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Expression of the human immunodeficiency virus frameshift signal in a bacterial cell-free system: influence of an interaction between the ribosome and a stem–loop structure downstream from the slippery site

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ABSTRACT

A –1 frameshift event is required for expression of the *pol* gene when ribosomes translate the mRNA of human immunodeficiency virus type-1 (HIV-1). In this study, we inserted the frameshift region of HIV-1 (a slippery heptanucleotide motif followed by a stem–loop) in a reporter gene coding for firefly luciferase. The ability of the corresponding mRNA, generated by *in vitro* transcription, to be translated in an *Escherichia coli* cell-free extract is the first demonstration that the HIV-1 frameshift can be reproduced in a bacterial cell-free extract, providing a powerful approach for analysis of the frameshift mechanism. The responses of the frameshift signal to chloramphenicol, an inhibitor of peptide bond formation, and spectinomycin, an inhibitor of translocation, suggest that the frameshift complies with the same rules found in eukaryotic translation systems. Furthermore, when translation was performed in the presence of streptomycin and neamine, two error-inducing antibiotics, or with hyperaccurate ribosomes mutated in S12, the frameshift efficiency was increased or decreased, respectively, but only in the presence of the stem–loop, suggesting that the stem–loop can influence the frameshift through a functional interaction with the ribosomes.

INTRODUCTION

A variety of viruses use a programmed –1 frameshift to synthesize their replicases. A well-known paradigm is human immunodeficiency virus type-1 (HIV-1), which uses a programmed –1 frameshift during translation of its mRNA to achieve synthesis of the Gag–Pol polyprotein, the precursor of the viral protease, reverse transcriptase and integrase (1–5). Standard translation of the same retroviral mRNA produces Gag, the precursor of structural proteins. The ratio of Gag to Gag–Pol proteins is critical for viral assembly and replication (6,7), which makes the ribosomal frameshift a potential anti-retroviral target (8). Several studies aimed at characterizing the ribosomal frameshifting signals in HIV-1 have been performed either in cultured eukaryotic cells or in a rabbit reticulocyte lysate *in vitro* translation system. In these studies, mRNAs encoded Gag and Gag–Pol, or, alternatively, the frameshift region of HIV-1 was inserted at the beginning of the coding region of a reporter gene, so that its expression was dependent upon the frameshift (9–15). It was found that the frameshift of HIV-1 occurs at a slippery heptamer, U UUU UUA, where the unshifted frame is indicated, followed at a distance of 7 nt by a potential stem–loop structure, whose existence was recently supported by enzymatic probing (16). This stem–loop does not appear to influence the frameshift in most *in vitro* studies, whereas it was found to stimulate the frameshift when the assays were performed in cultured cells. The HIV-1 frameshift was reproduced in yeast by Wilson *et al*. (17), who found that the stem–loop structure was dispensable, a conclusion challenged by Stahl *et al*. (15), who demonstrated a stimulatory effect of the stem–loop. The HIV-1 frameshift has also been reproduced in bacteria, using an appropriate reporter gene (18–20), but so far it has never been reproduced in a bacterial cell-free system.

The model that is currently favored to account for the ribosomal frameshift of HIV-1 is the so-called 'two-tRNA simultaneous slippage' (2,21). In this model, a minority of ribosomes bearing the aminoacyl-tRNA and the peptidyl-tRNA in the A and P sites, respectively, move back by 1 nt on the mRNA prior to peptide bond formation when they reach the slippery sequence. This model, initially proposed for retroviruses, also applies to a variety of other viruses, such as coronaviruses and the yeast L-A double-stranded RNA virus. Prokaryotic ribosomes use a large variety of mechanisms to promote frameshifting, depending upon the signals they encounter on the mRNA, and there are well-documented cases where they use the same mechanisms as eukaryotic ribosomes (reviewed in 3,4).

In this study, we show for the first time that the frameshift of HIV-1 can be reproduced in a bacterial cell-free extract and compare the frameshift efficiency in a bacterial cell-free extract and in bacteria. Second, using spectinomycin, an inhibitor of translocation, and chloramphenicol, an inhibitor of peptide bond formation, we show that the frameshift occurs before and not after peptide bond formation, thus indicating

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that it conforms to the rules that govern programmed –1 frameshifting in eukaryotes. Finally, we also show that under conditions that make the ribosomes either hyperaccurate or error-prone, the stem–loop structure downstream from the slippery sequence influences the frameshift.

MATERIALS AND METHODS

Escherichia coli strains

Escherichia coli RD100, which is wild-type with respect to ribosomal proteins (22), was a gift from Dr G. A. Mackie and was used for the preparation of bacterial extracts. *In vivo* studies were performed with *E.coli* K12 HMS174(DE3) (23), which contains the gene for T7 RNA polymerase under control of a *lac* promoter, inducible with isopropyl-β,D-thiogalactopyranoside (IPTG). The cloning experiments and the site-directed mutagenesis were done in *E.coli* DH5α (Gibco BRL) and in *E.coli* CJ236 (Bio-Rad), respectively.

