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Translation and M_1 Double-Stranded RNA Propagation: *MAK18* = *RPL41B* and Cycloheximide Curing

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***MAK18* is one of nearly 30 chromosomal genes of *Saccharomyces cerevisiae* necessary for propagation of the killer toxin-encoding M_1 double-stranded RNA satellite of the L-A double-stranded RNA virus. We have cloned and sequenced *MAK18* and find that it is identical to *RPL41B*, one of the two genes encoding large ribosomal subunit protein L41. The *mak18-1* mutant is deficient in 60S subunits, which we suggest results in a preferential decrease in translation of viral poly(A)-deficient mRNA. We have reexamined the curing of M_1 by low concentrations of cycloheximide (G. R. Fink and C. A. Styles, Proc. Natl. Acad. Sci. USA 69:2846–2849, 1972), which is known to act on ribosomal large subunit protein L29. We find that when M_1 is supported by L-A proteins made from the poly(A)$^+$ mRNA of a cDNA clone of L-A, cycloheximide does not decrease the M_1 copy number, consistent with our hypothesis.**

Saccharomyces cerevisiae is host to several double-stranded RNA (dsRNA) viruses (reviewed in references 42 and 43). Among them is the L-A virus and its satellite M_1, a 1.8-kb dsRNA species encapsidated in and replicated by the proteins encoded by L-A's single 4.6-kb dsRNA segment. The viral particles of L-A and M_1 are located in the cytoplasm of the yeast host. L-A and M_1 are very similar to the core particles of dsRNA viruses of higher eukaryotes, reoviruses and rotaviruses, in having replicase and transcriptase activities in the particle and perhaps in their structural symmetry (5).

As for the *Reoviridae*, replication of L-A and M_1 is conservative, with parental strands remaining associated, and sequential, with plus (i.e., coding) and minus strands synthesized at different points of the replication cycle. Viral plus strands are synthesized in the viral particle during a transcription step which uses the minus strand of the parent genome as a template. Newly synthesized plus strands are extruded into the cytoplasm, where they are translated and then encapsidated by viral proteins to form new virus particles. Minus-strand synthesis occurs inside the viral particle during a replication step, and mature viral particles result.

The L-A plus strand has two open reading frames (ORFs): ORF1 and ORF2 (16). ORF1 encodes the 76-kDa major coat protein, Gag. A combination of ORF1 and the overlapping ORF2 encodes the 180-kDa Gag-Pol fusion protein formed by a −1 ribosomal frameshift event (8, 14, 16). The Pol portion of this fusion protein is involved in the transcription and replication steps of the L-A and M_1 life cycles. The M_1 virus does not encode any proteins needed for its own propagation; however, it does encode a secreted protein toxin and immunity to that toxin (reviewed in reference 4). M_1 is dependent upon the protein products of L-A for its propagation (3, 44).

In addition to these two L-A-encoded proteins, propagation of L-A and M_1 is affected by an assortment of chromosomal genes. These genes are divided into two groups: the *SKI* genes and the *MAK* genes. The *SKI* genes repress the copy number of L-A and M_1 and the translation of their mRNAs (1, 21, 37, 46). These genes appear to constitute a host antiviral system. Mutants of these genes were first isolated on the basis of their superkiller (SKI) phenotype resulting from the increased copy number of M_1 (37). The *MAK* genes (for maintenance of killer) are defined by mutations that result in the loss of M_1. Approximately 30 *MAK* genes have been defined by mutation (reviewed in reference 42). Of these, only *mak3*, *mak10*, and *pet18* mutants lose L-A (33). How the *MAK* and *SKI* genes function in the host and how they affect viral propagation are important problems in understanding the basic mechanisms of viral replication.

The focus of this report is the *MAK* genes. These gene products include two ribosomal proteins (*MAK7*, *MAK8* [24, 45]), an *N*-acetyltransferase (*MAK3* [35]), DNA topoisomerase I (*MAK1* [36]), a membrane-associated protein that has homology with Cdc4p and β-transducin (*MAK11* [15]), a nuclear protein required to transit G_1 (*MAK16* [41]), and a protein needed for optimal growth on nonfermentable carbon sources with local similarities to the α subunits of T-cell receptors (*MAK10* [19]). While the individual properties of many of the *MAK* genes have been identified, except for *MAK3*, how these proteins contribute to the maintenance of M_1 was not understood.

