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Viruses of Fungi and Protozoans: Is Everyone Sick?

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I. INTRODUCTION

A. The Totiviruses

There are two extreme strategies for survival pursued by pathogens: kill the host and spread rapidly, and coexist with the host, sometimes providing a selective advantage. Most viruses have lifestyles that lie between these two extremes. Among the RNA viruses, the double-stranded RNA (dsRNA) viruses of protozoans and fungi represent the extreme of coexistence. This group of viruses, with few exceptions, has no infectious phase. The viruses exist as permanent persistent
infections, passing from cell to cell only by mating and cell division. In general, they seem to have no deleterious effects on their hosts, and in some cases they provide the host cells with a selective advantage, providing replication functions and encapsidation for satellite viruses that encode cellular toxins lethal to cells without the virus. In this respect, they have adopted a strategy similar to that of certain plasmids and DNA viruses of prokaryotes that encode toxins, restriction enzymes, or antibiotic resistance. This is the only example among RNA viruses of such a mutually beneficial symbiosis.

The Totiviruses comprise a subgroup of the fungal and protozoan viruses that is even more remarkable. With a single essential dsRNA, the Totiviruses are present in phyla separated by a billion years of evolution and are recognizably related to each other and to no other class of viruses (except possibly the partitiviruses of fungi). They have been discovered in at least nine genera of fungi (Saccharomyces, Ustilago, Helminthosporium, Gaeumannomyces, Mycogone, Yarrowia, Aspergillus, Thielaviopsis, and probably Agaricus) and at least four genera of protozoans (Leishmania, Eimeria, Giardiavirus, and Trichomonas) (Buck, 1986; Francki et al., 1991; Ghabrial, 1994; Roditi et al., 1994; Wang and Wang, 1991). The partitiviruses are also dsRNA viruses that exist solely as persistent infections. They have been discovered in an additional nine genera of fungi (Francki et al., 1991). There are limited sequence data available for the partitiviruses, so their relationship to the Totiviruses remains unclear. In almost all genera of protozoans and fungi in which a systematic search has been made, there are representatives of these related dsRNA viruses. Only in Candida (which lacks any known sexual cycle) and Neurospora have serious attempts at finding resident dsRNA viruses failed.

The prototypical Totivirus is the Saccharomyces cerevisiae virus L1 (or LA), abbreviated ScVL1 (or ScVLA). This is the most extensively studied fungal virus, and in many respects its genomic organization and life cycle are typical of the fungal and protozoan dsRNA viruses.

B. The ScV System

1. Gene Expression

Most laboratory Saccharomyces cerevisiae (yeast) strains contain virus particles in the cytoplasm (the Saccharomyces cerevisiae virus, or ScV), in which segmented double-stranded RNAs (dsRNAs) are separately encapsidated, and there is a single essential dsRNA. The large essential dsRNA of one viral family, L1, is 4580 base pairs (Diamond et al., 1989; Icho and Wickner, 1989) and encodes on the plus strand in reading frames that overlap by 130 bases a major capsid protein, Cap or P1 (Hopper et al., 1977), and an RNA-dependent RNA polymerase, or Pol
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(Diamond et al., 1989; Fujimura and Wickner, 1988a; Pietras et al., 1988), which are used by all dsRNAs of this family. The Pol protein is translated as a Cap–Pol fusion product by a frameshift event (Diamond et al., 1989; Dinman et al., 1991). The L1 Cap–Pol frameshift is similar to frameshifting in the retroviruses, both in site specificity and in RNA secondary and tertiary structure requirements (Dinman et al., 1991; Tzeng et al., 1992).

As with Coronavirus frameshifting (Brierley et al., 1989) and retroviral frameshifts (Chamorro et al., 1992), ScVL1 translational frameshifting requires a slippery site (GGGUAUA) and a 3' pseudoknot (Dinman et al., 1991; Tzeng et al., 1992). The yeast system has been very useful in understanding translational frameshifting (Dinman, 1995). It was in this system that it was first shown that +1 translational frameshifting in eukaryotes is due to rare tRNAs (Belcourt and Farabaugh, 1990; Farabaugh et al., 1993), and a number of nuclear genes affecting frameshifting efficiency have been identified in yeast (and in no other eukaryote) by two groups (Dinman and Wickner, 1994; Lee et al., 1995). Some of these are now known to affect mRNA stability (Cui et al., 1996). In the ScV system, our group first showed that ribosomes pause at a slippery site due to the presence of a downstream pseudoknot (Tu et al., 1992).

