Cell Host & Microbe
Article

Orientation-Dependent Regulation of Integrated HIV-1 Expression by Host Gene Transcriptional Readthrough

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DOI 10.1016/j.chom.2008.06.008

SUMMARY

Integrated HIV-1 genomes are found within actively transcribed host genes in latently infected CD4$^+$ T cells. Readthrough transcription of the host gene might therefore suppress HIV-1 gene expression and promote the latent infection that allows viral persistence in patients on therapy. To address the effect of host gene readthrough, we used homologous recombination to insert HIV-1 genomes in either orientation into an identical position within an intron of an actively transcribed host gene, hypoxanthine-guanine phosphoribosyltransferase (*HPRT*). Constructs were engineered to permit or block readthrough transcription of *HPRT*. Readthrough transcription inhibited HIV-1 gene expression for convergently orientated provirus but enhanced HIV-1 gene expression when HIV-1 was in the same orientation as the host gene. Orientation had a >10-fold effect on HIV-1 gene expression. Due to the nature of HIV-1 integration sites in vivo, this orientation-dependent regulation can influence the vast majority of infected cells and adds complexity to the maintenance of latency.

INTRODUCTION

Highly active antiretroviral therapy (HAART) can reduce HIV-1 viremia to below the limit of detection in many infected individuals (Perelson et al., 1997; Gulick et al., 1997; Hammer et al., 1997). However, HIV-1 persists in resting memory CD4$^+$ T cells as a stably integrated, transcriptionally silent provirus (Chun et al., 1995, 1997). These latently infected cells do not appear to produce any viral proteins and are thus unaffected by the antiretroviral drugs or the host immune system (Hermankova et al., 2003; Lassen et al., 2004b) . Nevertheless, upon cellular activation, replication-competent viruses can be quickly released. The decay rate of the latently infected resting CD4$^+$ T cells is extremely slow (Finzi et al., 1999; Siliciano et al., 2003). Replication-competent viruses can be recovered from this latent reservoir even in patients whose viral loads have been undetectable for 7 years (Siliciano et al., 2003). As such, this small pool of latently infected cells serves as a lifelong reservoir for the virus.

Understanding the molecular mechanisms by which HIV-1 latency is established and maintained is essential for developing strategies to "purge" the latent reservoir. The memory phenotype of cells harboring latent HIV-1 suggests that they arise from infected CD4$^+$ T cells that have reverted to a resting state (Pierson et al., 2000; Chun et al., 1997). Many proposed mechanisms of HIV-1 latency reflect aspects of the intracellular microenvironment that become suboptimal for HIV-1 gene expression in resting CD4$^+$ T cells (Lassen et al., 2004a). Mechanisms to explain HIV-1 latency at the transcriptional level include (1) proviral integration into sites that are or that become repressive for transcription (Jordan et al., 2001; Winslow et al., 1993); (2) the absence, in the nucleus of resting CD4$^+$ T cells, of crucial host transcription activators for HIV-1 expression (Bohnlein et al., 1988; Duh et al., 1989; Ganesh et al., 2003; Nabel and Baltimore, 1987; Tong-Starksen et al., 1987); (3) presence of cellular transcriptional repressors (Jiang et al., 2007; Tyagi, 2007; Williams et al., 2006; Coull et al., 2000; He and Margolis, 2002); (4) histone modifications that mediate repression of integrated HIV-1 gene expression (Williams et al., 2006; du Chene et al., 2007; Marban et al., 2007); and (5) premature termination of HIV-1 transcription due to the absence of viral protein Tat and Tat-associated host factors (Adams et al., 1994; Herrmann and Rice, 1995; Kao et al., 1987). The net effect of multiple mechanisms is the profound (but reversible) silencing of HIV-1 gene expression in resting CD4$^+$ T cells. Interestingly, although HIV-1 latency is not the sole result of any single mechanism, removing any one of the multiple restrictions on HIV-1 gene expression can lead to virus production in experimental settings, probably because of a strong positive-feedback loop involving HIV-1 Tat.

Because access of the transcriptional machinery to the integrated provirus is a prerequisite for HIV-1 gene expression, one widely discussed hypothesis is that latency reflects proviral integration into chromosomal sites that are, or that become, repressive for transcription (Jordan et al., 2001, 2003). This hypothesis is supported by analysis of integration sites in cell lines (J-Lat) selected for a latent phenotype (Jordan et al., 2003). In contrast, when cell lines are infected with HIV-1 in vitro without

selection for a latent phenotype, integration sites are generally found within actively transcribed host genes (Mitchell et al., 2004; Schroder et al., 2002). Further characterization of the HIV-1 integration sites in the inducible J-Lat cells revealed that alphoid repeats in the centromere, gene deserts, and highly expressed genes were favored in the inducible clones compared to the constitutively expressed clones (Lewinski et al., 2005). Importantly, in vivo studies of the bulk of HIV-1 integration sites in resting $CD4^+$ T cells from patients on suppressive HAART revealed that most latent viral genomes resided within the introns of active host genes, although technical limitations have restrained the separation of replication-competent proviruses from the much larger proportion of defective ones (Han et al., 2004). Notably, a new study of the integration sites of the expression-competent proviruses from infected primary cells from an in vitro latency model has shown preferential integration into gene regions at a similar frequency as that found in the in vivo study (data not shown). Therefore, latency is not simply due to the inaccessibility of the integrated proviruses to the transcriptional machinery.

A direct consequence of the nature of HIV-1 integration sites in vivo is that HIV-1 gene expression may be decreased by transcriptional interference (TI). TI is a *cis* effect of one transcriptional process on a second transcriptional process (Adhya and Gottesman, 1982; Callen et al., 2004; Eszterhas et al., 2002; Greger et al., 1998; Mazo et al., 2007; Shearwin et al., 2005; Petruk et al., 2006). In general, transcription from an upstream promoter suppresses gene expression from a downstream promoter. TI is observed in special situations in which transcription from the upstream promoter is not terminated before reaching the downstream promoter. In the case of integrated HIV-1 genomes, polymerase complexes initiating at the host gene promoters will be continuously running through the HIV-1 genome. This has two interesting consequences. First, the entire HIV-1 genome becomes incorporated into an intron of the primary transcript of the host gene from which it is spliced out and degraded (Han et al., 2004). Second, the readthrough transcription may alter the level of HIV-1 transcription initiated from the viral promoter and contribute to latency. In this study, we attempted to measure the influence of host gene readthrough transcription on expression of viral genes from the integrated HIV-1 provirus.

RESULTS

Strategy for Determining the Impact of Host Gene Transcriptional Readthrough on Expression of HIV-1 Genes from an Integrated Provirus

Our previous analysis of the sites of integration of HIV-1 proviruses in resting $CD4^+$ T cells from patients on HAART showed that HIV-1 DNA is usually integrated into introns of active cellular genes (Han et al., 2004). Therefore, HIV-1 is essentially a "genome within a gene," and the regulation of HIV-1 gene expression must be understood in this context. Based on studies in other systems, it is reasonable to assume that readthrough transcription of the host gene will impact HIV-1 gene expression. Effects of readthrough transcription have been analyzed for endogenous nested genes, integrated transgenes, and noncoding RNAs (Jaworski et al., 2007; Strathdee, 2006; Martianov et al., 2007). Proudfoot and colleagues have elegantly demonstrated that the binding of critical transcription factors to the HIV-1 long terminal repeat (LTR) is suppressed by transcriptional interference from a closely adjacent tandem HIV-1 promoter (Greger et al., 1998). However, since HIV-1 integration sites map throughout the length of the host genes and are not necessarily adjacent to a cellular promoter (Han et al., 2004), and since the average size of human genes is ~27 kb and some genes can be megabases in length (International Human Genome Sequencing Consortium, 2001), it is important to analyze the impact of readthrough transcription in a natural host-gene setting. Therefore, we established a model to measure HIV-1 expression levels under conditions where readthrough transcription could be turned on or off (Figure 1) in order to evaluate the hypothesis that readthrough transcription of the host gene affects HIV-1 gene expression. Given the extraordinary difficulty of carrying out mechanistic studies on the rare cells that constitute the latent reservoir in vivo, we set up an in vitro model using the HCT116 cell line. We inserted the HIV-1 genome in either orientation into a precisely determined intronic position about 20 kb downstream from the promoter of the hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) gene, an actively transcribed housekeeping gene, located on the X chromosome (Figure 2A). In male cells (such as HCT116), this gene is present in a single copy per cell. Karyotyping confirmed that the HCT116 cells had a single X chromosome (not shown). To generate matched sets of cells in which readthrough transcription of the *HPRT* gene either occurs (*Readthrough [+]*) or does not occur (*Readthrough [–]*), we included a triple repeat of a strong polyadenylation signal sequence and a spacer sequence to stop host gene transcription upstream of the HIV-1 genome. Notably, because recent work indicates that RNA polymerase II can continue past the polyadenylation site (West et al., 2004; Teixeira et al., 2004) and may move beyond 1 kb before falling off the DNA (Proudfoot et al., 2002; Dye and Proudfoot, 2001), a 1.5 kb segment of spacer DNA without homology to human sequences was introduced following the stop sequence to allow elongating polymerase to disengage before reaching the HIV-1 genome. RT-PCR analysis showed the nascent transcripts of the 3′ region of the spacer were significantly decreased in the readthrough (–) clone, confirming the effectiveness of the stop signal (see Figure S1 available online). This triple polyadenylation sequence was excised using Cre recombinase to generate *Readthrough (+)* cells. With the exception of the polyadenylation sequence and an adjacent *β-geo* selection cassette (Lobe et al., 1999), the *Readthrough (+)* and *Readthrough (–)* cells are identical. The internally deleted HIV-1 genome was derived from HIV-EGFP-HSAΔE vector (Reiser et al., 2000), which has two intact LTRs, the critical accessory genes like *tat* and *rev*, and GFP- and HAS-coding sequences in the *env* and *nef* genes, respectively (Figure 2B).

