens, 1992); capsids contain phosphorylated forms of the coat protein (see Section II,F,4). RDRP is present. In the yeast L-A virions RDRP occurs as one or two molecules of the 170-kDa fusion protein. The pol domain of the gag-pol fusion protein has an ssRNA binding activity (see below).

C. Genome Organization

The complete nucleotide sequence of the yeast L-A virus dsRNA (4579 bp) has been determined (Diamond et al., 1989; Icho and Wickner, 1989). The (+) strand RNA (4580 bases, as it contains an unpaired A residue at its 3' terminus) contains two ORFs, with no ORFs on the (-) strand RNA. The (+) strand RNA of the L-A virus has been identified by cell-free translation and sequencing of in vitro transcripts of virion dsRNA. The presence of two ORFs in the yeast L-A dsRNA is unusual for a dsRNA virus, since all viral dsRNAs, with the exception of the reovirus s1 dsRNA (Munemitsu et al., 1986), have single ORFs. It should also be mentioned in this respect that the yeast L-A virus, as well as other members of the family Totiviridae, is unique among dsRNA viruses in that it possesses an undivided genome.

The first ORF1 in the L-A (+) RNA strand starts with AUG at base 30 and terminates with UAA at position 2072 (Fig. 2). Thus, ORF1 (2043 bases) can encode a protein with a predicted size of 76 kDa (680 amino acid residues). Several lines of evidence strongly indicate that ORF1, designated cap (Diamond et al., 1989) or gag (Wickner, 1989),
codes for the major CP: (1) The principal translation product, in a cell-free system, of denatured L-A-dsRNA, or full-length transcripts of the genomic dsRNA, generated in vitro from purified virions, co-electrophoreses with authentic L-A coat protein and is specifically immunoprecipitated with antisera raised against L-A virions or CP. (2) The predicted size of the CP (76 kDa), based on ORF1 sequence is at the lower range of the 76–88 kDa of the reported estimates for the major CP, based on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analyses. Post-translation modification of totivirus CPs may contribute to their anomalous behavior in SDS–PAGE (see Ghabrial and Havens, 1992). (3) Yeast ribosomes are known to initiate at the first AUG, and even though there are other AUGs in frame with the first AUG, none of the potential ORFs is large enough to encode the CP.

ORF2, which is in the -1 frame with respect to ORF1, codes for an RDRP with features similar to the RDRPs of (+) ssRNA viruses and dsRNA viruses (Bruenn, 1991). The two ORFs together encode, via a -1 ribosomal frame-shifting event (Fig. 2), a gag–pol-like fusion protein (CP–RDRP) with a predicted size of 170 kDa. The 170-kDa fusion protein, which is resolved by SDS–PAGE as a 180-kDa minor protein.

![Diagram of L-A ORFs](image_url)

**Fig. 2.** Two L-A ORFs encode chimeric RNA polymerase–RNA-binding protein with major coat protein domain. Shown is the genome organization of the yeast virus L-A (+) strand RNA. ORF1 encodes the major capsid protein. ORF2 overlaps with ORF1 by 130 nt and is expressed, via a -1 ribosomal frame shift, as a fusion protein with a predicted size of 170 kDa. The ORF2 domain has ssRNA binding activity and contains conserved amino acid sequence motifs (SG...T...NT...N and GDD) characteristic of viral RNA-dependent RNA polymerases. The encapsidation signal [for virus binding site (VBS)] is present on the (+) strand 400 bases upstream of the 3' terminus. Overlapping with the VBS is the internal replication enhancer (IRE), which is necessary for full template activity of (+) strand RNA. (Courtesy of R. B. Wickner.)
in L-A capsids, shares a common antigenic domain with the major capsid polypeptide (Ridley et al., 1984; Fujimura and Wickner, 1988a). The 170-kDa protein also shares an antigenic domain with a sequence encoded by the 3' end of the L-A (+) strand RNA (Diamond et al., 1989; Fujimura and Wickner, 1988a). There are a number of cis-acting sites in the dsRNA molecule, including a viral binding site (VBS) believed to be involved in particle assembly, an internal replication enhancer (IRE), and a site controlling viral interference (INS). The cis-acting signals in the yeast L-A virus dsRNA genome are discussed in detail in Section II,E.

D. Replication Strategy and Virion Assembly

Because of the lack of suitable infectivity assays for mycoviruses, there is little information on whether virus dsRNA replication is synchronized with cell division. Although synchronized cultures of yeast can readily be obtained, there is disagreement on whether dsRNA synthesis occurs throughout the cell cycle or occurs through the G1 phase and ceases in the S phase, when DNA synthesis takes place (Newman et al., 1981; Zakian et al., 1981). In vivo studies involving density transfer experiments indicated that the replication of the yeast L-A dsRNA (as well as the M dsRNA) occurs conservatively in a sequential manner (Sclafani and Fangman, 1984). This mode of replication is analogous to that of the animal reoviruses. Thus, the parental dsRNA duplex remains intact, with the two strands of the progeny dsRNA molecules being synthesized asynchronously. The (+) strand RNA is synthesized first (on the parental dsRNA template), followed by (−) strand synthesis on the (+) strand, which is released from the first particle and now packaged in a separate particle.

With the exception of the yeast L-A virus, information on the replication cycle of totivirus dsRNA has mainly been derived from in vitro studies of virion-associated RNA polymerase activity and the isolation from infected cells of particles representing various stages in the replication cycle. For the yeast L-A virus, in vitro systems are available for studying in detail the various steps in the replication cycle (see below). In vitro reactions the virion-associated RNA polymerase activity of the yeast L-A virus (Herring and Bevan, 1977; Welsh et al., 1980; Bruenn et al., 1980; Fujimura et al., 1986), UmV-H1 (Ben-Zvi et al., 1984), or Hv190S virus (Ghabrial and Havens, 1989), isolated from lag phase cultures, catalyzes end-to-end transcription of dsRNA by a conservative mechanism to produce mRNA for capsid polypeptide, which is released from the particles. Purified virus preparations of the yeast L-A virus, isolated from log phase cells, contain a less dense class of
particles which package only (+) strand RNA (Fujimura et al., 1986; Fujimura and Wickner, 1987). In in vitro reactions these particles exhibit a replicase activity that catalyzes the synthesis of (−) strand RNA to form dsRNA. Thus, the (+) strand RNA is the species that is packaged to form progeny particles, and serves as the template for (−) strand synthesis. The mature progeny particles, which attain the same density as that of the dsRNA-containing virions isolated from the cells, are capable of synthesizing and releasing (+) strand RNA. This completes the replication cycle (Fig. 3). It is not known whether the (+) strand RNA associates with coat protein subunits or a preformed capsid (see below for a proposed model for virion assembly).

In the replication cycle of dsRNA viruses, like those of (+) strand RNA viruses and retroviruses, the viral (+) strands serve as mRNA, as the species packaged to form new virions and as a template for replication. It is thus essential that the packaging and replication steps have sufficient specificity to prevent propagation of nonviral RNAs at the expense of the virus (Wickner, 1989). The nature of the packaging and replication signals also gives clues about the interactions of the replication apparatus with its template, and knowledge of such signals is important for the development of vectors based on RNA viruses (see below).

Replication of the satellite M dsRNA associated with the yeast L-A virus also occurs conservatively (Williams and Leibowitz, 1987). However, the (+) strand RNA transcripts from M dsRNA may or may not be retained within the particle, depending on whether it contains one or two molecules of dsRNA (Fig. 3). The retained (+) strand serves as a template for (−) strand RNA synthesis to form a second molecule of dsRNA in the same particle (a headful replication mechanism). Particles containing one or two molecules of M dsRNA have been isolated from cells, as indicated earlier. The rationale for the headful mechanism is that the satellite dsRNAs are packaged in helper virus-encoded capsids designed to accommodate a much larger dsRNA molecule. Therefore, the particles may contain one or more molecules of satellite or defective dsRNAs, depending on when full capacity is attained (Esteban and Wickner, 1986).

Since the minor 170-kDa protein but not the major coat protein has a consensus amino acid sequence for RDRP (Pietras et al., 1988; Icho and Wickner, 1989) and for a ssRNA binding activity (Fujimura and Wickner, 1988b), the 170-kDa protein in empty particles is proposed to be responsible for these two activities. Present evidence supports the following virus assembly model, which is based on the nature of the 170-kDa protein: The ORF2 domain of the 170-kDa protein specifically recognizes and binds to the viral (+) ssRNA. The major coat protein
Fig. 3. Replication cycle of the yeast L-A dsRNA (top), its deletion mutant X dsRNA, and the associated satellite M dsRNA (bottom). Both (+) and (−) strand RNA syntheses take place within the virions. (+) Strand synthesis occurs conservatively from the dsRNA template, and the newly synthesized (+) strand RNA is extruded from the particles. The released (+) strands serve as mRNA, as the species that is packaged to form progeny virions, and as the template for (−) strand RNA synthesis. Mutants in the chromosomal MAK10 and PET18 genes have structurally unstable virions (Fujimura and Wickner, 1987). Both M and X dsRNAs are less than half the size of L-A dsRNA, and depend on it for viral proteins. As in the replication cycle of L-A dsRNA, the (+) strand RNA of X or M dsRNAs are packaged and replicated. Because the virions are designed to accommodate one L-A dsRNA molecule per particle, the newly synthesized (+) strand RNAs are not extruded. As a result, the retained (+) strand is copied to form a second dsRNA molecule in the same particle, and the process continues until the particle is full, hence the term “headful replication.” (Courtesy of R. B. Wickner.)
domain of the 170-kDa protein then primes capsid assembly by homologous association with free major coat protein subunits (Fujimura and Wickner, 1988a; Fujimura et al., 1992).

E. In Vitro Systems for Identifying cis-Acting Sites

To study the details of totivirus transcription and replication requires the development of in vitro systems in which it may be possible to separate the enzymes involved from the templates, modify the templates if desired, use alternative templates, and then recombine the reactants to reconstitute the activity. Such in vitro systems are now available for the yeast L-A virus and have allowed a detailed analysis of the template sites and the enzymes involved in these processes. Furthermore, because the yeast host is amenable to genetic manipulation, regulation of viral transcription, replication, and encapsidation can also be studied (for reviews see Wickner, 1989, 1991, 1993).

Empty L-A viral particles (produced following the release of dsRNA as a result of exposing mature virions to low-ionic-strength conditions) are used as the enzyme source in these in vitro systems. The empty particles can (1) transcribe added viral dsRNA to make (+) strand RNA conservatively (Fujimura and Wickner, 19891, (2) specifically bind to viral (+) strand RNA (Fujimura and Wickner, 198813; Esteban et al., 1988; Fujimura et al., 19901, and (3) replicate the viral (+) strand RNA to synthesize its dsRNA form in the presence of a host factor(s).

The satellite M dsRNA and the defective X and S dsRNAs, which are packaged, replicated, and transcribed in L-A-encoded capsids and which are expected to contain all of the cis-acting sites necessary for these processes (Esteban and Wickner, 1988), have provided convenient templates and valuable tools to define the cis-acting sites using these in vitro assays. M1 dsRNA (1.8 kbp), encoding a secreted protein toxin and immunity to that toxin (reviewed by Bussey, 1981; Bussey et al., 19901, is a satellite dsRNA, depending on L-A for its own replication (Bostian et al., 1980a; Sommer and Wickner, 1982). S14 dsRNA (793 bp) is derived from M1 by internal deletion (Bruenn, 1986); the deletion breakpoint is at position 253 of M1. Thus, S14 contains the 3′-terminal 540 nucleotides (nt) of M1. X dsRNA (530 bp), which is a deletion mutant of L-A (Esteban and Wickner, 1988), proved to be particularly valuable in these in vitro systems because it contains only the first 25 nt of the 5′ end of the L-A (+) strand RNA (Esteban et al., 1988); 490 nt of the remaining 505 bp are derived from the 3′ end of the L-A (+) strand RNA. Since X dsRNA is transcribed, encapsidated, and replicated in L-A viral particles (Esteban and Wickner, 1988), X RNA must include all of the cis sites necessary for these processes.
Transcription vectors containing cloned cDNA to X and M dsRNAs (Esteban et al., 1988) have been constructed and used to produce altered viral RNAs, which are then tested in the in vitro system.

The in vitro reaction to study the cis-acting sites required for replication (Fujimura and Wickner, 1988b) comprises empty particles, ssRNA template, polyethylene glycol (PEG), four nucleotide triphosphates, and a host factor fraction (a partially fractionated crude cell extract prepared from virus-free cells).

The in vitro system described by Fujimura and Wickner (1989) to study the cis-acting signals required for transcription is the first one known in which added dsRNA is transcribed into ssRNA. The reaction mixture used in this system is similar to the in vitro replication reaction discussed above, except that the template added is dsRNA and that there is a requirement for a much higher concentration (20% w/v) of PEG. The higher concentration of PEG may be necessary for the in vitro system to enhance the aggregation of the reactants and thus the accessibility of the template to the transcriptase.

It is believed that the 170-kDa CP-RDRP fusion protein is involved in both transcription and replication. Although it is not known how the same protein may catalyze two apparently different reactions, several possibilities have been discussed by Fujimura and Wickner (1989), including the hypothesis that different host-supplied proteins (host-encoded enzyme subunits) may be involved in the two reactions; these host proteins must be packaged in the particles in the process of encapsidation of the ssRNA template because the dsRNA-synthesizing particles (when mature) are converted in vitro (in the virion) to (+) strand RNA-synthesizing particles. In view of the latter finding, it is unlikely that irreversible modification of the 170-kDa protein is responsible for switching from replicase to transcriptase. Phosphorylation/dephosphorylation of the capsid protein of the totivirus Hv190S virus has been proposed to play a role in the switching of templates by the CP-RDRP fusion protein (Ghabrial, 1994; Ghabrial and Havens, 1992). Evidence for virion-associated protein kinase activity in the Hv190S virus has been reported, and the possibility that the kinase is host encoded has been discussed (Ghabrial and Havens, 1992).

1. Virus Binding Site (VBS)

The binding of empty L-A viral particles to viral (+) strands can be monitored by a gel retardation assay (Esteban et al., 1988, 1989). Using in vitro transcripts of cloned cDNA to X dsRNA and mutant derivatives, the binding site has been assigned to nt 121–154 (Esteban et al., 1988, 1989; Fujimura et al., 1990). Computer programs for secondary structure predict that this sequence should form a stem–loop
structure with a protruding A residue at the 5' side of the stem (Fig. 4). Fujimura et al. (1990) reported that the secondary structure, but not the nucleotide sequence of the stem, is essential for binding. Elimination of the protruding A residue or its substitution with U, C, or G destroyed binding. A similar structure, a stem–loop with a protruding A residue, has been reported to regulate viral genome expression and is believed to play a role in the initiation of assembly of several coliphages (Beckett et al., 1988). In these viruses the coat protein binds to the stem–loop and represses expression of viral replicase. Removal of the bulging A residue from the stem abolishes binding of the coat proteins.

The loop sequence 141–GAUCC–145 in X dsRNA (Fig. 4, black box) has also been determined to be essential to binding, since its substitution with AGCUU removed activity. Although the stem–loop structure (from nt 131–154) is adequate for binding, addition of 10 extra nucleotides 5' of the stem (nt 121–130) enhanced binding activity to that of the control containing the entire X sequence (Fujimura et al., 1990). Therefore, it is concluded that the 34 nt of X (+) strand RNA from nt 121–154 [corresponding to nt 4170–4203 of L-A (+) strand RNA] are sufficient for binding to empty viral particles. This enhancement is probably due to stabilization of the interaction with viral protein or host factor.

Using transcription vectors containing cloned M1 dsRNA and testing the generated transcripts for binding activity indicated that nt 1377–1406 of M1 are sufficient for binding empty viral particles. This region in M1 is similar in structure and location to the L-A site (Fig. 4). It is of interest that the loop sequence in X (+) strand RNA has only 1 nt mismatch with the loop sequence of M1's (Fig. 4, black boxes). When the X' loop sequence was mutated to M1's (the C at position 144 was changed to U), the modified RNA showed binding activity similar to that of the control (Fujimura et al., 1990).

2. The in Vitro VBS is the in Vivo Encapsulation Signal

To investigate in vivo encapsidation signals, cDNA fragments representing X dsRNA nt 64–448 that retain the VBS (nt 131–154) or only a 44-bp fragment (nt 121–164) were inserted into yeast expression vectors under the control of an inducible promoter (Fujimura et al., 1990). The plasmids were transformed into a yeast strain harboring the L-A virus. The results indicated that the heterologous transcripts containing X's binding site (derived from either the 425- or 44-bp insert) were packaged in vivo into L-A-encoded capsids. Likewise, a cDNA clone representing the 40-nt binding sequence of M1 dsRNA (nt 1377–1416) that contains the stem–loop sequence and 10 bases 3' and
FIG. 4. Nucleotide sequences of the viral binding site (VBS) and the internal replication enhancer (IRE) of X dsRNA (top) and the satellite M1 dsRNA (bottom). (Top) X dsRNA (530 bp) is derived from L-A dsRNA by internal deletion such that bases 43-530 of X's (+) strand are derived from the 3' terminus of L-A dsRNA. The sequence from nucleotides 131 to 154, which is potentially capable of forming a stem-loop structure with a protruding A residue on the 5' side of the stem, has been shown to be necessary for binding to opened empty particles in vitro (and to comprise the encapsidation signal in vivo). The boxed sequence is a 10- of 11-base direct repeat sequence within the IRE region. (Bottom) A structure in M1 resembling the internal site (combined VBS and IRE) in X dsRNA is found and was shown to have VBS activity. The loop sequences (black boxes) of X and M dsRNAs have only 1 nt mismatch and are interchangeable for VBS activity. (Courtesy of R. B. Wickner.)
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5' to the stem was inserted into the yeast expression vector. When expressed in vivo, the heterologous RNA with the M1 binding sequence was encapsidated in L-A-encoded capsids. Therefore, the 40-nt sequence in the M1 (+) strand or the 44-nt sequence in X RNA which are sufficient for binding to empty particles in vitro are also the in vivo signals for encapsidation in L-A-encoded capsids.

Because the VBS (nt 121–154) of X dsRNA and the IRE (nt 111–145, see below) partially overlap, it was necessary to establish the precise identity of the in vivo encapsidation signal. For this purpose, Fujimura et al. (1990) substituted the 5-nt 149–ATTTT–153 from the 3' side of the stem with 149–TAAAG–153 from the 5' side in the transcript expressed in vivo. This change, which is external to the IRE region but in the VBS, eliminated in vivo encapsidation. Thus, it is the VBS rather than IRE that corresponds to the in vivo encapsidation signal. The finding that the sequences in the L-A genome required for binding to empty particles in vitro are the in vivo encapsidation signal for L-A virus and its satellite M1 dsRNA should allow the definition of the region of the CP–RDRP fusion protein responsible for the site-specific binding (encapsidation) and the development of an RNA virus vector system using the known encapsidation, transcription, and replication signals.

3. cis-Acting Sites Required for Replication

By testing the template activity of T7 RNA polymerase transcripts of cloned cDNA to X dsRNA in the in vitro replication system, Esteban et al. (1989) have determined that both the 3'-end 30 bases and an internal site on the (+) strand are necessary for optimal replication [in vitro (−) strand synthesis]. Changing any one of the 3'-terminal 3 bases eliminates template activity, but the 3'-terminal 5 bases of M1 (+) strand can replace the 3'-terminal 4 bases of X (+) strand (Fig. 4). A subterminal stem–loop structure (within the 3'-end 30 nt) is also important for template activity (Fig. 4). The 3'-terminal 33 bases of M1 dsRNA can substitute for the 3'-end 30 bases of X dsRNA. The other is an internal site 400 nt away from the 3' end (IRE) that partially overlaps with the VBS (Esteban et al., 1988, 1989; Fujimura et al., 1990).

Evidence has been presented in support of the conclusion that VBS is the cis-acting encapsidation signal in vivo for the L-A virus (Fujimura et al., 1990). Since the IRE largely overlaps with the VBS, the segment including both the IRE and the VBS is referred to as the internal site (Fujimura and Wickner, 1992). A structure resembling X's internal site is found in M1 dsRNA (Fig. 4) and has been shown to have VBS activity (Wickner, 1989). Elimination of the internal site
reduces the template activity 5- to 10-fold. Thus, the enhancement of the replication reaction (which initiates at the 3' end of the template) is probably mediated by an increase in the local concentration of RDRP in the vicinity of the 3' terminus. When the internal site and the 3'-end site were split into two distinct RNA molecules, the internal site could no longer stimulate replication (no trans-activation). However, establishment of an intermolecular hydrogen bonding between these RNAs restored the replication-enhancing activity of the internal site (Fujimura and Wickner, 1992). This finding is consistent with a model in which L-A's RDRP interacts first with the internal site and then with the 3'-end site either by looping or by a local dissociation–reassociation mechanism. Furthermore, it clearly eliminates the anchored tracking and sliding models which require continuity of the RNA molecule between these two cis sites (Fujimura and Wickner, 1992). This conclusion is supported by the results of Esteban et al. (1989), who reported that deletions of up to 300 bases between the internal site and the 3' site had no significant effects on replication.

It is not known whether the VBS and the IRE, whose sequences largely overlap, are distinct but functionally related sites. Clearly, the VBS activity is prerequisite to the IRE activity. Fujimura and Wickner (1992) argued that the VBS-bound RDRP may be transiently activated by the IRE to interact with the 3' site on the same molecule, but not for trans action. Alternatively, the strong VBS binding necessary for encapsidation may impede the subsequent replication. The IRE may function in loosening this binding, allowing the RDRP to interact with the 3' site. The IRE includes a direct repeat (boxed, Fig. 4), the second of which forms part of the VBS stem. The first (5'-proximal) repeat could potentially participate in stem formation in place of the second (3'-proximal) repeat. This obviously leads to the abolishment of the VBS activity because of the importance, to the binding activity of VBS, of the A residue bulging from the stem, the loop sequence, and the stem–loop structure of the VBS (Fujimura et al., 1990).

4. cis-Acting Signals Required for Transcription

As discussed earlier, mature virions of totiviruses containing dsRNA have transcriptase activity and synthesize (+) strand RNA conservatively. Empty particles have transcriptase activity that uses added viral dsRNA as templates, and the transcription reaction in such an in vitro-reconstituted reaction also occurs conservatively (Fujimura and Wickner, 1989). It has been proposed that all of the cis-acting signals for transcription must reside in the 3'-terminal 25 nt of the L-A (-) strand RNA because these are the only ones present in the
deletion mutant X (−) strand that are derived from that region. Examination of the sequence of the 3' 25 nt of the L-A (−) strand RNA reveals that it is 88% AU. Other dsRNA viruses contain comparable AU-rich regions (Fig. 5). These AU-rich regions are suggested to facilitate the formation of the transcription–initiation complex by allowing the partial melting of the dsRNA. The mere presence of an AU-rich sequence at the 3' end by itself is not sufficient for recognition by the L-A transcriptase. For example, the φ6 dsRNAs were not transcribed by the L-A transcriptase (Fujimura and Wickner, 1989), even though 5 of 6 nt at the 3' ends are identical to those of M dsRNA (Fig. 5). This finding suggests that L-A transcriptase requires specific sequence or structure at the 3' ends of the (−) strands in addition to the AU-rich property.

The presence of the cis-acting signal for transcription at the 3' end of the (−) strand favors the propagation of only intact L-A genomes, as can be reasoned by the fact that all of the signals for encapsidation and replication are within 400 bases at the 3' end of the (+) strands. The 3'-end fragments that may result from partial degradation of L-A (+) strand RNA in vivo and that may be packaged and converted to dsRNAs are not expected to be transcribed, thus reducing the chances of generating defective particles.