The structure of the viral dsRNAs is outlined in Figure 1. A second dsRNA virus, ScVLa, is also present in most strains and has a single essential dsRNA of 4615 bp with a similar genomic organization and mode of expression (Park et al., 1996b). The L1 family of RNAs are packaged exclusively in particles with the L1 Cap protein, due to the highly specific recognition of these RNAs by the L1 Cap–Pol protein at a viral binding site (VBS) located in each of the RNAs (Fujimura et al., 1990; Shen and Bruenn, 1993; Yao and Bruenn, 1995; Yao et al., 1995, 1997).

Replication and transcription in this system are of general interest, since the Pol protein shares conserved domains with the RNA-dependent RNA polymerases (RDRPs) of the plus-strand RNA viruses (Bruenn, 1991; Diamond et al., 1989; Ichoh and Wickner, 1989; Pietras et al., 1988). L1 is also known as L-A and La as L-BC. In vivo expression of the genome is apparently by translation of the entire plus strand (Haylock and Bevan, 1981), although there is one report of a 5', but not a 3', subgenomic fragment (Bostian et al., 1983). There are no precedents for subgenomic mRNAs from dsRNA viral segments. Translation of the L1 plus strand synthesized by the nuclear DNA-dependent RNA polymerase (RNAP) from a cDNA expression vector is capable of producing the requisite viral gene products in appropriate proportions (Wickner et al., 1991).

The dsRNAs of ScV replicate conservatively. As in reovirus, transcription within viral particles is followed by extrusion of the newly synthesized plus strand, which then interacts with cellular and viral proteins. The plus strand is packaged in viral particles after recognition by the Cap–Pol fusion protein, and replicated
Fig. 1. Genome structure of L1, the one essential viral dsRNA. M1 = the toxin encoding dsRNA; S14 = a defective interfering dsRNA derived from M1 by internal deletion (dashed lines). Known coding regions are in italics. Arrows indicate cis-acting sites. Functional domains are shown in L1. VBS = viral binding site; IRE = internal replication enhancer; INS = interference sequence; vp3 refers to the region of L1 and La cap similar to vp3 of poliovirus.

by synthesis of minus strand, resulting in duplex formation (Bruenn, 1986; Fujimura et al., 1986; Nemeroff and Bruenn, 1986; Williams and Leibowitz, 1987). Although some viral particles may contain more than one copy of a viral dsRNA when transcription is not followed by extrusion of the new plus strand (Esteban and Wickner, 1986, 1988), new viral particles are only formed as described. That is, if a viral dsRNA is small enough and its transcript is not extruded from a particle after transcription, ensuing replication of the transcript will result in a particle with two molecules of the same dsRNA. This process may continue until multiple copies of very small dsRNAs are present in some particles (Esteban and Wickner, 1988).
2. Satellite Viruses

In k1 killer strains there is a second dsRNA, M1, of about 1.9 kb, which encodes a secreted toxin that kills sensitive cells (Bostian et al., 1980, 1984; Lolle and Bussey, 1986; Skipper et al., 1984). The M1 preprotoxin functions as an immunity protein (Boone et al., 1986; Hanes et al., 1986). This provides a convenient phenotype for determining the effect of viral and host mutations on the viral life cycle. Suppressive sensitive mutants (Somers, 1973) of ScV contain L1 and smaller, or S, dsRNAs (Sweeny et al., 1976; Vodkin, 1977). These are defective interfering versions of M1 that are derived from M1 by internal deletion, sometimes followed by tandem duplication (Bruenn and Kane, 1978; Bruenn and Brennan, 1980; Fried and Fink, 1978; Huan et al., 1991; Kane et al., 1979; Lee et al., 1986; Ridley and Wickner, 1983; Thiele et al., 1984). DI segments of rota-viruses have a similar structure (Scott et al., 1989). A second killer toxin, k2, is encoded by M2, a dsRNA that can also be packaged by L1 Cap (Dignard et al., 1991; E1-Sherbeini et al., 1984). The sequences necessary for interference (INSs) are located within an 89-bp region that includes two sequences homologous to the IRE-VBS in L1 (Huan et al., 1991).

