Title

HIV latency in isolated patient CD4+ T cells may be due to blocks in HIV transcriptional elongation, completion, and splicing

Journal

Science Translational Medicine, 10(430)

ISSN

1946-6234

Authors

Yukl, Steven A
Kaiser, Philipp
Kim, Peggy
et al.

Publication Date

2018-02-28

DOI

10.1126/scitranslmed.aap9927

Peer reviewed

HHS Public Access

Author manuscript

Sci Transl Med. Author manuscript; available in PMC 2018 August 28.

Published in final edited form as:

Sci Transl Med. 2018 February 28; 10(430): . doi:10.1126/scitranslmed.aap9927.

HIV latency in isolated patient CD4+ T cells may be due to blocks in HIV transcriptional elongation, completion, and splicing

Steven A. Yukl[*], **Philipp Kaiser**[†], **Peggy Kim**, **Sushama Telwatte**, **Sunil K. Joshi**[‡], **Mai Vu**, **Harry Lampiris**, and **Joseph K. Wong**

San Francisco Veterans Affairs Medical Center and University of California, San Francisco, 4150 Clement Street, 111W, San Francisco, CA 94121, USA

Abstract

Latently infected $CD4^+$ T cells are the main barrier to complete clearance of HIV infection, but it is unclear what mechanisms govern latent HIV infection in vivo. To address this question, we developed a new panel of reverse transcription droplet digital polymerase chain reaction (RT-ddPCR) assays specific for different HIV transcripts that define distinct blocks to transcription. We applied this panel of assays to $CD4^+$ T cells freshly isolated from HIV-infected patients on suppressive antiretroviral therapy (ART) to quantify the degree to which different mechanisms inhibit HIV transcription. In addition, we measured the degree to which these transcriptional blocks could be reversed ex vivo by T cell activation (using anti-CD3/CD28 antibodies) or latency-reversing agents. We found that the main reversible block to HIV RNA transcription was not inhibition of transcriptional initiation but rather a series of blocks to proximal elongation, distal transcription/polyadenylation (completion), and multiple splicing. Cell dilution experiments suggested that these mechanisms operated in most of the HIV-infected $CD4^+$ T cells examined. Latency-reversing agents exerted differential effects on the three blocks to HIV transcription, suggesting that these blocks may be governed by different mechanisms.

INTRODUCTION

HIV can establish latent infection in $CD4^+$ T cells, and these cells are thought to be the major obstacle to eradication of HIV (1–8). Latently infected cells do not produce virus constitutively but can be induced by T cell activation to produce infectious virus. The reversible lack of viral expression allows latent proviruses to escape detection by host defenses, allowing survival in long-lived $CD4^+$ T cells that can propagate the provirus during cell division (9, 10). No existing antiretroviral drug prevents HIV reactivation from latently

[*]Corresponding author. steven.yukl@ucsf.edu.
[†]Present address: Department of Infectious Diseases, Luzerner Kantonsspital, 6000 Lucerne 16, Switzerland.
[‡]Present address: Knight Cancer Institute, School of Medicine, Oregon Health and Science University, Portland, OR 97239, USA.

Author contributions: S.A.Y. and J.K.W. designed the study; P. Kaiser and S.A.Y. developed the assays and standards; J.K.W., S.A.Y., M.V., and H.L. recruited study participants; S.A.Y., P. Kaiser, P. Kim, S.T., and S.K.J. designed experiments; P. Kim, P. Kaiser, S.A.Y., S.T., and S.K.J. conducted experiments; S.A.Y., P. Kim, P. Kaiser, S.T., and S.K.J. analyzed data; S.A.Y. interpreted data and wrote the manuscript; S.A.Y., J.K.W., S.T., P. Kaiser, and H.L. edited the manuscript.

Competing interests: The authors declare that they have no competing interests.

Data and materials availability: The data sets generated and analyzed during the current study are included in the Supplementary Materials.

infected cells, which may contribute to the immune activation and organ damage that persist despite antiretroviral therapy (ART) and likely enable viral rebound when ART is interrupted (11).

Despite intensive study, it is unclear what determines whether an infected cell will progress to latent or productive infection. Multiple different mechanisms have been implicated in latent infection (12, 13), most of which involve blocks at various stages of transcription (14, 15). One study proposed that latency could be due to viral integration in transcriptionally silent regions (16), but subsequent studies have shown that HIV usually integrates into actively transcribed genes (17, 18). Some studies have suggested that epigenetic modifications (histone deacetylation and DNA methylation) can contribute to the establishment or maintenance of latency (19–27), whereas others found no evidence for such a role (28–30). It has been further suggested that latency results from low levels of host transcription initiation factors (NF-κB and NFAT) in resting cells [perhaps resulting from infection of $CD4^+$ T cells when they are in transition from an activated to a resting state (18, 31)] and/or from stochastic fluctuations in levels of Tat (32). Inhibition of HIV transcriptional initiation can also result from transcriptional interference, a process in which active transcription from a neighboring cellular gene “reads through” the HIV provirus and prevents binding of cellular initiation factors to the viral promoter (18, 28–30, 33, 34). Even with efficient initiation of HIV transcription, the RNA polymerase may stall just after the trans-activation response (TAR) region (35, 36). Such blocks to transcriptional elongation can be due to lack of host elongation factors (such as P-TEFb), the presence of host factors that inhibit elongation (such as NELF), nucleosome positioning, or insufficient viral Tat activity (12, 13, 35–41). When elongation fails, the transcription machinery may eventually disassemble, resulting in the accumulation of short, abortive TAR transcripts (35, 36). These transcripts have been detected in vivo and have been proposed as a marker for inhibition of elongation (13, 36, 40, 42–44). Other processes could also contribute to latency, including antisense transcription (45), low levels of Rev (46), a block in export of RNA from the nucleus (47), or RNA interference (48–51).

It is uncertain to what degree these mechanisms contribute to latent infection in vivo. Most published studies have focused on latency models consisting of HIV-infected cell lines or in vitro infected cells, and most have investigated only one mechanism at a time. To investigate the degree to which different mechanisms reversibly inhibit HIV transcription in cells from HIV-infected patients, we developed a new approach that uses a panel of reverse transcription droplet digital polymerase chain reaction (RT-ddPCR) assays to simultaneously quantify different HIV transcripts that define specific blocks to transcription or progression through these blocks (fig. S1). The intact HIV genome contains two long terminal repeat (LTR) regions (one each at the 5′ and 3′ ends) that play critical roles in RT and contain promoters for cellular transcription (15). In the provirus (DNA), these LTRs are identical and each can be subdivided into sequential regions: U3, R (the first part of which is the TAR region), and U5 (15). HIV transcription normally begins in the 5′ R region with transcription of the TAR loop and ends after the polyadenylation signal at the end of the 3′ R region so that a full-length genomic HIV RNA transcript contains two LTRs that differ from each other (RU5 and U3R) and from the LTRs in the HIV DNA (15). Transcriptional interference should result in “read-through” transcripts (18) that contain U3 sequences

upstream of the normal HIV transcription initiation site in the R region and thus can be distinguished from conventional HIV transcripts by the presence of the full U3-R-U5 sequence region. Because the TAR region is found in all HIV transcripts (twice in full-length transcripts) (15), the level of TAR-containing transcripts serves as a measure of HIV transcriptional initiation (35, 36, 44). Longer, processive transcripts can be detected using targets for downstream sequences ("long LTR") that measure the degree of transcriptional elongation, whereas an excess (more than twofold) of TAR over long LTR transcripts suggests inhibition of elongation (35, 36, 44). Polyadenylated ("polyA") HIV transcripts suggest completion of transcription (52), which facilitates nuclear export, RNA stability, and translation (53). Finally, multiply spliced Tat-Rev ("Tat-Rev") transcripts can be detected by an amplicon that spans the boundary between exon 1 and exon 2 of *tat* and *rev* (15). Because Tat protein stimulates initiation and elongation of HIV transcription, whereas Rev stimulates export of unspliced and incompletely spliced HIV RNA from the nucleus, the level of Tat-Rev transcripts may indicate the relative ability to overcome blocks to initiation, elongation, and nuclear export, which are three of the main proposed mechanisms of latency (15). Moreover, levels of multiply spliced but not unspliced HIV RNA in patients on ART predict the extent of viral rebound after therapy interruption so that these transcripts have been proposed as a surrogate marker for productive infection (54).

We reasoned that by simultaneously quantifying the levels of these different HIV transcripts in $CD4^+$ T cells from ART-treated patients, we could measure the degree to which HIV transcription was blocked by mechanisms operating at different stages of transcription. Moreover, we realized that it would be important to determine the degree to which each mechanism was reversed by T cell activation (the stimulus used to induce ex vivo reversal from latency and the presumed mechanism of reversal in vivo) and by different drugs that have been proposed to reverse latency. Therefore, we measured the level of each HIV transcript in aliquots of freshly isolated $CD4^+$ T cells from ART-suppressed patients as well as other aliquots that had been treated ex vivo with anti-CD3/CD28 antibodies or putative latency-reversing agents. We hypothesized that the main reversible block to HIV transcription would be at the stage of transcriptional initiation. Surprisingly, we found that the main reversible blocks to HIV transcription were not transcriptional interference or another block to initiation but rather blocks to proximal elongation, polyadenylation, and splicing.

RESULTS

The HIV transcriptional profile suggests blocks to elongation, completion, and splicing

RT-ddPCR assays were developed for different HIV sequence regions, including (i) U3-U5 (read-through; suggests transcriptional interference), (ii) TAR loop (total transcripts; indicates transcriptional initiation), (iii) R-U5/pre-Gag (long LTR; elongation beyond 5′ LTR), (iv) Pol, (v) Nef (distal transcription), (vi) U3-polyA (polyA; completion of transcription), and (vii) multiply spliced Tat-Rev (Tat-Rev). These seven HIV assays (fig. S1) performed similarly with respect to detection limit, dynamic range, linearity, and overall efficiency on HIV RNA standards (fig. S2, A to D). In $CD4^+$ T cells (day 0) freshly isolated from the blood of 15 ART-suppressed individuals, the relative abundance of HIV transcripts

(normalized to 1 μg of RNA or ~10^6 cells) was as follows: total (TAR; median, 25,020 copies/μg) > elongated (long LTR; median, 1816 copies/μg) > polyadenylated (polyA; median, 257 copies/μg) and read-through (median, 307 copies/μg) > multiply spliced Tat-Rev (Tat-Rev; median, 5 copies/μg) (Fig. 1A). Read-through transcripts were detected in all participants, suggesting transcriptional interference, but were 86-fold lower than total (TAR) transcripts (median across individuals of [read-through/TAR] = 0.012) and 7-fold lower than elongated transcripts (median [read-through/long LTR] = 0.15). Total transcripts were 10-fold higher than elongated transcripts (median [long LTR/TAR] = 0.097), indicating abundant transcriptional initiation but a substantial block to elongation. Elongated transcripts were sevenfold higher than polyadenylated transcripts (median [polyA/long] = 0.14), suggesting another block to completion of transcription. Moreover, polyadenylated transcripts were 28-fold higher than multiply spliced Tat-Rev transcripts (median [Tat-Rev/polyA] = 0.036), suggesting a block to multiple splicing. Freshly isolated (day 0) peripheral blood mononuclear cells (PBMCs) were available from 12 individuals and showed a similar pattern of HIV transcripts and blocks (Fig. 1B), except that the block to elongation was even greater than in $CD4^+$ T cells (median [long LTR/TAR] = 0.044; Wilcoxon signed-rank test, P = 0.0012).

To determine whether the block to completion represents more distal blocks to elongation between the long LTR (5′ pre-Gag) and polyA (3′ LTR) regions, additional RT-ddPCR assays were developed for HIV Pol (downstream of Gag) and Nef (just before the 3′ LTR), and all seven transcripts were quantified in six ART-suppressed individuals. A gradient was observed from long LTR to Pol to Nef (Fig. 1C), suggesting additional transcription stop points between the pre-Gag region and Nef. The median (Nef/long) was 0.097, indicating 10-fold lower levels of transcripts containing 3′ compared to 5′ sequence regions.

