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New Targets for Antivirals: The Ribosomal A-Site and the Factors That Interact with It

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Many viruses use programmed −1 ribosomal frameshifting to ensure the correct ratio of viral structural to enzymatic proteins. Alteration of frameshift efficiencies changes these ratios, in turn inhibiting viral particle assembly and virus propagation. Previous studies determined that anisomycin, a peptidyl transferase inhibitor, specifically inhibited −1 frameshifting and the ability of yeast cells to propagate the L-A and M_1 dsRNA viruses (J. D. Dinman, M. J. Ruiz-Echevarria, K. Czaplinski, and S. W. Peltz, 1997, *Proc. Natl. Acad. Sci. USA* 94, 6606–6611). Here we show that preussin, a pyrollidine that is structurally similar to anisomycin (R. E. Schwartz, J. Liesch, O. Hensens, L. Zitano, S. Honeycutt, G. Garrity, R. A. Fromtling, J. Onishi, and R. Monaghan, 1988. *J. Antibiot. (Tokyo)* 41, 1774-−1779), also inhibits −1 programmed ribosomal frameshifting and virus propagation by acting at the same site or through the same mechanism as anisomycin. Since anisomycin is known to assert its effect at the ribosomal A-site, we undertook a pharmacogenetic analysis of mutants of *trans*-acting eukaryotic elongation factors (eEFs) that function at this region of the ribosome. Among mutants of eEF1A, a correlation is observed between resistance/susceptibility profiles to preussin and anisomycin, and these in turn correlate with programmed −1 ribosomal frameshifting efficiencies and killer virus phenotypes. Among mutants of eEF2, the extent of resistance to preussin correlates with resistance to sordarin, an eEF2 inhibitor. These results suggest that structural features associated with the ribosomal A-site and with the *trans*-acting factors that interact with it may present a new set of molecular targets for the rational design of antiviral compounds.

Key Words: virus; HIV; frameshifting; ribosome; translation; drugs; elongation factors.

INTRODUCTION

Classically, anti-retroviral strategies have targeted virus-encoded proteins, e.g., reverse transcriptase and protease, on the theory that compromising these virus-specific functions will have minimal impact on the health of the host. Unfortunately, since viral populations evolve on time scales of weeks to months, drug resistance emerges rapidly. Thus, though the treatment of HIV-AIDS has been revolutionized through the development of highly active anti-retroviral therapy (HAART), multiple drug resistance has emerged threatening to undo the substantial advances that have been made (UNAIDS/WHO Global AIDS Statistics, 2001). An alternative strategy would involve targeting a host-encoded function that, if slightly altered, would have minimal effect on the host but be deleterious to the virus. As we have previously discussed, programmed −1 ribosomal frameshifting presents a promising candidate (Dinman *et al.,* 1998).

Many classes of RNA viruses utilize programmed ribosomal frameshifting (PRF) as a means to express multiple protein products while condensing genome size (reviewed in Brierley, 1995; Farabaugh, 1996). The efficiency of PRF helps to determine the ratio of viral structural to enzymatic proteins available for the viral particle morphogenic program, and even slight alterations in frameshift frequencies can severely inhibit virus propagation (reviewed in Dinman *et al.,* 1998). The identification of compounds that affect this process can be aided by integrating the models describing PRF into the context of the host-cellular process in which they occur, i.e., the translation elongation cycle. A summary of recent research shows that programmed −1 ribosomal frameshifting must occur during the translation elongation cycle after delivery of cognate aminoacyl-tRNA (aa-tRNA) to the ribosome and prior to the peptidyl transfer step (Dinman and Kinzy, 1997; Dinman *et al.,* 1997; Tumer *et al.,* 1998; Meskauskas and Dinman, 2001; Harger *et al.,* 2001). This "Integrated Model" of programmed ribosomal frameshifting previously led us to demonstrate that two peptidyl transferase inhibitors, anisomycin and sparsomycin, have antiviral properties (Dinman *et al.,* 1997). These drugs inhibit peptidyl transfer by distinct mechanisms (reviewed in Dinman *et al.,* 1997). By decreasing the affinity of ribosomes for the 3′ end of the aa-tRNA,

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anisomycin inhibits peptidyl transfer by blocking the "accommodation" step of translation, i.e., the active insertion of the 3′ end of the aa-tRNA into the A-site of the ribosomal peptidyl transferase center by eukaryotic elongation factor 1A (eEF1A). In contrast, sparsomycin inhibits peptidyl transfer both by increasing the affinity of the ribosomal P-site for the 3′ end of the peptidyl-tRNA and by altering its alignment relative to the peptidyl transferase active center. Here, we report that preussin, a natural product isolated from *Aspergillus ochraceus* that shares structural features with anisomycin (Schwartz *et al.,* 1988), has similar antiviral properties.

Building on the knowledge that anisomycin and preussin inhibit a ribosomal A-site-associated function, we also conducted a screen of known mutants of the *trans*-acting factors that interact with the A-site in an effort to identify potential new targets for antiviral compounds. eEF1A delivers aminoacyl-tRNAs (aa-tRNA) to the ribosomal A-site, and eukaryotic elongation factor 2 (eEF2) translocates the newly formed peptidyl-tRNA to the P-site (reviewed in Merrick and Nyborg, 2000). The prokaryotic homologs of these factors, EF-Tu, bound to aa-tRNA and EF-G, respectively, share a striking amount of overall structural similarity (Nissen *et al.,* 2000; Czworkowski *et al.,* 1994; al Karadaghi *et al.,* 1996), and the high degree of structural conservation between EF-Tu and eEF1A suggests a similar relationship between eukaryotic elongation factors (Andersen *et al.,* 2000). Molecular mimicry models of proteins resembling tRNA structures (reviewed in Nissen *et al.,* 2000) shared structural characteristics of A-site factors (see above), and overlapping binding sites on the ribosome (reviewed in Noller, 1997) indicate a rich array of targets for addressing A-site functions in viral maintenance. The emerging picture is that alteration of the accommodation step of translation is a mechanism that can severely impact on −1 PRF efficiency and virus propagation. Our finding that clusters of mutants in both eEF1A and eEF2 are resistant to both preussin and anisomycin suggest a new set of targets for the rational design of new antiviral agents.