5. The Frame-Shifting Site

Ribosomal frame-shifting is now recognized as an important means of translational control to produce two or more proteins at fixed ratios from coding domains with a single translation initiation site (Chamorro et al., 1992). The (+) strand RNA of the yeast L-A virus has two overlapping ORFs. The 5'-end ORF1 (gag) encodes the major viral coat protein (76 kDa), and the 3'-end ORF2 (pol) is expressed only by fusing the two ORFs to produce a 170-kDa protein with a ssRNA binding domain and a predicted RDRP domain (Fujimura and Wickner, 1988b;

\[
\begin{align*}
\text{L-A} & : \text{HO-} \text{CUUUU} \text{A} \text{A} \text{A} \text{A} \text{A} \text{U} \text{A} \text{U} \text{A} \text{A} \text{A} \text{U} \text{A} \\
\text{M1} & : \text{HO-} \text{CUUUU} \text{A} \text{U} \text{U} \text{C} \text{U} \text{U} \text{A} \text{C} \text{U} \\
\text{L-BC} & : \text{HO-} \text{CUUAAA} \text{A} \text{G} \text{G} \text{U} \text{G} \text{U} \\
\text{Hv190S} & : \text{HO-} \text{CUUCU} \text{A} \text{A} \text{U} \text{U} \text{A} \text{A} \text{A} \\
\phi 6 : \text{M or S} & : \text{HO-} \text{CCUUUU} \text{U} \text{U} \text{G} \text{A} \text{A} \text{A} \text{U} \text{A} \text{U} \text{A}
\end{align*}
\]

**Fig. 5.** The 3'-end sequences of the (−) strands of genomic and satellite mycoviral dsRNAs. The φ6 sequences are from Mindich et al. (1988). The L-A, M1, and BC sequences are from Fujimura and Wickner (1989). The Hv190S dsRNA sequence was obtained by direct RNA sequencing (unpublished observations).
Diamond et al., 1989; Icho and Wickner, 1989; Bruenn, 1991). Thus, the 170-kDa protein, a minor virion component, is a fusion protein with an N-terminal major coat protein domain and a C-terminal RDRP/RNA binding domain. The fusion mechanism is a −1 ribosomal frame-shift (Dinman et al., 1991; Tzeng et al., 1992), as is the case for the gag-pol fusion proteins of most retroviruses (Jacks and Varmus, 1985; Jacks et al., 1988b).

Frame-shifting in the −1 direction, first shown as a mechanism for the synthesis of Rous sarcoma virus pol gene products (Jacks and Varmus, 1985), is now known to affect expression of a variety of genetic elements in addition to the yeast L-A dsRNA, including most retroviruses (Hizi et al., 1987; Jacks et al., 1987, 1988a,b; Moore et al., 1987), coronaviruses (Brierly et al., 1987; Bredenbeek et al., 1990; den Boon et al., 1991), a plant luteovirus (Prufer et al., 1992), phage T7 (Dunn and Studier, 1983), bacterial transposons (Escoubas et al., 1991; Sekine and Ohtsubo, 1989), and the dnaX gene in bacteria (Blinkowa and Walker, 1990; Tsuchihashi and Kornberg, 1990). A +1 ribosomal shift has been noted in the retroviral-like transposon Ty-1 (Mellor et al., 1985; Clare et al., 1988), and recently predicted for the protozoal virus LRV1-1 (Stuart et al., 1992). Like the yeast L-A virus, LRV1-1 belongs to the family Totiviridae.

The signals responsible for −1 ribosomal frame-shifting, according to the simultaneous slippage model (Jacks et al., 1988a), include a “shift” or a “slippery” site heptamer, X XXY YYZ, in which the triplets represent the initial (or 0) reading frame, followed by a stem–loop structure that can form an RNA pseudoknot (a pseudoknot is a higher-order structure formed when bases in an RNA loop pair with a sequence 3’ of the stem–loop) (Pleij, 1990).

Studies in two laboratories (Dinman et al., 1991; Dinman and Wickner, 1992; Tu et al., 1992; Tzeng et al., 1992) provided strong evidence that fusing of the yeast L-A dsRNA ORF1 and ORF2 occurs by a −1 ribosomal frame-shifting, and that frame-shifting requires the predicted heptamer slippery site and a potential pseudoknot structure that involves a predicted stem–loop structure. In these studies a cDNA fragment comprising the putative frame-shift site in L-A dsRNA was inserted in expression and transcription vectors and tested for its ability to direct frame-shifting in both homologous and heterologous eukaryotic systems as well as in prokaryotes. Deletion analysis and site-directed mutagenesis indicated that a sequence of 71 nt (nt 1952–2022), including both the pseudoknot and slippery sites, is sufficient for frame-shifting.

Analysis of the slippery site (G GGU UUA) in L-A dsRNA indicated that any 3 identical bases (including C CC) in the first triplet were
sufficient for efficient frame-shifting (Dinman et al., 1991). This is unlike the model of Jacks et al. (1988a), which stipulates that X (in the heptamer X XXY YYZ) can only be A, U, or G. Furthermore, as long as base pairing was possible in the nonwobble bases, some frame-shifting was detected. The sequence requirements of the second triplet (U UU) were more stringent. Like the retroviruses, only triplets of A and U yielded efficient frame-shifting, and the identity of the 3 bases was required. Dinman et al. (1991) reasoned that inefficient frame-shifting with C CC or G GG in the second triplet is due to the higher energy required to unpair the tRNA that is properly paired to CCX or GGX rather than to a reluctance to repair in the shifted -1 frame. This explanation is supported by the finding that changing the seventh base in X XXC CCA to give X XXC CCC did not improve frame-shifting (Dinman et al., 1991).

The requirement for pseudoknot formation downstream of the slippery site was demonstrated by site-directed mutagenesis aimed at disrupting the two predicted base-paired stems (S1 and S2, Fig. 6) that

Fig. 6. Structural components of the yeast L-A dsRNA region that determines the -1 ribosomal frame-shifting. The slippery site GGGUUUA is enclosed in a black box. The potential pseudoknot structure (a pseudoknot is a higher-order structure formed when bases in an RNA loop pair with sequence outside the loop) 3' of the slippery site is also shown and involves bases 1969–2004. The pseudoknot is composed of two base-paired stems (S1 and S2) and two connecting loops (L1 and L2). S1, Bases 1967–1979 and 1994–2004; S2, bases 1984–1993 and 2013–2022. The two stems could stack coaxially to form a quasicontinuous helix according to the principle of Pleij et al. (1985). Data were from Dinman et al. (1991) and Tzeng et al. (1992).
constitute the predicted pseudoknot. The results of frame-shifting activity of various mutants indicated that both S1 and S2 are essential for frame-shifting and it is the secondary and tertiary structures rather than the primary sequence that are involved (Tzeng et al., 1992).

Tzeng et al. (1992) demonstrated that the yeast L-A frame-shifting signal can direct a −1 frameshifting in heterologous systems, including the wheat germ extract and rabbit reticulocyte lysate in vitro translation systems, and in Escherichia coli in vivo. Thus, ribosomes from prokaryotic and several different eukaryotic cells appear to respond to the yeast L-A signal in a similar manner. In this regard it should be mentioned that, whereas the minimal sequence of 71 nt has been shown to be sufficient to effectively direct frame-shifting in the wheat germ system in vitro and in yeast cells, it failed to function in E. coli. Tzeng et al. (1992) explained that the surrounding vector sequences may have interfered with the formation of the tertiary structure essential for impeding ribosomal movement (Tu et al., 1992) and subsequent efficient frame-shifting. The frame-shift signals in retroviruses are also known to function in heterologous systems (Jacks and Varmus, 1985; Wilson et al., 1988).

The levels of frame-shifting directed by the L-A frame-shift signal appear to differ with the system used to assay frame-shifting, as well as with the expression vector used in plasmid construction. Whereas the frame-shift efficiency in the wheat germ system has been reported as 3.5%, a much higher frame-shift efficiency (20–29%) was detected in the yeast system (Tzeng et al., 1992). Dinman and Wickner (1992), on the other hand, reported an efficiency of frame-shifting in yeast of 1.9%, and suggested there are two molecules of fusion proteins per virion, based on 120 capsid protein molecules per particle. The presence of two molecules of CP-RDRP, the protein with demonstrated RNA binding activity, is consistent with the recent finding of two adjacent VBS in the viral RNA (Shen and Bruenn, 1993).

6. Viral Interference Site (INS)

The phenomenon of viral interference (exclusion) has been observed with the yeast and the smut viruses. For example, in a cross between K1 yeast killer strains (containing L-A and M virions; L-A and M1 dsRNAs packaged separately in capsids encoded by L-A dsRNA) and suppressive-sensitive mutants (containing L-A and S virions; L-A and S dsRNAs separately encapsidated), the S virions can displace the M virions in the progeny (Ridley and Wickner, 1983). Thus, the S dsRNAs, which are derived from M1 by internal deletion, are analogous to defective interfering genomes of viruses of higher eukaryotes.

Huan et al. (1991) developed an in vivo assay in which the expression
of cloned S cDNA from an inducible promoter causes the loss of M1 particles, thus providing a convenient means to identify the cis-acting sequences involved in interference. These researchers mapped the INS to a region of 132 bp that contains two stem–loop structures similar to the VBS (Fig. 7), one of which is the VBS, previously identified in M1 dsRNA using an in vitro system. Transcripts containing the VBS alone without the second stem–loop structure are insufficient for interference. Shen and Bruenn (1993) reported that the second stem–loop structure is a VBS with affinity for viral particles but which has higher dissociation rate constant than the first VBS. Furthermore, the two adjacent VBS were shown to have additive INS activity and that binding to the two sites appeared to be independent.

In both S3 and S14 (+) strand RNAs (as well as in X dsRNA; Fig. 4), a long stem is topped with a loop with the sequence GAU(U/C)C. The GAUUC sequences present in the predicted loop 2 (Fig. 7) are sequences within a third direct repeat identical to the two previously indicated in the probable IRE–VBS site in M1 dsRNA (see Section II,E,3 and Fig. 4). Although a minimal sequence from bases 336–468 is necessary and sufficient for interference, as deduced from overlapping deletions (Huan et al., 1991), the minimal sequence whose expression did, in fact, eliminate M1 virions was from bases 165–468.

**F. Functional Domains in Proteins of Totiviruses**

Virions of totiviruses possess a number of enzymatic activities, including a transcriptase (Welsh et al., 1980; Ben-Zvi et al., 1984;
Ghabrial and Havens, 1989), a replicase (Fujimura et al., 1986; Nemeroff and Bruenn, 1986), a protein kinase (Ghabrial and Havens, 1992), a nucleotide phosphohydrolase (Dowhanick et al., 1994), a nucleotide phosphotransferase, a nucleotide kinase, and an inorganic pyrophosphatase (Georgopoulos and Leibowitz, 1987). Furthermore, the pol domain of the 170-kDa CP–RDRP fusion protein of the yeast L-A virus has an ssRNA binding activity (Fujimura and Wickner, 1988a). The association of a given enzymatic activity with virions is not considered evidence of being virus encoded. For example, it has yet to be determined whether the protein kinase activity associated with virions of the Hv190S virus is cellularly or virally encoded (Ghabrial and Havens, 1992).

Identification of functional domains requires first the identification and characterization of the mature protein possessing the enzymatic activity and then locating the active site within these polypeptides. Computer-assisted identification of functional domains in the yeast L-A proteins have located the conserved domains in the RDRP region, the RNA binding domain, and the putative domain of the nucleotide phosphohydrolase (Bruenn, 1991; Dowhanick et al., 1994). Thus, it seems that all enzymatic activities so far identified in the yeast L-A are associated with the CP–RDRP fusion protein. As for the CP domain, a central region of 245 amino acids within the sequence of the L-A CP was located that has significant similarity to the picornavirus VP3 (Bruenn et al., 1989). The presence of phosphorylated and nonphosphorylated forms of the capsid protein has recently been reported in virions of the Hv190S totivirus (Ghabrial and Havens, 1992). That CP heterogeneity in the Hv190S virions may reflect functional heterogeneity was suggested by the finding of apparent conformational differences between the phosphorylated and nonphosphorylated forms of the CP (see Section II,F,4).

1. The RDRP Domain

The yeast L-A pol ORF contains motifs conserved among RDRP of (+) strand RNA viruses as well as dsRNA viruses (Bruenn, 1991). Ribas and Wickner (1992) defined the regions surrounding the two most highly conserved RDRP consensus motifs [SG...T...NT..N (dots indicate any amino acid) and GDD] that are necessary for viral dsRNA replication. These workers showed that although these regions are highly conserved in primary and predicted secondary structures among a wide array of viruses, they are not interchangeable.

To define the essential regions in the L-A pol ORF, Ribas and Wickner (1992) adopted an “alanine [Ala] scanning mutagenesis” approach. Because Ala is not a helix-breaker, and is neither particularly hydroy-
phobic nor polar, mutagenesis to Ala is considered well suited for such studies. Ala was substituted for pairs of amino acids or single residues in the region containing the two conserved motifs (positions 526–602; the amino acid residues in the pol ORF are numbered starting with the Arg residue at base 1964, which is the first amino acid after the −1 ribosomal frame-shift). A yeast expression vector containing a full-length L-A cDNA clone (Wickner et al., 1991) was used, and following mutagenesis, the modified L-A expression vector was transformed into a yeast strain that is defective in MAK10 (Sommer and Wickner, 1982). In a mak10 host, L-A proteins expressed from a cDNA clone of L-A support the replication of M1 dsRNA but (for unknown reasons) do not support the multiplication of the L-A virus itself (Wickner et al., 1991). Thus, when L-A and M virions are introduced into the transformed strain via cytoplasmic mixing (cytoduction), only M1 virions are stably maintained. This provided an in vivo assay of the activity of the proteins encoded by the L-A cDNA clone in the absence of L-A virus itself. The presence or absence of M1 dsRNA in the cytoductants, as revealed by the killer activity assay, was used as the indicator of whether L-A proteins expressed from the mutant cDNA clone were active or inactive.

The results of these mutational analyses indicated that the most highly conserved residues, 544-SG, 549-T, and 579-GDD (the number refers to the first residue), were all essential for activity, but less strictly conserved 553-NT (which is NS, HT, or GT in some viruses) was 4% active when changed to AA, and 557-N (which is T, M, or S in other viruses) was 17% active when changed to A. The T residue at position 554 is often S in other viruses, and this works in L-A as well, but the N at position 557 cannot be changed to T. The eight nonconserved residues inside the SG...T...NT..N motif were essential, but the rates of loss of M1 virions were slower than for the conserved residues, except for the N at position 557. The boundaries of the two putative functional domains SG...T...NT.N and GDD are 21 amino acids (from 541-T to 561-M) and 29 residues (from 565-G to 593-V), respectively.

There are two GDD sequences in the L-A pol ORF, one starting at position 579 and the second at residue 707. Whereas any of the substitutions at the first GDD were lethal, there was no effect of changing 707-GDD to AEE. In this regard it seems unlikely that the two GDD motifs might be functional (one in transcription and the second in replication), as was previously proposed (Diamond et al., 1989).

Substitution of the nonconserved residues within the SG...T...NT.N motif, with corresponding residues from poliovirus and reovirus, essentially removed all activity (Ribas and Wickner, 1992). The presence
of highly conserved motifs in the RDRP sequences among such a diverse group of animal, plant, and fungal viruses suggests that these enzymes must have a consensus secondary structure in these regions. This was shown to be the case by the remarkable similarity of their computer-predicted secondary structures. The most conserved regions are predicted to have a β-sheet structure, with turns at the most conserved residue.

2. RNA Binding Domain

Unlike the DNA binding proteins, the RNA binding proteins are less well studied. Several sequence motifs for RNA binding domains have been proposed, including an arginine-rich RNA binding motif, “the arginine fork” (Calnan et al., 1991). An RNA recognition motif encompassing an octamer sequence that includes aromatic and basic amino acids (often arginine) has been observed by Query et al. (1989) to be conserved in many presumed RNA binding proteins. Dowhanick et al. (1994), who noted that the C-terminal region of all viral RDRPs has some similarity to the Query consensus (Query et al., 1989), expressed the C-terminal 100-amino-acid region of the pol ORF of the yeast L-A dsRNA as a β-galactosidase α-peptide fusion protein in E. coli. The fusion proteins were then tested for RNA binding activity in North-western assays. The results showed that this region of the protein does have RNA binding activity. Whereas the fusion protein bound efficiently to a probe containing both L-A dsRNA VBS and INS sites, the β-galactosidase α-fragment did not bind at all. However, as in North-western assays with the intact CP-RDRP fusion protein [and unlike those with whole virions (Fujimura and Wickner, 1988a)], no sequence specificity could be demonstrated (Dowhanick et al., 1994).

3. Nucleotide phosphohydrolase (NPH)

Highly purified L-A virions have NPH activity capable of removing the α, β, and γ-phosphates from nucleotides or from RNA or DNA (Dowhanick et al., 1994). It was proposed that the NPH activity may be responsible for removing the γ-phosphate from the transcription-initiating 5' GTP (Nemeroff and Bruenn, 1987; Dowhanick et al., 1994). NPH activity was demonstrated by the release of 32P from γ-32P-GTP-labeled S14 cDNA transcripts when used as substrate and added to full or empty virus particles. Furthermore, NPH activity was detected only in the CP-RDRP fusion protein band in renatured gels following SDS-PAGE of purified virions. In these experiments γ-32P-GTP-labeled S14 cDNA transcript was used as substrate, and was added to the gels at the polymerization step (Dowhanick et al., 1994).
4. CP Heterogeneity/Phosphorylation

Although the capsids of totiviruses are encoded by a single gene, and typically are composed of a single major polypeptide, CP heterogeneity has been observed in purified preparations of some totiviruses (Ghabrial et al., 1987). The origin of CP heterogeneity in the totivirus Hv190S virus was the subject of a recent comprehensive study. The capsids of Hv190S virus contain three polypeptides with molecular weights of 78,000, 83,000, and 88,000 (denoted p78, p83, and p88, respectively). Two of these polypeptides occur as major CPs, whereas the third polypeptide occurs as a minor component; p88 is always a major CP, while the relative abundance of p83 and p78 varies with the virus preparation (Ghabrial et al., 1987). The three CPs show similar peptide profiles when subjected to selective chemical cleavage at tryptophan residues or to limited proteolysis using V8 protease. No evidence could be obtained to support the idea that the smaller polypeptides are generated from p88 as a result of proteolysis during virus purification or storage (Ghabrial et al., 1987). Recently, evidence was presented that p83 and p88 are phosphoproteins (Fig. 8), whereas p78 is nonphosphorylated (Ghabrial and Havens, 1992). Furthermore, the presence of protein kinase activity, which utilizes ATP as a phosphoryl donor, in purified virions of Hv190S virus was demonstrated (Ghabrial and Havens, 1992). Recent phosphoamino acid analysis results indicated that the virion-associated protein kinase has serine/threonine protein kinase activity (1993, unpublished observations).

CP heterogeneity has also been reported for several other totiviruses; GgV-87-1-H, ScV-L-BC, and Y1V (Sommer and Wickner, 1982; Jamil and Buck, 1986; El-Sherbeini et al., 1987) are reported to possess two or more related capsid polypeptides of comparable size to those of the Hv190S virus. Although the origin of CP heterogeneity in these viruses is not known, posttranslational phosphorylation of a primary translation product, as in the case of the Hv190S virus, could account for the observed heterogeneity. Phosphorylation of the CP may be a common feature of viruses in the family Totiviridae, and may play a regulatory role in viral dsRNA transcription/replication.

Purified preparations of the Hv190S virus have been shown to separate into two closely spaced sedimenting components (190S-1 and 190S-2), distinguishable by CP composition, state of phosphorylation, and transcriptional efficiency (Ghabrial and Havens, 1992). The slower-sedimenting component (190S-1) contains p88 and p83 as the major CPs, and the faster component (190S-2) contains p88 and p78 (Fig. 9). It is unlikely that the differences in phosphorylation pattern between the 190S-1 and 190S-2 particles are responsible for their dif-
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**Fig. 8.** SDS–PAGE analysis of radiolabeled Hv190S virus, purified from 14-day-old stationary culture of *H. victoriae* isolate A-9, grown in the presence of [32P]phosphate; Coomassie blue-stained gel (lane B) and autoradiograph (lane D). Arrowhead (lane D) indicates the position of a radiolabeled 180-kDa protein expected to represent a fusion protein composed of the capsid protein and the putative RNA polymerase protein (analogous to the yeast L-A 170-kDa CP–RDRP fusion protein). Purified nonradioactive Hv190S virions were included as a control [stained gel (lane C) and autoradiograph (lane E)], and shows, in order of increasing electrophoretic migration, the three capsid proteins, p88, p83, and p78, respectively. Protein markers are shown in lane A, and their molecular weights are indicated to the left. (From Ghabrial and Havens, 1992.)

Differential sedimentation properties. It is proposed that the two particle types may represent various stages in the Hv190S virus replication cycle. Virus particles containing intermediates in the virus replication cycle (e.g., ssRNA or partially dsRNA molecules) have been detected in purified preparations of some dsRNA mycoviruses (Buck and Kempson-Jones, 1973; Buck, 1986). Studies on replication cycles of mycoviruses are complicated by the fact that mycoviruses are not amenable to conventional infectivity assays and that systems for obtaining synchronous infections with these viruses are not yet available (Buck, 1986).

The electrophoretic separation of the phosphorylated and non-phosphorylated forms of the CP in SDS–PAGE (Figs. 8 and 9) is probably due to differential binding of SDS to these proteins as a result of repulsion by protein-bound phosphates. The finding that the more highly phosphorylated capsid polypeptide, p88, migrated more slowly than the less phosphorylated or nonphosphorylated CPs, p83 and p78, respectively (Fig. 8), lends support to the conclusion that modification
of charge can affect electrophoretic mobility of proteins in SDS–PAGE (Tung and Knight, 1971). The differential migration of the phosphorylated peptides derived from p88 and p83 is also consistent with this idea (Ghabrial and Havens, 1992). The abnormal electrophoretic migration of intact phosphorylated viral proteins as well as of their cleavage products has been reported by several investigators (Marnell and Summers, 1984; Bell and Prevec, 1985; Hsu and Kingsbury, 1985). Furthermore, Hsu and Kingsbury (1985) reported that the anomalous behavior of the cleavage products of vesicular stomatitis virus NS phosphoprotein in SDS gels can be clearly revealed by performing electrophoresis in two different gel concentrations. This was also true for the largest phosphorylated cleavage product of p88 (Ghabrial and Havens, 1992).
Phosphorylation of the CP may serve to expand and modulate its properties and functions. There is strong evidence that phosphorylation of the structural proteins of certain viruses may be necessary for their interaction with viral nucleic acids and for subsequent assembly into virions (Leader and Katan, 1988). The finding that the phosphorylated, but not the nonphosphorylated, CPs are accessible to iodination in the intact virions (Fig. 10) suggests that they are conformationally different and thus may have specific roles in viral assembly and/or in the transcription/replication of the viral dsRNA genome. Using the proposed replication cycle of the yeast L-A virus (Fujimura and Wickner, 1988a; Wickner, 1989) as a model system for viruses belonging to the family Totiviridae, one would expect the virion-associated transcriptase and replicase activities of the Hv190S virus to be catalyzed by the same enzyme (the CP–RDRP fusion protein). In the yeast virus system the CP–RDRP protein catalyzes the synthesis of (−) strand RNA on (+) strand RNA templates in dsRNA-synthesizing particles of lighter density (replicase mode). When a full-length dsRNA molecule is synthesized in the now more dense mature particles, the CP–RDRP protein switches templates to catalyze the synthesis of full-length (+) strand transcripts (transcriptase mode). CP phosphorylation may modulate the switching of templates by the CP–RDRP fusion protein and

![Fig. 10. SDS–PAGE of ^125^I-labeled Hv190S virus capsid proteins. Intact (lanes 1 and 2) and dissociated (lanes 4 and 5) virions were labeled with ^125^I using Iodogen (Markwell and Fox, 1978), then disrupted (in the case of the intact virions), and analyzed by SDS–PAGE. (Top) Coomassie blue-stained gel. (Bottom) Autoradiograph. Lane 3 contains the molecular weight standard phosphorylase b (97 kDa). The positions of p88, p83, and p78 are indicated in the margin. (From Ghabrial and Havens, 1992.)](image-url)
the release of the full-length (+) strand transcripts from the mature particles.

Alternatively, phosphorylation/dephosphorylation may regulate viral dsRNA transcription. The finding that the more highly phosphorylated virions of the 190S-1 component were more efficient in transcriptase activity than those of the 190S-2 component is of interest in this regard. Assignment of multiple functions to the capsid polypeptides of viruses in the family Totiviridae may be justified, considering that the gene for the coat protein accounts for as much as half of the coding capacity of the viral genome (Ghabrial, 1988).

G. Biological Properties

1. Transmission

There are no known natural vectors for the transmission of viruses in the genus Totivirus. They are transmitted intracellularly during cell division, sporogenesis, and cell fusion. Although the yeast L-A virus is effectively transmitted via ascospores, the totiviruses infecting the ascomycetous filamentous fungi (e.g., GgV-87-1-H) are essentially eliminated during ascospore formation.

