



Origin, Adaptation and Evolutionary Pathways of Fungal Viruses

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Abstract. Fungal viruses or mycoviruses are widespread in fungi and are believed to be of ancient origin. They have evolved in concert with their hosts and are usually associated with symptomless infections. Mycoviruses are transmitted intracellularly during cell division, sporogenesis and cell fusion, and they lack an extracellular phase to their life cycles. Their natural host ranges are limited to individuals within the same or closely related vegetative compatibility groups. Typically, fungal viruses are isometric particles 25–50 nm in diameter, and possess dsRNA genomes. The best characterized of these belong to the family *Totiviridae* whose members have simple undivided dsRNA genomes comprised of a coat protein (CP) gene and an RNA dependent RNA polymerase (RDRP) gene. A recently characterized totivirus infecting a filamentous fungus was found to be more closely related to protozoan totiviruses than to yeast totiviruses suggesting these viruses existed prior to the divergence of fungi and protozoa. Although the dsRNA viruses at large are polyphyletic, based on RDRP sequence comparisons, the totiviruses are monophyletic. The theory of a cellular self-replicating mRNA as the origin of totiviruses is attractive because of their apparent ancient origin, the close relationships among their RDRPs, genome simplicity and the ability to use host proteins efficiently. Mycoviruses with bipartite genomes (partitiviruses), like the totiviruses, have simple genomes, but the CP and RDRP genes are on separate dsRNA segments. Because of RDRP sequence similarity, the partitiviruses are probably derived from a totivirus ancestor. The mycoviruses with unencapsidated dsRNA-like genomes (hypoviruses) and those with bacilliform (+) strand RNA genomes (barnaviruses) have more complex genomes and appear to have common ancestry with plant (+) strand RNA viruses in supergroup 1 with potyvirus and sobemovirus lineages, respectively. The La France isometric virus (LIV), an unclassified virus with multipartite dsRNA genome, is associated with a severe die-back disease of the cultivated mushroom. LIV appears to be of recent origin since it differs from its host in codon usage.

Key words: mycoviruses, hypoviruses, partitiviruses, totiviruses

Introduction

Fungal viruses or mycoviruses, despite their belated discovery, are now believed to be widespread in fungi and to have evolved at a very early stage in the phylogeny of their hosts. In general, mycoviruses are associated with latent infections of their hosts and are not known to have natural vectors. They are transmitted in nature intracellularly by hyphal anastomosis and heterokaryosis (lateral transmission), and are disseminated via spores (serial transmission). 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 (1,2).

Fungal viruses do not have an extracellular phase to their life cycles. Despite this, they are clearly very successful, being prevalent in all major taxa of fungi. Because of lack of infectivity as free particles, mycoviruses have been described as noninfectious, endogenous, or heritable viruses (3,4). The use of this terminology without ample explanation, however, may be misleading and inaccurate. Whereas the term noninfectious may be useful in differentiating between viruses that lack extracellular routes of infection and those that are infectious as free virions,

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it inaccurately implies structural deficiencies of the viruses (see section on mycovirus infectivity). The terms endogenous and heritable viruses imply that mycoviruses have the ability to integrate a DNA copy into host DNA. Although there is preliminary supportive evidence (S.M. Tavantzis, personal communication), this mode of mycovirus existence is not well documented for any group of fungal viruses. If true, this would be of considerable interest because it would mean that fungal viruses have evolved an ultimate intracellular existence.

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 isometric dsRNA viruses are classified into two families, *Totiviridae* and *Partitiviridae* (5,6). The complete nucleotide sequence of several of the totiviruses that infect fungi and parasitic protozoa have been determined and some of these viruses have been well characterized at the molecular level (7–15). Conversely, the complete nucleotide sequence of only one member of the family *Partitiviridae* has been reported (16).

In addition to the mycoviruses classified in the families *Totiviridae* and *Partitiviridae*, two other groups of dsRNA fungal viruses (17–19) have been more or less well characterized; these include the unencapsidated hypoviruses (family: *Hypoviridae*) and the La France isometric virus (LIV) associated with the La France disease of mushrooms (unclassified virus). This article will discuss some of the biological features of mycoviruses akin to their intracellular mode of transmission and coevolution with their hosts. The origin and evolutionary pathways of the isometric dsRNA mycoviruses will be emphasized.

Mycoviruses have Limited Host Ranges and are Often Associated with Symptomless Infections

As stated earlier, mycoviruses are transmitted intracellularly during cell division, sporogenesis and cell fusion. As a result of these intracellular modes of transmission, the natural host ranges of mycoviruses are limited to individuals within the same or closely related vegetative compatibility groups (20). Mycoviruses may be eliminated during sexual spore formation. Even though the yeast ssRNA and dsRNA viruses are effectively transmitted via ascospores, the

mycoviruses infecting the ascomycetous filamentous fungi are essentially eliminated during ascospore formation (1). On the other hand, ssRNA and dsRNA mushroom viruses are transmitted efficiently via basidiospores (21). Mixed infections with two or more unrelated viruses are common, probably also as a consequence of the ways by which fungal viruses are transmitted in nature. There are apparently no structural interactions between these viruses since heterologous encapsidation has not been reported in mixed infections (1,2). Accumulation of defective dsRNA and/or satellite dsRNA is a common feature of mycovirus infections, and the resulting complexity of dsRNA banding pattern has, in some instances, led to confusion in interpreting the nature and organization of the virus genomes. In these cases, molecular characterization of the virus in question is required to verify whether the essential genome is segmented, nonsegmented plus defective/satellite dsRNAs or whether the complexity is the result of mixed infections with more than one virus.

