Ribosomal protein L5 helps anchor peptidyl-tRNA to the P-site in *Saccharomyces cerevisiae*

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ABSTRACT

Our previous demonstration that mutants of 5S rRNA called *mof9* can specifically alter efficiencies of programmed ribosomal frameshifting (PRF) suggested a role for this ubiquitous molecule in the maintenance of translational reading frame, though the repetitive nature of the 5S rDNA gene (>100 copies/cell) inhibited more detailed analyses. However, given the known interactions between 5S rRNA and ribosomal protein L5 (previously called L1 or YL3) encoded by an essential, single-copy gene, we monitored the effects of a series of well-defined *rpl5* mutants on PRF and virus propagation. Consistent with the *mof9* results, we find that the *rpl5* mutants promoted increased frameshifting efficiencies in both the –1 and +1 directions, and conferred defects in the ability of cells to propagate two endogenous viruses. Biochemical analyses demonstrated that mutant ribosomes had decreased affinities for peptidyl-tRNA. Pharmacological studies showed that sparsomycin, a peptidyltransferase inhibitor that specifically increases the binding of peptidyl-tRNA with ribosomes, was antagonistic to the frameshifting defects of the most severe mutant, and the extent of sparsomycin resistance correlated with the severity of the frameshifting defects in all of the mutants. These results provide biochemical and physiological evidence that one function of L5 is to anchor peptidyl-tRNA to the P-site. A model is presented describing how decreased affinity of ribosomes for peptidyl-tRNA can affect both –1 and +1 frameshifting, and for the effects of sparsomycin.

Keywords: frameshifting; ribosome; virus

INTRODUCTION

Although the ribosome is exquisitely designed to maintain translational reading frame, there are several naturally occurring instances in which this process has been subverted. These exceptions to the rule provide us with a powerful set of tools that can be used to elucidate the molecular mechanisms underlying the normal maintenance of translational reading frame. Such “translational recoding” events can take many forms. The most common recoding events are cases where ribosomes are directed to slip by a single base in either the 5′ (–1) or 3′ (+1) direction: this is called programmed ribosomal frameshifting (PRF). Other

examples of translational recoding include prompting ribosomes to bypass a termination codon (termination suppression), making them “hop” over as many as 50 bases at a time, and “shunting” them around complex RNA tertiary structures (reviewed in Jacks, 1990; Farabaugh, 1996; Gesteland & Atkins, 1996). The great majority of examples of translational recoding have been characterized in viral systems. The critical role that these processes play in many pathogenic viruses infecting animals and plants make their study important to the field of virology (for reviews, see Brierley, 1995; Dinman, 1995; Gesteland & Atkins, 1996; Farabaugh, 1997). There are also documented examples in which translational recoding is utilized to regulate the expression of a growing list of both prokaryotic and eukaryotic chromosomally encoded mRNAs, suggesting that PRF may play a role in regulating the expression of a significant subset of chromosomally encoded mRNAs as well (see Hammell et al., 1999).

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Programmed ribosomal frameshifting in both the -1 and $+1$ directions is kinetically driven: *cis*-acting elements force ribosomes and their associated tRNAs to pause at specific mRNA sequences (reviewed in Dinman, 1995; Farabaugh, 1996). In -1 PRF, an elongating ribosome in which the A- and P-sites are occupied by aminoacyl- and peptidyl-tRNAs, respectively, encounters a defined RNA secondary structure such as an RNA pseudoknot, which directs it to pause over a special heptameric sequence called the "slippery site." The increased pause time over this sequence is thought to give the ribosome and the two ribosome-bound tRNAs the opportunity to slip one base in the 5' direction. Because of the nature of the slippery site, the non-wobble bases of the tRNAs can pair correctly with the -1 reading frame codons. This allows the ribosome to bypass the 0-frame (e.g., viral *gag* gene) termination codon upon resumption of elongation and continue translation to append a C-terminal extension to the protein (e.g., to synthesize viral Gag-pol fusion proteins). In the $+1$ PRF mechanism used by the yeast retrotransposable element *Ty1* to produce its Gag-pol protein, ribosomal pause times are dictated by tRNA abundance instead of mRNA structure, and the $+1$ shift occurs while only the P-site of an elongating ribosome is occupied by peptidyl-tRNA, that is, at a different step of the translation elongation cycle (Belcourt & Farabaugh, 1990). Here, the ribosome is forced to pause at a rare codon that corresponds to a single-copy tRNA. Upon slippage of the ribosome by one base in the 3' direction, the anticodon loop of the P-site peptidyl-tRNA can base pair to the new P-site codon in the $+1$ reading frame. The new A-site codon corresponds to an abundant aminoacyl-tRNA (aa-tRNA), which, when inserted into the A-site, establishes the $+1$ frameshift. Similar to programmed -1 ribosomal frameshifting, this mechanism also allows the ribosome to bypass the 0-frame (*TyA* gene) termination codon and continue translation to synthesize the viral *TyA-TyB* Gag-pol fusion protein.

We and others have demonstrated that the efficiency of viral PRF events determines the final stoichiometric ratios of viral structural to enzymatic proteins available for viral particle assembly, and that altering PRF efficiencies interferes with virus propagation (reviewed in Dinman, 1995; Dinman et al., 1998). In addition to the importance of PRF to the field of virology, the ability to monitor changes in frameshift efficiencies provides a powerful set of tools that can also be applied toward the elucidation of the *cis*-acting sequences and *trans*-acting factors that are normally involved in maintaining translational reading frame. We have used the endogenous yeast "killer virus" and *Ty1* systems to study -1 and $+1$ PRF, respectively. The efficiency of -1 PRF can be affected by changes in the *cis*-acting mRNA signal (Brierley et al., 1989, 1991, 1992; Dinman et al., 1991, 1997; Dinman & Wickner, 1992; Morikawa &

