Robust Growth of Human Immunodeficiency Virus Type 1 (HIV-1)

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ABSTRACT The persistence of human immunodeficiency virus type-1 (HIV-1) has long been attributed to its high mutation rate and the capacity of its resulting heterogeneous virus populations to evade host immune responses and antiviral drugs. However, this view is incomplete because it does not explain how the virus persists in light of the adverse effects mutations in the viral genome and variations in host functions can potentially have on viral functions and growth. Here we show that the resilience of HIV-1 can be credited, at least in part, to a robust response to perturbations that emerges as an intrinsic property of its intracellular development. Specifically, robustness in HIV-1 arises through the coupling of two feedback loops: a Rev-mediated negative feedback and a Tat-mediated positive feedback. By employing a mechanistic kinetic model for its growth we found that HIV-1 buffers the effects of many potentially detrimental variations in essential viral and cellular functions, including the binding of Rev to mRNA; the level of rev mRNA in the pool of fully spliced mRNA; the splicing of mRNA; the Rev-mediated nuclear export of incompletely-spliced mRNAs; and the nuclear import of Tat and Rev. The virus did not, however, perform robustly to perturbations in all functions. Notably, HIV-1 tended to amplify rather than buffer adverse effects of variations in the interaction of Tat with viral mRNA. This result shows how targeting therapeutics against molecular components of the viral positive-feedback loop open new possibilities and potential in the effective treatment of HIV-1.

INTRODUCTION

Although it is well established that the high mutation rate of human immunodeficiency virus type-1 (HIV-1), $10^{-4} \sim 10^{-5}$ mutations per base per replication round (1), provides an effective means for the virus to evade antiviral drugs (2) and host immune responses (3), it is not as widely appreciated that high mutation rates can have adverse effects on viral growth (4). Mutations may attenuate HIV-1 growth by altering, for example, interactions of Rev or Tat with their regulatory elements (5,6), RNA splicing of viral transcripts (7), and enzymatic activities of viral integrase (8), reverse transcriptase (9), or protease (10,11). Given the many ways that frequent deleterious mutations, acting alone or collectively, can impede growth, how does HIV-1 persist? When mutations inactivate some of its constitutive splice sites, HIV-1 can utilize alternative splice sites (7,12–15) or activate new cryptic sites (7). More generally, high recombination rates among different HIV-1 strains can create new mosaic strains that grow better than their parent precursors (16,17). We propose here an intrinsic mechanism for HIV-1 persistence that is defined by the network of virus-host interactions required for its intracellular growth. Specifically, we show how the coupling of Rev and Tat feedbacks in their effects on HIV-1 (post-) transcriptional regulation enable the virus to robustly respond to changes in essential virus and cell functions.

The post-transcriptional regulation of HIV-1 enables the virus to achieve a balanced expression of essential viral components and optimize the production of progeny virions (18–21). This regulation is mediated primarily by Rev, an HIV-1 regulatory protein that serves as a sequence-specific nuclear export factor for incompletely-spliced (full-length and singly-spliced) mRNAs (22–32), and a temporal regulator of their expression (18,21,33). Because this nuclear export of incompletely-spliced mRNAs removes the nuclear pool of precursors of fully spliced mRNA, causing a drop in the nuclear and cytoplasmic levels of fully spliced mRNA (29,34), the Rev activity creates over time a negative feedback loop, that at a late infection phase reduces the synthesis of viral regulatory proteins, including itself (34,35). Another regulatory protein, Tat, functions as a *trans*-activator. By stabilizing the elongating transcription complex (36,37), Tat dramatically increases the transcription of all viral mRNAs including its own. Hence, the Tat activity creates a positive feedback loop (38–40). Here we propose that these two feedback loops enable HIV-1 to adapt its growth to a diversity of conditions, including genetically or environmentally altered viral and cellular functions. Furthermore, in contrast to previous studies that have focused primarily on the regulatory role played by Rev, we find that the activity of Tat, when coupled with the Rev-mediated negative feedback, plays a significant role in maintaining the robustness of HIV-1 growth.

MATERIALS AND METHODS

Infection cycle of HIV-1

After HIV-1 enters its host cell, released viral RNAs are reverse-transcribed to produce a double-stranded DNA. This DNA is then transported into the nucleus and integrated into the host genome, forming a provirus (41). Fig. 1 *a* schematically shows events following the provirus formation, RNA

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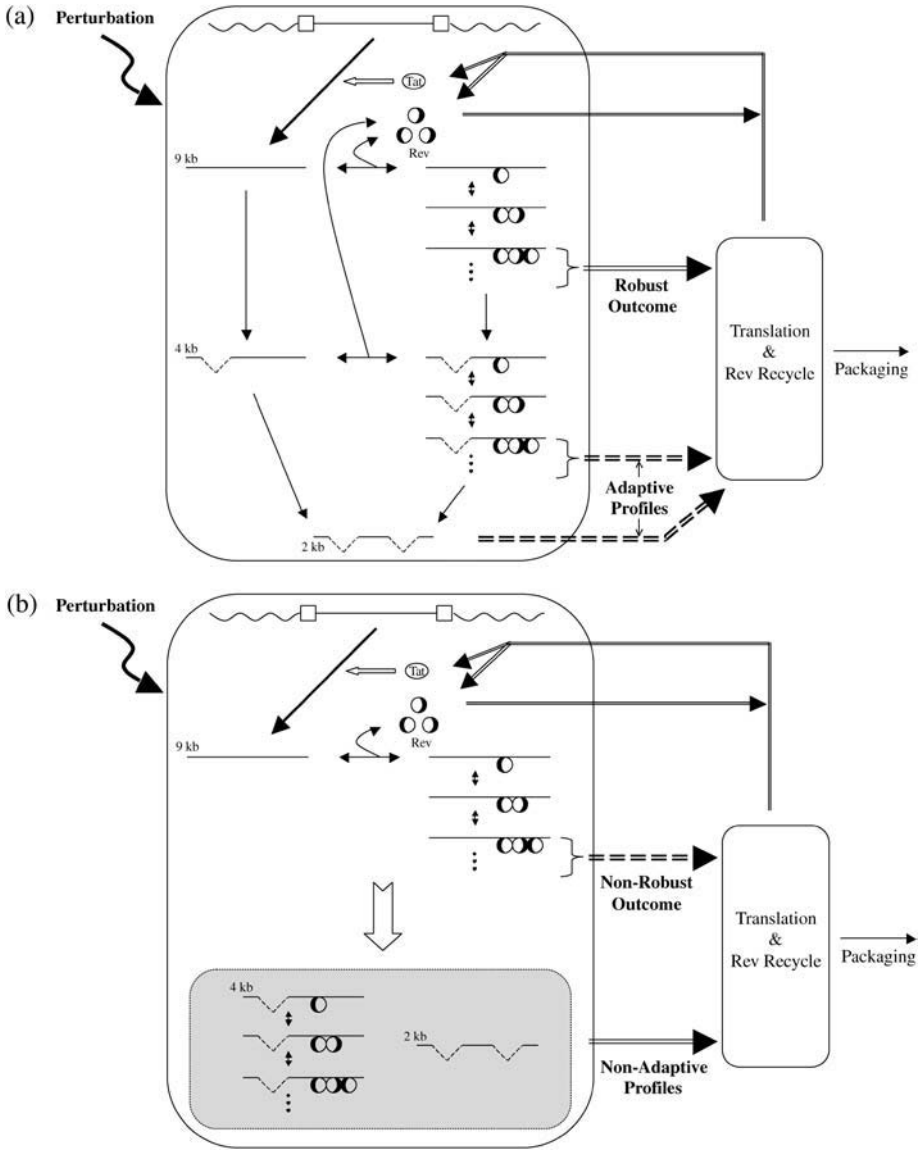

FIGURE 1 RNA splicing and (post-) transcriptional regulation processes of HIV-1 and its response to perturbations in viral and cellular functions. (*a*) Real virus. (*b*) Reference virus. Real HIV-1 adapts the cytoplasmic profiles of singly- and fully spliced mRNAs to a diversity of conditions, whereas the reference virus does not.

