In vitro and in vivo testing of conditionally replicating SIV and HIV-1 strains to study viral gene expression

van der Velden, G.J.

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An AUG codon upstream of rev and env open reading frames ensures optimal translation of the simian immunodeficiency virus Env protein

Gisela J. van der Velden, Monique A. Vink, Bep Klaver, Atze T. Das and Ben Berkhout

Laboratory of Experimental Virology, Department of Medical Microbiology, Center for Infection and Immunity Amsterdam (CINIMA), Academic Medical Center, University of Amsterdam

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Abstract

The mRNAs encoding the Rev and Env proteins of simian immunodeficiency virus (SIV) are unique because upstream translation start codons are present that may modulate the expression of these viral proteins. We previously reported the regulatory effect of a small upstream open reading frame (ORF) on Rev and Env translation. Here we study this mechanism in further detail by modulating the strength of the translation signals upstream of the open reading frames in subgenomic reporters. Furthermore, the effects of these mutations on SIV gene expression and viral replication are analyzed. An intricate regulatory mechanism is disclosed that allows the virus to express a balanced amount of these two proteins.
Introduction

Translation initiation on eukaryotic mRNAs generally occurs via ribosomal scanning [1], in which translation initiation factors interact with the 40S ribosomal subunit at the mRNA 5'-cap structure. The ribosomal subunit then scans along the 5'-untranslated region (UTR) until an AUG translational start codon is encountered. The efficiency of translation initiation depends on the sequence context surrounding the AUG start codon. In vertebrate cells, the optimal context is known as the Kozak consensus sequence: RCCAUGG, in which a purine at position -3, C at -1 and G at +4 have the strongest effects on the translation initiation efficiency [2]. If an AUG is not in a favorable context, translation initiation will be inefficient and the ribosome may continue scanning until it encounters an AUG start codon further downstream [2]. This mechanism of leaky scanning has been described for several viruses, including human immunodeficiency virus type 1 (HIV-1), and enables these viral mRNAs to produce more than a single protein [3–5]. For instance, the Vpu and Env proteins are encoded by the same HIV-1 mRNA, where a fraction of the ribosomes ignore the upstream Vpu start codon, which is in a weak Kozak context, and thus gain access to the downstream Env start codon [6–8]. Upstream AUG (uAUG) codons can also serve a regulatory role. For example, Rous sarcoma virus (RSV) uses upstream ORFs (uORFs) to regulate the level of Gag translation [9]. Other regulatory scenarios have also been described for uAUGs in other viruses and eukaryotes [1, 10–12].

The RNA genome of the simian immunodeficiency virus (SIV) displays a complex splicing pattern with several splice donor (SD) and splice acceptor sites (SA), which allows the expression of all structural (Gag, Pol, Env), regulatory (Tat, Rev) and accessory proteins (Vif, Vpx, Vpr, Nef) [13–15]. In principle, splicing ensures that the translational start codon of each protein-encoding ORF represents the first AUG on a dedicated mRNA. A major exception has been described for Rev and Env translation [16]. The mRNAs for both proteins are produced by splicing from SD1 to SA6, but the Rev mRNA requires a second downstream splicing event (Fig. 1). The single spliced mRNA is the unique source for Env translation, but in this mRNA Rev exon 1 is present upstream of the Env ORF. We recently reported the presence of an additional AUG codon immediately upstream of the Rev start codon on the Rev and Env mRNAs [16]. This uAUG (uAUG4 in Fig. 1) is highly conserved among different SIV strains [17] and we demonstrated that it has a regulatory role in SIV Rev and Env translation [16]. Our
data suggest that uAUG4 actively recruits ribosomes that subsequently encounter a translational stop codon just downstream of the Rev AUG, thus bypassing the Rev ORF. Here we investigate the intricate mechanism by which SIV regulates Rev and Env protein expression by modulating the strength of the competing upstream start codons uAUG4 and AUG-Rev. Because the short uORF that starts at uAUG4 (uORF4) terminates at a stop codon that overlaps with the Rev start codon, we also altered the position of this stop codon and tested for the impact on Rev and Env translation in subgenomic reporter constructs. In addition, we measured the effect of these modifications on SIV gene expression and virus replication.

