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Yeast Virus Propagation Depends Critically on Free 60S Ribosomal Subunit Concentration

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Over 30 *MAK* (maintenance of killer) genes are necessary for propagation of the killer toxin-encoding M_1 satellite double-stranded RNA of the L-A virus. Sequence analysis revealed that *MAK7* is *RPL4A*, one of the two genes encoding ribosomal protein L4 of the 60S subunit. We further found that mutants with mutations in 18 *MAK* genes (including *mak1* [*top1*], *mak7* [*rpl4A*], *mak8* [*rpl3*], *mak11*, and *mak16*) had decreased free 60S subunits. Mutants with another three *mak* mutations had half-mer polysomes, indicative of poor association of 60S and 40S subunits. The rest of the *mak* mutants, including the *mak3* (*N*-acetyltransferase) mutant, showed a normal profile. The free 60S subunits, L-A copy number, and the amount of L-A coat protein in the *mak1*, *mak7*, *mak11*, and *mak16* mutants were raised to the normal level by the respective normal single-copy gene. Our data suggest that most *mak* mutations affect M_1 propagation by their effects on the supply of proteins from the L-A virus and that the translation of the non-poly(A) L-A mRNA depends critically on the amount of free 60S ribosomal subunits, probably because 60S association with the 40S subunit waiting at the initiator AUG is facilitated by the 3′ poly(A).

Studies of viral propagation generally concentrate on the activities and interactions of virus-encoded proteins. However, the host environment and particular host components can play a crucial role in whether viral propagation is persistent or causes pathology to the host. This is amply demonstrated by studies of the roles of cellular components in bacteriophage replication and assembly.

Studies of the L-A double-stranded RNA (dsRNA) virus and its killer toxin-encoding satellite, M_1 dsRNA, have identified many genes of its host, *Saccharomyces cerevisiae*, which either repress viral propagation (the *SKI* genes, so named for the superkiller phenotype of mutants in these genes) or are necessary for M_1 propagation (*MAK* genes, for maintenance of killer). Recently, the *SKI2*, *SKI3*, and *SKI8* genes have been shown to act by specifically repressing the translation of non-poly(A) mRNAs, such as the L-A and M_1 mRNAs (28a). In this report, we examine the mechanism by which most of the *MAK* genes promote viral propagation.

The L-A dsRNA virus of *S. cerevisiae* has two open reading frames in its positive strand, the 5′ *gag* encoding its major coat protein (Gag) and the 3′ *pol* encoding the multifunctional Pol domain of the Gag-Pol fusion protein. M_1 is a dsRNA satellite of L-A, depending on the L-A-encoded Gag and Gag-Pol for its propagation. M_1 itself encodes no proteins required for its propagation but does encode the killer toxin, a secreted heterodimer protein processed out of a 32-kDa preprotoxin. The L-A genome lacks both 5′ cap and 3′ poly(A) (10, 42), and viral transcripts made in vitro lack both modifications, although a single uncoded A (or G) residue is found at the 3′ end of both viral strands (11, 42).

Mutants isolated by their inability to propagate the M_1 dsRNA identify about 30 chromosomal *MAK* genes. *MAK3* encodes an *N*-acetyltransferase whose acetylation of the N terminus of Gag is required by L-A and M_1 for viral assembly (39–41). *MAK1* (*TOP1*) encodes DNA topoisomerase I (44), *MAK8* (*TCM1*) encodes ribosomal protein L3 (55), *MAK11* encodes an essential membrane-associated protein with β-transducin repeats (22), and *MAK16* is an essential gene encoding a nuclear protein whose temperature-sensitive mutant arrests in G_1 (51). Some *GCD1*, *GCD10*, *GCD11*, and *GCD13* mutants also have a Mak^- phenotype (19). *GCD11* encodes the γ subunit of eIF2, while *GCD1* encodes one subunit of the guanine nucleotide exchange factor for eIF2, called eIF2B (12, 18). Except for *MAK3*, the role of these genes in viral propagation is not understood.

The RNA-dependent RNA polymerase of L-A (Pol) is formed by a −1 ribosomal frameshift event occurring in the region of overlap between the *gag* and *pol* open reading frames (13). The mechanism of this frameshift event (13, 14, 46, 47) is identical to that first described for retroviruses (24) and coronaviruses (8). The efficiency of the −1 ribosomal frameshift, and thus the ratio of Gag-Pol to Gag proteins, was found to be critical to viral propagation (14), and mutants with elevated frequency of −1 ribosomal frameshifting were accordingly found to lose M_1. But a survey of previously isolated *mak* mutants showed that none were affected in this process (14).

We show here that *MAK7* is *RPL4A* and that *mak7* mutants are deficient in 60S ribosomal subunits. Indeed, we find that most *mak* mutants, including several which are known not to encode ribosomal proteins, are similarly deficient. Mutants with mutations in several other *MAK* genes show half-mer polysomes, a phenomenon due to inefficient association of 60S subunits with the 40S subunits waiting at the initiator AUG. We suggest that a deficiency of 60S subunits puts L-A's poly(A)-deficient mRNA at a unique disadvantage compared with cellular $poly(A)^+$ mRNAs. This model is supported by the suppression of *mak* mutations by *ski* mutations (45), since the *SKI2*, *SKI3*, and *SKI8* genes are now known to specifically repress translation of poly(A)-deficient RNA (28a, 56).

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TABLE 1. Yeast strains used in this study

