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Regulation of expression of unintegrated and integrated HIV-1 DNA: keeping the wolves at bay

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The unintegrated HIV-1 DNAs formed by reverse transcription in the early hours after infection are subject to profound transcriptional silencing. The repression of expression of foreign DNA, as an aspect of the innate immune system, serves to restrict the activity of many invading pathogens. Newly formed retroviral DNAs are rapidly loaded with histones upon entry into the nucleus, and the repression of their expression is mediated by an array of host proteins that introduce histone modifications characteristic of heterochromatin, including histone methylation and histone deacetylation. Knockout or knockdown of expression or inhibition of these host factors can relieve the silencing, allowing for viral gene expression even in settings where HIV-1 DNA integration is blocked. When viral DNA integration is allowed, forming the integrated provirus, the silencing in most cases is dramatically relieved, leading to high levels of expression and formation of progeny virus. In some settings and cell types, silencing of the integrated DNA is maintained, or re-established, such that the infected cells retain a silent copy of the viral DNA without production of progeny virus. The basis for the typical switch from silent DNA to actively expressed DNA upon integration is not yet fully clear. This review will summarize the current understanding of the regulation of expression of unintegrated HIV-1 DNAs and the nature of the chromatin that is formed on the viral DNA, and will especially focus on the host machinery that establishes repressive heterochromatin-like structures on the unintegrated DNA. The activation of expression that normally occurs upon integration, and the special circumstances when viral DNA expression is not activated, will also be discussed. These cases can result in the formation of populations of infected cells carrying silent proviruses, which persist for decades in infected individuals in spite of antiviral therapy. This pool of latently infected cells can be stochastically reactivated to give rise to spreading virus whenever antiviral drugs are withdrawn, and constitute the barrier to a true "cure" of AIDS. The hope is that a deeper understanding of the regulation of expression of viral DNAs will lead to new means to prevent or control viremia and disease.

KEYWORDS

unintegrated DNA, HIV-1, transcriptional silencing, host factors, histone modifications

Introduction

One of the major strategies cells utilize to suppress virus infection – perhaps the most obvious to imagine – is to inhibit transcription of the viral genes. There are many approaches to achieve this inhibition. A very draconian approach is to shut off all transcription in the cell, leading to cell arrest or even death. But more targeted inhibition can also be used. Cells can inactivate or destroy viral RNA transcriptases, viral transcription factors, or components of RNA processing machinery involved in RNA capping, splicing, or nuclear export. But many host mechanisms for inhibiting viral gene expression target the viral DNA itself, rendering the viral DNA or the chromatin associated with the viral DNA inaccessible to transcription. These systems often act by targeting histones associated with the viral DNA, making covalent histone modifications that mark the chromatin for silencing, or condensing the DNA into heterochromatin-like structures that are poorly transcribed. This strategy is utilized to silence incoming DNAs and serves as an aspect of innate immunity, preventing infection by many pathogens, including DNA viruses, hepatitis B virus, and retroviruses such as HIV-1. In response, many of these viruses have evolved countermeasures that act to reverse the host defenses and allow for successful viral gene expression. In this review, we will discuss the host machinery involved in the silencing of incoming HIV-1 DNAs, focusing especially on the silencing of unintegrated DNA, and the dramatic change in regulation of transcription that occurs after DNA integration to form the provirus. We will also comment on the rare cases of proviruses that remain silent after integration, representing the reservoir of persistent DNA forms that can reactivate to reinitiate virus expression. These silent DNA copies are the major barrier to a true cure to AIDS.

Silencing of unintegrated retroviral DNAs

Retroviral infection begins with the reverse transcription of the single-stranded RNA genome to form a linear double-stranded DNA. This is a complex process, involving the synthesis of a minus-strand DNA, followed by the synthesis of a plus-strand DNA, coupled to the degradation of the initial RNA template (for a detailed description, see (1)). The linear DNA product includes two copies of the co-called Long Terminal Repeats (LTRs), sequence blocks which contain signals for forward transcription, and with short inverted repeats at their very termini. Upon entry into the nucleus, the linear DNA is integrated into the host genome by the viral integrase enzyme to form the provirus, a permanent component of the cell genome. The proviral DNA is retained through cell division and constitutes a new genetic element that can be transcribed by host RNA polymerase II to give rise to viral mRNAs, proteins, and virion particles. When infection occurs in germline cells, the provirus can become transmitted to progeny and so create a permanent mark of that phylogenetic lineage.

