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A Heptanucleotide Sequence Mediates Ribosomal Frameshifting in Mammalian Cells

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Ribosomal frameshifting is an essential requirement for replication of many viruses and retrovirus-like elements. It is regarded as a potential target for antiretroviral therapy. It has been shown that the frameshifting event takes place in the −1 direction within a sequence, the slippery sequence, which is usually followed by structured RNA. To distinguish between the basic sequence requirements and the modulating elements in intact cells, we have established a sensitive assay system for quantitative determination of ribosomal frameshifting in mammalian cell culture. In this assay system, the *gag* and *pol* genes of human immunodeficiency virus type 1 are replaced by the genes for the functional enzymes β-galactosidase and luciferase, respectively. The sensitivity of the test system allows us to demonstrate for the first time that the slippery sequence, a heptanucleotide, is sufficient to mediate a basal level of ribosomal frameshifting independent of its position within a gene. The stem-loop sequence serves only as a positive modulator. These data indicate that frameshifting could also occur during translation of cellular genes in which a slippery sequence is present within the reading frame. The resulting putative transframe proteins might have a functional importance for cellular processes.

Retroviruses and retrovirus-like elements from yeasts and other eukaryotes share some characteristics in their expression of Gag and Pol proteins. The production of the Pol proteins is mostly due to a translational frameshift event in the overlap region between *gag* and *pol*, giving rise to a Gag-Pol fusion protein (reviewed in references 8 and 11). The efficiency of the frameshift event determines the amount of the catalytic Pol proteins, which are needed in much lower quantities than the structural Gag proteins. A defined ratio of Gag-Pol and Gag seems to be a prerequisite for optimal assembly of the virus core particles (5, 6, 22).

Ribosomal frameshifting can operate in both directions, altering the reading frame in either the −1 or +1 direction. In eukaryotic retroviruses, the shift in the reading frame has been observed in the 5′ direction (−1) (2, 8, 11). The exact site of ribosomal frameshifting on the mRNA has been defined for several retroviral elements and is called the shifty or slippery sequence (10, 12, 13, 21). It consists of a 7-bp element with the consensus sequence X XXY YYZ (8, 11), where X and Y or Y and Z can be identical. Currently, the process of frameshifting is explained by the simultaneous slippage model (12), which comprises the translocation by one nucleotide of the aminoacyl-tRNA and the peptidyl-tRNA, located at the ribosomal A and P sites, respectively. The extent of translational frameshifting for different frameshift sites in naturally occurring viruses differs significantly. The estimated amounts are between 0.7 and 25% (1–4, 12, 13, 15, 17, 18, 23, 25, 28). On the primary transcript of some retroviruses, there are two frameshift sites, the first between *gag* and *pro* and the second between *pro* and *pol* (14, 18, 20, 21). In the case of mouse mammary tumor virus, the efficiency of the first frameshift is much higher than that of the second one (3). This implies that RNA sequence or structure influences the extent of frameshifting. Indeed, all defined retroviral shifty sequences are followed at a short distance (between 1 and 9 bases) by structured RNA, a stem-loop or a pseudoknot (8, 11). Experimental work has indicated that these RNA structures are required for efficient frameshifting (1, 3, 4, 12, 23).

The determination of frameshifting efficiency is rather difficult. The published data for human immunodeficiency virus type 1 (HIV-1) frameshifting range between 0.7 and 12% depending on the test system and the genetic constructs used as reporters (in vivo versus in vitro translation) (13, 17, 23, 25, 28). We have established an in vivo test system which is based on nonviral proteins, allowing an exact determination of relative frameshifting efficiency. In our assay system, the *gag* and *pol* genes of HIV-1 are replaced by the functional reporter genes for β-galactosidase and luciferase, respectively. Functional luciferase in the β-galactosidase–luciferase fusion protein (GAL-LUC) is only measurable when frameshifting in the −1 direction has taken place during translation of the mRNA. The test system is sensitive enough to determine frameshifting efficiency of less than 0.1% in certain high-expressing mammalian cell lines. Our results show that the HIV-1 shifty sequence is sufficient to mediate a basal level of ribosomal frameshifting, whereas the stem-loop of HIV-1 serves as a positive modulator.

