Comparative study of the effects of heptameric slippery site composition on -1 frameshifting among different eukaryotic systems

EWAN P. PLANT and JONATHAN D. DINMAN

Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, Maryland 20742, USA

ABSTRACT

Studies of programmed -1 ribosomal frameshifting (-1 PRF) have been approached over the past two decades by many different laboratories using a diverse array of virus-derived frameshift signals in translational assay systems derived from a variety of sources. Though it is generally acknowledged that both absolute and relative -1 PRF efficiency can vary in an assay system-dependent manner, no methodical study of this phenomenon has been undertaken. To address this issue, a series of slippery site mutants of the SARS-associated coronavirus frameshift signal were systematically assayed in four different eukaryotic translational systems. HIV-1 promoted frameshifting was also compared between *Escherichia coli* and a human T-cell line expression systems. The results of these analyses highlight different aspects of each system, suggesting in general that (1) differences can be due to the assay systems themselves; (2) phylogenetic differences in ribosome structure can affect frameshifting efficiency; and (3) care must be taken to employ the closest phylogenetic match between a specific -1 PRF signal and the choice of translational assay system.

Keywords: frameshifting; virus; ribosome; slippery site

INTRODUCTION

Ribosomes use messenger RNA as the template for making proteins. This process, called translation, is both highly efficient and very specific, resulting in few errors (for review, see Ogle and Ramakrishnan 2005). Aberrations include incorporation of the wrong tRNA, readthrough of termination codons, and alterations in reading frame. A number of viruses have been shown to contain programmed -1 ribosomal frameshift (-1 PRF) signals and frameshifting at these elements typically results in proteins with extended C termini encoding additional functions (Bekaert and Rousset 2005). Viruses that use -1 PRF infect a broad range of eukaryotic host cells, and the sequences that promote -1 PRF are diverse. Programmed -1 ribosomal frameshifting is an example of disruption, in a controlled manner, of ribosomal frame maintenance. The disruption is directional and limited to the repositioning of the tRNAs by a single nucleotide relative to the mRNA.

There are three major mRNA features that contribute to -1 PRF: a heptameric nucleotide sequence N NNW WWH (where the incoming reading frame is indicated by spacing), which permits the aminoacylated- and peptidyl-tRNAs to disengage from the mRNA and reengage codons in the -1 frame (Jacks et al. 1988); a stimulatory structure that is often an mRNA pseudoknot (for review, see Giedroc et al. 2000); and a spacer region between the slippery site and pseudoknot (Naphthine et al. 1999; Bekaert et al. 2003). The tRNAs that decode the slippery site are also integral to -1 PRF (Naphthine et al. 2003), and it has been proposed that modifications to these tRNAs could affect the stability of the codon:anticodon interaction altering frameshifting efficiency (Tsuchihashi and Brown 1992). It has also been observed that the species from which the ribosomes are derived can influence the type and frequency of frameshifting (e.g., Matsufuji et al. 1996; Sung and Kang 2003).

Examination of two decades of research reveals that both the absolute and relative frequencies of -1 PRF tend to vary depending on the sources of -1 PRF signals and ribosomes. However, to date, no systematic study of this phenomenon has been undertaken. The current study seeks to address this deficiency. Here, mutations were made in the slippery site of the frameshift signal from the SARS-associated coronavirus (SARS-CoV), and -1 PRF efficiencies determined using four different eukaryotic systems:

Reprint requests to: Jonathan D. Dinman, Department of Cell Biology and Molecular Genetics, Microbiology Building, Room 2135, University of Maryland, College Park, MD 20742, USA; e-mail: dinman@umd.edu; fax: (301) 314-9489.

Article published online ahead of print. Article and publication date are at <http://www.rnajournal.org/cgi/doi/10.1261/rna.2225206>.

Vero cells, reticulocyte lysates, wheat germ lysates, and yeast cells. This approach allowed for comparison of the effects of a single variable between multiple systems. HIV-1 mediated –1 PRF efficiencies were also compared between *Escherichia coli*, often used as an inexpensive and convenient assay system, and Jurkat cells, a transformed T-cell line more representative of the natural host cell. Assay system-specific differences observed in this study identify numerous issues that can affect –1 PRF efficiency and suggest that care should be used to find the most natural match between the source of assay system and specific –1 PRF signal to be analyzed.