Conventional infectivity assays for totiviruses using purified virus are not presently available. Attempts to infect mycelial cultures using purified virus have so far failed, even when treated cultures were shaken in the presence of abrasives (Hollings, 1978). The rigid cell wall in fungi, understandably, constitutes a barrier to virus uptake, and to damage or puncture the cell wall without killing the cell might not be possible. A microinjection approach for virus inoculation was used to infect the fleshy tissue of the cultivated mushroom, Agaricus bisporus. In this case partially purified virus preparations from diseased mushrooms were injected into developing sporophores (Hollings et al., 1963; Dieleman-van Zaayen and Temmink, 1968). However, the process was very inefficient and difficult to reproduce, and it is hard to be sure that chance contamination from airborne spores or spores in the virus preparations did not occur. Also, the finding that mushroom spawns can contain at least low levels of virus particles introduces the possibility that a latent infection may have been reactivated in the few cases when infection was detected (Buck, 1986).

To overcome the cell wall barrier, attempts have been made to infect fungal protoplasts with cell-free virus preparations; many of these have not been successful. The disease of H. victoriae was transmitted by inoculating protoplasts from a virus-free fungal isolate with purified virus in the presence of PEG. The frequency of infection and the
virus levels in the newly diseased colonies, however, were low, and verification of transmission was based on virus detection by immune electron microscopy (Ghabrial, 1986). Stanway and Buck (1984) achieved infection of 10% of protoplasts of *Gaeumannomyces graminis* incubated with purified virus particles in the presence of PEG. This result was unequivocal because (1) the recipient fungal strain was known to be completely free of virus particles, but known to be susceptible to virus infection, (2) the virus preparations were filter sterilized and completely free of fungal propagules, (3) inoculations were carried out under aseptic conditions (4) the viruses in the newly infected cultures were isolated and thoroughly characterized, and (5) the levels of viruses in the newly infected cultures were similar to those in the parent cultures and remained stable over three successive subcultures (Buck, 1986).

El-Sherbeini and Bostian (1987) used filter-sterilized virus particles prepared from appropriate yeast killer strains (K1 or K2) to inoculate spheroplasts of a standard sensitive uracil requiring (ura3-50) host strain, GG100-14D (containing only L-A and L-BC virions, as verified by the presence of the corresponding dsRNAs and CPs). The inoculation was done in the presence of a URA3-containing 2-μm-based plasmid and selection was made for URA3-regenerated spheroplasts. Uracil prototrophs were assayed for the killer phenotype by standard methods. With the inoculum from the K1 killer strain, 67% of the transformants assayed also contained M1 dsRNA. The infected colonies were highly stable killers with similar M1 dsRNA copy numbers to the donor strains and the same genotype as the host strain, with the exception of the uracil marker. As expected, infected yeast colonies containing M1 dsRNA showed the same profile of capsid polypeptides as the recipient host strain (i.e., all possessed L-A and L-BC CPs).

In a similar experiment the virions from a K2 killer strain were used to inoculate the same host strain, yielding slightly different results. Only 4% of the transformed colonies contained M2 dsRNA. Extracellular transmission of yeast viruses into yeast cells without the need to remove the cell wall was also demonstrated. Cells of strain GG100-14D were rendered competent by the lithium acetate procedure of Ito et al. (1983), and inoculated with virions from a K1 killer strain in the presence of the plasmid YEp24. As before, selection was made for URA3 transformants. Of the uracil prototrophs tested, 3% contained M1 dsRNA (Sturley et al., 1988).

Using a very similar procedure, Schmitt and Tipper (1990) obtained efficient transfection of the yeast strain 1938 (L dsRNA-free) by a virus preparation from strain 28 containing a mixture of L-28 and M-28 virions. A low efficiency of transmission, similar to that previ-
ously observed for inocula containing M2 virions (El-Sherbeini and Bostian, 1987), was obtained with strain GG100-14D. The reasons for the variation in transfection competence of different host strains for different M dsRNA species are unknown. They may reflect the efficiency of the helper functions of the resident L dsRNA species, the efficiency with which different (+) strand RNA transcripts of M dsRNA become encapsidated, initial selection against toxin expression, or other aspects of the complex host–virus interaction. In spite of this variation, all of the transfecants expressing K28 specificity were stable, all produced four times more K28 toxin than strain 28 (the source of the virus inoculum), and all contained correspondingly higher levels of M28 dsRNA. Western blotting and analyses of dsRNA patterns confirmed that the higher K28 toxin activities of transfected strains resulted from higher levels of toxin secretion and correlated with higher M28 dsRNA contents. Thus, the virions from strain 28 are sufficient to transfer the K28 phenotype. Since loss of this phenotype had been correlated with loss of M28 during curing, it is highly probable that the K28 phenotype is encoded by M28 dsRNA, just as M1 toxin and immunity are encoded by M1 dsRNA.

Extracellular transmission of the yeast L-A virus may also be possible during natural mating of yeast mating pairs (Buck, 1986; Sturley et al., 1988). Successful transfection of the protozoa Giardia lamblia has been accomplished via electroporation with (+) strand RNA transcribed in vitro from the G. lamblia virus (GLV) dsRNA (Furfine and Wang, 1990), an isometric dsRNA totivirus belonging to the genus Giardiaivirus (Wang and Wang, 1991). These recent accomplishments toward the development of efficient extracellular transmission assays are encouraging. Because of the multipartite nature of their genomes, the totiviruses are ideal for studies aimed at developing infectivity assays. Approaches along the lines of those discussed above for the yeast viruses and using inocula of purified virions, full-length in vitro transcripts of genomic dsRNA, or cloned cDNA to dsRNAs should be applicable to other totiviruses. Purified dsRNA is not expected to be infectious, as it cannot be translated in the fungal cell. Infectivity assays with dsRNA viruses must be performed using virions, as all dsRNA viruses possess virion-associated RNA polymerases that catalyze the transcription of dsRNA to messenger sense ssRNA.

2. Host Range

With one possible exception, there are no known experimental host ranges for the viruses in the genus Totivirus. Using the lithium acetate inoculation protocol, purified YIV, a totivirus infecting the yeast Yarrowia lipolytica, was introduced into S. cerevisiae strain GG100-14D
Analyses of the capsid polypeptide profiles and the dsRNA content revealed the presence of YIV at a concentration comparable to that in its natural host. The newly acquired dsRNA genome was stably maintained over numerous generations of cell growth. YIV was apparently adequately maintained by the MAK functions (see below) present in *S. cerevisiae* strain GG100-14D, to the extent that in certain instances YIV dsRNA excluded the endogenous L-A dsRNA.

As a consequence of their intracellular modes of transmission, the natural host ranges of totiviruses are limited to individuals within the same or closely related vegetative compatibility groups. Furthermore, mixed infections with two or more unrelated viruses are common, probably as a consequence of the ways by which fungal viruses are transmitted in nature. Examples of mixed infections involving totiviruses include the totiviruses ScV-L-A and ScV-L-BC, the totivirus AfV-S and the unclassified AfV-F, and the totivirus Hv190S virus and the possible partitivirus Hv145S virus. Apparently, there are no structural interactions between these pairs of viruses, since heterologous encapsidation has not been reported in mixed infections (Buck, 1986).

**H. Interaction with Host**

The yeast killer system, comprised of a helper totivirus and associated satellite dsRNA, is one of a very few known examples in which virus infection is beneficial to the host. The ability to produce killer toxins by immune yeast strains confers an ecological advantage over sensitive strains. The use of killer strains in the brewing industry provides protection against contamination with the adventitious sensitive strains.

Although the majority of fungal viruses have been reported to be avirulent and associated with latent infections of their hosts, it is becoming increasingly clear that phenotypic consequences of harboring specific mycoviruses or certain dsRNA molecules can range from symptomless to severely debilitating, and from hypovirulence to hypervirulence (Buck, 1986; Nuss and Koltin, 1990). The disease of *H. victoriae* (Ghabrial, 1986) and the La France disease of the cultivated mushroom, *A. bisporus* (van Zaayen, 1979), represent examples of pathological effects of fungal viruses. Although the present evidence for viral etiology in both examples is strong, it remains equivocal. Recent developments aimed at understanding the molecular basis of disease in the *H. victoriae* and *A. bisporus* systems are considered in Sections II, III, and V, respectively. The dsRNA-associated hypo-
virulence in the chestnut blight fungus is a well-documented example of a mycoviral-induced phenotype (see Section IV,E).

1. Host Genes Required for Maintenance of Viral and Satellite dsRNAs

Because of the extensive knowledge of yeast genetics and molecular biology, and because the killer phenotype can be readily scored, the yeast killer strain/virus system provides an excellent model system to study the effect of chromosomal genes on virus replication. There are over 30 chromosomal genes, termed MAK genes for maintenance of killer, whose products are necessary for replication of M dsRNA and are important or essential for cell growth (Wickner, 1978, 1979; Wickner and Leibowitz, 1976b, 1979). It is not surprising that viral genomes utilize essential (or at least important) host genes for their replication; otherwise, the host could easily eliminate viral infection by simply deleting a gene. Genes of known function that are required for M dsRNA (but not L-A dsRNA) maintenance include SPE2 and SPE10, which are required for polyamine synthesis; MAK8, which is identical to TCM1, the gene for trichodermin resistance, and encodes ribosomal protein L3; and MAK1, the gene encoding DNA topoisomerase I. MAK11, which has recently been shown to be essential for the host, is another member of the group of MAK genes that are necessary for the maintenance of M but not L-A dsRNA (Icho and Wickner, 1988), and whose mutations are suppressed by mutations in the antiviral SKI (superkiller) genes (see below).

The MAK11 gene has been cloned and sequenced, and its product is membrane associated. The sequence shows two structurally significant features: a hydrophobic N terminus and a lysine-rich C terminus (Icho and Wickner, 1988). The hydrophobic N terminus of the MAK11 product may serve to anchor the protein to the membrane, but it is not cleaved. The lysine-rich region may act to protect the M (+) strand RNA by functioning as an RNA binding domain (see below).

The MAK16 gene, whose mutation results in a loss of M1 dsRNA and also produces temperature-sensitive cell growth (Wickner and Leibowitz, 1979), has recently been cloned and sequenced (Wickner, 1988). The sequence of MAK16 suggested that its product (with a predicted size of 36 kDa) might be a nuclear protein, as two nuclear localization signals were found. This is supported by the finding that MAK16–LacZ fusion proteins that included these putative signals entered the nucleus (Wickner, 1988). mak16-1 mutants arrest at the nonpermissive temperature in G1 phase as unbudded mating competent cells. The sequence of MAK16 also reveals a highly acidic region, like those found in high-mobility-group nonhistone chromatin protein and
in the activating regions of the yeast GCN4 and GAL4 regulatory proteins (Wickner, 1988). Potential phosphorylation sites for serine/tyrosine protein kinases are also detected. It should be noted in this regard that mutants in the yeast CDC28 gene (CDC stands for cell division cycle), which is a serine/threonine protein kinase, arrest at G1, the same as mak16 mutants. It will be of considerable interest to determine the relationship of MAK16 to CDC28 and other CDC genes that act in G1 and to verify whether MAK16 protein is indeed phosphorylated.

Only three host genes (MAK3, MAK10, and PET18) are needed for the maintenance of L-A dsRNA. The MAK3 gene encodes an N-acetyltransferase that acetylates the N terminus of the major coat protein (Wickner et al., 1994). mak3-1 mutants cannot replicate L-A or M dsRNAs; the major CP in L-A and M virions is blocked (acetylated) in MAK+ strains, but unblocked in mak3-1 strains. The unblocked CP is apparently degraded and fails to assemble into virions. Whereas the MAK3 gene is not essential for cell growth, it is necessary for rapid growth on nonfermentable carbon sources (Wickner et al., 1994).

MAK10 expression is glucose repressed, with control sequences inside another gene. mak10 mutants cannot replicate L-A or M dsRNAs. The MAK10 gene was cloned and MAK10–LacZ chimeric constructs were used to study its expression, which was found to be low in glucose media and high in glycerol or ethanol media. As can be predicted, cells lacking mitochondrial DNA, and thus able to grow only on glucose, have undetectable levels of MAK10 expression. The previous finding that L-A virus replication is glucose repressed (Oliver et al., 1977) may now be explained based on the low expression in glucose-repressed cells of the MAK10 product which is necessary for L-A replication (Wickner et al., 1994).

In addition to being needed for L-A dsRNA replication, the chromosomal gene PET18 is needed for replication of mitochondrial DNA and for cell growth (Leibowitz and Wickner, 1978). Fujimura and Wickner (1986) determined that pet18 mutants, which are known to be temperature sensitive (ts) for M dsRNA replication (Wickner and Leibowitz, 1976b), are also temperature sensitive for L-A replication. The PET18 gene is involved in maintaining the structure of the L-A virions, but does not appear to affect the structure of the M virions. M1 and M2 virion-associated RNA polymerase activities have also been found to be more thermostable than those associated with L-A virions. Furthermore, pet18 mutations do not affect the RNA polymerase activity associated with M virions. L-A virion-associated RNA polymerase activity from pet18 cells, on the other hand, was more thermolabile than that from PET+ cells. Fujimura and Wickner (1986) studied the basis of
this differential thermolability and concluded that it is due to the instability of the virions from pet18 cells as a consequence of the release of template dsRNA from the virions.

In addition to pet18 mutations, mak10 mutations have also been shown to cause thermolability of the L-A virions (Fujimura and Wickner, 1986, 1987). Mature L-A virions as well as dsRNA-synthesizing L-A virions obtained from mak10(ts) mutants showed instability even though these particles were prepared from cells grown at the permissive temperature (Fujimura and Wickner, 1987). These results suggest that the MAK10 gene product itself is virion associated. Interestingly, the mature, but not the dsRNA-synthesizing, virions isolated from this mutant strain were not stabilized by 3 M CsCl. Fujimura and Wickner (1987) reasoned that the greater RNA content of the mature virions rendered them less stable than the dsRNA-synthesizing virions with lower RNA content. It is of interest in this regard that L-A, but not M, virions isolated from pet18 mutants showed thermoinstability.

The thermoinstability of L-A virions in pet18 cells is apparently due to the absence of PET18 products, since Toh-e and Sahashi (1985) have determined that two ORFs (MAK31 and MAK32) were necessary to complement the L-A maintenance defect of pet18 mutants and that all pet18 mutants have large deletions, including the entirety of MAK31 and MAK32. Thus, the complete deletion of these genes causes both temperature-sensitive maintenance of L-A dsRNA in vivo and unstable mature L-A virions in vitro. Possibly, the PET18 gene products are associated with L-A virions and their loss results in unstable virions, leading to the release of L-A dsRNA from the particles. Alternatively, the PET18 gene product may be necessary for the modification of other gene products (e.g., the MAK10 product) which are virion associated. The manner in which the product stabilizes the virion is not known. The cloned genes might be used to identify the gene products and test whether they are virion associated (Fujimura and Wickner, 1987).

The question of why L-A dsRNA replication requires only three chromosomal genes, whereas the replication of M dsRNA requires over 30 genes, remains unanswered. Transcription and replication [synthesis of (-) strand RNA] steps for both L-A and M dsRNAs occur within the virions. Because of the differences in size between the two templates, some of the M-specific chromosomal MAK genes may be involved in these steps. In this regard it is of interest to note that, like M dsRNA, a 530-bp deletion mutant (X dsRNA) that is derived entirely from L-A dsRNA requires all of the MAK genes tested. Both X and M dsRNAs replicate by the headful mechanism (see the replication cycle, Fig. 3), which entails the presence of multiple dsRNA copies per virus particle. It is also likely that many MAK genes are involved in the part
of the replication cycle that takes place outside the virions. For example, the M virions synthesize M (+) strand RNA, most of which is extruded from the virions. This M (+) strand RNA serves as mRNA for toxin-immunity protein synthesis. Alternatively or subsequently, M (+) strand RNA becomes packaged in new capsids. At this stage of the replication process, unlike the L-A (+) strand RNA, which may be efficiently and quickly protected by the CP it encodes, M (+) strand RNA may be particularly susceptible to degradation and the action of the host defense machinery. Because the product of the MAK11 gene has a C-terminal lysine-rich region, like many nucleic acid-binding proteins, it is possible that it interacts directly with M dsRNA or M (+) strand RNA, and thus plays a role in protecting M (+) strand RNA at this extraparticulate phase (Icho and Wickner, 1988).

Cell growth seems to modulate L-A dsRNA replication. When virions were purified from stationary phase cells, only mature virus particles were present and no dsRNA-synthesizing particles were detected (Fujimura et al., 1986). This suggests that this modulation occurs at the step of transcription or encapsidation of the transcripts into virions. Since the L-A dsRNA-synthesizing virions are known to become structurally and functionally mature in vitro by simply synthesizing the (−) strand RNA, it is more likely that the involvement of the chromosomal gene product, whose expression is controlled by cell growth, is at the encapsidation step.

2. The Yeast Antiviral System

Unlike the MAK genes, which regulate dsRNA replication through controlling the supply of host factors required for the replication function, the gene products of a set of six chromosomal superkiller (SKI) genes (SKI2, SKI3, SKI4, SKI6, SKI7, and SKI8) act as negative regulators (antiviral system) by lowering the copy number of M and L dsRNAs (Toh-e et al., 1978; Ridley et al., 1984). Mutations in any of these SKI genes lead to the development of the superkiller phenotype as a result of the increased copy number of M dsRNA. If the SKI genes are defective, the L-A virus, with the associated satellite M dsRNA system, becomes pathogenic, as cells become cold sensitive for growth.

The physiological importance of the SKI genes was first suggested by the finding that ski− mutants were cold sensitive at 8°C if an M replicon was present (Ridley et al., 1984). This effect was not related to the toxin or immunity functions encoded by M1 dsRNA, since deletion mutants of M1 (S dsRNAs), lacking most of the preprotoxin gene, also elicited the cold sensitivity for growth when introduced into ski− strains. It was not a matter of dsRNA concentration, however, since elimination of M dsRNA from a ski− cell containing both L-A and M
virions thus eliminating the repression of L-A dsRNA by M dsRNA) results in a 4-fold increase in total dsRNA but a loss of the cold-sensitive phenotype. Thus, the $SKI$ products are important to the cell to control M dsRNA copy number in order to prevent virally induced cytopathology.

The $SKI8$ gene was the first gene in this group to be cloned (Sommer and Wickner, 1987). The clone was used to construct a deletion mutant by gene disruption. In the absence of L-A and M dsRNAs, the disrupted strains showed slightly slower growth at $8^\circ C$ than the wild type. Introduction of M dsRNA resulted in complete inability to grow at $8^\circ C$. It seems that the only essential function of $SKI8$ is its role in repressing M dsRNA replication (Sommer and Wickner, 1987). $SKI3$, like $SKI8$, has also been shown to be essential to the cell only for this antiviral defense (Rhee et al., 1989). Sequencing data of the cloned $SKI3$ gene indicated that it could potentially encode a 163-kDa protein with a typical nuclear localization signal. The nuclear localization of the $SKI3$ product was supported by immunological analysis; an antibody produced to a fusion protein of $\beta$-galactosidase with a region of the $SKI3$ ORF reacts with a protein of about 165 kDa that copurified with nuclei (Rhee et al., 1989). Moreover, Hougan et al. (1989) demonstrated that mutant alleles of $SKI3$ resulted in an increased level of M1 dsRNA. These researchers did not detect any significant increase in the level of secreted toxin as a result of transforming a virus-free $ski3$ strain with a cloned cDNA to the K1 toxin, when compared with the wild-type $SKI$ strain transformed with the same plasmid. These findings indicate that the $SKI3$ gene product must act at the level of M1 dsRNA transcription or replication, rather than at the level of translation.

3. Natural Variants of the Yeast L-A Virus and Their Interactions with the Satellite M dsRNA: Helper and Exclusion Activities

Killer strains of $S. cerevisiae$ secrete a group of killer protein toxins ($K1, K2$, etc.) derived from a family of satellite dsRNAs ($M1, M2$, etc.), associated with the yeast L-A virus or variants thereof. M dsRNAs are dependent on the L-A virus for replication and encapsidation. Earlier genetic and biochemical studies have identified at least four types of natural variants of L-A dsRNA ($L-A-H, L-A-E, L-A-HN$, and $L-A-HNB$) that carry different combinations of non-Mendelian traits termed [HOKI], [EXLI], [NEX], and [B] (Wickner, 1986). From T1 fingerprints, Sommer and Wickner (1982) calculated that these L-A dsRNA variants share more than 99% sequence homology. Furthermore, virions containing these variant dsRNAs have identical protein profiles, as determined by SDS–PAGE analysis (Sommer and Wickner,
Thus, the expression of the different combinations of these traits by L-A dsRNA in different strains is due to minor sequence differences among these L-A dsRNA variants. [HOK], or helper of killer (H), is simply the function of L-A dsRNA needed by M1 or M2 dsRNA for its replication in a wild-type strain, and is assumed to be encoding the coat protein. The definitions of [EXL], or excluder of M2 (E), and [NEX], or nonexcluder of M2 (N), are related. [EXL] is the ability of certain L-A dsRNAs (L-A-E) to exclude M2 from strains that lack [NEX], but not from strains that have [NEX]. Thus, introducing L-A-E into a strain carrying L-A-H and M2 results in the loss of M2, but introducing L-A-E into a strain having L-A-HN and M2 does not result in loss of M2. Hannig et al. (1985) showed that L-A-E excludes M2 by lowering the copy number of L-A-H. However, L-A-E does not completely eliminate L-A-H. All wild-type K2 yeast killer strains contain L-A-H and M2 dsRNAs. L-A-H can support M1, and likewise, L-A-HN (the L-A variant present in all K1 wild-type killer strains) can maintain M2, indicating that essentially all L-A variants and M dsRNAs are interchangeable. Whereas L-A-E cannot maintain either M1 or M2 dsRNA in a wild-type nuclear genetic background, it can do so in a ski- host defective in the antiviral system. It should also be mentioned that M1 excludes M2 from any host strain independent of genotype (Wickner, 1983).

Now that the genome organization and expression strategy of L-A dsRNA have been elucidated and cDNA clones representing the entire dsRNA are available, it is possible to examine the molecular basis of the exclusion of M2 by L-A-E and other previously described biological phenomena involving the trilateral interaction among viral L-A dsRNA, satellite M-dsRNA, and host genes. For this purpose Wickner et al. (1991) constructed expression vectors containing full-length cloned cDNA of L-A dsRNA (pORF1 and -2) or cDNA representing only ORF1 (pORF1), and the constructs were transformed into different yeast strains with known genetic backgrounds. The expression of both ORFs from the cloned L-A cDNA was confirmed by demonstrating that the clone was able to maintain M1 in a maktO strain in the absence of L-A dsRNA. This finding proves that the L-A clone encodes biologically active proteins, since maintenance of M dsRNA is dependent on the expression of both ORF1 and -2.

L-A variants carrying [HOK] (L-A-H or L-A-HN) supplement L-A-E in supporting M1 in a wild-type host. The finding that either one of the two L-A expression vectors (pORF1 and -2 or PORF1) is sufficient to supply the [HOK] activity indicates that the defect in L-A-E is in the major coat protein and that it is either qualitative or quantitative. Comparing the nucleotide sequences of ORF1 of the L-A-E (when it
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becomes available) to the published sequence of L-A-HNB would be of interest in this regard. L-A-E, however, must be supplying a functionally competent CP–RDRP fusion protein. Since [HOK] is needed only in a SKI+ host, this suggests that one function of the major coat protein is to protect M1 dsRNA (and presumably L-A dsRNA as well) from the SKI antiviral system (Wickner et al., 1991).

[B], or bypassing MAK mutations, is the ability of certain L-A natural variants (termed L-A-HNB) to support M1 dsRNA in certain mak− hosts from which it would otherwise be lost. L-A-HNB dsRNA was the template used for cDNA synthesis and construction of the L-A expression vectors, mentioned above (Wickner et al., 1991). [B] suppresses many mak mutations, but not mak18-1 or mak27-1. The expression of [B] from the L-A expression vector requires only ORF1. Furthermore, the same pORF1 suppresses at least two mak mutations not normally suppressed by [B]. The finding that the expression of ORF1 suppresses many mak mutations is consistent with the idea that these genes are concerned with the efficiency of CP production from the L-A dsRNA transcripts. Alternatively, since these mak mutations are also those which are suppressed by ski mutations, they could be involved in M1 dsRNA defense against the SKI antiviral system or be one of the actual targets of the SKI system (Wickner et al., 1991).

