Tat has a dual role in simian immunodeficiency virus transcription

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Tat has a pivotal role in human and simian immunodeficiency virus (HIV and SIV) replication because it stimulates transcription by binding to the trans-activator response (TAR) element. In addition, several other Tat functions have been proposed. Most studies have focused on HIV-1 Tat and much less is known about SIV Tat. An SIVmac239 variant was constructed previously in which the Tat–TAR transcription mechanism is functionally replaced by the doxycycline-inducible Tet-On gene expression mechanism (SIV-rtTA). In this study, SIV-rtTA variants were used to analyse the functions of SIV Tat. It was shown that Tat-minus SIV-rtTA variants replicated efficiently in PM1 T-cells, ruling out an additional essential Tat function. Nevertheless, replication was suboptimal in other cells, and evolutionary pressure to repair Tat expression was documented. It was demonstrated that SIV-rtTA required Tat for optimal gene expression, despite the absence of the Tat–TAR axis. This Tat effect was lost upon replacement of the long terminal repeat promoter region by a non-related promoter. These results indicate that Tat can activate SIV transcription via TAR RNA and U3 DNA elements but has no other essential function in replication in cultured cells. The experiments were limited to cell lines and PBMCs, and did not exclude an accessory Tat function under specific conditions or in vivo.

INTRODUCTION

Transcription of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) is initiated by the binding of cellular factors to the 5′ long terminal repeat (5′LTR) promoter of the viral genome, which results in low levels of viral transcripts and allows production of the Tat trans-activator protein. Tat enhances transcription by binding to the trans-activator response (TAR) hairpin located at the 5′ end of the nascent RNA transcripts (reviewed by Bannwarth & Gatignol, 2005; Brady & Kashanchi, 2005) and recruitment of the positive transcriptional elongation factor (pTEFb) to the transcription complex at the 5′LTR promoter (Richter et al., 2002; Wei et al., 1998). pTEFb subsequently phosphorylates RNA polymerase II, which enhances transcription processivity (Bieniaz et al., 1999; Parada & Roeder, 1996). pTEFb also stimulates the assembly of new transcription complexes by recruiting TATA-box binding protein (Raha et al., 2005). Furthermore, Tat influences viral transcription by recruiting several chromatin-modifying proteins to remodel the promoter region (reviewed by Easley et al., 2010; Gatignol, 2007; Romani et al., 2010).

Several non-transcriptional functions have also been suggested for Tat (reviewed by Romani et al., 2010). For instance, Tat has been shown to influence RNA splicing (Jablonski et al., 2010), capping (Chiu et al., 2001, 2002; Zhou et al., 2003), translation (Braddock et al., 1990, 1993; Charnay et al., 2009; SenGupta et al., 1990) and reverse transcription (Apolloni et al., 2007; Harrich et al., 1997; Kameoka et al., 2001, 2002) of the viral RNA. It has also been proposed that Tat modulates the expression of multiple cellular genes (reviewed by Romani et al., 2010), interacts with a large number of cellular proteins (Gautier et al., 2009, and references therein) and inhibits the cellular RNA interference mechanism (Bennasser & Jeang, 2006; Bennasser et al., 2005; Haasnoot et al., 2007; Schnettler et al., 2009). Thus, in addition to its undisputed role in transcription activation, a large array of other functions has been proposed for Tat, although some of these have been disputed (Lin & Cullen, 2007; Sanghvi & Steel, 2011).

Analysis of the replication capacity of virus variants with a wild-type (wt) or mutated tat gene in cell culture is one of the best experimental models available to study the functions of Tat. Most Tat mutations severely impair virus replication because they inactivate the essential transcription function, making it difficult to study other processes that may be affected by the mutations. We recently used an HIV-1 variant in which the Tat–TAR transcription–activation mechanism was functionally replaced by the doxycycline (dox)-inducible Tet-On system to probe the importance of other Tat functions in virus replication (Das et al., 2011). In the context of this HIV-rtTA variant, it was possible to inactivate Tat expression without eliminating virus replication, but replication in cultured T-cells was
nevertheless stimulated by Tat. Further analysis revealed a TAR-independent interaction of Tat with the LTR promoter that stimulated viral gene expression and replication. These results indicated that Tat has a multi-faceted function in transcription but no other essential function in HIV-1 replication.

Most studies have focused on the function of HIV-1 Tat, and much less is known about the role of SIV Tat. Remarkably, mutational inactivation of Tat in an SIV-rtTA variant was found previously to block virus replication (Das et al., 2007a), whereas HIV-rtTA tolerated a very similar Tat mutation (Das et al., 2004a; Verhoef et al., 2001). We therefore re-examined the role of Tat in SIV replication by constructing and testing new SIV-rtTA variants in which Tat expression was inactivated by different mutations.