RESULTS AND DISCUSSION

Preussin specifically inhibits programmed −1 ribosomal frameshifting and cures yeast cells of the killer virus by the same mechanism as anisomycin

In a previous study we showed that agents with the property of being able to inhibit the peptidyl transferase reaction specifically altered programmed −1 ribosomal frameshifting and had antiviral activities (Dinman *et al.,* 1997). These studies also demonstrated that alteration of PRF was specific to these two agents and not a general property of translational inhibitors per se. One of the peptidyl transferase inhibitors, anisomycin, specifically affects the accommodation step of the translation cycle by inhibiting binding of the aa-tRNA 3′ stem to the acceptor site of the peptidyl transferase center (Grollman, 1967; Carrasco *et al.,* 1973). This in turn results in inhibition of the peptidyl transferase reaction. In searching for other compounds with comparable properties, we noted that the chemical structure of preussin is very similar to anisomycin (Fig. 1) (Schwartz *et al.,* 1988). The yeast strain JD88 was chosen for use in studies to examine the potential effects of this compound on −1 PRF and virus propagation because it is the same strain that was used in the anisomycin studies, thus allowing for direct comparison of these two drugs on PRF and virus propagation. JD88 harbors the yeast "Killer" virus, which provides a convenient marker for the effects of mutants and drugs on −1 PRF and virus propagation (reviewed in Dinman *et al.,* 1988). The killer virus is composed of the 4.6-kb dsRNA L-A virus (family *Totiviridae*), and a collection of 1.6- to 1.8-kb dsRNA satellite viruses termed M (M_1, M_2, M_3, $M_{28,}$ ···) (reviewed in Wickner, 1996). The L-A (+)-strand contains two open reading frames: *gag*, which encodes its major coat protein; and *pol*, encoding a multifunctional protein domain including the RNA-dependent RNA polymerase and domains required for packaging of viral RNA. *pol* is only translated as a Gag-pol fusion protein formed by a −1 ribosomal frameshift event. M_1, the most well-characterized satellite virus of L-A, is encapsulated and replicated inside of L-A encoded viral particles. Its translational product is a secreted toxin that promotes killing of non-virus-infected cells, while a killer toxin processing intermediate confers immunity on infected cells. Cells harboring L-A and M_1 have the Killer$^+$ phenotype, which is easily visualized by the halo of growth inhibition surrounding colonies of infected cells. Since the underlying "simultaneous slippage" mechanism of −1 PRF (Jacks *et al.,* 1988) is the same from L-A to HIV-1, the Killer virus provides us with a simple, inexpensive model with which to assay the genetic, biochemical, and pharmacological parameters governing −1 PRF (reviewed in Dinman *et al.,* 1998).

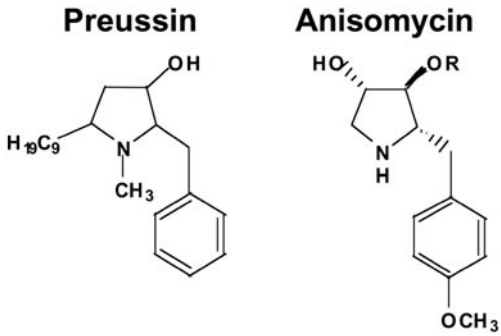

FIG. 1. Chemical structures of preussin and anisomycin.

Previous studies demonstrated that the most meaningful ranges of drug concentrations to use in this type of analysis were those at which cell growth was least affected, and at which overall translation efficiencies

were inhibited by <50% (Dinman *et al.,* 1997; Harger *et al.,* 2001). To determine a suitable range of concentrations to analyze the effects of preussin on PRF, cell growth was monitored by measuring the doubling times of JD88 cells grown in selective medium at various drug concentrations. We determined that cell growth was significantly inhibited at concentrations > 8 μg/ml (data not shown). To examine the relationship between preussin concentrations and overall translation, β-galactosidase (β-gal) activities were monitored in JD88 cells harboring the p0 plasmid grown in the presence of different concentrations of preussin for 5 h. The results demonstrated that at preussin concentrations between 5 and 6 μg/ml, overall translation in JD88 cells was inhibited by >50% (see Fig. 2A). These results led us to conclude that 0–4 μg/ml constituted an appropriate range of concentrations over which to monitor the effects of preussin on programmed ribosomal frameshifting. JD88 cells harboring the p0, p − 1, or p + 1 frameshift reporter plasmids were grown in the presence of preussin at concentrations of 1, 2, or 4 μg/ml for 5 h, after which PRF efficiencies were determined as described under Materials and Methods. These were compared to no-drug controls. The results show that preussin specifically inhibited L-A virus-directed −1 PRF, whereas it had no effect on Ty*1*-mediated +1 PRF (Figs. 2A, 2B, and Table 1). The effects were specific to −1 PRF as evidenced by the enhanced rate of decrease in β-gal activities in cells harboring p − 1 as compared to either p0 or p + 1 (Fig. 2A, Table 1).

Given the structural similarity between preussin and anisomycin, we investigated whether the two compounds were acting to inhibit programmed −1 ribosomal frameshifting through the same or different mechanisms. Accordingly, cells were grown in the presence of one or both agents for 5 h, after which −1 PRF efficiencies were determined. The results show that in the presence of both compounds, inhibition of −1 PRF was intermediate between anisomycin and preussin rather than additive (Fig. 2C). The lack of an additive effect suggests that the two drugs are acting through a similar mechanism or site of action. The ability of preussin to partially block the action of anisomycin supports this and suggests that it has a higher affinity of their mutual target.

It is possible that increasing concentrations of anisomycin or preussin preferentially destabilized the p − 1 reporter mRNA, resulting in an apparent but not real decrease in −1 PRF efficiency. To address this, RNase protection assays were used to monitor the steady-state abundances of the p0 and p − 1 transcripts from cells incubated with 0, 1, or 4 μg/ml of preussin, and 0, 1, or 10 μg/ml of anisomycin as described under Materials and Methods. The results of this analysis are shown in Fig. 2D and quantitated in Table 2. Examination of Table 2 shows that the p − 1 reporter mRNA was slightly stabilized with respect to the 0-frame control reporter mRNA in the presence of either of these compounds, suggesting that the actual decreases in −1 PRF were even greater than those calculated from the observed β-gal activities. These data demonstrate that the observed dose-dependent decreases in −1 PRF in response to these agents were not artifacts of changes in mRNA stabilities.

