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Chapter 3

An unintentional mutation in Env abolished SIV-rtTA-Tat$^{Y55A}$ replication

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Abstract

The SIV Tat protein has an essential function in virus replication because it stimulates transcription from the LTR promoter. We recently constructed SIV variants in which transcription is activated by an integrated Tet-On transcription system and demonstrated that replication of these SIV-rtTA variants is only slightly reduced upon knocking out Tat production. From this, it was concluded that Tat has no essential non-transcriptional role in SIV replication. However, a previously constructed SIV-rtTA variant with only a single amino acid substitution in the Tat protein (tyrosine-to-alanine at position 55) did not replicate. Because of these seemingly conflicting results, the replication defect of the SIV-rtTA-Tat^{Y55A} variant was analyzed. This search revealed that the Tat^{Y55A} virus had acquired an unintentional mutation in the Env region during construction. This mutation resulted in a truncated Env protein and abolished virus replication.
Introduction

Transcription of human and simian immunodeficiency virus (HIV and SIV) is initiated by the binding of cellular factors, including NFκB, Sp1, TATA-box binding protein and RNA polymerase II, to the promoter region in the 5’ long terminal repeat (LTR) domain of the viral genome. This transcription complex produces low levels of viral transcripts, which are subsequently spliced and translated. Among the early produced viral proteins is the Tat trans-activator protein, which enhances transcription by binding to the TAR hairpin located at the 5’ end of the newly formed RNA transcripts [1–5]. Upon the binding of Tat to TAR, the positive transcriptional elongation factor (pTEFb) is recruited to the transcription complex at the 5’ LTR promoter [6, 7]. The cyclin-dependent kinase 9 (CDK9) component of pTEFb subsequently phosphorylates the C-terminal domain of RNA polymerase II, which enhances its processivity [8, 9]. The assembly of new transcription complexes is also stimulated by pTEFb through the recruitment of TATA box binding protein to the LTR promoter [10, 11]. Furthermore, Tat recruits several chromatin modifying proteins to remodel the promoter region (reviewed in [11–13]) and we recently demonstrated that Tat can also stimulate transcription through a TAR-independent interaction with sequence elements in the U3 promoter region [14, 15].

Several other, non-transcriptional functions have been suggested for Tat during HIV-1 replication (reviewed in [11]). It has been proposed that Tat influences splicing [16], capping [17–19], translation [9, 20–22] and reverse transcription [23–26] of the viral RNA. It has also been suggested that Tat inhibits the cellular RNA interference mechanism [27–30]. Thus, in addition to its undisputed role in HIV-1 transcription activation, a large array of other functions has been proposed for HIV-1 Tat, although some of these roles have been disputed [31].

We previously described the construction of a doxycycline (dox)-dependent SIVmac239 variant in which the Tat-TAR mechanism of transcription control was replaced by the dox-inducible Tet-On gene expression system (Fig. 1) [32]. In this SIV-rtTA variant, the bulge and loop sequences in the stem-loop 1 (SL1) and stem-loop 2 (SL2) domains of TAR are mutated (TAR\textsuperscript{m}), which prevents Tat binding and subsequent activation of transcription [32]. Furthermore, the Nef gene was replaced by the gene encoding the rtTA transcriptional activator protein and dox-responsive tet operator (tetO) elements were inserted in the U3
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promoter region. In the absence of dox, rtTA cannot bind to the tetO sites and viral gene expression is “off”. Dox induces a conformational change in the rtTA protein that allows binding to the tetO sites and activation of transcription. Thus, viral gene expression and replication are switched “on” by dox administration.

We recently demonstrated that mutations in the Tat open reading frame (ORF) that prevent Tat production reduced SIV-rtTA replication slightly because transcription was no longer stimulated by the Tat-U3 interaction [15]. Tat inactivation did not, however, abolish SIV-rtTA replication, which demonstrates that Tat has no other essential function in SIV replication than its role in transcription. Remarkably, a previously constructed Tat<sup>Y55A</sup>-mutated SIV-rtTA variant did not replicate [32]. Because of these seemingly conflicting results, we set out to characterize the replication defect of the original SIV-rtTA-Tat<sup>Y55A</sup> variant.