RESULTS

Construction of Tat-deficient SIV-rtTA variants. Previously, we described the construction of a dox-dependent SIVmac239 variant in which the Tat–TAR mechanism of transcription control was replaced by the dox-inducible Tet-On gene expression system (Das et al., 2007a) (Fig. 1a). In this SIV-rtTA variant, the bulge and loop sequences in TAR are mutated (TARm) to prevent Tat binding and subsequent activation of transcription (Das et al., 2007a). Furthermore, the nef gene was replaced by the gene encoding the rtTA transcriptional activator protein, and tet operator (tetO) elements were inserted in the U3 promoter region. In the absence of dox, rtTA cannot bind to the tetO sites and viral gene expression is turned ‘off’. Dox induces a conformational change in rtTA that allows binding to the tetO sites and activation of transcription. Thus, viral gene expression and replication are switched ‘on’ by dox administration.

We constructed SIV-rtTA variants in which Tat was inactivated to study additional roles for Tat in SIV replication. As the tat ORF overlaps with other viral ORFs, we decided to minimally mutate the Tat sequence. For construction of the related HIV-rtTA variant, we previously introduced a Tyr→Ala substitution at position 26 (Y26A) in the cysteine-rich domain of HIV-1 Tat, which inactivated its TAR-dependent transcription function (Verhoef et al., 1997). HIV-1 and SIVmac239 Tat have a similar modular structure. The cysteine-rich domain is highly conserved and the tyrosine at position 26 in HIV-1 Tat corresponds to the tyrosine at position 55 in SIVmac239 Tat (Fig. 1b). We therefore introduced the equivalent Y55A mutation in SIV-rtTA, which did not affect any other ORF but nevertheless abolished SIV-rtTA replication (Das et al., 2007a). Because the Y55A mutation may have affected an unknown RNA sequence element that is critical for SIV replication, we constructed two new SIV-rtTA variants with different Tat-inactivating mutations. We mutated the translation start codon (AUG→AUG) to prevent Tat translation in the TatAUGmut variant. As we observed previously that a similar mutation did not completely block Tat translation in the HIV-1 context (Das et al., 2011), we also replaced the Leu codon at position 5 in the Tat ORF with a translation stop codon (UUG→UUG) so that residual translation would terminate prematurely (Fig. 1b). There are no alternative in-frame AUG codons in the Tat ORF. In the Tatstop variant, we replaced the Ser and Leu codons at positions 11 and 12 with translation stop codons (UCA→UAG and UUA→UAA, respectively).
respectively) so that only a 10 aa Tat peptide could be produced. These mutations were chosen carefully to be silent in the overlapping vpr gene.

To confirm that these mutations abolish Tat expression, we co-transfected 293T cells with the SIV-rtTA DNA constructs and a reporter construct in which the luciferase gene was under the control of a Tat-responsive LTR promoter. Cells were cultured with dox for 48 h and the intracellular luciferase level was measured to quantify Tat activity (Fig. 1c). A high luciferase level was observed with the SIV-rtTA-Tatwt construct, reflecting high Tat expression and activity, whilst a background level of luciferase activity was observed with the TatAUGmut and Tatstop mutants, confirming that Tat expression was blocked effectively (Fig. 1c).

**Tat deficiency reduces the replication capacity of SIV-rtTA.** Replication of the SIV-rtTA variants was tested in PM1 T-cells that were cultured with dox (Fig. 2a). The PM1 cell line is a clonal derivative of HUT78 that expresses CCR5 and allows replication of the parental SIVmac239 strain (Chen et al., 1998; Lusso et al., 1995). In agreement with previous results (Das et al., 2007a), SIV-rtTA-Tatwt replicated efficiently in these cells, whereas SIV-rtTA-TatY55A did not replicate. In contrast, the new Tat-deficient variants SIV-rtTA-TatAUGmut and SIV-rtTA-Tatstop replicated efficiently, although replication was slightly delayed compared with the Tatwt virus. The latter result demonstrated that SIV-rtTA can replicate without Tat but that the protein does have a stimulatory effect. It also indicated that the replication defect of SIV-rtTA-TatY55A was not due to the lack of an active Tat protein but was caused by another adverse effect.