As a rule, infections due to mycoviruses are both latent and persistent. Latency benefits the host for survival, and persistence benefits the virus in the absence of infectivity (extracellular mode of transmission). Fungal viruses thus appear to have coevolved in concert with their hosts (4). To ensure their retention, some fungal viruses have in fact evolved to confer some selective advantage upon their host (e.g. the killer phenotypes in yeasts and smuts). Nevertheless, mycoviruses persist and spread even without a selective advantage due to their efficient means of intracellular transmission.

Although a large number of the viruses that infect plant pathogenic fungi have been reported to be avirulent, 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 (2,22,23). Virus-induced diseases and virus-mediated attenuation of virulence in plant pathogenic fungi provide excellent opportunities for fundamental studies aimed at developing novel biological control measures. The hypovirulence phenotype in the chestnut blight fungus (*Cryphonectria parasitica*) is an excellent and well documented example for a mycoviral-induced phenotype that is currently being exploited for biological control (22,24). The debilitating disease of *Helminthosporium victoriae*, the causal agent of

Victoria blight of oats, is an example of pathogenic effects of fungal viruses (25).

Infectivity of Mycoviruses

Mycoviruses are unable to initiate infections of their hosts by extracellular routes and conventional infectivity assays using purified virions are not presently available. The rigid cell wall in fungi, understandably, constitutes a barrier to virion uptake, and to damage or puncture the cell wall without killing the cell might not be possible. To overcome the cell wall barrier, attempts have been made to infect fungal protoplasts with cell-free virion preparations. These assays, though successful in a few systems, are highly inefficient (2). Interestingly, the *Giardia lamblia* virus (GLV), a member of the family *Totiviridae* that infects the protozoan *Giardia lamblia*, is infectious as free virions as well as in transfection experiments involving electroporation with (+) strand RNA transcribed *in vitro* from virion dsRNA (26). Approaches using fungal protoplasts electroporated with full length *in vitro* transcripts of cloned cDNA to viral dsRNAs may also be applicable to mycoviruses.

GLV is similar in virion structure, genome organization and expression strategy to noninfectious totiviruses including those infecting protozoan and fungal hosts. This suggests that the lack of infectivity in those viruses is more likely related to host factors (membrane structure) than to defects in virus structure. *Giardia lamblia* is a primitive eukaryote with two nuclei, but no mitochondria, Golgi or endoplasmic reticulum. *G. lamblia*, but not other protozoan hosts of relatively more recent origin, must have natural cell surface proteins that may serve as virus receptors and allow the entire virion to enter the cell. For a dsRNA virus like GLV, entry of the entire virion is crucial since the virion-associated RNA-dependent RNA-polymerase (RDRP) must accompany the dsRNA template to initiate infection. For the noninfectious fungal totiviruses and partitiviruses as well as for the plant cryptoviruses (family *Partitiviridae*), there is no evidence for virus receptors in the surface membranes of fungal or plant protoplasts, after enzymatic removal of the impenetrable cell wall. These viruses gain entrance to the cell following intracellular transmission during cell division, sporogenesis and cell fusion.

dsRNA Mycoviruses Might Have Evolved to be Beneficial to their Hosts

Some dsRNA mycoviruses, like the DNA plasmids that confer antibiotic resistance upon their hosts, might have evolved to be beneficial to their hosts, e.g. the killer phenotypes in yeast and smuts (27,28). The discovery of the killer phenomenon in the sixties in the yeast (*Saccharomyces cerevisiae*) and in the smut fungus (*Ustilago maydis*) eventually led to the discovery of the isometric dsRNA mycoviruses with undivided genomes, presently classified in the genus *Totivirus* in the family *Totiviridae* (29). Yeast or smut killer strains secrete a protein toxin to which they are immune, but which is lethal to sensitive cells. Genetic and biochemical studies in yeast have conclusively shown that toxin production and immunity are cytoplasmically inherited, and that dsRNAs of viral origin comprise the cytoplasmic determinants. The killer toxin is encoded by a satellite dsRNA, denoted M-dsRNA, which is dependent on a helper virus with undivided dsRNA genome for encapsidation. The helper viruses [*Saccharomyces cerevisiae* virus- L-A (ScV-L-A) and *Ustilago maydis* virus-H1 (UmV-H1)], which are autonomously replicating viruses that do not require M-dsRNA for replication, also encode the RNA polymerase protein required for the replication of both the M-dsRNA and their own genomic monopartite dsRNAs. The helper virus dsRNA and M-dsRNA are separately encapsidated in identical capsids coded for by the helper virus.

Unlike the helper viruses associated with the yeast and smut killer systems, the member viruses in the genus *Totivirus* 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 suspected of being satellite or defective dsRNAs. It is surprising that the search for killer systems in plant pathogenic filamentous fungi is not being keenly pursued since killer toxins associated with such systems may have broad antifungal activity in order to confer selective advantage upon their hosts. The lethal activities of the killer toxins secreted by yeast and smut strains are limited to sensitive strains of yeast and smut, respectively.