4. The Capsid Protein Is the Target of the Host Defense Machinery: A Plausible Model to Explain the Interactions of Viral and Host Genes

Even though the precise target of the SKI antiviral system is not yet known, the proposal that the CP being the target would explain many of the reported interactions between host and viral genes. The findings that the defect in the natural variant L-A-E dsRNA, leading to loss of M1, is suppressed by ski mutations, and that it can be corrected by supplying the CP from a normal L-A dsRNA variant or from the expression vector pORF1 (see above), are consistent with the idea that the CP is the target of the SKI system or that the coat protein protects the actual target (genomic RNA).

In the presence of the L-A variant carrying [B], many MAK genes become dispensable for M1, and the same L-A maintains M1 at a higher than normal copy number (Uemura and Wickner, 1988). Wickner et al. (1991) demonstrated that the expression of the CP from pORF1 was sufficient to confer the [B] function onto the transformed cells. However, all of the mak mutations suppressed by the expression of pORF1 (or in the presence of [B]) are suppressed by ski mutations. If the target of the SKI system were the CP, then its overproduction by pORF1, or as a consequence of the high productivity of the L-A dsRNA
variant present (e.g., L-A-HNB), might swamp the SKI system, making the cell a ski phenocopy.

5. The Disease of H. victoriae

A major objective of studies on viruses that infect plant pathogenic fungi is to determine whether mycoviral dsRNAs or their specific gene products may perturb virulence expression in these fungi, and ultimately to utilize such knowledge in developing biological control measures for combating phytopathogenic fungi. In this regard the viruses infecting H. victoriae, the causal agent of Victoria blight of oats, are of special interest because they are associated with a disease of their fungal host (Lindberg, 1959, 1960; Ghabrial et al., 1979; Ghabrial, 1986) and may thus serve as a model system to investigate the pathological effects of fungal viruses and the molecular basis of disease in a plant pathogenic fungus.

Two isometric viruses, designated according to their sedimentation values as the 190 S and 145 S viruses, have been isolated from diseased isolates of H. victoriae (Sanderlin and Ghabrial, 1978). The two viruses have similar-sized particles, about 40 nm in diameter (Fig. 1). The Hv190S virus, a member of the family Totiviridae, contains a single dsRNA component, about 4.5 kbp in size. Four dsRNA species, 2.6, 2.8, 3.0, and 3.4 kbp in size, are associated with the 145 S virus. The following attributes of the H. victoriae system provide additional incentives to study the molecular basis of disease: (1) Disease symptoms, which include reduced growth, excessive sectoring, pronounced aerial mycelial collapse, and generalized lysis (Ghabrial, 1986), provide valuable selection markers for developing infectivity assays and for structure–function relationship studies of viral genes using DNA-mediated fungal transformation systems. (2) H. victoriae presents an excellent model system for a phytopathogenic fungus whose pathogenicity is dependent on the production of the host-specific toxin, “victorin” (Luke and Wheeler, 1955; Wolpert et al., 1988; Wolpert and Macko, 1989, 1991). Because diseased isolates of H. victoriae are hypovirulent and produce reduced levels of victorin, and because the genetics of victorin expression are partly known, it will be of interest to investigate the possible role of virus-encoded proteins in regulating victorin production. (3) Fungal isolates containing both the 190 S and 145 S dsRNAs secrete a broad-spectrum antifungal polypeptide (6–8 kDa), designated “victoriocin” (1993, unpublished observations). If victoriocin is dsRNA encoded, it may be analogous to the killer toxins in yeasts and smuts (Wickner, 1986). Diseased H. victoriae isolates may be analogous to “suicide” strains of yeast that are sensitive to their own toxin (Buck, 1986). The Hv190S virus shares many properties
with the yeast L-A virus. They are both members of the family Totiviridae, and they have similar-sized genomes and similar transcriptional and translational strategies (Ghabrial and Havens, 1989). It will be of considerable interest if victoriocin is indeed dsRNA encoded, since the killer phenomenon has not yet been found in filamentous fungi. The yeast killer protein is encoded by M dsRNA, a satellite dsRNA that is dependent on L-A dsRNA for replication and encapsidation (Wickner, 1986). Because the 145 S virus is not known to occur alone in *H. victoriae*, and has always been found in association with the Hv190S virus (Ghabrial, 1986), it is possible that the 145 S component represents a satellite virus or satellite dsRNAs. Characterization of the 145 S dsRNAs and their gene products is currently under way in our laboratory. The finding that victoriocin has broad-spectrum antifungal activity is not surprising, since the fungal host of the Hv190S virus is soil-borne, and in order for the killer toxin to confer ecological advantage it must have broad-spectrum activity.

I. The Protozoal Totiviruses

Definitive identification of viruses in protozoa has only been accomplished less than 10 years ago (Wang and Wang, 1986a,b). The first protozoal virus to be characterized was the *Trichomonas vaginalis* virus, (TVV), and shortly thereafter, a second dsRNA virus was isolated from *G. lamblia* (GLV). The dsRNA virus infecting *Leishmania braziliensis* (LRV1) was even a more recent discovery, and its presence became evident as a result of screening protozoal cells for the presence of RDRP activity (Tarr et al., 1988; Wildmer et al., 1989, 1990). Studies on the biochemical and molecular characterization of the monopartite dsRNA genomes of GLV and LRV1 are currently the major focus of at least three laboratories (for reviews see Patterson, 1990; Wang and Wang, 1991). All of the RNA viruses so far found in protozoal parasites appear to have monopartite dsRNA genomes, 5–7 kbp in size. The similarity of the dsRNA protozoal viruses to the dsRNA isometric fungal viruses in the genus *Totivirus* (Table II) has prompted the establishment of two new genera in the family Totiviridae to accommodate these viruses (see Section II,A).

The complete nucleotide sequence of the protozoal totivirus LRV1 has recently been determined. The 5284 nt contained two large ORFs and one small one, all in different phases on the same (+) strand RNA (Stuart et al., 1992). The ORF nearest the 3' end (ORF3; nt 2600–5233) predicts a protein with substantial homology to the RDRP of the yeast L-A virus. In addition, the LRV1 RDRP contained the sequence motifs found to be conserved among RDRPs from (+) strand and dsRNA vi-
ruses (Bruenn, 1991). Interestingly, the homology to the yeast viral RDRP extends beyond the domains that are conserved among all RNA RDRPs, suggesting a somewhat recent evolutionary divergence from the yeast virus. The dendrogram constructed by Stuart et al. (1992) to illustrate the degree of sequence similarity among viral RDRPs confirms that the RDRP from LRV1 is more closely related to L-A than to other viral RDRPs. The RDRPs of LRV1 and the yeast L-A virus are very similar in size and have somewhat similar hydropathy profiles and similar pIs (Table III), suggesting similar structure and function. The LRV1 may contain an ssRNA binding domain, as does the RDRP domain of the yeast L-A virus.

LRV1 ORF2 (nt 298–2670) predicts an 81.7-kDa protein. Although the predicted amino acid sequences show no detectable homology to the yeast L-A virus CP (nor to any other protein in the Swiss-Prot data base) and the hydropathy profiles are different, their sizes and pIs are somewhat similar (Table III), suggesting that ORF2 encodes the CP of LRV1 (Stuart et al., 1992). In addition, LRV1 ORF2 overlaps the RDRP ORF3 by 71 nt, resembling the organization of the yeast L-A genome. Thus, one may predict, by analogy to the L-A virus, that a gag–pol (CP–RDRP) fusion protein may be produced in LRV1 by translational frame-shifting. The predicted fusion protein would have similar size and pI to the yeast L-A CP–RDRP fusion protein (Table III). Analysis of the overlapping sequence between ORF2 (putative coat protein) and ORF3 (RDRP) predicts a +1 frame shift. Although the presence of the fusion protein has yet to be verified, its existence can be based on analogy to other viral systems. A sequence that could result in a +1

| Protein                  | No. of amino acids | Size (kDa) | pI  |
|-------------------------|--------------------|------------|-----|
| **RDRP**                |                    |            |     |
| LRV1-1 (ORF3)           | 874                | 98.3       | 8.24|
| ScV-L-A                 | 868                | 98.8       | 8.64|
| **Capsid protein**      |                    |            |     |
| LRV1-1 (ORF2)           | 741                | 81.7       | 6.52|
| ScV-L-A                 | 680                | 76.0       | 5.62|
| **Fusion protein**      |                    |            |     |
| LRV1-1 (ORF2–ORF3)      | 1575               | 175.9      | 7.64|
| ScV-L-A                 | 1504               | 170.4      | 7.49|

*Summarized from Stuart et al. (1992).*
frame-shift before peptide bond formation, such as that reported for the yeast Ty elements (Belcourt and Farabaugh, 1990), does occur in the ORF2/ORF3 overlap. Alternatively, the potential to form stable secondary structure within the region of overlap suggests that the frame shift may occur by a “long-jump” mechanism similar to that seen in the 50-nt ribosomal bypass of bacteriophage T4 gene 60 (Weiss et al., 1990). Analysis of this structure suggests that it could potentially form a pseudoknot, and that this and other intramolecular interactions might bring two GAU (Asp) codons into close physical proximity, making the long jump a “short hop” (Stuart et al., 1992).

Although the biological significance of the protozoal viruses to their hosts is poorly understood, there is considerable interest in using these viruses as molecular tools and models for the study of gene regulation in the parasitic protozoa and for developing gene transfer systems. The recent successful infection of G. Zamblia by electroporation of GLV (+) strand RNA transcripts (Furfine and Wang, 1990) and the synthesis of a cDNA library representing the entire LRV1 dsRNA genome (Stuart et al., 1992) are very encouraging steps toward these goals.

III. Totivirus Satellite dsRNAs That Encode Killer Toxins

The satellite dsRNAs associated with the totiviruses infecting the yeasts and smuts encode secreted toxins. Because the mature toxins are derived from larger precursors which are processed in the yeast (smut) secretory pathway, the yeast/virus/satellite system provides an excellent model system for investigating the molecular biology of protein export in eukaryotes. For example, the KEX1 and KEX2 genes, which were shown to be required for the production of the yeast mating pheromone α-factor (Dmochowska et al., 1987; Julius et al., 1984), have been originally identified because they are essential for the production of active K1 toxin (Wickner and Leibowitz, 1976a). The KEX2 gene encodes a membrane-bound endopeptidase (Kex2p) specific for cleaving the carboxyl side of pairs of basic amino acid residues (e.g., -Lys–Arg, –Arg–Arg). The KEX1 gene encodes a membrane-bound carboxypeptidase (Kex1p) specific for removal of the flanking basic amino acids from the processed intermediates (Dmochowska et al., 1987; Julius et al., 1984). Thus, studying the yeast killer system has allowed the definition of the components of the protease processing system, the first for any eukaryotes (Bussey et al., 1990). Detailed analysis of toxin processing in the yeast killer system has also indicated its remarkable similarity to that observed in the maturation of many eukaryotic hormones and neuropeptides. The recent demonstra-
tion that the \textit{KEX2/KEX1} gene products are functionally interchangeable between yeast and mammalian cells emphasizes the basic similarity of the prohormone (or killer protein precursor) processing machinery in yeast and neuroendocrine cells (Thomas \textit{et al.}, 1988, 1990).

There is considerable interest in the utility of the yeast preprotoxin in effecting the secretion of heterologous peptides and proteins from yeasts (Skipper \textit{et al.}, 1985). The prepro region of the \(\alpha\)-factor precursor has already proven useful in obtaining heterologous proteins as secreted products from yeast (Bitter \textit{et al.}, 1984; Brake \textit{et al.}, 1984; Miyajima \textit{et al.}, 1985; Mullenbach \textit{et al.}, 1986; Singh \textit{et al.}, 1986). Furthermore, the killer toxin gene has been explored as a dominant selectable marker in genetically manipulating the industrial yeasts (Bussey and Meaden, 1985). In addition to its intrinsic value as an anticontaminant for yeasts used in wine and beer fermentations, the yeast killer system has potential use in constructing transformation vectors to introduce desirable genes in yeasts (Boone \textit{et al.}, 1990; Vondrejs, 1987).

\subsection*{A. Yeast Killer System}

Killer strains of \textit{S. cerevisiae} secrete a group of protein toxins derived from a family of satellite dsRNAs (M1, M2, and M28), associated with the yeast L-A virus or strains thereof. M dsRNAs are dependent on the L-A virus for replication and encapsidation. While the yeasts that produce a given toxin are unaffected by it and are said to be immune, the toxin kills \textit{S. cerevisiae} strains lacking the satellite M dsRNA (for reviews see Tipper and Bostian, 1984; Wickner, 1986). Thus, toxin production and immunity to it is dependent on the presence of the satellite M dsRNA in cells coinfected with the helper L-A virus. At least three virus/satellite-based killer phenotypes have been reported for \textit{S. cerevisiae}: K1, K2, and K28. Each has a toxin-specific immunity system (Bussey \textit{et al.}, 1994; Schmitt and Tipper, 1990). Of the three toxin types, K1 is the most extensively characterized. A comparison among the three toxins is shown in Table IV.

\subsection*{1. Structure of M dsRNAs}

Although M1 and M2 dsRNAs lack significant sequence homology, they have similar organization (Dignard \textit{et al.}, 1991). They both contain an internal easily denatured AU-rich region "bubble" consisting of a 130- to 200-nt-long poly(A) tract on the (+) strand and a corresponding poly(U) tract on the (−) strand (Fried and Fink, 1978; Hannig \textit{et al.}, 1984, 1986; Hannig and Leibowitz, 1985). The AU-rich region
TABLE IV
COMPARATIVE PROPERTIES OF THE K1, K2, AND K28 KILLER TOXINS

| Property                  | K1                      | K2                      | K28                     |
|---------------------------|-------------------------|-------------------------|-------------------------|
| Genome                    | M1 dsRNA                | M2 dsRNA                | M28 dsRNA               |
| Genome size               | 1.8 kbp                 | 1.5 kbp                 | 2.1 kbp                 |
| AU-rich region            | Yes                     | Yes                     | No                      |
| 5' Hairpin region         | Yes                     | Yes                     | ND*                     |
| Precursor structure       | Pre-α–β, glycosylated  | Pre-α–β, glycosylated  | ND                      |
| Toxin structure           | α–β Heterodimer         | Glycosylated α–β       | ND                      |
| Optimum toxin activity    | pH 4.7                  | pH 4.3                  | pH 5.8                  |

aData are summarized from Whiteway et al. (1994) and Schmitt and Tipper (1990).

*ND, Not determined.

separates the 5’ toxin-encoding region (about 1 kbp) and the 3’-noncoding region containing the cis-acting sites essential for replication and encapsidation. Sequencing analyses of cDNA clones representing the preprotoxin coding regions of M1 and M2 dsRNAs indicated ORFs that encode proteins of 316 and 362 amino acid residues, respectively (Bostian et al., 1984; Dignard et al., 1991; Meskauskas and Citavicius, 1992). The calculated sizes of the proteins are 35 and 38 kDa, respectively. Full-length in vitro transcripts of virion M1 or M2 dsRNA can be purified to near-homogeneity by chromatography on oligo(dT)-cellulose. Although these transcripts lack a 3’-terminal poly(A) tract, they bind very tightly to oligo(dT)-cellulose or poly(U)-Sepharose due to its internal poly(A) tract (Hannig et al., 1986; Hannig and Leibowitz, 1985). The M28 dsRNA appears to lack such an AU-rich region, since oligo(dT)$_{15}$ could not be used to prime cDNA synthesis from either denatured M28 dsRNA or from in vitro-synthesized transcripts of virion M28 dsRNA (Schmitt and Tipper, 1990). Both M1 and M2 dsRNAs contain similar hairpin structures at their 5’ terminus of (+) strand (Fig. 11) in regions which also include the initiating AUG of the first long ORF (Hannig et al., 1984; Hannig and Leibowitz, 1985). The inability to obtain full-length cDNA clones of M dsRNA is probably due to the presence of such highly stable secondary structure at the ends of the molecules.

In a cell-free system the in vitro transcript of M1 dsRNA or denatured M1 dsRNA prime the synthesis of a 35-kDa polypeptide, denoted preprotoxin (Bostian et al., 1980a; Welsh and Leibowitz, 1982).
Hussain and Leibowitz (1986) developed a yeast translation system and showed that isolated (+) strand RNA or denatured M1 or dsRNA can be translated in a homologous system to produce preprotoxin. The yeast translation system was subsequently used to study the mechanism of translation initiation and the regulatory signals that may control gene expression at that stage.

Because M1 and M2 dsRNA molecules contain self-complementary sequences near the 5' termini, (+) strand RNA has extensive 5'-terminal stem–loop structures. The presence of such secondary structures has been confirmed by nuclease sensitivity studies (Hannig and Leibowitz, 1985). These secondary structures involve the regions surrounding the AUG initiating the first long ORF, which, in the case of M1, encodes the 35-kDa preprotoxin (Bostian et al., 1984). No such
secondary structure occurs around the 5'-proximal AUG in the (+) strand of L-A dsRNA (Thiele et al., 1984). Because the regions surrounding the 5'-terminal structures of the transcripts of M1 or M2 contain complementary sequences to the 3' termini of the yeast 18 S and 5.8 S rRNA molecules (Fig. 11), it was proposed that rRNA hybridization to these complementary sites could weaken the stability of the 5'-terminal hairpin, and as a consequence facilitate its function as a translational template (Leibowitz et al., 1988). Using the elongation inhibitor anisomycin in the yeast in vitro translation system, Leibowitz et al., (1988) demonstrated that the translation initiation complexes can be isolated and the possible role of RNA–RNA base pairing in these complexes may then be tested by cross-linking, an approach previously used with the wheat germ system.

2. Processing of Toxin

Expression of the cloned K1 or K2 killer precursor gene by the yeast ADH1 promoter in S. cerevisiae conferred the respective killer and immunity phenotypes on sensitive host yeast strains and facilitated studies of precursor processing and of toxin immunity and function (Lolle et al., 1984; Dignard et al., 1991). The secreted K1 toxin consists of two disulfide bond-linked subunits (α and β), which are released from a 42-kDa glycosylated precursor molecule following proteolytic processing (Bostian et al., 1983; Bussey et al., 1983; Dmochowska et al., 1987). The secreted K2 toxin appears to be a glycosylated molecule of 21.5 kDa (Whiteway et al., 1994). Toxin secretion is dependent on chromosomal KEX and SEC gene products as well as the action of enzymes sensitive to the chymotrypsin inhibitor, TPCK (Bussey et al., 1983; Lolle and Bussey, 1986; Tipper and Bostian, 1984; Wickner and Leibowitz, 1976a). Determination of the amino acid sequence of K1 toxin, deduced from the sequence of a cDNA copy of the M1 dsRNA (Bostian et al., 1984) and from the secreted toxin (Zhu et al., 1987), has revealed that the α-subunit is preceded by a 44-amino-acid leader peptide and that the α- (103 amino acids, 11.4 kDa) and β- (83 amino acids, 9.0 kDa) subunits are separated in the precursor by a glycosylated γ-peptide (Fig. 12). The precursor, targeted by its signal peptide to the endoplasmic reticulum (ER), enters the yeast secretory pathway, where it is glycosylated and proteolytically processed.

Studies involving the use of temperature-sensitive secretory defective sec mutants demonstrated that the toxin precursor followed the normal secretory pathway in yeast. No toxin was found to be secreted at the restrictive temperature in the sec mutants, and the precursor was found to accumulate at various stages of the pathway (see below), depending on the mutation (Bussey et al., 1983; Lolle and Bussey,
Fig. 12. Diagram of structure and processing of the K1 and K2 killer toxins. The probable signal peptidase (SPase) cleavage sites between alanine (A) and leucine (L) in K1, and between A and A in K2, are indicated by a vertical line in the shaded box (in case of K1), and by an arrow for K2. Sites for cleavage by KEX2 endopeptidase in the precursor (following basic residues which generate α- and β-toxin subunits) are indicated with the single-letter amino acid code followed by arrows. Removal of the C-terminal arginine (R) residues by the KEX1 gene product completes the processing. Glycosylation sites are indicated by the letter G below the preprotoxin open box. The shaded box represents the leader sequence in each toxin. Data were from Bussey et al. (1990) and Dignard et al. (1991).

The effects of various sec mutants on preprotoxin maturation indicate that it is classically processed via the ER, Golgi apparatus, and secretory vesicles (Bussey et al., 1983). Immunoprecipitation of killer cell extracts with antibodies raised against mature toxin identifies an intracellular 42-kDa precursor (protoxin), which differs from the 35-kDa preprotoxin molecule by incorporation of three mannose-based glycosylation units (Bostian et al., 1983; Bussey et al., 1983).

The use of the killer cDNA expression vector has allowed the overproduction of the toxin precursor from the ADH-1 promoter, which
Apparently overloads the entry process, causing precursor build-up. Three precursor species were identified (Lolle and Bussey, 1986): the 35-kDa preprotoxin, the primary translation product; a 33-kDa protoxin with the signal removed but unglycosylated; and the 42-kDa glycosylated protoxin. Pulse–chase kinetics, tunicamycin inhibition of glycosylation, endoglycosidase H treatment, and the use of ER-blocked sec mutants allowed an ordering of these precursors in the entry process. The 35- and 33-kDa precursors can be detected at the restrictive temperature in the early ER-blocked sec53 mutant, in which the 35-kDa species was chased to the 33-kDa precursor. At the later ER block caused by the mutation in sec18, the 35- and 33-kDa species were observed, and were chased to the 42-kDa species at the restrictive temperature. These findings, as well as those from the in vitro translation of denatured M1 dsRNA in a dog pancreas microsomal membrane system, which yielded the 33- and 42-kDa precursor species (Bostian et al., 1983), are consistent with posttranslational modification involving both proteolytic cleavage and glycosylation of the primary translation product.

Several lines of evidence suggest that the N-terminal leader or some part of it directs the precursor protein to the ER by functioning as a conventional signal sequence. When the entire 44-amino-acid residue toxin leader sequence plus the first 8 amino acids of the α-peptide were fused to a bacterial cellulase, the cellulase was directed to the extracellular medium when expressed in yeast. Little cellulase secretion was produced in the absence of the killer toxin leader, indicating a signal-like function for the sequence (Skipper et al., 1985). Structural analysis of the leader suggested that nested within the 44 amino acid residues there was an N-terminal 26-amino-acid residue signal sequence (Bostian et al., 1984). Work with deletions of this signal sequence and with an expression system and early sec mutants suggests that this signal peptide is necessary for targeting entry to the secretory pathway and that it is cleaved at the ER (Lolle and Bussey, 1986). The signal peptidase is believed to cleave between Ala26 and Leu27 of the preprotoxin (Fig. 12) (Lolle and Bussey, 1986). Cleavage at a single arginine site (Arg44) occurred by an as yet unidentified protease (Bostian et al., 1984). Recent studies on the expression of preprosomatostatin in yeast indicated that such activity (cleavage at a single Arg residue) is present in yeast (Bourbonnais et al., 1991). This activity is distinct from Kex2p, as the cleavage occurs in yeast kex2 mutants (Whiteway et al., 1994). The fate or function of the remaining N terminus, the 27- to 44-amino-acid segment, is unknown; presumably, it is removed in the Golgi apparatus, where further processing of the protoxin occurs.
In addition to the signal peptidase and the proposed leader cleavage, there are at least three proteolytic cleavage events involving the termini of α and the N terminus of β (Fig. 12). The primary event is an endoproteolytic cleavage following pairs of basic residues in the precursor by the product of the yeast KEX2 gene, an endopeptidase with homology to subtilisin (Julius et al., 1984; Bussey, 1988; Mizuno et al., 1988). The involvement of a dibasic endoprotease was realized on the identification of the γ–β junction at the peptide bond following Lys232 and Arg233 (Bostian et al., 1984). Although there are three other potential Kex2p sites, present evidence suggests that only the pair of Arg residues at positions 148–149 comprise a processing site that mark the α–γ junction in the precursor (Zhu et al., 1987). A subsequent event involves a carboxypeptidase B-like activity that removes the COOH-terminal basic residues of the α-subunit (Fig. 12) by the product of the KEX1 gene, a serine carboxypeptidase with homology to the yeast carboxypeptidase Y (Dmochowska et al., 1987). Following such processing the mature toxin is secreted via the constitutive secretory pathway to the growth medium.

Based on site-directed mutational analysis of putative processing sites in the K2 toxin coding region, Whiteway et al. (1994) concluded that processing of K2 toxin is consistent with a bipartite preprotoxin (unlike the tripartite precursor of the K1 toxin) with a single utilized Kex2p cleavage at KR-222, yielding an α-polypeptide of 172 amino acids which is N-glycosidated at two positions (N-177 and N-214), and with a β-polypeptide of 140 amino acids (Fig. 12) (Whiteway et al., 1994). The Kex1p carboxypeptidase is also required for processing of the KR residues at the terminus of the α-subunit (Fig. 12).