Viral particle assembly and virus propagation are especially sensitive to decreases in PRF efficiencies (reviewed in Dinman *et al.,* 1998; also see Harger *et al.,* 2001). To examine the *in vivo* effects of preussin on virus propagation, JD88 harboring L-A and M_1 were cultured in the presence of different concentrations of the compound and the effects on the ability of cells to maintain both viruses over time were assayed as described under Materials and Methods. Similar to our observations with anisomycin, preussin was also able to cure cells of the killer phenotype (Fig. 3A) in a manner that correlated with increased time of incubation with the compound (Fig. 3B). The observation that the rate of loss of the killer phenotype is independent of the extent of reduction in frameshifting efficiency suggests that elimination of the preexisting intracellular pool of killer viruses (estimated to be between 10^3 and 10^4 viral particles/cell, see Wickner, 1996) is the rate-limiting parameter, in this case as opposed to its being due to differences in the rates of decrease of viral particle replacement consequent to changes in frameshifting efficiencies. To determine the reason for loss of the killer phenotype, total cellular RNA was extracted, separated by electrophoresis, transferred to a membrane, and hybridized with both L-A and M_1 (−)-strand-specific probes, as described under Materials and Methods. The results demonstrate that, although preussin did not inhibit propagation of L-BC (Fig. 3C), a less well-characterized endogenous yeast dsRNA virus (reviewed in Wickner, 1996), neither the L-A nor the M_1 dsRNA genomes were present in cells grown in the presence of preussin (Fig. 3D). Thus, loss of the killer phenotype was a consequence of loss of both the L-A and the M_1 viruses.

To summarize, examination of the literature for small chemicals structurally similar to anisomycin led us to preussin. Though it is also able to specifically inhibit −1 PRF, by itself preussin is probably not an ideal antiviral compound. However, the work presented here is meaningful with regard to drug discovery because it establishes that at least two pyrollidines have the ability to inhibit programmed −1 ribosomal frameshifting and virus propagation. As such, it provides an opening to those who are actively involved in drug discovery to look toward new derivatives of pyrollidines as potential weapons in the antiviral arsenal.

Ribosomal A-site-specific *trans*-acting factors: A genetic analysis

By virtue of its ability to specifically inhibit accommodation of the acceptor stem of aa-tRNAs into the ribosomal A-site, anisomycin, and by inference preussin, can