Results

Design of subgenomic reporter constructs

The SIV genome yields a complex array of mRNA transcripts to express all viral proteins [13–15]. In principle, splicing ensures that the translational start codon of each protein-encoding ORF represents the first AUG on a dedicated mRNA. In the case of the Rev and Env mRNAs, this situation is more complex due to the fact that both mRNAs are produced by SD1 to SA6 splicing. The single spliced mRNA is the source for Env translation, whereas Rev translation is served from the doubly-spliced mRNA that is formed by additional SD3-SA7 splicing (Fig. 1). In fact, Rev forms a uORF on the Env mRNA that encodes 23 Rev amino acids.

Figure 1: The SIV genome and subgenomic Rev and Env mRNAs. The SIV DNA genome (on top) with a blow-up of the Tat/Rev/Env region and the Rev and Env mRNAs are shown. The relevant splice donor (SD3) and splice acceptor (SA6 and SA7) sites in SIV RNA are indicated. The regular AUG start codons are indicated (black circle) as well as the upstream AUGs (white circle).
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(exon 1) fused to 50 unrelated amino acids before a stop codon is reached. Furthermore, both ORFs face an AUG start codon (uAUG4) directly upstream of the Rev AUG. The short ORF that starts at uAUG4 (uORF4) terminates at a stop codon that overlaps with the Rev AUG (Fig. 1B). uAUG4 was found to have an impact on Rev and Env translation in subgenomic reporter constructs [16]. AUG-Rev has a negative impact on Env translation. In turn, uAUG4 has a suppressive impact on Rev translation and thus a positive effect on Env expression [16].

We set out to study the mechanism by which this regulation takes place in more detail. An array of mutants was made in which the Kozak motif (Fig. 2C) of uAUG4 was modulated to study the effects on Rev and Env translation (Fig. 2D, changes to uAUG4). Another set of mutants was made in which the uORF4 stop codon was shifted upstream or downstream (changes to uORF4 stop). A third set was constructed in which the strength of the Rev AUG, which represents a relatively weak Kozak motif, was increased (changes to AUG-Rev). Subgenomic rev-luciferase and env-luciferase reporter constructs were made, each encoding SIVmac239 sequences starting at SA6 (genomic position 6769), the splice site used for Rev and Env mRNA production (Fig. 2A). We purposely designed such minimal expression cassettes to avoid complexities present in the SIV genome, e.g. the Tat-LTR and Rev-RRE regulatory circuits. The luciferase reporter was fused to the intact second codon of the Rev and Env ORFs, leaving the sequence context around the AUG codon intact [33–36]. This creates a basic set of two SV40 early promoter-driven reporter constructs: SA6-rev-luc and SA6-env-luc (Fig. 2A). The constructs were transfected into 293T cells and the amount of luciferase protein produced was measured after two days as a measure of the translational efficiency, using renilla luciferase as an internal control.

Impact of uAUG4 strength on Rev and Env translation

As reported previously, SA6-rev-luc produces much less luciferase than the unrelated SV40-luciferase control vector and inactivation of uAUG4 (construct m4) results in a major stimulation of Rev translation compared to the wild-type (wt) control, consistent with active suppression of Rev translation by uAUG4 usage (Fig. 3A) [16]. The Kozak sequence motif of uAUG4 was weakened in construct k2, strengthened in k3 and k4 and made optimal in construct k5 [33, 37]. We measured improved Rev translation for k2 compared to wt, although the effect was not statistically significant (Fig. 3A). One would indeed expect a smaller effect
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Figure 2: Design of SIV mutations to probe the mechanism of translational regulation. (A) Schematic of the subgenomic reporter constructs. SIV sequences starting at SA6 were placed directly downstream of the SV40 promoter in the pGL3-control plasmid and the second codon of the Rev or Env ORF was fused to the luciferase gene. The uAUGs are depicted by a white circle and the regular start codons by a black circle. (B) The RNA sequence surrounding the rev AUG. The encoded Tat, Rev and uORF4 amino acids and their positions are depicted. (C) Alignment of the Kozak consensus sequence and the Kozak motifs of uAUG4, AUG-Rev and AUG-Env. (D) The introduced mutations and their effects. The mutant names are shown in the first column. The introduced mutations are underlined and the position of uAUG4 and AUG-Rev are boxed in grey. The mutations have been grouped by ‘Changes to uAUG4’, ‘Changes to uORF4’ and ‘Changes to AUG-Rev’. ‘Effect’ indicates the direct and indirect effects (KO is knock-out, Kozak +/- is strengthening/weakening, and shift marks changes to the stop codon position of uORF4). Under ‘additional effects’, additional changes in the Tat or Rev proteins are listed for the full-length SIV genome. ‘Construct background’ indicates in which viral backbone the mutation was introduced, either wild-type SIV or the modified SIV-rtTA genome.