Results

Inactivation of Tat

We previously described the construction of SIV-rtTA variants in which Tat activity was knocked out by mutation (Fig. 1B) [15]. Since the Tat open reading frame (ORF) partially overlaps with the Vpr, Rev and Env ORFs, all Tat mutations were carefully chosen not to affect any

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Figure 1: Inactivation of Tat. (A) The SIV-rtTA proviral genome. In SIV-rtTA, TAR was inactivated through several nucleotide substitutions in the bulge and loop sequences (TAR'), the Nef gene was replaced with the rtTA gene and tetO binding sites are present in the 5' and 3' LTR regions. (B) Mutations in Tat. The Tat protein sequence with the cysteine-rich, core and basic regions is shown. In the Tat<sup>AUGmut</sup> variant, the AUG start codon was replaced by the ACG codon for Thr and the UUG Leu codon at position 5 was replaced by the UAG stop codon. In the Tat<sup>stop</sup> variant, the UCA (Ser) and UUA (Leu) codons at position 11 and 12 were replaced by the stop codons UGA and UAA, respectively. The Tat<sup>Y55A</sup> variant contains a Tyr-to-Ala substitution (UAA to GCC) at position 55.
other protein. In the Tat\textsuperscript{Y55A} mutant, a tyrosine-to-alanine substitution at position 55 in the cysteine-rich domain of Tat effectively inactivated the transcription function [32, 44]. In the Tat\textsuperscript{AUGmut} variant, the translation start codon was mutated (AUG to ACG) to prevent initiation of Tat translation. Since we previously observed that a similar mutation did not completely block Tat translation in the HIV-1 context [14], the leucine codon at position 5 in the Tat ORF was replaced with a translational stop codon (UUG to UAG), which will lead to premature termination of any residual translation (Fig. 1B). In the Tat\textsuperscript{stop} variant, the serine and leucine codons at position 10 and 11 have been replaced with translational stop codons (UCA.UUA to UGA.UAA) so that only a short 10-amino acid Tat peptide can be produced. To confirm that the mutations abolish Tat activity, the SIV-rtTA DNA constructs were transfected into 293T cells together with a reporter construct in which the luciferase gene is under control of an LTR promoter that can be activated by SIV Tat [32]. Cells were cultured with dox for two days and the intracellular luciferase level was subsequently measured to quantify Tat activity (Fig. 2A). A high luciferase level was observed with the Tat\textsuperscript{wt} construct, which reflects high Tat activity. A background level of luciferase activity was observed with the Tat\textsuperscript{Y55A}, Tat\textsuperscript{stop} and Tat\textsuperscript{AUGmut} mutants, confirming that Tat expression was effectively blocked.

Replication of the Tat-mutated SIV-rtTA variants was tested in the PM1 T cell line, which expresses the CD4 and CCR5 receptors and allows replication of the parental SIVmac239 strain and the SIV-rtTA-Tat\textsuperscript{wt} derivative [36, 37] (Fig. 2B). Cells were transfected with the viral DNA constructs and cultured in the presence of dox. The Tat\textsuperscript{wt} virus replicated efficiently, which resulted in a high viral reverse transcriptase level in the culture medium and the appearance of virus-induced syncytia. In agreement with previous results, the Tat\textsuperscript{Y55A} variant did not replicate [15, 32], whereas the Tat\textsuperscript{stop} and Tat\textsuperscript{AUGmut} variants replicated efficiently [15], although slightly delayed compared to the Tat\textsuperscript{wt} virus (Fig. 2B).

