

Ribosomal Protein L3 Mutants Alter Translational Fidelity and Promote Rapid Loss of the Yeast Killer Virus

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Programmed –1 ribosomal frameshifting is utilized by a number of RNA viruses as a means of ensuring the correct ratio of viral structural to enzymatic proteins available for viral particle assembly. Altering frameshifting efficiencies upsets this ratio, interfering with virus propagation. We have previously demonstrated that compounds that alter the kinetics of the peptidyl-transfer reaction affect programmed –1 ribosomal frameshift efficiencies and interfere with viral propagation in yeast. Here, the use of a genetic approach lends further support to the hypothesis that alterations affecting the ribosome’s peptidyltransferase activity lead to changes in frameshifting efficiency and virus loss. Mutations in the *RPL3* gene, which encodes a ribosomal protein located at the peptidyltransferase center, promote approximately three- to fourfold increases in programmed –1 ribosomal frameshift efficiencies and loss of the M_1 killer virus of yeast. The *mak8-1* allele of *RPL3* contains two adjacent missense mutations which are predicted to structurally alter the Mak8-1p. Furthermore, a second allele that encodes the N-terminal 100 amino acids of L3 (called *L3Δ*) exerts a *trans*-dominant effect on programmed –1 ribosomal frameshifting and killer virus maintenance. Taken together, these results support the hypothesis that alterations in the peptidyltransferase center affect programmed –1 ribosomal frameshifting.

Programmed –1 ribosomal frameshifting is a mode of regulating gene expression used predominantly by RNA viruses and by a subset of bacterial genes to induce elongating ribosomes to shift the reading frame in response to specific mRNA signals (reviewed in references 16, 24, 27, and 30). Many viruses of clinical, veterinary, and agricultural importance utilize programmed frameshifting for the production of their structural and enzymatic gene products (reviewed in references 5, 6, 24, 27, 30, and 51). Thus, ribosomal frameshifting is a unique target with which to identify and develop antiviral agents (20, 41). Programmed –1 ribosomal frameshifting causes the ribosome to slip one base in the 5' direction and requires two *cis*-acting mRNA signals. The first sequence element is called the “slippery site,” which, in eukaryotic viruses, consists of a heptamer sequence spanning three amino acid codons, X XXY YYZ (the *gag* reading frame is indicated by spaces), where XXX can be any three identical nucleotides, YYY can be AAA or UUU, and Z is A, U, or C (8, 17, 21, 31). The second frameshift-promoting signal is usually a sequence that forms a defined RNA secondary structure, such as an RNA pseudoknot (7, 17, 36). This element is located approximately 4 to 8 nucleotides 3' of the slippery site and is thought to increase the probability that the ribosome will slip from the original reading frame in the –1 direction, in part by inducing ribosomes to pause at the slippery site (48, 53).

Based on the repetitive nature of the heptamer slippery sequence required for efficient programmed –1 ribosomal frameshifting, a simultaneous slippage model has been proposed to explain how ribosomes can be induced to change reading frames (31). A translating ribosome in which the A-

and P-sites are occupied by tRNAs is forced to pause over the slippery site as a consequence of the RNA pseudoknot. The increased pause time over this sequence is thought to give an opportunity for the ribosome and bound tRNAs to slip 1 base in the 5' direction. Because of the nature of the slippery site, this still leaves their non-wobble bases correctly paired with the mRNA in the new reading frame. Following the slip in the –1 direction, the ribosome continues translation in the new reading frame, producing the Gag-Pol polyprotein.

Since the simultaneous slippage model of programmed –1 ribosomal frameshifting requires that both the ribosomal A- and P-sites be occupied by tRNAs, it is implicit that this mechanism must occur after insertion of cognate aminoacyl-tRNA into the A-site, but prior to the translocation step of the translation elongation cycle. Furthermore, since programmed ribosomal frameshifting is driven by ribosomal pause events, mutations or agents that would serve to alter the amount of time that ribosomes are paused with both A- and P-sites occupied by tRNAs should specifically have an impact on the efficiency of programmed –1 ribosomal frameshifting. Since the peptidyl-transfer step in translation occurs while both the ribosomal A- and P-sites are occupied by tRNAs, we predicted that agents and mutations which alter the rate of this reaction would promote changes in programmed –1 ribosomal frameshift efficiencies and consequently would have antiviral properties.

In the yeast *Saccharomyces cerevisiae*, the L-A double-stranded RNA (dsRNA) virus utilizes a –1 ribosomal frameshift event for the production of a Gag-Pol fusion protein and has been an excellent model system with which to investigate this process (reviewed in references 12 and 20). M_1 , a satellite dsRNA virus of L-A that encodes a secreted killer toxin, is encapsidated and replicated by using the Gag and Gag-Pol gene products synthesized by the L-A virus (reviewed in reference 58). Maintaining the appropriate ratio of Gag to Gag-Pol is critical for maintenance of the M_1 virus (21). Alteration