Is dsRNA a Specially Appropriate Genome for the Persistently Intracellular Mycoviruses?

Buck (1) proposed that dsRNA might be a particularly suitable genome for a persistently intracellular virus and this could explain why dsRNA is so common in fungi compared to other organisms. He argued that a dsDNA would be too competitive for this role, unless integrated into host DNA, whereas dsRNA might be able to adapt some of the host dsDNA replication machinery for its own purposes (e.g. DNA topoisomerase I is essential for the replication of M dsRNA, a satellite dsRNA dependent on the totivirus ScV-L-A for replication and encapsidation).

It is well recognized, however, that the presence of viral dsRNA triggers many cellular responses to virus infection, probably through activation of dsRNA-dependent enzymes (30). Many viruses have evolved mechanisms to mask the effects of dsRNA on cells. Completely uncoated dsRNA has not been detected in cells infected with dsRNA viruses. The dsRNAs of hypoviruses that do not code for a coat protein are enclosed in host-encoded vesicles. Input viral dsRNA remains within the capsid throughout the life cycle and progeny genome is only synthesized after packaging of (+) strand progeny RNA into capsids. It is likely that the schemes that dsRNA viruses go through to prevent exposure of naked dsRNA in cells is a consequence of the profound effects that dsRNA has on the physiology of the cell.

dsRNA Mycoviruses Evolved in Close Association with their Hosts and are Able to Make Efficient use of Host Proteins for their Replication

The latent or symptomless relationship of most fungal viruses with their hosts is clear evidence that fungi and their viruses have coevolved and coadapted to a considerable degree. Many fungal viruses maintain only the genes that are essential for their survival (*RDRP* and *CP*, e.g. totiviruses and partitiviruses), but make efficient use of host proteins. The host cells have evolved to support only a defined level of virus replication beyond which virus infection becomes pathogenic. Virus pathogenesis and host cell death may lead to virus elimination since fungal viruses lack extracellular infectivity.

Because of amenability to genetic studies, the

yeast-virus system has provided significant information on the host genes required to prevent viral cytopathology (31). A system of six chromosomal genes, designated superkiller (or *SKI*) *SK12*, *SK13*, *SK14*, *SK16*, *SK17* and *SK18*, negatively control the copy number of the ScV-L-A totivirus and its satellite and defective dsRNAs, M and X, respectively. The only essential function of these genes is to block virus multiplication. 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. The *SKI* genes affect primarily the initiation of translation rather than the stability of mRNA, and are thus part of a cellular system that specifically blocks translation of non-polyadenylated mRNAs (like the (+) strand transcripts of L-A virus and its associated satellites). If the *SKI* genes are defective, the L-A virus with associated satellite M dsRNA system becomes pathogenic, as cells become cold sensitive for growth (31). About 30 chromosomal genes, termed *MAK* genes (for maintenance of killer), are required for stable replication of the satellite M dsRNA (31). Only three of these *MAK* genes are necessary for the helper virus (ScV-L-A) multiplication. Mutants defective in any of 20 *MAK* genes show a decreased level of free 60S ribosomal subunits. Since the *mak* mutations affecting 60S subunit levels are known to be suppressed by *ski* mutations and since the latter are now known to act by blocking translation of nonpolyadenylated mRNAs, the level of 60S ribosomal subunits is believed to be also critical for translation of nonpolyadenylated mRNAs (31).

The Hv190S totivirus that infects the plant pathogenic fungus *Helminthosporium victoriae*, utilizes host-encoded proteins (a protein kinase and a protease) for posttranslational modification of its CP. Phosphorylation and proteolytic processing of CP may play a role in regulating transcription and the release of (+) strand transcripts from virions (8,32). Hypoviruses (members of the family *Hypoviridae*) that infect the chestnut blight fungus have little or no effect on fungal growth but attenuate virulence by altering the G protein-linked cellular signal transduction processes (33). Hypovirus-infected fungal strains (hypovirulent strains) are able to colonize wound sites and form superficial cankers on infected chestnut trees. One may argue that hypovirulence in this system represents a compromise that is beneficial for the survival of both the virus and fungal host. Unlike

the virulent strains, hypovirulent strains do not kill the host trees, and furthermore, can convert virulent strains to hypovirulence.

The Evolutionary Relationships Among Viruses with dsRNA Genomes are Difficult to Ascertain

Six families of dsRNA viruses are presently recognized by the International Committee on Taxonomy of Viruses (ICTV, 34). They are differentiated based on host, number of genome segments and capsid structure (single or double shelled). Members of the family *Reoviridae* (10–12 segments packaged in double shelled virions) infect vertebrates, invertebrates and plants. The *Pseudomonas* phage $\phi 6$ (family *Cystoviridae*) is the only known dsRNA virus that infects bacteria; its genome is comprised of three segments that are packaged in enveloped virions. Members of the families *Birnaviridae* (hosts: vertebrates and invertebrates) and *Partitiviridae* (hosts: fungi and plants) have bipartite genome segments that are packaged together (birnaviruses) or separately (partitiviruses) in single shelled virions. The dsRNA viruses with undivided genomes (the family *Totiviridae*) package their monopartite genomes in single-shelled virions (hosts: fungi and parasitic protozoa). The sixth report of the ICTV lists a sixth family of dsRNA viruses, the family *Hypoviridae* (host: fungi), whose members lack conventional virions and their dsRNA molecules are enclosed in host-encoded vesicles. Whether the genomes of hypoviruses are dsRNA or ssRNA is still a matter of debate (see section on hypoviruses below).