Bishop, 1992; Dinman & Kinzy, 1997), and *in trans* by mutations in integral ribosomal components (Dinman & Wickner, 1995; Peltz et al., 1998), mutations in ribosome-associated factors (Lee et al., 1995; Cui et al., 1996, 1998a; Dinman & Kinzy, 1997; Ruiz-Echevarria et al., 1998), and by certain small molecules (Dinman et al., 1997; Hung et al., 1998). Efficiencies of $+1$ PRF can also be altered by changes in the *cis*-acting mRNA signal (Belcourt & Farabaugh, 1990), and by mutations which affect *trans*-acting factors such as relative abundance of specific tRNAs (Belcourt & Farabaugh, 1990), intracellular polyamine concentrations (Balasundaram et al., 1994a, 1994b), mutants of Elongation Factor-A (eEF-1A) (Dinman & Kinzy, 1997; Farabaugh & Vimaladithan, 1998), and by translocation inhibitors (Tumer et al., 1998; Hudak et al., 1999; Harger et al., 2001).

In the course of identifying *trans*-acting factors that affect programmed ribosomal frameshifting, we previously demonstrated that mutants of 5S rRNA, called *mof9*, promoted increased efficiencies of both -1 and $+1$ PRF (Dinman & Wickner, 1995). Though this work provided the first evidence for a functional role of 5S rRNA in translation, since the yeast genome contains 100–200 copies of the 5S rDNA gene, further genetic analyses were complicated by the presence of mixed populations of wild-type and mutant 5S rRNA species. 5S rRNA associates with the large ribosomal protein L5 (previously also known as L1 or YL3). In *Saccharomyces cerevisiae*, L5 is encoded by *RPL5*, a single-copy essential gene (Nazar et al., 1989; Nazar, 1991; Deshmukh et al., 1993). Given this 5S-L5 interaction and the amenability of *RPL5* to simple genetic manipulation, we tested the hypothesis that mutants of L5 may also be able to confer a *Mof*[−] phenotype on yeast cells. Here we report that some mutant *rpl5* alleles also confer this property upon cells. Biochemical and pharmacological analyses indicate that a defect in the interactions between the ribosome and peptidyl-tRNA is the root cause of the frameshifting and virus propagation phenotypes. Our findings are supported by the recent X-ray crystallographic study of the *Thermus thermophilus* 70S ribosome (Yusupov et al., 2001), and suggest that the peptidyl-tRNA plays a key role in maintaining translational reading frame.

RESULTS

The various *rpl5-HA* alleles affect both programmed -1 and $+1$ ribosomal frameshifting

Our prior genetic finding that mutations in 5S rRNA mutants could promote increased efficiencies in programmed ribosomal frameshifting were landmark in that they provided the first demonstration of a functional role in translation for this ubiquitous molecule. The problem, however, was that further progress in this field

was hampered by the highly repetitive nature of the 5S rDNA genes. Because 5S rRNA and L5 are known to physically interact, we reasoned that *rpl5* mutants would also affect PRF efficiencies. If this were true, the single-copy nature of the *RPL5* gene would render it amenable to genetic and biochemical manipulation, allowing us to circumvent the problem posed by 5S rRNA. To this end, we measured both -1 and $+1$ PRF in a series of previously described isogenic strains in which the chromosomal copy of *RPL5* had been deleted (*rpl5*- $\Delta 1$). In these strains, expression of this essential ribosomal protein was derived from a series of HA-tagged plasmid borne *rpl5* alleles (Deshmukh et al., 1993; Yeh et al., 1996). A summary description of the alleles and selected properties is presented in Table 1. The isogenic strains were transformed with a series of *URA3/CEN3*-based frameshift reporter plasmids (p0, p -1 , and p $+1$), and PRF efficiencies were determined. The results of these assays are summarized in Figure 1.

The efficiency of L-A promoted -1 PRF in cells harboring the wild-type *RPL5*-HA allele was 2%, comparable to previously measured wild-type levels in other strains (see Dinman et al., 1991). All of the *rpl5*-HA alleles that had previously been shown to alter ribosome function (i.e., *rpl5*-HA1 through -HA5; see Deshmukh et al., 1995) affected -1 PRF, albeit to differing extents (Fig. 1A). For example, -1 frameshifting efficiencies ranged from approximately 3.9-fold above wild-type levels in cells harboring the *rpl5*-HA2 allele (7.8%), to roughly 1.5-fold increases in cells harboring the *rpl5*-HA5 allele (3.1%). The effects of the different alleles on $+1$ PRF generally paralleled those observed with -1 frameshifting (Fig. 1B). The greatest effect on programmed $+1$ ribosomal frameshifting was also seen with the *rpl5*-HA2 allele (10.3%, >2 -fold above wild-type), whereas Ty1-directed $+1$ PRF ranged from 7.2 to 7.9% (approximately 1.5-fold greater than wild-type) in cells expressing the HA-1, HA-3, HA-4, and HA-5 alleles. The *rpl5*-HA9 allele (which lacks the C-terminal 2 amino acids of L5) did not significantly affect either -1 or $+1$ frameshifting efficiencies, commensurate with its lack of effect on ribosome function in vivo (Deshmukh et al., 1995).

TABLE 1. Description of the *RPL5* alleles used in this study (adapted from Deshmukh et al., 1995).

Allele	Description	5S rRNA binding	
		In vivo	In vitro
<i>RPL5</i> -HA	Wild-type RPL5 with C-terminal HA-tag	100	100
<i>rpl5</i> -HA1	K27E allele, ts at 37°C	65 $\pm$ 5	40 $\pm$ 5
<i>rpl5</i> -HA2	T28A allele, ts at 37°C	76 $\pm$ 8	60 $\pm$ 6
<i>rpl5</i> -HA3	V53G allele, ts at 37°C, cs at 13°C	17 $\pm$ 6	0
<i>rpl5</i> -HA4	G91R allele, ts at 37°C	83 $\pm$ 9	50 $\pm$ 5
<i>rpl5</i> -HA5	K289E allele, ts at 37°C	85 $\pm$ 2	ND
<i>rpl5</i> -HA9	Deletion of C-terminal 2 amino acids	ND	ND

ND: not determined.

