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Human Immunodeficiency Virus Type 1 *gag-pol* Frameshifting Is Dependent on Downstream mRNA Secondary Structure: Demonstration by Expression In Vivo

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The human immunodeficiency virus type 1 (HIV-1) Gag-Pol fusion polyprotein is produced via ribosomal frameshifting. Previous studies in vitro and in *Saccharomyces cerevisiae* have argued against a significant role for RNA secondary structure 3′ of the shift site, in contrast with other systems, in which such structure has been shown to be required. Here we show, by expressing the HIV-1 *gag-pol* domain in cultured vertebrate cells, that a stem-loop structure 3′ of the HIV-1 shift site is indeed important for wild-type levels of frameshifting in vivo.

Many retroviruses utilize translational frameshifting in order to generate *pol* gene products, leading to the production of a fixed ratio of Gag to Gag-Pol polyproteins (1, 7, 17, 18). This mechanism enables the *pol*-encoded gene products to be assembled into budding virions, as part of the Gag-Pol polyprotein, by virtue of signals contained in the *gag* region. In addition, frameshifting allows the virus to maintain a well-regulated ratio of Gag proteins to Gag-Pol proteins in infected cells, thereby ensuring efficient assembly of infectious particles (10, 32). Subsequent processing (primarily at the time of budding) by the viral protease generates the mature structural and enzymatic proteins found in virions (8, 36, 43).

The signals required to direct −1 ribosomal frameshifting have been well defined for some retroviruses (6, 19–22, 30), avian infectious bronchitis virus (2–4), the double-stranded RNA of *Saccharomyces cerevisiae* (9, 13), and *Escherichia coli dnaX* (11, 40, 41). For all of the −1 frameshifts described or proposed so far, a heptanucleotide sequence of the type XXXY YYZ can be found, followed by sequences which have the potential to form a secondary or even tertiary structure (18, 39). The heptanucleotide sequence appears to be important for the maintenance of two of three base pair formations between the codons and anticodons when the translating ribosome slips back one nucleotide; its sequence is also important for specifying the aminoacyl-tRNAs that mediate the shift (6, 19).

Of the frameshift sites that have been analyzed in detail so far in the genomes of viruses and viruslike particles, five have been shown to be dependent on RNA structural elements downstream of the frameshift site: Rous sarcoma virus (19), feline immunodeficiency virus (30), mouse mammary tumor virus (MMTV) *gag-pro* (6), avian infectious bronchitis virus (3), and yeast double-stranded RNA (9). In the latter four cases, the role of an RNA pseudoknot has been demonstrated and a structure more complex than a simple stem-loop is implied by deletion analysis of Rous sarcoma virus. In the case of human immunodeficiency virus type 1 (HIV-1), which also uses a −1 frameshift (20, 44), a stem-loop composed of 12 bp has been proposed (see Fig. 1), but no sequences complementary to those in the loop are present downstream, suggesting that a pseudoknot is not likely to form. Thus far, no role for the stem-loop structure in setting frameshifting efficiency has been demonstrated. In previous studies from this laboratory, chimeric mRNAs containing the HIV-1 shift site were used for in vitro translation in rabbit reticulocyte lysates (RRL); mutations of potentially base-pairing sequences produced no effect, but replacement of the downstream coding region, which removed the stem-loop, reduced frameshifting significantly (25). Another group, using chimeric constructs for both in vitro translation in RRL and in vivo expression in *S. cerevisiae*, found no effect of deletion of downstream sequences (44). Notably, no studies have yet been carried out in eukaryotic cells by using the HIV-1 shift site and stem-loop in its native context, i.e., as part of the complete *gag-pol* open reading frame. In this report, we describe such experiments, as well as some in vitro experiments, which demonstrate that the RNA secondary structure downstream of the shift site is necessary for wild-type levels of HIV-1 *gag-pol* frameshifting.