There are chromosomal genes (MAK) required for M1 and S maintenance, but usually not for L1 maintenance (Wickner, 1986, 1989). At least some of these are also necessary for an L1 DI mutant (Esteban and Wickner, 1988). The viral binding site (VBS), internal replication enhancer (IRE) (Esteban et al., 1989), and interference sequence (INS) (Huan et al., 1991; Shen and Bruenn, 1993) are probably synonymous, and consist of a 19–31-bp sequence with defined primary and secondary structure (Shen and Bruenn, 1993; Yao and Bruenn, 1995; Yao et al., 1995, 1997). The cis-acting sites of the viral dsRNAs are also outlined in Figure 1.

C. Host–Viral Interaction

ScV replication must be under direct or indirect host control, since the virus neither kills the cells nor fails to infect all progeny of infected cells. There are no DNA copies of the dsRNA genome present in host strains (Hastie et al., 1978; Wickner and Leibowitz, 1977). ScV replication may (Sclafani and Fangman, 1984; Zakian et al., 1981) or may not (Newman et al., 1981) be confined to the G1 phase of the cell cycle. Nothing is now known of the mechanism(s) of host regulation of ScV replication. However, there are many host genes known to affect maintenance of ScV: the MAK, SPE, SKI, KRB, MKT, and DET genes (Toh-e et al., 1978; Toh-e and Wickner, 1979, 1980; Wickner, 1986, 1987; Wickner and Leibowitz, 1977). Most of the MAK genes are necessary for ScV-M1 but not for
ScV-L1. The host functions, but not the viral functions, of some of these are known: MAK7 encodes ribosomal protein L4 (Ohtake and Wickner, 1995a); MAK8 encodes ribosomal protein L3 (Wickner et al., 1982), SPE2 encodes a product required for polyamine synthesis (Cohn et al., 1978); and MAK1 encodes DNA topoisomerase I (Thrash et al., 1984). Several of the MAK gene products (MAK3, MAK27, and MAK10) are necessary for maintenance of some ScVL1 viruses (El-Sherbeini and Bostian, 1987; Field et al., 1982; Sommer and Wickner, 1982), as well as for maintenance of ScVM1. MAK3 codes for an N-acetyltransferase responsible for blocking the N-terminus of P1 (Cap), which is apparently necessary to produce functional P1 (Tercero et al., 1993; Tercero and Wickner, 1992). Some 18 of the 30 known MAK genes affect the supply of free 60S ribosomal subunits, and hence the efficiency of translation of the uncapped unpolyadenylated L1 mRNA (Ohtake and Wickner, 1995b). ScV Cap de-caps cellular mRNAs, decoying the host degradation of uncapped mRNAs and thereby permitting translation of the uncapped viral mRNAs (Ohtake and Wickner, 1995b). Most of the interactions of ScV with host proteins appear to reflect the unusual requirements for translation of the uncapped unpolyadenylated viral RNAs, so it is not clear whether specific host functions are necessary for persistence. This was dramatized by the success in establishing brome mosaic virus as a persistent infection in yeast (Janda and Ahlquist, 1993; Quadt et al., 1995). However, a host factor does appear to be accessory to ScV replication (Fujimura and Wickner, 1988b).

II. RELATIONSHIPS AMONG dsRNA VIRUSES OF LOWER EUKARYOTES

A. Genome Expression

The Totiviruses, by definition, and Giardiaviruses encode all viral proteins on one strand of a single dsRNA. However, they all make a capsid polypeptide without Pol domains and must consequently have a mechanism by which to produce two polypeptides from one mRNA. ScV does this by -1 translational frameshifting, as described above. So does the Giardia lamblia virus G1V (Wang et al., 1993). However, Leishmania virus LrV1 and Trichomonas virus TvV use +1 translational frameshifting (Stuart et al., 1992; Su and Tai, 1996; Tai and Ip, 1995). Ustilago virus UmVP1H1 appears to use proteolytic processing of a Cap–Pol precursor (Park et al., unpublished), while Helminthosporium virus Hv190SV appears to use a “stop-and-go” translation (S. Ghabrial, personal communication) similar to that by which the chestnut blight fungus dsRNA virus
produces its RDRP (Shapira et al., 1991). Gene expression strategies in CyV and AbVL1 (see below) are not yet clear. In short, this family of closely related viruses uses a variety of strategies for gene expression — the end justifies the means.

