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Targeting frameshifting in the human immunodeficiency virus

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Abstract

Introduction—HIV-1 uses a programmed -1 ribosomal frameshift to generate Gag-Pol, the precursor of its enzymes, when its full-length mRNA is translated by the ribosomes of the infected cells. This change in the reading frame occurs at a so-called slippery sequence that is followed by a specific secondary structure, the frameshift stimulatory signal. This signal controls the frameshift efficiency. The synthesis of HIV-1 enzymes is critical for the virus replication and therefore, the -1 ribosomal frameshift could be the target of novel antiviral drugs.

Areas covered—This review examines various approaches used to select drugs interfering with the -1 frameshift of HIV-1. These include the selection and modification of chemical compounds that specifically bind to the frameshift stimulatory signal, the use of antisense oligonucleotides targeting this signal and the selection of compounds that modulate HIV-1 frameshift, by using bicistronic reporters where the expression of the second cistron depends upon HIV-1 frameshift.

Expert opinion—The most promising approach is the selection and modification of compounds specifically targeting HIV-1 frameshift stimulatory signal. The use of antisense oligonucleotides binding to the frameshift stimulatory signal is still questionable. The use of bicistronic reporters preferentially selects compounds that modulate the frameshift by targeting the ribosomes, which is less promising.

Keywords

-1 ribosomal frameshift; antiframe-shift agents; Gag-Pol/Gag ratio; HIV-1

1. The programmed -1 ribosomal frameshift in HIV-1

The human immunodeficiency virus of type 1 (HIV-1) is a retrovirus, which causes the acquired immunodeficiency syndrome (AIDS), a major health concern, and continues its deadly spread in spite of great progress in the current antiretroviral therapy. This situation makes it urgent to develop novel drugs against HIV-1 and to characterize novel targets in this virus. One such target is a ribosomal frameshift, a change in the reading frame that occurs during the translation of one of the mRNAs produced by the virus. Once the genome of HIV-1 is integrated into the DNA of the infected host, it generates, by transcription and splicing, a variety of mRNAs. Among them is the so-called full-length viral mRNA, which is unspliced and corresponds to the whole viral genome [1]. Translation of the HIV-1 full-length mRNA generates Gag, the precursor of the viral structural proteins and, via a programmed -1 frameshift, Gag-Pol, the precursor of the viral enzymes. This frameshift consists of a shift of the translational reading frame by one nucleotide in the 5' direction (-1

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frameshift), and is programmed since it is not random but occurs when ribosomes reach a specific sequence on the mRNA (Figure 1). This programmed change in the reading frame was discovered more than two decades ago [2] and, since that time, enormous progress has been made in the elucidation of the mechanism accounting for this frameshift.

The sequence where the shift occurs on the viral mRNA is the so-called slippery sequence, U-UUU-UUA (with codons presented in the zero frame). Nucleotides upstream of the heptanucleotide can also contribute to the frameshift efficiency (see below). The slippery sequence is followed by the frameshift stimulatory signal, which is an irregular helix made of a lower and an upper stem separated by a purine bulge. The structure of the frameshift stimulatory signal was first proposed from site-directed mutagenesis studies [3] and solved by NMR [4, 5] (Figure 2). These structural studies and the other studies reported in this review correspond to the HIV-1 group M subtype B, the group and subtype that prevail in the Western World. It is known that the group M of HIV-1 is responsible for the pandemic of AIDS [6]. The slippery sequence is conserved in all the subtypes of group M, but there are differences in the sequence of the frameshift stimulatory signal although this sequence is highly conserved within each subtype as shown by the Los Alamos HIV Sequence Database (<http://hiv-web.lanl.gov>). However, in all subtypes, the signal maintains the same bulged helix secondary structure [7].

