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The Sequences of and Distance between Two *cis*-Acting Signals Determine the Efficiency of Ribosomal Frameshifting in Human Immunodeficiency Virus Type 1 and Human T-Cell Leukemia Virus Type II In Vivo

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We have analyzed in cell culture the sequence elements that control the level of ribosomal frameshifting in the human T-cell leukemia virus type II (HTLV-2) *gag-pro* junction. The slippery sequence of HTLV-2 is sufficient to dictate a basal level of frameshifting. This level is enhanced by its upstream sequence context and by the downstream stem-loop structure which is located at an optimal distance of 7 bases. Frameshifting in human immunodeficiency virus *gag-pol* is similar to that of HTLV-2 *gag-pro*. However, experiments using hybrid cassettes of HTLV-2 and human immunodeficiency virus type 1 frameshift elements show that while the slippery sequence of HTLV-2 is less efficient, the stem-loop structure is a more efficient enhancer.

Many retroviruses, including the human retroviruses, make use of a −1 translational frameshifting to overcome the termination codon at the end of *gag* (1, 10, 11). In T-cell leukemia virus type II (HTLV-2), two ribosomal frameshift events lead to the synthesis of fusion proteins, Gag-Pro and Gal-Pro-Pol (7, 14, 21). These mechanisms of translational readthrough determine the ratio between the structural Gag protein and the catalytic proteins encoded by the *pro* and *pol* genes. Differential production of the fusion proteins with respect to Gag is a prerequisite for virus maturation (6, 9, 17). In vitro studies have demonstrated that two *cis*-acting sequence elements at the overlapping region between *gag* and *pol* enable retroviral ribosomal frameshifting (reviewed in reference 11): a slippery sequence, usually a heptanucleotide, and a 3′ adjacent RNA structure. Human immunodeficiency virus type 1 (HIV-1) and HTLV-2 are the only known retroviruses which have no pseudoknot RNA structure 3′ to the slippery sequence but exhibit a simple stem-loop structure. Recently we have shown that the slippery sequence of HIV-1, a heptanucleotide with the sequence U UUU UUA, is sufficient in vivo to mediate a basal level of frameshifting (20). The downstream stem-loop structure of HIV-1 serves as a positive modulator in the process. These findings are in accordance with the simultaneous-slippage model, which suggests that the actual frameshift takes place at the slippery sequence and is stimulated by the secondary structure (12). The distance between the two *cis*-acting elements seems to play an important role in the efficiency of ribosomal frameshifting in vitro (2, 16).

Ribosomal frameshifting in vivo mediated by *gag-pro* sequences from HTLV-2. We have used a highly sensitive and quantitative in vivo assay system (20) to characterize the influence of the sequence elements of the HTLV-2 *gag-pro* region on the efficiency of ribosomal frameshifting in BHK-21 cells. In the assay system the *gag* and *pro* genes of HTLV-2 are replaced by functional reporter genes encoding β-galactosidase (GAL) and luciferase (LUC), respectively (Fig. 1A). Functional LUC in the GAL-LUC fusion protein is measurable only when frameshifting in the −1 direction has taken place during translation of the polycistronic mRNA. Using this assay system we have previously shown that frameshifting activity facilitated by the *cis*-acting signal is similar in transient- and stable-expression experiments in a variety of mammalian cell lines (20).