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of the frameshift process by as little as two- to threefold promotes rapid loss of M_1 (21, 22). Genetic and biochemical analyses have identified a number of factors involved in determining the efficiency of programmed -1 ribosomal frameshifting (11, 12, 18, 19, 21–23, 34, 44). Based on this analysis, we have hypothesized that there is a surveillance complex which functions to monitor the A- and P-sites on the ribosomes to ensure that translation elongation occurs with high fidelity (15, 44). We suspect that this surveillance complex monitors the ribosome's peptidyltransferase activity to ensure maximal translational fidelity. Mutations that alter or inactivate the activity of this complex reduce translational fidelity at the A- and P-sites and allow increased levels of ribosomal frameshifting to occur. A set of *mof* (maintenance of frame) alleles were shown to increase programmed -1 ribosomal frameshifting efficiencies and promote loss of the killer virus (22). Furthermore, compounds that bind to the peptidyltransferase center on the ribosome and reduce translation fidelity can also modulate ribosomal frameshifting (19). Anisomycin and sparsomycin were shown to alter programmed -1 ribosomal frameshifting efficiencies both in cells and in *in vitro* translation extracts and to promote loss of the yeast L-A and its satellite dsRNA virus, M_1 (19). Taken together, these results indicate that modulation of the ribosomal peptidyltransferase center can alter the efficiency of programmed -1 ribosomal frameshifting and lead to inefficient virus propagation.

In the current study, we have genetically investigated the role of a ribosomal protein that is located at the ribosomal peptidyl-transfer center in modulating programmed frameshifting efficiencies. Previous results have shown that the yeast *RPL3* gene encoding the ribosomal protein L3 participates in the formation of the peptidyltransferase center (reviewed in references 38 and 39). Mutations in the *RPL3* gene (called *TCMI*) were initially identified by conferring resistance to the peptidyltransferase inhibitors trichodermin and anisomycin (32, 45). Independently, the *MAK8* gene (*MAK*, maintenance of killer) was identified by the inability of mutant alleles to maintain the M_1 satellite virus (59). Subsequent analysis demonstrated that *MAK8* is allelic to *RPL3* (60). Thus, a mutation in a ribosomal protein located in the peptidyltransferase center that cannot maintain the killer virus has been identified. We hypothesized that the underlying cause of killer virus loss observed in these cells may be a consequence of increased programmed -1 ribosomal frameshifting efficiency (i.e., that the *mak8* alleles may demonstrate a Mof^- phenotype). The results presented here demonstrate that strains harboring the *mak8-1* allele have increased programmed frameshifting efficiencies and strongly suggest that the loss of the killer virus is a due to alteration in translation fidelity. Furthermore, a *trans*-dominant *RPL3* allele has been identified that both increases programmed -1 frameshifting and interferes with the ability of yeast cells to maintain the M_1 dsRNA virus. Taken together, these results support the notion that modulation of the peptidyltransferase center results in alteration of programmed -1 ribosomal frameshifting efficiencies, promoting loss of the killer virus.

MATERIALS AND METHODS

Strains, media, enzymes, oligonucleotides, and drugs. *Escherichia coli* DH5 α and MV1190 were used to amplify plasmid DNA. The yeast strains used in this study are listed in Table 1. Transformations of yeasts and *E. coli* were performed as described previously (13). YPAD, YPG, SD, synthetic complete medium (H $-$), and 4.7MB plates for testing the killer phenotype were used as previously reported (22). Restriction enzymes were obtained from Promega, MBI Fermentas, Bethesda Research Laboratories, and Boehringer Mannheim. T4 DNA ligase and T4 DNA polymerase were obtained from Boehringer Mannheim, and precision *Taq* polymerase was obtained from Stratagene. Radioactive nucleo-

tides were obtained from NEN. Oligonucleotides used in these studies were purchased from IDT, and DNA sequence analysis was performed by the UMDNJ-RWJ DNA synthesis center. Anisomycin was purchased from Sigma, and sparsomycin was a generous gift from S. Pestka.

Plasmid constructs and programmed ribosomal frameshift assays. BlueScript KS plasmid was obtained from Stratagene. The pRS series of plasmids (10, 47) and pAS134 (1) have been previously described. Full-length *RPL3* and *mak8-1* were amplified from genomic DNA by PCR using the oligonucleotide primers $-300KpnI$ (5' CCCC GG TACCTCACGCACACTGGAATGAAT 3') and $+1300SacI$ (5' CCCC GAGCGCAACCTCCATTTGGACTTGG 3') and were cloned into the pRS300 series (pRS314, pRS315, and pRS316) digested with *KpnI* and *SacI* to make the pRPL3 and the *mak8-1* series of plasmids (Fig. 1A). To construct an *RPL3* gene disruption plasmid, the *KpnI-SacI RPL3* clone was subcloned into BlueScript KS (KS-RPL3), digested with *SphI*, the overhanging ends were filled with deoxynucleoside triphosphates using T4 DNA polymerase, and the clone was then digested with *XbaI*. Subsequently, pAS134 was digested with *XbaI* and *PvuII* to liberate the *hisG-URA3* cassette, which was subcloned into the *XbaI/blunt-ended KS-RPL3* to create pJD168 (Fig. 1B).

The original clone (YPF2-9) harboring the *L3 Δ* fragment was obtained as a high-copy suppressor of a *upf2* deletion strain as described previously (13). The active *L3 Δ* fragment was amplified and subcloned into the pRS series of vectors by using *Taq* DNA polymerase and the $-485BamHI$ (5' ATAGGATCCTTAA CCGGCGGACAGTAATA 3') and $+300BgIII$ (5' ATAGGATCCTTGTGTC ATCGTCGCTTGTAGTCTCAAAACCTTGGGGT 3') oligonucleotide primers to create pJD138 (low copy number, pRS315 based) and pJD139 (high copy number, pRS426 based) (Fig. 1C). The *PGK1* transcriptional terminator was subcloned 3' of the *L3 Δ* fragments as previously described (12).

