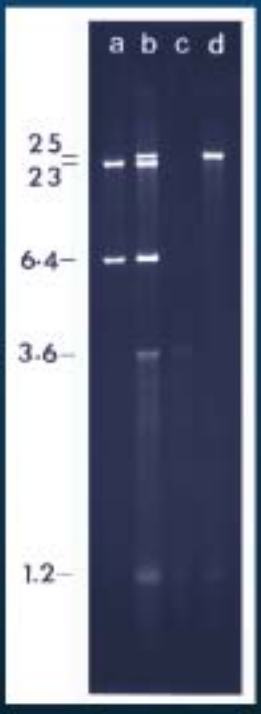
dsRNA GENETIC ELEMENTS

Concepts and Applications in Agriculture, Forestry, and Medicine



Edited by
Stellos M. Tavantzis

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Preface

The purpose of *dsRNA Genetic Elements: Concepts and Applications in Agriculture, Forestry and Medicine* is to compile and unify current knowledge on the biology of dsRNA moieties from yeast, filamentous fungi, nematodes, plants, and animals. The application of recombinant DNA techniques to dsRNA research has led to significant conceptual advances and paved a path for exciting technical developments and potential solutions to problems ranging from plant disease management to treating genetic conditions in humans. In the last few years, dsRNA research has opened new vistas in biology and offered new tools for studying gene expression and gene function.

dsRNA evokes a multitude of responses in a wide range of organisms ranging from protozoa to vertebrates. The dsRNA-activated protein kinase (PKR), expressed in plants and higher vertebrates, elicits the interferon antiviral and antiproliferative response, may activate the cell death program (apoptosis), and may have supporting roles in cytokine signaling and the immune response, cell differentiation, and transcriptional induction of dsRNA-regulated genes (see Chapter 2).

In contrast, recent studies have shown that dsRNA disrupts gene expression in a sequence-specific manner in several species of invertebrates and in young embryos of lower and higher vertebrates. In these experiments, the introduced exogenous dsRNA targets directly or indirectly the homologous cellular mRNA and activates a mechanism leading to its degradation (see Chapter 1). This phenomenon, called *RNA interference (RNAi),* is very similar to cosuppression or post-transcriptional gene silencing (PTGS) observed in plants. Interestingly, recent experimental evidence suggests that RNAi and PTGS may employ similar mechanisms. This dsRNA-related research has led to the development of a new, powerful reverse genetics tool that can be used to analyze gene function in a wide range of organisms (see Chapter 1). Furthermore, this technology can be adopted for many commercial applications in agriculture and medicine. In Chapter 1, you will find an outline of potential benefits as well as risks involved in the use of dsRNA-induced gene silencing in different groups of organisms.

As noted above, the dsRNA-induced PKR is involved in the interferon-induced antiviral response and may play important roles in response to cytokines and cellular stress, transcriptional activation, cell growth, regulation of cell differentiation, and cell death (apoptosis) (see Chapter 2). Thus, it is important that these PKR-induced responses be taken into consideration in sequence-specific gene-silencing studies using RNAi technology (see Chapter 1).

The dsRNA viruses from yeast (*Saccharomyces cerevisiae*) and the corn smut incitant, *Ustilago maydis*, and their killer toxins have been well characterized and can serve as models for the molecular characterization of other dsRNA mycoviruses or dsRNA elements. Moreover, studies on translation of yeast killer dsRNA and

post-translational processing have resulted in the discovery of numerous host (yeast) genes involved in the above as well as other cellular processes (see Chapter 3). In addition, investigations on the mode of action of killer toxins have led to the identification of genes involved in the biogenesis of cell wall. Finally, knowledge gained through studying killer toxins is currently being used to develop new approaches to treating yeast infections or to exclude yeast contaminants in fermentation.

Cloning of the *U. maydis* preprotoxin genes KP4 and KP6 and their expression in tobacco and maize led to tantalizing findings. The preprotoxins were processed to their respective functional forms identical to those of *U. maydis*, strong evidence of the existence of the kex2p protease pathway in plants. Furthermore, transgenic maize plants producing KP4 or KP6 toxins were resistant to *U. maydis* infection (see Chapter 4).

Since the discovery of virus-like particles in diseased mushrooms in 1962, scientists have shown that dsRNAs are ubiquitous in the fungal kingdom. Although fungal dsRNAs often occur in high concentrations, they do not always elicit an overt pathology in their fungal host. Perhaps this is one of the reasons why fungal dsRNA research has remained under-appreciated. Chapters 5 through 10 present cases in which direct or strong indirect experimental evidence has been presented showing that particular dsRNAs are associated with a wide range of biological responses. Chapters 5 through 9 focus on the most recent advances on dsRNA affecting filamentous fungi. The main reason for studying these dsRNAs is to understand how they or their products interfere with normal biological processes in their respective fungal hosts. To date, plant disease management relies heavily on the use of chemicals, leading to increased production costs, utilization of nonrenewable resources, water pollution, non-target effects, and development of tolerance by the target organism. Thus, understanding of genetic factors such as virulence-modulating fungal dsRNAs may lead to the development of biocontrol- or plant-genetic engineering-based strategies of plant disease management that fulfill the need for sustainable and nonpolluting agricultural practices.

Although most of the dsRNA systems described in Chapters 5 through 9 have been studied well, there remain technical problems related to launching the dsRNA from a cDNA clone. The hypovirus system of *Endothia parasitica*, the chestnut blight incitant, is the most highly developed dsRNA system of a plant pathogenic fungus. One of the main reasons for this progress has been the availability of a transfection system that launches the dsRNA from an infectious clone. This gene transfer system allowed the unravelling of the mechanism underlying the hypovirus-induced attenuation of virulence in *E. parasitica*, characterization of virus-encoded determinants responsible for altering the host genotype, and identification of host genes that are up- or down-regulated upon introduction of hypovirus dsRNA (see Chapters 5 and 6). Furthermore, transgenic hypovirulent strains of *E. parasitica* possess chromosomally integrated hypovirus cDNA and the derived, cytoplasmically replicating dsRNA. Thus, in contrast to naturally occurring hypovirulent strains that produce high percentages of dsRNA-free conidia and 100% dsRNA-free ascospores, essentially all of the spores produced by transfected strains have the integrated cDNA and the corresponding hypovirus dsRNA (see Chapter 5). This property is expected

to facilitate dissemination of hypovirulent inoculum and enhance the potential for biological control of the pathogen that devastated both the American and European chestnut in the beginning of the 20th century.

As noted above, lack of an effective gene transfer system has been a major obstacle in using the analytical power of reverse genetics in most of the dsRNA/plant pathogenic fungal systems. Recently, however, a novel gene transfer method involving *Agrobacterium tumefasciens* has been employed to efficiently transform yeast and several filamentous fungi (see Chapter 10). It is hoped that adoption of agrotransformation will allow development of practical gene transfer methods for several other fungi and, in turn, accelerate unveiling of the biological roles of dsRNAs found in these organisms.

Throughout the pages of this book, you will find examples of dsRNAs associated with perturbation of biological processes in their respective fungal hosts. In one of these cases, a hypovirulence-associated dsRNA (M2) converts a "disposable" pathway, the quinate pathway, from inducible to constitutive (see Chapter 8). The quinate pathway shares two intermediate substrates with the shikimate pathway, which leads to the synthesis of the three aromatic amino acids and other important metabolites. Thus, converting the quinate pathway to constitutive down-regulates the shikimate pathway and converts the host (*Rhizoctonia solani*) to hypovirulent. Quinate is one of the most prevalent phenolic compounds in composted leaf or bark litters and is used as a carbon source by soil bacteria and fungi. Interestingly, quinate converts a virulent, M2-lacking isolate of *R. solani* to hypovirulent and concurrently induces transcription and translation of a M2-specific gene. More importantly, the quinate-induced hypovirulence and M2 dsRNA expression persist even in the presence of virulence-enhancing amendments such as intermediates of the shikimate pathway or aromatic amino acids. Is it possible that quinate signals switching from parasitism to saprophytism in *R. solani* and perhaps other soil-borne plant pathogens? If so, would mature, quinate-rich leaf or bark compost become an effective biocontrol medium in the foreseeable future?

Plants are known to harbor bipartite, encapsidated, dsRNA-containing, symptomless cryptic viruses. In addition, several plant species have large-sized, cytoplasmic dsRNAs sequestered within membranous vesicles (see Chapter 11). These dsRNAs cause no visible symptoms and are transmitted only in a vertical manner, and their number of copies per cell is regulated by the nuclear background and developmental stage of the host cell. One of these large dsRNAs causes cytoplasmic male sterility (CMS) in broadbean (*Vicia faba*). Yield quality and quantity of this crop plant are low but could be improved by hybrid breeding, which depends on the availability of male sterile parental lines to prevent self-fertilization. Currently, the dsRNA-associated CMS in broadbean is generally unstable and gives rise to spontaneous revertants. It is tempting to speculate that practical methods will soon be developed for the creation of stable dsRNA-derived CMS in broadbean and other important crops.

Editor

Stellos M. Tavantzis, Ph.D., received an M.S. (1972) at the Agricultural University of Athens. In 1975, he joined the Department of Plant Pathology at the Pennsylvania State University where he received an M.S. (1977) and a Ph.D. (1980) working on the molecular characterization of the mushroom bacilliform virus, the first mycovirus shown to contain a single-stranded RNA genome. In 1980, he became a faculty member in the Department of Botany and Plant Pathology at the University of Maine. In the 1980s, he worked on characterization, replication, and epidemiology of potato viruses M, S, and X; molecular biology of the potato spindle tuber viroid; and characterization of "new" viroids occurring in different plant species. In 1986, he received an Alexander von Humboldt scholarship and spent over a year at the Max Planck Institute (MPI) for Plant Molecular Biology in Cologne as a visiting scientist. The work at MPI included engineering potato with the maize transposable element *En* and studying the behavior of this transposon in a dicotyledonous plant genome. Since the late 1980s, his research interests have focused on understanding the nature and biological roles of dsRNAs in the plant pathogenic basidiomycete *Rhizoctonia solani*. This work led to the discovery that certain dsRNAs are associated with attenuation of virulence in this fungal pathogen which, in turn, enhances the growth of the respective plant host. His current work revolves around the genetic and biochemical dissection of dsRNA-associated cytoplasmic hypovirulence in *R. solani* and its exploitation toward the development of sustainable biocontrol strategies against this pathogen.

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1 RNAi and Cosuppression: Double-Stranded RNA as an Agent of Sequence-Specific Genetic Silencing in Animals and Plants

Jeffrey A. Norman and Mary K. Montgomery

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1.1 INTRODUCTION

Double-stranded ribonucleic acid (dsRNA) elicits manifold responses in a phylogenetically diverse group of organisms. Recently, several species of invertebrates and a species of protozoan (see Table 1.1) have been shown to respond to the presence of dsRNA in a sequence-specific manner,[1] disrupting gene expression in a transient fashion (for most recent review, see Reference 2). In these cases, the introduced dsRNA is able to target endogenous messenger RNA (mRNA) for degradation.[3–5] This phenomenon has been called RNA interference or RNAi.

Mammals and perhaps all vertebrates appear to respond to dsRNA in at least two different ways. The presence of dsRNA can activate a global panic response that results in the degradation of all cellular mRNAs.[6] However, recent studies have demonstrated that very young embryos of both higher and lower vertebrates can respond to dsRNA by silencing endogenous genes in a sequence-dependent manner.[7–9]

A remarkably similar genetic silencing phenomenon called cosuppression or, more specifically, post-transcriptional gene silencing (PTGS), has been described in plants. In PTGS, an introduced exogenous transgene can trigger the post-transcriptional degradation of homologous cellular RNAs.[10] Although the chemical nature of the interfering agent has not been identified in all reported cases of PTGS, dsRNA has been shown to specifically interfere and cause a PTGS response in at least two species. Whether RNAi and PTGS represent two branches of an evolutionarily conserved pathway awaits additional study along with the identification of the molecular components that mediate the cellular responses. We describe the experimental approaches that are being taken to identify gene silencing pathway components and recent tantalizing results that suggest RNAi and PTGS may operate by very similar mechanisms.

The reverse genetics proffered by RNAi and PTGS now allows researchers in many different biological subdisciplines a relatively expedient means by which to analyze and manipulate the function of a wide assortment of genes. Herein we describe the initial discovery that dsRNA is an agent of sequence-specific genetic

TABLE 1.1
Summary of Results from RNAi Studies on Organisms Other Than *C. elegans*

Higher Taxon	Organism	Phylum (or Division)	Stage Tested[a]	Genes Targeted	dsRNA size (bp)	% Specific Phenotype[b]	Ref.
Protozoa	*Trypanosoma brucei* (trypanosome)	Protozoa	procyclic cells	α-tubulin	114	~90%	3
				β-tubulin	59	NR	
				actin	450	NR	
				PFR	450	NR	
Invertebrates	*Drosophila melanogaster* (fruit fly)	Anthropoda	syncytial blastoderm embryos	*fushi tarazu*	1936	72%	17
				even-skipped	1353	16%	
				tramtrack	857	90%	
			syncytial blastoderm embryos	*twist*	1470	86%	28
				engrailed	1200	79%	
				D-mef2	1760	72%	
				daughterless	3400	75%	
				S59 (NK1)	2900	75%	
					1529	76%	
				nautilus	500	<3%	
				white	NR	80%	
				nau-β-gal			
	Tribolium sp. (flour beetle)	Anthropoda	syncytial blastoderm embryos	*deformed*	1000	69%	31
	S. mediterranea (planaria)	Platyhelminthes	regenerating adults	myosin	1186	100%	32
				opsin	447	100%	
	Hydra	Cnidaria	whole polyps	*Ks1*	~650	85%	33

TABLE 1.1 (CONTINUED)
Summary of Results from RNAi Studies on Organisms Other Than *C. elegans*

Higher Taxon	Organism	Phylum (or Division)	Stage Tested[a]	Genes Targeted	dsRNA size (bp)	%Specific Phenotype[b]	Ref.
Vertebrates[c]	*Danio rerio* (zebrafish)	Chordata	1–2 cell embryos	*no tail*	685	27%[d]	7
				Zf-Pax2.1	354	31%[d]	
				floating head	721	36%[d]	
			1-cell embryos	*gfp*	187	97%	8
				Zf-T	321	71%	
				Pax6.1	298	28%	
	Mus musculus (mouse)	Chordata	oocytes and embryos	*Mmgfp*	714	93–100%	9
				c-mos	550	63%[c]	
				E-cadherin	580	100%	
Plants	*N. tabaccum* (tobacco)	Magnoliophyta	mature plants	potato virus Y protease	~850	44–54%	59
	O. sativa (rice)	Magnoliophyta	mature plants	β-glucoronidase reporter	NR	90%	

[a]dsRNA introduced by injection except in *T. brucei* and *Hydra* in which electroporation was used; and in plants in which dsRNA was produced *in vivo* from viral vectors expressing sense and antisense transcripts or hairpin structures.
[b]If loss-of-function mutant phenotype unknown, results reported are for % expected phenotype.
[c]Results reported are for most successful dsRNA concentrations used.
[d]A roughly equal % of nonspecific lethality also reported.
[e]Only 60-75% of *c-mos* genetic null mutants display a phenotype.
NR, not reported.

silencing in the nematode *C. elegans*, followed by a discussion of additional studies that provide testable models to explain the mechanism underlying silencing. We summarize the expanding literature demonstrating that dsRNA-mediated silencing is a widespread phenomenon within the animal and plant kingdoms. Finally, we address potential future applications as well as potential problems of the use of dsRNA technology in the realms of agriculture and human medicine.

1.2 DOUBLE-STRANDED RNA-MEDIATED GENETIC INTERFERENCE

1.2.1 RNAi IN THE NEMATODE *C. ELEGANS*

The nematode *C. elegans* has served as a model for the study of animal development over the past couple decades. Its life cycle of 3.5 days and development into self-fertilizing hermaphrodites or rare males enhance the researcher's ability to isolate and characterize genetic mutations. Its small size, transparency, and invariant lineage facilitated mapping of the entire cell lineage. Molecular techniques such as transgenic technology have helped to determine the function of hundreds of genes. *C. elegans* is also the first multicellular organism whose complete genome has essentially been sequenced. Although *C. elegans* must be one of the most widely studied organisms of all times, researchers continue to advance new methods by which to identify and analyze genes controlling its development, physiology, and behavior. Attempts to use antisense RNA as a reverse genetic technique led somewhat serendipitously to the discovery of dsRNA as a trigger of genetic silencing, opening up new avenues of research.

1.2.1.1 dsRNA as a Trigger of Sequence-Dependent Gene Silencing

The introduction of exogenous antisense RNA has been reported to interfere with and disrupt the activity of complementary loci in the endogenous genome of interest.[11] Initially, it was thought that this silencing mechanism depended on simple hybridization between the introduced single-stranded RNA (ssRNA) and the complementary endogenous sense or messenger RNA (mRNA). Theoretically, this pairing would blockade the mRNA from translational machinery and effectively silence the endogenous message.[12]

The use of antisense RNA to interfere with a gene's activity in *C. elegans* was first attempted by Su Guo and Ken Kemphues in 1995 to study the maternal effect gene *par-1*. Wildtype adult hermaphrodites injected with antisense RNA complementary to *par-1* produced embryos with a loss-of-function phenotype; however, control *par-1* sense RNA also produced the same loss-of-function phenotype.[13] These results were difficult to reconcile with the accepted model of how antisense RNAs were thought to target and degrade endogenous mRNAs. Accordingly, the *C. elegans* research community adopted the term "RNA interference" or RNAi[14] to refer to the method of injecting RNAs to interfere with a specific gene's activity.

In an attempt to gauge whether it was mere antisense-sense annealing that produced the observed phenotypes seen with RNAi, Fire et al.[1] investigated the effectiveness of gel-purified sense, antisense, and sense + antisense (ds) RNAs as RNAi triggers in *C. elegans.* RNAs corresponding to different maternal and zygotic genes with known mutant phenotypes were synthesized *in vitro* and introduced to the worms via microinjection into the syncytial gonad or other tissues. Fire and colleagues found that gel-purified single-stranded preparations did not produce robust RNAi effects, but that dsRNA produced more potent interference than either ssRNA alone. Fire et al.[1] suggested that phenotypes obtained with "sense" and "antisense" RNAs in previous reports were really due to the presence of small amounts of dsRNA. This dsRNA could result from ssRNA *in vitro* preps contaminated with RNA transcribed from the opposite strand due to low-level nonspecific binding of RNA polymerases. The term RNAi now commonly refers to the introduction of homologous dsRNA to block activity of a specific gene, resulting in null or hypomorphic phenotypes.

1.2.1.2 Hallmarks of RNAi in *C. elegans*

In addition to the initial identification of the molecular nature of the RNAi "trigger," several additional findings collectively characterize the RNAi effect and have begun to shed light on the mechanism that mediates the genetic interference. Most of the RNAi hallmarks summarized in this section appear to also apply to the other organisms for which dsRNA has been shown to elicit a sequence-dependent interference response. Double-stranded RNA, rather than single-stranded antisense RNA, is the interfering agent.[1] It is highly specific, targeting and disrupting gene activity in a sequence-specific manner (see section 1.2.3.4 on cross-interference for more details).[1] Gene activity is disrupted due to degradation of targeted mRNAs.[1,4] It is remarkably potent as only a few dsRNA molecules per cell are required for effective interference.[1] Notably, the interfering activity, and presumably the dsRNA, can cause interference in cells and tissues far removed from the site of introduction.[1,15,16] This last observation, although intriguing, does not represent a universal characteristic of RNAi, as this "spreading" effect is not observed in species of insects for which other aspects of RNAi appear to be conserved.[17] Another early hallmark of RNAi was that only dsRNAs complementary to sequences found in the mature message were effective at producing loss-of-function phenocopies, as no effect was seen when targeting promoter or intron sequences.[1,4] Of these observations, the substoichiometric potency of the introduced dsRNA proved particularly puzzling. This led to several questions, some of which could be addressed experimentally: What is the mechanism of the substoichiometric potency of the introduced dsRNA? Does dsRNA exert its effects post-trancriptionally or at the level of the gene? If post-transcriptionally, does the dsRNA work catalytically or is it amplified in the cell by a copy mechanism?

1.2.1.3 RNAi Operates Post-Transcriptionally

The substoichiometric potency of dsRNA could be explained if RNAi worked at the level of the endogenous targeted gene, either by causing mutations or by repressing

transcription. The former of these proposed mechanisms was dismissed early, as RNAi is a transient effect with the progeny of injected hermaphrodites eventually reverting back to wildtype, usually within the first or second generations.[1] Also, it was noted that RNAi was effective only if exonic sequences were targeted; dsRNA against promoter and intron sequences was ineffective at producing phenocopies. This result alone was not enough to disprove the mutational hypothesis, however, as a dsRNA adenosine deaminase feedback mechanism had been proposed that would have resulted in mutations in mRNA-encoding portions of the gene only.[18] Direct evidence against the mutational mechanism came from sequencing of a targeted gene *(unc-22)* from dsRNA-treated worms exhibiting an *unc-22* phenocopy; *unc-22* sequences from these affected worms were identical to wildtype.[4] Thus, RNAi does not appear to operate by directing mutations at the targeted locus.

Some cases of cosuppression in plants have been shown to result from methylation of the endogenous gene.[19] Methylation does not occur in *C. elegans,*[20] but some type of reversible covalent modification of the targeted locus could theoretically explain how RNAi exerts its effects. Evidence arguing against this latter mechanism came from RNAi studies of the *lin-15* operon by Montgomery et al.[4] Approximately 25% of genes in the *C. elegans* genome are organized into operons,[21] which has allowed for a relatively compact and efficient genome.[22] An operon is transcribed as a polycistronic message that then undergoes *cis*- and *trans*-splicing and additional RNA processing (see Figure 1.1). The *lin-15* operon comprises two genes: *lin-15b* and *lin-15a*. Genetic null mutations in either gene do not result in a phenotype; however, worms carrying a null mutation in both genes exhibit a multivulva phenotype.[23] Montgomery et al.[4] were able to obtain multivulva phenocopies if both *lin-15b* and *lin-15a* were targeted simultaneously but found that targeting the upstream or downstream genes individually produced few if any defective worms. This result indicated that targeting of the upstream gene in an operon had little to no effect on expression of downstream genes and vice versa. In the same study, nuclear transcripts of targeted transgenes could still be detected by *in situ* hybridization in dsRNA-treated embryos, although the levels of expression were reduced compared to untreated embryos. Cytoplasmic messages of the targeted transgenes were abolished. Together, these results argued that RNAi does not affect initiation and elongation of transcription but targets mRNAs directly such that they are degraded before they can be translated.

The *lin-15* operon RNAi results left open the question as to whether nuclear pre-mRNAs could be directly targeted by RNAi. The results indicated that at least some polycistronic messages were processed quickly enough such that, e.g., upstream processed mRNAs could be targeted for destruction while the downstream processed mRNAs were unaffected. Similar results have been reported for other operon-like clusters in that targeting of one gene product in the cluster does not seem to affect the activity of other genes within the cluster[24,25] (T. Blumenthal, personal communication). One notable exception was reported by Bosher et al.,[25] who analyzed the *lir-1/lin-26* operon. Their results demonstrate that a polycistronic message can be targeted and presumably degraded before processing and export to the cytoplasm. Specifically, whereas a *lir-1* genetic null mutant demonstrates no obvious phenotype, dsRNA targeting *lir-1* induced an embryonic lethal phenotype

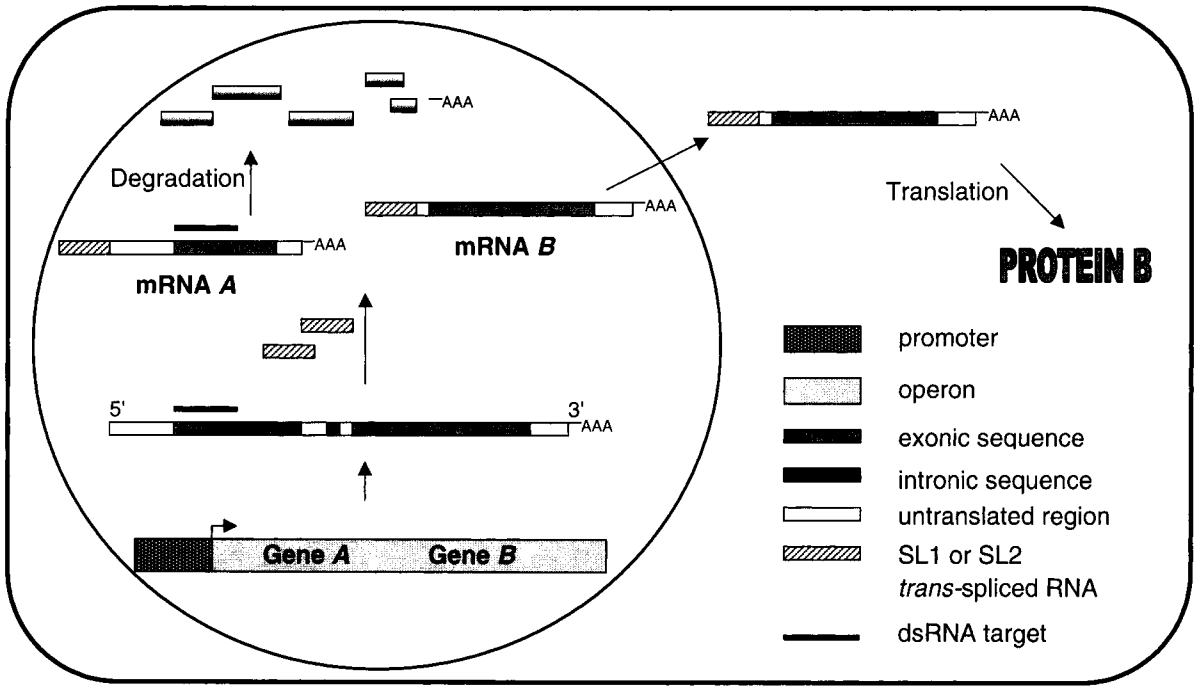

FIGURE 1.1 Double-stranded RNA can differentially target mRNAs within a polycistronic message. Many genes within the *C. elegans* genome are organized into operons that are transcribed as polycistronic messages. These pre-mRNAs undergo splicing and processing and are exported from the nucleus as mature mRNAs encoding single proteins. By introducing dsRNA that is complementary to only one member of an operon, it was possible to demonstrate that RNAi can target and degrade individual mature mRNAs without significantly affecting the activity of other genes within the operon.[4] This indicates RNAi operates post-transcriptionally. In one reported case, however, it has been demonstrated that targeting of an upstream component of an operon can lead to interference of downstream genes, presumably by causing degradation of the pre-mRNA prior to splicing (see text).

that is usually associated with *lin-26* loss-of-function mutants. Furthermore, the *lir-1* dsRNA-treated embryos showed a severe loss of *lin-26* expression as revealed by LIN-26 antibody staining. Embryos treated with dsRNA targeting introns of either *lir-1* or *lin-26* also exhibited a *lin-26* phenotype. Bosher et al.[25] postulated that *lir-1/lin-26* may be representative of unusually stable or slow-to-process pre-mRNAs and that these polycistronic messages could be particularly susceptible to RNAi targeting prior to processing. As it is unknown yet what percentage of operons fall within this class, phenotypes based on dsRNA targeting of individual genes thought to be organized within a single operon should be interpreted cautiously. Examining expression levels of other genes within an operon that are not being targeted should be considered, although conceivably loss of expression of one member of an operon may negatively regulate expression of the operon overall.

1.2.1.4 Resistant Genes and Tissues

Certain genes and some cell types in *C. elegans* have proven relatively resistant to RNAi when dsRNA is introduced by microinjection into the adult animal. In general, developmentally earlier acting genes such as maternal and early zygotic genes are more easily targeted than later expressing larval and adult genes in the F1 progeny of injected animals. Variable or negative results obtained for genes that are expressed during larval and adult stages may be due to dilution of the dsRNA during multiple rounds of cell division and/or degradation of the dsRNA over time. Some genes within this category have been successfully targeted by injecting dsRNAs directly into young larvae rather than one generation later (i.e., the adult hermaphrodite) (B. Harfe and A. Fire, personal communication).

Although the activities of dozens of maternally acting genes have been successfully disrupted using RNAi techniques, a small number of maternally expressed genes appear unaffected by dsRNA treatment. Typically, the maternal pool of a targeted message will be depleted within six hours of injection of dsRNA (MKM, unpublished data). In most cases of RNAi-resistant genes, it is not known if the targeted mRNAs have been degraded, as the observations are based on lack of the expected loss-of-function phenocopy. Conceivably, the lack of the expected phenotype might be due to perdurance of protein translated from maternal message prior to treatment with dsRNA. Examining dsRNA-treated animals with *in situ* hybridization and immunocytochemistry (if an antibody is available) for loss of gene expression would help determine if protein perdurance can explain such negative results.

Alternatively, some RNAs may be intrinsically resistant to the effects of dsRNA. Fire[2] has suggested that, because RNAi is thought to function by decreasing the half-life of targeted mRNAs, genes encoding mRNAs with very fast turnover rates might be able to escape targeting or may recover more quickly following targeting. Moreover, compensatory or feedback mechanisms may lead to upregulation (i.e., increased rates of transcription) for some targeted genes that could at least partially restore expression levels.[2]

Neurons in *C. elegans*, or a subset of them, appear to be particularly "RNAi-resistant." As mentioned earlier, dsRNA injected into one tissue of an adult animal

(e.g., the gut) can interfere with gene expression in other tissues within the same animal (e.g., muscle) as well as in cells of its progeny.[1] However, it has been exceedingly difficult to interfere with neuronal expression of some genes in both the injected animal and its progeny (J. Fleenor and A. Fire, personal communication) For example, loss of functional freen fluorescent protein (GFP) is observed within hours in most cells when *gfp* transgenic animals are injected with *gfp* dsRNA; however, GFP expression in many neurons persists indefinitely. This observation could be due to preferential perdurance of the GFP protein in neurons. Alternatively, some neurons may lack an ability to take up exogenous dsRNA (perhaps not surprising given their unique need to remain relatively insulated) or may lack the cellular machinery that mediates genetic interference. The latter explanation seems unlikely, as some previously "RNAi-resistant" neuronal genes have been successfully targeted using methods that produce dsRNA *in vivo* as opposed to being exogenously supplied (see the next section).

1.2.1.5 Methods for Delivery of dsRNA

Microinjection of dsRNA directly into the syncytial gonad or into the intestine of adult animals has been the most common method by which to introduce dsRNA into *C. elegans* and other nematode species. Although effective interference is typically obtained by this method, injections require a certain level of technical expertise, and the interference effects are short-lived. These relative drawbacks coupled with the observation that dsRNA can "spread" beyond the site of initial introduction led to attempts by some researchers to develop other methods for delivery of dsRNA into the animal's tissues.

Tabara, et al.[15] reported that RNAi phenotypes could be obtained by simply soaking animals for several hours in dsRNA. Presumably, the dsRNA enters cells initially by ingestion and absorption through the gut, although dsRNA might also enter through hypodermis exposed during molts. Timmons and Fire[16] were able to produce RNAi effects by culturing worms on transgenic *E. coli* expressing dsRNA targeting *C. elegans* endogenous genes. As with the soaking method, dsRNA is seemingly absorbed through the gut, in this case after the worms consume and digest the dsRNA-producing bacteria. Ingestion of dsRNA, as with the other introduction methods, does not result in any stable change in the host's genome, and the interference effect persists for only a relatively short time. (For a prolonged effect that lasts over multiple generations, worms can be transferred daily to plates containing freshly induced bacteria expressing the transgene.)

Methods for making dsRNA *in vivo* from transgenic lines containing sense and antisense constructs were initially somewhat discouraging;[16] such lines generally resulted in weaker RNAi phenotypes than seen with supplying preformed dsRNA exogenously. Possibly, RNA-binding proteins associate with nascent sense and antisense transcripts inhibiting their ability to anneal to one another when newly synthesized in the cell. An approach that seems to have averted this problem has been to design hairpin (or "snap-back") constructs that are expressed under a heat shock promoter[26] (See Figure 1.2); worms carrying these inverted repeat constructs demonstrate robust RNAi effects following heat shock treatment, even in neuronal cell

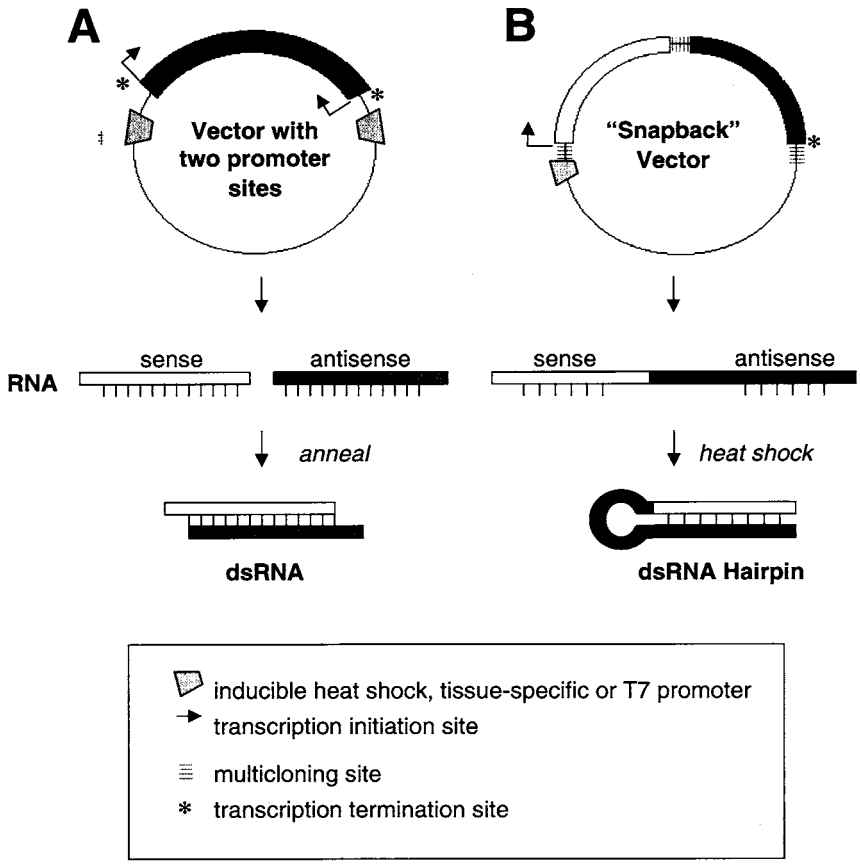

FIGURE 1.2 Transgenic vectors for producing dsRNA *in vivo*. Plasmid vectors have been designed to synthesize dsRNAs two ways. (A) The vector can contain promoter sequences at each end of a cDNA, such that both strands encoding the cDNA are transcribed. This approach relies on sense and antisense transcripts finding and annealing to each other in the molecularly dense environment of the nucleus. (B) An alternative approach is to design "snapback" constructs that generate dsRNA hairpin structures from a single promoter. This method results in more effective dsRNA formation. Both approaches can utilize a number of different promoters to control when and where expression occurs.

types. Furthermore, the use of an inducible promoter allows long-term maintenance of strains carrying such constructs, the generation of large numbers of "mutant" animals, and the ability to perform stage-specific RNAi. It should also be noted that transgenic bacteria expressing hairpin constructs rather than separate sense and antisense constructs also work more efficiently in "dsRNA feeding" protocols (L. Timmons, personal communication). The necessity for placing dsRNA-expressing constructs under tight regulatory control, whether through inducible or tissue-specific promoters, becomes obvious; if expression of such constructs cannot be tightly controlled, one might expect such transgenes to silence themselves.

1.2.1.6 RNAi-Based Genome-Wide Screens

One of the more innovative uses of RNAi technology has been its application to genome-wide searches for genes whose functions when eliminated by RNAi result in very specific phenotypes. This is truly the reverse genetic equivalent of the classic mutagenesis screen. Working from a cDNA library, dsRNAs are synthesized and injected as pools corresponding to a dozen or fewer genes. If an injected pool of dsRNAs produces the desired phenotype, dsRNAs corresponding to individual genes are injected separately until the specific sequence that is generating the phenotype is isolated and identified. Such an approach is being utilized by several laboratories to identify genes involved in embryonic development and regulation of the cell cycle.[27] With the sequence of the 97 Mbp *C. elegans* genome essentially complete, innovative approaches such as RNAi-based screens will be necessary for discerning the function of each of the approximately 19000 genes that make up the worm's genetic repertoire. The genome projects of other multicellular organisms are progressing, and RNAi-based screens may prove applicable here as well, particularly if the screens focus on identifying genes that function during early development.

1.2.2 RNAi in Other Invertebrates

Although the use of dsRNA to selectively knock down gene function was first utilized in the nematode *C. elegans,* it was not long until researchers tested the utility of dsRNA knockdown in other species. RNAi has now been shown to work in a phylogenetically diverse set of organisms, including insects, flatworms, cnidarians, and trypanosomes. Each of these early studies started with attempts to phenocopy known loss-of-function genetic mutations before applying the technique to the study of genes without genetically defined mutations.

1.2.2.1 *Drosophila*

Kennerdell and Carthew[17] used dsRNA corresponding to genes in the wingless pathway (*frizzled* and *frizzled 2*) to show that RNAi can elicit similar null or hypomorphic phenotypes. At the same time, Misquitta and Paterson[28] used RNAi to effectively knock down endogenous *nautilus* mRNA to determine its role in embryonic muscle formation. RNAi proved an invaluable technique in this study, as no *nautilus* loss-of-function mutants had been previously isolated. RNAi experiments targeting *Drosophila* genes with known mutant phenotypes produced results indistinguishable from the corresponding mutant alleles.[28]

Work in *Drosophila* supports a post-trancriptional mechanism for dsRNA-triggered gene silencing. For a few genes, dsRNAs targeting maternally provided mRNAs have been injected into very young embryos, with the resulting RNAi-induced phenotypes resembling maternal loss-of-function mutations. These results provide strong evidence that cytoplasmic RNAs can be directly targeted. Furthermore, because an entire pool of maternal mRNA can be wiped out by exposure to relatively few molecules of dsRNA, a catalytic or amplification model for RNAi is also supported. As with *C. elegans*, the RNAi effect in *Drosophila* is transient, with genes expressed at late stages of development more difficult to target than maternal

or early embryonic ones. Even so, a small percentage of embryos injected with dsRNA containing sequence corresponding to the *white eye* locus developed two weeks later into adult white-eyed flies, demonstrating that dsRNA and/or its effects can remain potent and long-lived.[28]

1.2.2.2 "Non-Model" Organisms

The handful of species that have served as models for animal development share some common characteristics, including short life cycles and the ability for researchers to perform forward genetic screens. Cloning and identification of mutated genes is relatively straightforward, and a vast number of genes from these organisms have been sequenced and deposited in the public databases. Indeed, now that both the *C. elegans* and *Drosophila* genomes have been almost completely sequenced,[29,30] both forward and reverse genetic approaches will be needed to determine the function of all these genes. RNAi will no doubt be one of several approaches used to accomplish this task.

But perhaps one of the most valuable uses of RNAi has been to open the door to reverse genetics for species genetically less tractable than *C. elegans* and *Drosophila*. Comparative studies of gene function between related species can tell us much about the evolution of developmental mechanisms, and RNAi may prove a valuable tool in this endeavor. We and several others within the *C. elegans* research community have used RNAi successfully to disrupt gene function in closely related nematodes such as *C. briggsae* and *C. remanei*. RNAi has been used to eliminate the maternal mRNA pool of *Drosophila* homologs in a few other insect species, including the flour beetle *Tribolium*[31] and the milkweed bug *Oncopeltus faciatus (*C. Hughes and T. Kaufman, unpublished data). Studies with both *Drosophila* and *Oncopeltus* have suggested that the technique works most effectively if early syncytial blastoderm stage embryos are injected,[17] (T. Kaufman, personal communication). Once embryos become cellularized, the dsRNA does not seem to spread throughout the embryo, and a mosaic pattern of affected cells is the most that can be achieved.

RNAi has also proven relatively successful in two species that occupy key phylogenetic positions that branch deep within the metazoa but nevertheless have not been genetically well characterized: the planarian flatworm[32] and the cnidarian *Hydra.*[33] Both species have been studied as models for regeneration, but lack the kind of genetics that facilitates forward genetic screens. Work on *Hydra* has also included studies of homologs of genes that function in axial patterning in higher organisms. The ability to disrupt the function of these homologs in the evolutionarily ancient cnidarians is a boon to researchers attempting to assess to what extent the functions of developmentally important genes have been conserved during the metazoan radiation.

1.2.3 RNAi in Vertebrates

1.2.3.1 dsRNA and PKR Activation

Probably the group of organisms that has generated the most interest and excitement concerning the potential utility of dsRNA-mediated genetic interference has been

the vertebrates. However, it has been proposed that using dsRNA to elicit a sequence-specific genetic silencing response may pose a problem, at least in the higher vertebrates.[2] Mammalian cells contain a cytoplasmic Ser/Thr protein kinase that becomes activated upon binding of viral or exogenous dsRNA. This dsRNA-dependant protein kinase (PKR, formerly kinase p68) regulates protein synthesis by phosphorylating the α subunit of eukaryotic protein synthesis initiation factor-2 (eIF2-α), which can lead to a general shutdown of the translational machinery. Paradoxically, PKR can also activate transcription factors such as NFκB,[34] which ultimately leads to interferon (INF) production and cellular apoptosis.[35] These cellular responses to the presence of dsRNA represent a global panic response that is thought to suppress viral replication by warning neighboring cells via an INF pathway prior to cellular suicide. Only one or a few molecules of dsRNA are needed to reach the threshold of INF induction,[36] which is reminiscent of the substoichiometric properties of RNAi observed in the invertebrates.[2,17] For an overview of dsRNA activation of PKR and the corresponding endogenous antiviral response, see Figure 1.3. Interestingly, it has been demonstrated that plants also have a dsRNA-dependent protein kinase (pPKR) that plays a similar antiviral role as its putative mammalian homolog.[37] It is not known yet whether more primitive vertebrates such as fish, amphibians, and reptiles, also contain a functional PKR ortholog, although a PKR-related gene has been identified in the puffer fish *Fugu rubripes.*[38]

1.2.3.2 Sequence-Specific Silencing in Zebrafish

Two reports have been published on the use of injected dsRNA to specifically interfere with the function of targeted genes in the zebrafish *Danio rerio*. Wargelius et al.[7] microinjected dsRNA corresponding to three genetically characterized genes with known loss-of-function mutant phenotypes into zebrafish embryos. Their results revealed that dsRNA treatment can induce gene-specific defects, although many embryos also displayed nonspecific lethality. The nonspecific lethality appears to be due to degradation of many, if not most, mRNAs. After injection, endogenous mRNA levels were reduced in experimental embryos as revealed by *in situ* hybridization; dsRNA-induced specific phenotypes were similar to those of genetically characterized mutants. However, these researchers also reported that the specificity and severity of the dsRNA-induced phenotypes were dependent on the amount of dsRNA injected. A range of phenotypes representing total and partial loss of function were observed along with non-anticipated phenotypic abnormalities. These observations suggested that the use of dsRNA to knock down gene function in a predictable and specific manner presents more technical difficulties in vertebrates than has been seen for many of the invertebrate species.

Li et al.[8] were also able to target a number of genes in zebrafish using dsRNA injections. Targeted genes included *gfp* expressed from a plasmid vector injected at the same time as the dsRNA; uninjected controls exhibited robust *gfp* expression, whereas those co-injected with *gfp* dsRNA exhibited weak or no fluorescence. Endogenous genes tested included a member of the T-box family of transcription factors (Zf-T) and a homolog of the Pax-*6/eyeless* genes. Targeting of a unique portion of Zf-T resulted in a *no tail (ntl)* phenotype, and corresponding mRNA levels

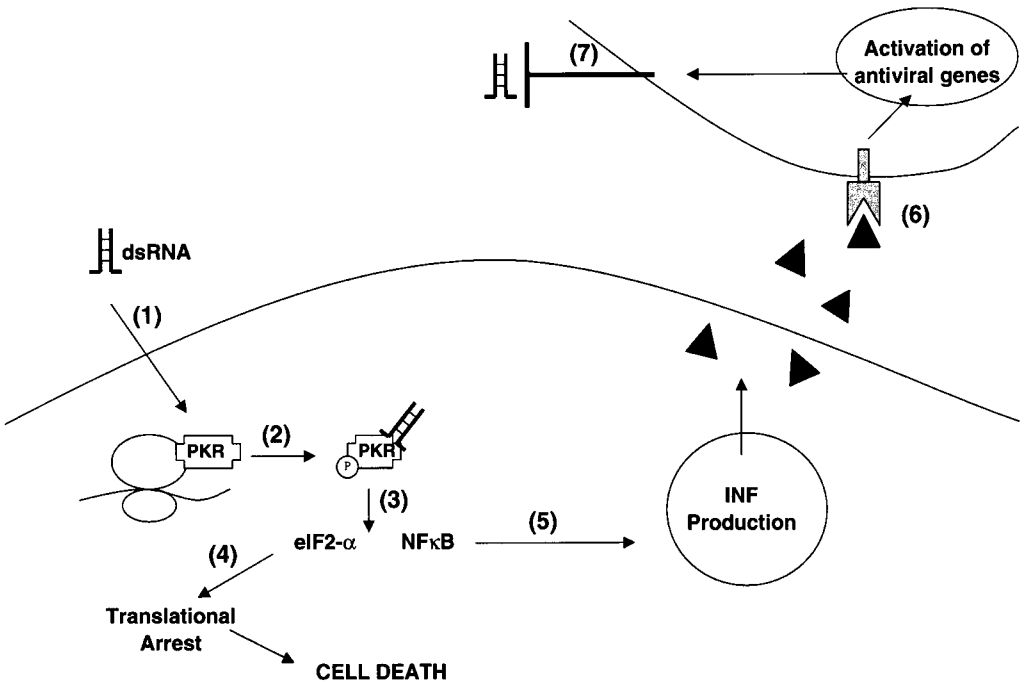

FIGURE 1.3 Cellular response to the activation of double-stranded RNA-dependent protein kinase (PKR). (1) dsRNA is introduced to the mammalian cell via viral invasion, expression from a vector, or directly via microinjection. (2) The dsRNA binds to PKR, causing PKR to dissociate from a ribosome and to undergo autophosphorylation, which activates it. (3) PKR is then able to phosphorylate its target eIF2-α, which leads to (4) translational arrest and ultimately cell death. PKR can also (3) activate transcription factors such as NFκB, which (5) induces the transcription of INF mRNA in the nucleus. Newly synthesized INF leaves the cell and (6) binds to extracellular surface receptors on neighboring cells. This binding activates a receptor-mediated signaling pathway that upregulates transcription of antiviral genes. Such INF-primed cells are now resistant to dsRNA expression.

were drastically decreased as revealed by *in situ* hybridization. The Zf-T dsRNA-treated embryos also resulted in altered expression patterns of *sonic hedgehog* and *floating head*; the patterns of misexpression of these two genes are consistent with those observed in Zf-T genetic mutants. The researchers argued that this result was further evidence that the T-box gene had been effectively and specifically targeted. Although no genetic mutation in the Pax-*6/eyeless* homolog was available, dsRNA-targeting of this gene in the zebrafish resulted in defects during eye development, which is consistent with the expected loss-of-function phenotype.

Similar to the findings of Wargelius et al., Li et al., also found that dsRNA injections resulted in variable phenotypes that ranged from partial to total loss of function. However, in contrast, Li et al. did not observe the nonspecific lethality reported by Wargelius et al. and several other zebrafish labs. Significantly, the dsRNA synthesized by the modified protocol devised by Li avoids triggering nonspecific lethality, even at relatively high concentrations of injected dsRNA. Embryos injected with control dsRNAs developed normally, and the expression patterns and levels of untargeted mRNAs resembled those of wildtype embryos. The control results suggest that the dsRNA is not catalyzing an antiviral response in the zebrafish embryos, which may not be surprising, as the experiments were carried out within a time period prior to the reported establishment of the zebrafish immune system. Furthermore, Li et al. stress that the levels of dsRNA used in their experiments were less than the concentration needed to elicit an INF-mediated cell response (see Reference 39). However, Li et al. did not address how long the exogenous dsRNA can perdure in the embryos. Also, one study has indicated that possibly as few as one molecule of dsRNA per cell is needed to induce an INF response.[36] If the injected dsRNA is stable and able to perdure in the zebrafish embryo, then theoretically it could induce INF production and be the culprit of the more severe and nonspecific phenotypes seen in older dsRNA-treated embryos.

1.2.3.3 Sequence-Specific Silencing in Mammals

To the scientific community at large, the greatest benefit that RNAi could have is its successful use in mammalian systems. Mice have been used as a model to understand the molecular, genetic, and physiological bases of many human biological processes. In numerous cases, they have been used to develop new treatments for diseases that afflict humans. One of the tools that have made mice good models for the study of conserved genes is the ability to perform homologous recombination in embryonic stem (ES) cells to generate true gene knockouts. These recombinant ES cells are then placed into young embryos that are implanted in the mouse uterus. If the recombinant ES cells are incorporated into the germline, a homozygous strain of mice carrying the knockout can be generated within a few generations, and the phenotype analyzed. Although this protocol has been used with great success, it is costly and typically requires up to a year to generate the homozygous strain carrying the knockout. If RNAi could be applied to mouse reverse genetics, a great savings in terms of time and cost could be gained.

Wianny and Zernicka-Goetz[9] were able to demonstrate gene specific interference by dsRNA in mouse oocytes and embryos. These researchers targeted maternal gene

function with dsRNAs corresponding to gfp, c-mos, and E-cadherin, which were produced *in vitro* and injected into mouse oocytes or one-cell embryos. Wianny and Zernicka-Goetz were able to silence both the gfp transgene and the two maternal genes without producing any significant cross-interference (Table 1.1). This success can be most likely attributed to targeting genes that are expressed very early in development. Injection of the dsRNA did not appear to activate the PKR pathway, because the experiments were performed in embryos prior to the development of a fully functional immune system.

Although these findings in zebrafish and mice are promising, RNAi has yet to be shown effective in juvenile or adult vertebrates. As mentioned previously, most mammalian cells express PKR which, upon activation by dsRNA, elicits a global panic response in its host cell and eventually leads to cell death. Prior to cell death is the release of interferons (INFs), which are able to warn neighboring cells and prime them to combat the foreign dsRNA. If RNAi is to work in adult vertebrates, it most likely will have to be delivered in such a way to limit the antiviral response of the host cell or in a suitable vector to circumvent the PKR/INF response altogether.[2] Another alternative is to perform RNAi experiments in PKR-mutant mice and cell lines, both of which are currently available.

1.2.4 Length Requirements and Cross-Interference

Some studies have attempted to elucidate the minimum length requirements for effective RNAi. Tabara et al.[15] have reported that dsRNAs corresponding from between 200 to 1000 bp appear to be effective to induce interference for a number of genes. Furthermore, Bosher and Labouesse report that using sequences greater than 500 bp is typically adequate.[27] Our strategy to determine the minimum length and sequence similarity required for effective RNAi has been to clone *C. elegans* genes with known loss-of-function phenotypes from a closely related species, *C. briggsae*. Clones with known length and sequence identity from both nematodes were then introduced via microinjection into *C. elegans* and *C. briggsae* adult hermaphrodites. Our observations indicate that dsRNAs as short as 60 bp can be used to substantially knock down activity of some maternal genes, but that longer sequences work more effectively (unpublished data).

Given the prevalence of gene duplication during the evolution of higher organisms, cross-interference with genes other than those the researcher desires to target is a potential problem. Cross-interference could be beneficial in some studies if the desired effect is to target an entire gene family (e.g., myosin). Otherwise careful attention must be paid in choosing unique sequences to enhance the chances of specifically targeting a single gene, and not all others that contain similar sequences. RNAi experiments targeting the gene *deformed (Dfd)* in *Drosophila* using the *Dfd* sequence from *Tribolium* did not result in interference, even though there is 84% sequence identity between the two homologs.[31] Our results using *C. elegans* and *C. briggsae* indicate that dsRNAs sharing as little as 80% sequence identity with endogenous genes are capable of effectively disrupting activity of those genes, although we have also observed differences in cross-interference susceptibility between nematode strains and species (unpublished data). Avoiding cross-interfer-

ence should be a relatively easy task for organisms such as *C. elegans* and *Drosophila,* whose genomes have been essentially completely sequenced, but may be a formidable problem in less genetically characterized species. Regardless, we recommend all RNAi experiments should be followed up by methods to confirm specific loss of the targeted message (e.g., *in situ* hybridization, Northerns, antibody staining), especially if no known null mutant is available for comparison or the targeted locus is a member of a large gene family.

1.2.5 Mechanism and Endogenous Functions

RNAi models

Any mechanism for RNAi needs to account for the potency and specificity of the process. Furthermore, RNAi appears to operate post-trancriptionally and results in degradation of targeted mRNAs. Evidence for this has been detailed in Sections 1.2.1.3 and 1.2.2.1 above and has been further supported by Ngo et al.[3] who demonstrated dsRNA-triggered silencing in the unicellular parasite *Trypanosoma brucei.* Based on data from Northern blots, targeted genes in *T. brucei* were still transcribed, but the nascent transcripts were degraded prior to their translation. This is consistent with similar results obtained from work with *C. elegans* and *Drosophila* and is reminiscent of the phenomenon of post-transcriptional gene silencing (PTGS) discussed later in Section 1.3. Although these results suggest that cytoplasmic mRNAs can be directly targeted and degraded during RNAi, there is also evidence demonstrating that mRNAs can be targeted and degraded before transport from the nucleus.[4,25] These latter results argue that RNAi does not directly affect the process of translation. The final stages of mRNA degradation may ultimately feed into more general RNA degradation pathways, but a definitive role for general RNA surveillance machinery in RNAi has not yet been demonstrated.

The specificity of RNAi suggests it is mediated by base pairing between the endogenous mRNA and the antisense strand of dsRNA. Experiments with heterologous RNAs in which mismatches between the antisense and sense strands were generated support this hypothesis (A. Fire, personal communication). RNAi in both *C. elegans* and *Drosophila* has been shown to require only a few molecules of dsRNA per cell to produce potent genetic interference.[1,17] These observations lead to the conclusion that the dsRNA must be amplified or must work catalytically.[1,2,4] In the catalytic model,[2,4] the dsRNA associates with cellular co-factors that partially unwind the dsRNA, allowing the antisense strand to base pair with a short region of the target endogenous message and marking it for destruction. "Marking" mechanisms could involve covalent modification of the target (e.g., by adenosine deaminase) or any number of other mechanisms. Potentially, a single dsRNA molecule could mark hundreds of target mRNAs for destruction before it itself is "spent." In the amplification model, the dsRNA is replicated by a dsRNA-dependent RNA polymerase.[2] Evidence supporting this latter model has recently emerged with the cloning and sequencing of the gene *ego-1,*[40] which was first genetically characterized on the basis of its role in certain germline functions. This gene encodes a homolog of an RNA-directed RNA polymerase (RdRP) implicated in genetic silencing phenomena in plants (see Section 1.3 on gene silencing in plants.) Notably, *ego-1* mutants are

RNAi-resistant for some genes expressed in the germline, indicating an essential role for RdRP in mediating RNAi. Additional RNAi co-factors are being identified through genetic and biochemical screens and the results to date are outlined in the following two sections.

Genetic Screens for RNAi-Deficient Mutants

Recently, genetic screens in *C. elegans* have been carried out to isolate mutant worms resistant to RNAi.[41] The goal of these screens has been to identify the protein (and possibly RNA) partners involved in mediating the cellular response to the presence of dsRNA as well as shed light on the true physiological role of the response. Intriguingly, two genes identified in two independent screens (*mut-7* and *rde-1*) appear to function in both dsRNA-mediated interference and suppression of transposon mobilization.[41,42] Both mutants, in addition to displaying RNAi resistance, exhibit mobilization of endogenous transposons. The gene *mut-7* (for mutator-7) has been found to encode a protein with sequence similarity to bacterial RNAseD and Werner syndrome helicase; these proteins exhibit exonuclease activity.[42] Ketting and colleagues[42] have proposed a model in which the wildtype function of the protein MUT-7 is to repress transposition via RNAi pathway components by degrading transposase mRNA, thus inhibiting both its translation into functional transposase protein and ultimately transposition events.

The gene *rde-1* (RNAi-deficient-1) is a member of a large gene family that includes the "founding" members *piwi* and *sting* from *Drosophila*, the plant genes *argonaute* and *zwille*, and the putative translation initiation factor eIF2C from rabbit.[41] Although the precise functions of *piwi, sting, zwille,* and *argonaute* are unknown, mutations in these genes have suggested roles in repeat-induced silencing and germline maintenance. The other homolog of rde-1, eIF2C, appears to function in translation initiation. Tabara et al.[41] speculate that RDE-1 protein might be brought to mRNA when associated with the interfering dsRNA, and then competitively inhibit eIF2C and thus translation initiation complex formation. This model suggests that the interfering RNA can directly interfere with translation of homologous mRNAs, which is not supported by observations that nuclear RNAs can be targeted. However, the authors note that this is only one possibility for RDE-1 function and that others exist, including the possibility that conserved domains between RDE-1 and eIF2C interact with factors whose functions are unrelated to the control of mRNA translation.

A connection between proper germline maintenance and dsRNA-induced silencing is further strengthened by the discovery that another gene involved in mediating RNAi, *ego-1*, also functions in germline development,[40] as noted previously. Such silencing phenomena appear to be a mechanism for combatting nucleic acid parasites, whether viral ("non-self") or of transposon origin ("selfish"). Suppression of viral replication or transposon mobilization may be particularly critical in the germline so that the deleterious consequences of such are not inherited by the progeny of the affected adult.

A Biochemical Approach

Phil Sharp, Ruth Lehman, and colleagues have developed an *in vitro* cell-free system that recapitulates many of the features of RNAi.[5] This system involves the use of

extracts of *Drosophila* syncytial blastoderm-stage embryos and was developed to complement genetic approaches to elucidate the biomolecular foundations of the RNAi response. Essentially, Tuschl et al.[5] tested the ability of dsRNA added to embryo extracts to block expression of reporter mRNAs. They found that the addition of dsRNA in their extracts caused interference that was sequence-specific, that dsRNA and not ssRNA worked as the interfering agent, and that interference resulted in degradation of the targeted mRNA. All of these results are consistent with features of RNAi observed *in vivo*. Some new insights gained from this approach include an observation that pre-incubation of the dsRNA with the embryo extract enhances its capacity to inhibit expression of the target mRNA, which is added subsequently to the cell lysate. The authors suggest that such an enhancement effect may be due to providing enough time for specific endogenous factors to either modify or associate with the exogenous dsRNA. Presumably, such endogenous RNAi co-factors would be present in limiting amounts and the addition of increasing amounts of dsRNA might not prove effective in increasing the potency of the interference. In support of this hypothesis, Tuschl et al. found that, upon introduction of *Drosophila nanos* dsRNA to the system, reporter mRNA levels increased, indicating that the addition of this *nanos* "competitor" dsRNA actually inhibited the potency of the reporter dsRNAs.

One hallmark of RNAi seen *in vivo* that is not reproduced in the *Drosophila in vitro* system is the potency of the dsRNA molecules at substoichiometric levels compared to their target mRNAs. The *in vitro* system requires an excess of dsRNA compared to corresponding mRNA targets. This disparity in the stoichiometric requirements of dsRNA to target mRNA *in vivo* and *in vitro* may be a result of the numerous problems encountered when designing an *in vitro* system to mimic *in vivo* processes; typically *in vitro* systems are less efficient than their *in vivo* counterparts.[5] However, the development of this system offers a biochemical approach to the identification of cellular RNAi co-factors and will most likely contribute significantly to our understanding of the cellular mechanism(s) mediating the sequence-specific interference response to dsRNA.

1.3 RNA-MEDIATED GENE SILENCING IN PLANTS

Since the late 1980s, it has been known that the introduction of transgenes into plants can result in silencing of the homologous endogenous locus and of the transgene itself instead of the expected result of overexpression of the introduced gene.[10] This phenomenon, termed "cosuppression" by the plant molecular biology community, has been likened to a phenomenon called "quelling" in filamentous fungi[43] and ciliates.[44] Some gene silencing in plants has been linked to DNA-DNA interactions, and others have been shown to be elicited as a result of the RNA product of the expressed transgene.

In plants, two types of RNA-mediated gene silencing have been described. The first, termed post-transcriptional gene silencing (PTGS), results in a significant reduction of the half-life of mRNA, effectively "silencing" the gene.[45] The second is called transcriptional gene silencing (TGS), which typically involves direct mod-

ification (e.g., methylation) of the chromatin template such that the gene is no longer expressed.[46]

Researchers who have been focusing on unveiling the mechanism(s) behind cosuppression have generated dozens of papers, and we refer readers seeking greater detail than we can offer here to recent reviews.[47,48] Below we summarize the most salient features of cosuppression and point out significant similarities and differences between cosuppression and RNAi.

1.3.1 Post-Transcriptional Gene Silencing (PTGS)

PTGS has been shown to play an immunological role in plants.[49–53] Evidence for this has come from several observations. First, viral RNAs can be targeted for PTGS.[54] Second, the PTGS response allows the host to mount a systemic defense instead of a site-specific response.[55] Third, several plant viruses have evolved proteins that can interfere with normal PTGS processes.[56] Finally, plants appear to be more susceptible to certain viral infections if their normal PTGS response is inhibited artificially.[56,57] Similar to recently proposed endogenous RNAi functions, it appears that PTGS plays an important role in suppressing replication and/or activation of viral as well as most likely other forms of "non-self" and "selfish" DNA.

Although many of the details regarding the mechanism by which PTGS functions *in vivo* are still a mystery, there is a wealth of experimental data, and several models have been proposed. Current models of PTGS initiation involve the production of aberrant RNA (abRNA). These putative abRNAs are thought to be produced via some abnormality in the transgene structure (e.g., cryptic flanking promoters, transcription through inverted repeat regions in the transgene inserts, or premature termination)[54] or by ectopic pairing of a transgene with a sequence-related endogenous gene[58] (reviewed in Reference 47). How the cell recognizes abRNA is not yet known, but a few have suggested that the abRNA might take the form of dsRNA and be recognized by dsRNA-binding proteins.[2,47]

Although studies have not addressed the precise concentrations of abRNA required to elicit PTGS in plants, replication of an effector molecule (tentatively RNA) has been proposed as a requirement for effective genetic silencing.[55,59] Two models have been proposed to explain the substoichiometric activity of dsRNA in PTGS and related phenomena. One proposes that dsRNA behaves catalytically[2,4,60] (Figure 1.4a) whereas the second model involves amplification of the triggering dsRNA[2,47] (Figure 1.4b). A model that includes a role for replication proposes that an RNA-dependent RNA polymerase (RdRP) generates antisense RNAs using abRNA or dsRNA as template. The short antisense transcripts generated by the RdRP might then hybridize to a complementary portion on nascent mRNA, sequestering it from endogenous transcriptional machinery and/or marking it for destruction (Figure 1.4b).[2,59,61] RdRP could also theoretically generate additional copies of dsRNA by replication and annealing of both strands and would thus provide a vehicle for dsRNA amplification.[2]

Definitive evidence for a role for RdRP in PTGS is still lacking, and abundant copy RNAs have not been detected in plant tissues displaying PTGS. However, an RdRP that is activated upon virus infection has been cloned from tomato,[62] and

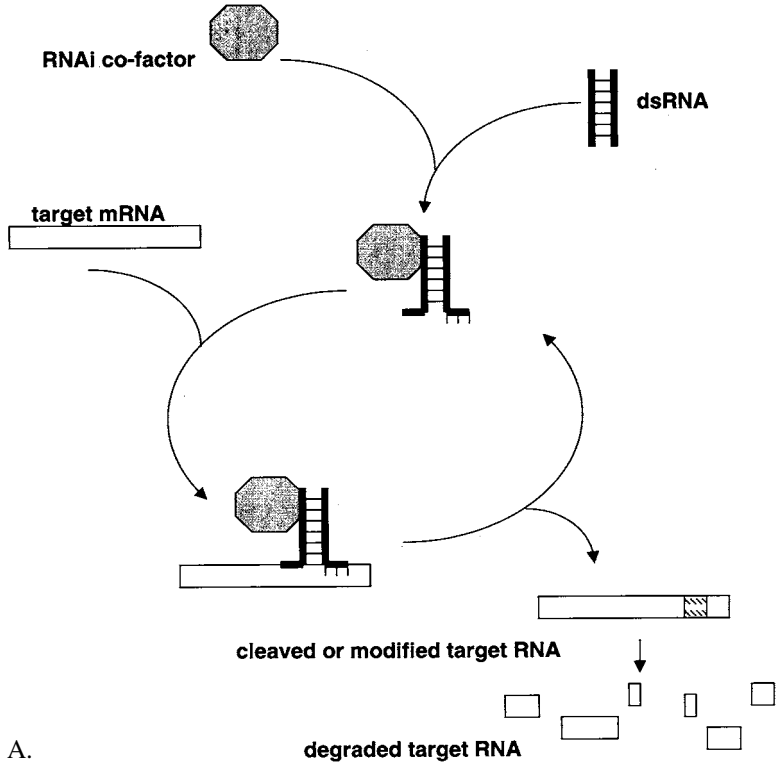

FIGURE 1.4 Two mechanistic models for double-stranded RNA-mediated genetic interference. (A) A catalytic model in which the dsRNA associates with cellular co-factors that target endogenous complementary mRNAs for degradation. The dsRNA partially unwinds and base pairs with the target "marking" it for destruction and then recycles to target additional messages.

putative homologs have been identified in additional plant species as well as in yeast and *C. elegans*. Recent genetic evidence indicating a role for an RdRP homolog in RNAi in *C. elegans* is particularly intriguing. The isolation and analysis of genetic mutants lacking RdRP activity in plant species will no doubt reveal whether this enzyme functions in mediating PTGS also. Encouragingly, several mutants defective in PTGS have been isolated in the flowering plant model *Arabidopsis* and in the fungus *Neurospora.*[54,63] These mutations await cloning and identification but should eventually help refine a mechanistic model for PTGS. It will be interesting to see whether any of these PTGS genes turn out to have conserved counterparts in RNAi pathway components that emerge from similar genetic screens being conducted in *C. elegans* and *Drosophila*. It is quite possible that RdRP may be the first such example.

The "aberrant RNA/RdRP" model outlined above is only one of several that have been proposed for PTGS function, most of which are not mutually exclusive.[2,4,47,64] Much debate still surrounds questions of how viral and transgene

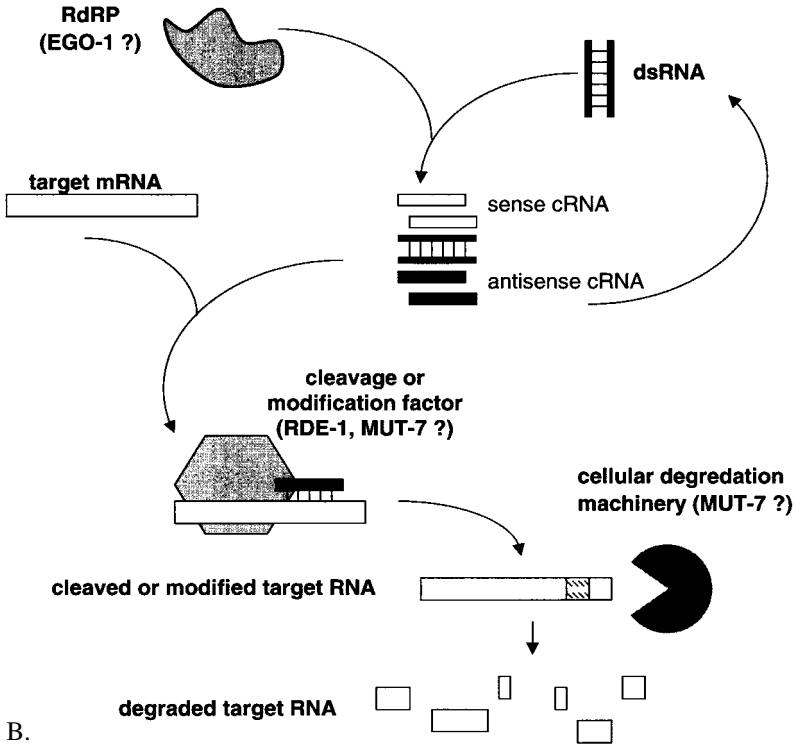

FIGURE 1.4 (CONTINUED) (B) A model for dsRNA amplification. An endogenous RNA-dependent RNA polymerase (RdRP) binds to dsRNA and synthesizes copy RNA (cRNA) from both strands, generating sense and antisense RNAs, which can anneal to generate additional copies of dsRNA. In this model, antisense RNA also anneals to endogenous mRNAs, and is the direct trigger for RNA degradation. Conceivably, the actual mechanism may incorporate components of both models, such as RdRP for dsRNA replication and direct base pairing between dsRNA and target mRNA. Putative RNAi pathway components that have been identified through genetic screens are shown. This figure is based on models suggested by References 2, 4, 42, 47, 59, and 61.

sequences trigger cosuppression, the molecular nature of the "trigger" as well as the "target," and the mechanism by which gene silencing can spread to surrounding tissues, including cells that do not contain the viral or transgene sequences. In a recent review, Grant champions the ectopic pairing model of PTGS initiation put forward by Baulcombe and English[58] in which direct DNA-DNA interactions are proposed to account for the production of abRNA. If this model is correct, and there is certainly good evidence supporting it, it would appear that PTGS occurs by a mechanism quite distinct from that operating in flies and worms. In flies and worms, RNAs can be both targets and triggers of RNAi, a form of PTGS in animals.[1,4,17,28] Most significantly, by targeting maternally provided mRNAs in fertilized embryos, RNAi has been shown to work in the absence of DNA-encoded material, indicating that cytoplasmic RNAs can be targeted (and degraded) directly.[17]

1.3.2 *De Novo* Methylation

Transgene-induced genetic silencing is often associated with hypermethylation of target genes, in some cases blocking RNA polymerase activity and inhibiting transcription.[19,65–67] This latter process is an example of transcriptional gene silencing (TGS). Whether or not RNA, single-stranded or double-stranded, is involved in the initiation of TGS is not clear, although there is evidence that supports an RNA trigger in at least some cases of TGS.[19,65,66] If involved, a large host of endogenous RNA enzymes (e.g., methylase, unwindase, etc.) must be utilized by any putative RNA-dependent system to produce the observed modifications to the plant's primary DNA sequence.

DNA methylation has been proposed to play a role in PTGS also. Methylation of cytosines within coding sequences has been proposed to result in the formation of abRNA that then triggers PTGS. Other researchers, however, have argued that methylation is not a prerequisite for abRNA formation but occurs as a consequence of PTGS.[61] We refer readers to a recent review by Fire[2] for a detailed discussion of possible mechanistic links between RNA-triggered chromosome modification and PTGS. Components of the PTGS and RNA-triggered methylation pathways should be revealed by the genetic screens mentioned in the section above and should help to determine to what extent these processes intersect.