of uAUG4 suppression than of complete uAUG4 inactivation as in m4 (Fig. 3A). We measured a subtle suppression of Rev translation upon further strengthening of uAUG4 in the k5 construct, whereas the k3 and k4 mutations had little effect. These results indicate that translation of uORF4 in wt SIV is providing nearly maximal Rev suppression that cannot be much
improved, which is consistent with the notion that uAUG4 is selected more frequently for translation than the Rev AUG [16].

SA6-env-luc produces a similar level of luciferase as the SV40-luc control, which is in agreement with previous observations [16]. Each of the mentioned uAUG4 changes has a slight to moderate negative impact on Env translation (Fig. 4A). We assume that this effect is composed of several effects: constructs m4 and k2 may translate less Env because ribosomes are diverted from uAUG4 to the Rev ORF, which suppresses Env translation. Constructs k3, k4 and k5 may exhibit reduced Env translation because more of the scanning ribosomes will be attracted to uORF4 and only a fraction of these ribosomes will be able to resume scanning upon uORF4 translation to reach and reinitiate at the Env ORF. There will likely also be less ribosomes scanning past both uAUG4 and AUG-Rev when the uAUG4 Kozak motif is strengthened, allowing for less leaky scanning directly toward the Env AUG.

Figure 3: Protein expression of subgenomic Rev reporters. (A-C) 293T cells were transfected with the indicated rev-luc reporter constructs that encode the firefly luciferase gene. Cells were cultured for two days and the intracellular firefly luciferase level (relative light units, RLU) was measured to quantify protein production, using renilla luciferase produced from the co-transfected pRL-CMV plasmid as an internal control. The ratio between firefly and renilla luciferase was calculated and the mean and SEM of 9 measurements are shown for (A) uAUG4 mutants, (B) uORF4 mutants and (C) AUG-Rev mutants. Statistical analysis performed by one-way ANOVA demonstrated that protein production of the m4, k9 and k10 constructs differed significantly from the wt (*, p<0.05). On top of the bars, the protein production is expressed as a percentage compared to the wt construct (set at 100%). Control, cells transfected with pGL3-control. Mock, cells transfected with pBluescript plasmid instead of firefly luciferase-encoding plasmid.
The role of the uORF4/AUG-Rev overlap

The uORF4/AUG-Rev overlap is intriguing because the uORF4 stop codon partially overlaps with the Rev start codon (Fig. 2B). We probed whether this genetic overlap has evolved to obtain maximal Rev suppression, e.g. because terminated ribosomes will not be able to rescan in the upstream direction to restart on the Rev start codon. Alternatively, the mere consumption of ribosomes at uORF4 may have a negative impact on the fraction of ribosomes that reach the Rev start. To distinguish between these two mechanisms, we moved the uORF4 stop codon further downstream, thus increasing the uORF size with 13 codons (k7; Fig. 2D) or a bit upstream, thus decreasing the uORF size with 1 codon (k6), theoretically allowing rescanning and almost direct reinitiation at the Rev AUG. In the former construct, the Rev Kozak motif is slightly weakened due to the stop shift mutation. Manipulation of the position of the uORF4 stop codon had surprisingly little effect on the level of Rev translation (Fig. 3B). Translation of Rev was slightly increased in k6, which is consistent with the idea that reinitiation is now possible because the uORF4 stop codon is slightly upstream of the Rev AUG. Translation of Rev was slightly decreased in k7, which is consistent with ribosome consumption by uAUG4 and a slightly weakened Kozak consensus for AUG-Rev (Fig. 2D). However, this effect is also small, which may be due to the already very efficient inhibition of Rev translation by the wt uORF4.