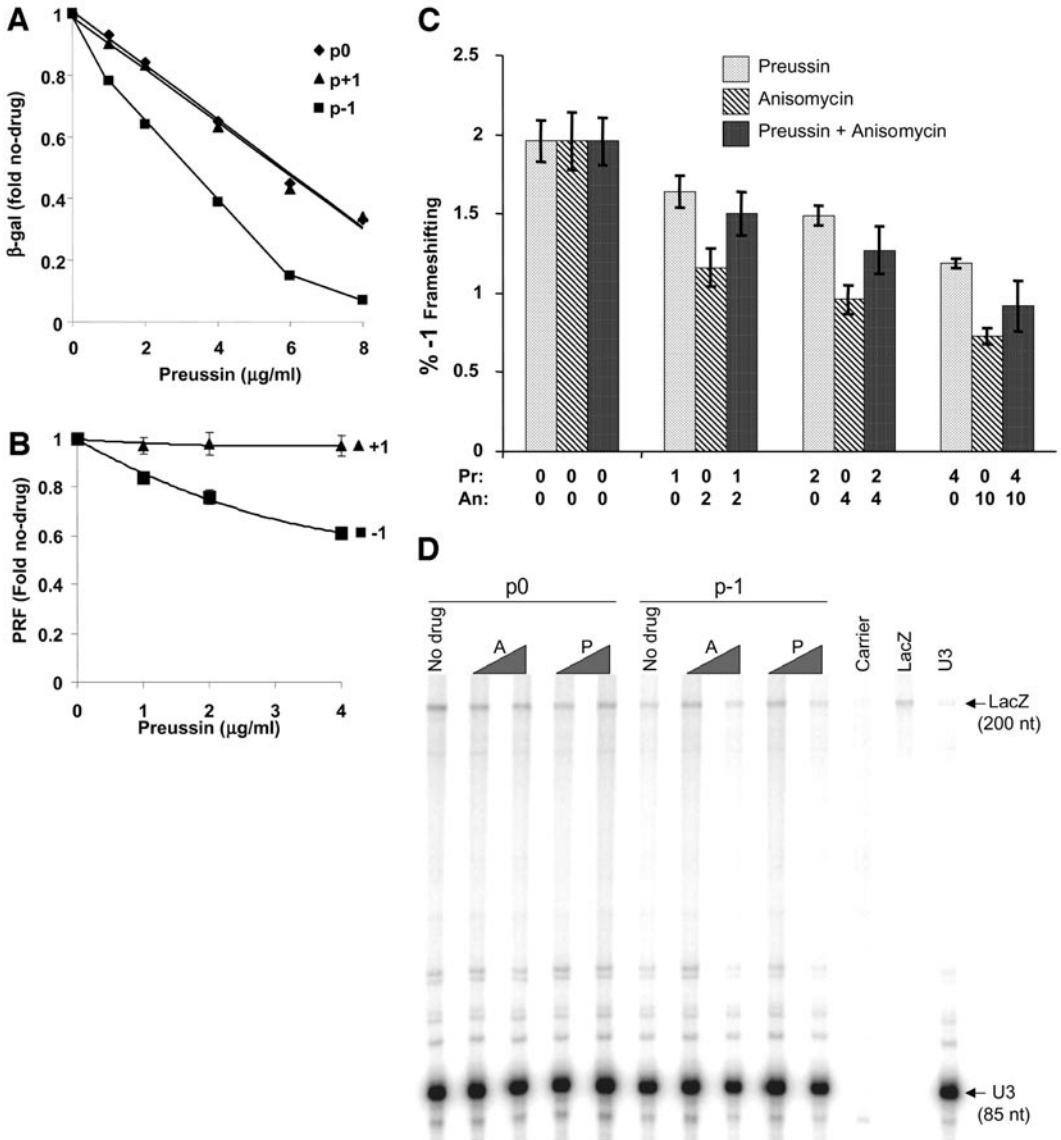

FIG. 2. Preussin specifically inhibits programmed −1 ribosomal frameshifting and cures yeast cells of the killer virus by the same mechanism as anisomycin. (A) Effects of preussin on translation. Preussin was added to midlogarithmically growing JD88 cells harboring p0, p − 1, or p + 1 at the indicated concentrations for 5 h after which β-gal activities were determined as described under Materials and Methods. Those from cells exposed to preussin were normalized to those obtained from no-drug controls. All assays were performed in triplicate. (B) Preussin specifically inhibits programmed −1 ribosomal frameshifting. Preussin was added to midlogarithmically growing yeast cells at the indicated concentrations for 5 h after which programmed −1 and +1 ribosomal frameshifting efficiencies were determined as described under Materials and Methods. All assays were performed in triplicate. Error bars indicate % error. (C) Preussin and anisomycin act through the same mechanism or at the same site. Programmed −1 ribosomal frameshifting efficiencies were determined in midlogarithmically growing yeast cells after 5 h of growth in the presence of preussin and/or anisomycin, as described above. Numbers indicate concentrations of the two compounds in μg/ml. (D) Differences in PRF efficiencies are not due to alterations in the steady-state abundances of the *Lac*Z −1 reporter mRNA. Cells harboring either p0 or p − 1 were grown for 5 h in the absence of either compound, 1 or 4 μg/ml of preussin, or 2 or 10 μg/ml of anisomycin, as indicated by ramps. Total cellular mRNA (20 μg) was extracted and subjected to RNase protection analysis using radiolabeled antisense probes corresponding to the 3′ ends of the *Lac*Z mRNA and U3 snRNA, as previously described (Harger *et al.,* 2001). The protected fragments are 200 and 85 nt, respectively, as indicated. Control lanes contained only tRNA and either *Lac*Z or U3 probes. Products were separated through a 6% PAGE urea denaturing gel and visualized using a Molecular Dynamics Typhoon phosphorimager.

be used as probes for ribosomal A-site specific functions. Capitalizing on our large collections of mutants of two A-site-specific *trans*-acting factors, eEF1A and eEF2, we undertook a series of genetic analyses designed to illuminate how the shared structural elements and ribosome binding properties of these two central translation elongation factors influence their functional interactions with the ribosomal A-site.

As a first step in this process, a series of strains bearing mutations in subunits of eEF1 were assayed for

TABLE 1

In vivo Effects of Preussin on Protein Synthesis

Preussin	β-Gal activity pO[a]	p − 1[a]	p + 1[a]	% −1 PRF[b]	% +1 PRF[c]
No-drug	87.50 ± 7.94 (100)	1.71 ± 0.14 (100)	5.16 ± 0.23 (100)	1.95 ± 0.12 (100)	5.9 ± 0.27 (100)
1 μg/ml	81.38 ± 4.88 (0.93)	1.33 ± 0.08 (0.78)	4.62 ± 0.20 (0.90)	1.64 ± 0.10 (0.84)	5.7 ± 0.25 (0.97)
2 μg/ml	73.50 ± 5.14 (0.84)	1.09 ± 0.06 (0.64)	4.26 ± 0.27 (0.83)	1.49 ± 0.09 (0.76)	5.8 ± 0.23 (0.98)
4 μg/ml	56.87 ± 3.98 (0.65)	0.67 ± 0.05 (0.39)	3.24 ± 0.24 (0.63)	1.19 ± 0.06 (0.61)	5.7 ± 0.24 (0.97)

[a] β-gal activities as a measure of the effects of preussin on translational competence. The β-galactosidase values given here are as previously defined (Dinman *et al.,* 1991). The results normalized to "fold no-drug" controls are shown in parentheses.

[b] Percent programmed −1 ribosomal fameshifting was calculated by multiplying the ratio of p − 1/p0 β-gal activities by 100%. The results normalized to "fold no-drug" controls are shown in parentheses.

[c] Precent programmed +1 ribosomal frameshifting was calculated by multiplying the ratio of p + 1/p0 β-gal activities by 100%. The results normalized to "fold no-drug" controls are shown in parentheses.

altered resistance or sensitivity to preussin. Preliminary studies to determine suitable drug concentrations showed that the MC213/MC214 strain background was slightly more sensitive to preussin than JD88. However, the isogenic nature of the yeast strains derived from the MC213/214 background allow for meaningful comparisons among those expressing wild-type and mutant forms of eEF1 subunits. The effects of mutations (1) in the GTP-binding domain of eEF1A (N153T, D156N, N153T+D156E, and E122K) (Carr-Schmid *et al.,* 1999; Dinman and Kinzy, 1997); (2) on both faces of the guanine nucleotide exchange factor eukaryotic elongation factor 1Bα (eEF1Bα, formerly eEF−1β) (F163A and K120RΔ-S121L122) (Carr-Schmid *et al.,* 1999; Andersen *et al.,* 2000); and (3) in cells lacking eEF1Bα and viable by overexpression of eEF1A (Kinzy and Woolford, 1995) were assayed for resistance to anisomycin and to preussin. The results show that mutations in the "NKXD" GTP-binding consensus element (i.e., N153T, D156N, N153T+D156E) resulted in increased resistance to preussin, as evidenced by their increased viability in the presence of 5 μg/ml preussin, as compared to cells expressing the wild-type form of eEF1A (Fig. 4A, compare top row with the next three rows down). Conversely, cells expressing the E122K mutation in eEF1A were slightly more sensitive to preussin, showing decreased viability in the presence of 5 μg/ml preussin, as compared to cells expressing the wild-type form of the protein (Fig. 4A, compare top and bottom rows). Interestingly, this mutant was also hypersensitive to anisomycin (Dinman and Kinzy, 1997), whereas this compound had no discernible effect on cells harboring the NKXD alleles (data not shown). We also observed that none of the eEF1Bα mutants showed any effect, i.e., those affecting its interaction with the G-domain of eEF1A (K120RΔ-S121L122), with domain II (F163A), and those entirely lacking the eEF1Bα protein (data not shown). This is consistent with our previous observations that alleles eEF1Bα had no effects on programmed ribosomal frameshifting (Dinman and Kinzy, 1997).

