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Ski6p Is a Homolog of RNA-Processing Enzymes That Affects Translation of Non-Poly(A) mRNAs and 60S Ribosomal Subunit Biogenesis

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We mapped and cloned *SKI6* of *Saccharomyces cerevisiae*, a gene that represses the copy number of the L-A double-stranded RNA virus, and found that it encodes an essential 246-residue protein with homology to a tRNA-processing enzyme, RNase PH. The *ski6-2* mutant expressed electroporated non-poly(A) luciferase mRNAs 8- to 10-fold better than did the isogenic wild type. No effect of *ski6-2* on expression of uncapped or normal mRNAs was found. Kinetics of luciferase synthesis and direct measurement of radiolabeled electroporated mRNA indicate that the primary effect of Ski6p was on efficiency of translation rather than on mRNA stability. Both *ski6* and *ski2* mutants show hypersensitivity to hygromycin, suggesting functional alteration of the translation apparatus. The *ski6-2* mutant has normal amounts of 40S and 60S ribosomal subunits but accumulates a 38S particle containing 5′-truncated 25S rRNA but no 5.8S rRNA, apparently an incomplete or degraded 60S subunit. This suggests an abnormality in 60S subunit assembly. The *ski6-2* mutation suppresses the poor expression of the poly(A)$^-$ viral mRNA in a strain deficient in the 60S ribosomal protein L4. Thus, a *ski6* mutation bypasses the requirement of the poly(A) tail for translation, allowing better translation of non-poly(A) mRNA, including the L-A virus mRNA which lacks poly(A). We speculate that the derepressed translation of non-poly(A) mRNAs is due to abnormal (but full-size) 60S subunits.

The 5′ end of eucaryotic mRNA has a cap structure of the form m7G5′ppp5′Xp, while the 3′ end is polyadenylated. Both of these structures are essential for efficient translation and mRNA stability. The requirement for cap and poly(A) structure for messenger expression constitutes a handicap for RNA viruses whose mRNA is not made by the cellular machinery. L-A virus mRNA lacks both 5′ cap and 3′ poly(A) structures (4, 52), and its cytoplasmic location makes it unlikely that it can use cellular enzymes to modify its mRNA. Indeed, translation plays a large role in the interactions of the L-A double-stranded RNA (dsRNA) virus with its host, *Saccharomyces cerevisiae* (reviewed in references 11, 22, 32, and 58). L-A has two overlapping open reading frames, *gag*, encoding the major coat protein, and *pol*, encoding the RNA-dependent RNA polymerase and packaging function. L-A uses a −1 ribosomal frameshift to make a Gag-Pol fusion protein, and the efficiency of this frameshift is critical for viral propagation (12–14).

Many viruses adopt a variety of tricks to acquire a cap or poly(A) structure. Influenza viruses and Bunyaviruses steal caps from cellular mRNAs; reoviruses, rhabdoviruses, and togaviruses encode their own capping enzymes; and *Picornaviridae* carry out cap-independent translation (15, 32). Picornaviruses, togaviruses, influenza viruses, and many other RNA viruses have an encoded sequence that is copied to produce mRNA with a 3′ poly(A) structure, while rhabdoviruses have no encoded poly(A) but add it enzymatically to their transcripts. However, reoviruses and many plant virus mRNAs lack a 3′ poly(A).

The apparent lack of 5′ cap and 3′ poly(A) does not prevent L-A from being highly expressed. However, this lack of both structures makes L-A mRNA expression sensitive to chromosomal mutations affecting functions related to cap or poly(A). Accordingly, studies of L-A have secondarily brought insights into the roles of cap and poly(A).

For instance, the *SKI* genes (named *SKI* for superkiller) were identified by the superkiller phenotype of mutants (45, 53). The L-A particles can separately encapsidate a satellite dsRNA, called M dsRNA, encoding a secreted protein toxin (the killer toxin). *ski* mutants have higher copy numbers of M_1 and L-A dsRNAs and so make more killer toxin (1). *SKI1* later proved to be *XRN1*, encoding the 5′→3′ exoribonuclease specific for uncapped RNA and responsible for the major pathway of mRNA decay (21, 23, 37, 50, 51). It is evident how the uncapped L-A mRNA would be affected by this protein. To subvert the Ski1p/Xrn1p nuclease, the L-A major coat protein has a decapping activity which decapitates some cellular mRNAs to partially distract the nuclease from working on the capless L-A mRNA (2, 3, 31).

While *SKI1* concerns RNA stability, *SKI2*, *SKI3*, and *SKI8* encode a system that blocks the translation of non-poly(A) [poly(A)$^-$] mRNA (31). *ski2*, *ski3*, or *ski8* mutants translated electroporated C^+ A^- [Cap^+ poly(A)$^-$] mRNA nearly as well as C^+ A^+ mRNA. Both physical and functional stabilities of the electroporated mRNA showed little effect from these mutations. Ski3p is a 163-kDa nuclear protein (44), while Ski2p is an RNA helicase with a glycine-arginine-rich domain typical of nucleolar proteins and is homologous to a human nucleolar protein (7, 28, 61). Ski8p has two copies of the β-transducin (WD) repeat sequence but otherwise has no close homologs (33). This suggested to us that the effects of *ski2* and *ski3* (and

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ski8) on expression of poly(A)$^-$ mRNAs might best be explained by a function taking place in the nucleus.

Strains with mutations in any of more than 20 genes resulting in deficiency of 60S ribosomal subunits are defective in viral propagation (5, 41). Mutations in *ski2*, *ski3*, or *ski8* suppress the effect of 60S subunit deficiency on viral propagation (31, 54). We adopted a model in which 60S interaction with poly(A) is a prerequisite for joining 40S subunits waiting at the initiator AUG (reviewed in reference 22), and these *SKI* products mediate this effect by a role in 60S subunit biogenesis (31, 41). This model explains why a deficiency of 60S subunits impairs viral propagation more than cell growth, why *ski* mutants show increased virus copy number and expression, and why *ski* mutations suppress mutations producing deficiency of 60S subunits.

SKI6 genetically resembles *SKI2*, *SKI3*, and *SKI8* (45). Here, we show that *SKI6* encodes an essential 246-residue protein homologous to several bacterial RNase PHs, an enzyme responsible for processing the 3′ end of pre-tRNAs. We find that *ski6-2* results in derepression of translation of non-poly(A) mRNA and hypersensitivity to the antibiotic hygromycin. The *ski6* mutation suppresses the effect of 60S subunit deficiency on viral propagation. Polysome gradients reveal a novel 38S particle in *ski6-2* mutants which contains a fragment of 25S rRNA and lacks 5.8S rRNA. These findings provide direct evidence that Ski6p is involved in 60S ribosomal subunit biogenesis and, perhaps through this, affects translation of non-poly(A) mRNA.