B. The Polymerase Domain in the dsRNA Viruses of Lower Eukaryotes

In contrast to the viral RDRPs as a whole (Bruenn, 1991), the Totivirus RDRPs are easily aligned with statistical significance (Bruenn, 1993). Eight conserved motifs were identified in five Totivirus RDRPs (Bruenn, 1993). Five more sequences not included in this collection but shown here (Table I; see also Fig. 4) are also 19–22% identical overall with this set, and show highly significant alignments as evaluated by BLAST. AbV1L1 is a viral segment from a dsRNA mycovirus in Agaricus bisporus that may or may not be a Totivirus (Van der Lende et al., 1996). There is no definitive evidence differentiating the possible presence of a number of Totiviruses and satellite RNAs (as in yeast or Ustilago) from the proposed multisegmented virus in Agaricus bisporus (Van der Lende et al., 1996). The sequence of the L1 segment (used here) is incomplete, and the open reading frame may extend at least 300 bases further. This would give at least 500 amino acids prior to the first conserved motif (LIGRR, motif 1), so that the encoded protein could be a fusion protein combining Cap with Pol and providing for cleavage of the precursor to generate a separate Cap, as appears to be the case with UmVP1H1 (Park et al., unpublished). CyV is an incomplete sequence derived (by RT-PCR) from a single-segmented dsRNA virus that was thought to be a plant virus (Coffin and Coutts, 1995), but it is probably a fungal virus contaminant (R. H. A. Coutts, personal communication). Another protozoan dsRNA virus that may be a Totivirus has been isolated from Eimeria nieschulzi and a partial sequence obtained from the region around motif 5 (Roditi et al., 1994). The tertiary structure of the polio RDRP suggests that motifs 3–7 are in the active site of the enzyme (S. Schultz, personal communication).

Figure 2 plots percentage identity across the entire RDRP alignment. Conserved motifs show up as peaks in identity, of which eight are numbered consecutively. The sequences of the conserved motifs and their positions in the proteins are shown in Table I. Although polio polymerase cannot be adequately aligned with the Totivirus RDRPs, it is possible to pick out all the conserved motifs of the Totiviruses in polio (with the possible exception of motifs 1, 2, and 8). Although it is clear that motifs 4, 5, and 6 are properly identified in polio, identification of motifs 7 and 8 from the structural determination will require careful consideration of all known polymerase structures (S. Schultz, personal communication).
# TABLE I