In this work, we report that *MAK18* encodes the ribosomal protein L41B and that *mak18* mutants are deficient in 60S subunits. These findings contributed to the general understanding that many of the *MAK* gene products are involved in the synthesis of 60S ribosomal subunits. We tested this idea by reexamining the curing of M_1 dsRNA with low concentrations of cycloheximide (11). Cycloheximide is known to act on the large ribosomal subunit protein, L29 (13, 34). We found that cycloheximide curing of M_1 dsRNA depends on the source of the L-A-encoded proteins. When these proteins are made from the viral poly(A)-deficient mRNA, M_1 is cured by cycloheximide (11); however, when Gag and Gag-Pol are synthesized from the poly(A)$^+$ mRNA of an L-A cDNA clone, cycloheximide does not decrease the M_1 copy number. These findings are consistent with our hypothesis that a deficiency in

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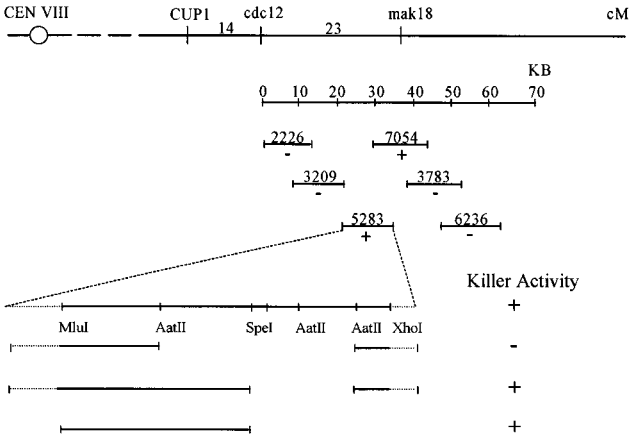

FIG. 1. Genetic map (top) and physical map (bottom) of the part of chromosome VIII including *MAK18*. Lambda clones of this region were tested for complementation of *mak18-1*, and only 5283 and 7054 were positive. Subcloning of 5283 showed that the region complementing *mak18-1* was the *Mlu*I-*Spe*I fragment shown.

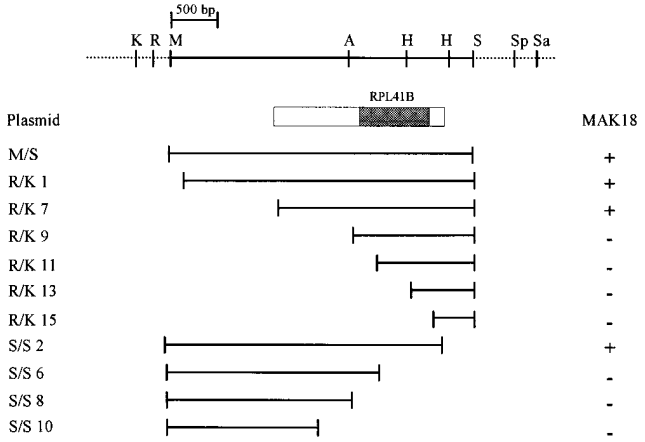

FIG. 2. Deletion mutants and sequence analysis of *MAK18*. The minimum extent of the *MAK18* gene (shaded box) was contained completely within the *RPL41B* open reading frame, and the maximum extent of the *MAK18* gene (open box) included all of *RPL41B*. K, *Kpn*I; R, *Eco*RI; M, *Mlu*I; A, *Aat*II; H, *Hpa*I; S and Sp, *Spe*I; Sa, *Sac*I.

60S subunits results in a preferential decrease in translation of the viral mRNA transcripts.

MATERIALS AND METHODS

Media and strains. Yeast media, including YPAD, YPG, SD, H-Ura, and 4.7 MB as described previously (31), were used. The *mak18-1* strain 3014 (*MAT***a** *his3,6 trp1 ura3 mak18-1 rho-o*) was used to test for *MAK18* complementing activity. Yeast strain 3380 (*MAT*α *kar1 ura2 leu2* L-A M_1) was used in cytoduction experiments to transfer L-A and M_1 into strain 3014. Strain 5X47 was the toxin-sensitive strain used as an indicator in the killer assay (26). Yeast strain 2907 (*MAT***a** *his3-200 leu2 trp1-901 ura3-52 ade2-10* L-A M_1) was used in the cycloheximide experiments. *Escherichia coli* DH10B was used for the propagation of plasmids.