It is possible that apparent changes in programmed frameshifting efficiencies were actually due to changes in the stability, and hence the steady-state abundance of the -1 and/or $+1$ frameshift LacZ reporter mRNAs relative to the 0-frame control, rather than to genuine changes in PRF efficiencies. RNase protection studies were carried out to address this possibility using the U3 RNA as an internal control. None of the *rpl5*-HA mutants had any significant effects on the ratios of U3 mRNA to 0-frame, -1 , or $+1$ frame LacZ mRNAs (Fig. 1C). Thus, the observed increases in PRF efficiencies in cells harboring the *rpl5*-HA alleles were not due to the preferential stabilization of the reporter mRNAs in either of these systems. In passing, it should be noted that both the -1 and $+1$ lacZ reporter mRNAs were equally as stable as the 0-frame control, even though these represent nonsense-containing mRNAs. This finding is in agreement with another recent report (Wang et al., 2001), reinforcing the notion that the bacterial LacZ transcript is immune to the eukaryotic nonsense-mediated mRNA decay apparatus (Dinman et al., 2000). In sum, the results of these experiments demonstrate that (1) like 5S rRNA mutants, alleles of *rpl5* can affect both -1 and $+1$ PRF efficiencies, and (2) there are allele-specific effects on these processes.

The *rpl5* alleles have differing effects on M₁ killer virus maintenance and Ty1 retrotransposition frequencies

We and others have previously demonstrated that alterations in programmed ribosomal frameshifting efficiencies impact on virus particle morphogenesis, and consequently on virus propagation (reviewed in Dinman et al., 1998). In light of the effects of the *rpl5* alleles on both -1 and $+1$ PRF, we monitored their impacts on the ability of yeast cells to propagate viruses that are dependent on these processes.

The M₁ killer virus was used to monitor the -1 PRF-specific viral effects because its propagation is affected by mutations that alter programmed -1 ribosomal frameshifting efficiencies in the L-A helper virus. L-A and M₁ were introduced into cells harboring the different *rpl5*-HA alleles by cytoplasmic mixing, and the ability of the cells to maintain the killer virus was monitored. There is a direct correlation between the severity of the -1 PRF defect and killer virus maintenance (Fig. 2). Specifically, the two *rpl5*-HA alleles having the greatest increases in -1 PRF efficiencies, that is, *rpl5*-HA2 and *rpl5*-HA4, could not maintain the killer phenotype at all (Fig. 2A), and were completely unable to maintain M₁ (Fig. 2B). These findings are consistent with our previous observations indicating that increases in -1 PRF that are greater than twofold above wild-type tend to severely impact on M₁ maintenance, whereas the effects of lesser increases are less onerous (reviewed in Dinman, 1995; Dinman et al., 1998).

FIGURE 1. Allele-specific effects of the *rpl5-HA* mutants on programmed ribosomal frameshifting. Cells harboring the *TRP1*-based wild-type *RPL5-HA* or the mutant *rpl5-HA* alleles were cotransformed with either p0, p-1 or p+1 (*URA3*-based vectors). Programmed ribosomal frameshifting was determined as described in the experimental procedures. **A:** Programmed -1 ribosomal frameshifting directed by the L-A virus signal. **B:** Programmed +1 ribosomal frameshifting directed by the Ty1 signal. Diamonds connote percent error. **C:** Differences in PRF efficiencies are not due to alterations in the steady-state abundances of the -1 or +1 reporter mRNAs. Total cellular mRNAs extracted from cells harboring either wild-type *RPL5-HA*, or the mutant *rpl5-HA* alleles and either p0, p-1, or p+1 were subjected to RNase protection analysis using radiolabeled antisense probes corresponding to the 3' ends of the LacZ mRNA and U3 snRNA. Products were separated through a 6% PAGE urea denaturing gel, and the image was visualized and quantitated by phosphorimager using a Typhoon 8600 phosphorimager and ImageQuant V5.2. software (Molecular Dynamics).

The dependence of the Ty1 retrotransposable element on +1 PRF was exploited as a means to monitor the +1 PRF-specific viral effects of the *rpl5-HA* alleles. To examine the effects of the differing *rpl5-HA* alleles on Ty1 retrotransposition, galactose was used to induce transcription of a *neo'*-tagged Ty1 mRNA from pJEF1105. The tagged Ty1 transcript is packaged and copied into Ty1 cDNA inside of Ty1 viruslike particles, which in turn enters the nucleus where the cDNA is integrated into a chromosomal locus. After curing cells of pJEF1105, the ability of the cells to grow in the presence of the drug geneticin (G418) is used as an indicator of Ty1 retrotransposition from the plasmid to a host cell chromosome. The process of retrotransposition is dependent on viral particle morphogenesis, which is in turn extremely sensitive to alterations in programmed +1 ribosomal frameshifting efficiencies (reviewed in Dinman, 1995). Thus, changes in Ty1 retrotransposition frequencies are indicative of the effects of changes in programmed +1 ribosomal frameshifting efficiencies. To quantitate these effects, cells

that had undergone galactose induction were grown in liquid medium, and spread onto medium containing geneticin at densities ranging from 10^4 to 10^8 CFU (see Materials and Methods). Ty1 retrotransposition frequencies were directly calculated by determining the ratio of *neo'* to the total number of CFUs per plate. Retrotransposition frequencies in control cells (pRPL5-HA) were 4.5×10^{-3} (Fig. 3). Inhibition of Ty1 retrotransposition paralleled the observed increases in +1 PRF promoted by the mutant *rpl5-HA* alleles, with affects ranging from negligible (*rpl5-HA9*) to greater than 99.99% inhibition (*rpl5-HA2*; Fig. 3).