Strain	Genotype	Source[a]
2552	*MAT*α *trp5 ura3 mak7-1 kar1-1* L-A-HN	
2907	*MAT***a** *his 3-200 leu2 trp1-901 ura3-52 ade2-10* L-A-o	K. Matsumoto
YOC76	*MAT***a** *his 3-200 leu2 trp1-901 ura3-52 ade2-10 rpl4a*::*LEU2* L-A-o	This study
YOC72	*MAT***a** *his 3-200 leu2 trp1-901 ura3-52 ade2-10 rpl4b*::*LEU2* L-A-o	This study
1074	*MAT***a** *leu1 kar1-1* L-A-HN M_1	
3164	*MAT***a** *arg1 kar1-1* L-A-HN M_1	
3165	*MAT*α *arg1 thr*(*1*, X) *kar1-1* L-A-HN M_1	
1886	*MAT*α *lys1 ade1 kar1-1 mak1-1* L-A-HN	
2404	*MAT*α *his4 kar1* [B] L-A-HNB M-o	
JR224	*MAT*α *kar1-1 ura2 leu2 trp1* L-A-o M_1 pI2L2	J. C. Ribas
D3-1C	*MAT*α *ura3* L-A-HN M_1	D. Masison
YOC198	*MAT*α *leu2* L-A-HN M_1	This study
2871	*MAT***a** *his5 ura3* L-A-HN M_1	
A364A	*MAT***a** *ade1 ade2 lys2 tyr1 his7 ura1 gal2* K+	
S37	*MAT*α *leu2-1 met5* K+	
RS190	*MAT***a** *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 top1-8*::*LEU2*	R. Sternglanz
3214	RS190/YCp50	R. Sternglanz
3215	RS190/pCC10 (*TOP1 URA3*)	R. Sternglanz
84	*MAT***a** *ade1 trp1 mak2-1*	
2321	*MAT***a** *aro7 leu2*$^{-}$ *his3*$^{-}$ *ura3-52 mak3-1*	
737	*MAT*α *thr1 lys2 mak4-1*	
755	*MAT*α *lys2 mak5-1*	
831	*MAT***a** *ade1 lys1 aro7 mak6-1*	
646	*MAT*α *ade1 ura1 thr1 mak8-1*	
1024	*MAT***a** *trp3 ade1 mak9-1*	
3197	*MAT*α *ura3-52 trp1*Δ *leu2*$^{-}$ *his6 mak10*::*URA3*	
2967	*MAT***a** *leu2 ura3 mak10*	
2747	*MAT*α *leu2 mak11-1* HOK-o B-o M-o	
2766	*MAT***a** *cdc16 ura3 ade1 mak11-1*	
YOC132	*MAT***a** *ura3 leu2 mak12-1*	This study
972	*MAT*α *trp3 mak13-1*	
1043	*MAT***a** *ade2 ura1 ?gal1 mak14-1*	
942	*MAT*α *ade2 mak15-1*	
2597	*MAT***a** *ade1 ura3-52 trp1-1 gal10 mak16-1*	
547	*MAT*α *leu2 met5 mak17-1*	
722	*MAT*α *ade1 mak20-1*	
716	*MAT*α *ade2 mak21-1*	
551	*MAT***a** *ade2 ura1 mak22-1*	
557	*MAT***a** *ura1 mak23-1*	
1299	*MAT***a** *arg1 mak24-1*	
530	*MAT***a** *ade2 mak25-1*	
1323	*MAT***a** *his2,6 ura3 mak26-1*	
1303	*MAT***a** *lys1 mak27-1*	
CY1746	*MAT***a** *ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 ssd1-d2 can1-100 sos1*::*LEU2*	T. Zhong
CY2509	*MAT*α *ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 ssd1-d2 can1-100 sos2*::*URA3*	T. Zhong
YAS282	*MAT***a** *ura3-52 his3*Δ *trp1*Δ *leu2-3,112 can*^{r} *rpl46*::*LEU2*	A. Sachs
JWY1410	*MAT***a** *rpl16A*::*TRP1 ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1-*Δ*101 ura3-52 can1*	J. L. Woolford
JWY1412	*MAT***a** *rpl16B*::*LEU2 ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1-*Δ*101 ura3-52*	J. L. Woolford
J771A	*MAT*α *rps28B*::*LYS2 met8-1 leu2-1 trp1-*α *lys2-BB his3-1 ura3-52*	J. R. Warner
J772A	*MAT*α *rps28A*::*TRP1 met8-1 leu2-1 trp1-*α *lys2-BB his3-1 ura3-52*	J. R. Warner
YOC195	*MAT***a** *his 3-200 leu2 trp1-901 ura3-52 ade2-10 rp51A*::*LEU2* L-A-o	This study
YOC197	*MAT***a** *his 3-200 leu2 trp1-901 ura3-52 ade2-10 rp51B*::*URA3* L-A-o	This study

[a] Strains for which no source is indicated are from our collection.

MATERIALS AND METHODS

Strains, media, and plasmids. The strains of *S. cerevisiae* used in this study are listed in Table 1. The strains in which M_1 is supported by an L-A cDNA clone, pI2L2 (52), were constructed by cytoduction from a *kar1-1* strain (JR5) harboring pI2L2 and M_1 (6). L-A-HN is the usual form of L-A found in most killer strains. H and N are genetic markers on L-A (38). L-A-HNB has the B (for bypass) trait that makes many *MAK* genes dispensable for propagation of M_1 (48). The strains containing only L-A and both L-A and M_1 were constructed by cytoduction with the *kar1-1* L-A-HN strain 1886, the *kar1-1* L-A-HNB strain 2404, and the *kar1-1* L-A-HN M_1 strain 1074 or 3165. The *rp51A* and *rp51B* disruptant strains were constructed from strain 2907 by using the disruptant plasmids pGOBLEU (2) and pUC9ΔB*Hinc*URA (1), respectively, as described previously (1, 2).

Escherichia coli DH5α [F^{-} ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 endA1 recA1 hsdR17* (r_k^{-} m_k^{+}) *doeR thi-1 supE44 gyrA96 relA1* λ^{-}] was used for DNA manipulations. *E. coli* JM109 [*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) F′ *traD36 proAB lacI*^{q} *lacZ*ΔM15] was used for production of single-stranded DNA for sequencing. Media used for growth of yeast strains and assay of the killer phenomenon were as described elsewhere (50).

Plasmid p6525 containing *MAK7* (*RPL4A*) (16) and pL4-1 containing the 6.7-kb *Hin*dIII fragment of *RPL4B* (57) were kindly provided by J. Erickson and M. Fried, respectively. Plasmid pRS316 (37) was used as a centromere-type vector plasmid for *S. cerevisiae*. Plasmids pUC18 and pBluescript (Stratagene) were used for the construction of *rpl4A*::*LEU2* and *rpl4B*::*LEU2* gene disruption plasmids, respectively (see below). Plasmids pTIC19 (*MAK11*) (22) and YCp50-1C (*MAK16*) (53) were used to transform the respective mutant strains. p596 is Bluescript KS+ with the 1.3-kb *Pst*I-*Sal*I fragment of pP-T_{316} (59) carrying M_1 sequences inserted between the *Pst*I and *Sal*I sites.

Genetic manipulation. Yeast strains were transformed by the lithium acetate method (23). Cytoductions and the killer assay were performed as described

previously (31). Other recombinant DNA procedures were carried out as described by Sambrook et al. (35).

Subcloning and sequencing of *MAK7*. Plasmid p6525 carried a yeast genome insert of approximately 38 kb (Fig. 1A). Several subclones were constructed by partial digestion with *Xba*I or *Bam*HI or by cloning into the centromere-type plasmid pRS316. The subclones were used to transform the *mak7-1* strain 2552. L-A and M_1 were introduced into the transformants by cytoduction using the *kar1-1* killer strain 1074. Cytoductants were tested for the killer phenotype. *MAK7* was localized to the 2.5-kb *Xba*I-*Eco*RI fragment (pYRC20), which was sequenced by making unidirectional deletions from both ends with exonuclease III and S1 nuclease (21).