DNA procedures. Plasmids were purified by an alkaline lysis procedure (2). Restriction enzymes, DNA polymerase I (Klenow fragment), calf intestinal phosphatase, and T4 DNA ligase were obtained from New England Biolabs. Transformation of yeast strains was achieved by the lithium acetate method (17). Transformation of *E. coli* was achieved by the calcium chloride procedure (30).

Phage. Bacteriophage lambda clones were obtained from the American Type Culture Collection. *E. coli* C600 was used to determine titers and prepare lambda DNA. Lambda DNA was prepared as follows. A 50-ml culture of C600 was grown in Luria broth plus 10 mM $MgCl_2$ overnight at 30°C. Two milliliters of the C600 culture was combined with 5×10^7 PFU of the virus, incubated for 10 min at 37°C, diluted into 100 ml of Luria broth plus 0.2% maltose and 10 mM $MgCl_2$, and grown at 37°C until lysis occurred. Solid NaCl was added to a final concentration of 1 M. The material was centrifuged, and the pellet was discarded. The supernatant was made to 10% (wt/vol) in polyethylene glycol 8000. After overnight incubation at 4°C and centrifugation at 6,000 rpm for 20 min, the pellet was resuspended in 3 ml of TM (50 mM Tris-HCl [pH 7.5], 8 mM $MgSO_4 \cdot 7H_2O$). Ten percent sodium dodecyl sulfate (0.3 ml) was added, the material was extracted with phenol-chloroform, and then chloroform and the lambda DNA were precipitated with ethanol and dissolved in TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]).

Cloning the *MAK18* gene. *MAK18* was genetically mapped to the right arm of chromosome 8, to the right of the *CDC12* gene (Fig. 1) (40). *MAK18* was cloned by an in vivo recombination technique (10), using the *E. coli*-yeast shuttle plasmid pBM2240 and lambda clones of yeast DNA. pBM2240 contains DNA fragments from the lambda vector which flank the cloned yeast insert. When a lambda clone and linearized pBM2240 are cotransformed into yeast, neither the linearized pBM2240 nor the lambda clone can propagate, but recombination between their homologous regions yields circular (and thus replication-competent) pBM2240 containing the yeast DNA insert from the lambda vector. To clone *MAK18*, strain 3014 (*mak18-1* ρ^o) was cotransformed with linearized pBM2240 and each of the six lambda clones covering this region to the right of the *CDC12* gene (25, 27). Colonies selected on H-Ura plates (three for each lambda clone) were tested for the presence of plasmids containing the *MAK18* gene as described below.

Testing plasmids for the *MAK18* gene. The *mak18-1* strain 3014 was transformed with the plasmid to be tested. The cytoplasmic material of a haploid strain can be transferred to another without diploidization or other change of nuclear genotype by transient heterokaryon formation (cytoduction) with the *kar1* mutant, which is defective in nuclear fusion. Recipient strain 3014 was made ρ^o by growing the cells on ethidium bromide to eliminate the mitochondrial DNA. Cytoplasm carrying the mitochondrial genome (ρ) as a cytoduction marker and the L-A and M_1 viruses was transferred to the recipient strain as described previously (26). The cytoduction mixture was streaked on H-Ura plates to select against the donor strain and for retention of the plasmid in the recipient strain. After 3 days, the colonies were replica plated onto SD, YPG, and 4.7 MB plates spread with a lawn of 5X47 cells, which are sensitive to the killer toxin. SD plates identify diploid cells, YPG plates identify diploids and cytoductants, and 4.7 MB killer test plates identify cells which are able to maintain M_1. A plasmid was determined to contain *MAK18* if the ρ^+ Ura^+ SD^- cytoductants were killers.