Ribosomes from the *rpl5-HA2* strain have decreased affinity for the 3' ends of the P-site (donor) tRNAs

The L5-5S rRNA ribonuclear particle (RNP) is stable in the absence of 60S ribosomal subunit assembly, and yeast cells contain a nuclear pool of L5-5S rRNA not associated with ribosomes (Deshmukh et al., 1993). 5S

FIGURE 2. The *rpl5*-HA2 and *rpl5*-HA4 alleles cannot maintain the killer virus. **A:** Killer assay of primary cytoductants. The killer virus was introduced by cytoplasmic mixing into *rpl5* Δ strains harboring plasmid-borne, HA-tagged *rpl5* alleles, and primary cytoductants were identified as described in the experimental procedures. These were replica plated onto a lawn of cells that are sensitive to the secreted killer toxin produced by the M₁ satellite virus of L-A. Killer activity was observed as a zone of growth inhibition around the colonies. **B:** Northern blot probing for L-A and M₁ killer virus. Total RNAs were isolated from the same strains and analyzed by northern blot for the presence of the L-A and M₁ viral RNAs as described in Materials and Methods.

rRNAs from HeLa and *Xenopus* cells compete less effectively than *S. cerevisiae* 5S rRNA for their ability to complex with *S. cerevisiae* L5 even though they differ from yeast 5S rRNA at only 13 of 120 positions (Brow & Geiduschek, 1987), demonstrating that changes in 5S rRNA sequence can affect its ability to complex with L5. Thus, one possible hypothesis is that the molecular underpinnings for what we phenotypically observe as *mof9* alleles may be due to changes in the interactions between the mutant 5S rRNAs and L5. However, this is not the case: there is no correlation between published L5-5S rRNA binding defects (see Table 1) and the severity of Mof⁻ phenotypes. This suggests that some other deficiency must account for the PRF defects.

Examination of the models describing programmed -1 and +1 ribosomal frameshifting reveals that a common feature of both is the slippage of peptidyl-tRNA. Thus, we reasoned that perhaps this tRNA was more slippery in the *rpl5* mutants. One way that this might come to pass could be if the interactions between mutant ribosomes and peptidyl-tRNAs were specifically weakened. The classical "fragment reaction" assays (reviewed in Pestka, 1974), which monitor the inter-

actions between tRNA 3' ends and purified ribosomes, were used to address this hypothesis. Purified wild-type or *rpl5*-HA2 ribosomes were incubated with either [¹⁴C]Phe-CACCA (acceptor) or acetyl-[¹⁴C]Phe-CACCA (donor) fragments, and binding reactions were performed as described in Materials and Methods. The results clearly show that the *rpl5*-HA2 ribosomes had a significantly lower affinity than wild-type ribosomes for the P-site specific acetyl-[¹⁴C]Phe-CACCA (donor) fragments (Fig. 4A). In contrast, the affinity of *rpl5*-HA2 ribosomes for the A-site-specific [¹⁴C]Phe-CACCA (acceptor) fragment was equivalent to wild-type ribosomes. These results clearly demonstrate that the L5-HA2 mutant has a peptidyl-tRNA specific binding defect, and are consistent with our prediction that L5 is involved in the binding of peptidyl-tRNAs with the ribosome.

Sparsomycin is an *rpl5*-HA2 antagonist

The biochemical analysis indicated that decreased affinity of the ribosomal P-site for peptidyl-tRNA correlates with the Mof⁻ phenotype of *rpl5*-HA2 cells. A potential problem with this however is that the biochemical studies are not physiological. A pharmacological approach using intact cells was thus used as an independent test of this hypothesis in a more physiologically relevant context. We reasoned that an agent that increases the affinity of the ribosome for this tRNA would potentially be able to compensate for the cellular lesion. Sparsomycin has this property: It stimulates binding of peptidyl-tRNA stem to the donor site of the peptidyl-transferase center (Hermer et al., 1969; Oen et al., 1974). Another peptidyltransferase inhibitor, anisomycin, which specifically inhibits binding of the 3' end of the aminoacyl-tRNA to the acceptor site of the peptidyltransferase center (Carrasco et al., 1973), provided an A-site-specific control for these studies. Previously, we demonstrated that these two peptidyltransferase inhibitors were capable of promoting altered efficiencies of programmed -1 ribosomal frameshifting and had antiviral effects at sublethal concentrations (Dinman et al., 1997). Additionally, the demonstration that programmed -1 ribosomal frameshifting was not affected by these drugs in cells harboring the *mak8-1* allele of *RPL3*, a gene whose product has been implicated in peptidyltransferase center formation, established the utility of these drugs as probes for altered ribosomal protein function (Peltz et al., 1998). Here, we have used these drugs to probe the nature of the defect in the *rpl5* mutants.

Owing to its strong effects on both -1 and +1 ribosomal frameshifting, the *rpl5*-HA2 allele was used to monitor the effects of sparsomycin and anisomycin on frameshifting. Figure 5A shows that whereas sparsomycin promoted an approximately 1.9-fold increase in -1 PRF efficiencies in wild-type cells (from 1.9% $\pm$ 0.15% to 3.6% $\pm$ 0.5%), it was antagonistic to the effect

FIGURE 3. Effects of the different *rpl5*-HA mutants on Ty1 retrotransposition. *rpl5* Δ strains harboring plasmid-borne, HA-tagged *rpl5* alleles were transformed with a galactose inducible, neo^r-tagged Ty1 cDNA plasmid (pJEF1105). Cells were grown at 20 °C on medium containing 2% galactose but lacking uracil for 4 days, replica plated at 25 °C onto rich media containing 2% dextrose, and then to nonselective media containing 100 μ g/mL of 5-fluoroorotic acid (5-FOA) and incubated at 25 °C to select for cells that had lost pJEF1105. Colonies of cells that were able to grow on 5-FOA were then assayed for Ty1 by spotting of 10-fold dilutions of cells onto medium containing 100 μ g/mL of Geneticin (G418). Precise measurements of retrotransposition frequencies (R.F.) were determined as described in Materials and Methods.