splicing, and (post-) transcriptional regulation. Transcription of the provirus produces full-length (9 kb) HIV-1 mRNAs. This primary RNA transcript then undergoes a series of splicing steps that yield the singly-spliced (4 kb) and fully (or multipally)-spliced (2 kb) classes of mRNAs, defined by more than 30 distinct mRNA species (7,13,14,42–44). The incompletely-spliced mRNAs primarily encode viral structural proteins. For example, full-length mRNA encodes Gag and Gag/Pol, and singly-spliced mRNA encodes Vif, Vpr, Tat, Vpu, and Env. In contrast, fully spliced mRNA primarily encodes viral regulatory proteins, such as Tat, Rev, and Nef. Initially, only fully spliced mRNA is exported to the cytoplasm, whereas full-length and singly-spliced mRNAs are retained in the nucleus and spliced to completion (23,25,29–31). After being expressed in the cytoplasm, Rev and Tat proteins are imported into the nucleus. Tat functions in the nucleus as a transactivator to increase synthesis of full-length mRNA. Once Rev is imported into the nucleus, it binds to each Rev response element (RRE) of incompletely-spliced HIV-1 mRNAs (30,45,46). A Rev monomer first binds to the high-affinity site of each RRE, after which additional Rev monomers assemble progressively along the length of the low-affinity sites (28,47–51). Once a critical threshold level of RRE-bound Rev is reached (28,49,52), the Rev-RNA complexes are actively exported to the cytoplasm. Thus, Rev functions in the nucleus as a positive regulator of the expression of intron-containing (or incompletely-spliced) viral messages. Once the Rev-RNA complex reaches the cytoplasm, either the RNA is translated to yield viral structural proteins or, in the case of full-length RNA, it may also be packaged into a progeny virion as a RNA genome (41, 53). The released cytoplasmic Rev is then recycled back to the nucleus for a subsequent round of export. Therefore, the level and timing of HIV-1 mRNA expression are regulated entirely at the (post-) transcriptional level by Tat and Rev proteins.

Mathematical model and computational methods

To develop insights into how HIV-1 achieves robust growth we employ here a mechanistic kinetic model. Models have been previously developed to study various aspects of HIV-1 intracellular growth (54–56), but they have lacked sufficient detail to offer insight into potential mechanisms of HIV-1 robustness. We have recently developed a detailed mechanistic model for HIV-1 growth with a focus on events that follow integration of the provirus (57). The model accounts for basal-level cellular transcription from proviral DNA, successive steps of RNA splicing, Rev-mediated and Rev-independent nuclear export of mRNAs, translation and shuttling of Rev and

Tat back to the nucleus, their respective feedbacks on transcription, a Rev threshold for nuclear export, and Rev-mediated inhibition of splicing, as shown schematically in Fig. 1 *a*. We employ the model here to probe how intracellular HIV-1 growth may be influenced by perturbations in viral and cellular functions, comparing the behavior of the simulated virus with the behavior of a reference virus. Perturbations to viral and cellular functions were implemented in silico as described previously (58,59). A detailed description of the model and a complete list of parameters used are provided in the Appendices.

To better appreciate the roles of the Rev and Tat regulatory feedbacks in HIV-1 growth, we also defined an open-loop reference virus where we suspended the Rev and Tat feedbacks. To simulate this reference virus, we first ran the full closed-loop simulation (including all feedbacks) using our base-case set of virus and host parameters. Next, we captured and saved from this full simulation the resulting dynamic profiles of singly-spliced and fully spliced HIV-1 mRNA accumulation in the cytoplasm. We then imposed these mRNA profiles on all open-loop simulations, independent of changes to virus or host parameters, as shown schematically in Fig. 1 *b*. Two points are important to note. First, the open-loop reference virus behaves identically with the closed-loop virus for base-case parameter values. Second, feedbacks are suspended at the step of nuclear export of singly- and fully spliced mRNA, so the cytoplasmic profiles of singly-spliced and fully spliced HIV-1 mRNA for the reference virus remain always the same.

We simulated two days of growth for both the closed-loop (real) and open-loop (reference) HIV-1, and we used these results to calculate the cumulative production of different HIV-1 mRNA or proteins, guided by the following rationale. To produce viral progeny, full-length mRNA and viral structural proteins expressed from full-length and singly-spliced mRNA must be packaged into relatively stable progeny virions before they are degraded in the cytoplasm. Therefore, time-cumulative yields of these incompletely-spliced mRNAs in the cytoplasm, rather than their levels at a fixed time point, should better correlate with actual yields of HIV-1 progeny virions. The dynamic behavior of Rev and Tat is more complex because Rev continuously shuttles between the nucleus and the cytoplasm, generally with faster rates of import than export (60–62), whereas Tat is imported to the nucleus, and they have different half-lives in different compartments (63). Thus, the levels of Rev and Tat in the nucleus will generally differ from their levels in the cytoplasm. Furthermore, for a given compartment, protein levels for different viral or cellular parameters will highly depend on the specific time-point considered. Therefore, we also characterized the overall behavior of Rev and Tat by their time-cumulative production rather than by their levels at a fixed time-point.

RESULTS AND DISCUSSION

Rev-mRNA binding constant and fraction of Rev-expressing mRNA

We first studied how HIV-1 would respond to mutations that alter the formation of protein-RNA complexes, specifically between the Rev protein and the Rev response element (RRE) of viral mRNA. We simulated the effects of such mutations by altering the Rev-mRNA binding constant over a range from 0.01 to 10 times its base-case value and by changing the fraction of the Rev-expressing mRNA (rev mRNA) in the fully spliced mRNA pool, a value that directly impacts the level of available Rev protein. We changed this fraction from 0.4 to 2 times its base-case value; the fraction of nef mRNA in the pool was correspondingly modified, whereas the fraction of tat mRNA was maintained at its base-case value. Fig. 2 shows the simulated behaviors of real and