We also measured the effect on Env translation in the Env-Luc context. Both the upstream (k6) and downstream shift (k7) have a negative impact on Env expression (Fig. 4B). The decrease of Env translation in k6 can be explained by the increase of Rev translation, which would suppress Env translation more effectively (compare Fig. 3B and 4B). K7 requires another explanation because reduced Rev translation (due to Kozak weakening) is expected to lead to increased Env translation. However, mutant k7 also extends uORF4, which normally ferries ribosomes to the Env AUG. Either lengthening of uORF4 triggers ribosomes to be less competent for resumed scanning [1] or the decreased size of the intercistronic region (24 to 11 codons) reduces the rescanning success. It has previously been reported that lengthening the intercistronic domain increases the chances of reinitiation [1, 38]. In mutant k7 the intercistronic length is decreased, therefore the chances of reinitiation would be diminished, explaining the significantly lower amount of Env-luciferase measured (Fig. 4B).
Impact of AUG-Rev strength on Rev and Env translation

Next we zoomed in on the inhibition of Env expression by translation of the upstream Rev ORF. We did so by improvement of the relatively weak Rev AUG Kozak sequence. The mutations varied from a minor improvement in k8 and k9 to a major improvement in k10. The k9 and k10 mutants also inactivate uAUG4, which is expected to further boost Rev translation. Indeed we measured a gradient of improved Rev expression from the rev-luc reporter, resulting in a 5-fold increase for k10 compared to wt (Fig. 3C). Rev-luciferase expression of this k10 variant approaches the activity measured for the control construct. When the same set of mutants was tested in the env-luc reporter we observed the opposite effect: a gradual extinction of Env translation (Fig. 4C). These combined results confirm the tight inverse regulation of Rev and Env translation. In other words, increased translation of the Rev ORF imperatively reduces Env translation from the Env mRNA. Therefore, SIV must have balanced the expression levels of these proteins for optimal replication.

Figure 4: Protein expression of subgenomic Env reporters. 293T cells were transfected with the indicated env-luc reporter constructs. Protein production was analyzed as described for Fig. 3. The mean and SEM of 6 measurements are shown for (A) uAUG4 mutants, (B) uORF4 mutants and (C) AUG-Rev mutants. Statistical analysis using one-way ANOVA indicated that protein production of the k2, k5, k7, k8, k9 and k10 constructs differed significantly from the wt construct (*, p<0.05). On top of the bars, the protein production is expressed as a percentage compared to the wt construct (set at 100%). Control, cells transfected with pGL3-control. Mock, cells transfected with pBluescript plasmid instead of firefly luciferase-encoding plasmid.
RNA stability

To rule out any differences in transcription or mRNA stability of the different luciferase constructs, total cellular RNA was isolated from transfected cells and the luciferase transcripts were analyzed by Northern blotting (Fig. 5). Differences in size between the two sets of luciferase constructs correlate with the expected transcript length (1828 nt for rev-luc, 1904 nt for env-luc, 1839 nt for control). Importantly, similar transcript levels were observed for the different constructs, which confirms that observed differences in protein production (Fig. 3 and 4) reflect differences in translation.

uORF4 changes in the full-length SIV genome

Inactivation of uAUG4 as in m4 is possible in the SIVmac239 genome without affecting the overlapping Tat ORF, but the new set of mutations do affect the Tat coding capacity (Fig. 2D, column ‘additional effects’). We therefore introduced these mutations in a modified SIVmac239 construct, in which the Tat-TAR axis of transcriptional control is replaced by the Tet-On system for doxycycline (dox)-inducible transcription [22]. The Tat function can be inactivated in this SIV-rtTA variant without impairing virus replication [23], thus allowing the introduction of mutations in the uORF4 region without causing a Tat-inactivation phenotype. Transcription and replication of SIV-rtTA is dox-dependent and we therefore added dox to the culture medium when virus production and replication was assayed.

Because the overlap of ORF4 with the Rev AUG is so intriguing, we elected to study
mutations m4, k6 and k7 in the context of the SIV-rtTA-Tatstop molecular clone, in which Tat was inactivated by the introduction of two consecutive stop codons in the beginning of the Tat ORF [23]. 293T cells were transfected with the parental and m4-mutated SIVmac239, the SIV-rtTA-Tatstop variant and its m4, k6 and k7 derivaties. These cells do not express the CD4 receptor that is required for SIV infection, but their high transfection competence allows us to measure intracellular protein expression and transient virus production. After culturing the cells (SIV-rtTA transfected cells with dox) for two days, intracellular Env protein production was analyzed by Western blotting. Using a monoclonal antibody against Env gp160/gp120, we measured a decreased amount of Env for m4 compared to wt SIVmac239 (Fig. 6A), confirming the results obtained with the env-luc constructs. The same trend was apparent for the SIV-rtTA-Tatstop set, where m4, k6 and k7 showed decreased Env levels when compared to wt (Fig. 6B), which corresponds with the expression levels measured with the env-luc constructs.