Conserved Motifs in RDRPs of *Totiviruses* Compared to Polio<sup>a</sup>

| RDRP | 1   | 2   | 3       | 4       | 5   | 6   | 7   | 8   |
|------|-----|-----|---------|---------|-----|-----|-----|-----|
| LRV1 | LGRG| 59  | WAAN.GS.HS| 44     | KLEH.GK..TRLL| 57  | DYDDPSQHT| 46  | TLMSGHRATSFINSVNLRA.YI| 11  | HVGDDILM| 33  | EFLRV| 9   | YLAR|
| TTV  | LGRG| 58  | WSKS.GS.HY| 41     | KLEH..GK..ERFY| 50  | DYTDPNSQHT| 43  | TLPSGRATHFIPFVNLWC.YT| 11  | CAGDVIL| 31  | EFLRK| 9   | YPCR|
| EnV  |     |     |          |         |         |     | TLMSGHRATTSFINSVNLAA.YV |
| Hv190| LQGRY| 64  | WCVN.GSQNA| 46     | KLEN.GK..DRAIF| 56  | DYDFNSQHS| 50  | TLMSGHRATFTNSVNLAA.YI| 14  | HAGDDVYL| 35  | EFLRL| 9   | YLR|
| ScVL1| LMNRG| 57  | WVPG.GSVHS| 46     | KYEW.GK..QRAIY| 52  | DYYDFNSQHS| 52  | TLLSGWRLTFPMNTVNLWA.YM| 15  | HNGDDVMI| 33  | EFLRV| 13  | YLSR|
| ScVLa| LENGV| 58  | IMPG.GSVHS| 46     | KYEW.GK..VRALY| 51  | DFDFNSQHS| 52  | TLPFSGWRLLTFNTALNYC.YL| 13  | HNGDDVFA| 33  | EFLRV| 11  | YLTR|
| UmVH1| LGRG| 66  | WLVS.GGSA| 55     | KNETGK.ARAIYI| 55  | DYPFDNSMHT| 63  | GLYSGDRTTLINTLNLIA.YA| 20  | CHGDDIIT| 34  | EYLRI| 10  | CLAR|
| UmVH2| PFNRV| 59  | RMPT.GSTVS|         | KYEW.GK..QRAIY |     |         |     |         |     |         |     |         |     |       |
| CyV  |     |     |         |         |         |     |         |     |         |     |         |     |         |     |       |
| AbV1L| LGRR| 79  | ADPSAGELIT| 44     | KHEV..GKNSRSW| 63  | DYANFNEQHS| 54  | GLLSGWRCTAYINNLINIAQY| 21  | TGGDDGCA| 35  | EFRPL| 10  | SVIR|
| GIV  | LGKV| 65  | WCTT.GSRY| 37     | KPEL..TK..VRAVI| 55  | DQSNFDRQPD| 59  | GLPSGWRKNTALLGLANT.QLL| 16  | VQGDDIAL| 33  | EFLR| 9   | YPA|
| Polio| VFGV| 64  | YGTG.GLEAL| 49     | KDEL.RSK..TKVEQ| 64  | DTGYDASLS| 42  | GMPSCGTSIFNSMINNL.II| 18  | AYGGDVIA| 38  | TFKLR| 9   | FLIH|

<sup>a</sup>The amino-acid sequences of the conserved motifs identified in the multiple sequence alignment of Figure 2 are shown in the standard single-letter amino-acid code. Periods indicate where insertions have been made to maximize alignment. Some sequences (e.g., EnV and UmVH2) are only partially determined in this region. Spacing between motifs (in amino acids) is indicated for each RDRP. Asterisks indicate residues identical in all *Totivirus* RDRPs. The numbers 1–8 indicate the conserved motifs. This alignment includes a region of about 400 amino acids (Fig. 2).
A number of studies have been done on mutations within viral RDRPs that generally confirm that the highly conserved motifs (such as 5 and 6 below) are required for enzyme function (Mills et al., 1988; Ribas and Wickner, 1992). Alanine substitutions have demonstrated the importance of motifs 3, 5, and 6 for replication or transcription in ScV (Ribas et al., 1994; Ribas and Wickner, 1992). Recently, the structure of most of the polio RDRP has been determined by X-ray diffraction, and its structure is quite similar to that of the DNA-dependent RNA polymerases, the DNA polymerases, and the reverse transcriptases (Kohlstaedt et al., 1992; Schultz et al., 1996). The five conserved motifs (3–7) appear in the “palm” of the polymerase (Hansen et al., 1997).

G1V, the *Giardia lamblia* virus, is distinctly different from the Totiviruses, although it is still close enough for accurate alignment. This is interesting, since it alone among the known protozoan dsRNA viruses is infectious to its host cells. Substitution of G1V motifs for ScVL1 motifs in the ScV RDRP demonstrates that motifs 3–7 are part of the polymerase function and that motifs 1, 2, and 8 have other functions (Routhier and Bruenn, 1998). As expected from previous results (Fujimura et al., 1990, 1992; Ribas et al., 1994; Yao et al., 1995), packaging is independent of motifs 1–8: all of these constructs gave normal packaging of VBS
containing transcripts in a two-plasmid assay (Routhier and Bruenn, 1998). Replication and/or transcription of ScV RNAs strongly requires the region of Pol represented in all the RNA-dependent RNA polymerases (motifs 3–7) (Routhier and Bruenn, 1998).