Yeast plasmid minipreparations. Cultures of yeast cells (2.5 ml) were grown overnight in H-Ura liquid media to an optical density at 550 nm (OD_{550}) of 1.0 to 2.0. A 1.5-ml portion of the overnight culture was spun down in a microcentrifuge and washed once with 600 μl of deionized water and once with 600 μl of 1 M sorbitol. The cells were resuspended in 600 μl of SCEM (1 M sorbitol, 0.1 M sodium citrate [pH 5.8], 10 mM EDTA, 30 mM 2-mercaptoethanol) containing 50 U of lyticase (Sigma) per ml. The cells were incubated at 30°C for 60 min. The spheroplasts were spun down and washed with 600 μl of 1 M sorbitol. At this point, the alkaline lysis plasmid preparation procedure was followed (2). The DNA was resuspended in 15 μl of TE, and 5 μl of the solution was used to transform Max Efficiency DH10B *E. coli* (BRL) selecting resistance to 20 μg of ampicillin per ml.

Sequence analysis. The gene for *MAK18* was subcloned into the *Sma*I site of vector pRS316 (32). The Erase-A-Base Kit (Promega) was used to generate two sets of unidirectional deletion plasmids. This kit uses exonuclease III, which digests insert DNA from a 5′ protruding or blunt-end restriction site but does not digest a 3′ end which has a 4-base overhang. For one set of deletion plasmids, pM/S was restricted with *Kpn*I and *Eco*RI. For the other set of deletion plasmids, *Sac*I and *Spe*I were used. These plasmids (Fig. 2) were tested for *MAK18* activity as described above and were used to sequence the *MAK18* gene. dsDNA sequencing of the *MAK18* gene was performed with the Sequenase Kit, version 2.0 (United States Biochemical).

Polysome preparation and analysis. Polysome preparation and analysis were performed as described previously (24).

Preparation of dsRNA. Yeast cells were grown to an OD_{600} of 1.0 to 2.0. dsRNA was prepared as described previously (12). Nucleic acid was resuspended in TE, and quantitation was by OD_{260}, where a value of 1.0 is equal to 42 μg/ml. Nucleic acid was resolved on 1.0% Tris-acetate-EDTA agarose gels containing 0.5 μg of ethidium bromide per ml.

Northern hybridization. Northern (RNA) hybridization was performed as described previously (24). An M_1 probe was prepared from p596 by transcription with T7 RNA polymerase and [α-^{32}P]UTP as described previously (24).

RESULTS AND DISCUSSION

Cloning of *MAK18*. *MAK18* was genetically mapped to the right arm of chromosome VIII, near *CDC12* (40) (Fig. 1). Riles et al. have constructed a physical map of chromosome VIII, using a bank of yeast DNA in a lambda vector (25, 27). As described in Materials and Methods, an in vivo recombination

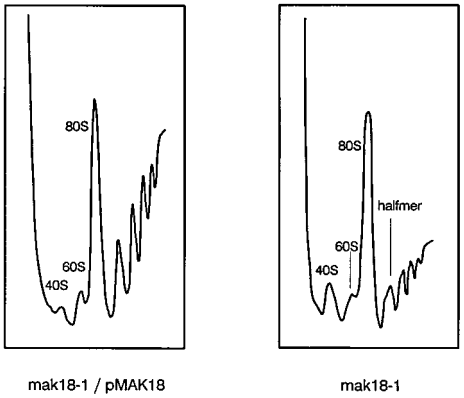

FIG. 3. Polysome profiles of isogenic *mak18-1* (3014) and wild-type (3014 with pM/S) strains.

technique (10) was used to test six lambda clones chosen on the basis of the genetic map location of *MAK18* (Fig. 1). L-A and M_1 were transferred by cytoduction into these cells. If the cell contained a recombinant *MAK18* plasmid, M_1 would be maintained and the cell would be a killer. Plasmids resulting from the in vivo recombination performed with lambda clones 7054 and 5283 complemented the *mak18-1* mutation. As can be seen in Fig. 1, the yeast inserts of these two lambda clones overlap.

Subcloning and sequencing of the *MAK18* gene. The recombinant *MAK18* plasmid derived from lambda 5283 recovered by transforming *E. coli* contained a yeast DNA insert of approximately 15 kb (Fig. 1). Subclones were tested for their ability to complement the *mak18-1* mutation (Fig. 1 and 2). The *MAK18* gene was located on an approximately 3.0-kb *Mlu*I-*Spe*I fragment. This fragment was inserted into the *Sma*I site of pRS316, generating the plasmid pM/S, whose complementation of the *mak18-1* mutation was confirmed.