***rpl4A* and *rpl4B* disruptions.** The 1.3-kb *Sph*I-*Xba*I fragment of *RPL4A* along with the *Xba*I-*Bam*HI linker region of pRS316 was inserted between the *Sph*I and *Bam*HI sites of pUC18. The *Xho*I-*Spe*I fragment of *RPL4B* on plasmid pL4-1 was cloned into pBluescript cut with the same enzymes. The 0.36-kb *Msc*I-*Sal*I fragment of the *RPL4A* coding region or the 0.40-kb *Msc*I-*Sal*I fragment of the *RPL4B* coding region was replaced with the *LEU2* gene on an *Hpa*I-*Sal*I fragment. The resultant plasmid pYRC42 (*rpl4A*::*LEU2*) and plasmid pYRC412 (*rpl4B*::*LEU2*) were digested with *Sph*I and *Xho*I, respectively, and used to transform strain 2907. Disruptions were confirmed by Southern hybridization (data not shown).

RNA preparation and Northern (RNA) hybridization. Total RNA was prepared by the glass bead method (25). To analyze the copy number of M_1 dsRNA, 50 μg of total RNA extracted from each strain was separated by electrophoresis on a 0.8% agarose gel, denatured, and blotted onto a nylon membrane as described previously (29). The M_1 RNA probe was prepared from plasmid p596 by transcription using T7 RNA polymerase and [α-^{32}P]UTP. For Northern hybridization, the prehybridization and hybridization were for 1 h at 55°C in 5× SSPE (0.9 M NaCl, 50 mM NaH_2PO_4 [pH 7.4], 5 mM EDTA)–50% formamide–0.1% sodium dodecyl sulfate (SDS)–5× Denhardt's solution–10 μg of denatured salmon sperm DNA per ml and for 15 h in the same buffer with the labeled RNA probe, respectively. Following the hybridization, the filters were washed twice with 2× SSPE–0.1% SDS for 15 min at 55°C, with 1× SSPE–0.1% SDS for 30 min at 55°C, and finally with 0.1× SSPE–0.1% SDS for 30 min at room temperature.

Polysome preparation and analysis. Polysome preparation and analysis were performed by minor modifications of a method described previously (20). Briefly, yeast cells grown at 30°C to an optical density at 600 nm (OD_{600}) of 0.5 in 200 ml of rich medium (YPAD) were harvested, washed once in 10 ml of distilled water, and incubated at 30°C (25°C for strain 2597) for 30 min in 10 ml of 1 M sorbitol containing 0.1 mg of Lyticase (Sigma) per ml. The spheroplast solution was mixed with 10 ml of 2× YPAD containing 0.8 M $MgSO_4$ and incubated at 30°C (25°C for strain 2597) for 1 h with gentle shaking. After cycloheximide was added to 100 μg/ml and the mixture was rapidly poured onto 5 ml of iced 1 M sorbitol containing 100 μg of cycloheximide per ml, the cells were harvested, washed once in 10 ml of 1 M sorbitol containing 100 μg of cycloheximide per ml, and resuspended in 1 ml of breaking buffer (10 mM Tris-HCl [pH 7.4], 100 mM NaCl, 30 mM $MgCl_2$, 100 μg of cycloheximide per ml, 200 μg of heparin per ml). Sodium deoxycholate was then added to 0.2%; after 5 min on ice, Nonidet P-40 was added to 0.5%; and the suspension was centrifuged for 10 min at 12,000 rpm at 4°C. The supernatant (25 OD_{260} units) was loaded onto a 10-ml linear 7 to 47% sucrose gradient in a buffer (50 mM Tris-acetate [pH 7.0], 50 mM NH_4Cl, 12 mM $MgCl_2$, 1 mM dithiothreitol) and centrifuged at 39,000 rpm for 2.5 h at 4°C in a Beckman SW41 rotor. The OD_{256} of the gradient was monitored continuously with a UA-5 UV monitor (ISCO).

Nucleotide sequence accession number. The sequence of the entire 2.5-kb *Eco*RI-*Xba*I fragment shown in Fig. 1A has been deposited in GenBank (accession number U17361).

RESULTS

***MAK7* is *RPL4A*, one of two genes encoding ribosomal protein L4.** *MAK7* was mapped on the left arm of chromosome VIII (54) and was cloned, using this information, on plasmid p6525 from λ6525 of Olson's lambda library (32) by Erickson and Johnston (16). The insert of p6525 was about 38 kb (Fig. 1A). Testing of subclones for complementation of *mak7-1* localized the *MAK7* gene to a 2.5-kb *Eco*RI-*Xba*I fragment (Fig. 1A), which was then subjected to sequencing analysis. This fragment has only one open reading frame greater than 100 amino acids, and it is identical to the *RPL4A* gene, one of the two genes encoding ribosomal protein L4 of the 60S subunit. Deletion mutants show that the *Sal*I site inside the *RPL4A* open reading frame is necessary for complementation of the *mak7-1* mutation, confirming that *RPL4A* is *MAK7*. This fragment also contains the upstream region of *SSB1* (26), which is 1.1 kb from the 3′ end of *MAK7* (Fig. 1A).

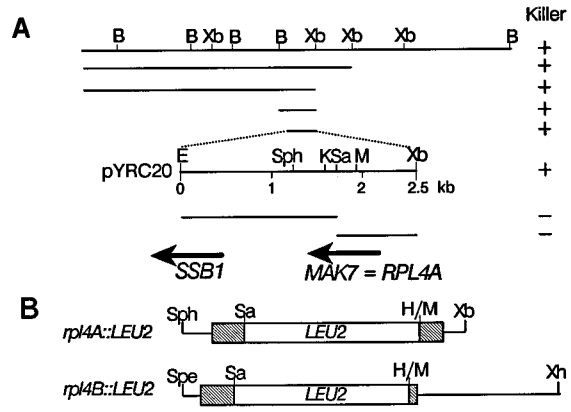

FIG. 1. (A) Location of the *MAK7* gene. The arrows indicate the coding regions of *MAK7* and *SSB1*. "Killer" shows ability of the indicated construct to complement *mak7-1*. Restriction sites shown: B, *Bam*HI; Xb, *Xba*I; E, *Eco*RI; Sa, *Sal*I; M, *Msc*I; Sph, *Sph*I; Spe, *Spe*I; Xh, *Xho*I; K, *Kpn*I; H, *Hpa*I. (B) Disruption plasmids for *RPL4A* and *RPL4B*. The open reading frames of the *RPL4A* and *RPL4B* genes are represented by the hatched boxes. The *LEU2* gene is represented by the open box.