TABLE 2

Quantitative Analysis of LacZ- and U3-Specific Bands from Figure 2C

	p0 LacZ/U3 ratio	p0 Fold 0 frame	p − 1 LacZ/U3 ratio	p − 1 Fold 0 frame
No drug	0.11	1	0.06	0.55
1 μg/ml anisomysin	0.08	1	0.08	1.0
10 μg/ml anisomysin	0.08	1	0.05	0.63
1 μg/ml preussin	0.07	1	0.07	1.0
4 μg/ml preussin	0.07	1	0.05	0.71

We previously demonstrated that expression of the E122K mutant form of eEF1A promoted elevated levels of −1 PRF and killer virus loss, but had no effect on +1 PRF (Dinman and Kinzy, 1997). Examination of PRF efficiencies in cells expressing the N153T, D156N, and N153+D156N mutants showed slight but repeatable reductions in −1 PRF efficiencies, promoting 84% (N153T), 79% (D156N), and 66% (N153T+D156E) of wild-type levels, while no changes in +1 PRF were observed (data not shown). Consistent with these observations, killer assays showed that the halos of growth inhibition were reduced in proportion to the extents of the −1 PRF defects in isogenic strains expressing these mutant forms of eEF1A (Fig. 4B). No effects on Ty*1* retrotransposition frequencies were observed in any of the eEF1A mutants (data not shown). This is consistent with the

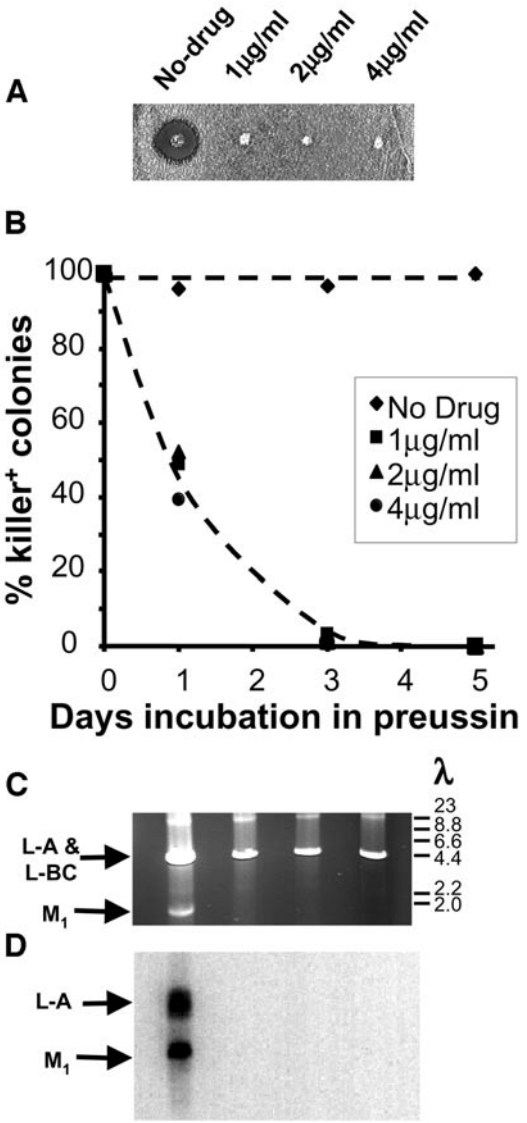

FIG. 3. Preussin promotes virus loss. JD88 cells were grown in rich medium in the presence of the indicated concentrations of preussin for 1–5 days; aliquots of cells were removed at 1, 3, and 5 days, streaked for single colonies onto rich medium lacking compounds, and replicated to killer indicator plates. (A) Killer indicator plate. The presence of the killer virus is indicated by the halo of growth inhibition around Killer$^+$ colonies. Loss of killer corresponds to the absence of the halo of growth inhibition. (B) Rates of killer loss were calculated as described under Materials and Methods. Preussin concentrations (μg/ml) are indicated. (C) Total nucleic acids were extracted from control (Killer$^+$) and non-Killer colonies from the 5-day time point, separated by agarose gel electrophoresis. The viral L-A, L-BC, and M_1 dsRNAs are indicated. (D) RNAs shown in C were transferred to nitrocellulose and hybridized with L-A and M_1 (+)-strand probes and visualized by autoradiography as described under Materials and Methods.

integrated model of programmed ribosomal frameshifting that predicts that alleles of *TEF2* should affect either −1 PRF and killer virus maintenance or +1 PRF and Ty*1* retrotransposition, but not both simultaneously (see Dinman and Kinzy, 1997).

As demonstrated above, preussin resistance correlated with decreased −1 PRF in cells expressing mutant forms of eEF1A and vice versa. What does this tell us about mechanism? Similar to N153 and D156, E122 is located in the GTP-binding domain of eEF1A. However, E122 lies in the fourth, nonconserved loop of this domain, physically distinct from the other two amino acid residues alleles. The E122K allele is unique among the original alleles isolated by Sandbaken and Culbertson (1988) in its dramatic effect on −1 PRF and killer virus maintenance (Dinman and Kinzy, 1997). One possible model is that decreased GTP hydrolysis in this mutant

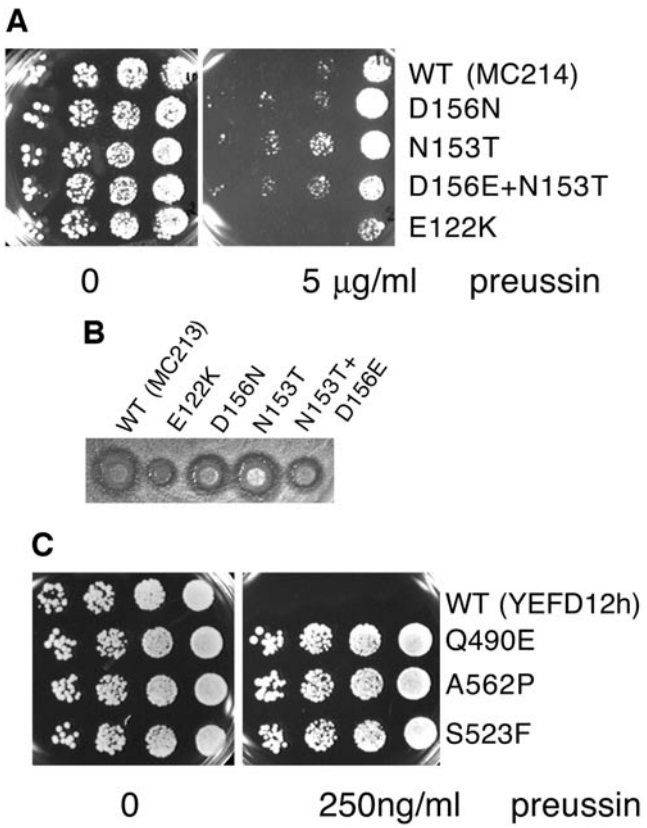

FIG. 4. Phenotypic analyses of yeast strains expressing mutant forms of eEF1A and eEF2. (A) Strains expressing eEF1A with mutations in the NKXD GTP-binding consensus element (D156N (TKY225), N153T (TKY226), and N153T+D156E (TKY229), another G-domain mutation of E122K (TKY252), and wild-type eEF1A (MC214)) were assayed on plates containing 5 μg/ml preussin as described under Materials and Methods. Increased or decreased colony numbers at successive fivefold dilutions are indicative of increased resistance or greater sensitivity to preussin, respectively. (B) Killer virus phenotypes of strains expressing wild-type eEF1A (MC213) or the mutants D156N (TKY278), N153T (TKY280), N153T D156E (TKY282), or E122K (TKY113) were monitored for viral maintenance by replica plating onto a lawn of 5x47-sensitive cells as described under Materials and Methods. Decreases in the diameter of the halos of growth inhibition are proportional to the extent of changes in −1 PRF efficiencies. (C) A subset of strains bearing eEF2 mutants (as described in Table 3) with IC_{50}s > 1.0 for preussin or wild-type eEF2 were assayed on plates containing 250 ng/ml preussin as described under Materials and Methods. The viability of cells in the presence of 250 ng/ml of preussin is indicative of resistance to this drug.