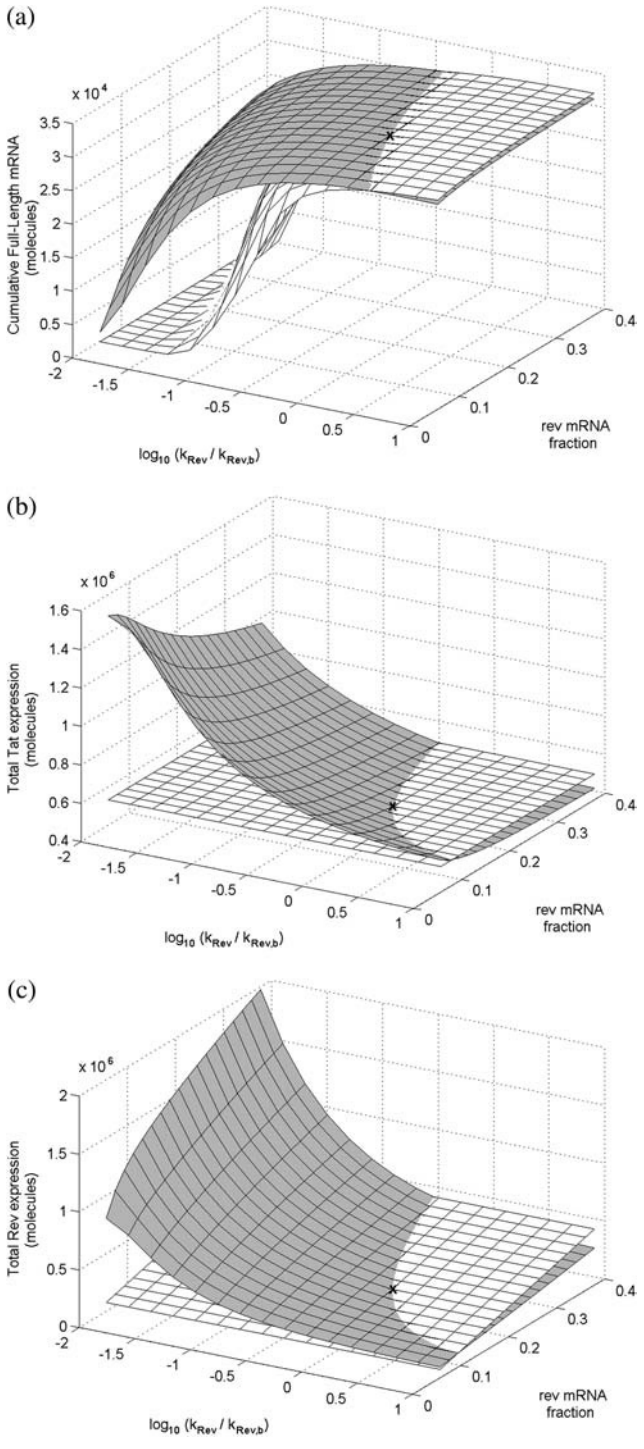

FIGURE 2 Responses of real and reference HIV-1 to perturbations in the Rev-mRNA binding affinity and the fraction of the rev mRNA in HIV-1 mRNA pool. (*a*) Time-cumulative yield of full-length mRNA in the cytoplasm. Time-cumulative production of (*b*) Tat and (*c*) Rev. Simulated behaviors for real and reference HIV-1 are indicated by solid and open surfaces, respectively, and the base-case behavior shared by both viruses is indicated by an × symbol. The results were obtained at two days' post-infection. The Rev binding affinity is plotted on the *y* axis, shown quantitatively as the base-10 log of the ratio of the Rev binding constant to its base-case value. Hence, the Rev binding constant ranges from 0.01- to 10-fold of its base-case value.

reference HIV-1, denoted by solid and open surfaces, respectively, where the base-case behavior shared by both viruses is indicated by an ''×'' symbol. Mutations that reduce the Rev binding constant or the fraction of rev mRNA act to directly reduce (cumulative) yields of full-length mRNA in the cytoplasm for both viruses (Fig. 2 *a*). By making productive Rev-mRNA interactions weaker or less frequent, they shift the dynamic equilibrium among free and Rev-bound mRNAs in the nucleus toward free mRNA. This enhances splicing of full-length mRNA, while also reducing the Rev-mediated transport of the full-length mRNA from the nucleus. In the case of real HIV-1, such mutations also foster production of singly- and fully spliced mRNAs in the nucleus by enhancing splicing of full-length mRNA. Although the mutations could delay the initiation of nuclear export of singly-spliced mRNA, the effect of its enhanced production in the nucleus dominates, increasing its cumulative yield in the cytoplasm. Similarly, enhanced production of fully spliced mRNA in the nucleus increases its cytoplasmic yield. Therefore, compared to reference HIV-1, expression of both Tat and Rev proteins is enhanced, as shown in Fig. 2, *b* and *c*, respectively. Feedback of Tat and Rev proteins to the nucleus then enhances synthesis of full-length mRNA and facilitates its export to the cytoplasm, respectively, compensating in part for direct depletion of full-length mRNA in the cytoplasm. Consequently, the cytoplasmic cumulative level of full-length mRNA for real HIV-1 responds less sensitively to these mutations than for the reference virus, as shown in Fig. 2 *a*.

In contrast, as the Rev binding constant or the fraction of rev mRNA increases to values above its base-case value, the dynamic equilibrium among free and Rev-bound mRNAs in the nucleus is shifted toward a higher Rev-bound mRNA. This reduces splicing of full-length mRNA, while also enhancing the Rev-mediated transport of the full-length mRNA from the nucleus, which directly enhances (cumulative) yields of full-length mRNA in the cytoplasm for both viruses. In the case of real HIV-1, however, such mutations also reduce production of singly- and fully spliced mRNAs in the nucleus. This generally reduces yields of both singly- and fully spliced mRNAs in the cytoplasm, although the nuclear export of singly-spliced mRNA is initiated slightly earlier, and expression of both Tat and Rev proteins is thereby reduced, as shown in Fig. 2, *b* and *c*, respectively. Reduced expression of Tat and Rev then act through regulatory feedbacks to reduce synthesis of full-length mRNA in the nucleus and decelerate its enhanced export to the cytoplasm, respectively, allowing its cumulative level in the cytoplasm to persist with less change than found for the reference virus, as shown in Fig. 2 *a*. This mechanism may help HIV-1 to prevent the overproduction of full-length mRNA in the cytoplasm (Fig. 2 *a*), while reducing the loss of singly-spliced mRNA in the cytoplasm (not shown). HIV-1 could thereby maintain the balance between the Gag and Env expression, a balance that is essential for HIV-1 growth (19,20).

mRNA splicing rate and Rev export threshold

We next investigated how HIV-1 responds to perturbations in the mRNA splicing rate and the threshold level (*Th*) of Rev required for nuclear export of full-length mRNA and singly-spliced mRNA. The splicing rate may be affected by a viral mutation that alters the splicing efficiency of a *cis*-acting viral sequence or induces an alternative splicing pattern with a different splicing efficiency. Moreover, the splicing rate could be also affected by a change in the availability or activity of the *trans*-acting cellular splicing machinery, reflecting the identity or physiological state of the host cell. A variation in the Rev export threshold may be mediated by perturbations in the availability and activity of the cellular exportin family. We varied the splicing rate over a range from 0.1 to 10 times its base-case value and the Rev export threshold over a range from 3 to 11 Rev monomers required per RRE (28,49).

As the splicing rate increases to values above its base-case value, the chance for full-length mRNA to exit the nucleus before being further spliced is reduced, giving lower cytoplasmic (cumulative) yields for both the real and the reference viruses (Fig. 3 *a*). In the case of real HIV-1, the production of singly-spliced mRNA in the nucleus also generally increases, leading to its higher cytoplasmic yield (not shown). However, for very high splicing rates, cytoplasmic yields of singly-spliced mRNA then fall, like the yields for the full-length species. Mechanistically, these yields fall because the negative effect of growing instability of singly-spliced mRNA in the nucleus dominates the positive effect arising from its enhanced production. Further, as the splicing rate increases, the production of fully spliced mRNA in the nucleus is initiated earlier and at a higher rate, due to a reduced stability of its precursor mRNAs, leading to an earlier rise and increased cytoplasmic yield (not shown). Therefore, the expression of both Tat and Rev are enhanced at higher splicing rates, as shown in Fig. 3, *b* and *c*. This is true even when splicing is excessively fast because the enhanced Tat expression from fully spliced mRNA compensates for the reduction in the Tat expression from singly-spliced mRNA. Enhancement of Tat and Rev expression then feed-back to exert compensatory effects on the dynamics of cytoplasmic full-length mRNA. Tat enhances its synthesis in the nucleus, and Rev facilitates its nuclear export and splicing inhibition. Here, as before, the effects of a mutation in real HIV-1 elicit direct and indirect system-level responses that, by affecting the cytoplasmic level of full-length mRNA in opposing directions, contribute to a robust response. This response is shown in Fig. 3 *a* for higher-than base-case splicing rates by the more gentle drop in cumulative full-length mRNA for real virus than reference virus.