Construction of plasmids containing an N-terminally modified luciferase gene

Plasmid pLucNWT is a derivative of pBluescriptSK– (Stratagene), in which an *Apa*I–*Sac*I fragment containing the luciferase gene from pGEM-*luc* (Promega) was inserted between the *Apa*I and *Sac*I sites, and where a *Nco*I site surrounding the initiator AUG triplet was created by the method of Kunkel *et al.* (24). Plasmid pSDLucNWT, a derivative of pLucNWT, was generated by inserting an oligonucleotide cassette containing a Shine–Dalgarno sequence, upstream from the initiator AUG. Plasmid pSDLuc–1(SHS + SL) was engineered by inserting a cassette encompassing the slippery heptanucleotide sequence (SHS) and the downstream stem–loop (SL) structure of the HIV-1 Gag–Pol region. The insertion is flanked by a *Bcl*I site 3′, so that the HIV-1 frameshift signals can be readily exchanged for other frameshift signals. Plasmid pSDLuc–1(SHS – SL) was derived from pSDLuc–1(SHS + SL) by deleting the stem–loop structure, using site-directed mutagenesis (24). In both pSDLuc–1 plasmids, luciferase expression requires a –1 frameshift. The in-frame corresponding controls, pSDLuc0(SHS + SL) and pSDLuc0(SHS – SL) were derived by adding an adenine immediately after the slippery heptanucleotide sequence (Fig. 1). Plasmid pLRCAT is a derivative of pBluescriptSK– (Stratagene), in which a fragment containing the chloramphenicol acetyltransferase (CAT) gene from pACY184 (25) was inserted in the *Cla*I site. The various plasmid constructs containing the luciferase or the pLRCAT plasmid were digested with *Sal*I and with *Bam*HI, respectively, and used for mRNA *in vitro* synthesis with T7 RNA polymerase following standard protocols. For *in vivo* studies, the *Apa*I site in the pSDLuc series was replaced with a *Bam*HI site and the *Bam*HI–*Sal*I fragment from these plasmids, containing the modified *luc* coding sequence plus the upstream Shine–Dalgarno sequence, was inserted into pET24 (Novagen), thus generating pETLuc–1(SHS + SL) and pETLuc–1(SHS – SL), as well as the corresponding in-frame controls. In all plasmid constructs, the sequences of the insertions were confirmed by dideoxy sequencing. For all constructs, a derivative was obtained by site-directed mutagenesis, where the slippery sequence, U UUU UUA, was substituted with a non-slippery sequence, U CCC GCG. These mutants were used as negative controls to demonstrate that the synthesis of luciferase from Luc–1 mRNAs was completely dependent upon a frameshift at the slippery sequence under the conditions of the assays.

Preparation of the *E.coli* cell-free expression system and *in vitro* translation

The protocol used for the preparation of the bacterial extract (S-30) was adapted from Mackie *et al.* (26), and *in vitro* translation was performed as described by Lesley (27), with minor modifications. All reactions were carried out for 25 min at 30°C in 50 µl samples containing 20 µl of S30 and the following components (final concentrations): 69 mM Tris–acetate, pH 7.9, 190 mM potassium glutamate, pH 8.0, 30 mM ammonium acetate, 2 mM dithiothreitol, 13 mM magnesium acetate, 18 mM potassium acetate, 2.25 mM ATP, 500 µM GTP (dilithium salt), 23 mM phosphoenol pyruvate (freshly prepared), 0.1 µg pyruvate kinase, 0.1 mg/ml *E.coli* tRNA, 35 mg/ml polyethylene glycol (8000 M_r), 20 µg/ml folinic acid, 13 U RNA guard® and 200 µM each of the 20 amino acids. The reaction was initiated by the addition of 2 µg, unless otherwise stated, of luciferase mRNA with and without various insertions from the HIV-1 Gag–Pol region, and 2 µg of native CAT mRNA as a competitor. The reaction was stopped by addition of EDTA at a final concentration of 6.5 mM. For experiments with antibiotics, translation reactions were incubated with or without the antibiotic at 30°C for 10 min before addition of mRNA. Luciferase activity was monitored in 10 µl aliquots from the translation mixtures with a Berthold Lumat LB 9507 luminometer, using a commercial luciferase assay system (Promega).

In vivo studies

To assess the efficiency of frameshifting of the bacterial ribosomes *in vivo*, the system of Studier *et al.* (23) was used, in which the reporter genes are expressed under control of a T7 promoter in a host strain containing an IPTG-inducible T7 RNA polymerase. The bacteria were transformed with each of the pET constructs containing the different derivatives of the *luc* gene. Stationary phase cultures, in M9 minimal medium containing 0.2% glucose, 0.4% casamino acids, 1 µg/ml thiamine and the antibiotics appropriate to maintain plasmid selection, were diluted 1/20 in fresh M9 medium, and expression of the *luc* gene was induced by adding 0.1–0.2 mM IPTG and the cultures were grown at 30°C to an optical density of 0.3–0.4 at 600 nm. Then, 45 µl aliquots of the cultures were processed and brought to 200 µl in a luciferase lysis buffer (25 mM Tris–phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% glycerol and 1% Triton X-100), as described in Promega Technical Bulletin 101, and 2.5 µl aliquots of the resulting lysates were used to monitor the luciferase activity as indicated above for the *in vitro* assays. The experimental values were normalized for equal numbers of cells, as assessed by the optical density at 600 nm.