The plasmids used to monitor programmed ribosomal frameshifting were previously described (11, 12, 18, 19, 54). Briefly, in all of these plasmids, transcription is driven from the yeast *PGK1* promoter into an AUG translational start site (Fig. 2). The *E. coli lacZ* gene serves as the reporter, and transcription termination utilizes the yeast *PGK1* transcriptional terminator. In the p0 plasmids, *lacZ* is in the 0-frame with respect to the translational start site, and measurement of β -galactosidase activity generated from cells transformed with these plasmids serves to represent the 0-frame controls. In the p -1 series, *lacZ* is in the -1 frame with respect to the translational start site and is 3' of the L-A-programmed -1 ribosomal frameshift signal, such that β -galactosidase can only be produced as a consequence of a programmed -1 ribosomal frameshift. Similarly, in the p $+1$ series, *lacZ* is in the $+1$ frame with respect to the translational start site and is 3' of the TyI-programmed $+1$ ribosomal frameshift signal, such that β -galactosidase can only be produced as a consequence of a programmed $+1$ ribosomal frameshift. The efficiency of programmed ribosomal frameshifting is calculated by determining the ratio of β -galactosidase activity produced by cells harboring either p -1 or p $+1$ divided by the β -galactosidase activity produced by cells harboring p0 and multiplying the result by 100%. Measurement of programmed -1 ribosomal frameshifting in the presence of anisomycin and sparsomycin was performed as described previously (19).

Construction of isogenic *mak8-1* and *RPL3* strains. Yeast strains JD100 and JD973 were mated, and the diploids were transformed with *PvuII*-linearized pJD168 and selected on H-Ura medium (22). Disruption of the *RPL3* locus on one chromosome was confirmed by Southern analysis as described below. Diploids were selected for loss of the chromosomal *URA3* insert by growth on 5-fluoroorotic acid (5-FOA). Ura $^-$ cells were transformed with pRPL3-Ura3, sporulated, and dissected onto YPAD medium. The resulting tetrads are from cross JD980. *rpl3 Δ* status was confirmed by the inability of spore clones to grow in the presence of 5-FOA. To construct isogenic *mak8-1* strains, cells were transformed with *pmak8-1-TRP1* and were subsequently grown in the presence of 5-FOA to select for loss of the wild-type pRPL3-Ura3 plasmid.

Killer assay. The killer virus assay was carried out as previously described (21). Briefly, yeast colonies were replica plated to 4.7MB plates (22) with a newly seeded lawn of strain 5X47 (0.5 ml of a suspension at 1 Unit of optical density at 550 nm per ml per plate). After 2 to 3 days at 20°C, killer activity was observed as a clear zone around the killer colonies. Loss-of-killer assays were performed with multiple wild-type and mutant strains.

Nucleic acid analyses. dsRNAs of L-A and M_1 viruses were prepared as described previously (25), separated by electrophoresis through 1.2% agarose gels, denatured in the gels in two changes of 30 min each of 50% formamide–9.25% formaldehyde–1 \times Tris-acetate-EDTA at room temperature, and transferred to nitrocellulose in 20 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate). L-A and M_1 negative strand RNA probes were labeled with [α - 32 P]UTP and hybridized to blots and washed as described in reference 22. RNase protection assays to determine the relative abundances of the *lacZ -1* frameshift reporter mRNAs and U3 small nuclear RNA in the isogenic wild-type, *mak8-1*, and *L3 Δ* strains were carried out as described previously (44).

RESULTS

The *mak8-1* allele of *RPL3* promotes increased programmed -1 ribosomal frameshifting efficiencies. Previous studies have demonstrated that peptidyltransferase inhibitors specifically affect programmed -1 ribosomal frameshifting efficiencies

TABLE 1. Yeast strains used in this study

Strain	Genotype ^a	Source
1906	<i>MATa leu2 mak8-1 K⁻ MKT⁺</i>	R. Wickner
5X47	<i>MATa/MATα his1/+ trp1/+ ura3/+ K⁻R⁻</i>	R. Wickner
2373	<i>MATa ura3 ski4-1 mkt1 K⁺⁺</i>	R. Wickner
2898	<i>MATa ura3 ade3 his(5,6) ski6-2 K⁺⁺</i>	R. Wickner
2413	<i>MATa ura3 cyh2 ski7-2 K⁺⁺</i>	R. Wickner
JD100	<i>MATa ura3-52 his3 trp1-δ1 K1⁺</i>	This study
JD973	<i>MATα ura3-SK1 LEU2::hisG TRP1::hisG lys2-SK1 ho::LYS2 ade3-210S</i>	This study
Cross JD980	JD100 \times JD973 with <i>RPL3::hisG</i> on 1 chromosome	This study
JD980-10C	<i>MATα lys2 his3 ura3 LEU2::hisG trp1-δ1 RPL3::hisG + pRPL3 or pmak8-1</i>	This study
JD13	<i>MATa his3 leu2 PEP4::HIS3 NUC1::LEU2 ura3 K1⁺</i>	This study
JD111	<i>MATα ura3-52 lys2-801 trp1-δ1 leu2⁻ his3⁻ K1⁺</i>	This study
JD890	<i>MATα ura3-52 trp1 leu2Δ1 his3Δ300 pbrΔ1-6 can1⁺ pep4::HIS3 SKI1::LEU2 [L-AHN M₁] K⁺⁺</i>	This study
JD2	<i>MATα ura3 trp1 ade8 ski2-2 K⁺⁺</i>	This study

^a --, molecularly undefined allele that at the genetic level does not revert, i.e., is a very strong mutant; ++, phenotypically strong killer strain.