MATERIALS AND METHODS

Genetic mapping of *SKI6*. M_2 dsRNA is a killer toxin-encoding satellite of the L-A virus, meaning that its coat protein and replication proteins are encoded by L-A. At temperatures of 32°C or higher, M_2 propagation depends on two genes, called *MKT1* and *MKT2* (maintenance of K_2), which are not needed by M_1 dsRNA (59, 60). Mutations in the *SKI* genes suppress this requirement of M_2 for *MKT* genes (45). Many laboratory strains carry *mkt1* mutations, and some have *mkt2* mutations. We crossed *mkt1 ski6* M_2 strains with a set of multiply marked strains designed for genetic mapping (18) which proved to also be *mkt1*. We found *ski6* tightly linked to *ade3* (parental ditype = 58, nonparental ditype = 0, tetratype = 10, 7.3 centimorgans) on the right arm of chromosome VII.

Cloning of *SKI6*. Strain RV493 (= *MAT***a** *ura3 ade3 his*$^-$ *leu2 trp1 ski6-2 mkt1* L-A-HN M_2) is stably K_2^+ at either 25 or 32°C, but if it becomes *SKI*$^+$, then it will be K_2^- after growth at 32°C. Several λ clones of yeast DNA located around *ADE3* (46) were tested by cotransformation of λ clone DNA and plasmid pBM2240 cut with *Eco*RI and *Xho*I (16). pBM2240 has homology with the arms of the λ clone, and the linearized plasmid can replicate only by recombining with the λ clone in such a way as to transfer the insert into the plasmid pBM2240 (16). The transformants were first isolated at 25°C, grown at 25 or 32°C, and then tested for killer activity. Only λ5047 gave recombinants which complemented *ski6-2* (see Fig. 1). The pBM2240 derivative carrying the insert of λ5047 was isolated, and subclones were made by cleavage with *Hin*dIII and ligation to pRS316 cut with the same enzyme. One of these subclones, 5047H8, complemented *ski6-2*. 5047H8 was cut with *Spe*I, *Sac*I, *Sal*I, *Cla*I, or *Xho*I, followed by ligation. The ends of several of these clones were sequenced and found to be included in the 9-kb region sequenced by Guerreiro et al. (20). Only the *Sal*I-cut and religated derivative of 5047H8 complemented *ski6-2*, suggesting that the open reading frame (ORF) designated YGR195w was *SKI6* (see Fig. 1). This was confirmed by cloning the 1,283-bp *Afl*II-*Afl*II fragment (blunt ended with Klenow fragment) containing YGR195w from the *Sal*I derivative of 5047H8 into pRS316 cut with *Sma*I and showing that the resulting plasmid, pSKI6, complements *ski6-2* (see Fig. 1).

Plasmid constructions. To make a disruption of *SKI6*, the *Afl*II-*Afl*II fragment containing *SKI6* was inserted in a Bluescript vector, making pSK+SKI6, and the *HIS3* gene on a *Sma*I-*Pvu*II fragment from pJJ215 (24) was inserted into pSK+*SKI6* cut with *Nde*I (blunt ended with Klenow fragment) and *Eco*RV, replacing a 369-bp segment of *SKI6* (Fig. 1). The resulting pSK+*ski6::HIS3* plasmid was digested with *Nae*I and *Sac*I and used to transform the diploid strain 2959 × 2966 (=*MAT***a**/*MAT*α *his3*/*his3 trp1*/*trp1 ura3*/+ +/*leu2* L-A-HN M_2). To confirm the disruption, chromosomal DNA was isolated, digested with *Hin*dIII, blotted onto a nitrocellulose filter, and probed with the 353-bp *Afl*II-*Nde*I fragment. The probe detected an 8.2-kb fragment in the normal sequence and a 2.4-kb fragment in the disrupted sequence. The disrupted diploid 2959 × 2966 *ski6::HIS3* segregated two viable His$^+$ spores with the undisrupted pattern to two inviable spores. Diploid disruptants showed both undisrupted and disrupted bands.

Expression of luciferase mRNAs. The luciferase mRNA expression plasmids T7 LUC [poly(A)$^-$] and T7 LUC50 [50-mer poly(A) tail] have been described previously (19). For RNA synthesis, T7 LUC was linearized with *Sma*I and T7 LUC50 was linearized with *Dra*I. Transcripts were synthesized with the Ambion MEGAscript transcription kit in the presence or absence of the cap analog m7GpppG in accordance with the manufacturer's instructions. After DNase I treatment followed by precipitation with LiCl, RNAs were passed over G-50 columns (5 Prime-3 Prime Inc. SELECT-B). RNA was quantitated both by measuring the optical density at 260 nm (OD_{260}) and by a comparison on agarose gels with known concentration standards.

RNA electroporation was done as described previously (17) with minor modifications. Cells for spheroplast preparations were grown in selective medium (H-Ura). After lyticase treatment, cells were incubated for 2 h in YPAD-sorbitol medium to make them metabolically active. Two micrograms of RNA was used for electroporation, and cells were assayed for luciferase activity after 2 h (or as indicated) of outgrowth at 30°C in YPAD-sorbitol medium. Luciferase activity was assayed as previously described (31) with a buffer containing coenzyme A.

Stability of electroporated RNA and luciferase accumulation. The method for determination of stability of electroporated RNA and luciferase accumulation is identical to that described previously (31) except that collected spheroplasts were quickly washed twice with 0.5 ml of 1 M sorbitol, and resulting pellets were frozen in a dry ice-ethanol mix. For every time and strain, two samples were collected. One pellet was used for a luciferase assay, and the other was used to extract RNA.

Yeast extracts, polysome preparation, and analysis. Cell lysis was done by a modification of a published protocol (40). Cycloheximide (100 μg/ml) was added to a 200-ml cell culture at 30°C in H-Ura at an OD_{600} of 0.4 or 0.5. For growth at the nonpermissive temperature, cells were grown first at 30°C to an OD_{600} of 0.05 to 0.1 and then at 39°C for 8 h. The culture was quickly cooled in ice water after cycloheximide was added and then centrifuged and washed in 20 ml of buffer A containing 20 mM HEPES (pH 7.5 with KOH), 10 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, and 100 μg of cycloheximide per ml (water was treated with diethylpyrocarbonate). After a quick centrifugation, the pellet was resuspended in 0.6 ml of buffer A. A 1.4-g amount of glass beads (Biospec Products) was added, and cell lysis was performed by bead beating twice for 30 s each time (with intermittent cooling) in a minibead beater (Biospec Products). After lysis, the cell extract was clarified for 5 min at full speed in a microcentrifuge. Twenty-five OD_{260} units of each lysate was centrifuged through 11 ml of 10 to 50% linear sucrose gradients containing buffer A without dithiothreitol or cycloheximide. For a plain polysome profile, gradients were centrifuged for 2.5 h at 39,000 rpm in an SW41 rotor at 4°C. To obtain better resolution of species around 40S, centrifugations at 27,000 rpm for 14 h were done. Gradients were run at 4°C through a UV ISCO type 6 monitor reading OD_{254}.