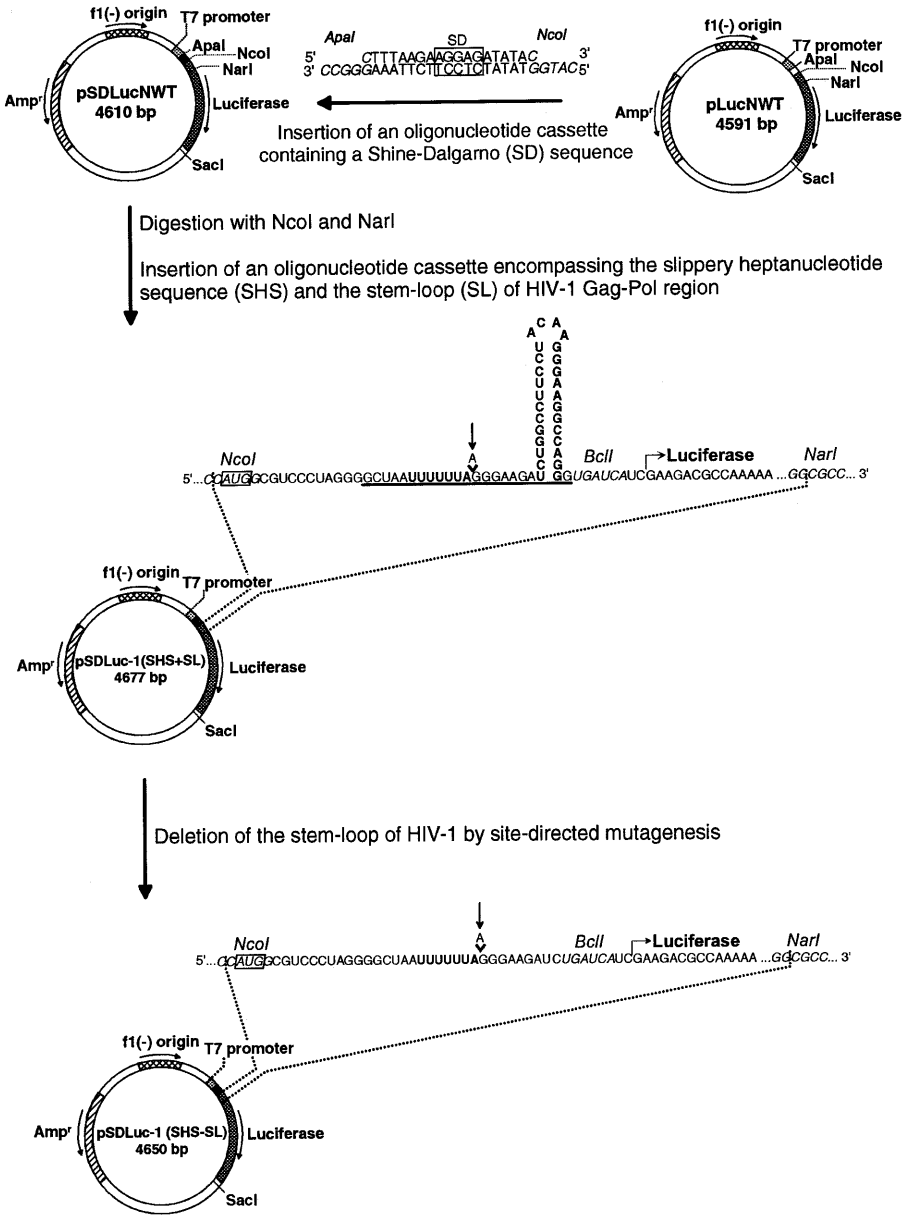

Figure 1. Structure of the luciferase expression vectors used in this study. Plasmid pLucNWT contains the luciferase reporter gene under control of a T7 promoter and a *Nco*I site surrounds the initiator AUG of the *luc* coding sequence. A derivative of pLucNWT, pSDLucNWT, was constructed by inserting a cassette containing a Shine–Dalgarno sequence (SD) upstream from the *Nco*I site. The HIV-1 frameshift region, encompassing a slippery heptanucleotide sequence followed by a stem–loop structure (in bold), was then inserted between the *Nco*I and *Nar*I sites, generating pSDLuc–1(SHS + SL). The sequence derived from HIV-1 is underlined. Plasmid pSDLuc–1(SHS – SL) was obtained by deleting the sequence coding for the stem–loop, by site-directed mutagenesis. Corresponding control plasmids, pSDLuc0(SHS + SL) and pSDLuc0(SHS – SL), where the luciferase coding region is in-frame with the insertion, were derived from the Luc–1 plasmids by adding an adenine residue immediately after the slippery sequence (indicated by an arrow). Details on the constructions are provided in Materials and Methods.

RESULTS

The HIV-1 frameshift can be reproduced in a bacterial extract

mRNAs encoding firefly luciferase, with an insertion that contains the slippery heptanucleotide sequence plus the stem–loop of the HIV-1 Gag–Pol region, were translated in an *E.coli* cell-free translation system. Equal amounts of Luc–1 and Luc0 mRNAs were used (0.2–2 μg), in the absence or presence of a constant amount of CAT mRNA (2 μg) that competes for the ribosomes but does not contain any slippery sequence. The frameshift efficiency was assessed by measuring the luciferase activity with the trans-frame mRNA (Luc–1) and the corresponding in-frame (Luc0) control, and, unless otherwise stated, expressed as the ratio of the trans-frame activity to that of the in-frame activity. The results are presented in Table 1. The luciferase activity was ~3-fold lower for Luc0 compared to Luc wild-type mRNA, which can be ascribed to the extension in the N-terminal region of the enzyme. The results show that the bacterial ribosomes can frameshift when they reach the

Table 1. Frameshift efficiency for decoding of the HIV-1 Gag–Pol frameshift region in an *E.coli* cell-free translation system