RESULTS

The absolute efficiency of –1 PRF varies depending on the species of ribosome

There are numerous examples in the literature where different groups using different translational assay systems obtained differences in –1 PRF efficiencies. For example, the percentage of –1 PRF promoted by the SARS-CoV frameshift signal was observed to vary widely in an assay system-dependent manner (Plant et al. 2005). These discrepancies have resulted in considerable confusion in the literature. To address this issue, the wild-type SARS-CoV frameshift signal, encompassing the U UUA AAC heptameric slippery site and three-stemmed pseudoknot, was cloned between the *Renilla* and firefly luciferase genes such that the firefly protein can only be expressed via a frameshift event (Plant et al. 2005). Programmed –1 ribosomal frameshifting efficiencies were measured as described in the methods using two in vivo (Vero and yeast cells) and two in vitro (lysates of rabbit reticulocytes and wheat germ) eukaryotic systems. The –1 PRF efficiency from this wild-type sequence was similar in Vero cells and reticulocyte lysates (12.8% and 17.9%), higher in wheat germ lysate (23%), and lower in yeast (3.1%) (Fig. 1).

To follow up on this observation, the slippery site was mutated to two other known slippery sequences: U UUU UUU and A AAA AAU. In both cases, the absolute amount of frameshifting was less than that of the wild-type sequence in the Vero cells and in vitro assay systems. However, while frameshifting from the wild-type sequence was higher in lysates of wheat germ compared to reticulocytes, the opposite was true when the slippery sequence A AAA AAU was used. This result suggested that –1 PRF efficiencies depended on both the slippery site sequence and the source of ribosomes. This observation prompted an in-depth analysis of the interactions between different ribosome sources and different nucleotides in the slippery site.

A-site codon effects: Influence of P-site codon context

To examine the affect of A-site codons on different ribosomes, a series of mutations was made to the se-

FIGURE 1. Frameshifting efficiency from individual slippery sites varies depending on the translational assay system. Luminescence from test constructs is expressed as a percentage of the nonframeshifting control for different slippery sites. The shading of the bars represents frameshifting stimulated by different translational systems: dark gray for Vero cells, light gray for reticulocyte lysate, white for yeast cells, and black for wheat germ lysate. Percentages of frameshifting and standard errors are indicated in both the figure and the table below.

quence of the wild-type SARS-CoV slippery site that the aminoacyl-tRNA anticodon base-pairs with after a –1 PRF event. Only A AAZ and U UUZ were examined, as triplets of G or C had previously been shown to not promote efficient frameshifting (Dinman et al. 1991; Brierley et al. 1992; Dinman and Wickner 1992), and in all cases the nucleotide in the zero frame wobble position (Z) was kept the same. In the first series of experiments, the wild-type SARS frameshift slippery site was altered from U UUA AAC to U UUU UUC and frameshifting was determined by dual luciferase assays. The relative frameshifting efficiencies promoted by U UUU UUC remained almost identical in Vero and yeast cells, and in reticulocyte lysates as compared to the wild-type sequence (Fig. 2A; Table 1). The exception was observed in wheat germ lysates, where there was a notable increase in frameshifting efficiency ($p = 7.6 \times 10^{-4}$). More dramatic differences were observed when the P-site contained a glycine codon (G GGA AAC vs. G GGU UUC). In this P-site context, frameshifting frequencies were consistently lower with UUU in the A-site. The biggest difference was a 66% decrease in yeast (P -value of 5.4×10^{-9}). These data suggest that in general U UUC is less preferable in the A-site than A AAC, and that the extent of that preference is dependent on the P-site codon context.