We also introduced the TatAUGmut and Tatstop mutations into the genome of a novel SIV-rtTA variant with an optimized LTR promoter and rtTA gene (SIV-rtTAopt). The optimized promoter configuration was obtained through SIV-rtTA evolution following long-term in vitro culture (unpublished data). The promoter changes included additional mutations in TAR, triplication of the nuclear factor κB (NF-κB)-binding site and the adjacent tetO element, and a deletion in the upstream U3 region (Fig. 3). The novel rtTA variant exhibited increased transcriptional activity and dox sensitivity and was obtained through in vitro evolution experiments with HIV-rtTA (Zhou et al., 2006). These promoter and rtTA modifications improved SIV-rtTA gene expression and replication but did not affect dox control (data not shown). Replication of the SIV-rtTAopt variants with a Tatwt, TatAUGmut or Tatstop gene was first tested in PM1 cells (Fig. 2b). All viruses replicated well, suggesting that improved transcription renders Tat redundant in these cells. However, when the SIV-rtTAopt variants were tested in 174xCEM cells (fusion of a B- and T-cell line; Fig. 2c) and in PBMCs isolated from cynomolgus macaques (Fig. 2d), replication of the TatAUGmut and Tatstop viruses was found to be delayed compared with the Tatwt virus. This reduced replication capacity of the Tat-deficient viruses indicated that Tat remains important for optimal SIV-rtTAopt replication in these cell types.

**Evolution of the Tat-deficient SIV-rtTA variants following long-term culture.** Inactivation of Tat did not significantly affect replication of the SIV-rtTAopt variants.
Hairpin (Fig. 4a). As a result, the out-of-frame AUG codon located introduced mutations but acquired a nucleotide insertion observed in culture 3. This virus stably maintained the whereas the mutations in culture 2 repaired wt protein 1 restored the production of an L5Q-mutated Tat protein, and SIV-rtTA, SIV-rtTAopt and SIV-rtTA resulted in 174xCEM cells. The virus was We also started long-term cultures of the Tat-deficient SIV-rtTAopt variants in 174xCEM cells. The virus was passaged onto fresh cells at the peak of infection when massive syncytia were observed. The period between infection and the appearance of syncytia decreased over time, and we could reduce the volume of the virus inoculum needed to start a new infection, which indicated that the virus replication capacity had improved. Sequence analysis of the viral tat gene after long-term culture revealed that the Tat^{AUGmut} virus acquired a C→U mutation that restored the Tat AUG start codon in two independent cultures (cultures 1 and 2, Fig. 4a). Moreover, in both cultures, an additional mutation was observed that removed the introduced stop codon (UAG→CAG in culture 1; UAG→UUG in culture 2), and in culture 2 a C→U mutation was found at position 6394, which was silent in the tat ORF. The combined mutations in culture 1 restored the production of an LSQ-mutated Tat protein, whereas the mutations in culture 2 repaired wt protein production (Fig. 4b). A different evolutionary path was observed in culture 3. This virus stably maintained the introduced mutations but acquired a nucleotide insertion further downstream (G, between positions 6314 and 6315; Fig. 4a). As a result, the out-of-frame AUG codon located at position 77 in the tat gene (bold and underlined at position 6282, Fig. 4a) was placed in register with the tat ORF. This frame-shift mutation allowed the production of a truncated Tat protein in which the N-terminal 35 aa were replaced by ten non-Tat residues (Fig. 4b). Moreover, this virus acquired a single nucleotide substitution (U→C at position 6398) that resulted in a F65L substitution in the Tat protein. This mutation did not affect other ORFs.

In three independent Tat^{stop} cultures, we observed a deletion of 15 or 24 nt in the tat gene, which included the introduced stop codons (Fig. 4c). These in-frame deletions allowed the production of Tat proteins lacking either aa 8–12 or 11–18 (Fig. 4d). In culture 3, a silent C→U mutation was observed at position 6308. In two of the three cultures, a point mutation at position 6444 that caused an R80Q substitution in the basic RNA-binding domain of Tat was also observed. The repeated selection of this mutation argues for an important role, for example in boosting Tat activity. The combined results showed that evolution of the Tat-deficient viruses in 174xCEM cells resulted in restoration of Tat production. These results confirmed that Tat has a positive function in SIV-rtTA replication.

Some of the evolved SIV-rtTA variants encoded a Tat protein with a deletion near the N terminus. It has been described previously that a truncated form of HIV-2 Tat lacking aa 8–33 can activate transcription from the viral LTR promoter (Berkhout et al., 1990). As HIV-2 and SIVmac239 are closely related, it seems likely that the truncated Tat proteins selected during virus evolution (removal of aa 1–35, 8–12 or 11–18) are transcriptionally active.