may slow down the accommodation step, increasing the amount of time that ribosomes are paused the slippery site, thus giving them more time to change frame. This would account for the increased −1 PRF efficiencies observed in cells expressing this form of the protein. Furthermore, addition of agents that inhibit this step would serve to exacerbate the intrinsic defect in the ability of the E122K mutant to effect accommodation of the aa-tRNA 3′ end into the ribosomal A-site, accounting for its hypersensitivity mutants to anisomycin and preussin. Conversely, increased aa-tRNA accommodation rates in the N153T, D156N, and N153+D156N mutants, e.g., through increased rates of intrinsic or facilitated GTP hydrolysis, could account for both their decreased rates of −1 PRF and their drug resistance. Though all of these mutants show reduced fidelity, suggestive of altered intrinsic GTPase activities, increased GTPase activity has only been directly demonstrated for the N153T mutant (Cavallius and Merrick JBC 1998). With regard to −1 PRF, increasing aa-tRNA accommodation rates would decrease the amount of time that ribosomes would be paused at the −1 frameshift signal, decreasing the likelihood of slippage. Furthermore, increasing the intrinsic ability of eEF1A to accommodate the aa-tRNA into the A-site of the peptidyl transferase center would allow these forms of the protein to partially overcome the blocks that anisomycin and preussin present at this step, thus accounting for the relative drug resistance of cells expressing the N153T, D156N, and N153+D156N mutants. While the link between reading frame maintenance and preussin sensitivity correlates with virus loss, it is also important to remember that several viruses recruit eEF1A for functions including RNA binding such as in West Nile virus and turnip yellow mosaic virus and association with viral proteins such as vesicular stomatitis virus RNA polymerase or HIV type I gag polyprotein (reviewed in Kinzy and Goldman, 2000). Additional roles for preussin on these functions remain to be determined.

The other elongation factor that interacts with the ribosomal A-site is eEF2. Though mutants of eEF2 have been shown not to affect −1 PRF efficiencies, since this protein interacts with the same region of the ribosome as eEF1A, it was reasonable to assume that preussin might also alter its function as well. Our testing of this hypothesis capitalized upon a series of isogenic strains *EFT1* and *EFT2* double-deletion strains harboring 14 different plasmid-borne alleles of the *EFT2* gene encoding eEF2 (Justice *et al.,* 1998). While the YEFD12h strain background for the eEF2 mutants was intrinsically more sensitive to preussin than the JD88 and MC213/214 backgrounds, the isogenic nature of these strains allowed for meaningful comparisons among cells expressing wild-type and mutant forms of eEF2. Interestingly, the preussin resistance profiles of these mutants closely paralleled those previously observed with the eEF2 inhibitor sordarin (Justice *et al.,* 1998) (Fig. 4C and Table 3). Sordarin allows eEF2 to bind ribosomes but inhibits GTP hydrolysis by inhibiting the transition from the pre-translocated ribosome•eEF2•GTP state to the post-translocated ribosome•eEF2•GTP state (Dominguez *et al.,* 1999). As discussed above, increased intrinsic GTP-hydrolysis rates by the drug-resistant mutants is also consistent with the observed correlation of preussin and sordarin resistance and suggests that, similar to sordarin, preussin may allow the elongation factors to bind to the ribosome, but inhibits their abilities to hydrolyze GTP.

TABLE 3

Preussin IC_{50} of eEF2 Mutant Strains

Plasmid[a]	Mutation in eEF2[a]	IC_{50} (μg/ml) preussin	IC_{50} (μg/ml) sordarin[a]
YCpEFT2	None	0.05	1
PSR13	R180G	0.66	15
PSR20	V187F	0.60	20
PSR8	Q490E	1.04	45
PSR16	Y521N	0.91	20
PSR27	S523F	1.06	>100
PSR15	S523P	1.16	>100
PSR6	I529T	0.88	30
PSR9	P559L	1.40	>100
PSR11	P559R	1.16	>100
PSR26	A562P	1.03	>100
PSR12	P727S	1.60	>100
PSR17	V774F	1.10	>100
PSR7	G790D	>2.0	>100

[a] Plasmids, mutations in *EFT2*-encoded protein, preussin, and sordarin resistance in the YEFD12h strain background from (Justice *et al.,* 1998).

To understand the relationship between the protein structure and the drug-sensitivity phenotypes, the residues that affect preussin sensitivity were mapped onto the structure of yeast eEF1A and a model of yeast eEF2 (Fig. 5). The mutations in eEF1A, shown on the yeast eEF1A structure of domain 1 (Fig. 5A) (Andersen *et al.,* 2000), are distinct from the switch regions but are near the nucleotide-binding site. For structure/function analyses of eEF2, the homologous residues were mapped in *Thermus thermophilus* EF-G (al Karadaghi *et al.,* 1996; Czworkowski *et al.,* 1994) based on a gap alignment using GCG, as the structure of eEF2 has not yet been solved. Examination of the EF-G structure reveals that the resistant mutations cluster predominantly around a cleft in domain 3, including two residues in domain 5 that contribute to this region (see Fig. 5B). In addition, two separate mutations in domains 1 and one in domain 5 form a separate, distinct cluster on the exterior of the protein. While some changes can be expected based on the differences between EF-G and eEF2, these likely serve as a starting point for identification of functional regions. Since eEF1A and eEF2 both bind the A-site, but at distinct steps in the elongation process, these results may indicate regions that are more conserved in the