Frameshifting is strongly dependent on the stem-loop structure in vivo. To test the idea that the stem-loop structure 3′ of the HIV-1 shift site is required for frameshifting in vivo, site-directed mutagenesis (45) was used to change the nucleotide sequence of regions predicted to be involved in base pairing (Fig. 1). The mutations were created such that a restriction site (*Bsp*EI) was generated on either side of the stem (i.e., mutants 5′ and 3′); when the mutations are combined, base pairing is restored (mutant 5′3′). The double mutant was also cleaved with *Bsp*EI and religated to make an in-frame deletion of 18 nucleotides, which effectively eliminates the potential to form a stable stem-loop (mutant ΔSL).

The full-length HIV-1 *gag-pol* genes were inserted downstream of the human cytomegalovirus enhancer-promoter, replacing *lacZ* sequences in pON249 (14). A *rev* response element (a *Bam*HI-*Bgl*II fragment from the HXB2 provirus) was placed 3′ of the *pol* termination codon but 5′ of the simian virus 40 polyadenylation sequences present in the vector, so that the expression of *gag* and *gag-pol* could be *trans* activated by *rev* (35). *rev* was supplied by expression of its cDNA from the cytomegalovirus-driven vector pcREV

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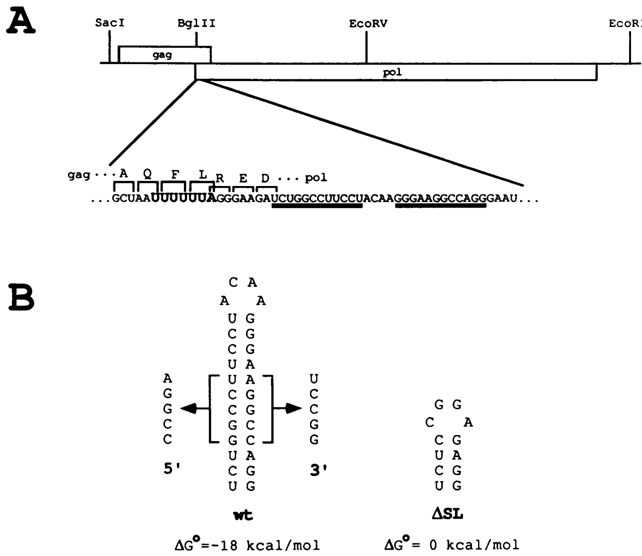

FIG. 1. HIV-1 *gag-pol* open reading frame arrangement and stem-loop mutations. (A) The overlapping reading frames encoding the HIV-1 Gag-Pol fusion polyprotein are shown as boxes above and below the line (not to scale). Relevant restriction sites are shown above the line. The nucleotide and amino acid sequence at the overlap is shown in detail below. Sequences proposed to base pair in the stem-loop structure are underscored. (B) Structure of the proposed stem-loop and mutants. The wild-type structure is drawn on the left, with the 5′ and 3′ mutations indicated; the 5′3′ mutant contains both of these mutations. Residual base-pairing possibilities in the deletion mutant ΔSL are shown on the right. Free energies of formation for both structures are indicated and were calculated by using Turner's rules (12, 42).

(26); high-level expression of *gag* and *gag-pol* was dependent on *rev*, as has been described by others (28, 29, 37). To eliminate the possibility that expression of the protease product might lead to processing of the 160-kDa polyprotein and thus make its detection and measurement of frameshift rates difficult, constructs were also prepared to encode a single glycine-to-alanine amino acid substitution at protease position 52, a mutation previously shown to inactivate protease activity (24).

Constructs were transfected into either avian (QT6, avian fibrosarcoma) (31) or simian (COS-7) (15) cells by calcium phosphate coprecipitation (16), followed by a shock with 10% dimethyl sulfoxide in phosphate-buffered saline for 1 (QT6) or 2 (COS-7) min. Two days later, cells were labelled with 0.25 mCi of [^{35}S]methionine and [^{35}S]cysteine (Trans-label; ICN) per ml for 4 h and lysed in phosphate-buffered saline containing 0.1% sodium dodecyl sulfate, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, 2 μg of leupeptin per ml, and 1 μg of pepstatin per ml. HIV-1 Gag and Gag-Pol proteins were immunoprecipitated with an anti-Gag (p24) monoclonal antibody (38) and electrophoresed in sodium dodecyl sulfate–10% polyacrylamide gels (23). Frameshifting efficiency was determined by scanning with a Phosphorimager (Molecular Dynamics).