Of note for purposes of our discussion here, methylation of the transgene itself has been linked to the somatic instability of PTGS in some cases. For example, in some plants variegated pigmentation has been associated with varying levels of functional PTGS.[68,69] In fact, the inherent instability and consequent unpredictability of transgene-induced phenotypes during PTGS is one of the limits to its practical application. The instability of PTGS may be a direct result of the vulnerability of the methylated transgene itself. Baulcombe[10] has proposed that the instability of the methylated transgene could lead to the loss or suppression of PTGS if, for example, there was a failure to maintain the methylated state of the transgene during mitosis, and if methylation of the transgene is required for PTGS.

1.3.3 Similarities between RNAi and PTGS

RNAi in animals and PTGS in plants share striking themes and similarities. RNAi and PTGS are cases of gene-specific genetic interference, catalyzed by the introduction of exogenous genetic material. In RNAi, dsRNA has been shown to be the instrument of interference. Likewise, dsRNA is capable of triggering a PTGS response in at least some plants.[59] Observations that the interference can spread from the point of introduction into a systemic event[1,70] is perhaps the most interesting mimesis between the two processes. In fact, an RNA molecule travelling through vascular tissue in the plant has been hypothesized as the synergist of the PTGS state.[71] This latter property is thought to be a mechanism to counteract the spread of viruses that may be the initial source of genetic material that the organism recognizes as foreign.

Genetic and biochemical screens are expected to aid in the identification of cellular co-factors that mediate RNAi and PTGS. Such approaches have already led

to the discovery of a number of these factors, including a putative RNAse and an RNA-dependent RNA polymerase (RdRP). Continued work in this area will eventually lead to a solid understanding of the mechanisms behind RNAi and PTGS and to what extent (if any) these processes are evolutionarily conserved.

Plants, like their mammalian counterparts, have a dsRNA-dependent protein kinase (pPKR) that phosphorylates plant eIF2-α, inhibiting *in vivo* protein synthesis.[72] Evidence suggests that it is the functional equivalent of mammalian PKR and may in fact be an ortholog. How is it that dsRNA can elicit a sequence-specific response in plants whereas mammals typically respond to the presence of dsRNA by a general shutdown of all translation? The answer to this question has yet to be elucidated.

1.4 FUTURE APPLICATIONS AND POTENTIAL PITFALLS

Much of the current emphasis in RNAi and cosuppression research has been to elucidate the mechanisms underlying these gene silencing phenomena and, in the process, gain an understanding of the intrinsic roles they play for their respective organisms. Furthermore, these techniques allow, with relative ease, an analysis of the function of many endogenous genes without the need for characterized mutant strains or alleles. However, do RNAi and cosuppression have any applications outside of those already mentioned? Currently, molecular geneticists from diverse fields are investigating this very question. In fact, procedures for RNA-induced gene silencing are now commonly used and a welcome addition to the battery of techniques used in various biological, commercial, and industrial laboratories. An example of the numerous (present and potential) commercial uses of these reverse genetic techniques is in the agriculture industry and has been thoroughly reviewed by Senior (1998).[73] Genetic transformation of many plant species has led to new crop varieties with favorable characteristics that could not normally be attained by conventional genetic approaches alone. Similarly, the control of certain traits, such as fruit ripening or the ability to channel energy stores from one pathway to another, are becoming commonplace in many global agribusiness circles.[74]

Whereas there are numerous cases in which genetic silencing may be the goal of those manipulating gene expression, there are certainly an equivalent number of situations in which silencing is not desirable. Cosuppression was first discovered because transgenes inserted into certain plant species were *not* behaving as expected. Transgenic technology that requires high expression levels from integrated transgenes has been utilized to enhance the genetic repertoire of plants, making them more disease- and pest-resistant. At this time, researchers cannot accurately predict which transgenes under which conditions will result in either over-expression of the introduced gene or silencing; although transgenes that are designed to generate inverted repeat structures will, it seems, in most cases result in silencing. A better understanding of the mechanisms that trigger cosuppression should lead to the ability of researchers to manipulate transgenic technology such that silencing mechanisms can be overcome if the goal is to obtain robust expression of introduced traits.

Like many technological innovations, the future potential of RNA-based gene silencing methods appears promising in many contexts but worrisome in others. Below we outline some potential agricultural and clinical applications as well as areas for concern.

1.4.1 Pest Control in Plants

A few biotech companies are expressing interest in the potential use of dsRNA-mediated genetic interference to fight eukaryotic plant parasites. Parasitic nematodes are the cause of several diseases affecting many commercially important crops[75] and might prove particularly susceptible to dsRNA-based treatment. The strategy would be to produce transgenic plants that express a dsRNA construct designed to target an essential gene in the parasite's genome that, when disrupted, would prove deleterious or lethal to the parasite. Ingestion of dsRNA has already been shown to be a fairly effective means for dsRNA delivery for certain free-living nematodes[16] and may also work for plant-parasitic species. This method, if it should prove capable of preventing parasitic species from gaining a foothold, would be a novel extension of transgenic technology already in use for warding off other types of plant diseases. However, it is worth noting that, even if such an approach works on an experimental basis, special caution should be exercised before applying such a technology to edible food plants, as the health risks (for humans or livestock) associated with ingestion of large amounts of dsRNA are unknown.

Regardless of whether such an approach fails or succeeds, it may be possible to use RNAi to identify essential genes of plant parasites, particularly parasitic nematodes, and thus design specific pharmacological strategies that target the biochemical pathways in which these genes function.

1.4.2 Parasite Treatment in Humans

As with plants, a number of parasitic nematode species plague humans worldwide.[76] Comparative genomics using information from the *C. elegans* and human genome projects as well as with information emerging from the filarial nematode sequencing projects could conceivably lead to the identification of nematode-specific essential genes. Targeting of such genes could be the basis for effective treatments that would rid the host of nematodes or disrupt the nematode's life cycle. There are also species of protozoa responsible for serious diseases afflicting humans, including malaria, African sleeping sickness, and Chagas' disease. In this regard, the use of dsRNA to disrupt the function of specific genes in the protozoan *Trypanosoma brucei*, a causative agent of African sleeping sickness, is significant.[3] It may be possible to develop dsRNA-based therapies that target parasite-specific genes, although any such therapy will have to avoid eliciting the mammalian PKR/INF pathway. The fact that PKR responds to small concentrations of dsRNA, but is inhibited by large concentrations of double-stranded nucleic acid,[34] may shed some light onto a delivery system tailored for mammalian systems. Another suggested alternative would be the development of chemical modifications to the dsRNA that would still allow it to function in gene-specific interference within the parasite while not inducing the PKR response in the host.[2]

1.4.3 Gene Therapy in Humans

The use of exogenous nucleic acids to augment disease therapy in humans has become one of the most heated and controversial biomedical topics. Two such approaches to gene therapy, antisense therapeutics and gene transfer, involve the production of exogenous ssRNAs *in vivo*. Consequently, these techniques may involve the subsequent production of dsRNA that may lead to activation of PKR and increased INF production. These pathways may have dramatic consequences if elicited accidentally, and their presence must be recognized and controlled in any drug or therapy that may induce, however inadvertently, the production of dsRNA.

1.4.3.1 Gene Transfer

Experiments in gene transfer began in 1993, and since then it has been used to combat a defective gene in cystic fibrosis (CF) patients.[77] Recently, Harvey et al.[78] administered an adenovirus vector to transfer a normal cystic fibrosis transmembrane conductance regulator (CFTR) into CF patients. This is accomplished by inserting the CFTR cDNA into the vector and introducing the vector to the airway via endobronchial spray. By using quantitative RT-PCR, Harvey and colleagues discovered that introduction of this vector via airway epithelia resulted in an increase of vector-derived transcripts to levels greater than 5% of normal CFTR mRNA concentration in CFTR patients. This increased level of CFTR mRNA is enough to rescue the function of the dysfunctional endogenous CFTR gene and results in decreased CF symptoms. This effect persists for only 30 days and is dose-dependent, but it is an encouraging testament to the potential power of introducing a wildtype gene to rescue a defective endogenous gene.

Currently, new vectors are being developed to circumvent or break through the stolid cellular defenses employed by airway epithelia to guard against penetration by foreign and potentially harmful moieties.[79] However promising the prospects of gene transfer therapy are, careful attention must be used in choosing or designing putative vectors (see Table 1.2) that will not result in accidental or unwanted production of dsRNA. This dsRNA could induce INF production, PKR activation, and subsequent cell death, which could be catastrophic for the patient.

1.4.3.2 Antisense Gene Therapy

The antisense approach to gene therapy involves the use of an exogenous gene to intervene in the normal transcriptional processes of a cell.[80] Antisense therapy may involve either the direct introduction of an exogenous antisense oligodeoxynucleotide or the production of antisense RNA from introduced plasmid vectors (Figure 1.5). In the first of these methods, a relatively small fragment of ssDNA is thought to bind to the mRNA of interest and effectively block endogenous cellular machinery from translating the message, thus preventing synthesis of the protein. This approach to gene therapy is less widely used than RNA antisense because of the difficulty of delivering the relatively high concentrations of exogenous DNA required to effectively Watson-Crick base-pair to the nanomolar concentrations of mRNA inside the cell. Even upon modification of the phosphodiester backbone to

TABLE 1.2
Characteristics of Commonly Used Vectors for asRNA Therapeutics[a]

Vector Type	Advantages	Disadvantages
Adenovirus	Accommodates large foreign sequences; transduces many mitotically active and nonreplicating cells; easy purification; episomal	Induction of strong host immune response
Adeno-associated virus	Transduces both mitotically active and nonreplicating cells; easy purification of large-titer virus preparations; episomal	Accommodates small foreign sequences; requires helper virus
Cationic lipids	Readily available; large variety available suitable for wide array of cell types	Toxic at high doses
Herpes simplex virus	Accommodates large foreign sequences; transduces nerve cells	Marked neurotoxicity
Lentivirus	Transduces nondividing cells; lack of expression of immunogenic viral proteins	Inherent pathogenicity of WT HIV virus
Molecular conjugates	Selective targeting of certain cells or subcellular structures	Difficult synthesis; large size; nonspecific binding
Nonviral plasmid DNA	Easy to produce; transfects both mitotically active and nondividing cells; does not integrate into host genome	Low transfection efficiency; unstable transgene expression
Retrovirus	Accommodates large foreign sequences; transduces many mitotically active cells	Difficult production; random integration into host's genome

[a]Based on information from Reference 83.

augment DNA penetration through cellular antiviral defenses, this technique appears to be problematic at best.[81,82]

The second approach to antisense gene therapy, and the one that involves direct production of transient antisense RNA, is through delivery of an exogenous gene via a suitable vector.[83] The basic principle underlying antisense gene therapy is to produce anti-parallel asRNA *in vivo* that can anneal to complementary mRNA targets. This base pairing would sequester the defective message and effectively silence the faulty gene. One of the most common impediments to antisense success during *in vivo* trials is the complexity needed in the construction of suitable vectors that are both able to traverse the cell membrane and able to be activated at a specific time and only in specific tissues. Currently, clinical trials of antisense therapeutic agents are widespread and biomedical firms are rapidly designing putative delivery vectors.[84]

Many types of vectors have been utilized in antisense trials, each with its own strengths and weaknesses[83] (see Table 1.2). The DNA encoding the asRNA can be naked or inserted into an RNA-generating vector. The latter method is the one most widely used and a combination of cis- and trans-acting regulatory elements can be integrated into the exogenous vector to control transcription of the asRNA. The addition of regulatory elements (e.g., transcription factors, exogenous promoters) is

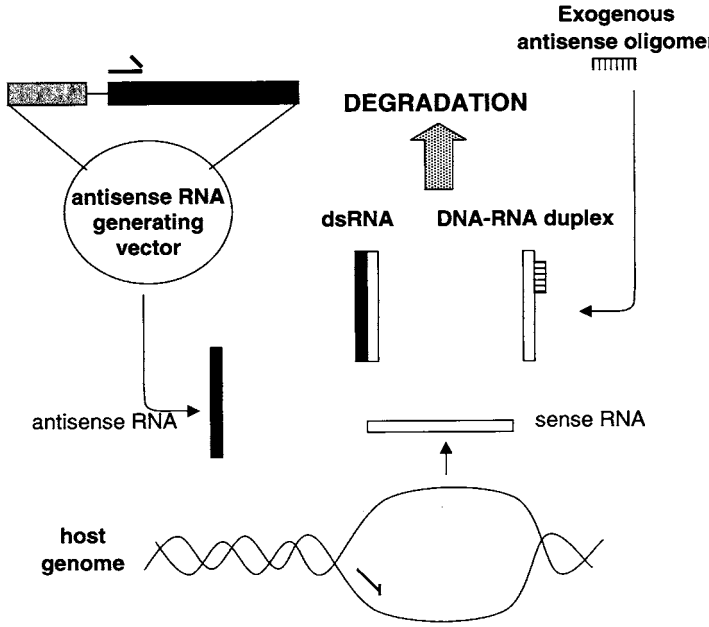

FIGURE 1.5 Strategies for antisense gene therapy. Antisense-generating plasmid vectors and antisense oligodeoxynucleotides are designed to inhibit gene function by blocking translation of complementary mRNAs. Note that the DNA pathway involves the direct stoichiometric binding of antisense DNA to a mRNA target whereas the use of plasmid vectors results in the formation of a dsRNA helix. Both pathways inhibit endogenous cellular machinery from translating targeted mRNA into functional protein, most likely by degrading bound messages.

virtually essential in successful treatment because activation of the asRNA gene can be controlled at relatively precise times and locations. These control elements limit the possibility of accidental spread of the asRNA and possible antiviral response from surrounding tissues and organ systems. Figure 1.6 shows a schematic representation of an idealized RNA-generating vector with intrinsic tissue-specific regulatory elements coupled with an inducible promoter. It is prudent to note that the final product is still a dsRNA duplex (asRNA generated from vectors hydrogen bonded with complementary endogenous mRNA) that can theoretically activate INF production and subsequent PKR activation. Vectors similar to the one described above, however, have been used effectively in some mammalian systems.[85,86]

1.4.3.3 Clinical Applications of dsRNA

Recently, dsRNA has been used to elicit intentional antiviral responses in dysfunctional cells. Poly(I):poly(C_{12}U), commercially called Ampligen, is one of these therapeutic agents that has been specifically designed to inhibit virus and tumor-cell growth *in vivo* through pleiotropic mechanisms. Ampligen has been reported to regulate two antiviral pathways: the 2,5 oligoadenylate synthetase/RNase L (2-5A synthetase/RNase L) pathway and the PKR-induced INF pathway.[87] Ampligen can

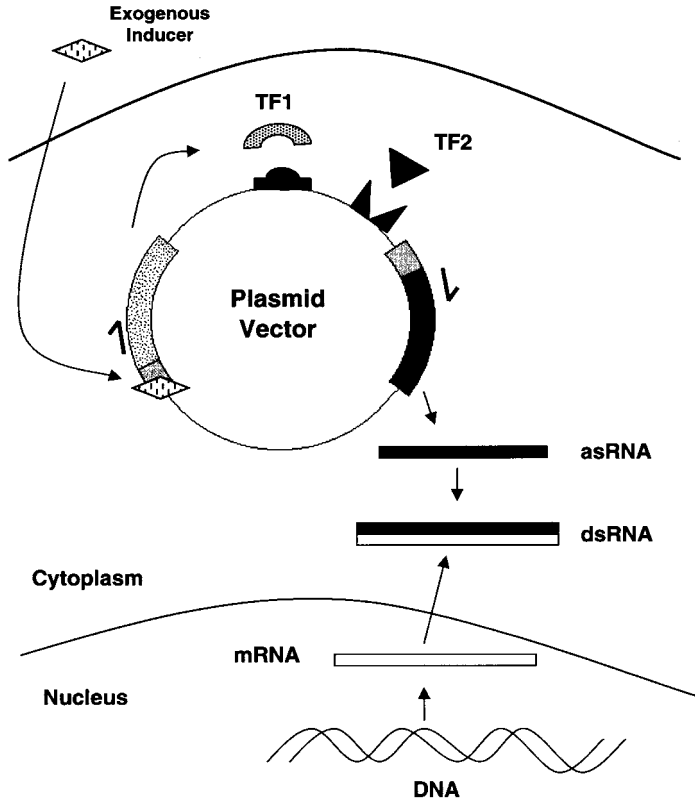

FIGURE 1.6 A vector for antisense RNA gene therapy containing multiple regulatory elements. A suitable vector containing cDNA sequences of the gene of interest, oriented in an antisense direction with respect to the basal promoter, is introduced into the cell. Transcription of antisense RNA (asRNA) is controlled by upstream elements that include binding sites for two separate transcription factors, one of which is also encoded within the vector (TF1), whereas the second is an endogenous tissue-specific transcription factor (TF2). TF1 is produced as a result of its activation by an exogenous inducer, and TF1 in turn induces the expression of asRNA in concert with TF2. By placing the sequence encoding the asRNA under the regulation of a promoter engineered so that it can only be activated in the presence of two transcription factors (one cell intrinsic and one foreign), it is possible to control expression of the asRNA in a given tissue at specific times.

induce the production of 2-5A synthetase independent of INF production and is also able to activate 2-5A and PKR pathways, whereas INF can only induce production but not activate these pathways. These differences in mechanisms between INF and Ampligen result in clinical and biological activities in interferon-intolerant or -resistant states. The 2-5A and PKR systems, upon activation, are able to perform their respective antiviral roles and, theoretically, destroy any virus infecting the cell that otherwise would have remained camouflaged to endogenous cell-mediated immunological defenses.

A study was conducted using healthy volunteers and Ampligen to gauge whether any side effects would result from the artificial induction of the immune system.[88] Several patients complained of "a mild flu-like syndrome" that lasted for 16 hours after poly(I):poly(C_{12}U) dosage, although few other side effects were seen at a 600 mg dosage. In the early 1990s, Ampligen was administered during clinical trials in the U.S., Canada, and Europe to patients suffering from chronic fatigue syndrome (CFS) and the human immunodeficiency virus (HIV).[87,89,90] However, clinical trials of Ampligen were halted by the FDA in the mid-1990s due to unexpected complications and fatalities in several of the clinical trials.

One explanation for the unexpected results of Ampligen introduction into patients may be a result of poly(I):poly(C_{12}U) being a type of mismatched dsRNA. Ampligen was designed to elicit a strong antiviral and cytoprotective effect and to increase production of T-cells. In fact, it has been shown that both free and immobilized poly(I):poly(C_{12}U) binds to PKR, resulting in autophosphorylation and activation of the kinase.[87] Furthermore, PKR was activated within a specific concentration range (10^{-7} to 10^{-6} g/mL). It is conceivable that, if administered incorrectly, Ampligen's induction of PKR could lead to rapid apoptosis of surrounding cells and immediate cell antiviral defense mechanisms that could produce flu-like symptoms or even more severe reactions. After modification and moderate success in Europe and Canada, Ampligen trials have resumed in the U.S. in FDA-approved phase III testing.[91]

Recent studies in *Drosophila* have isolated a protein named Dicer with RNAse III activity that cleaves introduced dsRNA molecules into 21–23 nt short interfering RNAs, or siRNAs.[92] These siRNAs appear to guide a protein complex containing RNAse activity to complementary target mRNA through base-pairing interactions. Similarly sized siRNA have also been found in *C. elegans*[93] and plants[94] undergoing RNAi/PTGS. Dicer activity is required for a robust RNAi response and thus may help explain the substoichiometric activity of longer dsRNAs. Homologs of *dicer* have been identified in *C. elegans* (Grishok et al., Cell, in press), and homologs of rde-1 have been found in *Drosophila, Neurospora,* and in the flowering plant *Arabidopsis thaliana*. The *Arabidopsis rde-1* homolog, AGO-1, is required for both PTGS and proper leaf differentiation,[95,96] indicating that RNAi/PTGS performs a regulatory function influencing development in addition to suppressing viral transposon expression. These studies and others provide additional evidence that RNAi and PTGS are essentially the same phenomenon. Of particular importance is the recent finding that the introduction of 21 bp dsRNAs into cultured mammalian cells can elicit gene-specific silencing.[97] The direct introduction of siRNAs into these cells appears to bypass the PKR response and may thus lead to safe gene-specific therapeutics for humans.

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2 The Double-Stranded RNA-Activated Protein Kinase PKR

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CONTENTS

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2.1 INTRODUCTION

Upon exposure to environmental stress, cells integrate multiple signaling pathways to elicit an adaptive response. If adaptation is not sufficient, then apoptosis occurs. The mechanism by which the cell chooses between adaptation and apoptosis is largely unknown. The immediate response to environmental stress results in a change in the capacity and specificity for cells to select mRNAs for translation. Most translational control occurs at the level of initiation. The rate of translation initiation depends on mRNA abundance, mRNA sequence and structure, the number of ribosomes, the availability of initiator $tRNA^{met}_i$, and the amount and activity of eukaryotic translation initiation factors (eIFs).[1] Reversible covalent modification of these eIFs is a fundamental mechanism that determines the rate of initiation. The most significant modification used for translational control in response to environmental stress is the reversible phosphorylation of eIF2. Phosphorylation of heterotrimeric GTPase eIF2 on the alpha subunit (eIF2α) rapidly reduces the level of functional eIF2 and limits initiation events on all cellular mRNAs. Reversible phosphorylation of eIF2α provides the cell with an efficient, rapid, and reversible means to respond to a variety of different stimuli.

Continued polypeptide chain synthesis requires the recycling of eIF2 between GTP- and GDP-bound states in a reaction catalyzed by the guanine nucleotide exchange factor, eIF2B.[2,3] eIF2 binds GTP and initiator $tRNA^{me}_i$ to deliver $tRNA^{met}_i$ to the small 40S ribosome to form a 43S complex. The 43S complex binds mRNA, and then the 60S ribosomal subunit joins with concomitant hydrolysis of eIF2-bound GTP to GDP. To promote another round of initiation, GDP bound to eIF2 must be exchanged for GTP in a reaction catalyzed by eIF2B, a pentameric guanine nucleotide exchange factor. Phosphorylation of eIF2α at residue serine 51 increases the affinity of eIF2 for GDP by 100-fold and thereby stabilizes the eIF2/GDP/eIF2B complex, preventing the GDP-GTP exchange reaction. Because the cellular levels of eIF2B are lower than the levels of eIF2,[4] the exchange process and translation initiation are inhibited when only a fraction (i.e., 20–30%) of eIF2α is phosphorylated because it sequesters eIF2B in the cell. In metazoan cells, there are four Ser/Thr protein kinases that phosphorylate eIF2α. These are the hemin-regulated inhibitor kinase (HRI),[5] the general control of amino amino acid biosynthesis kinase (GCN2),[6] the pancreatic eIF2α kinase or the PKR-endoplasmic reticulum (ER)-related kinase (PEK/PERK),[4,7,8] and the double-stranded (ds) RNA-activated protein kinase (PKR). This chapter reviews the structure and function of PKR.

PKR (previously known as p68, DAI, P1-eIF2 kinase) was originally characterized as an inhibitory activity present in reticulocyte lysate translation reactions.[9] PKR is expressed in the majority of cells in higher vertebrates and plants. Its transcription is induced by type 1 interferons (α and β).[10,11] PKR signals the interferon antiviral and antiproliferative response. In addition, PKR activation leads to induction of the cell death program, apoptosis.[12,13] Evidence also supports roles for

PKR in cell differentiation, cytokine signaling and the innate immune response, and transcriptional induction of dsRNA-regulated genes, such as interferon β.[14–18,19,20]

2.2 STRUCTURE AND MECHANISM OF PKR ACTIVATION

PKR is synthesized as a latent protein kinase of apparent mass 68 kDa in humans. The amino-terminus of PKR contains a dsRNA binding domain (dsRBD) and the Ser/Thr protein kinase domain is located in the carboxy-terminus[21,22] (Figure 2.1A). The dsRBD contains two dsRNA binding motifs (dsRBMs) of approximately 70 amino acid residues each. The dsRBMs are present in at least nine families of functionally distinct dsRNA binding proteins.[23] The most conserved region of the dsRBM resides in the amino terminus and is rich in basic amino acids. The first dsRBM (dsRBM1) has a better match to the consensus dsRBM and this correlates with its higher affinity for dsRNA demonstrated by mutagenesis and structural studies.[21,24] However, both dsRNA binding motifs are required for the specific and high affinity binding that is required for activation of PKR kinase activity.[25–30]

RNA molecules with a high degree of secondary structure activate PKR.[31–33] In addition to dsRNA and highly structured RNAs, polyanions such as heparin can activate PKR, although this activation does not require the dsRBD.[34,35] Binding of dsRNA to PKR exposes the ATP binding site[32,34] and induces dimerization (Figure 2.1B). Dimerization subsequently stimulates *trans*-autophosphorylation.[36,37] PKR autophosphorylation is essential to convert the kinase into a catalytically active form that can bind and phosphorylate eIFα.[34,38–40]

PKR is a ribosome-associated protein that is removed by a high salt wash.[41–43] Immunofluorescence demonstrated that PKR is localized on the surface of the rough endoplasmic reticulum as well as in the nucleolus, consistent with its ribosomal association.[44–46] Whereas PKR associated with the ribosome is monomeric, free cytosolic PKR is dimeric.[43] Interestingly, mammalian ribosomes inhibit PKR activation.[47] This leads to the hypothesis that upon activation, PKR dimerizes and is released from the ribosome. At least one 60S ribosomal protein, subunit L18, binds dsRBM1 in PKR and inhibits PKR activation by dsRNA in a competitive manner.[48] Although when human PKR was expressed in yeast it bound to the small 40S ribosomal subunit,[49] PKR expressed in COS-1 monkey cells was associated with the 60S ribosomal subunit, consistent with its interaction with L18.[48] Three ribosome-binding sites were identified, one in each dsRBM, and a higher affinity site in the PKR catalytic domain.[50]

2.2.1 PKR Structure and dsRNA Binding

PKR binds to RNA in an RNA-sequence independent manner as long as the RNA has an overall A-form geometry.[51] Viral dsRNA genomes (e.g., reovirus), replication intermediates, and mRNA transcripts with extensive secondary structure resembling dsRNA are potent activators of PKR. The activation curve for dsRNA is bimodal where low concentrations of dsRNA activate and high concentrations of dsRNA inhibit activation of PKR kinase activity.[9,39] Short RNA duplexes of 16 bp are capable

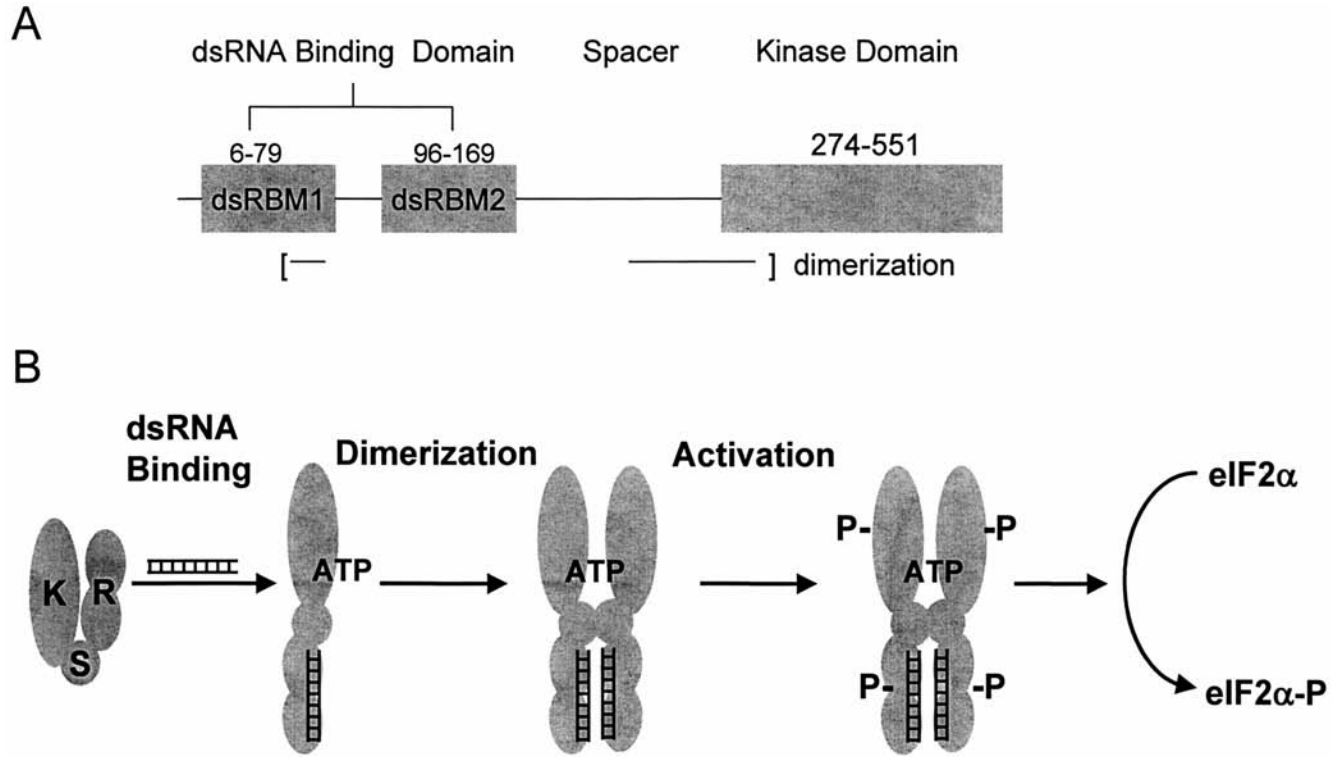

FIGURE 2.1 PKR Structure and Mechanism of Activation. The structure of PKR is depicted in A showing the dsRNA binding motifs and the regions that are proposed to mediate dimerization. The mechanism of PKR activation is depicted in B. The dsRNA binding domain (R), the spacer (S), and the protein kinase domain (K) are depicted. See text for details.

of binding PKR, although the dsRBD occupies an 11 bp segment.[24] However, the binding affinity and activation increase with length up to 85 bp and longer dsRNA molecules have no greater effect.[24,29,33] PKR binds dsRNA through electrostatic interactions and does not bind dsDNA or RNA-DNA hybrids.[9] This specificity is primarily due to interaction with 2'- hydroxyls and possibly some phosphate groups within the minor groove of dsRNA.[29] A solution structure of the 20 kDa amino-terminal dsRBD was determined by multidimensional nuclear magnetic resonance (NMR) spectroscopy.[52] The dsRBD adopts a dumb-bell shape in which the two dsRBMs are separated by a flexible linker of 22 residues. Each dsRBM contains an α–β–β–β–α fold in which the two α helices are stacked against a three-stranded antiparallel β sheet. Mutations in dsRBM1 that are known to disrupt dsRNA binding (Arg39, Phe41, Ser59, Lys60, Lys61, and Lys64)[28,30,53] form a positively charged surface for electrostatic interaction with negatively charged phosphate backbone oxygen atoms within the RNA. The interaction with the phosphate backbone can explain the dsRNA sequence-independent interaction. The NMR results suggest that the two dsRBMs wrap around the RNA duplex, which may account for the bimodal dependence on dsRNA for activation. At low concentrations of dsRNA, dsRBM1, with its higher dsRNA binding affinity, could facilitate dsRNA binding to the lower affinity dsRBM2. At higher concentrations of dsRNA, the binding may not be cooperative so that the two dsRBMs in PKR would bind to two different dsRNA molecules, thereby preventing a conformational change required for PKR activation.

2.2.2 PKR Dimerization

dsRNA binding induces PKR dimerization. Although one study suggests that dsRNA is required for efficient dimerization,[54] the consensus using different approaches supports the view that dsRNA binding is required for PKR activation but is not required for dimerization[36,55–57] PKR exists in an equilibrium between monomeric and dimeric forms.[43,57] Dimer formation increases upon addition of activator dsRNA, such as the HIV trans-activating region (TAR) RNA element.[57] At saturating concentrations of TAR RNA, an activation complex forms consisting of two molecules of PKR and one RNA molecule. Mutations that disrupt dsRNA binding do not affect dimerization.[56] Two dimerization motifs were identified in PKR. One resides in an amphipathic α-helix between residues 60 and 75 in the first dsRBM a region that may be influenced by dsRNA binding.[58] The other is localized between residues 244 and 296 outside of the dsRBD.[59]

Tryptophan fluorescence quenching demonstrated that HIV TAR RNA binding to intact PKR induces a conformational change in the catalytic domain.[57] Small angle neutron scattering demonstrated that a PKR dimer undergoes an extensive conformational change to an elongated form upon binding to one molecule of HIV TAR RNA. Deletion of the dsRBD (residues 1-227) yields a constitutively active apparently monomeric eIF2αkinase.[40] Based on these observations it was proposed that the dsRBD folds back to block the ATP binding site in the PKR catalytic domain (Figure 2.1B). Upon binding to dsRNA, the dsRBD extends out to elongate the molecule and makes accessible the ATP binding site within the catalytic domain. Support for this model is also derived from the observation that the amino-terminal

region of PKR can interact with the C-terminal kinase domain in a yeast two-hybrid assay.[60] This type of kinase regulation is reminiscent of pp60 Src in which the regulatory domain acts as an inhibitor of the kinase activity. The conformational change induced by dsRNA binding may also promote dimerization and subsequent trans-autophosphorylation;[40] however, dsRNA binding is not required for trans-autophosphorylation.

2.2.3 PKR Autophosphorylation

Autophosphorylation of PKR is the only mechanism known by which PKR becomes phosphorylated. Autophosphorylation of PKR occurs at many sites, although only two phosphorylation sites appear to significantly activate PKR. Numerous protein kinases are activated by phosphorylation of residues between kinase subdomains VII and VIII.[61] These activating phosphorylation sites are located at the N-terminal side of the conserved AlaProGlu (APE) motif in kinase subdomain VIII. Phosphorylation within this so-called activation loop may stimulate either (1) the binding of protein substrate or (2) the binding of ATP and the rate of phosphoryl transfer. PKR contains two Thr residues at positions 446 and 451 within this activation loop. Mass spectrometry analysis confirmed phosphorylation at Thr446, but not at Thr451, in PKR prepared from yeast.[62] Mutation of Thr446 to Ala substantially reduced PKR activity supporting a role for phosphorylation at this site for activation of PKR62. However, mutation at Thr551 completely abolished activity. Therefore, at present it is not known whether Thr551 is phosphorylated or if the Thr residue is functionally important for PKR activity.

A cluster of phosphorylation sites was identified between the dsRBD and the protein kinase domain. Mutation at Thr258 reduced the efficiency of autophosphorylation and substrate phosphorylation in vitro and partially inhibited kinase function *in vivo*.[63] However, mutations at two neighboring sites of autophosphorylation, Ser242 and Thr255, had little effect on kinase activity. The triple mutant retained significant activity, suggesting that other sites within PKR must be required for full activation. Mass spectrometry also detected phosphorylation at multiple residues from 88 to 97 between the two dsRBMs, although mutation of these residues had no effect.[62]

2.3 ROLE OF PKR IN THE INNATE IMMUNE RESPONSE

The innate immune response is initiated by the recognition of invariant molecules of infectious agents such as lipopolysaccharide (LPS) on Gram-positive bacteria or dsRNA, a common intermediate in viral replication. Binding of these molecules to specific cellular receptors activates distinct signaling pathways designed to limit or eliminate the pathogens. While the recognition of LPS is mediated by the Toll-family of extracellular or transmembrane pattern recognition receptors (i.e., CD14 and Toll-like receptor 4, respectively),[64] dsRNA binds to intracellular receptors such as PKR.

2.3.1 The Antiviral Actions of PKR

Eukaryotic viruses and their multicellular hosts have co-evolved complex interrelationships that permit virus reproduction without destruction of the host. The interferon-

induced cellular antiviral response is the first line of defense against viral infection. Upon viral infection, expression of interferon is induced at the transcriptional level. Interferon is secreted to protect adjacent cells from secondary infection, thereby limiting viral spread. Type I interferons are composed of the different types of interferon α that are produced in leukocytes and interferon β that is produced in fibroblasts and epithelial cells. Type I interferons bind to their receptors and activate signaling pathways to induce transcription of >30 genes.[10] Two of these genes encode latent enzymes, one being PKR and the other being Rnase L, that both require the presence of dsRNA for activation. Latent 2'-5'-oligoadenylate synthetase is activated by dsRNA to increase the synthesis of 2'-5'oligoadenylates that subsequently activate 2'-5'-A-dependent RNase L. Activated RNase L nonspecifically degrades single-stranded RNAs and thus limits virus production. Concomitantly, dsRNA binds to and activates PKR. This cellular antiviral response limits virus spread by preventing viral protein translation and induction of apoptosis. As a consequence, viruses have evolved multiple gene products that act through different mechanisms to inhibit this anti-viral response mediated by PKR. It is essential for viruses to inhibit PKR activity so as to replicate and/or establish their latent states. In addition to PKR activation, it is well known that viral infection activates other proapoptotic signaling pathways through down-regulation of Bcl-2, inactivation of the tumor suppressor p53, and inhibition of caspase activation.[65,66] It is now recognized that PKR, and to a lesser extent 2'-5'-A-dependent RNase L, are also cellular targets that viruses inactivate to prevent apoptosis.

PKR that is activated by dsRNA produced as a viral replication intermediate mounts an antiviral response through several different mechanisms that include (1) induction of interferon gene transcription through the interferon response factor IRF-1 and NFκB[67], (2) inhibition of viral protein synthesis at the level of initiation, and (3) induction of apoptosis with subsequent auto-digestion of the cell. These processes are discussed below. To prevent cell death and establish a productive infection, viruses must overcome the interferon-induced blockade on viral replication imposed by PKR. The level of PKR and its activation state in the cell can predict the outcome of a viral infection. For those viruses that do not have effective means for inactivating PKR, pathogenesis may be attributed to viral-induced host cell apoptosis. For example, overexpression of PKR renders cells resistant to encephalomyocarditis virus (EMCV) infection, an interferon sensitive virus, because cells undergo rapid apoptosis.[68] In addition, increased PKR levels sensitize cells to apoptosis induced by influenza virus infection.[69] In contrast, reduction in the PKR level converts a lytic EMCV infection into a persistent infection.[70]

The essential role for PKR in the innate antiviral response was demonstrated using PKR-deficient mice and mouse embryo fibroblasts (MEFs) created by homologous recombination in embryonic stem cells. In these mice and MEFs the antiviral effects of interferon-α against EMCV infection were impaired, although host responses to a number of viruses including vaccinia virus were described as normal.[71,72] More strikingly, mice lacking PKR were predisposed to lethal intranasal infection by the usually innocuous vesicular stomatitis virus and had increased susceptibility to influenza virus.[73] The role of PKR in viral infection is underscored by the observation that viruses, such as hepatitis C virus (HCV)[74–77] vaccinia virus[60,78,79] and possibly HIV,[80–82] have individually evolved multiple mechanisms to

inactivate the PKR pathway. The significance of PKR in viral replication provides a basis for rational drug design to prevent viral pathogenesis. Indeed, interferon-α is presently the most effective modality used to treat hepatitis C infection.

2.3.2 Viral Mechanisms to Inhibit PKR

Eukaryotic viruses have evolved diverse mechanisms to inhibit PKR function[74] (Figure 2.2). These mechanisms include (1) production of RNAs or proteins that act as dsRNA antagonists to prevent virus infection, (2) synthesis of proteins that bind and sequester dsRNA, (3) synthesis of proteins that prevent PKR dimerization, (4) production of proteins that interfere with PKR binding to substrate eIF2α, (5) production of proteins that direct phosphatase PP1 to dephosphorylate eIF2α and PKR, and (6) inhibition of PKR expression or enhanced degradation of PKR. The diverse mechanisms by which viruses have evolved to inhibit PKR support the essential role of PKR in the host antiviral response. Indeed, it appears that all interferon resistant viruses have evolved mechanisms to target PKR.

Many viruses prevent PKR activation by blocking PKR interaction with viral RNA activators. For example, the DNA tumor viruses adenovirus and Epstein Barr virus encode small RNA polymerase III transcripts that bind and prevent PKR activation by dsRNA.[83,84] The influenza NS1 gene product interacts with the PKR dsRBD to inhibit PKR activation and also binds dsRNA.[59,76,85] Hepatitis delta viral genomic RNA interferes with PKR activation in response to dsRNA.[86] Vaccinia virus E3L[60,83,87] and reovirus αF3[88] are dsRNA-binding proteins that bind and sequester dsRNA to prevent PKR activation. The herpes simplex virus type 1 Us11 protein is a ribosome-associated protein that binds RNA and can prevent PKR activation by interacting with the PKR dsRBD.[89]

Some viruses target the PKR kinase domain. Vaccinia virus K3L,[78,90] swine pox virus C8L,[91] and HCV E2[75] have homology to the eIF2α phosphorylation site and act as pseudosubstrates that bind and prevent PKR interaction with eIF2α. The baculovirus PK2 gene product is a truncated eIF2α kinase homologue that binds and prevents PKR autophosphorylation.[92] In contrast, HIV Tat acts as a substrate that is phosphorylated by PKR and functions as a competitive inhibitor of eIF2α phosphorylation.[80,93] The HCV NS5A protein binds to PKR and prevents dimerization.[94]

Finally, some viruses activate cellular gene products to prevent PKR signaling. For example, influenza virus activates the cellular PKR inhibitor P58^{IPK} to bind to and prevent PKR dimerization.[59] The herpes simplex virus (HSV1) protein (34.5 directs the cellular protein phosphatase 1 (PP1) to dephosphorylate PKR and eIF2α.[95] Poliovirus is reported to activate a protease that induces PKR degradation.[96] Finally, the large T antigen of simian virus 40 (SV40) induces a bypass of the translational block imposed by eIF2α phosphorylation, although the mechanism is unknown.[97]

2.4 ROLE OF PKR IN CELL CYCLE CONTROL AND APOTSOSIS

Numerous studies support that PKR activation inhibits cell growth. Overexpression of wild-type PKR is toxic to yeast, insect, and mouse cells.[92,98,99] Expression of a

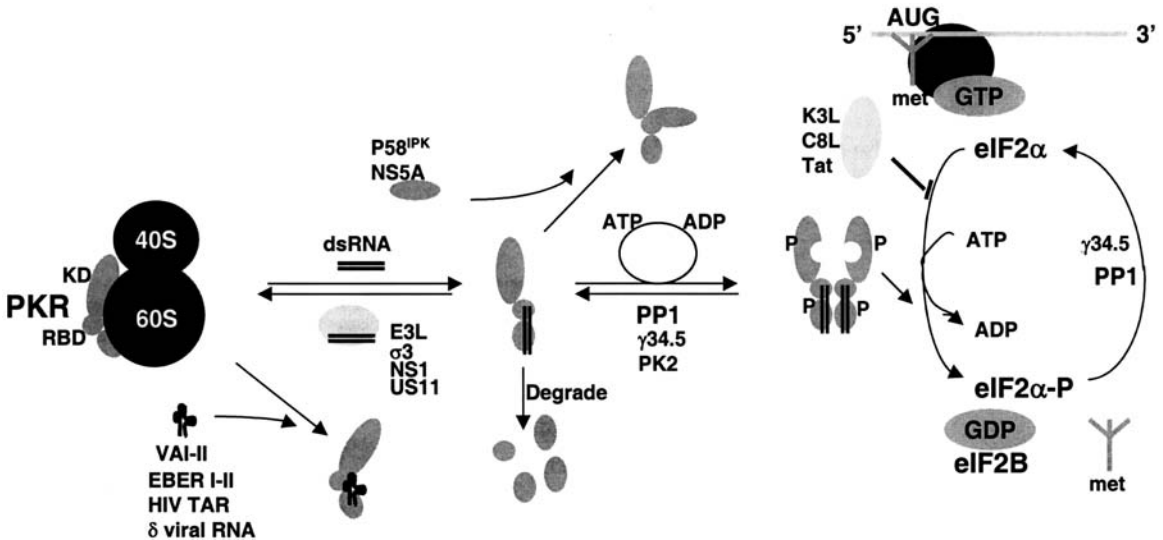

FIGURE 2.2 Viral Inhibitors of PKR. The mechanism of PKR activation is depicted showing the different sites for regulation by viral gene products. Viral inhibitors of the PKR pathway. PKR is depicted as a monomer with a kinase domain (KD) and two dsRNA binding domains (RBD) bound to the 60S ribosomal subunit. The binding of dsRNA induces a conformational change to promote PKR dimerization, autophosphorylation, and activation of the eIF2α kinase activity. Viral inhibitors that act through different mechanisms are depicted. Adenovirus VA RNAs, Epstein-Barr virus EBER RNAs, and HIV *trans*-activator responsive region TAR RNA bind and inhibit PKR and presumably displace the PKR from the ribosome. Numerous viral proteins, such as vaccinia virus E3L, influenza virus NS1, reovirus αF3, and Herpes simplex virus 1 (HSV-1) US11 bind and sequester dsRNA, thereby preventing activation by dsRNA. Vaccinia virus K3L, swine pox virus C8L, and HIV *trans*-activating transcriptional activator Tat act to inhibit PKR binding to eIF2α. Baculovirus PK2 is a truncated PKR homologue that binds and prevents PKR autophosphorylation. Protein phosphatase PP1 dephosphorylates phosphorylated eIF2α as well as phosphorylated PKR. HSV-1 encodes a protein α34.5 that targets and activates PP1. Hepatitis C virus (HCV) non-structural protein NS5A prevents PKR dimerization. In addition, influenza virus activates a cellular inhibitor $P58^{IPK}$ that also inhibits PKR dimerization. Poliovirus induces PKR degradation. See text for details.

catalytically inactive mutant PKR transforms NIH3T3 cells to form tumors in nude mice and promotes growth in HeLa cells.[100–102] This observation was ascribed to a *trans*-dominant inhibitory effect of the mutant enzyme on the endogenous wild-type PKR and implicated *PKR* as a tumor suppressor gene. In addition, overexpression of a non-phosphorylatable Ser51Ala mutant eIF″[103] as well as the cellular PKR inhibitors, such as p58IPK[104] and HIV TAR element binding protein (TRBP),[105] transforms NIH3T3 cells, similar to dominant negative mutants of PKR. A tumor suppressor activity for PKR is consistent with a rearrangement detected in the *PKR* gene in a lymphocytic leukemia cell line.[106] However, the levels of PKR activity observed in human breast cancer cells are controversial. Savinova and coworkers reported elevated expression of PKR in human breast cancer cells, although the actual level of PKR activity was reduced.[107] In contrast, Kim et al. observed elevated PKR protein, PKR activity and eIF2α phosphorylation in human breast cancer cells, suggesting a positive regulatory role for PKR in growth control.[108] At present, there is no compelling evidence that supports a tumor suppressor function for PKR in humans.

2.4.1 PKR Regulation of Cell Cycle and Induction of Apoptosis

Numerous studies demonstrated that the growth inhibitory activity of PKR could be attributed to apoptosis when overexpressed and/or activated in mammalian cells.[109–113] Transient or inducible overexpression of PKR activates apoptosis in different cell culture systems.[109–115] Overexpression of Bcl-2 protects cells from PKR-induced apoptosis, suggesting that Bcl-2 is downstream of PKR.[116] In addition, PKR induces apoptosis through Fas-associated protein with death domain (FADD)-mediated activation of caspase 8, although this response is not dependent on the Fas or TNFα (TNFR-1) receptors.[117]

Studies suggest that PKR activation induces cell cycle arrest.[118] Phosphorylation of eIF2α is required for the control of cyclin D1 translation and G1 cell cycle arrest that occur upon PKR activation.[119] When PKR expression was induced, cells accumulated in the G1 phase of the cell cycle and increased expression of effector molecules forming death-induced signaling complexes (DISC) such as Fas, TNFR-1, FADD and caspase 8. In contrast, expression of a dominant-negative PKR mutant reduced the transcription of genes encoding molecules in DISC complexes.[114,115] It is possible that PKR may directly activate transcription factors for genes encoding pro-apoptotic functions. Alternatively, activated PKR may affect the levels of specific transcription factors through an eIF2α phosphorylation-dependent pathway.(See Section 2.4.4 below).

PKR activation is also associated with differentiation in some cell culture systems. PKR activation occurs upon growth arrest of murine 3T3-F442A fibroblasts subsequent to their differentiation into adipocytes.[120–122] In the myogenic cell line L8 there is an association between the extent of PKR activation and the level of muscle-specific protein expression.[123] In this system, expression of a dominant-negative PKR mutant interfered with myogenesis.[124]

The localization of PKR to the rough ER membrane suggests that it might signal in response to ER stress. Indeed, activation of PKR occurs in response to calcium

depletion from the ER.[125] In addition, expression of a dominant-negative PKR mutant inhibited eIF2α phosphorylation and protected from translational inhibition in response to ER stress induced by calcium depletion or reduction of disulfide bonds mediated by dithiothreitol.[125,126] In contrast, translation and eIF2α phosphorylation were not affected upon induction of ER stress in PKR-deficient mouse embryonic fibroblasts (MEFs).[127] These results support that PKR does not mediate eFI2″ phosphorylation in response to ER stress and suggest that the dominant negative mutant PKR may not only inhibit PKR. Recently, it was shown that either deletion of PERK/PEK or expression of a PERK/PEK dominant-negative mutant prevented the translational attenuation induced by calcium depletion from the ER or from accumulation of unfolded proteins in the ER.[8,127] These results directly implicate a role for PERK/PEK in ER stress-induced phosphorylation of eIF2α. More recently, it was shown that ER stress induces eIF2α phosphorylation to upregulate the translation of factors required for transcriptional induction of genes encoding protein chaperone and additional adaptive functions.

2.4.2 The Phenotype of PKR-/- Null Mice

Based on the effect of PKR on the fundamental cellular processes of cell growth, differentiation and apoptosis, it was surprising that deletion of the PKR gene did not have a significant phenotype or lead to an increase in tumor incidence.[71,72] These observations bring into question the significance of the function of PKR as a tumor suppressor gene. The PKR-null mouse derived by Yang et al. deleted the N-terminal first two exons of the PKR gene. As a consequence, this deletion has the potential to produce a protein that, on the basis of site-directed deletion mutagenesis experiments,[101] may be expected to have residual PKR activity. However, Abraham et al. deleted the C-terminal region in the kinase domain of PKR and obtained a similar normal growth phenotype for the mouse.[72] In the mouse derived by Yang et al., the induction of type 1 interferon gene expression by dsRNA or by virus was not affected. However, the antiviral response induced by interferon-α and dsRNA toward EMCV was diminished.[71] In contrast, the C-terminal PKR deletion mouse did not show any effect on vaccinia virus (an interferon resistant virus) replication or on influenza virus (an interferon sensitive virus) lethality in the presence or absence of interferon-α/β treatment.[72] These studies support that another gene product may be functioning in a parallel pathway to PKR.

MEFs from the two strains of PKR-deleted mice displayed a low constitutive level of eIF2α phosphorylation, indicating that at least one additional eIF2α kinase is functioning.[72] MEFs homozygous for the PKR catalytic domain deletion had wild-type apoptotic responses to TNFα and influenza virus infection.[72] In contrast, MEFs prepared from the homozygous N-terminal PKR-null MEFs had an impaired apoptotic response to TNFα and dsRNA-mediated induction of type I interferon and activation of NFκB.[71] However, pretreatment with either type I or type II interferon restored the dsRNA responsiveness to one-half the level of that observed for wild-type MEFs.[71] Under these conditions, interferon treatment may activate transcription of the targeted PKR gene to increase the level of the N-terminal deleted PKR protein. Alternatively, these results might suggest that another interferon inducible gene

exists that encodes a function similar to PKR. Recently, it was suggested that interferon may activate cJun amino-terminal kinase (JNK) to restore dsRNA responsiveness.[67] At present it is not known what is responsible for the different phenotypes observed between the two PKR-null mouse lines. Because these PKR-null mice were back-crossed into different strains, this may contribute to the differences observed.

2.4.3 Actions Mediated Through eIF2α Phosphorylation

To elucidate the role of eIF2α phosphorylation in PKR-induced apoptosis, studies were performed with a Ser51Ala mutant eIF2α that is resistant to phosphorylation. Expression of this Ser51Ala mutant eIF2α protected cells from serum-deprivation-induced, TNFα-induced, and vaccinia virus-induced apoptosis.[110,128] Importantly, expression of a Ser51Asp mutant of eIF2α to mimic phosphorylated eIF2α inhibited protein synthesis and induced apoptosis in COS-1 monkey cells.[110] Apoptosis in these cells correlated with activation of caspase 3. These studies point to a direct role for eIF2α phosphorylation in promoting apoptosis under certain conditions.

More direct evidence was recently obtained through generation of a Ser51Ala mutation within the germline of the mouse by knock-in technology using Cre-Lox "in and out" homologous recombination. This approach was used to introduce the Ser51Ala mutation into the endogenous murine eIF2α gene without disrupting expression of the eIF2α gene.[129] This mutation prevents eIF2α phosphorylation in response to all known eIF2α kinases. Homozygous Ser51Ala mutant MEFs derived from these mice were resistant to a variety of apoptotic stimuli including treatment with type I interferon in the presence of poly rI:rC, treatment with TNFα, and serum deprivation. No effect was observed upon analysis of the heterozygous Ser51Ala MEFs. These results demonstrate that these treatments require eIF2α phosphorylation to effectively induce apoptosis.

The studies with cells harboring Ser51Ala mutant eIF2α strongly support that inhibition of translation initiation is required for some types of apoptosis. Further studies are required to elucidate how a reduction in protein synthesis initiation can induce an apoptotic cascade. Possible mechanisms include (1) translational inhibition of a short-lived anti-apoptotic gene product, such as an inhibitor of apoptosis (IAP)[130], (2) preferential translation of mRNA(s) encoding an effector(s) of apoptosis, or (3) an unidentified signaling mechanism from the translational apparatus into the apoptotic pathway. Support for the second hypothesis comes from the observation that the defective apoptotic response in the homozygous Ser51Ala eIF2α mutant MEFs can be complemented by protein synthesis inhibitors.

Surprisingly, mice that are homozygous for the Ser51Ala mutation displayed no significant phenotype. Therefore, although eIF2α phosphorylation at Ser51 is a ubiquitous regulatory step in protein synthesis that is conserved across all eukaryotic cells, it is not essential for mammalian development. These results support that the eIF2α kinases mediate environmental stress responses that do not occur during mammalian embryonic life. However, upon mating of the heterozygotes, homozygous Ser51Ala progeny were not detected. Upon close analysis, the homozygous mice did develop but died within 24 hr after birth.[129] The lethality in the homozygous

Ser51Ala mice was due to severe hypoglycemia. Further studies should elucidate how altered regulation of protein synthesis initiation may interfere with glucose metabolism.

2.4.4 Increased Translation of Selective mRNAs by eIF2α Phosphorylation

In yeast *S. cerevisiae*, it is known that eIF2α phosphorylation by the amino acid general control eIF2 kinase Gcn2p selectively increases the translation of *GCN4* mRNA.[6,131,132] Gcn2p is activated upon amino acid deprivation in yeast and mammalian cells.[133] In yeast, Gcn2p has a domain that binds uncharged histidyl tRNA and phosphorylates eIF2α to increase translation of *GCN4* mRNA by permitting readthrough of four open reading frames upstream of the AUG codon encoding GCN4. Gcn4p is a basic leucine zipper transcription factor that activates transcription of the amino acid biosynthetic genes. In a similar manner, it was shown that the translation the mRNA encoding the activating transcription factor ATF4 is dependent on GCN2 or PERK kinase activation and eIF2α phosphorylation.[129,133] Similar to *GCN4* mRNA, ATF4 mRNA has multiple upstream open reading frames, and it is proposed that under conditions of limiting eIF2, i.e., when eIF2α is phosphorylated, the ribosome scans through the upstream AUG codons to initiate at the authentic initiation codon encoding ATF4. It is possible that additional transcription factors may be regulated at a translational level through eIF2α phosphorylation. Based on these findings, it would not be surprising that PKR activation may also lead to the selective translation of some mRNAs.

2.5 PKR ACTIVITY REGULATED BY CELLULAR FACTORS (FIGURE 2.3)

Numerous cellular proteins can bind to and modulate PKR activation in vitro. However, there are few examples where these regulators have been unambiguously demonstrated to have physiological significance in vivo. The identity and functional significance of these PKR activator(s) in the absence of a viral infection are largely unknown. A number of proteins that contain dsRBMs are able to interact with PKR, presumably through bridging by binding to dsRNA, and alter its activation.

2.5.1 PACT/RAX

PACT was isolated as a PKR-interacting protein that has three dsRBMs.[134] RAX is the murine homologue of human PACT.[135] PACT/RAX and NF90, the large subunit of the nuclear factor for activated T cells (NFAT),[23] are the only known protein activators of PKR. PACT/RAX is a 35 kDa protein that binds the dsRBD of PKR in a manner that does not require dsRNA and elicits its activation. Overexpression of PACT activates PKR, increases eIF2α phosphorylation, and induces apoptosis.[136] Interestingly, upon treatment of cells with sodium arsenite, heat shock, or peroxide, or upon IL-3 deprivation from IL-3-dependent cell lines, RAX was rapidly phosphorylated and associated with PKR leading to PKR autophosphorylation and acti-

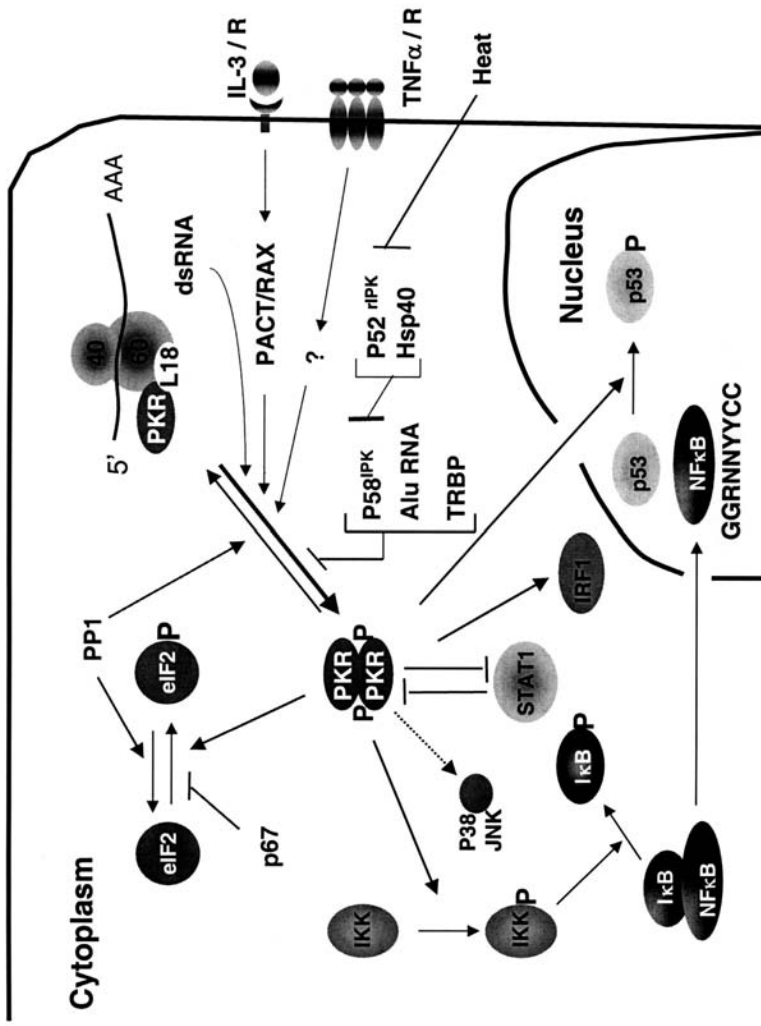

FIGURE 2.3 Cellular Regulators of PKR Signaling and Downstream Effectors. The mechanism of action for PKR cellular inhibitors is depicted in red. Pathways for PKR activation are shown in green. The major downstream effectors of PKR function are shown in bold arrows. Heat stress may signal through PACT/RAX or through p58^{IPK}.

vation.[135] Further studies are required to determine whether PACT/RAX couples membrane stress with regulation of protein synthesis through PKR activation.

2.5.2 CUG Repeats

Expanded trinucleotide repeats are a common feature of progressive genetic diseases such as myotonic dystrophy. These diseases are due to large expansions of trinucleotide repeats. Although the expansion causes nuclear retention of mRNAs, it is also possible that part of the mechanistic defect is due a cytoplasmic activity function. It was shown that CUG repeats can form stable hairpin structures that bind to PKR.[137] The threshold of binding was approximately 15 CUG repeats. Binding of CUG repeats to PKR was also shown to activate PKR *in vitro*. It is proposed that the disease could be, in part, due to a gain of function by expanded CUG repeats that bind dsRNA-binding proteins.

2.5.3 P58^{IPK}

PKR may also be regulated through numerous cellular inhibitors (Figure 2.3). P58^{IPK} was originally identified as a factor that is activated upon influenza virus infection that binds PKR, and prevents its dimerization and activation.[59] Through identifying protein-protein interactions and measuring the effect on PKR activation, Katze et al. proposed that P58^{IPK} might activate PKR in response to protein misfolding.[138] A model was proposed where P58^{IPK} functions as a co-chaperone and uses its J-domain to stimulate Hsp70 to modulate the conformation of PKR. In the absence of stress signals, P58^{IPK} is held in an inactive complex with Hsp40, Hsp70, and PKR. In response to stress, Hsp40 dissociates from P58^{IPK} so that P58^{IPK} may stimulate the ATPase activity of Hsp70 to promote refolding of PKR into a nonactive form.

2.5.4 HIV Tar-RNA Binding Protein (TRBP)

Any dsRNA-binding protein has the potential to regulate PKR activation. TRBP is cellular protein that binds the HIV TAR element, has a dsRNA-binding motif similar to PKR, and can inhibit PKR by preventing PKR dimerization.[54] Overexpression of either PKR inhibitor p58^{IPK} or TRBP in NIH3T3 cells inhibited apoptosis, similar to dominant-negative mutants of PKR.[104,105,139]

2.5.5 p67 Glycoprotein

p67 is an eIF2-associated 67 kDa glycoprotein that protects eIF2α from phosphorylation by binding to eIF2 and providing steric interference from PKR.[140] p67 was originally identified as a co-purifying component in eIF2 fractions. It contains multiple O-linked N-acetyl-β-D-glucosamine (GlcNAc) residues that are important to inhibit PKR. Overexpression of p67 inhibited PKR-mediated phosphorylation of eIF2α and reduced NFκB activation.[140,141] p67 transcription correlates with cell growth, and it was proposed that p67 may couple cell growth to translation via its inhibitory effect on eIF2α.[142]

2.5.6 Ribosomal Subunit Protein L18

L18 is a 60S ribosomal subunit protein that inhibits PKR activity *in vitro* and *in vivo*. Although L18 protein did not directly bind dsRNA, it did compete with dsRNA for binding to the first dsRBM of PKR.[48] L18 is frequently overexpressed in colorectal cancer tissue, and this may potentiate protein synthesis in the transformed cells.[48,143]

2.5.7 Alu RNAs

Alu RNAs are RNA polymerase III transcripts that are induced upon stress conditions and, at high concentrations, can inhibit PKR through binding to its dsRBD *in vitro* and *in vivo*.[144] At low concentrations, Alu RNA can activate PKR. Alu RNAs were proposed to facilitate tolerance to cell stress through reversing translational inhibition mediated by PKR activation.[144]

2.5.8 GADD34

GADD34 (also called MyD116) is a growth-arrest and DNA-damage inducible gene product of 34 kDa that has homology to HSV-1 α34.5. HSV-1 α34.5 and GADD34 both act to direct PP1 phosphatase to dephosphorylate eIF2α.[95] GADD34 can complement HSV-1 having a deletion in α34.5.[145] Because GADD34 is induced under stress conditions, it is believed that it functions to reverse the attenuation of protein synthesis upon recovery from the acute stress.

2.5.9 Less Well Characterized PKR Inhibitors

Several cellular modulators of PKR activity have been identified but poorly characterized. A 15 kDa protein inhibitor of PKR (dRF) was identified that is induced upon differentiation of 3T3-F442A cells into adipocytes.[120,121] An inhibitor was induced upon Ras transformation of BALB/C 3T3 fibroblasts,[146] which may also play a role in Ras-dependent inhibition of PKR that is required to permit viral protein synthesis in Ras transformed cells.[147] Future studies are required to identify the physiological significance of these inhibitors.

2.6 ROLE IN SIGNAL TRANSDUCTION AND TRANSCRIPTIONAL ACTIVATION

The function of PKR was originally identified in the context of a viral infection through its effect on eIF2α phosphorylation and translational inhibition. However, PKR also is reported to function in a wide range of cellular processes involving transcriptional induction and signaling pathways activated by specific cytokines, growth factors, dsRNA, and extracellular stress. Although the detailed mechanisms are largely unknown, PKR was shown to be required for optimal activation of several other protein kinases, such as P38, JNK, and IKK,[67,148–150] and transcription factors such as nuclear factor κB (NFκB), P53, signal transducer and activator of transcrip-

tion 1 (STAT1), activating transcription factor 2 (ATF2), and interferon regulatory factor 1 (IRF1).[15–17,19,151–153] Following is a brief description of some of these results.

2.6.1 Growth Factor Signaling

PKR is implicated in signaling from PDGF and IL-3 growth factors[154,155] (Figure 2.3). IL-3 deprivation from IL-3-dependent cells results in PKR activation and a decreased rate of protein synthesis.[155] Interfering with PKR expression disrupted PDGF signaling.[154] Finally, serum-deprivation induced apoptosis in a manner that required PKR and eIF2 phosphorylation.[110] However, the mechanism(s) by which PKR mediates these responses is not known.

2.6.2 Activation of Nuclear Factor κ for B Cells (NFκB)

PKR can lead to activation of the transcription factor NFκB that promotes a pro-inflammatory response.[15,16,18,156] The NFκB heterodimer is held in an inactive complex with its inhibitor IkB.[157] In response to activators, IkB is phosphorylated on serine residues 32 and 36 to induce its ubiquitin-dependent proteasomal-mediated degradation, allowing NFkB to translocate to the nucleus. I6B phosphorylation is mediated by a 700–900 kDa multimeric complex called I6B kinase (IKK). IKK contains two catalytic subunits IKK″ and IKKβ, that form homo- and heterodimers.[158] IKKβ is the major effector of I6B phosphorylation in response to cytokines. A third component of IKK is the NFκB essential modulator (NEMO), which interacts with IKKβ and regulates the activity of IKK.

Activation of PKR in cells leads to phosphorylation of IκB and activation of NFκB.[15] However, the role and requirement for PKR in phosphorylation of IκB is not known and somewhat controversial at present. First, PKR is not required for IκB phosphorylation, since PKR-/- null cells are not defective in cytokine-mediated IκB phosphorylation and NFκB activation.[67,150,158] However, cells defective in PKR cannot activate NFκB in response to dsRNA.[16,71] In addition, fibroblast and endothelial cellular responses to TNFα and macrophage responses to lipopolysaccharide (LPS) are defective in PKR-/- null cells, suggesting these molecules might signal through PKR in these cells.[115,112,156,159] However, the defect in NFκB activation in response to dsRNA or TNFα is not absolute in the PKR-/- null cells.[67] Several studies have shown that fibroblasts lacking either PKR or the β subunit of I6κB kinase (IKKβ) are defective in both dsRNA and VSV-mediated induction of interferon.[16,67,71] The differences in the requirement for PKR were suggested to depend on when the response was measured. PKR appears to increase the rate of NFκB activation.[67] Therefore, differences dependent on the presence of PKR may only be observed at early time points after stimulation. Finally, recent studies demonstrate that the protein kinase activity of PKR is not required activation of IκB *in vivo* and *in vitro* and that PKR interacts with IKKβ.[67,160] It is thought that PKR might provide a scaffold on which IKK activation may occur. Although NFκB provides primarily an anti-apoptotic function, numerous reports have correlated NFκB activation with apoptosis.[18] NFκB induces transcription of several death-promoting transcription factors including p53 and cMyc as well as other death-promoting genes, e.g., Fas, Fas ligand,

IRF-1, and caspase-1. Therefore, it is possible that signaling through PKR to activate NFκB might provide an apoptotic stimulus.

2.6.3 Tumor Suppressor P53

p53 is a transcription factor that regulates cell cycle in response to DNA damage induced by genotoxic stress. TNF″-induced apoptosis in U937 cells correlates with induction of p53.[161] PKR can bind and phosphorylate the carboxy-terminus of p53 at residue Ser392, although the significance of this phosphorylation is not known.[153] Both the transcriptional activity and cell cycle arrest functions of p53 are impaired in PKR-/- null cells.[19]

2.6.4 Signal Transducer and Activator of Transcription (STAT1)

PKR mediates interferon- and dsRNA- signaling pathways by modulating the function of the signal transducer and activator of transcription STAT1. PKR binds to STAT1 and inhibits its transcription activating function.[17] The interaction with STAT1 does not require the kinase activity of PKR. In addition, STAT1 binding to PKR inhibits PKR activation and phosphorylation of eIF2α.[162] Therefore, it appears that PKR may regulate STAT1 activity and STAT1 may also regulate PKR and translational control.

2.7 CONSIDERATIONS FOR THE FUTURE

Extensive information supports a fundamental role for PKR in the interferon-induced antiviral response. The structure of PKR and its mechanism of activation by dsRNA with subsequent inhibition of protein synthesis through phosphorylation of eIF2α are well characterized. In addition, recent results support that PKR signaling is fundamental in responses to cytokines and cellular stress, transcriptional activation, cell growth, regulation of cell differentiation and induction of apoptosis. Analyses of cells that express a non-phosphorylatable mutant eIF2α support that reduced translation initiation may be a primary mechanism to activate PKR-mediated apoptosis in response to a variety of stress conditions. It is not known how reduced translation initiation may activate apoptosis. Studies need to identify what mRNAs are preferentially translated upon eIF2α phosphorylation, similar to ATF4, and whether any of these encode functions important in the proapoptotic response. It is also important to identify which inhibitors of apoptosis having short half-lives and whether they are important in the PKR-mediated apoptotic response. The mild phenotype of the PKR-/- null mice support PKR provides primarily a stress-response-signaling pathway that is only activated upon appropriate signals, such as viral infection or heat shock or growth factor withdrawal. Therefore, to understand the full range of PKR functions, the PKR-/- null mice need to be carefully evaluated under a variety of different stress conditions.

The importance and mechanism of PKR activation and action in cellular processes other than translation initiation need to be elucidated. It is apparent that PKR

signals into multiple transcription pathways, however, the significance of PKR in regulation of these pathways needs to be characterized *in vivo*. To date, there have not been any physiological consequences of PKR gene deletion in the mouse other than responses to cytokines and viral infection. It will be important to carefully evaluate different responses in the PKR-deficient mice. Many mechanisms concerning the role of PKR in apoptosis have been proposed, so it will be necessary to identify how these mechanisms are integrated together as well as with the known mediators and executioners of cell death. Finally, the potential for pharmacological intervention in these processes makes PKR an attractive therapeutic target for drug discovery to reduce viral pathogenesis and should be explored.