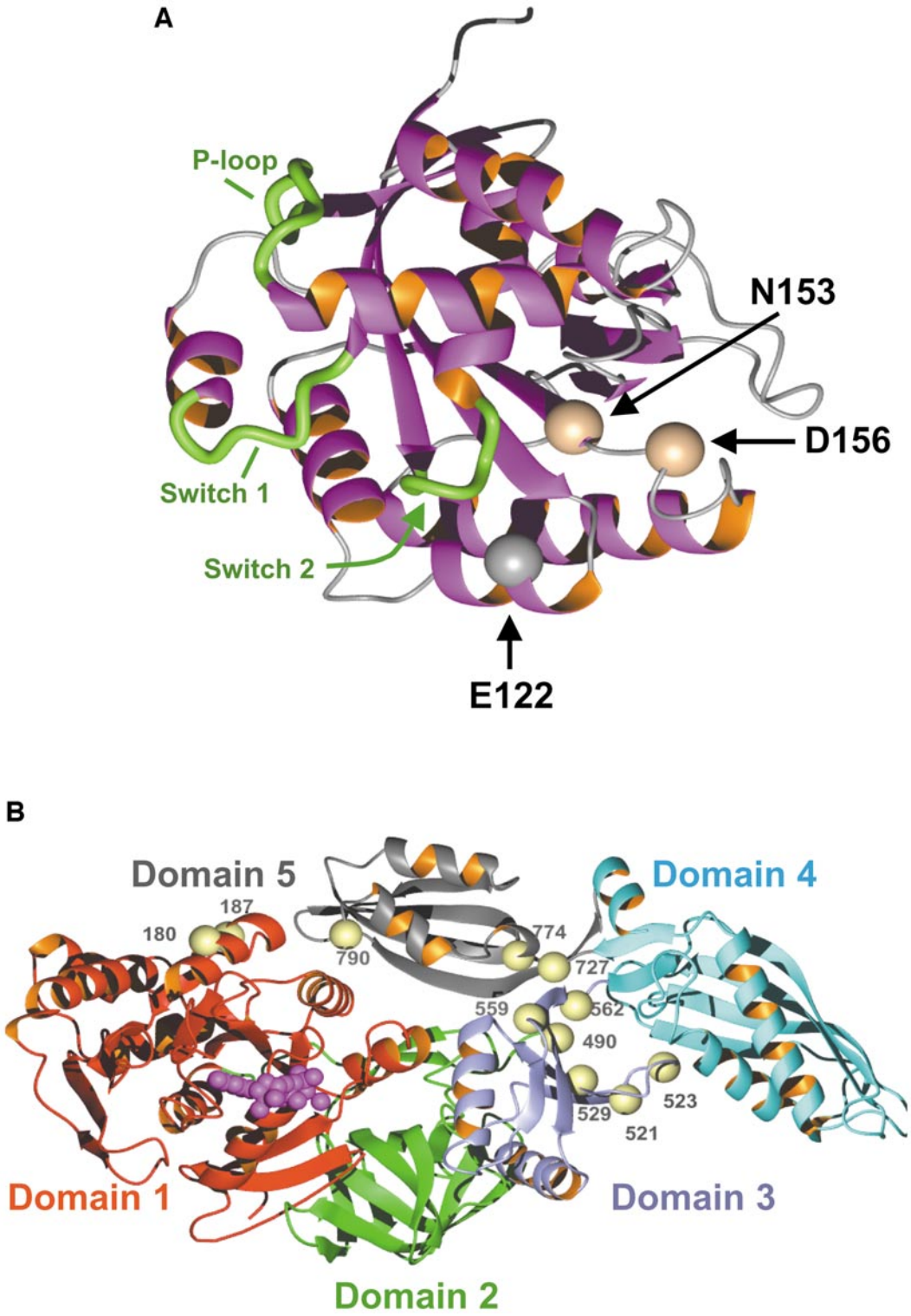

FIG. 5. Mapping drug-resistant alleles onto the crystal structures of eEF1A and the eEF2 homolog EF-G. (A) Location of preussin-sensitive mutants in the G-domain of yeast eEF1A (pdb entry 1F60). The residues N153 and D156 are marked with light spheres and E122 with a gray sphere. The thick green loops contain the P-loop, the switch 1, and switch 2 region involved in binding of the phosphates of GDP or GTP and Mg^{2+}. The figure was produced with MOLMOL (Koradi *et al.,* 1996). (B) Location of residues (large spheres) in EF-G (pdb entry 1FNM) corresponding to the preussin-sensitive mutants in eEF2 with amino acid residue numbers corresponding to eEF2 indicated. The structural domains in EF-G are shown in red, green, blue, cyan, and gray for domains 1,2, 3, 4, and 5, respectively. Small magenta spheres in domain 1 represent bound GDP.