Some mutations in the Tat region also affected the overlapping vpr and rev genes. The UAG→CAG mutation in Tat^{AUGmut} culture 1 resulted in an L5P substitution in Vpr (CUA→CCA codon change). The G nucleotide insertion between positions 6314 and 6315 in culture 3 caused a frame-shift in the vpr ORF after codon 87, resulting in a truncated 97 aa Vpr protein in which 14 C-terminal Vpr amino acids were replaced by ten non-Vpr residues. The 24 nt deletion observed in the Tat^{stop} cultures 1 and 2 caused deletion of Vpr aa 61–68, whereas the 15 nt deletion observed in culture 3 caused an A58G substitution plus deletion of aa 59–63. Although these mutations did not change the important α-helical region and HxRxG motifs (Mueller & Lang, 2002), it cannot be excluded that they affect Vpr activity. However, Vpr is an accessory SIV protein, and Vpr deletion does not prohibit virus replication in vitro (Gibbs et al., 1994) or in vivo (Gibbs et al., 1995; Hoch et al., 1995). The G→A substitution at position 6444 (observed in Tat^{AUGmut} cultures 1 and 3) caused an E5K substitution in Rev, which does not affect the critical Rev-responsive element RNA-binding domain or nuclear localization motif of the protein (Berchtold et al., 1994).
Tat affects SIV-rtTA expression. Transcription of SIV-rtTA is controlled by the inserted Tet-On system. To test whether or not Tat still contributes to SIV-rtTA gene expression, we analysed the effect of the Tat mutations on virus production. 293T cells were transfected with the Tat<sup>wt</sup>, Tat<sup>AUGmut</sup> and Tat<sup>stop</sup> SIV-rtTA<sub>opt</sub> constructs and

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Fig. 4. Evolution of Tat-deficient SIV-rtTA viruses. Mutations observed in Tat following long-term culture of SIV-rtTA<sub>opt</sub>-Tat<sup>AUGmut</sup> (a, b) and SIV-rtTA<sub>opt</sub>-Tat<sup>stop</sup> (c, d). In (a) and (c), the top line shows the nucleotide sequence of the 5' part of the wt tat gene, with the nucleotide numbers referring to the position in SIV-rtTA<sub>opt</sub>. As shown in Fig. 1(a), this part of the tat gene (translation start at position 6206) overlaps with the genes encoding Vpr (translation stop at position 6358) and Rev (translation start at position 6432). The codons that were mutated in the Tat<sup>AUGmut</sup> and Tat<sup>stop</sup> variants to knock out Tat are underlined. Sequence changes observed in three independent cultures are shown, with the culture number and days [d] of culture indicated on the left. Reversions back to wt sequence are highlighted with grey shading. insertions are indicated in bold, deletions are indicated with Δ, and the Vpr stop and Rev start codons are boxed. In (b) and (d), the corresponding amino acid sequences are shown. Stop codons are indicated with *.
cultured with dox for 48 h. These cells did not express the CD4 receptor required for SIV infection, but their high transfection competence allowed us to measure transient virus production by quantifying the viral capsid (CA)-p27 protein in the culture medium. Virus production was high for the Tat<sup>wt</sup> construct and was reduced to ~70 and ~50% of the wt level for the Tat<sup>stop</sup> and Tat<sup>AUGmut</sup> variants, respectively (Fig. 5a). These results indicated that Tat indeed stimulates SIV-rtTA gene expression. We demonstrated previously that TAR deletion does not reduce SIV-rtTA-Tat<sup>wt</sup> gene expression and replication (Centlivre et al., 2008), which indicates that Tat does not stimulate SIV-rtTA gene expression by residual binding to the mutated TAR<sup>m</sup> element.

To investigate whether Tat contributes to gene expression by enhancing SIV-rtTA<sub>opt</sub> promoter activity or by stimulating another gene expression step, the 5'LTR promoter region was replaced by the tetO-CMV promoter, which previously allowed us to generate a truly Tat-independent HIV-rtTA variant (Das et al., 2011). This dox-responsive promoter consists of three tetO elements coupled to a minimal TATA-box region from the CMV immediate-early promoter, and the transcript encodes an unrelated hairpin instead of TAR (Fig. 3). 293T cells were transfected with SIV-rtTA<sub>5'</sub>tetO-CMV variants with a Tat<sup>wt</sup>, Tat<sup>stop</sup> or Tat<sup>AUGmut</sup> gene, and virus production was measured after 48 h of culture with dox. We measured a similar level of virus production for the SIV-rtTA<sub>5'</sub>tetO-CMV-Tat<sup>stop</sup> and -Tat<sup>wt</sup> constructs (Fig. 5b). Thus, viral gene expression was no longer influenced by Tat when the SIV-rtTA<sub>opt</sub> U3–TAR region was replaced by an LTR promoter that lacked SIV-derived sequences. Because – as mentioned above – Tat does not influence SIV-rtTA expression via the TAR<sup>m</sup> element, this result indicated that Tat stimulates SIV-rtTA expression via the U3 promoter region. Moreover, this result indicated that Tat does not influence another gene expression step. The Tat<sup>AUGmut</sup> variant, however, still demonstrated a reduced level of virus production. This mutation may exert another effect (possibly the inadvertent mutation of an underlying RNA element), but we did not analyse this further.