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3 The Double-Stranded RNA Viruses of *Saccharomyces Cerevisiae*

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CONTENTS

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3.1 SUMMARY

There are two families of dsRNA viruses of yeast, L-A and L-BC, each having a single-segment genome of about 4.6 kb encoding the viral major coat protein (Gag) and the transcriptase-replicase (Pol). The latter is expressed as a Gag-Pol fusion protein formed by -1 ribosomal frameshifting. Satellite dsRNAs of L-A, called M_1, M_2,…, encode killer toxins—secreted protein toxins that kill other cells but not the secreting cell. The mechanisms by which L–A is transcribed, replicated, and packaged have been examined in considerable detail. Studies of expression of L-A and M information have led to the definition of hormone-processing enzymes (the Kex2 family), N-acetyltransferases (the Mak3 family), a system blocking expression of non-poly(A) mRNA (the Ski proteins), and proteins controlling maintenance of translational reading frame (Mof proteins and 5S rRNA). This system has applications in fermentation, in understanding the clinically important dsRNA viruses, and potentially in treatment of yeast infections.

3.2 DISCOVERY OF DSRNA VIRUSES IN YEAST

In 1963, Makower and Bevan reported a killer phenomenon in *Saccharomyces cerevisiae.*[1] Some strains secrete a toxin that is lethal to other strains but not to the secreting strain itself. The ability to secrete the killer toxin is called the K^+ phenotype, while the ability to resist the effects of the toxin are the R^+ phenotype (Figure 3.1). The toxin is a protein[2,3] encoded by a replicating dsRNA (M dsRNA).[4–6] Several killer toxin-immunity specificities have been described and these correspond to different M dsRNAs, called M_2, M_{28}, etc.[7–9] M dsRNA is a satellite RNA of the L-A dsRNA virus, meaning that L-A encodes the coat protein (Gag) that encapsidates M and the replicase-transcriptase (Pol) on which M depends.[10–13] The killer pheno-

FIGURE 3.1 The killer phenomenon. The K^+ strain secretes the killer toxin and kills the lawn of sensitive cells.

type was used for extensive genetic studies of this system, and the host functions revealed by this work have become of wide interest in their own right. Biochemical, molecular, and structural studies of the L-A virus have made it one of the best understood dsRNA viruses, although some of the most important questions remain unanswered. We will review the progress in our understanding of the L-A virus and the less intensively studied L-BC virus.

3.3 GENERALIZATIONS ABOUT FUNGAL VIRUSES AND RNA REPLICONS AND PRIONS

Strains of *Saccharomyces* harbour many different infectious elements (Table 3.1). In addition to the dsRNA viruses L-A (and its satellites) and L-BC, there are two naked (unencapsidated) cytoplasmic single-stranded RNA replicons, 20S RNA and 23S RNA, five retroviruses (or retrotransposons) and two prions (infectious proteins) (reviewed in Refs. 14–18). None of these elements pass outside of one cell and reenter another. Indeed, no fungal viruses are known to do so. All are infectious via cell-cell fusion, such as occurs during mating and hyphal anastomosis, and yet many are widely distributed. The retrotransposons are found in almost all strains, as are L-BC and 20S RNA. L-A and M dsRNAs and 23S RNA are also widely distributed. This can be explained by the frequent mating (of yeast) and hyphal fusion (of filamentous fungi) that occurs in nature.

Consistent with these intracellular life cycles, each of these replicons is associated with a structure resembling the core of the virion of the corresponding animal virus. The L-A structure has the same unusual symmetry properties and enzymatic activities as the cores of dsRNA viruses of animal cells. The Ty retroviruses have a structure similar to the cores of animal cell retroviruses, lacking the envelope structure. The 20S and 23S RNAs have no capsid per se but are associated in the cytoplasm with their respective replicases,[19,20] much like the intracellular phase of (+) ssRNA viruses.

While lytic viruses of bacteria and animals may lyse and leave their hosts, the viruses of yeast and other fungi must adapt to their host and establish a stable relationship. For example, Ty3 virion production is higher in haploid cells than in diploid cells and is turned on by exposure to the mating pheromones,[21] perhaps because mating is an opportunity for the virus to invade the potentially uninfected

TABLE 3.1
Infectious Elements of Saccharomyces Cerevisiae

Virus	Genome Size (kb)	Encoded Proteins		Features
dsRNA viruses				
L–A	4.6	Major coat protein (Gag)		-1 ribosomal frameshift
		RNA polymerase (Pol)		makes Gag-Pol
M_1, M_2, M_3, M_{28},…	1.6–1.8	Preprotoxin		Satellites of L–A; processing by Kex 1p, Kex2p
L–BC	4.6	Major coat protein (Gag)		
		RNA polymerase (Pol)		
ssRNA replicons				
20S RNA (=W dsRNA)	2.9	RNA polymerase		copy # induced by N-starvation or heat
23S RNA (=T dsRNA)	2.5	RNA polymerase		copy % induced by N-starvation or heat
Brome Mosaic Virus segment 3		CAT or URA3		Replicates dependent on segments 1 and 2 from cDNA clones
Flock House Virus		RNA polymerase, coat protein		Insect virus that replicates completely in *S. cerevisiae*
Retroviruses			**Group**	
Ty1, Ty2	5.8	Gag, Gag-Pol	*copia*	+1 ribosomal frameshift makes Gag-Pol
Ty3	5.3	Gag, Gag-Pol	*gypsy*	+1 ribosomal frameshift makes Gag-Pol
Ty4, Ty5	6.3	Gag, Gag-Pol	*copia*	
Prions	**Chromosomal gene**	**Normal form action**	**Prion phenotype**	
[URE3]	URE2	Ure2p—|Gln3p→Dal5p	USA uptake	
[PSI]	SUP35	Transl.release (w/Sup45p)	ter readthrough	

genome of the other mating partner. It is likely that the 10,000-fold induction of copy number of 20S RNA on certain media has a similar explanation.[22] L-A and L-BC viruses each maintain stable copy numbers at a level that does not detectably interfere with cell growth. At least part of this control is the repression of L-A copy number by the Ski proteins, their only essential function.

It is of interest that both the plant (+) strand RNA virus, brome mosaic virus (BMV), and the insect (+) strand RNA virus, Flock House virus, can replicate in yeast.[23,24] These are the "honorary yeast viruses," and these systems were developed to analyze host functions involved in viral replication, much as has been done for the dsRNA viruses that we will review here.

3.4 L-A VIRION STRUCTURE

Early sedimentation analysis indicated that L-A virions had about 120 Gag molecules per particle, a number incompatible with either a T = 1 or T = 3 icosahedral structure with a monomer of Gag as the asymmetric unit.[25] It was thus suggested that L-A was a T = 1 virus with an asymmetric dimer of Gag as the asymmetric unit.[25] Cryoelectron microscopy has defined the L-A virion structure with a resolution of 16 Å (Figure 3.2)[26,27] and demonstrates that it is indeed T = 1. Two different types of Gag molecules were observed with different environments (Figure 3.2).[26] This symmetry is identical to that subsequently found for the cores of rotavirus,[28] blue tongue virus,[29] cytoplasmic polyhedrosis virus,[30] phytoreovirus,[31] and bacteriophage ϕ6,[32] but it is not found outside the dsRNA viruses. Moreover, the many pores at several points in the structure appear suited for the entry of nucleotide triphophate substrates and exit of newly synthesized viral (+) strands.[26] It has been suggested that this unique structure may be an adaptation to the requirement, unique to the dsRNA viruses, that both (+) and (–) strand synthesis occur within the viral particles.[26] The RNA polymerase is fixed in position, because it is present as a Gag-Pol fusion protein, so the dsRNA must move past the polymerase for transcription to occur. The necessity to move around inside the particle requires that the RNA be less densely packed in the particle than if the particle were to be simply the carrier of information. In the case of dsRNA viruses of animal cells or bacteria, the core structure is the site of (–) strand synthesis during viral morphogenesis, and later, of (+) strand synthesis, after entry into the new cell, so it is suggested that the same functional requirements imply the same structure.

There is a striking radial variation of density within the particle suggestive of organized layers of RNA strands, but it has not yet been possible to visualize the RNA organization directly.[26] A similar structure has been found for rotavirus RNA within the particles.[33]

3.5 THE L-A VIRUS REPLICATION CYCLE AND THE HEADFUL REPLICATION MODEL

The L-A virus replication cycle is conservative,[34–37] meaning that the parental strands remain together after transcription (Figure 3.3). Both (+) and (–) strands are syn-

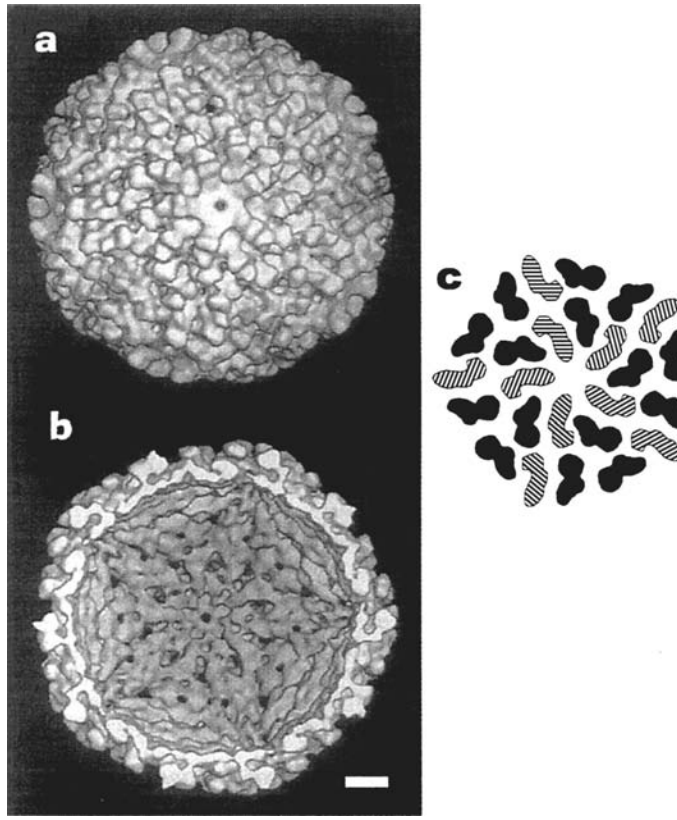

FIGURE 3.2 L-A viral particles. Three-dimensional reconstruction of the L-A virus capsid viewed from outside (a) or inside (b) along a five-fold axis.[26] The two different environments of individual Gag molecules are illustrated in (c). Bar = 50 Å.

thesized within viral particles,[38–40] a feature unique to dsRNA viruses. However, (+) and (–) strands are made at different points of the replication cycle (Figure 3.3), a character described as "asynchronous" synthesis.[38a,39,40] The (+) strands serve as mRNA, as the species packaged in new particles[41] and as the template for (–) strand synthesis (Figure 3.3).[39,40] There is no evidence for coordination of virus replication with the cell cycle.[34]

The L-A genome is present in intracellular viral particles that have an RNA polymerase activity.[10,38] All L-A virions have the same protein content, but their varied RNA content gives them variable densities, allowing clean separations on CsCl density gradients.[39,42] Thus, it was possible to separate viral particles containing only (+) strands from those with the genome's dsRNA form, and others with partially replicated molecules.

If viral particles are isolated from stationary phase cells, only dsRNA is present in the particles and only transcriptase [(+) strand synthesis] activity is observed.[38,39] However, if log phase cells are used, a lighter fraction of viral particles is found

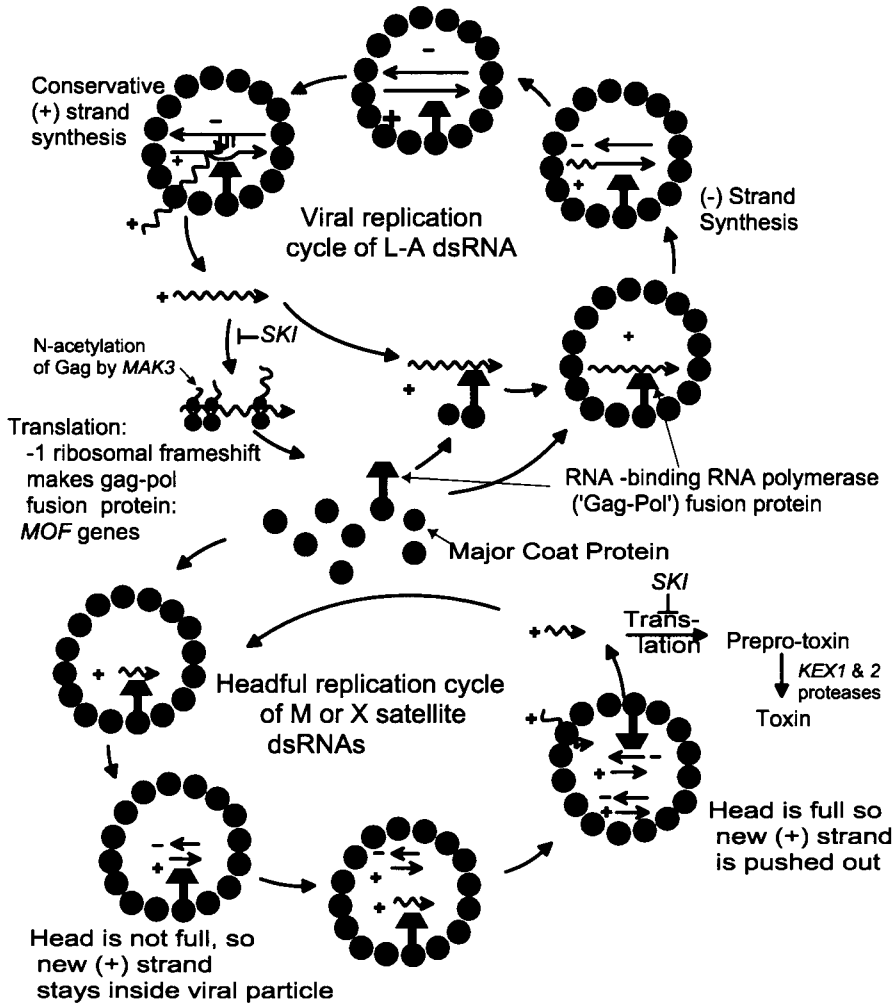

FIGURE 3.3 The L-A virus replication cycle. The sites of (+) and (–) strand synthesis of L-A and its satellites are shown along with the sites of action of chromosomal genes affecting replication and expression of viral RNAs.

that contains only viral (+) strands and carries out (–) strand synthesis, referred to as the *replication reaction*.[39] Having completed the (–) strand synthesis, these particles are then capable of carrying out the transcription reaction.[40] This indicates that the RNA synthetic reaction observed is a function of what RNA is in the particle. As will be seen below, this hypothesis was later confirmed by *in vitro* studies on opened empty particles.

Examination of viral particles containing M_1 dsRNA revealed that they can be separated into two different density classes.[42] The more dense particles have two M_1 dsRNA molecules per particle, and all of their (+) strand transcripts are extruded

from the particle. The less dense particles have only one M_1 dsRNA molecule per particle (hence their lower density) and retain 60% of their (+) strand transcripts within the particle.[42] These retained (+) strands can then be converted to dsRNA form. These results suggested the "headful replication model."[42] In contrast to the headful packaging model for T4 phage, this model suggests that a single (+) strand is packaged in the viral coat and is replicated until the capsid is full. Then, all new (+) strands are extruded from the particle.

Studies of a deletion mutant of L-A, called X dsRNA, retaining only 530 bases of its 4579 bp genome, provided strong support for the headful replication model.[25] Particles containing 1 to 8 copies of X dsRNA were found, as expected from the model: $530 \times 8 = 4240 < 4579$; $530 \times 9 = 4770 > 4579$. Finally, it was shown directly that a single molecule per particle of heterologous RNA containing the L-A packaging signal was packaged by the L-A virus[41] (see below).

3.6 L-A GENOME STRUCTURE

L-A has a linear dsRNA genome of 4579 bp with two overlapping open reading frames[13] (Figure 3.4). The 5' ORF encodes the major coat protein (Gag) while the 3' ORF has amino acid sequence motifs typical of RNA-dependent RNA polymerases (Pol).[13,43] Pol is expressed only as a fusion protein with Gag,[44] and this Gag-Pol fusion protein is expressed by a –1 ribosomal frameshift induced by a region of

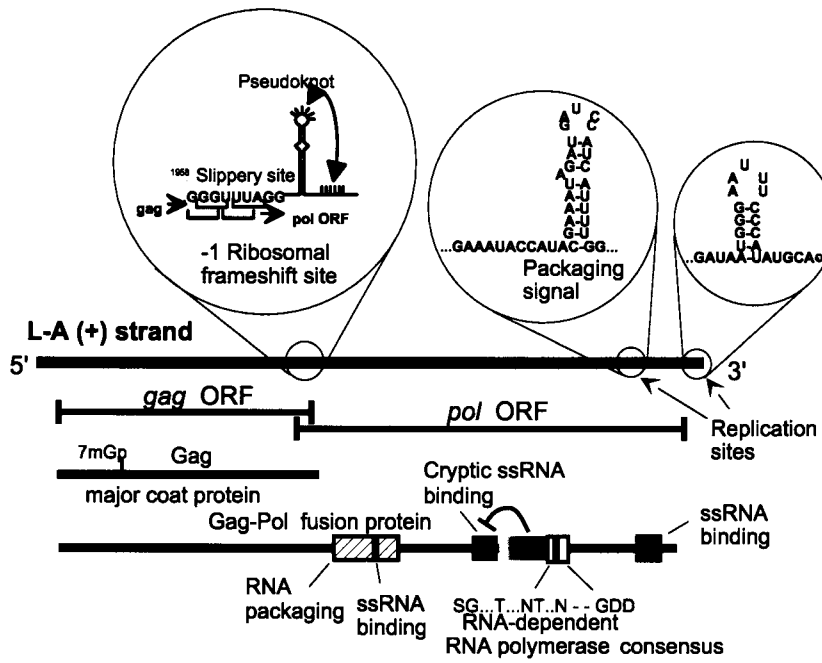

FIGURE 3.4 The L-A genome and sites important for RNA translation, packaging and replication.

overlap of the *gag* and *pol* open reading frames.[13,45,46] As discussed below in some detail, the mechanism of the –1 ribosomal frameshift is essentially the same as that of HIV and other retroviruses.

There is no detectable 5' cap structure for L-A,[47] and the 3' ends have an untemplated extra A residue,[48] but there is no poly(A) structure. As we will discuss below, these features have important implications for the factors controlling expression of the viral mRNAs.

3.7 *IN VITRO* SYSTEMS: REPLICATION, TRANSCRIPTION, AND RNA PACKAGING

Dialyzing L-A dsRNA-containing viral particles against low ionic strength buffer results in the particles releasing their RNA, possibly because the compact structure of the packaged RNA requires neutralizing ions.[49] These opened empty L-A particles specifically bind the (+) ssRNA of L-A, X, and M_1, exactly those RNAs that are packaged and replicated by L-A particles *in vivo*.[50]

3.7.1 The Packaging Site

The segment of the X (+) ssRNA necessary for specific binding to the opened empty particles was determined by deletion analysis and found to be a stem-loop structure equivalent to residues 4169–4204 of L-A[41,50,51] (Figure 3.4). The sequence of the stem was not important for binding activity, only that it be double stranded. An A residue protruding from the 5' side of the stem is necessary and must be an A, and the sequence of the loop is also important.[41] Computer analysis of the likely folding of the M_1 (+) strand showed that it has a stem-loop sequence (bases 1377–1416) with a 5' protruding A very similar to the X site-specific binding sequence[41] (Figure 3.5). This sequence proved to indeed serve as a sequence directing binding to the viral particles. The loop sequence of the L-A binding site is GAUCC, while that of M_1 is the similar GAUUC.

Proof that the site sufficient to specifically bind to the opened empty viral particles was the packaging site was obtained by introducing this site into a heterologous transcript and finding that, as a result, the heterologous transcripts were packaged in L-A viral particles *in vivo*.[41] The heterologous transcripts containing the packaging site were never found in particles containing L-A dsRNA, and the density of these viral particles indicated that only one heterologous transcript was packaged per viral particle. This constitutes strong support for the headful replication model described above.

Deletion mutants of M_1 dsRNA (called S mutants) lacking most of the toxin-coding region interfere with the propagation of the wildtype M_1 dsRNA,[52–55] A region of one of these, when expressed from a DNA plasmid, could weakly interfere with M_1 propagation.[56] This region includes the M_1 viral particle binding site described by Fujimura et al.,[41] and a second stem-loop structure that also has *in vitro* particle-binding and *in vivo* packaging activity[57] (Figure 3.5). This second M_1 packaging site resembles the others in being a stem-loop, with a similar loop sequence (GAUUC), and having an unpaired A residue at the same location relative

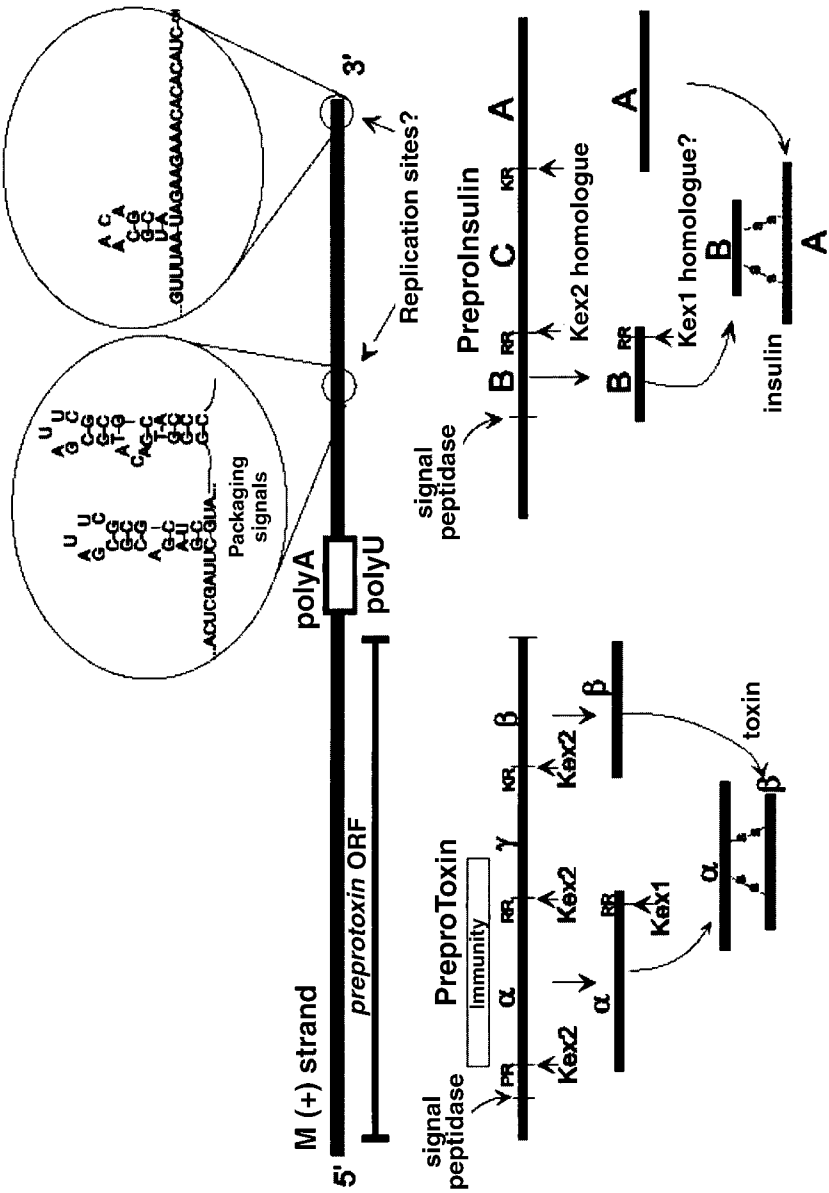

FIGURE 3.5 The M_1 genome, its sites for packaging replication and preprotoxin processing.

to the loop. However, the stem does not resume immediately below the unpaired A.[57]

Using *in vitro* selection for binding to viral particles of randomized sequences, the specificity of the binding site as described above was largely confirmed.[58] The selected sequences all had a protruding A, and the consensus loop was GAUUC. M_{28} dsRNA has a similar sequence that has been proposed to be a packaging signal.[59]

3.7.2 Protein Requirements for Packaging

The finding that the Pol domain of the Gag-Pol fusion protein had single-stranded RNA binding activity suggested a model for the mechanism of RNA packaging by the L-A virus.[44] It was proposed that the Pol domain bound the viral (+) strands, and that the Gag domain of the fusion protein associated with Gag protein monomers to assemble the virion, thus encapsidating a single (+) strand in each virion[44] (Figure 3.6). Further work led to the modification of minor aspects, but confirmation of the main features of this model. It was shown that the Pol domain was indeed necessary for packaging of viral (+) strands, but that the Gag domain was alone sufficient to form empty viral particles.[60]

However, the Pol domain actually has three single-stranded RNA binding regions, two of which are outside of the part of Pol that is necessary for RNA packaging.[61,62] One of the RNA binding domains extends from residues 172 to 190, entirely within the part of Pol (residues 67 to 213) that is necessary for RNA

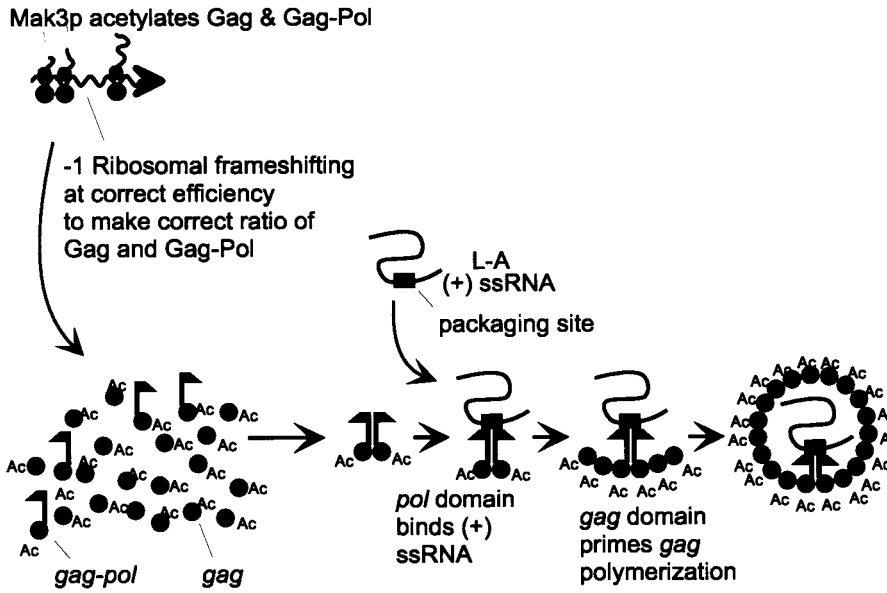

FIGURE 3.6 Packaging model for L-A virus particles. The Pol domain of the Gag-Pol fusion protein specifically binds to a viral (+) strand and the Gag domain interacts with Gag molecules to initiate capsid formation. Gag-Pol may be present as a dimer.

packaging.[60,61] Moreover, deletion of this N-terminal ssRNA binding domain prevents RNA packaging without affecting viral particle formation.[61] We thus believe that the 172–190 ssRNA binding site is responsible for viral (+) ssRNA packaging, but this binding is not specific for viral (+) strands *in vitro.*

It has not yet been possible to obtain soluble L-A RNA polymerase that is active in specifically binding, replicating, or transcribing suitable templates. It is possible that the L-A polymerase, which never naturally works outside of the viral particle, actually requires the particle structure for its activity. The rotavirus *in vitro* (–) strand RNA synthesis system is quite similar to the L-A system in that opened empty particles produced by low salt treatment enable replication of added templates.[63] In that system, it has been found that the *in vitro* activity of the RNA polymerase requires the major core structural protein, analogous to Gag.[64]

The ratio of Gag to Gag-Pol produced *in vivo* is determined by the efficiency of ribosomal frameshifting in the overlap region of the *Gag* and *Pol* reading frames (see below). Stable propagation of M_1 dsRNA, supported by viral proteins provided from a cDNA clone of L-A, requires an efficiency of ribosomal frameshifting within a factor of two of the normal ratio of about 1.9%.[65] This ratio provides the two Gag-Pol fusion protein molecules per particle actually found to be present.[66] Overproduction of Gag-Pol from an mRNA in which no frameshift event is necessary does not show such a specific requirement for the ratio of Gag to Gag-Pol.[66] Perhaps only when Gag and Gag-Pol are made from the same mRNA can they be incorporated into the same particles. This would explain the apparent conflict in the results, but there is no direct evidence for this idea.

3.7.3 Replication [(–) Strand Synthesis] by L-A Viral Particles

In vivo, the L-A virus particles replicate the (+) strands of L-A, X, and the various Ms. Adding any of these templates to the opened empty L-A particles in the presence of rNTPs and Mg^{2+} results in the synthesis of the corresponding (–) strands forming dsRNA.[49] Other ssRNA templates are not replicated. Using transcripts of a cDNA clone of X dsRNA and mutants of this clone the structural requirements for template activity were determined.[51]

Two sites on X (and therefore L-A) (+) strands are necessary for optimal template activity. The 3' end was essential, while deletion of an internal site, about 400 nucleotides from the 3' end and called the internal replication enhancer (IRE), reduced template activity about 10-fold.[51] The 3' end of X (+) strands consists of a stem-loop structure followed by AUGC3'OH (see Figure 3.4). Deletion of the 5' half of the stem loop virtually eliminated template activity as did mutation of either side of the stem to disrupt the base pairing. However, compensatory changes in the other side of the stem restored template activity proving that it was the stem as a structure, not the sequence that allowed the RNA to be replicated.[51] The final three bases were each changed to A residues, and this eliminated template activity in each case, as did deletion of the terminal C residue.[51] However, addition of an extra A residue had no effect.[51] This correlates with the fact that the L-A dsRNA is known to have an extra unpaired A residue on each 3' end.[48,67]

The IRE also includes a stem-loop structure that overlaps the viral binding site on the X (+) strands.[51] Several mutant transcripts seemed to differentiate the edges of the region needed for viral binding activity and for IRE activity, but the nonlinearity of the assays may have confounded the results. It remains possible that the VBS and IRE are the same site.

The mechanism of interaction of RNA polymerase with the internal and 3' end sites were investigated by examining a series of special templates.[68] The 3' end site alone was only poorly replicated, and, even in excess, competed poorly for replication with the intact template molecules. The IRE on a separate RNA molecule did not stimulate replication of the 3' end site, nor was it replicated. Rather, at only 2-fold molar excess, the IRE molecule inhibited conversion of the 3' end molecules to dsRNA form.[68] "Joint molecules," in which the IRE and 3' end are linked by hydrogen bonding, are replicated as well as are the normal molecules in which the continuous RNA chain connects these two functional regions.[68] The poorly replicated 3' ends lacking the IRE do not detectably bind to the opened empty particles (the RNA polymerase). When the IRE is attached, either as a continuous RNA molecule or as joint molecules, binding of the 3' end to the RNA polymerase is normal. These results indicate that the IRE promotes binding to the RNA polymerase. The 3' end is then in proximity to the polymerase and its replication is enchanced as a result. These results specifically rule out the idea that the RNA polymerase binds to the IRE and then tracks along the RNA to the 3' end. Rather, they suggest a model in which the 3' end loops to bind the polymerase (see Figure 3.7).[68]

After the *in vitro* replication reaction, the dsRNA product is released from particles. Of course, this contrasts with the *in vivo* situation in which the dsRNA product remains in the viral particles.

3.7.4 Protein Requirements for Replication

The most conserved amino acid residues of Pol are the most strictly required for propagating M dsRNA *in vivo*,[69] supporting the notion that Pol is indeed the RNA-dependent RNA polymerase. Interestingly, substituting these partially conserved domains from Sindbis virus or Reovirus into the L-A sequence produced a hybrid that was able to replicate M_1 dsRNA, although not stably.[69] Because of the similarities of the RNA polymerases of dsRNA viruses to those of (+) strand ssRNA viruses,[70] and the ability of these hybrid polymerases to partially replicate yeast viral RNA, it seems possible that screens could be developed using the killer system to find broad spectrum antiviral agents active against RNA dependent RNA polymerases.

There are three ssRNA binding domains in Pol, the most N-terminal of which (residues 172–190 of Pol) is necessary for packaging,[60,61] while the other two (residues 374–432 and 770–819) are dispensable for packaging but are necessary for M_1 propagation, presumably replication or transcription.[61,62] The ssRNA binding site within residues 374 to 432 is unusual in that its activity is detectable only after deletion of an adjacent region, residues 506–546.[62] Perhaps such an RNA binding region is responsible for conditional or temporary binding of viral RNA. None of the RNA binding activities of Pol made in *E. coli* are sequence specific.

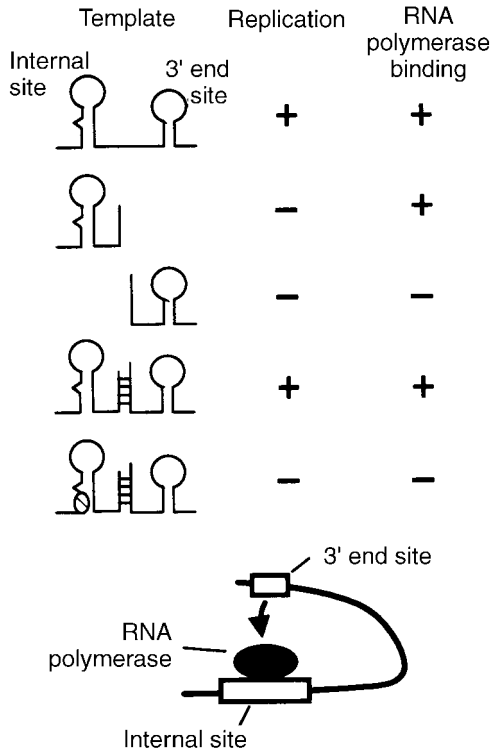

FIGURE 3.7 Model of replicase interaction with the internal replication enhancer site and 3' end. The data suggests that the RNA polymerase first binds to the internal site, and this brings it close to the 3' end where (–) strand synthesis begins.[68]

3.7.5 In Vitro Transcription [(+) Strand Synthesis] by L-A Viral Particles

L-A is the only dsRNA virus for which the template-dependent transcription reaction has been reconstituted *in vitro*.[37] The opened empty particles, when supplied with L-A, M_1 or X dsRNAs, synthesize the corresponding (+) ssRNAs. The enzyme does not have activity on L-BC, T or W dsRNAs from yeast, nor on bacteriophage ϕ6 dsRNA or rotavirus dsRNAs, indicating that it has the expected specificity.[37] Like the *in vivo* reaction, the *in vitro* template-dependent transcription reaction is conservative.[37] The transcriptase activity requires 40–80 µg/mL of dsRNA, while the replication reaction requires only 2 µg/mL of (+) ssRNA template.[37,49] Moreover, 20% polyethylene glycol is necessary to observe transcriptase activity, whereas 5% is optimal for the replicase. This indicates that the transcriptase has a lower affinity for its template than does the replicase for viral (+) strands. This may reflect the fact that the replicase has to pick out viral (+) strands from a sea of cellular mRNAs, whereas the template for the transcriptase is segregated in the viral particles and present at essentially infinite concentration.

3.8 TRANSLATION OF VIRAL mRNA AND POST-TRANSLATIONAL PROCESSING

The battleground between an RNA virus and its host is the translation apparatus. This can be seen in the utilization by Qβ replicase of Tu, Ts, and the S1 ribosomal protein, in the cleavage of eIF4G by poliovirus to take over translation with its uncapped mRNA, the cap-stealing by influenza viruses and bunyaviruses, and numerous other examples. The L-A virus of *S. cerevisiae* is constrained by its mode of propagation to avoid harming its host, but the lack of 5' cap and 3' poly(A) structure on its mRNA (see above) leaves it at a disadvantage in competition with cellular mRNAs. In addition, the requirements of particle assembly and the limited size of the L-A genome result in the virus using ribosomal frameshifting to make the Gag-Pol fusion protein.

3.8.1 Gag-Pol Fusion Protein Made by –1 Ribosomal Frameshifting

The 5' ORF of the L-A (+) strand encodes the major coat protein, called Gag.[13] The 3' ORF has the motifs typical of RNA-dependent RNA polymerases and is called Pol.[13] However, Pol is expressed only as a fusion protein with Gag, in the same way that retroviruses express their reverse transcriptase only as a fusion protein with their Gag.[13,44]

Ribosomal frameshifting is a process whereby a signal in the mRNA induces the ribosomes, at some frequency, to shift reading frame, resulting in a protein product that is encoded by two overlapping open reading frames (reviewed in Refs. 71–73). A +1 frameshift means that the ribosomes shift an extra base toward the 3' end of the mRNA, while a –1 frameshift means that the ribosomes shift one base toward the 5' end. Both +1 and –1 ribosomal frameshift signals are known, the former mostly in yeast retrotransposons[74] and the latter in retroviruses and some dsRNA and ssRNA viruses.

The region of L-A essential for ribosomal frameshifting is included in bases 1956 to 2027[45] (Figure 3.4). This region includes a classical slippery site, 5'G GGU UUA3', of the form X XXY YYZ (*gag* reading frame indicated) first shown by Jacks and Varmus to be responsible for ribosomal frameshifting by Rous sarcoma virus.[75] Ribosomal frameshifting occurs with the XXY sequence in the ribosomal P site paired with the tRNA carrying the partially completed *p*eptide, while the YYZ sequence is in the ribosomal A site paired with the *a*minoacyl tRNA. This unique sequence allows the P site and A site tRNAs to each slip back one base on the mRNA and re-pair with only the wobble base possibly mispaired. This is the "simultaneous slippage" model of –1 ribosomal frameshifting.[75] "X" can be any nucleotide, but "Y" can only be A or U, perhaps indicating that the tRNA-mRNA pairing in the A site of the ribosome must be loose to allow unpairing to occur.[45]

Following the slippery site is a stem-loop structure, whose loop can base-pair to a region further downstream,[45] an RNA pseudoknot. As in most other ribosomal frameshifting systems,[76] this pseudoknot structure is essential for efficient frameshifting by L-A.[46,77] Bruenn and coworkers showed that the pseudoknot serves to

make the ribosomes pause at the site of the frameshift,[77] a finding of general application to these systems.

As discussed above, the efficiency of ribosomal frameshifting determines the ratio of Gag to Gag-Pol produced and, in the case of L-A, this ratio appears to be critical (see detailed discussion above). Mutations in the slippery site[45,65] or in chromosomal genes affecting frameshift efficiency[65,78] (see below), or drugs that increase or decrease frameshift efficiency[79] each result in loss of M dsRNA and, in the last case, L-A as well. It has thus been proposed that these and other drugs affecting –1 ribosomal frameshiting efficiency are good candidates for antiviral drugs. Mutants affecting ribosomal frameshifting display a wide range of effects on different slippery sites,[78] suggesting that drugs could also be found with similar differential effects, i.e., high specificity for affecting only viral translation.

Chromosomal mutants affecting ribosomal frameshifting efficiency (called *mof* for maintenance of frame) were isolated using a vector in which expression of β-galactosidase required a change of reading frame.[78] Translation of this mRNA without a frameshift results in early termination, making these mRNAs potentially subject to degradation by the nonsense-mediated decay system. The first *mof* gene identified was affected in 5S rRNA indicating that this component of the 60S ribosomal subunit is involved in the proper maintenance of reading frame.[80]

mof4-1 is an allele of *UPF1*, a component of the nonsense mediated decay system that destabilizes mRNAs containing a premature stop codon.[81,82] The *mof4-1* allele is deficient in nonsense mediated decay, and this explains part of its effect on frameshifting. However, the *mof4-1* mutation shows a greater increase in frameshifting than does a *upf1*Δ mutant, suggesting that Mof4p/Upf1p has a direct effect on maintenance of frame.[82] Further evidence for a role of the Upf1-Upf2-Upf3 complex in controlling reading frame comes from the finding that *upf3* deletion mutations lead to increased ribosomal frameshifting at the L-A slippery site.[83] *upf2*Δ has the same effect on nonsense mediated decay as *upf3*Δ, but does not affect frameshifting, showing again that the two effects are distinct.

mof2-1 is an allele of *SUI1*, a gene controlling the accuracy of initiation at AUG codons.[84–86] In fact, *mof2-1* also affects nonsense-mediated decay and makes cells hypersensitive to paromomycin, indicating an effect on misreading in general.[84,85] It is suggested that Mof2p/Sui1p is part of a translation surveillance complex that controls the accuracy of initiation and elongation.[84]

Other components of the translation machinery found to affect ribosomal frameshifting include ribosomal protein L3, located at the peptidyltransferase center,[87] and elongation factor 1α.[88]

3.8.2 Ski1p/Xrn1p Is a 5'→3' Exoribonuclease Specific for Uncapped mRNAs

Mutants that overproduce the killer toxin are called *superkillers* and define seven genes[89,90] (Table 3.2). These mutants also result in the increase in copy numbers of L-A and L-BC dsRNAs and 20S ssRNA.[22,91] The *SKI1* gene[89] was also isolated as *XRN1,* the gene encoding a 5'→3' exoribonuclease specific for mRNAs lacking the 5' cap structure.[92,93] Ski1p/Xrn1p is the major mRNA degradation nuclease,[94–97] and

TABLE 3.2
Superkiller (SKI) Genes

Gene	Homology, Activity	Function	Ref.
SKI1	5'→3' exoribonuclease specific for uncapped RNA	mRNA turnover	(93)
SKI2	RNA helicase (homol)	block translation of non-poly(A) mRNA	(102)
SKI3	Repeat amino acid pattern	block translation of non-poly(A) mRNA?	(101)
SKI4	?	?	
SKI6	rRNA processing RNase	rRNA processing	(105)
SKI7	EF1-α homolog	block translation of non-poly(A) mRNA	(99)
SKI8	β-transducin repeats	block translation of non-poly(A) mRNA	(208)

Translation of Electroporated mRNAs

Strain	C+ A+	C+A–	C–A+	C–A–
	Translation of luciferase mRNA (light units/ μg protein)			
wildtype	24	0.41	1.4	0.13
ski2	45	14	1.3	0.56
ski3	22	11	0.79	0.51
ski8	90	41	5.8	3.2
Mutant/wt	**C+ A+**	**C+ A–**	**C–A+**	**C– A–**
		Ratio of Activities		
ski2 / +	(1.0)	18	0.5	2.3
ski3 / +	(1.0)	22	0.6	2.7
ski8 / +	(1.0)	34	1.2	5.0

the absence of a 5' cap on the L-A or M RNAs[47] provides a clear rationale for the sensitivity of L-A and M expression to Ski1p/Xrn1p.

3.8.3 Ski Proteins Inhibit Translation of Non-poly(A) mRNAs (Including Viral mRNA)

The *ski* mutants define six other genes, *SKI2, SKI3, SKI4, SKI6, SKI7,* and *SKI8* (Table 3.2).[89,90] Four of these genes, *SKI2, SKI3, SKI7,* and *SKI8,* encode proteins that appear to act together to block translation of mRNAs that lack the 3' poly(A) structure, such as the viral mRNAs.[98,99] Deletion of any of these four genes, or all four of them together, shows that they all are nonessential for growth in the absence of M dsRNA.[99–102] However, if the strain carries M dsRNA, the cells are cold sensitive for growth.[90] In the presence of another non-Mendelian genetic element, called [D] (for disease), *ski2* mutant strains are also high temperature sensitive and very slow growing at any temperature.[103] [D] is not located on L-A or M dsRNAs or mitochondrial DNA but has not been more precisely defined. Therefore, *SKI* genes function as an antiviral mechanism to prevent the growth defect that can result from an increase in copy number of L-A and M.

Several early clues suggested that the Ski2,3,7,8 system works by affecting the expression of viral mRNA. The *ski2* mutation raises the copy number of L-A, X, L-BC, and M dsRNAs and the 20S ssRNA replicon.[22,89,91] These are all cytoplasmic RNA replicons with no evidence of 5' caps or 3' poly(A) structures. In contrast, *ski2* does not affect retrotransposition by Ty1.[102] While Ski2p represses the copy number of M_1 and X when they are supported by the L-A virus, there is no such effect when either is supported by proteins supplied from the L-A cDNA clone (Table 3.3).[102] If the Ski2 system were directly inhibiting viral replication or transcription or degrading the viral dsRNA or (+) ssRNA, then it should lower M or X copy number in either case. One difference between support by the L-A virus and the L-A cDNA clone is that the L-A mRNA is capped and has poly(A) when made from the cDNA clone, but not when made by the virion.

TABLE 3.3
SKI2 Control of dsRNA Copy Number and Expression

Source of Gag and Gag-Pol	Satellite dsRNA	Toxin source	*SKI2*$^+$		*SKI2*Δ	
			Copy #	Toxin	Copy #	Toxin
L-A virus	M_1	M_1 dsRNA	+	+	++	+++
L-A cDNA	M_1	M_1 dsRNA	+++	++	++	+++
L-A virus	X	none	+		++	
L-A cDNA	X	none	++		++	
None	none	M_1 cDNA		++++		++++
None	none	M_1 cDNA		+		+

Deletion of SKI2 increases expression of toxin from M_1 dsRNA, but not from cDNA clones of M_1 information.[102] Note that copy number of M_1 dsRNA supported by the L-A cDNA clone goes down in *ski2* strains. However X dsRNA (which is not translated) supported by the L-A cDNA does not change its copy number in the *ski2* mutant.

When M_1 is supported by the L-A cDNA clone, a *ski2* mutation results in the expected increased killer toxin production but, surprisingly, a *decreased* M_1 dsRNA copy number is seen.[102] This finding again points to translation as the site of action of Ski2p. It is not consistent with Ski2p acting to degrade M_1 (+) ssRNA, since that would affect the copy number of M_1 dsRNA and toxin production in the same direction. The decrease in M_1 dsRNA in the *ski2* mutant may be due to competition between translation and packaging for M (+) strands: if translation of the (+) strands is increased by the *ski2* mutation, fewer are encapsidated and replicated. That Ski2p is not acting on the post-translational processing or secretion of the preprotoxin is shown by the absence of an effect of the *ski2* mutation on toxin made from a cDNA clone of M_1.[102]

The results above can be explained if the Ski2p system blocks translation of mRNAs that lack the cap or poly(A) structure. This hypothesis was tested by making an rDNA-*lacZ* fusion gene such that *lacZ* would be transcribed by RNA polymerase I and would lack both a 5' cap and 3' poly(A).[102] Expression of β-galactosidase from this construct was increased three-fold in the *ski2* mutant but was not affected by

ski2 if expression was driven by either of two RNA polymerase II promoters that would make capped poly(A)+ transcripts.[102]

Further examination of the effects of the Ski proteins on expression and its cap and poly(A) dependence utilized the RNA electroporation method developed by Daniel Gallie and co-workers.[104] Introducing luciferase mRNAs with or without cap (C+ or C–) and with or without 3' poly(A) (A+ or A–) showed that the presence or absence of poly(A) determined whether these Ski proteins affected expression of luciferase[98] (Table 3.2). The effect on C+A– mRNAs was a dramatic 20-fold or greater increase in expression in the *ski* mutants, but C–A+ mRNAs were essentially unaffected (Table 3.2). The magnitude of the effect on C– A– RNA was similar to the derepression of L-A copy number[91] or the effect on expression from the RNA polymerase I promoter.[102]

Although *in vivo* data indicate that the effect of Ski2p is on translation, not on RNA degradation, this question was re-examined using the electroporation method.[98,99,105] Following the electroporated pulse of mRNA, the kinetics of luciferase synthesis should distinguish between a primary effect on mRNA degradation and an effect on translation. An effect of the mutation on translation should increase the initial rate of luciferase appearance, while an effect on mRNA stability should increase the duration of synthesis without substantially affecting the initial rate. The kinetics show that the initial rate of appearance of luciferase from a C+A— mRNA is six-fold higher in a *ski2* strain than in the wildtype, and therefore that Ski2p affects translation.[98] The physical half-life of electroporated message was not affected significantly by a *ski2* mutation, but the functional half-life was increased as discussed below.

One remarkable conclusion of this work is that in the absence of the Ski2,3,8 system, translation does not require the poly(A) structure. In the *ski* mutants, translation of non-poly(A) mRNA is fully 1/3 as good as is translation of poly(A)+ mRNA[98] (Table 3.2).

Ski2p has the amino acid sequence motifs for an RNA helicase,[102] and its human homolog has been found in the nucleolus and on polysomes.[106,107] Ski3p is a nuclear protein with a repeat pattern (TPR repeats) of unknown significance.[101] These hints, and the critical dependence of dsRNA copy number on free 60S subunit concentration, suggested that the Ski2 and 3 proteins might be affecting translation by an effect on ribosome biogenesis,[98,108] but direct evidence for this hypothesis has not yet been obtained. Ski7p is homologous to elongation factor 1-α, but its localization has not yet been reported.

3.8.4 Effect of *SKI2* System on mRNA Turnover

The first electroporation studies of Ski2, Ski3 and Ski8 action showed that the duration of luciferase synthesis was affected, as well as the initial rates, indicating that both mRNA stability and translation were altered.[98] The mRNA turnover effect was suggested to be secondary to the translation effect. Parker's group has also found decreased mRNA turnover in *ski* mutants when the usual 5'→3' mRNA degradation pathway was blocked by either a poly(G) structure or by mutation of some element of the pathway.[109] These workers interpret the mRNA turnover effect as the primary

defect. However, it should be noted that the L-A and M mRNAs lack both cap and poly(A) structures and have no inherent block to 5'→3' degradation and yet are clearly affected by the Ski2, Ski3, Ski8 system as shown by the fact that these genes were first discovered by this effect.[89,90] Moreover, the electroporated messages used in the studies of Masison et al. and Benard et al. were likewise not blocked for 5'→3' degradation, and dramatic 20-fold or greater effects were seen.[98,99,105] Recently, Schwartz and Parker have shown that impaired translation, effected by mutations in translation initiation factors, results in increased mRNA turnover.[110] This again suggests that the Ski2 system may primarily affect translation. It is possible that by allowing translation of non-poly(A) mRNAs, the *ski2, 3,* and *8* mutations prolong the life of mRNAs by protecting them from degradation. It is also possible that there is an effect of these *ski* mutations on mRNA turnover that is in addition to its demonstrated effect on translation.

3.8.5 Ski6p, an RNAse Involved in rRNA Processing, Affects Translation

Unlike the other *SKI* genes, *SKI6* is an essential gene and encodes a homolog of RNase PH,[105] a highly specific *E. coli* enzyme responsible for trimming the 3' ends of pre-tRNAs.[111–113] Ski6p was also identified as a component of the exosome, a nuclear complex of 3' exonucleases involved in rRNA processing,[114] and Ski6p made in *E. coli* has been shown to have RNase activity.[114] The *ski6-1* mutant is ts for growth, but even at the permissive temperature for growth it produces an abnormal 38S ribonuclear particle containing a truncated form of 25S rRNA lacking sequences at its 5' end.[105] This particle is thus related to the 60S ribosomal subunit but lacks 5.8S rRNA, indicating that it is an incomplete or partially degraded 60S subunit.

ski6-1 mutants are hypersensitive to hygromycin B, suggesting that they are affected in the process of translation.[105] Electroporation of mRNAs with or without 5' cap or 3' poly(A) showed that, like mutants in the *ski2* group, translation of non-poly(A) mRNA was specifically derepressed about ten-fold. The kinetics of reporter enzyme expression and direct measurements of electroporated mRNA turnover indicated that translation was affected rather than message stability.[105] It was suggested that the altered translation properties of the *ski6-1* mutant is due to abnormal (but full size) 60S subunits.[105]

The properties of the *ski6-1* mutant and the presence of Ski6p in the nuclear exosome indicate that it is involved in rRNA processing and in fact affects the translation ability of the cell. Indeed, its derepressed translation of non-poly(A) mRNA explains its superkiller phenotype. Further work will be needed to understand why altered 60S subunits are specifically derepressed for translation of non-poly(A) mRNA; however, these results are consistent with a hypothesized specific interaction of 3' poly(A) and 60S subunits.[115]

3.8.6 Decapitation Activity of Gag Necessary for Toxin Expression

In the course of searching for activities in yeast extracts that bind the 5'-cap structure of mRNAs, Blanc et al. detected two protein species that covalently bound this

structure.[116] The major coat protein of both L-A and L-BC form a covalent complex with the cap from any donor mRNA. The 7methyl GMP from the cap attaches to his154 of the L-A Gag protein in a reaction that proceeds *in vitro* in the presence of Mg^{2+}.[116,117] By electroporating cap-labeled mRNAs into yeast cells, it was possible to demonstrate that the reaction occurs in cells.[98] Changing the site of cap attachment, his154, to asn, ser, or arg resulted in the inability of Gag to covalently attach the caps but left Gag able to bind to the cap structure noncovalently.[117]

The physiological role of this "decapitation" reaction was examined using the system in which the L-A viral proteins were expressed from the L-A cDNA clones and M_1 dsRNA was propagated by these proteins in the absence of the L-A dsRNA virus itself.[118] Mutating the Gag his154 on the L-A cDNA clone to prevent the decapitation reaction did not affect the efficiency of replication of the M_1 dsRNA, as shown by the unchanged copy number of this species.[117] However, the production of killer toxin, encoded by the M_1 dsRNA, was almost completely eliminated.[117] This indicates that the decapping activity is necessary for expression of dsRNA-encoded information.

The role of decapitation in expression of viral mRNA was suggested by the finding that mutation of the *ski1/xrn1* gene results in the restored ability of the M_1 dsRNA supported by the L-A cDNA clone carrying a mutation in his154 to produce active killer toxin.[98] The *SKI1/XRN1* gene encodes a 5' → 3' exoribonuclease specific for uncapped mRNAs and is involved in mRNA turnover.[92,93] Mutations in *ski2* or *ski8*, affecting expression of non-poly(A) mRNAs[98] (see above) had no effect. These results suggested that the decapping reaction was in some way protecting the uncapped viral mRNA from the Ski1/Xrn1 exoribonuclease and, indeed, measured levels of M_1 mRNA were consistent with this explanation.[98] It is likely that the decapping activity provides a decoy that diminishes the activity of Ski1p/Xrn1p on viral mRNAs by competition. The fact that *ski1* mutants were originally detected as superkillers[89] shows that such competition is not completely effective. Moreover, it may be only effective locally in the vicinity of the viral particles.

3.8.7 60S Ribosomal Subunits are Critical for M dsRNA Propagation

Twenty of the chromosomal genes whose mutation results in loss of M dsRNA but not of L-A dsRNA were found to be deficient in 60S ribosomal subunits.[108] These include genes encoding ribosomal proteins L3 *(MAK8/TCM1)*,[119] L4 *(RPL4A)*,[108] and L41 *(RPL41B)*.[120] Several other genes involved in 60S ribosomal subunit biogenesis have also been identified within this group, including *MAK1/TOP1* encoding DNA topoisomerase I,[121] *MAK21* encoding a homolog of a human CAATT binding protein,[122] *MAK5* encoding a DEAD-box helicase,[123] *MAK16* encoding an acidic nucleus-associated protein,[124] and *MAK11* encoding a membrane-associated protein.[125] Remarkably, among the nearly 30 genes whose mutants were isolated based on their loss of M dsRNA, none was deficient in 40S ribosomal subunits.

Ski6p is a subunit of the nuclear exosome, and its mutation has a superkiller effect. In contrast, Rrp6p is another 3'→5' exonuclease that is a subunit of the exosome, and mutation of its gene results in lower copy number of L-A.[126,127] The

authors attribute the *mak* effect of *rrp6* mutations to a decrease of 60S subunits in the mutants.

These results indicate that the level of free 60S subunits are somehow critical for M propagation, but the mechanism of this effect is unknown. As discussed above, viral mRNAs lack the 5' cap and 3' poly(A) structures, and *mak21* mutants show a modest but reproducible selective defect in translating mRNAs lacking either or both of these structures.[122] The requirement for normal levels of free 60S ribosomal subunits is suppressed by mutation of any of the *SKI* genes,[90,128] again tying the *SKI* genes to the translation process.

The *mak* mutants deficient in 60S ribosomal subunits lose M dsRNA, but their L-A copy number is only decreased a few fold.[108] X dsRNA, a deletion mutant of L-A retaining only *cis* signals necessary to be replicated, is, like M, dependent on L-A for encapsidation and replication.[25] X is completely lost from *mak* mutants deficient in free 60S ribosomal subunits like M.[25] This suggests that the deficiency of 60S subunits decreases the efficiency of translation of L-A mRNA, and that the L-A mRNA is packaged and replicated by the limiting coat proteins in preference to the X or M satellites.

3.9 N-ACETYLATION OF GAG BY THE MAK3P-MAK10P-MAK31P COMPLEX

The *mak3, mak10,* and *pet18* mutants are each unable to maintain and propagate either L-A dsRNA or M dsRNA.[4,129–131] The *pet18* mutation proved to be a large deletion on chromosome III between two Ty1 inserts, and only one of the genes deleted, *MAK31*, is involved in replication of L-A and M dsRNAs.[132] The sequences of *MAK31*[132] and *MAK10*[133] gave no clear hints about their roles in dsRNA replication. However, Mak3p has striking homology to a family of N-acetyltransferases.[134] The target of Mak3p acetylation was found to be the major coat protein, Gag, whose amino terminus is blocked in wildtype cells, but not in *mak3* mutant cells.[135] Unacetylated Gag is synthesized but is unable to assemble into viral particles, remains largely soluble, and is apparently degraded.[135]

Studies of the specificity of the Mak3p N-acetyltransferase showed that the N-terminal four amino acid residues (after the initiating methionine), LeuArgPheVal, have a dominant effect on preventing acetylation by other N-acetyltransferases and directing modification by Mak3p.[136] In their extensive studies of N-acetylation, Sherman's group has found similar specificity of Mak3p.[137] However, other than Gag, no natural substrates of Mak3p have been identified. We suggested that the slow growth of *mak3* mutants on glycerol[138] was due to their failure to acetylate one or more of α-ketoglutarate dehydrogenase, fumarate hydratase or Mrp1p, a mitochondrial ribosomal protein.[136] Each of the N-termini of these mitochondrial proteins is identical to that of Gag, MetLeuArgPhe. The failure of one or more of these proteins to be acetylated might impair their import into mitochondria and slow cell growth on glycerol. A slow growth on glycerol phenotype of *mak10* mutants has also been noted.[138]

Recently, evidence for a complex of Mak3p, Mak10p, and Mak31p has been reported.[139] This result, and the common properties of *mak3, mak10,* and *mak31,*

mutants suggests that Mak10p and Mak31p are needed for the N-acetyltransferase activity of Mak3p. Future studies may elucidate the role of the several subunits. Mak3p is apparently the catalytic subunit based on its homology with other N-acetyltransferases.

3.10 L-A PHENOMENA

The Gag protein is abundant in wildtype cells that carry L-A but, in several mutants, its levels (and L-A dsRNA) are further elevated. The *por1* gene encodes a voltage-dependent anion channel of the mitochondrial outer membrane.[140,141] Deletion of *POR1* results in slow growth on glycerol at 30C, with accumulation of high levels of L-A viral particles.[138,141] Growth on non-fermentable carbon sources is known to modestly elevate L-A copy number,[142] but the basis of the *por1* effect is unknown.

The *lcb2/scs1* gene encodes serine palmitoyltransferase, an enzyme in the pathway for sphingolipid biosynthesis.[143] Mutants in this gene, isolated as suppressors of Ca^{2+} sensitive mutants and characterized by their dependence on high levels of Ca^{2+} in the medium,[144] accumulate high levels of L-A virus.[145] Similar elevated L-A was seen in *scs2* mutants, which also have altered sphingolipid biosynthesis. The basis for this interesting effect remains to be elucidated.

NUC1 encodes the major mitochondrial nuclease, an enzyme associated with the mitochondrial inner membrane.[146,147] Nuc1p has nuclease activity on single- and double-stranded DNA and on single-stranded RNA, but not on dsRNA.[146] Deletion of *NUC1* results in dramatic increases in L-A copy number, again for reasons that are not yet evident.[148]

3.11 KILLER TOXIN PRODUCTION, SECRETION, AND ACTION

3.11.1 M dsRNAs Encode Preprotoxins

Strains carrying M_1 dsRNA secrete a protein toxin that can kill (K_1^+) other strains not carrying M_1. The killer strains are also immune (or resistant, R_1^+) to being killed by the K_1 killer toxin.[1] There are many killer systems in fungi including isolates of *Kluyveromyces lactis, Ustilago maydis, Pichia, Debaryomyces, Hansenula, Torulopsis, Willopsis,* and *Cryptococcus*,[8] reviewed in Refs. 149 and 150. Among *Saccharomyces* there are at least the K_1, K_2, and K_{28} systems determined by different M dsRNA satellites of L-A,[7,9,151] and the KHR and KRS systems with chromosomally encoded toxins.[152–154]

M_1 dsRNA encodes the prepro- form of the K1 killer toxin[6,155,156] reviewed in Refs. 157 and 158 (Figure 3.5). The 26 residue signal peptide is removed and, in the endoplasmic reticulum, the single polypeptide is cleaved to remove an internal segment, called "γ," that does not become part of the final heterodimeric toxin[6,155,156] reviewed by in Refs. 157 and 158.

The preprotoxin coding sequence begins at base 14 from the 5' end of M_1, an unusually short 5' non-coding sequence. The toxin gene ends at base 963 and is followed by a poly(A)•poly(U) region of about 200 bp, but varying in length as M_1

replicates.[159,160] Whether this sequence has a function in translation of M_1 (+) strands is not yet clear, but in spite of its presence, translation of M_1 (+) strands show an effect of the *ski2* mutation[102] suggesting it does not. The long 3' untranslated region contains the sites needed for RNA packaging and replication [(–) strand synthesis; see above].

3.11.2 Processing and Secretion of the Preprotoxin: *KEX* Genes and Prohormone Processing

The mechanism of processing of the prepro killer toxin is of particular interest, because it has led to important insights into the analogous (and homologous) processing of prohormones of mammals. The sequence of M_1 dsRNA combined with the sequence of the ends of the isolated toxin showed that cleavage occurred C-terminal to pairs of basic residues, or after PR[155,161,162] (Figure 3.5). Following this cleavage, the two basic residues were removed to give the sequence of the mature toxin. This pattern was similar to that known to be necessary for maturation of several mammalian prohormones, but the enzymes responsible for these processes were unknown (reviewed in Reference 163).

Two chromosomal genes were found to be necessary for secretion of an active killer toxin, but not for the propagation of the M dsRNA or for the immunity function to be expressed.[164–166] These genes were named *KEX1* and *KEX2* for killer expression. In addition to their defect in expressing the killer toxin, *kex2* mutants also fail to secrete the α pheromone,[166] a mating factor that strains of the α mating secrete to arrest their mating partner in the G1 phase of the cell cycle. This deficiency does not entirely explain the failure of mating by α cells, since supplying α pheromone in trans does not restore mating ability.[166] In addition to these defects, diploids homozygous for *kex2* arrest late in sporulation.[166] It was further found that *kex2* mutants had other biochemically defined differences in their secreted proteins and glycoproteins.[167]

Cloning and biochemical studies showed that Kex2p is a protease of the subtilisin group[168,169] that cuts specifically after pairs of basic residues. Kex1p is a carboxypeptidase, capable of removing the C-terminal basic residues left by Kex2p.[170–172] The processing of the α-factor precursor requires cleavages similar to those of the killer toxin (reviewed in Reference 173), thus explaining the failure of *MAT*α *kex2* strains to make this pheromone.[166]

Kex2p is a Ca^{2+}-requiring endopeptidase located in a late Golgi compartment, and attached to the membrane by a single trans-membrane domain.[169,174–177] Its *in vitro* specificity[178] accounts for its *in vivo* activities[155,166,179] as described above.

Knowing the structure of one enzyme with the specificity to process mammalian preprohormones, and which is responsible for the same function in yeast, it became possible to search for mammalian homologs experimentally (by selecting for cDNA clones complementing a *kex2* mutant) and electronically (by looking for matches with Kex2p in the database of mammalian sequences). These approaches have identified a series of homologous "kexins" including furin, PC1, PC2, PC4, PACE4, PC5A, PC5B, and PC7 (PC = prohormone converting enzyme, reviewed in Reference 163). These enzymes are also located in the transGolgi network or the immature

secretory granules and are responsible for processing both constitutive secreted proteins (like albumin) and inducible (hormonal) prohormone processing.[180,181] This is an example of an area of mammalian research whose development was absolutely dependent on earlier findings in yeast.

3.11.3 Mechanism of Killer Toxin Action

The K1 killer toxin has, in effect, two receptors. The K1 toxin binds specifically to (1→6)-β-D-glucan and can be purified on columns of this polymer.[182] Moreover, many mutants isolated based on their resistance to the K1 killer toxin (*kre*) are found to be defective in their synthesis of (1→6)-β-glucan.[183–186] The *kre1* mutants are defective in (1→6)-β-D-glucan synthesis, and the intact cells of such mutants are resistant to toxin action. However, spheroplasts of *kre1* mutants are still sensitive to killing by the K1 killer toxin.[187] In contrast, spheroplasts of cells carrying M_1 dsRNA are resistant to toxin action. These results indicate that there is a second level of toxin action at the membrane and that the immunity factor (perhaps the protoxin or a fragment thereof) binds to that receptor to make the toxin unable to act.[186]

The K1 killer toxin induces leakage of H^+ and K^+ ions from sensitive cells.[187,188] Purified killer toxin incorporated into artificial membranes induces the appearance of ion-permeable pores.[189] Although these results point to toxin formation of ion pores as the mechanism of killing, they do not suggest a mechanism of immunity.

Recent evidence indicates that the *TOK1*-encoded potassium channel affects sensitivity to the K1 killer toxin.[190] The *tok1* mutants were measurably more resistant to killer toxin, and cells in which Tok1p was overproduced were significantly hypersensitive. Moreover, artificially producing Tok1p in Xenopus oocytes made them susceptible to killer toxin-induced potassium channel activity. However, the *tok1* mutants show only a modest level of resistance to the K1 killer toxin, indicating that this is not the sole target of toxin activity.[190] In addition, no data has been presented on the effect of immunity on these phenomena.

The various killer toxins have very different targets (reviewed in Reference 150). Those directed against cell wall components, notably the K1 toxin against (1→6)-β-D-glucan, have been exploited by Bussey's group to define the genes involved in the biogenesis of this important structure (see references above). This may provide a general approach to genetic studies of cell wall constituents.

3.11.4 M_1-Encoded Resistance to the Killer Toxin

Killer strains are immune to the actions of the toxin they produce. The mechanism of immunity has been little studied and is poorly understood. Mutations in the α and γ regions of the protoxin from residues 85 to 177 result in loss of immunity, but deletions of all of β and the C-terminal half of γ leave immunity essentially intact.[191,192] The *kex* mutants are R^+ and make only the protoxin, suggesting that this component is the immunity protein.[191] A chromosomal gene, *REX1* (resistance expression) is necessary for immunity.[164] A 22 kDa peptide derived from the protoxin has been detected in normal killer strains.[193] This peptide reacts with antibodies to

both the killer toxin and to the γ-segment and is not found in *rex1* mutant cells, suggesting that it may be involved in the immunity process and that there may be alternate processing pathways of the protoxin.[193]

3.11.5 Application of Killer Toxins to Treatment of Yeast Infections

Polonelli and coworkers have devised an ingenious variant of the anti-idiotype method to develop a new approach to treatment of yeast infections (reviewed in Reference 150). Antibodies are generally complementary to the original antigen. Thus, an antibody to an antibody should be the complement of the complement and so should be similar to the original antigen. This approach has allowed development of antibodies with enzyme and other activities. If a killer toxin is the original antigen, then the anti-antibody should have killer toxin activity and protect the immunized animal against infection by toxin-sensitive yeast. Experiments of this type have now been carried out and are at least partially effective.[194–196]

3.11.6 Use of Killer Strains to Exclude Contaminants in Fermentation

Although many killer strains occur in nature, most strains are sensitive to a given killer toxin. Since yeast strains present in the original grapes (for example) can alter the flavor of the final product, their exclusion is desirable. One approach to this is to include L-A and M dsRNA viruses in the fermentation inoculum.[197] This was done by a series of cytoductions. First, mitochondrial DNA was transferred from the standard sake brewing strain to a *kar1* laboratory strain of *S. cerevisiae*. Then, the killer virus, along with mitochondrial DNA of the laboratory strain, was transferred to the brewing strain. Finally, the lab yeast mitochondrial DNA was eliminated from the brewing strain, and its own mitochondrial DNA was restored by a final cytoduction. Examination of the fermentation characteristics of the final strain showed that they were essentially the same as the initial sake brewing strain. This method could, in principle, be applied to any brewing strain if a haploid version of that strain could be obtained. Unfortunately, most brewing strains are polyploid and do not mate.

The experiments above were done using the K_1 killer, but most killer yeast isolated from fermentations carry the K_2 killer, perhaps because its toxin is more active and stable at the very acid pH of most fermentations.[7] The K_1 or K_2 killer virus can also be introduced using polyethylene glycol in a transformation protocol,[198] which has the theoretical advantages of not changing the genotype of the recipient strain and not requiring haploidy or mating competence. The method of El-Sherbeini and Bostian has been used by others in studies of variants of the L-A virus.[199]

3.11.7 L-A Genetics and Interference Phenomena

Several types of incompatibility between viral segments have been identified. M_1 and M_2 dsRNAs are incompatible, and M_1 usually wins the competition.[200,201] Dele-

tion mutants of M_1 (called S dsRNAs) eliminate M_1 itself.[5,52,53] X dsRNA, a deletion mutant of L-A, excludes M_1, and lowers the copy number of L-A,[25] as does M dsRNA.[91] The mechanisms for these exclusion phenomena are not known.

Several natural variants of L-A have been identified based on their interactions with M_1, M_2, and chromosomal genes (Table 3.4). L-A-E is unable (or unwilling) to support M_1 or M_2 in a wildtype host but can do so in a *ski2* host.[131,200] Since *ski2* derepresses translation of viral mRNA (see above), L-A-E may be a variant with particularly weak affinity for the translation apparatus. L-A-H is able to supply the function (called [HOK] for *h*elper *o*f *k*iller) needed by M_1 or M_2 for their propagation in wild type strains.[130] When L-A-E is introduced into a cell with L-A-H, the copy number of L-A-H is substantially reduced so that either M_1 or M_2 is lost from the cell. This is the *excl*usion trait of L-A-E (or [EXL]).[200,202]

TABLE 3.4
L-A Genetics and Exclusion Relations

Natural variant	Properties	Mnemonic
L-A-E	cannot maintain M in w.t. host can maintain M in *ski2* host lowers L-A-H copy #	E = excluder = [EXL]
L-A-H	can maintain M in w.t. host	H = helper of killer = [HOK]
L-A-HN	copy # not lowered by L-A-E eliminates M_2 in *mkt* host over 30C	N = non-excludable = [NEX]
L-A-HNB	maintains M in *mak11-1* host	B = bypass

Some natural variants of L-A-H are insensitive to copy number reduction by L-A-E, so that in a strain with this variant, M_1 or M_2 is *n*on*ex*cludable. This trait is called [NEX], and such L-As are denoted L-A-HN to indicate that they have both helper ([HOK]) and nonexcludable ([NEX]) properties. Most L-As in laboratory strains are of the L-A-HN type. All L-A-HNs examined to date have another property which distinguishes M_1 and M_2. In the presence of a null allele (or natural variant recessive alleles) of *MKT1* or *MKT2* (for maintenance of K2), strains with L-A-HN cannot propagate M_2 dsRNA at temperatures above 30C.[200] *MKT1* encodes a non-essential protein without substantial homology to known proteins.[203]

Another variant of L-A, having both the [HOK] and [NEX] traits, also makes M_1 no longer sensitive to the group of *mak* mutations that result in deficiency of free 60S ribosomal subunits.[199] This trait is called [B] for *b*ypass and could be due to increased affinity of the mRNA of this L-A-HNB for the translation apparatus. L-A-HNB also maintains M_1 at a higher copy number than does L-A-HN.