After transfection of these constructs, surviving HCT116 clones were selected with G418 (400 μg/ml) and 6-thioguanine (6-TG, 10 μg/ml) for the presence of *β-geo* and the disruption of *HPRT*, respectively. The frequency of correct homologous recombination events was extremely low (~1 per 2–6 million cells). For clones with correctly inserted HIV-1 genomes, an aliquot of cells was transfected with Cre recombinase to excise the polyadenylation signal and restore *HPRT* readthrough. These clones could be selected in HAT (hypoxanthine aminopterin thymidine) medium, which selects for *HPRT* expression, indicating HIV-1 sequences

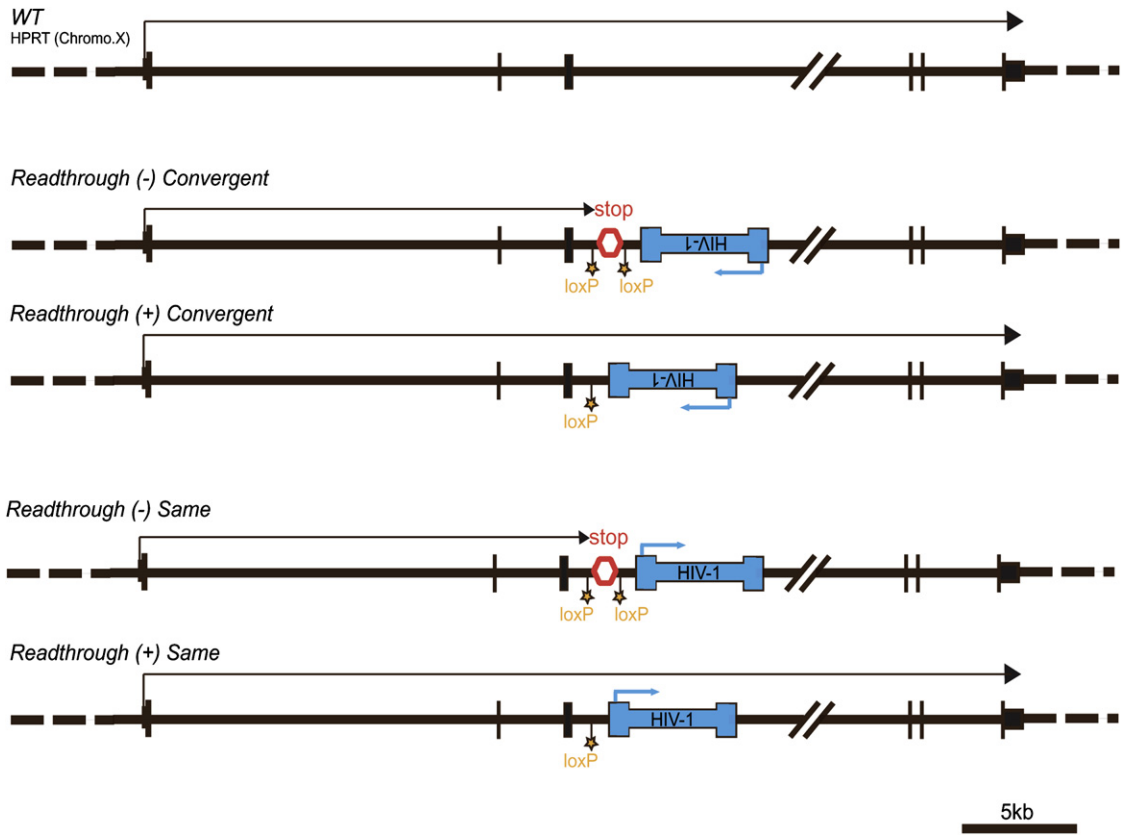

Figure 1. System for Measuring HIV-1 Expression Levels under Conditions Where Readthrough Transcription Can Be Turned On or Off
Using homologous recombination, the HIV-1 genome was inserted into the third intron of the *HPRT* gene on the X chromosome of the male cell line HCT116. Four clonal cell lines were generated that differed in the orientation of HIV-1 with respect to *HRPT* transcription (convergent or the same as the host gene *HPRT*) and on/off status of host gene readthrough due to the presence of a triple polyA signal (*Readthrough [+]* or *Readthrough [–]*). Host gene transcription was prematurely terminated by a "stop" site composed of an SV40 triple-polyA signal. The stop signal was flanked by two loxP sites so that it could be removed by Cre/loxP recombination to restore host gene transcriptional readthrough in the *Readthrough (+)* clones. Solid lines refer to the host gene *HPRT*, with vertical lines reflecting its exons. The direction of transcription of *HPRT* and HIV-1 are indicated with black and blue arrows, respectively.

do not efficiently truncate HPRT sequences. The end result was two sets of clonal cell lines in which readthrough transcription of the host gene either proceeds through or stops before reaching the HIV-1 genome, which was present in either the same or the reverse (convergent) orientation with respect to *HPRT*.

To verify that the cell clones obtained using the above selection protocols contained correctly inserted HIV-1 genomes, we used genomic DNA PCR (Figures 2A and 2C). PCR amplification using a 5′ primer in *HPRT* but outside the homology arm and a 3′primer in *β-geo* gave a band in *Readthrough (–)* cell lines but not the *Readthrough (+)* cells (Figures 2A and 2C). Using another 3′primer in the HIV-1 genome, PCR products of the correct size were obtained only for *Readthrough (+)* cells. Direct sequencing of joints provided further confirmation that the constructs were correct. We also carried out confirmatory Southern blot analysis (Figure S3). All four clones gave the expected pattern of bands. In addition, we carried out X-gal staining to verify the removal of *β-geo* by Cre/LoxP recombination in both *Readthrough (+)* cell lines (Figure 2D). Most importantly, we analyzed *HPRT* transcription to determine whether the stop cassette caused premature termination of upstream transcription (Figure 2E). Real-time RT-PCR from purified mRNAs was used to measure the *HPRT* mRNA levels in each clone. The real-time RT-PCR primers were placed in the two *HPRT* exons flanking the intron containing the HIV-1 genome. In the *Readthrough (–)* clones, *HPRT* transcription was not detected using the exon 3-exon 4 primer pair. In contrast, Cre-mediated excision of the stop signal in the *Readthrough (+)* clones restored *HPRT* transcription through this region to levels seen in wild-type cells (Figure 2E). It was thus possible to evaluate the effects of readthrough transcription on HIV-1 gene expression.