P-site codon effects: Ribosome source effects on frameshifting efficiency

The first three nucleotides of the slippery site were mutated from U UU to A AA, G GG or C CC, all of which have been

FIGURE 2. Influence of the A- and the P-site codons on -1 frameshifting in different translational systems. (A) Influence of the A-site codon. (B) Influence of the P-site codon. Frameshifting efficiencies were determined and expressed as fold change of the wild-type sequence (U UUA AAC) for each construct. The shading of the bars is the same as in Figure 1. *P*-values for fold change are in Table 1.

shown to support efficient -1 PRF (Dinman et al. 1991; Brierley et al. 1992; Dinman and Wickner 1992). In yeast, frameshifting was stimulated to the greatest extent with poly-A or poly-C in the P-site (Fig. 2B; Table 1). In contrast, -1 PRF was reduced in the metazoans (Vero cells and reticulocyte lysates) in the presence of poly-A, -G or -C (*P*-values from 1.8×10^{-9} to 8.8×10^{-22}), the lowest level of stimulation being produced by the poly-C sequence. In plants (wheat germ lysates) frameshifting efficiencies were

stimulated to a greater degree by pyrimidines than by purines in the P-site, with maximal enhancement by the poly-C sequence. In summary, the yeast, metazoan, and plant ribosomes all responded differently to the various P-site codon contexts.

Effects of the wobble position of the zero frame A-site codon are dependent on the identity of both the A- and the P-site codons

Previous studies showed that A, C, or U in the seventh position of the slippery site were able to stimulate efficient frameshifting in yeast and reticulocyte lysates (Dinman et al. 1991; Brierley et al. 1992; Dinman and Wickner 1992). To examine this parameter the seventh position was mutated to each of the four possible nucleotides in two different heptameric slippery site contexts: U UUA AAN and G GGU UUN. In yeast cells frameshifting was more efficient when the last nucleotide of the slippery site was A or U (Fig. 3A; Table 1), a trend that was more pronounced when the -1 frame P- and A-site triplets were poly-G and poly-U, respectively (Fig. 3B; Table 1). A cytosine in the seventh position also promoted efficient frameshifting when the A- and P-site codons were poly-U and poly-A, respectively (the wild-type U UUA AAC sequence) (Fig. 3A; Table 1). Frameshifting efficiency was diminished when the seventh position was a guanine no matter the A- and the P-site context. In wheat germ lysates, variations in fold change generally matched those of yeast except for the

TABLE 1. Fold change in frameshifting as determined in four different systems

Slippery site	Vero cells	Retic lysate	Yeast cells	Wheat germ
A-site changes (SARS-CoV slippery site)				
U UUA AAC	1	1	1	1
U UUU UUC	0.96 (5.6×10^{-1})	0.90 (2.8×10^{-1})	0.92 (2.5×10^{-1})	1.6 (7.6×10^{-4})
A-site changes (L-A slippery site)				
G GGA AAC	1	1	1	1
G GGU UUC	0.49 (4.7×10^{-5})	0.84 (1.9×10^{-2})	0.33 (5.4×10^{-9})	0.80 (3.3×10^{-1})
P-site changes				
U UUA AAC	1	1	1	1
G GGA AAC	0.31 (1.8×10^{-9})	0.61 (2.3×10^{-11})	0.93 (3.4×10^{-1})	0.65 (2.3×10^{-3})
C CCA AAC	0.13 (4.7×10^{-10})	0.14 (8.8×10^{-22})	1.15 (1.8×10^{-1})	1.28 (6.8×10^{-2})
A AAA AAC	0.34 (2.1×10^{-9})	0.44 (4.0×10^{-13})	1.25 (8.4×10^{-3})	0.10 (5.1×10^{-14})
Seventh position changes (SARS-CoV slippery site)				
U UUA AAC	1	1	1	1
U UUA AAU	0.25 (9.1×10^{-11})	0.60 (5.9×10^{-9})	1.10 (1.6×10^{-1})	1.45 (3.4×10^{-2})
U UUA AAA	0.22 (2.5×10^{-10})	0.63 (2.7×10^{-7})	1.05 (5.6×10^{-1})	1.24 (2.3×10^{-1})
U UUA AAG	0.13 (1.4×10^{-11})	0.26 (2.6×10^{-20})	0.17 (2.4×10^{-20})	0.82 (6.8×10^{-2})
Seventh position changes (L-A slippery site)				
G GGU UUC	1	1	1	1
G GGU UUU	0.86 (2.9×10^{-1})	0.84 (3.0×10^{-2})	2.43 (1.1×10^{-6})	1.76 (2.5×10^{-4})
G GGU UUA	1.84 (1.9×10^{-4})	1.17 (1.5×10^{-2})	1.69 (2.3×10^{-9})	1.98 (2.9×10^{-6})
G GGU UUG	0.30 (2.5×10^{-6})	0.45 (1.8×10^{-8})	0.41 (4.9×10^{-11})	0.45 (1.8×10^{-4})