Results from a representative experiment with QT6 cells are shown in Fig. 2; similar results were obtained with COS-7 cells (data not shown). The products of the *gag* gene, p55, p41, and p24/25, were strongly expressed from constructs containing an active protease (Fig. 2A, lane 1). When the protease was mutated, the main product was p55, with some p41 and p24/25 being produced by residual protease activity (Fig. 2A, lane 6, and Fig. 2B, lane 2). The Gag-Pol fusion protein, p160, was easily detected in both cases (Fig. 2A, lanes 1 and 6). Neither p160 nor any of the *gag* products was seen in immunoprecipitations from QT6 cells trans-

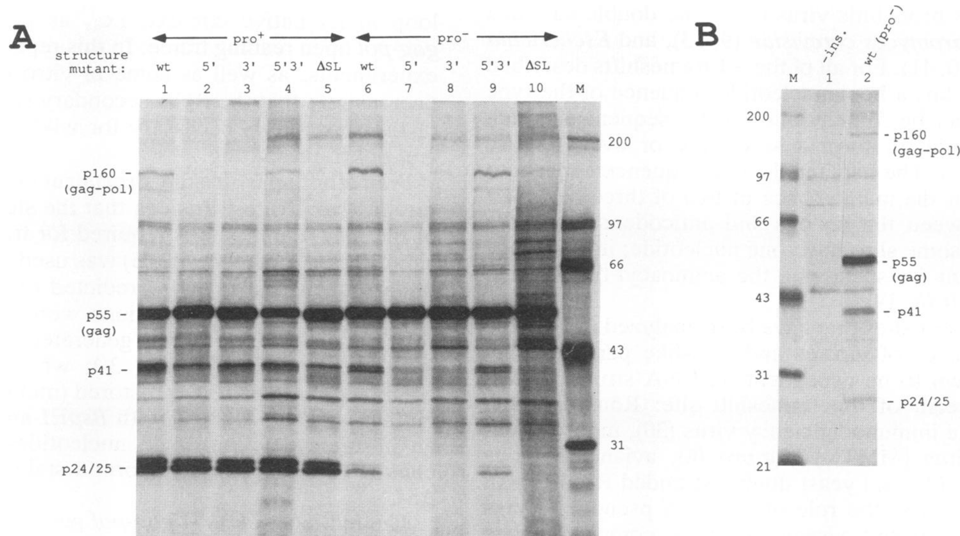

FIG. 2. HIV-1 *gag-pol* frameshifting in vivo is dependent on a downstream mRNA stem-loop structure. (A) Constructs containing the wild-type (lanes 1 to 5) or mutant (lanes 6 to 10) protease were cotransfected with pcREV (26) into QT6 cells, and ^{35}S-labelled HIV-1 Gag-containing proteins were immunoprecipitated with anti-p24 monoclonal antibody (38) and electrophoresed on a sodium dodecyl sulfate–10% polyacrylamide gel. The version of the stem-loop structure present in the construct used for transfection is indicated at the top of the figure (see Fig. 1B). Positions of relevant HIV-1 proteins are indicated on the left, and relative molecular masses of protein standards are indicated (in kilodaltons) on the right. (B) Immunoprecipitation from QT6 cells transfected with the cytomegalovirus vector lacking the HIV-1 Gag-Pol insert (lane 1) or containing the protease-deficient, wild-type structure insert (lane 2). The apparent increase in background in panel A compared with panel B is due to longer exposure time in panel A.