MATERIALS AND METHODS

Plasmid constructions. Plasmid pBgalluc-1 was constructed by insertion of a 3,050-bp fragment from a *lacZ* expression vector (pHβAPr1-β-Gal), extending from an *Hin*dIII site 5′ of the ATG to the *Eco*RI site at the 3′ end of the *lacZ* gene and an oligonucleotide of 55 bp (*Eco*RI-*Sal*I) reconstituting the 3′ end of *lacZ*, into plasmid pBFSLuc-1 (25) cleaved with *Hin*dIII and *Sal*I. Plasmid pBgalluc-1_{mut} was constructed by replacement of the *Sal*I-*Bgl*II fragment with a 24-bp DNA fragment (5′-T CGA CAA GCT AAC TTC CTC GGG AA-3′). pBgalluc0 was made by cleaving plasmid

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pBgalluc-1_{mut} at the *Bgl*II site, filling in, and religating. For construction of pBgalluc-1_{SL}, the stem-loop structure of pBgalluc-1 was eliminated by cleaving with *Bgl*II and *Bam*HI, filling in, and religating. Plasmid pBgalluc-1_{hepta} was constructed by replacement of the *Sal*I-*Bam*HI fragment in pBgalluc-1 with a 28-bp synthetic DNA fragment (5′-T CGA TCC CTT AGG CCT TTT TTA CAC GCG-3′) harboring only 7 nucleotides of the HIV shifty sequence in a non-HIV-1 context. pBgalluc-2_{SL} was made by digesting pBgalluc-1 with *Bam*HI and *Bgl*II, shortening the sticky ends, filling in, and religating. For construction of pBgalluc-1_{hepta^*}, the region encoding amino acids 272 to 275 of wild-type luciferase was mutated by polymerase chain reaction (9). The sequence G UUU UUA was changed to U UUU UUA, and by additional elimination of one nucleotide, the following luciferase-encoding region was put into the −1 frame. The mutations were made in a way that preserved the authentic amino acid sequence of the luciferase, provided that frameshifting takes place at the predicted codons.

Plasmids pBT7FSLuc-1, pBT7Luc-1_{SL}, and pBT7Luc-1_{hepta} correspond to pBgalluc-1, pBgalluc-1_{SL}, and pBgalluc-1_{hepta}, respectively (see Fig. 2). They were derived from previously described eukaryotic expression plasmids (25) by insertion of a T7 promoter oligonucleotide (23 nucleotides) into the *Xho*I site, which is located between the simian virus 40 promoter and the 5′ untranslated region. pBT7FSLuc0 was derived from pBT7FSLuc-1 by placing the luciferase gene in frame with respect to the start codon by a filling-in reaction. All plasmids were characterized by restriction endonuclease analysis and DNA sequencing of the relevant regions. The relevant sequences in these plasmids are shown in Fig. 2.

Cell culture and gene transfer. The cell lines used were 293, adenovirus type 5-transformed human embryonal kidney cells (ATCC CRL 1573); HeLa, a human epitheloid carcinoma cell line (ATCC CCL2); BHK-21, baby hamster kidney cells (ATCC CCL10); Ltk$^-$, mouse fibroblasts (16); and P3X63-Ag8.653, a mouse myeloma cell line (ATCC CRL1580). The cell lines were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transfection with plasmids was carried out by the calcium phosphate coprecipitation technique for adherent cell lines and by lipofection (GIBCO BRL) for P3X63 B cells. Stable transfectants were obtained by transfer of 5 μg of expression plasmid, 0.5 μg of puromycin resistance gene (pSpacΔp), and 4.5 μg of carrier DNA by the calcium phosphate coprecipitation technique as described earlier (25).