To test frameshifting in cells, the HIV-1 frameshift box (*Sal*I-*Bam*HI fragment) of pBgalluc-1 (20) was replaced by a synthetic 41-bp oligonucleotide comprising 39 bp from the HTLV-2 *gag-pro* overlapping region (7), giving rise to plasmid pBgalluc-2H. pBgalluc-1H was constructed by the introduction of 4 bp by filling in the *Bam*HI site which is located 3′ to the HTLV-2 sequence. This plasmid was used to quantify −1 frameshifting. All mutants were constructed by inserting synthetic DNA fragments with appropriate ends into the original plasmids. The 39-bp wild-type DNA sequence from the HTLV-2 genome, which is sufficient to mediate frameshifting in vitro (7), is able to induce ribosomal frameshifting in BHK-21 cells (pBgalluc-1H) (Fig. 1B). The activity of LUC expressed following transfection with a construct harboring an in-frame fusion between the open reading frames of *gal* and *luc* was set to 100%. The enzyme specific activities of GAL and LUC expressed from various constructs containing in-frame fusions of *gal* and *luc* but differing in the linker sequence are indistinguishable (data not shown). Frameshifting efficiency was calculated by relating the enzymatic activity expressed by the various vectors to that of the in-frame fusion constructs. Background levels (0.04%) were defined with plasmid pBgalluc-1H_{stop}, which contains two translation stop codons in different reading frames (Fig. 1B). Insertion of the 39-bp HTLV-2 frameshifting sequence in pBgalluc-1H induces 4% frameshifting, as measured by LUC activity (Fig. 1B).

In order to determine the *cis*-acting elements required for frameshifting in vivo, the 39-bp HTLV-2 *gag-pro* junction was subjected to mutational analysis. Deletion of the 3′ part of the stem-loop sequence leads to a more than sixfold reduction of frameshifting efficiency (pBgalluc-1HSL1 in Fig. 1B). None-

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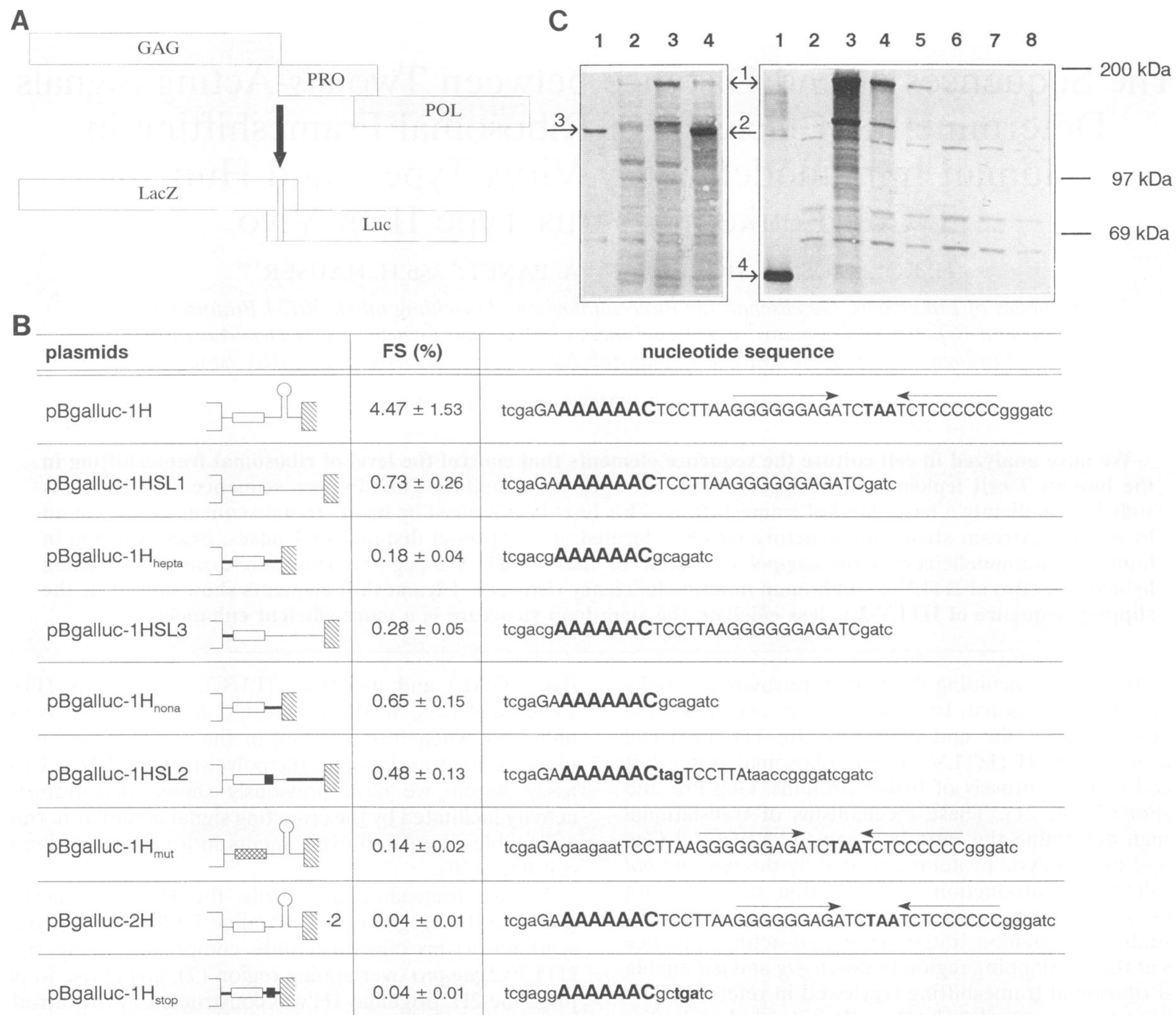