of the *rpl5*-HA2 allele on -1 PRF, promoting a ≈ 2 -fold reduction in these cells (from $6.0\% \pm 0.9\%$ to $2.8\% \pm 0.5\%$). Additionally as shown in Figure 5B, although sparsomycin had no effect on $+1$ PRF in wild-type

cells, it was also antagonistic to the effect of the *rpl5*-HA2 allele on this process as well, promoting an ≈ 1.6 -fold decrease (from $14.8\% \pm 2.5\%$ to $9.5 \pm 1.6\%$). In control experiments, in the presence of anisomycin -1

FIGURE 4. *rpl5*-HA2 ribosomes have decreased affinity for the peptidyl-tRNA fragment. Salt-washed ribosomes were incubated with either acetyl-[¹⁴C]Phe-CACCA (donor; **A**), or [¹⁴C]Phe-CACCA (acceptor; **B**) tRNA fragments in 500 μ L of a buffer containing 70 mM Tris-acetate, pH 7.2, 40 mM magnesium acetate, 0.4 M potassium acetate, and 50 mM NH₄Cl. Ethanol was added to a final concentration of 30%, and aliquots were taken during the time course at 24 °C. Samples were diluted to 1 mL with cold buffer, immediately precipitated onto a Millipore filter, washed, and incorporated radioactivity was quantitated in a scintillation counter. The reaction mix without ribosomes was used as control. All assays were performed in triplicate. Error bars represent standard error.

FIGURE 5. Sparsomycin is antagonistic to the *rpl5-HA2* frameshifting defect. Isogenic wild-type and *rpl5-HA2* cells harboring either p0, p-1, or p+1 frameshift indicator plasmids were grown in the presence of the indicated concentrations of sparsomycin or anisomycin for 5 h, after which programmed ribosomal frameshift efficiencies were determined as described in Materials and Methods. **A:** Effects of sparsomycin on -1 PRF in wild-type and *rpl5-HA2* cells. **B:** Effects of sparsomycin on +1 PRF in wild-type and *rpl5-HA2* cells. **C:** Effects of anisomycin on -1 PRF in wild-type and *rpl5-HA2* cells. **D:** Effects of anisomycin on +1 PRF in wild-type and *rpl5-HA2* cells. The PRF efficiencies are plotted on the Y axis. All assays were performed in triplicate. Error bars indicate percent error. **E:** The *rpl5-HA* mutants tend to be resistant to sparsomycin. Cultures of strains harboring the various *rpl5-HA* alleles were spotted in 10-fold dilutions onto rich medium containing sparsomycin (20 $\mu\text{g/ml}$) or anisomycin (15 $\mu\text{g/ml}$) and incubated at 30°C for 4 days. No-drug controls (YPAD) are also shown.

PRF efficiencies decreased in cells harboring both wild-type *RPL5* and the *rpl5-HA2* allele (Fig. 5C). Similarly, the lack of effect of anisomycin on +1 PRF in either wild-type or mutant cells (Fig. 5D) confirms that the *rpl5-HA2* has no impact at the ribosomal A-site. These results provide independent *in vivo* evidence that the effect of the Rpl5-HA2p is specific to the ribosomal P-site.

The response of cells to sparsomycin was used to address the question of whether the *Mof*[−] phenotypes of the remaining *rpl5-HA* alleles could also be explained by P-site-specific defects. Figure 5E shows that there was a direct correlation between the extent of the frameshifting defects and resistance to sparsomycin. There were no differences between wild-type and mutant cells in the presence of anisomycin. These results confirm the P-site-specific nature of the lesions, supporting the hypothesis that P-site-specific tRNA binding defects can account for the observed increases in both −1 and +1 PRF in cells harboring all of the mutant *rpl5-HA* alleles.

DISCUSSION

We have employed a series of previously identified alleles of the yeast gene encoding ribosomal protein L5 to shed light on ribosome structure and function. The mutants of L5 are similar to the *mof9* mutants of 5S rRNA in that they can affect both −1 and +1 programmed ribosomal frameshifting efficiencies. The biochemical basis for the effects of the *rpl5* mutants on PRF are specifically due to decreased affinity of mutant ribosomes for peptidyl-tRNA. This is supported by the observations that the P-site-specific peptidyltransferase inhibitor, sparsomycin, has a therapeutic effect on this P-site deficiency. The strong correlations between the effects of the *rpl5* mutants on ribosomal frameshifting efficiencies, virus propagation, and resistance to sparsomycin suggest an underlying defect in the same mechanism in all of the alleles, though to varying degrees. These findings suggest that L5 normally functions to anchor the peptidyl-tRNA to the P-site, and that it plays a key role in helping ribosomes to maintain reading frame. Our findings also suggest that assays of Ty1 retrotransposition frequencies provide a more sensitive monitor of the effects of changes in PRF efficiencies on virus propagation than does the killer virus.