Luc mRNA (μg)	In the absence of CAT mRNA			In the presence of CAT mRNA		
	Luciferase activity		Frameshift efficiency (%)	Luciferase activity		Frameshift efficiency (%)
	Luc0	Luc–1		Luc0	Luc–1	
0.2	1 473 387	46 207	3.1	861 581	27 456	3.2
0.5	3 039 864	89 328	2.9	1 587 279	41 671	2.6
1.0	5 323 968	145 579	2.7	2 763 206	77 866	2.8
2.0	8 666 365	255 867	3.0	4 429 701	136 077	3.1

Increasing amounts of Luc0 or Luc–1 mRNA were translated in a 50 μl translation mixture, in the presence or absence of 2 μg of chloramphenicol acetyltransferase (CAT) mRNA, which does not contain a frameshift sequence and competes for the ribosomes. Luciferase activity is expressed in relative light units, and results are the means of four independent experiments corresponding to 10 μl aliquots from the translation mixtures. The standard deviation of the means was ≤15%. Frameshift efficiency is expressed as described in the text (Results). When 2 μg of wild-type Luc mRNA, without a frameshift sequence, was translated in the absence or presence of CAT mRNA, luciferase activity was 19 242 460 and 12 395 641, respectively.

Table 2. Influence of the stem–loop structure on the frameshift efficiency of bacterial ribosomes

Conditions	Luciferase activity		Frameshift efficiency (%)
	Luc0	Luc–1	
In vitro			
Luc (SHS + SL) mRNA	4 429 701	136 077	3.1
Luc (SHS – SL) mRNA	6 817 324	193 285	2.8
In vivo			
Luc (SHS + SL) mRNA	6 645 265	907 483	13.7
Luc (SHS – SL) mRNA	8 719 208	550 541	6.3

Luc (SHS + SL) mRNAs contain the slippery heptanucleotide sequence followed 3′ by the stem–loop structure, whereas Luc (SHS – SL) mRNAs contain only the slippery heptanucleotide sequence. Luciferase activity is expressed in relative light units. Results are the means of at least four independent experiments and correspond to 10 μl aliquots for the *in vitro* assays and 2.5 μl aliquots from cell lysates processed as described in Materials and Methods. Representative values corresponding to cultures grown to an optical density of 0.3 at 600 nm, when Luc expression was induced with 0.15 mM IPTG, are shown for the *in vivo* assays. Standard deviations of the means were ≤15% and ≤20% for *in vitro* and *in vivo* assays, respectively.

HIV-1 frameshifting sequence. The frameshifting efficiency is ~3%, one-third of the value (9.7 ± 0.8) which we obtained with the same mRNAs translated in a rabbit reticulocyte lysate (data not shown). When the slippery sequence (U UUU UUA) was mutated to a non-slippery sequence (U CCC GCG), the frameshift efficiency was <0.1% (data not shown), demonstrating that the luciferase activity depends on a frameshift at this slippery sequence and not on a secondary initiation. The frameshift efficiency was independent of the Luc mRNA concentration and on the presence of the competitor CAT mRNA, at a concentration reducing translation efficiency by 50%. Further *in vitro* assays were performed with 2 μg of Luc mRNA, in the presence of 2 μg of CAT mRNA, since in bacteria a reporter mRNA is always translated in the presence of other mRNAs. Having established that the HIV-1 frameshift can be reproduced in a bacterial cell-free extract, we next assessed the role of the stem–loop structure following the shift site.

The downstream stem–loop structure is dispensable for frameshifting *in vitro*

The frameshift efficiency of bacterial ribosomes was investigated with Luc mRNAs containing the slippery sequence from HIV-1 but where the stem–loop structure following the slippery sequence was deleted (Fig. 1). Table 2 compares the frameshift efficiency of Luc mRNAs, with and without the stem–loop, in the bacterial cell-free system and in bacteria. The smaller size of the extension in the N-terminal portion of luciferase probably accounts for its higher activity when it is synthesized from Luc0 mRNA without the stem–loop, compared to Luc0 with the stem–loop. It can be seen that the frameshift efficiency is not significantly affected by absence of the stem–loop *in vitro*. Similarly, we observed that absence of the stem–loop did not affect the frameshift efficiency in a rabbit reticulocyte translation system (data not shown). *In vivo* the frameshift efficiency was ~14% with the construct containing the stem–loop, and mutagenesis of the shift site decreased the frameshift efficiency to <0.4% (data not shown), showing that the luciferase activity with the Luc–1 construct results from a frameshift at this site, as was shown *in vitro*. In contrast to what was observed *in vitro*, the frameshift efficiency was decreased ~2-fold in the absence of the stem–loop, which confirms the stimulatory effect of this structure under *in vivo* conditions.

Ribosomal mutations or antibiotics that bind to the ribosomes influence the frameshift efficiency

In addition to the characteristics of the shift region in the mRNA, it is likely that the ribosomes are also involved in the control of the efficiency of the frameshift process. To gain

Table 3. Effect of a ribosomal mutation on the frameshift efficiency in the presence or absence of the stem–loop structure in a bacterial cell-free system and in bacteria

Ribosomes	Relative frameshift efficiency	
	With the stem–loop structure	Without the stem–loop structure
In vitro		
Wild-type	23 ± 3	22 ± 3
With mutated S12	13 ± 2	21 ± 3
In vivo		
Wild-type	100	46 ± 8
With mutated S12	98 ± 12	50 ± 7