If mutations cause the splicing rate to fall relative to the base case, the chance for full-length mRNA to exit the nucleus before being further spliced increases, leading to its enhanced cytoplasmic yield, shown by the plateau region of

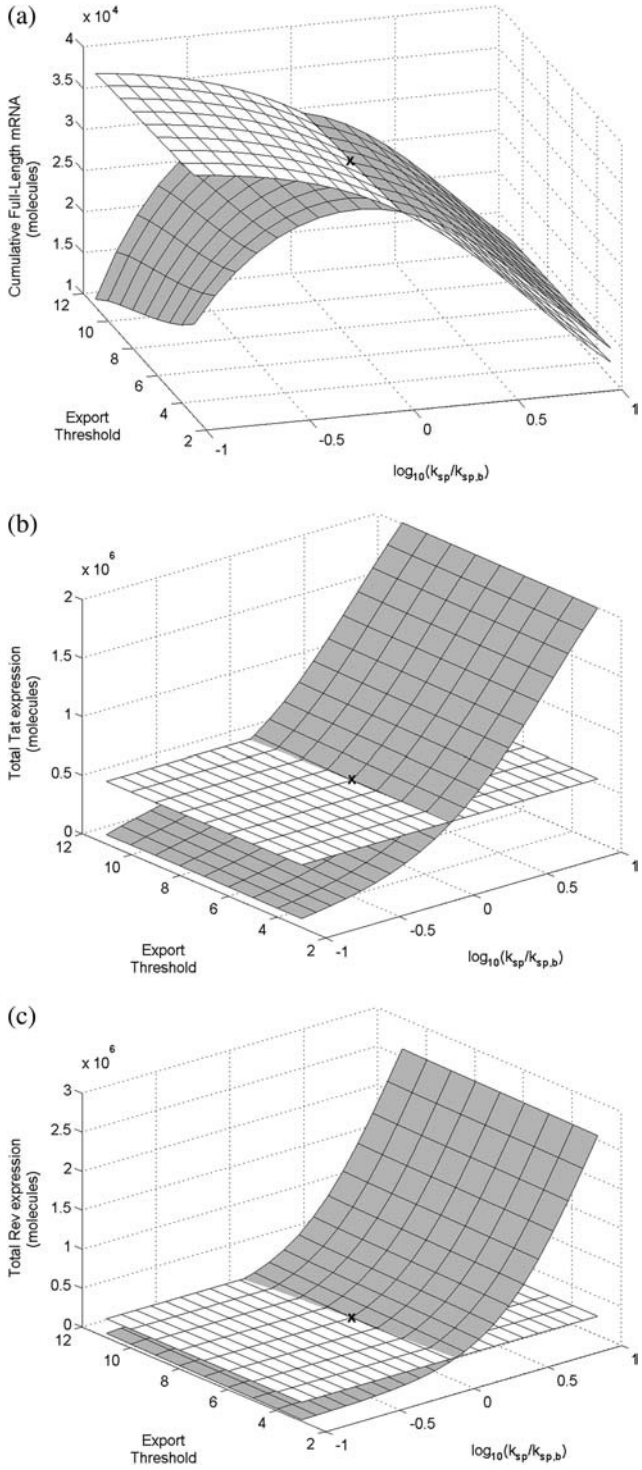

FIGURE 3 Responses of real and reference HIV-1 to perturbations in the mRNA splicing rate and the Rev export threshold. (*a*) Time-cumulative yield of full-length mRNA in the cytoplasm. Time-cumulative production of (*b*) Tat and (*c*) Rev. Simulated behaviors for real and reference HIV-1 are indicated by solid and open surfaces, respectively, and the base-case behavior shared by both viruses is indicated by an × symbol. The results were obtained at two days' post-infection. The mRNA splicing rate is plotted on the *x* axis, shown quantitatively as the base-10 log of the ratio of the splicing rate constant to its base-case value. Hence, the splicing rate constant ranges from 0.1- to 10-fold of its base-case value.

Fig. 3 *a*. In the case of real HIV-1, however, the production of singly- and fully spliced mRNA in the nucleus is simultaneously reduced, causing their cytoplasmic yields to drop. The resulting reductions in the expression of Tat and Rev (Fig. 3, *b* and *c*) then act to reduce synthesis of full-length mRNA in the nucleus and decelerate its enhanced nuclear export, respectively. If the mutation causes splicing to become excessively slow, however, the negative effects of a reduced nuclear export and synthesis of full-length mRNA dominate the positive effect of its enhanced stability, causing its cytoplasmic yield to drop (Fig. 3 *a*). These results suggest the existence of a range of splicing rates that will enable HIV-1 to optimize its growth in different host environments.

As the threshold for Rev export increases the cytoplasmic yield of full-length mRNA slightly but monotonously decreases for both the real and the reference viruses, as shown in Fig. 3 *a*, most apparent when splicing rates are below the base-case value. This relationship arises because more Rev is required for the export of full-length mRNA and more time is required for the higher Rev threshold to be reached, so full-length mRNA stays longer in the nucleus and is further spliced at the expense of its nuclear export. In the case of real HIV-1, the production of singly-spliced mRNA in the nucleus is simultaneously increased. However, for earlier stages of the HIV-1 life cycle, this increase is not directly translated into an enhanced cytoplasmic yield of singly-spliced mRNA because more Rev is required for its export. Nevertheless, singly-spliced mRNA eventually reaches its higher cytoplasmic level, and irrespective of the export threshold, its cumulative yields at later stages of the life cycle are quite similar (not shown). Due to the increased singly-spliced mRNA in the nucleus and its longer nuclear retention, however, the production of fully spliced mRNA always increases, leading to its enhanced cytoplasmic yield and therefore an enhanced expression of Rev (Fig. 3 *c*). In the case of Tat, because its expression depends on both singly- and fully spliced mRNA, higher export thresholds can slightly reduce its expression early in the growth cycle. However, its expression level rapidly recovers (Fig. 3 *b*), leading to an enhanced Tat transactivation, although the transactivation eventually saturates (not shown). Together, the enhanced expression of Tat and Rev then compensate for increased splicing of full-length mRNA by enhancing its synthesis and slightly facilitating its nuclear export, respectively, though this subtle effect is barely distinguishable in Fig. 3 *a*.

Nuclear import rate

Finally, we investigated the HIV-1 response to a perturbation in the import rate, where the same rate constants were assumed for both Tat and Rev. A variation in the import rate may be caused either by perturbations in the activity and availability of the cellular importin family, or by a viral mutation that directly alters the interaction of Rev and Tat with the importin family while indirectly changing the