The recent studies concerning the functions of Mak and Ski proteins make the interpretations of these phenomena plausible, but it will be important to define the alterations of L-A sequence that determine the [HOK], [NEX], [EXL], and [B] functions. The present inability to launch the L-A virus from a cDNA clone of L-A has, up to the present, prevented such studies.

3.11.8 [*KIL-d*], a New-Old Puzzle

A mutant with unusual properties was isolated in one of the early screens for strains affected in the killer system.[204] The original mutant had the K^-R^+ phenotype. Mating this mutant with any strain produced K^+R^+ diploids. Sporulation of the K^+R^+ diploids resulted in spore clones with a rainbow of phenotypes including K^-R^+, K^+R^-, K^-R^-, and variants with weak killer and/or resistance as well as some K^+R^+ segregants. Each of these segregants (including those that were K^+R^+) again formed K^+R^+ diploids when mated with any strain, and these gave rise to spore clones with the rainbow of phenotypes.[204] The 4:0 segregation of this phenomenon indicated that it was controlled by a non-Mendelian genetic element, and its connection with the killer phenomenon led to the assumption that this was a mutation of one of the killer dsRNAs, so this was denoted [KIL-d] (for diploid-dependent). Matings of the type *MATa* x *MATa* or *MAT*α x *MAT*α produced K^+R^+ diploids, suggesting that the phenomenon was not due to mating type heterozygosity.[204]

A recent reinvestigation of this phenomenon has substantially advanced our understanding, but its fundamental basis remains unclear.[205] [KIL-d] can be transferred by cytoduction, confirming that it is due to a nonchromosomal genetic element, but its properties make it clear that it is not a mutant of L-A or M dsRNA. Curing L-A and M_1 dsRNAs by growth at high temperature did not eliminate [KIL-d] as shown by a subsequent cross.[205] Moreover, passage of cytoplasm carrying [KIL-d] through a *mak3* or *mak10* mutant by cytoduction did not result in loss of [KIL-d] confirming this conclusion (Z. Talloczy and M. J. Leibowitz, personal communication). Curing mitDNA or the 2 micron DNA plasmid also did not result in loss of [KIL-d]. Making cells heterozygous for their mating type does not restore the normal phenotype, nor does changing their mating type using the *HO* gene.[205]

The results of a pair of reciprocal cytoductions were particularly revealing:

	Donor	Recipient	Cytoductants
A → B	*kar1* K^+R^+ →	[KIL-d] K^-R^+	[KIL-d] K^-R^+
B → A	[KIL-d K^-R^+ →	*kar1* K^+R^+	[KIL-d] K^+R^+

Transfer of normal killer cytoplasm into a [KIL-d] K^-R^+ strain did not change its phenotype, consistent with the authors' other evidence that [KIL-d] is not due to a mutation of the L-A or M dsRNAs. Transfer of cytoplasm from the [KIL-d] K^-R^+ strain to the normal strain also did not change its phenotype. But in this second cytoduction, the genotype of the recipients *was* changed. When these cytoductants were crossed, they gave rise to the spectrum of abnormal phenotypes in the meiotic segregants. Thus, the phenotype seems to be a manifestation of the state of the nucleus of the cell, while the genotype is a cytoplasmic trait. The nucleus seems to be "reset" to normal by mating, and the abnormality is revealed by meiosis-sporulation.[205]

Three intriguing puzzles about [KIL-d] are (1) what is the basis of its inheritance, that is, what is the non-chromosomal genetic element involved; (2) how does it produce the rainbow of phenotypes; and (3) how does mating restore the normal phenotype and meiosis produce the rainbow? Because [KIL-d] is lost and arises at

substantial frequencies, the authors suggest that it may be a "prion-like" phenomenon, but there is no concrete evidence as yet that points in this direction.

3.12 PROBLEMS AND GOALS

Although the L-A and M dsRNA virus system is probably the most highly developed of any dsRNA virus (largely because of the ease of manipulating *S. cerevisiae*), there remain a number of technical barriers and problems that need work. In spite of substantial efforts, a method has not yet been developed for launching the L-A virus from a cDNA clone.[206] Its early promise as a cloning vector has never materialized, in part because of the lack of a launching system. Studies of the natural variants of L-A and their interaction with M and host genes has also been limited by this problem. One need only read the work of Nuss and coworkers on the hypoviruses to see the potential value of having such a method available.[207]

Although the L-A Pol is homologous to the RNA-dependent RNA polymerases of picornaviruses and other (+) strand RNA viruses, it has not yet been possible to produce soluble active enzyme that would be suitable for structural studies, further mechanistic investigations, and potential design of general RNA-dependent RNA polymerase inhibitors for treatment of viral infections. This may be related to the fact that the rotavirus replicase requires the major structural protein of the viral cores (analogous to the L-A Gag) for its activity.[64] It is possible that the RNA polymerases of dsRNA viruses require the capsid structure (with which they are always associated) to be active.

Virus assembly could be better approached if a more detailed structure of L-A were available and if an *in vitro* assembly system were developed. One would like to know, for example, why N-acetylation of Gag is needed for assembly and whether there are special functional aspects to the pores through which ribonucleotides enter the virions and (+) strand transcripts leave. How does the decapitation activity of Gag work, and is the decapitation decoy model really correct?

The interactions of L-A and M dsRNAs with the various host gene products are partially understood, but more questions than answers are available. Translation is very important in the interactions of L-A and its satellites with the host. How do the Ski proteins work? Why are 60S subunits so critical? How is L-A translated rather well in spite of the absence of both 5' cap and 3' poly(A) structures? Are no host factors needed for replicase per se? What are the roles of *POR1, NUC1* and sphingolipids in regulating L-A copy number? What are the roles of the Mak10 and Mak31 subunits of the Mak3 N-acetyltransferase? What are [D] and [KIL-d] and how to they act? Of course, the studies of the genetic control of L-BC, 20S RNA and 23S RNA have just begun.

It is hoped that as L-A and the killer satellites become better understood, more applications of this system will be developed.

3.13 UPDATE

Slh1p is a DEVH-box RNA helicase with extensive homology to Ski2p beyond the helicase domains (Martegani et al., 1997). *slh1*Δ *ski2*Δ double mutants are

healthy (in the absence of viruses) and show normal rates of turnover of several cellular mRNAs, but they have very high L-A and L-BC copy numbers. The *slh1*Δ *ski2*Δ strains translate electroporated non-poly(A) mRNA with the same kinetics as polyA$^+$ mRNA (Searfoss and Wickner 2000). Thus, the translation apparatus is inherently capable of efficiently utilizing non-poly(A) mRNA, even in the presence of normal amounts of competing poly(A)+ mRNA, but is normally prevented from doing so by the combined action of the non-essential antiviral proteins Ski2p and Slh1p.

The enhancement of translation by the 3' poly(A) structure has been suggested to be due to an interaction of the poly(A) binding protein, Pab1p, with initiation factor eIF4G. However, mutation of eIF4G eliminating its interaction with Pab1p does not diminish the preference for poly(A)+ mRNA *in vivo*, indicating another role for poly(A) (Searfoss et al., 2001). The absence of Fun12p (eIF5B), a protein involved in 60S ribosomal subunit joining, specifically reduces translation of poly(A)+ mRNA, suggesting that poly(A) may have a role in promoting the joining step. The Fun12p step appears to be downstream of the Ski1p-Slh1p step. This suggests that Ski2p and Slh1p block translation of non-poly(A) mRNA by an effect on Fun12p, possibly by affecting 60S subunit joining, suggesting the model:

3'poly(A)-Pab1p ⊣ Ski2p-Slh1p ⊣ Fun12p-Tif5p → 60S subunit joining

The Ski2-Slh1 system functions both to block the expression of the non-poly(A) mRNAs made by viruses and those produced in the breakdown of cellular mRNA.

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4 The Double-Stranded RNA Viruses of *Ustilago Maydis* and Their Killer Toxins

Jeremy Bruenn

CONTENTS

Many fungi exhibit persistent infection with double-stranded RNA (dsRNA) viruses. Among these are the Totiviridae, dsRNA viruses in which a single segment of dsRNA encodes all necessary viral functions. There are also representatives of this family among the protozoans, and all are evolutionarily related.[1,2] One of the best-studied groups of totiviruses is that of the basidiomycete *Ustilago maydis.*

The *Ustilago maydis* viruses (UmV) are classified as Totiviridae, even though most infected cells have multiple segments of dsRNA. There are two origins for these multiple segments: some segments are satellite viruses that encode secreted polypeptides, some of which are toxins that kill sensitive cells (killer toxins); and some segments are the genomes of independent totiviruses. In both respects, the Ustilago viruses are similar to the dsRNA viruses of the yeast *Saccharomyces cerevisiae,* although they exhibit greater genomic diversity.

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4.1 THE VIRAL GENOME AND ITS ENCODED PRODUCTS

There are three distinct size groups of UmV dsRNAs. These are heavy (H), medium (M), and light (L). The three original isolates were P1, P4, and P6, after the killer toxins they encode. There may be as few as three viral dsRNAs, as in some P6 strains, or as many as seven viral dsRNAs, in some P4 isolates.[3–6] Eight of the UmV dsRNAs have been completely sequenced, and their coding regions and encoded polypeptides are summarized in Table 4.1. The H segments appear to encode the essential viral proteins; the M segments encode secreted polypeptides, usually killer toxins; and the L segments have neither coding regions nor defined functions.

TABLE 4.1
UmV Viral dsRNAs

RNA	Size	Coding region	Product (length)	Mature polypeptides (length)	Function
P1H1	6099	540–5999	Cap-Pol (1820)	Cap(?) Cap-Pol(?)	capsid pp RDRP
P1M1	1504	217–984	ppt(256)	α(103)	?
				β(83)	?
P1L	354	none			?
P1M2	1034	38–910	ppt(291)	α(120)	?
				β(117)	KP1 toxin
P4M2	1006	113–493	ppt(128)	KP4(105)	KP4 toxin
P4L	354	none			?
P6M2	1234	120–776	ppt(219)	α(79)	Kp6 toxin
				β(81)	KP6 toxin
P6L	355	none			?

4.1.1 The H Segments

The essential viral polypeptides are encoded on the H segments. As in other totiviruses, these are a capsid polypeptide and an RNA-dependent RNA polymerase (RDRP). Only one of the H1 segments has been entirely sequenced: P1H1 is 6099 bp, and encodes one open reading frame (540–5999), a polypeptide of 1820 amino acids with a putative capsid polypeptide in the N-terminal region and a typical RDRP sequence in the C-terminal region (Park, C.-M., and Bruenn, J.A., unpub.). This is reminiscent of the picornavirus genomes, which also have long polyproteins and long untranslated regions at the 5' ends of the plus strands. Some 490 bp of P6H1 have been sequenced, and there are only 10 base differences in this region between P1H1 and P6H1. These result in only two conservative amino acids substitutions in these 162 amino acids of the RDRP (Park, C.-M., and Bruenn, J.A., unpub). Another small region of P1H2 has been sequenced, which turns out to encode 58 amino acids from the amino terminal region of an RDRP, with 33% identity to the P1H1 RDRP

(Park, C.-M., and Bruenn, J.A., unpub.). This is most easily interpreted to mean that there are at least two totiviruses in *Ustilago* (H1 and H2). These would be similar to the ScVL1 (ScVL-A) and ScVLa (ScVL-BC) viruses that coexist in the same cells in *S. cerevisiae*.[1,2] Processing of the P1H1 polypeptide is proposed to occur by self-cleavage or by cleavage by a cellular enzyme. The nature of the UmV capsid polypeptides is discussed in Section 4.2.

4.1.2 The M Segments

Several M segments encode killer toxins. These are secreted proteins effective against species in the *Ustilaginaceae*, including those that are known as pathogens to maize, wheat, oats, and barley.[7]

There are three killer types: KP1, KP4, and KP6, which secrete KP1, KP4, and KP6 toxins, respectively. Correspondingly, there are three groups of resistant cells in which the resistance is determined by three independent recessive nuclear genes: p1r, p4r, and p6r.[8,9] The p1r, p4r, and p6r genes are thought to encode cellular (membrane or membrane-cell wall) receptors for the toxin (KP6) or targets for the toxin (KP4). Since these proteins are different in each case, there is no cross-resistance to the toxins. There are no single resistance alleles that confer simultaneous resistance to all three toxins.

4.1.2.1 Properties of the UmV Killer Toxins

All three *U. maydis* toxins have been purified.[10–15] All of these toxins consist of low molecular weight polypeptides.

The KP6 preprotoxin, encoded by the P6M2 segment of 1234 bp, is a protein of 219 amino acids. It is processed by removal of the signal peptide and Kex2p and Kex1p cleavages, resulting in the secretion of two polypeptides (α and β), which are not ionically or covalently associated as present in the medium.[16] They are 79 (α) and 81 amino acids (β) in length,[12] as recently confirmed by mass spectroscopy (Bruenn, unpublished) and amino terminal sequencing.[13] The KP6 preprotoxin appears[13] to be processed in a manner very similar to that of the *Saccharomyces cerevisiae* virus (ScV) k1 and k2 preprotoxins,[17–22] although the mature subunits of KP6 are not (like the k1 α and β subunits) joined by disulfide bonds.[16]

The X-ray structure of KP6 α has been determined.[23] It has four disulfide bonds stabilizing a compact structure consisting of a four-stranded anti-parallel β sheet with two α helices of opposite sense on one side of the sheet, a single β strand along one edge of the β sheet and a short N-terminal a helix on the other side of the β sheet (Figure 4.1).

The KP6 preprotoxin and other known killer preprotoxins are processed by two proteinases interchangeable among eucaryotes as disparate as yeast and mammals.[24–34] These proteinases (Kex2p and Kex1p) have not been directly demonstrated in plants, but both tobacco[35] and maize (J. Duvick and J. Bruenn, unpub.) correctly process the KP6 preprotoxin.

Both KP6α and KIP6β are necessary for KP6 toxin activity,[13,36,37] and it is possible to make them separately, using mutants that incorrectly process the preprotoxin, degrading that portion of the preprotoxin that encodes one of the mature

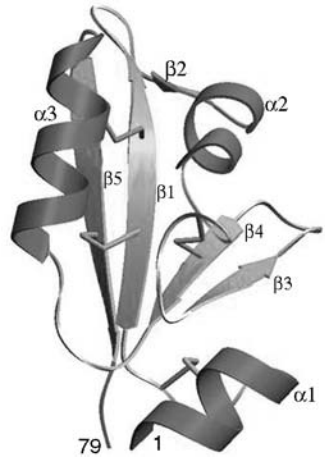

FIGURE 4.1 Ribbon diagram of KP6α structure (monomer). The four disulfide bonds are shown. (Reproduced by permission, courtesy of the American Society for Biochemistry and Molecular Biology.)

peptides.[38] Neither KP6α nor KP6β is glycosylated as secreted from Ustilago, even though KP6α does have a possible site for N-linked glycosylation, which is utilized in yeast.[13] In fact, none of the secreted polypeptides encoded by the UmV RNAs is glycosylated, despite numerous possible sites for N-linked glycosylation.[10,13]

The KP4 toxin is encoded by the P4M2 dsRNA, of 1006 bp, in a preprotoxin of 127 amino acids (13.6 kDa), from which the N-terminal 22 amino acids is removed by the signal peptidase.[12] The resultant toxin is 105 amino acids (11.1 kDa), with 10 cysteines and 5 disulfides, no N-glycosylation (and no possible sites for N-glycosylation), and no Kex2p cleavage.[11,12,14,39,40] KP4 toxin is the only known killer toxin that is not processed by a Kex2p-like enzyme. The KP4 toxin is properly produced in *S. cerevisiae kex2* mutants, while the KP6 toxin is not.[12]

The three-dimensional structure of the KP4 toxin is known (Figure 4.2). KP4 toxin appears to act by blocking necessary calcium channels.[40] The KP4 toxin, with five disulfides, belongs to the α/β sandwich family of proteins; it has seven β-strands and three α-helices, with the major secondary structural elements consisting of a β-sheet with five anti-parallel strands and two anti-parallel α-helices lying at approximately 45° to the strands in the β-sheet. *In vitro* mutagenesis has demonstrated that residue K42 is critical for function (T. Smith and J. Bruenn[85]).

The KP1 killer toxin has also been purified and amino acid sequence analysis performed to identify the toxin encoding dsRNA segment.[10] P1 cells generally have two M segments, M1 and M2, and there has been uncertainty about which encodes the KP1 toxin.[13,41] Genetic analysis of the P1 nonkiller deletion mutants showed a close relationship between KP1 expression and the presence of the P1M2 dsRNA.[5] One deletion nonkiller mutant, strain 3120, has M1 but not M2.[10] Western analysis using M1 polypeptide-specific antibodies showed that M1 polypeptides were equally expressed in both killer and some nonkiller cells. These observations suggested that

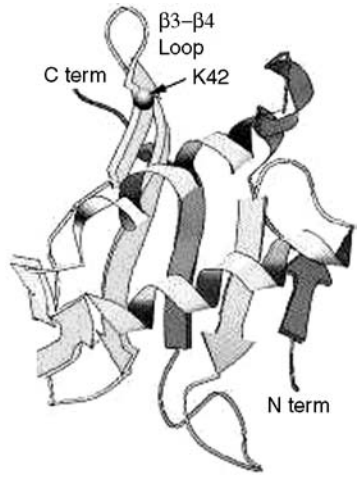

FIGURE 4.2 Ribbon structure of the KP4 toxin. (Courtesy of Tom Smith.)

M2 encodes the KP1 toxin or has a critical function for the expression of the active KP1 toxin. The KP1 killer toxin was purified and two internal tryptic peptides sequenced. These had sequences corresponding to those predicted from the P1M2 dsRNA. P1M2 is different from the other two toxin-encoding segments in that it does not have the L sequence at the 3' end of its plus strand. The two P1M1 polypeptides, which are secreted polypeptides present in the medium, have no known function (Park, C.-M., and Bruenn, J.A., unpub.).

P1M2 (1034 bp) encodes the KP1 preprotoxin (291 amino acids), which is subsequently processed by Kex2p into two polypeptides, α, of 120 amino acids, and β, of 117 amino acids. Only one of these (β) is involved in killing. KP1β is a basic toxin (pI 8.0) with a predicted size of 13,359 Da. The measured size of the KP1 toxin (12.4 kDa) is inconsistent with glycosylation. This is despite the existence of 2 possible N-linked glycosylation sites in KP1 β. Therefore, none of the UmV toxins is glycosylated. The one possible N-linked glycosylation site in KP6 α is utilized in *S. cerevisiae* but not in *U. maydis*.[13] Like the KP4 toxin with 10 cysteines (all in disulfides), the KP6 α polypeptide (8 cysteines, all in disulfides), and the KP6 β polypeptide (6 cysteines, probably all in disulfides), KP1 β has 6 cysteines.

4.1.2.2 Selective Advantage of Toxin-Producing Strains

Although otherwise compatible sensitive and killer strains will not form heterokaryons on laboratory medium, they may form dikaryons when injected into maize plants.[6] The apparent lack of effect of killer toxins during mating under laboratory conditions in maize may be due to insufficient amounts of toxin being present under these circumstances. Note that the KP4 and KP6 toxins are not denatured in or on plant tissues.[35,42] There must be some selective advantage to production of the killer toxins, since those dsRNA segments encoding them are not essential to maintenance of the viruses. We suppose that the toxin is effective against *U. maydis* and other

smuts during the normal course of infection, which does not take place (as it does in laboratory experiments) by hypodermic injection of plant tissues.

4.1.2.3 Mechanism of Action of the Toxins

The KP4 toxin is thought to block calcium channels in susceptible cells.[40] Fungi are now known to have calcium channels, with an α subunit very similar to that present in higher organisms. In *Saccharomyces cerevisiae*, this subunit is encoded by the CCH1 gene.[43] A second gene, MID1, encodes a second channel polypeptide.[44] The most likely cell membrane receptor for the KP4 toxin would therefore be one of these calcium channel polypeptides or a closely associated membrane protein. The very similar SMK toxin, from Pichia, which has two polypeptides in a structure almost identical to that of KP4,[45] does have a known membrane receptor in *S. cerevisiae* (which is susceptible to the toxin). This is an ATPase of unknown function.[46] However, *S. cerevisiae* is not susceptible to the KP4 toxin.

The KP6 toxin appears to act by forming its own K^+ or $NH4^+$ channel, as evidenced by electrophysiology with planar lipid bilayers (M. Zizi, personal comm.). KP6α does form a hexamer in crystals, with a very peculiar arrangement of monomers, highly reminiscent of a channel structure (Figures 4.3 and 4.4). Each trimer, held together by salt bridges, orients the phenylalanines of the N-terminal α helix to form a triangular structure that could trap an ion (Figure 4.3). Seen transversely (Figure 4.4), the hexamer forms a wasp-waisted structure that could be a channel, with dimensions (35 Å) appropriate for a cross-membrane assembly. The KP6 toxin also seems to require a cell membrane receptor, since spheroblasts of sensitive cells

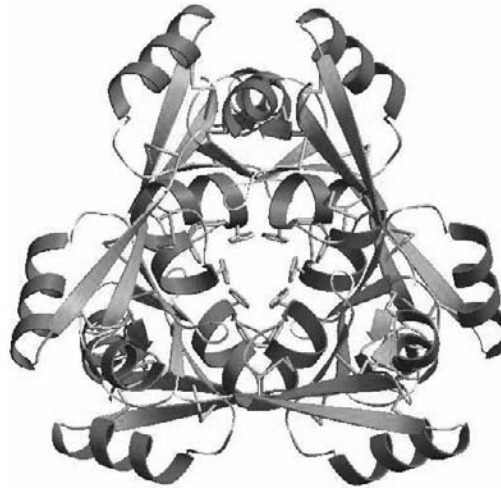

FIGURE 4.3 KP6α hexamer seen from above. The KP6 toxin appears to act by forming its own K^+ or $NH4^+$ channel, as evidenced by electrophysiology with planar lipid bilayers (M. Zizi, personal comm.). KP6α does form a hexamer in crystals, with a very peculiar arrangement of monomers, highly reminiscent of a channel structure. (Reproduced by permission, courtesy of the American Society for Biochemistry and Molecular Biology.)

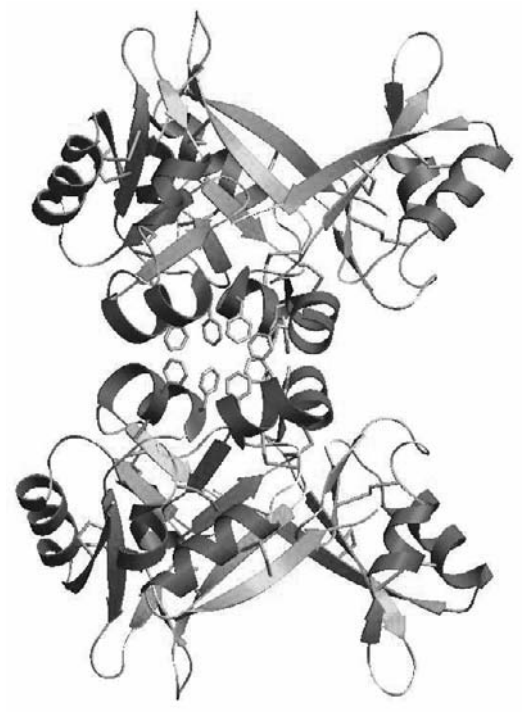

FIGURE 4.4 KP6α hexamer seen from the side. Phenylalanine side chains of the N-terminal α-helix are shown. Each trimer, held together by salt bridges, orients the phenylalanines of the N-terminal α-helix to form a triangular structure that could trap an ion (Figure 4.3). Seen transversely (Figure 4.4), the hexamer forms a wasp-waisted structure that could be a channel, with dimensions (35 Å) appropriate for a cross-membrane assembly. (Reproduced by permission, courtesy of the American Society for Biochemistry and Molecular Biology.)

remain sensitive and spheroblasts of resistant cells remain resistant.[37] If the toxin does act by forming an adventitious ion channel, it may require one or more membrane proteins as chaperones for insertion of the toxin polypeptides into the membrane.

4.1.2.4 Heterologous Expression of the UmV Toxins

Both the KP4 and KP6 toxins have been expressed from unaltered cDNAs in yeast, tobacco, and maize[12,35,42] (Duvick, J., and Bruenn, J.A., unpub.). Production of KP4 toxin in yeast is considerably more efficient than that of KP6 in yeast, reaching levels in the medium about one-fourth that produced by *U. maydis*. The toxin produced in yeast appeared identical to that produced in *U. maydis*.

Similarly, transgenic tobacco plants expressing the KP4 produced active KP4 toxin considerably more efficiently than they did KP6 toxin.[42] The toxin produced in plants appeared identical to that produced in *U. maydis*. So much KP4 toxin is made that it can be assayed directly by placing cut portions of unprocessed tobacco

leaves on sensitive *U. maydis* lawns. This is a promising strategy for producing maize plants resistant to *Ustilago* infection.

The KP6 toxin is synthesized and secreted in a functional form from transgenic tobacco plants. The specificity of this toxin is exactly the same as that of authentic KP6 toxin. Purified KP6α and β produced in the tobacco transgenics were characterized by westerns, by N-terminal sequencing, and by mass spectroscopy. Processing in tobacco is identical to that in *U. maydis*, except that the C-terminal Lys-Arg of the α precursor is removed in plants, while only the C-terminal Arg is removed in *U. maydis*.[35] This is the first strong evidence for the existence of a kex2p pathway in plants.[35]

4.1.2.5 Expression of the Toxin cDNAs in Maize

There are now expression vectors available for maize,[47–50] and it is possible to create transgenic maize plants[51,52] using microprojectile bombardment.[53] Preliminary results with maize plants producing the KP4 and KP6 toxins demonstrate increased resistance to *Ustilago* infection (Duvick, J., Bruenn, J.A., Gold, S., and Berry, J.O, unpub.).

4.1.3 The L Segments

As first shown for P6M2 and P6L,[54,55] the L segments of the viral dsRNAs are generally derived from the 3' ends of the plus strands of their cognate M dsRNAs. P1L is derived from P1M1, P4L from P4M2, and P6L from P6M2. In each case, the L segments have exactly the same sequence as the homologous regions of the M dsRNAs from which they are derived. Consequently, they have the same plus strand 3' ends, but different plus strand 5' ends. Since the 5' ends of the plus strands are diagnostic of fungal virus dsRNAs (Table 4.2) and probably reflect the sequence specificity of the viral RDRP for the 3' ends of the minus strands, it seems likely that the L segments are the result of processing of the M plus strands followed by packaging and replication. Since the 3' ends of the plus strands are preserved, replication of the L plus strands should be routine.

Considerable data support this model (Park, C.M., and Bruenn, J.A., unpublished). First, non-denaturing Northerns of total RNA from infected cells always show two plus strand RNAs from P1M1, P4M2, and P6M2: the cognate M plus strand and the L plus strand. Probes from the 5' region of the M plus strands show only the full-sized M plus strand, not a 5' fragment. This is true whether the RNAs are virally produced or are the result of RNAPII transcription of expression vector constructs containing full-sized M cDNAs. This latter experiment would seem to rule out internal initiation by the viral RDRP as an explanation for the L plus strands. In addition, the same pattern is observed even when expression of cDNAs is in heterologous systems such as yeast and tobacco. It has not been possible to demonstrate any self-cleaving activity of the M plus strands *in vitro*, so they do not appear to be ribozymes.

On the other hand, they do have a very peculiar predicted secondary structure, with a very long stem immediately preceding (by two bases in each case) the putative

TABLE 4.2
Ends of Fungal Virus RNA Plus Strands

	5' Ends	
UmVP1M1	GAAAAACUCU	UAAAAUAAAG
UmVP4M2	GAAAAACUUU	CUCAAAACAG
UmVP6M2	GAAAAACUCA	AUAAGAACGG
UmVP1M2	GAAAAAGAAA	AACAUAAAAC
UmVP1H1	GAAAAACUAU	CCCCGCUGGU
ScVL1	GAAAAAUUUU	UAAAUUCAUA
ScVLa	GAAUUUUUCG	GUGAACCGGA
ScVM1	GAAAAAUAAA	GAAAUGACGA
ScVM2	GAAAAAAUGA	AAGAGACUAC
ScVM28	GAAAAAAUUU	GAAUGGAGAG
Hv190sv	GAAAUUUAGG	GCACACACGA
	3' Ends	
UmVP1M1	AACGACUCAA	UAAAAUGCA
UmVP4M2	GGUGACUCAA	CAAAAUGCA
UmVP6M2	AGGUGACUAG	UAGAAUGCA
UmVP1M2	AGCCACUACU	GAGAAUGCA
UmVP1H1	ACUGGGGUG	GAAAAU CA
ScVL1	UGGGAAUUAC	CCAUAUGCA
ScVLa	GUACAUACGA	UACUACGCA
ScVM1	UAGAAGAAAC	ACCCAU CA

The UmV 5' and 3' ends are from a series of papers from the Bruenn lab[10,12,13,55] (Park, C.-M., and Bruenn, J.A., unpub.). The ScV sequences are from several labs.[65,66,77–80] The ScVM2 5' ends[81] and the ScVM28 5' end[82] are published. The Hv190sv sequence is from S.A. Ghabrial (pers. comm.)

cleavage site (Figure 4.5). This stem reaches the extreme of 23 perfect base pairs in P4M2, a really remarkable secondary structure possibly without precedent in any mRNA. If the three viral M plus strands that result in L plus strands are compared, they share a great deal of sequence similarity only in their 3' ends, with no similarity in their coding regions. The similarity is greatest right around the putative processing site, dropping off very rapidly 5' to this site (Figure 4.5). Preliminary experiments with rabbit reticulocyte lysates do demonstrate an enzyme activity capable of cleaving the M plus strands, leaving the L segment intact and degrading the resultant M 5' region, so that it does appear that a universal cytoplasmic enzyme is responsible for this processing event (Park, C.M., and Bruenn, J.A., unpublished).

However, the mystery is not so much the processing itself as its purpose: why this tremendous conservation of sequence to produce a non-coding L segment without demonstrable function? Synthesis of the L segments is not necessary for toxin production. Remember that production of the KP6 toxin, for instance, works perfectly from cDNA constructs in which the L portion of P6M2 is deleted, in *Ustilago* as well as in other eucaryotes.[13] Furthermore, the KP1 toxin is not produced

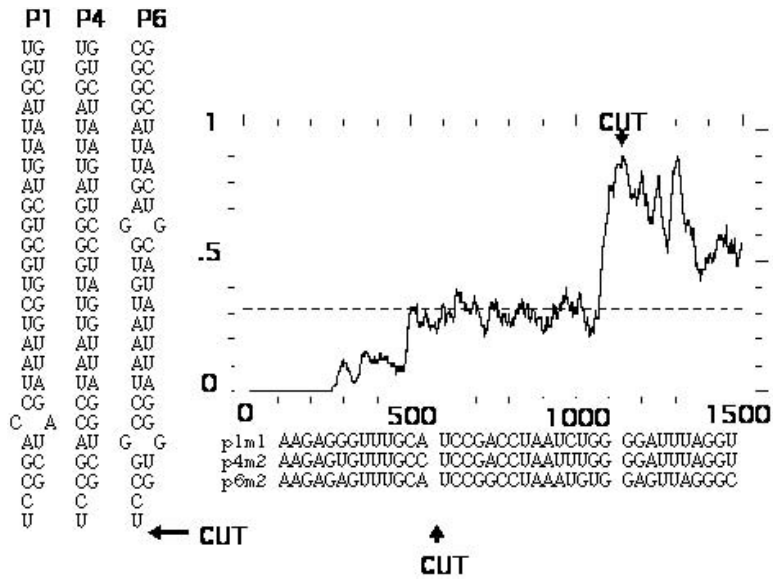

FIGURE 4.5 Primary and proposed secondary structure surrounding the regions of the M plus strand segments that become the 5' ends of the L plus strands. On the left is the proposed stem structure in this region. Lower right is the primary sequence showing the location of the 5' UCC and the sequence identities in this region. Upper right is a PLOTSIMILARITY[83,84] profile of the aligned sequences of P1M1, P4M2, and P6M2, showing the remarkable sequence identities near the proposed processing site.

from the M segment encoding L (P1M1), but rather from P1M2.[10] In some KP1 strains, there may be more than one L segment, and one of these may play a role in immunity to toxin.[56]

4.2 STRUCTURE AND ENZYMATIC ACTIVITIES OF THE VIRAL PARTICLES

4.2.1 Capsid Polypeptides and Capsid Structure

The capsid polypeptides of the *Ustilago* viruses are not well characterized. They do have molecular weights similar to those of ScV, approximately 75 kDa.[57,58] *In vitro* translation experiments demonstrate that H2, H3, and H4 encode capsid polypeptides.[59–61] In combination with the sequence of H1 and the fact that the H segments of different size have little sequence similarity,[55,61] this suggests that all the H segments encode essential viral polypeptides of a series of totiviruses present in the same cells, like ScVL1 and ScVLa.[62]

In two totiviruses, ScV and UmV, there is considerable information about the structure of the viral particles. In ScV, there are only two proteins in the capsid, the major capsid polypeptide (Cap) and the RDRP (Cap-Pol). Original calculations based on the density of ScV particles in CsCl predicted 120 copies of Cap per

particle.[63] This arrangement was confirmed by cryo-electron microscopy.[64] Careful measurements of the ratio of Cap-Pol to Cap indicated two copies of Cap-Pol per particle.[65,66] Consequently, viral particles appear to have two copies of Cap-Pol and 118 copies of Cap per particle. This is probably the case for UmV as well since, at least with P1H1, Cap-Pol is the primary translation product. In this case, the ratio of Cap-Pol to Cap would be controlled by cleavage frequency rather than –1 translational frameshifting frequency, as in ScV.[67]

The number of Cap domains present in viral particles was directly measured by cryo-electron microscopy of both UmV and ScV.[64] Both have 120 copies of the Cap domain present as asymmetric dimers. This is similar to the arrangement of the major capsid polypeptide of bluetongue virus, which also occurs as an asymmetric dimer, in which one subunit in each dimer adopts one configuration and the second adopts a second configuration.[68] This preserves the Caspar and Klug rules for assembly of icosohedral viruses,[69] in which a triangulation number (T) of 1 is allowed (60 capsid polypeptides) or a T of 3 (180 capsid polypeptides) but not a T of 2 (120 capsid polypeptides). The dsRNA icosohedral viruses are T = 1 viruses with two capsid polypeptides, each with 60 subunits, which differ only in their configuration, not in their primary sequence. These are assembled as asymmetric dimers in which the two monomers are shaped differently despite their identical primary sequence. All dsRNA viruses appear to have at least one such capsid polypeptide.[70]

4.2.2 Replication and Transcription of UMV RNAs

The dsRNA viruses have a unique life cycle in which transcription takes place in virions within the cell, transcript plus strands are extruded and either translated or packaged, and replication (minus strand synthesis) occurs in nascent virions. UmV particles do have transcriptase[71] and replicase activities and, at least in one case, M2, replication and transcription are semi-conservative.[72] This contrasts with the conservative transcription and replication in ScV.[73–75] Both conservative and semi-conservative modes of synthesis are represented among the fungal viruses.[76]

The initiation sites for replication (the 3' end of the viral plus strand) and for transcription (and 3' end of the minus strand) appear to have some sequence similarity in ScV and UmV RNAs (Table 4.2). The consensus sequence for plus strand initiation (as viewed in the plus strand) is GAAAAA and for the minus strand (as viewed in the plus strand) is AUGCA. It remains to be seen if these are the same among all fungal viruses.

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5 Molecular Basis of Symptom Expression by the *Cryphonectria* Hypovirus

Patricia M. McCabe and Neal K. Van Alfen

CONTENTS

5.1 INTRODUCTION

Cryphonectria parasitica is the causal agent of chestnut blight, a disease that devastated both the European and American chestnut populations during the first half of the twentieth century. The chestnut tree in North America has been eliminated by this pathogen from its historically dominant position in the Eastern deciduous forest. In Europe, however, a biological control of the fungus was found in chestnut plantations that were no longer succumbing to the disease. The biological control agent was found to be a fungal virus. Virulent strains of *C. parasitica* enter the chestnut tree at a wound site, where it forms a mycelial fan that grows though the inner bark of trees, destroying the phloem and cambium. Virus-infected strains of the fungus can colonize host wound sites but are able to penetrate bark to only a

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limited extent, resulting in a superficial non-girdling canker. This fungal virus has been credited with preventing the wholesale destruction of chestnut trees in Europe, as occurred in North America.

The best studied of the viruses responsible for biological control of the filamentous ascomycete, *C. parasitica,* is the *Cryphonectria hypovirus I* (CHV1). CHV1 infection perturbs normal developmental processes in this fungus; i.e., the fungus remains in a vegetative state, lacking normal sexual or asexual sporulation, and is unable to successfully invade its host plants. During normal development, *C. parasitica* produces a number of orange, yellow, and red pigments; young vegetative hyphae lack these pigments.[1] One consequence of infection by CHV1 is the lack of normal development resulting in reduced pigment production by the fungus. This phenotype provides a very useful visible marker to identify infected colonies of the fungus (Figure 5.1). As shown in Figure 5.1, the growth rate of the infected fungus in culture is normal.

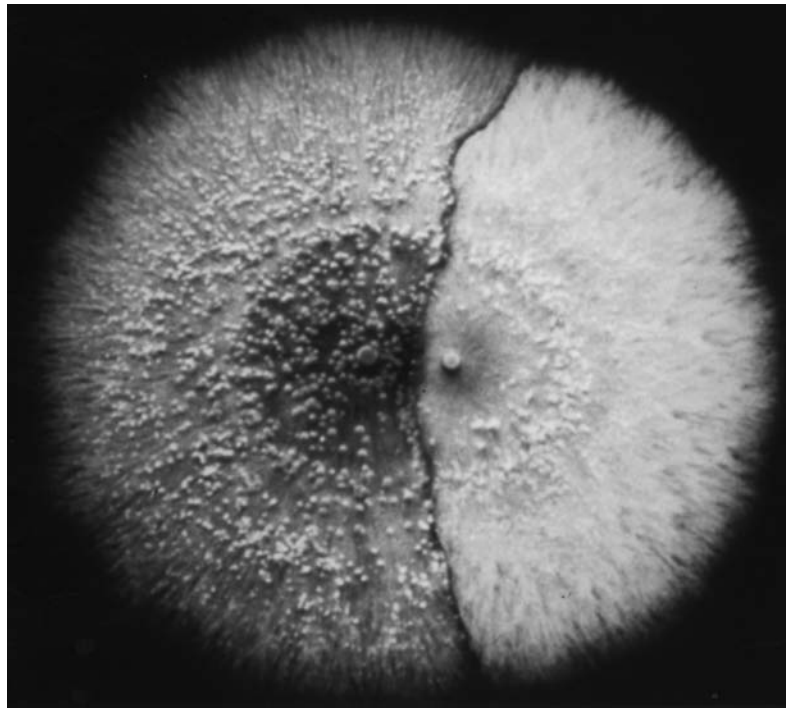

FIGURE 5.1 Phenotypic changes in *Cryphonectria parasitica* upon *Cryphonectria* hypovirus infection. The colony on the left is a wildtype strain. In addition to an orange/brown pigment, wildtype strains produce numerous conidia in asexual fruiting bodies. The colony on the right is a strain infected with CHV1. CHV1 infected strains are not pigmented; they remain white and produce very few asexual conidia in culture. (Photograph courtesy of Daniel Rigling. Reprinted from *Trends in Microbiology,* 7, McCabe, P. M., Pfeiffer, P., and Van Alfen, N. K., The influence of dsRNA viruses on the biology of plant pathogenic fungi, 377–381, ©1999, with permission from Elsevier Science.)

CHV1 is a defective virus that lacks both a protein coat and an infectivity cycle. Being limited to the cytoplasm of infected cells, the only mode of transmission of the virus is via hyphal anastomosis and cytoplasmic transfer. The few asexual conidia that are produced by infected strains can also carry the virus. Anastomosis is a common method of cytoplasmic and nuclear exchange between closely related strains of fungi. During this process of cytoplasmic exchange between different strains of the fungus, viruses can move into new hosts. The success of anastomosis depends on the similarity of alleles of the vegetative incompatibility (*vic*) genes in the fusing strains. *C. parasitica* has six characterized *vic* loci.[2] The alleles must be identical at all loci for successful compatibility to occur, and differences in a single locus often can result in post-fusion incompatibility, causing death of the fused cells. The nature of the incompatibility reaction varies both in a quantitative and qualitative manner according to the specific *vic* allele combinations.[2] This vegetative incompatibility reaction is the best known defense of *C. parasitica* to spread of the virus within populations of the fungus. Thus, at the population level, CHV1 is more successful in fungal populations that have few *vic* allelic differences.

CHV1 dsRNA is associated with fungal vesicles of approximately 100 nm in diameter.[3, 4] RNA dependent RNA polymerase activity co-purifies with these vesicles, and the putative viral RNA polymerase protein has been identified in preparations of these host vesicles.[5,6] Vesicle fractions totally lacking in dsRNA can be purified from uninfected *C. parasitica* strains and, in both cases, the contents of the vesicles are similar except for the presence of the viral genome in vesicles from infected strains. The presence of carbohydrates containing glucose, galactose, arabinose, and mannose in these vesicles, the sugars found in the fungal cell wall,[3] suggested that they might function as secretory vesicles in normal cells. CHV1 infection shows no changes in cell cytology except accumulation of these small vesicles;[4] all cell organelles appear normal. The normal vegetative growth of the infected fungus also suggests that the virus does not disrupt most host membrane systems.

5.2 STRATEGIES FOR STUDYING THE HYPOVIRULENCE ASSOCIATED SYMPTOMS

Interest in CHV1 stemmed from its potential as a biological control agent for chestnut blight in particular, but also as a means to study the basis of fungal virulence. Since CHV1 affects virulence expression without debilitating the fungus, it offers an opportunity to identify differentially expressed molecules that may be important for virulence of the pathogen that are independent of normal fungal growth. Virulence expression of *C. parasitica* involves the developmentally regulated mycelial fan that forcibly penetrates into host tissue killing host cells in advance of its growth. The organization of this specialized structure and the release of extracellular molecules that kill host cells show that developmental regulatory pathways and secreted enzymes or toxins are necessary for infection.[7] Strategies for the general study of fungal virulence have included genetic mutation combined with comparative molecular studies and general searches for toxic molecules secreted from virulent patho-

gens. Since the virus-infected strains are the functional equivalent of a mutation in a virulence controlling pathway, comparative molecular biology studies of virulent and isogenic hypovirulent strains are an obvious approach to study virulence.

The search for toxic molecules secreted from *C. parasitica* that could be involved in virulence have included extracellular enzymes and toxins. Extracellular enzymes potentially involved in the infection process were studied comparatively from virus-infected and uninfected strains of the fungus.[8–10] Such studies have shown that differences in expression of various enzymes exist between virulent and hypovirulent strains, but these studies have not successfully led to an understanding of the basis of virulence of this fungus. The same is true of the study of molecules reported to be extracellular toxins produced by *C. parasitica.*[11]

Since *C. parasitica* has proven to be a facile organism for molecular genetic manipulation, the comparative enzymatic studies of virulent and hypovirulent strains have been extended to include genetic level studies. Using sequences of genes of known extracellular enzymes implicated in virulence of other fungi, a number of homologous genes have been cloned from *C. parasitica* (Table 5.1). Preliminary studies have been carried out on a fungal cutinase, a cellulase and an endopolygalacturonase, all of which are down-regulated upon CHV1 infection,[12–14] and endothiapepsin, a rennin-like protease that is not down-regulated by CHV1.[15] Interestingly, deletion of the cellulase and endopolygalacturonase genes did not lead to a significant reduction in fungal virulence. Cutinase expression is inhibited by glucose and induced by cutin monomers.[16] Cutinase activity could be detected in cultures of *C. parasitica* only when grown under inducible conditions. However, in a pair of isogenic strains, the virus-infected strain showed significantly less cutinase activity than the virulent strains. Southern blot analysis showed that an identical cutinase gene was present in both the virulent and hypovirulent strains. However, there is no mRNA expression in CHV1 infected strains under inducible conditions.[12] There has been no deletion of the cutinase gene to examine the concomitant effect on activity and virulence. It is noteworthy that a single virulent strain, EP155, was infected with dsRNA of European and American origin via anastomosis with isogenic strains. In all cases, there was a significant reduction in cutinase enzyme activity and a corresponding decrease in mRNA and the cutinase protein as detected by Northern and Western hybridization, respectively.[12]

Although a number of proteins with a possible role in fungal pathogenicity have been characterized in some detail, these studies have provided few insights regarding how the virus causes symptoms. Many proteins probably act in concert to cause pathogenicity, and thus perturbation of a regulatory mechanism that controls expression of all of them is most likely the way the virus affects virulence. One of the recurring themes in recent studies of virulence of plant pathogens is the role of signaling between host and pathogen. It is expected that signals from the host affects expression of fungal virulence genes and that a possible mechanism of virus perturbation of virulence, and other developmental pathways of the fungus, is interference with the host's signal transduction pathway. Search for such viral effects on fungal signal transduction has been another approach used to understand how the virus causes symptoms. The extracellular enzyme laccase has been used as a simple marker for the effect of CHV1 on signal transduction.

TABLE 5.1
C. Parasitica* Genes Down-Regulated upon Infection with the *Cryphonectria Hypovirus

Gene	Function	Reference
crp	cell surface hydrophobin	Zhang et al., 1994
lac-1,	laccase	Rigling and Van Alfen, 1993
mf1-1	mating type specific pheromone	Zhang et al., 1998
mf1-2	mating type specific pheromone	Zhang et al., l998
mf2-2	mating type specific pheromone	Zhang et al., l998
cpg-1	G protein α subunit	Choi et al., l995
cpg-2	G protein α subunit	Choi et al, l995
cpgb-1	G protein β subunit	Kasahara and Nuss, l997
cbh-1	Cellobiohydrolase	Wang and Nuss, l995
enpg-1	Endopolygalacturonase	Gao et al., l996
	Cutinase	Varley et al., l992

5.2.1 Laccase as a Molecular Marker for Regulation of Gene Expression

Laccase is a copper-containing phenol oxidase and, although the function of this enzyme in *C. parasitica* is not known, its activity may be important in the infection process. Laccase activity has been implicated in a number of roles related to fungal development and virulence including degradation of lignin, formation of fruiting bodies, and pigment production.[17–19] It is known that virus-infected strains of *C. parasitica* are unable to penetrate into the tree, and it is possible that this lack of canker penetration is related to a reduced level of laccase.[20] Initial experiments showed CHV1 infection reduced laccase enzyme activity in Swiss strains of *C. parasitica.*[21] This stimulated interest in laccase as a molecular reporter for CHV1 infection. Characterization of the *C. parasitica* extracellular laccase showed it to be down-regulated at the mRNA level, resulting in less enzyme activity. There was a 75% reduction in laccase enzyme activity upon CHV1 infection without any decrease in biomass of the fungus.[22]

C. parasitica has three laccase genes; *lac1* and *lac3* are extracellular, and *lac2* is intracellular.[23, 24] The *lac1* gene has been sequenced and encodes for a polypeptide of 592 amino acids. The precursor peptide is processed to produce the mature protein.[20] Laccase is under dual regulation at the transcriptional level. A negative repressor acts to keep laccase levels relatively low,[20] and a positive regulator acts to increase laccase levels.[25] Expression of laccase varies greatly with the environment. Transcription levels are influenced by the growth medium, the age of the culture, and the amount of light available. Laccase repression can be overcome by the addition of low levels of protein synthesis inhibitors such as cyclohexamide or cyclosporin A.[20,26] Cyclohexamide is thought to prevent transcription of a negative regulator by blocking protein synthesis. Growth of cells in cyclohexamide or cyclosporin A increases levels of laccase transcript; with cyclohexamide, there is a

15–20-fold increase within a 24-hour period. This stimulation does not occur in CHV1 infected strains, suggesting the virus interferes with laccase regulation.[25,26] Inhibitors of calcium signaling such as the calcium chelators BAPTA and EGTA, and the calcium channel blocker lanthanum prevented the action of cyclohexamide and cyclosporin A, reducing laccase gene transcription to unstimulated levels. Inositol triphosphate (IP_3) activates calcium signaling, so laccase transcripts were examined in cells that had been exposed to lithium, which increases the amount of IP_3 in the cell, and neomycin, which prevents IP_3 production. As expected, cells exposed to lithium had increased laccase transcript levels, and those exposed to neomycin produced no laccase transcript. Interestingly, blockage of the calcium signaling pathway also prevents pigment production in wildtype strains, suggesting the virus may be acting via calcium mediated signaling pathway.[25]

Transformation of *C. parasitica* with the hygromycin gene fused to the laccase promoter showed that the promoter encoded all the information required for both induction and suppression of laccase on a 2.2 Kb region upstream of the laccase gene. Virus infection of the transformants switched off the reporter gene, indicating the virus acts on the laccase promoter.[25] Further analysis of the laccase promoter region identified a 111bp fragment to which it appeared multiple polypeptides could bind. Binding was further localized to a 22bp palindromic sequence near the center of the 111 bp binding sequence. Deletion of this sequence prevented protein binding indicating its importance. This 111 bp element was sufficient to restore cyclohexamide inducible transcriptional activation of laccase.[27]

5.3 ROLE OF SIGNAL TRANSDUCTION IN *C. PARASITICA* DEVELOPMENT

Development in fungi is the result of coordinated temporal and spatial expression of genes. For development to occur, many molecules and structures are required that have little or no role in fungal growth. The coordinated expression of these genes is somehow perturbed by CHV1 infection, with the result that asexual and sexual development as well as virulence expression in *C. parasitica* is largely prevented. The large array of genes necessary for development and virulence expression in *C. parasitica* probably remain repressed in virus-infected strains. Understanding how the virus prevents normal development will provide important insights into this process in fungi.

It is known that signal transduction pathways play an important role in the regulation of development. Development in microorganisms occurs as a result of internal and external signals. It is thus expected that effects of CHV1 infection on host signal transduction will be found. Signal transduction pathways were found to be important in laccase regulation, suggesting that CHV1 is interfering with a signal transduction process.[25] It is also known that CHV1 down-regulation of genes can be overcome by certain environmental stimuli like high light or nutrient availability,[28] again implicating signal transduction in virus-induced symptom expression.

Heterotrimeric G proteins are GTP binding proteins that consist of three subunits, α, β, and γ, and are known to be involved in signal transduction pathways.

Gα subunits have been cloned in a number of filamentous fungi and have been shown to be important in sporulation and development.[29–31] In most cases, it is the Gα subunit that transduces the signal. The first Gα subunit cloned from *C. parasitica*, *cpg-1,* showed 98% homology to a G protein α subunit from *N. crassa.*[32] *Cpg-1* is claimed to be down-regulated at the protein level upon virus infection,[13] but this could not be independently confirmed.[33] The phenotype that resulted upon *cpg-1* deletion (Δ*cpg-1*) is far more severe than that of virus infection. Like CHV1 infection Δ*cpg-1* had reduced canker size on dormant chestnut trees; however, the disruptant strains grew slowly, were highly pigmented (bright orange), and did not show a response to light, i.e., did not produce the usual light/dark growth rings. The deletion strain is severely debilitated, i.e., there is a 65–70% reduction in mycelial mass,[34] unlike the virus-infected strains in which growth in culture is normal. The lack of infection of dormant chestnut stems by the deletion mutant is probably a direct result of this debilitation. A second *C. parasitica* Gα subunit, *cpg-2,* which is 49% identical to *cpg-1*, has also been disrupted. However, the *cpg-2* disruption phenotype is mild; it only slightly decreased the growth rate of *C. parasitica* and had no effect on pigmentation, sexual and asexual sporulation, laccase transcription, or virulence.[34]

Gβ subunits have not been as well studied in fungi as Gα subunits; however, it is known that the Gβγ dimer is the signal molecule in the pheromone response pathway in *Saccharomyces cerevisiae* and *Cryptococcus neoformans.*[35,36] The first Gβ subunit from a filamentous fungus was cloned from *C. parasitica*, and its deletion results in increased linear growth on solid media but very little sporulation. Pycnidia were only produced in the center of the colony. However, there was a significant reduction in virulence (10% of wildtype), without a concomitant decrease in dry weight.[37] Thus, deletion of this G protein subunit does not result in general debilitation but a specific effect related to sporulation and virulence, suggesting involvement of the Gβ subunit in signal transduction pathways perturbed by CHV1.

Signal transduction pathways are responsible for most cellular processes, and the involvement of G proteins as mediators of the signal is well known. As described above, three G protein subunits have been cloned from *C. parasitica,* and deletion of each of these mimics some, but not all, of the CHV1 infection phenotype. Thus, G-proteins may transmit virus signals or prevent transmission of developmental signals. Deletion of the *C. parasitica* Gβ subunit most closely mimics the CHV1 infection phenotype, including increased growth and reduction in sporulation.[37] Although its effect on pheromone signaling and secretion of developmentally regulated proteins has not been examined, it is possible that this Gβ subunit is responsible for transmitting the signal to initiate development.

5.4 DIFFERENTIAL EXPRESSION COMPARISONS BETWEEN VIRUS-INFECTED AND UNINFECTED STRAINS

CHV1 infection prevents development and reduces virulence in *C. parasitica*, so it should reduce the level of expression of many of host genes, since most develop-

mentally regulated genes will not be highly expressed in infected strains. A number of methods can be used to distinguish between levels of gene expression between different strains of an organism or at different stages of fungal development. These methods do not begin with assumptions about the genes being regulated but identify anonymous genes or gene products that differ between the strains/stages of development. Powerful new technologies adopted from the microchip industry have recently been developed to optimize this strategy. Two older methods have been employed to study differential expression of genes between normal and hypovirulent strains of *C. parasitica* and have led to the identification of a number of genes that are expressed differentially in CHV1-infected and wildtype strains. In all studies, isogenic strains were examined, so the strains were identical except for CHV1 infection.

Powell and Van Alfen[38] used subtractive hybridization to identify genes that are differentially expressed. This method identified five cDNA clones that were down-regulated upon CHV1 infection. Several genes also appeared to be expressed only in CHV1 infected strains. One clone, V-2A-1, that was only expressed in virulent strains was further characterized. V-2A-1 has two genes associated with it that are 0.85 kb and 0.65 kb in length and were named *vir1* and *vir2,* respectively. Clone *vir2* also hybridized to the 0.85 kb mRNA of *vir1* suggesting the clones overlap. As these genes were only expressed in the virulent strain, they were thought to be important for pathogenicity (thus *vir* for virulence). *Vir 1* and *vir2* were expressed in two virulent strains, EP155 and Gramma, but were not expressed in strains infected with the European or North American hypoviruses. The American and European hypoviruses do not cross hybridize and produce different infection phenotypes; their only similarities are hypovirulence and reduced asexual sporulation.[38,39] As differential hybridization detects only high copy mRNAs, there are probably many more differences in gene expression upon CHV1 infection than were detected by this subtractive hybridization method.

A second method used to identify changes in gene expression was differential display. Differential display utilizes the polymerase chain reaction (PCR) to amplify arbitrary mRNA sequences using random primers and a degenerate oligo dT primer. PCR products are separated by PAGE, and the relative band intensities indicate the level of gene expression. Twenty arbitrary primers were used in a total of 80 combinations on mRNA from a virulent and hypovirulent *C. parasitica* strain. Examination of all primer pairs showed 139 PCR products that changed in intensity more than four-fold upon CHV1 infection. When increases of two-fold or greater were examined, there were more than 400 products identified. Expression of 296 PCR products was increased, and 127 PCR products decreased upon hypovirus infection.[40] These results are puzzling, since perturbation of development by CHV1 should result in many more genes being down-regulated than up-regulated upon infection by the virus. Differential display is a good method for detecting gene products that may be differently expressed in cells; however, false positives are often obtained, as any contaminating DNA will be amplified in addition to mRNA. As the method is PCR based, it is very sensitive and even miniscule amounts of contaminating DNA can lead to products. Also, minor variations in the quality of the RNA preparation can give misleading results. The method must be repeated and shown

to be highly reproducible before any conclusion can be reached. It is also important to confirm differences by Northern analysis using the differentially displayed band as a probe. Only two probes were used in Northern blots, and both showed down-regulation upon CHV1 infection.[40]

Differential expression was also examined at the protein level. Isogenic virulent and hypovirulent strains were examined for differential accumulation of polypeptides. Extraction of proteins from lyophilized mycelia showed differential accumulation of a number of proteins, the most prominent being a 24 KDa protein that was present in all wildtype strains but could not be detected in strains infected with the European or American hypovirus. Culture filtrates from both types of virus-infected strains showed similar changes in secreted protein profiles, indicating that the virus was affecting secreted proteins. Further characterization using two-dimensional gel electrophoresis identified eight proteins that were present only in wildtype strains; these ranged in size from 27–66 KDa. The results were consistent in two virulent and three hypovirulent strains, even though the dsRNA of each of the hypovirulent strains was unique. Less than 5% of the proteins detected showed differential expression.[41]

Although differential expression of both mRNA and proteins gave an indication of the level of down-regulation upon virus infection, further characterization of these genes is important to try to assign them a function. The relatively few proteins that were differentially expressed, as compared to the large number expected when development is perturbed, is probably the result of repression of developmental genes in the growth conditions used—shaking liquid culture.

5.5 CHARACTERIZATION OF GENES IDENTIFIED BY DIFFERENTIAL EXPRESSION

5.5.1 Pheromones

Differential hybridization of isogenic virulent and hypovirulent strains EP155 and UEP1 led to the identification of two genes thought to be important in virulence, *vir1* and *vir2*. Both genes were down-regulated upon CHV1 infection. Sequencing of *vir2* showed it to be similar in structure to known fungal pheromone genes, and deletion of this gene resulted in significant reduction in pycnidia and conidia production and perithicia were barren.[42] Colony morphology and growth rate of the *vir2* deletion strain is the same as wildtype, although a reduction in conidiation was observed. Significantly, there was no reduction in virulence of the strain. The *vir1* and *vir2* genes both have an 83bp open reading frame that encodes for a 23 amino acid peptide. These peptides have the hallmarks of fungal lipopeptide sex pheromones: a C-terminal CAAX box with an asparagine residue 8–11 amino acids in front of the box. CAAX is a prenylation signal, and farnesyl groups have been detected on the pheromones in other fungi. Based on this similarity and loss of sexual mating upon deletion of the genes, their names were changed to *mf2-1* and *mf2-2* (for *m*ating *f*actor).[33]

As *mf2-1* and *mf2-2* are so similar in structure to yeast pheromones, investigations were carried out to isolate the pheromone precursor gene of the opposite

mating-type strain. Subtractive hybridization was used to clone the pheromone gene, utilizing the fact that the pheromones are expressed in a mating-type specific manner. The gene isolated was shown to be similar to the *S. cerevisiae* α-factor pheromone, based not on sequence but on post-translational processing signals.[33,43] There is a single copy of this gene, and it was named *mf1-1*. Like *mf2-1* and *mf2-2,* it is expressed in a mating-type-specific manner. The *mf1-1* gene is encoded as a 530 amino acid polypeptide that is post-translationally modified to form a decapeptide that is the active pheromone. There are seven repeats of this decapeptide whose amino acid sequence is WCLFHGEGCW. All are preceded by the dipeptides XA or XP. The polypeptide is a preproprotein that is modified during secretion through the vesicle mediated secretory pathway (Figure 5.2). The decapeptide is produced by cleavage of the proprotein by three proteases. These proteases have been studied extensively in yeast and are named Kex2p, Kex1p, and Ste13p. Kex2p is a serine protease that cleaves following a pair of lysine or arginine residues at the amino end of the decapeptide.[44] Kex1 is a serine carboxypeptidase that processes the carboxy terminal of Kex2p processed proteins, removing the dibasic residues.[45] Ste13 is a membrane bound dipeptidyl aminoeptidase that cleaves the free N-terminus generated by Kex2p.[46] Digestion by all three peptidases is essential for production of the mature active pheromone. CHV1 infection down-regulates the pheromone precursor genes of both mating types and causes male sterility in the fungus; the strains will not mate.[33] CHV1 down-regulation of the pheromone genes was confirmed using other *C. parasitica* strains.[41] In all cases, virus infection decreased pheromone mRNA expression levels.

5.5.2 Cryparin

Comparison of the protein profiles of CHV1 infected and wildtype strains identified a number of proteins that were not present in CHV1 infected strains, the most striking being a highly abundant 24 KDa protein.[41] The protein, cryparin, was purified by extraction in 60% ethanol and HPLC.[47] Cryparin is an abundant cell-surface protein that belongs to the class of proteins known as hydrophobins. These proteins are highly conserved within the filamentous ascomycetes and are classified based on the spacing of eight cysteine residues in the polypeptide. The cysteines form disulfide bridges, resulting in hydrophobic loops. The only known property of these proteins is to increase the hydrophobicity of fungal spores and hyphae.[48,49] Cryparin was characterized through a combination of cDNA sequence analysis and N-terminal sequence analysis of the peptide. The carboxy-terminus of the protein is of particular interest as this region is conserved in structure between the various fungal hydrophobins. The most closely related hydrophobin to cryparin is the one produced by the Dutch elm disease pathogen, *Ophiostoma ulmi.*[50]

The cryparin gene has been cloned and encodes for a polypeptide of 9 KDa. Sequence analysis and examination of the N-terminus of the protein shows that cryparin is produced as a pre-proprotein that undergoes post-translational modification. Like the pheromone genes, cryparin is secreted via a vesicle mediated secretory pathway (Figure 5.2), and the mature protein is produced by cleavage of the prosequence by a Kex2p-like serine protease. Northern analysis shows that cryparin

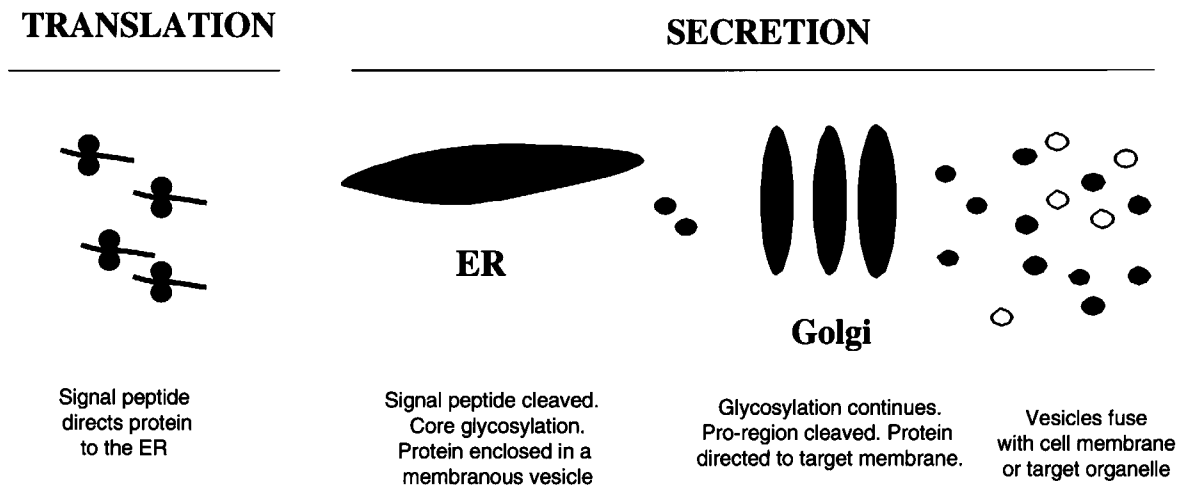

FIGURE 5.2 The fungal protein secretory pathway. Following translation, proteins are targeted to the endoplasmic reticulum (ER) by a signal peptide that is then cleaved. Core glycosylation may occur in the ER, and then the protein is enveloped in a membranous vesicle and transported through the Golgi system for further glycosylation. Further post-translational processing, e.g., removal of the prosequence by a Kex2p-like serine protease, occurs in the late Golgi. The vesicles then bud and move to the plasma membrane.

represents approximately 25% of the total mRNA in wildtype cells during exponential growth, and over 1% of the dry weight of the fungus when grown in liquid culture is the cryparin protein. The transcript is down-regulated 70% in CHV1 infected cells.[51]

Cryparin is secreted through the cell to the cell wall and culture medium where it reassociates with the cell wall.[52] Purified cryparin has lectin-like abilities. The protein will bind to the cell wall of a cryparin deletion strain and has the ability to agglutinate rabbit red blood cells.[47,52] The function of the protein has been examined through a knockout strategy. The knockout mutant was visually identical to the wildtype strain of the fungus. There were no alterations in asexual sporulation, sexual cycle, or virulence of the fungus after deletion of the cryparin gene. The hyphae lost their hydrophobicity in the mutant strain, however, and the shear strength of the hyphae was reduced. Based on these studies, we have concluded that cryparin plays an important role in maintaining the structural strength of the fungal cell wall. We have found that without cryparin pycnidia cannot erupt through the bark of American chestnut stems; thus, this protein plays an important role in asexual sporulation and in the biology of the fungus. This role clearly would provide an important selective advantage for preservation of this protein in the evolution of *C. parasitica.*[53]

5.6 THE ROLE OF CHV1 IN PERTURBATION OF PROTEIN SECRETION

Genes that have been identified based on differential expression between virulent and hypovirulent strains all appear to have a role in development. Laccase is thought to be involved in the formation of fruiting bodies,[54] cryparin is necessary for asexual sporulation, and fungal pheromones are essential for sexual sporulation.[33] The discovery that all genes identified by differential screening between CHV1-infected and uninfected strains code for secreted proteins is striking. Most virulence factors are thought to be secreted molecules, but there are also many molecules important for development that are likewise secreted from fungal cells (Figure 5.3).

The similarities in function of the differentially expressed genes led to further investigation into how the virus prevents development. The fact that CHV1 down-regulates a subset of proteins that are secreted without affecting growth suggests that there is more than one pathway for secretion in *C. parasitica*. Evidence for multiple secretion systems in eukaryotes has been described in the yeast *Yarrowia lipolytica,*[55] in which there are four distinct pathways. Each pathway is responsible for secretion of specific cargo to a particular destination. We hypothesize that CHV1 is targeting a subset of vesicles that are responsible for the secretion of developmentally important proteins (Figure 5.4), the equivalent of one of the pathways of *Y. lipolytica*. The hydrophobin cryparin was used to test this hypothesis as it is highly abundant. Cryparin co-purifies with a vesicle fraction isolated from *C. parasitica*[52] similar to that associated with CHV1 dsRNA.[5] Cryparin was identified in the vesicle fraction as the unprocessed form, which ran about 36 KDa on polyacrylamide, as well as the 24 KDa mature processed form, which is not glycosylated, confirming that these vesicles were used for protein secretion.[52] We hypothesize that the virus

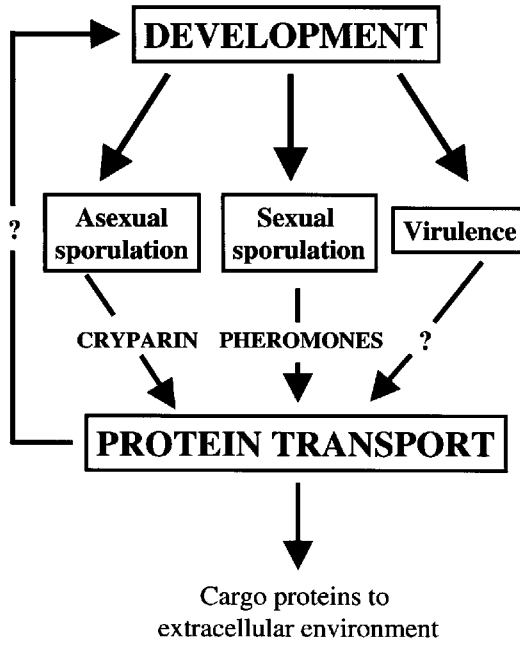

FIGURE 5.3 The relationship between fungal development and protein secretion in *Cryphonectria parasitica.*

targets for its replication a subset of vesicle types important for secretion of developmentally regulated proteins.

The proteins identified by differential expression that appear to be transported via a vesicle mediated secretory pathway are cryparin, laccase, and *mf1-1*. All are preproproteins whose mature peptide is formed after cleavage by a serine protease. Serine proteases are ubiquitous in eukaryotes, and those with specificity for lysine-arginine or arginine-arginine residues belong to the subtilisin superfamily. In yeast, this protease is called Kex2 and is responsible for maturation of the α-pheromone and toxins encoded by the killer viruses.[44] Filamentous fungi are known to have a similar protease responsible for maturation of secreted proteins and the homologous gene, *kexB*, has been cloned in *Aspergillus niger.*[56] Other viruses such as poliovirus are known to perturb host protein secretion. Poliovirus produces two proteins, 2B and 3A, which target the ER and Golgi, respectively. Protein 2B causes disassembly and fragmentation of the Golgi complex,[57] and protein 3A prevents ER to Golgi secretion, causing proliferation of ER membranes inside the cell.[58] Obviously, the effect of poliovirus on the cell is much more devastating than that of CHV1. Poliovirus prevents all protein secretion, killing cells very quickly, while CHV1 infection only partially disrupts protein secretion. We speculate that the lack of adverse effect of CHV1 on fungal growth is because the secretory vesicles used by the virus for replication are used only to secrete developmentally regulated proteins.