3. Mode of Action of Toxin

It is evident from the results of yeast transformation studies with the killer preprotoxin cDNA plasmid that the toxin and the component responsible for immunity are both encoded by the preprotoxin gene. Although the precise protein product that confers immunity has not been determined, site-directed mutagenesis of the preprotoxin gene maps the immunity domain within the region encoding the α-subunit (Boone et al., 1986; Sturley et al., 1986; Zhu et al., 1987). Since some of the mutations which fail to allow precursor processing retain immunity, it was suggested that the precursor can function as the immunity component (Boone et al., 1986; Sturley et al., 1986). In addition, strains defective in the processing proteases encoded by the KEX1 or KEX2 gene, both of which are required for the maturation of active toxin, retain immunity (Bussey et al., 1983). Models for conferring immunity hypothesize that immunity to the toxin occurs by the precursor or some...
derivative competing with the mature toxin for binding to a membrane receptor (Boone et al., 1986; Sturley et al., 1986). However, no convincing evidence for a membrane receptor exists, and the precursor component could directly interfere with the ability of the toxin to form ion channels (for reviews see Bussey et al., 1990, 1994).

Genetic and biochemical studies have shown that toxin action requires at least two steps. The toxin initially binds to a cell wall receptor which contains a \((1\rightarrow 6)-\beta-D\)-glucan (Al-Aidroos and Bussey, 1978; Hutchins and Bussey, 1983). Assembly of this glucan receptor requires a set of nuclear KRE (killer resistance) genes (Boone et al., 1990; Meaden et al., 1990). kre mutants are resistant to K1 toxin, but when kre mutant cells are converted to spheroplasts, they are sensitive to the toxin, suggesting the existence of a second step. Because the killer phenotype allows the selection of resistant mutants with glucan defects, Bussey and co-workers (Boone et al., 1990; Bussey et al., 1994; Meaden et al., 1990) are utilizing this system to explore synthesis of the cell wall receptor. The products of three genes, \(KRE1\), \(KRE5\), and \(KRE6\), are required for the sequential assembly of \((1\rightarrow 6)-\beta-D\)-glucan. Although the precise biochemical function of the KRE gene products remains obscure, \(\beta\)-glucan synthesis and assembly are complex and require many proteins in an apparently sequential process that traverses the yeast secretory pathway.

Physiological studies of K1 toxin action suggest that the toxin perturbs an energized plasma membrane state, causing ion leakage and subsequent cell death (de la Pena et al., 1981). By using the patch-clamp technique, it has recently been shown that the toxin forms voltage-independent cation channels in sensitive yeast spheroplasts and in artificial liposomes. Such channels are likely to be the basis of toxin action (Martinac et al., 1990). A possible functional assignment of domains of the K1 killer toxin has been suggested on the basis of the primary structure of the toxin subunits (Bostian et al., 1984). The \(\alpha\)-subunit contains two highly hydrophobic regions (residues 72–91 and 112–127) separated by a short hydrophilic region. This secondary structure suggested that the \(\alpha\)-subunit may be responsible for ion channel formation. In contrast, the \(\beta\)-subunit is hydrophilic and lacks potential membrane-spanning regions. Therefore, by analogy to the abrin and ricin classes of toxins (Olsnes and Phil, 1973), the \(\beta\)-subunit has been proposed (Bostian et al., 1984) to bind to the \((1\rightarrow 6)-\beta-D\)-glucan cell wall receptor. Published mutations in the toxin gene often lead to failure to secrete significant levels of toxin, making the analysis of phenotypes difficult (Boone et al., 1986; Sturley et al., 1986). The finding that mutations in the \(\beta\)-subunit may lead to a toxin which is inactive against whole cells but which kills spheroplasts is consistent with
a role of the β-subunit in binding to a cell wall receptor (Sturley et al., 1986).

Recently, extensive mutational analyses of both toxin subunits have been undertaken, and the results have indicated that whereas the α-subunit is necessary for channel formation, both the α- and β-subunits appear to be required for glucan binding (Zhu and Bussey, 1991). In this study regions encoding both toxin subunits were identified as affecting cell wall receptor binding. The inability of mutant toxins to interact with the β-glucan receptor was determined by both spheroplast killing and binding to a (1→6)-β-D-glucan column. Mutants that secreted a toxin inactive toward cells, but which retained the ability to kill spheroplasts, were interpreted as likely to be affected in a cell wall receptor binding domain. A large group of mutations localized to the α-subunit led to mutant toxins which failed to kill both cells and spheroplasts, suggesting that these mutations affected ion channel formation at least. Because both the α- and β-subunits are required for cell wall receptor binding, the A and B toxin model of ricin does not apply to the yeast killer toxin. The possibility that mutations in the α-subunit may indirectly perturb binding by the β-subunit cannot be ruled out, however (Zhu and Bussey, 1991).

The idea that the two hydrophobic regions flanking a central hydrophilic region in the α-subunit may be responsible for forming the transmembrane channel is supported by the mutational analysis by Zhu and Bussey (1991), who discovered that mutations in the regions encoding the two hydrophobic domains resulted in mutant toxins that were unable to kill spheroplasts (as would be predicted from the inability to form ion channels). One distinct characteristic of these mutations is that a charged amino acid such as Lys, Asp, or Arg, or an α-helix breaker residue, Gly or Pro, was introduced into the hydrophobic regions. These changes will reduce the hydrophobicity or perturb the α-helical structure of the two hydrophobic ion channel-forming regions (Zhu and Bussey, 1991). None of the mutations in the region encoding the β-subunit affects spheroplast killing activity, which suggests that this polypeptide is not involved in ion channel formation. Mutations altering the two hydrophobic regions of the α-subunit were found to be defective in both ion channel formation and immunity.

The organization of functional domains of yeast K1 toxin is distinct from the diphtheria toxin (which, like the yeast killer toxin, consists of two disulfide-linked peptide chains) and the abrin and ricin classes of A and B toxins. In the yeast K1 toxin the hydrophilic β-subunit does appear to be a B-type subunit involved in receptor binding. The α-subunit, in contrast, is multifunctional, having regions necessary for
ion channel formation, immunity, and cell wall receptor binding that appear to overlap in the polypeptide (Zhu and Bussey, 1991). The way in which the toxin is translocated from the β-glucan wall receptor to the site of action at the plasma membrane remains unknown.

4. The Immunity Domain

Yeast cells that secrete killer toxins are immune to the lethal action of their respective toxin. This immunity is distinguished from the resistance mediated by the host KRE genes, discussed earlier, by the fact that it is dependent on the presence of the M1 or M2 dsRNA. The finding that immunity was conferred by the toxin precursor gene led to studies mapping immunity to the α-subunit (Boone et al., 1986; Hanes et al., 1986; Lolle et al., 1984; Sturley et al., 1986). Mutations altering immunity define a region of the α-subunit that overlaps with the region that is believed to be involved in membrane channel formation. The dilemma of the immunity and toxin action being conferred by similar or overlapping domains of the protein was resolved by several models (Boone et al., 1986). These models propose that the toxin precursor, or some other α-containing nontoxic product derived from it, competitively interferes with mature toxin action. This could be by occupying a necessary receptor on the plasma membrane or directly by interfering with channel formation (Bussey et al., 1990). Because some mutants which fail to allow precursor processing and toxin secretion (as well as strains defective in the processing proteases Kex2p and Kex1p) retain immunity, it has been suggested that the precursor can function as an immunity component.

Mutations that affect immunity have been reported in the hydrophobic region near the N terminus of the K2 preprotoxin (Whiteway et al., 1994). Both K1 and K2 preprotoxins have extended regions of hydrophobicity, 40–50 amino acids long, at their N termini. The mutation that interferes with immunity resides at residues 38 and 39 of the K2 preprotoxin. The involvement of the leader sequence of both K1 and K2 preprotoxins in immunity should be investigated. Whiteway et al. (1994) postulated that the interaction with specific cellular targets is initially mediated by the leader sequence, with full immunity then being generated by subsequent interaction of the rest of the molecule. The location and identity of the cellular target for immunity remain to be elucidated.

B. Smut Killer System

Killer strains of the smut fungus U. maydis secrete proteins which are toxic to sensitive strains of the same or closely related species.
There are three distinct toxin specificities, KP1, KP4, and KP6, produced by different *U. maydis* strains (P1, P4, and P6, respectively); resistance to one toxin type does not confer resistance to the other two (for reviews see Koltin, 1986, 1988). Resistance to each toxin is conferred by a nuclear gene for each of the three toxins, and in the case of KP1, but not KP4 and KP6, a cytoplasmic immunity factor (dsRNA of viral satellite origin) may also be involved (Peery *et al.*, 1982; Puhalla, 1968). The alleles conferring resistance are recessive, indicating that a loss of function or a cellular component can lead to the inability to interact with the toxin (Koltin, 1988).

The smut killer system is more complicated than that of the yeast; as many as eight dsRNA segments can occur in an individual killer strain (Koltin *et al.*, 1978). The dsRNAs are packaged in 43-nm capsids which have a major CP with an estimated size of 75 kDa (Bozarth *et al.*, 1981). Each killer strain contains one or more representatives of three size classes of dsRNA segments: heavy (H), medium (M), and light (L). The original dsRNA patterns described for the three types of killer strains (Koltin *et al.*, 1978) were as follows: P1, with six segments (H1, H2, M1, M2, M3, and L); P4, with seven segments (H1, H2, H3, M2, M3, and L), and P6, with five segments (H1, H2, M2, M3, and L). Variations in these basic patterns of the smut killer strains have been noted as a result of the loss or addition of specific segments. As a consequence of such instability, some of the new strains isolated proved to be quite useful in mapping viral functions (Koltin, 1988). For example, the P6 strain that is currently in use in a major study involving the KP6 toxin contains only H1, M2, and L dsRNAs (Koltin, 1988). The H dsRNAs encode the CP and are packaged singly. The M segments which code for toxin production may be encapsidated singly or in combinations of multiple segments (Bozarth *et al.*, 1981). Present information on the origin of L (see below) suggest that it is separately encapsidated (Chang *et al.*, 1988).

Although none of the H dsRNAs have been cloned or sequenced, it is believed that some of the H dsRNAs represent the monopartite genome of totiviruses, *U. maydis* virus H (UmV-H). Assuming that the genome organization of UmV-H is similar to that of the yeast L-A virus, then only H1 and H2 dsRNAs (about 6.0 and 4.5 kbp in size, respectively) potentially have the coding capacity for the CP and a putative CP–RDRP fusion protein. Fungal strains or mutants that contain only H1 dsRNA have been found (Koltin *et al.*, 1978). The sizes of H3 and H4 dsRNA segments have been determined as 3.2 and 2.6 kbp, respectively (Shelbourn *et al.*, 1988), a genome size considerably smaller than would be expected for a virus member in the family Totiviridae (Buck and Ghabrial, 1991a). Although the H segments
within each subtype (P1, P4, or P6) lack sequence homology (Field et al., 1983), the major in vitro translation products in reactions primed with the individual denatured H1, H3, or H4 dsRNAs are immuno precipitable with antisera raised against UmV virions (Shelbourn et al., 1988). Whereas Podila et al. (1987a) reported that the H2 dsRNA from a P4 strain directed the synthesis of a 75-kDa polypeptide (the expected size of an authentic UmV capsid polypeptide) in a cell-free system that was immunoprecipitated by a capsid antiserum, Shelbourn et al. (1988) concluded from their in vitro translation and immunoprecipitation studies that H1, H3, and H4 segments (also from a P4 strain) encode capsid-related polypeptides in the range of 100–128 kDa. Apparently, the conditions used for denaturation or possibly the source of the reticulocyte lysates may influence the size of the translation products (Shelbourn et al., 1988). In general, it is difficult to reconcile the results of nucleic acid hybridization assays with the earlier genetic data as well as with those of in vitro translation and immunoprecipitation analyses (Koltin et al., 1978; Field et al., 1983; Podila et al., 1987a; Shelbourn et al., 1988). Some of these observations, however, may be explained if the killer strains examined were infected with two or more unrelated totiviruses (e.g., UmV-H1 and -H2), and the antisera used to react with the translation products of the individual segments were raised against virion preparations obtained from such mixed infections. This matter, however, will not be resolved until sequencing data become available and the genome organization of the individual H segments is elucidated.

Earlier genetic studies correlated the loss of the M segments with the loss of ability to produce active toxin (Koltin et al., 1978). In vitro translation studies have identified the M2 segment in a P4 killer strain as the toxin-encoding dsRNA (Podila et al., 1987a). The M1 dsRNA from P1 killer strains and the M2 from P6 strains are also translated in vitro to yield protein products immunoprecipitable with the respective antitoxin antibodies (Tao et al., 1990). Moreover, Podila et al. (1987b) presented evidence that the KP4 toxin was produced in vivo as a preprotoxin which was subsequently processed to a 10-12-kDa protein.

The highly purified KP6 toxin has been shown to consist of two polypeptides, initially designated VP10 and VP12.5 (later designated α- and β-polypeptides, respectively), that are not covalently linked (Peery et al., 1987). The interaction with the target is a sequential one in which the α-subunit must initiate the interaction. The β-subunit exerts its effect only if the cells were exposed first to the α-polypeptide. The cell wall is an important component, because spheroplasts are insensitive to the toxin that kills intact cells. Resistance to the toxin diminishes as a function of cell wall regeneration, suggesting the pres-
ence of receptors in the cell wall (Koltin, 1988). The M2 dsRNA that encodes KP6 has been cloned and sequenced (Tao et al., 1990). A single ORF was found that encodes a protein (preprotoxin) of 219 amino acids with a predicted size of 24.1 kDa. From the deduced amino acid sequence of the protein, the known NH₂ termini of the two subunits, and the predicted Kex2p-like cleavage sites, it was concluded that KP6 α- and β-polypeptides are processed from a preprotoxin in a manner similar to that of the yeast K1 toxin (Tao et al., 1990). Active KP6 was, in fact, secreted from yeast transformants expressing cloned cDNA to the KP6 preprotoxin. This finding indicates that the precursor was cleaved at the predicted dibasic residues, presumably by the KEX2 processing enzyme (Tao et al., 1990). Although the two secreted polypeptides were not glycosylated in U. maydis, the α-subunit was glycosylated in yeast. Both the yeast K1 toxin and KP6 toxins contain an additional processing site (Pro–Arg) which is cleaved by a protease activity following a single Arg residue, known to be present in yeast (see Section III, A, 2).

The sizes of the α- and β-subunits were estimated on the basis of N-terminal sequence analysis and the predicted C-terminal Kex2p-like cleavage at position 108 of the preprotoxin, followed by a predicted removal of the C-terminal Lys–Arg from the α-subunit by a Kex1p-like activity. The mature polypeptides are expected to have sizes of 78 amino acids, or 8.6 kDa (α-subunit), and 81 amino acids, or 9.1 kDa (β-subunit). These values are smaller than the 10 and 12.5 kDa estimated earlier by SDS–PAGE (Peery et al., 1987). Revised SDS–PAGE estimates for KP6 α- and β-subunits of 7.5 and 9.0 kDa have been reported (Tao et al., 1990).

Comparing the KP6 sequence with that of known killer toxins showed only similarity to the scorpion neurotoxins and cytotoxins. The KP6 α-polypeptide is of similar length to the neurotoxins (60–80 amino acids). Furthermore, the KP6 α-subunit, like the neurotoxins, has eight cysteines and is known to require intramolecular disulfide bridges for activity (Tao et al., 1990). KP6 α-polypeptide may also have a mode of action similar to that of the elapid cytotoxins (Rees et al., 1984), that is, by forming ion channels. Despite lack of similarity in primary structure, the hydrophobicity profiles of the β-subunits of KP6 and the yeast K1 toxins are very similar, and those of KP1 and the yeast K2 toxins are almost identical (Tao et al., 1990). Whereas the β-subunit of K1 is linked to the α-subunit by disulfide bonds, it acts as a monomer in the case of KP1 and KP6. Furthermore, the β-subunit is glycosylated in KP1 and K2, but not in K1 or KP6.

The KP4 toxin was recently purified to homogeneity and characterized (Ganesa et al., 1991). Unlike the bipartite KP1 and KP6 toxins, the purified KP4 toxin (7.2 kDa) is, surprisingly, comprised of a single subunit. Partial amino acids sequence analysis indicated a free N ter-
minus, and no homology to either the α- or β-subunit of KP6 was detected. Furthermore, the P4, but not the KP6, toxin is glycosylated (Ganesa et al., 1991).

The L dsRNA from a P1 strain has been sequenced, and analysis of the sequence revealed the absence of any long ORFs on either strand (Chang et al., 1988). The L is 3'-coterminal with the M dsRNA and is entirely derived from the 3' end. The 5' terminus of L dsRNA has no similarity to the highly conserved consensus GAAAAA, which is the 5' end of M1, as well as several other UmV dsRNAs (Field et al., 1983). The 3' end of the L (−) strand RNA is different from that of all other UmV dsRNAs (which is CA_{OH} or CG_{OH}). The GAA_{OH} of the L segment is derived from the internal sequence of M1 with a posttranscriptional addition of A at the 3' end. Thus, the L segments of UmV are unique in being derived from only one end of the larger M segment. In vitro translation of M1 results in a peptide whose size is consistent with its being encoded by the non-L region of M1. There are two likely mechanisms to give rise to L; internal initiation of transcription, or posttranscriptional cleavage of an M1 transcript (Chang et al., 1988). Thus, the origin of L is reminiscent of the generation of subgenomic RNA4 from RNA3 of (+) strand RNA viruses with tripartite genomes (e.g., bromoviruses and cucumoviruses), with the exception that these subgenomic RNAs encode the viral capsid polypeptide. Because L dsRNA lacks the consensus transcriptional start site present in M1, it is more likely that L is derived by cleavage of M1 (+) strand RNA.

The toxins secreted by killer strains of *U. maydis* are not related to their virulence or fitness as plant pathogens. The toxins have never been detected in infected plant tissues, and fungal strains lacking the toxin-encoding dsRNA segment are virulent (Nuss and Koltin, 1990). Because the toxins are not known to have deleterious effects on plant tissue, and because related species of *Ustilago* with various cereal hosts are sensitive to the toxins secreted by *U. maydis*, efforts are under way to produce transgenic plants that constitutively express the toxin as a means of biological control (J. A. Bruenn and Y. Koltin, 1993, personal communication).

IV. UNENCAPSIDATED dsRNA HYPOVIRUSES OF THE CHESTNUT BLIGHT FUNGUS

The chestnut blight fungus, *Cryphonectria (Endothia) parasitica* (Murr.) Barr, a devastating fungal plant pathogen, was introduced in North America from Asia at the turn of the century. It virtually elimi-
nated the American chestnut, *Castanea dentata* (Marsh.) Borkh., which was once a major component of the eastern deciduous forest. The tree now survives as stump sprouts, whose shoots successively become infected, are killed, and sometimes resprout from surviving stumps (for reviews see Anagnostakis, 1987; Van Alfen, 1982, 1986; Nuss and Koltin, 1990; Nuss, 1992).

Naturally occurring strains of the chestnut blight fungus, *C. parasitica*, that exhibit reduced levels of virulence (hypovirulence) have been shown to control disease progress in Europe (Van Alfen et al., 1975; Grente and Berthelay-Sauret, 1978; Anagnostakis, 1982, 1990). In addition to reduced virulence, these strains are characterized by reduced levels of sporulation and pigmentation (Anagnostakis, 1982, 1984; Elliston, 1985a), and by the presence of dsRNA genetic elements (van Alfen et al., 1975; Day et al., 1977). Conversion of dsRNA-free virulent strains to the hypovirulence phenotype is coincident with transmission of dsRNA during anastomosis with compatible hypovirulent strains, providing the basis for disease control (Van Alfen et al., 1975; Grente and Berthelay-Sauret, 1978; Anagnostakis and Day, 1979). There is considerable correlative evidence to support the conclusion that dsRNA is the cytoplasmic determinant for hypovirulence. A direct cause-and-effect relationship has recently been demonstrated for dsRNA and hypovirulence-associated traits (Choi and Nuss, 1992a,b; see Section IV,E). Because of the paucity of fungal systems in which specific phenotypes can be directly correlated with virus infection, the *C. parasitica*/dsRNA system presents an ideal system for investigating the molecular basis for hypovirulence in a plant pathogenic fungus. Furthermore, it may be possible to apply the knowledge gained to other plant pathogenic fungi for the purpose of biological control via the dissemination of hypovirulent fungal strains.

### A. Origin and Structure of dsRNA

The dsRNA isolated from hypovirulent strains of *C. parasitica* has been presumed to be of viral origin because the vast majority of known mycoviruses have dsRNA genomes (Buck, 1986). However, no virions typical of dsRNA viruses have been isolated to date; one exception to this is the recent preliminary report on reo-like virus particles isolated from strain C-18 (see Section IV,D). The dsRNAs are packaged in vesicles formed by the host (Hansen et al., 1985). The multiplicity of dsRNA species that may be present in the individual hypovirulent strains is an additional complicating factor to attempts to relate structure to function. L’Hostis et al. (1985) showed the lack of sequence homology between dsRNAs from European and American hypovirulent isolates.
These researchers proposed a number of possibilities for the origin of dsRNA multiplicity within the individual hypovirulent strains, including (1) a segmented genome of a single virus, (2) a mixed infection with two or more viruses, and (3) some of the dsRNA components may be satellite or deletion mutants. Examples of all three situations are presently known (Elliston, 1985b; Enebak et al., 1991; Shapira et al., 1991b).

Analysis of the terminal structures of the large dsRNAs of European and American origins (strains EP713 and GH2, respectively) has revealed that one terminus consists of a poly(A):poly(U) tract of variable length, referred to as the “homopolymer terminus” (Hiremath et al., 1986; Tartaglia et al., 1986). The other terminus, referred to as the “heteropolymer terminus” (Hiremath et al., 1986), has identical sequence in all five large dsRNA segments of EP713 [or L and M segments, according to the terminology of Shapira et al. (1991b), who designated the largest dsRNA as the L dsRNA and the other four collectively as M dsRNAs]. Likewise, the L and M dsRNA segments in GH2 have identical sequences at the heteropolymer terminus (Tartaglia et al., 1986). The results of Northern hybridization analysis, using a set of overlapping cDNA clones as probes, clearly indicated that the homology among the five large dsRNAs (L and M segments) in EP713 is extensive and not limited to the termini (Hiremath et al., 1988). Hiremath and co-workers suggested that the different segments may be produced by internal deletions of a single large dsRNA. Recent studies by Nuss and co-workers (Shapira et al., 1991a,b) have presented conclusive structural evidence to indicate that the dsRNA segments smaller than L dsRNA are indeed internally deleted forms of this dsRNA species (see Section IV,C).

B. Genome Organization and Expression Strategy

The L dsRNA present in the hypovirulent strain EP713 has been completely sequenced (12,712 bp) and its genome organization has been elucidated (Shapira et al., 1991a). The (+) strand RNA [the strand terminating with 3’ poly(A)] contained two contiguous coding domains, designated ORFs A and B, consisting of 622 and 3165 codons, respectively (Fig. 13) (Shapira et al., 1991a). The earlier report (Rae et al., 1989) that the genome of the L dsRNA is comprised of two overlapping ORFs (designated ORF1 and -2) should be discounted because of some sequencing errors (Choi et al., 1991b). The nucleotide sequence map derived from L dsRNA (Shapira et al., 1991a) has been confirmed by polymerase chain reaction (PCR) mapping analysis (Shapira et al., 1991b). The junction between ORFs A and B consists of the sequence
5'–UAAUG–3', where UAA functions as the termination codon for ORF A, and AUG comprises the 5'-proximal initiation codon within ORF B (Shapira et al., 1991a).