Readthrough Transcription in Convergent Orientation Inhibits HIV-1 Expression

Using this system, we first analyzed the HIV-1 gene expression in cell clones in which HIV-1 lies in the convergent orientation with respect to the host gene. We isolated mRNAs from each clone to measure the steady-state levels of mature HIV-1 transcripts, thus avoiding interference from HIV-1 sequences present in introns spliced from the host gene. Real-time RT-PCR amplification of a segment of the HIV-1 *gag* gene was carried out to quantify the amount of HIV-1 transcripts. 5′RACE using the same 3′primer confirmed the transcriptional start site at the HIV-1 LTR (Figure S4). By comparing the HIV-1 transcription levels in

the *Readthrough (+)* and *Readthrough (–)* clones, we discovered that HIV-1 transcription was decreased by approximately 4-fold when HIV-1 and the host gene were transcriptionally convergent (Figure 3A). The reduction was statistically significant ($p = 0.03$). In addition, the GFP-expression levels analyzed by flow cytometry showed a similar pattern of reduction by the host transcriptional readthrough (Figure 3B). Notably, the FACS plots suggested that even within clonal cells, the population of cells showed a broad expression profile. However, when the high- or low-expressing cells within the same cell population were sorted and subject to expansion, each population could reproduce the pattern of the entire population from which the cloned cell was sorted, suggesting that each clonal population exhibits significant variation in expression around a mean (Eszterhas et al., 2002).

Since HIV-1 transcription can be activated by TNF-α through NF-κB signaling (Swingler et al., 1994), we tested whether TNF-α could reverse this transcriptional interference. After 3 hr of TNF-α treatment, the nuclear NF-κB levels were significantly increased in treated cells (Figure S5), and HIV-1 gene expression was increased in both *Readthrough (+)* and *Readthrough (–)* clones (compare Figures 3A and 3B). However, a 3-fold inhibitory effect of transcriptional interference was still observed in the *Readthrough (+)* clones (Figure 3C). In both *Readthrough (+)* and *Readthrough (–)* clones, levels of HIV-1 gene expression were higher than in untreated cells (Figure 3A), indicating that TNF-α did have the expected upregulatory effect on HIV-1 transcription. However, TNF-α stimulation did not completely abrogate the effect of transcriptional interference. In addition, since the viral protein Tat is a crucial transcriptional activator, we analyzed whether addition of supplemental Tat by transient transfection could reverse the observed transcriptional interference. As was the case with TNF-α, supplemental Tat alone or in combination with TNF-α-stimulated HIV-1 transcription in both *Readthrough (+)* and *Readthrough (–)* clones but did not completely abrogate the effects of transcriptional interference (Figure 3C).

Readthrough Transcription in the Same Orientation Enhances HIV-1 Expression

Our analysis of HIV-1 integration in patients did not reveal any bias with regard to orientation (Han et al., 2004), and we expected a similar degree of transcriptional interference for HIV-1 genomes in the same orientation as the host gene. However, instead of transcriptional interference, we found that HIV-1 transcription was enhanced by ~4-fold when HIV-1 and the host gene were in the same orientation (Figure 4A). The enhancement was statistically significant ($p = 0.03$). Flow-cytometric analysis confirmed the increase in GFP expression in *Readthrough (+)* clones (Figure 4B) at the protein level. Again, we activated each cell clone with TNF-α, supplemental Tat, or both. In all conditions, the levels of gene HIV-1 expression were increased in each clone relative to levels observed in the absence of stimulation. However, the enhancing *cis* effect of readthrough transcription persisted even when the levels of HIV-1 gene expression were increased by TNF-α, Tat, or both (Figure 4C).

Promoter Occlusion at LTR by Host Transcriptional Readthrough

Promoter occlusion, originally defined by Adhya and Gottesman (1982) in λ phage, is the process through which one elongating complex blocks the assembly of another initiation complex. The interfering and the target promoters can be either convergent or in the same orientation (Shearwin et al., 2005). Because this mechanism is a general one, we set out to analyze the role of promoter occlusion in the orientation-dependent effect of readthrough transcription on HIV-1 gene expression.

Using chromatin immunoprecipitation (ChIP) followed by real-time PCR amplification of the HIV-1 promoter region (–116 to +4), we compared the binding of RNA polymerase II, general transcriptional factors, and the other transcription factors crucial for HIV-1 transcription to the HIV-1 promoter in *Readthrough (+)* and *Readthrough (–)* clones. The ChIP primers used capture both the 5′ and 3′LTRs and thus measure the net effect of readthrough on LTR occupancy. Figure 5A shows the results of ChIP in the set of cell clones in which HIV-1 and the host gene are in the convergent orientation. Total RNA polymerase II (RNAPII) can be readily detected at the HIV-1 promoter in the *Readthrough (–)* cells using the N20 antibody, which recognizes an epitope outside the carboxyl-terminal domain (CTD). Host gene readthrough reduced the occupancy of total RNAPII by 35%, consistent with the observed decrease in HIV-1 gene expression (Figure 3). As a control in this and other ChIP experiments involving RNAPII and general transcription factors, we examined occupancy at the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter and found no differences between *Readthrough (+)* and *Readthrough (–)* clones. Phosphorylation of the C-terminal domain, particularly at serine 5 within the heptad repeat, enhances early transcriptional elongation (Ho and Shuman, 1999). Using another RNAPII antibody that specifically recognizes phosphorylated serine 5 in the heptapeptide repeat sequence of the CTD, a 45% decrease in the binding of the active RNAPII was observed in the *Readthrough (+)* clones (Figure 5A). TATA box-binding protein (TBP) and transcription factor IIH (TFIIH) are two important general transcription factors. Using antibodies specific to TBP and p62 (a subunit of TFIIH), we demonstrated a similar reduction (32% for TBP and 42% for p62, respectively) in their occupancies on the HIV-1 promoter. ChIP of three other host proteins involved in HIV-1 transcription—specificity protein 1 (SP1), NF-κB p65, and cyclin-dependent kinase 9 (CDK9)—demonstrated reductions by 42%, 40%, and 36%, respectively, in *Readthrough (+)* clones. For these factors, controls were done by analyzing the occupancy of each factor at a control host promoter known to bind the relevant factor. In each case, occupancy was similar in *Readthrough (+)* and *Readthrough (–)* clones. Thus, the decrease in occupancy at the HIV-1 promoter in *Readthrough (+)* clones reflects a specific effect of readthrough transcription on the HIV-1 promoter. Such a consistent decrease in occupancy of multiple factors involved in HIV-1 transcription suggests that promoter occlusion plays an important role in the transcriptional interference caused by readthrough in the convergent orientation. The impaired accessibility of individual factors to the HIV-1 promoter can collectively inhibit the assembly of the initiation complex necessary for transcription.

Despite the consistent occupancy changes for multiple factors observed when the HIV-1 genome was in the convergent orientation, a more complex pattern was observed in the cells with HIV-1 in the same orientation as HPRT. As shown in Figure 5B, the amount of total RNAPII on the HIV-1 promoter