(19). Based on these and other observations (11, 14, 15, 43, 44, 55–57), we hypothesized that the surveillance complex monitors the ribosomal peptidyltransferase center and that mutations that affect either the surveillance complex or the peptidyltransferase center will affect programmed -1 ribosomal frameshifting efficiencies (19, 44). Thus, we predicted that yeast strains harboring chromosomal mutations affecting the peptidyltransferase center may also have defects in programmed -1 ribosomal frameshifting and killer virus maintenance. The *mak8-1* allele of ribosomal protein L3 initially presents a logical candidate with which to test this hypothesis, since strains harboring this mutation promoted loss of the killer virus. Programmed ribosomal frameshifting efficiencies were measured in vivo by using a series of *lacZ* reporter plasmids as described previously (12, 17, 19, 54) (see Fig. 2 for constructs). The p0 series of plasmids serve as the 0-frame controls, since *lacZ* is in the 0-frame with respect to the translational start site (Fig. 2). In the p -1 plasmid series, an L-A-

derived programmed -1 ribosomal frameshift signal is cloned into the polylinker, and the *lacZ* gene is in the -1 frame with respect to the translational start site (Fig. 2). Therefore, in these constructs, the *lacZ* gene can only be translated if the ribosome shifts the frame in the -1 direction. Similarly, the p $+1$ plasmid series contains at TyI a programmed $+1$ ribosomal frameshift signal cloned into the polylinker, and the *lacZ* gene is in the $+1$ frame with respect to the translational start site (Fig. 2). The TyI $+1$ reporter plasmid is used as a control to determine the specificity of the effect of the mutation on translation. The efficiencies of -1 and $+1$ ribosomal frameshifting are calculated by determining the ratio of β -galactosidase activities measured in cells harboring p -1 or p $+1$ to those harboring p0 and multiplying the result by 100%.

After cells (strain 1906 [Table 1]) harboring the *mak8-1* allele were transformed with p0, p -1 , or p $+1$, the efficiencies of programmed ribosomal frameshifting were determined. The results demonstrated that the programmed -1 frameshifting

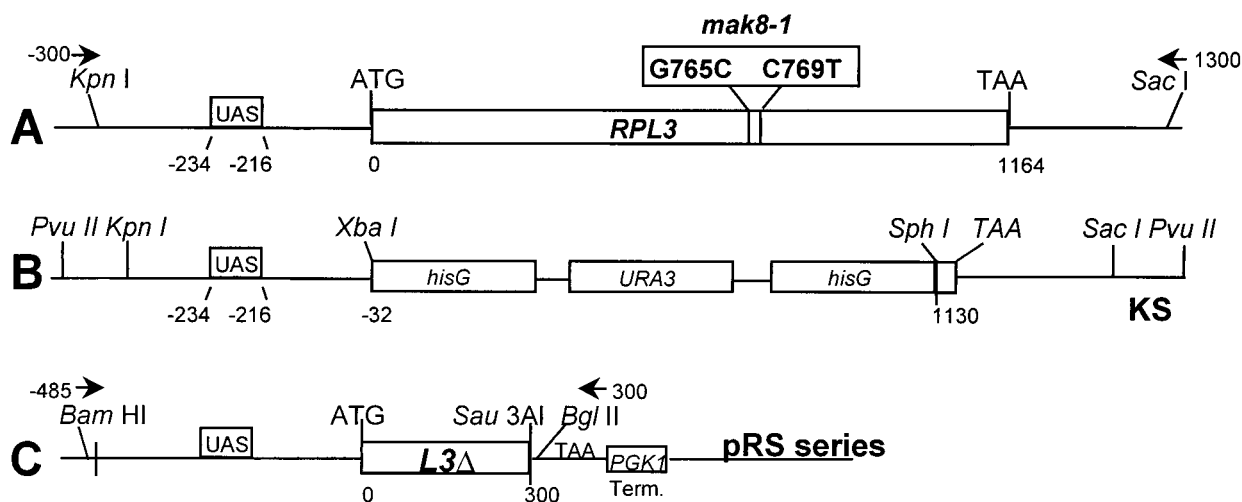


FIG. 1. (A) The full-length *RPL3* gene is located on chromosome XV in yeast cells and consists of an upstream activation signal (UAS) at bp -234 through bp -216 relative to the ATG start codon. The *RPL3* coding sequence is 1,164 bp long, and the gene encodes the 388-amino acid L3 protein. Restriction endonuclease sites are indicated. Full-length *RPL3* and mutant *mak8-1* alleles were cloned into the pRS series of plasmids by PCR with primers -300 KpnI and 1300 SacI as described in Materials and Methods. The sequence of *mak8-1* was determined from three independently isolated clones as described in Materials and Methods. The *mak8-1*-specific G765C (encoding a Trp-to-Cys change at amino acid residue 255) and C769T (encoding a Pro-to-Ser change at amino acid residue 257, which is a potentially significant change) mutations are indicated. (B) Map of the *RPL3-hisG-URA3* disruption plasmid. Based on BlueScript KS, a *hisG::URA3* marker was integrated into the *RPL3* locus between the *Xba*I site at -32 and an *Sph*I site at 1130. (C) Map of the *L3Δ* fragment. *L3Δ* contains the 5' noncoding sequence from position -485 plus the first 300 bp of *RPL3* (to the *Sau*3AI site). *L3Δ* was cloned into the pRS series of vectors by using primers -485 BamHI and 300 BglII. A *PGK1* terminator sequence is located downstream of the *L3Δ* coding region.