Northern hybridization. While gradients were read, fractions (0.5 ml) were collected and kept cool before an RNA extraction was performed. RNA was extracted by adding 50 μl of 10% sodium dodecyl sulfate (SDS), vortexing, and adding 550 μl of phenol previously equilibrated in buffer AE (50 mM Na acetate [pH 5.3], 10 mM EDTA). After vortexing, the tubes were incubated at 65°C for 4 min and then frozen in a dry ice-ethanol mix, followed by a 6-min centrifugation at room temperature. A second extraction with 600 μl of phenol-Tris-EDTA-chloroform was performed, RNA from 400 μl of supernatant was precipitated with 40 μl of 3 M Na acetate (pH 5.3) and 2.5 volumes of ethanol and centrifuged at 4°C for 30 min, and the pellet was washed in 80% ethanol. From each RNA pellet resuspended in diethylpyrocarbonate-water, one-fifth was loaded on a 1.4% agarose gel containing formamide. The size-fractionated RNA was blotted onto a Hybond-N membrane (Amersham). Hybridization to end-labeled oligodeoxynucleotide probes was carried out at 30°C overnight in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt's solution–0.5% SDS–1 mM EDTA. The filters were washed successively at 30°C in 5× SSC–0.1% SDS and then in 1× SSC–0.1% SDS (and 0.1× SSC–0.1% SDS if a background persisted).

Total RNA extracts were obtained from 30 ml of cell cultures (OD_{600} = 0.4) grown at 30°C. Cell pellets were resuspended in 400 μl of buffer AE, and RNA was extracted as previously mentioned. Northern blot analysis was performed as described above, except that 30 μg of RNA for each strain was loaded on a 4% agarose gel (Nusieve GTG; FMC BioProducts). The probes used were p18S (5′CGTCCTATTCTATTATTCCATG3′), p5.8S (5′TTTCGCTGGGTTCTTCATC3′), p25S (5′GCCCGTTCCCTTGGCTGTG3′) (complementary to 25S rRNA bases 2359 to 2377), p25S5′ (5′GCGGGTACTCCTACCTGATTTGAGGTC3′) (complementary to 25S rRNA bases 5 to 31), and p25S3′ (5′CAGCAGATCGTAACAACAAGGCTACTCTAC3′) (complementary to bases 3336 to 3365).

Drug hypersensitivity test. Isogenic wild-type and *ski6* cells growing in selective medium (H-Ura) in log phase were diluted to OD_{600}s of 0.15, 0.015, and 0.0015, and 5-μl aliquots were spotted on selective plates (H-Ura) with and without drug (the viabilities of both strains appeared to be identical and proportional to the measured OD on YPAD and H-Ura media). In the experiment shown, concentrations of hygromycin B, paromomycin, and cycloheximide of 50 μg/ml, 300 μg/ml, and 100 ng/ml (concentrations twofold higher prevented the growth of both strains), respectively, were used. The wild-type strain, 3221 (61), and an

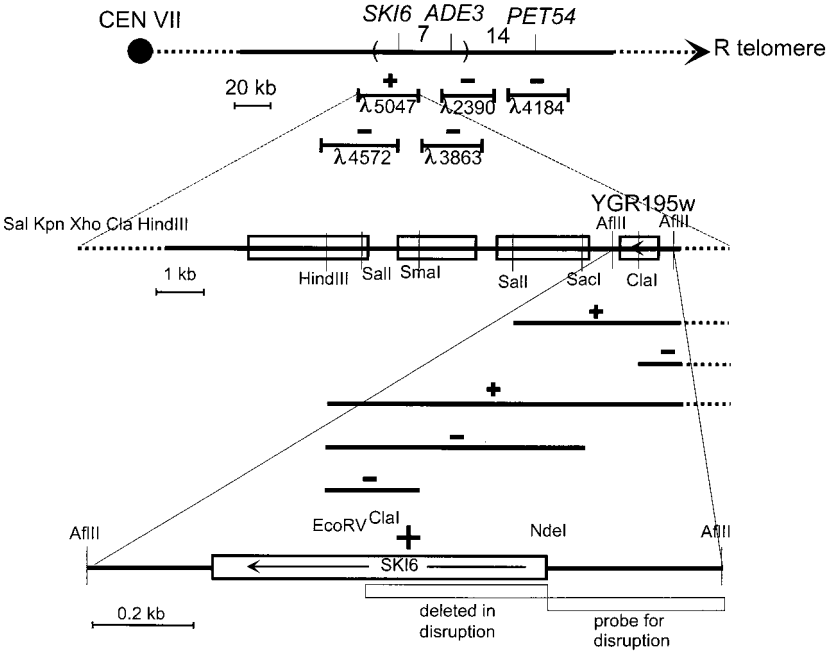

FIG. 1. Cloning of *SKI6*. The location of *SKI6* was determined on the linkage map, and genomic clones in this area were screened for complementation (see text). Complementation tests of subclones localized *SKI6* to YGR195w. The region deleted in the *ski6::HIS3* disruption and the probe used are shown.

isogenic *ski2* disruptant (*ski2::HIS3*) were tested in the same manner on YPAD plates with or without drug.

RESULTS

***SKI6* is an essential gene, and Ski6p is homologous to tRNA-processing enzymes and to proteins of *Caenorhabditis elegans* and *Schizosaccharomyces pombe*.** We genetically mapped *SKI6*, finding it tightly linked to *ADE3* on chromosome VII. We obtained λ clones (46) including this small area and cloned the gene from one of them (see Materials and Methods) (Fig. 1). Since the gene complementing the *ski6* mutation was obtained from DNA mapping close to *ADE3*, we have cloned *SKI6* and not a suppressor. Sequence analysis shows that the 246-residue Ski6 protein is most closely related to proteins encoded by uncharacterized ORFs found in *S. pombe* and *C. elegans* (Fig. 2). These proteins are similar through most of their sequences except for the *C. elegans SKI6* homolog, which has an N-terminal extension beyond the region of homology. Ski6p is also more distantly related to Mtr3p, a nucleolar protein required for mRNA export from the nucleus (25).

Ski6p also has homology to several tRNA-processing enzymes from bacteria, the RNase PH group. These are phosphate-dependent enzymes involved in the removal of the last few 3′ nucleotides from tRNA precursors (10, 26, 27). They are highly specific in their action and work together with other nucleases in trimming the 3′ end of the pre-tRNA molecule. They resemble polynucleotide phosphorylase in producing nucleoside diphosphates from RNA and phosphate and in their ability to synthesize RNA from nucleoside diphosphates (26). Ski6p has weaker similarity to *Bacillus subtilis* polynucleotide phosphorylase. We have not tested whether *ski6* mutations affect tRNA processing, but we present evidence below that they do affect ribosome biogenesis.