**Tat stimulates the transcriptional activity of the SIV-rtTA promoter.** To confirm that the LTR promoter in SIV-rtTA was Tat responsive, we constructed promoter-reporter plasmids in which expression of luciferase was controlled by the LTR promoter as present in SIV-rtTA, SIV-rtTA<sub>opt</sub> and SIV-rtTA<sub>tetO-CMV</sub>. 293T cells were transfected with these LTR–luciferase constructs together with an rtTA-expressing plasmid and increasing amounts of an SIV Tat-expressing plasmid. The intracellular luciferase level, which reflects promoter activity, was measured after culturing the cells with 0–100 ng dox ml<sup>–1</sup> for 48 h (Fig. 6a–c). All LTR promoters showed a low background activity in the absence of dox and the activity increased gradually upon dox titration, confirming their dox dependency. At every dox level, the activity of the SIV-rtTA promoter was indeed enhanced by Tat (up to 2.4-fold at 50 ng Tat; Fig. 6a). Similarly, the SIV-rtTA<sub>opt</sub> promoter was stimulated by Tat. However, this optimized promoter was more active than the original SIV-rtTA promoter at all dox levels, and its activity was enhanced by Tat to a lesser extent (up to 1.3-fold; Fig. 6b). As expected, the activity of the tetO-CMV promoter was not influenced by Tat (Fig. 6c), confirming the effect of Tat via U3 sequences. For comparison, we measured Tat–TAR-mediated activation of transcription by co-transfecting cells with an LTR–luciferase construct with a wt TAR element and an increasing amount of Tat (Fig. 6d). Transcription from this promoter was low in the absence of Tat and enhanced up to 25-fold by Tat co-transfection. Thus, the stimulatory effect of Tat on SIV-rtTA and SIV-rtTA<sub>opt</sub> promoter activity (up to 2.4- and 1.3-fold, respectively) was relatively small. Nevertheless, this TAR-independent Tat effect could explain the reduced

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**Fig. 5.** Tat mutations reduce SIV-rtTA gene expression. (a) 293T cells were transfected with the SIV-rtTA<sub>opt</sub> plasmid with either a wt or mutated tat gene and cultured for 48 h with dox. The CA-p27 levels in the culture supernatant are shown (mean±SEM; n=20). Statistical analysis (using one-way analysis of variance) demonstrated that the CA-p27 levels differed significantly between Tat<sup>wt</sup> and Tat-deficient viruses. *P<0.05. (b) Cells were transfected with the SIV-rtTA<sub>opt-tetO-CMV</sub> plasmids and cultured for 48 h with dox. The CA-p27 levels in the culture supernatant are shown (mean±SEM; n=22). Statistical analysis demonstrated that CA-p27 production differed significantly between the Tat<sup>wt</sup> and Tat<sup>AUGmut</sup> variants. *P<0.05. Mock indicates cells transfected with pBluescript.
virus production and replication of the SIV-rtTA variants following Tat inactivation.