Reporter gene assays and Western immunoblotting. β-Galactosidase and luciferase activities in stable transfectants of BHK-21 cells were determined as described previously (25). For Western blot analysis, 2×10^7 stably transfected cells were lysed with 250 μl of extraction buffer (140 mM NaCl, 10 mM Tris-HCl, 1 mM dithiothreitol [DTT], 2 mM phenylmethylsulfonyl fluoride, 0.5% [vol/vol] Nonidet P-40). The lysate was centrifuged at 14,000 × *g* for 15 min at 4°C. Aliquots (10 to 20 μl) of the resulting supernatants (equivalent to 150 μg of total protein) were separated by tricine-sodium dodecyl sulfate (SDS) gel electrophoresis (26). The proteins were transferred to filters (Millipore Immobilon) and incubated with rabbit sera and monoclonal antibody against luciferase or β-galactosidase. Immobilized antibodies were detected with a second alkaline phosphatase-conjugated antibody (Dianova). The antigen-antibody complexes were detected by an alkaline phosphatase assay system (Bio-Rad).

Determination of frameshifting in vitro. For determination of frameshifting efficiency in vitro, constructs analogous to those used for in vivo frameshifting but without β-galactosidase were used. In vitro transcription (19) was driven by the T7 promoter. The plasmids were linearized 3′ to the luciferase gene prior to transcription. Equal amounts of RNA (200 ng) were translated in a 20-μl rabbit reticulocyte lysate translation reaction (Amersham). The luciferase activity of a 2-μl aliquot was determined.

RESULTS

Test system for frameshifting efficiency. In order to investigate frameshifting in intact cells, the reporter construct pBgalluc-1 (Fig. 1A) and a corresponding reference plasmid, pBgalluc0, were constructed. In the reporter pBgalluc-1, the coding region for luciferase (without its own AUG) is fused in the −1 frame with respect to the coding region for β-galactosidase. Integrated between these two coding sequences is a 59-bp fragment of HIV-1 which contains the frameshift-mediating sequence of the *gag-pol* overlapping region. This viral sequence contains the shifty sequence, encoding U UUU UUA, followed by a palindrome which is thought to exhibit a stem-loop structure. Normal translation from the RNA of this construct leads to a translational stop shortly after the β-galactosidase region. Complete translation of the fusion protein GAL-LUC depends on ribosomal frameshifting at the HIV-1 sequence.

GAL-LUC exhibits the enzymatic activity of both proteins (Fig. 1). A comparison of the enzymatic activity and intensity of the bands in the Western blots shows that the fusion protein harbors the same specific activities as the nonfused proteins (data not shown). The relative enzymatic activities of luciferase and β-galactosidase therefore reflect the frameshifting efficiency. To quantify this ratio, the construct pBgalluc0 served as a reference. Since the coding regions for β-galactosidase and luciferase are in the same frame, translation of mRNA from this construct must lead exclusively to the fusion protein GAL-LUC. Frameshifting efficiency can be calculated by relating the enzymatic activities in pBgalluc-1-expressing cells to those in pBgalluc0-expressing cells.

Western blotting of the cellular products derived from the reporter constructs (Fig. 1B, lanes 3 to 6) shows that the fusion protein GAL-LUC (178 kDa) is synthesized (Fig. 1B, arrow 1). The intensity of the 178-kDa band corresponds to the amount of active luciferase determined by the enzymatic assay. In the −1 constructs, the major product expected is a protein that is somewhat larger than the wild-type β-galactosidase (Fig. 1B, arrow 3). This product is visible in lanes 4 to 7 in the anti-β-galactosidase antibody-treated Western blot (Fig. 1B, arrow 2). However, the Western blots also show that the fusion protein is degraded by cellular proteases. The cleavage sites are located in the region between the wild-type enzyme sequences and within the N-terminal part of the luciferase, giving rise to two anti-β-galactosidase-reactive and two antiluciferase-reactive degradation products (data not shown). The extent of degradation correlates with the amount of GAL-LUC. Furthermore, the β-galactosidase activities of the fusion protein and the shorter products are identical. Therefore, the calculation of the relative frameshifting efficiency is not altered.