Deletion derivatives of pM/S generated with exonuclease III were used for sequencing and for further localizing the *MAK18* gene by complementation in the *mak18-1* strain 3014 (Fig. 2). Analysis of the sequence and deletion data showed that *MAK18* is identical to *RPL41B* (18). Specifically, the region shown by the deletion mutants to be essential for complementing *MAK18* is entirely within the *RPL41B* open reading frame, and the region sufficient for *MAK18* complementation completely includes the *RPL41B* gene. The product of this gene is the 106-amino-acid 60S ribosomal subunit protein, L41. There are two L41 genes in *S. cerevisiae*, the other being *RPL41A* (18).

Polysome profile. In the *mak18-1* strain, the free 60S subunit peak was diminished relative to the free 40S subunit peak (Fig. 3). An appearance suggestive of half-mer polysome peaks, indicating that mRNA molecules containing ribosomes had both complete ribosomes with 60S and 40S subunits and 40S subunits alone, was also observed. When this strain was expressing Mak18p from plasmid pM/S, the free 60S subunit peak was restored, and half-mers were not found. This finding was the expected phenotype for a strain containing a mutation in a protein component of the 60S subunit (7, 23, 28).

Cycloheximide curing of M_1. Low levels of cycloheximide result in the selective curing of M_1 in yeast cells containing both L-A and M_1 (11), and cycloheximide is known to act on L29, another 60S subunit protein (13, 20, 34). Just as expression of the Gag and Gag-Pol proteins from an L-A cDNA plasmid suppressed the 60S subunit protein mutation *mak18-1* for M_1 propagation (44), under these conditions M_1 may not

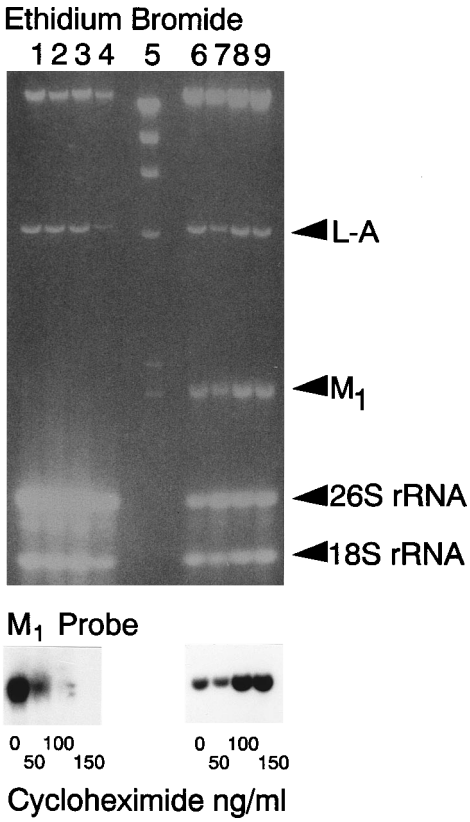

FIG. 4. Ineffective cycloheximide curing with M_1 supported by the L-A cDNA clone. Eight micrograms of nucleic acid from each strain was separated on a 1% agarose gel containing 0.5 μg of ethidium bromide per ml (top) prior to transfer to a filter for hybridization with the M_1 probe (bottom) as described in Materials and Methods. Lanes 1 to 4, strain 2907 grown with 0, 50, 100, and 150 ng of cycloheximide per ml, respectively. Lanes 6 to 9, strain 2907 containing the L-A cDNA plasmid pTIL131 grown with 0, 50, 100, and 150 ng of cycloheximide per ml, respectively. Lane 5, DNA standards.

be lost at the same level of cycloheximide which cured the cells of M_1 when it was supported by the L-A virus.

Strain 2907 with or without the L-A cDNA plasmid pTIL131 (44) was grown at various levels of cycloheximide. dsRNA was prepared from equal volumes of cells which had grown to an OD_{600} of 1.0 to 2.0, analyzed on an agarose gel (Fig. 4, top), blotted, and hybridized with the M_1 probe (Fig. 4, bottom). The Northern blot shows that M_1 was present in strain 2907 and was lost with increasing amounts of cycloheximide. At all levels of cycloheximide, cells containing the L-A cDNA plasmid maintained M_1. Furthermore, the copy number of M_1 was increased in cells containing the L-A cDNA plasmid such that this dsRNA species could be visualized by a very short exposure time.