Next we analyzed the impact of the m4, k6 and k7 mutations on viral gene expression by measuring CA-p27 levels in the culture supernatant using ELISA. A large increase was measured in CA-p27 production for the m4 variant compared to wt, both in the SIVmac239 and the SIV-rtTA context (Fig. 7A and B, respectively). In the SIV-rtTA context, the k6 mutation slightly decreased CA-p27 production and the k7 mutation increased production to almost the same level as m4. These opposite effects of k6 and k7 correlate with their opposite effects seen on Rev translation. More Rev as seen in k6 (Fig. 3B) could cause a premature switch to the export of full-length RNA to the cytoplasm before maximum

![Figure 6: Western blot analysis of Env production. 293T cells were transfected with plasmids encoding SIVmac239 or SIVmac239-m4 (A), SIV-rtTA-Tatstop or its m4, k6 or k7 derivative (B) or pBluescript (A, B mock) and cultured for 48 h (SIV-rtTA with dox). Intracellular proteins were analyzed by Western blotting using antibodies against gp160/gp120 (KK13) [29]. The position of the size marker proteins (in kDa) is shown on the left.](image-url)
transcriptional activation (in this case mediated by rtTA) has been achieved. This may cause the decrease in CA-p27 seen in k6. In k7 we see an opposite effect. Slightly less Rev is produced (Fig. 3B), which could lead to more rtTA production because of a delayed switch to full-length RNA export. Increased rtTA could mean more Gag and thus more CA-p27. These results highlight the complexity of the viral replication cycle.

**Virus replication and virus competition experiments**

We previously reported that inactivation of uAUG4 slightly impaired SIVmac239 replication. Sensitive virus competition studies indicated there was an approximate loss of 20% replication fitness, indicating that uAUG4 is necessary for optimal SIV replication [16]. Replication of the SIV-rtTA-Tat\textsuperscript{stop} variants was tested in the PM1 and 174xCEM cell lines that express the CD4 and CCR5 receptors [24–27]. Virus replication was monitored by measuring the virion-associated reverse transcriptase (RT) activity that accumulates in the culture supernatant. The SIV-rtTA-Tat\textsuperscript{stop} m4, k6 and k7 variants replicated efficiently in both cell lines. However, the m4 and k7 viruses showed delayed replication in 174xCEM cells, whereas the k6 virus replicated less efficiently in PM1 cells (Fig. 8).

Virus replication assays are often not sensitive enough to detect minor fitness differences. We therefore performed pairwise competition experiments with a mutant and wt SIV-rtTA-Tat\textsuperscript{stop} variant. An equimolar mixture of the wt and mutant plasmids was split
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and transfected into both PM1 and 174xCEM cells. At the peak of infection when massive syncytia were observed, the virus was passaged onto fresh cells and cell samples were taken for DNA isolation, PCR amplification and population-based sequence analysis. On both cell lines the wt virus outcompetes the m4, k6 and k7 mutant virus within the short timeframe of 14 days (Fig. 9). These results indicate that there is a significant loss of fitness upon changing uORF4, which we approximate to be around 20% [31, 39, 40]. Thus, although the mutants replicate efficiently on several cell lines, these results demonstrate that the uORF4 stop codon has to overlap with AUG-Rev for optimal viral replication.

Discussion

We set out to further investigate the mechanism employed by SIV to regulate Rev and Env translation by the presence of multiple upstream ORFs (uORF4 on the Rev mRNA, uORF4 and Rev on the Env mRNA). To that end, we modulated the strength of uAUG4 by weakening or strengthening its Kozak motif and studied the impact on Rev and Env translation using subgenomic reporter constructs. The results suggest that suppression of Rev translation by uAUG4 is nearly maximal, as further strengthening of the uAUG4 Kozak motif only minimally affected Rev translation. On the other hand, the levels of Env translation were moderately reduced, also when the uAUG4 Kozak motif was weakened. We propose that there are two
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possible routes for ribosomes to reach the Env AUG. One path is by reinitiation after having translated ORF4, thus bypassing the AUG-Rev. The second path is by double leaky scanning over uAUG4 and Rev-AUG. The more ribosomes uAUG4 absorbs, the less are able to reach Env by leaky scanning. Thus, the strength of uAUG4 seems designed to effectively suppress Rev translation and to allow optimal Env translation.