M_1 dsRNA propagation, L4 genes, and free 60S subunits. Arevalo and Warner (4) cloned and sequenced the *RPL4A* gene and showed that there are two yeast genes encoding ribosomal protein L4 (*RPL4A* and *RPL4B*). Yon et al. (57) subsequently cloned both genes and, comparing the two predicted protein sequences, found that there were 7 different amino acids out of 256. To test whether these genes might have different functions for killer (M_1 dsRNA) propagation, we constructed strains disruptant for *rpl4A* or *rpl4B* (Fig. 1B) and introduced L-A-HN and M_1 by cytoduction (Fig. 2A). Cytoductants of the *rpl4A*::*LEU2* disruptant did not show killer activity, while cytoductants of the isogenic *rpl4B*::*LEU2* disruptant showed weak killer activity. When we examined the copy number of M_1 dsRNA, we found that the *rpl4A*::*LEU2* disruptant lost the M_1 dsRNA, but the *rpl4B*::*LEU2* disruptant retained it at a very low level (Fig. 3). This difference parallels the difference in their killer activities. Next, we analyzed the ribosomal subunit profiles of the disruptant strains. The *rpl4A*::*LEU2* and *rpl4B*::*LEU2* strains have similar decreased levels of free 60S subunits (Fig. 4). The expression level of L4 in the *rpl4B*::*LEU2* strain may be at the lowest level consistent with propagation of M_1.

Yon et al. (57) reported that their *rpl4B* disruption mutant grew more slowly than their *rpl4A* disruption mutant, and our results confirm this finding. While the wild-type strain 2907 grew with a doubling time of 2.4 h in rich medium, the isogenic *rpl4A*::*LEU2* and *rpl4B*::*LEU2* strains had doubling times of 3.0 and 3.2 h, respectively.

Most *MAK* mutants have decreased free 60S ribosomal subunits. *MAK7* is the second *MAK* gene found to encode a 60S subunit protein, the first having been *MAK8* (*RPL3*) (55). We analyzed the ribosomal subunit profiles of 25 *mak* mutants (Fig. 5). Surprisingly, 18 *mak* mutants (*mak1*, *mak2*, *mak5*, *mak6*, *mak7*, *mak8*, *mak9*, *mak11*, *mak12*, *mak13*, *mak14*, *mak16*, *mak17*, *mak20*, *mak22*, *mak23*, *mak24*, and *mak27* mutants) have decreased free 60S subunits like the *mak7* disruptant. CEN plasmids carrying *MAK1*, *MAK7*, *MAK11*, or *MAK16* (22, 43, 51) introduced into the respective mutants restored the level of free 60S subunits to a normal level, showing that it was, in fact, the *mak* mutation in these strains that produced the 60S subunit deficiency (Fig. 6). These genes are thus likely to be involved in the biogenesis of 60S ribosomal

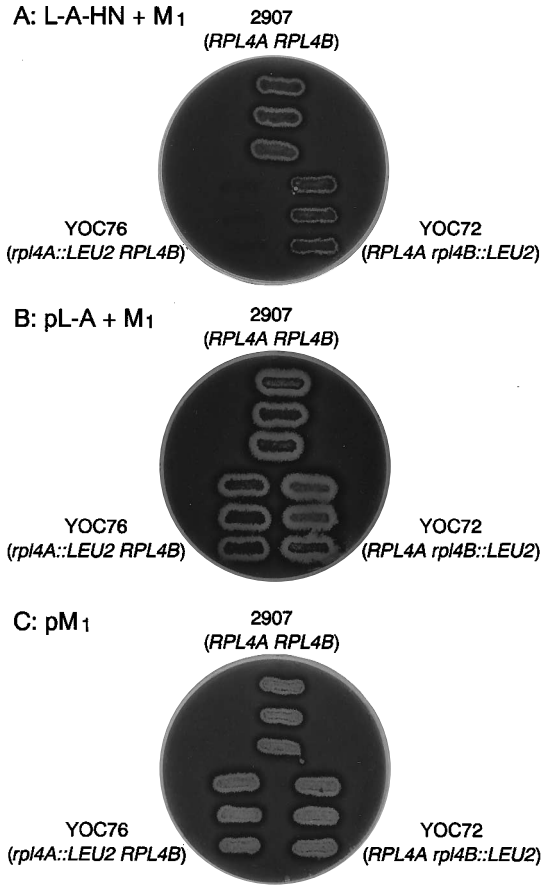

FIG. 2. Killer toxin assays of isogenic wild-type, *rpl4A*::*LEU2*, and *rpl4B*::*LEU2* disruptant strains. Plates were seeded with a lawn of a toxin-sensitive strain, and streaks of strains to be tested were applied (31). The width of the zone of killing surrounding the streak reflects the amount of killer toxin produced. (A) Killer activities of the cytoductants with L-A-HN and M_1. (B) Killer activities of cytoductants carrying M_1 supported by the L-A cDNA clone, pI2L2. (C) Killer activities of the transformants with the M_1 cDNA clone, pP-T_{316}.

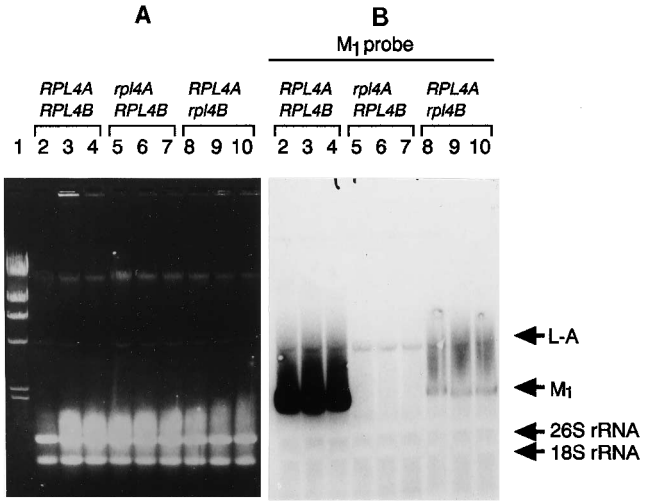

FIG. 3. The *rpl4A* disruptant lost M_1 dsRNA, while the *rpl4B* disruptant showed a markedly decreased M_1 copy number. Fifty micrograms of total RNAs was separated in a 0.8% agarose gel, stained by ethidium bromide (A), and hybridized with the M_1 probe (B) as described in Materials and Methods. Lanes: 1, λ *Hin*dIII fragments; 2 to 4, colonies of the parent strain 2907; 5 to 7, *rpl4A*::*LEU2* colonies; 8 to 10, *rpl4B*::*LEU2* colonies.

subunits, a conclusion evident for those encoding 60S subunit proteins. *MAK1* (topoisomerase I) is known from the studies of Sternglanz and coworkers (9, 36) to be involved in rRNA biosynthesis, but their differential deficiency of 60S subunits has not been previously reported.