HIV-1 frameshifting efficiency in cell culture and in vitro. The observed frameshifting is caused by the HIV-1 sequence. This is demonstrated by mutating the slippery sequence within the HIV-1 fragment (pBgalluc-1 to pBgal-

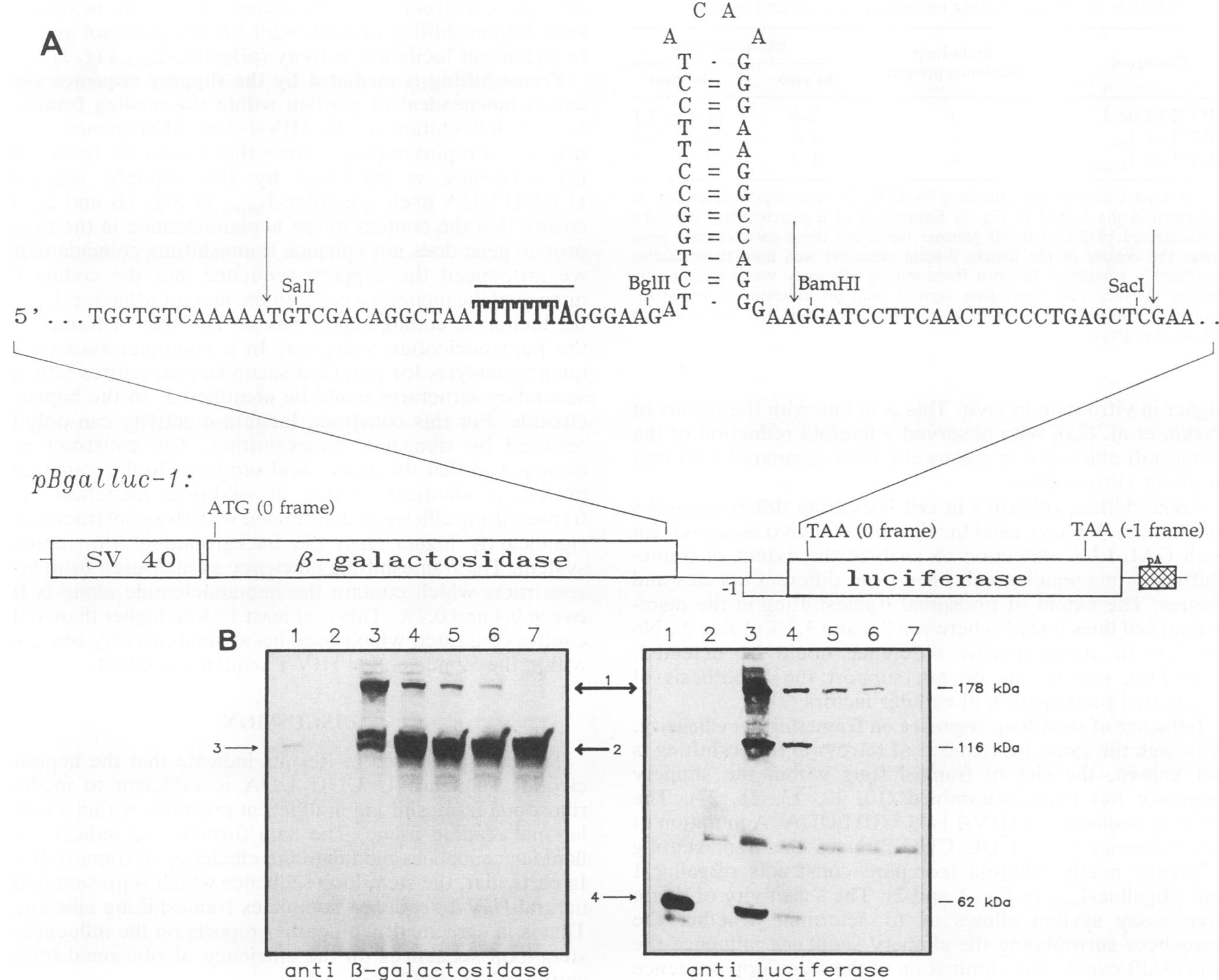

FIG. 1. Detection of ribosomal frameshifting in vivo by expression of the fusion protein GAL-LUC. (A) Expression construct pBgalluc-1, which allows quantification of frameshifting in vivo. The HIV-1 sequence (bp 2071 to 2130 of BH10) (24), flanked by synthetic *Sal*I (5') and *Bam*HI (3') sites, was integrated between two functional protein-encoding regions, those for β-galactosidase (5' to the left arrow) and firefly luciferase (3' to the right arrow). The sequence between the middle and the right arrow (*Bam*HI-*Sac*I) serves as a linker and replaces the AUG codon of the luciferase gene. The slippery sequence, which is indicated by boldface letters and an overline, is followed by a potential stem-loop structure. In this construct the luciferase gene is fused in the −1 frame with respect to the β-galactosidase reading frame. The translation of the full-length GAL-LUC fusion protein depends on ribosomal frameshifting at the HIV-1 slippery sequence. The in-frame translation product is terminated after six codons of the wild-type luciferase reading frame. The expression unit is driven by transcriptional control elements derived from simian virus 40. (B) Western blot analysis of translation products. Lanes 2 to 7 contain extracts of stably transfected BHK cells. The luciferase activity is given for each sample. Lane 2, mock transfected (2×10^2 light units); lane 3, pBgalluc0 (5×10^8 light units); lane 4, pBgalluc-1 (4×10^7 light units); lane 5, pBgalluc-1_{SL} (1.3×10^7 light units); lane 6, pBgalluc-1_{hepta} (1.1×10^7 light units); lane 7, pBgalluc-1_{mut} (5×10^5 light units). Lane 1 contains 100 ng of commercially available luciferase and 50 ng of β-galactosidase. Arrows: 1, fusion protein GAL-LUC; 2, in-frame translation product; 3, β-galactosidase; 4, luciferase.

luc-1_{mut}), which abolishes the production of the fusion protein (compare lanes 4 and 7 in Fig. 1B). The extent of ribosomal frameshifting is determined by comparing the luciferase and β-galactosidase activities of the −1 construct with those of an in-frame construct (pBgalluc0). According to this calculation, the frameshifting efficiency mediated by the HIV-1 sequence is about 3% in BHK cells. This differs significantly from the in vitro results (about 10%) obtained by other groups (13, 17, 28).

