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Double-Stranded RNA Viruses of *Saccharomyces cerevisiae*

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INFECTIOUS ELEMENTS OF *SACCHAROMYCES CEREVISIAE*

Two double-stranded RNA (dsRNA) viruses, two apparently naked single-stranded RNA (ssRNA) replicons, five retroviruses and, most recently, two putative prions have been found in the budding yeast, *Saccharomyces cerevisiae* (Table 1). In addition, retrotransposing mitochondrial introns have been found (76, 99) and segment 3 of brome mosaic virus can replicate in yeast cells (71). This review deals with the double-stranded RNA viruses, primarily the L-A virus and its killer toxin-encoding satellite dsRNAs and the cellular components that affect them. Recent reviews of the prions, ssRNA replicons, retroviruses (retrotransposons), and retrotransposing introns of *S. cerevisiae* are available (5, 10, 163, 167). Here, we emphasize those aspects of the study of L-A whose mechanisms have begun to be understood. Many other aspects have been reviewed recently (161).

Study of the yeast L-A virus system has provided insights into aspects of RNA transcription and replication, RNA packaging, virus structure, ribosomal frameshifting, protein N-acetylation, mRNA decapitation, translation of poly(A)$^-$ mRNA, proteolytic processing, and other virus-host and virus-virus interactions common among RNA viruses. These studies have been made possible by the classical genetic and molecular methods available for yeast studies in general and the special virus purification and in vitro RNA replication, transcription, and packaging systems developed to study L-A. As has been amply demonstrated since the early days of phage work, the study of viruses often sheds light on host processes. Moreover, it is no longer surprising to find that results obtained in yeasts have broad application in higher eukaryotes, a fact as true of yeast virology as it is of other areas.

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BIOLOGY OF THE YEAST dsRNA VIRUSES AND THE KILLER PHENOMENON

The two known dsRNA viruses of *S. cerevisiae* are called L-A and L-BC. Each is a family of structurally and functionally distinct viruses. Most yeast strains carry a member of one or both of these families, replicating stably in the cells, neither lysing them nor even detectably slowing their growth. This implies a balance between virus and host growth, some of the components of which will be discussed in this review. Like all known fungal viruses (reviewed in reference 163), L-A and L-BC spread by cell-cell fusion, a consequence of the mating process. In addition to the thick cell wall barrier to extracellular spread, it has been suggested that fungal viruses adopt this strategy because mating and hyphal fusion is very frequent in nature, making the extracellular route of spread dispensable. Indeed, nearly all yeast strains carry several of the RNA rep-

TABLE 1. Infectious elements of *S. cerevisiae*

Virus	Genome size (kb)	Encoding chromosomal gene and function	Encoded protein(s)	Group	Feature(s)	Reference(s)[a]
dsRNA viruses						
L-A	4.6		Major coat protein (Gag) RNA polymerase (Pol)		−1 ribosomal frameshift makes Gag-Pol	130
M_1, M_2, M_3, M_{28}, etc.	1.6–1.8		Preprotoxin		Satellites of L-A; processing by Kex1p, Kex2p	6, 97, 125
L-BC	4.6		Major coat protein (Gag) RNA polymerase (Pol)			130
ssRNA replicons						
20S RNA (= W dsRNA)	2.9		RNA polymerase		Copy number induced by N starvation, high temperature	153
23S RNA (= T dsRNA)	2.5		RNA polymerase		Copy number induced by N-starvation, high temperature	34, 153
Brome mosaic virus segment 3[b]			CAT or URA3[b]		Replicates dependent on segments 1 and 2A[b]	71
Retroviruses						
Ty1, Ty2	5.8		Gag, Gag-Pol	*copia*	+1 ribosomal frameshift makes Gag-Pol	10
Ty3	5.3		Gag, Gag-Pol	*gypsy*	+1 ribosomal frameshift makes Gag-Pol	121
Ty4	6.3		Gag, Gag-Pol	*copia*		136
Ty5			Gag, Gag-Pol	*copia*		149
Prions						
[URE3]		*URE2* (nitrogen catabolite repression)				162
[PSI]		*SUP35* (translation termination)				162

[a] References to original discovery or recent paper.
[b] Brome mosaic virus segment 3 replicates in yeast cells depending on the products of segments 1 and 2 supplied from cDNA expression clones. The chloramphenicol acetyltransferase (CAT) or *URA3* genes were inserted as markers (71).

licons. Mammalian viruses, such as human immunodeficiency virus and herpesviruses, also utilize this means of spreading, although they also have the extracellular route as an alternative. Seed-transmissible and pollen-transmissible plant viruses likewise spread without exiting the cell.

Many strains containing L-A also carry a satellite dsRNA of L-A, called M dsRNA, that encodes a secreted protein toxin (the killer toxin) and immunity to that toxin. There are actually several different M dsRNAs (M_1, M_2, M_3, M_{28}, etc.), each encoding a distinct toxin-immunity specificity. The production, processing, secretion, and action of these toxins have been studied in depth, and this phenotype has been used in the genetic analysis of the L-A virus system. Defective interfering derivatives of M_1 (44) and of L-A (39) have been described. These are deletion mutants encoding none of the proteins encoded by the normal forms but having the ability to interfere with their replication. X dsRNA, a 530-bp derivative of L-A, has been particularly useful.

L-A VIRUS STRUCTURE: $T = 1$ WITH 60 ASYMMETRIC Gag DIMERS

The L-A virions are icosahedral particles, about 39 nm in diameter, each containing a single 4.6-kb dsRNA molecule (Fig. 1). The virus has a single major coat protein of 76 kDa, called Gag, and a minor 180-kDa protein species, which is a Gag-Pol fusion protein (see below). Their hydrodynamic properties first suggested that each particle had 120 Gag monomers (37), a paradoxical result since the expected numbers for the two simplest icosahedral lattices, with triangulation numbers $T = 1$ and $T = 3$, are 60 copies and 180 copies, respectively (21). Scanning transmission electron micrographs confirmed the earlier estimate of 120 Gag molecules per particle (23). Cryoelectron microscopy and image reconstruction shows that the L-A viral particles have a single protein shell and that the particles have a $T = 1$ symmetry, with 60 equivalent asymmetric dimers of Gag (23). This structure is shared by the P4 dsRNA virus of the fungus *Ustilago maydis* (23).

dsRNA viruses of higher eukaryotes have two shells, with the outer shell removed in the lysosomes by cellular enzymes. The inner shell or core contains the viral genome and the transcription and replication enzymes and so is functionally similar to the L-A virus itself. Reovirus cores have 120 copies of the 155-kDa λ1 protein (123), and rotavirus cores have 120 copies of their major protein, the 94-kDa VP2 (113). This suggests that the structure of reovirus and rotavirus cores will resemble the L-A structure, a structure which is uniquely suited to the role of the fungal dsRNA virions and the cores of mammalian dsRNA viruses as an intracellular compartment for transcription and replication of the viral genome (23).

VIRAL REPLICATION CYCLES

Synthesis of both the plus and minus RNA strands occurs within the viral particles but at different points in the viral replication cycle (Fig. 2). Mature viral particles, which contain a single copy of the dsRNA genome, transcribe it in a conservative reaction. The plus strand transcripts produced are extruded from the viral particles, where they serve as mRNA for the synthesis of new viral proteins and as the species that is packaged by these proteins to form new viral particles. The new particles, containing the viral plus strands, then carry out minus-strand synthesis (the replication step) to form dsRNA, completing the cycle. This cycle closely resembles that of reoviruses and rotaviruses (123) except for the absence of the assembly of the outer shell and the extracellular phase.