The mapping of a cryptic RNA-binding domain between conserved motifs 2 and 4, which includes conserved motif 3 (Ribas et al., 1994), is probably indicative of a universal function of the polymerase, perhaps a conformational change during RNA translocation. One mutant tested by Wickner is a change of conserved motif 3 from KYEWGKQRAIY to AAEWGAQAAIY. This change obliterates support of ScVM1 (Ribas et al., 1994), consistent with G1V substitutions in this region (Routhier and Bruenn, 1998). This supports extension of the essential RDRP domain at least to motif 3.

A small region of the UmVP6H1 segment has been sequenced (C. M. Park and J. A. Bruenn, unpublished manuscript), and its comparison to UmVP1H1 shows only two conservative changes in 162 amino acids (total sequenced region of Pol in UmVP6H1). Over the sequenced region of UmVP1H2, UmVP1H2 is on average 34% identical to ScVLa and only 21.5% identical to UmVP1H1. In these same regions, ScVL1 and ScVLa are only 30.1% identical, so that UmVP1H2 and ScVLa are closer to each other than either is to a second virus present in the same cells. This is clear evidence of a common origin of UmVP1H2 and ScVLa prior to evolutionary separation of Ustilago and Saccharomyces. Similarly, the protozoan viruses EnV and LrV1, rather than the other fungal Totiviruses, are the closest relatives of fungal Totivirus Hv190SV. This relationship is confirmed by a similar analysis of the capsid polypeptide sequences; the capsid polypeptide of Hv190SV is clearly related to that of LrV1 (Fig. 3), but not to any of the other sequenced capsid polypeptides among the Totiviruses (not shown).

A phylogenetic analysis of motifs 2–8, combined with analysis of the sequenced regions of UmVP1H2 and EnV, gives a tree that summarizes these relationships (Fig. 4). Clearly, viruses present in the same cells may be much more closely related to viruses in cells evolutionarily separated by billions of years than they are to each other (e.g., UmVP1H2 to ScVLa and EnV to Hv190SV). Since these are noninfectious viruses, this can only be interpreted to mean that they arose very early in evolution and that their sequences have been well preserved for a very long time.

III. SATELLITE VIRUSES: KILLER TOXINS

Some of the dsRNA viruses of lower eukaryotes, like some plasmids and bacteriophages in the prokaryotes, confer a selective advantage on their hosts. In several genera of fungi, all of which are yeasts or have a yeastlike (nonfilamen-
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Fig. 3. Comparison of the Hv190SV capsid polypeptide sequence with that of Lrv1 by the GCG program COMPARE and plotted by the program DOTPLOT, using the default matrix, a window of 20 amino acids, and a stringency of 16. Arrows indicate regions of significant similarity.

Fig. 4. Phylogenetic tree of protozoan and fungal viruses with known RDRP sequences. Derived from motifs 2–8 and sequenced regions of UmVH2 and EnV by the protein parsimony algorithm of the PHYLIP programs (Felsenstein, 1989).
tous) mode of growth, satellite dsRNA viruses may encode secreted protein toxins that kill sensitive cells. In some cases (e.g., the *S. cerevisiae* k1 and k2 killer systems), the satellite virus itself provides the host cell with immunity to the toxin. This is the result of a function of the preprotoxin in the case of k1 (Boone et al., 1986). In some cases — for instance, with the *U. maydis* killer toxins — nuclear mutations are required to provide the host with resistance to the toxin (Finkler et al., 1992; Ginzberg and Koltin, 1994; Koltin and Kandel, 1978; Koltin et al., 1978). There are at least five genera of fungi in which such toxins have been well documented (Table II). Most of these are encoded by satellite dsRNA viruses, but some are encoded by DNA plasmids (*Kluyveromyces*) or by the nuclear genome (*Williopsis*). Most are processed by homologues of the Golgi enzymes (kex2p and kex1p) responsible for processing of serum proteins in mammals (Bloomquist et al., 1991; Brenner and Fuller, 1992; Fuller et al., 1988, 1989; Van de Ven et al., 1991; Zhu et al., 1992).