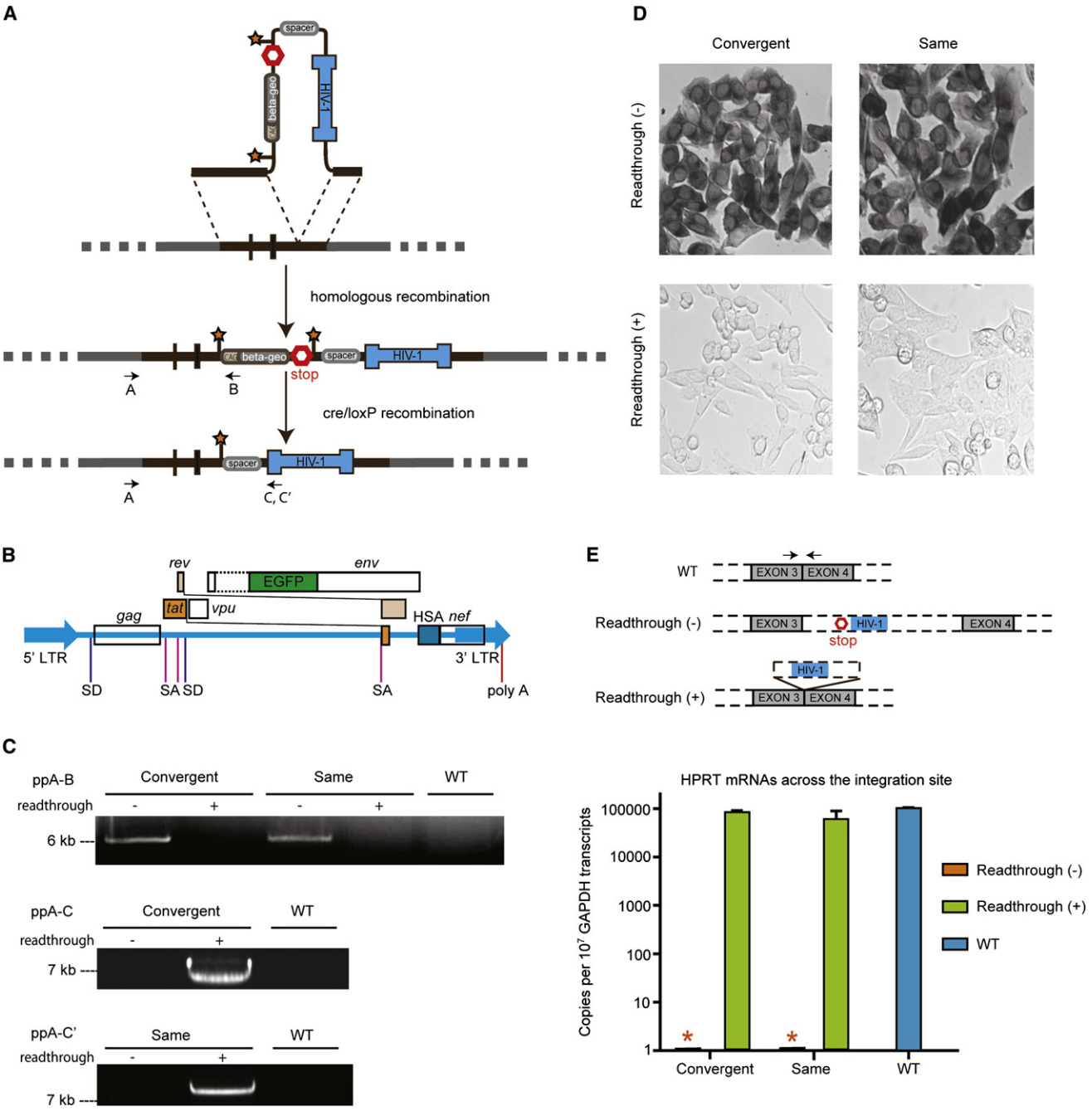

Figure 2. Establishment and Validation of Cell Clones Containing HIV-1 Provirus in the *HPRT* Gene

(A) Experimental outline for the recombination events. Step 1 shows homologous recombination. The constructs consisted of the following six parts: two flanking *HPRT* homology arms, an internally deleted HIV-1 genome (in either orientation), a selection cassette including a *β-geo* fusion gene, an SV40 triple polyA "stop" signal, and spacer DNA. They were introduced into the third intron of the *HPRT* gene by homologous recombination. Step 2 shows Cre/loxP recombination. The *β-geo* selection cassette and the stop signal were flanked by two loxP sites (marked by stars) and were removed by Cre recombinase to generate *Readthrough (+)* clones. Primers A, B, C, and C′ were used in the genomic DNA PCR to verify the recombination events.

(B) Map of the internally deleted HIV-1 genome inserted into *HPRT*. Two intact LTRs are marked as arrows. Exons of the functional accessory protein Tat are marked in orange. Coding regions for the marker genes EGFP and HSA are indicted in green and blue, respectively. Vertical lines point to major splicing sites (SA: splicing acceptor; SD: splicing donor). The distal HIV-1 polyA signal is indicated by a red line. All components are drawn to scale.

(C) Verification of recombination events using genomic DNA PCR. Primer sites are marked as in (A). "pp" refers to specific primer pairs used in the PCR. Bands of the correct size were obtained with the ppA-B for the *Readthrough (–) Convergent* and *Readthrough (–) Same* clones, indicating the presence of the *β-geo* selection cassette and the triple polyA stop signal (top panel). Using ppA–B, bands were not obtained using template DNA from the *Readthrough (+)* clones and the control WT HCT116 cells. Using ppA–C and A–C′, bands of the correct size were obtained for *Readthrough (+) Convergent* and *Readthrough (+) Same* clones, indicating successful excision of the *β-geo* selection cassette and the triple polyA stop signal (bottom panels).

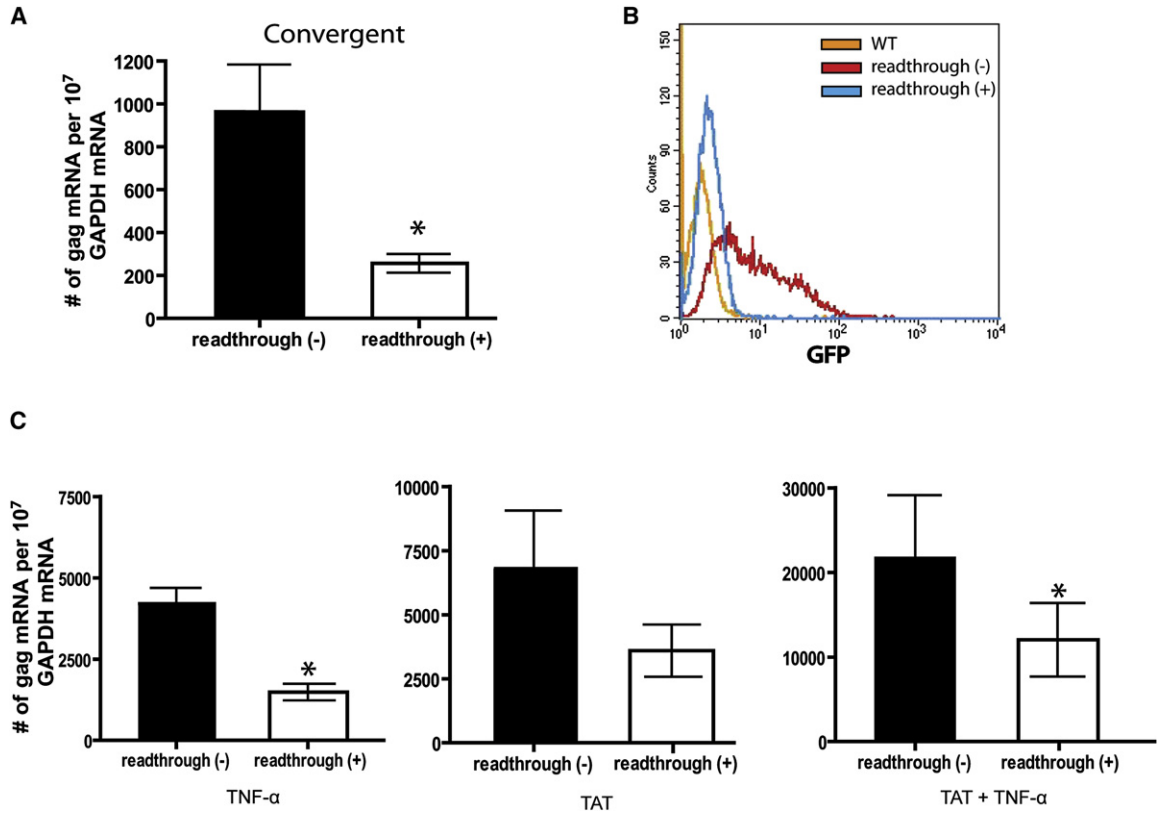

Figure 3. Effect of Readthrough Transcription on Expression of HIV-1 Proviruses Integrated in the Convergent Orientation

(A) HIV-1 transcription was measured by real-time RT-PCR amplification of a segment of the HIV-1 *gag* gene from the mRNA of the *Readthrough (–) Convergent* and *Readthrough (+) Convergent* cells. The amount of GAPDH mRNA was used to normalize the cell number in each sample. Values represent the mean ± SD of three independent experiments, each performed in duplicate. The * indicates statistical significance ($p < 0.05$) using one-tailed t test.

(B) FACS analysis of GFP expression in *WT, Readthrough (–) Convergent,* and *Readthrough (+) Convergent* cells.

(C) Quantification of the same segment of the HIV-1 *gag* gene in *Readthrough (–) Convergent* and *Readthrough (+) Convergent* cells following TNF-α activation, transfection with Tat, or both treatments.

was increased by approximately 2-fold in *Readthrough (+)* clones. This finding is consistent with the observed increases in HIV-1 gene expression. Some of the RNAPII captured in this assay may be part of elongation complexes transcribing the *HPRT* gene, although the occupancy of RNAPII at the spacer region upstream of the HIV-1 LTR was very low as revealed by ChIP experiments with primers in that region (data not shown), suggesting that the elongating RNAPII transcribing the host gene does not represent a major proportion of the amount of RNAPII observed around the HIV-1 promoter. The binding of CTD-phosphorylated active RNAPII was likewise increased by 2.1-fold. The occupancies of TBP and TFIIH showed increases of 1.6-fold and 1.3-fold, respectively. However, the occupancies of other host proteins involved in HIV-1 transcription showed a broader profile of changes, with a 15% decrease in SP1 occupancy, a 25% reduction in CDK9 occupancy, and relatively little change for NF-κB. Thus, promoter occlusion contributes to a decrease in HIV-1 gene expression when HIV-1 is present in the opposite orientation to the host gene but is not observed when HIV-1 is in the same transcriptional orientation.