plasmids	FS (%)	nucleotide sequence
pBgalluc-1H	4.47 ± 1.53	tcgaGA**AAAAAAC**TCCTTAAGGGGGGAGATC**TAA**TCTCCCCCCgggatc
pBgalluc-1HSL1	0.73 ± 0.26	tcgaGA**AAAAAAC**TCCTTAAGGGGGGAGATCgatc
pBgalluc-1H_{hepta}	0.18 ± 0.04	tcgacg**AAAAAAC**gcagatc
pBgalluc-1HSL3	0.28 ± 0.05	tcgacg**AAAAAAC**TCCTTAAGGGGGGAGATCgatc
pBgalluc-1H_{nona}	0.65 ± 0.15	tcgaGA**AAAAAAC**gcagatc
pBgalluc-1HSL2	0.48 ± 0.13	tcgaGA**AAAAAACtag**TCCTTAtaaccgggatcgatc
pBgalluc-1H_{mut}	0.14 ± 0.02	tcgaGAgaagaatTCCTTAAGGGGGGAGATC**TAA**TCTCCCCCCgggatc
pBgalluc-2H (-2)	0.04 ± 0.01	tcgaGA**AAAAAAC**TCCTTAAGGGGGGAGATC**TAA**TCTCCCCCCgggatc
pBgalluc-1H_{stop}	0.04 ± 0.01	tcgagg**AAAAAAC**gc**tga**tc

FIG. 1. Ribosomal frameshifting promoted by the HTLV-2 *gag-pro* overlapping region in vivo. (A) To determine frameshift efficiency in vivo, an expression system in which the HTLV-2 genes *gag* and *pro* are replaced by the genes encoding the enzymes GAL and LUC, respectively, was used. The shaded bar on the bottom symbolizes a cassette (41 bp), which comprises HTLV-2 sequences essential for −1 frameshifting. The system allows simple determination of frameshift efficiency since the LUC is fused in the reading frame that is −1 with respect to that of GAL. (B) Frameshifting (FS) efficiency of HTLV-2 in vivo was determined by measuring LUC and GAL activities in transient transfection experiments using the indicated plasmids as described earlier (20). GAL and LUC activity measurements were performed by standard assays detailed previously (19, 20). The values are obtained from six independent but parallel transient transfections of the pBgalluc plasmids in BHK-21 cells, and the standard deviation is indicated. LUC activity was normalized to the expression of GAL. The LUC gene of each construct is fused −1 relative to the GAL reading frame with one exception: the LUC gene of pBgalluc-2H is fused in the reading frame −2 relative to the GAL reading frame as indicated. The GAL open reading frame is depicted as an open box on the left end; a hatched box on the right end symbolizes the LUC open reading frame. Thin lines, HTLV-2 sequences; thick lines, mutated sequences of nonviral origin; open boxes, HTLV-2 heptanucleotide slippery sequence; cross-hatched box, mutated heptanucleotide slippery sequence; filled boxes, stop codon. The nucleotide sequence of each frameshift cassette is shown. Sequences derived from HTLV-2 are represented by capital letters; the slippery sequence is printed in boldface letters; the flanking stem-loop is marked by inverted arrows; stop codons are printed in boldface. (C) Western blot analysis of translation products. Extracts (10 μl each) of stably transfected BHK-21 cells were subjected to Western blotting as detailed previously (20). (Left) Immunostaining with antibodies directed against GAL; (right) LUC antibodies. The LUC activity (light units per 10 s) of cells transfected with pBgalluc plasmids is given in parentheses: lanes 1, 50 ng of LUC and 200 ng of GAL protein markers; lanes 2, mock-transfected cells; lanes 3, pBgalluc-HSL1 (10^8); lanes 4, pBgalluc-1H (1.8×10^7); lane 5, pBgalluc-1HSL1 (0.8×10^6); lane 6, pBgalluc-1H_{hepta} (0.4×10^6); lane 7, pBgalluc-1H_{mut} (1.6×10^5); lane 8, pBgalluc-1H_{stop} (10^5). The arrows indicate the positions of the following proteins: 1, fusion protein GAL-LUC; 2, in-frame translation product; 3, GAL; 4, LUC.