To determine how much of the observed difference between HIV transcripts could be due to proviral mutations (for example, internal deletions and APOBEC-induced hypermutations) corresponding to the primer/probe sequences, levels of the read-through (U3–U5), TAR, long LTR (R-U5/Gag), Pol, and Nef sequence regions were measured in the DNA extracted at the same time as the RNA (available for all but two participants) using the same primers/probes and ddPCR conditions that were used to measure each HIV RNA (note that the polyA and Tat-Rev assays do not detect HIV DNA). The read-through (U3–U5) and TAR HIV DNA were generally very similar and usually about twice that of the long LTR DNA (median [TAR/long LTR] = 2.11; mean [TAR/long LTR] = 2.00) (fig. S3), as expected. The long LTR DNA was slightly higher than that of Nef DNA (median [Nef/long LTR] = 0.79). To further evaluate how either deletions or hypermutations in the provirus could affect the observed HIV RNA measurements, all HIV RNAs except Tat-Rev were normalized to HIV DNA (both normalized to 10^6 cells using nucleic acid mass) measured by the same assay used for the RNA (except for polyA, which was normalized to the corresponding read-through assay, which shares the same forward primer and probe). The mean [TAR RNA/TAR DNA] was 10.1, corresponding to about 20 TAR transcripts per provirus. The differences between read-through, TAR, long LTR, polyA, and Nef transcripts were preserved even after normalization to the corresponding HIV DNA measures (Fig. 1, D and E), indicating that the observed blocks to transcriptional elongation and completion cannot be explained by either deletions or hypermutations in the proviruses.

T cell activation selectively reverses baseline blocks to splicing, elongation, and completion

HIV transcripts were quantified in freshly isolated (day 0) and ex vivo activated (anti-CD3/CD28; day 2) $CD4^+$ T cells from 12 individuals. When HIV transcripts were normalized to microgram of RNA (which expresses HIV transcription in relation to total cellular transcription), activation caused no net change in read-through or total transcripts, tended to increase elongated transcripts, and elicited greater increases in polyadenylated and multiply spliced Tat-Rev transcripts (Fig. 2, A to E). When normalized by DNA mass (a better surrogate for activated cell numbers), activation increased the numbers of all transcripts, but the increases in read-through (median [day 2/day 0] = 3.1) and TAR (median [day 2/day 0] = 2.4) did not exceed the global increase in cellular transcription, whereas long LTR transcripts tended to increase more (median [day 2/day 0] = 7.4), and the greatest increases were seen in polyA (median [day 2/day 0] = 21.8) and Tat-Rev (median [day 2/day 0] = 85.7) (Fig. 2, F to J). A similar pattern was observed when HIV RNA measures were normalized by cell counts (table S1) and also at day 1 of activation, before cells had begun to proliferate (table S1).

Ratios of one HIV transcript to another (which are independent of normalization to cell numbers) were used to evaluate levels in relation to total, elongated, or polyadenylated transcripts to measure the degree of transcriptional interference (read-through transcripts) or progression through blocks to elongation, completion, and multiple splicing. Activation did not change levels of read-through transcripts in relation to total HIV transcripts (Fig. 3A), although they constituted a lower proportion of elongated HIV transcripts (Fig. 3B). In contrast, activation increased the proportion of total HIV transcripts that were elongated (Fig. 3C), polyadenylated (Fig. 3D), and multiply spliced (Fig. 3E; median fold changes [day 2/day 0] of 2.7, 15.1, and 37.5, respectively); increased the fraction of elongated HIV transcripts that were polyadenylated (Fig. 3F) and multiply spliced (Fig. 3G; median fold changes of 5.9 and 14.1, respectively); and increased the proportion of polyadenylated HIV transcripts that were multiply spliced (Fig. 3H; median fold change of 3.0). Using the relative levels of total versus elongated, elongated versus completed, and completed versus multiply spliced HIV transcripts, we also calculated the proportion of HIV transcripts blocked at the stages of elongation, completion, and splicing (see the “HIV RNA quantities in freshly isolated and ex vivo activated $CD4^+$ T cells” section under Materials and Methods). Freshly isolated $CD4^+$ T cells were characterized by strong blocks to elongation (present in median 80% of transcripts), completion (87%), and multiple splicing (96%) (Fig. 3I). Activation reduced the proportion of transcripts blocked at the stages of elongation, completion, and splicing (median changes from day 0 to day 2 of 34, 61, and 6.5%, respectively; Fig. 3I).

HIV transcriptional blocks are present in most infected cells

Replicate terminal cell dilutions were used to determine the frequencies of freshly isolated (day 0) and ex vivo activated (day 2) $CD4^+$ T cells that contained each HIV transcript. When normalized to cell counts, the amount of each HIV transcript showed surprisingly little variation across replicates or cell dilutions until terminal dilution was achieved (fig. S4). The relative order of cell frequencies positive for the five transcripts mirrored the HIV RNA

quantities in the bulk cell population (Fig. 4, A and B). Moreover, 89 to 100% of HIV-infected cells contained TAR transcripts (Fig. 4B). Activation increased the frequency of cells containing elongated ($P = 0.040$), completed ($P = 0.0016$), and especially multiply spliced Tat-Rev transcripts ($P = 9.6 \times 10^{-6}$; all Wilcoxon signed-rank tests; Fig. 4, C to F). By comparing the relative frequencies of cells containing total versus elongated, elongated versus completed, and completed versus multiply spliced HIV transcripts (see the "Cellular distribution of HIV transcripts" section under Materials and Methods), we also calculated the proportion of cells with blocks to elongation (median, 93% of TAR^+ cells), completion (76% of long LTR^+ cells), and multiple splicing (>99% of $polyA^+$ cells) (Fig. 4G). Activation decreased the frequency of cells with blocks to elongation, completion, and splicing (median changes from day 0 to day 2 of 21, 20, and 13%, respectively) (Fig. 4G).

Latency-reversing agents exert differential effects on the blocks

Amounts of each HIV transcript were measured after ex vivo latency-reversing treatment of $CD4^+$ T cells from five ART-suppressed individuals (Fig. 5 and figs. S5 and S6). No agent induced read-through transcripts more than 1.7-fold (median change, 0.98). JQ1 and disulfiram induced no HIV transcript by more than 2.1-fold, and chaetocin caused less than 2-fold changes in all but one individual. Panobinostat and romidepsin increased total (median, 2.9- and 4-fold) and elongated transcripts (median, 3.7- and 4.6-fold) but had less effect on polyadenylated (median, 1.9- and 1.5-fold) or multiply spliced transcripts (median, 0.7- and 1.2-fold). In contrast, ingenol mebutate (PEP005) increased polyadenylated (13-fold) and multiply spliced (21-fold) transcripts more than total (2.6-fold) or elongated transcripts (3.2-fold). The differential effects of these agents suggest that the mechanisms underlying the blocks to completion and splicing may differ from those that mediate the blocks to initiation and elongation.

DISCUSSION

A "transcription profiling" approach was used to infer the degree to which HIV transcription is reversibly blocked by different mechanisms in vivo. Aside from differences in HIV transcription, consideration must be given to other factors that can affect the quantity of each transcript. One such factor is the performance characteristics of the various assays, especially the overall efficiency of detection. However, the relatively small differences in assay efficiency (fig. S2) do not explain the large differences observed between quantities of different HIV RNA transcripts. Internal deletions and APOBEC-induced hyper-mutations are found in a high percentage of proviruses (55, 56) and could contribute to lower detection of some RNA sequence regions, especially Pol and Tat-Rev. Although deletions tend to affect the interior of the provirus and spare the LTRs (55, 56), proviruses with deletion of one LTR ("single LTR") or everything but one LTR ("solo LTR") have also been described (10, 57). We found that measures of the read-through (U3–U5) and TAR HIV DNA were generally very similar and usually about twice that of the long LTR (R-U5/Gag) DNA (median [TAR/long LTR] = 2.11; mean [TAR/long] = 2.00), suggesting that in most patients, most proviruses have two intact LTRs and one long LTR (R-U5/Gag) region. Moreover, the differences between read-through, TAR, long LTR, polyA, and Nef transcripts were preserved even after normalization to the corresponding HIV DNA measures (Fig. 1, D and

E), indicating that the observed blocks to transcriptional elongation and completion cannot be explained by either deletions or hypermutations in the proviruses. Proviral mutations affecting Tat (if they do not prevent transcription), Rev, or splice junctions could contribute to the measured difference between quantities of polyA and multiply spliced Tat-Rev RNA. However, activation and PEP005 caused dramatic increases in multiply spliced Tat-Rev RNA, and activation caused a greater than 10-fold increase in the frequency of cells that contain multiply spliced Tat-Rev, indicating that in these cells, there is still a reversible block to splicing that cannot be explained by any deletions or hypermutations in the proviruses.

Alternatively, differences in RNA stability could also contribute to the patterns observed. However, previous studies on HIV latency have almost exclusively implicated mechanisms that involve HIV transcription or nuclear export (14, 15), which makes it very unlikely that HIV latency is due to degradation of HIV RNA. In addition, the presence of a polyA tail should confer increased stability in eukaryotic cells (53), yet polyadenylated HIV transcripts constituted only a small fraction of all the HIV transcripts. Moreover, differences in RNA stability do not explain the differential effects of activation or latency-reversing agents. Therefore, it is likely that the observed differences between the quantities of various HIV transcripts, and the changes with activation or latency-reversing agents, primarily reflect blocks at distinct stages of HIV transcription.

HIV transcriptional initiation can be inhibited by different mechanisms, including transcriptional interference. Here, the minimal U3–U5 amplicon necessary to distinguish read-through from canonical HIV transcripts was detected in all participants. However, read-through transcripts constituted only 1% of total and 15% of elongated transcripts, and activation did not decrease the ratio of read-through/total transcripts, suggesting that transcriptional interference plays a relatively modest role in reversible inhibition of HIV transcription in vivo. Recent work in latency models has also suggested that antisense HIV transcripts may contribute to latency (45). Antisense transcripts could be detected by our assays, and although these assays are unable to distinguish whether any given transcript is sense or antisense, the 10-fold excess of long LTR (5′ only) over Nef (3′ only) suggests that ≤10% of all LTR-extended transcripts originate from the antisense direction.

The degree of HIV transcriptional initiation was assessed by the amount of RNA containing the TAR region, which is found in all HIV transcripts, in relation to other amplicons and to HIV DNA. Total TAR-containing HIV transcripts were detected by our polyadenylation-RT-ddPCR assay at quantities much higher than previously reported for other HIV transcripts. This is likely because 90% of these are prematurely terminated transcripts that are difficult to reverse transcribe using conventional priming strategies, because they form a tight hairpin that is too short for random hexamers and hinders binding of hexamers or gene-specific reverse primers (44). Moreover, terminal cell dilution experiments revealed that 89 to 100% of HIV-infected cells harbor TAR-containing transcripts. These findings suggest that the extent of HIV transcriptional initiation is much greater than previously recognized. Because presumably only one TAR loop can bind to the proviral DNA at any given time and we observed an average of 20 TAR copies per provirus, most of these TAR transcripts likely represent previous HIV transcriptional initiation events. In the presence of a sustained block to elongation, the TAR loop transcript may dissociate from the proviral DNA, allowing for a

new initiation event and accumulation of these transcripts in the cell over repetitive cycles of HIV transcriptional initiation. Regardless of the half-life of these TAR loop transcripts, their accumulation suggests a dynamic process of repetitive cycles of HIV transcriptional initiation, which differs from the prevailing model of a relatively static, quiescent cell in which HIV is transcriptionally silent. Activation increased total (TAR) transcripts, but the increase did not exceed the global increase in human cellular transcription and was small compared to the absolute or proportional increase in elongated, completed, or multiply spliced transcripts. These results suggest that inhibition of transcriptional initiation is not the main mechanism that reversibly inhibits HIV transcription in vivo.