Half-mer polysomes with normal amounts of the free 40S and 60S subunits were observed in another three *mak* mutants (*mak4*, *mak21*, and *mak26* mutants), indicating poor association of 60S and 40S subunits. Half-mer polysomes were also observed in mutants with mutations of the translation initiation factor eIF-2B (*gcn3 gcd2* double mutant) (17) and eIF-5A (*tif51a* and *tif51b* double mutant) (27). The *gcd1* (γ subunit of eIF-2B) and *gcd11* (γ subunit of eIF-2) mutants were also reported as *mak* mutants by Harashima and Hinnebusch (19). These data indicate that translation initiation, especially the association of 60S subunits, might be very important for the propagation of the virus. *MAK10* encodes a glucose-repressed protein that is also essential for both the L-A helper virus and M_1 satellite (28, 38). The two *mak10* strains examined each showed some deficiency of 40S subunits, but complementation with the *MAK10* gene on a CEN plasmid (28) did not change the profile, indicating that the 40S defect was due to some other mutation(s) in the strains. The rest of the *mak* mutants (*mak3*, *-15*, and *-25* mutants) showed normal profiles. *MAK3* encodes an *N*-acetyltransferase whose modification of the N terminus of the L-A coat protein is essential for viral assembly (39–41). These genes might not be involved in the translation machinery.

Interestingly, none of the 25 *mak* mutants examined is deficient in the 40S subunits. These data suggest that the concentration of free 60S subunits is particularly critical for maintenance of M_1 dsRNA virus.

The *rps28B* 40S subunit protein disruptant and *rpl16B* and *rpl46* of the 60S subunit are *mak* mutants. We tested the killer phenotype of some 40S and 60S protein gene disruptant mutants by cytoduction or tetrad analysis (Table 2). Mutants with *rpl16B* (33) and *rpl46* (34) disruptions in 60S subunit protein genes lost the killer phenotype, but *rpl16A* (33), *sos1*, or *sos2* (58) mutants did not. The *rpl16A* mutants are less deficient

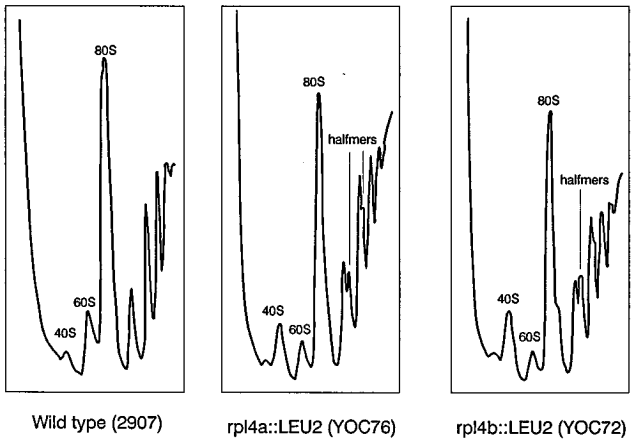

FIG. 4. The *rpl4A* and *rpl4B* disruptions lower the concentration of the free 60S ribosomal subunits. Polysome gradients of the indicated isogenic strains were carried out as described in Materials and Methods. The peaks of free 40S, 60S, and 80S subunits and half-mer polysomes are indicated by arrows. Analysis was carried out as described in Materials and Methods.

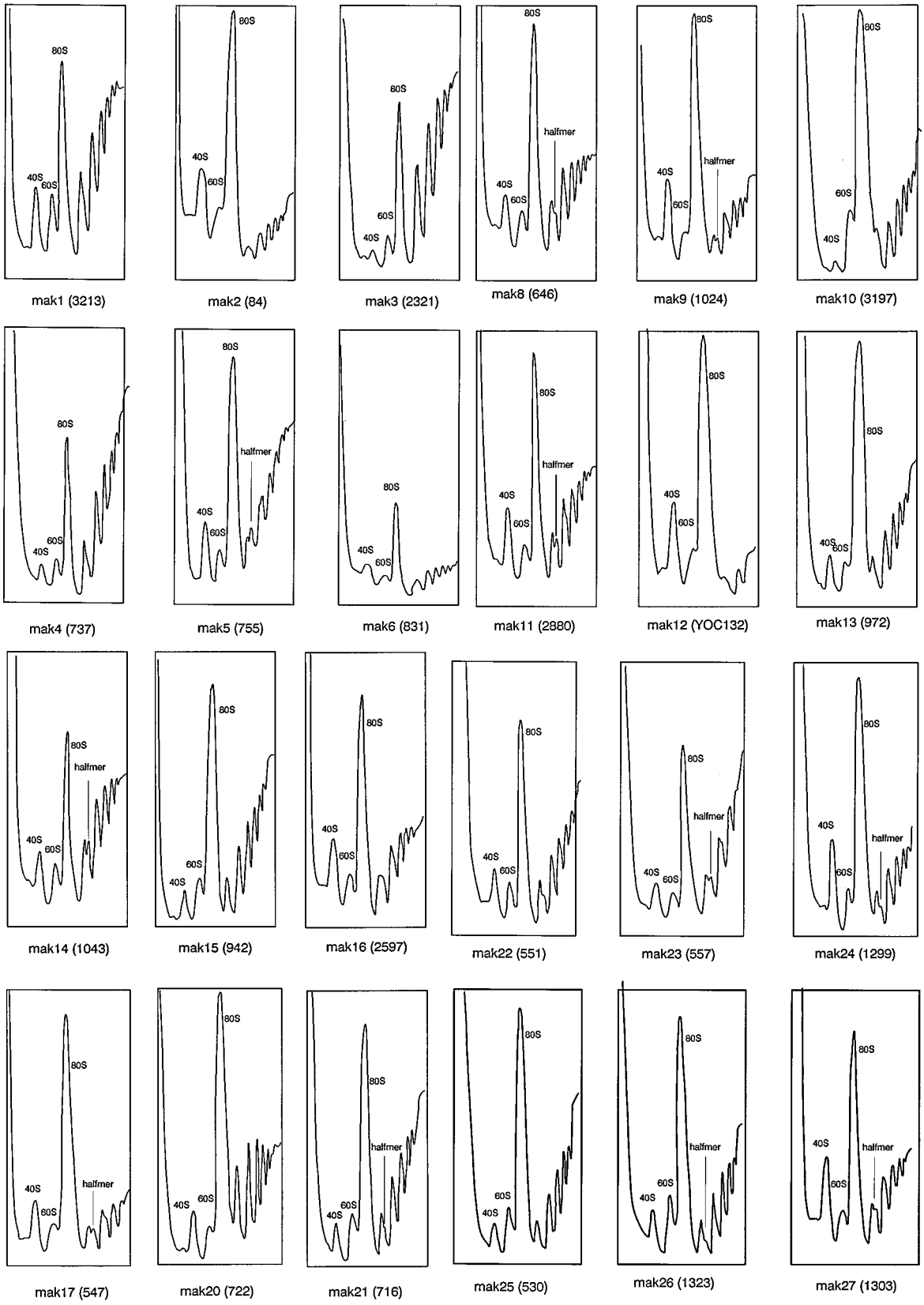
40S
60S
80S
halfmer
mak1 (3213)
mak2 (84)
mak3 (2321)
mak8 (646)
mak9 (1024)
mak10 (3197)
mak4 (737)
mak5 (755)
mak6 (831)
mak11 (2880)
mak12 (YOC132)
mak13 (972)
mak14 (1043)
mak15 (942)
mak16 (2597)
mak22 (551)
mak23 (557)
mak24 (1299)
mak17 (547)
mak20 (722)
mak21 (716)
mak25 (530)
mak26 (1323)
mak27 (1303)

FIG. 5.