The lower stem of the frameshift stimulatory signal is unstable [4, 5] and easily unwound by ribosomes translating the viral mRNA. On the other hand, the upper stem is extraordinarily stable, interferes with the movement of the ribosomes and constitutes the effective frameshift stimulatory signal [4, 5, 8]. After peptide bond formation, normal translocation during translation elongation allows the deacylated tRNA and the peptidyl-tRNA to move from the P and A sites to the E and P sites, respectively, dragging the mRNA by three nucleotides in the process [9, 10] (Figure 3A). A current attractive but still hypothetical model for programmed -1 ribosomal frameshifts is that the frameshift is triggered by an incomplete translocation of two nucleotides instead of three, due to the resistance of the upper stem of the frameshift stimulatory signal to unwinding. This incomplete translocation, which occurs in the elongation cycle prior to the occupancy of the P and A sites by the slippery sequence, prevents the deacylated tRNA and peptidyl-tRNA from occupying the standard E and P sites. As a result, the two tRNAs occupy intermediate sites, displaced by only two nucleotides from their initial position. Note that this incomplete movement occurs only for a small proportion of ribosomes, as the frameshift efficiency in cultured human cells is approximately 10% [11]. Therefore, the majority of ribosomes succeed in unwinding the upper stem and continue translation in the zero frame. Because of the incomplete movement of the two tRNAs, the last codon of the slippery sequence, UUA is positioned in an intermediate entry site that will be occupied by the incoming aminoacyl-tRNA^{Leu}. After the arrival of this tRNA, the three tRNAs unpair from the mRNA and move sequentially by one nucleotide in the 5' direction and re-pair to the mRNA in the -1 frame (Figure 3B). The movement of these tRNAs into the -1 frame is presumably driven by the many favourable contacts between the tRNAs and the standard ribosomal A, P and E sites [12, 13]. The ribosomes then unfold the upper stem and translation continues in the -1 reading frame. Also, it is possible that ribosomes use more than one mechanism for changing the reading frame [reviewed in 13, 14]. For example, an incomplete two-base translocation that triggers a -1 frameshift could occur for a small proportion of ribosomes in the following elongation cycle, when the slippery sequence occupies the P and A sites [15]. One still unsolved question concerns the fact that only a small proportion of the ribosomes make a -1 frameshift. There are two basic hypotheses that could explain this phenomenon. The first is based on the idea that translating ribosomes are processive Brownian motors [16]. This model posits that translating ribosomes experience stochastic thermal fluctuations that produce a Boltzmann distribution of energetic states, with some ribosomes in a more stable

state and others in a less stable state. If frameshifting involves overcoming an energetic barrier, then only the small fraction of ribosomes that are in a favourable energetic state may shift into the -1 frame. A second, alternative hypothesis is that a small population of ribosomes are specialized to frameshift with high efficiency. An example of such specialized ribosomes would be those that lack a ribosomal protein or differ in posttranscriptional or posttranslational modifications from the majority of the ribosome population [17]. This latter hypothesis suggests that frameshifting may be modulated by cellular conditions, which could explain why reported measurements of HIV-1 frameshift efficiencies vary widely depending upon the source of translating ribosomes [18]. Recently, a novel mechanism was proposed for a murine leukaemia virus recoding by readthrough of a stop codon, suggesting that an equilibrium-dependent switch between two conformations of the readthrough stimulatory signal controls the readthrough efficiency. However, this switch does not appear to exist for HIV-1 frameshift stimulatory signal [19].

The frameshift efficiency depends upon the kinetics of translation elongation [14]. Also, since the frameshift stimulatory signal is unwound after the passage of a ribosome, whether the next ribosome is close or at a distance will provide less or more time for the signal to refold before encountering this ribosome. This influences the probability of frameshifting, since no frameshift occurs in the absence of the frameshift stimulatory signal. Therefore, although the frameshift occurs during the elongation phase of translation, it has been shown that translation initiation can modulate the frameshift efficiency by influencing the distance between translating ribosomes [20, 21]. Cellular factors could also affect the frameshift. An example found so far is the release factor eRF1 [22]. Depletion of this factor was shown to enhance HIV-1 frameshifting about two-fold but the mechanism accounting for this effect is unknown. In the same trend, it can be hypothesized that some cellular kinases and phosphatases could also affect the frameshift efficiency by modulating the activity of various factors involved in translation.