In order to ensure that the different results are due to the expression system used and not to the choice of the reporter gene, the frameshifting efficiency was also determined in vitro. For these experiments, a shorter construct was used. This construct is the same as pBgalluc-1 except that it lacks the β-galactosidase part. The remaining part, encoding an N-terminally extended luciferase (25), is driven by combined promoters from simian virus 40 and T7. This construct was used to determine the efficiency of frameshifting in a reticulocyte lysate system and in transfected BHK-21 cells. The frameshifting efficiency in the in vitro system is 11%, whereas in BHK cells the efficiency is 2 to 4% (Table 1). The results show that frameshifting efficiency is three times

TABLE 1. Frameshifting efficiency in vivo and in vitro[a]

Construct	Stem-loop sequence present	Efficiency (%)	
		In vivo	In vitro
pBT7FSLuc-1	+	2–4	11.4 ± 2.1
pBT7Luc-1_{SL}	−	1–4	3.5 ± 0.1
pBT7Luc-1_{hepta}	−	1–3	3.0 ± 0.5

[a] In vivo frameshifting efficiency in BHK-21 cells was determined as described in the legend to Fig. 2. Expression of β-galactosidase from the cotransfected pHβAPr1-β-Gal plasmid harboring the β-galactosidase gene under the control of the human β-actin promoter was used to normalize transfection efficiency. In vitro frameshifting efficiency was calculated by relating the luciferase expression derived from the indicated in vitro-transcribed and -translated plasmids to that from pBT7FSLuc0. The standard deviation is given.

higher in vitro than in vivo. This is in line with the results of Parkin et al. (23), who observed a fivefold reduction of the frameshift efficiency in eukaryotic cells compared with that in the in vitro system.

Frameshifting efficiency in cell lines from different species and tissues. We have used the described in vivo assay system with GAL-LUC detection to analyze the extent of frameshifting in mammalian cell lines from different species and tissues. The extent of ribosomal frameshifting in the mammalian cell lines tested is between 2.2 and 3.2% (Table 2). No species- or tissue-specific difference could be detected. Therefore, our results do not support the hypothesis of differential participation of cellular factors (7).