Comparative analysis of the amino acid sequences of proteins encoded by dsRNA viruses revealed surprisingly little similarity between viruses of different genera, even those belonging to the same family, e.g. those belonging to the family *Reoviridae*. Even though the RNA-dependent RNA polymerases (RDRPs) are the most highly conserved genes among RNA viruses, phylogenetic analysis of the RDRPs did not suggest a monophyletic origin for dsRNA viruses. The dsRNA viral RDRPs tend to group with different subdivisions (supergroups) of the (+) strand RNA viruses (35). Koonin (35) concluded from such phylogenetic studies that dsRNA viruses could have originated at multiple events in evolution from different supergroups of (+) strand RNA viruses. Clustering of (+) strand RNA viruses in three

supergroups, however, received little support in a recent phylogenetic study to reevaluate the higher taxonomy of RNA viruses based on RNA polymerase (36). These authors concluded that it is more appropriate to present the evolutionary relationships between RNA viruses as a set of distinct subtrees, the links between which are unclear, rather than a single and resolved phylogenetic tree. Because of the lack of conservation in primary sequence and size among polymerases, it was suggested that the requirements for polymerase function can be fulfilled by diverse means supporting the notion of polyphyletic origins. Convergent evolution could then explain the presence of many of the conserved motifs among RDRPs.

The Isometric dsRNA Totiviruses Infecting Simple Eukaryotes are Probably Monophyletic

The isometric dsRNA totiviruses infecting fungi and protozoa are unique among dsRNA viruses in that their genomes are undivided whereas the genomes of all other dsRNA viruses are segmented. The complete nucleotide sequences of eight totiviruses belonging to three genera in the family *Totiviridae* have been published (Table 1). The genome organization and expression strategy of these viruses are similar; each virus contains two genes; the 5' proximal encodes a coat protein (CP) and the 3' proximal encodes an RDRP. Except for LRV2-1, the RDRP ORF overlaps the CP ORF and is in the -1 frame (ScV-L-A, ScV-La, Hv190SV and GLV) or in the $+1$ frame (LRV1-1, LRV1-4, and TVV) with respect to the CP ORF. The RDRP ORF of LRV2-1 does not overlap the CP ORF (Table 2).

Expression of RDRP as CP-RDRP fusion protein via -1 ribosomal frameshifting has been well documented only for ScV-L-A. Virion-associated CP-RDRP has been detected as a minor protein in case of ScV-L-A, ScV-La (L-BC) and GLV. The RDRP of Hv190SV (and possibly that of LRV1-4) is present as a nonfused separate virion-associated minor protein and is proposed to be expressed via an internal initiation mechanism. Although no CP-RDRP fusion proteins were ever detected *in vivo* or associated with virions of TVV, LRV1-1, LRV1-4, or LRV2-1, expression of RDRP as a fusion protein by $+1$ ribosomal frameshifting (TVV, LRV1-1 and LRV1-4) or ribosomal hopping (LRV2-1) has been proposed.

Table 1. Viruses in the family *Totiviridae* whose complete nucleotide sequences have been reported

Genus	Virus (Alternative Name)	Abbreviation	Accession Number	Ref.
<i>Totivirus</i>	Saccharomyces cerevisiae virus L-A	ScV-L-A	J04692	9
	(Saccharomyces cerevisiae virus L1)	ScVL1	X13426	7
	Saccharomyces cerevisiae virus La	ScV-La	U01060	10
	(Saccharomyces cerevisiae virus L-BC)	ScV-L-BC		
	Ustilago maydis virus H1	UmV-H1	V01059	3
	Helminthosporium victoriae 190S virus	Hv190SV	U41345	8
<i>Giardiavirus</i>	Giardia lanblia virus	GLV	L13218	15
	Trichomonas vaginalis virus*	TVV	U08999	14
<i>Leishmaniavirus</i>	Leishmania RNA virus 1-1	LRV1-1	M92355	13
	Leishmania RNA virus 1-4	LRV1-4	U01899	11
	Leishmania RNA virus 2-1	LRV2-1	U32108	12

*Tentative member.

Sequence comparison analysis of the predicted amino acid sequences of totivirus RDRPs indicated that they share significant sequence similarity and contain characteristic eight conserved motifs (3 and Table 3). This sequence similarity was common to the totiviruses that infect the yeast and smut fungi as well as those infecting parasitic protozoa. The RDRP of Hv190SV, a recently characterized totivirus that infects a filamentous ascomycetous fungus, was also found to contain the same 8 conserved motifs (8). The sequence similarity among the RDRPs of these viruses infecting simple eukaryotes extends beyond the highly conserved 8 motifs (Tables 3 and 4). Of the 70 amino acid positions contained in these conserved motifs, the Hv190SV RDRP is identical in 48, 47, 46, 40, 38, and 21 positions, respectively, to the RDRPs of LRV1, TVV, ScV-L-A, UmVH1, ScV-La and GLV.