Both ORFs A and B of L dsRNA encode polyproteins that undergo autoproteolytic processing during translation (Choi et al., 1991b; Shapira and Nuss, 1991). ORF A has been shown to encode two polypeptides, p29 and p40 (Fig. 13), which are generated from a nascent polyprotein by an autocatalytic event mediated by p29 (Choi et al., 1991b). Mutational analysis of p29 revealed that residues Cys162 and His215 are essential for autocatalytic cleavage and confirmed results of microsequence analysis which had indicated that cleavage occurred between Gly248 and Gly249 (Choi et al., 1991a,b). Furthermore, kinetic analysis suggested that p29 autoproteolysis occurs cotranslationally (Choi et al., 1991a). A similar autocatalytic event was also observed during the translation of ORF B, resulting in the release of a 48-kDa protein, designated p48, from the N-terminal domain of the ORF B-encoded polyprotein (Shapira et al., 1991a). Using a combination of in vitro and in vivo expression studies coupled with mutational and microsequence analyses, Shapira and Nuss (1991) were able to locate the autocatalytic domain within the C-terminal region of p48, and to identify Cys341 and His388 as residues essential for autoproteolysis. Moreover, autoproteolytic processing by p48 was also demonstrated when expressed in E. coli, and microsequence analysis of the generated C-terminal cleavage product indicated that cleavage occurred between Gly418 and Ala419. Thus, both proteolytic activities resemble virus-encoded papainlike proteases, and the similarities between p29 and p48 suggest that their coding domains could have arisen as a result of a gene duplication event (Choi et al., 1991b; Shapira and Nuss, 1991).

Domains that contain putative RDRP and RNA helicase motifs have also been identified (Shapira et al., 1991a; Koonin et al., 1991). Due to
the similarity in genetic organization and expression strategy of the hypovirulence-associated dsRNAs to those of several viral genomes, the ICTV has recently approved the establishment of a new family, "Hypoviridae," to include these unencapsidated viruses (Hillman et al., 1994). The genus Hypovirus was also approved as the only recognized genus under the family Hypoviridae, and the virus (of European origin) infecting C. parasitica strain EP713, designated hypovirus 713, or HYPV-713, was established as the type species (Hillman et al., 1994).

1. The Hypovirus Infecting Strain NB58 Is Related to HYPV-713

The virus infecting C. parasitica strain NB58, isolated from a recovering chestnut tree in New Jersey, is of special interest because of the similarity of its dsRNA sequence to that of viruses of European (but not North American) origin. Furthermore, the NB58 virus does not appear to affect pigmentation and spore production (Hillman et al., 1992). It is believed that strain NB58 was isolated from a region in New Jersey where hypovirulent C. parasitica isolates of European origin were released for the purpose of biological control. Strain NB58, however, like North American hypovirulent fungal strains, is pigmented and produces asexual spores. Moreover, only a single dsRNA species (about 12.5 kbp in size) is associated with virus-infected single conidial isolates of strain NB58. This is unlike most viruses isolated from European strains, in which multiple dsRNA segments are usually associated with virus infection. The finding that the NB58 viral genome is comprised of a single dsRNA is consistent with the idea that the genomes of members of the family Hypoviridae are undivided. The NB58 virus is hereafter referred to as HYPV-58.

A cDNA library representing HYPV-58 dsRNA has been synthesized and mapped, and cDNA clones representing the 5' and 3' termini of the (+) strand RNA have been sequenced (Hillman et al., 1992). Like the 3' terminus of the HYPV-713 (+) strand, the 3' end of the analogous strand of HYPV-58 contains a short poly(A) tail. The 5' leader sequence of HYPV-58 dsRNA is also similar to that of HYPV-713 in that it inhibits downstream gene expression in cell-free systems (Rae et al., 1989; Hillman et al., 1992). Like the HYPV-713, approximately the first 500 nt of HYPV-58 (+) strand RNA lack long ORFs (>120 nt). Whereas the AUG that initiates the first ORF of HYPV-713 dsRNA is preceded by seven other AUG codons (Rae et al., 1989), the corresponding number for HYPV-58 is nine AUG codons (Hillman et al., 1992). It seems unlikely that any of the small upstream ORFs is translationally active because of the relative randomness of ORFs in the leader sequences of HYPV-713 and HYPV-58 dsRNAs. The similarity in leader
structure between the two viruses suggests that they utilize similar translational strategies.

The results of nucleic acid hybridization assays and limited sequencing data indicated that the regions of similarity between the dsRNAs of HYPV-713 and HYPV-58 are clustered at the termini and a large internal region (Hillman et al., 1992). It is of interest to note that the divergence of these sequences in the 5'-proximal coding regions of the two viral genomes involves a region in HYPV-713 that encodes several polypeptides, including a viral proteinase p29. The finding that expression of cloned cDNA to HYPV-713 ORF A (including the region of sequence divergence) in a pigmented virulent strain resulted in the reduction of pigmentation is consistent with the fact that strain NB58 that harbors HYPV-58 (with nonhomologous ORF A) is pigmented. Recent sequencing and translation data also support the idea that this region is not conserved between the two viruses (B. I. Hillman, 1993, unpublished observations). Optimal alignments of the 5'- and 3'-proximal sequences of the two viruses revealed an overall similarity of 60–65% and 65–70%, respectively. Stretches of greater than 90% identity between HYPV-713 and HYPV-58 at the 3' termini have also been located. The presence of strongly conserved sequences at the termini of these two viruses is probably because these sequences are required for replication (Hillman et al., 1992).

2. Sequence Similarities between Hypoviruses and Positive-Sense RNA and dsRNA Viruses

Computer-assisted analysis of the putative polypeptide products encoded by ORFs A and B of HYPV-713 L-dsRNA revealed five distinct domains with significant similarity to known conserved domains within plant potyvirus-encoded polyproteins (Koonin et al., 1991). These included the putative RDRP, RNA helicase, two papain-like Cys proteases related to the helper component protease (HC-Pro), and a Cys-rich domain of unknown function similar to the N-terminal region of the HC protein (Fig. 14).

Alignments of the RDRPs of the yeast L-A virus as well as of other dsRNA viruses, RDRPs of supergroup I-positive strand viruses, and RDRP of HYPV-713 indicated that the overall similarity between HYPV-713 and the L-A was less pronounced than that between HYPV-713 and the potyviruses. Another interesting feature of the HYPV-713 RDRP is the substitution for Gly in the highly conserved GDD motif. Analogous substitution occurs in the RDRPs of coronaviruses, toroviruses, several (-) strand RNA viruses, and the dsRNA bacteriophage φ6 (Koonin et al., 1991). Tentative phylogenetic trees were generated based on the RDRP alignment using three inde-
Fig. 14. Comparison of the organization of the polyproteins of HYPV-713 and potyviruses. Related domains are highlighted by identical shading. The boundaries of HYPV-713 ORFs A and B are indicated by horizontal arrows. Cleavage sites are indicated by vertical lines and vertical arrows. CP, Capsid protein; POL, RNA polymerase; HEL, helicase; PRO, protease. [Adapted from Koonin, et al. 1991.]

The computer-assisted analysis of Koonin et al. (1991) also indicated that the HYPV-713 helicase-like sequence detected in the C-terminal region of ORF B showed some similarity to the putative helicase of tobacco vein mottling potyvirus, and it largely conformed to the consensus pattern of conserved amino acid residues typical of the so-called helicase superfamily I (Gorbalenya et al., 1989). Tentative phylogenetic trees revealed the grouping of the putative helicase of HYPV-713 with those of (+) strand RNA viruses.

As mentioned earlier, HYPV-713 encodes two Cys proteases and an additional domain related to the HC protein of potyviruses. Like the potyvirus-encoded HC-Pro, the HYPV-713-encoded proteases resemble papainlike proteases (Oh and Carrington, 1989; Choi et al., 1991b; Shapira and Nuss, 1991). Additional similarities between p29 and HC-Pro include the occurrence of conserved amino acid sequences around the essential Cys and His residues, the nature of the cleavage dipeptides, and the distances between the essential residues and the cleavage sites. The sequence surrounding the essential Cys residues for both proteases is GYCY, while the consensus sequence flanking the essential His residue of HC-Pro is HVV/LD, and that for p29 is HV-VVD (Fig. 15). The positions of the essential residues relative to the
cleavage sites are also similar for the two proteases: C-(72 aa)-H-(40 aa)-G/G for HC-Pro, C-(52 aa)-H-(32 aa)-G/G for p29, and somewhat similar to p48 [C-(46 aa)-H-(29 aa)-G/A]. In all five proteases of potyviruses and hypoviruses shown in Fig. 15, a conserved Gly residue is located two positions to the amino side of the essential Cys position, and an aromatic amino acid (Tyr/Phe) is found on the carboxyl side. It is of interest that an alignment of the amino acids deduced from the N-terminal region of HYPV-713 ORF B, which encodes p48, with the homologous region of HYPV-58 reveals conservation of the Cys and His (as well as the Gly residue indicated above), residues, which have been shown by Shapira and Nuss (1991) to be required for autocatalytic activity. The Gly/Ala cleavage site is also conserved (Fig. 15) (B. I. Hillman, 1993, personal communication).

Tartaglia et al. (1986) speculated that the dsRNA associated with hypoviruses are analogous to the replicative form of an ancestral ssRNA virus. Considering the relative organization of the conserved domains within the HYPV-713 and potyvirus-encoded polyproteins (Fig. 14), Koonin et al. (1991) proposed that HYPV dsRNA might have evolved by rearrangement of a (+) strand RNA potyvirus-like genome. The following events were perceived to have taken place: (1) transposition of the helicase gene; (2) duplication of the sequence encoding the protease domain of the HC; (3) deletion of the sequence encoding the protease domain of the nuclear inclusion protein Nla; (4) deletion of the CP gene; and (5) emergence of the termination codon separating the two ORFs. Because of their intracellular mode of transmission, fungal viruses can dispense with an extracellular route of infection and the required packaging function. In the absence of a CP, the ratio of the ssRNA genome to the replicative form could have been altered.

### Alignment of the cysteine and histidine residues and the cleavage sites for p29 and p48 of HYPV-713, and the helper component protease encoded by two potyviruses.

**TEV-HC-Pro**
- IANEGCYMNI- 64 -KTMHVLD- 31 -KTYNVG GMNR

**TVMV-HC-Pro**
- IAKEGCYINI- 64 -KTIHVVD- 31 -AQYKVG GLVY

**HYPV-713-p29**
- QFGQQGCVL- 45 -HVVVHD- 23 -PLARIG GRLN

**HYPV-713-p48**
- PVEEGC- 39 -QCVHIVA- 20 -PDILVG AEAG

**HYPV-58-p50**
- PVKEGEC- 40 -GLVHCEP- 21 -EPCVVG A---

Fig. 15. Alignment of the cysteine and histidine residues and the cleavage sites for p29 and p48 of HYPV-713, and the helper component protease encoded by two potyviruses. The essential residues are indicated by bold vertical lines, while conserved residues are indicated by asterisks. The arrow indicates the autocatalytic cleavage site. Data were summarized from Shapira and Nuss (1991). The data for HYPV-58-p50 were supplied by B. I. Hillman (1993, unpublished observations).
so that the dsRNA form predominated. Because gene module shuffling has been recognized as a major trend in the evolution of (+) strand viruses, Koonin et al. (1991) proposed that a similar process may account for the evolution of a dsRNA virus-like genetic element from a (+) strand RNA virus. Arguments against this proposal would include the fact that the predicted 12.5-kb single-stranded genomic RNA has never been detected in the infected cells. Because hypoviruses are typically devoid of capsids, it will be difficult to determine whether the hypovirus dsRNA is equivalent to the genomic RNA in mature virions of a dsRNA virus or represents an intermediate phase in the replication of a ssRNA virus.

C. Defective dsRNAs and Their Role in Hypovirulence

In addition to the L and M dsRNAs, *C. parasitica* strain EP713 contains several smaller dsRNA segments, ranging in size from 0.6 to 1.7 kbp. PCR mapping analysis provided evidence that both the M and S dsRNAs are generated from L dsRNA by internal deletion events (Shapira et al., 1991b). Sequence analysis of cloned cDNA of S dsRNA also confirmed that these elements retained both the heteropolymer and homopolymer domains found in L dsRNA. Sequencing data from three different S dsRNA species indicated a single deletion breakpoint and retained 149, 155, or 156 bp of the terminus, corresponding to the 5' end of the coding strand and 440, 447, or 449 bp of the other terminus. Two of the S dsRNA species (S2 and S3) contained, within the boundaries of the breakpoint, additional sequence information consisting of 42 or 95 bp that appeared unrelated to the L dsRNA. Shapira et al. (1991b) reasoned that the characteristics of the S1 dsRNA breakpoint are consistent with a copy-choice model for the generation of S dsRNA in which the synthesis of a nascent RNA strand is interrupted, possibly by secondary structure constraints. This is followed by dissociation of the complex, reassociation with a second template, mediated by the sequence homology between the 3' end of the nascent RNA and a region of the template 3' of the breakpoint, and completion of the synthesis of the nascent RNA strand. The internally deleted RNA then serves as a template for subsequent RNA synthesis.

This model for S dsRNA generation is based on the assumption that the replication strategy of L dsRNA involves ssRNA intermediates, and is complicated by the presence of nonviral sequences between the recognized breakpoints of S2 and S3 dsRNAs. The nonhomologous sequences could represent cellular RNA that was introduced as a result of a recombination event between cellular and viral RNAs (Shapira et al., 1991b). The finding that insertion of 54 nt corresponding to a
region in the 28 S ribosomal RNA into the influenza virus hemagglutinin gene resulted in enhanced viral pathogenicity (Khatchikian et al., 1989) is of interest in this regard. Thus, an understanding of how the defective dsRNAs are generated and acquire nonhomologous sequence information may provide valuable insight into the mechanisms of L dsRNA replication and expression.

The studies of Shapira et al. (1991a,b) suggest that the entire genetic information required for maintaining the hypovirulence phenotype resides in the L dsRNA. Structurally, the M and S dsRNAs resemble defective interfering RNAs described for other viral systems, including the yeast L-A virus killer system (Bruenn, 1986). The influence of M and S dsRNAs on the replication and expression of L dsRNA and their contributions to the hypovirulence phenotype are difficult to assess because of the lack of conventional infectivity assays. While the S dsRNA species consist exclusively of noncoding sequences, the larger M dsRNAs are potentially capable of directing the synthesis of certain encoded polypeptides (p29 and p40 of ORF A). PCR mapping indicated that about 3.5 kbp of each terminus appeared to be conserved intact (Shapira et al., 1991b). Thus, the presence of M dsRNA should not affect the accumulation of ORF A-specified polypeptides, but would be expected to impact on the synthesis and processing of ORF B-specified polypeptides. On the other hand, the accumulation of S dsRNAs may interfere with the synthesis of L and M dsRNAs by outcompeting these dsRNAs for the limited RNA polymerase sites. However, examples in which the S dsRNAs are the predominant species present in the host cells are not known. The impact of the dynamic competition between genomic and defective dsRNAs on hypovirulence expression has not been investigated.

Since the defective S dsRNAs replicate efficiently in the presence of L dsRNA, they must contain all of the cis-acting signals required for dsRNA replication. Sequence analysis of cloned cDNAs to S dsRNAs indicated that these regulatory elements reside within the 5'-terminal 155 nt and within the 3'-terminal 450 nt at the homopolymer end (Shapira et al., 1991b). Future studies will undoubtedly define precisely the location of the cis-acting sites, and when available, such information should be valuable in constructing expression vectors to introduce desired foreign genetic information into hypovirulent strains of the chestnut blight fungus.

D. Reo-like Viruses Associated with Hypovirulence

Recent careful examination of the dsRNA complement present in two hypovirulent strains (C-18 and 9B-2-1) isolated from cankers on
chestnut trees in West Virginia revealed the presence of 11 or 10 dsRNA segments, respectively, none of which was larger than 5 kbp in size (Fig. 16). The cultural characteristics of these two strains, such as colony morphology, pigmentation, and conidia production, were similar to those of other hypovirulent strains from North America (Enebak et al., 1991). However, their dsRNA content was different, as indicated, since North American hypovirulent strains typically have a single species of high molecular weight (Dodds, 1980). The 11 segments of C-18 appeared to occur in equimolar amounts when examined in stained gels (Fig. 16). Furthermore, Northern hybridization analysis using cloned cDNA probes revealed that the 11 dsRNA segments have unique sequences (B. I. Hillman, 1993, personal communication). Partially purified virus preparations from strain C-18 contained viruslike particles 60 nm in diameter, and the set of 11 dsRNA segments could be resolved from fractions containing such particles. Moreover, single conidial isolates from C-18 showed an all-or-none pattern of transmission of the 11 dsRNA segments, suggesting that the 11 dsRNA segments represent the multipartite genome of a single virus (B. I. Hillman, 1993, personal communication).

The properties of the C-18 virus described so far are reminiscent of viruses in the family Reoviridae (Holmes, 1991). The sizes of genome segments from the C-18 virus are in the same range as those of the

![Fig. 16. Silver-stained 8% polyacrylamide gels of dsRNAs from C. purasitica strains C-18 and 9B-2-1, and the plant reovirus, wound tumor virus (WTV). The dsRNA segments of WTV range in size from 851 bp to approximately 5 kbp. (Courtesy of B. I. Hillman.)](image-url)
wound tumor virus, a member of the Phytoreovirus genus of the family Reoviridae (Fig. 16). The significance of the finding that a reo-like virus is associated with hypovirulence lies not only in providing an additional avenue for exploiting hypovirulence as a means of biological control, but also in opening the possibility of finding natural vectors or alternative hosts for fungal viruses. Although phytoreoviruses, like fungal viruses, are not mechanically transmitted, they are transmitted by leafhoppers in a propagative manner. Furthermore, wound tumor virus grows in insect cell lines derived from embryonic tissue of vectors, thus providing an excellent experimental system for studies on virus replication and molecular biology (for a review see Nuss and Dall, 1990).

E. Molecular Basis of Hypovirulence

With the recent progress in elucidating the basic genetic organization of HYPV-713 and the development of a DNA-mediated transformation system for C. parasitica (Hillman et al., 1989; Churchill et al., 1990), the question of whether the phenotypic traits exhibited by hypovirulent C. parasitica strains are the result of a general response of the host to the physical presence of replicating dsRNAs, or whether these traits are dependent on functions encoded by specific hypovirus sequences, has been addressed. Transformation of an isogenic virulent, dsRNA-free C. parasitica strain with a cDNA copy of ORF A was shown to confer some of the hypovirulence-associated traits, including reduced pigmentation, reduced laccase accumulation, and suppressed conidiation (Choi and Nuss, 1992a). Although virulence was not reduced this report demonstrated that uncoupling of hypovirulence from associated traits seems to be possible.

In another study, Choi and Nuss (1992b) reported that the complete hypovirulence phenotype can be conferred by transformation with a full-length cDNA copy of HYPV-713 dsRNA. Such engineered hypovirulent transformants contain both a chromosomally integrated cDNA copy of the viral genome and a resurrected cytoplasmically replicating dsRNA form. Chen et al. (1993) demonstrated stable physical and functional transmission of integrated viral cDNA to both conidia and ascospore progeny, thus presenting possibilities for novel modes of transmission not previously known for hypoviruses. These studies establish a direct cause and effect relationship between hypovirulence-associated traits and specific viral coding domains.

Powell and Van Alfen (1987a,b) have demonstrated that specific host poly(A)^+ RNAs and polypeptides are down-regulated in dsRNA-containing strains. Hypovirus infection is believed to perturb the ex-
pression of specific developmentally regulated fungal host genes at the mRNA level. Some of these genes have recently been cloned (e.g., laccase) and their function and nature of down-regulation are being investigated (see below).

The extracellular laccase, a copper-containing phenol oxidase known to occur in many plant and fungal species has attracted interest because its production has been reported to be reduced in hypovirulent strains of \textit{C. parasitica} (Rigling \textit{et al.}, 1989). The biological function of this enzyme remains obscure, even though the genes encoding laccases from four fungal species have been cloned and sequenced (Germann \textit{et al.}, 1988; Aramayo and Timberlake, 1990; Kojima \textit{et al.}, 1990; Choi \textit{et al.}, 1992). Laccase activity has been implicated in sporulation, pigment production, lignin degradation, and pathogenesis (Ander and Eriksson, 1976; Law and Timberlake, 1980; Leatham and Stahmann, 1981; Anagnostakis, 1987; Bar-Nunn \textit{et al.}, 1988). Infection with hypoviruses has been reported to down-regulate laccase biosynthesis, and this has been attributed to the reduction in laccase mRNA accumulation (Rigling \textit{et al.}, 1989; Rigling and Van Alfen, 1991; Choi \textit{et al.}, 1992). Because hypovirulent strains are known to induce only superficial cankers, unlike the deep necrotic cankers elicited by virulent strains, Choi \textit{et al.} (1992) speculated that this deficiency in hypovirulent strains may be related to the reduced level of laccase production.

Another developmentally regulated gene that is affected by virus infection is that which encodes a highly abundant cell surface protein, cryparin (Carpenter \textit{et al.}, 1992). This polypeptide (18.6 kDa) was purified to homogeneity and used to prepare specific polyclonal sera. Cryparin was found in aerial hyphae and fruiting bodies, it has lectinlike properties, and its N-terminal end has a Gly–Ser-repeating sequence. The properties of cryparin suggest that it is a structural protein associated with fungal development. The role of this virally regulated polypeptide, if any, in virulence expression in \textit{C. parasitica} is unknown. It is of interest that cryparin resembles, in physical properties and sites of accumulation, the putative phytotoxin cerato-ulmin produced by the Dutch elm disease fungus (Stevenson \textit{et al.}, 1979; Takai and Hiratsuka, 1980).

Future studies on the molecular basis of hypovirulence will undoubtably involve the molecular dissection of newly described viral strains that are structurally related but biologically distinct from well-characterized strains (e.g., HYPV-58 and HYPV-713, which are associated with pigmented and nonpigmented phenotypes in their host, respectively), as well as of biologically similar but structurally unrelated viruses (e.g., the two unrelated viruses, the reo-like virus C-18 and
HYPV-58, which are associated with similar hypovirulent and pigmented phenotypes in their hosts). These studies will be facilitated by the availability of cDNA libraries representing the complete sequence of HYPV-713 and the partial sequence of HYPV-58, as well as by the available information on the expression strategy of the HYPV-713 genome (Choi et al., 1991a,b; Koonin et al., 1991; Shapira et al., 1991a,b; Shapira and Nuss, 1991). The recent cloning of a number of C. parasitica genes (Choi and Nuss, 1990; Rigling and Van Alfen, 1991; Choi et al., 1992), some of which may be the target of hypovirus-mediated modulation, and the development of an efficient transformation system for C. parasitica (Hillman et al., 1989; Churchill et al., 1990) should provide valuable tools and material for such studies.

V. Evidence for a Viral Etiology for the LA France Disease of the Cultivated Mushroom Is Stronger 30 Years Later

In 1948 a serious disease of the cultivated mushroom A. bisporus, characterized by malformed fruiting bodies and loss of crop, was first reported in a mushroom house owned by the La France Brothers of Pennsylvania (Sinden and Hauser, 1950). The disease was termed La France disease, and similar afflictions were reported soon afterward from England, France, The Netherlands, Italy, Denmark, Japan, and Australia. Different designations, such as “X-disease,” “watery stipe,” “brown disease,” and “die-back,” were given to essentially the same disease as the La France disease, first observed in Pennsylvania (reviewed by van Zaayen, 1979). The significance of the 1948 report lies in that it led to the discovery of fungal viruses. Hollings (1962) observed at least three types of virus particles in diseased mushroom sporophores. This was the first report of virus particles in association with a fungus. Because the nature of the genomes of the viruses involved was not elucidated, and because apparently healthy mushrooms did harbor viruses, earlier infectivity assays were difficult to interpret. Furthermore, precautions against contamination with exogenous spores were not taken. Today, 30 years later, the evidence for viral etiology is stronger, but remains equivocal.

Mushrooms from diseased crops contain virus particles as well as up to 10 major dsRNA species (Marino et al., 1976; Wach et al., 1987; Harmsen et al., 1989). The dsRNAs and the isometric particles copurify from diseased mushrooms (Barton and Hollings, 1979). Recent dsRNA analysis demonstrated a close association between a conserved set of nine dsRNAs (Fig. 17) and the disease in mycelial
cultures (Koons et al., 1989) and sporophores (Romaine and Schlagnhauffer, 1989; Wach et al., 1987). Bacilliform (19 × 50 = nm) particles and isometric virus particles 25 and 34–36 nm in diameter are most commonly isolated from diseased mushrooms (Passmore and Frost, 1979; van Zaayen, 1979). Symptomless healthy mushrooms from high-yielding crops in The Netherlands (Harmsen et al., 1989) always contain one major dsRNA (L6 dsRNA, Fig. 17), or else totally lack dsRNA (Marino et al., 1976). The 10 dsRNAs range in size from 0.8 to 3.6 kbp (Table V), and each apparently has unique sequences, since they do not cross-hybridize when cloned dsRNAs are used as hybridization probes under stringent conditions (Harmsen et al., 1989).