TABLE 1. Quantitation of frameshifting efficiencies in vivo and in vitro

Construct	Frameshifting efficiency (%)			
	In vivo[a]		In vitro (RRL)[b]	
	QT6	COS-7	*Eco*RV RNA	*Eco*RI RNA
wt pro$^+$	0.65	0.63	3.6	2.2
5′ pro$^+$	0.095	0.29[c]	1.3	0.78
3′ pro$^+$	0.091	0.26[c]	1.5	0.62
5′3′ pro$^+$	0.73	1.0[c]	3.4	2.0
ΔSL pro$^+$	0.15	0.20	1.5	1.3
wt pro$^-$	0.70	0.65		
5′ pro$^-$	0.13	0.18		
3′ pro$^-$	0.13	0.17		
5′3′ pro$^-$	0.84	1.1		
ΔSL pro$^-$	0.12	0.25		

[a] Quantitated by scanning with a Phosphorimager (Molecular Dynamics) and corrected for methionine and cysteine content; values represent an average from two to four experiments.
[b] Quantitated by scintillation counting of bands cut out from dried gels and corrected for methionine content; values represent an average from two experiments.
[c] These values were derived from a single experiment.

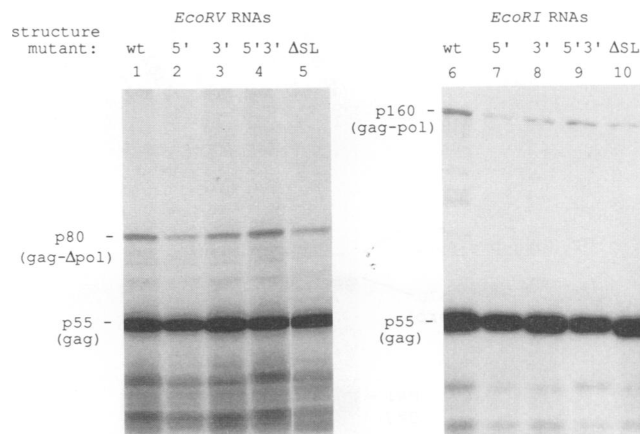

FIG. 3. Frameshifting in RRL is modestly affected by stem mutations. SP6 constructs were linearized with *Eco*RV (lanes 1 to 5) or *Eco*RI (lanes 6 to 10) and used for in vitro translation as described previously (6, 20, 27). The positions of the Gag (p55), truncated Gag-Pol (p80), and Gag-Pol (p160) proteins are indicated on the left. The stem-loop structure is indicated at the top of the figure (see Fig. 1B).

fected with the vector lacking an insert (Fig. 2B, lane 1). The pattern of Gag and Gag-Pol protein expression in transfected QT6 cells resembles that seen in other cell types used to express p160 (29, 33, 36, 37).

Mutation of either side of the stem (Fig. 2A, lanes 2, 3, 7, and 8) or deletion of the stem-loop structure (lanes 5 and 10) reduced the frameshifting efficiency by a factor of four- to sevenfold in QT6 cells (Table 1). The compensatory mutant, 5′3′, restored frameshifting to wild-type levels (Fig. 2A, lanes 4 and 9). (The decreased yield of p160 in lane 4 is paralleled by a similar decrease in p55, implying a lower transfection efficiency in this sample.) Reduced yields of p160 are also observable indirectly by the increased yield of p55 and the reduced amount of p24/25 when frameshifting is below wild-type levels (Fig. 2A, lanes 2, 3, and 5); this effect is likely a result of lower production of the protease (derived from p160). A similar pattern is also seen for the constructs containing the mutant protease (note the complete absence of p24/25 in lanes 7, 8, and 10).

The deleterious effects of disruption of the stem-loop on frameshifting were also seen in COS-7 cells, although the magnitude of these effects was apparently lower (Table 1). However, this may be related to difficulties in quantitation of the very low levels of p160 expressed in COS cells (data not shown).