Monitoring of Ty1 retrotransposition frequencies provides a more sensitive means to assess the impact of changes in programmed ribosomal frameshifting efficiencies than does the killer assay

Our findings demonstrate that smaller changes in +1 PRF efficiencies have more severe impacts on the ability of Ty1 to retrotranspose than similar changes in −1

PRF efficiencies have on killer virus maintenance. For example the 1.6–1.7-fold increases of the *rpl5-HA3*, *-HA4*, and *-HA5* alleles (7.2%–7.9% +1 PRF) promoted >97% inhibition of Ty1 retrotransposition. We believe monitoring for changes in Ty1 retrotransposition frequency is more sensitive to changes in ribosomal frameshifting efficiencies than the killer assay for two reasons. The first is due to the fact that cells contain a large preexisting pool of L-A and M₁ killer virus, and thus, this pool must be diluted out over many generations before loss of the killer phenotype can be observed. In contrast, because there is no preexisting pool of neo^r-tagged Ty1, the effects of anything that acts to block its propagation will be immediately noticeable. The second reason that Ty1 is more sensitive to changes in PRF efficiencies is because the process of Ty1 viral particle assembly is more complex than that of L-A, requiring a highly coordinated sequence of protein-processing events. In this respect, viral particle morphogenesis of Ty1 is more akin to retroviruses, suggesting that very small changes in PRF efficiency may severely impact retrovirus propagation, giving further credence to programmed ribosomal frameshifting as a target for antiviral agents.

A biochemical basis for altered frameshifting efficiencies

We have been investigating the effects of mutations in *trans*-acting factors on efficiencies of programmed ribosomal frameshifting as tools to probe ribosome function and structure relationships (reviewed in Dinman et al., 1998). In so doing, we have developed a model of PRF that describes how alterations in the kinetics of each of the subreactions of the elongation program specifically affect either −1 or +1 PRF, but not both simultaneously. Thus, the abilities of the *mof9* (5S rRNA) and *rpl5* mutants to alter both −1 and +1 PRF make these mutants particularly interesting. It is notable that there is no correlation between magnitudes of the frameshifting and the 5S rRNA binding defects of the *rpl5-HA* mutants. For example, although the *rpl5-HA2* allele promoted the greatest increases in programmed frameshifting efficiencies and had the most significant impact on both killer and Ty1, its reported ability to bind 5S rRNA both *in vivo* and *in vitro* (60–76% of wild type) was significantly better than either the *rpl5-HA1* or *rpl5-HA3* alleles (40–65% and 0–17% of wild-type, respectively; see Table 1). Thus, quantitative changes in the interactions of L5 with 5S rRNA cannot account for the observed effects on PRF. On the contrary, our results suggest that L5 must be able to interact with 5S rRNA in order to manifest its influence on frameshifting.

Examination of the models describing programmed −1 and +1 ribosomal frameshifting reveals one commonality: they both require slippage of peptidyl-tRNAs. This led us to test the hypothesis that the P-sites of

FIGURE 6. Model describing how a decreased affinity of ribosomes for peptidyl-tRNA can promote increases in both -1 and $+1$ ribosomal frameshifting, and how the defects can be "cured" by sparsomycin. Left: Model describing L-A virus-directed programmed -1 ribosomal frameshifting. Ribosomes with both A-site bound aa-tRNA and P-site bound peptidyl-tRNA are forced to pause over the slippery site by the downstream RNA pseudoknot. Upon slippage of one base in the 5' direction, the non wobble bases of the tRNAs re-pair to the -1 frame codons. Top: Wild-type ribosomes slip at wild-type rates, indicated by light gray, thin arrow. Middle: decreased affinity of the *rpl5*-mutant ribosomes for the peptidyl-tRNA (dotted) allows for increased frequencies of ribosomal slippage (black, heavy arrow). Bottom: Sparsomycin increases the affinity of ribosomes for peptidyl-tRNA, almost completely counteracting the effects of the *rpl5* mutations on -1 PRF (dark gray, medium width arrow). Right: model describing programmed $+1$ ribosomal frameshifting in Ty1 (from Belcourt & Farabaugh, 1990). Ribosome with a peptidyl-tRNA in the P-site and an empty A-site and which is forced to pause over the slippery site by the presence of the "hungry" AGG codon, which corresponds to a single-copy Arg-tRNA^{Arg}. Upon slippage of one base in the 3' direction, the peptidyl-tRNA is able to perfectly base pair to the $+1$ frame codon. Top: Wild-type ribosomes slip at wild-type rates, indicated by light gray, thin arrow. Middle: Decreased affinity of the *rpl5*-mutant ribosomes for the peptidyl-tRNA (dotted) allows for increased frequencies of ribosomal slippage (black, heavy arrow). Bottom: By increasing the affinity of ribosomes for peptidyl-tRNA, sparsomycin almost completely counteracts the effects of the *rpl5* mutations on $+1$ PRF (dark gray, medium width arrow).

ribosomes containing mutant L5 proteins might have decreased affinities for this class of tRNAs, increasing their chances of slipping. This model is graphically presented in Figure 6. Our data support the model, demonstrating that ribosomes isolated from cells bearing the *rpl5*-HA2 allele have significantly lower affinities for the P-site specific tRNA donor fragment. The pharmacological analyses provide independent and physiologi-

cally relevant support for our model. Sparsomycin stimulates binding of peptidyl-tRNA stem to the donor site of the peptidyl-transferase center, stimulating the formation of inert complexes between the peptidyl end of peptidyl-tRNA and ribosomes, and perturbing the ribosome donor stem complex (Herner et al., 1969; Carrasco et al., 1973). In wild-type cells, we have suggested that by slowing the rate of the peptidyl transfer

reaction, sparsomycin may increase the amount of time that a ribosome is paused over the frameshift signal with both its A- and P-sites occupied by tRNA, increasing the likelihood of -1 ribosomal frameshifting (Dinman et al., 1997). Further, because the $+1$ frameshift occurs independently of the peptidyltransfer reaction, sparsomycin has no effect on this process in wild-type cells. In the *rpl5* mutants however, both -1 and $+1$ frameshifting efficiencies are elevated because the peptidyl-tRNA is less tightly bound to the ribosome, and is thus more inclined to slip. In this context, by increasing the affinity of the ribosome for this tRNA, sparsomycin is able to partially correct the defect, bringing the peptidyl-tRNA-ribosome kinetic interaction back to near wild-type status. Phenotypically, this is reflected by the ability of this drug to partially cure the -1 and $+1$ PRF defects in the *rpl5*-HA2 strain. Further, because the extent the frameshifting and virus propagation defects in all of the *rpl5* mutants closely followed their sparsomycin-resistance profiles, we rationalize that they all have P-site specific peptidyl-RNA binding defects, and that the differences in frameshifting and virus propagation defects are a consequence of allele-specific differences in the individual defects.