Bruenn (3) questioned why the noninfectious dsRNA viruses of lower eukaryotes are much more

closely related to each other than are the dsRNA viruses at large. The most likely explanation, according to Bruenn, is that these viruses are monophyletic and that the progenitor was a non-infectious virus in a single cell type, and that cell type gave rise to both protozoa and fungi. Our recent finding (Table 4 and Fig. 1) that Hv190SV is more closely related to the leishmaniaviruses (LRV1 and LRV2) than to the yeast viruses (ScV-L-A and ScV-La) supports this hypothesis and suggests that Hv190SV and LRVs existed prior to the divergence of fungi and protozoa.

With the recent availability of the complete nucleotide sequence of Hv190SV dsRNA (8), it was of interest to carry out sequence comparison analyses of all known totivirus sequences (three fungal and four protozoal totiviruses). For this purpose, the programs GAP (for pairwise sequence alignment and statistical tests) and PILEUP (for multiple

Table 2. Genome size, length of 5' UTR and overlap regions, and RDRP expression strategy of totiviruses

Virus	dsRNA Size (bp)	5'UTR (nt)	Overlap (nt)	RRDRP Expression*
ScV-L-A	4,579	29	130	- 1 frameshifting
ScV-La	4,615	24	154	- 1 frameshifting
Hv190SV	5,178	289	16	internal initiation
GLV	6100	368	220	- 1 frameshifting
TVV	4,647	287	14	frameshifting
LRV1-1	5,284	447	71	+ 1 frameshifting
LRV1-4	5,283	449	71	+ 1 frameshifting
LRV2-1	5,241	340	none	ribosome hopping

*Mechanism of RDRP expression has only been documented for ScV-L-A.

Table 3. The eight conserved motifs in RDRPs of dsRNA viruses of simple eukaryotes*

	1	2	3	4	5	6	7	8
LRV1†	LLGRG 59	WAANGS . HS 49	GKTRLLL 57	DYDDFNSQHT 46	TLMGHRATSFINSVLNRAYI 11	HVGDDILM 33	EFLRV 9	VLAR
TVV	LLGRG 58	WSKSGS . HY 45	GKERFIY 50	DYDDFNSQHT 43	TLPSCGHRATTFINPVLNWCYI 11	CAGDDVIL 31	EFLRK 9	VPCR
ScVLa	LMNRG 57	WVPGGSVHS 50	GKQRAIY 52	DYDDFNSQHS 52	TLLSGWRLLTTFMNTVLNWAYM 15	HMGDDVMI 33	EFLRV 13	YLSR
ScVLa	LENGV 58	IMPGGSVHS 50	GKVRALY 51	DFDDFNSQHS 52	TLFSGWRLLTTFNTALNYCYL 13	HNGDDVFA 33	EFLRV 11	YLTR
Hv190S	LQGRY 61	WCVNGSQNA 46	GKDRAIF 55	DYDNFNSQHS 45	TLMGHRATTFNSVLNAAYI 15	HAGDDVYL 34	EFLRL 9	YLCR
UmVH1	LYGRG 66	WLVSGSSAG 61	GKARAIY 55	DYPDFNSMHT 63	GLYSGDRDTLLINTLLNIAYA 20	CHGDDIIT 34	EYLRI 10	CLAR
	*	**	** *	* **** *	* ** * * * ** *	***	* ** *	* **
GLV	LLGKV 65	WGTGSGYI 41	TKVRAYI 55	DQSNFDRQPD 59	GLPSGKWKTALLGALINTQLL 16	VQGGDIAL 33	EFLRR 13	MMIR
BCV		GPPGGETHM 44	TKVRGVW 52	DWSSFDSST 52	GLPSGSYTSIVGSVYVNRRLRI 16	TQGGDLSLI 35	TFLGR 9	SLDR

*Data compiled from Bruenn (3) and Huang and Ghabrial (8). Residues identical in all sequences are indicated by asterisks.

†See Table 1 for virus abbreviations.

Table 4. GAP evaluation of totivirus CP and RDRP sequence alignments

	L-A	La	190S	LRV1	LRV2	GLV	TVV
L-A*		7†	0	0	2	0	0
La	26		0	0	1	0	0
190S	27	11		9	7	0	0
LRV1	17	11	47		49	0	1
LRV2	26	10	26	79		0	1
GLV	6	3	3	4	6		2
TVV	12	7	13	25	36	5	

* See Table 1 for virus abbreviations.

† Values represent the number of standard deviations that separate the quality score of the actual GAP-generated alignment from the mean of randomized alignments (values less than 3 is considered not significant). Evaluations of the significance of CP alignments are shown above diagonal; those of the RDRP are shown below diagonal.

sequence alignments) of the UWGCG package (version 8) were used. Furthermore, maximum parsimony analyses of the deduced amino acid sequence of the RDRP ORF region of each dsRNA were performed to estimate the phylogenies of each RDRP. The partitivirus *Atkinsonella hypoxylon virus* (AhV, 37) was included as an outgroup to determine the correct root placement. Branch and bound searches from the RDRP ORF region of each