Fold changes were calculated for each type of slippery site. *P*-values shown in parentheses were determined as previously described in Jacobs and Dinman (2004).

unexpectedly efficient -1 PRF promoted by the U UUA AAG slippery site.

Though the trends using reticulocyte lysates and Vero cells differed somewhat from those of yeast cells and wheat germ lysates, they were generally similar with each other with some exceptions. For example, the effects of the seventh base positions were less pronounced in reticulocyte lysates, and frameshifting efficiencies were generally higher in reticulocyte lysates when the -1 frame A- and P-site triplets were poly-G and poly-U (Fig. 3B; Table 1). Additionally, when the A- and the P-site triplets were poly-U and poly-A, -1 PRF was stimulated to a greater degree by A or U in the seventh position in reticulocyte lysates as compared to Vero cells (Fig. 3A; Table 1). There was a slight preference for A over U or C when the P-site codon was GGG (Fig. 3B; Table 1). As with the yeast cells, frameshifting efficiency was minimal when guanine was the seventh nucleotide regardless of the A- and the P-site codons.

Comparison of HIV-1 mediated -1 PRF between a human T-cell line and *E. coli* expression systems

Assay systems are often chosen for cost and convenience. For example, many experiments analyzing the HIV-1 frameshift signal have been performed using relatively inexpensive and convenient *E. coli*-based translational assay systems instead of the natural host cell, i.e., human T-cells. To investigate whether this might affect measurements of frameshifting HIV-1 promoted -1 PRF was assayed in both in *E. coli* and a human T-cell line (Jurkat cells)-based assay systems. Frameshifting as measured by the dual luciferase assay was threefold higher in the Jurkat cells ($P = 4.2 \times 10^{-4}$) (Table 2). These findings suggest that there are significant functional differences between the two systems and raises the question of the utility of heterologous assay systems for mechanistic and pharmacological investigations.

FIGURE 3. Effects of the seventh position of the slippery site on -1 frameshifting in different translational systems. (A) Effects with a SARS-like slippery site. (B) Effects with a (L-A)-like slippery site. Frameshifting efficiencies were determined and expressed as fold change of the SARS wild-type sequence (U UUA AAC) for each construct. The shading of the bars is the same as in Figure 1. P -values for fold change are in Table 1.

TABLE 2. Analysis of HIV-1 frameshifting in *E. coli* and Jurkat cells

	HIV-1-directed frameshifting		
	% Frameshifting	Fold change	P -value
<i>E. coli</i>	2.4		
Jurkat cells	9.6	3.1	4.2×10^{-4}

Fold changes were calculated for the HIV-1 frameshift signal in a prokaryote cell and in a human T-cell. P -values were determined as previously described in Jacobs and Dinman (2004).

DISCUSSION

Over the past two decades, the number of known -1 PRF signals has increased exponentially. However, assays to monitor frameshifting from these signals have often utilized translational systems derived from sources very distant from the species that the viruses normally infect. We show here that, even within a kingdom, different ribosomes frameshift with different efficiencies in both ribosome and frameshift signal-dependent manners. For example, frameshifting at the infectious bronchitis virus -1 PRF signal in *E. coli* was pseudoknot-independent and also appeared to require slippage of only a single tRNA, in direct contrast to the requirement for pseudoknots and dual-tRNA slippage in rabbit reticulocyte lysates (Brierley et al. 1997). Thus, while analyses using “mixed” systems may highlight the importance of host features, mechanistic conclusions about the viral frameshift signal could be compromised. Our studies demonstrating significant differences in -1 PRF between *E. coli* and Jurkat cells (a human T-cell line) sustain this view (Table 2). This is further supported by studies from other groups highlighting differences in frameshifting mechanism between prokaryotic and eukaryotic systems (Garcia et al. 1993; Sung and Kang 2003). These considerations cast a shadow on the interpretation of mechanistic studies utilizing such “mixed” systems, e.g., those examining HIV-1 frameshifting in *E. coli* (Weiss et al. 1989; Yelverton et al. 1994; Horsfield et al. 1995; Leger et al. 2004) and should be seriously considered in designing anti-viral drug screens.