Effect of Readthrough Transcription on Histone Modification at HIV-1 Promoter

In an attempt to find another mechanism to explain the impact of orientation, we used ChIP to analyze the histone modifications in

(D) Verification of excision of the β-*geo* selection cassette and the triple polyA "stop" signal by X-gal staining. Cells were stained before and after the Cre/loxP recombination. *Readthrough (–)* cells have the β-*gal* selection marker and were therefore stained blue (dark in the picture). β-*gal* was removed by Cre/loxP recombination in *Readthrough (+)* cells, as indicated by negative X-gal staining.

(E) Verification of the termination of *HPRT* transcription by the triple polyA signal. *HPRT* mRNA levels were measured by real-time RT-PCR. PCR primers (marked as arrows) were placed in the two *HPRT* exons (exons 3 and 4) flanking the intron containing the HIV-1 genome. The probe (marked as a short line) was located at the junction of the two exons. The upper panel shows the predicted processing of *HPRT* mRNAs, with removal of the third intron by splicing in the wild-type (WT) *Readthrough (+)* cells and termination of transcription in the *Readthrough (–)* clones. The lower panel shows real-time RT-PCR results from the sample and control cells. Values represent the mean ± SD of three independent experiments, each performed in duplicate. In both *Readthrough (–) Convergent* and *Readthrough (–) Same* cells, *HPRT* mRNA was undetectable (*) using primers in exons 3 and 4.

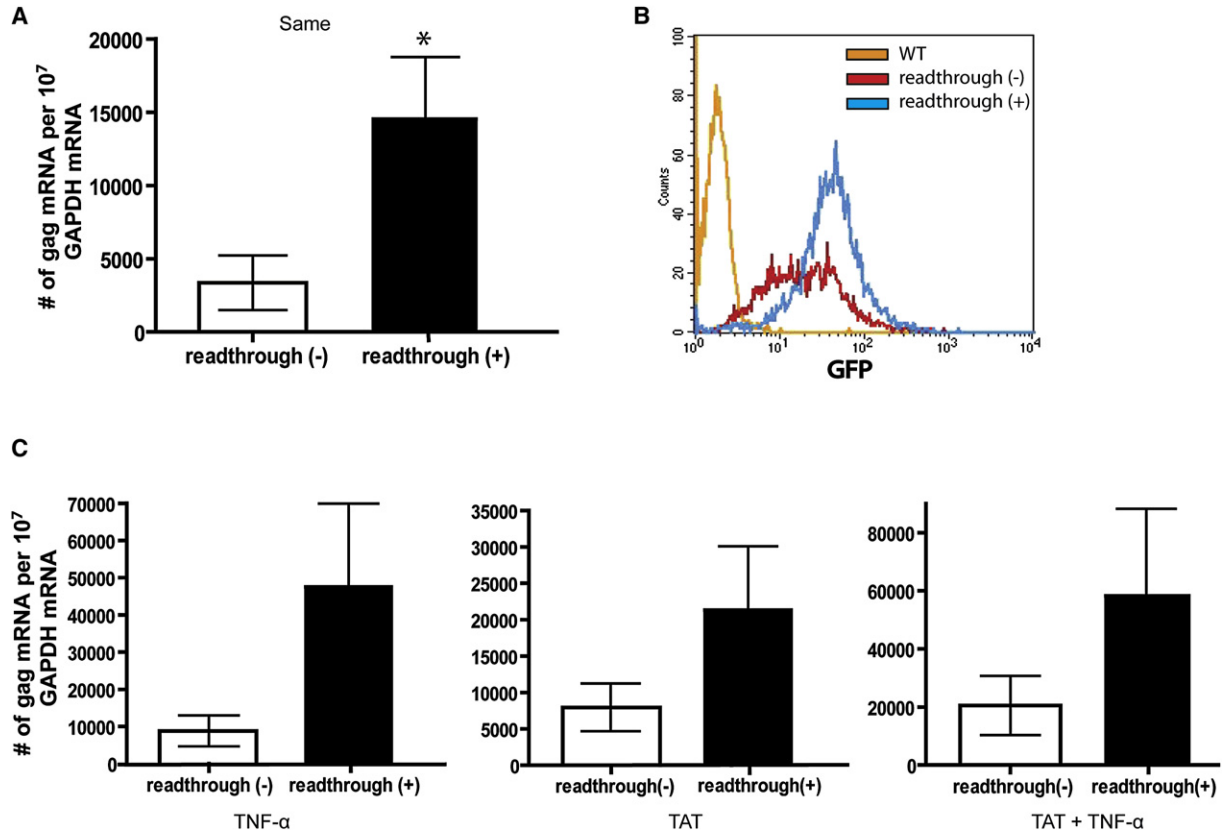

Figure 4. Effect of Readthrough Transcription on Expression of HIV-1 Proviruses Integrated in the Same Orientation as *HPRT*

(A) Quantification of HIV-1 mRNA in the *Readthrough (–) Same* and *Readthrough (+) Same* cells. The amount of GAPDH mRNA was used to normalize the cell number in each sample. Each bar represents results from three independent experiments, and error bars, which represent the standard error of the mean, were generated accordingly. The * indicates statistical significance ($p < 0.05$) using one-tailed t test.

(B) FACS analysis of GFP expression in *WT, Readthrough (–) Same,* and *Readthrough (+) Same* cells.

(C) Quantification of the same segment of the HIV-1 *gag* gene in *Readthrough (–) Same* and *Readthrough (+) Same* cells following TNF-α activation, transfection with Tat, or both treatments.

the vicinity of the integrated provirus. Several recent studies have identified particular histone modifications as landmarks for the transcriptional status of genes. For example, H3K9,14Ac was found enriched around the promoter of active genes (Guenther et al., 2007), H3K36me3 was associated with elongation (Guenther et al., 2007), and H3K27me3 was linked to silencing (Schuettengruber et al., 2007). The nucleosome positions around the HIV-1 transcriptional start site have been well characterized by Verdin and colleagues (Verdin et al., 1993). Since the nucleosome located at the HIV-1 promoter-enhancer region could be disrupted during transcriptional activation (Verdin et al., 1993), we examined the region (+437 to +543) downstream of the transcriptional start site of HIV-1.

In the convergent orientation, *Readthrough (+)* cells exhibit higher levels of histones bearing the H3K36me3 modification (Figure 6). Histones bearing the H3K9,14Ac and H3K27me3 modifications were increased, as well. When HIV-1 was inserted in the same transcriptional orientation as the host gene, there were similar increases for the H3K9,14Ac and H3K27me3 modifications, whereas the amount of H3K36me3 was little affected by the readthrough (Figure 6). These results suggest that changes in histone modification may not be a major contributor to the orientation-dependent regulation we observed.

Orientation-Dependent Regulation of HIV-1 Gene Expression by Host Readthrough Transcription

Our system allows quantitation of the effect of host readthrough transcription on the integrated HIV-1 genome in each orientation with or without readthrough. Because HIV-1 is generally integrated within actively transcribed host genes, transcriptional readthrough is the normal state. For HIV-1 genomes located at an identical site within an actively transcribing host gene, orientation relative to the host gene has a >10-fold effect and is statistically significant ($p = 0.02$) on the steady-state level of HIV-1 transcription (Figure 7A). LTR-driven GFP expression demonstrated similar magnitude of difference (Figure 7B). Therefore, host gene transcriptional readthrough regulates the expression of integrated HIV-1 proviruses in an orientation-dependent manner (Figure 7C). When HIV-1 and the host gene are in the same orientation, host readthrough enhanced the integrated HIV-1 expression, while in the convergent orientation, such an effect in *cis* is inhibitory.