The effect of the Tat-inactivating mutations was also tested in the context of a novel SIV-rtTA variant with an optimized LTR promoter configuration and rtTA gene (SIV-rtTA\textsubscript{opt} [15]). The optimized promoter configuration was obtained in recent \textit{in vitro} evolution studies with SIV-rtTA-Tat\textsuperscript{wt} and includes additional mutations in TAR, triplication of the NFKB binding site and the adjacent tetO element, and a deletion in the upstream U3 region [15]. The novel rtTA variant exhibits increased transcriptional activity and dox-sensitivity and was obtained through \textit{in vitro} evolution experiments with HIV-rtTA [34]. These promoter and rtTA modifi-
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Figure 2: Tat activity and replication of SIV-rtTA variants. (A, C) Tat activity in SIV-rtTA variants. C33A cells were transfected with the SIV-rtTA (A) and SIV-rtTA<sub>opt</sub> (C) constructs together with a reporter construct in which the luciferase gene is under control of the wild-type HIV-1 LTR promoter, which is activated by SIV Tat [32]. Cells were cultured with dox for two days and the intracellular luciferase level (relative light units, RLU) was subsequently measured to quantify Tat activity. The mean and SEM of 4 (A) and 12 (C) measurements is shown. Mock, cells transfected with pBluescript instead of an SIV-rtTA construct. (B, D) Replication of the SIV-rtTA variants. PM1 cells were transfected with 5 µg of the SIV-rtTA (B) or SIV-rtTA<sub>opt</sub> (D) plasmids with a wt or mutated Tat gene. Cells were cultured with 1 µg/ml dox and replication was monitored by measuring the RT level in the culture supernatant. No virus replication was observed in the absence of dox (data not shown).

...cations improve transcription from the SIV-rtTA LTR promoter and SIV-rtTA-Tat<sup>wt</sup> replication, but do not affect dox-control (data not shown).

Tat activity measurements with the SIV-rtTA<sub>opt</sub> construct confirmed that the introduced mutations abolished Tat activity (Fig. 2C). The Tat<sup>stop</sup> and Tat<sup>AUGmut</sup> variants replicated efficiently and with kinetics similar to the Tat<sup>wt</sup> variant in PM1 cells (Fig. 3D). The Tat<sup>Y55A</sup> variant did not replicate, which demonstrates that the replication defect introduced by the Y55A mutation in Tat could not be overcome by improving viral transcription.

The Tat<sup>Y55A</sup> mutation does not affect viral gene expression

Transcription of SIV-rtTA and SIV-rtTA<sub>opt</sub> is controlled by the integrated components of the
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Tet-On system. To test whether or not the Tat\textsuperscript{Y55A} mutation affects viral gene expression, we analyzed virus production of the Tat\textsuperscript{wt} and Tat\textsuperscript{Y55A}-mutated SIV-rtTA and SIV-rtTA\textsubscript{opt} constructs upon transfection of 293T cells. These cells do not express the CD4 receptor that is required for SIV infection, but their high transfection competence allows us to measure transient virus production by quantifying the viral CA-p27 protein in the culture medium. In the absence of dox, the viral promoter was not active and no detectable CA-p27 was produced (data not shown). When the cells were cultured in the presence of dox, virus production was comparably high for the Tat\textsuperscript{wt} and Tat\textsuperscript{Y55A} constructs (Fig. 3A, C), which indicates that the Tat\textsuperscript{Y55A} mutation does not affect viral gene expression.

We also quantified the production of rtTA as an alternative measure for viral gene expression. 293T cells were transfected with the viral constructs and a tetO-luciferase

Figure 3: Gene expression is not affected in the Tat\textsuperscript{Y55A} variant. (A, C) CA-p27 production. 293T cells were transfected with the SIV-rtTA (A) or SIV-rtTA\textsubscript{opt} (C) plasmids with either a wild-type or Y55A-mutated tat gene and cultured for two days in the presence of dox and the CA-p27 level in the culture supernatant was measured. The mean and SEM of 4 measurements is shown. Statistical analysis (using student t-test) demonstrated that the CA-p27 levels did not differ significantly between Tat\textsuperscript{wt} and Tat\textsuperscript{Y55A} variants. (B, D) rtTA production. Cells were transfected with the SIV-rtTA (B) or SIV-rtTA\textsubscript{opt} (D) plasmids and a reporter construct in which the luciferase gene is under the control of the LTR-tetO-promoter as present in SIV-rtTA\textsubscript{opt} [32]. Cells were cultured for two days in the presence of dox and the intracellular luciferase level was measured to quantify rtTA production. The mean and SEM of 6 (B) and 14 (D) measurements is shown. Statistical analysis demonstrated that the rtTA activity did not differ significantly between the Tat\textsuperscript{wt} and Tat\textsuperscript{Y55A} variants. Mock, cells transfected with pBluescript. In the absence of dox, there was no detectable CA-p27 or rtTA production (data not shown).
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Figure 4: Knocking out Tat\textsuperscript{Y55A} production does not restore virus replication. Replication of the SIV-rtTA\textsubscript{opt} variants with a wt or mutated tat gene was tested in PM1 cells. Cells were transfected with 5 µg of the virus plasmids and were cultured with 1 µg/ml dox. Replication was monitored by measuring the RT level in the culture supernatant. No virus replication was observed in the absence of dox (data not shown).