2. Do programmed -1 ribosomal frameshifts occur during translation of cellular genes?

So far, two genes of retroviral origin that use a programmed -1 ribosomal frameshift have been found in humans: PEG10 [23], which is highly expressed during development and is also expressed in a limited number of adult tissues, and could play a role in cancer [24], and the human paraneoplastic antigen Ma3 gene, whose ectopic expression in tumor cells is associated with serious neurological phenotypes [25]. The tumor suppressor APC, a regulator of intestinal stem cell proliferation, is also proposed to use a programmed -1 frameshift in humans [26]. In yeast, it has been shown that operational -1 frameshifts destabilize mRNAs, thus controlling their abundance [27, 28], and it is suggested that this type of regulation could be found in all eukaryote genomes. (Note that these frameshifts are not called programmed because they do not generate a distinct protein. However, they share the characteristics of programmed -1 frameshifts i.e. a slippery sequence and a downstream frameshift stimulatory signal.) Also, it is known that ribosomal frameshifts can contribute to epitopes for an expanded repertoire of cytotoxic CD8 T-cells, but the mechanisms accounting for these frameshifts are not known [29]. The occurrence of programmed translational frameshift events in humans has consequences for the development of novel retroviral drugs.

3. The frameshift as a target

The frameshift efficiency in HIV-1 controls the Gag-Pol/Gag ratio that appears critical for virus assembly and maturation. It is commonly assumed that a deviation of this ratio, whether a decrease or an increase, would handicap viral replication. This notion originated

from assays using a double-stranded RNA virus in yeast whose replication requires a programmed -1 frameshift [30]. However, this virus can use a range of frameshift efficiencies from 1.3 to 3.5% for its replication, showing that doubling a basal value does not interfere with viral replication. Subsequent studies directed at the HIV-1 frameshift site supported the idea that a decrease or, depending upon its extent, an increase in frameshift efficiency inhibits viral replication (see below).

A small decrease (30 to 60%) of HIV-1 frameshift efficiency was observed in response to sequence changes in the slippery site or the frameshift stimulatory signal, either as the result of natural variations [31] or via laboratory mutations [11]. Such sequence changes indeed handicap viral replication and it has been proposed that even a small shortage of Gag-Pol can inhibit the assembly of functional virions. But what happens when there is an increase in HIV-1 frameshift efficiency, therefore an excess of Gag-Pol? Interestingly, a two-fold increase in HIV-1 frameshift efficiency, obtained with either the ribosome-targeting drug sparsomycin [32] or a hydrazide derivative [33], resulted in an increase in viral replication. This observation can be explained if the Pol proteins are limiting during viral assembly, and is in agreement with the observation mentioned above for the yeast virus, confirming that there is a window of values for HIV-1 frameshift efficiencies that are compatible with the virus replication. Shehu-Xhilaga et al. [34] co-transfected cultured 293 T cells with HIV-1 proviral DNA and an HIV-1 Gag-Pol expression vector. With this procedure, a range of Gag-Pol/Gag ratios was artificially obtained and it was found that an increase of about four-fold or more of the basal Gag-Pol/Gag ratio actually interfered with viral replication. However, no change was seen in the proportion of Gag-Pol versus Gag within the virions. The authors related the problem caused by the increased Gag-Pol/Gag ratio to a reduction of the RNA dimer stability within the virions produced under those conditions. One can also wonder what happens if the stability of the frameshift stimulatory signal is increased, thus increasing its resistance to unwinding and its interference with translocation. It had been observed that decreasing the stability of the upper stem of HIV-1 frameshift stimulatory signal decreases the frameshift efficiency, but increasing this stability also decreases this frameshift efficiency [35]. More recently, Sorensen and his colleagues investigated how artificial frameshift stimulatory signals with an increased stability influence frameshifting in bacteria. They found that, beyond a given threshold, a fraction of the ribosomes that frameshifted as well as a fraction of those that did not frameshift were stalled at the signal, preventing them from continuing translation [36]. Thus, if this is applied to HIV-1, an increased stability of the frameshift signal would handicap HIV-1 replication by interfering with translation of Gag and Gag-Pol. Altogether, these results show that a drug targeting the -1 ribosomal frameshift of HIV-1 must bring the frameshift efficiency outside the window of efficiencies that are acceptable for the virus. Increasing the basal frameshift value presents the danger that it may be insufficient to handicap viral replication and may even stimulate it, whereas decreasing the basal frameshift value is generally able to interfere with viral replication, even if this decrease is small.