A. Molecular Characterization of Disease-Specific dsRNAs

The M2 (1.3 kbp), M1 (1.55 kbp), and L3 (2.8 kbp) dsRNAs (Table V) have been cloned and their nucleotide sequences were determined (Harmsen et al., 1991). Putative coding sequences for proteins with predicted sizes of 38, 40, and 87 kDa were found for M2, M1, and L3.
TABLE V
PROPERTIES OF THE DISEASE-SPECIFIC dsRNA SEGMENTS FROM Agaricus bisporus*  

| dsRNA | Size (kbp) | In vitro translation product (kDa) |
|-------|------------|---------------------------------|
| L1    | 3.6        | 130b (129)                      |
| L2    | 3.0        | 84?                             |
| L3c   | 2.8        | 88 (87, L3p)                    |
| L4    | 2.7        | 84?                             |
| L5    | 2.5        | NDc                             |
| L6c   | 2.4        | ND                              |
| M1c   | 1.55       | ND (40, M1p)                    |
| M2c   | 1.4        | 45 (44, M2p)                    |
| S1    | 0.9        | 28                              |
| S2    | 0.8        | 28                              |

aData are summarized from Harmsen et al. (1989, 1991).

bA 130-kDa protein was detected among the translation products of total dsRNA. This protein was assigned to L1 because the latter is the only dsRNA species with sufficient coding capacity. The detection of a minor protein of 129 kDa in dsRNA-containing fractions of cesium sulfate gradients is of interest in this regard (Goodin et al., 1992).

cThe complete nucleotide sequences of these dsRNA segments have been determined; each has a single ORF. The predicted sizes of the putative proteins L3p, M1p, and M2p are indicated in parentheses.

cThe L6 dsRNA segment is believed to be associated with the 25-nm isometric particles isolated from symptomless mushrooms (see Fig. 17).

dND, Not determined.

dsRNAs, respectively. The average G+C content of these dsRNAs was 43%, close to that of A. bisporus nuclear DNA. The nucleotide sequences, as well as the deduced amino acid sequences, appear to be unique, as no matching sequences could be found among data bases (Harmsen et al., 1991). No large repeated sequences were present in the three dsRNAs, except for a 14-base repeat (AACAUACGUAGUGU) in the 3'-noncoding region of L3 dsRNA (Harmsen et al., 1991). A mutual comparison of all three dsRNAs for short identical sequences yielded a hendecameric sequence (AACGGCUAGUU) that was present in the
5'-noncoding region of M2 dsRNA and of L3 dsRNA. The presence, by chance, of identical hendecameric sequences in two different nucleic acid molecules is statistically very small. Thus, the hendecameric consensus found in M2 and L3 dsRNAs could be involved, for example, in the formation of complexes between RNA and the CP or between RNA and the RDRP. However, the consensus VBS known for the yeast L-A dsRNAs (Esteban et al., 1988) was not found in any of these three dsRNAs.

A small dsRNA of 0.39 kbp, designated S3, is occasionally found in large amounts in diseased mushrooms. Because it is largely comprised of the noncoding ends of M2 dsRNA, S3 has been proposed to represent an internally deleted variant of that dsRNA. The mushroom S3 is apparently stably maintained in A. bisporus; it is unlikely that it represents a processed product from M2 dsRNA. More likely, S3 resembles the defective interfering S dsRNAs associated with the yeast L-A virus. No translation products were detected when denatured S3 was used to direct protein synthesis in a cell-free system (Harmsen et al., 1991).

Full-length transcripts of dsRNAs L3, L6, M1, M2, S2, and S3 were detected in total ssRNA preparations from diseased mushrooms by hybridization with cloned probes (Harmsen and Wessels, 1994). These results showed that at least six of the dsRNAs were actively transcribed in diseased sporophores. Whether these transcripts serve as mRNA or act as templates for dsRNA synthesis, or both, is not known.

The coding capacity of the dsRNAs was determined by in vitro translation of denatured total dsRNA as well as of individually purified dsRNA segments. In general, seven major in vitro translation products were resolved; these were designated p28a, p28b, p45, p59, p84, p88, and p130, according to their apparent molecular weights (Harmsen and Wessels, 1994). The in vitro translation products of the individually isolated dsRNAs M2, S1, and S2 yielded single major polypeptides with sizes corresponding to p45, p28a, and p28b, respectively. Several translation products were detected for L3, but the largest had the size of p88. Inconclusive results were obtained with the translation of other individually isolated dsRNAs, L1, L2, L4–L6, and M1. Only L1 (3.6 kbp) has sufficient coding capacity to encode a polypeptide of 130 kDa, as resolved in translations of total dsRNA (Table V). Based on its size, the 130-kDa polypeptide is a candidate to comprise the viral RDRP. It is of interest in this regard that a minor protein of 129 kDa has been reported to copurify with the 36-nm virions from diseased mushrooms (Goodin et al., 1992; see below).
B. Packing of Disease-Specific dsRNAs

Using a purification procedure that involves chloroform extraction, PEG-NaCl precipitation, differential centrifugation, and equilibrium centrifugation in cesium sulfate gradients, Goodin et al. (1992) were able to obtain from diseased sporophores purified virus preparations highly enriched in 36-nm isometric particles and which contained only minor amounts of the 25-nm isometric particles and the $19 \times 50 = \text{nm}$ ssRNA bacilliform virus. The cesium gradient fractions that contained the 36-nm particles also contained the nine disease-specific dsRNAs of 0.8–3.6 kbp. The 36-nm isometric virus was designated La France isometric virus (LIV) (Goodin et al., 1992). Two major polypeptides of 63 and 66 kDa and a minor one of 129 kDa were detected by SDS-PAGE of the dsRNA-containing fractions of the cesium gradients. Although the nine dsRNAs have unique nucleotide sequences (Harmsen et al., 1989), three of the segments (M1, S1, and S2) occur in submolar concentrations and could be satellite dsRNAs (Fig. 17). The bacilliform particles of the mushroom bacilliform virus (MBV) are not likely to package any of the nine dsRNAs. Highly purified virions of MBV do not contain dsRNA, but rather a 4.4-kb ssRNA that shares no sequence homology with the disease-specific dsRNAs (Romaine and Schlagnhafer, 1991). Moreover, MBV has been shown to have a single CP of 24.5 kDa (Tavantzis et al., 1980). The 25-nm isometric particles, on the other hand, are present in concentrations too low to accommodate the dsRNAs. It is noteworthy that the isometric 25-nm particles and the 2.4-kbp dsRNA (L6 segment, Table V and Fig. 17) have been reported to occur in healthy tissues (Hicks and Haughton, 1986; Harmsen et al., 1989; Romaine and Schlagnhafer, 1989).

Although present evidence strongly suggests that the nine disease-specific dsRNAs are encapsidated in 36-nm isometric virus particles (Goodin et al., 1992), it is not clear whether the dsRNAs are encapsidated individually, in various combinations, or all nine segments are packaged in single particles. Examination of the cesium sulfate gradient profile and results of dsRNA and protein analyses of the gradient fractions reveal that at least two broad-density components were resolved: a lighter component of empty capsids and a nucleoprotein component (Goodin et al., 1992). Judging from the size of the particles (36 nm in diameter), it is highly unlikely that all dsRNAs are packaged together in single particles. More likely, LIV represents a multiparticle system in which the various particle classes have similar densities. Packaging of the dsRNA segments probably occurs by a "headful-type"
mechanism, in which either various combinations of different dsRNA segments, or single/multiple copies of the individual dsRNAs, dependent on size of segment, are packaged together. The various particle classes are thus predicted to have similar densities, and this is consistent with the results of cesium sulfate density gradient analysis of the purified virions (Goodin et al., 1992).

C. Etiology of the La France Disease and Future Studies

Although considerable progress has been made toward an understanding of the etiology of La France disease of the cultivated mushroom, the role, if any, of the bacilliform MBV in pathogenesis remains a mystery. MBV has not been detected alone in infected mushrooms, and is always found in association with the isometric virus LIV. In the absence of reliable infectivity assays, a molecular approach that combines elucidating the organization and expression strategies of all nine disease-specific dsRNAs of LIV and using transformation vectors to transfer individual viral genes to virus-free host strains may be possible. The recent isolation and characterization of the glyceraldehyde-3-phosphate dehydrogenase gene from A. bisporus (Harmsen and Wessels, 1994), and the possible use of the promoter and terminator sequences of this highly expressed gene in constructing transformation vectors, are encouraging in this regard. When the CP gene is identified and cloned, it may then be possible to extend the "coat protein-mediated approach," a strategy that worked well in protecting plants against several plant viruses (Beachy et al., 1990), to the cultivated mushroom for the purpose of producing transgenic mushrooms with virus resistance. The satellite nature of segments M1, S1, and S2 should be verified, and their possible role in disease development or modulation of disease symptoms should be investigated.

VI. Concluding Remarks

Considerable progress has been made during the past 5 years in our understanding of the molecular biology of two genera of mycoviruses, Totivirus and Hypovirus. The well-characterized yeast/virus killer system, comprised of a helper totivirus (L-A virus) and a family of satellite dsRNAs, and the hypoviruses infecting the chestnut blight fungus have served and continue to serve as valuable molecular tools and model systems for the elucidation of viral and eukaryotic gene expression and regulation. The yeast L-A virus is unique among RNA viruses in that template-dependent in vitro systems are available to examine
the intricate details of viral gene expression, replication, and encapsidation. Furthermore, because *S. cerevisiae* is amenable to genetic manipulation, and the killer phenotype can be easily scored, the yeast killer system is helping to address questions related to viral interaction with host genes (virus maintenance and antiviral defense mechanisms in eukaryotic cells), protein transport, processing, and secretion, as well as the molecular biology of cell wall synthesis. The isolation of the yeast genes required for viral replication or for antiviral activities has led to the search for counterparts in other systems. For example, the study of processing of yeast killer toxin precursors has allowed the isolation of genes for the mammalian processing enzymes which are not amenable to isolation by other means.

In the next 5 years I expect that more mycoviruses (particularly those in the family Totiviridae and potential satellites) will be characterized at the molecular level. Virologists will realize that even though the biological significance of mycoviruses is not immediately apparent, these viruses, thanks to the yeast killer model system, can be exploited as valuable molecular tools to dissect many of the cellular processes involved in gene expression and regulation which are currently not understood. I expect that more information will be generated on whether the products of certain yeast MAK genes are virion associated and on how these gene products may stabilize the virions. More antiviral SKI genes will be isolated and characterized, and more will be learned about how they repress virus replication. PCR technology will be exploited in searching genomic libraries of both mammalian and plant systems for the presence of counterparts to the yeast antiviral genes.

The importance of exploiting mycoviruses in the development of molecular approaches for the control of fungal diseases will come into focus. With the recent progress in elucidating the genome organization of the hypoviruses and the development of efficient DNA-mediated transformation systems, an understanding of the molecular basis of hypovirulence in the chestnut blight fungus will be forthcoming. It is anticipated that the information gained from studying the chestnut blight fungus/hypovirus system will be applicable to other systems. The search for totiviruses and associated satellites and killer phenotypes among plant pathogenic fungi should intensify. The strategy of producing transgenic plants in which killer toxins are expressed is currently being pursued as a means of biological control against the smut fungi. If killer systems are found in other plant pathogenic fungi, this strategy should be applicable. Because of recent technological developments in molecular biology and plant transformation and regeneration, the use of molecular approaches for disease control is a
reality. At the present time control of plant pathogenic fungi is a formidable task due to the lack of appropriate disease control strategies. In addition to the health hazards and the risks to the environment, the use of fungicides is often cost prohibitive. The need for biocontrol measures to combat fungal diseases cannot be overstated.

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REFERENCES

Adler, J., Wood, H. A., and Bozarth, R. F. (1976). *J. Virol.* 17, 472–476.
Al-Aidroos, K., and Bussey, H. (1978). *Can. J. Microbiol.* 24, 228–237.
Anagnostakis, S. L. (1982). *Science* 215, 466–471.
Anagnostakis, S. L. (1984). In “The Ecology and Physiology of the Fungal Mycelium” (D. H. Jennings and A. D. M. Rayner, eds.), pp. 353–366. Cambridge University Press, Cambridge, England.
Anagnostakis, S. L. (1987). *Mycologia* 79, 23–37.
Anagnostakis, S. L. (1990). *For. Sci.* 36, 113–124.
Ander, P., and Eriksson, K. E. (1979). *Phytopathology* 69, 1226–1229.
Ander, P., and Eriksson, K. E. (1976). *Arch. Microbiol.* 109, 1–8.
Aramayo, R., and Timberlake, W. E. (1990). *Nucleic Acids Res.* 18, 3415.
Bar-Nunn, N., Tal-Lev, A., Harel, E., and Mayer, A. M. (1988). *Phytochemistry* 27, 2505–2509.
Barton, R. J., and Hollings, M. (1979). *J. Gen. Virol.* 42, 231–240.
Beachy, R. N., Loesch-Fries, S., and Tumer, N. (1990). *Annu. Rev. Phytopathol.* 28, 451–474.
Beckett, D., Wu, H. N., and Uhlenbeck, O. C. (1988). *J. Mol. Biol.* 204, 939–947.
Belcourt, M. F., and Farabaugh, P. J. (1990). *Cell* 62, 339–352.
Bell, J. C., and Prevec, L. (1985). *J. Virol.* 54, 697–702.
Ben-Zvi, B. S., Koltin, Y., Mevarech, M., and Tamarkin, A. (1984). *Mol. Cell. Biol.* 4, 188–194.
Bevan, E. A., and Makower, M. (1963). *Proc. Int. Congr. Genet. 11th* 1, 202.
Bevan, E. A., Herring, A. J., and Mitchell, D. J. (1973). *Nature (London)* 245, 81–86.
Bitter, G. A., Chen, K. K., Banks, A. R., and Lai, P. H. (1984). *Proc. Natl. Acad. Sci. U.S.A.* 81, 5330–5334.
Blinkowa, A., and Walker, J. R. (1990). *Nucleic Acids Res.* 18, 1725–1729.
Boone, C., Bussey, H., Greene, D., Thomas, D. Y., and Vernet, T. (1986). *Cell* 46, 105–113.
Boone, C., Sommer, S. S., Hensel, A., and Bussey, H. (1990). *J. Cell Biol.* 110, 1833–1843.
Bostian, K. A., Hopper, J. E., Rogers, D. T., and Tipper, D. J. (1980a). *Cell* 19, 403–414.
Bostian, K. A., Sturgeon, J. A., and Tipper, D. J. (1980b). *J. Bacteriol.* 143, 463–470.
Bostian, K. A., Jayachandran, S., and Tipper, D. J. (1983). *Cell* 32, 169–180.
Bostian, K. A., Elliot, Q., Bussey, H., Burn, V., Smith, A., and Tipper, D. J. (1984). *Cell* 36, 741–751.
Bourbonnais, Y., Danoff, A., Thomas, D. Y., and Shields, D. (1991). *J. Biol. Chem.* **266**, 13205–13208.

Bozarth, R. F., Koltin, Y., Weissman, M. B., Parker, R. L., Dalton, R. F., and Steinlauf, R. (1981). *Virology* **113**, 492–502.

Brake, A. J., Merryweather, J. P., Goit, D. G., Heberlein, U. A., Masiarz, F. R., Mullennbach, M. S., Urdea, P., Valenzuela, P., and Barr, P. (1984). *Proc. Natl. Acad. Sci. U.S.A.* **81**, 4642–4646.

Bredenbeek, P. J., Pachuk, C. J., Noten, A. F. H., Charite, J., Luyster, W., Weiss, S. R., and Spaan, W. J. M. (1990). *Nucleic Acids Res.* **18**, 1825–1832.

Buck, K. W. (1986). In “Fungal Virology” (K. W. Buck, ed.), pp. 85–108. CRC Press, Boca Raton, Florida.

Buck, K. W., and Ghabrial, S. A. (1991a). In “Classification and Nomenclature of Viruses, Fifth Report of the International Committee on Taxonomy of Viruses” (R. I. B. Francki, C. M. Fauquet, D. L. Knudson, and F. Brown, eds.), pp. 208–211. Springer-Verlag, New York.

Buck, K. W., and Ghabrial, S. A. (1991b). In “Classification and Nomenclature of Viruses, Fifth Report of the International Committee on Taxonomy of Viruses” (R. I. B. Francki, C. M. Fauquet, D. L. Knudson, and F. Brown, eds.), pp. 203–205. Springer-Verlag, New York.

Buck, K. W., and Kempson-Jones, G. F. (1973). *J. Gen. Virol.* **18**, 223–235.

Bussey, H. (1981). *Adv. Microb. Physiol.* **22**, 93–122.

Bussey, H. (1988). *Yeast* **4**, 17–26.

Bussey, H., and Meaden, P. (1985). *Curr. Genet.* **9**, 285–291.

Bussey, H., Saville, D., Greene, D., Tipper, D. J., and Bostian, K. A. (1983). *Mol. Cell. Biol.* **3**, 1362–1370.

Bussey, H., Boone, C., Zhu, H., Vernet, T., Whiteway, M., and Thomas, D. Y. (1990). *Experientia* **46**, 193–200.

Bussey, H., Boone, C., Brown, J., Cooper, A., Hill, K., Roemer, T., Sdicu, A. M., and Zhu, H. (1994). In “Viruses of Simple Eukaryotes: Molecular Genetics and Applications to Biotechnology and Medicine” (M. J. Leibowitz, Y. Koltin, and V. Rubio, eds.). The University of Delaware Press, Newark, Delaware. In press.

Calnan, B. J., Tidor, B., Biancalana, S., Hudson, D., and Frankel, A. D. (1991). *Science* **252**, 1167–1171.

Carpenter, C. E., Mueller, R. J., Kazmierczak, P., Zang, L., Villalon, D. K., and Van Alfen, N. K. (1992). *Mol. Plant–Microbe Interact.* **4**, 55–61.

Chamorro, M., Parkin, N., and Varmus, H. E. (1992). *Proc. Natl. Acad. Sci. U.S.A.* **89**, 713–717.

Chang, T.-H., Banerjee, N., Bruenn, J., Held, W., Peery, T., and Koltin, Y. (1988). *Virus Genes* **2**, 195–206.

Chen, B., Choi, G. H., and Nuss, D. L. (1993). *EMBO J.* **12**, 2991–2998.
Choi, G. H., and Nuss, D. L. (1990). *Nucleic Acids Res.* 18, 5566.
Choi, G. H., and Nuss, D. L. (1992a). *EMBO J.* 11, 473–477.
Choi, G. H., and Nuss, D. L. (1992b). *Science* 257, 800–803.
Choi, G. H., Pawlyk, D. M., and Nuss, D. L. (1991a). *Virology* 183, 747–752.
Choi, G. H., Shapira, R., and Nuss, D. L. (1991b). *Proc. Natl. Acad. Sci. U.S.A.* 88, 1167–1171.
Choi, G. H., Larson, T. G., and Nuss, D. L. (1992). *Mol. Plant–Microbe Interact.* 5, 119–128.
Churchill, A. C. L., Ciuffetti, L. M., Hansen, D. R., Van Etten, H. D., and Van Alfen, N. K. (1990). *Curr. Genet.* 17, 25–31.
Clare, J. J., Belcourt, M., and Farabaugh, P. J. (1988). *Proc. Natl. Acad. Sci. U.S.A.* 85, 6816–6820.
Dalton, R. E., Podila, G. K., Lurkey, W. H., and Bozarth, R. F. (1985). *Virus Res.* 3, 153–163.
Day, P. R., Dodds, J. A., Elliston, J. E., Jaynes, R. A., and Anagnostakis, S. L. (1977). *Phytopathology* 67, 1393–1396.
de la Pena, P., Barros, F., Gascon, S., Lazo, P. S., and Ramos, S. (1981). *J. Biol. Chem.* 256, 10420–10425.
den Boon, J. A., Snijder, E. J., Handschke, O. P., de Vries, A. A. F., Horzinek, M. C., and Spaan, W. J. M. (1991). *Mol. Cell. Biol.* 11, 2910–2920.
Diamond, M. E., Dowhanick, J. J., Nemeroff, M. E., Pietras, D. F., Tu, C., and Bruenn, J. A. (1989). *J. Virol.* 63, 3983–3990.
Dieleman-van Zaayen, A., and Temmink, J. H. M. (1968). *Neth. J. Plant Pathol.* 74, 48–51.
Dignard, D., Whiteway, M., Germain, D., Tessier, D., and Thomas, D. Y. (1991). *Mol. Gen. Genet.* 227, 127–136.
Dinman, J. D., and Wickner, R. B. (1992). *J. Virol.* 66, 3669–3676.
Dinman, J. D., Icho, T., and Wickner, R. B. (1991). *Proc. Natl. Acad. Sci. U.S.A.* 88, 174–178.
Dmochowska, A., Dignard, D., Henning, D., Thomas, D. Y., and Bussey, H. (1987). *Cell* 50, 573–584.
Dodds, J. A. (1980). *Phytopathology* 70, 1217–1220.
Dowhanick, J. J., Shen, Y., Tu, C. L., Tzeng, T. H., and Bruenn, J. A. (1994). In “Viruses of Simple Eukaryotes: Molecular Genetics and Applications to Biotechnology and Medicine” (M. J. Leibowitz, Y. Koltin, and V. Rubio, eds.). The University of Delaware Press, Newark, Delaware. In press.
Dunn, J. J., and Studier, F. W. (1983). *J. Mol. Biol.* 166, 477–535.
Elliston, J. E. (1985a). *Phytopathology* 75, 151–158.
Elliston, J. E. (1985b). *Phytopathology* 75, 170–173.
El-Sherbeini, M., and Bostian, K. A. (1987). *Proc. Natl. Acad. Sci. U.S.A.* 84, 4293–4297.
El-Sherbeini, M., Bostian, K. A., LeVitre, J., and Mitchell, D. S. (1987). *Curr. Genet.* 11, 483–490.
Enebak, S. A., Hillman, B. I., MacDonald, W. L., and Abad, A. R. (1991). *Phytopathology* 81, 1153.
Escoubas, J. M., Prere, M. F., Fayet, O., Salvignol, I., Galas, D., Zeribih, D., and Chandler, M. (1991). *EMBO J.* 10, 705–712.
Esteban, R., and Wickner, R. B. (1986). *Mol. Cell. Biol.* 6, 1552–1561.
Esteban, R., and Wickner, R. B. (1988). *J. Virol.* 62, 1278–1285.
Esteban, R., Fujimura, T., and Wickner, R. B. (1988). *Proc. Natl. Acad. Sci. U.S.A.* 85, 4411–4415.
Esteban, R., Fujimura, T., and Wickner, R. B. (1989). *EMBO J.* 8, 947–954.
Field, L. J., Bruenn, J. A., Chang, T. H., Pinchasi, O., and Koltin, Y. (1983). *Nucleic Acids Res.* **11**, 2765–2778.

Fried, H. M., and Fink, G. R. (1978). *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4224–4228.

Fujimura, T., and Wickner, R. B. (1986). *Mol. Cell. Biol.* **6**, 404–410.

Fujimura, T., and Wickner, R. B. (1987). *Mol. Cell. Biol.* **7**, 420–426.

Fujimura, T., and Wickner, R. B. (1988a). *Cell* **55**, 663–671.

Fujimura, T., and Wickner, R. B. (1988b). *J. Biol. Chern.* **263**, 454–460.

Fujimura, T., and Wickner, R. B. (1989). *J. Biol. Chern.* **264**, 10872–10877.

Fujimura, T., and Wickner, R. B. (1992). *J. Biol. Chern.* **267**, 2708–2713.

Fujimura, T., Esteban, R., and Wickner, R. B. (1986). *Proc. Natl. Acad. Sci. U.S.A.* **83**, 4433–4437.

Fujimura, T., Esteban, R., Esteban, L. M., and Wickner, R. B. (1990). *Cell* **62**, 819–828.

Fujimura, T., Ribas, J. C., Makhov, A. M., and Wickner, R. B. (1992). *Nature (London)* **359**, 746–749.

Furfine, E. S., and Wang, C. C. (1990). *Mol. Cell. Biol.* **10**, 3659–3663.

Ganesa, C., Flurkey, W. H., Randhawa, Z. I., and Bozarth, R. F. (1991). Arch. Biochem. Biophys. **286**, 195–200.

Georgopoulos, D. E., and Leibowitz, M. J. (1987). *Yeast* **3**, 117–129.

Germann, U. A., Muller, G., Hunziker, P. E., and Lerch, K. (1988). *J. Biol. Chem.* **263**, 885–898.