Tat\textsuperscript{Y55A} does not have a trans-dominant negative effect on virus replication

Whereas the Tat\textsuperscript{stop} and Tat\textsuperscript{AUGmut} mutations block Tat production, the Tat\textsuperscript{Y55A} mutation results in the production of a modified Tat protein. If this Tat\textsuperscript{Y55A} protein has a trans-dominant negative effect on viral replication, knocking out Tat expression should restore virus replication. Therefore, an SIV-rtTA\textsubscript{opt} variant was constructed in which the Tat\textsuperscript{Y55A} mutation was combined with the Tat\textsuperscript{AUGmut} mutation. Replication of this SIV-rtTA\textsubscript{opt}-Tat\textsuperscript{AUGmut/Y55A} double mutant was tested in PM1 cells (Fig. 4). Like the Tat\textsuperscript{Y55A} variant, the Tat\textsuperscript{AUGmut/Y55A} double mutant did not replicate. This result demonstrates that the replication defect of the Tat\textsuperscript{Y55A} virus is not due to a trans-dominant negative effect of the Tat\textsuperscript{Y55A} protein, because this protein is not produced in the Tat\textsuperscript{AUGmut/Y55A} variant.

Differences in splicing between the Tat\textsuperscript{wt} and Tat\textsuperscript{Y55A} SIV-rtTA\textsubscript{opt} variants

Next we analyzed the effect of the Tat\textsuperscript{Y55A} mutation on viral RNA production and processing. 293T cells were transfected with the SIV-rtTA\textsubscript{opt} Tat\textsuperscript{wt} and Tat\textsuperscript{Y55A} constructs and intracellular viral transcripts were analyzed by Northern blotting (Fig. 5). No quantitative or qualitative differences were apparent for the unspliced transcripts. However, the bands resulting from the partially and fully spliced transcripts were more diffuse for the Tat\textsuperscript{Y55A} variant (arrows in Fig. 5). This result indicates that the Tat\textsuperscript{Y55A} mutation affects viral RNA splicing. To characterize the differences in spliced RNAs in more detail, the viral RNA was reverse
transcribed and the resulting cDNA was PCR amplified with primer combinations that specifically detect fully spliced transcripts, partially spliced transcripts or both fully and partially spliced transcripts (Fig. 6A and B). Several PCR products that correspond to differently spliced mRNAs were obtained with all primer combinations (Fig. 6C). The PCR products were isolated from gel and sequenced to characterize these mRNAs. Comparison of the SIV-rtTAopt-Tatwt and TatY55A products demonstrated an increased level of the mRNAs resulting from splicing from the major splice donor site (SD1) to splice acceptor site 5 (SA5). Spliced RNAs resulting from SA5 usage have been reported previously [45] as a minor SIV mRNA species. Like the transcripts spliced at SA6, the SA5 RNAs encode Rev (fully spliced) or Env (partially spliced). The SA5 RNAs, however, carry several additional AUG codons upstream of the Rev and Env AUGs, which may affect Rev and Env translation.