Influence of stem-loop sequence on frameshifting efficiency. Although the exact mechanism of retroviral frameshifting is not known, the site of frameshifting within the slippery sequence has been determined (10, 12, 13, 21, 27). The slippery sequence of HIV-1 is U UUU UUA. A mutation of this sequence to C UUC CUC reduces the frameshifting efficiency nearly 100-fold (compare constructs pBgalluc-1 and pBgalluc-1_{mut} in Fig. 1 and 2). The sensitivity of the in vivo assay system allows us to determine whether the sequences surrounding the slippery sequence influence the frameshift event. The elimination of the stem-loop sequence reduces frameshifting efficiency about threefold in vivo (pBgalluc-1_{SL}) (Fig. 2) and in vitro (pBT7Luc-1_{SL}) (Table 1). These data show the quantitative influence of the sequence which has been reported to form a stem-loop (23) on frameshifting activity. However, the influence of the stem-loop is less drastic than with other viruses, in which destabilization of the RNA structure leads to a more than 10-fold reduction in frameshifting efficiency (1, 3, 4, 12).

According to the currently discussed mechanistic models, frameshifting on the HIV-1 slippery sequence should only, take place towards −1 (12, 27). Indeed, another stem-loop

TABLE 2. Frameshifting in different mammalian cell lines[a]

Cells	Species	Cell type	Frameshifting efficiency (%)
293	Human	Embryonal kidney	2.2 ± 0.5
HeLa	Human	Cervical carcinoma	3.0 ± 0.9
BHK	Hamster	Kidney	3.2 ± 0.7
Ltk^-	Mouse	Fibroblasts	3.0 ± 0.7
P3X63-Ag8.653	Mouse	B cells	2.6 ± 0.1

[a] Frameshifting efficiency was calculated as described in the legend to Fig. 2 after transient transfection of plasmids pBgallucO and pBgalluc-1. Values represent averages for at least three independent transfection experiments ± standard deviation.

deletion construct in which luciferase activity is only restored upon shifting into the −2/+1 frame does not give rise to significant luciferase activity (pBgalluc-2_{SL}, Fig. 2).

Frameshifting is mediated by the slippery sequence alone and is independent of position within the reading frame. A further delimitation of the HIV-1-derived sequence in the pBgalluc-1 reporter genes shows that significant frameshifting efficiency is mediated by the slippery sequence U UUU UUA itself (pBgalluc-1_{hepta} in Fig. 1B and 2). To ensure that the context of the heptanucleotide in the fusion protein gene does not enhance frameshifting coincidentally, we introduced the slippery sequence into the coding sequence of the luciferase gene. In the mutant pBgalluc-1_{hepta*}, the luciferase coding region was set into the −1 frame 3′ to the heptanucleotide sequence. In a computer-assisted sequence analysis for potential secondary structures (29), no secondary structures could be identified 3′ to the heptanucleotide. For this construct, luciferase activity can only be restored by ribosomal frameshifting. The construct was designed so that the amino acid sequence in the transframe protein is identical to that in wild-type luciferase. The frameshifting efficiency determined with this construct is still significantly higher than the background of the reporter system. The frameshifting efficiency as measured from both constructs which contain the heptanucleotide alone is between 0.4 and 0.7%. This is at least 10-fold higher than in the control construct, which contains a mutated shifty sequence within the 59-nucleotide HIV-1 sequence context.

DISCUSSION

The data presented in Results indicate that the heptanucleotide sequence U UUU UUA is sufficient to mediate ribosomal frameshifting in different positions within a translational reading frame. The data furthermore indicate that flanking sequences modulate the efficiency of frameshifting. In particular, the stem-loop sequence which is present in the natural HIV-1 sequence stimulates frameshifting efficiency. This is in agreement with earlier reports on the influence of stem-loop sequences on the efficiency of ribosomal frameshifting (1, 3, 4, 12, 23).

A comparison of frameshifting efficiencies in cell lines from different species and tissues indicates that −1 frameshifting, which is directed by defined nucleotide sequence information, is an inherent property of the translational apparatus whose efficiency is not influenced by cell-specific factors. We have not been able to determine the extent of ribosomal frameshifting in the natural host cells of HIV-1 with this assay system, since the expression of transfected genes in T cells is not sufficient. However, CD4-expressing 293 cells, which represent an excellent host for HIV-1 replication (unpublished), show the same efficiency as the other cell lines tested.