Ghabrial, S. A. (1980). *Annu. Rev. Phytopathol.* **18**, 441–461.

Ghabrial, S. A. (1986). In “Fungal Virology” (K. W. Buck, ed.), pp. 163–176. CRC Press, Boca Raton, Florida.

Ghabrial, S. A. (1988). In “Viruses of Fungi and Simple Eukaryotes” (Y. Koltin and M. J. Leibowitz, eds.), pp. 353–369. Dekker, New York.

Ghabrial, S. A. (1994). In “Viruses of Simple Eukaryotes: Molecular Genetics and Applications to Biotechnology and Medicine” (M. J. Leibowitz, Y. Koltin, and V. Rubio, eds.). The University of Delaware Press, Newark, Delaware. In press.

Ghabrial, S. A., and Havens, W. M. (1989). *J. Gen. Virol.* **70**, 1025–1035.

Ghabrial, S. A., and Havens, W. M. (1992). *Virology* **188**, 657–665.

Ghabrial, S. A., Sanderlin, R. S., and Calvert, L. A. (1979). *Phytopathology* **69**, 312–315.

Ghabrial, S. A., Bibb, J. A., Price, K. H., Havens, W. M., and Lesnaw, J. A. (1987). *J. Gen. Virol.* **68**, 1791–1800.

Goodin, M. M., Schlaghauser, B., and Romaine, C. P. (1992). *Phytopathology* **82**, 285–290.

Gorbalenya, A. E., Koonin, E. V., Donchenko, A. P., and Blinov, V. M. (1989). *Nucleic Acids Res.* **17**, 4456–4469.

Grente, J., and Berthelay-Sauret, S. (1978). In “Proceedings of the American Chestnut Symposium” (W. L. MacDonald, F. C. Cech, J. Luchok, and H. C. Smith, eds.), pp. 30–34. West Virginia University Press, Morgantown, West Virginia.

Hanes, S. D., Burn, V. E., Sturley, S. L., Tipper, D. J., and Bostian, K. A. (1986). *Proc. Natl. Acad. Sci. U.S.A.* **83**, 1675–1679.

Hannig, E. M., and Leibowitz, M. J. (1985). *Nucleic Acids Res.* **13**, 4379–4400.

Hannig, E. M., Thiele, D. J., and Leibowitz, M. J. (1984). *Mol. Cell. Biol.* **4**, 101–109.

Hannig, E. M., Leibowitz, M. J., and Wickner, R. B. (1985). *Yeast* **1**, 57–65.

Hannig, E. M., Williams, T. L., and Leibowitz, M. J. (1986). *Virology* **152**, 149–158.

Hansen, D. R., Van Alfen, N. K., Gillies, K., and Powell, W. A. (1985). *J. Gen. Virol.* **66**, 2605–2614.

Harmsen, M. C., and Wessels, J. G. H. (1994). In “Viruses of Simple Eukaryotes: Molecular Genetics and Applications to Biotechnology and Medicine” (M. J. Leibowitz, Y. Koltin, and V. Rubio, eds.). The University of Delaware Press, Newark, Delaware. In press.
Harmsen, M. C., van Griensven, L. J. L. D., and Wessels, J. G. H. (1989). *J. Gen. Virol.* **70**, 1613–1616.

Harmsen, M. C., Tolner, B., Kram, A., Go, S. J., de Haan, A., and Wessels, J. G. H. (1991). *Curr. Genet.* **20**, 137–144.

Herring, A. J., and Bevan, E. A. (1977). *Nature (London)* **268**, 464–466.

Hicks, R. G. T., and Haughton, K. L. (1986). *Trans Br. Mycol. Soc.* **86**, 579–584.

Hillman, B. I., Rae, B., Tartaglia, J., and Nuss, D. L. (1989). In "Molecular Biology of Plant Pathogen Interactions," Proc. UCLA Symp. Vol. 101, pp. 59–70.

Hillman, B. I., Shapiro, R., and Nuss, D. L. (1990). *Phytopathology* **80**, 950–956.

Hillman, B. I., Tian, Y., Bedker, P. J., and Brown, M. P. (1992). *J. Gen. Virol.* **73**, 681–686.

Hillman, B. I., Fulbright, D. W., Nuss, D. L., and Van Alfen, N. K. (1994). In "Classification and Nomenclature of Viruses, Sixth Report of the International Committee on Taxonomy of Viruses" (F. A. Murphy, ed.). Springer-Verlag, New York. In press.

Hiremath, S., L’Hostis, B., Ghabrial, S. A., and Rhoads, R. E. (1986). *Nucleic Acids Res.* **14**, 9877–9896.

Hiremath, S., Nuss, D., Ghabrial, S. A., and Rhoads, R. E. (1988). *J. Gen. Virol.* **69**, 2441–2453.

Hizi, A., Henderson, L. E., Copeland, T. D., Sowder, R. C., Hixson, C. V., and Oroszlan, S. (1987). *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7041–7045.

Hollings, M. (1962). *Nature (London)* **196**, 962–965.

Hollings, M. (1978). *Adv. Virus Res.* **22**, 1–53.

Hollings, M., Gandy, D. G., and Last, F. T. A. (1963). *Endeavour* **22**, 112–117.

Holmes, I. H. (1991). In "Classification and Nomenclature of Viruses, Fifth Report of the International Committee on Taxonomy of Viruses" (R. I. B. Francki, C. M. Fauquet, D. L. Knudson, and F. Brown, eds.), pp. 186–199. Springer-Verlag, New York.

Hougan, L., Thomas, D. Y., and Whiteway, M. (1989). *Curr. Genet.* **16**, 137–144.

Hsu, C.-H., and Kingsbury, D. W. (1985). *J. Biol. Chem.* **260**, 8990–8995.

Huan, B., Shen, Y., and Bruenn, J. A. (1991). *Proc. Natl. Acad. Sci. U.S.A.* **88**, 1271–1275.

Hussain, I., and Leibowitz, M. J. (1986). *Gene* **46**, 13–23.

Hutchins, K., and Bussey, H. (1983). *J. Bacteriol.* **154**, 161–169.

Icho, T., and Wickner, R. B. (1988). *J. Biol. Chem.* **263**, 1467–1475.

Icho, T., and Wickner, R. B. (1989). *J. Biol. Chem.* **264**, 6716–6723.

Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983). *J. Bacteriol.* **153**, 163–168.

Jacks, T., and Varmus, H. E. (1985). *Science* **230**, 1237–1242.

Jacks, T., Townsley, K., Varmus, H. E., and Majors, J. (1987). *Proc. Natl. Acad. Sci. U.S.A.* **84**, 4298–4302.

Jacks, T., Madhani, H. D., Masiarz, F. R., and Varmus, H. E. (1988a). *Cell* **55**, 447–458.

Jacks, T., Power, M. D., Masiarz, F. R., Luciw, P. A., Barr, P. J., and Varmus, H. E. (1988b). *Nature (London)* **331**, 280–283.

Jamil, N., and Buck, K. W. (1986). *J. Gen. Virol.* **67**, 1717–1720.

Julius, D., Brake, A., Blair, L., Kunisawa, R., and Thorner, J. (1984). *Cell* **37**, 1075–1089.

Khatchikian, D., Orlich, M., and Roth, R. (1989). *Nature (London)* **340**, 156–157.

Kojima, Y., Tsukuda, Y., Kawai, Y., Tsukamoto, A., Sugirra, J., Sakaino, M., and Kita, Y. (1990). *J. Biol. Chem.* **265**, 15224–15230.

Koltin, Y. (1986). In "Fungal Virology" (K. W. Buck, ed.), pp. 109–143. CRC Press, Boca Raton, Florida.

Koltin, Y. (1988). In "Viruses of Fungi and Simple Eukaryotes" (Y. Koltin and M. J. Leibowitz, eds.), pp. 209–242. Dekker, New York.

Koltin, Y., Mayer, I., and Steinlauf, R. (1978). *Mol. Gen. Genet.* **166**, 181–186.
Koltin, Y., Levine, R., and Peery, T. (1980). *Mol. Gen. Genet.* 178, 173–178.

Koonin, E. V., Choi, G. H., Nuss, D. L., Blapura, R., and Carrington, J. C. (1991). *Proc. Natl. Acad. Sci. U.S.A.* 88, 10647–10651.

Koons, K. C., Schlaghaufer, B., and Romaine, C. P. (1989). *Phytopathology* 79, 1272–1275.

Law, C. J., and Timberlake, W. E. (1980). *J. Bacteriol.* 144, 509–517.

Leader, D. P., and Katan, M. (1985). *J. Gen. Virol.* 69, 1441–1464.

Leibowitz, M. J., Hussain, I., and Williams, T. L. (1988). In "Viruses of Fungi and Simple Eukaryotes" (Y. Koltin and M. J. Leibowitz, eds.), pp. 133–160. Dekker, New York.

L'Hostis, B., Hiremath, S. T., Rhoads, R. E., and Ghabrial, S. A. (1985). *J. Gen. Virol.* 66, 133–160.

Lindberg, G. D. (1959). *Phytopathology* 49, 29–31.

Lindberg, G. D. (1960). *Phytopathology* 50, 457–460.

Lolle, S. J., and Bussey, H. (1986). *Mol. Cell. Biol.* 6, 4274–4280.

Luke, H. H., and Wheeler, H. E. (1955). *Phytopathology* 45, 453–458.

Marder, R., Saksena, K. N., Schuler, M., Mayfield, J. E., and Lemke, P. A. (1976). *Appl. Environ. Microbiol.* 31, 433–438.

Markwell, M. K., and Fox, C. F. (1978). *Biochemistry* 17, 4807–4817.

Marnell, L. L., and Summers, D. F. (1984). *J. Biol. Chem.* 259, 13518–13524.

Martinac, B., Zhu, H., Kubalski, A., Zhou, X.-L., Culberson, M., Bussey, H., and Kung, C. (1990). *Proc. Natl. Acad. Sci. U.S.A.* 87, 6228–6232.

Meaden, P., Hill, K., Wagner, J., Slipetz, D., Sommer, S. S., and Bussey, H. (1990). *Mol. Cell. Biol.* 10, 3013–3019.

Mellen, J., Fulton, S. M., Dobson, M. J., Wilson, W., Kingsman, S. M., and Kingsman, A. J. (1985). *Nature (London)* 313, 243–246.

Meskauskas, A., and Citavicus, D. (1992). *Gene* 111, 135–139.

Mindich, L., Nemhauser, I., Gottlieb, P., Romantschuk, M., Carton, J., Frucht, S., Strassman, J., Bamford, D. H., and Kalkkinen, N. (1988). *J. Virol.* 62, 1180–1185.

Miyajima, A., Bond, M. W., Otsu, K., Arai, K. I., and Araki, N. (1985). *Gene* 35, 155–161.

Mizuno, K., Nakamura, T., Oshima, T., Tanaka, S., and Matsuo, H. (1988). *Biochem. Biophys. Res. Commun.* 156, 429–434.

Moore, R., Dixon, M., Smith, R., Peters, G., and Dickson, C. (1987). *J. Virol.* 61, 480–490.

Mullenbach, G. T., Tabrizi, A., Blacher, R. W., and Steiner, K. S. (1986). *J. Biol. Chem.* 261, 719–722.

Munemitsu, S. M., Atwater, J. A., and Samuel, C. E. (1986). *Biochem. Biophys. Res. Commun.* 140, 508–514.

Nemeroff, M. E., and Bruenn, J. A. (1986). *J. Virol.* 57, 754–758.

Nemeroff, M. E., and Bruenn, J. A. (1987). *J. Biol. Chem.* 262, 6785–6787.

Newman, A. M., Elliott, S. G., McLaughlin, C. S., Sutherland, P. A., and Warner, R. C. (1981). *J. Virol.* 62, 1180–1185.

Nuss, D. L. (1992). *Microbiol. Rev.* 56, 561–576.

Nuss, D. L., and Dall, D. J. (1990). *Adv. Virus Res.* 38, 249–306.

Nuss, D. L., and Koltin, Y. (1990). *Annu. Rev. Phytopathol.* 28, 37–58.

Oh, C. S., and Carrington, J. C. (1989). *Virology* 171, 692–699.

Oliver, S. G., McCready, S. J., Holm, C., Sutherland, P. A., McLaughlin, C. S., and Cox, B. S. (1977). *J. Bacteriol.* 130, 1303–1309.

Olives, S., and Phil, A. (1973). *Eur. J. Biochem.* 25, 179–185.

Passmore, E. L., and Frost, R. R. (1979). *Phytopathol. Z.* 80, 85–87.
Patterson, J. L. (1990). Exp. Parasitol. 70, 11–113.
Peery, T., Koltin, Y., and Tamarkin, A. (1982). Plasmids 7, 52–58.
Pietras, D. F., Diamond, M. E., and Bruenn, J. A. (1988). Nucleic Acids Res. 16, 6225.
Pleij, C. W. A. (1990). Trends Biochem. Sci. 15, 143–147.
Pleij, C. W. A., Rietveld, K., and Bosch, L. (1985). Nucleic Acids Res. 13, 1717–1731.
Podila, G. K., Bozarth, R. F., and Flurkey, W. H. (1987a). Biochem. Biophys. Res. Commun. 149, 391–397.
Podila, G. K., Flurkey, W. H., and Bozarth, R. F. (1987b). J. Gen. Virol. 68, 2741–2750.
Powell, W. A., and Van Alfen, N. K. (1987a). Mol. Cell. Biol. 7, 3688–3693.
Powell, W. A., and Van Alfen, N. K. (1987b). J. Bacteriol. 11, 5324–5326.
Prüfer, D., Tacke, E., Schmitz, J., Kull, B., Kaufmann, A., and Rohde, W. (1992). EMBO J. 11, 1111–1117.
Puhalja, J. E. (1968). Genetics 60, 461–474.
Query, C. C., Bentley, R. C., and Keene, J. D. (1989). Cell 57, 89–101.
Rae, B. P., Hillman, B. I., Tartaglia, J., and Nuss, D. L. (1989). EMBO J. 8, 657–663.
Rees, B., Samma, J. P., Thierry, J. C., Gilbert, M., Fischer, J., Schweitz, H., Reilly, J. D., Bruenn, J., and Held, W. (1984). Biochem. Biophys. Res. Commun. 121, 619–625.
Rhee, S. K., Icho, T., and Wickner, R. B. (1989). Yeast 5, 149–158.
Ribas, J. C., and Wickner, R. B. (1992). Proc. Natl. Acad. Sci. U.S.A. 89, 2185–2189.
Ridley, S. P., and Wickner, R. B. (1983). J. Virol. 45, 800–812.
Ridley, S. P., Sommer, S. S., and Wickner, R. B. (1984) Mol. Cell. Biol. 4, 761–770.
Rigling, D., and Van Alfen, N. K. (1991). J. Bacteriol. 173, 8000–8003.
Rigling, D., Heiniger, U., and Hohl, H. R. (1989). Phytopathology 79, 219–223.
Romaine, C. P., and Schlagnhauber, B. (1989). Mycologia 81, 822–825.
Romaine, C. P., and Schlagnhauber, B. (1991). Phytopathology, 81, 1336–1340.
Sanderlin, R. S., and Ghabrial, S. A. (1978). Virology 87, 142–151.
Schmitt, M. J., and Tipper, D. J. (1990). Mol. Cell. Biol. 10, 4807–4815.
Sclafani, R. A., and Fangman, W. L. (1984). Mol. Cell. Biol. 4, 1618–1626.
Sekine, U., and Ohtsubo, E. (1989). Proc. Natl. Acad. Sci. U.S.A. 86, 4609–4613.
Shapira, R., and Nuss, D. L. (1991). J. Biol. Chem. 266, 19419–19425.
Shapira, R., Choi, G. H., and Nuss, D. L. (1991a). EMBO J. 10, 731–739.
Shapira, R., Choi, G. H., Hillman, B. I., and Nuss, D. L. (1991b). EMBO J. 10, 741–746.
Shelbourn, S. L., Day, P. R., and Buck, K. W. (1988). J. Gen. Virol. 69, 975–982.
Shen, Y., and Bruenn, J. A. (1993). Virology 195, 481–491.
Simon, A. E. (1988). Plant Mol. Biol. Rep. 6, 240–262.
Sinden, J. W., and Hauser, E. (1950). Mushroom Sci. 1, 96–100.
Singh, A., Lugovoy, J. M., Kohr, W. J., and Perry, L. J. (1986). Nucleic Acids Res. 12, 8927–8939.
Skipper, N., Southerland, M., Davies, R. W., Kilburn, D., Miller, R. C., Warren, A., and Wong, R. (1985). Science 230, 958–960.
Sommer, S. S., and Wickner, R. B. (1982). Cell 31, 429–441.
Sommer, S. S., and Wickner, R. B. (1987). Virology 157, 252–256.
Stanway, C. A., and Buck, K. W. (1984). J. Gen. Virol. 65, 2061–2066.
Stevenson, K. J., Stater, J. A., and Takai, S. (1979). Phytocochemistry 18, 235–238.
Stuart, K., Weeks, R., Guilbride, L., and Myler, P. (1992). Proc. Natl. Acad. Sci. U.S.A. 89, 8596–8600.
Sturley, S. L., Elliot, Q., LeVitre, J., Tipper, D. J., and Bostian, K. A. (1986). EMBO J. 5, 3381–3389.
Sturley, S. L., El-Sherbeini, M., Kho, S. H., LeVitre, J. L., and Bostian, K. A. (1988). In "Viruses of Fungi and Simple Eukaryotes" (Y. Koltin and M. J. Leibowitz, eds.), pp. 179–208. Dekker, New York.
Takai, S., and Hiratsuka, Y. (1980). Can. J. Bot. 58, 663–668.
Tao, J., Gineberg, I., Banerjee, N., Held, W., Koltin, Y., and Bruenn, J. A. (1990). Mol. Cell. Biol. 10, 1373–1381.
Tarr, P. I., Aline, R. F., Smiley, B. L., Scholler, J., Kethly, J., and Stuart, K. (1988). Proc. Natl. Acad. Sci. U.S.A. 85, 9572–9575.
Tartaglia, J., Paul, C. P., Fulbright, D. W., and Nuss, D. L. (1986). Proc. Natl. Acad. Sci. U.S.A. 83, 9109–9113.
Tavantzis, S. M., Romaine, C. P., and Smith, S. H. (1980). Virology 105, 94–102.
Thiele, D. J., Hannig, E. M. and Leibowitz, M. J. (1984). Mol. Cell. Biol. 4, 92–100.
Thomas, G., Thorne, B. A., Thomas, L., Allen, R. G., Hruby, D. E., Fuller, R., and Thomas, J. (1988). Science 241, 226–230.
Thomas, L., Cooper, A., Bussey, H., and Thomas, G. (1990). J. Biol. Chem. 265, 10821–10824.
Tipper, D. J., and Bostian, K. A. (1984). Microbiol. Rev. 48, 125–156.
Toh-e, A., and Sahashi, Y. (1985). Yeast 1, 159–172.
Toh-e, A., Guerry, P., and Wickner, R. B. (1978). J. Bacteriol. 136, 1002–1007.
Tsuchihashi, Z., and Kornberg, A. (1990). Proc. Natl. Acad. Sci. U.S.A. 87, 2516–2520.
Tu, C., Tzeng, T.-H., and Bruenn, J. A. (1992). Proc. Natl. Acad. Sci. U.S.A. 89, 8636–8640.
Tung, J. S., and Knight, C. A. (1971). Biochem. Biophys. Res. Commun. 42, 1117–1121.
Tzeng, T.-H., Tu, C.-L., and Bruenn, J. A. (1992). J. Virol. 66, 999–1006.
Uemura, H., and Wickner, R. B. (1988). Mol. Cell. Biol. 8, 938–944.
Van Alfen, N. K. (1982). Annu. Rev. Phytopathol. 20, 349–362.
Van Alfen, N. K. (1986). In "Fungal Virology" (K. W. Buck, ed.). CRC Press, Boca Raton, Florida.
Van Alfen, N. K., Jaynes, R. A., Anagnostakis, S. L., and Day, P. R. (1975). Science 189, 890–891.
van Zaayen, A. (1979). In "Viruses and Plasmids in Fungi" (P. A. Lemke, ed.), pp. 239–324. Dekker, New York.
Vondrejs, V. (1987). Microbiol. Sci. 4, 313–316.
Wach, M. P., Sriskantha, A., and Romaine, C. P. (1987). Phytopathology 77, 1321–1325.
Wang, A. L., and Wang, C. C. (1986a). Mol. Biochem. Parasitol. 21, 269–276.
Wang, A. L., and Wang, C. C. (1986b). Proc. Natl. Acad. Sci. U.S.A. 83, 7956–7960.
Wang, A. L., and Wang, C. C. (1991). Annu. Rev. Microbiol. 45, 251–263.
Weiss, R. B., Huang, W. M., and Dunn, D. M. (1990). Cell 62, 117–126.
Welsh, J. D., and Leibowitz, J. M. (1982). Proc. Natl. Acad. Sci. U.S.A. 79, 786–789.
Welsh, J. D., Leibowitz, M. J., and Wickner, R. B. (1980). Nucleic Acids Res. 8, 2349–2363.
Whiteway, M., Dignard, D., Vernet, T., and Thomas, D. Y. (1994). In "Viruses of Simple Eukaryotes: Molecular Genetics and Applications to Biotechnology and Medicine" (M. J. Leibowitz, Y. Koltin, and V. Rubio, eds.). The University of Delaware Press, Newark, Delaware. In press.
Wickner, R. B. (1978). Genetics 88, 419–425.
Wickner, R. B. (1979). Genetics 92, 803–821.
Wickner, R. B. (1983). Mol. Cell. Biol. 3, 654–661.
Wickner, R. B. (1986). Annu. Rev. Biochem. 55, 373–395.
Wickner, R. B. (1988). Proc. Natl. Acad. Sci. U.S.A. 85, 6007–6011.
Wickner, R. B. (1989). FASEB J. 3, 2257–2265.
Wickner, R. B. (1991). In "The Molecular Biology of the Yeast Saccharomyces" (J. R. Broach, E. W. Jones, and J. R. Pringle, eds.), pp. 263–296. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
Wickner, R. B. (1993). J. Biol. Chem. 268, 3797–3800.
Wickner, R. B., and Leibowitz, M. J. (1976a). Genetics 82, 429–442.
Wickner, R. B., and Leibowitz, M. J. (1976b). J. Mol. Biol. 105, 427–443.
Wickner, R. B., and Leibowitz, M. J. (1979). J. Bacteriol. 140, 154–160.
Wickner, R. B., Icho, T., Fujimura, T., and Widner, W. R. (1991). J. Virol. 65, 155–161.
Wickner, R. B., Dinman, J. D., Tercero, J. C., Widner, W. R., Lee, Y.-J., and Matsumoto, Y. (1994). In "Viruses of Simple Eukaryotes: Molecular Genetics and Applications to Biotechnology and Medicine" (M. J. Leibowitz, Y. Koltin, and V. Rubio, eds.). The University of Delaware Press, Newark, Delaware. In press.
Wildmer, G., Comeau, A. M., Furlong, D. B., Wirth, D. F., and Patterson, J. L. (1989). Proc. Natl. Acad. Sci. U.S.A. 86, 5979–5982.
Wildmer, G., Keenan, M. C., and Patterson, J. L. (1990). J. Virol. 64, 3712–3715.
Williams, T. L., and Leibowitz, M. J. (1987). Virology 158, 231–234.
Wilson, W., Braddock, M., Adams, S. E., Rathjen, P. D., Kingsman, S. M., and Kingsman, A. J. (1988). Cell 55, 1159–1169.
Wolpert, T. J., and Macko, V. (1989). Proc. Natl. Acad. Sci. U.S.A. 86, 4092–4096.
Wolpert, T. J., and Macko, V. (1991). Plant Physiol. 95, 917–920.
Wolpert, T. J., Macko, V., Acklin, W., and Aigoni, D. (1988). Plant Physiol. 88, 37–41.
Wood, H. A., and Bozarth, R. F. (1973). Phytopathology 63, 1019–1021.
Zakian, V. A., Wagner, D. W., and Fangman, W. L. (1981). Mol. Cell. Biol. 1, 673–679.
Zhu, H., and Bussey, H. (1991). Mol. Cell. Biol. 11, 175–181.
Zhu, H., Bussey, H., Thomas, D. Y., Gagnon, J., and Bell, A. W. (1987). J. Biol. Chem. 262, 10728–10732.
Zuker, M., and Stiegler, P. (1981). Nucleic Acids Res. 9, 133–148.