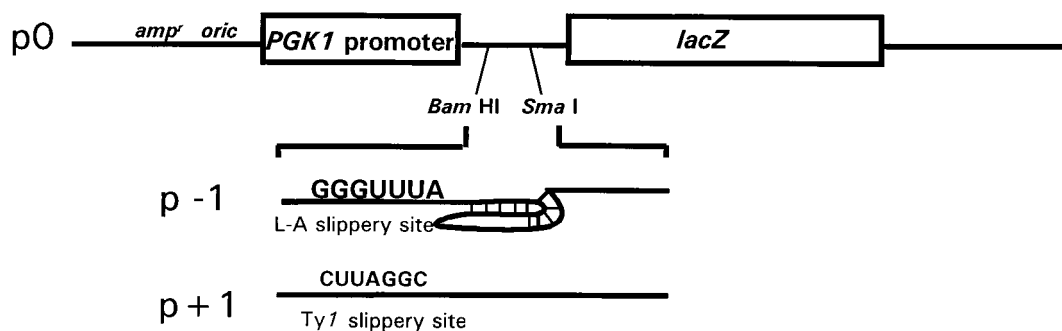


FIG. 2. Vectors used to measure programmed ribosomal frameshifting efficiencies. The 0-frame control reporter plasmid p0 and the -1 ribosomal frameshift test plasmid p-1 are described in references 12 and 17, and the $+1$ ribosomal frameshift test plasmid p+1 is described in references 3 and 58. In these constructs, transcription is driven from the constitutive phosphoglycerol kinase 1 (*PGK1*) promoter. The efficiencies of programmed ribosomal frameshifting are determined by dividing the β -galactosidase activities produced from the frameshift reporters (p-1 or p+1) by those produced from the p0 control and multiplying the resulting ratios by 100%.

efficiency in the *mak8-1* strain was 5.2%, approximately three-fold greater than the 1.7 to 2.0% normally observed in wild-type strains (Table 2). To confirm that the change in programmed -1 ribosomal frameshifting efficiency was solely due to the *mak8-1* allele, isogenic wild-type and *mak8-1* strains were constructed, and programmed -1 frameshifting in these cells was determined as described above (cross JD980 [Table 1]). In isogenic backgrounds, the *mak8-1* allele of *RPL3* promotes an approximately 2.5-fold increase in programmed -1 ribosomal frameshift efficiency ($\approx 4.9\%$ in *mak8-1* compared to $\approx 1.9\%$ in the isogenic wild-type strain [Table 2]). The *mak8-1* allele was also unable to maintain the M_1 killer virus (Table 2). However, *mak8-1* had no effect on programmed $+1$ ribosomal frameshifting (Table 2). Taken together, these results demonstrate that the *mak8-1* allele causes an alteration in programmed -1 ribosomal frameshift efficiencies. Thus, the *mak8-1* allele is also a *mof* mutant, in that these strains demonstrate increased programmed -1 ribosomal frameshifting efficiencies and loss of the killer virus (11, 12).

Characterization of the *mak8-1* lesion. The *mak8-1* allele was amplified by PCR from genomic DNA harvested from strain 1906, and the DNA sequence was obtained from three independently isolated clones (see Materials and Methods). The results demonstrated that the *mak8-1* allele harbors two separate mutations spaced four nucleotides apart (Fig. 1A). The G765C mutation encodes a Trp-to-Cys change at amino acid residue 255. The C769T mutation changes a proline at residue 257 to serine, a potentially significant structural change.

Strains harboring the *mak8-1* allele are resistant to the effects of peptidyltransferase inhibitors on programmed -1 ribosomal frameshifting. We previously demonstrated that peptidyltransferase inhibitors specifically alter programmed -1 ribosomal frameshifting efficiencies (19). It has been previously demonstrated that cells harboring mutant alleles of *rpl3* are resistant to the cytotoxic effects of peptidyltransferase inhibitors (28, 32, 45, 60). These include strains harboring the *mak8* and the *tcml* classes of *RPL3* alleles. Thus, we asked whether members of this class of agents affect programmed -1 ribosomal frameshifting in strains harboring *mak8-1*. To examine this, *mak8-1* and wild-type cells harboring either p0 or p-1 frameshift indicator plasmids were grown in the presence of various concentrations of either anisomycin or sparsomycin for 4 h, and programmed ribosomal frameshifting efficiencies were determined as described above. The results demonstrated that both anisomycin and sparsomycin altered ribosomal frameshifting in wild-type cells (Fig. 3). In contrast, neither anisomycin nor sparsomycin had any further effect on programmed

-1 ribosomal frameshifting in *mak8-1* strains (Fig. 3). These results provide strong evidence that a defect affecting the peptidyltransferase center is responsible for the observed increase in programmed -1 ribosomal frameshifting in *mak8-1* cells.