DISCUSSION

We used an SIV-rtTA variant in which the Tat–TAR transcription activation mechanism is functionally replaced by the integrated rtTA-tetO components of the Tet-On system to study the role of Tat in SIV replication. Firstly, we showed that the new Tat-deficient SIV-rtTA variants (TatAUGmut and Tatstop mutants) were replication competent and that a previously tested Tat inactivation (Y55A) that yielded a non-replicating virus was not representative. This mutation may have affected an as yet unidentified underlying sequence element in the viral RNA and thus caused the replication defect, but this was not investigated further. Importantly, we showed that SIV-rtTA can replicate without Tat, which demonstrates that Tat plays no essential role in SIV replication besides its transcription function. Secondly, by introducing an optimized LTR promoter and a more active rtTA, we obtained fast-replicating Tat-minus SIV-rtTAopt variants that nevertheless exhibited reduced replication capacity compared with the Tat-positive virus. Thirdly, evolution experiments with the Tat-deficient SIV-rtTAopt variants documented significant pressure to restore Tat translation, which confirms that Tat stimulates SIV-rtTA replication. Fourthly, we demonstrated that SIV-rtTA gene expression was enhanced by Tat. It seems unlikely that Tat exerts this function through the TAR motif, because this element is inactivated by multiple mutations that eliminate Tat binding (Das et al., 2007a). In line with this idea, TAR deletion does not reduce SIV-rtTA gene expression and replication (Centlivre et al., 2008). We succeeded previously in making a truly Tat-independent HIV-rtTA variant by substitution of the viral U3 sequences by non-related promoter elements (Das et al., 2011). Following transplantation of the LTR promoter from this HIV-rtTAtetO–CMV into SIV-rtTA, we reached the point where viral gene expression was no longer influenced by Tat. Unfortunately, these SIV-rtTAtetO–CMV variants (with either a wt or mutated tat gene) were not replication competent, probably due to unrelated adverse effects of the insertion of HIV-optimized sequences into SIV (see below).

Promoter activity assays demonstrated that the activity of the SIV-rtTA and SIV-rtTAopt promoters was dependent on dox and was further enhanced by Tat. This TAR-independent stimulatory effect of Tat (up to 2.4- and 1.3-fold on the SIV-rtTA and SIV-rtTAopt promoters, respectively; Fig. 6a, b) was relatively small when compared with the stimulatory effect of Tat on the activity of a TARwt-containing promoter (up to 25-fold; Fig. 6d). In fact, in the presence of a wt TAR element, the TAR-independent stimulatory effect may be reduced further due to the high affinity of the Tat–pTEFb complex for TAR (reviewed by Bannwarth & Gatignol, 2005). Nevertheless, this

Fig. 6. The SIV-rtTA LTR promoter is stimulated by Tat. (a–c) 293T cells were transfected with promoter-reporter plasmids in which expression of firefly luciferase was controlled by the LTR promoter as present in SIV-rtTA (a), SIV-rtTAopt (b) and SIV-rtTAtetO–CMV (c) (LTR promoter configurations are shown in Fig. 3). The cells were co-transfected with an rtTA-expressing plasmid and 0–60 ng SIV Tat-expressing plasmid. After culturing the transfected cells with 0–100 ng dox ml\(^{-1}\) for 48 h, the intracellular luciferase level (RLU) was measured (mean ± SEM; n=3). (d) Cells were transfected with an SIV-rtTA LTR–luciferase construct with a wt TAR element and 0–50 ng Tat plasmid. After culturing the cells for 48 h (no dox), the intracellular luciferase level was measured (mean ± SEM; n=3).
Tat-independent Tat effect could explain the reduced virus production (Fig. 5a) and reduced replication (Fig. 2) of the SIV-rtTA variants following Tat inactivation. The SIV-rtTAopt promoter was more active and less influenced by Tat than the SIV-rtTA promoter, which is probably due to the presence of additional tetO and NF-kB sites. This difference in activity and Tat stimulation explains why SIV-rtTAopt replication is less affected by Tat inactivation than SIV-rtTA replication (Fig. 2a, b). Substitution of the LTR promoter by the tetO-CMV promoter that lacked SIV-derived U3 and TAR sequences allowed us to obtain truly Tat-independent gene expression. This SIV-rtTA_{tetO-CMV} variant subsequently allowed the introduction of the Tat_{stop} mutation without a decrease in gene expression. We observed previously that TAR deletion does not reduce SIV-rtTA-Tat_{wt} gene expression and replication (Centlivre et al., 2008), which indicates that Tat does not stimulate SIV-rtTA gene expression by residual binding to the mutated TAR element. These results therefore indicated that Tat stimulates SIV-rtTA gene expression through a direct or indirect interaction with U3 elements. It has been shown previously that U3 sequences influence Tat-mediated activation of transcription (Ilyinskii & Desrosiers, 1996; Pöhlmann et al., 1998), but because the constructs used in these studies contained a wt TAR element, it was not possible to discern whether this stimulation was TAR dependent or independent. Our constructs lacked a functional TAR element, suggesting that the Tat–U3 effect does not depend on the Tat–TAR interaction. In agreement with this, we recently showed that HIV-rtTA gene expression is also stimulated by Tat through a TAR-independent interaction with the U3 promoter region (Das et al., 2011). Detailed analysis demonstrated that HIV Tat required the Sp1-binding sites in the U3 region for this stimulatory effect. The SIV U3 region contains four Sp1 sites, which may similarly be involved in the Tat–U3 interaction, but a role of other U3 domains cannot be excluded.