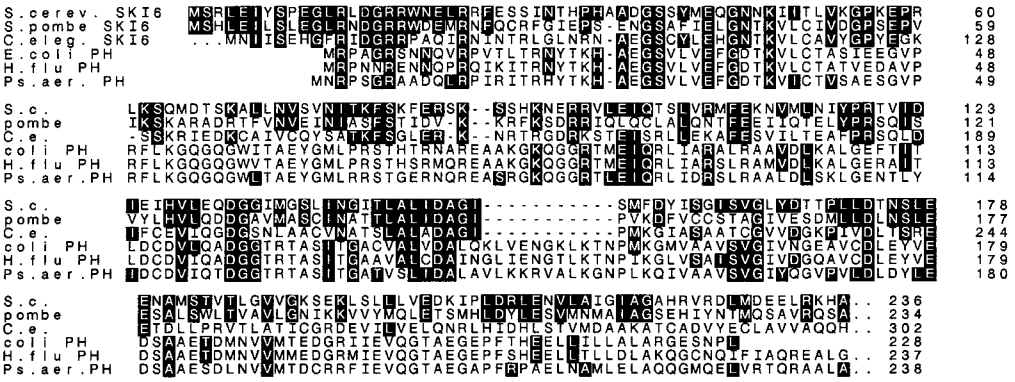

FIG. 2. Ski6 protein is homologous to tRNA-processing enzymes of bacteria (9, 27) and proteins encoded by uncharacterized ORFs of *Schizosaccharomyces pombe* (pombe; GenBank accession no. D89141) (64) and *Caenorhabditis elegans* (C. eleg. or C. e.; EMBL accession no. Z49909) (62). S.c. or S. cerev., *S. cerevisiae*; coli or E. coli, *Escherichia coli*; H. flu, *Haemophilus influenzae*; Ps. aer., *Pseudomonas aeruginosa*.

TABLE 1. Translation of electroporated luciferase mRNAs[a]

Strain	Expt no.	Luciferase activity[b] for phenotype of:			
		C^+ A^+	C^+ A^-	C^- A^+	C^- A^-
ski6-2 pSK16 (*SKI*$^+$)	1	39.5	1.22	9.05	0.46
		39.6	1.07	9.22	0.38
		39.8	1.14	9.15	0.37
	2	39.6	1.35	11.0	0.43
		39.8	1.32	11.4	0.45
		39.8	1.31	11.4	0.45
	3	41.4	1.17	8.9	0.34
		42.5	1.23	9.2	0.32
	Avg	40.4	1.22	9.8	0.39
ski6-2 pRS316 (*ski6*)	1	32.9	9.3	11.1	2.31
		33.1	9.3	11.1	2.29
		33.1	9.4	11.1	2.20
	2	29.4	10.5	14.3	2.26
		27.5	10.5	14.0	2.35
		27.5	10.5	14.0	2.35
	3	30.4	8.6	9.6	2.28
		31.2	9.0	10.1	2.31
	Avg	30.6	9.6	11.7	2.29
ski6/*SKI*$^+$ ratio[c]		0.76 (1.0)	7.9 (10.4)	1.19 (1.56)	5.9 (7.8)

[a] Strain RV493 (K_2^-) carrying either pSKI6 (*SKI*$^+$) or the vector pRS316 (*ski6*) was grown at 30°C in H-Ura and electroporated with 2 μg of luciferase mRNAs prepared with (C^+) and without (C^-) 5′ cap and with (A^+) or without (A^-) 3′ poly(A) as described in Materials and Methods.

[b] Luciferase activity is expressed in light units per microgram of protein. The blank is <0.01 light unit.

[c] This row of data shows the *ski6*/*SKI*$^+$ ratio normalized on the assumption that this ratio is 1.0 for C^+ A^+ mRNA.

A deletion-substitution mutation was produced by replacing the *Nde*I-*Eco*RV fragment of *SKI6* extending from just upstream of the AUG to codon 122 with the *HIS3* gene. The meiotic tetrads produced two viable His$^-$ spores:two inviable spores (15 of 16 tetrads examined), indicating that *SKI6* is an essential gene. The *ski6-2* mutant strains are temperature sensitive for growth at 39°C, and the temperature sensitivity is complemented by our clone of *SKI6*. In liquid culture, *ski6-2* cells gradually stop growth after about three doublings, with no unique morphology of the arrested cells. Revertants do not appear on plating a *ski6* mutant at 39°C on rich medium.

Thus, *SKI6*, whose mutation, like *ski1*, *ski2*, *ski3*, and *ski8*, increases expression of the uncapped, nonpolyadenylated viral mRNAs, is an essential gene with similarities to sequences corresponding to known 3′→5′ exonucleases. Ski6p might act on stability of mRNAs (on the Ski1p model) or on translation (like Ski2p, Ski3p, and Ski8p).

Ski6p blocks translation of non-poly(A) mRNA. We used electroporation of luciferase mRNAs to study the effect of a *ski6-2* mutation (Table 1). In a wild-type strain, cap$^+$ poly(A)$^+$ mRNA was translated 33 times better than cap$^+$ poly(A)$^-$ mRNA (Table 1). In the isogenic *ski6-2* strain, the poly(A)$^+$ mRNA was translated only three times better than poly(A)$^-$ mRNA. Thus, the *ski6-2* mutation increases the efficiency of translation of C^+ A^- mRNA 10-fold. A similar effect on C^- A^- mRNA translation was also seen, with an eightfold increase in translation in the *ski6-2* strain (Table 1). In contrast, there is no effect of the *ski6-2* mutation on translation of C^- A^+ mRNA (Table 1).

Since the poly(A) structure is important in both translation and stability (reviewed in reference 22), we examined luciferase mRNA stability by direct assay of both structural integrity (Fig. 3) and functional integrity (Fig. 4). Radiolabeled mRNAs were electroporated into cells and incubated as they were for translation, samples were taken periodically, the cells were washed, and RNA was extracted. The amount of intact mRNA remaining was determined by electrophoresis and autoradiography (Fig. 3). Quantitation of the autoradiograms showed that each form of the luciferase mRNA was, if anything, more stable in the wild type than in the *ski6-2* mutant, suggesting that mRNA turnover was not the basis of better expression in the mutant. However, since we could not determine into what compartments the electroporated mRNAs had been delivered, we examined the functional integrity of the luciferase mRNAs by measuring the kinetics of luciferase synthesis with the same samples (Fig. 4). This tests the stability of those mRNA molecules with access to the translation apparatus. For the poly(A)$^+$ mRNAs, the kinetics of synthesis by mutant and wild type were similar. For the mRNAs lacking poly(A), synthesis was greater in the *ski6* strain from the earliest time points, indicating a difference in translation rather than in mRNA stability. Plotted as percent maximal activity, the wild-type strain showed the same pattern as did the *ski6* strain. If mRNA instability in the wild type were the cause of the reduced expression of the non-poly(A) mRNAs, it would quickly reach 100% maximal expression and then stop, while the *ski6* mutant would continue expression (19). This was not observed (Fig. 4).