Total HIV transcripts were greater than elongated transcripts (median, 10-fold) in every individual, suggesting a block to HIV transcriptional elongation. An excess of short transcripts has been described in latency models and blood cells from some patients (13, 36, 40, 42), but the polyadenylation-RT-ddPCR assay used here may be able to detect short transcripts with an efficiency and accuracy that was not previously possible (44), and the results suggest that the block to elongation is greater and more pervasive than previously recognized. T cell activation increased the ratio of elongated to total transcripts and the frequency of cells with elongated transcripts, suggesting that the block to elongation is partially reversed by activation.

Elongated HIV transcripts were greater than polyadenylated HIV transcripts in all but one individual, and the median difference was about sevenfold. The low ratio of polyadenylated to elongated transcripts was surprising and implicates another block to completion of HIV transcription. Although the polyA assay will detect read-through–5′ LTR–polyadenylated transcripts, the sevenfold excess of long LTR over polyA transcripts in the unstimulated cells suggests that if read-through–5′ LTR–polyA transcripts are present, they constitute less than or equal to one-seventh of the total. Although previous studies have reported the presence of non-polyadenylated HIV transcripts (13), low quantities of polyadenylated HIV transcripts (13, 52), and/or regulation of HIV transcription by 3′ end processing (58, 59), this block to completion has not been well described as a mechanism of latency, in part due to the lack of quantitative comparison between elongated and polyadenylated transcripts. Activation increased the absolute number of polyadenylated transcripts, the ratio of polyadenylated/total and polyadenylated/elongated transcripts (the latter often approached 1), and the frequency of cells with polyadenylated transcripts. When the blocks to HIV transcription were expressed as a proportion of transcripts, the greatest effect of activation was to reduce the block to completion. These results suggest that the block to completion is one of the main reversible blocks to HIV transcription and a new, important mechanism of latency.

This block to completion could represent additional barriers to transcriptional elongation (distal to the TAR loop) and/or a block to polyadenylation. We observed a gradient from long LTR to Pol to Nef, suggesting additional transcription stop points between the pre-Gag region and Nef. Nef RNA/DNA tended to be higher than polyA RNA/DNA, which could indicate transcriptional pausing at the 3′ LTR and/or a block to polyadenylation. End processing of the viral RNA could be inhibited by pausing at the 3′ TAR stem-loop or low expression of HIV-1 Vpr, which modulates polyadenylation activity (60), or human cellular factors such as CDK11 (58, 59) and polyadenylation factors. Because polyadenylation

facilitates nuclear export, stability, and translation (53), the lack of polyadenylated HIV RNA could contribute to the previously observed block in nuclear export of HIV RNA (47) and to low amounts of HIV protein.

Multiply spliced Tat-Rev transcripts were also lower than polyadenylated transcripts (typically by >10-fold) in all participants, suggesting a specific block to multiple splicing. As described by others, Tat-Rev was very low in the unstimulated cells (61, 62). The lack of Tat protein could contribute to the block to proximal elongation, and lack of Rev could contribute to the previously described block to nuclear export of RNA (47). The block to splicing was partially reversed by activation, which increased the amount of Tat-Rev, the ratios of Tat-Rev to total, elongated, and polyadenylated transcripts, and the frequency of cells with Tat-Rev. In absolute terms, the greatest effect of activation was to increase the amount of Tat-Rev and the frequency of cells with Tat-Rev. These results suggest that the block to splicing contributes strongly to the reversible inhibition of HIV transcription. The reversible component could involve activation-induced differences in human genes such as spliceosome components, SR proteins (such as SF2/ASF), MATR3, and PSF (15, 63). At the same time, amounts of Tat-Rev were low relative to polyA even after activation, suggesting a nonreversible component of this block, which may reflect HIV sequence-specific elements [multiple inefficient splice donor and acceptor sites (15), intronic and exonic splicing silencers (15), or proviral mutations affecting Tat, Rev, or splice junctions (55, 56)] or negative feedback by Rev (15, 63).

The baseline amounts of different HIV transcripts and HIV DNA regions also have important implications for deciding which HIV RNA or DNA sequence region to quantify to answer a given study question. HIV transcripts containing 5′ regions (especially TAR) were present at much higher copy numbers and may prove especially sensitive to detect HIV sequences. However, many of these total or elongated HIV transcripts may never produce polyadenylated HIV or protein. Although present at much lower copy numbers, polyA and Tat-Rev transcripts may have more functional significance and may serve as markers for HIV protein and productive infection, respectively (54). Multiple published assays target Gag or Pol, which indicate unspliced RNA but may be intermediate in terms of their copy numbers and functional significance.

Replicate terminal cell dilution was used to investigate the cellular distribution of the different HIV RNA transcripts and blocks to transcription. The pattern of the five different HIV transcripts observed in bulk unstimulated cells was surprisingly consistent across replicates until a given transcript reached terminal dilution, and the frequency of cells containing the different HIV transcripts mirrored the quantities of each transcript in the bulk population. These results suggest that the observed transcriptional blocks operate in most infected cells. Total transcripts were detectable even at cell replicates predicted to contain one to two copies of HIV DNA, suggesting that almost every HIV-infected cell contains TAR transcripts. This suggests that some degrees of transcriptional initiation and block to elongation exist in almost every HIV-infected $CD4^+$ T cell in the blood. TAR transcripts may thus serve as a marker for HIV-infected cells and a means to target them, given their short, structured sequence and higher copy numbers with respect to the provirus. Finally, the cell

dilution experiments suggest that the effects of activation are not limited to one or a few rare cells.

In addition to cell activation, different latency-reversing agents were tested for their ability to reverse the different blocks to HIV transcription. No substantial induction of read-through transcripts was observed with any latency-reversing agent, in contrast to one published study (52), and most classes of latency-reversing agents caused little if any induction of polyadenylated transcripts, in agreement with the aforementioned study (52). Panobinostat and romidepsin increased total and elongated transcripts but had less or no effect on polyadenylated and spliced transcripts, whereas PEP005 strongly induced completed and spliced transcripts but had lesser effects on initiation and elongation. The differential effects of these latency-reversing agents suggest that the mechanisms underlying the blocks to completion and splicing may differ from those that mediate the blocks to initiation and elongation.

These results have important implications for rational design of therapies aimed at reversing latency. Given the selective effect of different latency-reversing agents, optimal reversal of latency may require a combination of drugs that can reverse all the different blocks to HIV transcription. Because polyadenylation facilitates RNA nuclear export, stability, and translation (53), polyadenylated HIV transcripts may be important for translation into HIV protein, which serves as the source for the peptide antigens required for T cell recognition and killing of infected cells. Given the functions of the polyA tail and the fact that multiply spliced transcripts serve as the primary template for translation of Tat and Rev, both polyadenylation and multiple splicing may be important for expression of Tat and Rev proteins, which facilitate the transformation from latent to productive infection (15). The selective ability of disulfiram and histone deacetylase (HDAC) inhibitors to increase total and elongated transcripts, with relatively little effect on polyadenylated or multiply spliced transcripts, may explain why these agents increased cell-associated HIV RNA in human trials but did not result in measurable decreases in HIV DNA or latently infected cells, suggesting that they did not lead to killing of reactivated cells (64–69).

Although most of the HIV RNA that we detected was incomplete, and thus noninfectious, we were unable to determine how much of this RNA originated from replication-competent proviruses. However, several caveats should be acknowledged. First, it is difficult to demonstrate both inducibility and infectivity in the same provirus without T cell activation, but activation also changes the cell’s gene expression and precludes analysis of the basal transcription patterns. Second, much regulation of HIV transcription may depend on cellular factors that are independent of viral sequence. Third, noninfectivity may be a consequence of sequence changes throughout the provirus, but only those that create functional defects in the LTRs or Tat would be expected to affect transcription. Therefore, the factors that regulate HIV transcription of many noninfectious proviruses may not differ from those that regulate transcription of infectious viruses. In direct support of this hypothesis, one study found that the frequency of peripheral blood cells that could be induced to transcribe HIV RNA correlated precisely with the frequency that could be induced to express infectious virus (70). Fourth, the cell dilution experiments from this study suggest that the blocks to HIV transcriptional elongation, completion, and splicing operate in most HIV-infected cells. For

these reasons, it is likely that the reversible blocks to HIV transcription described in this study also serve as the main mechanisms of latency in circulating $CD4^+$ T cells.

Our findings challenge the prevailing model that HIV latency is due to a lack of transcriptional initiation and suggest that latent infection of blood $CD4^+$ T cells is due to blocks to elongation, distal transcription/polyadenylation, and multiple splicing. It is unclear if these mechanisms vary in different latency models, cell/tissue types, or patients with different phenotypes and the degree to which they are reversed in vivo by existing latency-reversing agents. The transcription profiling approach described in this study should be used to investigate these questions. As confirmation that these mechanisms operate in cells with replication-competent provirus, the methods used here should be applied to latency models that use primary cells and infectious virus. However, because latency models may not recapitulate what happens in vivo, the ultimate proof may require new technologies or approaches. Meanwhile, the mechanisms described in this study provide a framework to investigate new cellular factors involved in HIV latency. Our findings should help in the design and evaluation of new therapies that can silence or kill latently HIV-infected cells. The approach that we have developed could also be applied to investigate transcriptional blocks in other viruses (particularly those that can cause latent infections) and in other organisms.

MATERIALS AND METHODS

Study design

The study was a pilot, cross-sectional study. The primary study objectives were (i) to quantify the degree to which HIV transcription was inhibited by transcriptional interference or other blocks to HIV transcriptional initiation, transcriptional elongation, and subsequent transcriptional processes, including completion of transcription and splicing, in $CD4^+$ T cells from patients on suppressive ART and (ii) to determine the degree to which these blocks were reversed ex vivo by T cell activation. The study participants were HIV-infected adult patients on suppressive ART. A total of 23 blood samples were obtained from 18 study participants (table S2); three participants were sampled twice, and one was sampled three times to obtain blood for different experiments. The 18 study participants were recruited prospectively and sequentially from the Infectious Diseases Clinic at the San Francisco Veterans Affairs Medical Center from May 2015 to December 2016. Inclusion criteria included (i) infected with HIV-1, (ii) on ART, and (iii) last viral load <50 copies/ml. The study was approved by the local Institutional Review Board of the University of California, San Francisco and the San Francisco Veterans Affairs. All participants provided informed consent.

Blood cell isolation

PBMCs were recovered by Ficoll density gradient centrifugation (44) from fresh venous blood. PBMCs were used to isolate total $CD4^+$ T cells by negative selection using the Untouched Human $CD4^+$ T Cell Kit (Thermo Fisher Scientific). In 12 individuals for whom the cell count of PBMCs was high enough that not all of them were needed for $CD4^+$ T cell

isolation, aliquots of freshly isolated PBMCs were immediately frozen as cell pellets (not cryopreserved).

HIV RNA quantities in freshly isolated and ex vivo activated CD4+ T cells

Aliquots of $CD4^+$ T cells (typically at least 6×10^6) were frozen as cell pellets (not cryopreserved cells) immediately after isolation (day 0 or unstimulated) or after ex vivo activation for 2 days (12 individuals) with anti-CD3/CD28 antibody-coated beads (25 μl per million cells) and interleukin-2 (IL-2; 20 U/ml). Antiretrovirals (nevirapine and indinavir) were used to prevent new infection. When the cell count of isolated $CD4^+$ T cells was high enough to permit additional conditions, aliquots of cells were activated ex vivo for 1 day (9 of the above 12 individuals) or cultured for 1 to 2 days with antiretrovirals but neither anti-CD3/CD28 nor IL-2 (8 of the above 12 individuals). Cells were cultured at 10^6 cells/ml in RPMI with 10% fetal bovine serum, penicillin/streptomycin (10 U/ml), and L-glutamine (2 mM). Read-through, total (TAR), elongated (long LTR), completed (polyA), and multiply spliced (Tat-Rev) transcripts in unstimulated and activated $CD4^+$ T cells were measured by RT-ddPCR and normalized to 1 μg of RNA [corresponding to about 10^6 unstimulated cells (71)] using the known RNA input into the RT and the fraction of the RT used for each ddPCR well. To determine whether the observed difference between levels of the long LTR and polyA transcripts was due to an additional, more downstream block to elongation between these two sequence regions (5′ LTR/pre-Gag and 3′ LTR/polyA tail), we developed additional RT-ddPCR assays for two intermediate sequence regions: Pol (downstream of Gag but present only in unspliced transcripts) and Nef (not removed by splicing and located at the 3′ end just before the 3′ LTR). All seven HIV transcripts (see fig. S1) were quantified in six additional samples of freshly isolated (day 0) $CD4^+$ T cells from ART-suppressed individuals.