Frameshift efficiency was assessed by measuring luciferase activity generated by translation of the different types of Luc mRNA (with a –1 frame and in-frame with the Luc coding sequence and where the slippery heptanucleotide sequence is followed or not by the stem–loop structure) in an *E.coli* cell-free translation system and in bacteria. A value of 100 was arbitrarily ascribed to the frameshift efficiency *in vivo* with wild-type ribosomes and Luc mRNA containing the slippery heptanucleotide sequence followed by the stem–loop. Results are the means ± SD of five independent experiments.

further insights into the role of the ribosomes, the frameshift efficiency was investigated *in vitro* and *in vivo* with bacteria harboring a mutation in the 30S protein S12. These bacteria, obtained by selecting spontaneous mutants resistant to streptomycin, are known to contain hyperaccurate ribosomes (28), and we verified that these ribosomes have an increased translational fidelity by determining that they restrict the read-through of nonsense codons (data not shown). In a cell-free bacterial extract, we found that the frameshift efficiency was decreased 2-fold with hyperaccurate ribosomes, but only in the presence of the stem–loop. In contrast, *in vivo* the frameshift efficiency was not affected by the S12 mutation whether the stem–loop was present or not on the reporter mRNA (Table 3). Identical results were obtained with different S12 mutants selected independently (data not shown).

The frameshift efficiency was also assessed in a bacterial cell-free translation system with wild-type ribosomes in the presence of various inhibitors of protein synthesis: streptomycin and neamine (two aminoglycoside antibiotics that stimulate translational misreading), chloramphenicol (an inhibitor of peptidyl transferase activity), and spectinomycin (an inhibitor of translocation) (29–32). The concentrations of antibiotics used were sub-inhibitory and decreased overall translation by <50%. The effects of the different drugs are summarized in Figure 2. This figure compares the luciferase activity at different concentrations of drugs, with in-frame or –1 frame mRNAs, which contain HIV-1 slippery sequence followed or not by the stem–loop structure. It can be seen that in the presence of streptomycin or neamine, luciferase activity with the Luc–1 mRNA decreased less than that with the Luc0 mRNA, indicating that frameshift efficiency increases under these conditions. This effect was modest but reproducible, and was observed only when the stem–loop was present. Chloramphenicol also increased the frameshift efficiency, but independently of the presence or absence of the stem–loop. Again, the changes were modest but significant. Spectinomycin did not affect the frameshift, whether the stem–loop was present or absent.

DISCUSSION

The HIV-1 programmed –1 frameshift occurs efficiently in an *E.coli* cell-free system

In this study, we demonstrated that prokaryotic ribosomes can efficiently recode the HIV-1 Gag–Pol frameshift sequence inserted in the mRNA of a firefly luciferase reporter gene. Under the conditions of our *in vitro* assays, the frameshift efficiency was ~3%. This is comparable to the frameshift efficiency observed for the same sequence with mammalian ribosomes, which was reported to vary between 3 and 10% in a rabbit reticulocyte lysate, depending upon the system used to monitor the frameshift efficiency (11,14 and references therein). Differences between the tRNA populations (33,34) also likely contribute to differences in frameshift efficiency between bacterial and mammalian translation systems. In bacterial cultures, the frameshift efficiency increased to ~14%. The rate of translation is higher in bacteria than in a cell-free bacterial extract, and we suggest that this higher rate of translation accounts for the higher frameshift efficiency observed *in vivo*. This suggestion is in agreement with previous observations from Falk *et al*. (35), showing with eukaryotic ribosomes that frameshift efficiency is elevated in high translation rate systems, and also from Garcia *et al*. (36), who found that the frameshift efficiency for ribosomes encountering a plant viral slippery sequence is higher in a reticulocyte lysate than in a wheat germ extract, a less efficient translation system. In previous studies with bacteria where a reporter gene contained the slippery sequence from HIV-1 with (18) or without (19,20) the downstream stem–loop, the frameshift efficiency was 2 and 1%, respectively, lower than the value of 14% that we observed in the present study. The reason for this discrepancy is not clear, but it likely results from differences in the experimental conditions and/or in the stability and abundance of the reporter mRNA used.

The stem–loop structure downstream from the heptanucleotide slippery sequence of HIV-1 is dispensable for the *in vitro* frameshift

We found that the frameshift efficiency with bacterial ribosomes was unaffected by the presence of the stem–loop