theless, unlike the situation in vitro (7), significant frameshifting is observed even in the absence of the stem-loop. Interestingly, the frameshifting level mediated by the HTLV-2 heptanucleotide sequence alone is further reduced more than sixfold (Fig. 1B). This indicates that the HTLV-2 nucleotide context neighboring the slippery sequence increases frameshifting efficiency. Frameshifting was further reduced when the heptanucleotide in the HTLV-2 context was mutated (pBgal luc-1H_{mut}, Fig. 1B). This level of expression is only slightly higher than the background levels measured for plasmid

pBgalluc-1H$_{stop}$. Frameshifting towards −2 or +1 cannot be mediated by the HTLV-2 sequence, since the measured efficiency from pBgalluc-2H is identical to the pBgalluc-1H$_{stop}$ background level (Fig. 1B). From these experiments we conclude that the measurable basal frameshifting efficiency is promoted by the HTLV-2 heptanucleotide sequence alone while a further increase in frameshifting efficiency is due to the presence of the RNA stem-loop sequence. The frameshift results, measured by LUC activity, correlate to results of protein analysis by Western blot (immunoblot) of cell extracts of stable transfectants using antibodies directed against either LUC or GAL (Fig. 1C). The GAL-LUC fusion protein with the expected molecular mass of 178 kDa is expressed in pBgalluc0HSL1 (lane 3) and in pBgalluc-1H (lane 4) cells. Although an exact quantitation cannot be obtained by means of Western blotting, the data are consistent with the measured LUC enzyme activities (compare Fig. 1B and C).

Within the same nucleotide sequence context, frameshifting efficiency measured in vitro is usually higher than that in vivo (18, 20). This also is true for the frameshift efficiency promoted by the HTLV-2 *gag-pro* region. As pointed out by Falk et al. (7), the translation rate of the reading frame upstream of the frameshift site might influence frameshifting efficiency in a direct correlation. We assume that the values of frameshifting determined in each experimental system (4, 13, 18, 23) represent the efficiency of the frameshifting site in a certain base context and given particular translation rates. However, the results from all assays for the measurement of frameshifting efficiency give rise to the same conclusions concerning relative frameshift rates in response to different sequence conditions. An exception seems to be the fact that no frameshift is detectable in the in vitro systems when the stem-loop structure is absent, while a significant level of activity is measured in vivo. We believe that the difference between the in vitro and the in vivo results may reflect differences in the sensitivity of the detection methods.