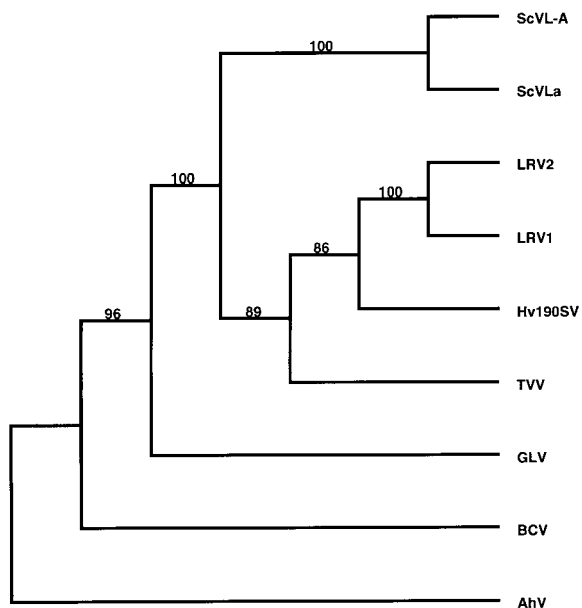


Fig 1. Phylogeny estimates of totiviruses derived from aligned deduced amino acid sequences of RDRP. The resulting consensus tree of 100 bootstrap replicates is shown; the number above each node indicates the percent of bootstrap replicates in which that node was recovered. Tree was outgroup rooted to the partitivirus *Atkinsonella hypoxylon virus* (AhV). See Table 1 for totivirus abbreviations. BCV = beet cryptic virus.

dsRNA were done using the maximum parsimony methods as implemented in PAUP version 3.1.1 (38).

Results of GAP evaluation of the significance of the sequence alignments of the totivirus CP and RDRP ORFs are shown in Table 4. The sequence similarity in the CP ORF between the two leishmaniviruses LRV1 and LRV2 was highly significant (percent identity 37% with a quality score 49 standard deviations above the mean of random alignments). The only other CP sequence similarities that were probably significant in the GAP evaluation test were between the two yeast viruses, ScV-L-A and ScV-La (percent identity 21% with a quality score 7 standard deviations above the mean of random alignments) and between Hv190SV and the two leishmaniviruses (identities of 21 and 23%, and quality scores of 7 and 9 standard deviations above the mean of random alignments, respectively). All other combinations of CP sequence alignments were not significant (Table 4). Highly significant sequence similarities, however, were found in the RDRP ORF between all combinations of totivirus sequences with the possible exception of those involving GLV sequence. The percent identity between GLV and any of the other totiviruses was about 20% with quality scores of 3–6 standard deviations above the mean of random alignments. This suggests that GLV RDRP is possibly similar to the other totivirus RDRPs. Of interest in this regard was the finding that Hv190SV RDRP is more closely related to the leishmaniviruses (percent identity of 30% and quality scores of 25–49 standard deviations above the mean of random alignments) than to the yeast viruses (percent identity of 24% and quality scores of 11–27).

Phylogeny estimates derived from multiple alignments of totivirus RDRP ORFs (Fig. 1) indicated that

the two leishmaniaviruses LRV1 and LRV2 (percent sequence identities of 46%) are most closely related to each other. This is also true for the two yeast viruses ScV-L-A and ScV-La (identity of 32%). It is of considerable interest that Hv190SV, a mycovirus infecting a filamentous fungus, was found to be a sister clade to the leishmaniaviruses and not to the yeast viruses (Fig. 1). This is in agreement with the GAP evaluation results of the CP and RDRP sequence alignments, discussed above (Table 4).

The RDRPs of the Partitiviruses Infecting Fungi and Plants Have Sequence Similarity to Totiviruses

The ICTV has recently grouped together the fungal partitiviruses (family *Partitiviridae*) and the plant cryptoviruses (family *Cryptoviridae*) into one family (*Partitiviridae*) and the family name *Cryptoviridae* was dropped. Viruses in the new family *Partitiviridae* are classified into four genera: viruses belonging to the genera *Partivirus* and *Chrysovirus* infect fungi and those belonging to the genera *Alphacryptovirus* and *Betacryptovirus* infect plants (6). The partitivirus genome is comprised of two linear dsRNA segments, the smaller codes for the CP and the larger codes for the virion-associated RDRP. Each of the genomic dsRNA segment is monocistronic, but additional dsRNA segments (satellite and/or defective) may be present. The complete nucleotide sequence of only one partitivirus (AhV) is available; the RDRP sequence of the beet cryptic cryptovirus (BCV) is also available. The RDRP sequence of AhV was more closely related to BCV than to any of the totiviruses. However, the RDRP sequences from these two viruses were more closely related to totiviruses than to any of the (+) strand RNA viruses (37).

Hypoviruses Have Sequence Similarity to (+) Strand RNA Plant Viruses

Hypoviruses (members of the family *Hypoviridae*) do not encode a coat protein and their genetic information is found predominantly in the form of linear dsRNA (10–13 kbp) associated with pleomorphic membranous host-encoded vesicles. The positive (coding) strand of the type species, *Cryphonectria hypovirus 1-713* (CHV1-713) is polyadenylated at

the 3'-terminus with an average tail length of approximately 20–24 residues. The 5'-terminus of the positive strand appears to be blocked, although the blocking group is unknown. A 5'-leader of approximately 500 nucleotide residues, including several AUG triplets, precedes the AUG codon that initiates the first long ORF, ORF A. The ORF A product may or may not be autocatalytically cleaved, depending on the virus. The UAA termination sequence at the end of ORF A is part of the pentanucleotide UAAUG in all hypoviruses investigated to date, with the AUG of the UAAUG pentanucleotide initiating the other long ORF, ORF B. The N-terminal product of ORF B is a papain-like cysteine protease that autocatalytically releases from the growing polypeptide chain. No further processing *in vitro* has been demonstrated for the remaining 300 kDa polypeptide from this ORF (18).