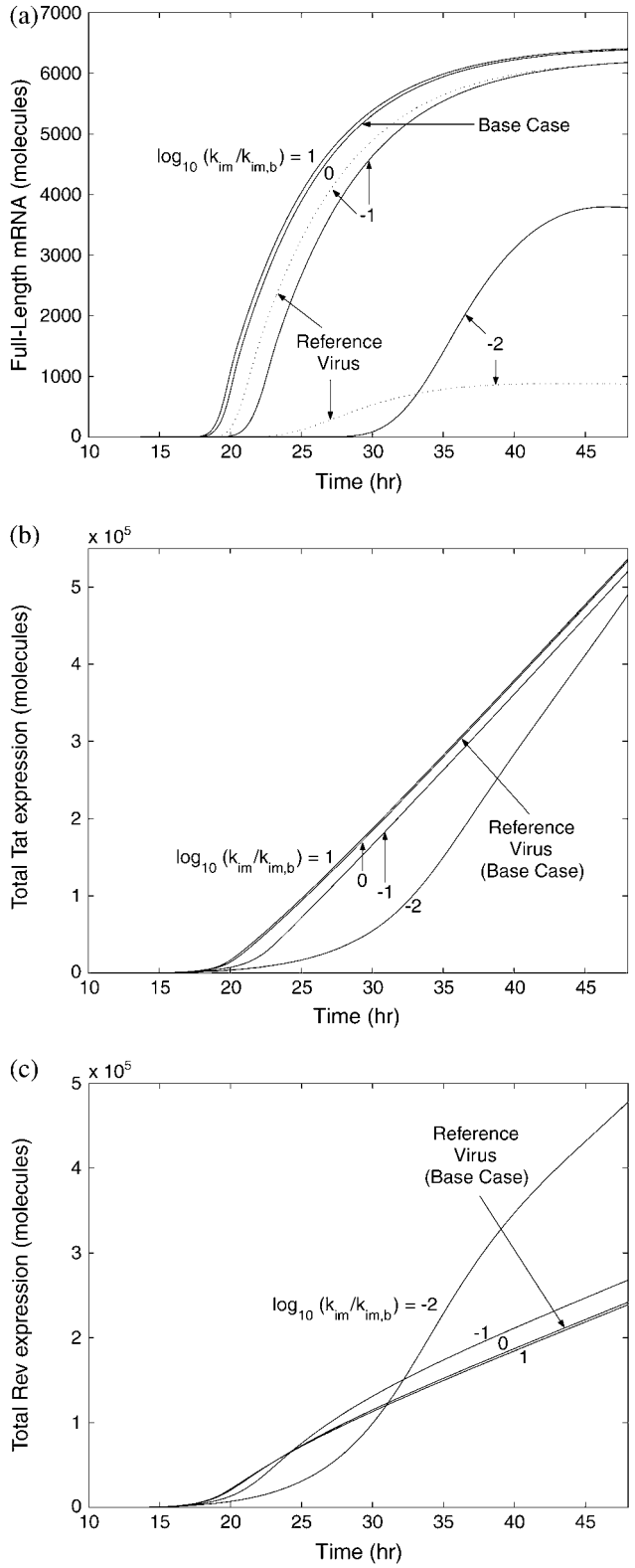

FIGURE 4 Responses of real and reference HIV-1 to perturbations in the import rate of Tat and Rev. (*a*) Time profiles for the level (not cumulative yield) of cytoplasmic full-length mRNA. Time-cumulative production of (*b*) Tat and (*c*) Rev. Simulated trajectories for real and reference HIV-1 are indicated by solid and dotted lines, respectively. The reference virus always exhibits the same cumulative production profiles for Tat and Rev (*b* and *c*), corresponding to those obtained at the base-case value. The number on each curve denotes the base-10 logarithm of the ratio of the import rate constant to its base-case value. Hence, the import rate constant ranges from 0.01- to 10-fold of its base-case value.

import rate. Fig. 4 *a* shows the time profiles for the level (not cumulative yield) of cytoplasmic full-length mRNA for different import rates. Simulated trajectories for real HIV-1 and the reference virus are indicated by solid and dotted lines, respectively. The reference virus always exhibited the same cumulative production profiles for Tat and Rev (Fig. 4, *b* and *c*), corresponding to those obtained at the base-case value, since the cytoplasmic accumulation profiles of singly- and fully spliced mRNA remain the same, irrespective of the import rate.

As the import rate decreases, the cytoplasmic yields of full-length mRNA for both real HIV-1 and the reference virus monotonically decrease, with a delayed initial rise, as shown in Fig. 4 *a*. Here, slower import of Rev to the nucleus restricts the nuclear export and splicing inhibition of full-length mRNA, providing it more time to be spliced. In addition, a slower import of Tat delays and reduces Tat transactivation. In the case of real HIV-1, however, the production of singly-spliced mRNA in the nucleus is less inhibited, though its initial rise is slower due to the reduced Tat supply. Therefore, lower import rates cause a delay in the initial rise of cytoplasmic singly-spliced mRNA (Rev effect) and slow its subsequent rise (both Rev and Tat effect); however, it eventually attains a higher cytoplasmic level than it would at the base-case import rate (not shown). Similarly, despite a slower initial rise of fully spliced mRNA in the nucleus it eventually attains higher cytoplasmic levels relative to the base case (not shown). Accordingly, as shown in Fig. 4, *b* and *c*, lower import rates cause slower initial rises of both Tat and Rev; however, their rise rates eventually become faster than their corresponding values for the base-case import rate. These then compensate for the initial reduction in the synthesis of full-length mRNA and facilitate the nuclear export and splicing inhibition of full-length mRNA. Furthermore, because Tat and Rev are much more stable in the nucleus than in the cytoplasm (63), their losses by degradation increase as the import rate decreases. The enhanced production of Tat and Rev may also compensate for these losses. Consequently, as shown in Fig. 4 *a*, although the initial rises of cytoplasmic full-length mRNA for real HIV-1 are delayed compared to those for the reference virus, real HIV-1, with its responsive feedback of Tat and Rev, eventually yields higher levels of cytoplasmic full-length mRNA.

For higher import rates, the cytoplasmic yields of full-length mRNA for both the real and reference HIV-1 monotonically increase, with an earlier initial rise, as shown in Fig. 4 *a*. As in the previous cases, real HIV-1 acts to maintain its growth robustness by decelerating an enhanced nuclear export of full-length mRNA and also reducing its new synthesis. However, at the current base-case value of the import rate

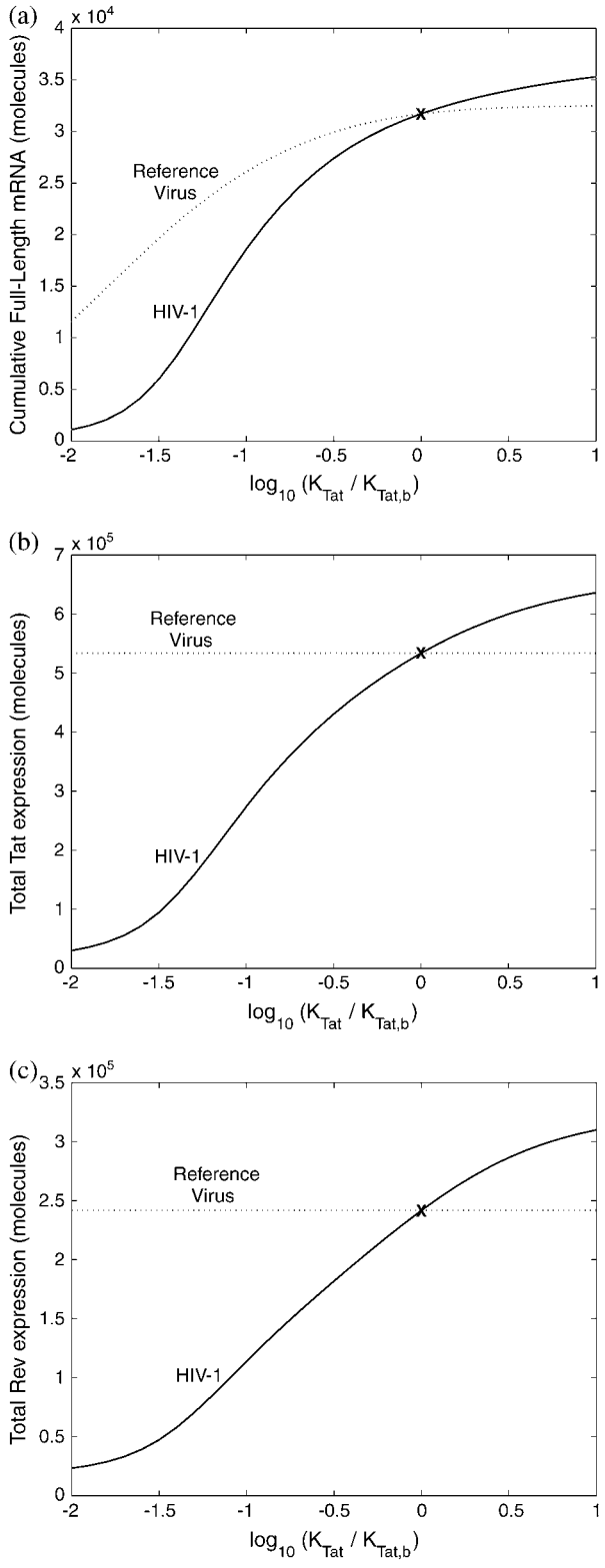

FIGURE 5 Responses of real and reference HIV-1 to perturbations in the Tat-mRNA binding affinity. (*a*) Time-cumulative yield of full-length mRNA in the cytoplasm. Time-cumulative production of (*b*) Tat and (*c*) Rev. Simulated behaviors for real and reference HIV-1 are indicated by solid and dotted lines, respectively, and the base-case behavior shared by both viruses is indicated by an × symbol. The results were obtained at two days' post-infection. The Tat binding affinity is plotted on the *x* axis, shown quantitatively as the base-10 log of the ratio of the Tat equilibrium constant to its base-case value. Hence, the Tat equilibrium constant ranges from 0.01- to 10-fold of its base-case value.

constant, equal to 10 times the export rate constant, the effect of this robustness mechanism is negligible.