4. Approaches that target HIV-1 frameshift

The importance of the frameshift efficiency in the control of the Gag-Pol/Gag ratio encouraged the development of various approaches attempting to discover small molecules that could modulate HIV-1 frameshift efficiency. Several of these approaches took advantage of the fact that HIV-1 frameshift efficiency can be recapitulated in yeast and bacteria [37, 38]. Dinman et al. [39] first showed that drugs targeting the ribosome such as sparsomycin and anisomycin altered the efficiency of a programmed -1 frameshift when they were used at sub-lethal doses. A pioneering work was performed by Hung et al. [40], who used a reporter firefly luciferase with the frameshift region of HIV-1 inserted in the coding sequence of luciferase, such that only ribosomes that make a -1 frameshift in this

region synthesize the C-terminal part of luciferase and therefore a functional protein. A control reporter was made where the two portions of luciferase were in frame and where the slippery sequence was inactivated by mutagenesis. The reporter mRNAs were translated in a rabbit reticulocyte lysate and high-throughput screening of a library of 56,000 chemical agents selected a benzene derivative, RG501, which increased HIV-1 frameshift efficiency two-fold with the frameshift reporter while having a minimal effect on the translation of the control reporter, and was found to interfere with HIV-1 replication. It was recently demonstrated by the group of Butcher that the selected compound does indeed interact with HIV-1 frameshift stimulatory signal. However, it also interacts non-specifically with other RNAs, and its structure suggests that it can bind to any RNA helix of nine base pairs or greater in length [41]. This is consistent with the data of Hung et al. [40], indicating that RG501 modulates the efficiency of a large number of programmed -1 frameshifts. The observed lack of specificity suggests that RG501 can in fact target the ribosome, a rich source of helical RNA structures [41]. Furthermore, its interference with viral replication was observed at doses much lower than those altering the frameshift efficiency, casting a doubt on a direct relationship between this interference and the change in frameshift efficiency [42]. In order to better control for non-specific effects on translation in frameshift assays, bicistronic reporters were developed where expression of the second cistron depends upon the HIV-1 frameshift. Among these were bicistronic luciferase reporters with Renilla luciferase as the first cistron and firefly luciferase as the second cistron [43, 44]. Fluorescent bicistronic reporters (with the Red Fluorescent Protein and Green Fluorescent Protein) were also developed (see Figure 4), which have been utilized in bacteria [45], in cultured mammalian cells [46] and in yeast [47]. The bacterial system [45] was used to screen a library of arginine-rich peptides and successfully selected a small number of peptides that decreased HIV-1 frameshift efficiency about two-fold. Two out of ten peptides were shown to decrease HIV-1 frameshift efficiency by about 40% when coexpressed with an appropriate dual-luciferase reporter in cultured mammalian cells, but these peptides have not yet been tested for their effect on HIV-1 replication. Also, the peptides were not specific for the HIV-1 frameshift and modulated other programmed -1 frameshifts, suggesting that their target is the ribosome, as in the study of Hung et al. [40]. Recently, Dulude et al. [33] screened a library of 80,000 chemical compounds, using a dual-luciferase reporter expressed in cultured mammalian cells. They selected a hydrazide derivative that increased HIV-1 frameshift efficiency about two-fold. However, this derivative did not interfere with HIV-1 replication but stimulated it, as mentioned above. This stresses the fact that a window of frameshift values is compatible with HIV-1 replication. The selected compound was not specific for the HIV-1 programmed -1 frameshift, also suggesting that its target was the ribosome.

Two independent research groups, the group of Butcher and that of Miller focused on the selection by high-throughput screening of drugs interacting with HIV-1 frameshift stimulatory signal and on the synthesis of modified drugs susceptible to interact specifically with that structure. Butcher and coworkers found that guanidoneomycin, obtained by modifying neomycin [48], and doxorubicin, selected by high-throughput screening of 34,500 compounds [49], interact with HIV-1 frameshift signal. These compounds however lack specificity and bind to other targets. They obviously need to be optimized, but set the stage for the development of improved drugs that could efficiently and specifically target HIV-1 frameshift signal. As mentioned above, the Butcher group also studied the interaction between HIV-1 frameshift stimulatory signal and RG501, the compound selected by Hung et al. [40] in the first high-throughput screening attempting to target the HIV-1 frameshift event [41]. This study provides useful information for the characterization of potential modifications that may increase specificity of binding to the HIV-1 frameshift stimulatory signal.