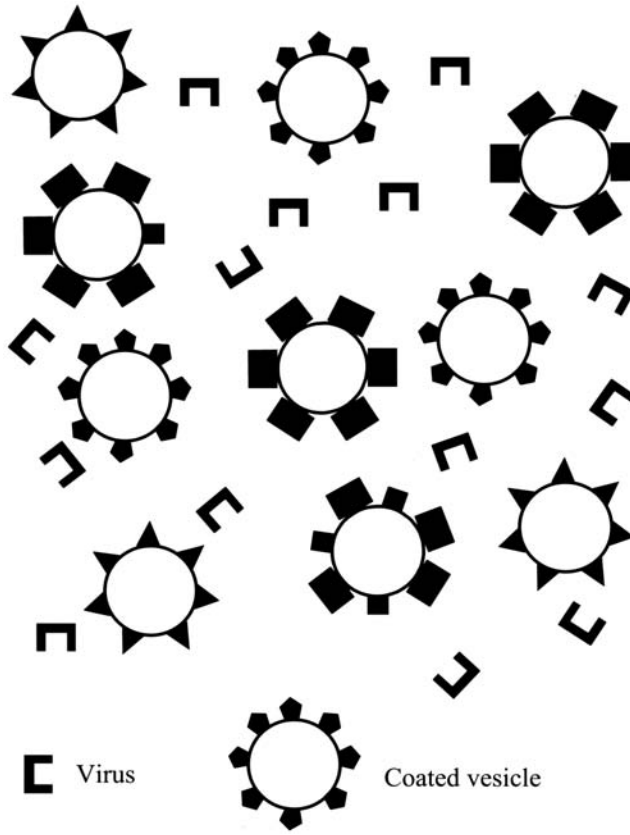

FIGURE 5.4 Virus targeting of fungal vesicles. We hypothesize that there are multiple vesicle types present in the cell, each of which has different surface proteins or coats. We hypothesize that CHV1 targets a specific subset of vesicles, possibly due to its coat protein, and utilizes them for replication. The virus does not affect other vesicle types.

5.6.1 Regulation of Protein Secretion

The hypothesis that CHV1 stops fungal developmental by perturbation of secretion of developmentally regulated proteins remains to be demonstrated. Although so many of the differentially expressed proteins that were identified by this non-biased method proved to be processed and secreted by the same pathway, and although there is evidence that the virus uses vesicles of this pathway for its replication, the evidence is still circumstantial. The most difficult aspect of this hypothesis to reconcile with known effects of the virus on its host is the down-regulation of transcription of these secreted protein precursor genes.[59] By our hypothesis, the block in secretion of these Kex2 processed proteins must send a signal to reduce the transcription of these and other developmentally regulated genes. We know of no current precedent for such signaling in fungi or other eukaryotes. It would certainly be unusual, however, if

transcription and secretion of the same gene/gene products were each independently affected by the virus. Since we know that they are both affected, we are seeking the interaction between secretion and transcription that is predicted by the simplest explanation of our data. This putative signaling could be mediated by the G proteins thought to have a role in virus-symptom induction. G proteins are involved in regulation of protein transport in eukaryotes,[60] but their specific action differs between organisms. Of particular interest is the *S. cerevisiae* Gβ subunit, STE4p. STE4p is the molecule that transmits the pheromone response signal.[35] Even more closely related is the *Aspergillus nidulans sfaD* gene, which encodes for a Gβ subunit that promotes vegetative growth and represses sporulation,[61] symptoms that are very similar to the developmental defects observed in *C. parasitica*.

5.7 CONCLUSION

CHV1infection of *C. parasitica* results in an effective biological control of chestnut blight and holds great promise for forestry and agriculture if it, or similar fungal viruses, can be utilized in other plant pathogenic fungi. Infection by this virus does not kill the fungus, but it affects transcription of a specific subset of host genes and secretion of host proteins important for development while leaving the fungus in a healthy juvenile state of growth. Understanding the basis of the interaction between CHV1 and *C. parasitica* could lead to a better understanding of how fungi cause disease and result in novel disease control strategies that could be widely applicable.

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6 Engineering Hypoviruses for Fundamental and Practical Applications

Donald L. Nuss, Baoshan Chen, Lynn M. Geletka, Todd B. Parsley, and Nobuhiro Suzuki

CONTENTS

6.1 INTRODUCTION

The concept of using a parasite of a parasite for disease control is appealing from both an intellectual and an ecological perspective. The phenomenon of transmissible hypovirulence, in which virulence of the chestnut blight fungus, *Cryphonectria parasitica*, is attenuated by double-stranded RNA viruses in the family Hypoviridae, is often cited as a classic example of this approach to biological disease control. Progress in the development of an infectious cDNA-based reverse genetics system for hypoviruses has provided the means for engineering these viral agents for both fundamental and practical applications, e.g., as a tool for probing signal transduction processes underlying fungal pathogenesis and for enhanced biocontrol potential.[1,2,3] This chapter describes the basic techniques used for manipulating hypovirus genomes and provides specific examples of how they are being applied to identify virus-encoded determinants responsible for altering fungal host phenotype, to gain insights into dispensable and essential elements of viral replication, and to fine tune the interaction between a fungal pathogen and its plant host.

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6.2 BASIC TECHNIQUES USED FOR ENGINEERING HYPOVIRUSES

The utility provided by the hypovirus reverse genetics system can be more fully appreciated after considering some of the distinguishing features of this virus group. As is true generally for fungal viruses,[4] hypoviruses have no extracellular route of infection. Thus, infection cannot be initiated by exposing uninfected hyphae to a cell extract prepared from an infected strain. Rather, hypovirus transmission is limited to dissemination via conidia (asexual spores) or through cytoplasmic mixing following hyphal anastomosis (fusion of hyphae). Although recognized by the International Committee on Viral Taxonomy,[5] hypoviruses do not encode a coat protein and do not form discrete virus particles. Instead, hypovirus genetic information is found predominantly in the form of double-stranded RNA (dsRNA) associated with cytoplasmic membranous vesicles.[6–8]

A key milestone in the development of an infectious hypovirus cDNA was the elucidation of the complete nucleotide sequence of the prototypic hypovirus CHV1-EP713.[9] Completion of the nucleotide sequence not only provided the first view of the genome organization of a hypovirus, it allowed Choi and Nuss[10] to construct a full-length cDNA copy of the 12.7 kbp CHV1-EP713 genomic RNA and install that viral cDNA into the chromosome of virus-free *C. parasitica* strains under the transcriptional control of a *C. parasitica* gene promoter using a transformation plasmid vector that also conferred hygromycin resistance (Figure 6.1). Resulting hygromycin resistant transformants exhibited phenotypic traits that were indistinguishable from strains naturally infected with the CHV1-EP713 virus, including hypovirulence. Moreover, Chen et al.[11] showed that these "transgenic" hypovirulent strains contained cytoplasmically replicating viral dsRNA that originated as large cDNA-derived nuclear transcripts that were subsequently trimmed of nonviral vector nucleotides.

Even with the complete CHV1-EP713 nucleotide sequence in hand, development of the hypovirus reverse genetics system would not have progressed as rapidly had it not been for the parallel development of a facile *C. parasitica* DNA-mediated transformation system.[12] Additional refinements have resulted in the design of a variety of plasmid transformation vectors that provide transformation frequencies in excess of 1,000 transformants/μg DNA and allow ectopic expression of endogenous or foreign genes.[13,14,15] Additional attractions of the *C. parasitica* transformation system include the ability to preserve frozen viable, competent spheroplasts for long time periods and to achieve nuclear homogeneity by simply selecting uninucleate single conidial isolates from transformed colonies. Because the *C. parasitica* genome is haploid, targeted gene disruption is an effective and routine procedure for functional analyses.[14,16–20] Gene mapping and *in vivo* structure/function analyses also benefit from the recent development of efficient genetic complementation vectors.[20,21] Thus, the hypovirus/*C. parasitica* experimental system provides the capability for readily manipulating the genomes of both a eukaryotic virus and its host.

The demonstration that cDNA-derived hypovirus dsRNA present in transgenic hypovirulent *C. parasitica* strains was processed from a nuclear transcript[11] sug-

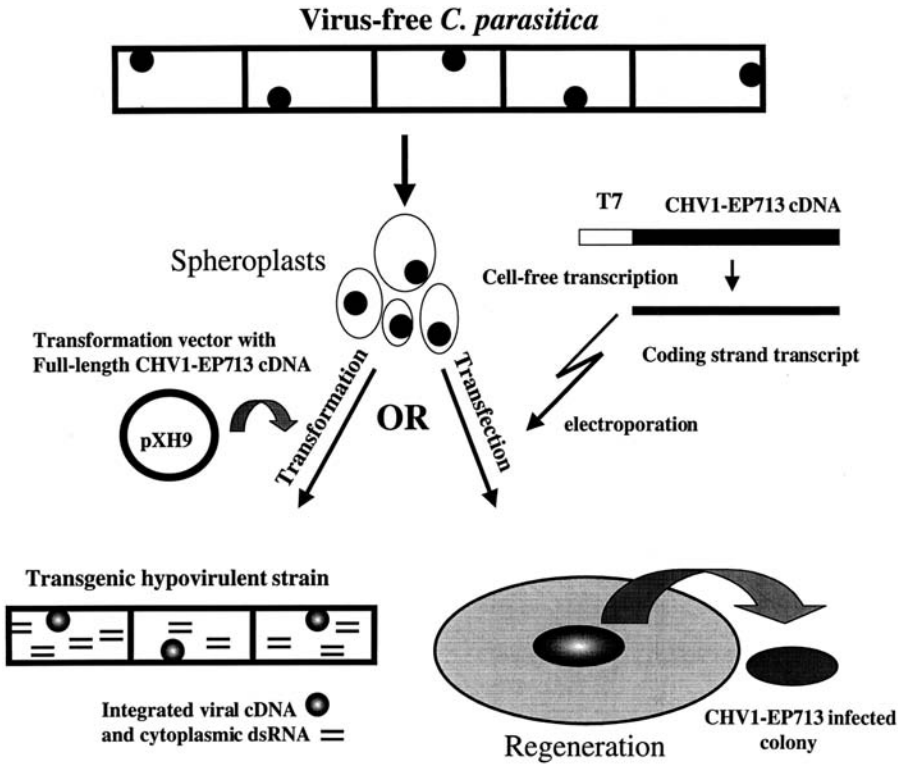

FIGURE 6.1 Diagram illustrating the basic elements of the *C. parasitica* transformation (left) and transfection (right) protocols. For both protocols, cell wall-free spheroplasts are generated from virus-free *C. parasitica* strains.[12] For transformation, a transformation plasmid containing a full-length hypovirus cDNA copy, e.g., plasmid pXH9, is introduced into the spheroplasts by DNA-mediated transformation.[10] The viral cDNA in such transformation vectors is fused downstream of the *C. parasitica* GPD promoter and fused upstream of the GPD terminator. The transformation plasmid also contains the *Escherichia coli* hygromycin B phosphotransferase gene as a selectable marker flanked by the *Aspergillus nidulans trpC* promoter and terminator domains. Transformants containing chromosomally integrated pXH9 DNA are selected following cell-wall regeneration and growth in the presence of 40 μg/mL hygromycin B. Characterization of the resulting transformants revealed the presence of chromosomally integrated pXH9 and cDNA-derived cytoplasmically replicating L-dsRNA (=).

The hypovirus transfection system relies on synthetic transcripts corresponding to the 12.7 kb coding strand of the prototypic hypovirus CHV1-EP713 that are synthesized in a T7 polymerase-dependent cell-free transcription reaction programmed with an appropriately linearized plasmid (pLDST) that contains a full-length cDNA copy of CHV1-EP713 L-dsRNA fused to the T7 promoter sequence. Resulting transcripts are introduced into fungal spheroplasts by electroporation. A portion of the transfected spheroplasts are then placed in the middle of a Petri dish and gently surrounded by molten regeneration agar. Since the hyphal structures that are regenerated from the transfected spheroplasts fuse at a very high frequency, the resulting dense colony is essentially a large cytoplasmic network. Consequently, replicating hypovirus RNA is able to effectively migrate unobstructed throughout the colony. Infected mycelia can thus be readily obtained by transferring a portion of the colony to a new plate.

gested the possibility that hypovirus infection could also be initiated by introducing a synthetic copy of the viral coding strand into spheroplasts. Chen et al.[22] confirmed this prediction with the development of a successful hypovirus transfection protocol that involved electroporation of full-length *in vitro* synthesized CHV1-EP713 transcripts into spheroplasts prepared from virus-free *C. parasitica* (Figure 6.1). A key element of the hypovirus transfection system is the propensity of *C. parasitica* to undergo hyphal anastomosis. Since hyphae fuse with their neighbors as they regenerate from the electroporated spheroplasts after plating in hyperosmotic regeneration medium, replicating hypovirus RNA present in successfully transfected spheroplasts can readily spread throughout the colony. Consequently, virus-infected mycelia can be obtained even at a low transfection efficiency and without reliance on a selectable marker. This versatile method has been used to effectively extend hypovirus infection to four fungal species taxonomically related to *C. parasitica*[22,23] and to begin identifying virus-encoded symptom determinants as discussed in Section 6.4.

Several fundamental and practical implications arising from the development of an infectious hypovirus cDNA are described in the following sections. As a general comment, especially within the context of this book, it is noteworthy that reverse genetics systems have been developed for only three groups of dsRNA viruses. In addition to the hypoviruses, these include bacteriophage $\phi 6$[24] and two birnaviruses; Infectious Bursal Disease Virus (IBDV) of poultry[25] and Infectious Pancreatic Necrosis Virus (IPNV) of fish.[26] Although viruses are widely distributed in fungi[27] and several are associated with interesting phenotypic traits, including hypovirulence, hypoviruses are the only viral agents for the entire kingdom Fungi for which an infectious cDNA has been developed and, consequently, the only fungal virus for which Koch's postulate has been rigorously completed.

6.3 ENGINEERING HYPOVIRUSES FOR ENHANCED BIOLOGICAL CONTROL POTENTIAL

Recent reports have confirmed earlier observations that hypovirus-mediated hypovirulence effectively contributes to the control of chestnut blight in European forests and orchards.[28,29] In contrast, hypovirulence has been generally ineffective when introduced into North American forest ecosystems. Factors contributing to this lower efficiency are thought to include barriers to cytoplasmic spread of hypoviruses imposed by a genetic system that determines the ability of different *C. parasitica* strains to anastomose; the vegetative compatibility (VC) system. Fungal strains with identical alleles at all or most of the genetic loci that control VC readily anastomose allowing transmission of cytoplasmically replicating hypoviruses. As the number of dissimilar alleles increases, the ability of fungal strains to fuse decreases, hampering hypovirus transmission. It follows, therefore, that the efficiency of dissemination of a hypovirus that is associated with an introduced hypovirulent strain would be inversely related to the VC diversity of the *C. parasitica* population. Field surveys have clearly shown that VC diversity is much greater for *C. parasitica* populations in North American forest ecosystems relative to that found in Europe.[2,29–31] An

additional limitation to spread of hypoviruses infecting natural hypovirulent strains concerns the rate of virus transmission through asexual spores. Depending on the combination of fungal and hypovirus genomes, between 10 and 90% of the conidia formed by these strains are virus-free,[23,32,33] resulting in the continual production of virulent inoculum. A third limitation to hypovirus dissemination arises from the fact that hypoviruses are not transmitted to ascospore progeny of a sexual cross involving a natural hypovirulent strain.[34]

Because transgenic hypovirulent strains contain a chromosomally integrated viral cDNA, it was predicted that viral genetic information would be inherited in a Mendelian fashion by ascospore progeny of a sexual cross. Additionally, these hypovirus-containing progeny should represent a spectrum of different VC types due to allelic rearrangement at the VC genetic loci. Furthermore, essentially all asexual spores generated from a canker containing a transgenic hypovirulent *C. parasitica* strain should carry the integrated viral cDNA and the derived, cytoplasmically replicating viral RNA. These trangenic propagules have the potential to serve as spermatia in a sexual cross or, following germination, to convert a canker infected with a vegetatively compatible virulent strain, or possibly initiate a new, independent hypovirulent canker. These combined transmission properties were predicted to circumvent natural transmission barriers leading to enhanced dissemination of the hypovirulence phenotype and potentially enhanced biological control of *C. parasitica* populations having high VC diversity.

Chen et al.[35] subsequently confirmed that transgenic hypovirulent strains did indeed exhibit the predicted novel mode of hypovirus transmission in the laboratory, setting the stage for testing of these strains in the field. Under conditions specified in United States Department of Agriculture (USDA) Biotechnology Permit 94-010-01, a transgenic hypovirulent *C. parasitica* strain containing the CHV1-EP713 cDNA was introduced into a Connecticut forest site in July of 1994 as a single-season, limited environmental release. Since the transgenic hypovirulent strain contained genetic and molecular markers specific for the fungal and hypovirus genomes, respectively, it was possible to monitor the independent dissemination of the input hypovirulent strain and the cDNA-derived hypovirus RNA. Evidence was obtained for cytoplasmic spread of the cDNA-derived RNA to nontransgenic endogenous *C. parasitica* strains.[2] Hypovirus transmission from transgenic hypovirulent strains to ascospore progeny was also confirmed under actual field conditions. However, persistence of the released hypovirulent strain was limited to two years after introduction. This level of persistence raises important issues previously discussed by MacDonald and Fulbright,[36] and further addressed in Section 6.7, concerning the need to balance ecological fitness and the level of virulence attenuation for effective biological control. The generally positive results of the limited release trial provided a firm basis for extending field studies of transgenic hypovirulent *C. parasitica* strains to include a population replacement strategy. This protocol attempts to replace indigenous virulent strains with nonlethal transgenic counterparts through an intense, multi-year deployment of multiple transgenic hypovirulent strains representing both fungal mating types and several different VC types in a small area with an abundance of native American chestnut sprouts (the release was initiated in 1998 under USDA Biotechnology Permit # 96-275-01).

6.4 MAPPING VIRUS-ENCODED SYMPTOM DETERMINANTS

The availability of an infectious hypovirus cDNA also provides the means for mapping virus-encoded determinants responsible for the interesting changes in host phenotype that accompany virus infection. Although different hypoviruses cause different constellations of phenotypic changes, the symptoms caused by a specific hypovirus are stable and generally consistent in the background of different *C. parasitica* strains. For example, phenotypic changes associated with CHV1-EP713 infection include reduced orange pigmentation, reduced asexual sporulation, loss of female fertility, and significantly reduced virulence.[1,3,34,36] CHV1-EP713 infection has also been shown to down-regulate the accumulation of transcripts encoded by a number of specific fungal genes, e.g., the genes for laccase,[37] a sexual pheromone,[14] a cell wall hydrophobin,[38] a cellobiohydrolase,[39] a cutinase,[40] and a polygalacturonase.[18,41] In a broader study using differential mRNA display analysis, Chen et al.[42] reported that hypovirus infection results in a stable and extensive alteration of the host gene expression profile. The pleiotropic nature of these changes suggested the possibility that hypovirus infection results in the perturbation of one or more key regulatory pathways. In this regard, hypovirus infection has been reported to alter fungal G-protein-mediated signal transduction (reviewed in Reference 3) and cellular protein secretory pathways (reviewed in Reference 43). As with most complex biological systems, it is likely that hypoviruses encode multiple symptom determinants that target multiple cellular components or regulatory pathways.

Efforts to map virus-encoded symptom determinants have relied on three primary approaches used independently or in combination: (a) cellular expression of viral coding domains independent of virus infection, (b) mutagenesis of a hypovirus infectious cDNA clone, and (c) the construction of recombinant chimeras from hypoviruses that differ in their influence on host phenotype. The first two approaches will be illustrated in this section by reviewing the results leading to the identification of the CHV1-EP713-encoded papain-like protease p29 as a symptom determinant. The utility of a comparative virology approach employing recombinant chimeras to identify symptom determinants and to fine tune the interaction between a pathogenic fungus and its plant host will be addressed in Section 6.5.

Hypovirus protein p29 is one of two papain-like proteases that are involved in processing of polyproteins encoded by the two large open reading frames contained within the CHV1-EP713 genome, ORF A and ORF B. As indicated in Figure 6.2, the polyprotein, p69, encoded by ORF A is processed by an autocatalytic event mediated by a papain-like protease domain located near the N-terminus to release p29 and p40.[44,45] ORF B encodes a large polyprotein that is also autocatalytically processed in cis by a second papain-like protease domain near the N-terminus liberating a protein designated p48.[46] While additional processing events are predicted for the ORF B-encoded polyprotein, protein products other than p48 and details of processing pathways and mechanisms remain to be elucidated.

Even before the development of the CHV1-EP713 infectious cDNA clone, Choi and Nuss[10] were able to demonstrate conclusively that a subset of the phenotypic changes exhibited by hypovirus-infected *C. parasitica* was caused by a viral coding

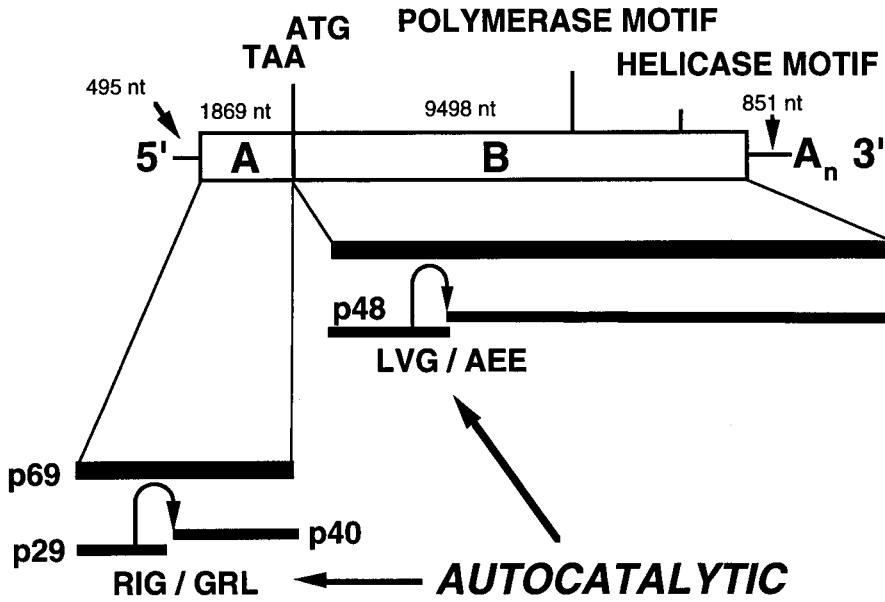

FIGURE 6.2 Hypovirus genetic organization and basic expression strategy. The coding strand RNA of the prototypic hypovirus CHV1-EP713 consists of 12,712 nt, excluding the poly(A) tail. The 5'-proximal coding domain, ORF A (622 codons), encodes two polypeptides, p29 and p40, that are released from a polyprotein, p69, by an autocatalytic event mediated by p29. Cleavage occurs between Gly-248 and Gly-249 during translation and is dependent upon Cys-162 and His-215 within the putative catalytic site.[44,45] Expression of ORF B (3,165 codons) also involves an autoproteolytic event in which a 48 kDa polypeptide, p48, is released from the N-terminal portion of the encoded polyprotein. In this case, cleavage occurs between Gly-418 and Ala-419 and is dependent upon essential residues Cys-341 and His-388.[46] Both p29 and p48 resemble papain-like proteases and one proteolytic domain may have arisen as a result of a duplication event of the other.[49] The junction between ORF A and ORF B consists of the sequence 5'-UAAUG-3'. Translational mapping studies have indicated that the UAA portion of the pentanucleotide serves as a termination codon for ORF A, while the AUG portion is the 5'-proximal initiation codon for ORF B. Computer assisted analysis revealed five distinct domains within the CHV1-713 RNA coding regions that showed significant sequence similarity to previously described domains within plant potyvirus-encoded polyproteins.[49] These include putative RNA-dependent RNA polymerase and RNA helicase motifs located in the approximate portions of ORF B indicated by vertical lines. Adapted with permission from Reference 9.

domain and not the result of a general reaction of the fungal host to the presence of the replicating viral RNA. Transformation of virus-free strain EP155 with a cDNA copy of ORF A under the transcriptional control of the *C. parasitica* glyceraldehyde 3-phosphate dehydrogenase (GPD) promoter conferred several traits that were similar to those exhibited by the same strain infected with CHV1-EP713; specifically, reduced orange pigmentation, reduced asexual sporulation, and reduced accumulation of laccase activity. Significantly, these transformants were not reduced in virulence, indicating an uncoupling of several hypovirulence-associated traits from hypovirulence.

Craven et al.[47] subsequently mapped the activity responsible for these phenotypic changes to the p29 coding domain. The ability of p29 to alter fungal phenotype was shown to be dependent on its release from the p69 polyprotein precursor but independent of its intrinsic proteolytic activity. These investigators further tested the consequences of deleting p29 in the context of the CHV1-EP713 infectious cDNA clone. A deletion mutant, Δp29, lacking 88% of the p29 coding domain, was found to retain full infectivity. However, the fungal colonies infected with this mutant exhibited a near restoration of orange pigment production, a moderate increase in conidiation and a slight, but measurable, increase in laccase production relative to wildtype CHV1-EP713-infected colonies. Deletion of the p29 coding domain did not alter the level of virus-mediated hypovirulence. It was concluded from these studies that, although not essential for either viral replication or hypovirulence, p29 contributed to virus-mediated reductions in fungal pigmentation, asexual sporulation and laccase accumulation. Additionally, the construction of the p29 deletion mutant demonstrated for the first time the feasibility of engineering infectious hypovirus cDNA to produce a hypovirulent fungal strain with specific phenotypic traits.

Building on the results of Craven et al.,[47] Suzuki et al.[48] were able to map the p29 symptom determinant domain to a region extending from Phe-25 to Gln-73 by a gain-of-function analysis following progressive repair of the Δp29 mutant cDNA. Interestingly, this region of p29 had previously been identified by Koonin et al.[49] as having a moderate level of sequence similarity with the N-terminal portion of the HC-Pro papain-like protease encoded by the plant-infecting potyviruses. The similarity extended to four conserved cysteine residues, Cys-38, Cys-48, Cys-70, and Cys-72, that made obvious targets for further mutational analysis. While substitution of a glycine for either Cys-38 or Cys-48 was silent, mutation of Cys-70 resulted in a very severe phenotype that included significantly reduced mycelial growth and profoundly altered colony morphology. In contrast, substitution for Cys-72 resulted in a symptom phenotype less severe than that caused by wildtype CHV1-EP713, approaching that observed for the Δp29 mutant. The reduction in symptom expression associated with the substitution of a glycine for Cys-72 is consistent with a positive role for this conserved residue in p29-mediated symptom expression. Interpretation of the more severe phenotype observed for the Cys-70 substitution mutant is less straightforward. One possible explanation is that the severe phenotype is a consequence of the constitutive activation or deactivation of regulatory pathways, perhaps as a result of an altered physical interaction of the mutated p29 with a specific pathway component. If so, this mutant may provide a particularly useful reagent for efforts to identify corresponding cellular targets of p29 action.

The relationship between the fungus-infecting hypoviruses and the plant-infecting potyviruses, particularly the structural and functional similarities for p29 and HC-Pro, deserves further comment. The first indications of a possible evolutionary relationship between hypoviruses and potyviruses surfaced during the early characterizations of the CHV1-EP713-encoded p29 and p48 proteases. Choi et al.[44] noted striking similarities between the p29 and the HC-Pro proteases in the form of conserved amino acid sequences around essential catalytic cysteine and histidine residues, the composition of the cleavage dipeptides and the distance between the essential catalytic residues and the cleavage sites. Shapira and Nuss[46] reported a

similar pattern for the p48 protease. These observations led Koonin et al.[49] to perform a detailed computational analysis of the CHV1-EP713 coding domains leading to the identification of five distinct hypovirus domains with significant sequence similarities to previously described conserved domains among potyvirus polyproteins. In addition to the two protease domains, these included an RNA-dependent RNA polymerase, an RNA helicase domain, and the aforementioned cysteine-rich domain with similarity to the N-terminal portion of HC-Pro that we now know contains the p29 symptom determinant described above. Based on these similarities and related considerations, Koonin et al.[49] proposed a common ancestry for hypoviruses and plant potyviruses.

Similarities between HC-Pro and p29 also extend to their multifunctional nature. In addition to its classical role in facilitating aphid transmission,[50,51] HC-Pro has been reported to proteolytically process the viral-encoded polyprotein,[52] stimulate vascular-dependent long-distance movement,[53] promote potyvirus genome amplification,[54,55] and support transactivation of heterologous virus multiplication in mixed infections.[56] As discussed above, functions assigned to p29 range from autoproteolysis to suppression of host asexual sporualtion. Moreover, p29 was found to have a differential impact on the different cellular processes in different fungal hosts.[42] Recent studies suggest that many of the functions tentatively assigned to HC-Pro may be a manifestation of suppression of post-transcriptional silencing.[57] Given the proposed evolutionary relationship between HC-Pro and p29, it is tempting to speculate that these two viral proteins may modulate regulatory processes by interacting with ancestrally related regulatory pathways in their respective hosts. The identification of a defined p29 symptom determinant domain and the availability of p29 mutant alleles, such as the Cys-70 substitution mutant, will facilitate efforts to test this possibility.

6.5 COMPARATIVE VIROLOGY AND HYPOVIRUS ENGINEERING

Surveys of European and North American *C. parasitica* field isolates have revealed considerable variability in virulence and morphological traits.[32,36,58,59] Interest in understanding the basis of this diversity has now lead to the cloning and complete sequence determination of three hypoviruses representing three separate species[9,60,61] and the partial sequence analysis of several others.[62–65] Recent detailed studies have revealed considerable differences in the spectrum and severity of hypovirulence-associated symptoms, even for *C. parasitica* strains harboring hypoviruses that are related at the nucleotide level.[60,62] The observation by Craven et al.[47] that deletion of p29 partially relieved only a subset of the symptoms associated with CHV1-EP713 infection clearly implicated the involvement of multiple viral-encoded factors in symptom expression. In an effort to define these additional symptom determinants, Chen and Nuss[66] recently exploited the diversity among natural hypovirus isolates by developing a comparative virology system based on the construction of an infectious cDNA clone of a second hypovirus, CHV1-Euro7.

The decision to clone CHV1-Euro7 was based on reports from Dr. William MacDonald and coworkers (personal communication) that the *C. parasitica* strain

harboring this hypovirus, designated Euro7, disseminated at a high rate after introduction into several North American forest ecosystems. Subsequent direct comparisons of strain Euro7 with CHV1-EP713-infected *C. parasitica* strain EP713 and virus-free strains derived from Euro7 and EP713, Euro7(-v) and EP155, respectively, revealed a number of interesting phenotypic differences.[66] Euro7 actually grows faster than the corresponding virus-free strain on synthetic solid medium, while strain EP713 grows more slowly than isogenic strain EP155. Strain EP713 is considered highly debilitated on chestnut tissue, forming small, superficial cankers that contain few, if any, spore-forming stromal pustules. In contrast, infection of chestnut tissue by Euro7 is characterized by aggressive canker expansion early after inoculation that eventually slows or ceases, concomitant with heavy callus formation at the canker margins. Moreover, these cankers are covered by stromal pustules containing viable asexual spores. Cankers initiated by either of the corresponding isogenic virus-free strains EP155 and Euro7(-v) expand unchecked and produce copious amounts of stromal pustules covering the canker face.

By constructing an infectious cDNA clone of CHV1-Euro7, it was possible to examine whether the differences in phenotypic traits exhibited by strains Euro7 and EP713 were due to the relative contributions of the two viral genomes or to additional contributions by the genomes of the two fungal hosts. This was accomplished by independently transfecting the corresponding virus-free strains Euro7(-v) and EP155 with synthetic transcripts of the two viruses.[66] The results clearly showed that differences in canker morphology, canker expansion, and asexual sporulation on chestnut stem tissue exhibited by the two hypovirus-infected strains is controlled to a much greater extent by the hypovirus genome than by the genome of the fungal host. Using an analogy to plant viruses, Chen and Nuss[66] suggested that CHV1-EP713 and CHV1-Euro7 can be viewed as severe and mild hypovirus strains, respectively.

Because CHV1-Euro7 and CHV1-EP713 have a high level of nucleotide sequence similarity (Figure 6.3), studies were initiated to test whether it would be possible to construct viable recombinant chimeras from the two infectious cDNA clones. This capability would provide a potentially powerful tool for mapping viral determinants responsible for the differences in hypovirulence levels and associated symptoms conferred by the two viruses. The feasibility of the approach was initially demonstrated by interchanging the two viral polyprotein coding domains, ORF A and ORF B.[66] The chimeric transcript containing the CHV1-Euro7 ORF A and ORF B from CHV1-EP713 behaved like the severe hypovirus strain, i.e., transfectants produced colonies with morphologies similar to that produced by EP713 and caused small superficial cankers with few, if any, stromal pustules. In stark contrast, the chimeric viruses that contained ORF A from CHV1-EP713 and ORF B from CHV1-Euro7 had all of the characteristics of strain Euro7. It was concluded from these initial studies that the determinants responsible for the differences in fungal colony morphology, sporulation, and canker morphology conferred by the two viruses reside predominantly within ORF B.

Chen et al.[67] have extended the initial CHV1-EP713/CHV1-Euro7 chimera study to show that it was possible, by swapping specific domains within ORF B, to generate chimeric hypovirus-infected *C. parasitica* isolates that exhibit a spec-

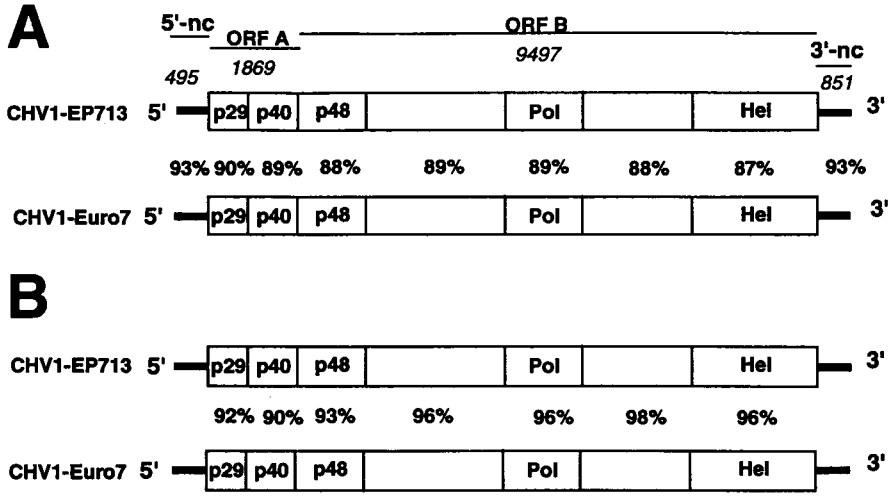

FIGURE 6.3 Comparison of the CHV1-Euro7 cDNA sequence information with that of the prototypic hypovirus CHV1-EP713. Similarities at the nucleotide levels are shown in Panel A. Previously identified protein coding regions are indicated within the open boxes representing the viral genome (Shapira et al., 1991). The lengths in nucleotides for the 5'- and 3'-noncoding regions and ORFs A and B for CHV1-EP713 are indicated at the top. The number of nucleotides for comparable regions of CHV1-Euro7 are 494, 844, 1869, and 9494, respectively. The percent nucleotide identity for different coding and non-coding regions is indicated between the two viral genome diagrams. Similar information at the deduced amino acid level is presented in Panel B.

trum of defined colony and canker morphologies. Several of the severe strain traits were observed to be dominant. However, these authors showed that it was possible to uncouple the severe strain traits of small canker size and suppression of asexual sporulation. By appropriately mixing and matching specific domains, it was possible to engineer hypovirulent fungal strains that formed small cankers similar in size to that caused by CHV1-EP713-infected isolates but with the capacity for producing asexual spores at levels approaching that observed for fungal isolates infected with the CHV1-Euro7 mild strain. These results demonstrated that hypoviruses can be engineered to fine-tune the interaction between a pathogenic fungus and its plant host.

6.6 HYPOVIRUSES AS GENE EXPRESSION VECTORS

In addition to providing the means for examining the consequences of mutating specific portions of the viral genome, a reverse genetics system also provides the opportunity to explore the potential for development of hypoviruses as fungal gene expression vectors. Potential applications include the incorporation of a visual reporter gene to provide a convenient means for monitoring hypovirus movement from cell to cell during anastomosis or long distance through a fungal colony or

even a fungal population. In this regard, we have been able to successfully adapt the green fluorescent protein (GFP) gene from *Aequorea victoria* as a reporter system in *C. parasitica*.[68] It might also be possible to enhance ecological fitness of hypovirulent *C. parasitica* strains by expressing nuclear genes that are normally downregulated by hypovirus infection, e.g., viral expression of the mating pheromone gene Mat-2[69] might relieve virus-mediated female infertility. Hypovirus host range might be extended to a number of pathogenic fungi by incorporating *C. parasitica* host-range determinants, once identified, within recombinant viral constructs. It is also conceivable that the phenotypic changes caused by hypovirus infection could be further modified by incorporating foreign genes that might alter specific host metabolic or signaling pathways.

The observation by Craven et al.[47] that 88% of the p29 coding domain in the CHV1-EP713 infectious cDNA clone was dispensable for viral replication suggested the possibility that one could replace the deleted portion with heterologous sequences. Additionally, since CHV1-EP713 RNA is not encapsidated, constraints on the size of heterologous inserts were expected to be minimal. In an effort to develop a hypovirus gene expression vector, Suzuki et al.[68] recently modified the CHV1-EP713 cDNA clone to generate twenty different vector candidates. Although transient expression was achieved for a subset of vectors that contained the GFP gene sequence, long-term expression was not observed for any vector construct. Analysis of viral RNAs recovered from transfected fungal colonies revealed that the foreign genes were readily deleted from the replicating virus, although small portions of foreign sequences were retained by some vectors after months of replication. However, characterization of the viable vector progeny provided unexpected new insights into essential and dispensable elements of hypovirus replication. The nucleotide sequence corresponding to the first 24 codons of p29 were required for viral replication, while the remaining 598 codons of ORF A, including all of the p40 coding region, were dispensable. Substantial alterations were also tolerated in the pentanucleotide UAAUG that contains the ORF A termination codon and the overlapping putative ORF B initiation codon. For example, replication competence was maintained following either a frame-shift mutation that caused a two codon extension of ORF A or a modification that produced a single-ORF genomic organization.

Insertions of foreign gene sequences into the CHV1-EP713 cDNA have, so far, been restricted to ORF A. It is conceivable that regions within ORF B will be more tolerant of gene insertions. An alternative vector strategy is suggested by the observation that the 5'-terminal portion of the p29 coding domain is required for CHV1-EP713 replication. Shapira et al.[70] previously characterized internally deleted dsRNAs recovered from strain EP713 that retained only the terminal noncoding regions of the CHV1-EP713 dsRNA: ~150 bp from the terminus corresponding to the 5'-end of the coding strand and ~ 450 bp from the other terminus. Although these small dsRNAs replicated in the presence of the full-length viral RNA, efforts to use these terminal sequences to construct a helper virus-dependent replicon as a gene expression vector were unsuccessful (Chen and Nuss, unpublished results). The absence of the N-terminal p29 coding domain in those constructs may have been responsible for the failure to observe expression of the inserted genes. It is anticipated that continued efforts to develop hypovirus expression vectors by gene insertion in ORF

B or the use of defective RNA platforms will lead to additional unexpected revelations about hypovirus molecular biology.

6.7 FUTURE DIRECTIONS

This chapter has reviewed only a limited set of the potential applications to be derived from the hypovirus reverse genetics system. These ongoing efforts will continue in directions that the reader can logically anticipate and need little additional discussion. It is clear from the combined results obtained with the recombinant CHV1-EP713/CHV1-Euro7 chimeric viruses that multiple viral domains contribute to differences in the severe and mild phenotypes and that different sets of determinants contribute to canker and colony morphologies. Although these studies have provided a firm foundation for future refined mapping and mechanistic studies, additional detailed information concerning ORF B polyprotein processing will be required for a clear understanding of the phenotypic contributions of processing intermediates and/or viral protein-protein interactions.

The comparative virology approach also has the potential for providing detailed insights into mechanisms by which hypoviruses alter host phenotype. We have suggested that hypovirus-mediated alterations of cellular signal transduction pathways compromise the ability of the invading fungus to respond appropriately to molecular and environmental cues during the infection process, thereby impeding penetration and canker expansion.[3,71] If this model is correct, it would follow that differences in canker morphology observed for isolates infected with the mild and severe hypovirus strains might result from differences in the degree to which the two viruses impact cellular signal transduction. We anticipate that the mapping of domains responsible for the differences in symptoms exhibited by the two viruses will likely lead to the identification of viral determinants responsible for altering specific cellular signaling pathways. Preliminary results using pathway-specific promoter/reporter transformation plasmid constructs indicate that the chimeric viruses can be used to correlate virus-induced changes in cellular signaling with virus-induced alterations in fungus-host pathogenic interactions (Parsley, Geletka, Chen and Nuss, unpublished results).

Two longer-term experimental directions with potentially broad implications deserve more detailed comment: extending hypovirus host range and enhancing biological control potential.

The successful use of the hypovirus transfection protocol to establish hypovirus infection in fungal species related to *C. parasitica*[22,23] raises the question of just how far the hypovirus host range can be extended. The answer to this question is influenced by several actual and perceived technical deficiencies associated with both the transfection and transformation protocols. The hypovirus transfection system depends on the ability of the fungal recipient to undergo extensive anastomosis after cell wall regeneration to permit efficient spread of the introduced replicating viral RNA. Many important medical and agricultural fungal pathogens are dimorphic. That is, they exhibit a filamentous or yeast-like morphology, depending on the environment. Anastomosis occurs only when the fungus assumes the filamentous morphology. Unfortunately, the regeneration medium currently utilized in the trans-

fection protocol promotes a yeast-like morphology for all dimorphic fungi tested (Chen and Nuss, unpublished observation).

The hypovirus transformation system was found to be less effective than the transfection system in extending host range. Two of the fungal species that readily supported hypovirus infection upon transfection with the synthetic viral transcript, *C. cubensis* and *Endothia gyrosa*, consistently failed to develop stable infections when transformed with the infectious hypovirus cDNA clone.[23] Since we know that cDNA-derived hypovirus RNA undergoes pre-mRNA splicing events at one of five potential cryptic splice sites,[11] we have suggested that the failure to launch viral dsRNA after transformation of some fungal species may be related to deficiencies in processing or translocation of the viral transcript to the cytoplasm. In addition, the transformation system depends on promoter-dependent transcription of the viral cDNA insert. It is possible that the *C. parasitica* GPD promoter used in the current transformation vector fails to function in a variety of other fungal species.

Three approaches to overcome these technical deficiencies immediately present themselves. The two issues associated with the transformation protocol, potential faulty processing of cDNA-derived viral transcripts and promoter function can be addressed by site directed mutation of all cryptic splice sites in the viral cDNA and incorporation of readily available homologous fungal promoters into the transformation plasmid, respectively. Finally, it may be possible to improve the transfection protocol for extending host range by biolistic delivery of synthetic hypovirus transcripts directly into fungal hyphae, a process for which there is some precedent in *C. parasitica.*[72] The potential utility of hypoviruses for understanding and controlling fungal pathogenesis in other filamentous fungi justifies further exploitation of these alternative approaches for extending hypovirus host range. Recent progress in the molecular characterization of virulence-modulating viruses from other pathogenic fungi[73–77] also suggests that reverse genetic systems for other interesting mycoviruses might become available in the near future.

MacDonald and Fulbright[36] have noted that most attempts at biological control of chestnut blight in North America have employed highly hypovirulent *C. parasitica* strains that harbored viruses with properties similar to CHV1-EP713, i.e., strains that, while highly curative, were quite debilitated. Moreover, these authors argued that successful hypovirulence-mediated biological control is likely to require a balance between ecological fitness and virulence attenuation. To persist and spread, a hypovirulent isolate must be able to effectively colonize and produce spores on chestnut bark. As noted above, CHV1-Euro7-infected *C. parasitica* strains differ from strains infected with CHV1-EP713 in precisely these properties. Additionally, a recent survey of European hypoviruses[78] indicated a wide distribution and dominance of CHV1-Euro7-related isolates and a low incidence of CHV1-EP713-related isolates. This result is consistent with the greater ecological fitness predicted for the former group of hypoviruses. It may also be possible to further enhance biological control by tempering the ability to colonize with a reduced capacity for canker expansion. The small canker, dense pustule production phenotype exhibited by isolates infected with several CHV1-EP713/CHV1-Euro7 chimeras would appear to meet these criteria. By using the full-length cDNA clones of CHV1-Euro7 or CHV1-EP713/CHV1-Euro7 chimeras to construct transgenic hypovirulent *C. parasitica*

strains, it will be possible to combine properties of enhanced colonization and spore production with a novel mode of transmission to ascospore progeny. As has been emphasized in previous reviews and publications from this laboratory, successful implementation of this transgenic approach to control of chestnut blight requires a multidisciplinary effort involving cooperation from forest pathologists, ecologists, molecular biologists, and regulatory agencies.

ACKNOWLEDGMENTS

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7 Viruses of the Dutch Elm Disease Fungi

Kenneth W. Buck and Clive M. Brasier

CONTENTS

7.1 INTRODUCTION

The biology of the Dutch elm disease fungi will first be described to provide a basis for understanding the role of their viruses in the epidemiology of the disease and the potential of the viruses for development as biological control agents.

7.1.1 The Dutch Elm Disease Cycles

Dutch elm disease (DED) is caused by the ascomycete fungi, *Ophiostoma* (formerly *Ceratocystis*) *ulmi* and *O. novo-ulmi*.[1–9] Both species are heterothallic (obligatorily outcrossing) with two mating types, designated A and B. DED is transmitted by scolytid elm bark beetles of which *Scolytus scolytus* and *S. multistriatus* are the

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most important. *S. scolytus* is so far confined to Europe and central Asia, but *S. multistriatus* now occurs in both Europe and North America. The annual disease cycle is characterized by saprotrophic and pathogenic phases. At the start of the saprotrophic phase in summer and autumn, bark of diseased trees is colonized by beetles carrying spores of *O. ulmi* or *O. novo-ulmi*. Breeding galleries are carved out, around which the fungi multiply by mycelial growth between autumn and spring with a distinct sequence of sporulation, involving formation of conidia, synnemata, and ascospores.[10–11] In the spring, newly hatched beetles, carrying fungal spores, emerge and fly to healthy elm trees where they cut feeding grooves in the crotches of twigs. In so doing, the beetles contaminate feeding grooves with the fungus which, after a brief mycelial phase, enters the xylem where it multiplies as yeast-like cells (blastospores). In this pathogenic phase, the fungus produces a number of wilt toxins, including the hydrophobin cerato-ulmin (CU), which together with the elm's anti-fungal responses, results in blockage of the xylem and death of the tree. The fungus in the xylem is released into the bark by the activities of the bark breeding beetles. The beetles also introduce a range of fungal genotypes directly into the bark at this time. In addition to the pathogenic-saprotrophic (xylem-bark) cycle, there is also an overlapping completely saprotrophic (bark-bark) cycle with vector bark beetles transferring fungal inocula from the breeding galleries of one diseased tree to the breeding galleries of another diseased tree. Hence, the fungus often establishes in the bark of diseased trees as a mosaic of different genotypes. The fungus frequently does not kill the entire root system of infected trees, so that root suckers can arise around the base of dying or felled trees. When these develop to a certain size (ca. 4 to 6 m), their bark is again thick enough to be colonized by the bark beetles, if they become infected with the Dutch elm disease fungus. In the U.K., there are many millions of such suckers growing in hedges and these represent a major genetic resource for the eventual re-establishment of the elm. However, at present, the disease is continually recycling in these sucker populations.

7.1.2 The Two Twentieth Century Pandemics of Dutch Elm Disease

There have been two pandemics of Dutch elm disease in the northern hemisphere this century.[1–5] The first, caused by the ascomycete fungus *Ophiostoma ulmi* (Buisman) Nannf., began in northwest Europe (The Netherlands, France, Belgium, Germany) around 1910 and spread rapidly to other parts of western and southern Europe, overseas to Britain and North America in the 1920s, eastward through the Balkans, into Turkey and across the Ukraine in the 1930s, and appeared in Tashkent in Soviet Central Asia in 1939. *O. ulmi* is relatively weakly pathogenic toward the four European elm species, *Ulmus procera* (English elm), *U. carpinifolia* (smooth-leaved elm), *U. glabra* (Wych elm), and *U. laevis* (white elm), and in Britain it caused 10 to 20% mortality. In Europe, the first pandemic started to decline in the 1940s, but a similar decline was not observed in North America, where the predominant elm species, *U. americana*, is more susceptible to *O. ulmi*.[6–9]

The current or second pandemic has been caused by the appearance of a new, more aggressive species, *O. novo-ulmi* Brasier[5] (formerly called the aggressive sub-

group of *O. ulmi*), which causes near 100% mortality in mature elms. Two races of *O. novo-ulmi* have been distinguished, the Eurasian (EAN) race and the North American (NAN) race, both with different geographical origins. The EAN race is believed to have appeared in the Ukraine-Moldavia-Romania region during the 1940s and has subsequently spread into Hungary and across Yugoslavia to northern Italy during the 1950s and 1960s, into Czechoslovakia and Poland in the early to middle 1970s, and into Denmark, eastern Germany and southern Sweden in the middle to late 1970s. It had also reached Volgograd in the north Caspian by the late 1960s, northern Iran in the 1970s, Turkey and Greece in the 1970s and 1980s, and central Asia in the 1980s. Interestingly, the EAN race has appeared in southwest Ireland but is probably absent from Great Britain (England, Scotland, and Wales). The NAN race probably appeared in North America around the Great Lakes area also in the 1940s and has spread across the U.S.A. and Canada, reaching both the east and west coasts during the 1970s to 1980s; it was introduced into Britain in the late 1960s, probably through importation of infected Rock elm (*U. thomassi*) from Canada, and has subsequently spread eastward and southward into France, The Netherlands, Belgium, Switzerland, Italy, Germany, Denmark, Sweden, Portugal, and Spain. In areas that represent early centers of the current epidemics such as the American Midwest, Britain, and Romania, extremely heavy disease losses have occurred. In Britain alone, over 25 million elms have died since 1970. In countries where the NAN or EAN race has arrived more recently, such as Spain and Sweden, equivalent losses are occurring, and worldwide elm losses are over 1 billion. The probability is that, as a result of the second pandemic, a majority of the mature field elms will be killed from the Rocky mountains to the east coast of North America and throughout Europe to central Asia.[9]

7.1.3 Distinguishing Features of *O. Ulmi* and the EAN and NAN Races of *O. Novo-Ulmi*

O. ulmi and *O. novo-ulmi* may be distinguished by a variety of morphological and physiological properties.[5] The two species have distinctive perithecial dimensions, colony morphologies, growth rates, and temperature optima for growth. *O. ulmi* is a weak pathogen on elms of moderate resistance and has poor bark colonizing ability, whereas *O. novo-ulmi* is a strong pathogen with good bark colonizing ability. Furthermore, *O. ulmi* and *O. novo-ulmi* produce low and high amounts of fitness or pathogenicity products, such as the hydrophobin wilt toxin CU[12] and cell wall-degrading enzymes respectively. A particularly significant difference is the unidirectional reproductive barrier that exists between the two species. *O. novo-ulmi* as female strongly rejects *O. ulmi* as male mating partner. Moreover, hybrids produced from laboratory crosses of the two species are of generally low fitness. Thus, F1 progenies show a wide range of unique colony patterns; exhibit a strong negative interaction for characters such as pathogenicity, growth rate, and cerato-ulmin production; and many are female sterile.[13] At current epidemic fronts, *O. novo-ulmi* is steadily migrating into areas previously occupied only by *O. ulmi*. Interspecies hybrids occur rarely and probably transiently in natural populations of *O. ulmi* and *O. novo-ulmi*.[14] However, the distinctive biological characteristics of *O. novo-ulmi* are maintained, and *O. ulmi* is being rapidly replaced by *O. novo-ulmi*.

The EAN and NAN races of *O. novo-ulmi* may be distinguished on the basis of colony morphology, growth rate, and a dimorphism in the EAN, which has the ability to alternate between a wildtype and a distinctly different uniform powdery mutant (up-mut) colony form. They may also be distinguished by a unidirectional fertility barrier manifest by the partial rejection of the NAN as male partner by the EAN.[15] Despite these differences, the two races appear to be closely related and are not reproductively isolated. In countries, such as Italy, Austria, and The Netherlands, where the EAN and NAN races now occur together, there is evidence for the formation and survival of EAN/NAN hybrids.

O. ulmi and *O. novo-ulmi*, and the EAN and NAN races of *O. novo-ulmi*, can also be distinguished by a wide variety of molecular methods.[16–23]

7.1.4 A Dutch Elm Disease Fungus from the Himalayas, *Ophiostoma Himal-Ulmi*

Although the appearance and spread of *O. novo-ulmi* are well documented, its origin remains a mystery. Of the various hypotheses that have been put forward,[4,24] the most likely is that *O. novo-ulmi* was introduced into Europe and North America from another biogeographic region where it is endemic. China was for many years regarded as a possible source of *O. novo-ulmi*, but this hypothesis has been largely discarded as a result of surveys in that country.[4] More recently, a new *Ophiostoma* species, *O. himal-ulmi* Brasier and Mehrotra, was isolated from the Himalayas where it appears to be endemic without causing severe disease in the native elm populations.[25] Although its biological and molecular properties indicate that *O. himal-ulmi* is a distinct species and unlikely to have been involved in the recent origin of *O. novo-ulmi*,[22,25] it is of interest because of its ability to cause severe disease on European and North American elms,[25] illustrating the existence of hidden Dutch elm disease pathogens in Himalayan forests. Furthermore *O. himal-ulmi* is more closely related to *O. ulmi* and *O. novo-ulmi* than are other *Ophiostoma* species that are tree pathogens or sapstain fungi, such as *O. piceae*, *O. quercus,* and *O. stenoceras*, which have previously been considered as candidates for a role in the evolution of *O. novo-ulmi.*[24,26]

7.1.5 Early Reports of Double-Stranded RNA in *O. Ulmi* and *O. Novo-Ulmi*

Double-stranded RNA (dsRNA) was first reported in *Ophiostoma* by Pusey and Wilson,[27,28] who noted that "less aggressive" isolates (probably *O. ulmi*) usually contained multiple segments of dsRNA in the size range 2.9 to 0.6 kbp, whereas "more aggressive" isolates (probably *O. novo-ulmi*) were generally dsRNA-free or contained fewer dsRNA segments. Later, Hoch *et al.*[29] found no clear association between the presence of dsRNA, or specific segments of dsRNA ranging in size from 3.9 to 0.9 kbp, and degree of aggressiveness in a range of aggressive (probably *O. novo-ulmi*) and nonaggressive (probably *O. ulmi*) isolates. Subsequently, it has become clear that the differences in pathogenicity between *O. ulmi* and *O. novo-ulmi* are determined largely by differences in multiple nuclear genes[5,13,30] and that

many isolates of the highly aggressive species *O. novo-ulmi* contain dsRNA. However, transmissible diseases have now been described in both *O. ulmi* and *O. novo-ulmi,* [31,32] which in at least some cases are closely associated with specific segments of dsRNA[33–34] (see also Section 7.2.3) and which can reduce the ability of the fungus to cause disease.[32]

7.2 TRANSMISSIBLE DISEASES OF *O. NOVO-ULMI*

A transmissible disease was first described in an *O. novo-ulmi* NAN race isolate from France (H321).[31] H321 grew more slowly than its healthy counterparts, and this phenotypic trait could be transmitted from H321 to compatible healthy isolates. The genetic determinant of the disease was called a disease-factor (d-factor), and H321 was said to be d-infected. Many d-infected isolates have since been obtained from natural populations of the NAN and EAN races of *O. novo-ulmi* and of *O. ulmi*. When a colony of a diseased isolate is grown alongside that of a healthy isolate, spontaneous fusion of hyphae from opposing diseased and healthy colonies results in a "d-reaction," characterized by the formation of morphologically distinct, slow-growing sectors arising from the originally healthy colonies. Isolates derived from these sectors exhibit the diseased phenotype. Experiments with isolates carrying a single gene nuclear marker (tolerance to methyl 2-benzimidazole carbamate (MBC) fungicides) showed that d-factors are extranuclear.

The phenotypic effects of d-infection vary and d-factors may differ qualitatively. D-factors have been numbered depending on the order of discovery. Hence, the d-factor in H321 was called the d^1-factor, and H321 was renamed H321d^1. Although most d-infected isolates have been obtained by recognizing abnormal phenotypes in collections of isolates from natural populations of *O. novo-ulmi* and *O. ulmi*, the d^2-factor was identified during a field experiment.[11,32] An elm tree was inoculated in the autumn with an *O. novo-ulmi* NAN isolate (W2*tol1*), a spontaneous MBC-fungicide-tolerant mutant of a healthy English NAN isolate W2. During the winter and spring, the isolate spread from the xylem to the bark, which also became colonized by beetles carrying wild (MBC-sensitive) *O. novo-ulmi* genotypes. Analysis of MBC-tolerant isolates subsequently made from the inner bark surface of logs cut from the dead tree showed that some of these, designated Log1/3-7d^2, Log1/3-8d^2, Log1/3-13d^2, and Log1/3-15d^2 (hereafter named L7d^2, L8d^2, L13d^2, and L15d^2, respectively) were diseased having apparently acquired the d^2-factor from a wild *O. novo-ulmi* genotype.

Apart from slow growth, the phenotypes of d-infected isolates include irregular "amoeboid" colonies, reduced conidial viability and probably reduced survival during beetle flight, reduced CU production[35] and, when growth is severely impaired, a reduced level of protoperithecial and therefore ascospore production.[32] D-infection can also reduce the ability of the fungus to cause disease in elm trees. This is probably a result of poor spore germination and the slow growth of the germ tubes, which makes a xylem infection more difficult to establish. In field experiments using artificial beetle feeding grooves, 1,000 spores of a healthy *O. novo-ulmi* isolate were sufficient to cause xylem infection of English elm *U. procera*, whereas about 50,000 spores of a d^2-infected isolate were needed for infection.[36,37] This will be discussed

further in Section 7.3, on biological control. In d^2-, d^5-, d^{10}-, and d^{12}-infected isolates, the levels of cytochrome oxidase (measured spectrophotometrically as the levels of cytochrome aa_3) were found to be reduced compared to those of the corresponding healthy isolates,[38,39] suggesting that these d-factors affect the mitochondria causing them to become respiratory-deficient. The degree of reduction in cytochrome oxidase varied, being greatest for the d^2-factor, intermediate for the d^{12}-factor, and lowest for the d^5- and d^{10}-factors.[39] d^1-infected isolates, however, had similar cytochrome oxidase levels to those of the corresponding healthy isolates, indicating that the d^1-factor has a different mode of action to the d-factors which affect the mitochondria.

7.2.1 Transmission of D-Factors

The main mode of transmission of d-factors from one fungal genotype to another is by hyphal fusion (anastomosis). This probably occurs mostly in the saprotrophic (bark) phase of DED when the fungus is in the mycelial form and many fungal genotypes are in close proximity. Viable anastomoses generally are confined to isolates of the same species, so that transmission of d-factors is likely to be largely within species, although transitory fusions between hyphae of different species may lead to rare interspecies transmission. Within a species, anastomosis is controlled by vegetative incompatibility (*vic*) genes. There are at least seven different *vic* genes in *O. novo-ulmi*, some of which may be multi-allelic.[40] If all the *vic* genes of two fungal genotypes are identical, the genotypes are vegetatively compatible, and viable anastomoses can form. Vegetatively compatible genotypes are placed in the same vegetative compatibility (vc) group. If one or more *vic* genes of two genotypes are different, the genotypes are vegetatively incompatible and hyphal anastomosis leads to a post-fusion incompatibility reaction, resulting in the death of the fused cells. The severity of incompatibility reactions depends on the number of *vic* gene differences, qualitative and quantitative differences in the effects of different *vic* genes, and epistatic effects between *vic* genes. Between individual colonies of *O. novo-ulmi*, several vegetative incompatibility reaction types have been observed:[40] wide (w), narrow (n), line-gap (lg), and line (l). W-reactions are the most severe, and differences in *w* genes are dominant over other *vic* genes. W-reactions are controlled by alleles at one[40] or two[41] *w* loci. Similar, but not identical, incompatibility reactions occur between different *vic* genotypes of *O. ulmi*.[42] Vegetative incompatibility reactions can greatly restrict the transmission of d-factors from one fungal genotype to another, depending on the severity of the incompatibility reaction.[32,40]

D-factors can be transmitted into *O. novo-ulmi* asexual spores (conidia), although a proportion of conidia also lose d-factors, reverting to the healthy phenotype.[32–34] The frequency of transmission of d-factors into conidia is difficult to determine precisely, because expression of d-factors is reversibly suppressed in a high proportion of single conidial isolates, a phenomenon that has been called *latency*.[34,43,44] Latently d-infected single conidial isolates initially have a phenotype similar to that of healthy isolates but after, a period of growth in culture, a second cycle of conidiogenesis or sometimes storage at 4°C, these isolates invariably reverted to an overtly d-infected phenotype. Of 50 viable single conidial isolates derived from the diseased *O. novo-ulmi* isolate L8d^2, 12 were found to have permanently lost the d^2-

factor, 8 were seen to be overtly d-infected immediately after conidial germination, and 20 were latently d-infected, i.e., initially appeared to be healthy, but later reverted to the d^2-phenotype. Taken with the observation that d^2-infection reduces conidial viability by about 40%,[32] it would appear that the d^2-factor is transmitted to the majority of single conidial progeny. Transmission of the d^2-factor when the fungus multiplies as yeast-like cells appears to be similar with some cells having lost the d^2-factor, but the majority being either overtly or latently d-infected.[32–34,43,44] In contrast, the d^2-factor is consistently lost from infected *O. novo-ulmi* isolates during a sexual cross. In several experiments, all single ascospore isolates were healthy even when both parents of the cross were d^2-infected.[32,33]

7.2.2 Effect of D-Factors on the Population Biology of *O. Ulmi* and *O. Novo-Ulmi*

In European *O. ulmi* populations (now becoming extinct), the A and B mating types are equally represented and the population is very heterogeneous with respect to vc types.[42] Only about 15% of European *O. ulmi* populations are d-infected, consistent with restricted d-factor transmission by vegetative incompatibility reactions (Section 7.2.1). Circumstantial evidence suggests that d-infections may have been partly responsible for the decline in elm disease levels in Europe in the 1940s.[45] It is possible that, prior to the 1940s, there were fewer vc groups in European *O. ulmi* populations and that d-infection became widespread. Vc gene mutants may then have been selected on account of their greater resistance to d-factor transmission, which, with subsequent sexual recombination, resulted in the current high level of vc diversity and a lower frequency of d-infection. In North American *O. ulmi* populations (also becoming extinct), the A mating type is predominant. There is also one predominant vc group, termed the *O. ulmi* American vc supergroup, with only a small remainder of the population (*ca.* 6%) being of other vc types. The American vc supergroup also corresponds to the most common *O. ulmi* vc type in Europe, suggesting that *O. ulmi* genotypes of this vc type may have been introduced into North America in the 1920s.[42] D-infections are more common in the North American *O. ulmi* population (ca. 60%), consistent with the preponderance of a single vc supergroup. Because the American elms are more susceptible to *O. ulmi* than are the European elms, it is possible that the widespread d-infection in the populations still did not provide sufficient selection pressure for accumulation of mutant vc groups. Also, the predominance of the A mating type may have reduced the opportunity for generation of new combinations of *vic* genes by sexual reproduction.

European populations of *O. novo-ulmi* at epidemic fronts tend to be near clonal, consisting of only a single vc group of B mating type. Genotypes carrying the most common and widespread EAN vc group (termed the *EAN vc supergroup*), or the most common NAN vc group (termed the *European NAN vc supergroup*), are presumably responsible for the highest proportion of the *O. novo-ulmi* epidemics in Europe, although other vc groups have been found at epidemic fronts in various locations. Generally, *O. novo-ulmi* xylem isolates from epidemic fronts are free from overt d-infection. However, d-infections soon appear and become common in *O. novo-ulmi* bark isolates at epidemic fronts (both EAN and NAN) (50 to 90% of

isolates), presumably because d-factor transmission is aided by the presence of a single vc clone.[3,32,45]

The source of the d-infection is not known. It is possible that some *O. novo-ulmi* genotypes in the xylem at epidemic fronts were latently d-infected and changed to an overtly diseased phenotype on reaching the bark. Alternatively, d-factors could have been transmitted from *O. ulmi* to *O. novo-ulmi* in the bark phase. When *O. novo-ulmi* enters a new region in which *O. ulmi* was endemic, there is an explosion of the *O. ulmi* population, probably because the newly diseased trees provide new breeding grounds for the local beetles, which introduce predominantly *O. ulmi* inoculum into the bark.[1] Hence, in newly diseased trees at *O. novo-ulmi* epidemic fronts, there is likely to be more *O. ulmi* than *O. novo-ulmi* in the bark. Hyphae of *O. ulmi* and *O. novo-ulmi* would not be expected to form viable anastomoses, but transitory fusions between hyphae may be sufficient to enable d-factors to be transmitted from *O. ulmi* to *O. novo-ulmi*, which would then spread rapidly through the clonal *O. novo-ulmi* population. D-factor transmission as a result of rare sexual crosses between *O. ulmi* and *O. novo-ulmi*[14] seems less likely in view of the absence of d-factors from ascospore progeny of crosses between *O. novo-ulmi* isolates.[32,33]

Within a few years (by mid-epidemic), *O. novo-ulmi* populations in Europe become highly heterogeneous with respect to vc groups, the A mating type appears, and the level of d-infection drops to about 5% of the population.[3] It is possible that *vic* genes, and the A mating-type gene, have introgressed from *O. ulmi* into *O. novo-ulmi*. Although *O. ulmi* and *O. novo-ulmi* are largely reproductively isolated, rare *O. ulmi*/*O. novo-ulmi* hybrids have been isolated.[14] *O. novo-ulmi* genotypes with newly acquired *vic* genes may be relatively resistant to d-factor transmission and hence may outgrow d-infected individuals of the original vc clone. Hence, d-factors may act as selective agents for the accumulation of new *vic* genotypes. Furthermore, the introduction of the A mating type would enable sexual crossing to occur, generating more combinations of *vic* genes. Such genotypes would be expected to be d-factor-free and resistant to d-factor transmission. Hence, the lower level of d-infection in post-epidemic populations is readily explained. *O. ulmi*/-*O. novo-ulmi* hybrids would be expected to be relatively unfit compared to their parents.[13] However, further crossing between the transient hybrids and *O. novo-ulmi* could eliminate undesirable *O. ulmi* genes, resulting in new *O. novo-ulmi* genotypes containing introgressed, beneficial *O. ulmi vic* and mating-type genes that help the population to resist deleterious d-factor diseases.

A different situation exists in New Zealand, where *O. novo-ulmi* (European NAN vc supergroup, B-mating type, with no overt d-infection) was introduced about 10 years ago.[46,47] No d-infections, new vc types, or the opposite (A-) mating type have so far appeared in the *O. novo-ulmi* population. *O. ulmi* is absent from New Zealand, and hence there is no opportunity for introgression of genes or transmission of d-factors from *O. ulmi* to *O. novo-ulmi* as is thought to have occurred in Europe.

Another contrasting situation is found in North America. There are three dominant vc groups in North American *O. novo-ulmi* populations (all of which are NAN race), the North American NAN vc supergroup (58%), the second American vc supergroup (20%), and the European NAN vc supergroup (10%); the last two of these were introduced into Europe in the 1960s. The B mating type is predominant

in these three supergroups (61 to 82%). The remaining component of the population (12%) is highly diverse for vc groups with equal numbers of A and B mating types.[48,49] However, the potential for generation of new vc types in the population via sexual recombination is very high,[41] and the scarcer vc types are apparently arising continually in nature via sexual recombination but are outcompeted by the dominant vc types.[49] The level of d-infection is very low (< 2%), despite the relatively few vc groups found. It has been suggested that this low level of d-infection would not provide sufficient selection pressure for the emergence of new *vic* genotypes.[49] This is a reasonable explanation, because as in Europe *O. novo-ulmi* in North America enters new regions in which *O ulmi* is already present, and the opportunities for hybridization and introgression of beneficial genes from *O. ulmi* to *O. novo-ulmi* would appear to be similar in the two continents. However, the low level of d-infection in North American *O. novo-ulmi* populations raises an enigma. If d-infection has spread from *O. ulmi* to *O. novo-ulmi* in Europe, as seems likely (see above), why has this not occurred to the same degree in North America, where *O. novo-ulmi* vc clones are widespread and the resident *O. ulmi* populations have a higher frequency of d-infection than in Europe?

7.2.3 The Molecular Nature of D-Factors

7.2.3.1 The d^2-Factor

Association of Three Mitochondrial dsRNA Segments with the d^2-Factor

Analysis of isolates L7d^2, L8d^2, and L15d^2 by polyacrylamide gel electrophoresis (PAGE) indicated the presence of 10 dsRNA segments, with approximate sizes (kbp) of 3.49 (RNA-1), 3.03 (RNA-2), 2.69 (RNA-3), 2.43 (RNA-4), 2.33 (RNA-5), 2.21 (RNA-6), 0.95 (RNA-7), 0.89 (RNA-8), 0.48 (RNA-9), and 0.33 (RNA-10).[33] Isolate L13d^2 also had 10 dsRNA segments of the same size, except for RNA-7, which was 0.92 kbp. Further analysis of L8d^2 dsRNA using a higher-resolution PAGE system showed that RNA-1 and RNA-3 could each be resolved into two segments, designated 1a and 1b, 3a and 3b, respectively, giving 12 segments in total.[50] W2*tol1*, the healthy progenitor of L8d^2 (see Section 7.2) was shown to contain RNA-1a, RNA-1b, and RNA-8. Therefore, L8d^2 must have acquired nine dsRNA segments and its diseased phenotype from a wild *O. novo-ulmi* isolate.

Transmission of the disease from L8d^2 to dsRNA-free, healthy *O. novo-ulmi* isolates in the same vc group was accompanied by transmission of all the dsRNA segments found in L8d^2.[33] Following conidiogenesis, no loss of dsRNA segments was observed in single conidial isolates that were either overtly or latently d-infected. However, the minority of single conidial isolates that had reverted to the healthy phenotype had lost from 3 to 11 dsRNA segments. The segments that were lost always included RNA-4, RNA-7, and RNA-10.[33,50] Healthy isolates recovered from elm xylem after inoculation of elm trees with L8d^2 or other d^2-infected *O. novo-ulmi* isolates were also found to have lost variable numbers of dsRNA segments. These again always included RNA-4, RNA-7, and RNA-10.[33] These results suggest that the d^2-factor consists of one or more of these three RNA segments, although it

is also possible that some or all of the other nine dsRNAs are also needed for expression of the d^2-phenotype. Unequivocal proof will require transfection of dsRNA-free *O. novo-ulmi* isolates with full-length RNA transcripts derived from cloned cDNAs of RNA-4, RNA-7, and RNA-10. However, it is clear that RNAs 1a, 1b, 2, 3a, 3b, 5, 6, and 9, in the absence of RNAs 4, 7, and 10, have no overt deleterious effects on *O. novo-ulmi*.