Antibiotic sensitivity of *ski6* mutants. Many mutations affecting components of the translation apparatus produce hypersensitivity to hygromycin B (6, 49) and other drugs that increase translational errors (30). We found that *ski6-2* strains are hypersensitive to hygromycin B (Fig. 5), supporting the notion that they affect translation. We also found that *ski2* strains are hypersensitive to hygromycin B and slightly hyper-

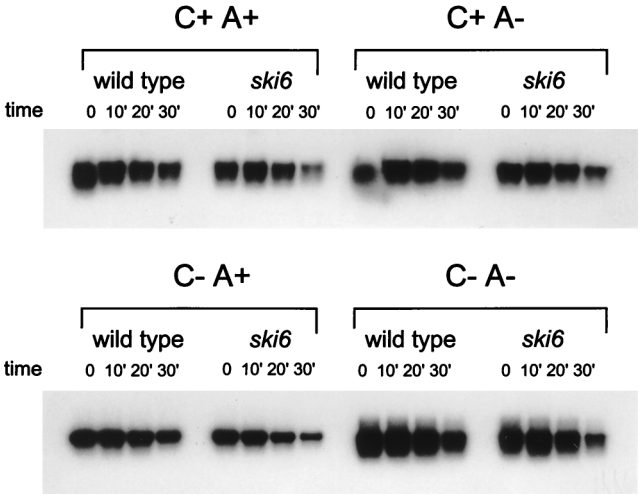

FIG. 3. *SKI6* does not affect stability of electroporated luciferase mRNA. Labeled mRNA was electroporated into wild-type and *ski6-2* cells. The kinetics of luciferase synthesis (Fig. 4) and mRNA degradation were determined on portions of the same samples taken at 0, 10, 20, and 30 min. Extracted RNA was analyzed by agarose gel electrophoresis and autoradiography as described previously (31).

sensitive to cycloheximide (Fig. 5). Neither *ski2* nor *ski6* mutants are hypersensitive to paromomycin (data not shown).

***ski6* mutants have a novel 25S rRNA-containing particle.** At 30°C, growth rates of the isogenic *ski6* and wild-type strains are almost identical, although the *ski6* mutant shows a greater delay in leaving stationary phase than does the wild type. The amount of polysomes in *ski6* strains is consistently lower than that in the wild-type (Fig. 6). The normal growth rates imply that translation is proceeding at an essentially normal rate, although, as shown above, non-poly(A) mRNAs are more translatable in *ski6* cells under these conditions. In cells grown at 30°C, the polysome gradients reveal the existence of an extra peak in the *ski6* strain, sedimenting slightly slower than the 40S subunits (Fig. 6 and 7). This 38S extra peak is most clearly distinguished from the 40S ribosomal subunit in longer centrifugation runs (Fig. 7). The amount of this extra peak is increased by a shift of temperature to 39°C, the nonpermissive temperature, and the mutant stops growing after three more generations. The ratio of polysomes in *ski6-2* cells to those in SKI^+ cells is similar at 39°C to the ratio at 30°C (Fig. 6).

We examined the distribution of rRNAs by Northern blot analysis of gradient fractions (Fig. 6). Fractions containing the extra peak in the mutant, close to the 40S peak, give the expected signal with an 18S rRNA probe for both strains. A probe specific for 25S rRNA detects a species in the novel peak that is smaller than 25S rRNA and does not appear in the wild type (Fig. 6). A probe specific for 5.8S rRNA shows that this species is absent from this part of the gradient in both the mutant and wild type (Fig. 6). Longer centrifugation allows better separation of this extra peak (about 38S) from the 40S subunit peak and reduces contamination of free 60S subunits (Fig. 7A). Northern blot analysis confirms again in these fractions the presence of an RNA of about 20S, whose origin is the 25S rRNA. This 20S RNA might be a product of degradation of the 25S rRNA that is poorly protected in an incomplete 60S subunit. The ratio of 18S rRNA in the *ski6* mutant to 18S rRNA in the wild type is 1.04, while the ratio of full-length 25S rRNA in the *ski6* mutant to 25S rRNA in the wild type is 1.05. Thus, the ratios of free ribosomal subunits are similar.

Probing the rRNA in the 38S peak with probes specific for the 5′ end or the 3′ end of 25S rRNA shows that the 25S-related species lacks the normal 5′ end but has sequences close to the 3′ end (Fig. 7B). The 25S-related sequence in the 38S peak can be distinguished both by size and by hybridization specificity from breakdown products of 25S rRNA found in the 60S peak of both mutant and wild-type strains (Fig. 7B). The latter hybridizes with both 5′ and 3′ probes, suggesting that they are a mixture of randomly broken molecules, while the former hybridizes with the 3′ probe but not with the 5′ probe.

Comparison of total 5.8S rRNA in isogenic mutant and wild-type strains shows a 1.9-fold decrease in the *ski6-2* mutant cells, with 18S rRNA as the control (Fig. 8).

ski6-2* suppresses the effect of 60S subunit deficiency on L-A mRNA translation.** The *SKI* genes were discovered based on their derepression of virus expression. Mutations in genes needed for 60S ribosome biogenesis, including 60S subunit protein genes, show reduced copy number of L-A dsRNA and loss of the killer toxin-encoding satellite M_1 dsRNA (41). The *mak7-1* mutation is deficient in ribosomal protein L4, has decreased free 60S ribosomal subunits, and shows halfmers, due to polysomes with a 40S subunit which is awaiting 60S joining (41). These mutants lose M_1 dsRNA, but we find that *ski6-2 mak7-1* double mutants propagate M_1 normally. The *mak7-1 ski6-2* strain 4566-2C (=*MATa** *trp1 leu2 ura3 ski6-2 mak7-1 his*$^-$ *ade3*) was transformed with either pSKI6 or pYRC50 (41) or both to make isogenic strains defective in one or both genes.