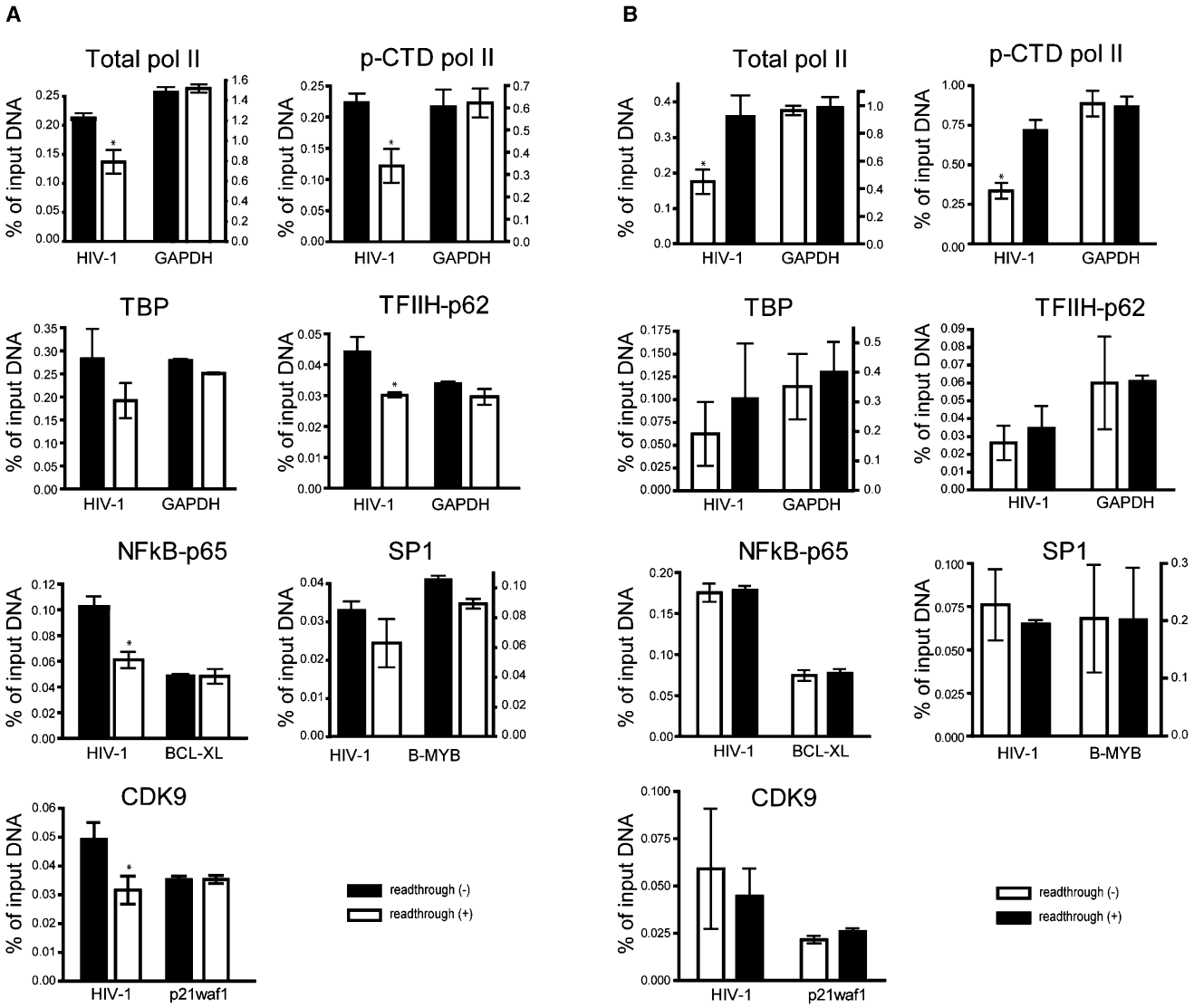

Figure 5. ChIP Analysis of the Effect of Readthrough Transcription on Occupancy of the HIV-1 Promoter

(A) Promoter occupancy for HIV-1 in the convergent orientation. The occupancy of the HIV-1 promoter by the components crucial for HIV-1 transcription was reduced by host gene transcription readthrough when HIV-1 and the host gene were in the convergent orientation. ChIP assays were carried out using specific antibodies to total RNAPII, phosphorylated RNAPII, TBP, TFIIH (p62), NF-κB, SP1, and CDK9. After crosslinking and immunoprecipitation, the −116 to +4 region corresponding to the HIV-1 promoter was amplified from *Readthrough (–) Convergent* cells (black bars) and *Readthrough (+) Convergent* cells (white bars). The occupancy of each protein on the relevant control promoter was measured simultaneously. Each bar represents results from three independent experiments, and error bars, which represent the standard error of the mean, were generated accordingly. The * indicates statistical significance ($p < 0.05$) using one-tailed t test. (B) Promoter occupancy for HIV-1 in the same orientation as *HPRT*. ChIP analysis for the HIV-1 promoter occupancy in the *Readthrough (–) Same* cells (white bars) and *Readthrough (+) Same* cells (black bars) was performed as described above.

DISCUSSION

Using a system in which HIV-1 proviruses are inserted in precisely the same position within an active host gene in either orientation with and without readthrough transcription, we demonstrate that there is orientation-dependent *cis* regulation of transcription of integrated HIV-1 by the readthrough transcription of the host gene. Transcriptional interference is observed when HIV-1 is inserted in the opposite orientation as the host gene, while enhancement of viral gene expression occurs when HIV-1 is in the same orientation as the host gene. Because of the nature of HIV-1 integration sites in vivo (Han et al., 2004; Liu et al., 2006), HIV-1 gene expression in the vast majority of infected cells is likely to be influenced by this interaction between the host gene and the provirus at the transcriptional level.

Transcriptional interference between convergent promoters has uniformly been found to be inhibitory (Callen et al., 2004; Elledge and Davis, 1989; Eszterhas et al., 2002; Hongay et al., 2006; Imamura et al., 2004; Prescott and Proudfoot, 2002; Ward and Murray, 1979). Similarly, we found a 4-fold reduction in the HIV-1 gene transcription by the host gene readthrough when HIV-1 was in the opposite orientation as the host gene.

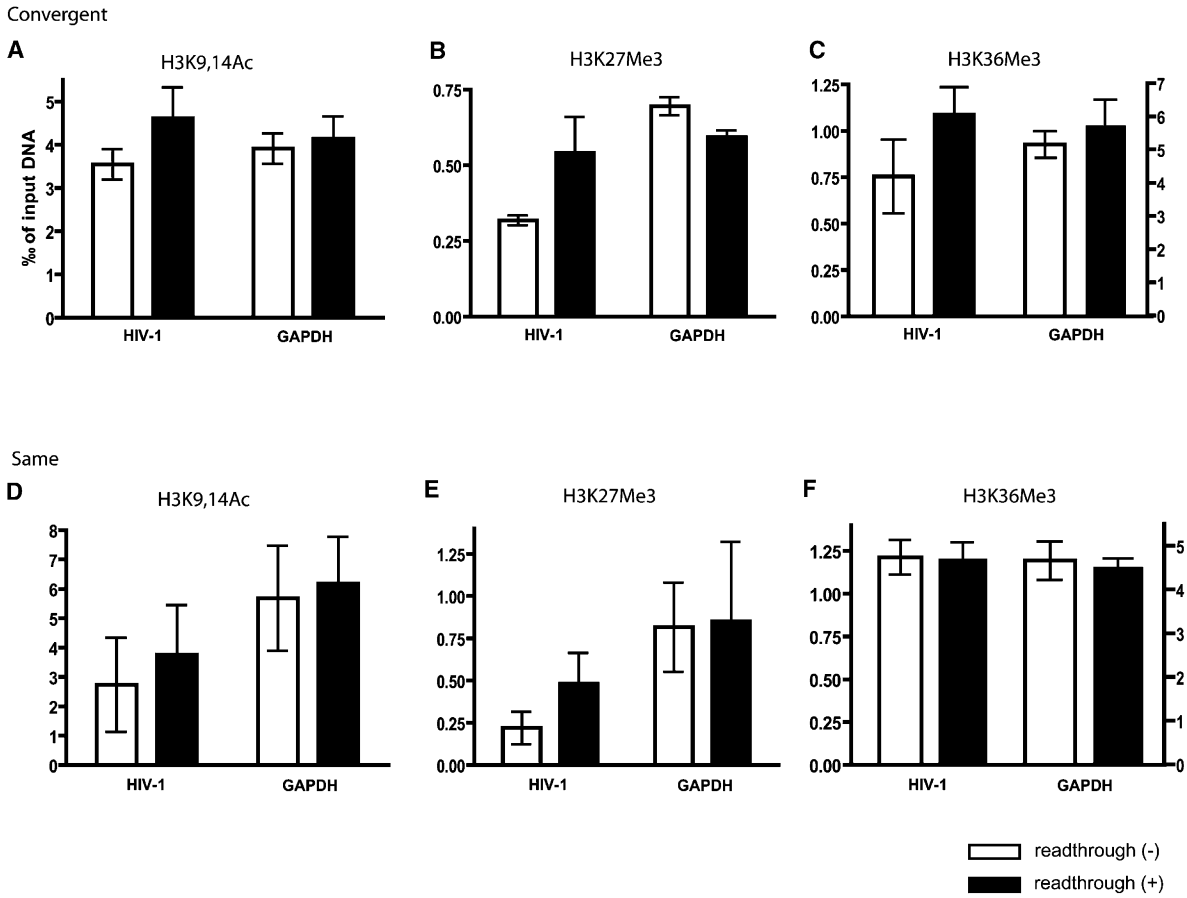

Figure 6. Effect of Readthrough Transcription on Histone Modifications at the HIV-1 LTR

Histone modifications in the vicinity of the integrated provirus were analyzed by ChIP assays using antibodies to H3K9,14Ac, H3K27me3, and H3K36me3 for precipitation followed by amplification of the region (+437 to + 543) downstream of the transcriptional start site of HIV-1.