Electron microscopic analysis of subcellular fractions of *O. novo-ulmi* L8d^2 prepared by differential and density gradient centrifugation failed to detect isometric or other types of virus-like particles found in a range of fungi,[51] or pleomorphic membrane-bound vesicles of the type associated with dsRNA in *Cryphonectria parasitica*, the causative agent of chestnut blight.[52] In contrast, the dsRNA segments of L8d^2 were found to copurify with the mitochondria.[38] Subsequently, it was found that, in addition to the dsRNA segments, L8d^2 contains a mitochondrial DNA "plasmid" derived from the mitochondrial DNA. This is not a "true" plasmid, because "true" plasmids are autonomously replicating DNAs that have sequences distinct from that of the host genome, including the mitochondrial DNA. Many linear and circular "true" plasmids have been found in fungal mitochondria,[53,54] although not so far in *O. novo-ulmi*. The L8d^2 "plasmid" exists predominantly as concatemers of a 2.2 kbp monomer.[55] Transmission of the L8d^2 dsRNA segments and the d^2-phenotype to healthy isolates by hyphal anastomosis was not accompanied by transmission of either the mitochondrial DNA or the DNA "plasmid," but new DNA "plasmids" derived from different regions of the mitochondrial DNA were produced *de novo* in the newly d^2-infected recipients.[55] Hence, these "plasmids" are probably a symptom, rather than a determinant, of the d^2-infection. Nucleotide sequence comparisons of the L8d^2 "plasmid" and the mitochondrial DNA indicated that the "plasmid" was derived by recombination between two long repeat sequences in the mitochondrial large ribosomal RNA gene.[56] Transmission of dsRNA from L8d^2 to healthy isolates probably occurs as a result of transient fusions of mitochondria, which are common in fungi. Transmission of a *C. parasitica* mitochondrial dsRNA was accompanied by mitochondrial DNA recombination,[57] indicating somatic fusion of mitochondria. However, transmission of fungal mitochondrial DNA plasmids, independently of the mitochondrial DNA, has also been reported.[58] Transmission of the *O. novo-ulmi* mitochondrial dsRNA occurs independently of both the mitochondrial DNA and the mitochondrial DNA "plasmid."

In a sexual cross between *O. novo-ulmi* L8d^2 acting as the female (protoperithecial) partner and a healthy dsRNA-free and "plasmid"-free isolate acting as the male (conidial) partner, out of 20 randomly selected single ascospore progeny that were all healthy, 19 were free from dsRNA, and one contained only RNA-2.[33] In similar crosses, it was shown that ascospore progeny were free from the DNA "plasmid" whether L8d^2 acted as the female or male partner of the cross.[55] These results were unexpected, because mitochondrial DNA in ascomycete fungi, including *O. novo-ulmi*,[55,59] is known to be maternally inherited. Elimination of dsRNA as a result of sexual crossing has been found in other fungi. Cytoplasmic (non-mitochondrial) virus dsRNAs were not transmitted, or only infrequently transmitted, to ascospore progeny in a number of filamentous ascomycete fungi, such as *Gaeumannomyces graminis*, the causative agent of take-all disease of cereals,[60] and *C.*

parasitica.[61] There is evidence that host proteins are needed for the replication of many RNA viruses,[62–64] and it is possible that any *O. novo-ulmi* proteins needed to replicate the mitochondrial dsRNAs may become limiting during ascospore formation.[51] However a 2.7 kbp mitochondrial dsRNA in isolate NB631 of *C. parasitica* was transmitted to ascospore progeny to a variable extent. In a mating between a mitochondrial dsRNA-containing isolate and a dsRNA-free isolate of *C. parasitica*, transmission into ascospores was only obtained when the dsRNA-containing isolate acted as the female parent. The efficiency of dsRNA transmission was 43%. In another cross between the same dsRNA-containing female parent and another dsRNA-free isolate, the efficiency of dsRNA transmission was only 8%.[57] Another possibility for the lack of transmission of dsRNA into *O. novo-ulmi* ascospores is that mitochondria in L8d^2 may be a heterogeneous population, with some mitochondria that contain dsRNA and DNA "plasmid" being respiratory deficient due to reduction in cytochrome oxidase levels, and other "healthy" mitochondria that are dsRNA-free and "plasmid"-free with normal cytochrome oxidase levels being respiratory competent. During ascospore formation, there may be selection for the "healthy" dsRNA-free and plasmid-free mitochondria. In the case of *C. parasitica*, although the mitochondrial dsRNA is associated with a moderate reduction in pathogenicity,[57] there is no evidence for reduced cytochrome oxidase levels, and hence there may be less selection against mitochondria containing dsRNA during ascospore formation.

Sequence Analysis of the Three d^2-Factor-Associated dsRNAs

RNA-4 from *O. novo-ulmi* isolate L8d^2 was shown to consist of 2,599 nucleotides that contained one long open reading frame (ORF) with the potential to encode a protein of 783 amino acids (calculated molecular mass 92,244 Da).[65] The ORF contained 11 UGA codons, which in the mitochondria of most ascomycete fungi do not act as stop codons but code for the amino acid tryptophan.[66,67] Only two UGG tryptophan codons were present in the ORF. Furthermore, the RNA had 73.3% A + U residues, and there was a preference for codons with A or U in the third position. Both of these features are also characteristic of mitochondrial DNA genomes.[68–70] The putative protein encoded by RNA-4 contains amino acid motifs characteristic of RNA-dependent RNA polymerases (RdRp) of RNA viruses.[71,72] The 5' nucleotides 1-28 and the 3' nucleotides 2574–2599 were each able to be folded independently into potentially stable stem-loop structures. The 5' nucleotides 1-39 and 3' nucleotides 2561–2599 showed a high degree of inverted complementarity to each other, which could potentially form a stable panhandle structure. These putative structures may act as recognition sites for the RdRp to enable it bind to and replicate the RNA.

RNA-7 (1057 nucleotides) was shown to be derived from RNA-4 by three internal deletions.[65] The four regions in RNA-7 that corresponded to regions of RNA-4 (percent sequence identity to RNA-4 is shown in parentheses) were: nucleotides 1–300 (95.0%), 301–348 (91.7%), 349–828 (92.9%), and 829–1057 (94.8%). Because of the extensive deletions within the RdRp ORF, it is unlikely that RNA-7 could encode a functional protein and its replication is likely to be carried out by the RdRp encoded by RNA-4. The 5' and 3' sequences that were proposed to act as RdRp recognition sites in RNA-4 were retained in RNA-7 with near identity. The

lack of complete identity between the other homologous regions of RNA-4 and RNA-7 suggests that RNA-7 has not arisen recently from RNA-4. Since very little polymorphism was found in the RNA-7 sequence, it is likely that it has evolved from the primary deletion product to achieve the most efficient replication. It is also possible that RNA-7 acts as a defective-interfering RNA, and it is noteworthy that the molar amount of RNA-7 in isolate L8d[2] was about ten times that of RNA-4.

The sequence of RNA-10 (317–330 nucleotides) indicated that it could be derived almost entirely from nucleotides 2-86 of RNA-4 or RNA-7 by a series of direct and inverted repeats of this sequence. A mechanism for the formation of RNA-10, involving replicase-driven strand switching between minus-strand and plus-strand templates, followed by utilization of the nascent strand as a primer and template to form a snap-back RNA, has been suggested.[73] Like RNA-7, RNA-10 is likely to be replicated by the RdRp encoded by RNA-4. Possibly nucleotides 2-86 of RNA-4 are the only *cis*-acting sequences needed for replication by this RdRp. RNA-10 showed more sequence polymorphism than RNA-7, accounting for the range of sizes, and may be continuously evolving to achieve maximum replication. In isolate L8d[2], the molar amount of RNA-10 was about 50 times that of RNA-4, hence RNA-10 may also act as a defective-interfering RNA. The likely dependence of both RNA-7 and RNA-10 for their replication on the RdRp encoded by RNA-4 explains why the loss of RNA-4 (which can occur in *O. novo-ulmi* L8d[2] either during conidiation or during multiplication as yeast-like cells) is always accompanied by loss of RNA-7 and RNA-10.

Multiple Independently Replicating RNAs in O. Novo-Ulmi Isolate L8d[2]

Sequence analysis of RNA-3a (2,617 nucleotides), RNA-5 (2,474 nucleotides), and RNA-6 (2,343 nucleotides) of *O. novo-ulmi* isolate L8d[2] indicated that, like RNA-4, each was rich in A + U residues (68.5%, RNA-3a; 73.2%, RNA-5; 70.7%, RNA-6). Also, each of these RNAs contained a long ORF with the potential to encode RdRp-like proteins of 718 amino acids (80,274 Da), 729 amino acids (86,181 Da), and 695 amino acids (80,087 Da), respectively.[65,74] These ORFs also contained multiple UGA tryptophan codons and were rich in codons ending in A or U residues. Western blot analysis using antibodies raised against a conserved RdRp region detected a protein of ca. 80 kDa in extracts of a single conidial isolate (sci 31, derived from *O. novo-ulmi* isolate L8d[2]), which contained only RNA-6. However, no such protein was detected in a dsRNA-free *O. novo-ulmi* isolate.[75] 80 kDa is close to the predicted size of the RNA-6 encoded RdRp, and the results are consistent with the use of UGA tryptophan codons in its synthesis. RdRp activity was also detected in this isolate, but not in a dsRNA-free *O. novo-ulmi* isolate. The reaction products corresponded to the double-stranded and single-stranded forms of RNA-6. Hence, it is likely that the 80 kDa protein is the RdRp encoded by RNA-6.

Nucleotide sequence identities between RNAs 3a, 4, 5, and 6 were in the range 42.9 to 54.8%, and amino acid sequence identities of their encoded RdRps were in the range 20.3 to 33.3%.[65] Since *O. novo-ulmi* isolate sci31 contains only RNA-6, it is clear that this RNA is able to replicate independently of the other RNAs that coexist in isolate L8d[2]. The degree of divergence of sequences between RNAs 3a,

4, 5, and 6 and their encoded RdRps suggest that each of these RNAs replicates independently of the others and that each RdRp specifically recognizes its own RNA. As with RNA-4, the 3' and 5' terminal sequences of RNAs 3a, 5, and 6, can each be folded into potentially stable stem-loop structures, and the 3' and 5' sequences of RNA-6 can potentially form a panhandle structure. However the differences in lengths and sequences of these putative structures may provide a basis for them to be distinguished by the different RdRps.

It is also likely that RNAs 1a, 1b, 2, and 3a are each able to replicate independently. Cloned cDNA probes from RNAs 3a, 4, 5, and 6 did not hybridize to RNAs 1a, 1b, 2, and 3b in Southern blots. A single ascospore isolate derived from *O. novo-ulmi* isolate L8d[2] was found to contain only RNA-2.[33] *O. novo-ulmi* isolate W2*tol1*, the progenitor of L8d[2] (see Section 7.2), contains only RNAs 1a, 1b, and 8, and single conidial isolates derived from L8d[2] have been found which lack RNA-1a, but retain RNA-1b.[50] Similarly single conidial isolates have been found which lack either RNA-3a or RNA-3b. RNA-8 (0.89 kb) and RNA-9 (0.48 kb) are probably too small to encode an RdRp and, like RNAs 7 and 10, are probably defective RNAs derived from one of the other RNAs. It is likely that RNA-8 is derived from RNA-1b, since it is present with only RNA-1a and RNA-1b in isolate W2*tol1*, it is always lost in single conidial isolates derived from L8d[2] whenever RNA-1a and RNA-1b are lost, but is retained in single conidial isolates which have lost RNA-1a, but retained RNA-1b. By similar deductions, RNA-9 could be derived from RNA-2 or RNA-6. It is noteworthy that the loss of dsRNA segments which occurs in a small proportion of single conidial or yeast-like cells from *O. novo-ulmi* isolate L8d[2] is non-random. The most stable RNAs were RNA-2 and RNA-6, which were retained in nearly all single conidial and yeast-like cell isolates.[33,50] All eight independently replicating RNAs in L8d[2] are present together in one cell, because most single conidial isolates contain all these RNAs. However whether they are all present in one mitochondrion is not known.

Taxonomy and Evolution of Mitochondrial RNA Viruses (Mitoviruses)

RNAs 3a, 4, 5, and 6 of *O. novo-ulmi* isolate L8d[2] were found to be related to a 2,728 bp mitochondrial dsRNA in isolate NB631 of *C. parasitica*. Nucleotide sequence identities ranged from 43.0 to 45.3%. The *C. parasitica* dsRNA has a long ORF with the potential to encode an RdRp-like protein of 809 amino acids with multiple UGA tryptophan codons.[76] Recently a new family of viruses with naked RNA genomes, the *Narnaviridae*, comprising two genera, *Narnavirus* and *Mitovirus*, has been established by the International Committee on Taxonomy of Viruses (ICTV).[77] The *Narnavirus* genus contained the yeast cytoplasmic 20S and 23S RNAs, which were designated *Saccharomyces cerevisiae narnavirus* 20S RNA (ScNV-20S) and *S. cerevisiae narnavirus* 23S RNA (ScNV-23S) respectively.[77,78] The *Mitovirus* genus contained the *C. parasitica* mitochondrial dsRNA which was designated *C. parasitica mitovirus* 1-NB631 (CpMV1-NB631).[77,78] The mitochondrial location of *O. novo-ulmi* L8d[2] RNAs 3a, 4, 5, and 6[38], their sequence relationships and similar genome organization to CpMV1-NB631, and the ability of the four L8d[2] RNAs and CpMV1-NB631 RNA to be folded into potentially stable stem-

loop structures at their 5' and 3' ends, suggested that they should also be assigned to the *Mitovirus* genus.[65] As the degrees of sequence divergence between L8d[2] RNAs 3a, 4, 5, and 6 are similar to those between these RNAs and the *Cryphonectria* virus CpMV1-NB631, and each of the four L8d[2] RNAs appears to replicate independently, it has been proposed that each of these RNAs constitutes a separate mitovirus species. These have been designated *O. novo-ulmi mitovirus* 3a-Ld (OnuMV3a-Ld), *O. novo-ulmi mitovirus* 4 (OnuMV4-Ld), *O. novo-ulmi mitovirus* 5-Ld (OnuMV5-Ld), and *O. novo-ulmi mitovirus* 6 (OnuMV6-Ld), respectively. The designation of L8d[2] RNAs 1a, 1b, 2, and 3b as separate mitovirus species must await determination of their nucleotide sequences.

The *Cryphonectria* and *Ophiostoma* mitoviruses have been shown to be distantly related to yeast narnaviruses, ScNV-20S and ScNV-23S, and more distantly related to positive-stranded RNA bacteriophages of the *Leviviridae* family.[65,74,76] In phylogenetic trees based on conserved RdRp amino acid sequences, viruses in the *Mitovirus* genus formed a cluster that was distinct from viruses in the *Narnavirus* genus and *Leviviridae* family (100% bootstrap support).[65,74] Furthermore, formation of a larger cluster consisting of the *Narnavirus* and *Mitovirus* genera and the *Leviviridae* family, which was distinct from other families of cytoplasmic RNA fungal viruses (*Totiviridae*, *Partitiviridae*, *Hypoviridae*, *Barnaviridae*), was moderately well bootstrap supported (76%). Mitoviruses and narnaviruses exist in the cell as double-stranded and single-stranded (positive-stranded) forms, with the single-stranded form often predominating.[65,74,76,79] It is therefore possible that mitoviruses and narnaviruses have both evolved from the positive-stranded RNA bacteriophages (*Leviviridae*) or from a common progenitor.[65,74,76]

A 3,570 bp dsRNA (the M2 dsRNA) has been described from the basidiomycete fungus *Rhizoctonia solani* (a causative agent of root diseases of many crop plants),[80] which is related to the *Ophiostoma* and *Cryphonectria* mitoviruses.[65,74,76,80] Nucleotide sequence identities between M2 dsRNA and the *Ophiostoma* and *Cryphonectria* mitovirus RNAs ranged from 41.0 to 43.7%. Like the *Ophiostoma* and *Cryphonectria* mitovirus RNAs, the 5' and 3' sequences could be folded into putative stem-loop structures, although the 3' stem-loop did not extend right to the 3' terminus as with the mitovirus RNAs. There was inverted complementarity between 9 nucleotides at the 5' and 3' termini, allowing a putative panhandle structure to be formed, although much shorter than those described for OnuMV4-Ld and OnuMV6-Ld. The *R. solani* M2 dsRNA contains a long ORF with the potential to encode an RdRp-like protein of 754 amino acids. Amino acid identities between the M2 RdRp and those of the *Ophiostoma* and *Cryphonectria* mitovirus RNAs were in the range 19.2 to 26.2%, and there were sufficient numbers of identical amino acids to allow an accurate alignment of all six sequences. The highest amino acid sequence identities were in the conserved motifs, II–VI,[65] typical of RdRps of RNA viruses.[71,72] However, there was also a moderately high degree of homology in a region immediately upstream, designated motif I and suggested to be specific to RdRps of mitoviruses.[65] The RdRp-like protein encoded by ORF A of M2 dsRNA also has some sequence similarity to two domains of the *S. cerevisiae* AROM polypeptide. This catalyses reactions in the shikimic acid pathway, which leads to the synthesis of aromatic amino acids, such as phenylalanine. It has been suggested that these domains of the

ORF A polypeptide might interfere with the shikimic acid pathway, and hence with the production of phenylalanine and its metabolite phenylacetic acid, which is thought to be involved in the pathogenicity of *R. solani*. The M2 dsRNA-containing isolate of *R. solani* is known to be hypovirulent and to produce lower levels of phenylacetic acid than virulent isolates.[80] However, the sequence similarity of the ORF A polypeptide to the AROM polypeptide is not maintained in the corresponding regions of the RdRps of the *Ophiostoma* and *Cryphonectria* mitoviruses. M2 dsRNA is longer than CpMV1-NB631 RNA or any of the *Ophiostoma* mitovirus RNAs which have been sequenced. The extra length is downstream of the RdRp ORF (ORF A) and contains a second short ORF (ORF B) with the potential to encode a protein of 95 amino acids. Whether this is expressed is not known. It is noteworthy that dsRNAs 1a and 1b of *O. novo-ulmi* isolate L8d[2] are a similar size to the *R. solani* M2 dsRNA. It would therefore be interesting to determine if these have an ORF equivalent to the M2 ORF B.

Unlike the *Ophiostoma* and *Cryphonectria* mitoviruses, which are located predominantly, and probably exclusively, in the mitochondria, only a small proportion of the *R. solani* M2 dsRNA copurified with the mitochondria and most of it was found in the cytosol.[80] Furthermore, M2 dsRNA ORF A contains no UGA tryptophan codons; its 13 tryptophan codons were all UGG, despite an overall bias toward codons ending in U or A residues. It is likely that M2 dsRNA replicates in the cytoplasm, but the possibility that it replicates in both the cytoplasm and the mitochondria cannot be excluded. Whether the *R. solani* M2 dsRNA should be assigned to the *Mitovirus* genus or a new sister genus within the *Narnaviridae* family will require further study.

Fungal viruses are transmitted predominantly if not exclusively by intracellular routes, the main method of transmission between different fungal genotypes being by hyphal anastomosis.[51,81,82] This would limit transmission to the same or closely related fungal species (see Section 7.2.1). Hence, it may be expected that fungal viruses, including mitoviruses, may coevolve with their hosts.[81,82] RNA viruses have the potential to evolve much more rapidly than their hosts, because the virus-encoded RNA-dependent RNA polymerases have no proof-reading activities, unlike the host DNA-dependent DNA polymerases.[83] Mitoviruses have compact genomes, with most if not all of the genome being composed of the RdRp ORF and sequences in the 5' and 3' untranslated regions needed for RNA polymerase recognition and translational signals. Hence, the rate of mitovirus evolution may be limited by structural and functional constraints. Coevolution of a mitovirus with its fungal host may occur if virus RNA replication involves specific interactions between viral proteins or RNA and host proteins. Bacteriophages of the *Leviviridae* family, from which mitoviruses may have evolved (see above), require four bacterial proteins as well as the phage-encoded RdRp for replication of their RNA genome,[84] and there is evidence for the involvement of host proteins in the replication of eukaryotic RNA viruses also.[51,63,64] A mutation in a host protein required for virus replication may need a compensatory mutation in a viral protein or RNA to maintain an interaction. However, the coexistence of eight highly divergent mitoviruses in *O. novo-ulmi* L8d[2] suggests that host proteins may not play a major role in mitovirus evolution.

Nevertheless, if coevolution of mitoviruses and their fungal hosts has occurred over a long period of time, it may be expected that sequence divergence of different viruses would reflect the divergence of their hosts. In this case, viruses of ascomycete fungi (*O. novo-ulmi* and *C. parasitica*) should be more closely related to each other than to a virus of a basidiomycete fungus (*R. solani)*. This is clearly not so, since the sequence diversity between OnuMV3a-Ld, OnuMV4-Ld, OnuMV5-Ld, and OnuMV6-Ld is as great as between these viruses and CpMV1-NB631 or the *R. solani* mitovirus-like M2 dsRNA, or between CpMV1-NB631 and the *R. solani* M2 dsRNA. One possible explanation is that rare horizontal transmission of mitoviruses has occurred resulting from (a) transient fusion between hyphae of ascomycete and basidiomycete fungi, (b) hyphal anastomosis within a species of an ascomycete fungus in the presence of virus released from a lysed basidiomycete fungus in the same environment, or (c) the chewing activities of mycophagous animals such as mites. However, a more likely explanation is that the different lineages of mitoviruses emerged before the divergence of ascomycetes and basidiomycetes. This would be analogous to gene duplication and evolution of paralogous gene lineages in host chromosomal DNA. If all the eight *O. novo-ulmi* mitoviruses, CpMV1-NB631, and the *R. solani* mitovirus-like M2 dsRNA diverged from a common ancestor prior to the divergence of ascomycetes and basiomycetes, these paralogous lineages will all have been evolving for the same length of time. This would account for their similar levels of divergence and the relatively low levels of similarity.

Another factor that may have influenced the evolution of the eight mitoviruses in *O. novo-ulmi* L8d[2] is that closely related mitoviruses may be incompatible if they replicate in the same mitochondrion. The ability of different mitoviruses to replicate in the same mitochondrion could require a minimum sequence divergence. Mitovirus RNAs that are closely related and can be replicated by the same polymerase may compete with each other, leading to elimination of the less efficiently replicated RNA. Similar explanations have been suggested for incompatibility of closely related plasmids in bacteria[85] and cytoplasmic dsRNA viruses of the *Totiviridae* family in the basidiomycete fungus, *Ustilago maydis*.[86]

The *Ophiostoma*, *Cryphonectria,* and *Rhizoctonia* mitovirus or mitovirus-like RdRps have been found to be related to putative RdRps encoded by two ORFs in the mitochondrial genome of the plant *Arabidopsis thaliana*.[65,74,87] The *Arabidopsis* mitovirus-like RdRps were only about one-third the size of the fungal mitovirus RdRps, and there is no evidence as to whether they are expressed or functional. The homologous sequences corresponded mainly to conserved RdRp motifs II to VI, but they also included part of motif I, which is considered to be specific to mitovirus RdRps.[65,74,87] A shorter mitovirus-like RdRp sequence, again corresponding to conserved motifs, was also found in the bean, *Vicia faba*.[87] It has been suggested that these results are indicative of horizontal transfer of nucleic acids between fungi and plants.[87] Plant pathogenic fungi and mycorrhizal fungi often come in close contact with their host plants, and rare virus transmission between fungi and plants is a possibility. However, an alternative explanation is that fungal and plant mitoviruses have evolved from a mitovirus of an ancestral host, prior to the divergence of fungi, plants, and animals. Mitochondrial dsRNA elements have been described in a number of plants, e.g., alfalfa,[88] maize,[89] and sugarbeet,[90] and mitoviruses could be wide-

spread in plants. Similar dsRNAs have been described in insects.[91] Sequences of a much wider range of mitoviruses and mitovirus-like RNA elements, in fungi, plants, animals, and protists will be required to obtain definitive answers to these interesting questions.

Mode of Action of the d²-Factor

The sequences of the three RNAs associated with the d²-factor provide few clues as to how they may cause the symptoms of d-infection, such as reduced cytochrome oxidase production, aberrant growth, and low conidial viability. OnuMV4-Ld encodes an RdRp (Section 7.2.3.1, *Sequence Analysis of the Three d²-Factor-Associated dsRNAs*). It is possible that replication of this mitovirus RNA (and the more abundant derived defective RNAs, RNA-7, and RNA-10) also requires an essential mitochondrial protein whose concentration is limiting. If this were the case, it would be necessary to postulate either that (a) the replication of all the other mitoviruses found in *O. novo-ulmi* L8d², which have no overt effect on the fungus, does not require this essential mitochondrial protein or that (b) the expression of the d²-factor requires the cumulative effects of the replication of all the mitovirus and associated RNAs for the level of the postulated mitochondrial protein to drop below a critical threshold. Other d-factors associated with reductions in cytochrome oxidase levels, such as the d⁵-, d¹⁰-, and d¹²-factors, are also associated with multiple dsRNA segments,[23,39,44] which is consistent with this hypothesis—although it does not prove it.

Another possibility is that there is sequence similarity between OnuMV4-Ld RNA (and RNAs 7 and 10) and a region of the *O. novo-ulmi* mitochondrial DNA. These may act as antisense RNAs to suppress the expression of essential mitochondrial genes, e.g., cytochrome oxidase subunits. Sequencing of the mitochondrial coding regions of the *O. novo-ulmi* cytochrome oxidase (*cox*) subunit I, II, and III genes has not revealed any significant sequence similarity with the sequences of OnuMV4-Ld (or OnuMV3a-Ld, OnuMV5-Ld, or OnuMV6-Ld),[92] but mitochondrial intron sequences within the *cox I* or *cox II* genes or sequences upstream of the *cox I*, *cox II,* or *cox III* genes, which may include transcriptional regulatory sequences, have not been determined. It is clear that much further work will be needed to elucidate how the d²-factor-associated mitovirus RNAs affect cytochrome oxidase levels and cause all the other symptoms of d²-infection.

7.2.3.2 The d¹-Factor

O. novo-ulmi isolate H321d¹ was found to contain 7 dsRNA segments with sizes (kbp) of 1.87 (RNA-1), 1.59 (RNA-2), 1.51 (RNA-3), 0.80 (RNA-4), 0.73 (RNA-5), 0.67 (RNA-6), and 0.55 (RNA-7). Analysis of single conidial isolates that had retained the d¹-factor showed that only RNAs 1, 2, and 5 were consistently associated with the d¹-factor.[34,44] Subcellular fractionation has shown that the dsRNA in *O. novo-ulmi* isolate H321d¹ is not associated with the mitochondria.[93] This is consistent with the observation that there is no reduction in cytochrome oxidase levels in H321d¹ (Section 7.2). RNAs 1 and 2 of H321d1 are in the size range of viruses in the *Partitivirus* genus of the *Partitiviridae*, a family of viruses with genomes of two dsRNA segments.[82] Recently, a partitivirus has been found in *O. himal-ulmi*.[94] It is

possible that the d^1-factor is associated with a partitivirus. The size of RNA-5 suggests that it could be a defective RNA derived from RNA-1 or RNA-2. The mode of action of the d^1-factor is unknown.

7.3 VIRUSES AS POTENTIAL BIOLOGICAL CONTROL AGENTS FOR DUTCH ELM DISEASE

The potential of d-factor-associated viruses as biological control agents for Dutch elm disease has been extensively reviewed,[32,37,46,95,96] and the effects of d-factors on the population biology of *O. ulmi* and *O. novo-ulmi* were discussed in Section 7.2.2. While d-factors may have contributed to the decline of *O. ulmi*,[45] it is clear that they have not prevented the widespread destruction of elm populations by *O. novo-ulmi*. To develop d-factor-associated viruses as biological control agents, it is necessary to understand why a natural biological control has not occurred. The main way in which d-factor-associated viruses reduce the ability of *O. novo-ulmi* to cause disease is by greatly increasing the numbers of conidia required to initiate an infection in an elm tree. In field experiments using artificial feeding grooves, infection of the highly susceptible *U. procera* required about 50,000 conidia of a d^2-infected isolate, but only 500 to 1,000 conidia of a healthy isolate.[36,37,97] Infection of the moderately resistant *U.* x *hollandica* cv. "Commelin" required 50,000 to 500,000 conidia of d^2- and d^9-infected isolates, compared to 5,000 conidia of a healthy isolate.[97] Other less severe d-factors had more moderate effects.[97]

If elm trees are inoculated with sufficient numbers of conidia to initiate a xylem infection, there is little difference in the amount of disease caused by d-infected and healthy isolates.[32] In nature, whether an infection of an elm tree sufficient to cause disease will occur will depend on the number of conidia carried by a vector beetle, the numbers of beetles feeding on the tree, the relative resistance of the elm, the proportion of d-infected conidia, the severity of effects on the fungus caused by the d-factor, and various environmental factors. If the beetles feeding carry insufficient conidia to initiate a xylem infection, the disease cycle will be broken.[11] About 60% of individuals of the largest European beetle vector, *S. scolytus,* carry the minimum 500 spores needed to cause disease on *U. procera* by a healthy *O. novo-ulmi* isolate. Only 25% of *S. scolytus* individuals carry more than 5,000 spores and about 15% carry more than 10,000 spores.[11,36,37,98] At epidemic fronts, when only one major vc group is present, up to 90% of bark populations of *O. novo-ulmi* in Europe can be d-infected (see Section 7.2.2). However, this implies that at least 10% of conidia of such bark populations of *O. novo-ulmi* are likely to be healthy, and therefore 25% of *S. scolytus* populations could carry the minimum 500 healthy spores needed to cause disease in *U. procera*. This is clearly sufficient to enable the continued spread of Dutch elm disease, even when d-infection levels are high. The viability, and hence the infectivity, of the inoculum may increase in the post-epidemic period, when more vc types appear, leading to restricted transmission and lower frequencies of d-factors (see Section 7.2.2).

In North America, where only the smaller European elm bark beetle, *S. multistriatus*, with its much smaller spore load, is present, the potential impact of d-

factors may be greater, although this may be partly offset by the high disease susceptibility of American elms.[46,48,97] Furthermore, the American *O. novo-ulmi* population has a higher frequency of A mating types. Since ascospores are produced at various times during the bark saprotrophic stage,[10] and are free from d-infection,[32,33] inocula coming from newly ascospore-derived colonies would be highly infective irrespective of the prior level of d-infection in the *O. novo-ulmi* bark population.

Overall, it is clear that, if d-factor-associated viruses are to be developed as biological control agents, it will be necessary to infect a much larger proportion (ideally 100%) of the *O. novo-ulmi* population and to prevent the loss of the d-factor viruses that occurs in ascospore formation and, to a lesser extent, through conidiation and multiplication as yeast-like cells in the xylem. Both these objectives could be achieved by incorporating cDNA copies of the d-factor virus RNA genomes, linked to appropriate transcriptional control signals, into the mitochondrial DNA (for mitoviruses) or nuclear DNA (for cytosolic viruses). This has been achieved for a *C. parasitica* hypovirus, and the hypovirus cDNA incorporated into the *C. parasitica* nuclear genome has been shown to be mitotically and meiotically stable over several generations.[61] Integration of virus cDNA copies into the mitochondrial or nuclear genomes would enable the virus to be introduced into a wide range of *vic* genotypes either by direct transformation or sexual crossing. This, in turn, would enhance the potential for virus spread in *O. novo-ulmi* populations by hyphal anastomosis. The phenomenon of latency (Section 7.2.1) could affect the efficacy of a virus-based control agent, and more work is needed to understand the relationship between latency and overt expression of d-factors before they can be exploited to maximum advantage.

There has been much discussion about which d-factors may be the most efficacious for release as biological control agents. Webber[37] suggested that the best choice may be d-factors that have only moderate effects on fungal growth rate and conidial viability, enabling the d-infected fungus to be maintained in the saprotrophic bark-bark cycle and regularly transmitted by vector bark beetles. Sutherland and Brasier,[97] agreeing with this assessment, considered that the severe d-factors d^2, d^3, and d^9 might be considered too debilitating and d-factors, causing more moderate effects, such as d^5, d^6 and d^8, might be more suitable as control agents. Alternatively, it was suggested that isolates containing a range of d-factors could be released. Using a stochastic, spatially extended model, Swinton and Gilligan[99] deduced that predictions of success based on high competitive ability (i.e., vertical transmission or the ability of the d-infected fungus to establish new colonies) are likely to be more robust than those based on the high degree to which the d-infected fungus can transmit d-factors to healthy isolates (horizontal transmission). In contrast, Taylor et al.[100] concluded that the most effective biocontrols require high horizontal transmission. Since high vertical transmission and high horizontal transmission are not mutually exclusive, it is likely that both these attributes could contribute to a successful biocontrol agent. However, the factors that contribute to high vertical transmissibility may also contribute to the disease-producing capacity of the fungus, and *vice versa*. The best compromise may be a d-factor that causes moderately deleterious effects with a reasonably high degree of vertical transmissibility of the infected fungus but with a reduced ability to cause Dutch elm disease. This is consistent with the suggestions

of Webber[37] and Sutherland and Brasier[97] for deployment of d-factors causing moderate effects. A high degree of horizontal transmissibility, possibly leading to 100% infection of *O. novo-ulmi* bark populations, would also be desirable and might eventually lead to the local elimination of *O. novo-ulmi*.

In the future, it may be possible to genetically modify *O. novo-ulmi* viruses for the control of Dutch elm disease. For example, two-component mitovirus vectors could be constructed. The first component would be the wildtype mitovirus RNA that encodes the RdRp. The second component would include *cis*-acting sequences required for RNA replication from the first component linked to antisense RNA sequences corresponding to an essential mitochondrial gene, such as a cytochrome oxidase subunit gene. Replication of the two-component vector system in *O. novo-ulmi* mitochondria could suppress expression of mitochondrial genes, leading to phenotypes similar to those produced by the d^2-factor, but without the complication of latency. Experiments of this type are in progress.[92] It may also be possible to modify cytoplasmic RNA viruses to reduce the pathogenicity of *O. novo-ulmi.* The *C. parasitica* hypoviruses specifically reduce the pathogenicity of their host fungus by reducing the levels of heterotrimeric G proteins, which in turn greatly reduces the levels of pathogenicity genes induced when the fungus makes contact with its host plant.[101] G protein-coupled signal transduction pathways may also be involved in the pathogenicity of a range of plant pathogenic fungi, including *O. novo-ulmi*. Hence, a cytoplasmic virus vector (analogous to the mitovirus vectors described above) could be designed to reduce G protein expression (using antisense RNA or gene silencing technology) and hence pathogenicity in *O. novo-ulmi.* Such a vector may have an advantage over wildtype d-factor viruses in that only pathogenicity would be affected. Since the fungus would not be debilitated with regard to saprotrophic growth, it should have a high degree of vertical transmissibility in the bark-to-bark cycle. Integration of cDNA copies of the vector into the fungal chromosomal DNA should improve the persistence of such vectors in the wild.

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8 Double-Stranded RNA Elements Modulating Virulence in *Rhizoctonia Solani*

Stellos M. Tavantzis, Dilip K. Lakshman, and Chunyu Liu

CONTENTS

8.1 INTRODUCTION

To date, plant disease management has relied heavily on the use of chemicals leading to increased production costs, utilization of fossil-fuel energy, water pollution, non-target effects, and development of tolerance by the target organism. Understanding of the factors regulating virulence in the pathogen will lead to the development of biocontrol- or genetic-engineering-based strategies of plant disease management that will fulfill the need for sustainable and nonpolluting agricultural practices.

Species of *Rhizoctonia* cause economically important diseases on most of the world's major field and vegetable crops, fruit and forest trees, turfgrasses, and ornamental plants.[1] Thus, characterization of genetic factors regulating virulence in *R. solani* could have a significant impact on a wide spectrum of plant-related human endeavors.

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Isolates of *R. solani* are grouped on the basis of hyphal anastomosis reactions into at least 13 anastomosis groups (AGs). In general, AGs are genetically isolated noninterbreeding populations with distinct host ranges.[2] Our work is focused on AG 3, because it is the major cause of rhizoctonia disease of potato in N. America, including Maine.[3] Rhizoctonia disease occurs wherever potato is grown but is most severe where soils are moist and cool (16 to 23°C). Quantitative and qualitative yield losses vary[1] and could be quite high.[4] Seed potato treatment with pentachloronitrobenzene (PCNB) is routinely used to control rhizoctonia disease in Maine and elsewhere. PCNB is one of the most persistent soil fungicides and has a wide spectrum of antimicrobial activity.[5] It is harmful to beneficial microorganisms living in the rhizosphere of potato or other crop plants.[6] Colonization of potato plants with growth-promoting rhizobacteria resulted in a 17% yield increase.[7] Thus, biocontrol of rhizoctonia diseases could restore the plant rhizosphere ecosystem and improve crop performance, including yields. As a soil-borne pathogen, *R. solani* is well suited for biocontrol-based strategies of disease management. Seed (tuber) coating with a preparation of hypovirulent (or nonpathogenic) propagules is feasible and requires no carriers or vectors to spread hypovirulence.

The form genus *Rhizoctonia* DC includes species that are beneficial to plants through the formation of mycorrhiza.[8] Moreover, in addition to their biocontrol properties, nonpathogenic isolates of *R. solani*[9,10] and binucleate *Rhizoctonia* isolates[11] have been shown to promote plant growth and can be used in a number of sustainable agricultural practices.[12] Thus, understanding how virulence is brought about in *Rhizoctonia* could lead to a cascade of advances in sustainable agriculture we can barely envision at present.

Our work revolves around the concept of hypovirulence (less than normal disease-producing capacity) attributed to cytoplasmic double-stranded RNA (dsRNA) elements. dsRNAs occurring in hypovirulent fungal isolates can be transmitted to virulent strains by hyphal anastomosis and converted to hypovirulent.[13]

8.2 CYTOPLASMIC HYPOVIRULENSE IN *R. SOLANI*

A cytoplasmically controlled degenerative disease of *R. solani* was reported in 1978.[14,15,16] The above condition was characterized by loss of mycelial pigmentation, reduced growth rate and sclerotia production, presence of dsRNA, and hypovirulence similar to that of *C. parasitica*.[17] Three out of 13 strains had dsRNA and were hypovirulent, whereas the 10 virulent isolates contained no detectable dsRNA.[16] Subsequent studies, however, were not in accordance with these findings.[18,19,20,21] In the late 1980s and early 1990s, our research group generated several lines of evidence suggesting that although the mere presence of dsRNA was not associated with specific phenotypes, particular dsRNA elements might down- or up-regulate virulence in *R. solani*.[22,23,24,25] For example, when one or more dsRNAs were removed by hyphal-tip isolation, the virulence of the resulting cultures was increased dramatically (Figure 8.1). More importantly, we showed that dsRNAs from *R. solani* isolates obtained from the same field, as well as those from different continents, were quite diverse genetically.[23,24] This work shed some light to the controversy, as it became evident that dsRNA elements, carrying different genetic information and occurring

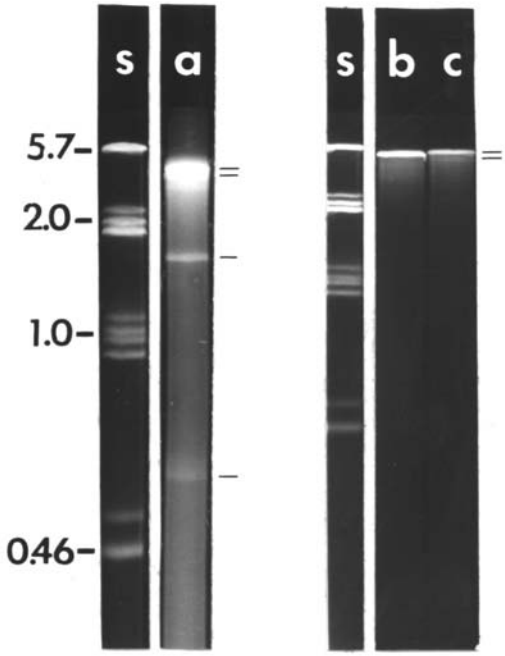

FIGURE 8.1 Agarose gel electrophoresis of dsRNAs from the AG 3 isolate Rhs 41 (lane a), and its hyphal-tip derivatives Rhs 41-2 (lane b) and Rhs 41-3 (lane c). Numbers on the left indicate molecular weight of standard-size dsRNAs from *Helminthsporium* maydis (Hm 9, 5.7×10^{-6}), *Penicillium chrysogenum* (PeV, 2.0×10^{-6}), and *P. stoloniferum* (1.0×10^{-6}, and 0.46×10^{-6}) (lane s). Lines on the right show the position of the dsRNA bands found in the three cultures. Virulence, determined as percent of lesioned stem tissue, was 4.0 (cd), 6.5 (bcd), and 16.2 (a) for Rhs 41, Rhs 41-2, and Rhs 41-3, respectively. The letters in parenthesis indicate levels of significance according to Duncan's multiple range test.

in different fungal genotypes, might have varying effects, direct or indirect, on the virulence of the respective hosts.

So, we set out to study a genetic model[26] involving a particular set of dsRNAs, some of which appeared to be associated with distinct phenotypes. Rhs 1AP is a virulent field isolate belonging to AG 3. Over a period of years, we selected three morphologically distinct sectors of Rhs 1AP, which gave rise to the hypovirulent (essentially nonpathogenic) cultures Rhs 1A1, Rhs 1A2, and Rhs 1A3. Rhs 1AP (P stands for parental) possesses two dsRNAs of 23 kb (L2) and 6.4 kb (M1) (Figure 8.2). Rhs 1A1 has three dsRNAs in addition to the two dsRNAs found in the "parental" Rhs 1AP. The apparent sizes of the novel dsRNAs are 25 kb (L1), 3.6 kb (M2), and 1.2 kb (S1). In contrast, Rhs 1A2 and Rhs 1A3 lack the two dsRNAs (L2 and M1) of Rhs 1AP but have M2 and S1, and L1 and S1, respectively. Northern blot hybridization and sequencing analyses showed that dsRNAs with corresponding sizes (L2 and M1) occurring in Rhs 1AP and Rhs 1A1 are genetically identical, but the five dsRNAs are not related to one another. The phenotype and dsRNA content of the above four type cultures are stable. The three dsRNAs that were not detectable

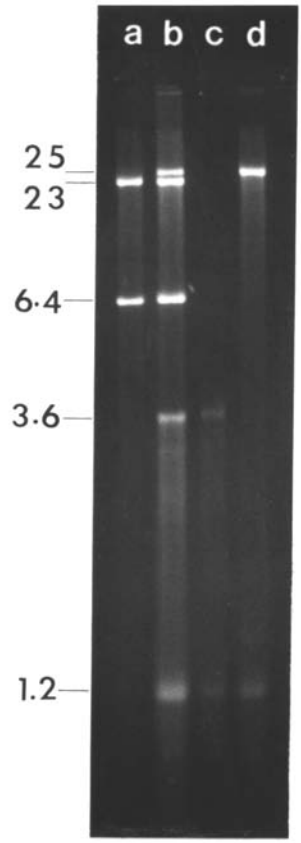

FIGURE 8.2 Electrophoretogram of dsRNAs from the *R. solani* AG 3 isolates Rhs 1AP (lane a), Rhs 1A1 (lane b), Rhs 1A2 (lane c), and Rhs 1A3 (lane d) on an ethidium bromide-stained, 1% agarose gel. Numbers on the left indicate the sizes of the dsRNAs in kilobases. (From Lakshman and Tavantzis, 1994).

by ethidium bromide staining or northern blot hybridization in Rhs 1AP were found in DNA or RNA form using PCR or RT-PCR, respectively.[26,27]

Due to the absence of sporulation of *R. solani in vitro,* dsRNA segregation studies cannot be conducted. So, we paired the above four cultures (Rhs 1A P, 1, 2, and 3) in selected combinations aimed at transmitting particular dsRNAs into cultures in which they were previously undetectable. The goal was to generate groups of essentially isogenic isolates having the same dsRNA content or a-single-dsRNA difference and compare their respective virulence. In addition, a few hyphal-tip cultures cured from one dsRNA (M1) were obtained. The study centered on the potential impact of the M1 (6.4 kb) and M2 (3.6 kb) elements. Groups of as many as six independently (different pairings) produced cultures possessing the same dsRNA content had the same degree of pathogenicity (Table 8.1) and cultural characteristics. Removal of M1 by hyphal tipping resulted in loss of virulence,

TABLE 8.1
Association of the M2 dsRNA (3.6 kb) with Hypovirulence, and the M1 dsRNA (6.4 kb) with Virulence in *Rhizoctonia Solani*

M1 dsRNA M2 dsRNA	Present Absent	Present Present	Absent Present
Isogenic cultures	Rhs 1AP, P3-3a, P3-3b, P3-Pe1, P1-Pb2, P3-Pa, P3-Pc2	P1-Pa, P1-P6, Rhs 1A1	Rh2 1A2, 23-3b, 13-3c, 23-2a, 21-2b2, Phs 1A1HT, 21-2a, Rhs 1A3
Virulence[a]	20–30	10–16	0–10

[a]Virulence expressed as percentage of sprout stem area covered by lesions over the entire area of an 8-cm-long segment extending 4 cm on either side of the point of inoculation.

whereas its acquisition by hyphal anastomosis brought about a dramatic increase in virulence.[28] In contrast, transmission of the M1 dsRNA resulted in a significant increase in virulence of the recipient culture (Figure 8.3). Moreover, transmission of small amounts of the M2 (3.6 kb) dsRNA resulted in a dramatic attenuation of the virulence conferred by M1 (Table 8.1). RFLP analysis of mitochondrial DNA (mtDNA) from paired and "derivative" cultures showed no evidence of mtDNA migration from the donor to the "recipient" cultures. Gobbi and coworkers[29] also showed no evidence of mtDNA migration between vegetatively compatible strains of *C. parasitica*. The above work has provided strong indirect evidence suggesting that a single culture of *R. solani* may harbor two dsRNAs that have diametric effects on the same trait (virulence).

The dsRNA transmission data provided a glimpse of the biological importance of dsRNA in *R. solani,* but it has been apparent that a transfection system is needed to generate unequivocal evidence regarding the precise biological roles of specific dsRNA moieties in particular genotypes of the pathogen. A number of research groups have tried unsuccessfully to transform *R. solani.* However, a novel method involving *Agrobacterium tumefasciens* has been used recently to transform a number of fungi that have been recalcitrant to transformation in the past.[30] We believe that adoption of the above method will enable "rhizoctonia researchers" to genetically engineer *R. solani* in the near future.

8.3 HYPOVIRULENCE-MEDIATED BIOCONTROL OF *R. SOLANI*

We conducted field experiments to determine if a hypovirulent AG 3 isolate, Rhs 1A1, could reduce rhizoctonia disease caused by virulent AG 3 isolates. Rhs 1A1 induces a slight discoloration on potato stems at the point of entry, unlike the dark, sunken necrotic lesions caused by virulent isolates.[26] In plants inoculated with both Rhs 1A1 and virulent isolate Rhs 27, disease was significantly ($P = 0.05$) reduced by 56% as compared with that of plots inoculated with the virulent Rhs 27 alone.[10] Interestingly, plants inoculated with Rhs 1A1 alone exhibited a greater ($P = 0.05$)

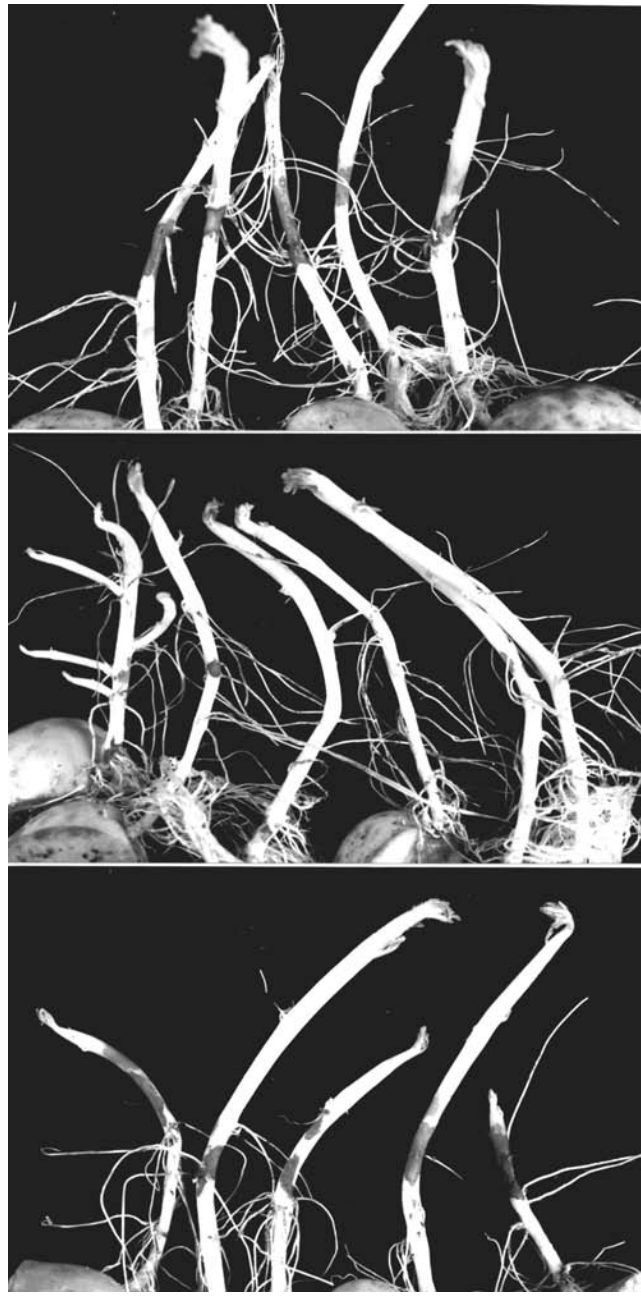

FIGURE 8.3 Association of the M1 dsRNA (6.4 kb) with an increase in virulence in *R. solani.* Sprouts of potato cultivar "Katahdin" infected with virulent Rhs 1AP (top panel), hypovirulent Rhs 1A3 (middle panel), and P3-3b (bottom panel). P3-3b was derived from Rhs 1A3 after pairing of Rhs 1AP with Rhs 1A3 and transmission of the M1 dsRNA from the former to the latter culture. (From Jian et al., 1997.)

growth response (Figure 8.4), expressed as (1) a 4-fold increase in the dry weight of stolons (yield predictor), (2) an 1.7-fold increase in the dry weight of stems (including foliage), and (3) full bloom, and onset of tuberization 7–10 days earlier than their respective control.[10] Sneh and coworkers[9] also reported increased plant growth response induced by nonpathogenic *R. solani*.

The increased growth response of potato to the hypovirulent isolate Rhs 1A1 appeared to be phytohormone-induced. Earlier work showed that *in vitro* cultures of *R. solani* produce phenylacetic acid (PAA), which is capable of causing the same disease symptoms on potato as the pathogen itself.[31] Experimental evidence implicating PAA in determining the rhizoctonia disease reaction is quite convincing. PAA application induces the rhizoctonia disease syndrome in the absence of physical contact between the host and the pathogen.[32,33,34,35] PAA, and its metabolites from plant sources and *R. solani*, acts as a plant growth regulator (auxin) at low concentrations,[36,37] but at high concentrations it acts as a toxin.[31] Indeed, we showed that the amount of PAA (μg/g dry weight of mycelium) produced by the hypovirulent isolate Rhs 1A1 was only 10% of that produced by virulent AG 3 isolates.[25,38] We confirmed the earlier studies by conducting *in vitro* experiments to examine the effect of PAA concentration on potato plantlet growth. Axenically produced potato stem cuttings were placed on potato propagation agar media amended with different concentrations of PAA and allowed to develop into plantlets. Potato plantlets grown on media amended with a PAA concentration similar to that produced by a hypovirulent *R. solani* culture were significantly larger than control plantlets, whereas those grown on PAA concentrations similar to those synthesized by virulent isolates exhibited growth inhibition (Bandy and Tavantzis, unpublished data).

8.4 NATURE OF dsRNA ELEMENTS IN *R. SOLANI*

Some of the dsRNAs found in *R. solani* are components of typical mycoviruses.[20,39] Isometric virus particles, 33 nm in diameter, from a dsRNA-containing AG 2 isolate, Rhs 717, contained two dsRNAs of 2.4 kb and 2.2 kb.[39] A mixture of the two dsRNAs encoded two major polypeptides of 71 kDa and 77 kDa *in vitro*. Both translation products were specifically immunoprecipitated by antibodies against intact virions. A virion-associated RNA polymerase activity was characterized.[39] The bisegmented genome of the dsRNA virus from Rhs 717 was characterized.[40] The larger segment, dsRNA 1, is 2,363 bases long, whereas the smaller segment, dsRNA 2, has 2,206 bases. The 5' ends of the coding strands of dsRNA 1 and dsRNA 2 are highly conserved (100% identity over 47 bases). Analysis of the coding potential of each of the two segments showed that dsRNAs 1 and 2 could code for polypeptides of 730 amino acids (bases 86 to 2275; molecular mass 86 kDa) and 683 amino acids (bases 79 to 2130; molecular mass 76 kDa), respectively. The 86 kDa polypeptide has all the motifs of dsRNA RNA-dependent RNA polymerases (RDRP)[41] and has a significant homology with putative RDRPs of partitiviruses from *Fusarium poae* (46% identities)[42] and *Atkinsonella hypoxylon* (39% identities).[43] The 76 kDa protein shows homology with the putative capsid proteins (CP) of the same viruses, consistent with the *in vitro* translation data. No subgenomic RNAs were found in Rhs 717, in congruence with the fact that the long open reading frames (ORF) coding

FIGURE 8.4 Field plots of potato cv. "Katahdin" plants inoculated with grain colonized by the hypovirulent *R. Solani* culture Rhs 1A1 (left panel) or sterile grain control (right panel). Plants inoculated with Rhs 1AP exhibited significantly increased vigor and gave rise to flowers and stolons seven to ten days earlier than their control counterparts.

for the putative RDRP and CP cover the entire length of the respective dsRNAs. Although the dsRNAs from Rhs 717 and other isolates[20] are of typical viral nature, unencapsidated dsRNAs have been found in *R. solani* by our group (M1 and M2 dsRNAs, see below) and Finkler and coworkers.[44]

8.5 dsRNAs ASSOCIATED WITH CHANGES OF VIRULENCE IN *R. SOLANI*

As mentioned previously, the dsRNA transmission study showed that the M1 dsRNA (6.4 kb) is associated with virulence, whereas the M2 dsRNA (3.6 kb) causes an attenuation of virulence.[28] Subsequently, we focused on characterizing these two genetic elements.

The M1 dsRNA has been sequenced to completion (6,398 bp).[45] M1 possesses ORF's longer than 100 amino acids on three different reading frames (1, 3, -1). *ORF 2* is on frame 1 and comprises 1,747 amino acids. The putative polypeptide of *ORF 2* has a significant sequence similarity with the 1A protein of bromoviruses, a large plant virus group.[45] Some regions, including all six helicase motifs, are highly conserved. The *ORF 2* polypeptide is also phylogenetically related to ORF 1a of the lactate dehydrogenase virus (LDV), which causes a persistent infection in mice.[45] Infected mice exhibit chronically elevated levels (5- to 10-fold) of seven serum enzymes due to a decrease in the rate of enzyme clearance. It is possible that M1 causes a similar increase in the activity of enzymes involved with electron transport, thus increasing vigor and virulence in *R. solani*.

The polypeptide product of *ORF 5*, located on frame -1 of M1, is phylogenetically related to the cytochrome ubiquinol c oxidase assembly factor (CytCAF). The two polypeptides share 35% amino acid identities and 27% conservative substitutions over a stretch covering 80% of the CytCAF sequence.[45] The region of homology between the two polypeptides includes conserved regions and various functional domains for CytCAFs. It is interesting, from an evolutionary point of view, that a putative viral helicase (*ORF 2* polypetide) and an electron transport enzyme have such a relatively close genetic relatedness.

The M1 dsRNA lacks an ORF encoding a RNA-dependent RNA polymerase (RDRP).[45] However, M1 is always associated with a 23 kb dsRNA (L2). Cultures possessing M1, also contain L2, although the reverse is not true.[45] Moreover, the two dsRNAs are associated with the same subcellular fraction. Interestingly, L2 has an ORF encoding an RDRP, which is genetically related (38% identities, 38% conservative substitutions in a stretch of 474 amino acids) to an "enigmatic" dsRNA from Japonica rice,[46] and the dsRNA associated with the "447" cytoplasmic male sterility in *Vicia faba* (see chapter by P. Pfeiffer in this book) (Figure 8.5). RNA viruses are known to evolve at a rapid rate, and this causes a considerable sequence divergence even among related viruses. Thus, the degree of relatedness between three proteins encoded by RNA genomes occurring in a fungus, a monocotyledonous, and a dicotyledonous plant is remarkable (Figure 8.5).

The genetic information carried by the M2 dsRNA is also found in DNA form in both the virulent Rhs 1AP and the hypovirulent Rhs 1A1, but the replicating dsRNA and its full-length transcript are detectable only in Rhs 1A1.[26] A major

FIGURE 8.5 Alignment of putative RNA-dependent RNA polymerases (RDRPs) from the L2 dsRNA (23 kb) of Rhs 1AP (L2), the rice enigmatic dsRNA (ENIGMA), and the dsRNA associated with the "447" cytoplasmic male sterility in broadbean *(Vicia faba)* (FABA). Identical amino acids are black-boxed, and similar amino acids are gray-boxed.

portion of the M2 dsRNA is located in the cytoplasm, whereas a smaller amount is found in mitochondria. The carboxy-terminal part of *ORF A* contains the consensus motif (GDD)[41] and the three conserved regions A, B, and D, of an RNA dependent RNA polymerase (RDRP). *ORF A* has a significant sequence similarity with a protein encoded by a hypovirulence-associated mitochondrial dsRNA from strain NB631 of *Cryphonectria parasitica*, incitant of chestnut blight.[47] Furthermore, this polypeptide possesses three transmembrane helices at positions 413–434, 468–486, and 552–571, respectively.[27]

A stretch (positions 190 to 517) of the *ORF A* polypeptide is genetically related to two enzyme domains of the pentafunctional polypeptide AROM from yeast (*S. cerevisiae*), which is a mosaic of five individual domains, and carries out steps 2 to 6 of the shikimate pathway in yeast and filamentous fungi.[48] More importantly, the *ORF A* polypeptide is genetically related to the QUTR protein of the quinic acid pathway.[49] The genetic relatedness between the gene products of *arom*, *qutR,* and *ORF A* will be further discussed below.

8.6 THE QUINATE-SHIKIMATE PATHWAY CONNECTION

The pentafunctional AROM protein, which is active as a dimer of identical subunits, converts 3-deoxy-D-arabino-heptulosonic acid-7-phosphate (DAHP) into 5-enolpyruvyl shikimate-3-phosphate (EPSP).[50] This conversion involves steps 2 to 6 of the prechorismate (shikimate) pathway.[51] The AROM protein occurs in yeast, filamentous fungi, and *Euglena,*[49,52] and is the product of the *arom* gene, which evolved by fusing five separate monofunctional genes encoding the equivalent enzymatic activities in prokaryotes and plants.[51,53] The two intermediates, 3-dehydroquinate (DHQ) and dehydroshikimate (DHS), that are intercorverted by the 3-dehydroquinase activity of the AROM protein are also intermediates of the quinate utilization (qut) pathway, which is responsible for the catabolism of quinate to protocatechuic acid (PCA) in many fungi and bacteria.[51] Quinate comprises approximately 10% (w/w) of decaying leaf matter and, therefore, is an abundant carbon source for many soil microorganisms. The eight genes of the *qut* gene cluster are upregulated at the transcriptional level positively by the QUTA protein (activator) and downregulated by the QUTR protein (repressor).[54] Thus, QUTR up modulates the shikimate pathway by allowing a flux of DHQ and DHS from the quinate into the shikimate pathway.

Comparison of the predicted amino acid sequences of the *arom, qutA,* and *qutR* genes showed that *qutA* and *qutR* evolved from splitting of a duplicated copy of the *arom* gene with the two N-terminal domains (DHQ synthase, and EPSP synthase) comprising *qutA*, and the three C-terminal domains [shikimate synthase (SK), dehydroquinase (DQse), and shikimate dehydrogenase (SDH)] giving rise to *qutR*. The *qutA* gene evolved further by acquiring a zinc binuclear cluster domain that allows it to bind to specific upstream sequences of the promoters of the *qut* genes.[55,56] The repressing effect of QUTR is brought about through stoichiometric binding of QUTR to the QUTA protein.[57,58]

Strong experimental evidence has shown that the two identical polypeptides comprising the native AROM protein fold into a tight globular conformation in which the N- and C-terminal domains, DHQS and SDH, respectively, are closely linked.[50] Hawkings and coworkers[53] pointed out that these data suggest a mechanism regarding the specific binding of the activator QUTA by the repressor QUTR. As in the native AROM protein, QUTA and QUTR bind to each other so that the N-terminus of the activator, DQHS-like domain, is in close proximity with the C-terminus, SDH-like domain, of the repressor. This conformation appears to allow the DQse-like and SK-like domains of QUTR to block the interaction between the surface acidic amino acids located near the C-terminus of QUTA and the transcription initiation complex.[59]

Genetic and molecular evidence suggests that the inducer quinic acid, the activator (QUTA in *A. nidulans*, qa in *N. crassa*), and the repressor (QUTR in *A. nidulans*, qs in *N. crassa*) interact to control expression of all *qut* (or *qa*) genes at the transcriptional level.[54,55] The activator binds to a conserved 16-bp site in the 5' flanking regions of all *qa* genes.[60] Each gene has at least one such recognition site, including the gene encoding the activator, which possesses at least four functional domains: the DNA-binding domain, a dimerization domain, a transcription activation domain, and a domain for interaction with the repressor.[57] The DNA-binding domain, located at the N-terminal of the activator, is a conserved 28 residue region containing a six-cystein zinc-binding motif.[61] The transcription activation domain consists mostly of acidic residues, and is located at the C-terminal of the activator.[62] Deletion mutations involving this domain result in noninducible *qa*-IF- mutants. The domain for interaction with the repressor overlaps with the transcription activation domain at the C-terminal of the activator.[57]

The overall sequence similarity between the QUTA protein of the *A. nidulans* and the AROM protein is 23% (identities), and as high as 35% in certain regions. A similar genetic relatedness exists between QUTR and AROM.[53,57] It is important to note here that, in a region of 343 amino acids, the N-terminal of the putative polypeptide A of the M2 dsRNA has a comparable relatedness with the DQse-like and SDH-like domains of AROM[27] and QUTR (Lakshman and Tavantzis, unpublished data). Thus, both the repressor QUTR and the M2 polypeptide A possess DQse-like and SDH-like domains, but polypeptide A lacks the portion of the EPSP-like and the SK-like domains that comprise the N-terminal of QUTR. It is this N-terminal of QUTR that occludes the negatively-charged region of QUTA and prevents it from interacting with the transcription initiation complex. This important difference between polypeptide A and QUTR is the basis of the hypothesis we have put forward to explain the potential mechanism used by the M2 dsRNA to interfere with a specific (and essential) metabolic pathway, thus affecting the degree of pathogenicity in *R. solani*.

8.7 THE PSEUDOREPRESSOR HYPOTHESIS

As discussed earlier, polypeptide A of the M2 dsRNA has two of the three domains of the repressor protein. The missing SK-like and a portion of the EPSP-like domains are thought to occlude the rich in acidic residues transcriptional activation domain

of the activator.[49] We have proposed that in cultures containing the M2 dsRNA ($M2^+$), the *ORF A* polypeptide binds to the *R. solani* QUTA analog (QUTA-RS) but, since it lacks the SK-like domain, it still allows the transcription activation domain of QUTA-RS to interact with the transcription initiation complex. According to this hypothesis, the M2-encoded polypeptide A would act as a truncated suppressor of the quinate pathway, thus converting this pathway from inducible to constitutive. The self-replicating ability of M2 would allow the *ORF A* polypeptide to reach high concentrations and outcompete the *R. solani* QUTR analog (QUTR-RS) in binding QUTA-RS. This interaction between the ORF A polypeptide and QUTA-RS would result in a constitutive expression of the quinate pathway in the absence of quinate and, in turn, down-modulation of the shikimate pathway, aromatic amino acid, and PAA synthesis and, therefore, hypovirulence in M2-containing isolates of *R. solani*. Huiet[63] and Lamb and coworkers[64,65] have shown that, in *N. crassa*, dysfunctional QUTR causes constitutive expression of the quinate pathway genes. Lamb and coworkers[65] also demonstrated that, if expression of the quinate pathway genes persists in the absence of environmental quinate, dehydroquinate and dehydroshikimate will flux from the shikimate into the quinate pathway and drastically reduce aromatic amino acid synthesis. So, important predictions of the above hypothesis have been demonstrated experimentally in other fungal species.

Recently, the focus of our work has been to determine whether the above hypothesis is valid. The role of the M2-encoded polypeptide A is central in this hypothesis. The size of polypeptide A is 83 kDa, so we use the designation p83 for referring to it. We produced p83-specific antibodies and used them in western blot analyses of protein samples from M2-containing, hypovirulent, and M2-lacking virulent cultures. The data showed a protein band binding the anti-p83 antibodies and having the expected migration rate. Moreover, this protein was present in $M2^+$ but absent from M2-lacking cultures. Subsequent experiments (see below) showed that the mRNA encoding this protein has a sequence identical to that of the M2 dsRNA (Liu, Lakshman, and Tavantzis, unpublished data).

Culturing the virulent isolate Rhs 1AP on defined media amended with quinate (either at induction or carbon utilization level) reduced its virulence in a dramatic manner. We raised the question as to whether this switch to hypovirulence was associated with the presence of polypeptide p83 *(ORF A)* encoded by the M2 dsRNA. In ensuing work, we showed that the quinate-mediated conversion of Rhs 1AP to hypovirulence is associated with induction of an M2-specific, polysome-associated RNA, and the presence of the M2-encoded p83. Total protein from quinate-induced and uninduced Rhs 1A1 (hypovirulent, possessing M2) and Rhs 1AP was fractionated by SDS-PAGE and subjected to western blot analysis using protein A (p83)-specific antibodies. As expected, p83 was present in uninduced and induced Rhs 1A1. The virulent ($M2^-$) uninduced Rhs 1AP had no detectable p83, but the quinate-induced, converted-to-hypovirulent Rhs 1AP contained p83 (Liu, Lakshman, Tavantzis, unpublished data).

Polysomes from uniduced and quinate-induced Rhs 1AP were immunopurified using purified IgG from p83-specific antisera. RNA from column-eluted polysomes were analyzed by RT-PCR using M2-specific primers as well as a combination of oligo(dT) and the least-degenerate primer representing the custom-made peptide

(located near the 3' end of *ORF A* of M2) that was used to raise the p83-specific antibodies. Results showed that polysomal RNA from quinate-induced Rhs 1AP contains M2-specific RNA. Moreover, sequencing of the 1.17-kb RT-PCR product showed that it is identical to the respective region of M2. The oligo(dT)-degenerate primer combination gave no RT-PCR products (Liu, Lakshman, Tavantzis, unpublished data). Since the M2 ssRNA transcript has no poly(A) tail, this result is congruent with the data of the western blot analysis. These experiments demonstrated that (1) p83, which was found to be specifically associated with $M2^+$ cultures of *R. solani* is encoded by the M2 dsRNA (*ORF A*), and (2) the quinate-induced hypovirulence in the virulent, $M2^-$ Rhs 1AP is associated with the induction of M2-specific polysomal RNA, and the polypeptide (p83) encoded by this RNA.

To monitor the level of expression of the shikimate and quinate pathways in $M2^+$ and $M2^-$ cultures and perform dihybrid assays (protein-to-protein interactions), we set out to clone the *arom* gene (shikimate pathway) as well as the activator and the suppressor genes of the quinate pathway of *R. solani*. RT-PCR of total RNA from *R. solani* (Rhs 1AP) was performed, using degenerate primers derived from consensus sequences of known AROM proteins, to clone and subsequently sequence the *R. solani arom (arom-rs)* mRNA and gene. The *arom-rs* mRNA of *R. solani* consists of 5,459 bases and terminates into a poly(A) sequence (Lakshman, Liu, Tavantzis, unpublished data). This is the first *arom* gene to be sequenced from a basidiomycete. It has regions of high homology with *arom* genes from other fungi but contains five introns as opposed to one intron found in other *arom* genes. The RT-PCR/degenerate primer strategy has not been successful in cloning the *quta* or *qutr R. solani* analogs. We shall use the cDNA differential-display approach (quinate induction vs. non-induction) to clone these genes.

We have also conducted enzyme activity assays for quinate dehydrogenase (quinate pathway) and shikimate kinase (shikimate pathway) to determine the level of expression of the respective pathways in uninduced and quinate-induced M2-containing (hypovirulent) and M2-lacking (virulent) isolates. The results showed that when uninduced, the M2-containing Rhs 1A1 had a high quinate dehydrogenase (QDHase) activity, several-fold higher than that of uninduced M2-lacking Rhs 1AP. Upon quinate induction, there was a several-fold increase in QDHase activity in Rhs 1AP, as compared to that of the uninduced state, whereas QDHase activity in induced Rhs 1A1 increased to a lesser degree but remained significantly higher than that of induced Rhs 1AP (Liu, Lakshman, Tavantzis, unpublished data). Clearly, the quinate pathway is constitutive in the M2-containing hypovirulent Rhs 1A1. This is in congruence with the hypothesis described above regarding the *modus operandi* of the M2 dsRNA.

Unlike the QDHase, the shikimate kinase (SKase), one of the AROM enzyme activities, was not inducible by quinate in *R. solani*. However, when uninduced (untreated), the virulent, M2-lacking Rhs 1AP has a significantly higher SKase activity and therefore a higher level of shikimate pathway expression than the M2-containing, hypovirulent Rhs 1A1 (Liu, Lakshman, Tavantzis, unpublished data). These results are consistent with the notion that the virulent Rhs 1AP has a higher level of PAA, the determinant of virulence. In fact, all of the data described in this section are congruent with the hypothesis that a polypeptide product of a gene

located on a dsRNA element (M2) is associated with induction of hypovirulence in *R. solani*.

8.8 PLANT COMPOSTS, QUINATE AVAILABILITY, AND MANAGEMENT OF RHIZOCTONIA DISEASE

One of the most interesting findings of our recent work is the dramatic reduction of virulence in induced by quinate in *R. solani.* The ramifications of this phenomenon, in terms of managing rhizoctonia disease under commercial field conditions, could be quite exciting. Quinic acid is one of the most prevalent phenolic compounds in composted leaf or bark litters. It may be a constituent of up to 2 to 10% of dry weight in coniferous needles[66,67] and in the leaves of woody angiosperms.[68] It is important to briefly review the literature concerning the composting process and the use of compost amendments in the soil to manage plant diseases incited by *R. solani*.

The process of organic material (leaves or bark) decomposition takes place in three stages.[69] In the first phase, soil bacteria and fungi utilize sugars and other easily biodegradable components of the plant material. During this time, compost temperature rises to 40–60°C. During the second step, cellulose and other similar polymers are degraded, and temperature rises to 40–70°C. Glucose becomes available at this stage. The third or curing phase involves break down of lignins and other complex polymers that decompose very gradually. Phenolic compounds, including quinate, appear during this last phase.