Codon context of the slippery sequence. To further elucidate the role of the nucleotides surrounding the slippery sequence in enhancement of frameshifting activity, the regions flanking the HTLV-2 slippery sequence were modified individually by heterologous sequences. Elimination of the HTLV-2 sequence 3′ to the heptanucleotide (pBgalluc-1H$_{nona}$) or introduction of a stop codon 3′ to the slippery sequence (pBgalluc-1HSL2) decreased frameshifting efficiency only slightly (about 30%) compared with that of the HTLV-2 frameshifting sequence which includes the neighboring 3′sequences but cannot form the stem-loop (pBgalluc-1HSL1). The replacement of the HTLV-2 short flanking sequence 5′ to the slippery sequence by changing 2 nucleotides adjacent to the 5′ end of the heptanucleotide had a stronger effect (pBgalluc-1HSL3). Frameshifting activity was reduced to a third of the levels determined by the HTLV-2 frameshift sequence, which harbors the adjacent 3′ HTLV-2 sequences but lacks part of the stem-loop (pBgalluc-1HSL1). This result indicates that the 2 nucleotides 5′ to the HTLV-2 heptanucleotide are involved in the frameshifting process. The basic slippery sequence for HTLV-2 therefore seems to be an octa- or a nonanucleotide. This might point to the importance of the ribosomal E site (15) for the frameshift process (8, 22).

Influence of distance between the slippery sequence and secondary structure on frameshifting efficiency. The enhancement of frameshifting efficiency by the HTLV-2 stem-loop sequence is restricted to an optimal distance from the slippery sequence. The results in Fig. 2 show that the frameshifting efficiencies are a function of the distance between the heptanucleotide and the 5′ end of the stem-loop sequence. Modulation of the spacing between the two elements indicates that the optimal spacing is 5 to 8 nucleotides (in accordance with the distance between these two elements in HTLV-2, 7 bases). Since we have demonstrated that the base composition 3′ to the slippery sequence of HTLV-2 does not have a significant influence on frameshifting (Fig. 1B), we conclude that the effects on the efficiencies are primarily attributable to the changes in the length of the spacer region.

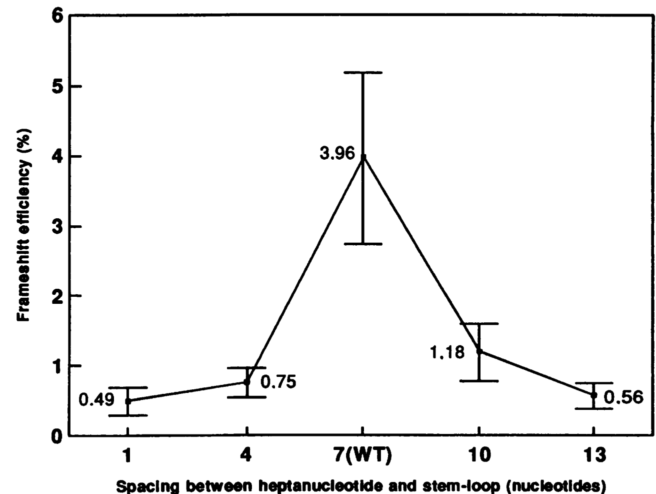

FIG. 2. Variation of the spacer between the heptanucleotide and stem-loop. The frameshift efficiency was determined as described for Fig. 1B after transient transfection of plasmids with different sizes of the spacer regions between the heptanucleotide and stem-loop from HTLV-2. Values represent averages from at least six independent transfection experiments. Standard deviations are indicated by bars. Sequence of the spacer between the HTLV-2 heptanucleotide and the wild-type stem-loop structure: for pBgalluc-1HSP1, T; for pBgalluc-1HSP4, TCC T; for pBgalluc-1HSP10, TCC TTA ATA A; and for pBgalluc-1HSP13, TAG TCC TTA ATA A.