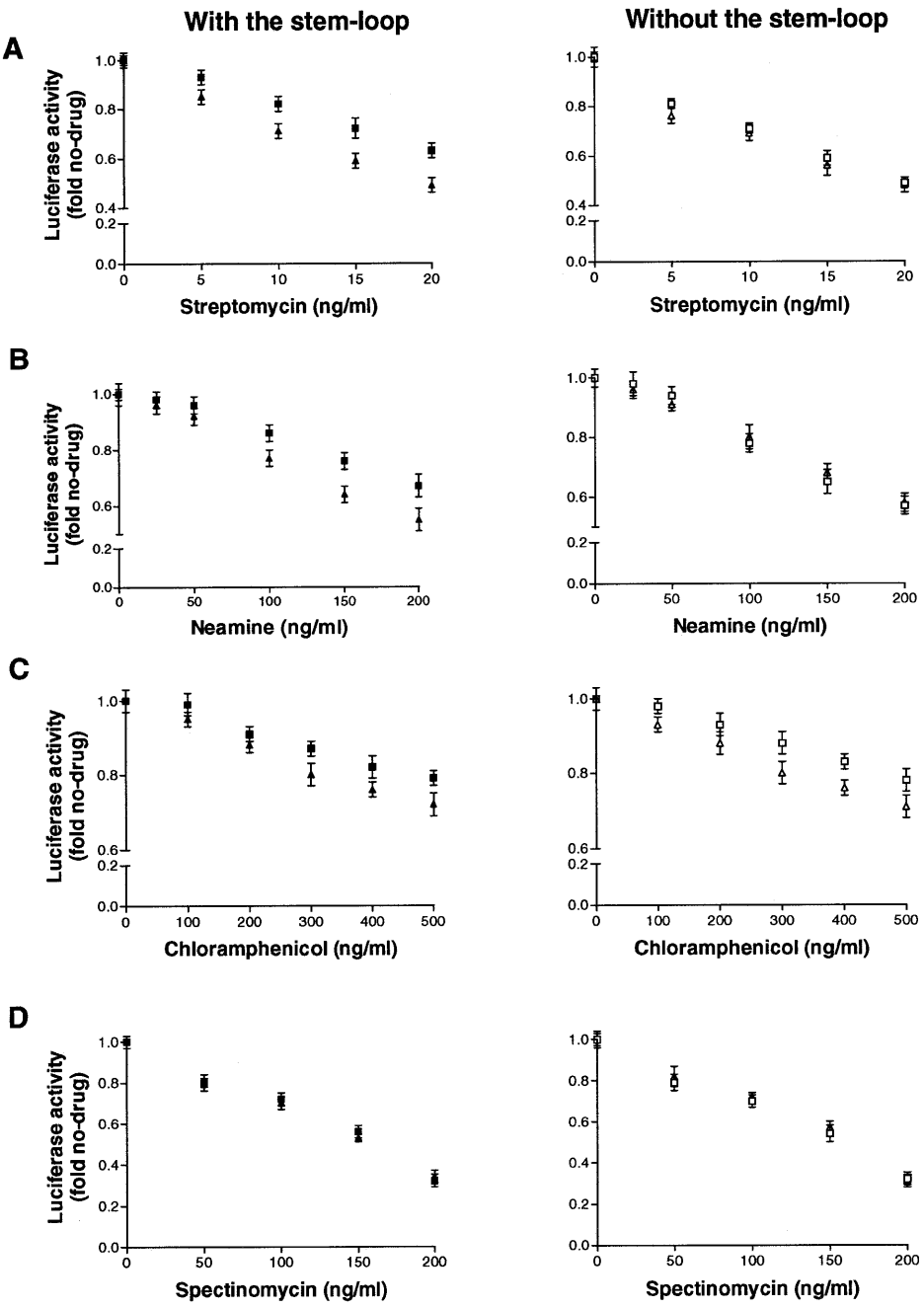

Figure 2. Effect of antibiotics inhibiting specific steps of the elongation cycle on the HIV-1 programmed –1 ribosomal frameshift. The Luc–1 and Luc0 mRNAs, containing either the slippery heptanucleotide sequence plus the stem–loop of the HIV-1 frameshift region (left) or only the slippery heptanucleotide sequence (right), were translated in an *E.coli* cell-free translation system, in the absence or presence of different antibiotics. A value of 1 was arbitrarily ascribed to luciferase activity in the absence of antibiotic. The results represent the averages of at least five independent experiments. Highest and lowest values observed are indicated by error bars. (Filled triangle), Luc0(SHS + SL) mRNA; (filled square), Luc–1(SHS + SL) mRNA; (open triangle), Luc0(SHS – SL) mRNA; (open square), Luc–1(SHS – SL) mRNA.

structure downstream from the slippery sequence in a cell-free system, whereas it is stimulated ~2-fold by the same stem–loop *in vivo*. A similar observation has been made with eukaryotic ribosomes, where the frameshift efficiency for the HIV-1 slippery sequence was much more dependent on the presence of the stem–loop in cultured cells than *in vitro* (10,11,14 and references therein). *In vivo* ribosomes move along the mRNA at a high rate and we suggest that when they contact a stem–loop downstream of a slippery sequence, as is the case in the HIV-1 frameshift region, they are slowed down during the period of time required for their associated helicase activity to melt the stem–loop. Indeed, the stimulatory effect on frameshifting of a stem–loop following the HIV-1 slippery sequence was directly related to the stability of this stem–loop, when assayed in yeast and in cultured mammalian cells (37). However, *in vitro* ribosomes move at a slower rate and our

results suggest that the necessity of melting the stem–loop does not appear to further slow down the ribosomes. This would account for the lack of effect or the weak effect of the stem–loop on frameshift efficiency, as shown by our results and in agreement with the literature (10,11,14). However, this does not apply to pseudoknot structures at the sites of ribosomal frameshift, which are very stable (38) and likely more difficult to unwind. Assays in a eukaryotic *in vitro* translation system demonstrated that a slippery sequence followed by a pseudoknot causes the ribosomes to pause, which increases the probability of a frameshift (39,40). Interestingly, when the HIV-1 slippery sequence was inserted into a reporter mRNA followed by a pseudoknot structure from mouse mammary tumor virus or from a coronavirus, the *in vitro* frameshift efficiency increased ~3- to 5-fold compared to the construct containing the stem–loop, demonstrating a substantial stimulatory effect of the pseudoknots (41,42).