The process of reverse transcription is initiated soon after entry into the cytoplasm, and for many years was thought to be completed in the cytoplasm. This notion was based on the observation that avian retroviruses could carry out reverse transcription even in enucleated cells (2), and that murine leukemia viruses could similarly synthesize viral DNA in nondividing cells, where entry into the nucleus was blocked (3). But recently this dogma has been upended. Studies with HIV-1 have demonstrated that reverse transcription continues after nuclear entry, is completed in the nucleus, and indeed may be fully completed only very soon before integration occurs (4, 5).

Integration is not a highly efficient process, and a significant portion of the incoming linear DNA does not successfully integrate in wild-type infections. While the efficiency of integration is likely to vary with cell type, setting, and multiplicity of infection, various experiments suggest that only about 10-30% of the total viral DNA in cells infected *in vitro* is integrated to form proviral DNA (6–8). Whenever integration does not occur, a portion of the linear DNA gives rise to two circular DNA forms: one-LTR circles formed by homologous recombination between the LTRs, and two-LTR circles formed by non-homologous end joining (9, 10). The integration reaction can be specifically blocked in several ways: cells can be treated with potent inhibitors of the integrase enzyme such as raltegravir; or infection can be performed with viral mutants carrying point mutations in the catalytic site of the integrase. Blocking integration, either pharmacologically or by mutation, increases the levels of the circular forms. The circular DNAs do not contain origins of DNA synthesis, and do not replicate. They can persist for a period of time, but eventually disappear with dilution as the cells grow and divide. The 2-LTR circles can be quite stable in some settings, with a long lifetime in nondividing cells (11–13).

The ability of the unintegrated retroviral DNAs to serve as templates for transcription by the host RNA polymerase has been studied for many years. The murine leukemia virus DNAs were found to be very poorly expressed in the absence of integration (14). Analysis of various integration-defective HIV-1 mutants revealed similarly low expression compared to integration-competent virus (15–17). The level of expression varied considerably across cell lines (18). Some cell lines showed only modest levels of silencing of unintegrated HIV-1 DNA relative to integrated DNA, while some lymphoid cell lines showed dramatic levels of silencing (19). The silencing of incoming DNA is not limited to retroviral DNAs, but is a common response to many viral DNAs (20) and even transfected DNAs (21, 22), and is an aspect of innate immunity to defend against pathogens.

What factors might be mediating the silencing? An important clue to the mechanism of this silencing was the observation that histone deacetylase inhibitors such as trichostatin A dramatically relieved this silencing and enhanced expression from unintegrated DNAs, including expression of transgenes delivered by integrase-defective vectors (23–25), suggesting that histone modifications might be involved. Analysis of the composition of the DNAs present in the PreIntegration Complexes (PICs) by Chromatin ImmunoPrecipitation (ChIP) revealed that the DNAs of MLV (26) and HIV-1 (27, 28) were rapidly loaded with histones upon nuclear entry. Both the linear and circular forms were found to be loaded with histones before integration, and this occurred even

when integration was blocked pharmacologically or by mutation. The histones soon acquired covalent marks of silent DNAs, including low levels of acetylation and high levels of H3 trimethylation on lysine 9 (H3K9me3). Analysis of the DNA by micrococcal nuclease protection assays suggested that the histones were present in the form of nucleosomes, and were well phased and positioned relative to the viral promoter sequences in the LTR (28). These findings suggested that nucleosomes are positioned on the unintegrated DNA relatively early, and that the positions are roughly maintained after integration. It should be noted that the loading is unusual in at least one way: it is occurring on naked DNA that has not previously seen nucleosomes, unlike the usual situation occurring at replication forks where nucleosomes are placed onto both leading and lagging strands of the newly copied DNA. Notably, the DNAs of the PICs contained high levels of the noncanonical or variant histone H3.3 (27), which are often associated with promoter regions of both active and inactive genes (29). H3.3 is typically loaded by the distinctive chaperone complexes HIRA and DAXX, and can be deposited on DNA in a replication-independent manner, consistent with the setting of the PICs. The so-called "linker histones" H1a/b were also present (27), suggesting that the viral DNA was condensed into heterochromatin-like structures. All these findings suggest that the incoming HIV-1 DNA is organized into condensed chromatin that is poorly accessible to RNA polymerase II.