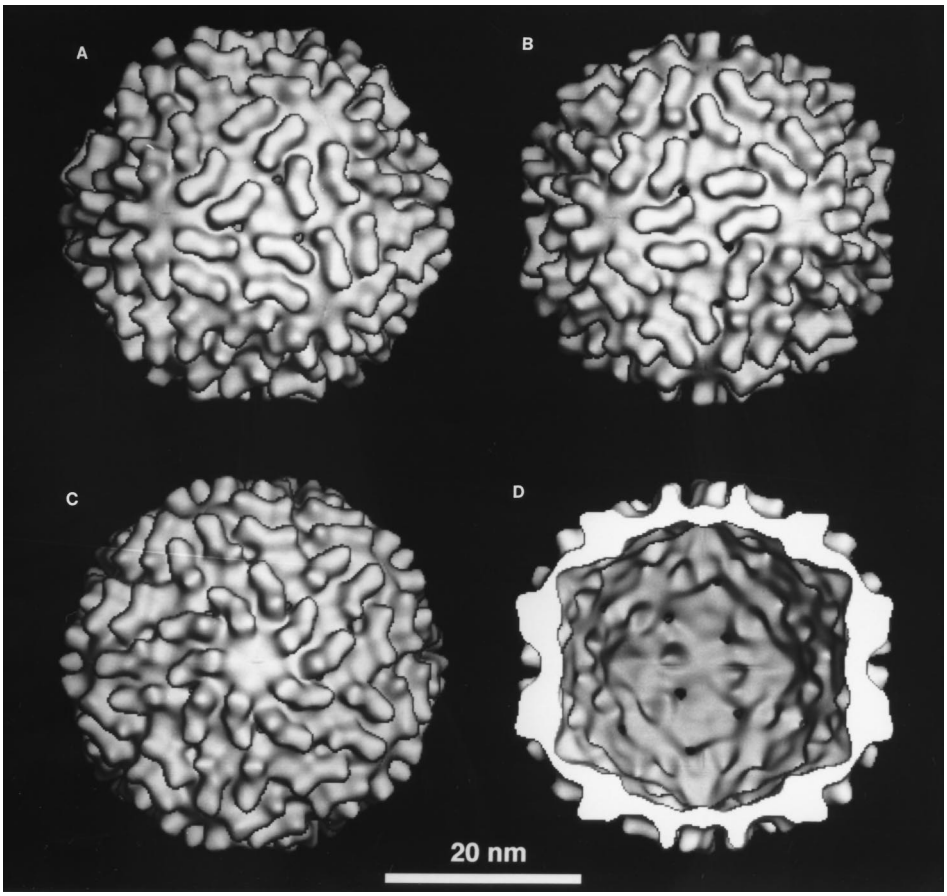

FIG. 1. Three-dimensional structure of empty L-A virus at a resolution of 26 Å (2.6 nm) (23). Surface-shaded representations of the outer surfaces of empty capsids, viewed along fivefold (A), threefold (B), and twofold (C) axes of symmetry, are shown. A model with the front half of the protein shell removed is also shown (D). L-A virions have 60 holes about 1.5 nm in diameter that extend through the capsid wall. These holes, three per facet and spaced about 6 nm apart, might be the channels through which nucleoside triphosphates enter and nascent plus-strand ssRNA is extruded into the cytoplasm. Photo courtesy of J. R. Caston, B. L. Trus, and A. Steven.

The replication cycle of the toxin-encoding satellites of L-A (M_1, M_2, etc.) and the defective interfering mutants of L-A (such as X dsRNA) is similar to that of L-A itself, except that not all plus-strand transcripts are extruded from the particle (Fig. 2, bottom). These satellites and defective interfering particles are encapsidated in separate particles from those containing L-A dsRNA (11). Particles containing either one or two M_1 dsRNA molecules are found, consistent with the fact that M_1 is less than half the size of L-A (37). Particles with two M_1 dsRNA molecules per particle are full and eject all of the new plus-strand transcripts from the particle. Over 60% of those with only one M_1 dsRNA molecule per particle retain the new plus strand within the particle, where it can be converted to a second dsRNA molecule (37). This mechanism is called headful replication, to distinguish it from the headful packaging mechanism found for some DNA bacteriophages. Each new particle packages a single plus strand, which replicates inside the viral particle until it is full. Particles containing X dsRNA (530 bp) also have the properties expected from a headful replication mechanism. Particles having 1 to 8 (4,600/530 = 8.6) X dsRNA molecules per particle are found (39). As further confirmation of this idea, it has been shown directly that L-A virus particles encapsidate one molecule per particle (47). These results imply that, as for other isometric virus particles, the capacity of the particle is determined by the structure of the coat protein and not by the size of the genome (as is true for filamentous viruses). The particle capacity is enough for about one L-A dsRNA molecule. That this mechanism is not unique to L-A is suggested by the finding of particles containing one, two, or three molecules of a dsRNA segment in one of the killer-associated viruses of *U. maydis* (13).

L-A ENCODES Gag AND Gag-Pol

The proteins encoded by L-A plus-strand ssRNA and the special sites on the RNA are shown in Fig. 3. There are two open reading frames (ORFs), overlapping by 130 nucleotides, with the 3′ ORF in the −1 frame relative to the 5′ ORF (66). The 5′ ORF encodes the major coat protein, while the 3′ ORF includes the consensus amino acid sequence patterns typical of the RNA-dependent RNA polymerases (RDRPs) of plus-strand ssRNA and dsRNA viruses. This evident analogy with retroviruses led us to name the 5′ ORF *gag* (and its 76-kDa protein product Gag) and the 3′ ORF *pol* (and its protein product Pol). Pol is expressed only as a 180-kDa Gag-Pol fusion protein formed by a −1 ribosomal frameshift event executed by about 2% of the ribosomes translating the region of overlap of *gag* and *pol* (29, 50, 66). This system has been used in extensive studies of factors affecting ribosomal frameshifting, motivated in part by the importance of this process in the propagation of HIV and other retroviruses (see below).

Gag not only plays a structural role in forming the compart-

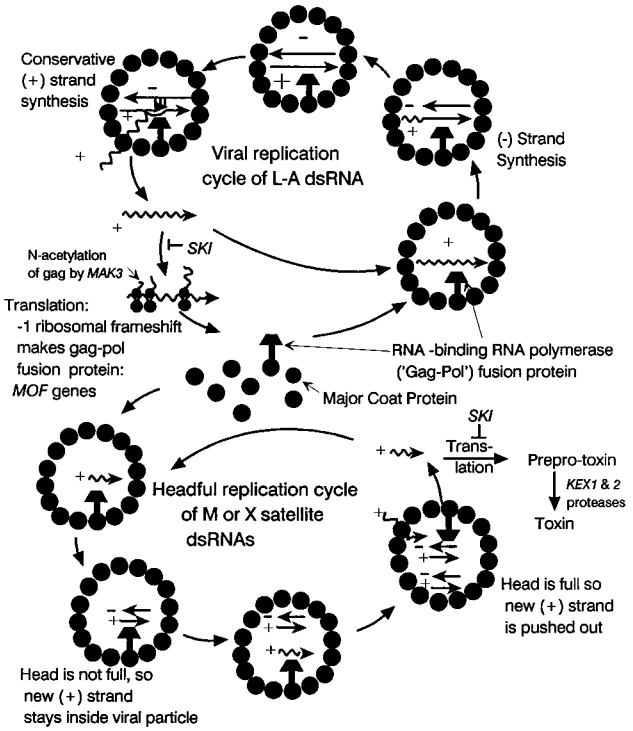

FIG. 2. Replication cycles of L-A and its satellites.

ment in which the dsRNA genome is protected from other cellular components while it is replicated and transcribed but also plays a catalytic role. Gag can remove the 5′ cap structure, ^{7m}GMP, from cellular mRNAs and attach it to itself at His-154 (8, 9). This activity is important in expression of viral mRNAs, as discussed below.

The similarity of the RDRP domain of the L-A Pol to that of other plus-strand ssRNA and dsRNA viruses has motivated a detailed study of its functional domains. The importance of the most highly conserved regions for virus propagation has been explored in detail by "alanine scanning mutagenesis" (118). As expected, the residues most highly conserved among plus-strand and dsRNA viruses were those most strictly required to propagate the M_1 satellite. Remarkably, substitution of the 75 amino acid residues in the L-A Pol corresponding to the SG. . .T. . .NT. .N and GDD domains with the reovirus or Sindbis virus sequence allowed significant RNA polymerase activity, although many alanine substitution mutations within these regions were almost completely inactive.

Pol has three ssRNA-binding domains (Fig. 3), each necessary for virus propagation (116, 117). The central ssRNA-binding domain (residues 374 to 432 of Pol) is unusual in that it is detected only after deletion of a part of Pol C-terminal to it (residues 506 to 546). This "cryptic" RNA binding may be part of the replication apparatus. There are several steps in the viral propagation process at which the polymerase must bind and then release the viral RNA, and this conditional binding may be reflected in the observed cryptic activity. The central cryptic ssRNA-binding domain includes one of the domains conserved among RDRPs (82), suggesting that this binding site may function in polymerization. The proximity of the C-terminal ssRNA-binding domain to other consensus RDRP do-

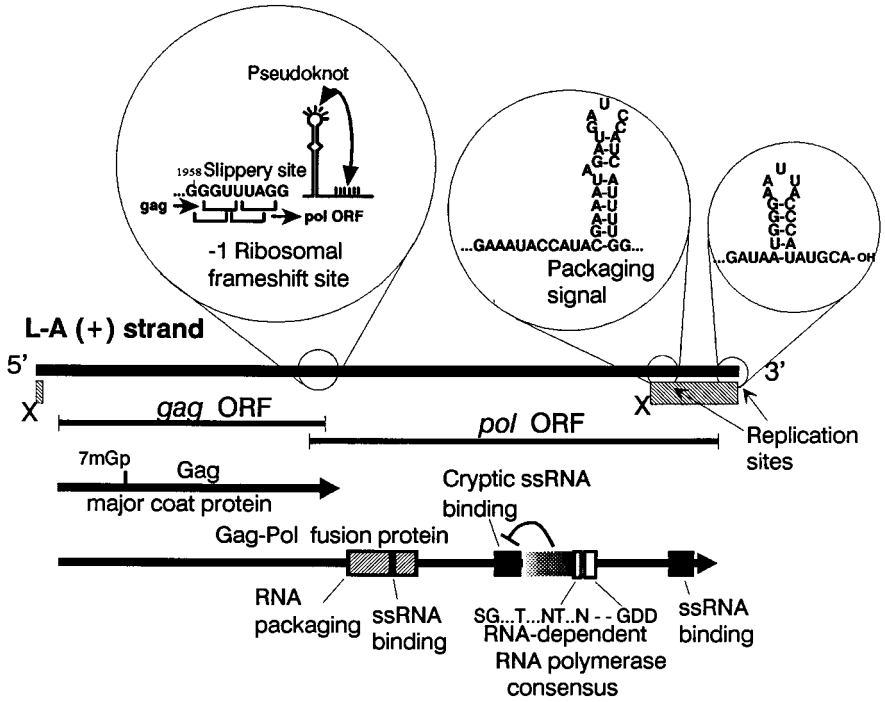

FIG. 3. L-A plus-strand coding information and sites of −1 ribosomal frameshifting, packaging, and replication. The replication reaction (minus-strand synthesis on a plus-strand template) requires the 3′-end site shown at the right and the internal replication enhancer (IRE) that largely overlaps but may be distinct from the packaging signal. The *gag* ORF encodes the major coat protein, Gag, which can remove the ^{7m}GMP from capped mRNAs and covalently attach it to His-154. Pol includes the RDRP, three ssRNA-binding sites (the central one cryptic and inhibited by a region C-terminal to it), and a region necessary for packaging viral plus-strand ssRNA. The 5′ and 3′ regions retained by the X dsRNA (a deletion mutant of L-A) are shown by the hatched boxes labeled X.