TABLE 4

S. cerevisiae Strains Used in This Study

Strain	Genotype	Source
5x47	*MATa/MATα his1/+ trp1/+ ura3/+ K-R-*	(Dinman and Kinzy, 1997)
JD88	*MATa ura3-52 lys2-801 ade2-10 trp1-Δ1* [L-AHNB M_1]	Dinman Lab
MC213	*MATα ura3-52 leu2-3, 112 his4-713 trp1Δ1 tef2Δ tef1::LEU2 lys2-20 met2-1 pTEF2-URA3*	(Sandbaken and Culbertson, 1988)
MC214	*MATα ura3-52 leu2-3, 112 his4-713 trp1Δ1 tef2Δ tef1::LEU2 lys2-20 met2-1 pTEF2-TRP1*	(Sandbaken and Culbertson, 1988
TKY113	*MATα ura3-52 leu2-3, 112 his4-713 trp1Δ1 tef2Δ tef1::LEU2 lys2-20 met2-1 pTEF2-4-URA3* (E122K)	(Dinman and Kinzy, 1997)
TKY225	*MATα ura3-52 leu2-3, 112 his4-713 trp1Δ1 tef2Δ tef1::LEU2 lys2-20 met2-1 ptef2-17-TRP1* (D156N)	(Carr-Schmid *et al.,* 1999)
TKY226	*MATα ura3-52 leu2-3, 112 his4-713 trp1Δ1 tef2Δ tef1::LEU2 lys2-20 met2-1 ptef2-19-TRP1* (N153T)	(Carr-Schmid *et al.,* 1999
TKY229	*MATα ura3-52 leu2-3, 112 his4-713 trp1Δ1 tef2Δ tef1::LEU2 lys2-20 met2-1 ptef2-18-TRP1* (N153T/D156E)	(Carr-Schmid *et al.,* 1999
TKY235	*MATα ura3-52 leu2Δ1 met2-1 trp1Δ101 his4-713 lys2-801 met2-1 tef5:::TRP1 pTEF5 LEU2*	(Carr-Schmid *et al.,* 1999)
TKY243	*MATα ura3-52 leu2Δ1 met2-1 trp1Δ101 his4-713 lys2-801 met2-1 tef5::TRP1 ptef5-7 LEU2* (K120RS121ΔI122Δ)	(Carr-Schmid *et al.,* 1999
TKY252	*MATα ura3-52 leu2-3, 112 his4-713 trp1Δ1 tef2Δ tef1::LEU2 lys2-20 met2-1 pTEF2-4-TRP1* (E122K)	This study
TKY278	*MATα ura3-52 leu2-3, 112 his4-713 trp1Δ1 tef2Δ tef1::LEU2 lys2-20 met2-1 ptef2-17-URA3* (D156N)	(Carr-Schmid *et al.,* 1999)
TKY280	*MATα ura3-52 leu2-3, 112 his4-713 trp1Δ1 tef2Δ tef1::LEU2 lys2-20 met2-1 ptef2-19-URA3* (N153T)	(Carr-Schmid *et al.,* 1999
TKY282	*MATα ura3-52 leu2-3, 112 his4-713 trp1Δ1 tef2Δ tef1::LEU2 lys2-20 met2-1 ptef2-18-URA3* (N153T + D156E)	(Carr-Schmid *et al.,* 1999
TKY298	*MATα ura3-52 leu2Δ1 met2-1 trp1Δ101 his4-713 lys2-801 met2-1 tef5::TRP1 pTEF2 URA3*	(Andersen *et al.,* 2001)
TKY370	*MATα ura3-52 leu2Δ1 met2-1 trp1Δ101 his4-713 lys2-801 met2-1 tef5::TRP1 ptef5-23 LEU2* (F163A)	(Andersen *et al.,* 2000)
TKY128	*MATα* alpha *ade2 lys2 leu2 his3 trp1 ura3 eft1::HIS3 eft2::TRP1 pEFT2-LEU2*	(Justice *et al.,* 1998)
YEFD12h	*MATa ade2 ura3 his3 leu2 trp1 eft1::HIS3 eft2::TRP1* + YCpEFT2	(Justice *et al.,* 1998

nature of their interactions with the ribosome. Together, the information on both proteins and their effects in the presence of these compounds suggest new ways to think about the control of gene expression and the rational design of compounds that, by modulating it, possess antiviral activities.

MATERIALS AND METHODS

Strains, genetic methods, drugs, and media

Escherichia coli strains DH5α and MV1190 were used to amplify plasmids, and *E. coli* transformations were performed using standard calcium chloride method as described previously (Sambrook *et al.,* 1989). Preussin was purified as previously described (Schwartz *et al.,* 1988), and anisomycin was obtained from Sigma Chemical Corp. (St. Louis, MO). All eEF2 mutant strains were prepared by transformation of the indicated plasmid in Table 3 into YEPD12h followed by growth on 5-fluoroorotic acid. Yeast strains are given in Table 4 and 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 and Leibowitz, 1976). Cytoduction of L-A and M_1 from *kar1-1* donor strains into rho-0 acceptor strains were as previously described (Dinman and Wickner, 1994). Determination of IC_{50} values for preussin for the eEF2 mutants were as previously described (Justice *et al.,* 1998). Plate assays for preussin and anisomycin sensitivity/resistance were performed by growing the indicated strains overnight in YPAD at 29°C. Cells were diluted to A_{600} of 0.05 and serial fivefold dilutions (4 μl) were spotted onto YPAD plates containing preussin at 5 μg/ml for the eEF1 mutant strains and 250 ng/ml for the eEF2 mutant strains. The plates were incubated at 29°C for 5–7 days.

Programmed ribosomal frameshift and RNase protection assays

The plasmids p0, p − 1, and +1 used to monitor PRF have been described previously (Harger *et al.,* 2001). In these plasmids, transcription is driven from the yeast *PGK1* promoter; the enzymatic reporter is composed of the *E. coli lacZ* gene, and transcription termination utilizes the yeast *PGK1* transcriptional terminator. In the p0 plasmids, *lac*Z is in the 0-frame with respect to the translational start site, and measurement of β-gal activity generated from cells transformed with these plasmids serve as the 0-frame controls. In the p − 1 series, *lac*Z 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-gal can only be produced as a consequence of a −1 PRF event. Similarly, in the p + 1 series, *lac*Z is in the +1 frame with respect to the translational start site and is 3′ of the Ty*1* +1 PRF signal. The efficiency of programmed ribosomal frameshifting is calculated by determining the ratio of β-gal activity produced by cells harboring either p − 1 or p + 1 divided by the β-gal activity produced by cells harboring p0 and multiplying by 100%. All assays were performed in triplicate on three different occasions. Measurements of the effects of preussin and anisomycin on programmed ribosomal frameshifting were performed as described previously (Dinman *et al.,* 1997). Briefly, 0.5 ml of overnight cultures of JD88 cells were diluted into 2 ml of fresh medium containing either of the two compounds, or drug-free controls (starting $O.D._{595} \approx 0.3–0.5$). The final concentrations of anisomycin were 2, 4, or 10 μg/ml, and the final concentrations of preussin were 1, 2, or 4 μg/ml. Cultures were incubated at 30°C for 5 h, after which frameshifting efficiencies were determined as described above. RNase protection assays were performed as previously described probing for *Lac*Z mRNA steady-state abundances, which were normalized to U3 snRNA (Harger *et al.,* 2001).

Virus propagation assays

The killer assay was done by replica-plating *Saccharomyces cerevisiae* colonies onto 4.7-MB plates (Wickner and 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 M_1 viruses were prepared as described (Liermann *et al.,* 2000), separated by electrophoresis through 1.0% agarose gels, and visualized by ethidium bromide staining. The abundance of L-A and M_1 dsRNAs were monitored by RNA blotting as previously described (Dinman and Wickner, 1994). Rates of loss of killer virus were monitored as previously described (Dinman *et al.,* 1997). Briefly, cells were grown in rich medium containing the same concentrations of preussin as described above and were passaged into fresh compound-containing medium every 24 h. Aliquots of cells were taken after 1, 3, and 5 days and plated onto rich media to allow growth of single colonies for 2–3 days, after which they were assayed for killer activity. The frequency of killer loss was measured by the ratio of $Killer^-$/total colonies. A minimum of 100 individual colonies were assayed for each compound concentration and each time point. Assays to monitor Ty*1* retrotransposition frequencies were performed as previously described (Harger *et al.,* 2001).

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