Host machinery involved in the silencing of unintegrated HIV-1 DNA

What host machinery is responsible for the silencing of unintegrated DNAs, and for the histone loading and marking? No KD screens have yet found circumstances that completely prevented the loading of histones on PICs *per se*, suggesting that there may be redundant complexes capable of loading histones. Different cell types may contain a distinctive set of complexes performing the loading. But a number of factors involved in chromatin organization have been identified that play roles in silencing (30). The histone methyl transferase SETDB1/ESET is involved, introducing H3K9 trimethyl marks. A variety of histone deacetylases, including HDAC1 and 4, are likely to be involved. Knockout of a large DNA binding protein, NP220, was found to substantially relieve the silencing of HIV-1 DNA (30). This protein recognizes short polypyrimidine sequences in the LTR and may tether additional factors to the DNA to mediate the silencing. While NP220 interacted with the so-called HUSH complex (31) to silence MLV, knock down of HUSH had little effect on the silencing of unintegrated HIV-1 DNA (30, 32), indicating that other factors may be recruited by NP220 in the case of HIV-1.

A scan of histone chaperones in HeLa cells suggested that two chromatin modifiers, CHAF1A and CHA1B, play a major role in silencing (33). KD of either of these two factors led to a substantial loss of silencing of unintegrated HIV-1 DNA. Perhaps these factors help position the nucleosomes, or otherwise organize the nucleosomes into a heterochromatin-like state that is poorly transcribed. Although the CHAF1A/B factors are known to act in some settings as subunits of the canonical CAF-1 complex, their activity on HIV-1 DNA was independent of the RBBP4 subunit of that complex. It was further interesting that KD of these two factors had no impact on silencing of unintegrated DNA of MLV, indicating that distinct arrays of factors are active on distinctive retrovirus genomes.

Genome-wide screens have identified additional factors that are important for silencing. Such screens have discovered that SLF2, a factor involved in the localization of the SMC5/6 complex (for Structural Maintenance of Chromosomes), is important for silencing (32, 34). SMC5/6 is a loop-extruding motor complex, and is essential for the maintenance of cellular DNA repeat regions (35, 36). Knock down of these factors resulted in a modest but significant relief of the silencing of unintegrated HIV-1 DNA. Several other known players in DNA repair were not found to be needed in silencing the viral DNAs, suggesting that SMC5/6 served a special role in this activity. SMC5/6 proteins were found to use a surprising enzymatic activity to induce silencing: they mediated the SUMOylation of unintegrated chromatinized HIV-1 DNA, and inhibiting SUMOylation by mutation of an E3 SUMO ligase, or pharmacologically, relieved silencing (37). The target and consequences of the SUMOylation remain to be determined. One possible mechanism of action of the SMC5/6 complex is to mediate the localization of the viral DNA to SUMO-rich nuclear condensates, such as PML bodies.

Yet another screen for silencing factors, by siRNA knockdown, identified a number of factors including PolE3, which together with PolE4, forms the POLE holoenzyme functioning as a histone H3 and H4 chaperone (38). PolE3 was found to help maintain unintegrated HIV-1DNA in a repressive chromatin state (39). Depletion of PolE3 enhanced early virus expression, but it actually reduced the overall efficiency of virus replication, suggesting that premature expression of the viral genome could prevent proper establishment of proviruses, or even be toxic to cells. In this context, the silencing of unintegrated DNA may enhance the recovery of cells acquiring silent integrated proviruses, and thus the establishment of the long-lived reservoir of latently infected cells.

Yet another genome-wide CRISPR-Cas9 knock-out screen has very recently identified an essential role of the PTEN gene (for phosphatase and tensin homolog) in the silencing of unintegrated HIV-1 DNA (40). PTEN's phosphatase activity negatively regulates the PI3K-Akt pathway which otherwise would promote transcription from unintegrated HIV-1 DNA. The knockout of PTEN, or the inhibition of PTEN's phosphatase activity by point mutagenesis, increased Akt phosphorylation, activated its kinase activity, and thereby enhanced transcription from unintegrated HIV-1 DNA. The activated Akt kinase utilized several transcriptional factors (NF-κB, Sp1, and AP-1) to promote viral expression.