Ribosome structure and L5 function

In light of the recent breakthroughs in our understanding of prokaryotic ribosomal structure, the studies presented here help to further illuminate how structure relates to function. The recent X-ray crystallographic analysis at 5.5 Å of the *T. thermophilus* 70S ribosome containing bound mRNA and tRNAs revealed that the L5 molecule from this organism (tL5) directly contacts the T-loop of the peptidyl-tRNA (Yusupov et al., 2001). However, a soon-to-be-published cryo-EM reconstruction of the yeast 80S ribosome at ≈ 12 Å shows that yeast L5 (yL5) is structurally analogous to tL5, and that the structural analog of tL5 is yL11 (R. Beckmann, pers. comm.). The yL5/tL5 molecules are at the top of the central protuberance on the solvent side of the large subunit. These proteins do not appear to have any direct contacts with the peptidyl-tRNA, but rather are only in indirect contact with the yL11/tL5-peptidyl-tRNA interaction through 5S rRNA. Thus, it appears that the yL5 plays an indirect, rather than a direct, role in helping to anchor the peptidyl-tRNA to the ribosome. Further, the structural data explain the requirement for 5S rRNA binding of the *rpl5*-HA mutants to exert their effects on programmed ribosomal frameshifting: 5S rRNA must act as a bridge to connect yL5 to yL11 and the peptidyl-tRNA. These studies also suggest that mutants of yL11 and also mutants affecting this region of the 5S rRNA, for example, in the Loop B–Helix III–Loop C region of the molecule, might also affect peptidyl-tRNA/ribosome interactions, programmed ribosomal frameshifting, and virus propagation.

MATERIALS AND METHODS

Strains, sequencing, genetic methods, and media

E. coli strains DH5 α and MV1190 were used to amplify plasmids, and *E. coli* transformations were performed using the standard calcium chloride method as described previously (Sambrook et al., 1989). Dr. John Woolford kindly provided us with a set of *RPL5* gene disruption strains (*rpl5* Δ = *MAT* α , *ura3-52 trp1* Δ 101 *leu2 his3* Δ 200 *ade1 rpl5* Δ 1) in which all L5 is produced from haemagglutinin-tagged (HA-tagged) *RPL5* clones on a *LEU2 CEN* vector. The strains in this set harbor either the wild-type *RPL5*-HA gene or a series of *rpl5*-HA mutants that have been defined as having assembly defects with 5S rRNA (Deshmukh et al., 1993). Yeast were transformed using the alkali cation method (Ito et al., 1983). YPAD, YPG, SD, synthetic complete medium (H-) and 4.7-MB plates for testing the killer phenotype were used as described previously (Wickner & Leibowitz, 1976). Cytoduction of L-A and M₁ from strain JD758 into rho-o (*RPL5*-HA and *rpl5*-HA mutant) strains were as previously described (Dinman & Wickner, 1994). Plasmids were rescued from yeast by glass bead lysis, and transformation into *E. coli*. DNA sequence analysis was performed by the Robert Wood Johnson Medical School DNA Synthesis and Sequencing Laboratory. Assays to monitor the ability of cells to grow in the presence of anisomycin or sparsomycin were as previously described (Dinman & Kinzy, 1997).

Programmed ribosomal frameshift assays

Plasmids p-1 and p0, which were used to monitor PRF, have been described previously (Dinman et al., 1991; Dinman & Wickner, 1995; Cui et al., 1996, 1998a; Tumer et al., 1998). To make a new pTy1+1 series of vectors, the synthetic oligonucleotides 5'-CCCCGGATCCACAATGTATCCACATCTAATAACT-3' and 5'-CCCCGGTACCAGATCTTATAAGGGTTCTGTATG-3' were used to amplify the native Ty1 frameshift signal beginning 102 nt upstream, and ending 111 bases downstream of the Ty1 CTTAGGC slippery site. This sequence was amplified by PCR from pEJF1105 (Boeke et al., 1988). Briefly, in all of these plasmids, transcription is driven from the yeast *PGK1* promoter into an AUG translational start site. The *E. coli lacZ* gene serves as the enzymatic 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 serve as 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 -1 PRF signal such that β -galactosidase can only be produced as a consequence of a -1 PRF event. Similarly, in the pTy1+1 series, *lacZ* is in the $+1$ frame with respect to the translational start site, and is 3' of the Ty1 +1 PRF signal. The efficiency of programmed ribosomal frameshifting is calculated by determining the ratio of β -galactosidase activity produced by cells harboring either p-1 or pTy1+1 divided by the β -galactosidase activity produced by cells harboring p0, and multiplying by 100%. All assays were performed in triplicate on three different occasions. Anisomycin and sparsomycin were obtained from Sigma

Chemical Company (St. Louis, Missouri), and measurement of programmed ribosomal frameshifting in the presence of these drugs was performed as described previously (Dinman et al., 1997).

Virus propagation assays

The killer assay was done by replica plating *S. cerevisiae* colonies onto a 4.7-MB plates (Wickner & Leibowitz, 1976) with a freshly 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 2–3 days at 20 °C, killer activity was observed as a zone of growth inhibition around the killer colonies. dsRNA of L-A and M₁ viruses were prepared as described (Fried & Fink, 1978) and separated by electrophoresis through 1.0% agarose gels and visualized by ethidium bromide staining. The abundance of L-A and M₁ dsRNAs were monitored by RNA blotting as previously described (Dinman & Wickner, 1994).