Examination of absolute -1 PRF efficiencies shows that in vitro assay systems consistently produced higher values than did the in vivo systems (Fig. 1; Table 1). Examination of the raw data (data not shown) also reveals that while the ratios of firefly to *Renilla* luciferase activities for the readthrough control were similar for the in vivo analyses (yeast = 0.29, Vero cells = 0.30), they were significantly lower in the in vitro cell lysate-based systems (reticulocyte lysate = 0.11, wheat germ lysate = 0.0003). These findings suggest that a higher fraction of translational events were incomplete in the in vitro systems and may reflect gross differences in fidelity between in vitro and in vivo assay systems. However, presentation of the analyses between systems as differences in fold change was used to control for such differences in translation efficiency. The very low activity observed in

wheat germ lysates may also reflect differences in the codon usage of the luciferase genes and of wheat genes. Lower frameshifting efficiencies in wheat germ lysates compared to reticulocyte lysates has been previously observed (Garcia et al. 1993). However, codon usage at the slippery site did not correlate with observed differences in frameshifting for each of the systems tested (data not shown).

Examination of absolute frameshift efficiencies may also illuminate how phylogenetic differences in ribosome structure may affect function. It has long been known that the size of ribosome subunits from metazoans are greater than those from fungal and plant ribosomes ("The Comparative RNA Web Site," <http://www.rna.icmb.utexas.edu>), and cryo-electron microscopic studies are revealing kingdom-specific differences on a finer scale. For example, a comparison of the architecture of ribosomes from humans and from yeast reveals significantly more mass around the periphery of the human ribosome, mostly attributable to the presence of additional expansion segments, and the *E. coli* ribosome is even smaller (Spahn et al. 2004). It is possible that these differences may qualitatively affect the interaction between the ribosome and the pseudoknot, affecting the phasing of the slippery site relative to the A- and the P-sites. By this theory, the HIV-1 and SARS-CoV -1 PRF signals would have evolved to optimize frameshifting efficiency in the context of the larger metazoan ribosome.

Analyses of the fold changes for each slippery site in a particular lysate relative to a wild type slippery sequence can be used to highlight different predilections of the various systems for different -1 frame P- and A-site codons and for the wobble nucleotide in the seventh position of the slippery site. For example, regardless of the translation efficacy, the trends for the two metazoan systems, reticulocyte lysate and Vero cells, were similar (Figs. 2, 3; Table 1). The exception was the increase in frameshifting efficiency in reticulocyte lysate when poly-G was in the -1 frame P-site (Figs. 2B, 3, cf. A and B). We do not know if this difference was a result of reduced efficacy of the in vitro system or if it represents real differences in slippery site specific interactions between monkey (Vero cells) and rabbit (reticulocyte lysate) ribosomes.

In comparing the -1 frame A-site variants, A AAC decoded by tRNA^{Asn} versus U UUC decoded by tRNA^{Phe}, there was an indication of some dependence on the P-site sequence (Fig. 2A; Table 1). The lower frameshifting efficiencies with the glycine codons in the P-site observed in each system suggests that the strength of the base-pairing of the codon:anticodon interaction in the P-site may be an important determinant of frameshifting. The A-site tRNAs, tRNA^{Asn} and tRNA^{Phe}, each decode two synonymous codons (AAC/AAU and UUC/UUU, respectively) (Marck and Grosjean 2002). The tRNA^{Asn} will be mismatched at the wobble position after repositioning on the -1 AAA codon, whereas tRNA^{Phe} will not. The lack of difference between -1 PRF efficiencies from the U UUA AAC and U UUU UUC signals in Vero cells, yeast cells, and reticulocyte lysates suggests that the ability to repair at the wobble position

in the A-site after -1 PRF does not contribute to frameshifting efficiency.