A significant difference between the frameshifting efficiency in in vitro systems and in intact cells has been observed for the wild-type sequence (Table 1). A similar observation regarding the differences in frameshifting efficiency in vitro and in vivo was made by Parkin et al. (23). These authors found a much lower efficiency (0.7%) when they expressed the native HIV-1 *gag-pol* domain in cultured avian and simian cells. The difference in frameshifting efficiency between our reporter gene assay system and the native HIV-1 sequence in vivo and in vitro may be explained by technical differences or by biological specificities such as differential translational efficiency.

Our data suggest that the heptanucleotide sequence

plasmids	luciferase frame	luciferase expression in %
pBgalluc0 -CAaGCTAAcTTccTcGGGAAgatcGA GAA-	0	**100**
pBgalluc-1 -CAGGCTAA**TTTTTTA**GGGAAGA GAA-	-1	**3.2** ±0.7
pBgalluc-1_{SL} -CAGGCTAA**TTTTTTA**GGGAAGATC-	-1	**0.9** ±0.3
pBgalluc-1_{hepta} -tcccttaggcc**TTTTTTA**cacgc-	-1	**0.7** ±0.4
pBgalluc-1_{hepta*} -aagaagagct**TTTTTTA**gatcc-	-1	**0.4** ±0.2
pBgalluc-1_{mut} -CAaGCTAAcTTccTcGGGAAGA GAA-	-1	**0.04** ±0.03
pBgalluc-2_{SL} -CAGGCTAA**TTTTTTA**GGGAAGAga-	-2	**0.03** ±0.02

FIG. 2. Delimitation of nucleotide sequences required for frameshifting in BHK-21 cells. Enzymatic quantification of frameshifting by transient expression of the bifunctional fusion protein GAL-LUC. Relative luciferase expression normalized to β-galactosidase activity from the indicated expression constructs is shown. The luciferase activity in extracts of cells transfected with the −1 fusions is due to ribosomal frameshifting, resulting in the bifunctional GAL-LUC protein. The absolute amount of enzyme activity from 2×10^5 pBgalluc0-expressing cells is 2×10^5 light units (from luciferase) and an optical density at 420 nm of 0.25 (10-min reaction) (from β-galactosidase). The β-galactosidase activity from the −1 mutants and pBgalluc0 does not differ significantly. The luciferase expression of the indicated mutants is normalized to the β-galactosidase activity and related to the amount of light units with pBgalluc0, which is defined as 100%. Values for frameshift efficiency were obtained by at least six independent transfection experiments. The standard deviation is indicated. The β-galactosidase gene and a *Sal*I linker are depicted as an open box; hatched boxes represent the *Bam*HI-*Sac*I linker sequence and the luciferase gene (see also Fig. 1A). Sequences derived from HIV-1 are shown by large capital letters, and the shifty sequence is printed in boldface capitals. Whereas in all other constructs the shifty sequence is between the reading frames for β-galactosidase and luciferase, in pBgalluc-1_{hepta*} the shifty sequence has been placed into the coding region for the luciferase gene.

U UUU UUA positioned correctly at any site within a reading frame is sufficient to mediate frameshifting. Because of the calculated frequency of that heptanucleotide in the genome, frameshifting should also take place in nonviral mammalian genes. We have performed a computer-assisted search of human sequences from the EMBL data base (release 29, December 1991). While the frequency of the heptanucleotide sequence within flanking and intronic gene sequences is three times higher than statistically expected, it occurs much less frequently in the reading frame of human genes. The heptanucleotide sequence in the appropriate reading frame is nearly fivefold underrepresented. We found 19 different genes from 11,256 human sequence files in which the motif U UUU UUA should lead to transframe proteins. The length of the putative transframe extensions is from 4 to 50 amino acids. The combination of the heptanucleotide sequence with an adjacent secondary structure was found in several cases. We conclude that during evolution, the frameshift sites that were not needed or neutral were eliminated. We predict that the remaining transframe proteins play a distinct role in cellular metabolism.

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