The origin of the toxin genes encoded by dsRNA satellite viruses is probably the nuclear genome of the host. Two lines of evidence justify this conclusion. First, the dsRNA genome of the k1 killer toxin gene (M1) in *S. cerevisiae* includes an internal polyA sequence of variable length (Hannig et al., 1986). The origin of this sequence is not viral: none of the dsRNA viruses have polyadenylated RNAs, although the viral polymerase commonly adds one nontemplated A or G residue at the 3' end of its transcripts, both plus and minus strands (Brennan et al., 1981; Diamond et al., 1989). The location of this sequence in M1 is highly significant: it comes immediately after the coding sequence for the preprotoxin (Hannig et al., 1986) and before the 3' region necessary for packaging of viral plus strands (Fujimura et al., 1990; Huan et al., 1991; Shen and Bruenn, 1993; Yao et al., 1995, 1997). The most likely explanation for this structure is that the viral replicase switched from its normal template to an erroneously packaged cellular mRNA after copying the several hundred 3' bases of L1. Subsequent selection may have resulted in duplication of the 3' packaging signal in M1 (Shen and Bruenn, 1993) and the addition of the 5' terminal GAAAAA RNA sequence to the M1 plus strand, as well as loss of all L1 sequences not essential for packaging. The other two dsRNA-encoded killer toxins in *S. cerevisiae* (k2 and k28) also have internal polyA sequences in their genomic dsRNAs (Dignard et al., 1991; Hannig et al., 1984; Schmitt, 1995; Schmitt and Tipper, 1990; Skipper, 1983), so that it is possible that all arose from one initial miscopying event.

Many of the killer toxins may have arisen from a common ancestor. Most of the killer toxins have two polypeptides (α and β) that may be separate (*U. maydis* KP6), noncovalently associated in solution (*Pichia farinosa* KK1), or linked by intermolecular disulfides (*S. cerevisiae* k1). The exceptional toxin, which is a single polypeptide (*U. maydis* KP4), appears from its structure to be a tandem duplication of one sequence that has subsequently diverged (Gu et al., 1995), and
| Organism               | Type | Peptide | Size (aa) | Genome | Mechanism of action | Processing | 3D structure | References                          |
|-----------------------|------|---------|-----------|--------|---------------------|------------|--------------|--------------------------------------|
| Saccharomyces cerevisiae | k1  | α       | 103       | dsRNA  | Channel             | kex2p, kex1p | no           | Bostian et al., 1984; Martinac et al., 1990; Skipper et al., 1984 |
|                       | β    |         | 83        | dsRNA  |         |             | no           |            |
| S. cerevisiae         | k2  |         | 178       | dsRNA  |         | kex2p, kex1p   | no           | Dignard et al., 1991                |
|                       | β    |         | 159       | dsRNA  |         | kex2p, kex1p   | no           | Schmitt, 1995                       |
| Ustilago maydis       | KP1  | β       | 117       | dsRNA  |         | kex2p, kex1p   | no           | Park et al., 1996a                  |
| U. maydis             | KP4  | α       | 105       | dsRNA  | Ca²⁺ inh.        |             | α-β sandwich | Gu et al., 1995; Park et al., 1994 |
|                       | KP6  | β       | 79        | dsRNA  | Channel           | kex2p, kex1p | 4-strand β/2α | Li et al., 1997; Tao et al., 1990 |
| Williopsis mrakii     | WmKT|         | 88        | DNA    | β-glucan inh.     | kex2p 2x4 strand β sheet |             | Antuch et al., 1996; Kimura et al., 1993 |
| (Hansenula mrakii)    | (HMK)|         |           | DNA    | β-glucan inh.     | kex2p 2x4 strand β sheet |             |                                      |
| Pichia farinosa       | KK1  | α       | 63        | DNA    |         | kex2p, kex1p   | like KP4     | Kashiwagi et al., 1997; Suzuki and Nikkuni, 1994 |
| Pichia inositavora    | NRRL18709 | β  | 77        | DNA    |         |         | no           | Hayman and Bolen, 1991               |
| Pichia kluveri        | ?    |         | ?         | DNA    | Channel   | ?           | no           | Kagan, 1983                           |
| Kluyveromyces lactis  | α    |         | 865       | DNA    |         | kex2p, kex1p   | no           | Stark et al., 1990; Stark and Boyd, 1986 |
|                       | β    |         | 252       | DNA    |         | kex2p, kex1p   | no           | Stark et al., 1990; Stark and Boyd, 1986 |
|                       | γ    |         | 229       | DNA    |         | kex2p, kex1p   | no           | Stark et al., 1990; Stark and Boyd, 1986 |