The degree of compost maturation appears to be the most important factor determining the effect compost will have on plant infection by *Rhizoctonia* species. Fresh plant amendments generally increase rhizoctonia disease severity,[70] whereas mature compost suppresses the pathogen.[71] It has been reported that colonization of the compost by *Trichoderma* sp. is necessary for the suppression of *R. solani*.[72] However, fresh hardwood bark did not suppress rhizoctonia damping-off, in spite of the high density of *Trichoderma* inoculum.[73] In contrast, composted bark suppressed the pathogen, even though the *Trichoderma* inoculum was two orders of magnitude lower than that of the fresh bark. It appears that suppresiveness of the composted bark coincided with the breakdown of lignins and the formation of quinate. Furthermore, when exogenous cellulose, equivalent to the amount contained in fresh bark, was added to composted hardwood bark, the suppressive effect of this amendment on *R. solani* was negated.[72] Apparently, the cellulosic substrate gave rise to glucose, which increased the pathogen's virulence. This is in agreement with our findings showing that the virulence of *R. solani* is proportional to the amount of glucose added to the growth media (Liu, Lakshman, Tavantzis, unpublished data).

Most composts suppress *Pythium* and *Phytophthora* root rots, but only 20% of the composts tested suppress *Rhizoctonia* damping-off.[73] Composts prepared from ligno-cellulosic substrates such as tree barks suppress *Rhizoctonia* more effectively than composts prepared from low ligno-cellulosic and high sugar substrates.[74] The former group of composts contain higher levels of quinate than the latter group during their *Rhizoctonia* suppressive phase. We propose that quinate concentration and availability is an important determinant of the effect composts may have on

Rhizoctonia disease incidence under field or greenhouse conditions. In fact, a review of the literature indicates that disease suppression can be attributed, at least in part, to modifications of the abiotic soil environment (including nutrients) affecting the degree of pathogenicity of *Rhizoctonia*.[75]

8.9 FUTURE PERSPECTIVES

One of our most significant recent findings has been the fact that in the virulent, M2-lacking isolate Rhs 1AP, quinic acid reduces virulence dramatically and, at the same time, induces replication and expression (mRNA and respective protein product) of the M2 dsRNA. This process is similar to that taking place in the hypovirulent, M2-containing isolate Rhs 1A1, which originated as a sector (spontaneous mutant) of Rhs 1AP. In Rhs 1A1, the quinate pathway is constitutively expressed, and M2 and its transcription and translation products are abundant. Moreover, chorismic acid, end-product of the shikimic acid pathway and precursor of the aromatic amino acids, enhances the virulence of Rhs 1AP dramatically. In the presence of quinate, however, not only the effect of chorismate on virulence is nullified, but virulence is reduced to a level as low as that brought about by quinate alone (Liu, Lakshman, Tavantzis, unpublished data). Thus, quinate initiates a process that overrides the effect of chorismate (e.g., increased aromatic amino acid and, in turn, PAA synthesis). It is important to determine if the above two phenomena [permanent (spacial) hypovirulence of Rhs 1A1 vs. induced (temporal) hypovirulence in Rhs 1AP] represent essentially the same process. This will be accomplished by comparing the genes that are expressed differentially (as compared to Rhs 1AP) in Rhs 1A1 and quinate-induced Rhs 1AP. More importantly, these cDNA differential-display experiments will allow identification of genes involved in hypovirulence (genes that are switched on or induced in Rhs 1A1 and quinate-induced Rhs 1AP) or virulence (genes that are down regulated in the above two cases or up-regulated in the presence of chorismate).

Several fungi that have not been possible to transform in the past have been transformed recently using a simple and effective protocol involving *Agrobacterium tumefasciens*.[30] We plan to adopt this method to genetically engineer *R. solani*. We plan to transfect virulent *R. solani* with M2-related sequences, such as the *qutR*-related region as a palindrome (iRNA), *ORF A* (p83) or the full-length sequence. The goal is to determine their effect on the quinate and shikimate pathways, M2 expression, and virulence. Availability of a transformation system for *R. solani* will also allow us to study genes of interest from the differential display experiments.

Marc Cubeta and collaborators at the North Carolina State University (NCSU) have genotyped over 300 field isolates of *R. solani* from Maine, North Carolina, and a number of other states and Canadian provinces. They have used a cassette of several genes to assess the degree of genetic diversity of this soil-borne fungus. In a collaborative project, we are using this database to help us understand the significance of the M2 dsRNA in the biology of *R. solani*. Some of the goals of this work are to determine the frequence of occurrence of M2 in field populations, the genetic variability of M2, whether it is always associated with hypovirulence, if particular

genotypes are more likely to harbor M2, or if environmental factors such as soil temperature affect its expression. Several isolates in this collection have been characterized in regard to their vegetative compatibility with one another (Cubeta and coworkers, unpublished data). It will be interesting to determine the distribution of the M2 dsRNA among these isolates and their ability to serve as donors or recipients of M2. This work will help us understand phenomena related to horizontal transmission of M2 under field conditions and thus design the best possible biocontrol strategy for this cosmopolitan plant pathogen.

We believe that the dramatic reduction in virulence induced by quinate in laboratory experiments warrants further studies to determine the effect of quinate on the virulence of *R. solani* under greenhouse and field conditions. We plan to use heat-treated compost (to eliminate interference by microorganisms) high in lignocellulosic compounds and evaluate its suppressiveness against *R. solani* at different stages of maturation. Quinate concentrations will be monitored during all phases of the greenhouse experiments. These studies might lead to effective management strategies against the multiple diseases caused by *R. solani* and may have a significant impact against other soil-borne plant pathogens.

Finally, the potential of hypovirulent isolates of *R. solani* to increase plant vigor and yields warrants further studies focusing on the (1) availability of hypovirulent strains representing several vegetative incompatibility groups in AG 3 and other agronomically important AGs and (2) biological properties of these isolates under field conditions.

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9 Molecular Genetics of the Viruses Infecting the Plant Pathogenic Fungus *Helminthosporium Victoriae*

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CONTENTS

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9.1 INTRODUCTION

9.1.1 The Viruses that Infect Plant Pathogenic Fungi

Viruses are widely distributed in fungi. Typically, fungal viruses have isometric particles 25 to 50 nm in diameter and possess dsRNA genomes. Depending on whether the genome is monopartite or bipartite, the isometric dsRNA viruses are classified into two families, *Totiviridae* or *Partitiviridae*.[1–4] In addition to the totiviruses and partitiviruses, the unencapsidated hypoviruses (family: *Hypoviridae*) and the La France isometric virus (LIV) (synonym, Agaricus bisporus virus 1; AbV1) associated with the La France disease of mushrooms (unclassified virus) also have dsRNA genomes.[5] Furthermore, two families of positive sense single stranded RNA mycoviruses have recently been described, *Barnaviridae* and *Narnaviridae*.[6,7]

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.[5,8–11] 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.[12] The debilitating disease of *Helminthosporium victoriae*, the causal agent of Victoria blight of oats, the diseased phenotype of the Dutch elm disease fungus *Ophiostoma novo-ulmi*, the die-back disease (La France) of the cultivated mushroom are examples of pathogenic effects of fungal viruses.[13–15]

A major objective of studies on viruses that infect plant pathogenic fungi is to unravel the mechanisms by which mycoviral dsRNAs or their specific gene products may perturb virulence expression in their fungal hosts and ultimately to utilize such knowledge in developing biological control measures for combating phytopathogenic fungi. In this regard, the dsRNA viruses infecting *Helminthosporium victoriae* (telomorph: *Cochliobolus victoriae*) are of special interest because they are associated with a debilitating disease of their fungal host and may serve as a model system to investigate the molecular basis of disease in a plant pathogenic fungus.[13,16,17]

9.1.2 The Diseased Phenotype of *Helminthosporium Victoriae*

H. victoriae was first described in 1946 as the causal agent of a new disease in oats called Victoria blight, named after the parent cultivar, Victoria.[18] The Victoria blight disease arose after the introduction of resistance (Pc-2 gene) to a completely different disease, crown rust of oats, which was genetically linked to susceptibility to a previously unknown soil fungus, *H. victoriae*.[19] The disease caused by *H. victoriae* rose to epidemic proportions in 1947 and 1948 and resulted in serious yield losses in most oat-growing regions of the United States.[13]

Virulence in *H. victoriae* is correlated with the production of the host specific pathotoxin "victorin."[20,21] Certain abnormal or "diseased" isolates of *H. victoriae*

that produce reduced amounts of victorin inflict little or no deleterious effects in the susceptible cultivar "Victorgrain".[13,16] The diseased phenotype is transmitted via hyphal anastomosis, and diseased isolates are characterized by reduced growth, excessive sectoring, aerial mycelial collapse, and generalized lysis.[13,22] The diseased phenotype of *H. victoriae* is of special interest not only because diseased isolates are hypovirulent but also because they harbor two isometric viruses with dsRNA genomes, designated according to their sedimentation values as the *Helminthosporium victoriae 190S virus* (Hv190SV) and Hv145S virus.[23] Because disease severity appears to correlate with the concentration of Hv145S virus in diseased mycelium, and because the Hv145S virus has always been found associated with Hv190S virus, it has been suggested that the Hv145S virus or a mixed infection with the two viruses is the cause of the disease.[17] Since no fungal isolate was ever found that contained the Hv145S virus alone, the possibility has been raised that the 145S sedimenting component may represent a satellite virus or satellite dsRNA that is dependent on the Hv190S virus for replication and encapsidation.[17,23] Results of recent studies indicated that Hv145SV encodes its own RNA-dependent RNA polymerase, but it has yet to be determined whether the Hv145SV may be dependent on the Hv190SV for encapsidation.[24]

The diseased phenotype was transmitted by inoculating protoplasts from the virus-free fungal isolate (isolate 408) with purified virions of both the Hv190S and 145S viruses. The frequency of infection and stability of the newly diseased colonies, however, were very low, and verification of transmission was based on virus detection by immune electron microscopy.[25] A molecular approach based on DNA-mediated transformation of *H. victoriae* with full length cDNA clones of viral dsRNAs is currently being pursued to verify the viral etiology of the diseased phenotype (see Section 9.2.5). Using such a molecular approach, it has been unequivocally established that viral dsRNA is the causal agent of hypovirulence in the chestnut blight fungus.[26,27]

9.2 HELMINTHOSPORIUM VICTORIAE 190S VIRUS

Helminthosporium victoriae 190S virus (Hv190SV) is a member of the genus *Totivirus* in the family *Totiviridae*. Other genera in the family are *Giardiavirus* and *Leishmaniavirus*, the members of which infect parasitic protozoa. The *Saccharomyces cerevisiae L-A virus* (ScV-L-A) is the type member of the family.[1] The complete nucleotide sequence of several totiviruses has been determined, and some of these viruses have been well characterized.[28] The Hv190S virus has been well characterized biochemically and at the molecular level.[23,29–34] Like other members of the family *Totiviridae*, the Hv190S virus has isometric particles, 40 nm in diameter, containing an undivided dsRNA genome (5.2 kbp in size), which is slightly larger than that of the yeast L-A virus (4.6 kbp).[1,3]

9.2.1 Genome Organization

The complete nucleotide sequence of the Hv190SV dsRNA genome (5,178 bp) has been determined and the sequence deposited in the GenBank database (accession

#U41345). Sequence analysis showed that Hv190SV dsRNA genome, like that of other totiviruses, is composed of two large overlapping open reading frames (ORFs) (Figure 9.1). The 5' proximal ORF (ORF 1) codes for the capsid protein (CP), whereas the 3' ORF (ORF 2), which is in the –1 frame relative to ORF 1, contains the consensus RNA-dependent RNA polymerase (RDRP) motifs.[32] Present evidence indicates that translation of ORF1 starts at the AUG at position 290. This initiation codon, however, resides in an unfavorable sequence context (UCCAUGU). Although the next in frame AUG is at position 503 and is in a strong context (GGCAUGG),

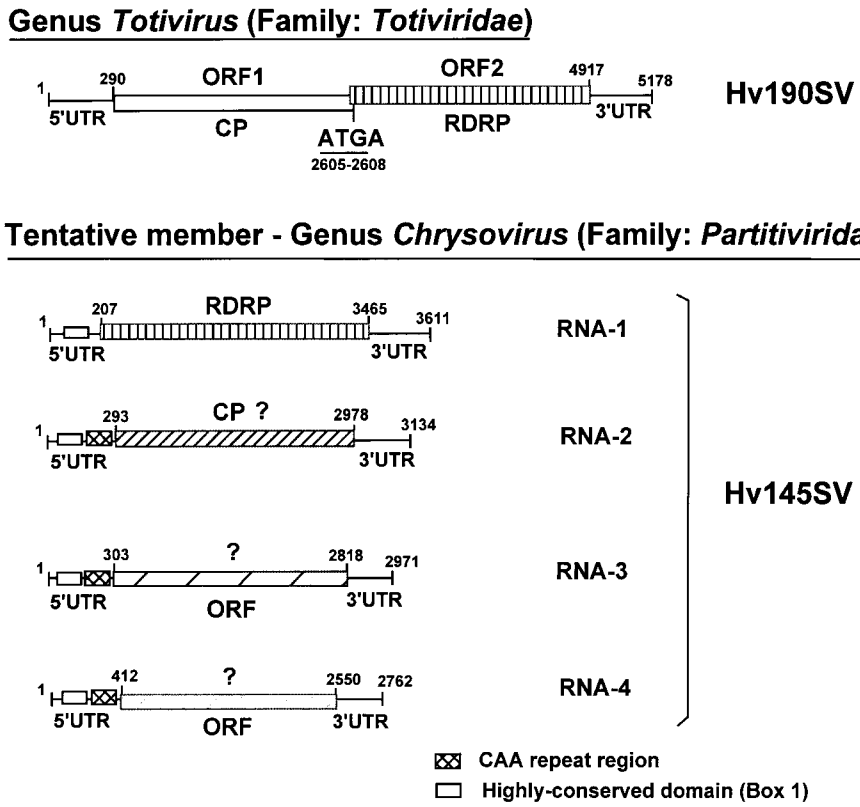

FIGURE 9.1 Genome organization of the totivirus *Helminthosporium victoriae 190S virus* (Hv190SV) dsRNA and of the chrysovirus-like Hv145S dsRNAs. Hv190SV: Two large overlapping open reading frames (ORFs) with the 5' proximal ORF encoding a capsid protein (CP) and the 3' ORF encoding an RNA-dependent RNA polymerase (RDRP). Note that the termination codon of the CP ORF overlaps the initiation codon (underlined) of the RDRP ORF in the tetranucleotide sequence AUGA. Hv145SV: The four dsRNAs associated with the 145SV particles have recently been completely sequenced. dsRNA 1 (3.6 kbp) contains a single, large ORF and encodes for the RDRP (125 kDa); dsRNA 2 potentially encodes for a single, large polypeptide of 100 kDa, presumably a coat protein; dsRNAs 3 and 4 are also both monocistronic and code for unidentified proteins. Sequences at the 5'- and 3' UTRs are highly conserved in all four Hv145S dsRNAs.

bacterial expression from the AUG at 503 resulted in a product that migrates significantly faster in SDS-PAGE gels than p88, the primary translation product of the CP ORF.

The 5' end of the positive strand of the dsRNA genome is uncapped and highly structured and contains a relatively long (289 nucleotides) 5' untranslated region (5' UTR) with two non-initiator AUGs. These structural features of the 5' UTR of Hv190SV positive sense RNA predict that the CP ORF (with its AUG present in suboptimal context) is translated via an internal ribosome entry site (IRES) mechanism.[32]

In addition to the UGA at position 2606–2608, the predicted termination codon of ORF1, there are two other out of frame UGAs in close proximity. It was therefore important to verify that the UGA at position 2606–2608 (Figure 9.2) is the stop codon for ORF1. For this purpose, site-directed mutagenesis of the predicted CP stop codon as well as the upstream UGA was carried out. Bacterial expression of the construct in which the upstream UGA was mutated yielded a product indistinguishable in size from wildtype p88. On the other hand, mutating the putative CP stop codon yielded a fusion product of estimated molecular mass of 97 kDa (Figure 9.2). These results verified that the stop codon at position 2606–2608 is indeed the termination codon for the CP ORF. Because mutating the CP stop codon did not result in the production of a CP-RDRP fusion protein, expression of RDRP as a CP-RDRP fusion protein by a read-through mechanism can be ruled out.[32]

The downstream ORF of Hv190V dsRNA genome, encoding the RDRP, is in a –1 frame with respect to the CP ORF and its translational start codon (nt positions 2605–2607) overlaps the stop codon for the upstream CP ORF (nt positions 2606–2608) in the tetranucleotide sequence 2605-AUGA-2608 (Figure 9.1). Hv190SV RDRP is detectable as a virion-associated minor component, and by analogy to the ScV-L-A, the type species of the genus *Totivirus*, it may be present in the virions as 1–2 molecules compared to 120 CP molecules.[32]

9.2.2 Genome Expression

9.2.2.1 Capsid Protein ORF

Although Hv190SV capsid is encoded by a single gene, it contains three closely related capsid polypeptides (CPs), p78, p83, and p88 (Figure 9.2).[29] Purified Hv190S virion preparations contain two types of particles, 190S-1 and 190S-2, that differ slightly in sedimentation rates and in capsid composition. The 190S-1 capsids contain p88 and p83, occurring in approximately equimolar amounts, and the 190S-2 capsids are composed of similar amounts of p88 and p78.[31] *In vivo* and *in vitro* phosphorylation studies indicated that p88 and p83 are phosphoproteins, whereas p78 is nonphosphorylated.[31] Results of *in vitro* translation studies with either the denatured Hv190SV dsRNA or the full length *in vitro* transcript of genomic dsRNA indicated that p88 is the major translation product.[29,30]

Expression studies in bacterial and eukaryotic systems have confirmed that p88 is the primary translation product of the CP ORF of Hv190SV and that it is cleaved at its C-terminal region to generate p78.[32,33] Because *in vivo* phosphorylation experiments using the Hv190SV-infected fungal host indicated that virion p78 is non-

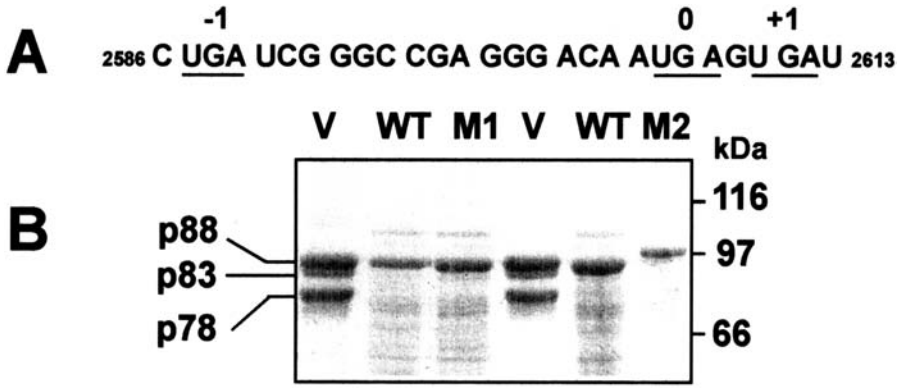

FIGURE 9.2 Expression of wildtype and mutagenized Hv190SV CP gene (ORF1) in *E. coli*. The nucleotide sequence (nt 2586-2613) flanking the predicted stop codon (UGA, 0) of ORF1 is shown in panel A; the presence of two additional UGAs (in the –1 and +1 frames relative to ORF1) is indicated by horizontal lines under the respective triplets. A Coomassie-stained SDS polyacrylamide gel of bacterially expressed wildtype and mutated CP is shown in panel B. Lanes V, purified Hv190S virions; lanes WT, bacterially-expressed products from wildtype CP construct; lanes M1 and M2, expressed products from constructs in which the UGA triplet in either the –1 frame or the 0 frame, respectively, were separately mutated to a sense codon. The positions of virion polypeptides p88, p83, and p78 are indicated to the left, and the positions of protein size standards are shown to the right. (From Huang, S., and Ghabrial, S.A., *Proc. Natl. Acad. Sci.* U.S.A. 93, 12541, 1996, with permission.)

phosphorylated, it was proposed that both the sites for phosphorylation and proteolytic cleavage reside in the C-terminal region of p88 and that proteolytic processing of p88 to yield p78 leads to dephosphorylation of the CP.

The nucleotide sequencing data predicts that ORF1 of Hv190SV dsRNA (2,319 bp) can code for a coat protein of 772 amino acid residues with calculated molecular mass of 81.2 kDa and pI of 5.85. The reasons why an estimate of 88 kDa was obtained by SDS-PAGE for the CP with predicted 81 kDa remain unknown. Interestingly, most estimates by SDS-PAGE of the CP size of ScV-L-A is about 88 kDa, even though its predicted molecular mass is 76 kDa.[35]

Phosphorylation of the Hv190SV CP is predicted to be mediated by a cellular kinase (see Section 9.4). Phosphorylation may serve to expand and modulate the properties and functions of the CP. 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.[36] The finding that the phosphorylated, but not the nonphosphorylated, capsid proteins are accessible to iodination in the intact virions suggests that they are conformationally different and thus may have specific roles in the transcription/replication of the viral dsRNA genome.[31] Capsid protein phosphorylation may modulate the switching of templates by the RDRP protein and 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.[31]

9.2.2.2 RNA-Dependent RNA Polymerase

In Western blots using an antiserum to bacterially expressed RDRP, the product of ORF 2 was identified as a separate virion-associated minor polypeptide with an *M*r value of 92,000.[32] The Hv190S virus thus differs from other totiviruses that only express their RDRP as a fusion protein (CP-RDRP) by fusing ORFs 1 and 2 via a translational frameshifting.[37] The overlap region (16 nt) between the two ORFs of Hv190SV dsRNA (Figure 9.1) is considerably smaller than that in the totiviruses that infect the yeast and the parasitic protozoa *Giardia lamblia* and *Leishmania brasiliensis* (71 to 130 nt). The overlap region of these totiviruses contains sufficient information (structures necessary for ribosomal frameshifting including a slippery site and a pseudoknot structure involving a predicted stem-loop structure) to promote fusion of ORF1 and ORF2 *in vivo*. The overlap region in Hv190SV dsRNA genome, on the other hand, lacks a heptamer slippery site, and a potential pseudoknot structure cannot be predicted from the secondary structure of the sequences flanking the 3'-terminal region of the CP gene. These observations suggest that expression of RDRP occurs by a mechanism different from translational frameshifting. The finding that the termination codon of the CP ORF (nucleotide position 2605-AUG-2607) overlaps with the predicted start codon for the RDRP ORF (2606-UGA-2608) in the sequence AUGA (Figure 9.1) suggests that RDRP is translated by an internal initiation mechanism. We have recently presented evidence that Hv190SV RDRP is separately expressed from its downstream ORF in dicistronic constructs by a coupled termination-reinitiation mechanism.[38]

The recent report that two totiviruses (SsRV1 and SsRV2) infecting the filamentous fungus, *Sphaeropsis sapinea*, are similar to Hv190SV in genome organization and predicted expression strategy is of considerable interest. Like Hv190SV, SsRV1 and SsRV2 have a short overlapping region between the two ORFs that lacks both a slippery site and a predicted pseudoknot.[39] Likewise, these two viruses may not synthesize CP-RDRP fusion proteins. The finding that totiviruses infecting filamentous fungi differ from those infecting the yeast in the way they express their RDRP has interesting implications in regard to phylogeny and the nature of the cellular factors that regulate totivirus replication/transcription.

9.2.3 Double-Stranded RNA Replication

Information on the replication cycle of Hv190SV dsRNA has been derived mainly from *in vitro* studies of virion-associated RNA polymerase activity and the isolation of particles representing different stages in the replication cycle.[30,31] In *in vitro* reactions, the RNA polymerase activity associated with Hv190S virions catalyzes end-to-end transcription of dsRNA by a conservative mechanism to produce a full-length transcript, which is released from the particles.[30] Purified Hv190S virions contain a less dense class of particles which package (+) strand RNA only. In *in vitro* reactions, these particles exhibit a replicase activity that catalyzes the synthesis of (–) strand RNA to form dsRNA.[40]

A proposed life cycle of Hv190SV is depicted in Figure 9.3. Three host-encoded proteins, a protein kinase, a protease, and an RNA-binding protein (Hv-p68; see Section 9.4) are proposed to play a role in dsRNA replication and packaging. The protein kinase and protease have been shown to be involved in posttranslational modification of the CP.[31,33,34] As discussed before, phosphorylation of CP may regulate dsRNA transcription/replication. Proteolytic processing and cleavage of a C-terminal peptide leads to dephosphorylation and the conversion of p88 to p78; this may play a role in the release of the (+) strand RNA transcripts from virions (Figure 9.3). The RNA-binding cellular protein Hv-p68 (see Section 9.4) is proposed to mediate the binding of RDRP to its RNA template and is predicted to be packaged along with RDRP and the RNA template. It is expected that the virion-associated transcriptase and replicase activities of the Hv190S virus to be catalyzed by the same RDRP protein.

9.2.4 Capsid Assembly

Expression of the CP ORF in insect cells was found to generate both p78 and p88, which assembled into virus-like particles morphologically indistinguishable from the empty capsids often observed in purified Hv190S virions.[23,33] Furthermore, it has been demonstrated that the N-terminal, but not C-terminal, CP deletions are incompetent for assembly.[33] The fact that the bacterially expressed unmodified p88 is competent for assembly into virus-like particles indicates that neither phosphorylation nor proteolytic processing of CP is required for capsid assembly.[34]

Because of the inability to obtain synchronous infections with mycoviruses, it is not known whether posttranslational modification of CP takes place prior to or after capsid assembly. For clarity, we propose that the capsids are initially assembled from p88 (Figure 9.3), even though virus capsids that are totally composed of only p88 have never been isolated from infected fungal isolates.[40]

9.2.5 A Transformation System for *H. Victoriae*

A transformation vector for *H. victoriae* based on the hygromycin B resistance marker has recently been constructed and used to transform *H. victoriae* virus-free isolates with a full-length cDNA clone of Hv190SV. Expression of Hv190SV genes is controlled by *Cochliobolus heterostrophus GPD1* promoter and *Aspergillus nidulans trpC* terminator signals. The vector contains an engineered hygromycin resistance gene under the control of *A.* nidulans *trpC* promoter. The hygromycin-resistant transformants expressed the Hv190SV CP, as determined by Western blotting analysis. Whether viral dsRNA is generated and replicated in those transformants has yet to be determined.[40]

9.3 THE HELMINTHOSPORIUM VICTORIAE 145S VIRUS

Unlike the well characterized Hv190SV, the Hv145SV has only been subjected to limited biochemical characterization.[23] The Hv145S virions are 40 nm in diameter

and are indistinguishable in size or appearance from the Hv190S virions. Four dsRNAs (2.7, 2.9, 3.1, and 3.6 kbp in size) are associated with the Hv145S virus (Figure 9.4). Because of the similarity in size of dsRNA segments between the Hv145S and the chrysovirus *Penicillium chrysogenum virus* (PcV), type member of the genus *Chrysovirus* in the family *Partitiviridae*, the Hv145S virus was tentatively classified as a member of the genus *Chrysovirus*.[2,4] Results of Northern hybridization assays using cloned cDNA probes to the 190S and 145S dsRNAs indicated the lack of sequence homology between the Hv145S dsRNAs and those of the Hv190S and PcV dsRNAs (Figure 9.4). Furthermore, using cloned cDNA probes for the individual 145S dsRNA showed that each of the four 145S dsRNAs has unique sequences.

Unlike the Hv190S virus, the capsid of the Hv145SV is not well characterized. Protein and dsRNA analyses of highly purified 145S virions (pooled from several preparations and subjected to two-three cycles of sucrose density gradients) revealed that they contained the four 145S dsRNAs, and their capsids were essentially those typical of the 190S virions (p78, p83, and p88). In addition, two polypeptides of *M*r 110 and 135×10^3 were also detected as minor components. These results suggest that: (a) the 145S dsRNAs may be packaged in capsids encoded by the Hv190S virus (consistent with the putative satellite nature of the Hv145SV), or (b) the capsids of the 145SV comprise the 110/135 kDa polypeptides but the capsid are unstable and dissociate during purification.[40]

Molecular cloning and sequencing of all four dsRNAs of the Hv145S virus have recently been completed. The four Hv145S dsRNAs share common, highly conserved domains at their 5' and 3' termini, suggesting all four dsRNAs may be replicated by the same RDRP (Figure 9.4).

9.3.1 Genome Organization

The genome organization and distinctive nucleotide sequence features of the 145SV dsRNAs are depicted in Figure 9.1.

9.3.1.1 The 5' and 3' Untranslated Regions

The 145SV dsRNAs are not capped at their 5'-ends. The 5' UTRs are relatively long, between 200 and 400 nts in length, and their sequences have the potential to form extensive secondary structures. Direct comparison of the nucleotide sequences have revealed that, in addition to the highly conserved 5' and 3' termini, there are regions of high sequence similarity within the 5' UTRs and 3' UTRs among all four dsRNAs (Figure 9.1). A highly conserved 75-nt region with almost 90% identity is present in the 5' UTR of all four dsRNAs (designated "Box 1"). A second region of strong sequence similarity is present in dsRNAs 2, 3, and 4 and is positioned immediately downstream of the highly conserved Box 1. This region consists of a stretch of 30–35 nucleotides (with sequence similarity above 80%) and is composed of a reiteration of the sequence "CAA," which depicts a strong resemblance to the $(CAA)_n$ repeats present in the 5'- UTRs of tobamoviruses. The poly (CAA) region of tobamoviruses has been reported to function as translational enhancers.[41–43] The 5' UTR of dsRNA 1 contains only a short 10- to 12-base stretch of the "CAA" sequence,

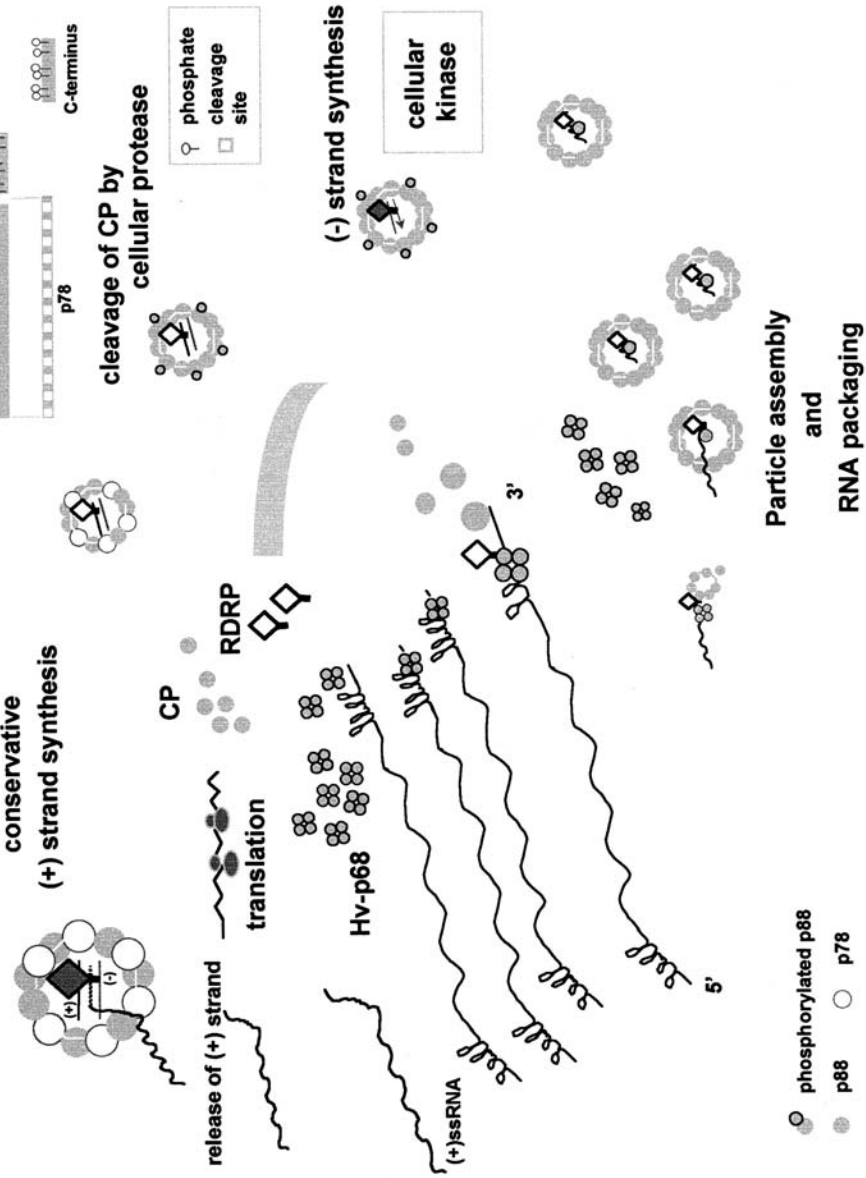
conservative
(+) strand synthesis
(+)
(-)
release of (+) strand
translation
CP
RDRP
Hv-p68
(+)ssRNA
cleavage of CP by
cellular protease
p88
p78
C-terminus
phosphate
cleavage
site
(-) strand synthesis
cellular
kinase
3'
5'
Particle assembly
and
RNA packaging
phosphorylated p88
p88
p78

FIGURE 9.3 A proposed model for replication of Hv190SV dsRNA. Mature virions contain a single dsRNA molecule and their capsids are composed entirely or primarily of the capsid protein (CP) p88. Virions representing different stages of the virus life cycle can be purified from the infected fungal host, *Helminthosporium victoriae*, including the well characterized 190S-1 and 190S-2 virions.[31] These two types of virions differ in sedimentation coefficient, phosphorylation state, and CP composition; 190S-1 capsids contain p88 and p83, whereas the 190S-2 capsids contain p88 and p78 (p88 is the primary translation product of the CP gene; p83 and p78 represent posttranslational proteolytic processing products of p88 at the C-terminus). p88 and p83 are phosphorylated, whereas p78 is nonphosphorylated. Transcription occurs conservatively, and the newly synthesized dicistronic (+) strand RNA transcript is released from the virions and translated using the cellular protein-synthesizing machinery. The released (+) strand RNA is translated into CP and RDRP and packaged in capsids assembled from the primary translation product p88; the cellular RNA-binding protein Hv-p68 (see Section 9.4.1) is proposed to mediate the binding of RDRP to the (+) strand RNA template. Synthesis of (–) strand RNA occurs on the (+) strand RNA template inside the virion. Phosphorylation of CP is catalyzed by a host kinase and is proposed to play a regulatory role in transcription/replication. A host-encoded protease catalyzes proteolytic processing of phosphorylated p88; this occurs in two steps leading first to p83 (the generation of the 190S-1 virions) and then to p78 (190S-2 virions). p83, an intermediate between p88 and p78, is not included in the diagram for clarity. It is not known whether p88 is phosphorylated prior to or following assembly. Synthesis of (–) strand RNA occurs on the (+) strand RNA template inside the virion.

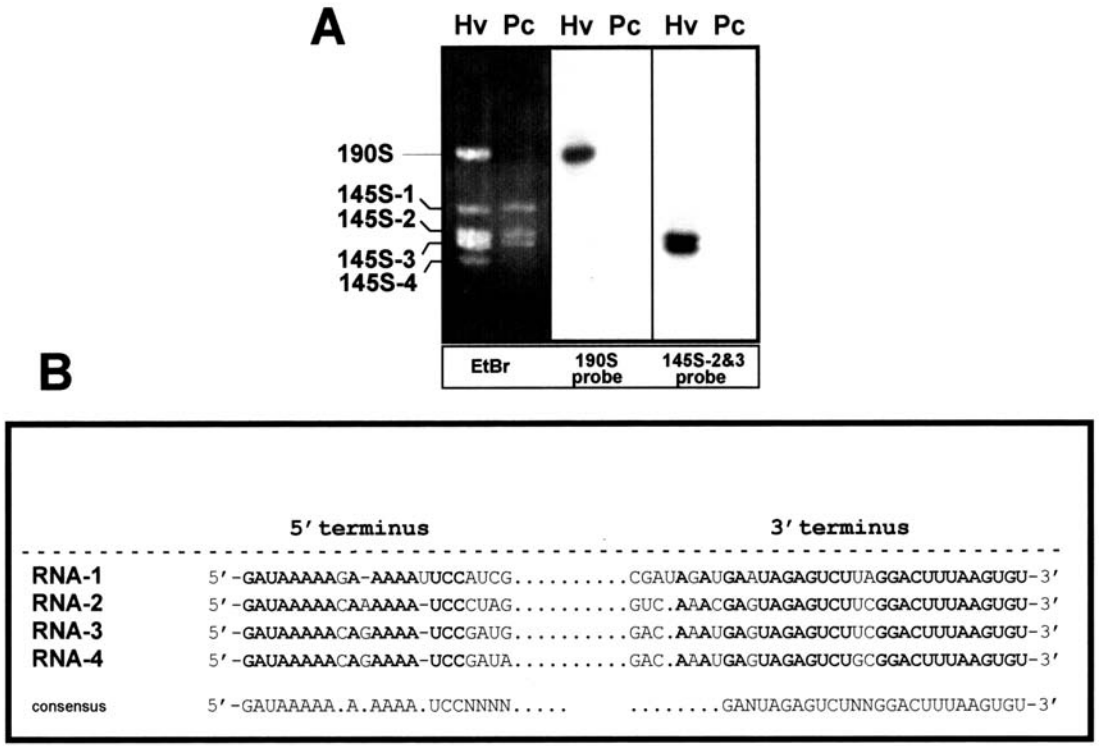

FIGURE 9.4 (Panel A). Northern hybridization analysis of the Hv190SV and Hv145 dsRNAs using cloned cDNA probes. The Hv145SV dsRNA segments each contain unique sequences and are distinct from the dsRNAs from Hv190SV and the chrysovirus *Penicillium chrysogenum virus* (Pc). (Panel B). Conserved sequences at the 5' and 3' termini of all four 145SV dsRNAs.

and it may therefore represent an "imperfect CAA" repeat. Whether the Hv145SV "CAA" repeats have a similar function as the translational regulatory elements of tobamoviruses has yet to be determined. It is of interest to note that translational enhancement in *Tobacco mosaic virus* (TMV) has been proposed to be mediated by a cellular protein that binds to the poly (CAA) region in TMV RNA.[42] A highly conserved sequence region with similarity above 90% over a stretch of 65 nts is also present at the 3' UTR of all four 145SV dsRNAs.[24]

9.3.1.2 Coding Regions

Sequence analysis indicated that all four dsRNAs are monocistronic, each containing a single, large ORF (Figure 9.1). Database homology searches with both nucleotide and deduced amino-acid sequences for dsRNAs 2, 3, and 4 revealed no significant similarities to known sequences. dsRNA 2, however, is predicted to code for a CP, as is characteristic of members of the family *Partitiviridae*.[44] The single ORF present in RNA 1 is predicted to code for a polypeptide of 125,000 kDa. The deduced amino-acid sequence revealed the presence of the eight highly conserved motifs diagnostic of the RDRPs of double-stranded and positive-strand RNA viruses (Table 9.1).[24,28,44] Sequence comparison analysis indicate that the 145SV RDRP is more closely related to members of the genus *Totivirus* than to those of the genus *Partitivirus*. Paired sequence alignments using the GAP program indicate that Hv145S RDRP shares significant sequence similarity to the RDRPs of the totivirus *Ustilago maydis* virus H1 (UmVH1) (32% similarity, 23% identity) and to that of the unclassified virus La France isometric virus (synonym: Agaricus bisporus virus 1; 36% similarity, 25% identity)[24,50]. GAP values obtained with RDRPs from viruses in the families *Partitiviridae* and *Totiviridae* (except for UmVH1) are not statistically significant.[24]

9.4 VIRUS-HOST INTERACTIONS

It has recently become increasingly evident that cellular factors play important roles in the transcription and replication of RNA viruses.[51] The genomes of fungal viruses in the families *Totiviridae and Partitiviridae* are of very limited sizes; probably the minimal required to be designated as true viruses. They comprise two genes that encode a capsid protein (CP) and an RNA-dependent RNA polymerase (RDRP). The CP and RDRP genes are either present on the same dsRNA molecule (totiviruses) or on separate dsRNA segments (partitiviruses). It is thus not surprising that these viruses subvert host proteins for their own use. We have already reported that host enzymes (a protein kinase and a protease) are predicted to be involved in post-translational modification of the Hv190SV CP.[31,33,34]

9.4.1 The Cellular Protein HV-P68

Viral dsRNAs, mostly that of the Hv145SV, has recently been shown to co-purify with a top component that is resolved near the meniscus when purified virions preparations from diseased isolates are subjected to sucrose density gradient centrifugation (Figure 9.5). As previously described, purified virus preparations from *H. victoriae* are resolved into several sedimenting components including the

TABLE 9.1
Comparison among the Conserved Motifs of RDRPs of dsRNA Mycoviruses including the putative RDRP encoded by Hv145SV dsRNA1.[a]

	1[b]	2	3	4	5
SsRV1	LLGRA (61)	WCVNGSQND (47)	KL-EHG-KTRAIFACDTRSY (47)	LDFDDFNSHHS (45)	TLPSGHRGTTIVNSVLNAAYI (14)
Hv190SV	LQGRY (61)	WCVNGSQNA (42)	KL-ENG-KDRAIFACDTRSY (47)	LDYDNFNSQHS (45)	TLMSGHRATTFTNSVLNAAYI (14)
SsRV2	LQGRA (64)	WAVNGSQSG (46)	KL-EHG-KTRAIFACDTLNY (47)	LDYDDFNSHHS (46)	TLMSGRRGTTYISSVLNEVYL (14)
UmVH1	LYGRG (66)	WLVSGSSAG (55)	KLNETGGKARAIYGVTLWHY (47)	YDYPDFNSMHT (64)	GLYSGDRDTTLINTLLNIAYA (20)
Hv145SV	LLGRR (73)	WMTKGSLVS (56)	KLNENGHKDRVLLPGGLLHY (44)	YDWANFNVQHS (49)	GLYSGWRGTTWDNTVLNGCYM (20)
	* **	* ::**:	** * * * *::::::: *	:*:::**: *:	:* ** *:** :::** :*
FpV1			SDRDGILKQRPVYAVDDLFL (47)	IDWSGFDQRLP (72)	GVPSGMLNTQFLDSFGNLFLL (19)
RhsV 717			SKRDGTLKVRPVYAVDELFL (47)	IDWSGYDQRLP (71)	GVPSGMLLTQFLDSFGNLYLI (19)
Ah2HV			SKRD-NLKVRPVYNAPMIYI (47)	IDWSRFDHLAP (92)	GVPSGILMTQFIDSFVNLTIL (19)
BcV3			ADLREKTKVRGVWGRAFHYI (48)	LDWSSFDSSVT (50)	GIPSGSYYTSIVGSVVNRLRI (15)
FsV1			SPRD-DPKTRLAWIYPSEML (47)	LDFSSFDTKVP (61)	GVPSGSWWTQLVDSVVNWILV (14)
			: :: :*:*::: :	:*:* :* :	*:*** : *:: :*::*: :

	6		7		8
SsRV1	LHTGDDVYIRA	(18)	RINPAKQSVGFGTGEFLRM	(8)	GYLARSVASFVSGNW
Hv190SV	LHAGDDVYLRL	(18)	RMNPTKQSIGYTGAEFLRL	(8)	GYLCRAIASLVSGSW
SsRV2	IHVGDDVYLGV	(18)	RMNPMKQSVGHTSTEFLRL	(8)	GYLARAVASTISGNW
UmVH1	LCHGDDIITVH	(18)	KGQESKLMIDHKHHEYLRI	(9)	GCLARCVATYVNGNW
Hv145SV	DQGGDDVDQEF	(18)	EATKSKQMIG-RNSEFFRV	(8)	ASPVRGLATFVAGNW
	: :***::		: :: *:::: *::*		::: * :*: ::*:*
FpV1	FIMGDDNSAFT	(26)	SKTKSIITTLRHKIETLSY	(8)	RPIGKLVAQLCFPER
RhsV 717	FIMGDDNSIFT	(26)	SKTKSVITTLRSKIETLSY	(8)	RDVEKLIAQLVYPEH
Ah2HV	FIMGDDNVIFT	(26)	NISKSAVTSIRRKIEVLGY	(8)	RSISKLVGQLAYPER
BcV3	YTQGDDSLIGE	(20)	NPDKTEYSTDPGYVTFLGR	(8)	RSLDKCLRLLMFPEY
FsV1	RVLGDDS-AFM	(21)	SDEKSISVEDATELKLLGV	(8)	RETEEWFKLALYPEG
	:::***: :::		: *: :: : ::: *::		* :: :: :**

[a] Blocks 1 through 8 correspond to regions in the deduced amino acid sequence of Hv145SV dsRNA 1 with high similarity to the eight conserved motifs of RNA polymerases. Amino-acid positions corresponding to blocks 1 and 2 are not well defined for the partitivirus RDRPs, and therefore these sequence blocks are not presented in the table.

[b] Multiple sequence alignments were obtained using Clustal W with RDRP amino acid sequences of totiviruses (top): ScRV1 and *ScRV2 (Sphaeropsis sapinea RNA virus 1* and *2*, respectively),[39] Hv190SV (*H. victoriae 190S virus*),[32] UmV-H1 (*Ustilago maydis virus H1*),[44] and, partitiviruses (bottom): FsV1 (*Fusarium solani virus 1*),[45] FpV1 (*Fusarium poae virus 1*),[46] RhsV 717 (*Rhizoctonia solani virus 717*),[47] and Ah2HV (*Atkinsonella hypoxylon 2H*).[48] BCV3 (*Beet cryptic virus 3*),[49] an alphacryptovirus (*Partitiviridae*) infecting a plant species is also included for the comparison.

Asterisks indicate identical residues; colons indicate a plurality of at least three identical residues within subgroups. Numbers in parenthesis correspond to number of amino-acid residues separating the blocks.

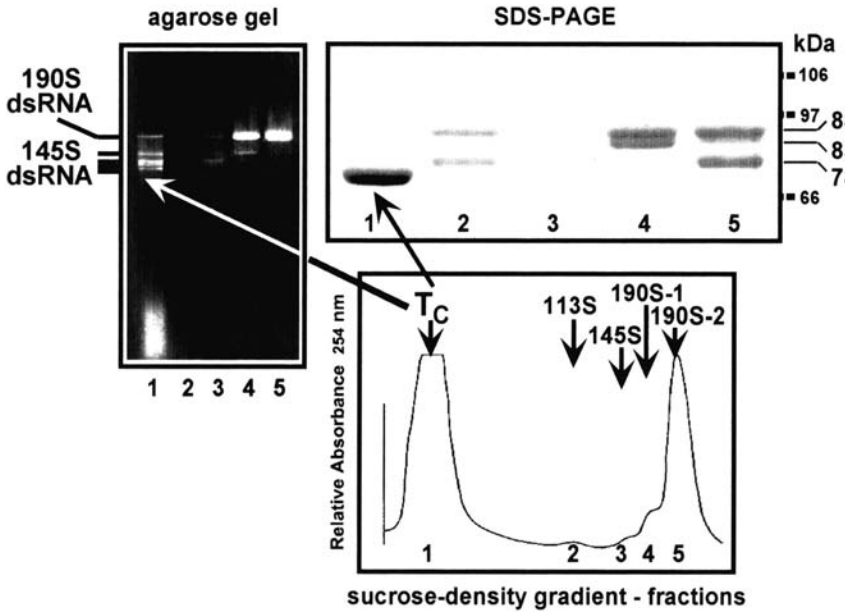

FIGURE 9.5 Sucrose-density gradient profile of a purified virus preparation from a diseased isolate of *H. victoriae.* Gradient fractions 1–5 were analyzed for protein (SDS-PAGE) and dsRNA contents (agarose-gel electrophoresis). (From Soldevila, A., Havens, W.M., Ghabrial, S.A., *Virology* 272, 183, 2000, with permission.)

Hv190S-1 and Hv190S-2 components (Figure 9.5, fractions 4 and 5, respectively), the Hv145SV and the 113S component containing empty capsids (Figure 9.4, fractions 3 and 2, respectively). SDS-PAGE analysis of the top component revealed a single major protein of *M*r 68,000, denoted Hv-p68 (Figure 9.5). RNA analysis by agarose gel electrophoresis showed that the top component contained predominantly the 145S dsRNAs (Figure 9.5). Western blotting analysis using polyclonal antibodies to Hv-p68 indicated that Hv-p68 is overproduced in virus-infected isolates compared to virus-free ones.[52]

The gene encoding Hv-p68 has been isolated from a cDNA library generated in lambda phage using mRNA from a virus-infected fungal isolate. The complete nucleotide and deduced amino-acid sequences of Hv-p68 have been determined.[53] Sequence comparison analysis revealed that Hv-p68 belongs to the large family of flavin-adenine dinucleotide (FAD)-dependent glucose-methanol-choline (GMC) oxidoreductases (Figure 9.6) with 67 to 70% sequence identity to the alcohol oxidases of methylotrophic yeasts.[53]

A molecular mass estimate of 550 kDa has been obtained for the native-size of Hv-p68 using gradient-purified Hv-p68 and high performance size-exclusion chromatography. Based on a molecular-mass of 68 kDa, as determined by SDS-PAGE, the native-size estimate of 550 kDa suggested an octameric subunit conformation for Hvp68.[52] The oligomeric arrangement of Hv-p68 has been confirmed by electron microscopic examination (Figure 9.7, panel 1), which revealed the presence of octad

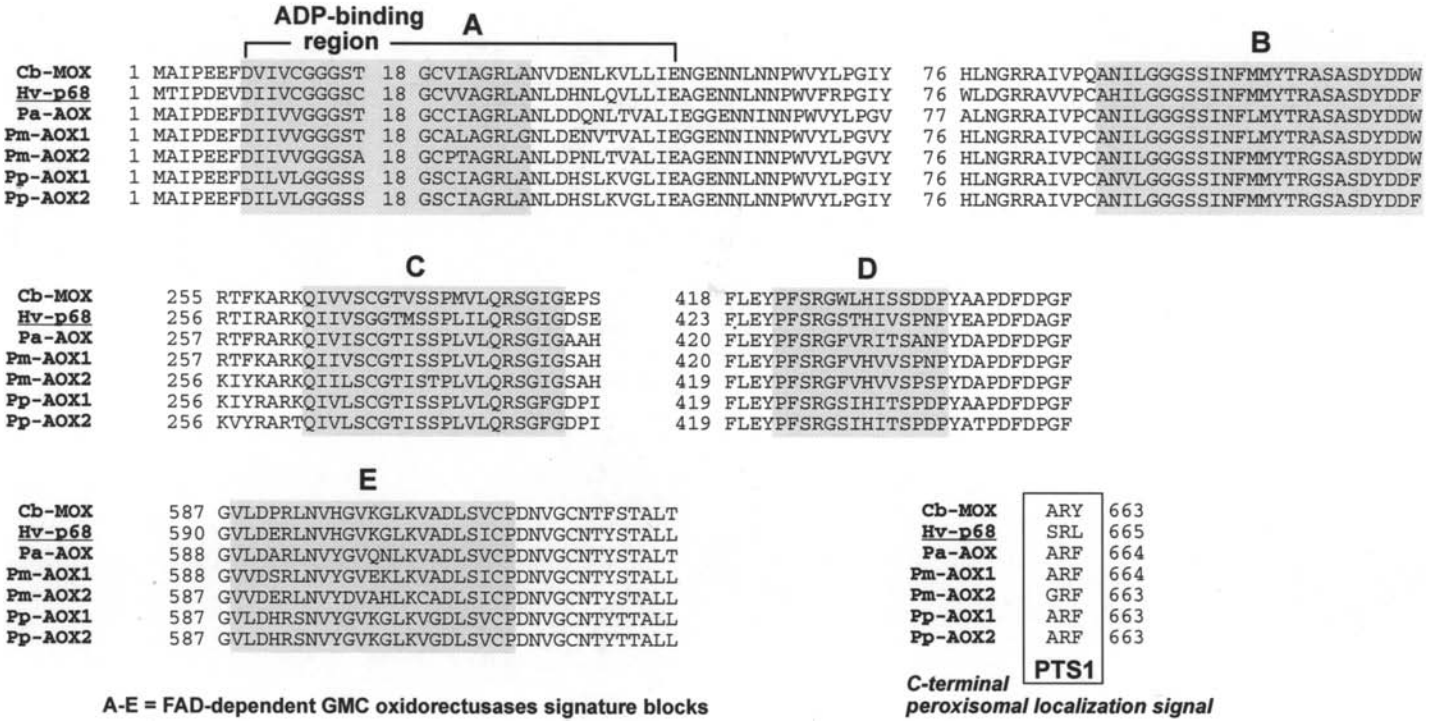

FIGURE 9.6 Amino acid sequence comparison between Hv-p68 and alcohol oxidases of methylotrophic yeasts. Multiple sequence alignments, generated with Clustal W, showed that Hv-p68 contains the conserved signature blocks (blocks A-E; shaded areas) characteristic of FAD-dependent GMC oxidoreductases. A C-terminal peroxisomal targeting signal (PTS1) is present in all alcohol oxidases and Hv-p68. Alcohol oxidases (MOX or AOX) from Cb = *Candida boidinii,* Pa = *Pichia angusta,* Pm = *Pichia methanolica,* and Pp = *Pichia pastoris*. (From Soldevila, A.I., and Ghabrial, S.A., *JBC* 276, 4652, 2001, with permission.)

aggregates of approximately 15–20 nm in diameter comprising two tetragons face to face. The combined results of SDS-PAGE, size exclusion chromatography, and electron microscopy strongly suggest that Hv-p68, like some other oxidases, is present *in vivo* as an oligomeric protein consisting of eight identical subunits.[52]

Expression of the Hv-p68 gene at the transcriptional level has been examined. Elevated levels (10- to 20-fold) of the Hv-p68 transcript were found in the virus-infected fungal isolates compared to the virus-free ones (Figure 9.7, panel 2). This finding is in agreement with the higher levels of protein detected by ELISA and Western analysis in the virus-infected isolate.[52,53] Unlike the alcohol oxidases from methylotrophic yeasts, the Hv-p68 purified from fungal extract shows low levels of methanol oxidizing activity. Furthermore, the levels of Hv-p68 transcripts are not significantly different in methanol-supplemented versus glucose-supplemented cultures (Figure 9.7, panel 2) indicating that Hv-p68 transcription is neither inducible by methanol nor repressed by glucose. All together, the data suggest that the Hv-p68 promoter may not be regulated in the same fashion as the promoters for the alcohol oxidases of yeast and that the Hv-p68 promoter is up-regulated during the course of viral infection of *H. victoriae*.

Hv-p68 has been examined for RNA-binding activity by gel retardation and Northwestern analysis. When the ^{32}P end-labeled 190S and 145S dsRNAs were incubated with increasing amounts of Hv-p68 followed by agarose gel electrophoresis, a band of free dsRNA and a band of retarded dsRNA (RNA-protein complex) were detected at the lower protein concentrations. However, at higher protein concentrations, only the band of retarded dsRNA was observed (Figure 9.7, panel 3).[52] DsRNA, ssRNA, and yeast t-RNA in molar excess amounts were tested as competitors in binding reactions. As shown in Figure 9.7, panel 3, a 5- to 10-fold molar excess of the unlabeled 190S/145S dsRNAs or the unlabeled ssRNA from the comovirus *Bean pod mottle virus* ssRNA, when added to the standard binding reaction, completely abolished binding to the probe. Yeast t-RNA at 250-fold molar excess only partially competed with binding to the dsRNA probe. Retardation of the radiolabel is completely attributable to the binding of the probe to Hv-p68 and not to binding of unincorporated radiolabeled nucleotides, since both the free and retarded radiolabeled dsRNA are not detected when RNase A is added to parallel binding reactions (Figure 9.7, panel 3).[52]

The RNA-binding domain of Hv-p68 has been mapped the to the N-terminus of the Hv-p68 protein (ADP-binding domain) using Northwestern analysis and deletion mutants of bacterially expressed Hv-p68 (Figure 9.7, panel 4). Southern analysis of genomic DNA from several species of the genus *Cochliobolus* (anamorph, *Helminthosporium*) indicated that a single copy of the *Hv-p68* gene is present in all species of the genus *Cochliobolus* examined. The *Hv-p68* gene, however, was not detected in *Penicillium chrysogenum* or in two non-methylotrophic yeasts, *S. cerevisiae* and *S. pombe*.[53]

9.5 CONCLUDING REMARKS

Considerable progress has recently been made in the molecular characterization of the dsRNA genomes of the viruses that infect *H. victoriae* and in elucidating their

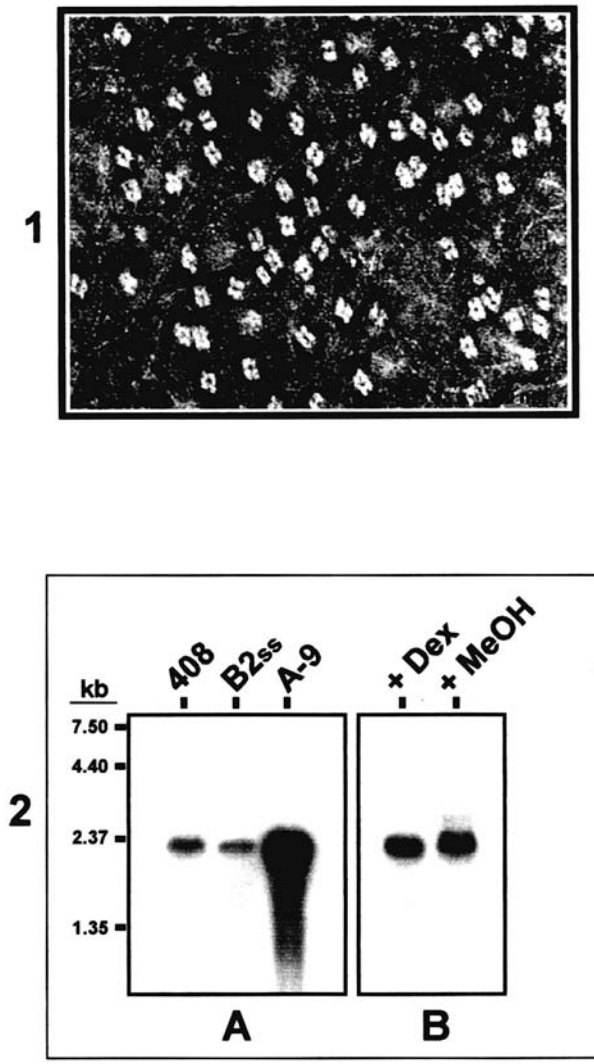

FIGURE 9.7 Properties of the cellular protein Hv-p68. Panel 1: The multimeric conformation of Hv-p68 is demonstrated by electron microscopy of gradient-purified protein. Octad aggregates comprising two tetragons face to face are visible in the electron micrograph. Panel 2: Expression of Hv-p68 transcripts. (A) Northern blot analysis of total RNA from virus-infected and noninfected isolates showing significantly higher levels of the Hv-p68 transcript in the virus-infected isolate A-9 (10- to 20-fold) than in noninfected isolates (408 and B2ss). (B) Expression level of Hv-p68 transcript in cultures supplemented with dextrose (Dex) was similar to that in cultures supplemented with methanol (MeOH) as the sole carbon source. *(continues)*

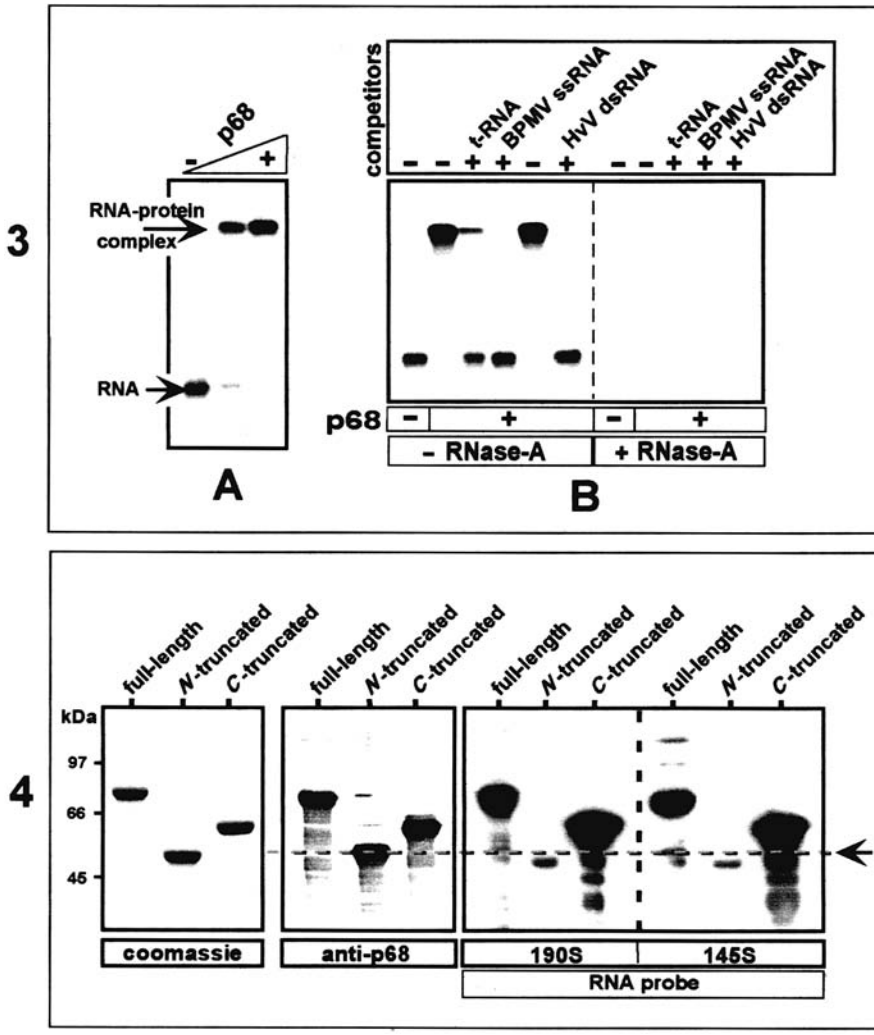

FIGURE 9.7 *(continued)* Properties of the cellular protein Hv-p68. Panel 3: Gel-retardation assay for analysis of RNA-binding activity of purified Hv-p68. (A) In the presence of Hv-p68, the radiolabeled dsRNAs were shifted to the slower-migrating protein-RNA complexes. (B) Specificity of binding of Hv-p68 in the presence of competitors was tested by adding excess molar amounts of unlabeled viral dsRNA, ssRNA and yeast t-RNA to the binding mixture. DsRNA and ssRNA completely displaced binding to the radiolabeled dsRNA probe, whereas yeast t-RNA partially inhibited binding. Panel 4: Northwestern analysis of binding activities of bacterially-expressed full-length Hv-p68 in comparison with N-terminal or C-terminal deletions. Similar RNA binding was observed for either the full-length or a C-terminal deletion of Hv-p68. The N-terminal truncated Hv-p68, however, did not show any detectable binding to the radiolabeled probes. Arrow and dotted line across autoradiographs indicate position of the N-terminal truncated Hv-p68. (From Soldevila, A.I., Havens, W.M., and Ghabrial, S.A., *Virology* 272, 183, 2000; Soldevila, A.I., and Ghabrial, S.A., *JBC* 276, 4652, 2000, with permission.)

genome organization and expression strategy. With the availability of full-length cDNA clones of the viral dsRNAs and of a DNA-mediated transformation system for *H. victoriae*, it is now possible to provide unequivocal evidence for the viral etiology of the diseased phenotype in virus-infected *H. victoriae*. This is particularly important in view of the fact that the vast majority of mycoviruses have been reported to be avirulent and have no deleterious effects on their hosts.[5]

Our studies on the *H. victoriae*-virus system led to the discovery of a novel cellular oxidase that copurifies with viral dsRNAs and exhibits RNA-binding and phosphotransferase/kinase activities.[40] Cellular proteins that bind to viral RNA may serve as components of RDRP or may serve to bring various regions of a viral RNA template together to form transcription or replication complexes.[51] It is generally accepted that protein phosphorylation provides a now-classical mechanism for signal transduction in eukaryotic cells. Thus, proteins (like Hv-p68) with diverse activities including phosphotransferase/kinase activities may fulfill a fundamental role in normal cell function. Deviations in the function of such proteins (e.g., overexpression due to virus infection) may provide intrinsic mechanisms underlying cell pathologies.

Investigations of cellular proteins (e.g., Hv-p68) that may play a functional/regulatory role in virus life cycle could provide us with targets that could be used to counteract fungal invasion and reveal ways in which continued growth and development of the pathogenic fungus could be effectively blocked. For example, should we demonstrate that overexpression of Hv-p68 in virus-free fungal isolates induces the diseased phenotype in the absence of virus infection, we may then investigate the effects of certain extracellular stimuli that may activate the transcription of the Hv-p68 gene (or homologues in other fungi). Although we do know the substrate for the oxidase activity of Hv-p68, the structurally similar alcohol oxidases from methylotrophic yeasts and filamentous fungi oxidize alcohols (aliphatic or aromatic) irreversibly to aldehydes that are toxic. A buildup of such toxic intermediates when Hv-p68 is overexpressed in virus-infected isolates may lead to the lytic/diseased phenotype. The *H. victoriae*-virus system may thus serve as a model system for investigating the molecular basis of disease in a plant pathogenic fungus. The information gained from such a study may be applicable to economically important plant pathogenic fungi. At present, 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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10 Unraveling the Viral Complex Associated with La France Disease of the Cultivated Mushroom, *Agaricus bisporus*

C.P. Romaine and M.M. Goodin

CONTENTS

10.1 HISTORICAL PERSPECTIVE

In 1962, a landmark paper published in *Nature* (London) describing virus-like particles (VLPs) in diseased mushrooms,[1] *Agaricus bisporus*, created a new branch of science called mycovirology (fungal virology). Besides setting a precedent for the previously unconsidered notion that a mere fungus could be infected by a virus, it established a new line of research to elucidate the etiologic agent of a devastating malady affecting the mushroom industry. This disorder, which is most commonly referred to as La France disease, was first reported in 1950 by Sinden and Hauser[2]

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following a severe episode on the La France mushroom operation located in southeastern Pennsylvania. For more than a decade, the cause of La France disease baffled scientists, who had searched endlessly for the presumptive causal bacterium, fungus, or nematode, but to no avail.

The prospect of a virus being the underlying agent of a malady of commercial mushrooms created a new and exciting dimension for scientific exploration. Scientists were inspired to investigate other fungi for the relevancy of viral infection. The outcome of this exploration, which continues today, is an appreciation for how widely viruses are distributed throughout the realm of fungi, and how their interaction with the host, although often highly productive in terms of viral replication, seldom culminates in an overt pathology.[3,4] Yet, the biological significance of viral infection has been compellingly shown for a number of fungi but probably remains grossly under appreciated within the fungal kingdom as a whole. Double-stranded RNA (dsRNA) genome viruses, which represent the vast majority of those infecting fungi and, more broadly, dsRNA genetic elements or sequences, are now known to mediate a diversity of biological responses. Phenomena engendered by dsRNA molecules range from the interferon-inducing factor in *Penicillium stoloniferum*, to the killer trait in *Saccharomyces cerevisiae* and *Ustilago maydis*, hypovirulence in *Cryphonectria parasitica* and *Ophiostoma novo-ulmi,* expression of virulence in *Rhizoctonia solani* and *Helminthosporium victoriae*, cytoplasmically-inherited male sterility in French bean, and gene silencing (i.e., posttranslational gene silencing, RNAi, cosuppression, quelling) (refer to the other chapters in this book).

During the 1970s, La France disease occurred at epidemic proportions in North America and Europe and was a major factor limiting the commercial production of mushrooms.[5–7] Its staggering economic impact created an urgent need to identify the causal virus and pinpoint the sources of infection for effective disease intervention. However, the generally innocuous nature of viruses emerging from the investigation of other fungi precipitated controversy centered on the validity of the purported viral nature of La France disease.[8] This concern seemed warranted based on electron microscopic evidence supporting the prevalence of apparently the same viruses in both healthy and diseased tissues.[9,10] Furthermore, speculation was heightened by the irreproducibility of studies claiming disease induction by cell-free transmission of the VLPs.[5,8]

The application of dsRNA analysis[11,12] as an investigative tool was a major contributing factor in advancing a viral etiology for La France disease. This method exploited the fact that most fungal viruses have dsRNA genomes and that each virus will have a characteristic dsRNA profile composed of different numbers and sizes of segments. DsRNA analysis succeeded where electron microscopy failed at discriminating between morphologically similar, but genetically distinct, viruses and between *bona fide* viruses and normal cellular components. Clearly, dsRNA analysis afforded a level of resolution and molecular authentication of viral infection unparalleled by electron microscopy.

In 1976, Marino et al.[13] were the first to disclose the presence of numerous dsRNA molecules in a diseased isolate of *A. bisporus*. The dsRNA isolation technique used by this group was too burdensome for large-scale etiological studies. However, an abbreviated protocol[14] permitted the analysis of large populations of

mushrooms for the purpose of exploring the prevalence and diversity of dsRNA viruses in *A. bisporus*. Surveys carried out during the 1980–90s on cultivated mushrooms in the U.S. and Europe revealed the coincidence of a specific set of dsRNA molecules and La France disease.[6,15–20] This highly conserved dsRNA pattern was distinct from the one associated with healthy mushrooms,[6,15,19–21] indicating that a genetically disparate virus was unique to diseased tissues. Moreover, the association of the same dsRNA pattern with abnormalities from different parts of the world provided strong evidence for a single major disease with one viral etiology plaguing cultivated mushrooms.

Precisely 30 years after the association of VLPs with La France disease, Goodin et al.[22] succeeded in isolating the suspected primary causative virus. The name La France isometric virus (LIV) was coined to describe this virus composed of 36 nm isometric particles, which encapsidated the full complement of the disease-specific dsRNAs. And, it is this strict association between its encapsidated dsRNAs and La France disease that distinguishes LIV from the plethora of other VLPs and viruses in *A. bisporus* for which a pathological relevance had not been established.[5,23–26] In the last two decades, significant progress has been made in advancing our understanding of the composition, genome organization, and replication of the *Agaricus* viruses,[27–33] although many critical questions remain unanswered.

Today, we view the etiology of La France disease with far greater clarity than a few decades ago but recognize that all of the available evidence establishes a correlative, but not a causal, relationship for LIV. A systematic molecular dissection of the viral complex in *A. bisporus* suggests pathogenesis is mediated by LIV acting alone amidst other seemingly benign viruses. Our knowledge of these other viruses includes a *Hypoviridae*-like dsRNA element prevailing in healthy mushrooms[21] and a single-stranded RNA (ssRNA) *Mushroom bacilliform virus* (MBV) that is frequently found coinfecting with LIV in diseased tissues.[27,30,34] Future research may reveal that these viruses, or yet to be discovered viruses, do, in fact, have a biological effect on *A. bisporus*. Indeed, novel dsRNAs have been implicated in a newly recognized pathology ("patch disease") occurring on cultivated mushrooms in Europe.[35,36] The recent advent of a practical genetic transformation system for *A. bisporus* is an exciting development,[37] which situates us on the brink of a critical analysis of the *Agaricus* viruses and the exploitation of molecular biotechnological strategies for disease resistance.[38–50]

10.2 BIOLOGY OF LA FRANCE DISEASE

La France disease is among the most serious infectious pathologies on commercially cultivated Agaric mushrooms. Since its initial description more than 50 years ago in the U.S.,[2] the disease is now known to be endemic to most mushroom-growing countries.[5–7] La France disease occurs in an episodic fashion and causes significant crop loss annually. All cultivated varieties of *A. bisporus* succumb to the disease,[51] rendering the use of hygiene as the only means for disease control, and molecular genetic approaches for resistance an alluring prospect.