The group of Miller synthesised and screened in situ an 11,325 member resin-bound dynamic combinatorial library, based on a core structure inspired by nucleic acid binding, bis-intercalating natural products [50]. A set of disulfide-containing peptides was obtained whose stability was enhanced by incorporating an olefin bioisostere of the disulfide moiety [51]. These promising products are not toxic, have binding affinities (K_d) in the high nanomolar to low micromolar range, and display some specificity towards the HIV-1 frameshift stimulatory signal. However, they have not yet been assayed against HIV-1.

A completely different approach consists of using modified oligonucleotides that could affect ribosomal -1 frameshifting by binding to an mRNA downstream of a slippery sequence. Initially, it was shown that the binding of these oligonucleotides, in the absence of the authentic frameshift stimulatory signal, created a duplex that interferes with the movement of the ribosome along the mRNA [52-54]. Among the modified oligonucleotides used were antisense morpholino RNA oligonucleotides or nucleotides bearing locked nucleic acid (LNA) modifications that resist degradation by cellular nucleases and have a high affinity for their complementary sequence. Interestingly, Oh and coworkers recently showed that an antisense peptide-oligonucleotide complementary to a portion of the frameshift stimulatory signal decreased the frameshift efficiency of the coronavirus that causes the severe acute respiratory syndrome (SARS) [55] and interfered with the virus replication. They hypothesized that the binding of the antisense oligonucleotide altered the structure of the frameshift stimulatory signal and increased its resistance to the movement of the ribosomes along the RNA. As discussed above, increasing the stability of the frameshift stimulatory signal can stall the ribosomes on the slippery sequence, accounting for the observed decrease in the frameshift efficiency. However, so far, no antisense oligonucleotide was found that could interfere with HIV-1 frameshifting. In one case, an oligonucleotide complementary to a portion of the frameshift region was crosslinked to its target [56]. This blocked translation of the viral messenger, but such procedure is not appropriate for the development of anti-HIV drugs. Moreover, a potential problem is that antisense oligonucleotides can induce dramatic immune modulation by binding to Toll-like receptors [57].

5. Conclusion

A large number of drugs against HIV-1 have been developed and are successfully used to target the viral enzymes or entry [58, 59]. However, the rapid emergence of drug-resistant variants underlines the necessity to envisage other targets and to continue the search for new drugs against HIV-1. The HIV-1 frameshift constitutes a target that has not been exploited so far, but is very promising for the development of novel antiretroviral drugs [see also 60].

6. Expert opinion

After a survey of the present research on targeting the HIV-1 frameshift event, it appears that the use of bicistronic reporters preferentially selects for drugs interacting with the ribosomes, which contain a very large variety of RNA structures. Our opinion is that drugs targeting the ribosomes should not be favoured, in light of the key role played by ribosomes in cellular life and the high probability that such drugs would cause a number of toxic effects. Moreover, the frameshift events that occur in human genes could be affected under these conditions, with unknown and potentially dangerous side effects. The same comment applies to drugs targeting cellular factors that could modulate programmed -1 frameshifts. As to the oligonucleotides targeting frameshift stimulatory signals, their use is based on a simple principle and one successful case has been reported with the SARS coronavirus. Whether a similar approach can be applied with success to the HIV-1 frameshift stimulatory signal is however still questionable.

In light of the current results, our opinion is that the most promising approach is the one that selects compounds specifically targeting the frameshift stimulatory signal of HIV-1. The power of chemistry makes it possible to select, modify and improve compounds satisfying the conditions of specificity, high affinity for the target and lack of toxicity. Also, as suggested by the group of Butcher [41], the use of “RNA-focused” fragment libraries [61] could be highly beneficial for this search. Such RNA-focused libraries would contain compounds that are complementary to the chemical composition of RNA, e.g. planar aromatic groups for stacking on nucleobases, positively charged groups that can interact with phosphates, and polar, peptide-like groups that can hydrogen bond to various functional groups on the RNA [51]. There are still many hurdles in this approach but it appears to have an enormous potential. As mentioned above, there are variations in the sequence of the frameshift stimulatory signal between the subtypes of HIV-1 group M [7], suggesting that different compounds may need to be developed against the different subtypes. The expertise obtained with drugs targeting HIV-1 group M subtype B, which has been the subject of the studies targeting the ribosomal frameshift to date, should be readily transferrable to the search for drugs targeting other subtypes. However, it is likely that the virus will be able to escape by mutation drugs targeting the frameshift stimulatory signal, which means that these drugs should be used in combination therapy, as it is usually the case for anti-HIV-1 drugs. This raises a concern for a combination with protease inhibitors. Indeed, mutations causing resistance to protease inhibitors are known to evolve in and around the frameshift stimulatory signal. These mutations do not alter the frameshift efficiency, ruling out suggestions that an increase in frameshift efficiency, hence in the synthesis of Gag-Pol, the protease precursor, could counteract the effect of protease inhibitors [62–64]. It cannot be excluded, however, that these mutations cause subtle changes in the structure of the frameshift stimulatory signal, interfering with the binding of drugs that target this signal. Therefore, the danger exists that a single mutation could cause resistance to both the protease inhibitors and the frameshift-targeting drugs. This should be carefully considered in any future drug development efforts targeting the frameshift site.