-1 PRF efficiencies differed significantly between the systems depending on the identity of the P-site codon (Fig. 2B; Table 1). The order of preference for poly-N in the P-site for Vero cells and reticulocyte lysates was U > G > A > C, the same as that determined for the avian infectious bronchitis coronavirus frameshift signal in reticulocyte lysates (Brierley et al. 1992). A similar trend was observed when the BWYV frameshift signal was analyzed in reticulocyte and wheat germ lysates, with poly-U eliciting higher levels of frameshifting than poly-G (Garcia et al. 1993). In contrast, yeast cells displayed very little nucleotide preference for poly-U or poly-G at the P-site in the context of the SARS-CoV. Frameshifting efficiency had previously been shown to be more than sixfold higher with poly-U compared to poly-G in the context of the L-A frameshift signal G GGU UUA (Dinman et al. 1991). This result, and the difference seen between the G GGA AAC and G GGU UUC slippery sites (Fig. 2A; Table 1), may stem from the different tRNA^{Gly} decoding each P-site. These results do not correlate with the strength of P-site hydrogen bonding suggested above but rather suggest that the tRNAs themselves modulate -1 PRF. This could be due to interactions between the peptidyl-tRNA and the ribosome (e.g., see Meskauskas and Dinman 2001), and/or perhaps interactions between the peptidyl- and aminoacyl-tRNAs. The latter seems quite likely when the results from mutations to the seventh position of the slippery site are considered. The identity of peptidyl-tRNA has also been shown to affect the out of frame binding of the A-site tRNA in Ty3 frameshifting (Vimaladithan and Farabaugh 1994).

Similar trends within each system were recorded when the seventh position was mutated and the P-site sequence was either U UUA or G GGU (Fig. 3; Table 1). The exceptions were decreased frameshifting in Vero cells with G GGU UUC compared to U UUA AAC, and increased frameshifting in wheat germ lysates when the seventh position was A or U and the P-site was G GGU rather than U UUA. These data clearly indicate that the specific interactions of different ribosomes with particular tRNAs in the A-site affects frameshifting efficiency. The A-site codon has also been shown to affect the rate of P-site frameshifting in eubacteria (Barak et al. 1996). Interestingly, even though the asparagine and phenylalanine codons (AAU/AAC and UUU/UUC, respectively) are each likely decoded by the same tRNA (Marck and Grosjean 2002), frameshifting was different for these codons in each system tested (Fig. 3A, cf. U UUA AAC and U UUA AAU; 3B, cf. G GGU UUC and G GGU UUU). It is possible that these differences stem from the different stability of the codon:anticodon interaction. Modification of the tRNA around the anticodon modulates the stability of this interaction, and conversely, the affinity of these tRNAs for the ribosome (discussed in Agris 2004). It is unclear what effects these modifications have on

–1 PRF. tRNAs lacking modifications at position 34 of the tRNA have been shown to promote higher levels of frameshifting in vitro (Carlson et al. 1999, 2000). However, cos 7 (monkey kidney) cells cultured in media with or without queuine, required for a position 34 modification, showed no differences in –1 PRF (Marczinke et al. 2000). Similarly, yeast strains deficient in modifications at position 37 of the tRNA also had no effect on –1 PRF (Dinman and Wickner 1994; Urbonavicius et al. 2003). The different frameshifting frequencies stimulated by tRNA^{Asn} and tRNA^{Phe} may be due to changes in the tRNA structure resulting from the stabilization of the codon:anticodon interaction after the initial selection that then affects ribosomal fidelity.

In conclusion, the data presented here show that there are differences in frameshifting efficiencies in the different systems assayed. These differences may be due to a number of factors, including (1) differences in fidelity between in vivo and in vitro systems; (2) differences in the slipperiness of individual tRNAs; (3) differences in the structure of the tRNAs after decoding; and (4) differences in the structure of different ribosomes. Because of these differences, caution should be taken to match the source of translational systems to as closely as possible to the –1 PRF signal to account for host ribosome specificities.