We also tested replication of the new SIV-rtTA variants with the tetO–CMV promoter configuration in PM1 and 174xCEM cells. In these viruses, the U3–TAR region in both the 5′- and 3′LTR region was replaced by the tetO–CMV sequences to avoid strand-transfer difficulties during reverse transcription of the viral genome. Unfortunately, none of the SIV-rtTA_{tetO-CMV} variants showed any replication in multiple independent experiments, and we were unable to obtain replication-competent variants by spontaneous virus evolution (data not shown). The tetO–CMV promoter configuration was obtained through evolution of an HIV-rtTA_{tetO-CMV} variant in SupT1 T-cells (Das et al., 2011). This configuration may be optimal for HIV-rtTA replication in SupT1 cells but insufficient for SIV-rtTA replication in PM1 and 174xCEM cells. The tetO–CMV promoter may, for example, require a transcription factor that is present only in SupT1 cells. We also tested SIV-rtTA_{tetO-CMV} replication on SupT1 cells (data not shown) but were repeatedly unable to detect replicating virus, which is probably due to the absence of the CCR5 co-receptor on SupT1 cells.

Here, we demonstrated that Tat activates SIV LTR transcription not only via TAR but also via elements in the U3 promoter region. Besides this dual role in transcription, Tat seems to have no other essential function in SIV replication. These results were obtained in several cell types (293T, PM1, 174xCEM and macaque PBMCs), but we cannot exclude the possibility that Tat may exhibit another important function in other cell types or in vivo.

METHODS

Construction of SIV-rtTA variants

The SIV-rtTA plasmids with a wt Tat gene (pSIV-rtTA-Tat_{wt}) or Y55A-mutated Tat gene (pSIV-rtTA-Tat_{Y55A}) have been described previously (Das et al., 2007a). The plasmid pSIV-rtTA-Tat_{Y55A} was constructed by mutagenesis PCR (Mikaelian & Sergeant, 1992) on a pKP-5′SIV template (Das et al., 2007a). PCRs were performed with primers SIV-Tat-AUGmut (5′-CATAATAGACAGGAGACCC-CTTGAAGGAGCA-3′; mismatching nucleotides underlined) plus pLAI3 Seq (5′-TGTCTCATGAGGGGATATA-3′), and with SIV-Tat-2 (5′-GGGAACCACCCCTGGGATATG-3′) plus pKP-3′mut (5′-AGACCGGTATAGGGTGCCGCA-3′). The fragments were purified, mixed and PCR amplified with SIV-Tat-2 and pLAI3 Seq. The product was digested with HindIII and Xhol and ligated into the corresponding sites of pKP-5′SIV, producing pKP-5′SIV-rtTA_{AUGmut}. The Nar1–SpII fragment from this plasmid was used to replace the corresponding fragment of pSIV-rtTA to construct pSIV-rtTA-Tat_{Y55A} and pSIV-rtTA-Tat_{stop} was constructed as described for pSIV-rtTA-Tat_{AUGmut} but with primer SIV-Tat-stop (5′-GAGGAGACAGGAGAATCATGAAGATCCATGCA-3′) instead of Tat_{AUGmut}.

In plasmid pSIV-rtTAopt, an optimized promoter configuration is present in the 5′- and 3′LTR. This configuration was obtained through evolution of SIV-rtTA-TAR+78_{CMV} (Das et al., 2008) following long-term culture in 174xCEM cells (unpublished data). The 63 nt sequence encompassing the NF-kB site and adjacent tetO element (5′-TCGCTGAAACAGCAGGACTTTCCACAGGATCCACG-5′; NF-kB site underlined and tetO site in bold) was trippedlicated and a 289 nt upstream U3 sequence was deleted (positions 976–1006 in the SIVmac239 sequence; GenBank accession no. M33262). In addition, this virus contained a TAR +43_{CMV} mutation. These mutations did not affect dox control, nor did they restore Tat responsiveness. Moreover, pSIV-rtTA_{opt} contained the rtTA-A16 gene (Zhou et al., 2006). The Nar1–SpII fragment of pSIV-rtTA-Tat_{Y55A} and pSIV-rtTA-Tat_{stop} was ligated into the corresponding sites of pSIV-rtTA_{opt}, resulting in pSIV-rtTA_{tetO-CMV} and pSIV-rtTA_{tetO-CMV}.