Design of anti-HIV therapies

Our results have implications for the design of anti-HIV therapies. Specifically, they suggest one seek to identify and target molecular components that are not controlled by, or perhaps even act against, the robustness function. Consider, for example, how HIV-1 would respond to mutations that alter the equilibrium constant between the Tat protein and the Tat (or transactivation) responsive element (TAR) of the viral mRNA. As the Tat equilibrium constant decreases to values below its base-case value, the level of Tat transactivation and thus the synthesis of full-length mRNA in the nucleus are reduced, leading further to reduced cytoplasmic (cumulative) yields of full-length mRNA for both the real and the reference viruses, as shown in Fig. 5 *a*. Here simulated behaviors for real and reference HIV-1 are indicated by solid and dotted lines, respectively, and the base-case behavior shared by both viruses is indicated by an ''×'' symbol. In the case of real HIV-1, such mutations also reduce the production of both singly- and fully spliced mRNAs in the nucleus. Reduced production of fully spliced mRNA then reduces its yield in the cytoplasm and thereby reduces Rev expression (Fig. 5 *c*), which further restricts the nuclear export of full-length and singly-spliced mRNAs and thereby reduces their cytoplasmic yields. Then reduced cytoplasmic yields of both singly- and fully spliced mRNA reduce the expression of Tat (Fig. 5 *b*). Feedback of Tat and Rev proteins to the nucleus then reduces further synthesis of full-length mRNA and its export to the cytoplasm, respectively, and thereby creates a positive feedback loop. This response is shown in Fig. 5 *a* for a lower-than base-case equilibrium constant by the more rapid drop in cumulative full-length mRNA for the real virus than for the reference virus. Similarly, HIV-1 would respond to any antiviral action that reduces the Tat equilibrium constant in a way that would aggravate its growth by amplifying the decrease in the expression of Tat and Rev. Hence, targeting viral or cellular molecular components that induce positive-feedback loops may well exhibit more potent antiviral activities than strategies that target robustly maintained functions. Further, such a strategy should have potential for the treatment of a wide range of viruses, with an emphasis on those that have a similar regulatory mechanism.

CONCLUSIONS

Our results here suggest that HIV-1 may have evolved to maintain its stable growth against mutational- or environmentally

caused variations in viral and cellular parameters, and its (post-) transcriptional regulation process, mediated by both Tat and Rev, is responsible for this robustness of HIV-1 growth. Given a relatively small-sized genome and a limited number of molecular components, HIV-1 may have evolved this compact but multi-purpose mechanism to simultaneously satisfy a range of survival criteria including, but not limited to, a balanced expression of each viral component, optimal virion production, and growth robustness.

Robustness is a potentially ubiquitous feature of living systems that enables them to reliably perform essential tasks in the face of the perturbations in their internal parameters or their external environments (64–67). This property may arise from the complex interconnection of intracellular genetic and biochemical networks (64,65). However, we have shown here that a minimal network in HIV-1 growth, defined by interactions between only two feedback loops, one negative (Rev) and one positive (Tat), exhibits robust behavior. Moreover, unlike cellular robustness, where an understanding of the underlying molecular interactions is limited, our analysis of HIV-1 post-transcriptional regulation reveals how networks defined by a small number of components and interactions can exhibit robustness. By identifying and characterizing robustness in other minimal networks, one may begin to unveil its principles and mechanisms in more complex biological systems.

APPENDIX 1: MATHEMATICAL MODEL FOR HIV-1 INTRACELLULAR GROWTH

$$dF_N/dt = (Tc_b + Tc_{add} \times K_{Tat} \times T_N/(1 + K_{Tat} \times T_N)) \times PV + k_d^{(1)} \times FR_N^{(1)} - (k_{sp}^F + k_{deg,N}^{RNA} + k_a^{(1)} \times R_N) \times F_N. \quad (1)$$

$$dS_N/dt = k_{sp}^F \times F_N + k_d^{(1)} \times SR_N^{(1)} - (k_{sp}^S + k_{deg,N}^{RNA} + k_a^{(1)} \times R_N) \times S_N. \quad (2)$$

$$dM_N/dt = k_{sp}^S \times S_N + \sum_{i=1}^{sn} \left((1 - d^{S,(i)}) \times k_{sp}^S \times SR_N^{(i)} \right) - (k_{exp}^M + k_{deg,N}^{RNA}) \times M_N. \quad (3)$$

$$dF_C/dt = \sum_{i=1}^{sn} \left(k_{exp}^{F,(i)} \times FR_N^{(i)} \right) - k_{deg,C}^{RNA} \times F_C. \quad (4)$$

$$dS_C/dt = \sum_{i=1}^{sn} \left(k_{exp}^{S,(i)} \times SR_N^{(i)} \right) - k_{deg,C}^{RNA} \times S_C. \quad (5)$$

$$dM_C/dt = k_{exp}^M \times M_N - k_{deg,C}^{RNA} \times M_C. \quad (6)$$

$$dFR_N^{(i)}/dt = k_a^{(i)} \times R_N \times FR_N^{(i-1)} + k_d^{(i+1)} \times FR_N^{(i+1)} - (k_d^{(i)} + k_a^{(i+1)} \times R_N + k_{exp}^{F,(i)} + (1 - d^{F,(i)}) \times k_{sp}^F + k_{deg,N}^{RNA}) \times FR_N^{(i)} \quad (\text{for } i = 1 \ldots sn). \quad (7)$$

$$dSR_N^{(i)}/dt = k_a^{(i)} \times R_N \times SR_N^{(i-1)} + k_d^{(i+1)} \times SR_N^{(i+1)} + (1 - d^{F,(i)}) \times k_{sp}^F \times FR_N^{(i)} - (k_d^{(i)} + k_a^{(i+1)} \times R_N + k_{exp}^{S,(i)} + (1 - d^{S,(i)}) \times k_{sp}^S + k_{deg,N}^{RNA}) \times SR_N^{(i)} \quad (\text{for } i = 1 \ldots sn) \quad (8)$$

$$dR_C/dt = f_{Rev} \times Tr \times f_{rev}^M \times M_C + k_{exp}^R \times R_N + \sum_{i=1}^{sn} \left(i \times (k_{exp}^{F,(i)} \times FR_N^{(i)} + k_{exp}^{S,(i)} \times SR_N^{(i)}) \right) - (k_{imp}^R + k_{deg,C}^{Pro}) \times R_C. \quad (9)$$

$$dT_C/dt = f_{Tat} \times Tr \times \left(f_{tat}^S \times S_C + f_{tat}^M \times M_C \right) + k_{exp}^T \times T_N - (k_{imp}^T + k_{deg,C}^{Pro}) \cdot T_C. \quad (10)$$

$$dR_N/dt = k_{imp}^R \times R_C + \sum_{i=1}^{sn} \left(k_d^{(i)} \times (FR_N^{(i)} + SR_N^{(i)}) \right) + \sum_{i=1}^{sn} \left(k_{deg,N}^{RNA} \times i \times (FR_N^{(i)} + SR_N^{(i)}) \right) + \sum_{i=1}^{sn} \left((1 - d^{S,(i)}) \times k_{sp}^S \times i \times SR_N^{(i)} \right) - \left(\sum_{i=1}^{sn} \left(k_a^{(i)} \times (FR_N^{(i-1)} + SR_N^{(i-1)}) \right) + k_{exp}^R + k_{deg,N}^{Pro} \right) \times R_N. \quad (11)$$

$$dT_N/dt = k_{imp}^T \times T_C - (k_{exp}^T + k_{deg,N}^{Pro}) \times T_N. \quad (12)$$