**Replication defect of TatY55A virus is due to a frame-shift mutation in Env**

Since the TatY55A variant demonstrated a difference in splicing of the Rev and Env transcripts, we analyzed Env production by the SIV-rtTA variants. SIV produces Env as a precursor protein of 160 kD (gp160) that is subsequently cleaved into a surface protein (SU) of 120 kD (gp120) and a transmembrane protein (TM) of 41 kD (gp41). 293T cells were transfected with the SIV-rtTAopt constructs and the parental SIVmac239 as control. After culturing the cells with dox for two days, the intracellular proteins were analyzed by Western blotting (Fig. 7). When we used the KK13 monoclonal antibody that binds the gp120 domain [42], the SIVmac239
An unintentional mutation in Env abolishes SIV-rtTA-TatY55A replication

Figure 6: The TatY55A virus demonstrates increased SD1-SA5 splicing. (A) The SIV-rtTA RNA genome. The splice donor (SD) and splice acceptor (SA) sites as identified in the SIVmac239 genome [45] are indicated. Total cellular RNA isolated upon transfection of 293T cells with the SIV-rtTAopt variants (as described in Fig. 5) was reverse transcribed and the resulting cDNA was PCR amplified using primers combinations 1+2, 1+3 and 1+4 (primers indicated with black triangles) to amplify partially+fully spliced, partially spliced and fully spliced transcripts, respectively. (B) Schematic representation of the partially and fully spliced viral transcripts as identified by RT-PCR and subsequent sequence analysis (see panel C). The exons are indicated in black and the introns are indicated with grey dashed lines. (C) The PCR products were size-separated by agarose gel electrophoresis and stained with ethidium bromide. The identity of the different products is indicated on the right. Sequence analysis was used to identify the products from primer combinations 1+2 and 1+3. The products from primer combination 1+4 were not sequenced, but their identity was based on the splice site usage observed with primer combinations 1+2 and 1+3.
An unintentional mutation in Env abolishes SIV-rtTA-Tat\\textsuperscript{Y55A} replication

The sample demonstrated the expected gp120 and gp160 proteins. The same proteins were observed with the SIV-rtTA Tat\\textsuperscript{wt} construct. Surprisingly, the Tat\\textsuperscript{Y55A} and Tat\\textsuperscript{AUGmut/Y55A} variants showed only one band with a size of ~140 kDa. We also analyzed the protein samples with the KK41 antibody that was supposed to be monoclonal and target the gp41 domain [43]. However, this antibody detected not only gp41 and gp160 in the SIVmac239 and SIV-rtTA-Tat\\textsuperscript{wt} samples, but also gp120, which may indicate that it is not monoclonal. In agreement with the results obtained with KK13, analysis with KK41 revealed the presence of a ~140 kD protein in the SIV-rtTA-Tat\\textsuperscript{Y55A} and Tat\\textsuperscript{AUGmut/Y55A} samples. These analyses indicate that Env production is disturbed in the Tat\\textsuperscript{Y55A} and Tat\\textsuperscript{AUGmut/Y55A} viruses, which can explain their replication defect. Because both viruses did not produce the gp160 precursor protein and the gp41 product, we sequenced the gp41 domain in SIV-rtTA\\textsubscript{opt}-Tat\\textsuperscript{Y55A}. This analysis revealed the presence of a 2-nt deletion (nt 8459 and 8460 in SIV-rtTA\\textsubscript{opt}; Fig. 8) downstream of the Rev-responsive element (RRE) that caused a frame-shift mutation in the Env ORF. The Env protein that can be produced by this virus will consist of the gp120 domain, 126 gp41 amino acids and 16 non-gp41 amino acids (Fig. 8). Apparently, this truncated precursor protein is not processed into gp120 and truncated gp41 proteins, as only a ~140 kD protein was detected on the Western blot. The absence of gp120 and gp41 explains the replication defect of SIV-rtTA-Tat\\textsuperscript{Y55A}. This Env mutation was also introduced in the SIV-rtTA-Tat\\textsuperscript{AUGmut/Y55A} construct that was derived from the Tat\\textsuperscript{Y55A} construct, which explains the replication defect of this double mutant.