If sequence variations or mutations (deletions and APOBEC-induced hypermutations) in the HIV DNA corresponding to the primer/probe regions do not preclude transcription, they could be transcribed as HIV RNAs that either lack primer/probe sequences (thus preventing detection) or contain sequence mismatches that could potentially reduce detection efficiency. To determine how much of the observed difference between HIV transcripts could be due to mutations in the HIV proviral sequence regions corresponding to the primers/probes, levels of the read-through (U3–U5), TAR, long LTR (R-U5/Gag), Pol, and Nef sequence regions were measured in the DNA extracted at the same time as the RNA (except for participants 116 and 118 from whom DNA was not available). DNA regions were measured using the same primers/probes and ddPCR conditions that were used to measure each HIV RNA. Because the polyA and Tat-Rev assays are specific for RNA and do not detect an HIV DNA standard containing an intact provirus (pNL4-3), these assays were not applied to the cellular DNA. However, the HIV sequence targeted by the polyA assay is entirely contained within the U3–U5 region of the read-through assay, and these assays share the same forward primer and probe, so the read-through assay can also be used to assess for most proviral deletions that would affect the polyA assay. To further evaluate how either deletions or hypermutations in the provirus could affect the observed HIV RNA quantities, all HIV RNAs except Tat-Rev were normalized to amounts of HIV DNA (both HIV RNA and DNA were normalized to 10^6 cells using nucleic acid mass) measured using the same assay used

for the RNA (except for polyA, which was normalized to the corresponding read-through assay containing the same forward primer and probe).

Although the normalization to RNA mass captures the change in each HIV transcript in relation to human cellular transcription, we observed that activation caused large but variable increases in the yield of total cellular RNA in relation to DNA (by three- to fourfold or greater), suggesting that activation dramatically increases the amount of total RNA per cell so that RNA mass is not a good surrogate for cell number in the activated cells. We also investigated the effect of activation on different housekeeping transcripts that have been studied in T cell activation (72, 73), including RPLP0, 18*S* ribosomal RNA (rRNA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), but activation increased the amounts of all of them in relation to total cellular RNA, suggesting that these were also not a good surrogate for cell numbers. As two other means to normalize the HIV transcript quantities to numbers of activated cells, we normalized the HIV RNA measurements in the unstimulated and activated cells by the cell equivalents recovered in the extracted DNA (available for 11 of 12 participants) or the cell counts before extraction (available for 6 of 12 participants).

We also analyzed the ratios of one HIV transcript to another, which do not depend on normalization to cell numbers. These ratios were used to express the levels of different HIV transcripts as a proportion of total (ratio to TAR), elongated (ratio to long LTR), or polyadenylated (ratio to polyA) HIV transcripts. The degree of transcriptional interference was assessed by levels of read-through transcripts (which themselves suggest a block) in relation to total transcripts (read-through/ TAR), whereas progression through blocks to elongation, completion, and splicing was measured by the ratio of postblock to preblock transcripts (elongated/total, polyadenylated/elongated, and multiply spliced/ polyadenylated, respectively).

We also calculated the proportion of transcripts that are blocked at the stages of transcriptional elongation (1 − (2*elongated/total]), completion (1 − [polyadenylated/ elongated]), and multiple splicing (1 − [Tat-Rev/polyadenylated]) in each sample. Note that calculating the block to elongation is complicated because any given elongated transcript can have either two TAR regions (if transcription has proceeded through the TAR region of the 3′ LTR) or one (if transcription does not proceed to the 3′ TAR region), but the formula (1 − [2*long LTR/TAR]), which assumes that all elongated transcripts are complete, provides a minimum estimate of the block to elongation.

Cellular distribution of HIV transcripts

To measure the frequency of $CD4^+$ T cells containing each transcript, freshly isolated (day 0) $CD4^+$ T cells from three additional study participants were counted (average of two manual counts and automated counts using the Bio-Rad TC20), subjected to serial, replicate five-fold cell dilutions (from 5×10^6 to 320 cells), and snap-frozen. For two of these participants, another aliquot of $>13 \times 10^6$ $CD4^+$ T cells was activated in bulk by culture for 2 days with anti-CD3/CD28 beads and IL-2 (conditions as above, including antiretrovirals to prevent spreading infection) and then subjected to the same replicate serial fivefold cell dilutions and freezing. Read-through, total (TAR), elongated (long LTR), completed (polyA), and multiply spliced Tat-Rev transcripts were measured in each replicate and then

normalized to cell numbers using the cell counts, the proportion of RNA going into each RT reaction (one-half of the total), and the proportion of each RT used for ddPCR.

The frequencies of $CD4^+$ T cells containing each HIV RNA transcript were calculated using the total number of replicates and the number of positive replicates at each cell count using the method of extreme limiting dilution analysis (74). The frequencies of HIV-infected cells containing each transcript were calculated in similar fashion by using the number of HIV-infected cells in each dilution [inferred from measurement of HIV DNA by ddPCR for the long LTR region (75) in the 5×10^6 and 1×10^6 cell replicates, using the assumptions of one long LTR HIV DNA copy per provirus and one provirus per cell] and the proportion of positive replicates at each dilution. *P* values (see table S1, sheet 7) were calculated using the website for extreme limiting dilution analysis (http://bioinf.wehi.edu.au/software/elda) (74). Note that the estimated frequencies are minimums, because very low quantities of a given transcript may be missed because of aliquoting the RNA for different ddPCR assays (see below). We also calculated the proportion of cells in which HIV transcription reached a given stage and was subsequently blocked by taking the difference between the frequencies of cells containing pre- and postblock HIV transcripts and dividing by the frequency of cells with preblock transcripts:

$$\begin{aligned}
&\text{Proportion of HIV-infected cells that initiate HIV transcripts} = \\
&f(\text{TAR in HIV-infected CD4}^+\text{ T cells}) \\
&\text{Proportion of cells in which there is HIV transcriptional initiation} \\
&\text{but not elongation} = [f(\text{TAR}) - f(\text{long LTR})]/f(\text{TAR}) = 1 - [f(\text{long LTR})/f(\text{TAR})] \\
&\text{Proportion of cells in which there is elongation but not completion} = \\
&[f(\text{long LTR}) - f(\text{polyA})]/f(\text{long LTR}) = 1 - [f(\text{polyA})/f(\text{long LTR})] \\
&\text{Proportion of cells in which there is completion but not multiply spliced} \\
&\text{Tat-Rev} = [f(\text{polyA}) - f(\text{Tat-Rev})]/f(\text{polyA}) = 1 - [f(\text{Tat-Rev})/f(\text{polyA})]
\end{aligned}$$

where $f(x)$ represents the frequency of cells containing HIV transcript x.

Effect of latency-reversing agents

To examine the effect of ex vivo treatment with latency-reversing agents, peripheral $CD4^+$ T cells from five study participants were aliquoted for culture (5×10^6 cells per well), rested overnight in the above medium with antiretrovirals, and then treated for 24 hours with different controls or latency-reversing agents. Controls included medium alone, DMSO (negative control), and anti-CD3/28 plus IL-2 (positive control). Latency-reversing agents were used at previously published concentrations (52) and included the histone methyltransferase inhibitor chaetocin (50 nM), the HDAC inhibitors panobinostat (30 nM) and romidepsin (40 nM), the BET bromodomain inhibitor JQ1 (2 μM), disulfiram (1 μM), and the protein kinase C agonists ingenol 3,20-dibenzoate (20 nM) and PEP005 (12 nM). Because of variable and sometimes insufficient yields of $CD4^+$ T cells, a few agents were not tested in all individuals. Cell counts and viabilities were measured in each well before harvesting, as were the yields of nucleic acid (RNA and DNA) after extraction. To examine the effect of latency-reversing agents, quantities of each HIV RNA transcript in the treated cells were expressed as copies per microgram of RNA and also divided by the corresponding

amounts in the negative control (DMSO) well (also in copies per microgram of RNA) to express the fold change relative to DMSO.

Nucleic acid extraction

For efficient recovery of short RNAs, nucleic acid extraction was performed on the basis of differential solubility using Trireagent (Molecular Research Center Inc.). Total cellular RNA and DNA were isolated using Trireagent per the manufacturer's instructions, including the use of polyacryl carrier (2 to 3 μl), except that the optional back extraction buffer was used to isolate DNA. RNA and DNA concentrations were quantified using ultraviolet (UV) spectrophotometry (NanoDrop 1000). RNA quality was assessed in a subset of samples using the Agilent Bioanalyzer 2100 and showed no evidence of cellular RNA degradation. One aliquot of total RNA (1 μg for most experiments or one-half of the total RNA for cell dilution experiments) was applied to the polyadenylation-RT reaction used to generate complementary DNA (cDNA) for the TAR assay, whereas another aliquot of RNA (4 to 5 μg for most experiments, but up to 7 μg when needed to measure all seven transcripts or one-half of the total RNA for cell dilution experiments) was applied to a separate RT reaction used to generate cDNA for the other ddPCR assays.

Reverse transcription

To prepare cDNA for all assays except the TAR assay [which requires previous polyadenylation for efficient RT of short transcripts (44)], an aliquot of RNA was used in a common RT reaction. A combination of random hexamers and poly-dT was used to avoid bias toward RT of the 3′ end (as can be seen with poly-dT), the 5′ end (as can be seen with random hexamers), or any one gene (as occurs with gene-specific primers); the use of a shorter poly-dT (dT15) with lower annealing temperature helped prevent inhibition of the subsequent ddPCR (44). This common RT was performed in 50 μl containing 5 μl of 10× SuperScript III buffer (Invitrogen), 5 μl of 50 mM $MgCl_2$, 2.5 μl of random hexamers (50 ng/μl; Invitrogen), 2.5 μl of 50 μM dT15, 2.5 μl of 10 mM deoxynucleoside triphosphates (dNTPs), 1.25 μl of RNAseOUT (40 U/μl; Invitrogen), and 2.5 μl of SuperScript III RT (200 U/μl; Invitrogen). For additional measurement of Pol and Nef transcripts, all reagents were increased proportionally for a final RT volume of 70 μl. Control RT reactions were established using RNA from HIV^- donor cells (negative control), "no RT" controls containing patient RNA but no superscript (when RNA yields were sufficiently high), and HIV RNA standards (positive controls; see below). RT reactions were performed in a conventional thermocycler at 25.0°C for 10 min, 50.0°C for 50 min, followed by an inactivation step at 85.0°C for 5 min. For best comparison of different transcripts, aliquots from the same common RT reaction were used in subsequent ddPCR assays for read-through, long LTR, polyA, Tat-Rev, and (in some cases) Pol and Nef regions.