Chloramphenicol but not spectinomycin increases HIV-1 ribosomal frameshifting

The availability of the bacterial *in vitro* frameshift assay allowed us to take advantage of various well-characterized inhibitors of the bacterial translational machinery. Two drugs were first investigated for their effect on HIV-1 ribosomal frameshifting in an *E.coli* cell-free translation system: chloramphenicol (which interferes with peptide bond formation), and spectinomycin (which blocks translocation). Spectinomycin did not affect the ribosomal frameshift, whether the stem–loop was present or absent. This does not support a previous suggestion by Weiss *et al*. (18) that the HIV-1 programmed frameshift could occur after peptide bond formation. Indeed, if this had been the case, an inhibitor of translocation would have increased frameshift efficiency, by leaving more time for the ribosomes to shift. In agreement with our observations, Tumer *et al*. (43) observed that an inhibitor of translocation did not affect the programmed –1 frameshift in the yeast L-A double-stranded RNA virus. In contrast to spectinomycin, chloramphenicol, an inhibitor of peptide bond formation, was found to slightly but reproducibly increase frameshift efficiency, whether the stem–loop was present or not. The classic two-tRNA slippage model (2,21) proposes that the ribosomal frameshift occurs prior to peptide bond synthesis with the ribosome bearing the aminoacyl-tRNA and the peptidyl-tRNA in the A and P sites, respectively. An inhibitor of peptide bond synthesis such as chloramphenicol should therefore increase frameshift efficiency, as shown by our results. The same observation was made by Dinman *et al*. (44), when investigating the effect of sparsomycin, a peptidyl transferase inhibitor, that increased the programmed –1 frameshift in the yeast L-A double-stranded RNA virus. One could argue here that Dinman *et al*. (44) showed that, in contrast to sparsomycin, anisomycin, another peptidyl transferase inhibitor, decreased the programmed –1 frameshift. However, as discussed by these authors, this decreased frameshift likely results from the fact that translation preferentially aborts on frameshifted ribosomes in the presence of anisomycin.

A refinement of the classic two-tRNA simultaneous slippage model

Our results with chloramphenicol and spectinomycin appear to support the hypothesis that HIV-1 frameshifting follows the rules of the classic two-tRNA slippage model (2,21) in bacterial extracts. However, a drawback of this model is that peptide bond formation takes place as soon as the aminoacyl-tRNA reaches the A site, which does not leave much time for the frameshift to occur. The incoming aminoacyl-tRNA is delivered to the ribosome as a ternary complex (aminoacyl-tRNA·GTP·EF-Tu), which first interacts with an entry site (A/T), from where it moves to the A site after GTP hydrolysis and release of EF-Tu·GDP (reviewed in 45,46; Fig. 3A). This step is rate limiting in protein synthesis (46). To solve the problem raised by the transient character of the state where the two-tRNA slippage was proposed to occur, we suggest that it could occur before occupancy of the A site (Fig. 3B), with the peptidyl-tRNA in the P site and the aminoacyl-tRNA complexed to EF-Tu in the A/T site, either before or after GTP hydrolysis. This suggestion, which was also envisaged by Farabaugh (4), is supported by the characterization of mutants in the yeast counterpart of EF-Tu that increase programmed –1 frameshifting in the yeast L-A double-stranded RNA virus, probably because of interference with GTP hydrolysis (47). However, our results do not exclude the possibility that, for some of the ribosomes that shift the reading frame, the aminoacyl-tRNA occupies the A site, as proposed by the classic two-tRNA slippage model. Moreover, one-tRNA slippage, with ribosomes bearing the peptidyl-tRNA in the P site but having an empty A site (19,20; reassessed in 3), could also contribute to the frameshift.

The stem–loop structure downstream from the heptanucleotide slippery sequence of HIV-1 influences the frameshift by interacting with the ribosome

As discussed above, the frameshift efficiency of wild-type ribosomes traversing the HIV-1 Gag–Pol region is not affected by absence of the stem–loop in a cell-free system. Interestingly, when we performed the *in vitro* translation assays with hyperaccurate ribosomes harboring an altered 30S protein S12, the frameshift efficiency was decreased, but only in the presence of the stem–loop structure. In the same line, in the presence of the error-inducing drugs streptomycin and neamine, the frameshift efficiency was increased, but again only in the presence of the stem–loop. Our interpretation of these results, in the light of the refined model of two-tRNA slippage, is that several parameters modulate the frameshift efficiency with mutant ribosomes or in the presence of the error-inducing drugs. With hyperaccurate ribosomes, occupancy of the A site is slowed down (48). According to the refined model for frameshifting, we predict that this should increase the frameshift efficiency by providing more time for the ribosomes to shift before A site occupancy and instantaneous formation of the peptide bond. On the other hand, hyperaccurate ribosomes weaken the codon–anticodon interaction at the A site and consequently at the A/T site (49), which contributes to their increased capacity to correct translation errors. Since codon–anticodon interaction for the aminoacyl-tRNA in the post-slippage state relies on the interaction of two bases rather than three as in the pre-slippage state, we predict that this weakening of codon–anticodon interaction likely disfavors the post-slippage state. The converse situation is encountered with ribosomes in the presence of streptomycin or neamine, which strengthen the codon–anticodon interaction in the A site (49). This strengthening, by stabilizing binding of the tRNA in the