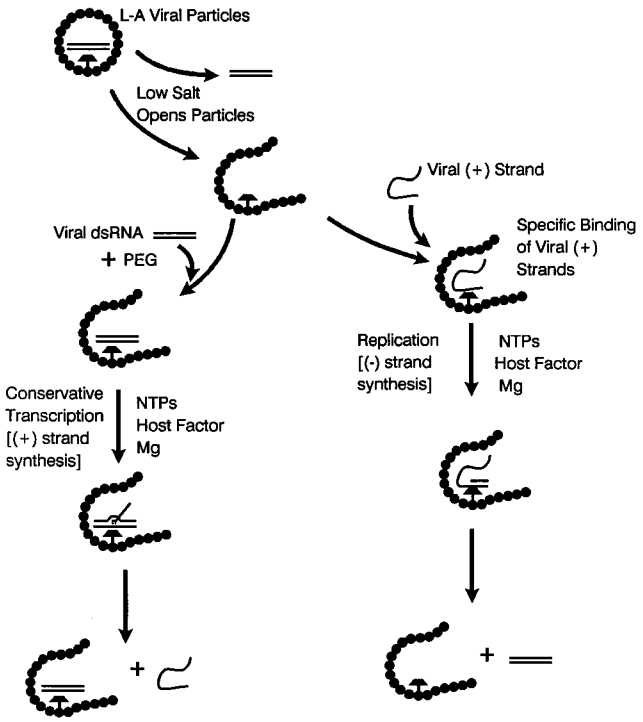

FIG. 4. In vitro systems; the template determines the reaction. Purified L-A viral particles release their dsRNA when dialyzed against low-salt solutions. Added viral plus-strand ssRNA is then specifically bound and can be replicated. Alternatively, if viral dsRNA is added, along with high concentrations of polyethylene glycol (PEG), transcription takes place. NTP, nucleoside triphosphate.

mains likewise suggests that this may be its function. The N-terminal ssRNA-binding domain (residues 172 to 190 of Pol) is within the region shown to be essential for packaging of viral RNA (residues 67 to 213 of Pol), and for this reason it is believed to be the part of the protein that binds the packaging site on the RNA. This is the only ssRNA-binding domain whose deletion eliminates packaging ability, supporting this assumption (116, 117).

REPLICATION AND TRANSCRIPTION OF VIRAL RNA: IN VITRO SYSTEMS

Dialysis of L-A dsRNA containing viral particles against low-salt solutions results in these particles losing their RNA (Fig. 4). However, added viral plus-strand ssRNA is specifically bound and converted to the dsRNA form by these opened empty particles (51). This replicase activity utilizes any of the templates known to be replicated by L-A, including L-A plus strands, M_1 plus strands, and plus strands of X. Artificial transcripts of a cDNA clone of X dsRNA were used to determine the sequences and structures recognized by the replicase activity. Recently, in vitro template-dependent replication of rotavirus has been achieved by applying this method to rotavirus core particles (22). This should enable the complete determination of the replication signals for this important pathogen.

The replicase reaction absolutely requires a correct 3′-end sequence and structure, but either the L-A or M_1 3′ end will suffice, and these are substantially different (36, 51, 53) (Fig. 3; also see Fig. 5). The 3′-terminal three bases of the L-A plus strands (. . . .AUGC-OH) are each necessary for template activity. The transcriptase also often appends an extra untemplated A or G residue on the 3′ end of the plus strands (17, 60). The replicase works equally well with or without the presence of an extra untemplated A residue at the 3′ end of L-A (+) strands (. . .AUGCA-OH) (36). The 3′ five bases of M_1 (. . .ACAUG-OH) can be substituted for the 3′ four bases of L-A, and the resulting template is fully active. The stem-loop structure 4 nucleotides from the 3′ end of L-A is also necessary for template activity: the stem structure, but not its sequence, is recognized. Although M_1 plus strands are as active a template as L-A plus strands, the closest stem-loop structure in M_1 is 16 bp from the 3′ end.

While the correct 3′-end sequence and structure is sufficient for minimal template activity, a second region, 400 nucleotides from the 3′ end, is necessary for full activity (36). This internal replication enhancer (IRE) overlaps the packaging site in the RNA (see below and Fig. 3). The 3′ end of the M_1 plus-strand ssRNA can replace the L-A 3′ end, indicating that there is no direct specific interaction (such as base pairing) between the internal site and the 3′ end (Fig. 5). The relation of the IRE to the 3′-end site was investigated in detail by placing the two sites on separate molecules (53). A molecule with the correct 3′ end but lacking the IRE was replicated at 10% of the normal rate. Addition of a second molecule without the normal 3′ end but carrying the IRE failed to stimulate replication of the molecule with the 3′-end site, instead inhibiting the residual level of synthesis, presumably by binding the polymerase to a molecule whose 3′ end was not suitable for initiating synthesis. When the two sites were on separate molecules which could hydrogen bond to each other (IRE ⌋≡⌊ 3′ end), stimulation of synthesis was observed, and only the molecule with the correct 3′ end was converted to dsRNA form (53). These results indicate that the polymerase binds first to the internal site and that this brings it into proximity to the 3′ end, where synthesis is to begin. It does not track down the RNA chain to find the 3′ end but probably binds to the 3′ end while still bound to the internal site, forming a loop. Alternatively, binding to the IRE may facilitate initiation by increasing the concentration of polymerase at the 3′ end. The central and C-terminal RNA-binding sites may be involved in these processes (116, 117).

Having converted the plus strands to dsRNA, the L-A viral particles switch to carrying out a conservative transcription reaction (plus-strand ssRNA synthesis on the dsRNA template) (48, 104, 126), producing transcripts that lack a 5′ cap structure and a 3′ poly(A), although, as mentioned above, a single untemplated A or G residue is found at the 3′ end of plus strands (17, 60).

The L-A opened empty particles used for the in vitro replication reaction were also used to develop an in vitro template-dependent dsRNA transcription system (52) (Fig. 4). The transcriptase activity of this preparation requires a very high dsRNA concentration and 20% polyethylene glycol but is specific for the L-A dsRNA or other normal templates such as X or M_1 dsRNAs. Because large amounts of template are necessary and the activity is not high, the precise features required for a dsRNA template to be recognized by the transcriptase have not yet been established. However, X dsRNA includes only 25 bp from the 5′ end of L-A, and it is actively transcribed; therefore, it is assumed that the transcription recognition signal is within this region. A similar activity of L-BC opened empty particles prefers L-BC dsRNA as the template (118a).

TRANSLATION OF VIRAL mRNA

The natural battleground between an RNA virus and its host is the translation apparatus. Many RNA viruses turn off host protein synthesis (poliovirus, influenza virus, vesicular stoma-