Polyadenylation–reverse transcription

Although conventional approaches to RT (such as random hexamers, poly-dT, or different specific reverse primers) can efficiently detect the TAR region on longer HIV RNA molecules (read-through or virion standards), we found that these approaches were markedly inefficient (<3%) at detecting short transcripts containing only the TAR loop (short standard), likely due to its short length and a tight hairpin structure that hinders primer

binding (44). In contrast, we found that addition of a polyA linker (using polyA polymerase) allowed for subsequent RT of the TAR loop with a high efficiency that did not differ between short and long standards (44). Therefore, a second aliquot (typically 1 μg) of cellular RNA was used in a polyadenylation-RT reaction to prepare cDNA for the TAR assay (44). Polyadenylation was carried out in a volume of 20 μl containing 3 μl of 10× SuperScript III buffer (Invitrogen), 3 μl of 50 mM $MgCl_2$, 1 μl of 10 mM adenosine 5′-triphosphate (Epicentre), 2 μl of polyA polymerase (4 U/μl; Epicentre), and 1 μl of RNAseOUT (40 U/μl; Invitrogen) at 37.0°C for 45 min. Subsequently, the remaining RT reagents were added in a volume of 10 μl containing 1.5 μl of 10 mM dNTPs (Invitrogen), 1.5 μl of random hexamers (50 ng/μl; Invitrogen), 1.5 μl of 50 μM oligo dT15, and 1 μl of SuperScript III reverse transcriptase (200 U/μl; Invitrogen). RT was performed in a conventional thermocycler at 25.0°C for 10 min, 50.0°C for 50 min, followed by an inactivation step at 85.0°C for 5 min.

Droplet digital PCR

Primer and probe sequences are listed in table S3. ddPCR was used because it enables “absolute” quantification, it is relatively less dependent on PCR efficiency (which may be reduced by sequence mismatches or inhibitors), and it may be more precise than conventional quantitative PCR (qPCR) at low copy numbers (76). ddPCR was performed using the QX100 Droplet Digital qPCR System (Bio-Rad). Samples were tested in duplicate, and each reaction consisted of a 20-μl solution containing 10 μl of ddPCR Probe Supermix (no deoxyuridine triphosphate), 900 nM of primers, 250 nM of probe, and 5 μl of undiluted RT product or 500 ng of cellular DNA (fragmented using a QIAshredder column) (75). Droplets were amplified using a 7900 Thermal Cycler (Life Technologies) with the following cycling conditions: 10 min at 95°C, 45 cycles of 30 s at 95°C and 59°C for 60 s, and a final droplet cure step of 10 min at 98°C. Droplets were read and analyzed using the QuantaSoft software in the absolute quantification mode.

Assay validation and performance characteristics

To ensure that there is no substantial bias for or against any one transcript, a series of specific HIV standards was prepared, quantified by independent means (UV spectrophotometry and/or the Abbott RealTime assay), and used to test each assay for overall efficiency, detection limit, dynamic range, linearity, sensitivity to inhibition by background cellular RNA, and specificity (absence of false positives). Validation experiments were performed using HIV plasmid DNA (pNL4-3) and four different HIV RNA standards: (i) a “short” 58-nucleotide TAR loop sequence (HXB2 positions 456 to 513) corresponding to abortive transcripts (44); (ii) a read-through standard (positions 326 to 2844) (44); (iii) a “virion” standard containing full-length HIV genomic RNA (44); and (iv) a “six-assay” standard consisting of a synthetic read-through, multiply spliced, polyadenylated HIV RNA.

The short, read-through, and virion standards were prepared as described previously (44). The six-assay standard was prepared by constructing a plasmid (pNL43_RT_MS_polyA) containing synthetic HIV pNL4-3 sequences (GeneArt, Life Technologies) from the beginning of the 5′ U3 region to the first splice donor site (1 to 743), the splice acceptor in

exon 4 to the end of *tat/rev* exon 1 (5777 to 6044), the beginning of *tat/rev* exon 2 to the 3′ LTR polyA signal (8369 to 9636), and a polyA tail of 100 adenosine nucleotides. These sequences were cloned into pcDNA3.1(+) from the Nhe I site to the Xba I site by the providing company. The plasmid was linearized with Xba I (confirmed by gel electrophoresis) and then purified of protein and ribonucleases by digestion with Proteinase K followed by extraction with buffered phenol/chloroform/isoamyl alcohol (25:24:1), extraction with chloroform, and ethanol precipitation. Templates for the short, read-through, and six-assay standards were transcribed using the T7 MEGAscript Kit (Invitrogen). In vitro transcribed RNA standards were treated with deoxyribonuclease (DNase) I, purified using Qiagen's miRNeasy kit (short and read-through standard) or RNeasy kit with on-column DNase (six-assay standard), and quantified on the basis of molecular weights and replicate measurement of RNA mass using the NanoDrop 1000 spectrophotometer. The correct length and integrity of each standard were confirmed using the Agilent RNA 6000 Nano assay.

Standards were diluted to the appropriate copy numbers and mixed with cellular RNA extracted from donor PBMCs. Efficiencies of detection were expressed as ratios of absolute cDNA copies measured by ddPCR to the expected copies per ddPCR well, as calculated from the input of HIV RNA standard into the RT reaction, the fraction of the RT reaction used per ddPCR well, and the fact that the virion and six-assay standards contain two identical TAR regions. The overall efficiency of each assay was calculated by plotting measured versus expected copies and determining the slope using linear regression analysis.

The variability between cell aliquots and reproducibility of the extraction, RT, ddPCR, and activation was tested using replicate cell pellets of 5×10^6 unstimulated (day 0) PBMCs, replicate cell pellets of 5×10^6 unstimulated (day 0) $CD4^+$ T cells, and replicate wells of 5×10^6 activated (day 2) $CD4^+$ T cells that were obtained from the same participant on the same day (fig. S2E). Additional cell dilutions and replicates were performed as part of the terminal dilution experiments used to measure the frequency of cells containing each HIV transcript (fig. S4).

Note that because RNA extracted from a given sample was aliquoted for two separate RT reactions, and only a fraction of each RT reaction was used for a given assay, it is possible that these methods may fail to detect a given transcript if present at very low amounts in the starting sample. Tat-Rev transcripts were detected in 19 of 23 samples (from 14 of 18 individuals) of unstimulated $CD4^+$ T cells from which we had $\geq 5 \times 10^6$ cells, whereas the remaining transcripts were detected in all these samples. However, this limitation should be considered when interpreting the cell dilution experiments, particularly when diluted down to one to two HIV-infected cells.

Statistical analysis

The Wilcoxon signed-rank test was used to compare measures of different HIV RNA transcripts or transcriptional blocks and to compare measures of the same transcript or block between unstimulated and activated cells. All statistics were performed using GraphPad Prism (version 7) or the website for extreme limiting dilution analysis (http://bioinf.wehi.edu.au/software/elda) (74).

With the Wilcoxon signed-rank test, a *P* value of 0.05 can be obtained with a minimum of six pairs if differences between the paired values are consistent. For the primary study measures (amounts of five HIV transcripts and blocks to transcription in unstimulated and activated $CD4^+$ T cells), a sample size of 12 (twice that required) was chosen to achieve better power to detect reasonably consistent differences. For extension of the primary findings to secondary study questions requiring much higher numbers of $CD4^+$ T cells and assays (cell frequencies, effect of latency-reversing agents), smaller sample sizes were chosen in cases where the trends were clear and/or recapitulated findings from the primary study outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the study participants and the staff at the Infectious Diseases Clinic of the San Francisco Veterans Affairs Hospital. We also thank H. Günthard and A. Rice for helpful discussions. The reagent pNL4-3 was obtained through M. Martin at the NIH AIDS Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH.

Funding: This work was supported by the U.S. Department of Veterans Affairs [IK2 CX000520-01 (S.A.Y.) and I01 BX000192 (J.K.W.)], the National Institute of Diabetes and Digestive and Kidney Diseases at the NIH [1R01DK108349-01 (S.A.Y.)], the National Institute of Allergy and Infectious Diseases at the NIH [1R01AI132128 (S.A.Y. and J.K.W.), R56AI116342 (J.K.W.), R33AI116218 (J.K.W.), R56AI091573 (J.K.W.), and U19AI096109 (J.K.W.)], the American Foundation for AIDS Research (amfAR) Institute for HIV Cure Research [109301 (S.A.Y. and J.K.W.)], and the Swiss National Science Foundation [PBZHP3_147260 (P. Kaiser)].

REFERENCES AND NOTES

1. Chun TW, Finzi D, Margolick J, Chadwick K, Schwartz D, Siliciano RF. In vivo fate of HIV-1-infected T cells: Quantitative analysis of the transition to stable latency. Nat Med. 1995; 1:1284–1290. [PubMed: 7489410]
2. Chun TW, Carruth L, Finzi D, Shen X, DiGiuseppe JA, Taylor H, Hermankova M, Chadwick K, Margolick J, Quinn TC, Kuo YH, Brookmeyer R, Zeiger MA, Barditch-Crovo P, Siliciano RF. Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. Nature. 1997; 387:183–188. [PubMed: 9144289]
3. Wong JK, Hezareh M, Günthard HF, Havlir DV, Ignacio CC, Spina CA, Richman DD. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. Science. 1997; 278:1291–1295. [PubMed: 9360926]
4. Finzi D, Hermankova M, Pierson T, Carruth LM, Buck C, Chaisson RE, Quinn TC, Chadwick K, Margolick J, Brookmeyer R, Gallant J, Markowitz M, Ho DD, Richman DD, Siliciano RF. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. Science. 1997; 278:1295–1300. [PubMed: 9360927]
5. Finzi D, Blankson J, Siliciano JD, Margolick JB, Chadwick K, Pierson T, Smith K, Lisziewicz J, Lori F, Flexner C, Quinn TC, Chaisson RE, Rosenberg E, Walker B, Gange S, Gallant J, Siliciano RF. Latent infection of $CD4^+$ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. Nat Med. 1999; 5:512–517. [PubMed: 10229227]
6. Ramratnam B, Mittler JE, Zhang L, Boden D, Hurley A, Fang F, Macken CA, Perelson AS, Markowitz M, Ho DD. The decay of the latent reservoir of replication-competent HIV-1 is inversely correlated with the extent of residual viral replication during prolonged anti-retroviral therapy. Nat Med. 2000; 6:82–85. [PubMed: 10613829]
7. Strain MC, Günthard HF, Havlir DV, Ignacio CC, Smith DM, Leigh-Brown AJ, Macaranas TR, Lam RY, Daly OA, Fischer M, Opravil M, Levine H, Bacheler L, Spina CA, Richman DD, Wong JK.