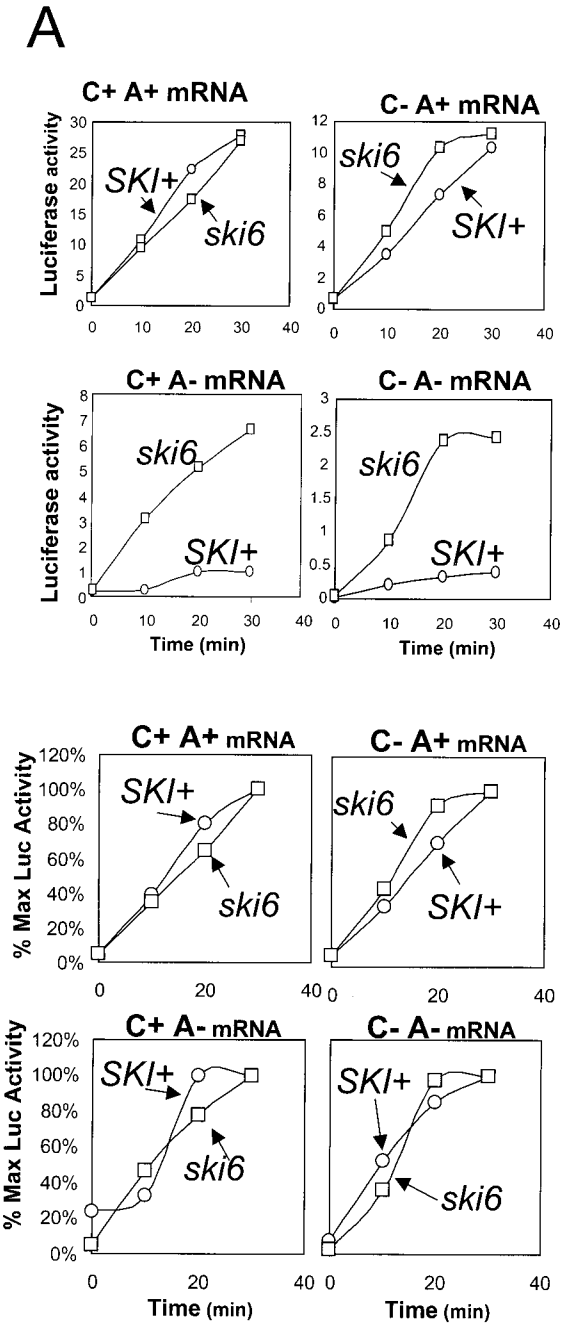

FIG. 4. Kinetics of luciferase accumulation in *ski6-2* and SKI^+ cells indicates an effect on translation rather than mRNA degradation. (A) Kinetics of luciferase activity accumulation are plotted in the upper panels (luciferase activity in light units per microgram of protein), and percent maximal luciferase activity (% Max Luc Activity) is plotted in the lower panels. (B) Comparison of *ski6-2* and SKI^+ cells in translation of C^+ A^- mRNA over a 90-min time course. If accumulation were low in wild-type cells for C^+ A^- or C^- A^- mRNAs because of mRNA degradation, then activity would accumulate rapidly and stop at later time points. The plateau (100%) would be expected early. In fact, the kinetics as percent maximal activity are similar for SKI^+ and *ski6* strains for all types of mRNA. The differences in rates of accumulation must be due to differences in translation rate. The percentage was calculated as follows: 100 {[(value at time t) − (value at t = 0)]/[30-min value]}.

Polysome profiles were obtained as described in Materials and Methods, and the ability of the double mutant to propagate M_1 was examined. The *mak7-1* strain lost M_1, but the isogenic wild type and the *ski6-2 mak7-1* double mutant propagate M_1 nor-

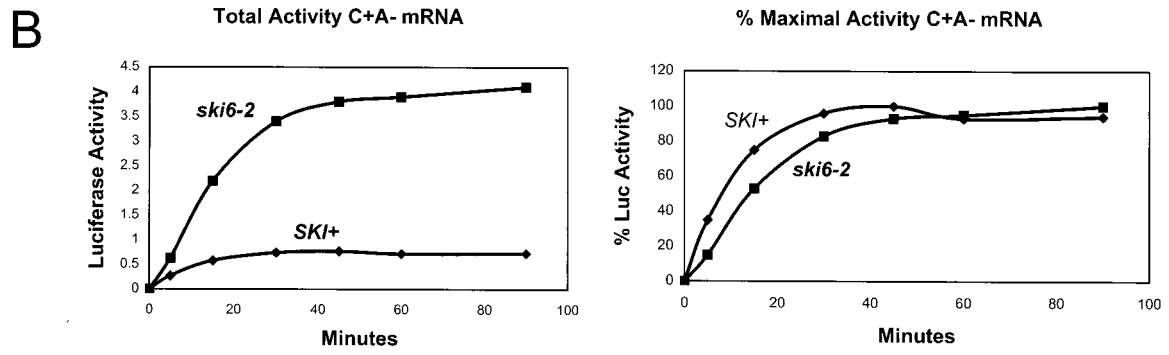

FIG. 4—*Continued.*

mally. This indicates that translation of the L-A mRNA [which lacks poly(A)] is improved as a result of the *ski6-2* mutation. Moreover, the halfmer peak is absent in polysome gradients of the double mutants (Fig. 9), suggesting an alteration in the 60S joining reaction.

DISCUSSION

Ski6p is involved in 60S ribosome assembly. Mutations in ribosomal protein genes result in decreased rates and extents of ribosome assembly as expected from their being essential components of the final structure (36, 63). Other components that are not part of the finished product but are necessary for its construction have also been identified (29, 47, 55, 57).

Ski6p is clearly not one of the ribosomal proteins, but we show that a *ski6* mutant, even at the permissive temperature, accumulates a novel particle that contains a fragment of 25S rRNA

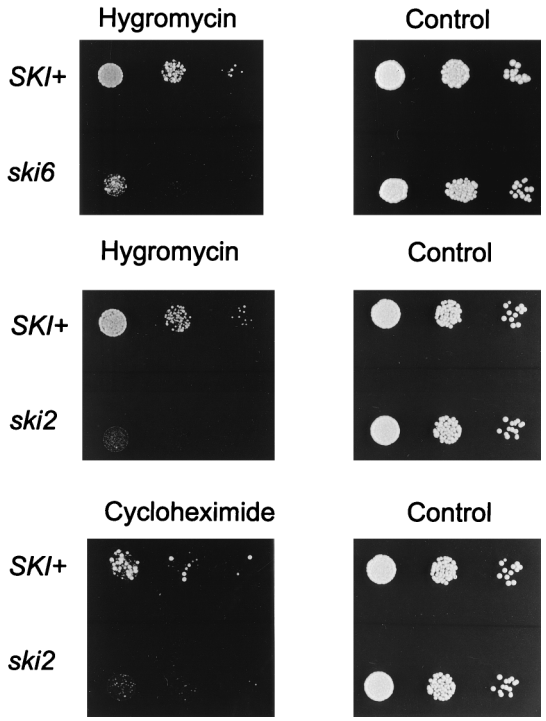

FIG. 5. Drug sensitivity assay. Different dilutions of log-phase cultures were tested for their sensitivities to hygromycin B or cycloheximide (see Materials and Methods).