Three-dimensional structures determined by NMR or X-ray crystallography (3D structure) are abbreviated α for alpha helix and β for beta sheet. "No" indicates no structural determination has been done. In the "mechanism of action" column, "inh." indicates inhibition. The "processing" column lists the enzymes (other than signal peptidase) known or suspected to be involved in processing of the preprotoxin.
it could easily have arisen from two polypeptides processed by kex2p by a single deletion of the genomic sequence encoding the intervening sequence and the cleavage sites in the prepropolypeptide. This model is supported by the tertiary sequence identity between KP4 and the KK1 toxin, in which α and β together have the structure of the single polypeptide in KP4 (Kashiwagi et al., 1997). Models for KP6 β assume that it arose by a tandem duplication of the ancestral form of the KP6 α sequence followed by divergence as well (Li et al., 1997). Further structural analysis of killer toxins may help derive an evolutionary tree for them.

The second line of evidence indicating that the dsRNA killer toxins arose from nuclear genes is the relationship between the *U. maydis* KP4 killer toxin and the *Pichia farinosa* KK1 toxin. These have no evident primary sequence similarity, but their tertiary structures are essentially identical (Gu et al., 1995; Kashiwagi et al., 1997; Park et al., 1994; Suzuki and Nikkuni, 1994), even though KP4 is a single polypeptide and KK1 is two polypeptides. The KP4 toxin is encoded by a satellite dsRNA virus in *U. maydis* and the KK1 toxin by the nuclear genome of *P. farinosa*. The obvious explanation for this data is that the two toxins retain the same function, in which a few key residues inhibit the Ca$^{2+}$ channel when held in appropriate position by the protein scaffolding, so that only the tertiary structure of the toxin is conserved by evolution. Since one of these toxins is encoded by a nuclear gene, it may be this gene that is the prototype for all the killer toxins.

The existence of dsRNA segments encoding killer toxins may partially explain the prevalence of endogenous dsRNA viruses in the fungi, since these segments clearly provide a selective advantage to the cells harboring their parental viruses. However, the majority of isolates of fungi in which killer toxins have been discovered lack the dsRNAs encoding the toxins but still have the parental viruses.

IV. OTHER VIRAL SYMBIONTS IN FUNGI

In addition to the Totiviruses and Partitiviruses, fungi are replete with a number of other viruslike symbionts. The hypovirulence-associated dsRNA virus of chestnut blight fungus is an endosymbiont like the Totiviruses but has no capsid polypeptide, instead being enclosed in a membrane (Koonin et al., 1991; Shapira et al., 1991). There are two single-stranded RNA elements in *S. cerevisiae* encoding their own RDRPs but without any capsid or membrane (Garcia-Cuellar et al., 1995; Matsumoto et al., 1990; Matsumoto and Wickner, 1991; Widner et al., 1991). None of these elements is related to the Totiviruses as judged by genome structure, expression, or RDRP sequence, but they are related to other viral families (Koonin et al., 1991). Similarly, there are numerous retrotransposons in the fungi, the best characterized of which is the Ty element. Ty encodes a single
capsid polypeptide and a reverse transcriptase (Clare and Farabaugh, 1985); its single-stranded RNA genome is encapsidated within *S. cerevisiae* cells; and its reverse transcriptase, which is related to those of the retroviruses, is synthesized by a +1 translational frameshift followed by proteolytic processing (Belcourt and Farabaugh, 1990; Farabaugh, 1996; Farabaugh et al., 1993).

This collection of viruses and degenerate viruses testifies to the ubiquity of viruses present very early in evolution, some of which appear to have been trapped in the fungi and protozoans, where the only sure strategy for survival is to become permanent residents of host cells.

In summary, most of the fungi and protozoans may have dsRNA virus symbionts, and this symbiosis predates differentiation of the single-celled organisms, so that it is a symbiosis of very ancient origin.

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