MATERIALS AND METHODS

Strains, genetic methods, and programmed ribosomal frameshifting assays

E. coli strain DH5 α was used to amplify plasmids, and *E. coli* transformations were performed using the high efficiency transformation method of Inoue et al. (1990). Yeast strain JD932 (*MATa ade 2–1 trp1–1 ura3–1 leu2–3,112 his3–11,15 can1–100*), Jurkat cells, and African green monkey Vero cells were used for in vivo measurements of –1 PRF. YPAD and synthetic complete medium (H–) were used as described previously in Dinman and Wickner (1994) for yeast culture. Yeast cells were transformed using the alkali cation method (Ito et al. 1983). African green monkey Vero cells and Jurkat cells were cultured in DMEM with L-glutamine or RPMI-1640 with L-glutamine and 25 mM HEPES, respectively (BioWhittaker), supplemented with 10% FBS at 37°C in 5% CO₂. Cells cultured without antibiotics were transfected with plasmid DNA using Amaxa Nucleofector solution according to the manufacturer's instructions (Amaxa). Dual luciferase assays for programmed ribosomal frameshifting in yeast were performed as previously described in Harger and Dinman (2003). Dual luciferase assays using Vero cells or Jurkat cells were performed the day following transfection with cells lysed with the Passive Lysis Buffer (Dual-Luciferase Reporter System, Promega) as previously described in Plant et al. (2005). *E. coli* cells were cultured in LB media with carbenicillin for plasmid maintenance overnight. They were diluted 1:20 in fresh LB with isopropyl- β -D-thiogalactopyranoside to a final concentration of 1 mM to induce dual luciferase production and cultured for an additional 3 h. Cells were harvested by centrifugation and lysed for 15 min in Passive Lysis Buffer prior to the luciferase assay. Wheat germ and rabbit reticulocyte lysates containing tRNAs from Ambion were

used to monitor frameshifting in vitro using synthetic mRNA transcripts (Ambion mMACHINE mMACHINE transcription kit), generated with T7 polymerase from plasmids that had been digested with SspI, Proteinase K-treated, phenol/chloroform- and chloroform-extracted, and ammonium acetate-precipitated. At least three readings were taken for each assay, and the assays were repeated ($n = 2$ –12) until the data were normally distributed to enable statistical analyses both within and between experiments (Jacobs and Dinman 2004).

Plasmid construction, oligonucleotides, and mutagenesis

The SARS-CoV frameshift signal has previously been characterized and dual luciferase plasmids containing the sequence responsible were described in Plant et al. (2005). Three sets of plasmids were used in this work: the first for expression in yeast, the second set for analyses in tissue culture and cell lysates, and the third for expression in *E. coli*. The yeast zero-frame control plasmid pJD474 has the frameshift signal cloned such that the luciferase coding regions are in frame with respect to each other, while test plasmid pJD465 requires a frameshift event for the expression of the downstream firefly protein, which is in the –1 frame with respect to the *Renilla* open reading frame. The second set of plasmids, the zero frame control pJD464 and –1 PRF test construct pJD502, contain the SV40 early promoter, T7 promoter, and SV40 late polyadenylation signal for expression in mammalian tissue culture and transcription of RNAs for in vitro analyses in cell free lysates. pJD465 and pJD502 were used as templates for site-directed mutagenesis of the slippery site. Vectors with the HIV frameshift signal cloned into the dual luciferase plasmids for transfection into mammalian cells have previously been described (Grentzmann et al. 1998). PCR primers were used to amplify the dual luciferase cassette from both the control and test plasmid such that the cassette could be cloned into pBlue-script II SK+ as a HindIII-BclI fragment. Expression of the dual luciferase proteins was induced in *E. coli* as described above. These are now summarized in Table 3.