The uORF4 stop codon overlaps with the Rev ORF start codon. This arrangement seems to be optimal for maximal Env expression, as an upstream or downstream shift of the uORF4 stop codon negatively impacted Env translation. A premature stop could allow the ribosomes to initiate translation on AUG-Rev instead of AUG-Env. A late stop could reduce Env expression by two means. First, ribosomes that translate an extended uORF4 may be less likely to reinitiate at AUG-Env [1]. Second, a reduction of the intercistronic region between uORF4 and Env may also reduce the potential for resumed scanning and reinitiation. The latter effect is consistent with previous studies [1, 38]. By gradually

Figure 9: Virus competition experiments. Virus competition experiments were conducted in PM1 and 174xCEM cells. Pairwise competitions were started by transfecting an equimolar mixture of SIV-rtTA-Tatstop wt and m4 (A), k6 (B) or k7 (C) viral DNA constructs as previously described [30, 31, 40]. The proviral DNA was analyzed by sequencing at the peak of infection when the virus was passaged onto fresh cells. Analysis of the original input DNA (day 0) revealed a double signal (arrow), which confirms the presence of both the wt and mutant virus. The first nucleotide named above each double peak is the nucleotide present in the wt sequence (green: A; red: U; black: G; blue: C).
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strengthening the Kozak motif of AUG-Rev, we demonstrate that Rev translation down-regulates Env translation in a direct manner, which is in agreement with our previous observations [16].

Whereas Rev translation can easily be boosted up to 5-fold with a stronger Kozak motif, this is likely not allowed in the full-length SIV genome because it has a direct negative effect on Env translation. Apparently the restricted Rev translation suffices for optimal export of singly-spliced and unspliced RNA in SIV replication. Increased Rev levels may not only disturb the balanced production of spliced and unspliced mRNAs, but may also be detrimental due to cell toxicity [41]. Although the m4, k6 and k7 mutations did not block virus replication, the sensitive virus competition assays demonstrated a loss in replication fitness. All mutants also showed changes in viral gene expression, as seen by Env Western blot and CA-p27 ELISA analyses. Interestingly, the k6 and k7 mutations both reduced Env production, but had an opposite effect on CA-p27 production. Whereas the results of the subgenomic reporter constructs are relatively simple to interpret, the results obtained with the virus constructs are more complicated due to – among other things – the intrinsic complexities of the transcription circuit (controlled by Tat in SIVmac239 and by rtTA in SIV-rtTA) and the Rev-dependent nuclear export of the viral mRNAs. An increase in Rev could mean a premature switch to nuclear export of unspliced and single-spliced SIV RNA, which could result in reduced Tat/rtTA expression from double-spliced SIV RNA and thus an overall reduced gene expression. Thus, balanced gene expression instead of maximal expression seems key to support efficient SIV replication.

Taken together it seems that SIV has evolved to allow maximal Env protein translation, despite the fact that its ORF represents the third ORF on the dedicated Env mRNA. A complex regulatory mechanism that utilizes two uORFs to achieve high Env translation and at the same time moderate Rev levels. We previously described the striking parallel in Env protein translation scenarios between SIV and HIV-1. Both Env mRNAs have a uORF (Vpu in HIV-1, Rev in SIV) and a uAUG very close to the start of that uORF [6, 16]. Thus, the Env mRNA seems uniquely suited among the many HIV and SIV transcripts to allow a complex level of regulation at the level of mRNA translation.
**Methods**

**Construction of subgenomic reporter plasmids.** The construction of pSIV-SA6-rev-luc, pSIV-SA6-rev-luc-4, pSIV-SA6-env-luc and pSIV-SA6-env-luc-4 was previously described [16]. Mutations in the pSIV-SA6-rev-luc plasmid were created by PCR using forward primer (FP) SIV-rev-SA6-luc (Table 1) and one of the reverse primers (RP) from the set GV013-GV022 (Table 1). The PCR product was digested with HindIII and NarI and ligated into the corresponding sites of pGL3-control (Promega) to create pSIV-SA6-rev-luc variants k2 to k10.