It is very likely that more host factors involved in silencing are yet to be found. The presence of H3.3 histones offers some possibilities; in particular, H3.3 is responsive to distinctive "readers", including ZMYND11, which recognizes the H3.3K36me3 mark to suppress both transcription and RNA splicing (41).

While the unintegrated HIV-1 DNAs are often silent, it is also true that in many cell lines and settings, there is substantial

expression (42–44). Some nondividing cells were especially capable of expression from unintegrated DNA (45, 46). An important observation is that this early viral expression is strongly promoted by Vpr, a virion-associated accessory protein (17, 47, 48). Vpr is an enigmatic protein with many functions. It promotes the nuclear import of the preintegration complex (PIC); and it induces a strong G2 cell cycle arrest. At least some of the activities involve its utilization of the Cul4A/DDB1 complex to induce ubiquitinylation and degradation of a number of target proteins, including CCDC137 (49). The identity of the targets most important for unintegrated DNA expression, however, are not completely clear. They may include SLF2, the tethering factor for SMC5/6 (32). The magnitude of the effects of Vpr are large, and though the level of expression may not be as high as from an integrated provirus, it can be sufficient to even allow for the completion of a full replication cycle of integrase-defective viruses (18, 50). This expression from nonintegrating lentiviral vectors is sufficiently high that such vectors have been promoted for use in gene therapy applications, avoiding the potentially oncogenic consequences of insertional activation caused by provirus formation (51–53). Such expression, however, would last only as long as the lifetime of the unintegrated DNA.

A very surprising factor that activates HIV-1 expression from unintegrated DNAs is the HTLV-1 Tax protein. Tax is the transactivator protein for the HTLV-1 virus, which recruits the transcription factors CREB and CPB/p300 to viral CREB-response elements (vCRE) located in the U3 region of the 5′ LTR (54) as well as the general transcription factors TFIIA and TFIID. Cells infected by HTLV-1 and expressing Tax were found to support expression of unintegrated HIV-1 DNA (55, 56). Presumably Tax activation of these various transcription factors is sufficient to overcome the normal repression of expression of the HIV-1 DNAs. While Tax is not likely to be functioning very often in the case of HIV-1 infection in patients, it does indicate that there can exist activated cell states that permit early expression. Rare cells that are activated by nonviral means may be especially susceptible to HIV-1 infection by this route.

Is the silencing of unintegrated DNA significant in any way to inhibit virus replication in humans? The importance is likely to be limited to a short window of time, because upon integration of the viral DNA, the block to transcription is largely relieved (see below). Tests of the course of MLV infection in mouse cell lines after KO of the silencing machinery did reveal a noticeable increase in the rate of virus spreading (30), and though the increase was modest, this could be important in the setting of virus transmission from patient to patient. HIV-transmission is very inefficient, and is typically due to a single virus termed the transmitted/founder virus. This suggests that there is a tight bottleneck at this early stage (57), so even small increases in virus infectivity could be very significant. It is unclear what characteristics of the virus are most important to pass through this bottleneck (though it is known that almost all transmitted virus utilize the CCR5 coreceptor). Some studies suggest that the transmitted/founder viruses are especially resistant to inhibition by type 1 interferon (IFN-a) (58), though other studies did not detect a similar resistance to IFN in the transmitted viruses of particular clades (59). It has been pointed out that even minor differences in replication efficiency could promote transmission through the bottleneck (60). The incoming Vpr protein, as noted above, could be one means for the virus to inactivate the silencing machinery. Whatever feature of the virus is most heavily responsible for relieving the silencing, early expression from the unintegrated DNA could increase the efficiency of successful provirus formation, or the establishment of a spreading infection. If expression were high enough, it could even induce the early formation of new virion particles before the later high-level of virion formation from the integrated provirus.