APPENDIX 2: NOMENCLATURE

F_N, F_C	Full-length mRNA in the nucleus and cytoplasm, respectively.
S_N, S_C	Singly-spliced mRNA in the nucleus and cytoplasm, respectively.
M_N, M_C	Fully (or multipally)-spliced mRNA in the nucleus and cytoplasm, respectively.
R_N, R_C	Rev in the nucleus and cytoplasm, respectively.
T_N, T_C	Tat in the nucleus and cytoplasm, respectively.
$FR_N^{(i)}$	(i) Rev-bound form of full-length mRNA in the nucleus, where $FR_N^{(0)} = F_N$.
$SR_N^{(i)}$	(i) Rev-bound form of singly-spliced mRNA in the nucleus, where $SR_N^{(0)} = S_N$.
sn	Maximum number of Rev monomers per RRE.
Th	Threshold number of Rev for nuclear export of Rev-RNA complexes.
Tc_b	Basal transcription rate for HIV-1.
Tc_{add}	Increase in HIV-1 transcription by Tat transactivation.
Tr	Steady-state translation rate.
k_{sp}^F	Splicing rate constant for the first splicing (from full-length to singly-spliced mRNAs).
k_{sp}^S	Splicing rate constant for the second splicing (from singly-spliced to fully spliced mRNAs).
$d^{F,(i)}$	Splicing delay factor for the splicing from $FR_N^{(i)}$ to $SR_N^{(i)}$.
$d^{S,(i)}$	Splicing delay factor for the splicing from $SR_N^{(i)}$ to M_N.
$k_{exp}^{F,(i)}$, $k_{exp}^{S,(i)}$, k_{exp}^M	Nuclear export rate constants for $FR_N^{(i)}$, $SR_N^{(i)}$, and M_N, respectively.
k_{exp}^R, k_{exp}^T	Nuclear export rate constants for Rev and Tat, respectively.
k_{imp}^R, k_{imp}^T	Nuclear import rate constants for Rev and Tat, respectively.
$k_a^{(i)}$	Association constant for the binding of Rev and $FR_N^{(i-1)}$ (or $SR_N^{(i-1)}$).

$k_d^{(i)}$	Rev dissociation constant for $FR_N^{(i)}$ and $SR_N^{(i)}$.
$k_{deg,N}^{RNA}$, $k_{deg,C}^{RNA}$	RNA degradation rate constants in the nucleus and cytoplasm, respectively.
$k_{deg,N}^{Pro}$, $k_{deg,C}^{Pro}$	Protein degradation rate constants in the nucleus and cytoplasm, respectively.
f_{tat}^{S}, f_{tat}^{M}	Fraction of tat mRNA in singly- and fully spliced mRNAs, respectively.
f_{rev}^{M}	Fraction of rev mRNA in fully spliced mRNA.
f_{Rev}	Probability for rev mRNA to encode Rev.
f_{Tat}	Probability for tat mRNA to encode Tat.

APPENDIX 3: MODEL DESCRIPTION

The model describes the RNA splicing and (post-) transcriptional regulation processes of HIV-1 shown schematically in Fig. 1 *a*. Events that precede the initial round of HIV-1 transcription, such as reverse-transcription of viral RNAs, nuclear transport of the viral DNA, and its integration into the host genome, are described by reported fixed-time delays based on experimental observations (68–70). In our simulation we assumed one provirus per cell was produced 12 h after entry.

Full-length mRNA (F_N) is first synthesized in the nucleus by cellular base-level transcription, and its synthesis is accelerated by Tat transactivation as the level of nuclear Tat (T_N) increases (Eq. 1). The base-level transcription rate (Tc_b) was approximated by dividing a typical eukaryotic RNA polymerase elongation rate, 40 bases/s (71), by the length of HIV-1 RNA genome, 9500 bases (72). We used Michaelis-Menten expressions to describe the Tat transactivation and its saturation kinetics, caused either by the limitation in the number of polymerases simultaneously attached to a single provirus, or by limiting host co-factors of Tat. We assumed transcription rates could be activated up to 100-fold (73–75). Full-length mRNA undergoes a series of alternative splicing events in the nucleus to yield singly-spliced mRNA (S_N) and fully spliced mRNA (M_N). We modeled the RNA splicing as a two-step process that first converts full-length mRNA to a singly-spliced intermediate (the first splicing), which is then further converted to fully spliced mRNA (the second splicing), as shown in Fig. 1 *a*. First-order kinetics are assumed for both the first and second splicing processes (Eqs. 2 and 3), and the same splicing rate constants ($k_{sp}^{F} = k_{sp}^{S}$) are assumed unless otherwise specified. It has been reported that the overall RNA splicing process of HIV-1 is completed within 2 h (76), which corresponds to $k_{sp} > 1$ molecule/h. However, due to the lack of direct measurements of the splicing rate constant, observations made on adenovirus type-2 have been adopted, which is one molecule per 20–30 min (or 2–3 molecules/h) (54,77). This value may serve as an upper bound for HIV-1 splicing rate constant, since HIV-1 encodes intrinsically inefficient splice sites (30,78,79) that make processing of HIV-1 RNA less efficient than the processing of cellular or other viral RNAs. The incompletely-spliced mRNAs (F_N and S_N) are in dynamic equilibrium with their corresponding 1:1 Rev-RNA complexes, $FR_N^{(i)}$ and $SR_N^{(i)}$, as shown in Eqs. 1 and 2. The superscripts of $FR_N^{(i)}$ and $SR_N^{(i)}$ denote the number (i) of Rev monomers bound to the full-length and singly-spliced mRNAs, respectively. We assumed that up to 12 Rev monomers ($sn = 12$) can bind to each RRE (28,49). Fully spliced mRNA is derived not only from free singly-spliced mRNA (S_N), but also from its various Rev-bound forms, $SR_N^{(i)}$ (where, $i = 1, \ldots, sn$), as shown in Eq. 3. A splicing delay factor (d, $d^{F(i)}$, or $d^{S(i)}$) describes the extent of (direct or indirect) splicing inhibition mediated by the binding of Rev to RNA containing the RRE, and its value ranges from 0 to 1. For $d = 0$ there is minimal inhibition of splicing and Rev has no effect on the stability of the RNA in the nucleus; instead, Rev enhances cytoplasmic expression solely by facilitating nuclear export relative to splicing. For $d = 1$ there is almost complete inhibition of splicing; levels of Rev-RNA complexes in the nucleus fall due to nuclear export or RNA degradation, not because of splicing. Fully spliced mRNA is also subject to nuclear export (Eqs. 3 and 6), whereas free incompletely-spliced mRNAs (F_N and S_N) are assumed to remain in the nucleus. The cytoplasmic pools of incompletely-spliced RNAs (F_C and S_C) are instead generated by nuclear export of the corresponding nucleic Rev-RNA complexes ($FR_N^{(i)}$ and $SR_N^{(i)}$), as shown in Eqs. 4 and 5 (also Eqs. 7 and 8). Unless otherwise specified, we assume that only Rev-RNA complexes above a fixed Rev threshold level (Th) are subject to nuclear export ($k_{exp}^{F,(i)} = k_{exp}^{S,(i)} = 0$, for all $i < Th$), where $Th = 7$ was applied as its base-case value, and this threshold is the same for complexes containing full-length and singly-spliced mRNAs. Furthermore, first-order kinetics and the same export rate constants are assumed for all the export processes ($k_{exp}^{M} = k_{exp}^{F,(i)} = k_{exp}^{S,(i)}$, for all $i \geq Th$). In the nucleus the i^{th} Rev-bound forms of incompletely-spliced mRNAs ($FR_N^{(i)}$ and $SR_N^{(i)}$) are in dynamic equilibrium with the corresponding $(i - 1)$ and $(i + 1)$ Rev-bound forms (Eqs. 7 and 8), where the $(i = 0)$ species ($FR_N^{(0)}$ and $SR_N^{(0)}$) correspond to the free mRNAs (F_N and S_N). Here we implemented high- and low-affinity Rev bindings by applying different Rev association ($k_a^{(i)}$) and dissociation ($k_d^{(i)}$) constants (51) for each (i) Rev-bound form of incompletely-spliced mRNA. Unless otherwise specified, we assumed that only the first Rev binding is a high-affinity Rev binding, and the same rate constants are applied to all low-affinity Rev bindings. Each (i) Rev-bound form of full-length mRNA ($FR_N^{(i)}$) is spliced, but with a delayed kinetics characterized by $d^{F(i)}$, to yield the corresponding singly-spliced mRNA ($SR_N^{(i)}$) (Eqs. 7 and 8). Then each $FR_N^{(i)}$ ($i = 1, \ldots, sn$) is further spliced, with delay $d^{S(i)}$, to yield fully spliced mRNA (M_N) (Eqs. 3 and 8). In the simulation, unless otherwise specified, the same value was assumed for all the splicing delay factors, and $d = 0.8$ was applied as its base-case value. All the nucleic and cytoplasmic mRNAs, including both free and Rev-bound forms, are subject to RNA degradation, and the same degradation rate constants were assumed for all the mRNAs.