TABLE 3. Summary of plasmids and slippery sites

Slippery site	Plasmid (yeast)	Plasmid (other ^a)
U UUA AAC	pJD465	pJD502
U UUA AAU	pJD678	pJD681
U UUA AAG	pJD679	pJD682
U UUA AAA	pJD680	pJD683
G GGU UUC	pJD576	pJD648
G GGU UUU	pJD698	pJD658
G GGU UUA	pJD575	pJD647
G GGU UUG	pJD577	pJD649
G GGA AAC	pJD572	pJD646
C CCA AAC	pJD696	pJD656
A AAA AAC	pJD697	pJD657
A AAA AAU	pJD654	pJD584
HIV-1 frameshift indicator	N.A.	pJD786 (<i>E. coli</i>)
HIV-1 control	N.A.	pJD787 (<i>E. coli</i>)

Plasmids used to monitor frameshifting in yeast are as indicated.

^aUsed to indicate plasmids used to monitor frameshifting in Vero cells, rabbit reticulocyte lysates, and wheat germ lysates.

TABLE 4. Synthetic oligonucleotides employed for site-directed mutagenesis

slipmut1F	GCTCCGGATCCGTTTTTGAAMGGGTTTGCGGTGTAAGTG
slipmut1R	CACCTACACCGCAAACCCCTTCAAAAACGGATCCGGAGC
slipmut2F	GCGCTCCGGATCCGTTTTTTTTYGGGTTTGCGGTGTAAGTGCAG
slipmut2R	CTGCACTTACACCGCAAACCCRAAAAAAACGGATCCGGAGCGC
slipmut3F	GCGCTCCGGATCCGTTGGGAAACGGGTTTGCGGTG
slipmut3R	CACCGCAAACCCGTTTCCCAACGGATCCGGAGCGC
slipmut5F	GCTCCGGATCCGTTGGGTTTGGGTTTGCGGTGTAAGTGC
slipmut5R	GCACTTACACCGCAAACCCSAAACCAACGGATCCGGAGC
slipmut6F	GATCTAGCGCTCCGGATCCGTTAAAAAATGGGTTTGCGGTGTAATGTC
slipmut6R	GCACCTACACCGCAAACCCATTTTAAACGGATCCGGAGCGCTATGTC
SARSuadF	CGTTTTTAAADGGGTTTGCGGTGTAAGTGCAGC
SARSuahDLR	AACCHTTTTAAAAACGGATCCGTCGACATTG
SARSguuF	CGTTGGGTTTTGGGTTTGCGGTGTAAGTGCAGC
SARSguuDLR	AACCCAAAACCAACGGATCCGTCGACATTG
SARSaacF	CGTTAAAAAACGGGTTTGCGGTGTAAGTGCAGC
SARSaacDLR	AACCCGTTTTTAAACGGATCCGTCGACATTG
SARSscacF	CGTTCCCAAACGGGTTTGCGGTGTAAGTGCAGC
SARSscacDLR	AACCCGTTTGGGAACGGATCCGTCGACATTG
Hind II Fluc	TTCAAGCTTACAATTGGACTTTC
Bcu I RLuc	CCACTAGTAATGACTTCGAAGTTATG

In general, the slippery site can be defined as N NNW WWH, where N is any three identical bases; W is A or U; and H is A, C, or U (the frame of the initiator AUG is indicated by the spacing) (Dinman et al. 1991; Brierley et al. 1992; Dinman and Wickner 1992). The SARS wild-type slippery site was mutated from the native UUUAAC sequence to an assortment of variations that conform to the known N NNW WWH requirements. Mutagenesis was performed using the Stratagene QuikChange XL II kit using oligonucleotides synthesized and purified by IDT (Table 4). All mutations were confirmed by sequencing. Sequences of genes encoding tRNAs and known tRNA were obtained from the Web site <http://www.uni-bayreuth.de/departments/biochemie/sprnzl/trna/> (Sprinzl et al. 1998).

ACKNOWLEDGMENTS

We thank members of Dinman lab for invigorating discussion and Dr. J. DeSefano for the Jurkat cells. This work was supported by a grant to J.D.D. from the NIH (GM58859).

Received September 13, 2005; accepted December 20, 2005.

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