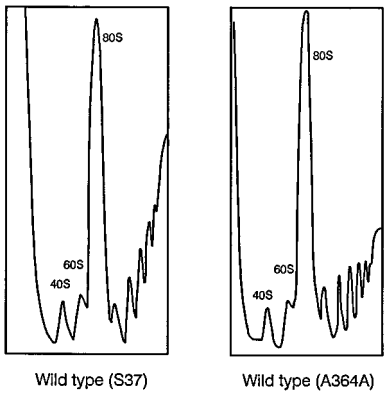

FIG. 5. Most *mak* mutants have decreased free 60S ribosomal subunits. Polysome profiles were obtained as described in Materials and Methods.

TABLE 2. Killer phenotype of 40S and 60S subunit protein-deficient mutants

Ribosomal subunit	Strain	Genotype	Killer phenotype[a]
60S	JWY1410	*rpl16A*	+
	JWY1412	*rpl16B*	−
	YAS282	*rpl46*	−
	CY1746	*sos1*	+
	CY2509	*sos2*	+
40S	J772A	*rps28A*	+
	J771A	*rps28B*	−
	YOC195	*rp51A*	+
	YOC197	*rp51B*	+

[a] Killer phenotypes of YAS282 (*rpl46*), J771A (*rps28B*), and YOC197 (*rp51B*) were tested by tetrad analysis with the strains YOC198, A364A, and D3-1C, respectively. Killer phenotypes of the other strains were tested by cytoduction with either strain 3164 or strain 3165.

than the *rpl16B* mutants (33), and this is presumably the explanation for why the former are not *mak* mutants while the latter are. Among the genes tested encoding 40S subunit proteins, only the *rps28B* (3), not the *rps28A* (3), *rp51A* (2), or *rp51B* (1), disruptant lost the killer phenotype (Table 2). Interestingly, the *rp51B* disruptant keeps the killer phenotype, even though it grew more slowly, with a doubling time of 4.0 h in rich medium, than the isogenic *rpl4A* disruptant (3.0 h). This finding supports the view that the 60S subunits are generally more necessary for viral propagation than the 40S subunits, as does the absence of 40S subunit-deficient mutants among the *mak* mutants.

Disruption of *rpl4A*, *rpl4B*, *mak11*, or *mak16* lowers the copy number of L-A. The *mak7-1*, *mak11*, and *mak16* mutants lose the M_1 killer virus but not the L-A helper virus. To determine whether these mutations have effects on only the M_1 virus or

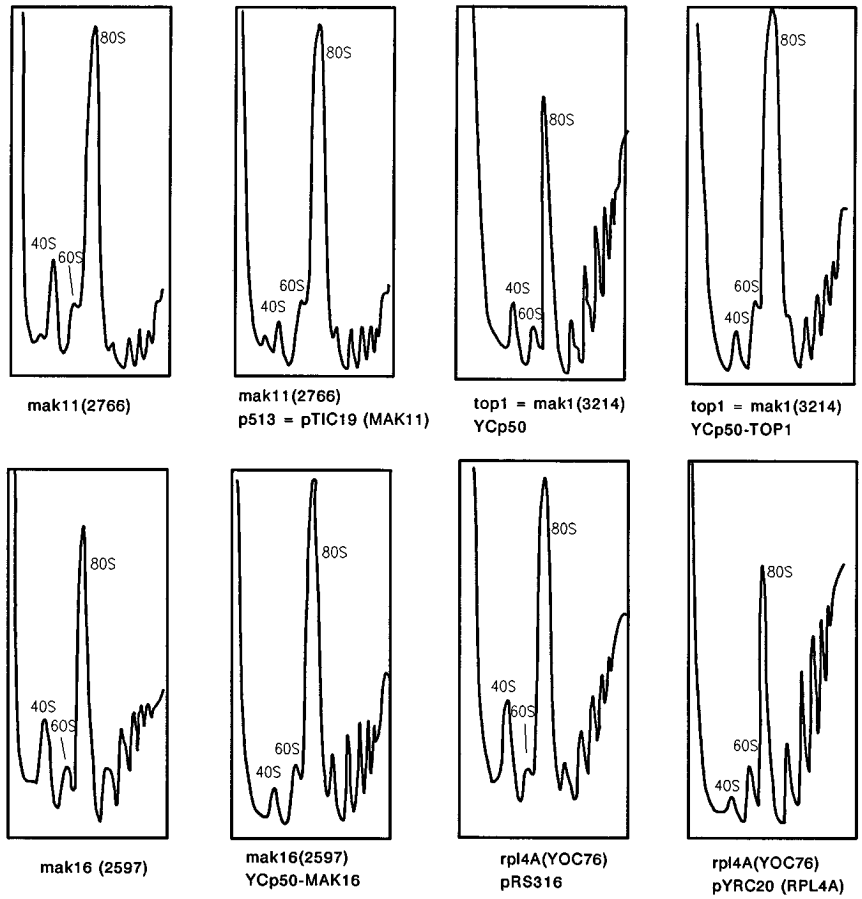

FIG. 6. The free 60S subunits were restored by single-copy *MAK1*, *MAK7*, *MAK11*, or *MAK16* in the respective mutant. Polysome profiles were obtained as described in Materials and Methods. These results show that the deficiency of free 60S subunits in *mak1*, *mak7*, *mak11*, and *mak16* strains was due to the respective *mak* mutation.