To measure Ty1 retrotransposition frequencies, cells harboring the different *rp15-HA* alleles were transformed with pJEF1105 (Boeke et al., 1988) and selected on H-ura, leu with 2% dextrose. Transformants were then grown on H-ura, leu with 2% galactose at 20 °C for 4 days. Patches of cells were subsequently replica plated back onto nonselective media containing 2% dextrose, and then to nonselective media containing 100 µg/mL of 5-fluoroorotic acid (5-FOA) and incubated at 25 °C to select for cells that had lost pJEF1105. Colonies of cells that were able to grow on 5-FOA were then assayed for Ty1 using both qualitative and quantitative assays. Visual inspection of cell growth after spotting of 10-fold dilutions of cells onto medium containing 100 µg/mL of Geneticin (Sigma) provided a qualitative assessment of Ty1 retrotransposition competence. To quantitatively measure Ty1 retrotransposition frequencies, cells taken from 5-FOA plates were grown in YPAD liquid media overnight, OD₅₅₀ were determined, and 10-fold dilutions of cells ranging from 10¹ to 10⁵ colony forming units (CFU) were seeded onto YPAD medium containing 100 µg/mL of Geneticin. Retrotransposition frequencies were calculated by dividing the number of Geneticin-resistant (neo^r) colonies by the total number of CFU seeded onto the plate (as empirically determined by counting colonies on YPAD plates without Geneticin).

Nuclease protection assays

Total cellular RNA was isolated from cells harboring the various *rp15-HA* alleles and frameshift test vectors using methods previously described (Cui et al., 1996). RNase protection assays measuring LacZ mRNA abundances relative to a U3 snRNA control were performed as previously described (Cui et al., 1998b; Tumer et al., 1998). The image was visualized and quantitated by phosphorimager using a Typhoon 8600 phosphorimager and ImageQuant V.5.2. software (Molecular Dynamics).

Preparation of donor and acceptor fragments

Yeast tRNAs were charged with [¹⁴C]phenylalanine as previously described (Harris & Pestka, 1973). Briefly, a 400-mL re-

action mix composed of 200 µg of yeast tRNA (Sigma), 25 mM Tris-HCl, pH 7.5, 20 mM MgCl₂, 10 mM ATP, 50 nmol phenylalanine (313 mCi/mmol, NEN), 50 mL aminoacyl-tRNA synthetase (Sigma, 9 U/mL) was incubated for 25 min at 37 °C. After addition of 40 mL of 3 M sodium acetate, pH 5.0, the mixture was extracted twice with an equal volume of water-saturated phenol and once with chloroform. It was then precipitated with 2.5 vol of ethanol at –20 °C for 1 h. After centrifugation for 10 min, the pellet was resuspended in 50 mL of 2 mM potassium acetate, pH 5.0. The charged tRNAs were acetylated as previously described (Haenni & Chapeville, 1966). Briefly, [¹⁴C]Phe-tRNA^{Phe} was resuspended in 200 mL of 0.2 M sodium acetate, pH 5.0, followed by addition of 2.5 mL of acetic anhydride. After 1 h incubation on ice, another 2.5 mL of acetic anhydride was added and incubation at 0 °C continued for an additional hour. The tRNA was precipitated by addition of 2.5 vol of ethanol. The [¹⁴C]Phe-tRNA^{Phe} and acetyl-[¹⁴C]Phe-tRNA^{Phe} were subsequently digested with 500 U of RNase T1 in 200 mL of 0.3 M sodium acetate, pH 5.0, for 1 h at 37 °C, and the reaction mixtures were purified using DEAE Sephadex as previously described (Pestka et al., 1970). The resulting [¹⁴C]Phe-CACCA (acceptor) and acetyl-[¹⁴C]Phe-CACCA (donor) fragments were used as substrates for the tRNA fragment binding assays.

Purification of ribosomes and tRNA fragment binding assays

Salt-washed ribosomes were purified as previously described (Merrick, 1979). Briefly, yeast cells were grown in 0.5 L of YPAD overnight, collected by centrifugation, and washed twice with buffer A (20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA, 0.25 M sucrose). The cell pellet was suspended in 20 mL of buffer A, 30 g of glass beads (0.45 mm diameter) were added, and cells were disrupted by vortexing. The yeast lysate was centrifuged twice for 15 min at 15,000 rpm in a Sorvall S34 rotor, and the supernate was pelleted at 100,000 × *g* for 3 h. The pellet was suspended in 6 mL of buffer B (20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA, 0.25 M sucrose, 0.5 M KCl), and placed on a cushion of 3 mL of buffer C (20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA, 1 M sucrose, 0.5 M KCl). After centrifugation at 50,000 rpm for 4 h (SW55 Ti), the pellet was dissolved in 1 mL of buffer A. After a clarifying spin for 1 min in a microfuge, OD₂₆₀ readings were taken (1 A₂₆₀ unit = 19 pmol ribosomes; see Abraham & Pihl, 1983). Protein content of ribosomes was also estimated using Protein Assay Reagent (Biorad). Ribosomes were suspended in buffer A at concentration 4 pmol/mL, and stored at –70 °C.

The fragment binding assays followed a modification of the previously published protocol (Harris & Pestka, 1973). Briefly, ribosomes (400 pmol) were incubated with 800 pmol of either donor or acceptor fragments in 500 mL of a buffer containing 70 mM Tris-acetate, pH 7.2, 40 mM magnesium acetate, 0.4 M potassium acetate, and 50 mM NH₄Cl. Ethanol was added to a final concentration of 30%, and 20-µL aliquots were taken during the time course at 24 °C. Samples were diluted to 1 mL with cold buffer (50 mM Tris-HCl, pH 7.2, 0.4 M KCl, 40 mM MgCl₂, 30% ethanol), immediately precipitated onto a Millipore filter, washed with 1 mL of the dilution buffer, and counted in a scintillation counter. The reaction mix without

ribosomes was used as control. All assays were performed in triplicate.

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