The wild-type frameshifting efficiency (percent of counts in p160 compared with total counts in p160 and p55, adjusted for methionine and cysteine content) was approximately 0.7% (Table 1). This value is significantly lower than the 1.5 to 5% values recently obtained from in vivo experiments, in which frameshift-dependent reporter constructs, containing the HIV-1 shift site and stem-loop, were expressed in other cell types (29, 34). To our knowledge, the ratio of Gag to Gag-Pol polyproteins in HIV-1-infected cells has not been accurately measured; the ratio in cells infected with other retroviruses is usually estimated to be 20:1 to 50:1 (8). The efficiency observed here is also lower than the 5 to 10% values previously obtained from in vitro experiments with HIV-1-derived RNAs in RRL or with expression in *S. cerevisiae* (20, 44). The low values obtained here may be a result of technical differences in quantitation methods, premature translation termination of p160, or differential stability or immunoprecipitation of the p160 and p55 proteins. Nevertheless, the level of expression of p160 in this system is sufficiently high to quantitate the effects of the stem-loop structure. Interestingly, the low frameshifting efficiency in mammalian cells seen with the HIV stem mutants (0.1%) is similar to the value obtained with a reporter construct lacking the HIV stem-loop (5).

Influence of the stem-loop structure on frameshifting in vitro. One explanation for the strong dependence of frameshifting in vivo on the stem-loop structure, in contrast to previous results (25, 44), is our use of the native Gag-Pol coding region in the present experiments. We therefore tested the frameshifting efficiency of wild-type and mutant HIV-1 *gag-pol* RNAs in RRL. RNAs were synthesized in vitro by using SP6 RNA polymerase to transcribe templates containing the SP6 promoter 5′ of the HIV-1 *gag* initiator AUG (20, 27). Constructs were linearized prior to transcription with *Eco*RV, to make a truncated RNA, or with *Eco*RI, to make mRNA with full-length *gag-pol*. In vitro translation of the *Eco*RV mRNA in the presence of [^{35}S]methionine generates the 55-kDa Gag product and a truncated Gag-Pol product of 80 kDa (Fig. 3, lane 1); translation of the *Eco*RI mRNA produces the 55-kDa Gag and 160-kDa Gag-Pol proteins, as well as a number of other intermediate- and smaller-sized polypeptides that may arise as a result of premature termination or initiation at alternate start codons (lane 6). These results are similar to those obtained previously by Jacks et al. (20).

The efficiencies of frameshifting during translation of the *Eco*RV and *Eco*RI mRNAs were found to be 3.6 and 2.2%, respectively, as determined by scintillation counting of bands cut out from the dried gel (Table 1). This small difference in frameshifting efficiencies between the two mRNAs probably reflects reduced yields of the 160-kDa protein from the *Eco*RI mRNA, for the reasons mentioned above. The frameshifting efficiencies on the stem-loop mutant mRNAs (5′,3′ and ΔSL) were all found to be reduced by a factor of two- to threefold relative to the corresponding wild-type mRNA (Fig. 3, lanes 2, 3, 5, 7, 8, and 10; Table 1).