For construction of the SIV-rtTA_{tetO-CMV} variant, mutagenesis PCR was performed with primers SIV-CMV2loop-Fwd (5′-GCTCCCGGTTAATAAGGATCTTAC-3′) and pLAI3 Seq. The PCR product was digested with XhoI and XbaI and ligated into the corresponding sites of pSIV-rtTA_{opt}, resulting in pSIV-rtTA_{tetO-CMV} and pSIV-rtTA_{tetO-CMV}.
on the pSIV-rtTAopt template. The products were purified, mixed and PCR amplified with primers SIV-CMV2loop-Fwd and pLA133Seq. The resulting fragment was digested with XmnI and AatII and ligated into the corresponding sites of pSIV-rtTAopt. The resulting plasmid pSIV-rtTAopttetO-CMV contained the tetO–CMV promoter in the 3′ LTR. To introduce the tetO–CMV promoter into the 5′ LTR, the 3′ LTR sequence was PCR amplified with primers SIV-LTR-4 (5′-AGGCTTAGAGCCGCGCTGGAAGGGATTATATACTAGTGAAG3′; NotI site underlined) and SIV-LTR-5 (5′-ATCCGGCTGATGACGTGCTAATCGGCAATCTGCTAGG-GATTTTCCTGCT-3′; NotI site underlined). The product was digested with NotI and NotI and used to replace the corresponding 5′ LTR fragment in pSIV-rtTAopt and pSIV-rtTAopttetO-CMV, resulting in pSIV-rtTAopttetO-CMV and pSIV-rtTAopttetO-CMV, respectively.

Cell and virus cultures

293T, PM1 and 174xCEM cells were transfected with 1 (293T) or 5 μg (PM1 and 174xCEM) SIV-rtTA plasmid and cultured with 1 μg dox ml⁻¹, as described previously (Das et al., 2004b, 2007a). Cell-free culture supernatants were harvested and virus production was quantified by CA-p27 ELISA (Advanced Bioscience Laboratories). PBMCs isolated from cynomolgus macaques were infected with equal amounts, and virus production was measured in a dual-luciferase assay (Promega). Tat and promoter activity assays

The Tat activity of the SIV-rtTA variants was measured after transfection of 293T cells with 1 μg SIV-rtTA plasmid, 20 ng pBlue3′ LTR-luc (plasmid in which expression of the firefly luciferase gene is controlled by the wt HIV-1 LTR promoter) and 0.5 ng pRL-CMV, as described previously (Das et al., 2011). After culturing the cells for 48 h with 1 μg dox ml⁻¹, firefly and Renilla luciferase production was measured in a dual-luciferase assay (Promega). Tat activity was calculated as the ratio between the firefly and Renilla luciferase activities and corrected for between-sesssion variation (Ruijter et al., 2006).

To measure the Tat responsiveness of the SIV-rtTA LTR promoter variants, 293T cells were transfected with 20 ng pSIV-rtTAopttetO-LTR-Luc (Das et al., 2007a), pSIV-rtTAopttetO-Luc or pTet-O-CMV-LTR-Luc, 0.4 ng pCMV-rtTA-V16 (Zhou et al., 2006), 0.5 ng pRL-CMV, 0–50 ng pcDNA3-SIV-Tat (Das et al., 2007a), 0–50 ng pcDNA3 (empty vector; the total amount of pcDNA3-SIV-Tat and pcDNA3 was kept at 50 ng) and 930 ng pBluescript as carrier DNA, as described previously (Das et al., 2007a). In the plasmid pSIV-rtTAopttetO-LTR-Luc, expression of the firefly luciferase gene is controlled by the SIV-rtTA LTR promoter (Das et al., 2007a). For the construction of pSIV-rtTAopttetO-Luc, the Xhol–XmnI LTR fragment of SIV-rtTAopt was used to replace the corresponding sequence in pSIV-rtTAopttetO-LTR-Luc. In pTet-O-CMV-LTR-Luc, expression of firefly luciferase is controlled by the tetO–CMV promoter, as present in SIV-rtTAopttetO-CMV and HIV-rtTAopttetO-CMV (Das et al., 2011). For the construction of pTet-O-CMV-LTR-Luc, the 5′ LTR region of HIV-rtTAopttetO-CMV was PCR amplified with primers U3XbaNot and AD-AUG (Das et al., 2011). The product was digested with EcoRV and HindIII and ligated into the corresponding sites of pHIV-LTR-ΔtetO-X/H-luc (Das et al., 2007b). TAR-mediated activation of transcription by Tat was measured as described previously (Das et al., 2007a). After transfection, the cells were cultured for 48 h with 0–100 ng ml⁻¹, the firefly and Renilla luciferase activities were measured and the promoter activity was calculated as the ratio between the firefly and Renilla luciferase activities and corrected for between-session variation (Ruijter et al., 2006).

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