M_1 propagation and L-A mRNA translation. The M_1 satellite encodes no proteins required for its own propagation; instead, it relies on the Gag and Gag-Pol proteins encoded by L-A. Therefore, the *mak* mutations may affect M_1 propagation by their effect on the translation of the Gag and Gag-Pol transcripts. The L-A mRNA transcripts of Gag and Gag-Pol are synthesized by the viral transcriptase, and they differ from the majority of cellular messages in that they lack both the 5′ cap and 3′ poly(A) tail structures. Interestingly, if M_1 is maintained by an L-A cDNA clone instead of by the L-A virus, several *mak* mutations, including *mak18-1*, do not result in the loss of M_1 (44). The Gag and Gag-Pol transcripts of the cDNA

clone do have the 5′ cap and 3′ poly(A) tail structures. This result suggests that the absence of these structural features on the L-A transcripts is a critical feature for the propagation of M_1.

We show here that *MAK18* is one of the genes for the large ribosomal protein L41, *RPL41B*. Stimulated by this result and the knowledge that *MAK18* is *TCM1* encoding large ribosomal protein L3 and that *MAK7* is *RPL4A* (24), we carried out an analysis of the ribosomal profiles of all the *mak* mutants. This analysis showed that mutants with mutations in 18 *mak* genes have decreased levels of free 60S ribosomal subunits (24). Mutants with mutations in another three *mak* genes had normal levels of the 60S and 40S ribosomal subunits but had half-mer polysomes indicative of the decreased ability of the 60S and 40S subunits to interact on an mRNA molecule. We propose that this decrease in the concentration of free 60S subunits is the basis of the loss of M_1 by many *mak* mutants.

The growth of cells on low concentrations of cycloheximide results in the preferential curing of M_1 dsRNA (11). This effect of cycloheximide mimics the effect of a *mak* mutation. Cycloheximide is known to affect the large ribosomal subunit, L29 (13, 34), and cycloheximide-resistant mutants with mutations in the L29 gene (*cyh2*) are indeed resistant to curing of M_1 by this drug (20). Just as supplying the L-A-encoded proteins from a poly(A)$^+$ transcript of an L-A cDNA clone suppressed the effect of 60S subunit deficiency produced by *mak18-1* on M_1 propagation (44), these same circumstances prevented even a decrease in M_1 copy number when the cells were exposed to cycloheximide. This observation supports our view of the critical dependence of translation of viral non-poly(A) mRNAs on the pool of 60S subunits. Low levels of cycloheximide have an inhibitory effect on the initiation of translation (6), pointing to the possibility that initiation of viral protein translation is the sensitive step.

Why are 60S subunits critical for viral propagation? The 5′ cap and 3′ poly(A) structures possessed by most cellular messages are involved in the efficient initiation of translation of an mRNA molecule. When translation commences, the 40S ribosomal subunit binds to the cap structure at the 5′ end of the mRNA and moves down the message to the initiator AUG codon where it awaits the arrival of the 60S subunit. The 3′ poly(A) structure is believed to facilitate the 60S subunit's association with the 40S subunit, perhaps by the action of the poly(A) binding protein (22, 29). The *mak* mutations that produce a deficiency of free 60S ribosomal subunits probably put the viral poly(A)-deficient messages at a disadvantage relative to cellular poly(A)$^+$ mRNAs because the latter are better able to attract 60S subunits.

While L-A provides M_1 with the proteins Gag and Gag-Pol, it has been suggested that L-A may do so only after its own protein requirements have been met (9, 24, 39). This mechanism can be viewed as preferential *cis* packaging. A decrease in the 60S subunit concentration, by reduction of the efficiency of L-A plus-strand translation, could thus result in the selective loss of M_1.

The antiviral action of the six chromosomal *SKI* genes is mediated by their ability to limit the translation of poly(A)-deficient mRNA (21). Thus, translation efficiency in a *ski* mutant is nearly indifferent to the presence of the 3′ poly(A). This predicts that the *ski* mutations should suppress the *mak* mutations that produce a deficiency in 60S subunits, as has long been known to be true (38). In a *ski mak* double mutant, although 60S subunits are deficient (because of the *mak* mutation), these subunits are nearly indifferent to the presence or absence of poly(A) on the mRNA, so the viral poly(A)-deficient mRNAs are not at a disadvantage relative to the poly(A)$^+$ cellular mRNAs.

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