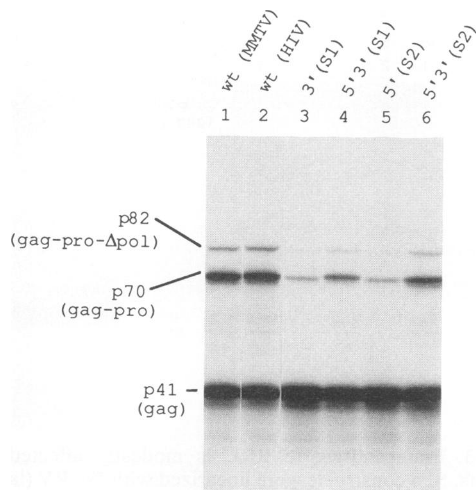

FIG. 4. The HIV-1 shift site is responsive to structural signals in a heterologous context. The MMTV shift site (A_6C) in the chimeric construct pMGPP, containing the MMTV *gag*, *pro*, and *pol* genes fused to the 5′ portion of RSV *gag* downstream of the SP6 promoter (21), was mutated to U_6A as previously described (6). Similar constructs containing the pseudoknot-destabilizing mutations were also prepared. SP6 constructs were linearized with *Bgl*II (located in the *pol* frame) and used for in vitro transcription to generate chimeric MMTV RNAs containing the HIV-1 shift site, which were used for translation in RRL. The positions of the Gag (p41), Gag-Pro (p70), and truncated Gag-Pro-Pol (p82) proteins are indicated on the left. Lane 1, wild-type MMTV; lane 2, wild-type MMTV containing the HIV-1 shift site; lane 3, MMTV mutant 3′(S1) (3′ side of stem 1 of the pseudoknot mutated); lane 4, MMTV mutant 5′3′(S1) (stem 1 reformed by compensatory changes on the 5′ side); lane 5, MMTV mutant 5′(S2) (5′ side of stem 2 of the pseudoknot mutated); lane 6, MMTV mutant 5′3′(S2) (stem 2 reformed by compensatory changes on the 3′ side). All of the constructs used for lanes 2 to 6 contain the HIV-1 shift site. The MMTV structure mutants are described in more detail elsewhere (6).

The double mutant, 5′3′, in which base pairing is restored, did not display these small but consistent reductions in frameshifting efficiency (lanes 4 and 9). Thus, the predicted stem-loop structure has a small but reproducible effect in promotion of frameshifting in vitro in RRL when frameshifting occurs in its native context.

The HIV-1 shift site can respond to heterologous structural signals. To determine whether frameshifting at the HIV-1 shift site is influenced by structural signals in a heterologous context and is not inherently more slippery than other sites, the MMTV *gag-pro* shift site (A AAA AAC) was replaced with the HIV-1 site (U UUU UUA). Frameshifting at the MMTV *gag-pro* site in RRL is very efficient and is strongly dependent on an RNA pseudoknot 3′ of the shift site (6). The shift-site switch led to an increase in frameshifting (to 20%, Fig. 4, lane 2) compared with that seen for the HIV-1 site in its own context (3.6%, Fig. 3), a result of the influence of the MMTV pseudoknot (6); this value is equal to that observed for the MMTV shift site in its native context (lane 1). The downstream pseudoknot was disrupted by altering the sequence of one side of either of its two stems, S1 and S2 (6). These structural mutations had drastic effects on frameshifting at the HIV-1 site, decreasing *gag-pro* frameshifting over 10-fold to 1.5 to 2% (Fig. 4, lanes 3 and 5). Frameshifting efficiency was restored to 7 (for S1) or 12% (for S2) by compensatory changes that should reform the pseudoknot (lanes 4 and 6); similar findings were previously reported with constructs containing the MMTV shift site (6). Interestingly, the 1.5 to 2% values obtained with the stem mutants, which disrupt the pseudoknot but leave a relatively small stem-loop intact, are close to the 3.6% value seen with wild-type HIV-1.

The experiments presented in this paper demonstrate that HIV-1 *gag-pol* frameshifting in vivo is indeed dependent on a structural element in the mRNA downstream of the frameshift site. Similar but less marked effects were also observed in vitro, when stem mutants were placed in the context native to HIV-1 RNA. This result is consistent with all other cases of −1 ribosomal frameshifting examined to date. We cannot readily explain the previously reported lack of effects of the stem-loop in heterologous contexts (25, 44) or the enhanced dependence on this structure in vivo compared with that in vitro. One explanation might be that the translational kinetics are very different between expression systems, such that different steps, such as elongation, frame slippage, tRNA availability, and pausing at the secondary structure, are rate limiting. Another possibility is that a *trans*-acting factor present in QT6 and COS-7 cells either stabilizes the secondary structure or sensitizes the ribosome to its presence and that this factor is more limiting in RRL and absent from yeast cells. With this in mind, it would be interesting to examine frameshifting in T lymphocytes or other cell types known to be targets for HIV-1 infection.

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