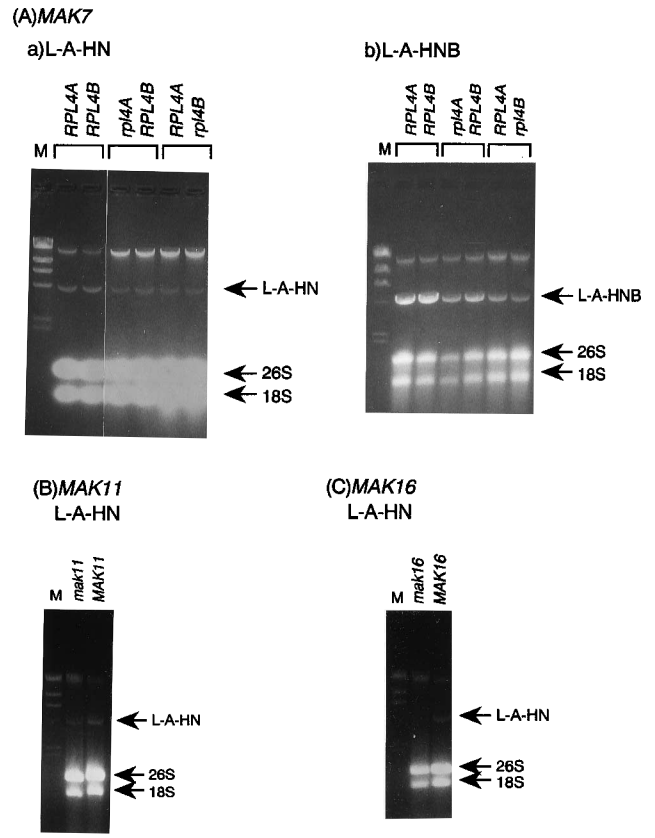

FIG. 7. Disruption of *rpl4A*, *rpl4B*, *mak11*, or *mak16* mutations lowers the L-A copy number. (A) Disruptants of *rpl4A* (YOC76) and *rpl4B* (YOC72) and the parent strain (2907). (B) The *mak11* mutant (2766) with or without the single-copy plasmid carrying *MAK11*. (C) The *mak16* mutant (2597) with or without the single-copy plasmid carrying *MAK16*. In each case, 20 μg of nucleic acids extracted from each strain was analyzed by electrophoresis on a 1% agarose gel and stained with ethidium bromide. Lane M is λ *Hin*dIII fragments. L-A-HN and L-A-HNB are genetic variants of L-A (see text).

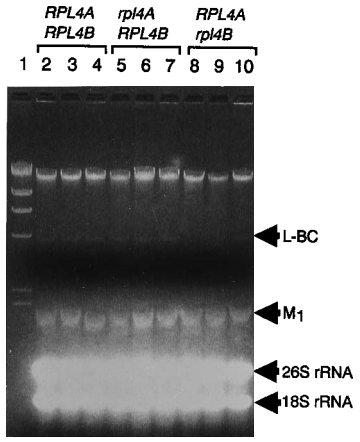

FIG. 8. M_1 copy number of the parent and disruptant strains in which M_1 is supported by the L-A cDNA clone, pI2L2. Twenty micrograms of nucleic acids extracted from each strain was analyzed by electrophoresis on a 1% agarose gel and stained with ethidium bromide. Lanes: 1, λ *Hin*dIII fragments; 2 to 4, colonies of the parent strain 2907; 5 to 7, *rpl4A*::*LEU2* strains; 8 to 10, *rpl4B*::*LEU2* strains.

on both the L-A and M_1 viruses, we examined the copy number of L-A dsRNA. When only the L-A virus was introduced into the mutant strains, we found that the copy number of L-A decreased in the *rpl4A*::*LEU2* and *rpl4B*::*LEU2* disruptants and in the *mak11* and *mak16* mutants (Fig. 7). Further, the amount of the L-A major coat protein in these strains was lower than in the parent strain (data not shown).

Effect of *rpl4A* and *rpl4B* disruptions on M_1 supported by pL-A. The L-A cDNA clone suppresses several *mak* mutations (*mak10*, *mak11*, *mak18*, and *mak27*) (52). We found that the *mak7* disruption is also suppressed by the L-A cDNA clone. The L-A cDNA clone, pI2L2, in the absence of the L-A virus, maintained the M_1 satellite at the same copy number in *rpl4A*::*LEU2*, *rpl4B*::*LEU2*, and parent strains (Fig. 8). The results of Fig. 7 and 8 suggest that *mak* mutations affect M_1 propagation by their effect on the supply of proteins from the L-A virus. Toxin was produced normally in the parent or the *rpl4B*::*LEU2* disruptant strain but was slightly lower in the *rpl4A*::*LEU2* disruptant (Fig. 2B). Comparison of the same strains lacking L-A and M_1 but making toxin from an M_1 cDNA clone, pP-T_{316} (59), showed toxin production essentially the same in the three strains (Fig. 2C). Thus, *rpl4A*::*LEU2* does not affect processing or secretion of the toxin.

Thus, the *rpl4A*::*LEU2* disruption has a greater effect on M_1 viral expression than the *rpl4B*::*LEU2* disruption, and both affect M_1 by their effect on L-A virus expression, since their effects are suppressed by expressing L-A proteins from the cDNA clone.

DISCUSSION

The basis of the loss of M_1 dsRNA by the many *mak* mutants has been unclear, except for the case of *MAK3*, which encodes an *N*-acetyltransferase whose modification of the N terminus of the L-A coat proteins is necessary for viral assembly. Our finding that *MAK7* encodes ribosomal protein L4 and that *MAK18* encodes ribosomal protein L41 (11a), along with the known identities of *MAK8* and *RPL3* (55) and the involvement of *MAK1* (*TOP1*) in rRNA biosynthesis (9, 36), led us to examine the ribosomal profiles of all of the *mak* mutants. In mutants of 18 of the 25 *MAK* genes examined, free 60S subunits were deficient compared to 40S subunits, and no *mak* mutations produced 40S subunit deficiency. Mutants in a further three genes showed apparently normal levels of 40S and 60S subunits but showed half-mer polysomes, indicative of poor ability of 60S subunits to associate with the 40S subunit waiting at the initiator AUG.

While a clear deficiency of free 60S subunits is observed in many *mak* mutants, most of the 60S subunits are present as polysomes or monosomes paired with 40S subunits. Thus, the absolute deficiency of 60S subunits is more modest than that observed for free 60S subunits. Nonetheless, it is for the free subunits that the viral and cellular mRNAs compete in initiation. Our experiments measure only the ratio of free 60S to 40S subunits, not the absolute amounts of either. It is unlikely that mutations in 60S ribosomal proteins increase the amount of 40S subunits, but this remains a possibility in other cases.