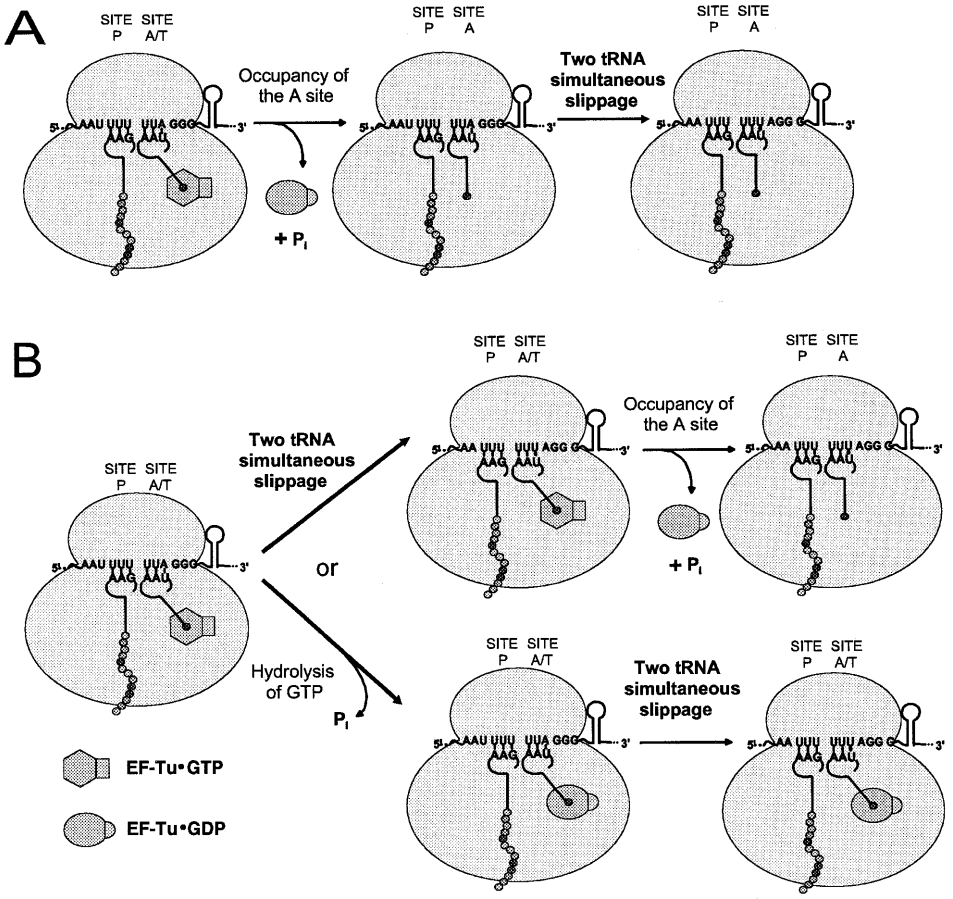

Figure 3. Models of the two-tRNA simultaneous slippage for the programmed –1 frameshift. (**A**) Classic model of two-tRNA simultaneous slippage according to Jacks *et al*. (21): the ribosome-bound peptidyl-tRNA^{Phe} in the P site and aminoacyl-tRNA^{Leu} in the A site slip back by 1 nt after occupancy of the A site by the aminoacyl-tRNA and prior to peptide bond formation. (**B**) Refined model of simultaneous slippage where the ribosome-bound peptidyl-tRNA in the P site and aminoacyl-tRNA in the entry site (A/T), complexed to EF-Tu bound to either GTP or GDP, slip back by 1 nt before occupancy of the A site by the aminoacyl-tRNA.

post-slippage state, could favor the shift. However, occupancy of the A site is accelerated by streptomycin or neamine (48), which should disfavor the shift by leaving less time for the ribosomes to shift before peptide bond formation. We propose that these antagonistic effects account for the lack of influence of S12 mutations or error-stimulating antibiotics on frameshift efficiency in the absence of the stem–loop structure.

However, in the presence of the stem–loop, frameshift efficiency is decreased by S12 mutations whereas it is increased in the presence of the error-inducing drugs. The binding of streptomycin or neamine or the presence of mutations in S12 are known to induce conformational changes in the ribosomes (50,51 and references therein). One attractive hypothesis is that when ribosomes mutated in S12 encounter the stem–loop, they undergo a transition to a conformation which is less prone to slip. Conversely, ribosomes bound to streptomycin or neamine would undergo a transition to a conformation which is more prone to slip. This hypothesis therefore suggests that there is a functional interaction between the ribosome and the stem–loop structure, and not merely a physical contact. In line with this suggestion, Tinoco and co-workers (52,53), who studied the conformation of pseudoknots that influence frameshifting by NMR, found that a specific bent conformation is required for these pseudoknots to stimulate the frameshift, which implies that they do not just act as a barrier to movement of the ribosome but could specifically interact with the ribosome. However, *in vivo* the frameshift efficiency was unaffected when ribosomes were hyperaccurate. As discussed above, ribosomes move along the mRNA at a higher rate *in vivo* than *in vitro*. This enables the downstream stem–loop to stimulate the frameshift, but more subtle effects detected when ribosomes move at a slower pace, such as those resulting from ribosomal mutations, can be masked. Altogether, our results indicate that the cell-free translation system appears more appropriate to identify the ribosomal components involved in the interaction with the stem–loop.

CONCLUSION

The *E.coli* cell-free translation system constitutes a powerful system to gain further insights into the mechanisms that control the programmed –1 ribosomal frameshift of the Gag–Pol region of HIV-1. Our results suggest that ribosomes interact with the stem–loop structure 3′ to the slippery sequence and show that ribosomes with an altered control of translational accuracy are good candidates to further investigate

this interaction. A variety of mutations in ribosomal proteins or RNA have been described that either increase or decrease the control of translation accuracy (28,54,55). These mutants will probably provide important information on the interaction between the ribosome and the stem–loop, which constitutes a suitable target for the development of novel anti-retroviral drugs.

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