Heterogeneous clearance rates of long-lived lymphocytes infected with HIV: Intrinsic stability predicts lifelong persistence. Proc Natl Acad Sci USA. 2003; 100:4819–4824. [PubMed: 12684537]

8. Siliciano JD, Kajdas J, Finzi D, Quinn TC, Chadwick K, Margolick JB, Kovacs C, Gange SJ, Siliciano RF. Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting $CD4^+$ T cells. Nat Med. 2003; 9:727–728. [PubMed: 12754504]
9. Chomont N, El-Far M, Ancuta P, Trautmann L, Procopio FA, Yassine-Diab B, Boucher G, Boulassel MR, Ghattas G, Brenchley JM, Schacker TW, Hill BJ, Douek DC, Routy JP, Haddad EK, Sékaly RP. HIV reservoir size and persistence are driven by T cell survival and homeostatic proliferation. Nat Med. 2009; 15:893–900. [PubMed: 19543283]
10. Maldarelli F, Wu X, Su L, Simonetti FR, Shao W, Hill S, Spindler J, Ferris AL, Mellors JW, Kearney MF, Coffin JM, Hughes SH. HIV latency. Specific HIV integration sites are linked to clonal expansion and persistence of infected cells. Science. 2014; 345:179–183. [PubMed: 24968937]
11. Joos B, Fischer M, Kuster H, Pillai SK, Wong JK, Böni J, Hirschel B, Weber R, Trkola A, Günthard HF. Swiss HIV Cohort Study, HIV rebounds from latently infected cells, rather than from continuing low-level replication. Proc Natl Acad Sci USA. 2008; 105:16725–16730. [PubMed: 18936487]
12. Lassen K, Han Y, Zhou Y, Siliciano J, Siliciano RF. The multifactorial nature of HIV-1 latency. Trends Mol Med. 2004; 10:525–531. [PubMed: 15519278]
13. Lassen KG, Bailey JR, Siliciano RF. Analysis of human immunodeficiency virus type 1 transcriptional elongation in resting $CD4^+$ T cells in vivo. J Virol. 2004; 78:9105–9114. [PubMed: 15308706]
14. Marcello A. Latency: The hidden HIV-1 challenge. Retrovirology. 2006; 3:7. [PubMed: 16412247]
15. Karn J, Stoltzfus CM. Transcriptional and posttranscriptional regulation of HIV-1 gene expression. Cold Spring Harb Perspect Med. 2012; 2:a006916. [PubMed: 22355797]
16. Winslow BJ, Pomerantz RJ, Bagasra O, Trono D. HIV-1 latency due to the site of proviral integration. Virology. 1993; 196:849–854. [PubMed: 8372452]
17. Schroder ARW, Shinn P, Chen H, Berry C, Ecker JR, Bushman F. HIV-1 integration in the human genome favors active genes and local hotspots. Cell. 2002; 110:521–529. [PubMed: 12202041]
18. Han Y, Lassen K, Monie D, Sedaghat AR, Shimoji S, Liu X, Pierson TC, Margolick JB, Siliciano RF, Siliciano JD. Resting $CD4^+$ T cells from human immunodeficiency virus type 1 (HIV-1)-infected individuals carry integrated HIV-1 genomes within actively transcribed host genes. J Virol. 2004; 78:6122–6133. [PubMed: 15163705]
19. Bednarik DP, Mosca JD, Raj NBK. Methylation as a modulator of expression of human immunodeficiency virus. J Virol. 1987; 61:1253–1257. [PubMed: 3469417]
20. Bednarik DP, Cook JA, Pitha PM. Inactivation of the HIV LTR by DNA CpG methylation: Evidence for a role in latency. EMBO J. 1990; 9:1157–1164. [PubMed: 2323336]
21. Schulze-Forster K, Götz F, Wagner H, Kröger H, Simon D. Transcription of HIV1 is inhibited by DNA methylation. Biochem Biophys Res Commun. 1990; 168:141–147. [PubMed: 2327994]
22. Singh MK, Pauza CD. Extrachromosomal human immunodeficiency virus type 1 sequences are methylated in latently infected U937 cells. Virology. 1992; 188:451–458. [PubMed: 1585628]
23. Gutekunst KA, Kashanchi F, Brady JN, Bednarik DP. Transcription of the HIV-1 LTR is regulated by the density of DNA CpG methylation. J Acquir Immune Defic Syndr. 1993; 6:541–549. [PubMed: 8496786]
24. Van Lint C, Emiliani S, Ott M, Verdin E. Transcriptional activation and chromatin remodeling of the HIV-1 promoter in response to histone acetylation. EMBO J. 1996; 15:1112–1120. [PubMed: 8605881]
25. Sheridan PL, Mayall TP, Verdin E, Jones KA. Histone acetyltransferases regulate HIV-1 enhancer activity in vitro. Genes Dev. 1997; 11:3327–3340. [PubMed: 9407026]
26. Pearson R, Kim YK, Hokello J, Lassen K, Friedman J, Tyagi M, Karn J. Epigenetic silencing of human immunodeficiency virus (HIV) transcription by formation of restrictive chromatin structures at the viral long terminal repeat drives the progressive entry of HIV into latency. J Virol. 2008; 82:12291–12303. [PubMed: 18829756]

27. Taube R, Peterlin M. Lost in transcription: Molecular mechanisms that control HIV latency. Viruses. 2013; 5:902–927. [PubMed: 23518577]
28. Jordan A, Defechereux P, Verdin E. The site of HIV-1 integration in the human genome determines basal transcriptional activity and response to Tat transactivation. EMBO J. 2001; 20:1726–1738. [PubMed: 11285236]
29. Pion M, Jordan A, Biancotto A, Dequiedt F, Gondois-Rey F, Rondeau S, Vigne R, Hejnar J, Verdin E, Hirsch I. Transcriptional suppression of in vitro-integrated human immunodeficiency virus type 1 does not correlate with proviral DNA methylation. J Virol. 2003; 77:4025–4032. [PubMed: 12634362]
30. Duverger A, Jones J, May J, Bibollet-Ruche F, Wagner FA, Cron RQ, Kutsch O. Determinants of the establishment of human immunodeficiency virus type 1 latency. J Virol. 2009; 83:3078–3093. [PubMed: 19144703]
31. Ghose R, Liou LY, Herrmann CH, Rice AP. Induction of TAK (cyclin T1/P-TEFb) in purified resting $CD4^+$ T lymphocytes by combination of cytokines. J Virol. 2001; 75:11336–11343. [PubMed: 11689614]
32. Weinberger LS, Burnett JC, Toettcher JE, Arkin AP, Schaffer DV. Stochastic gene expression in a lentiviral positive-feedback loop: HIV-1 Tat fluctuations drive phenotypic diversity. Cell. 2005; 122:169–182. [PubMed: 16051143]
33. Lewinski MK, Bisgrove D, Shinn P, Chen H, Hoffmann C, Hannenhalli S, Verdin E, Berry CC, Ecker JR, Bushman FD. Genome-wide analysis of chromosomal features repressing human immunodeficiency virus transcription. J Virol. 2005; 79:6610–6619. [PubMed: 15890899]
34. Lenasi T, Contreras X, Peterlin BM. Transcriptional interference antagonizes proviral gene expression to promote HIV latency. Cell Host Microbe. 2008; 4:123–133. [PubMed: 18692772]
35. Kao SY, Calman AF, Luciw PA, Peterlin BM. Anti-termination of transcription within the long terminal repeat of HIV-1 by *tat* gene product. Nature. 1987; 330:489–493. [PubMed: 2825027]
36. Adams M, Sharmeen L, Kimpton J, Romeo JM, Garcia JV, Peterlin BM, Groudine M, Emerman M. Cellular latency in human immunodeficiency virus-infected individuals with high CD4 levels can be detected by the presence of promoter-proximal transcripts. Proc Natl Acad Sci USA. 1994; 91:3862–3866. [PubMed: 8171003]
37. Emiliani S, Van Lint C, Fischle W, Paras P Jr, Ott M, Brady J, Verdin E. A point mutation in the HIV-1 Tat responsive element is associated with postintegration latency. Proc Natl Acad Sci USA. 1996; 93:6377–6381. [PubMed: 8692823]
38. Emiliani S, Fischle W, Ott M, Van Lint C, Amella CA, Verdin E. Mutations in the *tat* gene are responsible for human immunodeficiency virus type 1 postintegration latency in the U1 cell line. J Virol. 1998; 72:1666–1670. [PubMed: 9445075]
39. Sonza S, Mutimer HP, O'Brien K, Ellery P, Howard JL, Axelrod JH, Deacon NJ, Crowe SM, Purcell DFJ. Selectively reduced *tat* mRNA heralds the decline in productive human immunodeficiency virus type 1 infection in monocyte-derived macrophages. J Virol. 2002; 76:12611–12621. [PubMed: 12438587]
40. Lin X, Irwin D, Kanazawa S, Huang L, Romeo J, Yen TSB, Peterlin BM. Transcriptional profiles of latent human immunodeficiency virus in infected individuals: Effects of Tat on the host and reservoir. J Virol. 2003; 77:8227–8236. [PubMed: 12857891]
41. Yukl S, Pillai S, Li P, Chang K, Pasutti W, Ahlgren C, Havlir D, Strain M, Günthard H, Richman D, Rice AP, Daar E, Little S, Wong JK. Latently-infected CD4+ T cells are enriched for HIV-1 Tat variants with impaired transactivation activity. Virology. 2009; 387:98–108. [PubMed: 19268337]
42. Adams M, Wong C, Wang D, Romeo J. Limitation of Tat-associated transcriptional processivity in HIV-infected PBMC. Virology. 1999; 257:397–405. [PubMed: 10329550]
43. Core LJ, Lis JT. Transcription regulation through promoter-proximal pausing of RNA polymerase II. Science. 2008; 319:1791–1792. [PubMed: 18369138]
44. Kaiser P, Joshi SK, Kim P, Li P, Liu H, Rice AP, Wong JK, Yukl SA. Assays for precise quantification of total (including short) and elongated HIV-1 transcripts. J Virol Methods. 2016; 242:1–8. [PubMed: 28034670]

45. Landry S, Halin M, Lefort S, Audet B, Vaquero C, Mesnard J-M, Barbeau B. Detection, characterization and regulation of antisense transcripts in HIV-1. Retrovirology. 2007; 4:71. [PubMed: 17910760]

46. Pomerantz RJ, Seshamma T, Trono D. Efficient replication of human immunodeficiency virus type 1 requires a threshold level of Rev: Potential implications for latency. J Virol. 1992; 66:1809–1813. [PubMed: 1738210]

47. Lassen KG, Ramyar KX, Bailey JR, Zhou Y, Siliciano RF. Nuclear retention of multiply spliced HIV-1 RNA in resting $CD4^+$ T cells. PLOS Pathog. 2006; 2:e68. [PubMed: 16839202]

48. Omoto S, Ito M, Tsutsumi Y, Ichikawa Y, Okuyama H, Brisibe EA, Saksena NK, Fujii YR. HIV-1 nef suppression by virally encoded microRNA. Retrovirology. 2004; 1:44. [PubMed: 15601474]

49. Bennasser Y, Le SY, Benkirane M, Jeang KT. Evidence that HIV-1 encodes an siRNA and a suppressor of RNA silencing. Immunity. 2005; 22:607–619. [PubMed: 15894278]

50. Weinberg MS, Morris KV. Are viral-encoded microRNAs mediating latent HIV-1 infection? DNA Cell Biol. 2006; 25:223–231. [PubMed: 16629595]

51. Huang J, Wang F, Argyris E, Chen K, Liang Z, Tian H, Huang W, Squires K, Verlinghieri G, Zhang H. Cellular microRNAs contribute to HIV-1 latency in resting primary $CD4^+$ T lymphocytes. Nat Med. 2007; 13:1241–1247. [PubMed: 17906637]

52. Bullen CK, Laird GM, Durand CM, Siliciano JD, Siliciano RF. New ex vivo approaches distinguish effective and ineffective single agents for reversing HIV-1 latency in vivo. Nat Med. 2014; 20:425–429. [PubMed: 24658076]

53. Guhaniyogi J, Brewer G. Regulation of mRNA stability in mammalian cells. Gene. 2001; 265:11–23. [PubMed: 11255003]

54. Fischer M, Joos B, Hirschel B, Bleiber G, Weber R, Günthard HF. Swiss HIV Cohort Study. Cellular viral rebound after cessation of potent antiretroviral therapy predicted by levels of multiply spliced HIV-1 RNA encoding *nef*. J Infect Dis. 2004; 190:1979–1988. [PubMed: 15529263]

55. Ho YC, Shan L, Hosmane NN, Wang J, Laskey SB, Rosenbloom DI, Lai J, Blankson JN, Siliciano JD, Siliciano RF. Replication-competent noninduced proviruses in the latent reservoir increase barrier to HIV-1 cure. Cell. 2013; 155:540–551. [PubMed: 24243014]

56. Bruner KM, Murray AJ, Pollack RA, Soliman MG, Laskey SB, Capoferri AA, Lai J, Strain MC, Lada SM, Hoh R, Ho YC, Richman DD, Deeks SG, Siliciano JD, Siliciano RF. Defective proviruses rapidly accumulate during acute HIV-1 infection. Nat Med. 2016; 22:1043–1049. [PubMed: 27500724]

57. Hughes SH, Coffin JM. What integration sites tell us about HIV persistence. Cell Host Microbe. 2016; 19:588–598. [PubMed: 27173927]

58. Valente ST, Gilmartin GM, Venkatarama K, Arriagada G, Goff SP. HIV-1 mRNA 3′ end processing is distinctively regulated by eIF3f, CDK11, and splice factor 9G8. Mol Cell. 2009; 36:279–289. [PubMed: 19854136]