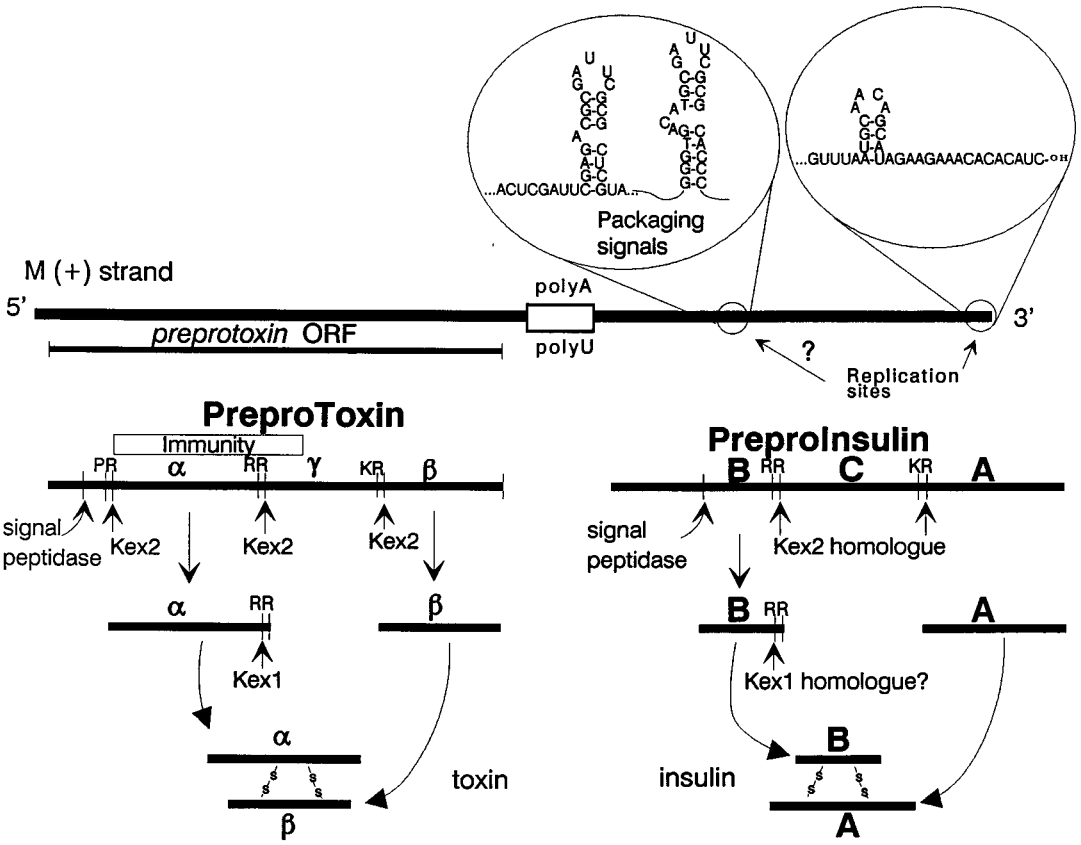

FIG. 5. M_1 plus-strand coding and packaging and replication sites. The killer preprotoxin encoded by M_1 is processed in a manner that is analogous to preproinsulin processing by homologous enzymes. The packaging signals of M_1 and the 3′ replication site are shown. It is not known whether there is an IRE for M_1. The internal poly(A) sequence encoded on the M_1 plus strand apparently does not substitute for a 3′ poly(A) structure in translation, since the translation of this mRNA is sensitive to the Ski2p, Ski3p, Ski8p system that specifically blocks non-poly(A) mRNA translation.

titis virus, Sindbis virus, coronaviruses, etc.) but can ignore the DNA replication, transcription, and cell cycle apparatus, and most other macromolecular systems. L-A does not shut off host protein synthesis but must deal with the host requirement that mRNAs have 5′ caps and 3′ poly(A). This requirement has forced RNA viruses to adopt a variety of tricks. Influenza virus, replicating in the cell nucleus, cleaves the methylated 5′ cap structure from nascent cellular mRNAs (112), and the viral RNA polymerase uses them as primers for synthesis of viral mRNA (12). The 3′ poly(A) structure is added by the viral polymerase repeatedly copying a 3′ subterminal oligo(U) sequence in the viral RNA (reviewed in reference 79). Bunyaviruses also appropriate caps from cellular mRNAs in the same manner (7, 110). Members of the family *Reoviridae* encode their own capping enzymes (24, 55) but lack 3′ poly(A). Sindbis virus has a 5′ cap, synthesized by viral protein nsP1 (122), and has an encoded 3′ poly(A). Picornaviruses have an encoded 3′ poly(A) and lack 5′ caps but have developed an internal ribosome entry site or ribosome landing pad, a special 5′ structure that induces the ribosome to ignore the absence of a 5′ cap and start translation far from the 5′ end of the mRNA (72, 111). This mechanism is apparently also utilized by a coronavirus for translation of one of its mRNAs (91).

The L-A virus lacks both the 5′ cap structure and the 3′ poly(A), and yet its major coat protein, Gag, may form up to several percent of total cell protein. Recent work has made clear that translational mechanisms involving the cap and poly(A) structures are important in determining the level of expression and efficiency of propagation of L-A and its satellites.

The *SKI2,3,8* System Blocks the Translation of Non-Poly(A) mRNA

A system of six chromosomal genes, *SKI2*, *SKI3*, *SKI4*, *SKI6*, *SKI7*, and *SKI8* (for superkiller), negatively controls the copy number of L-A and its satellites, M and X dsRNAs (3, 119, 144). At least *SKI2* and *SKI8* also lower the copy numbers of L-BC dsRNA (3) and the ssRNA replicon, 20S RNA (96).

When M_1 or M_2 dsRNA is present, mutation of any of these *ski* genes leads to cold sensitivity of cell growth (119). An additional non-Mendelian element, called [D] for disease, makes *ski* mutants carrying M dsRNA unable to grow at elevated temperatures as well and grow slowly even at 30°C (38). The [D] genome has not been identified, but it is not located on L-A, M, or mitochondrial DNA; however, it appears to depend on L-A for its propagation (38). Two lines of evidence show that the cold sensitivity of *ski* mutants is not due to overproduction of the killer toxin: (i) the S dsRNA deletion mutants of M_1 (44) make *ski* cells cold sensitive, although they are deleted for the toxin gene; and (ii) an M_1 cDNA expression clone makes cells produce much more toxin than a *ski* mutation but does not make the cells cold sensitive. Thus, the *SKI* genes are essential to prevent viral cytopathology. However, a deletion of *ski2*, *ski3*, or *ski8* resulted in normal growth in the

TABLE 2. *SKI2*, *SKI3*, and *SKI8* block translation of non-poly(A) mRNA[a]

Mutant/ wild type	Luciferase production relative to C^+A^+ cells[b]			
	C^+A^+	C^+A^-	C^-A^+	C^-A^-
ski2/+	(1.0)	18	0.5	2.3
ski3/+	(1.0)	22	0.6	2.7
ski8/+	(1.0)	34	1.2	5.0

[a] Spheroplasts were transfected with luciferase reporter mRNAs by electroporation, and luciferase activity was measured after outgrowth.

[b] Values are from at least two independent transfections of two different spheroplast preparations for each strain. All strains lacked L-A and M_1 viruses and L-A cDNA clones, except strain 2601 and its *ski3*::*URA3* derivative, which carried L-A and M_1 dsRNAs. C^+, capped; C^-, uncapped; A^+, poly(A)$^+$; A^-, poly(A)$^-$. Data are from reference 95.

absence of M dsRNA, indicating that the only essential function of these genes was in blocking viral propagation.

As described in more detail below, the chromosomal *MAK* genes are necessary for the propagation of M_1 and M_2 dsRNAs and in some cases L-A as well. Mutations in many of these genes are suppressed by *ski* mutations (146). M_1 propagation can be supported by L-A viral proteins supplied from a cDNA clone of L-A, but under these circumstances (just as when cells are *ski*), many of the *MAK* genes necessary for propagation of M_1 are no longer needed (165). Therefore, the effect of *SKI2* on propagation of M_1 supported by the L-A cDNA clone was examined (170). It was found that while a *ski2* mutation increased the copy number of M_1 dsRNA when its replication was supported by the L-A virus, there was no such increase when the L-A cDNA clone was the sole source of viral proteins. Although the copy number of M_1 did not increase in the *ski2* mutant, the amount of toxin produced (made from M_1 viral mRNA) did increase. The *ski2* mutation did not, however, affect toxin production from a cDNA clone of M_1, indicating that this was not an effect on the processing or secretion of the toxin protein. It was suggested that since the viral mRNA lacked both 5′ cap and 3′ poly(A) structures but the mRNA made from the cDNA clones contained both structures, the *SKI2* gene might be acting by blocking the translation of mRNAs lacking one or both of these modifications (170).

This hypothesis was directly tested by examining the translation of luciferase from synthetic mRNAs, with or without these modifications, that were electroporated into cells (95) (Table 2). The critical feature was found to be the 3′ poly(A) structure, with capped, non-poly(A) mRNA producing 20- to 40-fold more luciferase in *ski2*, *ski3*, or *ski8* cells than in isogenic wild-type cells. mRNAs lacking both cap and poly(A) were translated two- to fivefold better in the *ski* mutants than in the wild type, corresponding to the level of derepression of copy number of L-A, L-BC or 20S RNA. The kinetics of luciferase synthesis and direct measurements of M_1 mRNA levels indicate that these *SKI* genes affect primarily the initiation of translation rather than the stability of the mRNA (95). Thus, the *SKI2*, *SKI3*, and *SKI8* genes are part of a cellular system that specifically blocks the translation of non-poly(A) mRNAs.

M_1 Propagation Depends Critically on Free 60S Ribosomal Subunit Levels

Nearly 30 chromosomal genes, called *MAK* (for maintenance of killer), required for stable propagation of M_1 dsRNA have been defined genetically (155, 156, 166) (Table 3). Among these, only *MAK3*, *MAK10*, and *PET18* were found necessary for L-A propagation (130, 169). *PET18* is actually a large deletion between two copies of Ty1 (145). Careful examination of several of these mutants showed that although they did not lose L-A dsRNA, the levels of L-A (and Gag) were decreased two- to fourfold compared with those in isogenic wild-type strains (107).