Except for *mak3*, *mak10*, and *pet18*, all *mak* mutants were believed to affect only M_1. We find here that all *mak* mutations tested result in decreased L-A copy number, although, as previously shown, L-A is not lost. When M_1 is maintained by proteins produced from an L-A cDNA clone, its copy number is not affected by these *mak* mutations. This finding indicates that these *mak* mutations affect M_1 propagation by affecting the supply of L-A-encoded proteins. Indeed, we find that mutations in *mak7*, *mak11*, or *mak16* substantially reduce the

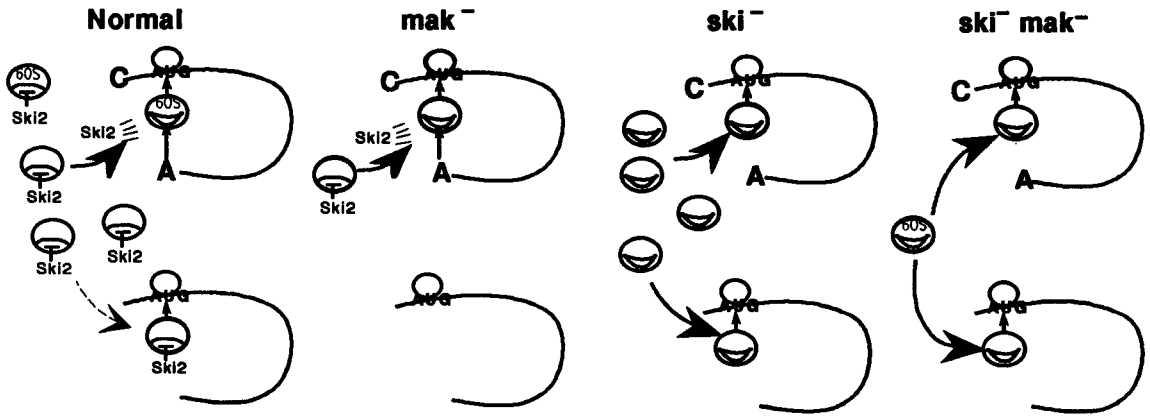

FIG. 9. Model of the effects of certain *MAK* and *SKI* proteins on translation of viral non-poly(A) mRNA. The association of 60S subunits with 40S subunits is believed to require the 60S subunits to interact with the 3′ poly(A) structure (30). Ski2p, Ski3p, and Ski8p-controlled proteins (shown as Ski2) may mediate this requirement, as their mutation nearly eliminates the preference in translation for poly(A)$^+$ mRNA. This poly(A) requirement gives cellular mRNAs an advantage over viral non-poly(A) mRNAs. This advantage is even greater when 60S subunits are scarce because of certain *mak* mutations. The advantage is nearly eliminated by a *ski* mutation, resulting in suppression of *mak* mutations by *ski* mutations (45).

supply of L-A-encoded proteins and the copy number of L-A dsRNA.

Do *mak* mutations affect M_1 more than L-A because of *cis* packaging? It is known that M dsRNA encodes no proteins important for its own replication and relies entirely on L-A for viral proteins (7). The diminished supply of L-A-encoded proteins results in a decreased copy number of L-A but complete loss of M_1. We suggest that L-A can preferentially utilize its encoded proteins and that only excess proteins can be used by M, a limited form of *cis* packaging.

The same notion has been used to explain the selective loss of M_1, but not L-A, from strains in which the efficiency of ribosomal frameshifting was altered producing an altered ratio of Gag-Pol to Gag proteins (15). It was argued that, under conditions of excess Gag-Pol, the L-A positive strand being packaged could be translated to make up the deficiency of Gag, and this would preferentially be used locally. Likewise, when Gag-Pol was less abundant than normal, it might be most likely to bind to the L-A positive strand from which it was just translated. Certain aspects of exclusion of the L-A virus by the L-A cDNA clone were also explained by invoking a partial preference for *cis* packaging (49). Thus, while no direct experiments demonstrating preferential *cis* packaging exist, several lines of indirect evidence point to this possibility.

How does a decreased level of 60S subunits selectively affect translation of viral mRNA? A general decrease in the rate of protein synthesis is not sufficient to explain the loss of M_1 dsRNA or the decreased copy number of L-A. If all mRNAs were translated at half the normal rate, the cells would grow at half the normal rate and the virus would be stably maintained at its normal copy number. The finding that *mak* mutants affect 60S subunits but not 40S subunits indicates that 60S subunit deficiency must selectively affect the translation of L-A mRNA. This view is supported by the fact that although the disruption in the 40S subunit protein gene, *rp51B*, affects cell growth more severely than that in the 60S subunit protein gene, *rpl4A*, the former disruptant does not lose M_1 while the latter does.

In translation initiation, the 40S subunit, with the initiator tRNA and initiation factors, loads on the 5′ end of the mRNA and moves to the first AUG. There it waits for the 60S subunit to arrive. The 60S subunit's association with the waiting 40S subunit is believed to be facilitated by its interaction with the 3′ poly(A), possibly through the action of the poly(A)-binding protein (reviewed in references 30 and 34). We suggest that a deficiency of free 60S subunits should preferentially affect initiation of protein synthesis from non-poly(A) mRNAs, since this interaction of 3′ poly(A) with the 60S subunits would attract them to poly(A)$^+$ mRNAs (Fig. 9).

Ski2p, Ski3p, and Ski8p are part of a cellular system that represses expression of dsRNA viral information. It has recently been found that these proteins act by blocking translation of non-poly(A) mRNA, such as that produced by L-A and M_1 (28a, 56). It was suggested that these Ski proteins act in ribosome biogenesis to make the 60S subunits require the interaction with the 3′ poly(A) structure in order to complex efficiently with the 40S subunits waiting at the initiator AUG. The 60S subunits of a *ski2*, *ski3*, or *ski8* mutant are thus relatively indifferent to the presence or absence of poly(A) (28a), and the effects on M_1 propagation of deficiency of 60S subunits produced by *mak* mutations are thus suppressed by these *ski* mutations (45) (Fig. 9).

Unexplained features. Although the explanation for most of the *mak* mutants (isolated by their inability to propagate M_1) appears to be via their effects on 60S subunit levels, we found that a single 40S subunit protein deletion mutation, *rps28B*, also produced the Mak$^-$ phenotype. This mutant may be affected in the interaction of 40S subunits with 60S subunits. Alternatively, its Mak$^-$ phenotype may be related to the deficiency of 5′ cap structure in L-A mRNA or to another aspect of translation, such as the efficiency of the −1 ribosomal frameshifting critical to produce the correct Gag-Pol/Gag ratio (14). Moreover, several of the *mak* mutants tested here had normal levels of free 60S subunits and normal polysome profiles, indicating that our model does not apply in these cases. For reasons that we do not yet understand, the *sos1* and *sos2* mutants, although deficient in 60S subunits (58), propagate L-A and M_1 normally (Table 2).

L-A-HNB (B for bypass) is a natural variant of L-A which makes dispensable for M_1 propagation many of the *MAK* genes found here to cause deficiency in 60S ribosomal subunits (5, 48). The mechanism of this effect is not yet evident but could be due to the presence in this L-A variant of sequences which promote 60S association in place of the 3′ poly(A) or to a lower preference for *cis* packaging. We find that *rpl4A*::*LEU2* and *rpl4B*::*LEU2* have the same effect on L-A-B copy number

as they do on an L-A lacking B (Fig. 7), indicating that the former mechanism is unlikely.

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