Could the silencing of expression early in infection actually be of any benefit to the virus? This is not clear, but it is possible that it helps the virus evade detection by the innate immune system early in infection. HIV-1 typically achieves entry, reverse transcription, and integration of the viral DNA, without any dramatic activation of the innate immune responses (61). In part this is due to the sequestration of the viral RNA and DNA within the virion particle, preventing its recognition by host factors such as MDA-5 or RIG-I. Mutations in the viral capsid that destabilize the particles can allow for activation of innate immunity and inhibition of infection (62, 63). High expression from unintegrated DNA might similarly trigger antiviral responses, and the lack of such expression could therefore promote the evasion of detection and successful progression through the early stages of infection. Once the establishment of the provirus in a permissive cell has occurred, the subsequent high-level expression likely will trigger innate immune responses, but at that time it is too late for the cell to fully block infection: the provirus is not readily removed, and expression can follow at any later time to induce new virion release.

Activation of expression upon viral DNA integration

Upon integration of the HIV-1 DNA in permissive cells, there follows a robust transcriptional activation of the provirus from the enhancer and promoter sequences of the viral LTR. This expression is to some extent promoted by the integration site preference of the virus for actively expressed regions of the genome. Transcription is initiated by RNA Polymerase II of the host at the U3-R junction of the LTR, producing 5' capped mRNAs that can either be unspliced or spliced to encode the various viral proteins. Initial transcription typically pauses until binding of the Tat protein to the TAR element near the 5' end of the transcripts. Tat acts to recruit the P-TEFb factor, which directs phosphorylation of the RNA polymerase C-terminal tail to allow elongation down the provirus (64–67) (for recent review see (68)). It is notable that the activation of proviral expression is subject to dramatic positive feedback by virtue of the transactivation by Tat leading to production of more Tat. Thus any early transcription that results in even low levels of expression of the Tat protein will result in dramatic increases in subsequent transcription events. Tat is extremely potent and active at very low concentrations.

How integration of the DNA triggers the dramatic increase in transcription, as compared with the minimal expression of the unintegrated DNA, is not known. The nucleosomes established on the unintegrated DNA are largely retained on the provirus, with the

important exception that the single nucleosome at the transcription start site is evicted (28). There are also minor shifts in the positioning of nearby nucleosomes, and additional nucleosome-free regions appear upon induction of expression (69). Tat binding recruits histone acetyltransferases that modify nucleosomal histones present on the proviral DNA (70). Tat also interacts with chromatin remodelers that may be important in altering nucleosome mobility around the promoter (71, 72). The activation of expression does not seem to require the displacement of histones that occurs during the transit of a replication fork through the provirus, because expression is observed even with infection by lentiviral vectors in arrested cells (73, 74). It is clear that integration is associated with a dramatic switch in the histone marks that are present on the viral nucleosomes (75). While the histones on unintegrated DNAs are marked by low acetylation and high H3K9 trimethylation, the integrated proviral DNA histones show high acetylation and low H3K9methylation. Whether this is a cause or a consequence of the high expression is not completely clear, but enhancing acetylation by HAC inhibitors can profoundly increase proviral expression (76). One hypothesis for the mechanism of the change in chromatin status is the "spreading" of the active chromatin from the host flanking sequences into the inactive chromatin of the previously unintegrated DNA. Chromatin spreading has been described in other settings (77–80), and can be imagined to occur by the recognition of active histone marks on one nucleosome mediating the placing of a similar active mark on an adjacent nucleosome. Thus, a "reader" of an active mark would recruit a "writer" to place an active mark nearby; a protein recognizing an acetylated histone could recruit a histone acetyltransferase to modify another histone. Which machinery might be performing this function in a typically HIV-1-infected T cell is not certain.

The site of integration may play a role in determining the activation of expression, and the level of expression. The integration site profile and the preference for integration into active or open chromatin has been extensively studied and is too large a topic be covered here (for a recent review, see (81)). There is a trend for HIV-1 DNA integration to target expressed genes, uniformly across the transcribed sequences and not exclusively at the transcription start sites (as is the case for MLV). These preferences involve interaction of the viral integrase IN with host factors including the transcription factor LEDGF, an IN cofactor, and probably other transcription factors. The C-terminal sequences of IN, in particular, interact with many host proteins and nucleic acids to modulate sites of integration (82). The integration profile is also affected by the capsid protein CA, which interacts with host factor CPSF6 and controls the depth of progression of the PIC into the nucleus before integration (83). CA retained on the PIC may affect the extent and timing of the activation of expression. Another interesting finding is that the viral IN protein can "jump start" the transition from silence to active expression. A mutant IN engineered to prevent the normal acetylation of the IN C-terminal tail was able to mediate normal integration with a normal integration site selectivity, but exhibited a delay in the transition to active expression (84). The results suggest that the wild-type IN may remain at the site of integration to stimulate transcription, perhaps by recruiting positive transcription factors to the provirus.