Rev and Tat proteins are expressed from their corresponding cytoplasmic mRNAs (Eqs. 9 and 10), and Rev continuously shuttles between the nucleus and the cytoplasm, generally with faster rates of import than export (60–62), whereas Tat is imported to the nucleus (Eqs. 9–12). The steady-state translation rate (Tr) was obtained by dividing a typical eukaryotic ribosome elongation rate, 100 ~200 AA/min (2 AA/s was applied here) (80), by the space between eukaryotic ribosomes, ~1 ribosome/80 bases (or 1 protein product/80 bases) (71,81), in poly-ribosomal states. It has been reported that Rev-expressing mRNA (rev mRNA) encodes both Rev and Nef proteins (7,12,42,44), where the fraction of Rev (or probability for rev mRNA to encode Rev), f_{Rev}, is ~0.5–0.7 (44). In contrast, Tat-expressing mRNA (tat mRNA) encodes practically Tat protein only, where the fraction of Tat (or probability for tat mRNA to encode Tat), f_{Tat}, is >0.998 (44). $f_{Rev} = 0.5$ and $f_{Tat} = 1$ were assumed in the simulation. Unless otherwise specified, the same export rate constants were assumed for Rev and all HIV-1 mRNAs ($k_{exp}^{R} = k_{exp}^{M} = k_{exp}^{F,(i)} = k_{exp}^{S,(i)}$, for all $i \geq Th$), whereas the export rate of Tat was assumed to be zero ($k_{exp}^{T} = 0$). However, we assumed the same import rate for both Rev and Tat ($k_{imp}^{R} = k_{imp}^{T}$). Rev and Tat in both compartments (nucleus and cytoplasm) are subject to protein degradation. It has been reported that the half-life of Rev is significantly shorter in the cytoplasm (4 h) than in the nucleus (16 h) (63). We assumed that the half-life of Tat was the same as the half-life of Rev in each of the compartments (63,82). In the cytoplasm the release of Rev from Rev-RNA complexes contributes to a cytoplasmic pool of Rev. Because the HIV-1 growth dynamics is insensitive to the rate of the Rev release unless it is excessively low, we modeled the Rev release as an instantaneous process, and therefore variables for Rev-RNA complexes in the cytoplasm were not explicitly included in the model (Eqs. 4, 5, and 9). In the nucleus, Rev is in dynamic equilibrium with the different Rev-bound forms of incompletely-spliced mRNA ($FR_N^{(i)}$ and $SR_N^{(i)}$, $i = 0, \ldots, sn$) (Eq. 11). Rev in the nucleus is much more stable than HIV-1 mRNAs (25,63,83), so we assumed that Rev is recycled from all the Rev-RNA complexes ($FR_N^{(i)}$ and $SR_N^{(i)}$, $i = 1, \ldots, sn$) after RNA degradation, and also from Rev-bound forms of singly-spliced mRNA as they react to form fully spliced mRNA (Eq. 11). Finally, we took that the diameter of a T-cell nucleus to be 6 μm (54,84) when performing unit conversion from molar concentration to the number of molecules in the nucleus.

APPENDIX 4: KINETIC CONSTANTS AND PARAMETERS

Number of provirus (PV):	$PV = 1$ copy/cell (69,85).
(Eukaryotic) basal transcription rate:	<40 nts/s (71). $Tc_b \approx 40$ nts/s $\times$ 1 HIV genome/9500 nts ≈ 0.25 transcripts/min for HIV.
Increase in transcription by Tat transactivation (Tc_{add}):	25 ~100-fold (73–75). $Tc_{add} = 25 - 0.25 = 24.75$ transcripts/min (100-fold in the simulation).
Equilibrium constant of Tat with TAR (K_{Tat}):	28.57 ~100/μM (86). $K_{Tat} = 28.57/\mu$M in the simulation.
Association and dissociation constants of Rev with RRE (51)	
A. High-affinity binding:	$k_a^{(1)} = 2.5 \times 10^5/(\text{Ms})$, $k_d^{(1)} = 3.0 \times 10^{-5}$ 1/s.
B. Low-affinity binding:	$k_a^{(i)} = 4.4 \times 10^5/(\text{Ms})$, $k_d^{(i)} = 3.8 \times 10^{-2}$ 1/s, where $i \geq 2$. $k_a^{(sn+1)} = k_d^{(sn+1)} = 0$.
Maximum number of Rev per RRE (sn):	$sn = 12$ Revs/RRE (49).
Splicing rate constants (k_{sp}):	2 ~3 molecules/h for adenovirus type-2 (54,77). $k_{sp}^F = k_{sp}^S = 2.5$ molecules/h in the simulation.
Export rate constant (k_{exp}):	In vivo Rev export half life $\approx$ 20 min (61). $k_{exp}^{F,(i)} = k_{exp}^{S,(i)} = k_{exp}^M = k_{exp}^R = \ln(2)/20\,\text{min} = 0.0347/\text{min}$, $k_{exp}^T = 0$ in the simulation.
Import rate constant (k_{imp}):	0.303 $\pm$ 0.099 /min for Tat (60). $k_{imp}^T = k_{imp}^R = 10 \times k_{exp}^R = 0.347/\text{min}$ in the simulation.
mRNA degradation rate (k_{deg}^{RNA}):	Half-life in the nucleus or cytoplasm $\approx$ 4 h (25,83,87). $k_{deg,N}^{RNA} = k_{deg,C}^{RNA} = \ln(2)/4\,\text{h} = 0.0029/\text{min}$ in the simulation.
(Eukaryotic) steady-state translation rate (Tr) (71,80,81):	$Tr =$ (1 protein/80 nt) $\times$ (3 nt/1 AA) $\times$ (2 AA s) $\times$ (60 s/min) ≈ 4.5 proteins/min.
Protein degradation rates (k_{deg}^{Pro})	
A. Half-live of Rev in the nucleus: >16 h (63)	$k_{deg,N}^{Pro} = \ln(2)/16\,\text{h} = 0.0433/\text{h}$ in the simulation.
B. Half-life of Rev (or Tax (82)) in the cytoplasm: <4 ~4.5 h (63,82)	$k_{deg,C}^{Pro} = \ln(2)/4\,\text{h} = 0.1733/\text{h}$ in the simulation.
Fraction of rev mRNA in fully spliced mRNA (f_{rev}^M):	0.19 ~0.34 (7,13), $f_{rev}^M = 0.19$ in the simulation.
Fraction of tat mRNA in singly- and fully spliced mRNAs (f_{tat}^S, f_{tat}^M):	f_{tat}^S, = 0.05 (7), $f_{tat}^M = 0.02$~0.09 (7, 13), f_{tat}^S, = $f_{tat}^M = 0.05$ in the simulation.
Probability for rev mRNA to encode Rev (f_{Rev}):	0.5 ~0.7 (44), $f_{Rev} = 0.5$ in the simulation.
Probability for tat mRNA to encode Tat (f_{Tat}):	>0.998 (44), $f_{Tat} = 1$ in the simulation.

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