(A–C) Differences between the *Readthrough (–) Convergent* and the *Readthrough (+) Convergent* cells in levels of H3K9,14Ac, H3K27me3, and H3K36me3, respectively.

(D–F) Differences between the *Readthrough (–) Same* and the *Readthrough (+) Same* cells in H3K9,14Ac, H3K27me3, and H3K36me3, respectively. The white bars refer to the *Readthrough (–)* cells, and the black bars refer to the *Readthrough (+)* cells. Each bar represents results from three independent experiments, and error bars, which represent the standard error of the mean, were generated accordingly.

In addition, ChIP analysis revealed a consistent 30%–40% reduction in the occupancy of RNAPII and other relevant transcription factors at the HIV-1 LTR in this situation. Although the recruitment of various proteins of the preinitiation complex still occurred, it was significantly downregulated by readthrough transcription. In addition to a reduced occupancy of critical transcription factors at the HIV-1 promoter, other mechanisms might also be involved. For example, collisions between converging elongation complexes can lead to the premature termination of the transcriptional progress of one or both complexes, with the distance between the two convergent promoters affecting the strength of the interference (Callen et al., 2004). In certain experimental systems, the ability of an elongating polymerase to read through a DNA-bound protein roadblock is enhanced by increasing the number of elongation complexes (Epshtein et al., 2003). Since we did not detect an equal reduction in the steady-state level of *HPRT* transcripts (Figure 2E) when HIV-1 was in the opposite orientation to *HPRT*, collision does not appear to be a major contributor to the observed interference. However, it is possible that such a mechanism may not be reciprocal (Callen et al., 2004). In principle, due to the convergent direction of the two promoters, the generation of both sense and anti-sense RNAs may induce the degradation of HIV-1 sequences, resulting in a decrease in the steady-state level of HIV-1 transcripts. To test this possibility, we transiently transfected the cell lines with an siRNA targeting Dicer-1, a critical component of the RNA interference machinery. No obvious change in the steady-state HIV-1 transcripts level was observed by transiently knocking down Dicer-1 (Figure S6).

In spite of the universal inhibitory effect between two active convergent promoters, results for two promoters transcribing in the same direction vary in different systems. Inhibition of the transcription from downstream promoters has been widely observed in models involving closely adjacent tandem promoters or in tandemly associated transgenes (Yahata et al., 2007; Eszterhas et al., 2002; Greger et al., 1998; Corbin and Maniatis, 1989; Eggermont and Proudfoot, 1993; Yahata et al., 2007). Proudfoot et al. (2002) demonstrated that in cell lines with two

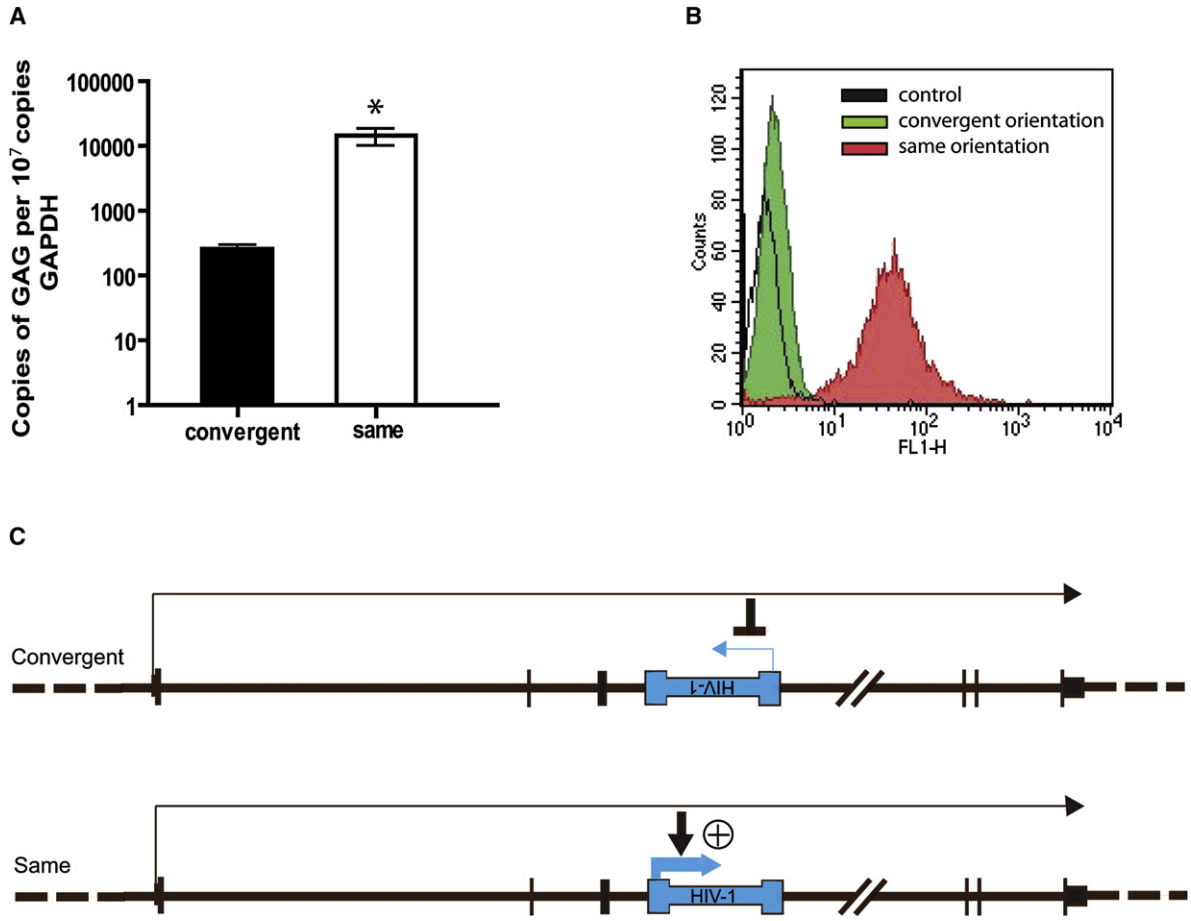

Figure 7. Summary of the Orientation-Dependent Regulation of HIV-1 Gene Expression by Host Readthrough Transcription

(A) Quantification of HIV-1 gene expression at an identical site within an actively transcribing host gene (*HPRT*) by real-time RT-PCR. Orientation relative to the host gene has a >10-fold effect on the steady-state level of HIV-1 transcription. Each bar represents results from three independent experiments, and error bars, which represent the standard error of the mean, were generated accordingly. The * indicates statistical significance ($p < 0.05$) using one-tailed t test.

(B) FACS analysis showed a >1 log difference in the mean intensity of the LTR-driven GFP expression between *Readthrough (+) Same* and *Readthrough (+) Convergent* cells.

(C) Host gene transcriptional readthrough mediates orientation-dependent regulation. It is inhibitory when HIV-1 and the host gene are in the convergent orientation and enhancing when they are in the same orientation.

tandemly integrated HIV-1 promoters, transcription from the upstream LTR had a negative impact on the downstream one (Greger et al., 1998). However, an unexpected finding of our system is that upstream transcription could indeed enhance HIV-1 gene expression for HIV-1 proviruses that are in the same orientation as the host gene. Interestingly, consistent with our results, several studies have shown that upstream transcription can increase the transcription from the downstream promoter. For instance, introducing an active promoter upstream to the silent human endogenous retrovirus (HERV)-K18 promoter activates its transcription in *cis* (Leupin et al., 2005). In addition, noncoding transcription from the upstream mouse T cell receptor-α locus can activate Jα promoters located several kilobases downstream, and the blockage of the upstream elongation abolished the downstream transcription (Abarrategui, 2007). Furthermore, it has been reported that the same transgene inserted at the same position can be expressed in one orientation but silenced in the other orientation in insect, mouse, and cultured human cell lines, although the underlying mechanisms were not clearly known (Alami et al., 2000; Francastel et al., 1999; Sabl and Henikoff, 1996).