Mutations in the pSIV-SA6-env-luc plasmid were created by PCR with FP SV40-seq and RP GV022 (Table 1) and a second PCR with a FP from the GV023-GV031 set and RP TA016-luc (Table 1). Both PCR products were then mixed and served as template for a third PCR with FP SV40-seq and RP TA016-luc (see [18] for more information on the method). The resulting PCR product was digested with HindIII and NarI and ligated into the corresponding sites of pSIV-SA6-env-luc to create pSIV-SA6-env-luc variants k2 to k10. All construct sequences were verified by sequencing.

**Luciferase assay.** To quantify protein production of each reporter construct, 293T cells were cultured to 60% confluency in 2-cm² wells and transfected with 100 ng luciferase reporter construct, 0.5 ng pRL-CMV and 900 ng pBluescript (as carrier DNA) as previously described [19]. The plasmid pRL-CMV (Promega), in which the expression of renilla luciferase is controlled by the cytomegalovirus (CMV) immediate-early enhancer/promoter, was co-transfected to allow for correction of differences in transfection efficiency. The firefly and renilla luciferase production was measured after culturing the cells for 48 h. Protein production was calculated as the ratio between firefly and renilla luciferase activities and corrected for between-session variation [20].

**Isolation of RNA.** For RNA analysis, 293T cells were cultured in 2-cm² wells and transfected with 1 µg of the subgenomic reporter construct by calcium phosphate precipitation [21]. Cells were washed with phosphate-buffered saline (PBS) after 48 h, lysed in 350 µl RLT buffer (Qiagen) and homogenized with a QIAshredder column (Qiagen). Total cellular RNA was isolated with the RNeasy kit (Qiagen) and contaminating DNA was removed on the column with the RNase-free DNase kit (Qiagen).

**Northern blot analysis of RNA.** After electrophoresis of 5 µg RNA in a 1% agarose gel in 1x morpholinepropanesulfonic acid (MOPS) buffer (40 mM MOPS, 10 mM sodium acetate, pH 7.0) with 6.5 % formaldehyde at 100 volt, RNAs were transferred onto a positively charged nylon membrane (Boehringer Mannheim) overnight by means of capillary force. RNAs were linked to the membrane in a UV cross-linker (Stratagene). The NcoI-XbaI luciferase fragment of the pGL3-control plasmid (Promega) was heated at 96°C for 10 min and labeled with [α-32P]dCTP by use of a High Prime DNA labeling kit (Roche). Pre-hybridization and hybridization of the membrane with the probe were done in ULTRAhyb buffer (Ambion) at 60°C for 1 and 16 h, respectively. The membrane was then washed twice for 15 min at room temperature in low-stringency buffer (2x SSC [1 x SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0], 0.2% sodium dodecyl sulfate [SDS]) and twice for 30 min at 60°C in high-stringency buffer (0.1x SSC, 0.2% SDS). Images were obtained using the Storm 860 phosphorimager (Amersham Biosciences) and data analysis was performed with the ImageQuant software package. The RNA was stained with ethidium bromide to identify the 18S and 28S rRNA bands. The size of the luciferase transcripts was estimated using 18S and 28S rRNAs as markers. The amount of 18S and 28S rRNA was similar for all samples.
A uAUG codon ensures optimal translation of Env in SIV

Construction of SIV-rtTA mutants. The SIVmac239 proviral DNA genome (Genbank accession number MM33262.1) was used as wild-type virus. The inactivation of uAUG4 in SIVmac239 was previously described [16], as was the construction of the modified molecular clone SIV-rtTA [22] and its derivative SIV-rtTAopt-Tatstop [23]. To inactivate uAUG4 in SIV-rtTA-Tatstop, separate PCRs were performed on SIV-rtTAopt-Tatstop with FP SIV-Tat-1 (GGTATGGGGTTCTGGAAGA) plus RP SIV-Tat-Splice-2 (GTTGGATATGGTTTGTGATGCAAGATGTATT) and mutagenic FP GV032 (AAAAAGCTTGGGGATTTTGTATGACAAATCAC; mismatching nucleotides underlined) plus RP SIV-Env-8-GV (GTTGCTGCACTCCAGCC). The products were mixed and served as template for a third PCR with FP SIV-Tat-1 and RP SIV-Env-8-GV. This product was digested with SphI and Kpn2I and ligated into the corresponding sites in SIV-rtTAopt-Tatstop to generate SIV-rtTAopt-Tatstop-m4. Construction of SIV-rtTAopt-Tatstop-k7 was performed in the same manner using FP GV034 (TGGGATATGTTATGCTCAAATGCAGAAGAA) as mutagenic primer. Introduction of the k6 mutations (see Fig. 2D) was performed as described above using FP GV033 (TGGGATATGTTATGACAAATGAGCAAT) as mutagenic primer. The final PCR product for SIV-rtTAopt-Tatstop-k6 construction was digested with Apel and ClaI and the fragment was ligated into the corresponding sites of SIV-rtTAopt-Tatstop. All construct sequences were verified by sequencing.