The high-level expression of the integrated provirus is driven by DNA sequences in the U3 region of the 5'LTR, which include the binding sites for a number of positive transcription factors (85). The LTRs are highly variable among the various HIV-1 clades and even within a clade. Common features in the core promoter include sites for binding by NFkB, a potent activator of expression; multiple sites recognized by SP1, a ubiquitously expressed zinc-finger protein; and the TATA box bound by TFIID, a large complex consisting of TATA binding protein (TBP) and a number of TBP associated factors (TAF). Upstream of these core elements are an array of enhancer elements that include binding sites for the basic-leucine zipper proteins C/EBP and AP-1, as well as USF, Ets-1, and LEF-1. Some of these factors are T cell specific and contribute to the cell type specificity for vigorous virus replication. The ultimate level of expression of the integrated provirus is thus the result of a complex interplay between the various LTRs of the virus and the array of host factors expressed by the particular infected cell. The presence of the multiplicity of binding sites for positive acting transcription factors gives the virus a wide range of cell types that offer some potential for replication, though the activated T cell is perhaps the most permissive host.

Silent proviruses in latently infected cells

While the majority of proviral DNAs integrate in active euchromatin and are highly expressed, there is always a minority that integrate in heterochromatin or that otherwise remain silent. Some proviruses that are initially transcribed may subsequently go silent. Cells carrying these proviruses collectively will ultimately give rise to the latent pool of infected cells that persist in persons living with HIV-1 (PLWH). The factors that determine the choice between high expression and latency are not fully known, but the data suggest that the decision is largely bimodal – either "on" or "off" (86–88). Those cells that actively produce virus are either killed by the virus directly or recognized by the adaptive immune system and killed by CD8-positive T cells. There is thus a selection over time for cells with silenced proviruses. The few surviving cells that keep the proviral DNA silent are invisible to the immune system, and these cells can have a very long lifetime – often these are memory T cells, but they can include other cell types. When these cells are put in culture and activated, for example by PMA and ionomycin or by stimulation by anti-CD3, viral expression is induced and infectious virus is produced. The ability of the virus to establish latent infections may be highly beneficial to the virus in the long run; it may constitute a successful mechanism of survival, and may have been selected over evolutionary times as a "bet-hedging" strategy (89).

It is not known what special features of these latently infected cells promote the silence of their proviral DNAs. There may be more than one mechanism by which the cells can maintain silence. Some could be due to location of integration, and some experiments suggest that there is an enrichment for proviruses located in heterochromatic chromatin, as in centromeric regions, over long periods of time on antiviral therapy (90, 91). Some mechanisms could be by imposition of unusual repressive histone marks, DNA

marks, or chromatin conformations placed on the proviruses. Methylation of cytosines in the proviral DNA may be involved in repression (92), though recent work suggests this is not a major player (93). Some mechanisms could involve selection for unusual transcriptional factor profiles in the infected cell – either loss of a positive factor, or presence of a repressor.

There is much interest in the very rare cohort of infected patients that can maintain low or undetectable levels of virus without antiviral therapy – so-called "elite controllers" or "transient elite controllers". These individuals can maintain the integrated proviruses in a state of silence, or suppress replication of any low-level virus production before high level replication occurs, even without antiviral therapy. It is unclear if they all carry proviruses in unusual chromosomal locations, but there is evidence that at least some have proviruses in centromeric satellite DNA or zinc-finger genes, both associated with heterochromatin features (94–96). In addition, they may have unusual immune responses to the virus that are capable of suppressing virus spread (97). Inducing such a state of control over provirus expression would be a major step toward an effective cure of AIDS – the so called "block and lock" approach. Ensuring that such control is strong and long-lasting, acting on all the proviruses in the pool of infected cells, will be challenging.