The severity of La France disease is influenced by host genotype, time of infection, cultural conditions, and other unknown factors. Symptoms range from a chronic infection involving almost imperceptible yield loss to an acute infection associated with malformed fruit bodies and total crop failure. In severe episodes, the first indication of the disease is a delayed emergence of the fruit bodies, which is followed by the development of barren zones throughout the production beds (Figure 10.1). Fruit bodies surrounding the bare areas typically assume a "drumstick" phenotype involving elongated stems and small misshapen caps. Affected fruit bodies show a weakened development of rhizomorphs, as evidenced by the ease with which they can be removed from the growing substrate. There is also a tendency for diseased fruit bodies to mature prematurely, with the discharge of prodigious numbers of spores. This event has important epidemiological consequences, because spores are thought to be the primary mode of spread for the causal agent.[51,52]

Vegetative cultures derived from spores or tissue of symptomatic fruit bodies also manifest an abnormal phenotype. In comparison to healthy cultures, the growth of diseased cultures is markedly slower, and the mycelium is appressed, has an irregular border, and shows an intense brown-colored pigmentation (Figure 10.1). However, it has been noted that monosporus cultures originating from diseased fruit bodies may display a normal vegetative phenotype.[5,52] Diseased cultures are usually debilitated to the extent of either producing a meager yield of fruit bodies exhibiting the characteristic disease symptoms or failing to fruit altogether.

10.3 DOUBLE-STRANDED RNAS IMPLICATED IN LA FRANCE DISEASE

Fungal viruses typically have dsRNA genomes,[3,4] and so the detection of duplex RNA molecules in symptomatic tissues constitutes the most convincing evidence for the involvement of viruses in La France disease. Several lines of evidence implicate a specific set of six major dsRNAs and several minor segments in the etiology of the disease as follows:

1. The dsRNAs are consistently found in fruit bodies and mycelial cultures manifesting disease symptoms.[6,15–20]
2. In *in vivo* radiolabeling studies, they are the only duplex RNA molecules found actively replicating in diseased tissue.[18]
3. The dsRNAs are lost in concert from mycelial cultures with a loss of disease symptoms.[5,18]
4. They are transmitted efficiently through the sexual spores of *A. bisporus*,[52] which agrees with the known mode of spread of the causal agent.[51]

The fact that the dsRNAs co-segregated exclusively during sporogenesis was taken as evidence for interdependently replicating molecules, perhaps comprising the genome of a single virus.[52]

The dsRNA pattern most frequently associated with La France disease consists of six major dsRNAs: L1 (3.8 kb), L2 (3.1 kb), L3 (3.0 kb), L4 (2.8 kb), L5 (2.6 kb), and M2 (1.3 kb), and three minor dsRNAs, which occur in submolar amounts, M1

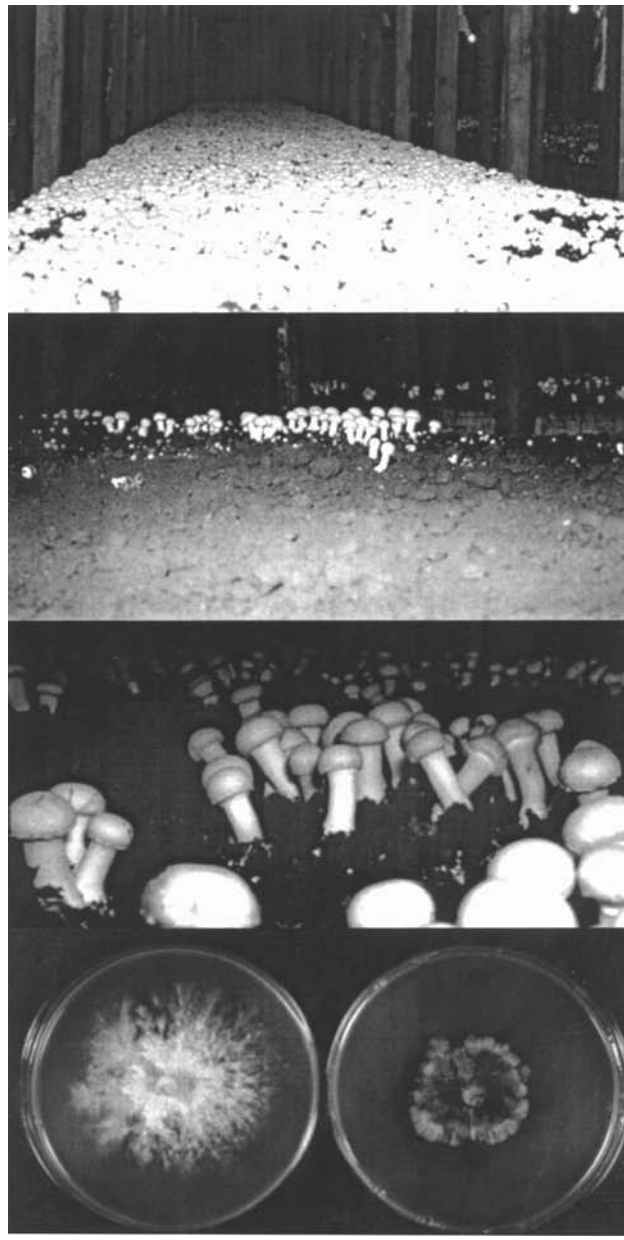

FIGURE 10.1 Characteristic symptoms of La France disease of cultivated mushrooms. From top to bottom: a healthy mushroom crop developing as an even carpet of fruit bodies; a crop affected by La France disease showing large barren zones in the production beds; the classical "drumstick syndrome" of the disease involving fruit bodies having small misshapen caps and elongated stems; a healthy mycelial culture of *A. bisporus* (left) and a diseased culture (right) exhibiting reduced vigor, appressed mycelium, and an increased brown discoloration.

(1.7 kb), S1 (0.9 kb), and S2 (0.8 kb) (Figure 10.2).[6, 13, 17, 19, 20] Aside from the nine commonly encountered dsRNAs, several other minor disease-specific dsRNAs have been isolated and include S3 (0.50 kb), S4 (0.48 kb), S5 (0.40 kb), and S6 (0.38 kb).[17] Extremes in the variation of the pattern of nine dsRNAs, using gel analysis and ethidium bromide staining, ranged from one disease isolate containing all 13 dsRNAs to numerous isolates lacking S1-S6 and two isolates showing deletions of either M1 or M2.[17] Small discrepancies in the sizes reported for the various dsRNAs exist among different research groups and probably reflect an electrophoretic artifact rather than actual differences in the RNAs.

10.4 LA FRANCE ISOMETRIC VIRUS (LIV)

The complete set of nine disease-specific dsRNAs was found to co-purify with isometric proteinaceous virus particles using a protracted procedure entailing PEG-NaCl precipitation, differential centrifugation, and isopycnic centrifugation in cesium sulphate.[22] This virus, LIV, is the principal suspected causal agent of La France disease, which is predicated on exhaustive evidence supporting an association

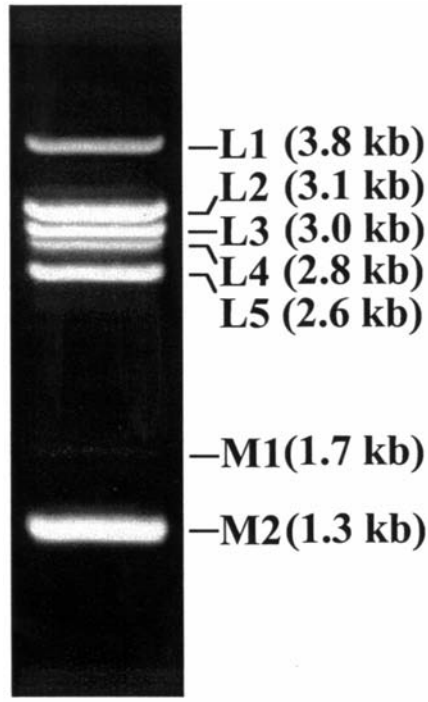

FIGURE 10.2 Gel electrophoretic analysis of the dsRNAs associated with La France disease. Shown is the highly conserved pattern of the six major segments, L1–L5 and M2, and the minor segment, M1. The size of each dsRNA is indicated. Minor segments S1 and S2 are not shown.

between the presence of its encapsidated dsRNAs in mushrooms[6,13,17,19,20] and mycelial cultures[15,18] and the manifestation of the disease. This association is corroborated by a wealth of electron microscopic data establishing the relationship between the presence of isometric particles and the disease.[5,7,9]

LIV is composed of 36-nm isometric particles encapsidating the six major dsRNAs, L1–L5 and M2 (3.8–1.3 kb) and three minor dsRNAs, M1 (1.7 kb), S1 (0.9 kb), and S2 (0.8 kb).[22,53] Minor segments S3–6, which have detected in the total dsRNA fraction of some diseased mushroom isolates,[17] were not associated with purified virus. It is unclear if this was because they are not encapsidated or if they were simply absent from the mushroom isolates used as a source of virus for purification.

The nine most prevalent disease-related dsRNAs, L1–L5, M1, M2, S1, and S2, showed no cross hybridization under stringent conditions,[19] suggesting that each has a unique sequence and is not a defective remnant[54–59] or subgenomic RNA.[60,61] The most probable interpretation of the data in hand is that the six major dsRNAs represent the genome of LIV, whereas the minor segments, present in submolar amounts, have characteristics consistent with satellite RNAs.[62–66]

In all likelihood, LIV is a multicomponent virus whereby the dsRNAs are packaged using a "head-full" mechanism in an undetermined arrangement among several particles.[4] This is based on the prediction that a 36-nm particle could not accommodate the full complement of dsRNA. The virions are composed of two major polypeptides of Mr 63K and 66K, and a minor polypeptide with an estimated mass of 129K (p129),[22] and 115K.[53] Presumably, p129 and the 115K polypeptide are identical, and the apparent difference in size reflects variation in electrophoretic analyses. The 63K and 66K polypeptides appear to be proteolytic end products of two larger polypeptides of Mr 90K (p90) and 120K (p120), respectively. Proteolytic cleavage of these polypeptides evidently had no effect on the morphology, sedimentation rate, or dsRNA content of the virions. Also, p90, p120, and p129 were shown to be serologically unrelated.[53] Considering their molar ratios, p90 and p120 could be construed as major structural polypeptides comprising the capsid, while p129 might be the expected RNA-dependent RNA polymerase (RdRp).[3]

The presence of a minor polypeptide, p129, in the particles suggested that LIV might possess a virion-associated RdRp. Existence of such a polymerase was predicted by sequence analysis of the L1 dsRNA (3.8 kb), which revealed significant homology with RdRps.[31] Additionally, amino acid analysis of tryptic digests of p129 showed that it was encoded by L1 dsRNA.

Direct enzymatic evidence for a virion-associated RdRp came with the demonstration of an RNA polymerase activity that was engaged in the synthesis of RNA transcripts corresponding to each of the dsRNA segments.[67] This activity existed in virus-enriched subcellular fractions and in more highly purified LIV preparations obtained by rate-zonal centrifugation in sucrose density gradients. This enzyme displayed the RNA template dependency, requirements for catalysis, and sensitivity to inhibitors expected of a RdRp. Analysis of the *in vitro* synthesized reaction products by denaturing gel electrophoresis revealed three major products of 1.8, 1.3, and 0.95 kb instead of full-length transcripts. Despite the inability to synthesize full-length transcripts *in vitro*, it was shown that transcripts of only one polarity were

synthesized *in vitro* and *in vivo*. Using reverse transcription (RT) PCR with a single primer targeting either the sense or antisense strand during the reverse transcription step, only the coding strand of M2 dsRNA was detected in a ssRNA fraction from diseased mushrooms, suggesting that the LIV RdRp synthesizes viral messenger RNAs *in vivo*.

10.4.1 Genome Organization of LIV

Nucleotide sequence information is available for five (L1, L3, L5, M1, and M2) of the nine predominant LIV-associated dsRNAs.[29,31] Each segment is a unique sequence containing a single putative open reading frame (ORF). A 23 nt sequence (GGCAACGGCUAGUUGGCCAAAUU) is common to the 5'-terminus noncoding regions of the positive strands of L5 and M2 and is reflected in part (AACGGC-UAGUU) in the 5'-terminus noncoding region of the positive strand of L3. L1 dsRNA apparently lacks this sequence, although it might reside in the ~200 nt of undetermined sequence upstream of the ORF. A feature common to the four major segments, L1, L3, L5, and M2, is the use of the termination codon UAA, which is not shared by the minor segment, M1. M1 dsRNA also lacks the 5'-proximal sequence found in L3, L5, and M2. The available sequence data could be construed as further evidence in favor of the major dsRNAs composing the genome of a single virus, and the minor segments being satellite in nature.

The real or putative coding assignments for the sequenced dsRNAs are as follows:

1. *L1 dsRNA*. A sequence of 3396 nt of a predicted 3600 nt contains a single large ORF starting at nucleotide 13 and ending in nucleotide 3249 with the codon UAA. This ORF has the coding capacity for a putative protein of Mr 122K, but considering that the entire sequence has not been determined, it could have a maximal mass of ~129K. As mentioned earlier, L1 encodes for a virion-associated RdRp by virtue of its significant homology with the sequence of RdRps of dsRNA viruses and amino acid sequence of p129 in virions.
2. *L3 dsRNA*. A 2748 nt sequence of an estimated 2800 nt has an ORF initiating at nucleotide 246 and ending at nucleotide 2609 with a UAA stop codon. This dsRNA segment contains a 15 nt poly (A) tail at the 3'-terminus of the positive strand, similar to L-dsRNA evoking hypovirulence in *C. parasitica*.[56] L3 dsRNA encodes for a predicted protein of Mr 87K, which based on amino acid analysis of tryptic digestion products is p90, one of the major virion-associated polypeptides.[31] A comparative analysis of the sequence data with available sequences in databases failed to show significant homology with other proteins.
3. *L5 dsRNA*. A 2455 nt sequence, which was thought to represent the entire segment, was shown to have an ORF starting at nucleotide 125 and ending at nucleotide 2299 with the codon UAA. This sequence encodes a putative Mr 82K protein having no noteworthy homology with known proteins.

4. *M1 dsRNA.* A sequence of 1402 nt of an expected 1550 nt contains a single ORF initiating at nucleotide 164 and terminating at nucleotide 1241 with a UAG. The putative ORF encodes a protein of Mr 40K. *In vitro* translation using denatured M1 dsRNA failed to produce a discrete polypeptide.[29] The hypothetical protein showed no significant similarities with proteins in databases.
5. *M2 dsRNA.* Of an estimated 1350 nt, 1306 nt has been sequenced and contains a ORF initiating at nucleotide193 and terminating at nucleotide 1212 with a UAA. M2 encodes a nonstructural protein of Mr 38K found accumulating in the cytoplasm of infected cells.[31] This protein showed no extensive homology with other proteins.
6. *S3 dsRNA.* This is a minor dsRNA detected in some diseased mushroom isolates. Based on its sequence (390 nt), it is a terminally-conserved internal deletion mutant of M2.[29]

The taxonomic position of LIV is an unsettled issue, because the complexity of its genome has not been resolved. Assuming the six major dsRNA segments compose the genome, then LIV would bear little resemblance to dsRNA fungal viruses belonging to the only recognized families, *Totiviridae*, *Partitiviridae*, and *Hypoviridae*.[68] Members of these taxa are characterized by genomes composed of one or two molecules of dsRNA.

10.5 MUSHROOM BACILLIFORM VIRUS (MBV)

Among the several types of morphologically distinct VLPs originally associated with La France disease[1] was a 19 × 50 nm bacilliform particle bearing a striking resemblance to *Alfalfa mosaic virus* (Family *Bromoviridae*).[68] This virus, MBV, was isolated in the early 1980s and found to be unique in that it possessed a positive-sense, ssRNA genome, a feature common to plant viruses.[27,28] Interestingly, amino acid sequence analysis of the putative replicase and the coat protein suggested an evolutionary relationship between MBV and plant viruses, particularly members of the luteoviruses and carmoviruses.[32] MBV is recognized as the sole member of the genus *Barnavirus,* belonging to the family *Barnaviridae* ("*Barna*" derived from *ba*cilliform-shaped *RNA* virus).[69,70] The key features distinguishing the family *Barnaviridae* from other taxa of fungal viruses is a ssRNA genome and a bacilliform-shaped virion.

10.5.1 Genome Organization of MBV

The complete nucleotide sequence of the MBV genome has been determined.[32] The genome is composed of a linear, positive-sense, single-stranded RNA, which is 4009 nucleotides in length. The RNA contains four major putative ORFs and has 5'-terminus and 3'-terminus noncoding regions of 60 nucleotides and 250 nucleotides, respectively (Figure 10.3). The RNA is neither capped at the 5'-terminus nor polyadenylated at the 3'-terminus. ORF1 commences at the first AUG codon at nucleotide 61 and ends with UAA at nucleotide 598. ORF2 is in the +1 reading frame, com-

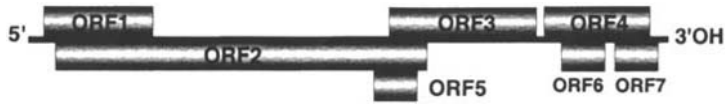

FIGURE 10.3 Genome organization of *Mushroom bacilliform virus* (MBV) (4009 nt) (reprinted from *Virus Taxonomy,* p. 992, Fig. 2, ©2000 Academic Press).

mencing at nucleotide 68 and terminating at nucleotide 2039 with UAG. ORF3 is in the same reading frame as ORF1 (–1 relative to ORF2), beginning at nucleotide 1882 and ending with a UAA codon at nucleotide 3144. An overlap of 259 nucleotides exists between ORFs 2 and 3. ORF4 initiates at nucleotide 3162, 18 nucleotides downstream of the ORF 3 termination codon in the same reading frame, and terminates at nucleotide 3757 with the codon UGA.

ORFs 1 through 4 are capable of encoding polypeptides of Mr 20K (p20), 73K (p73), 47K (p47), and 22K (p22), respectively. ORF3 encodes a putative RdRp, and ORF4 the capsid protein. The deduced amino acid sequences of both ORFs 2 and 3 contain putative helicase-like motifs. Three minor ORFs potentially encode polypeptides of Mr 8K, 6.5K, and 6K, respectively. The putative polypeptides encoded by ORF1 and the three minor ORFs did not show extensive homology with known polypeptides.

In a cell-free system, MBV RNA directs the synthesis of a major polypeptide of Mr 77K, and possibly several minor polypeptides of Mr 21-37K.[28] However, the possible relationship between the 77K *in vitro* translation product and putative p73 encoded by ORF 2 has not been determined. Similarly, whether any of the low molecular weight translation products represent the p24 capsid protein or putative p20 encoded by ORF1 is not known.

In vivo analysis was used to identify the subgenomic RNA (sgRNA) of MBV that encodes the capsid protein.[33] When a digoxigenin-labeled RNA complementary to the 3'-terminal 130 nt of MBV was used as a probe in Northern hybridization analysis of RNA isolated from MBV infected tissue, a 0.9 kb RNA transcript was detected, in addition to the viral genomic RNA. Primer extension analysis mapped the 5' end of the 0.9 kb subgenomic RNA to nt 3117 of the MBV genome. This position is 892 nt from the 3'-end of the genome and 47 nt upstream from the translation initiation codon of the coat protein gene. In accordance with the proposed evolutionary relationship between MBV and plant viruses, the sequence ACAAAA at the transcriptional start site of the sgRNA is identical to that for the C-strain of *Southern bean mosaic virus* and the Wageningen strain of *Potato leafroll virus.*

10.6 NATURE OF THE ASSOCIATION BETWEEN LIV AND MBV

Based on electron microscopic analysis, LIV can occur as a single infection, whereas MBV occurs only in mixed infections with spherical VLPs, particularly ones resembling LIV.[5] Furthermore, a 1.4 kb cDNA clone of MBV RNA failed

to hybridize with the LIV dsRNAs *in toto*.[71] This pattern of infection and lack of extensive homology led to speculation that MBV might be a satellite virus whose replication is dependent on LIV as the helper virus.[62–66] However, this relationship has been invalidated by the identification of mushroom isolates singly-infected by MBV.[30] Moreover, infection by MBV without evidence of LIV, based on dsRNA analysis and RT-PCR amplification with a sensitivity of <10 fg, persisted through the vegetative and fruiting stages of *A. bisporus*.[72] Thus, MBV evidently replicates autonomously, at least in the absence of LIV. Sequence analysis suggesting the MBV genome encodes a RdRp is consistent with the capacity for self-replication.[32]

MBV is commonly found as a double infection with LIV in La France-affected mushrooms. Using RT-PCR analysis, an estimated 60% of diseased mushroom isolates collected in North America during a 13-year period were found to be doubly-infected by LIV and MBV.[30] The fact that the disease was absolutely correlated only with the presence of LIV argued in favor of MBV being nonessential for pathogenesis. In further support of this hypothesis, no obvious phenotypic anomalies were observed during the vegetative and reproductive development of an *Agaricus* culture singly-infected by MBV.[72]

The mere idea that LIV and MBV are independently replicating viruses does not exclude the possibility of a synergistic or antagonistic relationship. In fact, the incidence of MBV was found to be 12-fold higher in mushroom isolates infected by LIV than in isolates without evidence of LIV infection.[30] This would seem to imply that MBV derives a survival advantage through its association with LIV. Still, a direct analysis is needed to draw definitive conclusions about the pathogenicity of MBV, the possible modulating effect of MBV on the symptoms associated with LIV infection, and any interplay of LIV transactivation of MBV.[73]

10.7 DOUBLE-STRANDED RNAS IN HEALTHY MUSHROOMS

Several dsRNAs have been detected in apparently healthy fruit bodies and mycelial cultures of *A. bisporus*.[6,15,19–21] Among the most common are two major dsRNAs, L-RNA (>13 kb) and S-RNA (2.4 kb), and a minor dsRNA, M-RNA (5.2 kb) (Figure 10.4).[21] The three dsRNAs associated in an RNAse-resistant fashion with ~75 nm diameter fungal membrane vesicles, reminiscent of the viruses in the family *Hypoviridae*.[68] It is not known if the dsRNAs compose the genome of one or several viruses. It is known, however, that they lack sequence homology, as determined by hybridization analysis, with the LIV dsRNAs, ruling out their role as progenitor molecules.[21]

Because an overt pathology has not been associated with the vesicle-bound dsRNAs, they are presumed to be innocuous, typifying the generally benign nature of fungal viruses. However, this virus is worthy of further investigation in view of its prevalence in commercial mushroom spawns (i.e., mushroom seed) and structural similarity to a family of viruses whose members have a pathological impact on their fungal host.

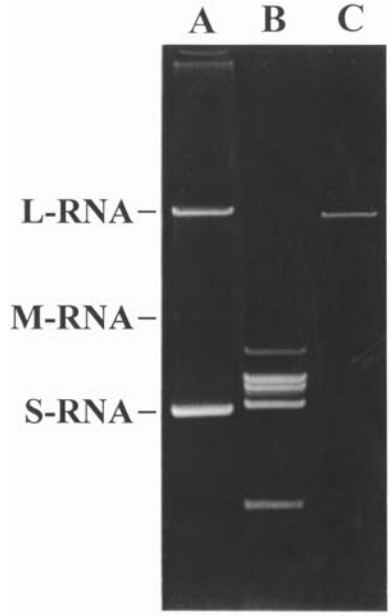

FIGURE 10.4 Gel electrophoretic analysis of the dsRNAs from healthy fruit bodies. Lane A, L-RNA (>13 kb), M-RNA (5.2 kb), and S-RNA (2.4 kb) associated with a membrane vesicle-enriched subcellular fraction of healthy tissues; lane B, the La France disease-specific dsRNA segments L1-L5 (3.8-2.6 kb) and M2 (1.3 kb); lane C, a dsRNA (13.1 kb) isolated from barley. (Reprinted from *Current Genetics* 25, p. 131, Fig. 3, ©1994 by Springer-Verlag.)

10.8 PATCH DISEASE: A NEWLY SUSPECTED DOUBLE-STRANDED RNA-RELATED DISORDER ON MUSHROOMS

A newly recognized infectious disorder on cultivated *A. bisporus* referred to as "patch disease" (a.k.a. "virus X") was first noted in 1996 on a British farm.[35] Within three years, persistent episodes of the disease were occurring with some regularity throughout the British industry.[36] Patch disease continues to be a source of consternation for the British mushroom industry, recently being a factor in the demise of three farms. There have been confirmed cases of the disease in Ireland, Holland, and the United States.[74]

Crops affected with this disorder develop barren areas in the production beds (i.e., patches). These areas show an arrested development of pins or a delayed formation of fruit bodies, often with an overproduction of normal appearing fruit bodies at the peripheries. The bare areas can develop randomly throughout the beds or assume sharply demarcated zones or patterned undulations and swirls. However, the name "patch" does not describe the full scope of symptoms. In another syndrome, fruit bodies show a premature opening of the veil and, in yet another, a brown discoloration. The disease can strike at any stage in the cropping cycle and generally intensifies with time.

The infectious nature of the disease was demonstrated by the transmission of the causal agent to healthy mushrooms using *A. bisporus*-colonized compost from an affected area as inoculum.[36] When compared to healthy cultures, *Agaricus* cultures originating from patch areas grew slower, had irregular margins, and showed a more intense brown-colored pigmentation. Generally, the phenotypic changes in cultures with patch disease were less severe than those afflicted with La France disease.

Several novel dsRNAs have been detected in fruit bodies growing at the edges of patch areas (Figure 10.5) and in *Agaricus* cultures originating from within affected areas.[36,72] Surprisingly, the same dsRNAs could be isolated from apparently healthy fruit bodies growing in normally productive areas of diseased crops.[72] Generally, the patch-related dsRNAs occur at considerably lower concentrations than the LIV dsRNAs in La France disease, which is based solely on the quantity of fruit body tissue that is required to visualize them in ethidium bromide-stained gels. The positive correlation between the presence of the dsRNAs and patch disease was upheld in a broad survey of British mushroom farms.[36,74]

The discovery of unique dsRNAs in symptomatic tissues, particularly in the absence of other candidate pathogens, suggests the involvement of a new virus(es) in the patch disorder. However, a more rigorous testing of the proposed cause-and-effect relationship is warranted. Research efforts should address several immediately answerable questions. Are the dsRNAs exclusively associated with anomalies, or

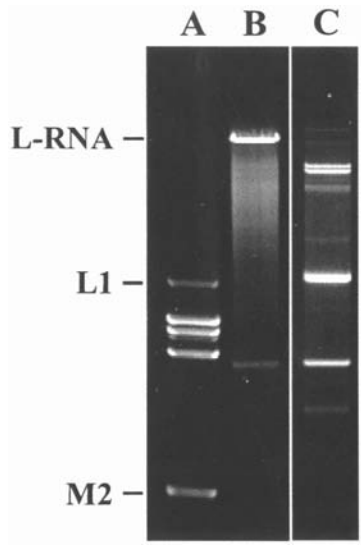

FIGURE 10.5 Relative electrophoretic mobilities of the dsRNAs associated with patch disease on commercial mushrooms. Lane A, L1 (3.8 kb)-L5 and M2 (1.3 kb) dsRNAs isolated from fruit bodies with La France disease. Lane B, L-RNA (>13 kb) and S-RNA (2.4 kb) isolated from healthy fruit bodies. Lane C, the dsRNA pattern correlated with the occurrence of patch disease.

will an exhaustive search reveal their presence at a high frequency in healthy tissues? Do diseased cultures produce symptomatic fruit bodies? Do the different symptom syndromes correspond to specific RNA profiles? That being said, as with La France disease, only circumstantial evidence for a viral nature will have been gathered, with definitive proof awaiting the outcome of studies involving the expression of viral sequences in *A. bisporus* following their introduction by transfection[75–80] or genetic transformation.[81–83]

10.9 RECENT ADVANCES IN THE GENETIC TRANSFORMATION OF *AGARICUS BISPORUS*

A recent noteworthy breakthrough is the development of a genetic transformation system for the mushroom holding the promise of a powerful analytical tool for the molecular investigation of La France disease.[37] The unavailability of a practical gene transfer system has been a major obstacle precluding the critical analysis of the *Agaricus* viruses and, more broadly, the genetic improvement of this commercially important fungal species.

The new gene transfer system for *A. bisporus* is based on the *Agrobacterium*-mediated transformation (Agro-transformation) procedure originally described for the yeast, *S. cerevisiae,*[84,85] and later extended to other fungi.[86] Although the Agro-transformation method proved more convenient than the existing protoplast-based scheme,[87,88] it suffered from a comparably low efficiency of transformation for *A. bisporus* (~0.00003%). However, it was subsequently shown that transformation using a plasmid vector having a homologous promoter, rather than an *Aspergillus* promoter, and cocultivation of *Agrobacterium* with fruit body gill tissue, instead of spores, led to extraordinary efficiencies.[87]

Transformation was carried out with a plasmid vector (pBGgHg) composed of a pCAMBIA1300 plasmid backbone with the hygromycin B phosphotransferase (*hph*) gene controlled by the *A. bisporus* glyceraldehyde 3-phosphate dehydrogenase promoter. After cocultivation of *Agrobacterium* and *A. bisporus* for 3 days, hygromycin-resistant cultures appeared after 7 to 28 days of incubation from up to 95% of the gill tissue pieces (Figure 10.6). Transformants showed up to four copies of the *hph* gene integrated at random sites in the genome (Figure 10.6). Using this procedure, transgenic cultures could be generated in less than two weeks, and mature fruit bodies eight weeks later. The antibiotic resistance trait was shown to be stably maintained in *A. bisporus*, being expressed by the first-generation fruit bodies and spores.[37]

The newly acquired ability to introduce foreign genes with relative ease into *A. bisporus* creates exciting prospects of obtaining succinct answers to questions regarding the pathogenicity, replication, and interactions of the *Agaricus* viruses. At the same time, this technique should prove invaluable in exploiting molecular strategies for incorporating viral disease resistance in cultivated mushrooms[38–50] and as a tool for the molecular genetic analysis of biological processes in this species.

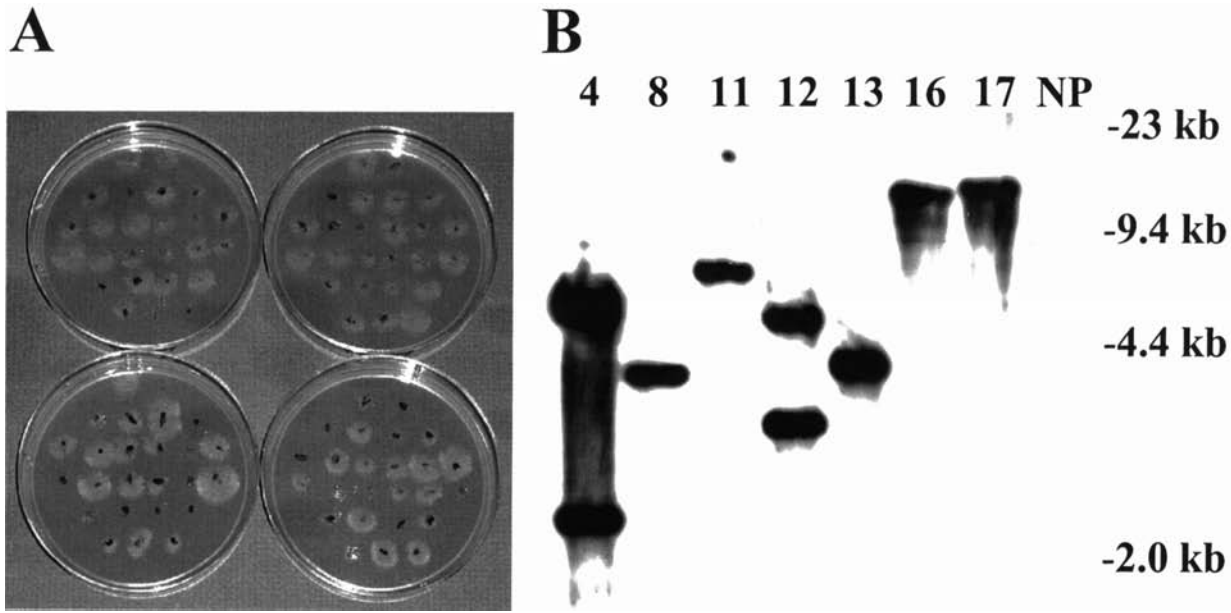

FIGURE 10.6 Agro-transformation of *Agaricus bisporus*. A, selection of putative hygomycin-resistant transformants after 2 weeks on a hygomycin-amended medium (30 μg/mL). B, Southern blot analysis of DNA isolated from six putative hygromycin-resistant transformants (4, 8, 11, 12, 13, 16, and 17), and the nontransformed parental culture (NP). Genomic DNA (5-10 μg) was isolated from broth cultures, digested with *Sac*I, and probed with a biotin-labeled 1-kb *hph* gene sequence. The positions of molecular DNA size markers are indicated.

10.10 FUTURE PROSPECTS

The discovery of VLPs in mushrooms afflicted with La France disease marked an important milestone in the field of virology, because it provided the first shred of evidence for the existence of viruses infecting fungi. Within the last two decades, considerable progress has been made in unveiling the nature of the viral complex in *A. bisporus*. Pathogenesis in La France disease apparently results from infection by a multipartite dsRNA virus. Other presumably benign viruses found infecting *A. bisporus* could conceivable modulate symptoms associated with infection by this virus or be pathogenic as well. However, at this time, the latter two hypotheses are highly conjectural.

Undoubtedly, an essential goal for the future study of La France disease is sequencing the complete set of nine dsRNAs encapsidated by virions of LIV. Yet to be determined are the sequences of the L2, L4, S1, and S2 dsRNAs. Future sequencing projects must emphasize capturing the termini of these molecules. Several controls should be conducted to ensure that critical sequences have not been overlooked. The termini of dsRNAs typically contain secondary structures that may not be cloned using standard techniques and, thus, their sequence may not be readily determined. When the sequences of dsRNAs have been re-examined using (1) direct RNA sequencing, (2) tailing dsRNA with UTP or CTP in addition to ATP, and (3) adding poly (dG) to the products of primer-extension in 5'-RACE, up to 177 nucleotides of new sequence have been recovered.[89,90] When completed, the sequence of the dsRNAs should provide critical information to (1) resolve outstanding taxonomic issues, (2) provide insight into the replication strategies executed by LIV, (3) dissect the molecular basis of La France disease, and (4) develop novel dsRNA-based expression vectors.[91]

A key element in understanding the viral-host interaction in La France disease is to determine (1) the minimal number of dsRNA segments required for replication, (i.e., is L1 dsRNA capable of autonomous replication), (2) the contribution of each dsRNA to the disease phenotype, and (3) the ability of the minor dsRNAs to modulate symptoms. Achieving these goals will not only satisfy Koch's postulates for LIV but will identify targets for the development of genetically-engineered virus resistance mechanisms. In a strategy analogous to that used to develop resistance to plant viruses, yeast strains have been cured of viral infection by over expression of the ScVLa coat protein[41] or expression of *gag* and *gag-pol*.[40] Now, with Agro-transformation of *A. bisporus* a reality, and the assignment of the p90 LIV coat protein to dsRNA L3, strides can be taken to develop genetically-engineered viral resistant mushrooms.

In addition to addressing questions concerning the replication of LIV per se, the tools are now available to explore the interaction of LIV with other *Agaricus* viruses. For example, investigating the interaction of MBV with LIV may provide insight into a synergism between fungal viruses as well as determining whether MBV derives a survival advantage from LIV. Finally, the nature and significance of the 25 nm VLPs, which are commonly observed in cell-free extracts of diseased fruit bodies[5] or as a minor contaminant in LIV preparations,[22] have yet to be determined. This is particularly crucial when one considers that 25 nm particles, but not 36 nm

particles, were described from diseased mushrooms in the seminal publication on fungal viruses![1]

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11 Large dsRNA Genetic Elements in Plants

and the Novel dsRNA Associated with the "447" Cytoplasmic Male Sterility in *Vicia Faba*

Pierre Pfeiffer

CONTENTS

11.1 INTRODUCTION

In contrast to fungi, which frequently harbor noninfectious dsRNAs genetic elements of viral origin, the great majority of viruses that infect plants have a single-stranded RNA genome of positive polarity. During the replicative cycle, a double-stranded form of the genomic RNA is formed transiently; it is then used as a template by the

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virus-encoded RNA-dependent RNA polymerase (RDRP) to generate many copies of positive strand RNA, which are translated, amplified, and encapsidated to form virions. Thus, such infections are characterized by a vast excess of plus strand RNA over dsRNA: the double-stranded form is only transient, and transcription is highly asymmetrical.

A few species of plants have been identified that contain resident dsRNA viruses causing no visible symptoms and are therefore dubbed "cryptic viruses." Such viruses have generally a bipartite genome, with one RNA segment coding for the polymerase and the other for the coat protein that encapsidates separately the two genomic dsRNAs. Like with other dsRNA viruses, the polymerase present in the virus particles transcribes the genomic dsRNAs to generate positive strand transcripts that are used as mRNAs for translation of the virus-encoded proteins and/or packaged by the coat protein together with a molecule of polymerase. Second strand synthesis (the actual replication step) occurs in the previrions to yield mature virus particles. Cryptic viruses lack a movement protein and are therefore unable to move from cell to cell within the plant that contains them. They are exclusively transmitted via a vertical mode through the ovule and pollen but are nevertheless considered as *bona fide* viruses despite their lack of infectivity.[1] In a plant containing a cryptic virus, virus-free sectors may therefore arise during development if the rate of cell division outcompetes the pace of virus multiplication.

In addition to cryptic viruses and a few phytoreoviruses, some ill-defined, symptomless cytoplasmic resident dsRNA elements of high molecular weight have been detected and studied in several species of plants: pepper,[2] cultivated rice,[3–5] and wild rice,[6–7] bean,[8] barley,[9] and in the "447" male-sterile line of *V. faba.*[10,11] They are generally symptomless and are transmitted exclusively on a vertical mode, much like the cryptic viruses. These large dsRNAs have a plasmid-like lifestyle, with the number of copies per cell being tightly regulated by the nuclear background and the developmental stage of the host cell.[12] Several of them have now been sequenced and shown to have only one coding strand: they use the universal genetic code to produce a single long polyprotein (400 to 600 kDa), which is probably processed into functional moieties by embedded protease function(s), a strategy shared by many plant viruses.[13] Like all other dsRNA genetic elements, they must somehow be topologically confined in a structure that isolates them from the cytoplasm to avoid activating dsRNA-responsive enzymes such as PKR, a protein kinase that phosphorylates a translation initiation factor. In dsRNA viruses, the genomic dsRNA is contained within the virions, and some small unencapsidated dsRNAs in fungi and plants have been shown to be sequestered in the mitochondria. We believe that all cytoplasmic high molecular weight dsRNAs found in plants are contained within membraneous vesicles, as has been unambiguously demonstrated for the dsRNA of *V. faba,*[10] where membraneous structures very similar to those of the hypovirulence-associated virus CHVl of the chestnut blight[14,15] were identified.

Such dsRNA elements have now been shown to derive from a common ancestor belonging to the alpha-like supergroup of viruses. They can be considered as coat-protein deficient viruses that survived as their replicative form and have therefore been suggested the name of Endornaviruses.[16] We shall here review the peculiarities of the dsRNA associated with the male-sterile "447" *Vicia faba* line and draw the

parallel with other dsRNAs sharing similar characteristics and occurring in several species of higher plants.

11.2 THE WHIMSICAL "447" CYTOPLASMIC MALE STERILITY OF *VICIA FABA* AND ITS ASSOCIATED "CYTOPLASMIC SPHERICAL BODIES"

As a nitrogen-fixing legume with a high nutritional value, broadbean (*Vicia faba* L.) is an interesting crop for both animal feed and human consumption. In recent years, however, the acreage devoted to this crop has been decreasing steadily, mainly due to its low and rather irregular yields. Productivity and crop quality could be improved by taking advantage of the good degree of heterosis, or hybrid vigor of this plant, which could be exploited at its best by hybrid breeding. This calls, however, on the availability of male sterile parental lines to prevent self-fertilization and streamline the production of hybrid seeds. To date, there is no predictable and reliable way of obtaining CMS lines, and these are generally obtained as the result of mutagenesis, wide crosses, and interspecific crosses in breeding programs.

In *Vicia faba,* Bond et al.[17] described in 1966 a cytoplasmic male sterility, termed "447," which resulted from the interaction of the 447 cytoplasmic factor with a recessive nuclear gene (rf) (cytoplasmic male sterility would therefore be better described as nucleocytoplasmic male sterility). However, this male sterile line (447/rf.rf) proved rather unstable, with spontaneous revertants appearing in response to increased plant age and adverse environmental factors, thus hampering its large-scale use for breeding. In addition, such reversion occasionally affected only part of the plant, but the loss of male-sterility was then permanent in the progeny raised from revertant seeds.

Finally, permanent restoration was an additional unusual feature of this line: when the male-sterile line was pollinated by a restorer (Rf.Rf), fertility was fully restored in the F1 progeny (447/rf.Rf). Unexpectedly, self-fertilization of this F1 yielded only fully fertile plants, although in principle 25% of the plants should now carry the (rf.rf) genotype in a "447" cytoplasmic background. Not a single male-sterile plant then reappeared in all subsequent selfing and backcrosses, as if the "447" cytoplasmic factor had been definitely lost.

These observations should be placed in the historical context of the 1970s, at a time where the molecular basis of CMS was not yet unravelled: it was then thought that this condition resulted from a virus infection and that male sterility was just another symptom like mosaics, ringspots, necroses, and other manifestations of damage caused by viruses to the plant, and after which these viruses generally gained their names (e.g., tobacco mosaic virus, tomato aspermy virus, and tomato bushy stunt virus, to name a few). In 1976, the electron microscopic observations of Edwardson et al.[18] revealed indeed that the cytoplasm of the "447" male-sterile plants contained "cytoplasmic spherical bodies" or CSB (also called virus-like particles or VLPs) that seemed to lack a proper capsid but instead resembled vesicles of rather monodisperse size (about 70 nm in diameter), limited by a peripheral unit membrane and often featuring a star-shaped and dark staining core. These cytoplasmic vesicles

were seemingly found in greater abundance in ovules, and dissected flowers were therefore first used as starting material for biochemical studies. We found out later that all tissues of the plant contain such particles,[11] and can be therefore used as starting material for their purification.

11.3 CYTOPLASMIC SPHERICAL BODIES CONTAIN A DOUBLE-STRANDED RNA OF HIGH MOLECULAR WEIGHT

The apparent correlation between the presence of CSB and the CMS trait led Scalla et al.[19] to attempt purification of these structures. Since these particles seemed to be associated with a membrane, these authors were careful to avoid detergents and organic solvents (both of which are often used in plant virus purification protocols to improve recovery and purity of virus preparations) and were able to demonstrate the presence of such CSB in the post-mitochondrial or polysomal fraction. In addition, they found that these particles were absent from maintainer lines, and that they irreversibly disappeared upon restoration, thus confirming the results of electron microscopic studies. They also identified that these CSB contained RNA as genetic material and that this RNA was partially resistant to RNAse treatment. Using a different approach that did not preserve the structure of the CSB (n-butanol/urea/TX-100 extraction), Grill and Garger[20] demonstrated that this high molecular weight RNA was indeed associated with the "447" CMS and established its double-stranded nature by thermal denaturation, solubility at high salt, and CF-11 cellulose chromatography. They also demonstrated that the dsRNA gave rise to three molecular species after denaturation and suggested that one of the strands was interrupted.

The presence of CSB, as detected by electron microscopy, paralleled the occurrence of dsRNA, and subsequent studies confirmed that there was an absolute correlation between the CMS trait, the presence of the CSB, and the dsRNA. Although Grill and Garger[20] originally claimed that they had succeeded in transmitting this dsRNA and hence the CMS by a dodder bridge, many subsequent attempts by Scalla and his colleagues,[19,21] among others, to repeat this transmission disproved this statement, which was retracted in a later paper.[22]

The behavior of the CSB is indeed very reminiscent of that of cryptic viruses, a family of dsRNA viruses that replicate in plant cells without causing any apparent phenotype. By contrast with classical plant RNA viruses, these viruses do not move from cell to cell, because they lack the movement protein. This protein encoded by plant viruses is required to facilitate the passage of infectious material through modified plasmodesmata either as a movement protein-viral PNA complex or as virus particles travelling through tubules formed by the movement protein.[23,24] Lacking such movement protein, cryptic viruses are exclusively transmitted vertically by both egg and pollen, and their distribution into daughter cells after cell division determines their maintenance there. Any cell that has lost its contents of cryptic virus will, if part of a meristem, lead to the formation of a shoot devoid of cryptic virus. No virus particles will subsequently diffuse from the other parts of the plant: in the absence of reinvasion of the "cured" shoot, chimaeric plants are

thus obtained in which some parts still contain cryptic virus and others do not. The same holds true for the CSB associated with the "447" CMS: once a shoot of *V. faba* has reverted to fertility (instability is strongly influenced by environmental factors), no CSB will diffuse into this shoot from the rest of the plant, which remains sterile despite perfect tissue continuity. Analysis of dsRNA confirmed that it was absent from reverted parts of the plant while still present in the shoots that had remained male-sterile.

11.4 PURIFICATION OF THE CSB

To gain a better understanding of how the presence of these CSB may induce disruption of the normal pollen maturation pathway, Dulieu and coworkers[25] set out to purify these particles and raise antibodies directed against their antigenic determinants. CSB were first semipurified by differential centrifugation and further purified on sucrose gradients. It was found later that CSB were present in all tissues of the plant[11] and that their purification from the post-mitochondrial fraction could be expedited by PEG precipitation, a technique routinely used for concentrating many plant viruses. Anti-CSB antibodies were raised and could be used in an ELISA test to screen plantlets very early in their development instead of having to wait for the floral stage. Confirming previous observations, an absolute correlation was obtained between a positive ELISA response indicative of the presence of the CSB and the male sterility trait; in addition, increased levels of CSB paralleled a better stability of the CMS. These antibodies were also used for immunochromatography purification to further study these CSB: silver staining and western blot analysis led Desvoyes and Dulieu[26] to conclude that the CSB contained very little protein. The viral polymerase known to be associated with the CSB (see below) could not be detected immunologically or by staining. Finally, the major antigenic determinants were found to be resistant to proteases but were destroyed by periodate treatment and thus are likely to correspond to sugar residues.

11.5 RNA-DEPENDENT RNA POLYMERASE ACTIVITY IS ASSOCIATED WITH THE CSB

All double-stranded viruses have an associated polymerase activity and share the same life cycle: transcription of the coding strand is mediated by the co-encapsidated polymerase and occurs in the particle. The single-stranded mRNA thus synthesized is released into the cytoplasm where it is translated to generate coat protein subunits (for *bonafide* viruses) and the polymerase, which in some cases exists as a coat protein-polymerase fusion protein. The single-stranded RNA is subsequently co-encapsidated with its polymerase by coat protein subunits to form previrions in which second strand synthesis occurs. This scheme is common to plant, animal, and fungal viruses: dsRNA is never found roaming freely in the cytoplasm, since its presence there would activate the dsRNA-mediated cascade of cellular responses. We reasoned that, although apparently devoid of coat protein, the CSB should have an associated RNA polymerase activity. Indeed, addition of labeled nucleotide triphosphates to a semipurified preparation of CSB resulted in significant incorporation

into dsRNA[10]). RNA synthesis depended on the presence of all four NTPs and magnesium, and was insensitive to inhibitors of cellular DNA-dependent RNA polymerase such as actinomycin D and alpha-amanitin. The membranous vesicles efficiently protected the dsRNA from added RNAse but seemed to be quite permeable to NTPs, since addition of nonionic detergent resulted only in a modest increase of the incorporation. This contrasted with the situation encountered with the membranous vesicles of the *Cryphonectria* hypovirulence-associated virus where such detergents boosted RNA synthesis by three- to four-fold.[15] Finally, the distribution of dsRNA along the gradient paralleled that of RNA-dependent RNA polymerase activity: all CSB seemed therefore to be functionally equivalent and active in RNA synthesis rather than being, for instance, distributed into two classes of particles containing either mature dsRNA or intermediates of replication.

The RNA synthesized was in a double-stranded form, as judged by its resistance to RNAse at high salt, and this strongly suggested a semiconservative transcription by a strand displacement mechanism. Agarose gel analysis of the neosynthetized RNA after denaturation with methylmercury hydroxide revealed that, even after long periods of pulse followed by a chase, the labeled RNA reached a limit size that appeared to be short (in the range of 4.5 kb) compared to the full-length molecule, then estimated at 16.7 kbp.[22] No reinitiation of transcription seemed to occur *in vitro*. By analogy with other RNA plant viruses, such as bromoviruses that derive subgenomic RNAs from the 3' distal part of polycistronic messengers, it seemed logical to assume that the short labeled RNA, which had no double-stranded counterpart, corresponded to partial transcription of the full-length dsRNA. The polymerase would initiate at an internal nick (hence, the three molecular species appearing in denaturing gels) and terminate at the 3' terminus of the (+) strand of the dsRNA: this preferentially labeled RNA would therefore correspond to a subgenomic messenger.

11.6 THE UNEXPECTED STRUCTURE OF THE "447" DSRNA

To further analyze the mechanisms of transcription and replication of the dsRNA and to generate complete cDNA molecules using terminal primers, we attempted to sequence directly the free 3'OH termini of the dsRNA by "wandering spot analysis." These were labeled *in vitro* by T4-RNA ligase-mediated addition of [^{32}P]pCp, and submitted to partial hydrolysis followed by 2-D analysis.[27] The short RNA species was quasi-exclusively labeled and found to terminate very accurately, with less than 10% of the molecules lacking the last nucleotide. The full-length complementary strand of 16.7 kb was also labeled, but not the expected ca. 12 kb RNA covering the rest of the strand. The same situation occurred again when free 3' OH termini were polyadenylated *in vitro* by yeast poly(A) polymerase. Preferential labeling of the short RNA species seemed to confirm a preferential accessibility, consistent with its anticipated mapping at the 3' end of the dsRNA molecule. By contrast, the 3' end of the species making up the rest of the same strand on the dsRNA molecule would be poorly accessible to modification enzymes at the level of the nick, or even blocked as a cyclic nucleotide unable to support addition of further nucleotides. The positive

polarity of the 4.5 kb RNA was established by northern blotting experiments, which also finally revealed the existence of its expected ca. 12 kb counterpart. The vast majority of the dsRNA molecules (>99%) featured this single-stranded interruption in the coding strand, but a small population of dsRNA molecules had a continuous (+) strand, as required to allow completion of the replication cycle. Interestingly, the signal obtained with the 4.5 kb RNA was consistently much more intense than that of the other molecular species, suggesting that this molecule may exist in more than stoechioetric amount in the dsRNA. Thus, dsRNA molecules appear to feature several molecules of polymerase in the process of synthesizing subgenomic RNA by a strand displacement mechanism.

11.7 MOLECULAR CLONING OF THE "447" DSRNA

Using *in vitro* polyadenylated dsRNA, it was now possible to generate oligo(dT) primed cDNA and to progressively construct overlapping cDNA clones of the dsRNA. At later stages, RT-PCR was used to bridge the gaps and to accurately map the 5' termini of the dsRNA molecule by taking advantage of the added poly(A) stretch on the opposite strand. This led to the surprising discovery[11] that a ca. 2.8 kb self-PCR product was formed when oligo(dT) alone was used as a primer: such molecules were generated by amplification between the preferentially polyadenylated 3'OH terminus of the short RNA of positive polarity and the polyadenylated 3'OH terminus of the minus strand. The cloned product was found to be 2735 nucleotide long (excluding the added homopolymer tails) and contained a coding sequence starting at AUG 42–44 but devoid of a stop codon to terminate translation. This led to a reappraisal of the size and respective arrangement of this small RNA, which could not be considered as "subgenomic" any more, since it lacked a translation termination signal. All determinations confirmed an accurately positioned nick at NT 2735, with a few clones lacking the terminal nucleotide, and the sequence matched (within a few uncertainties) that determined by direct sequencing of *in vitro* labeled RNA.

Several imperfect repeats were detected on the complete cDNA, which may correspond to gene duplication events that occurred during the evolution of the dsRNA. The most striking feature remained however the single ORF found on the plus strand and which ran uninterrupted across the nick. The potentially 5803 aa long protein it encodes is certainly processed *in cis* by embedded protease function(s), a strategy used by many plant viruses to derive a set of functional polypeptides from a single precursor.[13] The typical motifs of helicase and RNA-dependent RNA polymerase common to all RNA viruses were detected and confirmed the viral origin of the dsRNA.

11.8 SEQUENCE COMPARISONS REVEAL THE RELATION WITH OTHER HIGH-MW DSRNA SPECIES IN PLANTS

Due to their large size and the absence of deleterious symptoms, only few of these high molecular weight dsRNAs have been sequenced completely. In addition, their

double-stranded nature interferes with the generation of long cDNAs due to rapid reannealing of both RNA strands following denaturation. For the dsRNA found in the "Black Turtle Soup" cultivar of *Phaseolus vulgaris,*[8] a short partial sequence was reported, and this study was discontinued after it was demonstrated that this dsRNA was not associated with CMS in bean as had been thought previously.[28] Similarly, later studies showed that the dsRNA detected in male-sterile lines of cultivated rice[3] was not associated with the CMS trait, since it could also be detected in male-fertile lines.

In a systematic study, Fukuhara and coworkers[6,7,12] analyzed and sequenced several dsRNAs isolated from rice lines from diverse origins, some of which were used for the breeding of cultivated rice, *Oriza sativa* L. Many *japonica* cultivars of *O. sativa* were found to contain such dsRNA (designated J-dsRNA in the temperate lines and T-dsRNA in the tropical lines), as well as one strain of *0. rufipogon* (W-dsRNA), a wild rice considered as an ancestor of the cultivated rice. Computer comparisons revealed readily that all these rice dsRNAs not only shared a high degree of similarity between themselves[6] (and therefore probably evolved from a common ancestor before the various species of rice diverged), but also that they shared extensive similarities with the "447" dsRNA of *V. faba,*[11,16] especially in the beginning of the helicase and in the RDRP domains.

All these large dsRNA were found to be linear and had a size ranging from ca. 14 kbp for rice to 17.6 kbp in *V. faba.* They all shared a common structure and organization: only one strand contained a single ORF encoding a long protein featuring the typical helicase and RDRP signatures of RNA viruses, and which is probably processed by embedded protease function(s). Interestingly, J-dsRNA, T-dsRNA, and W-dsRNA all had, like the "447" RNA of *V. faba,* a nick that interrupted the positive strand and thus the ORF at a precisely defined, but different, position. The general organization of these various large dsRNA replicons from plants is given in Figure 11.1, along with that of Hepatitis Virus E, a single-stranded RNA virus of the alpha-viruses supergoup whose replicase gave the next highest BLASTP score value. Maximum amino acid sequence homology was found among the different rice dsRNAs, both in the region of the nick and in the RDRP stretch of the polyprotein. Another interesting feature of the rice dsRNAs resided in the high degree of sequence homology with conserved stem and loop structures both in the 5' noncoding regions and in the position of the start codon,[16] which probably represent cis-acting elements involved in replication and translation.

11.9 IN RICE, THE HOST CELL ESTABLISHES A *MODUS VIVENDI* WITH RESIDENT DSRNAS

These structural features are certainly related to the maintenance and replication of such dsRNA species; indeed, these replicons were found at a low but constant number of copies per cell (ca. 100 molecules per cell) in all tissues of the plant. However, when cell suspensions were derived from plant tissue, the dsRNA contents increased ten-fold in all lines tested. This points to a control of the copy number by a regulatory system that is conserved among the various rice strains, even though these diverged

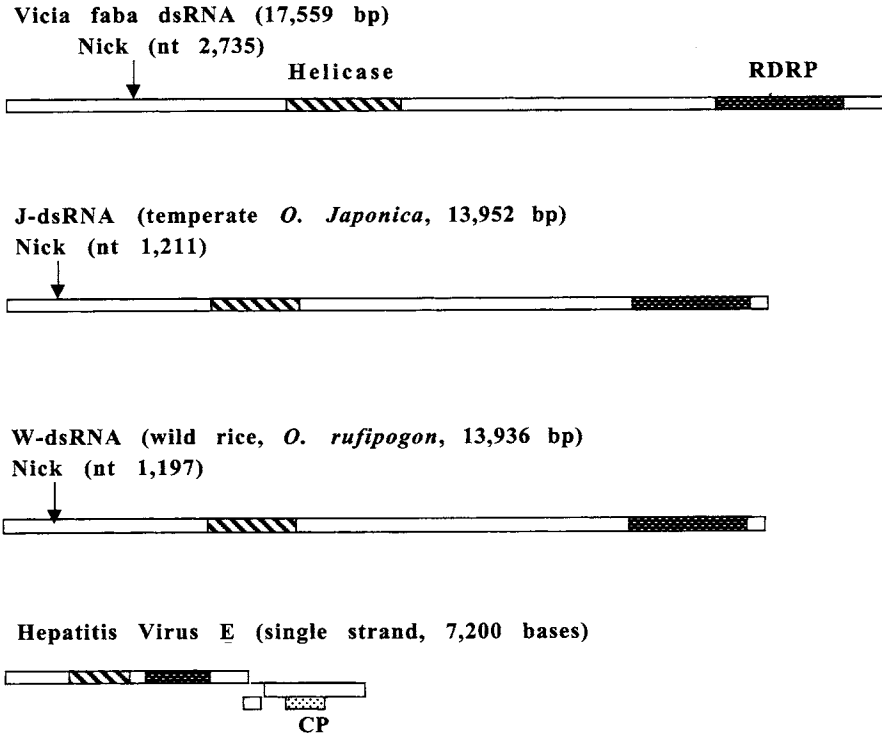

FIGURE 11.1 Similarities in the genetic and structural organization of large dsRNA replicons from plants. To date, all replicons of this type are characterized by a single, long open reading frame interrupted by a nick in the coding strand. However, a small proportion of these dsRNA molecules feature a continuous plus strand that is required for replication and may also be used for translation. Although no protease activity has been described to date, it is very likely that the long polypeptide is processed by embedded protease function(s) as in many other viruses. These dsRNA replicons originate probably from ancestral alpha-like single-stranded RNA viruses that have lost initially their coat protein (CP) gene and later their movement protein. Interestingly, the same arrangement of the helicase and RNA-dependent RNA polymerase (RDRP) genes was retained. The genome of Hepatitis E Virus, whose RDRP was found most similar to that of the V *faba* dsRNA, is shown for comparison.

several thousand years ago. In addition, the copy number of dsRNA per cell increased ten-fold in pollen grains for all lines; like the other large dsRNA replicons found in various plants, rice dsRNAs are noninfectious and cannot be transmitted horizontally but are transmitted exclusively in a vertical mode both through the egg and pollen. In view of the limited amount of cytoplasm present in a pollen grain, the higher copy number of dsRNA (1000 copies/cell) accounts for the highly efficient paternal inheritance (up to 98%).

While the sequence variations showed that these dsRNAs had evidently co-evolved with their host, the stability of dsRNA copy number among the various strains and the identical increase of dsRNA content in cultured cells pointed to a

highly conserved and stringent mechanism for copy number control. Therefore, Moriyama et al.[7] set out to determine whether the dsRNA maintenance system had similarly evolved by performing reciprocal crosses between the wild rice *(O. rufipogon,* containing W-dsRNA) and temperate *japonica* rice (containing J-dsRNA). They found out that several situations could be encountered where incoming W-dsRNA would either exclude, be excluded by, or coexist with the resident dsRNA in *the japonica* background. In those F1 plants harboring both dsRNA species, the copy number of the total population of dsRNA was maintained at about 100 copies per cell. When the self-pollinated progeny of an F1 plant harboring both dsRNA was analyzed, these were found to segregate away from each other in F2 plants; these now contained either W-dsRNA or T-dsRNA, or none at all, but no plant was found to contain both dsRNA species. However, both plants that were originally devoid of dsRNA and those F2 plants that contained no dsRNA after self-pollination of the F1 could support replication of new incoming dsRNA. Thus, the absence of dsRNA in rice did not result from a lack or loss of a specific maintenance factor. This situation contrasts with that observed in the "447" line of *V. faba* where selfing of the progeny obtained after a restoration cross never yielded any male-sterile plants. In *V. faba,* the "447" dsRNA was irreversibly lost after restoration, probably because this results in the elimination of such dsRNA maintenance factor(s). Similarly, crosses between temperate and tropical *japonica* lines (containing the J-dsRNA and T-dsRNA, respectively) yielded progeny containing both dsRNA species. However, when *O. rufipogon* was the recipient, resident W-dsRNA tended to exclude any other incoming dsRNA. It would be of interest to determine whether these two dsRNA species compete for limiting host-encoded factors for their replication and maintenance, or whether the wild rice cytoplasmic background does not support the presence and replication of other dsRNAs.

11.10 THE RICE DSRNAS ARE ALSO ASSOCIATED WITH A SPECIFIC RDRP AND CONTAINED IN MEMBRANOUS VESICLES

Based on their apparent association with CMS and enrichment in partially purified preparations of these organelles, large dsRNA replicons such as the LBN RNA found in maize[29] or the *Brassica* dsRNAs[30] were originally thought to be intramitochondrial. However, when better separation techniques, such as Percoll gradients, were used, it became clear that such dsRNA merely copurified with mitochondria, and this led Monroy et al.[31] to conclude that the occurrence of dsRNA in mitochondria may be less widespread than suggested in the literature.

Similarly, the large dsRNAs present in some rice cultivars were originally described as associated with cytoplasmic male sterility and thus with mitochondria,[3] but this was later disproved, since similar dsRNAs were also found in male fertile plants.[4,5] In addition, Fukuhara and colleagues (personal communication) have now found evidence that the rice dsRNA replicons are also associated with their specific RDRP and contained within membranous vesicles very similar to those containing the dsRNA genome ChV-1[15] and the "447" dsRNA of *V. faba.*[10]

11.11 HIGH MOLECULAR WEIGHT dsRNA REPLICONS IN PLANTS DERIVE FROM THE ALPHA-LIKE VIRUS SUPERGROUP

The first large unencapsidated dsRNA replicon to be sequenced was CHV-1, the virus associated with transmissible hypovirulence in chestnut blight.[32] Because it was found to encode two polyproteins that were processed by embedded cysteine-proteinase functions very reminiscent of that of potyviruses,[33] it was hypothesized that this dsRNA replicon had evolved from a potyvirus that had lost its coat protein and survived as the replicative form in a fungal cytoplasmic environment.[34]

When the first dsRNA from rice was sequenced, parallels were drawn with CHV-1 based on their apparent similarity.[5] It became however rapidly clear from further sequence comparisons that the polyproteins encoded by dsRNA replicons isolated from various plants were only distantly related to CHV-1 and potyviruses. In addition to the extensive intra-group homologies detected in two specific regions of the various rice dsRNAs, the helicase and the RDRP domains specific to RNA viruses, the next highest degree of similarity was found with the RDRP sequences of closteroviruses, Hepatitis E virus and Alfalfa mosaic virus, all of which belong to the "alpha-like supergroup" of single-stranded RNA viruses.[11,16] No significant homology was found with dsRNA viruses, which confirms that these large unencapsidated dsRNA replicons derive from single-stranded RNA viruses rather than dsRNA viruses. Studies with mutants of single-stranded RNA viruses showed that absence or loss of function of the coat protein resulted in a dramatic decrease of accumulation of positive strand RNA.[35–37] Similarly, *Umbraviruses* do not encode a coat protein. They depend on a helper luteovirus for encapsidation[38] and accumulate preferentially as dsRNA. These dsRNAs are most likely contained in virus-like particles (membranous vesicles) associated with the tonoplast of the cell vacuole.[39]

11.12 CONCLUSIONS

The long enigmatic, "endogenous" or "indigenous" unencapsidated large dsRNA replicons that had been identified in various plants have now revealed part of their secrets and allowed a common picture to emerge. Like any dsRNA molecule, they have to be topologically isolated from the cytoplasm and, since no virus particles could be isolated, they were for a long time suspected to be intramitochondrial. This contention was, however, increasingly challenged with improved purification techniques, and molecular cloning has now demonstrated conclusively that they use the universal code to express their genetic information. This contrasts with the few dsRNA plasmids or viruses that have been positively identified as being intramitochondrial and use the mitochondrial code instead (see the chapters by Tavantzis, Lakshman and Liu, and Buck and Brasier in this book).

The first unencapsidated large dsRNA replicon (CHV-l) conclusively demonstrated to be packaged in cytoplasmic membranous vesicles was the chestnut blight hypovirulence-associated, viral dnRNA.[14,32] Similarly, the *V. faba* "447" dsRNA was shown to be contained within membranous vesicles as a complex with its replicase,[10]

and a similar association was demonstrated to exist in CHV-l.[15] Thus, these structures are functionally equivalent to replicative complexes of positive strand viruses, which are known to be membrane-associated. Molecular cloning and sequencing confirmed this interpretation by demonstrating that these dsRNA replicons were much more closely related to ssRNA viruses (Potyviruses for CHV- 1, and Alphaviruses for the dsRNAs from *V. faba, O. sativa, O. rufipogon* and *P. vulgaris*) than to dsRNA viruses. This is consistent with the different strategy the latter use for their replication; plus-strand synthesis is directed by the RDRP contained in the virus particles and is followed by translation of the coat protein and the polymerase. The ssRNA and the polymerase are then encapsidated into previrions where second strand synthesis (i.e., the actual replication) occurs. Here again, no free dsRNA is ever found in direct contact with the cytoplasm.

One of the striking features shared by all large cytoplasmic dsRNA replicons sequenced so far is the presence of a single long open reading frame and its apparent interruption by a nick in the coding strand. Although it was tempting to draw the parallel with CHVl where ORFA and ORFB are translated by a stop-restart process,[32] the absence of a stop codon at the end of the 5' proximal small RNA rules out that it is used as a subgenomic messenger for expressing an equivalent ORFA. On the other hand, a small proportion of continuous positive strand has been detected in the "447" dsRNA[27] and now also in rice dsRNA (Fukuhara, personal communication). This limited population of uninterrupted positive strand, which is at any rate required for replication, may also be used for translation of the long polyprotein. The accurate but different position of the nick in all these dsRNAs indicates that it is certainly relevant to the life cycle of these replicons, although we have no indication of the mechanism by which it is generated.

This structural feature is unique to these large cytoplasmic dsRNA replicons found in plants and reinforces the contention that they derive from a common ancestor, most probably an infecting ssRNA virus whose coat protein gene was either lost or inactivated. Having established a new relation with the host plant, in which the rate of dsRNA synthesis paralleled the rate of cell division, the movement protein gene being no longer required became in turn dispensable and was probably lost. Indeed, no homology to movement proteins was detected in these large dsRNAs. Consequently, they cannot be transmitted horizontally by any of the classical inoculation procedures but are transmitted efficiently in a vertical manner through both egg and pollen. Nevertheless, such defective viruses retained two basic functions: the RNA-dependent RNA polymerase with its associated helicase, and the gene(s) that induce(s) the formation of the replicative vesicles from various endomembrane compartments[40] to accommodate their dsRNA genome.

The life cycle of these dsRNA replicons is essentially plasmid-like: their copy number is controlled by the cellular background and may be influenced by development.[6] They have established a long-standing relationship with their host with which they have co-evolved and are essentially devoid of detrimental effect. A 13.2 kb dsRNA found in the "Barsoy" cultivar of barley could be traced to an ancestral progenitor imported from Japan at the beginning of this century. Zabalgogeazcoa et al.[9] showed by pedigree analysis that this dsRNA had been maintained for over 90 years of selection. Similarly, cultivated lines of rice still contain dsRNA

after centuries of breeding, a strong indication that such dsRNAs do not cause any adverse effects on their host plants.

The case of the "447" cytoplasmic in *V. faba* is, however, exceptional for two reasons: first, the presence of the dsRNA has in this instance a phenotypic effect, namely pollen abortion, and second, it is the only case where a CMS phenotype seems to be associated with the presence of a large dsRNA replicon. Although the entire sequence of this dsRNA has been established[11] and its viral origin confirmed,[16] the mechanism by which one or several of the gene products encoded by this replicon cause CMS in this case remains unknown. Indeed, pollen formation is a highly energy-demanding process, and CMS results from a mitochondrial dysfunction in anther tissues, particularly in the cells of the tapetum layer that provide energy and metabolites to the differentiating microspores. Essentially, any perturbation of proper mitochondrial biogenesis (the number of these organelles increases 20- to 40-fold in tapetum cells during pollen formation) and function may lead to pollen abortion.

As a general rule, CMS can be traced to rearrangements in the mitochondrial DNA leading to the formation of chimaeric, nonfunctional polypeptides that perturb normal mitochondrial function. These may, for instance, compete with their normal counterparts in the multisubunit energy-generating enzymatic complexes in mitochondria as the pcf gene in petunia[41] or, as for the Texas CMS-associated *T-urfl3* in maize, assemble as pore-forming oligomers in the inner mitochondrial membrane.[42] Recently, cloning of fertility restorer genes has shed new light on the possible mechanisms of CMS. In some cases, fertility restoration could be assigned to modified transcription patterns through the use of alternative transcription initiation sites. Similarly, differential processing and/or editing of transcripts after restoration can also introduce new translation start and stop codons.[43] In all cases, this results in a decreased accumulation of the CMS-associated polypeptides. On the other hand, restoration of the Ogura male sterility in radish was correlated with an altered post-translational regulation. While transcription levels and translation patterns were unchanged, the decreased stability of the CMS-associated mitochondrial membrane protein resulted in its reduced accumulation after restoration. Finally, the *rf2* gene required for fertility restoration of the T-CMS in maize was recently shown to encode a putative aldehyde dehydrogenesase.[44] In this case, an altered mitochondrial function in anther tissues of male sterile plants may result in increased production of aldehydes which can be detoxified by the RF2 protein.[45]

The viral origin of the "447" dsRNA suggests additional possible mechanisms for this CMS. In fact, several dsRNA viruses infecting fungi, e.g., the killer virus of yeast and of *Ustilago maydis,* encode protein toxins. These polypeptides assemble in the plasma membrane of susceptible cells to form pores that damage them by causing efflux of ions and small electrolytes. It is tempting to speculate that the "447" dsRNA may encode such a protein toxin that would either be expressed selectively in anther tissues or activated by anther-specific compounds such as flavonoids, much like the *T-urf13* in maize.

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