Episomal expression of the N-terminal 100 amino acids of ribosomal protein L3 increases programmed frameshifting and loss of the killer virus. We have recently identified a second *RPL3* allele that affects both programmed -1 ribosomal frameshifting and virus maintenance. This allele was isolated by its ability to abrogate the capability of cells harboring the *upf2-1* allele to allow the expression of nonsense-containing mRNAs (13). Previous results have demonstrated that mutations in the *UPF1*, *UPF2*, and *UPF3* genes can affect several aspects of translational fidelity (12, 15, 44, 55, 56). In particular, a specific mutation in the *UPF1* gene (*mof4-1*) and a deletion of the *UPF3* gene both increase programmed -1 ribosomal frameshifting efficiencies and lead to loss of the killer virus (12, 44). Characterization of the antisuppressor of *upf2-1*, YPF2-9, demonstrated that it encodes the N-terminal 100 amino acids of the *RPL3* (see Materials and Methods). This allele was renamed *L3 Δ* (Fig. 1C). Based on this connection between the *upf* mutants and L3, we examined the effect of episomal expression of *L3 Δ* on programmed ribosomal frameshifting. Episomal expression of *L3 Δ* protein in wild-type cells from either low- or high-copy plasmids promoted an approximately threefold increase in the efficiency of programmed -1 ribosomal frameshifting, but had no effect on programmed $+1$ ribosomal frameshifting (Fig. 4A). Expression of *L3 Δ* in wild-type cells also promoted high rates of killer phenotype loss (Fig. 4B). RNA hybridization analysis revealed that loss of the killer phenotype was a consequence of failure to maintain the M_1 satellite dsRNA virus (Fig. 4C).

TABLE 2. Assays of programmed -1 ribosomal frameshifting and the killer phenotype in yeast cells harboring the wild-type *RPL3* gene or the *mak8-1* allele

Strain	% Ribosomal frameshift ^a		Killer phenotype ^b
	-1	$+1$	
1906 (<i>mak8-1</i>)	5.18 \pm 0.12	4.12 \pm 0.16	-
980-10C pRPL3	1.93 \pm 0.18	5.34 \pm 0.18	+
980-10C + pmak8-1	4.85 \pm 0.12	5.47 \pm 0.11	-

^a The percent -1 ribosomal frameshifting was calculated by multiplying the ratio of p-1/p0 β -galactosidase activities by 100%. Absolute error is shown.

^b Killer phenotype was determined as described in Materials and Methods.

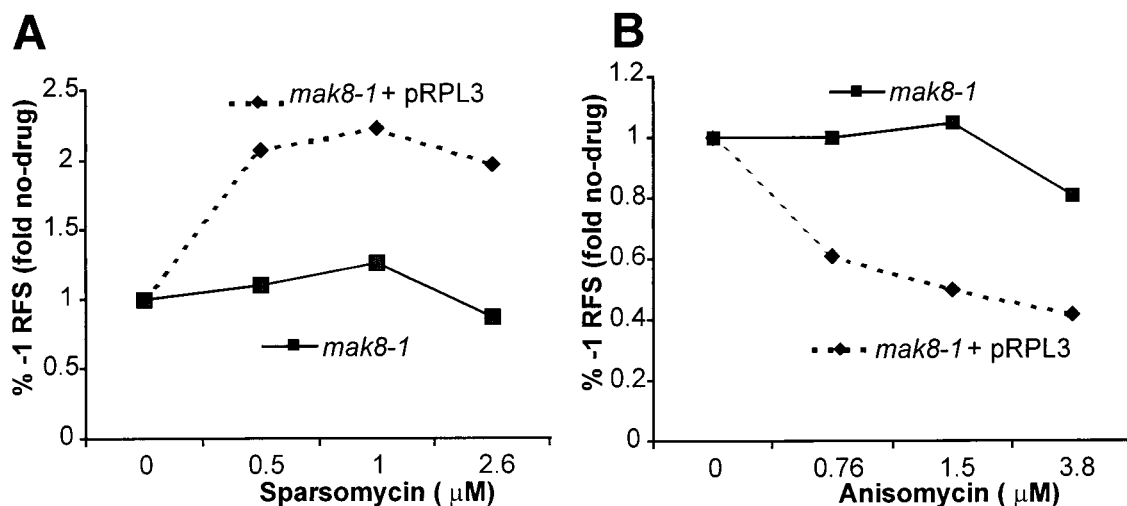


FIG. 3. Programmed -1 ribosomal frameshifting (-1 RFS) in a *mak8-1* strain is not further affected by peptidyltransferase inhibitors. Isogenic wild-type and *mak8-1* cells harboring either p0 or p -1 frameshift indicator plasmids were grown in the presence of the indicated concentrations of sparsomycin (A) or anisomycin (B) for 4 h, after which programmed -1 ribosomal frameshifting efficiencies were determined as described in Materials and Methods. In the absence of drugs, wild-type cells promote approximately 2% efficiency of programmed -1 ribosomal frameshifting, whereas this value is approximately 5% in cells harboring the *mak8-1* allele. The fold changes in programmed -1 ribosomal frameshifting efficiencies are plotted on the y axis.