Reactivation from latency

Though the proviral DNAs in the reservoir in patients treated with effective antiviral therapies are largely silent, the reappearance of replicating virus upon withdrawal of therapy is surprisingly rapid, suggesting that low levels of expression may be continuing. There is evidence that the pattern of expression is in stochastic transcriptional "bursts" – short blips of expression that can initiate full-blown viremia if not brought under control (98). Expression can be thought of as "noisy" and acting in random cells at random times (99, 100), driven by a variety of host factors (101).The bursts would be amplified by the Tat activation, as if the provirus existed in a hair-trigger state that could be switched from off to on whenever a threshold level of expression is crossed. There is great interest in devising means to enhance the silencing of integrated proviruses, or suppress these blips and so prevent the reemergence of replicating virus (102). One approach would be to inhibit Tat, and indeed small-molecule inhibitors of Tat have been identified (103, 104). Such inhibitors can significantly extend the virus-free period after cessation of antiviral therapy (105), though not indefinitely. Inhibition of any host factors utilized by Tat could similarly impact reactivation. Unfortunately, these factors are important for the expression of many cellular genes, and so inhibition of Tat in this way without global effects is not plausible. Targeting them only when they are present in the context of a Tat complex might be a means to reduce toxicity. The presence of the multiple binding sites for so many positive transcription factors in the viral LTR, however, suggests that there are many ways by which virus can be reactivated, and inhibiting them all will be very challenging.

Intentionally inducing relief from silencing, followed by clearance of the infected cells, is one conceptual approach to a cure of AIDS (the "shock and kill" approach). The pool of infected cells is probably not a monolithic population, will carry proviruses in many genomic locations, and may be silent for a diversity of mechanisms. Because of this potential heterogeneity, it may be very hard to uniformly and completely activate all the silent copies in a person (106). And the retention of only a few, or perhaps even one copy of a replication-competent provirus in a small number of cells may permit reactivation and reinitiation of viremia. Such cells may even reside in selective tissues and organs that will be hard to access (107, 108). The "shock and kill" route may be a very challenging means to eliminate the reservoir (109).

The ultimate means to prevent reversal from latency, of course, is to eliminate the viral DNA itself – to clear all the infected cells that carry latent proviruses. These cells, however, carry no distinctive mark that we know of that differentiate them from uninfected cells, since they do not express viral proteins, and we know of no truly selective bullet to kill them. There is a shotgun approach of killing the entire lymphoid cell population and replacing it with a bone-marrow transplant of virus-resistant cells – as with cells of CCR5-mutant donors, or genetically engineered stem cells – and while this approach has been successful in a small number of patients, it is extremely demanding, risky, and expensive. Devising a way to target the proviral DNA *in situ*, without ex vivo manipulations, perhaps using CRISPR-based gene editing, may someday provide a true cure that is scalable to all PLWH. In cell culture proviruses can be excised with engineered restriction enzymes or RNA-guided CRISPR nucleases (110–113), but never with complete efficiency, and *in vivo* any residual proviruses would always have the potential for reactivation and renewed viremia.

Discussion and closing thoughts

The study of the silencing of HIV-1 DNAs, both early in infection and at later times has taught us much about the regulation of gene expression in general, and about the roles of histone modifications and chromatin structure in silencing incoming DNAs. The findings have suggested new mechanisms and new targets that may allow for better control of HIV-1 replication and pathogenicity. There may especially be useful new means to reduce virus transmission from patient-to-patient, a particularly vulnerable stage in the virus life cycle. The brief window time after infection – the first 6-12 hours of infection – is a critical period when virus is susceptible to restriction, and targeting the unintegrated DNA is the best way to interrupt the viral life cycle before it is too late, after the provirus has been formed. There may also be new ways to deal with late-stage infections, when proviruses have been established in large numbers of cells scattered throughout the body. We may ultimately find ways to activate expression and clear cells carrying proviruses, or alternatively to permanently suppress proviral expression and provide an effective cure for AIDS, even without truly eliminating the virus. But these mechanisms are going to require new tools not yet in our toolbox. What may be a path forward is the establishment of a pool of virus-resistant cells in a patient, providing normal cellular immunity even when virus is present. Bone marrow transplants from rare donors lacking the CCR5 coreceptor have effectively cured a

handful of patients, providing proof in principle of the approach. What will be needed is a means to create such virus resistance *in situ*, without the need for bone marrow transplant or ex vivo gene engineering. New gene editing tools, introduced by new gene delivery methods, may one day provide such cures at acceptable cost.

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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