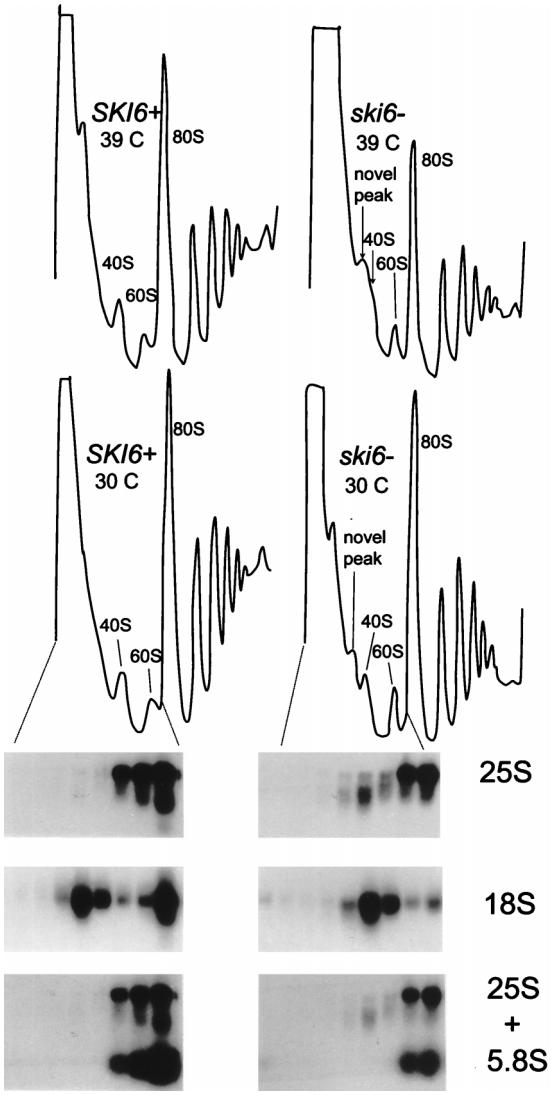

FIG. 6. *ski6-2* cells show a novel species of rRNA whose origin is 25S rRNA. Polysome profiles of *ski6-2* (*ski6*−) and wild-type (*SKI6*+) cells grown at either 30 or 39°C are shown. The A_{260} is plotted on the vertical axis. Northern analysis of fractions from polysome profiles at 30°C were made by using probes for 25S (p25S), 18S (p18S), or 5.8S (p5.8S) rRNAs (see Materials and Methods).

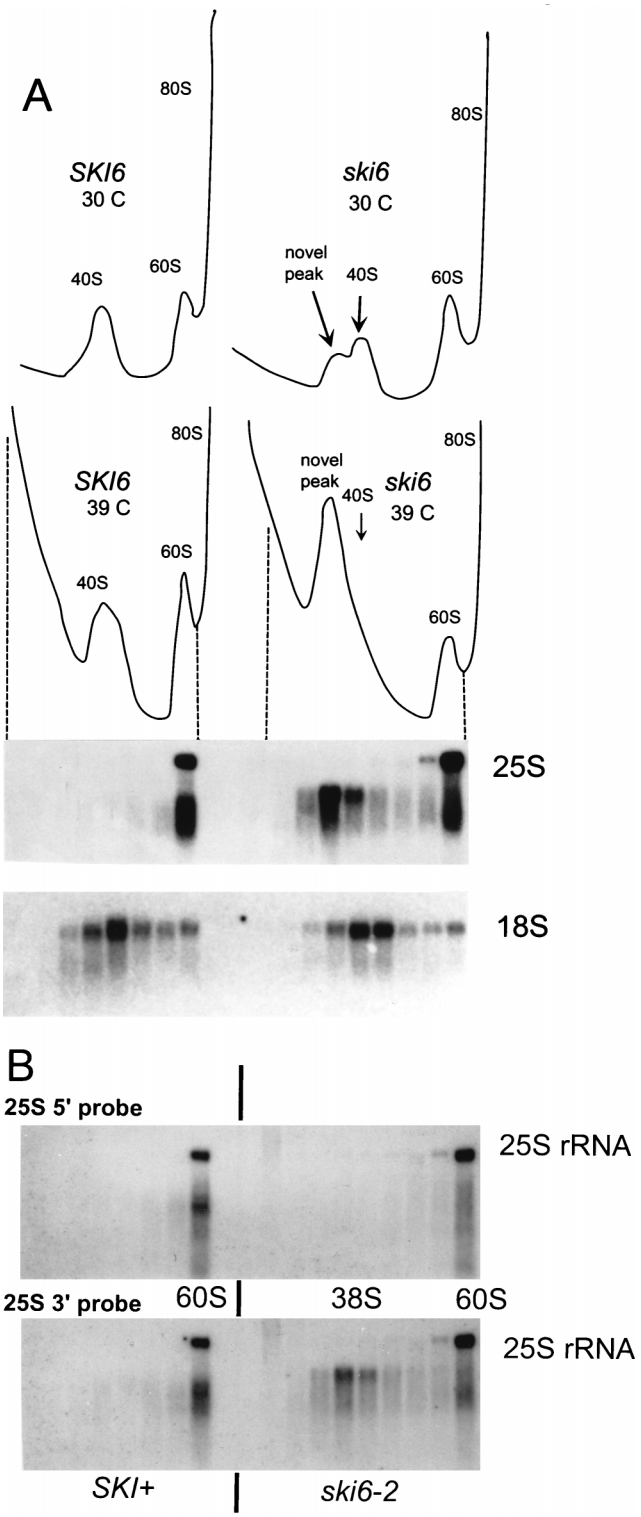

FIG. 7. (A) Long-term centrifugation on sucrose gradients allows a better separation of the novel 38S species from the usual 40S peak. The A_{260} is plotted on the vertical axis. Northern analysis of fractions from polysome profiles at 39°C was done by using probes against the 25S (p25S) and 18S (p18S) rRNAs (see Materials and Methods). The dashed lines show the points on the UV scan corresponding to the fractions analyzed by Northern hybridization. (B) Hybridization of the blots from panel A with probes specific for either the 5′ end (p25S5′) or 3′ end (p25S3′) of 25S rRNA.

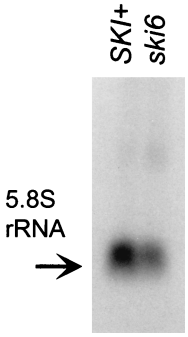

FIG. 8. A *ski6-2* strain shows a deficiency in 5.8S rRNA accumulation. Northern blot analysis was done with 30 μg of total RNA extracted from cells grown at 30°C. The same blots were hybridized with 18S rRNA as a control (data not shown). Blots of each were quantitated by scanning.

lacking the 5′ end of the normal 25S rRNA. This is likely to be either a misassembled 60S subunit or a misassembled and then partially degraded 60S particle. This particle lacks 5.8S rRNA, and the cells show some deficiency of total 5.8S rRNA in comparison to an isogenic wild-type strain. There is no change in the relative levels of free 60S or 40S particles. It is unlikely that the 38S particle carries out any of the reactions of protein synthesis since it lacks both 5.8S rRNA and part of 25S rRNA.