MAK8 is identical to *TCM1*, encoding ribosomal protein L3 (168), while *MAK7* is identical to *RPL4A* (107), one of two genes encoding ribosomal protein L4, and *MAK18* is identical to *RPL41B* (20), encoding ribosomal protein L41. That all three of these genes encode 60S ribosomal subunit proteins led us to examine the ribosomal subunit profiles of all *mak* mutants (107). Mutants defective in any of 20 *MAK* genes showed decreased levels of free 60S subunits; none showed changes in levels of 40S subunits (20, 107). Cycloheximide acts on ribosomal protein L29 (45, 135) and cures M_1 dsRNA (43).

The above results indicate that the supply of free 60S subunits is critical for M_1 propagation and affects the L-A copy number to a more modest extent. Although the *mak* mutations result in loss of M_1 dsRNA when M_1 relies on the L-A virus for its supply of viral proteins, these mutations do not cause the loss of M_1 or even decrease its copy number when viral coat proteins are supplied from an L-A cDNA clone (20, 107, 165). Cycloheximide likewise does not cure M_1 from a cell in which it is supported by the L-A cDNA clone (20). These results again suggest a relation to the absence of 5′ cap and 3′ poly(A) on viral mRNAs. The *mak* mutations affecting 60S subunit levels have long been known to be suppressed by *ski2* and *ski3* mutations (146). Since these genes are now known to act by blocking translation of non-poly(A) mRNAs (95) (see above), the possibility arose that the level of 60S subunits was similarly critical for translation of non-poly(A) mRNA.

The determination that *MAK7* is *RPL4A* led to an under-

TABLE 3. Chromosomal genes needed for propagation of M dsRNA

MAK gene(s)	Needed by	Encoded protein or function	Reference(s)
MAK3	M, L-A	*N*-Acetyltransferase modifying Gag	139, 140
MAK10	M, L-A	Respiration, no homology	27, 88
PET18 (=*MAK30* + *MAK31*)	M, L-A	Unknown	90, 145
MAK1 (=*TOP1*)	M	DNA topoisomerase I	141
MAK2, *MAK5*, *MAK6*, *MAK9*, *MAK12*, *MAK13*, *MAK14*, *MAK17*, *MAK20*, *MAK22*, *MAK23*, *MAK24*, *MAK27*	M	60S subunit biosynthesis	107
MAK4, *MAK15*, *MAK21*, *MAK25*, *MAK26*	M	Unknown	
MAK7 (=*RPL4A*)	M	60S subunit protein L4	107
MAK8 (=*TCM1*)	M	60S subunit protein L3	168
MAK11	M	60S subunit biosynthesis	65, 107
MAK16	M	60S subunit biosynthesis	160, 107
MAK18 (=*RPL41B*)	M	60S subunit protein L41	20
MAK19	M	60S subunit biosynthesis	20
MKT1, *MKT2*	M_2 only	Unknown	157, 159

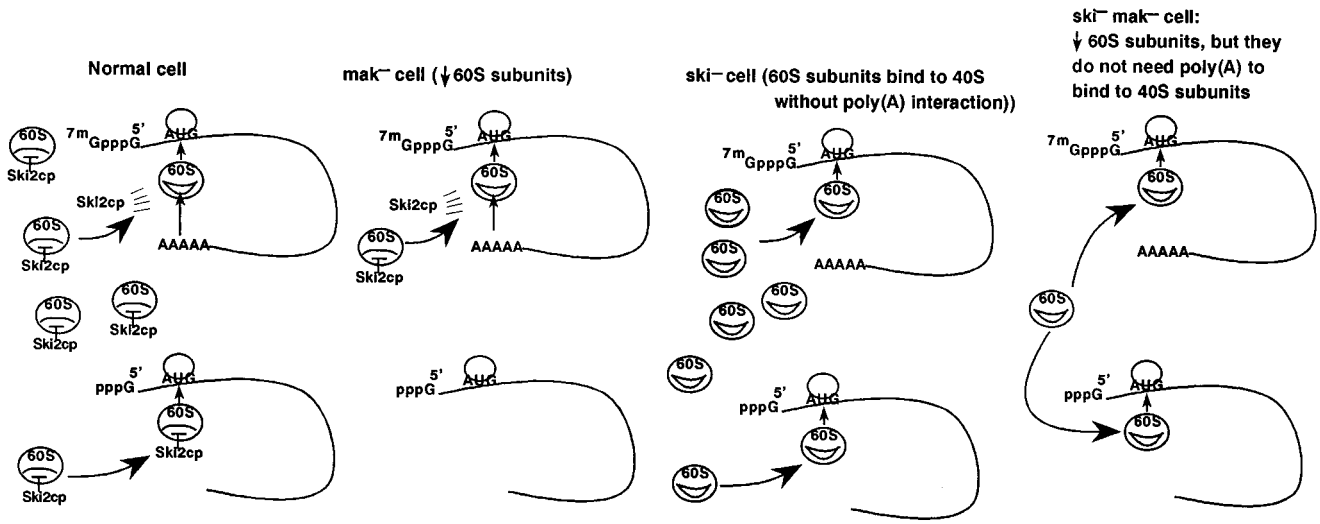

FIG. 6. The *SKI2,3,8* system appears to make 60S subunits require interaction with 3′ poly(A) before they will join the 40S subunits. This model explains the stringent requirement of M_1 for normal levels of free 60S ribosomal subunits: in 60S-deficient cells, viral transcripts compete poorly with poly(A)$^+$ cellular mRNAs. The *ski2*, *ski3*, or *ski8* mutations suppress *mak* mutations without restoring normal levels of 60S subunits.

standing of *KRB1*, a dominant suppressor of *mak7-1* which, although tightly centromere linked, defied mapping to the 16 known chromosomes (164). *KRB1* is, in fact, a stable extra chromosome formed from a fragment of chromosome XII on which lies a second copy of *RPL4B* (106). While a single copy of *RPL4* is sufficient for cell growth (albeit with low levels of 60S subunits), two copies are necessary for propagation of M_1 dsRNA. This can be either *RPL4A* on VIII and *RPL4B* on XII or two copies of either gene (106). This result again shows the importance of the level of free 60S subunits for M_1 viral propagation.

Do *SKI2*, *3*, and *8* Determine 60S Subunit Interaction with Poly(A)?

Translation in eukaryotes begins with the 40S subunit and associated initiation factors binding to the 5′ cap structure and moving to the initiator AUG. There it awaits the 60S subunit. Munroe and Jacobson (102) have suggested, on the basis of biochemical evidence, that the 60S subunit must interact with the 3′ poly(A) structure before it can join with the 40S subunit waiting at the initiator AUG. If the *SKI2*, *SKI3*, and *SKI8* genes mediated this requirement, it would provide a unified explanation for many of the data discussed here (Fig. 6).

The *ski2-2* mutation suppresses *mak7-1* (= *rpl4A*) without restoring the level of 60S subunits to normal (95). Presumably, the 60S subunits are qualitatively changed so that they no longer require interaction with the 3′ poly(A) before they will join with the 40S subunit waiting at the AUG. This implies that ribosome biogenesis is affected by the *SKI2,3,8* system. Consistent with this idea, it was found that Ski3p is nuclear (115) and Ski2p contains a glycine-plus-arginine-rich region typical of nucleolar proteins (170). Two close mammalian homologs of Ski2p have been identified (26, 86, 105). One of these, called 170A (86) or *SKI2W* (26), has been localized to the nucleolus of HeLa cells.

Members of the *Reoviridae* (including reoviruses, rotaviruses, and orbiviruses) have a 5′ cap structure but lack 3′ poly(A). Mammalian cells contain close homologs of *SKI2*, which might function similarly to the yeast *SKI2* system. How do members of the *Reoviridae* ensure the efficient translation of their mRNAs?

Gag Makes Decapitated Decoys To Distract the *SKI1*/*XRN1* Exoribonuclease

While many of the *MAK* genes affect 60S subunit biogenesis and the *SKI2,3,8* group appears to involve 3′ poly(A), the *SKI1* gene is concerned with the 5′ cap structure of mRNAs. The *ski1-1* mutation results in the superkiller phenotype and also produces a slow-growth phenotype (144). Stevens identified a 5′ → 3′ exoribonuclease specific for uncapped mRNA (133, 134). The properties of mutants deleted for the gene encoding this enzyme (*XRN1*) showed that it was not essential but that it was involved in mRNA degradation, because mRNAs lacking cap and/or poly(A) accumulated in mutant cells (63). Detailed studies of the mRNA turnover pathways for several genes have shown that Xrn1p is involved in the degradation of mRNAs whose poly(A) has been substantially shortened (100, 101).

The *xrn1* mutants have many phenotypes, some no doubt as a result of their affecting the turnover of most mRNAs in the cells (63, 78, 80, 85, 133, 143). Recently, Johnson and Kolodner mapped *xrn1* and, finding it located near the known position of *ski1* on chromosome VII, proceeded to show that *xrn1* and *ski1* are allelic (73), a result which we have confirmed (95a). The known specificity of the Xrn1p exoribonuclease for uncapped RNAs implies that it should target the viral mRNAs, particularly in view of their lack of a 3′ poly(A) structure.