Figure 7: Western blot analysis of Env. 293T cells were transfected with plasmids encoding SIVmac239, SIV-rtTA\\textsubscript{opt}-Tat\\textsuperscript{wt}, SIV-rtTA\\textsubscript{opt}-Tat\\textsuperscript{Y55A}, SIV-rtTA\\textsubscript{opt}-Tat\\textsuperscript{AUGmut/Y55A} or pBluescript (mock control), cultured for 48 h with dox and lysed in 2x SDS gel loading buffer. The samples were analyzed by Western blotting using antibodies against gp160/gp120 (KK13) [42] and against gp160/gp41 (KK41) [43]. The position of the size marker proteins (in kDa) is shown on the left.
An unintentional mutation in Env abolishes SIV-rtTA-Tat<sup>Y55A</sup> replication

**Discussion**

Here we demonstrate that the SIV-rtTA-Tat<sup>Y55A</sup> construct carries an unintentional mutation in the Env gene that prevents the production of the gp120 and gp41 Env proteins. SIV requires both Env proteins for infection of cells and it seems obvious that this Env deficiency was responsible for the replication defect of SIV-rtTA-Tat<sup>Y55A</sup>.

Construction of the SIV-rtTA-Tat<sup>Y55A</sup> plasmid required multiple cloning steps and oligonucleotide-directed mutagenesis PCR reactions [32]. However, none of the PCR amplified fragments included the region in gp41 in which we now identified a 2-nt deletion (Δ2nt; nt 8459 and 8460 in SIV-rtTA<sub>opt</sub>). In the lower panel, the nucleotide sequence of the wt and mutated Env gene is shown (from nt 8416-8511) with the resulting amino acid sequence on top (wt) or below (Y55A; numbers corresponding to amino acid position in gp41). The 2-nt deletion observed in the Y55A virus results in a translational frame-shift after gp41 amino acid 126 and translation will stop at the UGA stop codon after amino acid 142.

![Figure 8: The Env gene in the Tat<sup>Y55A</sup> viruses is mutated.](image)
late transcription via the U3 elements. In HIV-rtTA, Tat also stimulates transcription through an interaction with sequence elements in the U3 promoter region. We recently observed that this Tat-U3 interaction was not affected by a tyrosine-to-alanine mutation at position 26 in HIV-1 Tat [14]. The tyrosine at position 55 in SIV Tat corresponds to the tyrosine at position 26 in HIV-1 Tat and both are located in the highly conserved cysteine-rich domain of Tat. Thus, the Y55A mutation in SIV Tat and the Y26A mutation in HIV-1 Tat reduce TAR-mediated activation of transcription (Fig. 2; [32, 44, 47]), but do not affect U3-mediated stimulation of transcription. This position in Tat is possibly involved in binding to TAR RNA but not in the interaction with the U3 DNA. We previously showed that the Tat-U3 interaction does not require TAR binding [14].

When compared to SIV-rtTA<sub>opt</sub>-Tat<sup>wt</sup>, the SIV-rtTA<sub>opt</sub>-Tat<sup>YSSA</sup> variant showed an increase in splicing from SD1 to SA5 (Fig. 6). It seems unlikely that this difference in splicing is due to the inactivated Tat<sup>YSSA</sup> protein because the SIV-rtTA<sub>opt</sub>-Tat<sup>AUGmut</sup> variant that does not produce any Tat demonstrated a wild-type splicing pattern (data not shown). The Tat<sup>YSSA</sup> mutation may have affected splicing by the inadvertent mutation of an underlying splice enhancer or suppressor sequence element in the viral RNA, or by changing the local RNA structure, which may influence the use of such a sequence element. In HIV-1, mutations that alter the secondary structure of the Tat-coding RNA region have been shown to affect splicing [48] and we recently observed a similar effect in Tat-mutated HIV-rtTA variants (unpublished results). Although we cannot exclude that the identified deletion in Env is responsible for the splice differences, this scenario is less likely because of the large distance between the SA5 site and the position of the 2-nt deletion.