59. Pak V, Eifler TT, Jäger S, Krogan NJ, Fujinaga K, Peterlin BM. CDK11 in TREX/THOC regulates HIV mRNA 3′ end processing. Cell Host Microbe. 2015; 18:560–570. [PubMed: 26567509]

60. Mouland AJ, Coady M, Yao XJ, Cohen ÉA. Hypophosphorylation of poly(A) polymerase and increased polyadenylation activity are associated with human immunodeficiency virus type 1 Vpr expression. Virology. 2002; 292:321–330. [PubMed: 11878934]

61. Fischer M, Wong JK, Russenberger D, Joos B, Opravil M, Hirschel B, Trkola A, Kuster H, Weber R, Günthard HF. Swiss HIV Cohort Study. Residual cell-associated unspliced HIV-1 RNA in peripheral blood of patients on potent antiretroviral therapy represents intracellular transcripts. Antivir Ther. 2002; 7:91–103. [PubMed: 12212929]

62. Fischer M, Joos B, Niederöst B, Kaiser P, Hafner R, von Wyl V, Ackermann M, Weber R, Günthard HF. Biphasic decay kinetics suggest progressive slowing in turnover of latently HIV-1 infected cells during antiretroviral therapy. Retrovirology. 2008; 5:107. [PubMed: 19036147]

63. Kula A, Gharu L, Marcello A. HIV-1 pre-mRNA commitment to Rev mediated export through PSF and Matrin 3. Virology. 2013; 435:329–340. [PubMed: 23158102]

64. Spivak AM, Andrade A, Eisele E, Hoh R, Bacchetti P, Bumpus NN, Emad F, Buckheit R III, McCance-Katz EF, Lai J, Kennedy M, Chander G, Siliciano RF, Siliciano JD, Deeks SG. A pilot

study assessing the safety and latency-reversing activity of disulfiram in HIV-1infected adults on antiretroviral therapy. Clin Infect Dis. 2014; 58:883–890. [PubMed: 24336828]

65. Archin NM, Bateson R, Tripathy MK, Crooks AM, Yang KH, Dahl NP, Kearney MF, Anderson EM, Coffin JM, Strain MC, Richman DD, Robertson KR, Kashuba AD, Bosch RJ, Hazuda DJ, Kuruc JD, Eron JJ, Margolis DM. HIV-1 expression within resting $CD4^+$ T cells after multiple doses of vorinostat. J Infect Dis. 2014; 210:728–735. [PubMed: 24620025]
66. Elliott JH, Wightman F, Solomon A, Ghneim K, Ahlers J, Cameron MJ, Smith MZ, Spelman T, McMahon J, Velayudham P, Brown G, Roney J, Watson J, Prince MH, Hoy JF, Chomont N, Fromentin R, Procopio FA, Zeidan J, Palmer S, Odevall L, Johnstone RW, Martin BP, Sinclair E, Deeks SG, Hazuda DJ, Cameron PU, Sékaly RP, Lewin SR. Activation of HIV transcription with short-course vorinostat in HIV-infected patients on suppressive antiretroviral therapy. PLOS Pathog. 2014; 10:e1004473. [PubMed: 25393648]
67. Rasmussen TA, Tolstrup M, Brinkmann CR, Olesen R, Erikstrup C, Solomon A, Winckelmann A, Palmer S, Dinarello C, Buzon M, Lichterfeld M, Lewin SR, Østergaard L, Søgaard OS. Panobinostat, a histone deacetylase inhibitor, for latent-virus reactivation in HIV-infected patients on suppressive antiretroviral therapy: A phase 1/2, single group, clinical trial. Lancet HIV. 2014; 1:e13–e21. [PubMed: 26423811]
68. Elliott JH, McMahon JH, Chang CC, Lee SA, Hartogensis W, Bumpus N, Savic R, Roney J, Hoh R, Solomon A, Piatak M, Gorelick RJ, Lifson J, Bacchetti P, Deeks SG, Lewin SR. Short-term administration of disulfiram for reversal of latent HIV infection: A phase 2 dose-escalation study. Lancet HIV. 2015; 2:e520–e529. [PubMed: 26614966]
69. Sogaard OS, Graversen ME, Leth S, Olesen R, Brinkmann CR, Nissen SK, Kjaer AS, Schleimann MH, Denton PW, Hey-Cunningham WJ, Koelsch KK, Pantaleo G, Krogsgaard K, Sommerfelt M, Fromentin R, Chomont N, Rasmussen TA, Østergaard L, Tolstrup M. The depsipeptide romidepsin reverses HIV-1 latency in vivo. PLOS Pathog. 2015; 11:e1005142. [PubMed: 26379282]
70. Derdeyn CA, Kilby JM, Miralles GD, Li LF, Sfakianos G, Saag MS, Hockett RD, Bucy RP. Evaluation of distinct blood lymphocyte populations in human immunodeficiency virus type 1—Infected subjects in the absence or presence of effective therapy. J Infect Dis. 1999; 180:1851–1862. [PubMed: 10558941]
71. Fischer M, Huber W, Kallivroussis A, Ott P, Opravil M, Lüthy R, Weber R, Cone RW. Highly sensitive methods for quantitation of human immunodeficiency virus type 1 RNA from plasma, cells, and tissues. J Clin Microbiol. 1999; 37:1260–1264. [PubMed: 10203467]
72. Mane VP, Heuer MA, Hillyer P, Navarro MB, Rabin RL. Systematic method for determining an ideal housekeeping gene for real-time PCR analysis. J Biomol Tech. 2008; 19:342–347. [PubMed: 19183798]
73. Ledderose C, Heyn J, Limbeck E, Kreth S. Selection of reliable reference genes for quantitative real-time PCR in human T cells and neutrophils. BMC Res Notes. 2011; 4:427. [PubMed: 22011438]
74. Hu Y, Smyth GK. ELDA: Extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. J Immunol Methods. 2009; 347:70–78. [PubMed: 19567251]
75. Yukl SA, Kaiser P, Kim P, Li P, Wong JK. Advantages of using the QIAshredder instead of restriction digestion to prepare DNA for droplet digital PCR. Biotechniques. 2014; 56:194–196. [PubMed: 24724845]
76. Strain MC, Lada SM, Luong T, Rought SE, Gianella S, Terry VH, Spina CA, Woelk CH, Richman DD. Highly precise measurement of HIV DNA by droplet digital PCR. PLOS ONE. 2013; 8:e55943. [PubMed: 23573183]

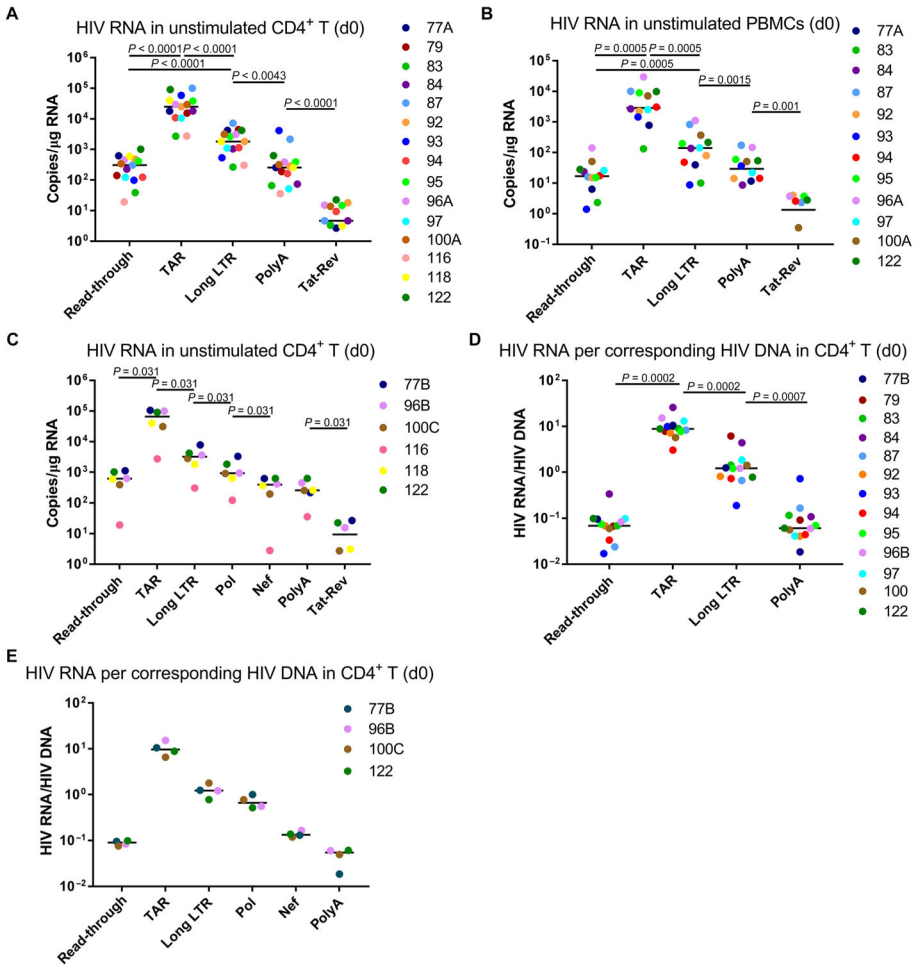

Fig. 1. HIV transcription profiles in freshly isolated CD4$^+$ T cells and PBMCs from HIV-infected patients on ART suggest blocks to HIV transcriptional elongation, completion, and multiple splicing

(**A** to **C**) Total RNA from unstimulated [day 0 (d0)] CD4$^+$ T cells (A and C) and PBMCs (B) was used for a polyadenylation-RT-ddPCR assay for the TAR loop (found in all HIV transcripts) and RT-ddPCR assays for HIV sequence regions suggesting transcriptional interference (read-through transcripts), transcriptional elongation (long LTR), completion of transcription (polyA), and multiple splicing (Tat-Rev). Each transcript was normalized to 1 µg of cellular RNA (~10^6 cells) and plotted on a log scale. (C) To determine whether there are blocks to more distal elongation beyond the long LTR region, additional assays were developed for HIV Pol and Nef, and all seven transcripts were measured in six HIV-infected patients on ART. (**D** and **E**). To evaluate how deletions or hypermutations in the provirus could affect the measured HIV RNA quantities, HIV DNA was measured using the same ddPCR assays (except Tat-Rev and polyA, which are RNA-specific), and each HIV RNA was normalized to the corresponding HIV DNA (polyA was normalized to the read-through assay, which uses the same forward primer/probe). Bars indicate the median. Comparisons between transcripts were performed using the Wilcoxon signed-rank test.