Although the $+1$ frameshift and killer loss data strongly suggest that programmed -1 ribosomal frameshifting is elevated in cells harboring the *mak8-1* and *L3Δ* alleles, the formal possibility exists that specific stabilization of the LacZ -1 mRNA relative to the 0-frame control mRNA may account for an apparent increase in programmed -1 ribosomal frameshift efficiencies and that M_1 loss is a consequence of some other defect. The level of the LacZ -1 frameshift reporter mRNA was not affected either by cells harboring the *mak8-1* allele or by expression of *L3Δ* (data not shown). These data are consistent with previous results demonstrating that expression of *L3Δ* (YPF2-9) did not affect the levels of a nonsense-containing *Cyh2* precursor mRNA in a *upf2-1* strain (13). Thus, *L3Δ* also behaves like a *mof* mutant in that it inhibits virus propagation as a consequence of increased programmed -1 ribosomal frameshifting.

***L3Δ* is dominant to the *ski* mutants.** The L-A and M_1 mRNAs present poor translational substrates because they do not possess either the 5' m⁷G5'ppp5'Xp cap or 3' poly(A) tails (9, 52). The lack of these structures does not prevent their expression in wild-type cells, but does make their expression sensitive to mutations which affect translation (e.g., the *mak* mutants) (4, 35, 40). Conversely, L-A and M_1 copy numbers are increased in cells harboring mutations in chromosomal genes which are involved in recognition of 5' caps or 3' poly(A) tails and in the degradation of mRNAs lacking these structures (2, 4, 35). However, since the ratio of Gag to Gag-Pol is critical for viral particle morphogenesis, we predicted that mutations which change this ratio by altering frameshift efficiencies should be dominant to the effects of the *ski* mutants, because although the L-A and M_1 mRNAs are either stabilized or translated more efficiently by the *ski* mutations, the ratios of Gag to Gag-Pol should still be altered as a consequence of the *mof* mutations.

To test this hypothesis, a plasmid harboring the *L3Δ* allele was introduced into cells harboring mutations in a series of *SKI* genes. These included the *SKII/XRN1* gene, which encodes the major 5'→3' exoribonuclease that degrades uncapped RNAs (29, 33, 37, 49, 50), the *SKI2* and *SKI6* genes, which play a role in ribosome biogenesis, and which are both required for effi-

cient translation of poly(A) mRNAs, and the 3'→5' exonuclease activity of the exosome (2, 4, 35), *SKI4*, and *SKI7* (42). The results demonstrated that episomal expression of *L3Δ* promoted rapid loss of the killer phenotype, which was due to loss of the M_1 virus (Fig. 4C).

DISCUSSION

Mutations affecting ribosomal protein L3 promote loss of the M_1 killer virus by altering the efficiency of programmed -1 ribosomal frameshifting. The mechanism governing programmed -1 ribosomal frameshifting suggests that drugs and mutations which affect the peptidyl-transfer reaction may alter programmed -1 ribosomal frameshift efficiencies and have antiviral effects (19). We previously used peptidyltransferase inhibitors to demonstrate the validity of this model (19). The results presented here have shown that two alleles encoding mutant forms of ribosomal protein L3, which was previously implicated in formation of the peptidyltransferase center, also alter programmed -1 ribosomal frameshift efficiencies and have antiviral effects. Taken together, these results support the hypothesis that the peptidyltransferase center may present a novel target for antiretroviral therapeutic agents.

It has long been known that cells harboring *mak8* alleles cannot propagate the M_1 satellite virus (59). Additional alleles of *RPL3*, named *tcm1*, were also characterized based on their resistance to the peptidyltransferase inhibitor trichodermin (26, 28, 32, 45, 46). These alleles also have the Mak^- phenotype (60). However, the precise mechanism responsible for killer virus loss in this class of mutants was not determined. Previous results suggested that *mak8-1* did not affect programmed -1 ribosomal frameshifting efficiencies (22). However, the interpretation of those results was incorrect in that only changes in overall β -galactosidase activities generated from a frameshift reporter construct by using sister spore clones were examined. The present study rectifies those defects by directly measuring programmed ribosomal frameshifting efficiencies in isogenic strains. The results presented here demonstrate that alterations in programmed -1 ribosomal frameshifting efficiencies are responsible for the inability of cells