Would a “true” Tat<sup>YSSA</sup>-mutated SIV-rtTA (that lacks the mutation in Env) replicate efficiently? It most likely would. Knocking out Tat production does slightly reduce SIV-rtTA replication because transcription is no longer stimulated by the Tat-U3 interaction. Tat<sup>YSSA</sup> can, however, activate transcription via the U3 region (see above) and it thus seems likely that the corresponding virus replicates efficiently. The mutation may affect splicing, but we observed normal production of CA-p27, rtTA and full-length RNA genomes, which makes it unlikely that replication of SIV-rtTA-Tat<sup>YSSA</sup> would be significantly affected. Nevertheless, the construct has to be made and tested to answer this question and exclude unforeseen effects.
Methods

Construction of SIV-rtTA variants. Construction of the SIV-rtTA plasmids with a wild-type Tat gene (SIV-rtTA-Tat<sup>wt</sup>) or Y55A-mutated Tat gene (SIV-rtTA-Tat<sup>Y55A</sup>) was previously described [32]. In the plasmid pSIV-rtTA<sub>opt</sub>, an optimized promoter configuration is present in both the 5’ and 3’ LTR. This configuration was obtained through evolution of SIV-rtTA-TAR+78<sup>CU</sup> [33] upon long-term culturing in 174xCEM cells (to be described elsewhere). The 63-nt sequence encompassing the NFκB site and adjacent tetO element (TCGCTGAAACAGCAAGGGACTTTCCACAGGATCCCTATCAGTGATAGAGAAATACCACTCCCTA; NFκB site underlined; tetO site in bold) was triplicated and a 289-nt U3 sequence upstream of the NFκB site was deleted (corresponding to positions 9776-10064 in the SIVmac239 proviral genome sequence in GenBank/EMBL; accession number M33262). In addition, this virus contained a TAR+43<sup>UC</sup> mutation. These mutations did not affect dox-control, nor did they restore Tat-responsiveness. Moreover, pSIV-rtTA<sub>opt</sub> contained the more active and dox-sensitive V16 variant of the rtTA gene that was obtained in HIV-rtTA evolution studies [34]. The SphI-NheI fragment (which contains Vpr, Tat, Rev and Env coding sequences) of pSIV-rtTA-Tat<sup>Y55A</sup> was ligated into the corresponding sites of pSIV-rtTA<sub>opt</sub>, which resulted in pSIV-rtTA<sub>opt</sub>-Tat<sup>Y55A</sup>.

Cell and virus cultures. Human embryonic kidney (HEK) 293T cells and C33A cervical carcinoma 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 MEM non-essential amino acids (Gibco). For the production of virus particles, 293T cells were cultured to 60% confluency in 2-cm<sup>2</sup> wells and transfected with 1 µg of the SIV-rtTA plasmids by calcium phosphate precipitation as previously described [35]. Transfected cells were cultured with 1 µg/ml doxycycline (dox, Sigma D-9891) for 48 h. Cell-free culture supernatants were harvested and virus production was quantified by CA-p27 ELISA (Advanced Bioscience Laboratories).

The PM1 T cell line [36, 37] was cultured at 37°C and 5% CO<sub>2</sub> 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 [35] and cultured in 5 ml medium with 1 µg/ml dox. 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 a standard [32, 38].

Tat activity assay. To quantify the Tat activity of the different SIV-rtTA variants, C33A cells were cultured to 60% confluence in 2-cm<sup>2</sup> wells and transfected with 1 µg of the SIV-rtTA plasmids (with variable Tat gene), 20 ng pBlue3’LTR-luc and 0.5 ng pRL-CMV as previously described [32]. The plasmid pBlue3’LTR-luc contains the complete Tat-responsive LTR promoter of the wild-type HIV-1 LAI strain coupled to the firefly luciferase reporter gene [39]. 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 correction for differences in transfection efficiency. After culturing the cells for 48 h with 1 µg/ml dox, the cells were lysed in passive lysis buffer and the firefly and renilla luciferase production was measured with a dual-luciferase assay (Promega). The Tat activity was calculated as the ratio between the firefly and renilla luciferase activities and corrected for between-session variation [40].
rtTA activity assay. To quantify rtTA production by the different SIV-rtTA constructs, 293T cells were cultured to 60% confluency in 2-cm² wells and transfected with 20 ng pSIV-LTR-Δ15tetO-TARₘ-luc construct, 0.5 ng pRL-CMV and 1 µg pSIV-rtTA construct. In the pSIV-LTR-Δ15tetO-TARₘ-luc plasmid, the expression of firefly luciferase is under the control of the dox-rtTA responsive LTR-Δ15tetO promoter of SIV-rtTA [41]. After transfection, the cells were cultured for 48 h with 1 µg/ml dox and subsequently lysed in passive lysis buffer. The firefly and renilla luciferase activities were determined and the rtTA activity was calculated as the ratio between the firefly and renilla luciferase activities and corrected for between-session variation [40].