However, the *ski6* mutants plainly do have alterations of the translation apparatus itself. They are hypersensitive to hygromycin B, a phenotype typical of mutants in components of the translation apparatus. *ski2* mutants show the same phenotype. This suggests that, in addition to the 38S defective 60S ribosomal subunits that accumulate in *ski6* strains, the normal-sized 60S subunits are probably also functionally abnormal, perhaps by containing improperly processed rRNA.

Mitchell et al. have found that Ski6p is in a complex with other RNA-processing exoribonucleases (34), suggesting that it has a similar function. One of these exoribonucleases, Rrp4p, is known to be involved in 5.8S rRNA processing (35), and *ski6* (renamed *rrp41*) mutants are likewise defective in 5.8S rRNA processing (34).

Does Ski6p derepress translation of non-poly(A) mRNAs via alterations of full-sized 60S subunits? Ski6p, like Ski2p, Ski3p, and Ski8p, is necessary for blocking the translation of non-poly(A) mRNAs, such as the viral mRNAs whose overexpression formed the basis of the original mutant isolations. The evidence points to translation, rather than mRNA turnover, as the basis of the derepressed expression of non-poly(A) mRNAs. The kinetics of luciferase accumulation and direct measurements of mRNA turnover show the pattern expected for a translation effect.

The *ski2*, *ski3*, *ski6*, and *ski8* mutants translate non-poly(A) mRNAs nearly as well as they do poly(A)$^+$ mRNAs (31; also this work), showing that the translation apparatus is inherently able to use non-poly(A) mRNAs. Indeed, it had already been shown that poly(A)-deficient mRNAs are found on polysomes in strains lacking the *SKI1/XRN1* exoribonuclease that degrades uncapped mRNAs (21) and in a poly(A) polymerase temperature-sensitive mutant shifted to the nonpermissive temperature (43).

Elucidating the means by which Ski proteins block translation of non-poly(A) mRNA requires consideration of the role in translation of the 3′ poly(A) structure of eucaryotic mRNA, an area which remains controversial (reviewed in references 22 and 48). Translation of electroporated mRNAs is stimulated by the 5′ cap and 3′ poly(A) structures by 12- and 200-fold, re-

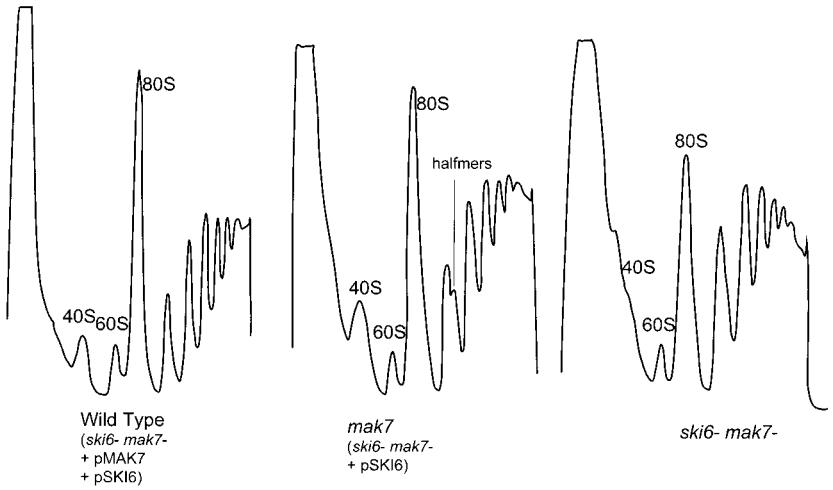

FIG. 9. *ski6-2* suppresses *mak7-1* without relieving the 60S ribosomal subunit deficiency of *mak7-1* strains. The *mak7-1 ski6-2* strain 4566-2C (=*MAT***a** *trp1 leu2 ura3 ski6-2 mak7-1 his*$^-$ *ade3*) was transformed with either pSKI6 or pYRC50 (41) or both to make isogenic strains defective in one or both genes. Polysome profiles were obtained as described in Materials and Methods.

spectively (19, 56). The 3′ poly(A) structure is known to affect mRNA turnover (for an example, see reference 8), but the kinetics of reporter synthesis show that there is a substantial effect on synthesis, independent of mRNA stability differences (19).

One role suggested for poly(A) is to promote the joining of the 60S subunit to the 40S subunit waiting with associated initiation factors at the initiator AUG (38; for reviews, see references 22 and 39). This was proposed to occur by an interaction between the poly(A) and the 60S subunit, forming a circular mRNA, at least at the time of initiation. Our results support this model (31, 41; also this work). A deficiency of free 60S ribosomal subunits (in strains with mutations in any of 20 *mak* genes) results in poor translation of the viral mRNAs because they lack poly(A) and so compete poorly with the poly(A)$^+$ cellular mRNAs for limiting free 60S subunits (41). Indeed, limitation of 40S or 60S subunits in a strain temperature sensitive for poly(A) polymerase showed greater discrimination against non-poly(A) mRNA when 60S subunits were limiting (42). Mutations in the *ski2*, *ski3*, *ski6*, and *ski8* genes improve viral mRNA translation [since they improve the translation of all non-poly(A) mRNAs] and suppress the effect of 60S subunit deficiency (without restoring the levels of 60S subunits) (31; also this work). Another model for the role of poly(A) in translation involves the association of the poly(A) binding protein with eIF-4G (48). However, this model does not suggest an explanation of our data.

We suggested that the Ski2, Ski3, and Ski8 proteins prepare 60S subunits so that they have the requirement for interaction with the 3′ poly(A) structure before they will join with the 40S subunit waiting at the AUG (31, 41, 58). We had no direct evidence that these proteins have these effects by altering 60S biogenesis besides the fact that Ski3p is nuclear (44) and that Ski2p is highly homologous to the mammalian Ski2p (28, 61) which has been localized to the nucleolus (28). In the case of *ski6*, we have shown directly that 60S subunits are altered.

Conclusions. We find that *SKI6* encodes a homolog of bacterial tRNA-processing enzymes and is necessary for a system that specifically blocks translation of non-poly(A) mRNA. We see a novel species in *ski6* mutants that includes a fragment of 25S rRNA but lacks 5.8S rRNA, suggesting that in these strains 60S subunits are not properly formed. Hygromycin B hypersensitivity and suppression of halfmers in a mutant deficient in the ribosomal protein L4 also support the idea that a subtle modification exists in the functional ribosomes and suggests that this modification bypasses the requirement of a 3′ poly(A) for translation.

The results presented here and previously (31) suggest that the default for translation is efficient translation of mRNA independent of poly(A) and that, in a wild-type cell, the specificity for poly(A) mRNA is accomplished (by the Ski2, Ski3, Ski6, and Ski8 proteins) by repressing translation of poly(A)$^-$ mRNA.

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