In an apparently unrelated line of study, in searching for cap-binding yeast proteins that might be involved in translation, Blanc et al. found that the major coat proteins of L-A and L-BC could each covalently bind the 5′ cap in vitro (8). The reaction could also be demonstrated in vivo (95). The L-A Gag protein cleaves the ^{7m}GMP from the cap structure and covalently attaches it to His-154 of Gag in a reaction that requires only Mg^{2+} and proceeds to the extent that a substantial proportion of Gag molecules become so modified (9). The physiological role of this reaction was examined by studying the effect of changing His-154 of Gag in an L-A cDNA expression plasmid and examining the effect on the replication and ex-

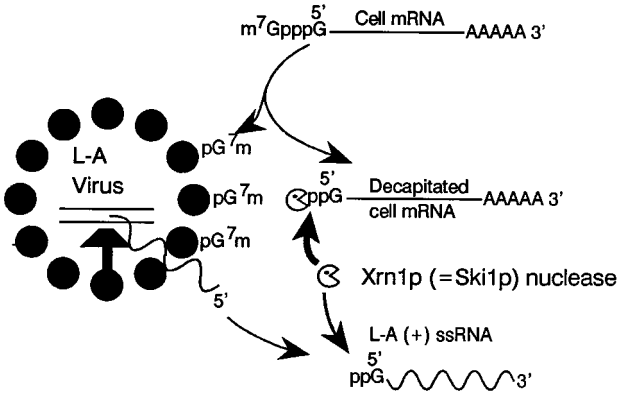

FIG. 7. Decapitation decoy model. Gag removes the caps from cellular mRNAs, creating substrates for the Ski1p/Xrn1p 5′ → 3′ exoribonuclease specific for uncapped mRNAs. The exoribonuclease is partially diverted from attacking the viral mRNA, which also lacks a cap.

pression of M_1 dsRNA dependent on this plasmid in the absence of the L-A virus. It was found that while the copy number of M_1 dsRNA was unaffected, almost no killer toxin was produced (9). Thus, expression of viral information was primarily affected.

These two lines of study came together when it was found that the requirement for the cap-binding activity for expression of viral information was no longer evident if the cell was deleted for *ski1/xrn1* (95). M_1 mRNA and toxin production were nearly undetectable if the L-A expression clone had a mutation in His-154 but were restored to normal by a *ski1/xrn1* mutation (but not by a *ski8* mutation as expected from the hypothesized role of Ski8p in affecting translation initiation rather than mRNA degradation [see above]). One possible explanation was that Gag was using the stolen cap to cap L-A and M_1 transcripts, but it proved impossible to detect such a reaction, even at a very low level (95). The alternative explanation was that the purpose of removal of the cap was not to utilize the cap but, rather, to produce uncapped cellular mRNA, the other product of the reaction. We suggested that these decapitated cellular mRNAs could serve to partially distract the Ski1p/Xrn1p exoribonuclease so that expression of viral mRNAs can proceed (95) (Fig. 7). Clearly, the nuclease is not completely swamped by the decapitated mRNAs, because *shi1/xrn1* mutants were, in fact, superkillers. Since L-A is typically present at 1,000 or more copies per cell and there are 120 Gag molecules per particle, even if each Gag only reacted once, a substantial amount of decoy RNA could be generated. However, the effect might be local, producing decoy uncapped mRNA only around viral particles with little impact on overall mRNA turnover.

Lethality of *ski1 ski2* and *ski1 ski3* double mutants. The *ski1/xrn1* system degrades decapped mRNAs and is not essential for cell growth. The *ski2,3,8* system blocks translation of non-poly(A) mRNAs and is likewise nonessential. Double mutants defective in both of these systems are lethal (73), perhaps because the elevated levels of partially degraded mRNAs are more readily translated, producing more aberrant products than when either system is defective alone. The authors ruled out any role of L-A or M in this lethality (73), but it remains possible that the lethality is due to derepression of L-BC or 20S RNA, whose copy numbers are also known to be repressed by the *SKI2,3,8* system (3, 96). Since L-BC and 20S RNA are both cytoplasmic, they probably lack the 5′ cap structure and so should be repressed by *SKI1/XRN1* as are L-A and M dsRNAs. Synergistic effects would not be surprising.

Gag-Pol Fusion Protein Formed by a −1 Ribosomal Frameshift: How and Why

The 5′ *gag* and 3′ *pol* genes of L-A overlap by 130 bp (66), and Pol is expressed only as a Gag-Pol fusion protein (50). The mechanism of formation of the fusion protein is −1 ribosomal frameshifting (29, 66), a mechanism identical to that shown by Jacks et al. to be responsible for producing the retroviral Gag-Pol fusion protein (70). Unlike the classical frameshift mutations, in which addition or deletion of one or more bases produces a defective protein, ribosomal frameshifting occurs when the structure and sequence of the mRNA induce the ribosome to change reading frame with some frequency. Many retroviruses, dsRNA viruses, and plus-strand ssRNA viruses use essentially the same mRNA structure to induce host ribosomes to shift back 1 base on the mRNA, producing, at a defined efficiency, a fusion protein encoded by parts of two ORFs. A recent review of ribosomal frameshifting in yeasts has appeared (28), as have several more general reviews of the subject (1, 41, 62, 69).

Why make a Gag-Pol fusion protein? The Pol domain of L-A includes a region necessary for the packaging of the viral plus-strand ssRNA (49, 117). Assuming that the Gag domain of the fusion protein anchors the fusion protein in the capsid by its interaction with Gag molecules, the existence of the fusion protein ensures packaging of the viral genome and inclusion of the RNA polymerase in the particle. The latter role is also played by the Gag-Pol fusion protein in retroviruses.

Mechanism of −1 ribosomal frameshifting. The simultaneous-slippage model of −1 ribosomal frameshifting is based on the structural requirements of the mRNA for efficient slipping (70) (Fig. 3). The "slippery site" is a sequence of the form X XXY YYZ, where the 0 (unshifted) reading frame is indicated. X can be any nucleotide, but all three X bases must be the same for optimal efficiency. Y can only be A or T. The X XXY YYZ slippery-site sequence enables the tRNAs in the P and A sites to re-pair in the −1 frame with correct pairing for each of their nonwobble bases. Detailed analysis has shown that the efficiency of re-pairing is a prime determinant of the efficiency of frameshifting (70). The requirement for Y to be A or U has been interpreted to mean that the frequency of unpairing of the tRNA at the A site of the ribosome is also important in determining the efficiency of −1 frameshifting (29).

The second structural element necessary for frameshifting is generally an RNA pseudoknot, a stem-loop structure in which the loop is base paired to a sequence 3′ to the base of the stem. When the ribosome encounters this strong secondary structure in the mRNA, it pauses with the slippery site in the A and P sites (131, 147). It is striking that a simple stem-loop of equal stability is much less efficient at promoting frameshifting (14, 15, 131). The structure of the pseudoknot may better promote the precise arrest of the ribosomes, with the slippery site in the position allowing the −1 shift. The pairing of the loop with the single-stranded region 3′ to the base of the stem restricts turning of the stem as the ribosome attempts to unwind it. Alternatively, there may be some specific effect of the pseudoknot on the ribosomes themselves or on some translation factor.

Why make the fusion protein by ribosomal frameshifting? Retroviruses, plus-strand ssRNA viruses, and dsRNA viruses use their full-length plus strands for three purposes: (i) as mRNA, (ii) as the species encapsidated to form new viral particles, and (iii) as a template for replication. If, for purposes

of translation, one of these viruses were to splice or edit some of their plus strands to make a fusion protein, they would be forming mutant genomes, unless at the same time they removed a site necessary for packaging or replication (66). Thus, RNA splicing and editing is unknown among the many plus-strand ssRNA and dsRNA viruses, and splicing in retroviruses (to make *env*) results in removal of the packaging sequence (called Ψ), thus preventing the propagation of the spliced product as a mutant. Fusion proteins are made instead by ribosomal frameshifting or by readthrough of termination codons, both mechanisms that leave the plus strands unchanged. Splicing is well known among minus-strand RNA viruses and among DNA viruses which do not have this limitation.

How critical is the efficiency of frameshifting? The efficiency of −1 ribosomal frameshifting determines the ratio of Gag to Gag-Pol proteins made in the cell. For both the dsRNA and retroviruses which use this mechanism, it is to be expected that the efficiency of frameshifting will be important for viral propagation. This has been tested for the L-A dsRNA virus satellite M_1 and for the Ty1 retrotransposon of *S. cerevisiae* which uses a +1 ribosomal frameshifting event to form its Gag-Pol fusion protein. Propagation of M_1 dsRNA in L-A virions can be supported by an L-A expression cDNA clone (165). Modification of the slippery site of this clone enabled adjustment of the efficiency of frameshifting to almost any desired value. Mutations were also isolated in chromosomal genes affecting the efficiency of ribosomal frameshifting (*MOF*, for maintenance of frame [see below]) and used in these experiments. It was found that an increase or decrease of twofold in frameshifting efficiency would be tolerated by M_1 but that beyond these limits, M_1 was lost (30).