Cell culture. Human embryonic kidney (HEK) 293T cells were cultured in Dulbecco’s modified eagle medium supplemented with 10% fetal calf serum (v/v, Gibco), 40 units/ml penicillin, 40 µg/ml streptomycin and 0.1 mM minimal essential medium non-essential amino acids (Gibco). For the production of virus particles, 293T cells were cultured to 60% confluency in 2-cm² wells and transfected with 1 µg of the SIVmac239 or SIV-rtTA plasmids by calcium phosphate precipitation as previously described [21]. Cells transfected with SIV-rtTA constructs were cultured with 1 µg/ml doxycycline (dox, Sigma D-9891). Cell-free culture supernatants were harvested after 48 h and virus production was quantified by CA-p27 enzyme-linked immunosorbent assay (Advanced Bioscience Laboratories).

The PM1 T cell line [24, 25] and the 174xCEM cell line [26, 27] were cultured at 37°C and 5% CO2 in Advanced RPMI 1640 containing 1% fetal bovine serum (v/v, Gibco), 2 mM L-glutamine (Gibco), 15 units/ml penicillin and 15 µg/ml streptomycin. To assay virus replication, cells were transfected with 5 µg of the SIV-rtTA constructs by electroporation [21] and cultured in 5 ml medium. PM1 cells were cultured with 10 ng/ml dox and 174xCEM cells were cultured with 100 ng/ml dox. For all replication curves, the virus level in the culture medium was determined with a real-time PCR-based reverse transcriptase (RT) assay, in which AMV RT was used as standard [22, 28].

Western blot analysis. 293T cells were transfected with 1 µg SIVmac239 or SIV-rtTA construct in 2-cm² wells by calcium phosphate precipitation [22]. Cells were cultured for 48 h (SIV-rtTA transfected cells with 1 µg/ml dox) and lysed in 2x SDS gel loading buffer (100 mM Tris-HCl, pH 6.8; 4% SDS; 0.2% bromophenol blue; 20% glycerol; 200 mM β-mercaptoethanol). 10 µl of the lysate was subjected to SDS-PAGE and transferred onto Immobilon-P membrane (Millipore). For immunochemochemical detection of Env protein, membranes were incubated with mouse ascites containing anti-gp160/gp120 antibodies KK13 [29]. Bound antibodies were visualized with peroxidase-linked antimouse IgG and the ECL+ kit (Amersham Biosciences) and analyzed with a Luminescent Image Analyzer LAS-3000 (Fujifilm).

Virus competition experiments, proviral DNA isolation and sequencing. Virus competition experiments were conducted in PM1 and 174xCEM T cells to determine the replication fitness of the start/stop codon mutants relative to the “parental” wt SIV-rtTA-Tatstop virus. Pairwise competitions were started by transfecting equal amounts of each
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viral construct as previously described [30–32]. A sample of the equimolar input mixture was kept as a control.

For proviral DNA analysis, infected cells were pelleted by centrifugation at 1,500 g for 4 min and washed with PBS. DNA was solubilized by resuspending the cells in 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, and 0.5% Tween20, followed by incubation with 200 µg/ml of proteinase K at 56°C for 60 min and subsequently at 95°C for 10 min. For the analysis of Rev-coding exon 1, proviral DNA sequences were PCR amplified from total cellular DNA with primers SIV-Tat-2 (GGGAACCATGGATGAATG) and SIV-Env-4 (CCCTGTCATGTGAATTTACGCT). The PCR product was subsequently sequenced using the BigDye Terminator cycle sequencing kit (Applied Biosystems) and primer SIV-Tat-1 (GGTAGTGAGGTCTGGAG). The sequences were aligned using the CodonCode Aligner software package.

**Funding**

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Table 1: Primers used for construction of subgenomic reporters. Mismatching nucleotides are capitalized in the forward primers (FP) and reverse primers (RP).
References


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