Isolation of RNA. 293T cells were transfected with 1 µg of the SIV-rtTA constructs by calcium phosphate precipitation and cultured in 2-cm² wells [35]. 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 an RNeasy kit (Qiagen) and contaminating DNA was removed on the column with RNase-free DNase kit (Qiagen).

Northern blot analysis of RNA. After electrophoresis of 5 µg RNA on 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 V for 4 h, 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 SalI-XmaI rtTA fragment of the pSIV-rtTAopt plasmid was labeled with [α-³²P]dCTP with the High Prime DNA labeling kit (Roche). Pre-hybridization and hybridization of the membrane with the probe were done in ULTRAhyb buffer (Ambion) at 55°C for 1 and 16 h, respectively. The membrane was 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], 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 a Storm 860 phosphorimager (Amersham Biosciences). The RNA was stained with ethidium bromide to identify the 18S and 28S rRNAs. The amount of these rRNAs was similar for all samples.

RT-PCR analysis of viral RNA. RNA was reverse transcribed with ThermoScript reverse transcriptase at 50°C (Invitrogen) using oligo(dT)₁₂ and random hexamers as primers. The cDNA product was used as template in a PCR assay with primers SIV-LTR-7 (CCCTCTTCAATAAGCTGCCAT; nt 567-588 in pSIV-rtTAopt) and SIV-Tat-Splice-2 (GTTGGATATGGGTTTGTTTGATGCAGAAGATGTATT; nt 6479-6514) to detect partially and fully spliced RNAs, with SIV-LTR-7 and SIV-Env-4 (CCCTGTCATGTTGAATTTACAGCT; nt 7006-7029) to detect partially spliced RNAs, and with SIV-LTR-7 and tTA-Rev-4 (GGAAGGCAGGTTCGGCTC; nt 9719-9702) to detect the fully spliced RNAs. The cDNA was denatured at 94°C for 5 min and PCR-amplified in 30 cycles of 1 min 95°C, 1 min 55°C, 2 min 72°C and a final extension time of 7 min at 72°C. The PCR products were visualized on a 1% agarose gel stained with ethidium bromide. To identify the splicing products, the DNA fragments were purified and sequenced with the BigDye Terminator cycle sequencing kit (Applied Biosystems) and the same primers that were used in the PCR.

Western blot analysis. 293T cells were transfected with 1 µg SIV-rtTAopt construct in 2-cm² wells by calcium phosphate precipitation [32]. Cells were cultured for 48 h 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 immunochemical detection of Env protein, membranes were incubated with mouse ascites containing anti-gp160/gp120 antibodies.
An unintentional mutation in Env abolishes SIV-rtTA-TatY55A replication

KK13 [42] or anti-gp160/gp41 antibodies KK41 [43]. Bound antibodies were visualized with peroxidase-linked anti-mouse IgG and the ECL+ kit (Amersham Biosciences) and analyzed with a Luminescent Image Analyzer LAS-3000 (Fujifilm).

Env gene sequence analysis. Sequence analysis of the Env gene in SIV-rtTAoptTatwt and TatY55A constructs was performed using the BigDye Terminator cycle sequencing kit (Applied Biosystems) and primers SIV-Env-4-AD (CCCTGTCATGTTGAATTTACAGCT; nt 7006–7029), SIV-Env-5-AD (GGTTTGACCTTGCTTCTTGGAT; nt 8549–8570), mut-Env-3-AD (GTCTCCCACAAGTCTCCCCA; nt 9052–9071), myt-Env-4-AD (GCTCCACCCGTATTGTAGGTA; nt 8971–8991) and rtTA-28-rev (CGTCAGGCCCTCGATACCGACT; nt 9217–9238). The sequences were aligned using the CodonCode Aligner software package.

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