That the ratio of Gag-Pol to Gag is critical for M_1 propagation was interpreted in view of our packaging model (see Fig. 8). It was proposed that an excess of fusion protein results in initiation of assembly of too many viral particles, so that Gag runs out with few of them completed. A deficiency of fusion protein is equivalent to an excess of Gag, which might result in closure of viral particles before a plus strand has been bound by Pol (30).

The efficiency of the +1 ribosomal frameshifting of the retrotransposon Ty1 is also critical for retrotransposition (75, 171). The mechanism of the Ty1 +1 shift is the shift of $tRNA^{Leu}_{UAG}$ from the 0 frame to the +1 frame at a hungry codon waiting for a $tRNA^{Arg}_{CCU}$ to arrive (4). Frameshifting efficiency can be adjusted by over- or undersupplying the $tRNA^{Arg}_{CCU}$. In each case, a change in frameshifting efficiency adversely affects retrotransposition. Polyamine deficiency also elevates frameshifting efficiency and thus inhibits retrotransposition (2).

Chromosomal genes affecting the efficiency of frameshifting. Mutants altered in chromosomal genes affecting −1 ribosomal frameshifting have been isolated by a colony assay that measures β-galactosidase activity from a vector in which its expression requires a −1 frameshift at the L-A site (31). The nine genes identified are called *MOF* for maintenance of frame, their presumed normal function (Table 4). A similar screen has been done with a retroviral site (87). The large number of genes is not unexpected, because many ribosomal proteins and elongation factors might influence the process of maintaining proper reading frame. Mutants increased the efficiency of frameshifting 2.7- to 9-fold above the normal 1.9%. Mutants with the greatest increases in frameshifting produced loss of M_1 dsRNA. Several mutants showed temperature dependence of elevated frameshifting, with normal efficiency at 20°C and elevated efficiency at 30°C. The *mof2-1*, *mof5-1*, and *mof6-1* mutations also produced cell cycle arrest phenotypes, presumably because of production of an abnormal protein from some cellular gene (31).

TABLE 4. Properties of *mof* mutants[a]

Mutant	Frameshifting (fold relative to WT)[b]	M_1 dsRNA	Growth[c]	Arrest phenotype	Petite
mof1-1	3.3	−	+		+
mof2-1	7.9	−	*ts*	Dumbell	Pet
mof3-1	2.9	+	+		+
mof4-1	4.0	−	+		+
mof5-1	4.0	−	*ts*	Multibud	Pet
mof6-1	2.5	−	*ts*	Large, unbudded	+
mof7-1	2.6	+	+		+
mof8-1	2.7	+	+		+
mof9-1	3.0	+	+		+

[a] *MOF9* is 5S rRNA.
[b] WT, wild type.
[c] *ts*, temperature sensitive.

The product of the *MOF9* gene has been identified as 5S rRNA (32). Two point mutations of 5S rRNA studied by Van Ryk and Nazar (148), G99A and C98G, each produced a similar elevation of −1 ribosomal frameshifting to the *mof9-1* mutant. This result is of interest, because it is the first evidence for the specific function of 5S rRNA in translation.

Can −1 ribosomal frameshifting be used as a target of antiviral drugs? Since the efficiency of ribosomal frameshifting is critical to the propagation of at least M_1 and the yeast retrovirus Ty1 and since the *mof* mutants show that substantial alterations of the efficiency of frameshifting can be tolerated by the host, it seems likely that drugs which increased or decreased ribosomal frameshifting and thereby blocked viral propagation could be developed. The *mof* mutants show significant specificity for different slippery sites, suggesting that drugs with similar specificity and hence limited toxicity could be developed. Yeast vectors and strains suitable for this purpose have been developed (30).

POSTTRANSLATIONAL PROCESSING

MAK3 N-Acetyltransferase Modification of Gag Is Necessary for Assembly

The *MAK3* gene is necessary for propagation of both M_1 and L-A (130). Mak3p has substantial homology with several *N*-acetyltransferases (139), particularly with the *Escherichia coli rimI* protein, which acetylates the N terminus of ribosomal protein S18 (68, 172). Gag from normal viral particles has a blocked N terminus, as does that made from an L-A cDNA clone in a wild-type host. However, Gag made from an L-A cDNA clone in a *mak3* mutant is unblocked and does not assemble into particles (140). The unblocked Gag has the N-terminal sequence expected from the cDNA clone with the initiator methionine residue intact. The N-terminal 4 amino acid residues of Gag (Met-Leu-Arg-Phe-) are sufficient to make β-galactosidase N acetylation be dependent on *MAK3* (138). This indicates that this sequence both prevents acetylation by the *NAT1* and *NAT2* systems (84, 129) and suffices as a signal recognized by Mak3p.

Like L-A, the major coat proteins of Rous sarcoma virus, tobacco mosaic virus, turnip yellow mosaic virus, alfalfa mosaic virus, and potato X virus are N-terminally acetylated (61, 83, 98, 103, 108). However, the enzymes responsible for these modifications and the necessity for these modifications are not known.

The *mak3* mutants also grow slowly on nonfermentable carbon sources (27), an effect independent of the loss of L-A (139). The Mak3p recognition sequence (MLRF. . .) is also found at the N terminus of several chromosomally encoded mitochondrial proteins, suggesting that their precursors may require acetylation for transport; this may explain the slow growth of *mak3* cells on nonfermentable carbon sources (139).

Killer Preprotoxin Is Processed To Form Mature Toxin

Extensive studies by H. Bussey, D. J. Tipper, K. A. Bostian, D. Y. Thomas, and their collaborators have defined the processing and secretion pathway of the preprotoxin encoded by M_1 dsRNA (Fig. 5). The production and action of the killer toxins are not detailed here, but are reviewed in references 18, 19, and 142. Briefly, the immunity factor appears to be the protoxin itself, but the detailed mechanism of immunity has not been defined. The K_1 toxin binds to β(1-6) glucan in the cell wall (64) and then produces proton pores in the cell membrane (94). In contrast, the K_{28} toxin binds to cell wall mannan and finally acts by blocking DNA synthesis (124).

KEX1 and *KEX2* Processing Proteases and Mammalian Prohormone Processing

It has long been known that most mammalian prohormones are processed by cleavage C-terminal to a pair of basic residues followed by removal of the basic residues themselves. However, the proteases responsible for this process were not known. The *KEX1* and *KEX2* genes were discovered by their requirement for the production of active killer toxin, although they are dispensable for propagation of the L-A and M_1 dsRNAs (154). The *KEX2* gene was also found to be necessary for mating by strains of the α mating type, in part because it was required for production of the α pheromone (89), a peptide produced by *MAT*α cells that prepares *MAT***a** cells for mating. Kex2p was shown to be a Golgi protease that cleaves C-terminal to pairs of basic residues (74), and Kex1p was shown to be the activity removing the remaining pair of basic residues (25, 33, 150). This allowed the identification by homology of several mammalian prohormone-processing enzymes (reviewed in reference 132).

RNA PACKAGING: IN VITRO AND IN VIVO

L-A opened empty viral particles have a high affinity in vitro for the plus-strand ssRNAs of the replicons that they normally package, including L-A, M_1, and X (a deletion mutant of L-A) (35). The binding site on X plus-strand ssRNA was localized by this assay and proved to be a stem-loop structure about 400 nucleotides from the 3′ end of the X plus strands with an A residue protruding from the 5′ side of the stem (Fig. 3) (35, 36, 47). The stem structure is important for the binding, as is the sequence of the loop and the protruding A residue (36, 128). Since the viral particles have to pick out viral plus-strand ssRNA from a sea of cellular mRNAs, it was hypothesized that this binding activity corresponded to the RNA-packaging reaction. This was confirmed by showing that inclusion of the binding site in a transcript from an unrelated plasmid (artificial packaging substrate) resulted in the packaging of that transcript in L-A viral particles in vivo (47). A similar stem-loop structure with a protruding A residue was found in the M_1 sequence by computer analysis and shown to serve as a packaging signal (47) (Fig. 5). Another nearby structure with a similar loop sequence can also serve as a packaging site (128) (Fig. 5). The L-A packaging site substantially overlaps with the IRE (see above). While several mutant RNAs show differential effects on binding activity and replication activity (36), these differences may reflect nonlinearities of the two assays rather than any inherent difference in the sites.

The artificial packaging substrate was also found to be packaged in viral proteins made from the L-A cDNA expression clone, allowing definition of the protein requirements for RNA packaging. It was found that amino acid residues 67 to 213 of Pol were necessary for packaging (49, 117). This region includes one of the ssRNA-binding domains of Pol, suggesting that it is responsible for recognizing and binding the viral plus strands in the process of packaging (117).

The production of a new viral particle includes both the joining of the viral proteins and their packaging of a viral plus strand. The L-A headful replication phenomenon shows that the particle size is determined by the structure of the major coat protein, Gag, rather than by the size of the encapsidated genome (37). Gag made in a *mak3* mutant lacks the normal N-terminal acetylation and fails to assemble into viral particles (139, 140). The normal frameshift efficiency of 1.9% suggests that each virion has two Gag-Pol fusion protein molecules (29). The icosahedral $T = 1$ structure of L-A virions with a dimer of Gag molecules as the asymmetric unit suggests that a dimer of Gag-Pol molecules may be formed as well (23). New L-A virions package a single plus strand per particle (47), so we suppose that it is the packaging domains of a dimer of Gag-Pol that actually recognize the viral plus strands. The current model for the assembly and packaging process of the L-A virus is shown in Fig. 8.