Base composition and stability of the secondary structure are responsible for enhancing frameshifting efficiency. To investigate the influence of the stem-loop on the efficiency of ribosomal frameshifting, stem-loop mutants were tested. As shown in Fig. 3A, the results give rise to the following conclusions: mutations in both arms of the stem-loop (pBgal luc-1HA and -1HB) which destroy base pairing lead to a loss of frameshifting enhancement. The residual activity from these two plasmids is similar to the frameshifting derived from the stem-loop deletion mutant (compare pBgalluc-1HA and -1HB in Fig. 3A with pBgalluc-1HSL1 in Fig. 1B). Compensatory mutations which restore base pairing of the secondary structure, but not the stability (ΔG, −8.5 kcal [ca. 36 kJ]/mol compared with −13 kcal [ca. 44 kJ]/mol), do not restore the frameshifting efficiency (pBgalluc-1HAB, Fig. 3A). Other mutations in the stem, as well as the deletion of the stop codon located in the loop, whereby the strength of the secondary structure was retained, facilitate frameshifting, although with somewhat lower efficiency compared with that of the wild type (compare pBgalluc-1H with pBgalluc-1H$_{Loop}$, -1HCD, and -1HLS in Fig. 3A).

The results which are based on enzyme activity were confirmed by Western blot analysis of the protein extracts from a single stable transfection experiment (Fig. 3B). The appearance of the GAL-LUC fusion protein (arrow 1) correlates with the extent of frameshifting measured by enzymatic activities. These results are consistent with those from in vitro frameshifting experiments. It thus seems that although stability of

pBgalluc-1	H	H_{Loop}	HCD	HAB	HB	HA	HLS
stem-loop	wild-type	loop mutant	mutant 1	mutant 2 compens.	3' arm mutant	5' arm mutant	mutant 3 mirrored wt
FS efficiency (%)	3.96±1.23	2.68±0.81	2.67±0.77	0.68±0.20	0.65±0.17	0.47±0.12	3.54±0.52
ΔG (kcal/mol)	-13.0	-13.0	-13.0	- 8.5	- 2.8	- 2.8	-13.0

FIG. 3. Mutational analysis of the stem-loop structure of HTLV-2 in vivo. (A) Influence of stem-loop sequence and stability on frameshift efficiency. The frameshift (FS) efficiency was determined as described before (see Fig. 1B legend). Values for frameshifting efficiency ± standard variations were obtained by six or more independent transfection experiments. The free energy of the stem-loop sequences was calculated with the computer program of Zuker et al. (24) at 37°C (1 kcal = 4.184 kJ). wt, wild type. (B) Western blot analysis of translation products of the stem-loop mutants. Lanes 2 to 10 contain extracts of stably transfected BHK-21 cells. Lanes 1 to 5 correspond to lanes 1 to 5 of Fig. 1C. Light units per 10 s are given in parentheses: lane 6, pBgalluc-1H_{Loop} (0.7×10^7); lane 7, pBgalluc-1HCD (1.0×10^7); lane 8, pBgalluc-1HAB (1.2×10^6); lane 9, pBgalluc-1HB (2.3×10^6); lane 10, pBgalluc-1HA (1.2×10^6). See the legend to Fig. 1C for proteins identified by arrows.

the stem may play an important role in frameshifting, an additional factor which depends on the base context of the secondary RNA structure plays a role in the enhancement of frameshifting. This might be due to differential binding of cellular proteins stabilizing the secondary structure. That conclusion is also supported by the fact that some base changes which retain the free energy of the HTLV-2 stem-loop or mutations in the loop (Fig. 3A) (7) reduce the efficiency of frameshifting. Thus, the three-dimensional structure of the stem-loop sequence could play an additional role.