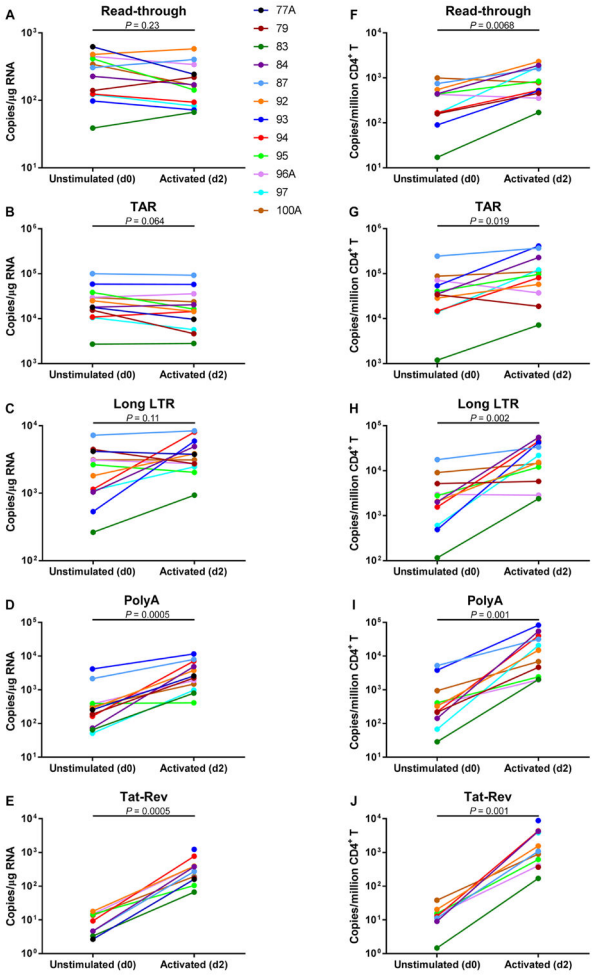

Fig. 2. Activation of freshly isolated CD4$^+$ T cells from HIV-infected patients on ART results in successive increases in elongated, polyadenylated, and multiply spliced HIV transcripts

(**A** to **J**) Using RT-ddPCR, read-through (A and F), total (B and G), elongated (C and H), polyadenylated (D and I), and multiply spliced Tat-Rev (E and J) HIV transcripts were measured in unstimulated CD4$^+$ T cells (day 0) and ex vivo activated CD4$^+$ T cells (day 2) from 12 ART-suppressed individuals. (A to E) Effect of activation, as normalized by RNA mass. Measures of each HIV transcript were normalized to 1 μg of cellular RNA (~10^6 unstimulated cells) to show how activation changes amounts of each HIV transcript in relation to global cellular transcription. (F to J) Effect of activation, as normalized by DNA mass. HIV RNA measures in the unstimulated and activated cells were also normalized to 10^6 cells using the cell equivalents recovered in the extracted DNA (a surrogate for cell numbers in the activated cells; available for 11 of 12 participants). Each graph shows the change in HIV RNA from day 0 (unstimulated) to day 2 (activated) on a log scale; each color represents a separate individual; comparisons were performed using the Wilcoxon signed-rank test.

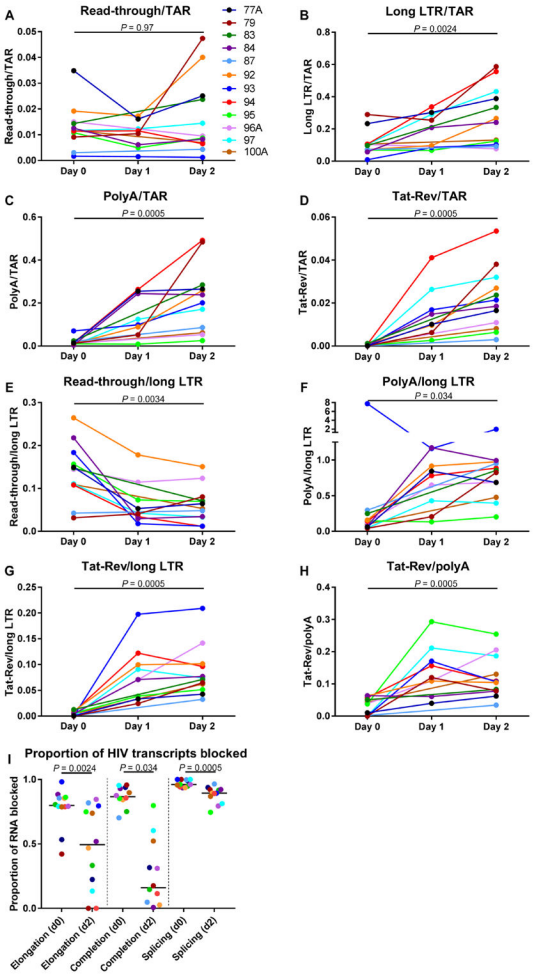

Fig. 3. Activation of freshly isolated CD4$^+$ T cells from HIV-infected patients on ART selectively reverses baseline blocks to splicing, elongation, and completion

The effects of activation were also measured using the ratio of one HIV transcript to another, which is independent of normalization to cell numbers. (**A** to **D**) Ratios to total (TAR) transcripts. HIV transcript measures in the unstimulated (day 0) and activated (days 1 and 2) CD4$^+$ T cells were normalized to TAR to express the proportion of all HIV transcripts that are read-through (A), elongated (B), polyadenylated (C), and multiply spliced Tat-Rev (D) transcripts. (**E** to **G**) Ratios to long LTR. HIV transcript measures were normalized to long LTR to express the proportion of elongated HIV transcripts that are read-through (E), polyadenylated (F), and multiply spliced Tat-Rev (G) transcripts. (**H**) Ratio to polyadenylated. The ratio of Tat-Rev to polyA was used to express the proportion of completed transcripts that get multiply spliced to produce two-exon Tat-Rev. (**I**) Proportion of HIV transcripts blocked at the stages of transcriptional elongation, completion, and multiple splicing in unstimulated (day 0) and activated (day 2) CD4$^+$ T cells. Each color represents a separate individual; comparisons were performed using the Wilcoxon signed-rank test.

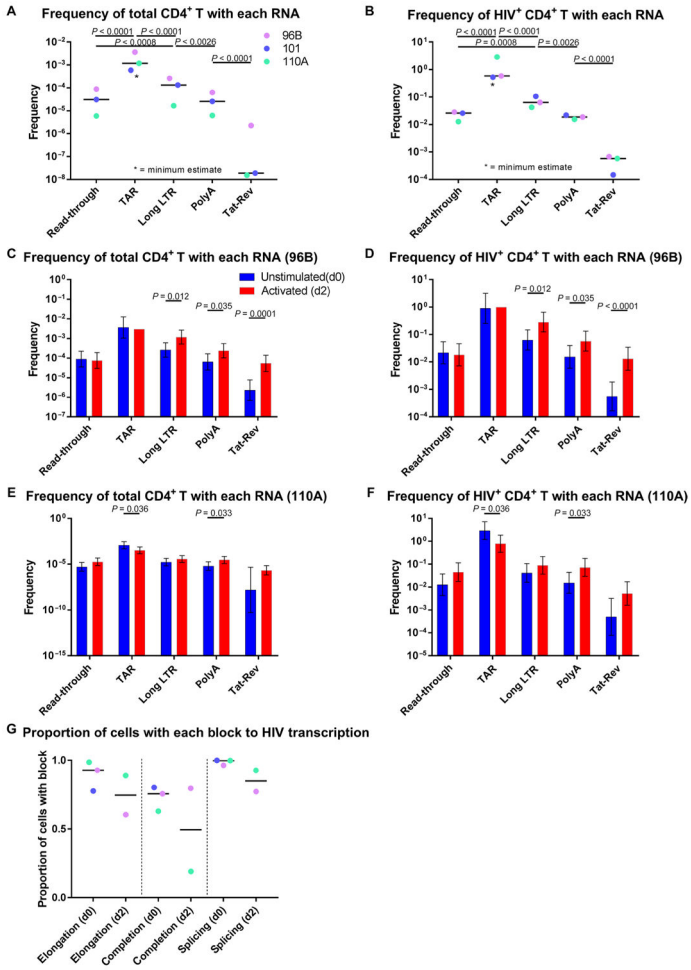

Fig. 4. The frequency of CD4$^+$ T cells containing each HIV transcript reflects the levels of each HIV transcript in the bulk cell population

Unstimulated (day 0) CD4$^+$ T cells from three ART-suppressed HIV-infected individuals were counted and subjected to serial, replicate (≤6) fivefold cell dilutions. For two of these participants, another aliquot of cells was activated for 2 days with anti-CD3/CD28 antibody-coated beads (antiretroviral drugs were used to prevent new infection) and then serially diluted. Read-through, TAR, long LTR, polyA, and Tat-Rev transcripts were measured in each dilution and replicate. The number of HIV-infected cells in each replicate was inferred by measuring HIV DNA in the bulk cells. (**A** and **B**) The frequencies of unstimulated total CD4$^+$ T cells (A) and HIV-infected CD4$^+$ T cells (B) containing each HIV transcript were calculated using the proportion of positive replicates at each cell number using the method of extreme limiting dilution analysis (74). Bars indicate the median. (**C** to **F**) Effect of activation on the frequency of total CD4$^+$ T cells (C and E) and HIV-infected CD4$^+$ T cells (D and F) containing each transcript. Blue columns, unstimulated (day 0); red columns, activated (day 2). Bars represent 5 to 95% confidence intervals. *P* values were calculated using the website for extreme limiting dilution analysis (74). (**G**) Proportion of cells in which HIV transcription was blocked at the stages of elongation, completion, and splicing (of cells with TAR, long LTR, and polyA, respectively). Bars indicate the median.

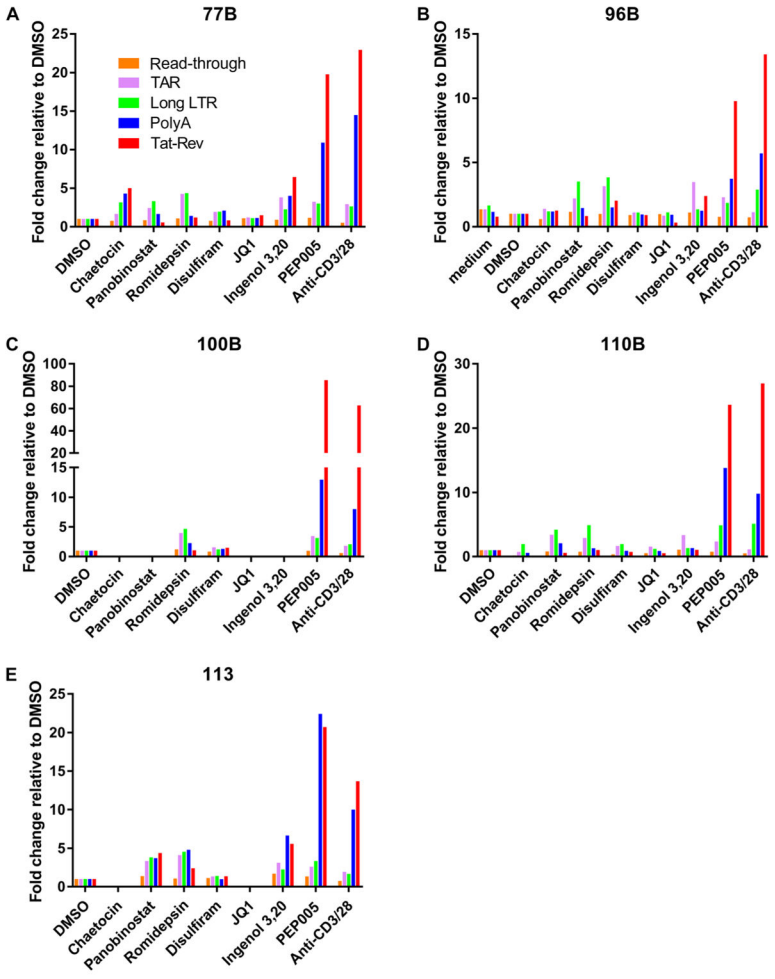

Fig. 5. Putative latency-reversing agents exert differential effects on the different blocks to HIV transcription

(**A** to **E**) Total $CD4^+$ T cells from five ART-suppressed HIV-infected individuals (A to E) were divided into aliquots of 5×10^6 cells and cultured for 24 hours with dimethyl sulfoxide (DMSO) (negative control), different latency-reversing agents, or anti-CD3/ CD28 antibody-coated beads. Latency-reversing agents were used at concentrations that can be obtained in the plasma or those used in previous ex vivo studies. After 24 hours, cells were harvested from each well and used to measure read-through (orange), total (pink), elongated (green), polyadenylated (blue), and multiply spliced Tat-Rev (red) HIV transcripts using RT-ddPCR. Amounts of each transcript were divided by those in the negative control (DMSO) well to show the fold change (*y* axis) in each transcript (different colors) for each latency-reversing agent or control on the *x* axis. Ingenol 3,20, ingenol 3,20- dibenzoate; PEP005, ingenol mebutate (ingenol-3-angelate).