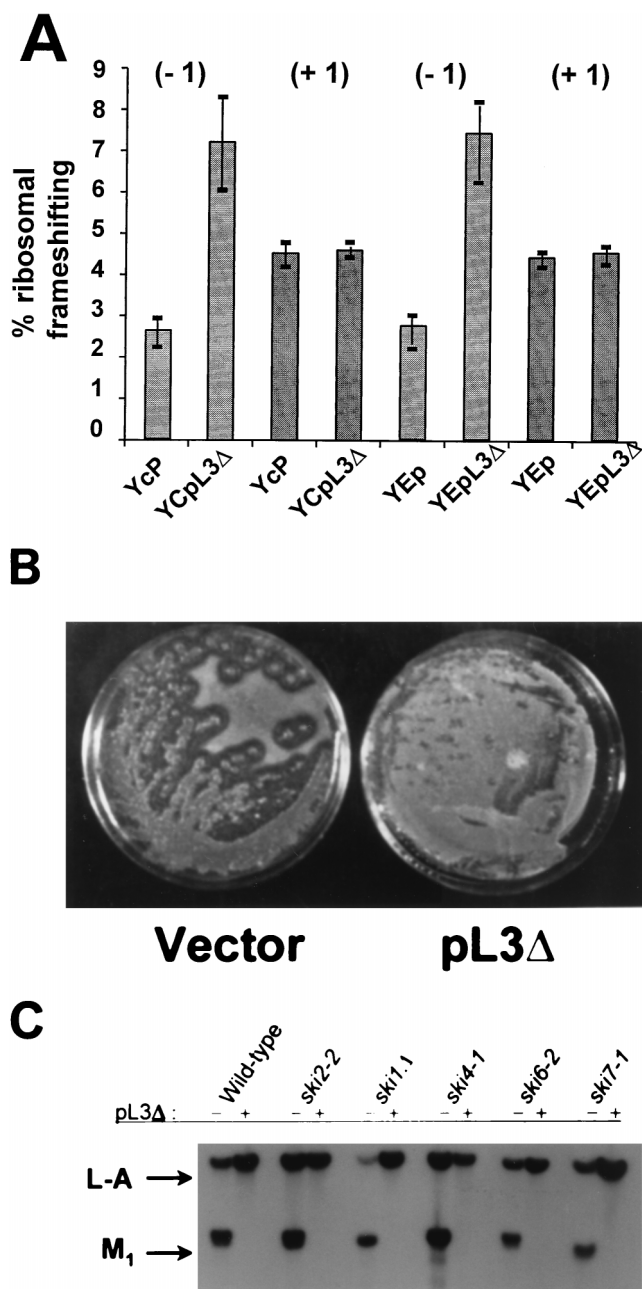


FIG. 4. The *L3Δ* allele of RPL3 confers a dominant-negative *Mof*⁻ phenotype. (A) Wild-type cells were transformed with *L3Δ* cloned into either low-copy-number (YcP) or high-copy-number (YEp) vectors, and programmed -1 and +1 ribosomal frameshifting efficiencies were monitored with the following results: programmed -1 ribosomal frameshifting, YcP = 2.63% ± 0.34%, YCpL3Δ = 7.22% ± 1.13%, YEp = 2.74% ± 0.64%, and YEpL3Δ = 7.42% ± 1.13%; programmed +1 ribosomal frameshifting, YcP = 4.52% ± 0.29%, YCpL3Δ = 4.60% ± 0.14%, YEp = 4.42% ± 0.18%, and YEpL3Δ = 4.53% ± 0.22%. (B) Killer phenotypes of wild-type cells (JD890) transformed with YCpL3Δ or vector. (C) Total nucleic acids were extracted from cells transformed with vector alone (-) or from cells transformed with YCpL3Δ (+), and RNA was transferred to nitrocellulose and hybridized with L-A- and M₁-specific negative-strand probes. L-A- and M₁-hybridizing bands are indicated.

harboring this mutation to maintain the M₁ dsRNA virus. Given the previous demonstration that peptidyltransferase inhibitors promote virus loss by altering programmed -1 ribosomal frameshift efficiencies, as well as the role of the L3

protein in peptidyltransferase center formation, our results indicating that mutations in *RPL3* affect programmed -1 ribosomal frameshifting are consistent with the view that alteration of peptidyl-transfer activity affects this process. In addition, the finding that episomal expression of the *L3Δ* allele confers a dominant negative *Mof*⁻ phenotype provides a novel tool that can be used to probe the contribution of L3 to translational fidelity. The *trans*-dominance of the L3 peptide illuminates the importance of programmed -1 ribosomal frameshifting in the viral life cycle. The fact that *L3Δ* is dominant to the *ski* mutants demonstrates that even when viral RNAs and proteins are in excess, these mutants cannot overcome the imbalance in the ratio of Gag to Gag-Pol proteins as a consequence of altered programmed -1 ribosomal frameshifting efficiency. Thus, the *trans*-dominance of the *L3Δ* allele with respect to the *ski* mutants supports the hypothesis that the efficiency of programmed ribosomal frameshifting plays a critical role in the viral particle morphogenetic process by ensuring the correct ratio of viral structural to enzymatic proteins.

We envision two models to explain the role of the L3 protein in programmed -1 ribosomal frameshifting. In one, we suggest that the incorporation of defective L3 protein (either Mak8-1p or L3Δp) into ribosomes would result in suboptimal L3 function, yielding the observed translational fidelity defect. Alternatively, it is possible that expression of these alleles results in a subpopulation of L3-deficient ribosomes. Since it is thought that the large rRNA is responsible for peptidyltransferase activity (38, 61), these L3-deficient ribosomes would retain a small amount of peptidyltransferase activity. In both scenarios, defects in peptidyltransferase activity are predicted to slow the rate of translation elongation while both the ribosomal A- and P-sites are occupied. In the context of frameshifting, this would result in a longer ribosomal pause at the programmed -1 ribosomal frameshift signal, increasing the likelihood of a successful frameshift. If this model is true, then the observed increases in programmed -1 ribosomal frameshifting efficiencies promoted by these alleles should represent the sum of programmed frameshifting promoted by normal plus defective ribosomes.

In sum, we have demonstrated that two genetically defined alleles encoding L3, a ribosomal protein which has been demonstrated to participate in formation of the peptidyltransferase center, both affect the efficiency of programmed -1 ribosomal frameshifting and promote loss of the killer virus. These studies are consistent with our pharmacologically based observations that peptidyltransferase inhibitors specifically affect programmed -1 ribosomal frameshifting efficiencies and demonstrate the utility of using programmed ribosomal frameshifting as an assay to probe the mechanisms which regulate the process of protein translation.

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