Comparison of the sequence elements involved in frameshifting in HIV-1 and HTLV-2. The HTLV-2 slippery sequence is less efficient in mediating ribosomal frameshifting compared with the heptanucleotide of HIV-1 (Fig. 1B and 4). On the other hand, the frameshifting efficiency of the HTLV-2 39-base *gag-pro* overlap sequence gives rise to a frameshifting activity equal to that of the 59-base *gag-pol* overlap RNA sequence of HIV-1. This would imply a stronger enhancement by the secondary structure from HTLV-2 in comparison with that by the stem-loop structure of HIV-1. We have tested this assumption by combining the two elements, the slippery sequence and secondary structure, of both viral sequences in different combinations. The results are depicted in Fig. 4. Clearly, in all of the combinations used, the HIV-1 slippery sequence (black bars) is more efficient than the corresponding sequence of HTLV-2 (open bars). On the other hand, the secondary-structure sequence of HTLV-2 is much more efficient in enhancing frameshifting. Therefore, the most efficient frameshifting is observed with a sequence cassette comprising the HTLV-2 stem-loop and HIV-1 heptanucleotide. This efficiency of about 17% is reached only by frameshift sequences containing pseudoknots (3, 5). The mutated stem-loop which only slightly affected frameshifting in the HTLV-2 base context (HLS) is more than two times less efficient than the HTLV-2 wild-type stem-loop when translocated to the HIV-1 slippery sequence (Fig. 4). Moreover, the stem-loop sequence of HIV-1 does not stimulate the frameshifting promoted by the less efficient HTLV-2 heptanucleotide. This suggests that enhancement of frameshifting at the heptanucleotide sequence AAA AAAC of HTLV-2 depends on more stringent requirements for sequence, stability, and structure of the stem-loop than does that at the HIV-1 UUUUUUA frameshift site. Further-

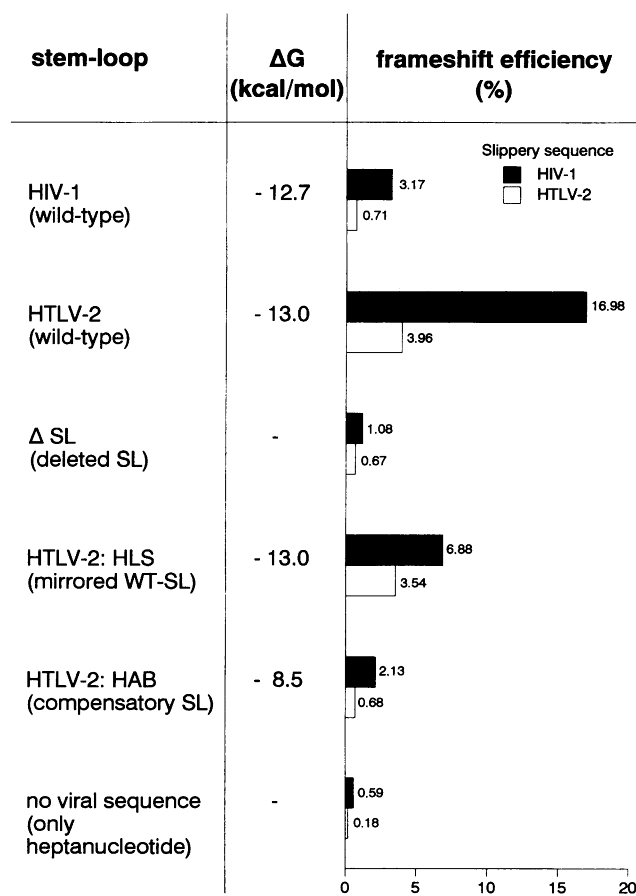

FIG. 4. Comparison of slippery sequence and stem-loop structure of HIV-1 and HTLV-2. The frameshift efficiencies were determined as described before (Fig. 1B). The values were obtained from at least six independent transient transfection experiments. The free energy was calculated as described in the Fig. 3 legend. The sequences of the stem-loops in the mutants are depicted in Fig. 1B and 3.

more, the stimulation of frameshifting by the stem-loop at the HTLV-2 site is stringently dependent on the natural distance of about 7 nucleotides (Fig. 2) between the slippery sequence and the stem-loop. On the other hand, the distance from the more efficient slippery sequences UUUUUUA (as in HIV-1) to the stem-loop need not to be so stringently maintained; it may vary from 3 to 9 bases (reviewed in reference 10). This finding was also confirmed by a further experiment in which a synthetic stem-loop structure positioned 13 bp downstream of the slippery sequence UUUUUUA of HIV-1 significantly enhanced frameshifting activity (10a).

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