Sequence analysis of the putative polypeptide products encoded by ORF A and B of CHV-713 L-dsRNA revealed five distinct domains with significant similarity to known conserved domains within plant potyvirus-encoded polyproteins (39). These included the putative RDRP, RNA helicase, two papain-like cysteine proteases related to the helper component protease (HC-Pro), and a cysteine-rich domain of unknown function similar to the N-terminal region of the HC protein.

Alignments of the RDRPs of the yeast L-A totivirus as well as of other dsRNA viruses, RDRPs of supergroup 1 (+) strand viruses and RDRP of CHV1-713 indicated that the overall similarity between CHV1-713 and the yeast L-A was less pronounced than that between CHV1-713 and the potyviruses. Another interesting feature of the CHV1-713 RDRP is the substitution for Gly in the highly conserved GDD motif. Analogous substitution occur in the RDRPs of coronaviruses, toroviruses, several (-) strand RNA viruses, and the dsRNA bacteriophage $\phi 6$ (39). Tentative phylogenetic trees were generated based on the RDRP alignment using three independent algorithms. Regardless of the method used, the results suggest the grouping of the CHV1-713 RDRP with those of the potyviruses and (+) strand RNA viruses, and not with those of dsRNA viruses (39).

Sequence comparisons also indicated that the CHV1-713 helicase-like sequence detected in the C-terminal region of the 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 II (40). Tentative phylogenetic trees revealed the grouping of the putative helicase of CHV1-317 with those of (+) strand RNA viruses. As mentioned earlier, CHV1-713 encodes two cysteine proteases and an additional domain related to the helper-component protein of potyviruses. Like the potyvirus-encoded helper-component protease (HC-Pro), the CHV1-713-encoded proteases resemble papain-like proteases (41–43). Additional similarities between CHV1-713 p29 and HC-Pro include the occurrence of conserved amino acid sequences around the essential cysteine and histidine residues, the nature of the cleavage dipeptides, and the distances between the essential residues and the cleavage sites.

Tartaglia et al. (44) speculated that the dsRNA associated with the unencapsidated hypoviruses are analogous to the replicative form of an ancestral ssRNA virus. Considering the relative organization of the conserved domains within the CHV1-713-encoded and potyvirus-encoded polyproteins, Koonin et al. (39) proposed that CHV1-713 dsRNA might have evolved by rearrangement of a positive strand RNA potyvirus-like genome. The following major events were perceived to have taken place: a) transposition of the helicase gene; b) duplication of the sequence encoding the protease domain of the HC; c) deletion of the sequence encoding the protease domain of the NIa; d) deletion of the capsid protein gene; and e) 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 capsid protein, the ratio of the ssRNA genome to the replicative form could have been altered so that the dsRNA form predominated. Because gene module shuffling has been recognized as a major trend in the evolution of positive strand viruses, Koonin et al. (39) propose that a similar process may account for the evolution of a dsRNA virus-like genetic element from a (+) strand RNA virus (see Fig. 2, pathway 7 for possible origin of hypoviruses from supergroup 1 (+) strand RNA viruses).

The Mushroom Bacilliform Barnavirus has Sequence Similarity to (+) Strand RNA Sobemo- and Carmoviruses

The mushroom bacilliform virus (MBV), the type species of the family *Barnaviridae*, has bacilliform virions resembling those of alfalfa mosaic virus. MBV is often found in association with an isometric virus (La France isometric virus or LIV) in cultivated mushroom exhibiting symptoms of La France disease, a severe die-back disorder of mushrooms (17,19,46). The virus has a (+) strand RNA genome of 4 kbp, and thus differs from the majority of fungal viruses which contain dsRNA genomes. The genome contains four large ORFs of which the putative RDRP displays sequence homology with proteins encoded by viruses with sobemovirus lineage in supergroup 1 of (+) strand viruses (48). The coat protein, however, shows homology with a coat protein encoded by a virus with a carmovirus lineage in supergroup 2 of (+) strand RNA viruses (48).

Evolutionary Pathways of the dsRNA Mycoviruses

Totiviruses and Partitiviruses

Totiviruses that infect fungi and parasitic protozoa are believed to be of ancient origin since protozoa and fungi diverged very early in evolution, at the time of divergence of animals and fungi. It was hypothesized that the progenitor virus infected a single cell type, and that cell type gave rise to both protozoa and fungi (3). This hypothesis is supported by our finding that the totivirus Hv190SV that infects a filamentous fungus is more closely related to the leishmania-viruses LRV1 and LRV2 (protozoal totiviruses) than to the yeast viruses suggesting that Hv190S and LRVs existed prior to the divergence of fungi and protozoa.

Three theories are usually advanced when the origin of viruses is considered. These theories entail that viruses originated from either degenerate forms of intracellular parasites, self-replicating cellular mRNA, or from a prebiotic self-replicating RNA molecules (48). The theory of self-replicating cellular mRNA as the origin of totiviruses is attractive because of their apparent ancient origin, simplicity of genomes, close relationship among the RDRPs and ability to use host proteins efficiently. A cellular

mRNA (e.g. the mRNA encoding a DNA-dependent RNA polymerase) might have acquired the ability to replicate itself by obtaining an origin of replication. Provided this self-replicating mRNA could acquire a gene for a coat protein, then a very simple (+) strand RNA virus would be generated. Replication of (+) strand RNA involves the synthesis of a (-) strand RNA intermediate, and it is possible that dsRNA viruses may have arisen from (+) strand RNA viruses (35). Alternatively, cellular translational control mechanisms involving the production of antisense RNA, could also lead to the generation of dsRNA (Fig. 2, pathway 1).