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highlights

- The programmed -1 frameshift that occurs upon translation of the full-length mRNA of HIV-1 generates the precursor of the viral enzymes.
- This frameshift occurs at a specific sequence on the viral mRNA, the slippery sequence, which is followed by an irregular stem-loop, the frameshift stimulatory signal. This signal controls the efficiency of the frameshift, which is critical for the virus replication.
- Altering the stability of the frameshift stimulatory signal can change the frameshift efficiency.
- Small chemical compounds were found to bind to the frameshift stimulatory signal and to modulate the frameshift efficiency.
- An absolute requirement is to increase the specificity of binding of these compounds. Those that satisfy this requirement will constitute promising novel anti-HIV-1 drugs.

Figure 1.

Translation of HIV-1 full-length mRNA. Conventional translation of this mRNA by the majority of ribosomes generates the Gag protein, the precursor of the virus structural proteins (matrix, capsid, nucleocapsid) while a minority of ribosomes make a -1 frameshift at a specific slippery sequence within the frameshift region (see the text) and generate the Gag-Pol protein. The situation can be described as if these ribosomes were reading a base doublet and not a base triplet when translating this slippery sequence. Before the shift, the sequence of Gag-Pol is identical to that of Gag but, after the shift, the ribosomes translate a completely different sequence of amino acids, the Pol part, which is the precursor of the viral enzymes (protease, reverse transcriptase, integrase). Note that the viral mRNA is highly structured but is represented as a straight line for the sake of simplicity.

Figure 2.

The HIV-1 frameshift stimulatory signal. A. The complete signal is a bulged helix whose lower part is easily unwound by the ribosomes while the upper part resists unwinding and acts as the effective signal during translation. B. Structure of the complete HIV -1 stimulatory signal as determined by NMR [5]. The Data Bank accession codes of the atomic coordinates from the RCSB Protein Data Bank are 1Z2J.

Figure 3.

Normal translocation versus incomplete translocation followed by a -1 ribosomal frameshift. A. Normal translocation. After peptide bond formation, the deacylated tRNA and the peptidyl-tRNA move by three nucleotides pulling the mRNA along, so as to occupy the E and P site, respectively. This step is followed by the binding of the next aminoacyl-tRNA to the entry (A/T) site. The deacylated tRNA is ejected from the E site and the aminoacyl-tRNA then occupies the A site. B. Incomplete translocation triggering a -1 frameshift. Translocation is incomplete because of the presence of a frameshift stimulatory signal downstream of a slippery sequence. This signal (here, the upper stem-loop of HIV-1 frameshift stimulatory signal) causes a resistance to the movement of the mRNA. The deacylated tRNA and peptidyl-tRNA, which have moved by two instead of three nucleotides, occupy intermediate sites (E* and P* sites), and the incoming aminoacyl-tRNA occupies an intermediate entry (A*/T*) site. The three tRNAs move then by one nucleotide so as to occupy their standard binding sites on the ribosome. This is followed by the ejection of the deacylated tRNA and the occupancy of the A site by the aminoacyl-tRNA. Translation continues in the -1 frame.

Figure 4.

Scheme of bicistronic reporter mRNAs used to screen for compounds modulating HIV-1 frameshift efficiency. The two cistrons are separated by the HIV-1 frameshift region. The first cistron is translated according to conventional rules while translation of the second cistron is made only by ribosomes that make a -1 frameshift in the HIV-1 frameshift region. Rluc= Renilla luciferase, Fluc= firefly luciferase, RFP=Red Fluorescent Protein, GFP= Green Fluorescent Protein.