Although this model is consistent with the known facts, it has been noted that the in vivo transcripts of an L-A cDNA expression clone are not packaged in the L-A particles, even though these transcripts carry the intact L-A packaging site (118a). While it is possible that the presence of 5′ cap or 3′ poly(A) on these transcripts somehow poisons them as packaging substrates, such structures should also have been present on the artificial packaging substrate in vivo transcripts used in the definition of the packaging site on the RNA and the packaging domain of Pol.

The packaging model for L-A contrasts with the mechanism demonstrated for the dsRNA bacteriophage φ6. Elegant experiments by Mindich and Bamford and their colleagues have shown that φ6 preformed core particles can specifically package and replicate phage plus strands to ultimately produce infectious particles (see, for example, references 46 and 58).

Evidence for *cis* Packaging by L-A

M_1 is more sensitive than L-A to changes in frameshift efficiency (30, 31) and to deficiency of 60S ribosomal subunits (*mak* mutations [3, 107]). This suggests that limiting viral proteins are utilized preferentially by L-A over M_1. This could be because M_1 has a lower affinity for these proteins than does L-A or because these proteins preferentially package their mRNA, the L-A plus strands. This latter possibility is referred to as *cis* packaging. The X dsRNA mutant of L-A is composed entirely of L-A sequences, and so one assumes that it has the same inherent affinity for viral proteins as does L-A itself. Nonetheless, X dsRNA propagation depends on at least *MAK16*, *MAK18*, *MAK21*, *MAK26*, and *MAK27* (39). X resembles M_1 in this regard and differs from L-A, suggesting that the *cis*-packaging hypothesis is correct.

Does Packaging Control Translation?

Clearly, a viral plus strand that has been packaged can no longer be translated; conversely, packaging of viral plus strands is limited by how much protein has been translated from them.

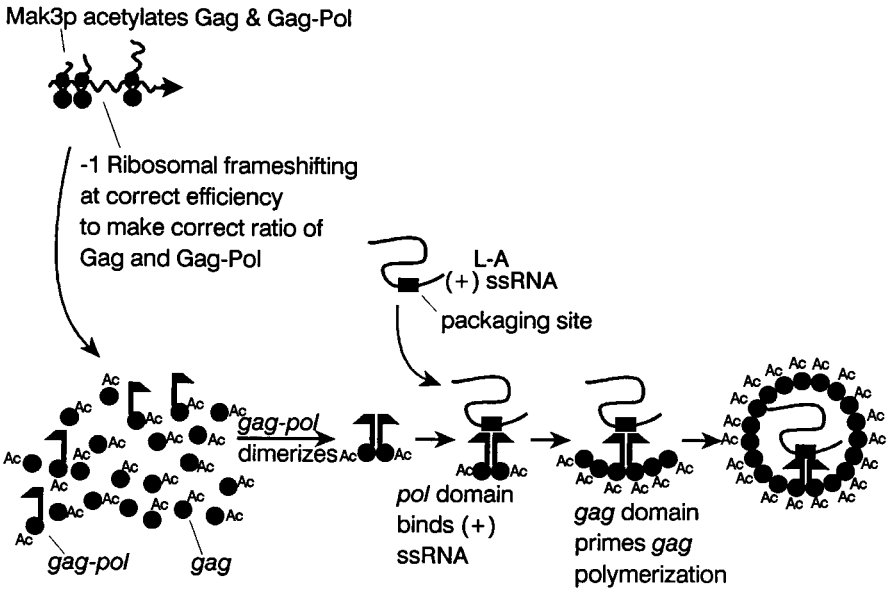

FIG. 8. Packaging model for L-A. The recognition of the packaging site by Pol, the initiation of assembly by the fusion protein, and the covalent attachment of Gag and Pol ensure the packaging of a single viral plus-strand ssRNA and of the RNA polymerase in each particle. It is known, however, that assembly of morphologically normal particles can proceed with Gag alone (49).

Thus, if there is a single pool of unpackaged plus strands, a natural control mechanism would ensue.

L-BC IS CLOSELY RELATED TO L-A

L-BC has long lived in the shadow of L-A because of both its 10-fold-lower copy number and its lack of an associated (killer or other) phenotype. The designation L-BC is derived from the finding that there were at least two viral entities whose propagation was independent of *MAK3*, *MAK10*, and *PET18* (and thus distinct from L-A) (130, 169). These were called L-B and L-C and were distinguishable based on the RNase T_1 fingerprints of their genomic dsRNA (130). Other workers referred to various L-As as L1 and L2, reflecting their hypothesis that L1 could maintain only M_1 dsRNA and L2 could maintain only M_2 dsRNA (42). Although this hypothesis proved to be incorrect (59, 158), the names have persisted. Bruenn and coworkers now refer to L-A as L1 and to L-B and L-C as L_a or even L-A.

L-BC has a replication cycle much like L-A (48), and in vitro RNA-binding and template-dependent replication and transcription systems for L-BC have now been described (118b). While the L-BC replicase prefers L-BC plus-strand ssRNA, it uses X plus strands surprisingly well, with the same requirements for the 3′-terminal 4 bases and the adjacent stem-loop structure that the L-A replicase has. Neither of these structures is found in the L-BC 3′ end. The binding of viral plus strands by the opened empty particles is, however, completely specific for L-BC plus strands, and the transcriptase reaction strongly prefers L-BC dsRNA.

Like L-A, the L-BC copy number is lowered severalfold by the *SKI2,3,8* system (3). It is likely that L-BC lacks a 5′ cap structure, and so it is expected that expression of L-BC is repressed by *SKI1/XRN1* as well. The complete 4,615-bp sequence of L-BC (called L-A in the GenBank record [no. SCU01060]!) reveals a striking similarity to that of L-A (109), particularly in the RDRP consensus domains (16). The ORFs homologous to *gag* and *pol* of L-A are similarly at the 5′ and 3′ ends of the L-BC plus strands and overlap by 154 bp, with *pol* in the −1 frame relative to *gag*.

CONCLUSIONS AND PROSPECTS

The wide range of genetic and biochemical methods available have made L-A one of the best-studied dsRNA viruses. Nonetheless, there are important methods that are not available in this system and important problems that have not been satisfactorily addressed. Transfection with viral RNA (presumably plus strands) has not yet succeeded, although methods for transient expression have been reported (40, 120). Although viral proteins expressed from an L-A cDNA clone can support the propagation of M_1 or X dsRNAs, RDRP activity from such clones has not been obtained. It is possible that "assembly" of the RDRP requires the presence of a viral genome that can be replicated, as has been shown for brome mosaic virus in *S. cerevisiae* by Quadt et al. (114). The assembly of the virion is a promising area, which would benefit from knowing the X-ray structure and having an in vitro assembly system.

With the finding that most of the chromosomal genes involved in viral propagation and its regulation are actually genes determining properties of the translation system [ribosome biogenesis, blocking of translation of non-poly(A) mRNA, degradation of capless mRNA, and efficiency of ribosomal frameshifting], methods of in vitro translation become critical for further progress. Recent improvements in this area (67) should be useful. It remains surprising that the uncapped nonpoly(A) L-A transcripts are so well translated. For example, the 5′ cap is needed for translation initiation, not only for stabilization of the mRNA. A recent report of an internal ribosome entry site in the 5′ end of the *Leishmania* virus plus strand may provide a clue (93).

The properties of L-A have proven to be typical of a rapidly growing family of viruses of fungi and parasitic microorganisms, the *Totiviridae*. These are single-segment dsRNA viruses with a replication cycle resembling that of L-A, having a single capsid layer and making a Gag-Pol fusion protein by ribosomal

frameshifting or another translational trick (reviewed in reference 163). These include L-BC of *S. cerevisiae*; viruses of the protozoans *Leishmania* spp. (137), *Giardia lamblia* (54, 152), *Trichomonas vaginalis* (77, 151), and *Eimeria* spp. (127); and fungal viruses (reviewed in reference 56) from *U. maydis* (81), *Helminthosporium victoriae* (57), *Aspergillus foetidus*, *Yarrowia lipolytica*, *Gaeumannomyces graminis*, and *Zygosaccharomyces baili*. Their study is revealing unique and unexpected features. In addition to those mentioned above are, for example, a site-specific RNase associated with *Leishmania* viral particles (92), a protein kinase associated with *Helminthosporium* virions that phosphorylates the major coat protein (57), and a *Trichomonas* virus-related alteration of cell surface antigens (151).

In the same way that DNA phages and then DNA viruses were important tools in the study of host DNA replication and transcription mechanisms, as well as important objects of study in their own right, RNA viruses, including those of yeasts, are playing an important role in studies of host functions. Moreover, the importance of RNA viral infections in diseases of humans, animals, and plants guarantees continuing interest in their mechanisms of propagation and interaction with their hosts.

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