The yeast 20S and 23S RNA replicons (31) and their replicative forms (RFs) T and W dsRNAs may represent fossils of the ancestral RNA replicons that gave rise to the single and double stranded RNA viruses with simple genomes (Fig. 2, pathways 2 and 3). Based on tentative phylogeny of RDRP of (+) strand RNA viruses, Koonin and Dolja (48) grouped the 20S/23S RNAs (or their RFs, T/W dsRNAs) with those of phage lineage under supergroup 2 of (+) strand RNA viruses. The regressive theory of virus origin (47) may account for the occurrence of the 20S RNA replicons and similar RNA replicons of prokaryotic (phage) lineage in fungi. Because the totivirus RDRPs were grouped with those of supergroup 1 of (+) strand RNA viruses (48), one may envision that the totiviruses have arisen from 20S-like RNA replicons with supergroup 1 lineage.

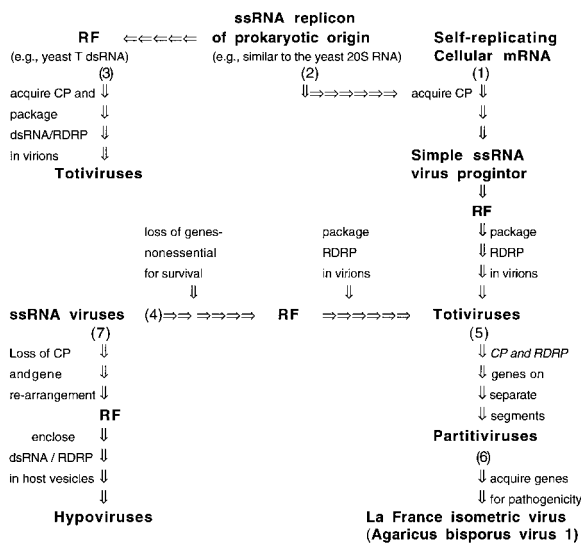


Fig 2. Diagram showing possible pathways in the evolution of mycoviruses.

Totiviruses could also have evolved from progenitors with more complex genomes via reduction/loss of genes not essential for survival (see pathway 4, Fig. 2). Those who favor this pathway present as evidence known examples of closely related dsRNA viruses that differ in the number of dsRNA segments (4). Although very few segmented dsRNA viruses have been characterized at the molecular level, it appears that the lost segments in question represent defective or satellite dsRNAs. Thus, no viral sequences (genes) were lost since the defective dsRNAs represent redundant sequences and satellite dsRNAs contain sequences unrelated to the virus.

The partitiviruses may have evolved from totiviruses by dividing their genomes between two dsRNA segments (pathway 5, Fig. 2). Like the totiviruses, the partitiviruses have simple genomes comprised of two genes (*CP* and *RDRP*) but, unlike the totiviruses, each gene is carried on a separate dsRNA segment (bipartite genomes). Whereas many of the totiviruses express their RDRP as a CP-RDRP fusion protein by fusing the CP and RDRP ORFs, the partitiviruses express their CP and RDRP as separate proteins from their divided genomes. The totiviruses (e.g. Hv190SV) that express their RDRP as a separate nonfused protein, possibly via an internal initiation mechanism (8), may be intermediates in the evolution of partitiviruses from ancestral totiviruses.

La France Isometric Virus (LIV)/Agaricus Bisporus Virus 1 (AbV1)

The LIV (or AbV1), an unclassified virus, is associated with a severe die-back disease (named La France disease) of cultivated mushroom. The virions isolated from diseased fruit bodies and mycelium are isometric 34–36 nm in diameter and co-purify with 9 dsRNAs (referred to as disease-associated dsRNAs). The size of dsRNA segments varies from 3.6 kbp to 0.78 kbp, three of which are believed to be satellites (2,49). Although present evidence strongly suggests that the 9 disease-specific dsRNAs are encapsidated in 34–36 nm isometric virus particles (50), 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 sulphate gradient profile and results of dsRNA and protein analyses of the gradient fractions reveals that at least two broad density components were resolved, a lighter component of empty capsids and a

nucleoprotein component (50). Judging from the size of the particles (34–36 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 sulphate density gradient analysis of the purified virions (50).

The amino acid sequences have now been deduced from the nucleotide sequences of five of these dsRNAs, and only one of the predicted proteins (RDRP) shows homology to proteins in current databases (19,49). The LIV RDRP is most closely related to those of totiviruses and partitiviruses. However, unlike the totiviruses and partitiviruses, LIV is believed to be of recent origin since its codon usage differs from that of its host *Agaricus bisporus* (19). Because of its genome nature (segmented dsRNA genome) and RDRP sequence similarity, it may be possible that LIV arose from a partitivirus by acquiring additional genes (via reassortment/recombination) that are essential for its spread in the mushroom fleshy tissue and for pathogenicity (see pathway 6, Fig. 3).

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