Several mechanisms may explain how readthrough transcription increases expression of HIV-1 present in the same orientation as the host gene. One potential mechanism is a change in histone modification of chromatin (Williams et al., 2006; du Chene et al., 2007; Marban et al., 2007). However, when we analyzed three histone markers (H3K9, 14Ac, H3K36me3, and H3K27me3) that are characteristic of promoter activation, active elongation, and silencing (Guenther et al., 2007; Schuettengruber et al., 2007), we did not observe major differences in post-translational modification of histones whether HIV-1 was in the same or opposite orientation as the host gene. Other potential explanations can also be considered. The integration site of HIV-1 in our system was in the third intron of *HPRT*, about 300 bp downstream from the end of the third exon of *HPRT*. Although HIV-1 is present at the identical site within the host gene in both

sets of cells, the relative distances between the HIV-1 promoter and the adjacent *HPRT* exon are different due to the length of the HIV-1 construct itself. As mRNA processing reactions—such as splicing—occur cotranscriptionally, the splicing complex might have an effect on an adjacent promoter. In addition, removal of repressors upstream of the HIV-1 transcription start site might occur differentially depending on orientation. Finally, alterations in DNA topology upon readthrough elongation might positively effect transcription from other promoters in the same orientation.

Since HIV-1 randomly inserts into different positions within active host genes (Han et al., 2004), it is possible that the level of the orientation-dependent regulation will be variable and may depend on the relative rate of HIV-1 promoter clearance and the rate of the host gene elongation (Callen et al., 2004; Mazo et al., 2007). Nevertheless, our results reveal another layer of complexity of the HIV host gene interaction that is likely to affect the vast majority of integrated proviruses and that may play a role in HIV-1 latency and in the regulation of HIV-1 gene expression in productively infected cells. Orientational preferences were not apparent in the bulk of HIV-1 integration sites in patients' resting $CD4^+$ T cells (Han et al., 2004) and inducible J-Lat cells (Lewinski et al., 2005). However, since integration in a convergent orientation inhibits HIV-1 expression, it will be interesting to determine whether there is a bias toward such an orientation in the subset of infected, resting $CD4^+$ T cells that harbor replication-competent HIV-1, thus contributing to HIV-1 latency in vivo. Interestingly, in the human genome, the activities of human endogenous retroviruses (HERV) are very low (Smit, 1999). For those that reside within genes, HERVs in the convergent orientation with respect to the host gene are overrepresented by 5-fold. The orientation-dependent positive and negative effects on HIV-1 gene expression were not abrogated by stimulation with TNF-α or Tat. Thus, in activated cells, the enhancing effect may lead to greater HIV-1 gene expression in activated T cells that have a provirus integrated in the same orientation as the host gene. At the population level, this may compensate for transcriptional interference observed in cells with proviruses integrated in the convergent orientation.

EXPERIMENTAL PROCEDURES

Constructs

The forward and reverse orientation constructs used for homologous recombination, p203MJHIV-F and p203MJHIV-R, respectively, were assembled in multiple steps as described in the Supplemental Experimental Procedures.

Development of Cell Lines

Wild-type HCT116 cells (ATCC) were transfected with either p203MJHIV-R or p203MJHIV-F by Lipofectamine 2000 (Invitrogen). *Readthrough (–)* clones, with HIV-1 in the convergent (p203MJHIV-R) or same (p203MJHIV-F) orientation, were generated by homologous recombination as described in the Supplemental Experimental Procedures. To isolate *Readthrough (+)* cells, a vector encoding Cre (a gift from Dr. Randall Reed), was transfected into each *Readthrough (–)* clone by nucleofection (Program D-32, VCA-1003, AMAXA). Transfected cells were subjected to selection for restored-*HPRT* gene expression in the base media plus 1 × HAT supplement (GIBCO). Individual colonies were picked and further expanded as above and underwent X-gal staining (Gene Therapy Systems) to confirm excision of the *β-gal* cassette by Cre recombinase. RT-PCR was carried out for measuring the effectiveness of the disengagement of upstream elongating polymerase in conditions described in the Supplemental Experimental Procedures.

Genomic DNA PCR

Genomic DNA from each cell clone was extracted using the Gentra Puregene Kit. PCR was performed with genomic DNA to verify the correct recombination events as described in the Supplemental Experimental Procedures.

Southern Blot Analysis

Genomic DNA was isolated from 3×10^6 cells from the four cell lines and wild-type HCT 116 cells. A double-restriction digest with 25 μg genomic DNA for each of the cell lines was carried out overnight at 37°C with SphI and BlpI. The digests were then analyzed by Southern blotting (Supplemental Experimental Procedures).

5′RACE

mRNAs from each cell clone were isolated using QIAGEN Oligotex mRNA Extraction Kit. 5′RACE was performed according to the FirstChoice RLM-RACE Protocol (AMBION). HIV-1- specific primers used in the nested PCRs were the following: first round, 5′ TGAAGGGATGGTTGTAGC 3′; nested, 5′ AGCTCCCTGCTTGCCCATA 3′.

Quantification of Steady-State mRNA Levels

Reverse-transcription reactions from isolated mRNAs were carried out using random hexamers (SuperScript III, Invitrogen). Real-Time PCR was performed to quantify the steady-state mRNA levels with ABI7000, 7300, or 7900 Real-time PCR Systems (Applied Biosystem), as described in the Supplemental Experimental Procedures. A GAPDH-expression assay (Hs00266705_g1, Applied Biosystems) was used to normalize the cell number in each sample.

Activation Assays

Cells were treated with TNF-α (R&D Systems) at 10 ng/ml for 3 hr and then lysed for mRNA extraction or assayed for nuclear levels of NF-κB expression. The HCT116 nuclear fraction was extracted using the Nuclear Extract Kit (Active Motif), and the level of NF-κB p65 determined using the TransAM NF-κB p65 Kit (Active Motif). Supplemental Tat was introduced by transfecting pcDNA-TAT-86 (a gift from Dr. Avi Nath) into each cell clone, and HIV-1 expression was measured 48 hr later using real-time PCR. Transfection efficiency was measured by SYBR Real-Time RT PCR (Applied Biosystems), using primers tat86 F: 5′AGTGTTGCTTTCATTGCC3′ and tat86 R: 5′GGTGGGTTGCTTTGATAG3′.

ChIP Assays

Cells were treated with 1% formaldehyde for 10 min, and chromatin was isolated using ChIP-IT Express Kit (Active Motif). Nuclei were sonicated using 15 bursts of 10 s (Output 4, Sonicator 3000, Misonix) to produce DNA fragments ranging from 200 to 600 bp. Immunoprecipitation of specific proteins and DNA elution were performed as described in the ChIP-IT Kit. A complete description of the antibodies used, PCR conditions, and analytical methods is given in the Supplemental Experimental Procedures.

siRNA Knockdown Experiments

Predesigned siRNA specific to Dicer-1 (137012, AMBION) was transfected into cells by Lipofectamine RNAiMAX (Invitrogen). Cells were lysed, and mRNAs isolated 48 hr after the transfection. Knockdown efficiency was verified by the Taqman expression assay (Hs00998582_g1) for Dicer-1.

Statistical Analysis

One-tail t test was performed in Excel to calculate the statistical significance with $\alpha = 0.05$.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures and six figures and can be found online at http://www.cellhostandmicrobe.com/cgi/content/full/4/2/134/DC1/.

ACKNOWLEDGMENTS

We would like to thank Dr. Sarah Wheelan, Dr. Jeffrey Corden, and Dr. Stephen Desiderio for valuable advice. This work was supported by NIH grant 43222 and by the Howard Hughes Medical Institute.

Received: January 17, 2008
Revised: April 9, 2008
Accepted: May 23, 2008
Published: August 13, 2008

REFERENCES

Abarrategui, I.K.M.S. (2007). Noncoding transcription controls downstream promoters to regulate T-cell receptor alpha recombination. EMBO J. *26*, 4380–4390.

Adams, M., Sharmeen, L., Kimpton, J., Romeo, J.M., Garcia, J.V., Peterlin, B.M., Groudine, M., and Emerman, M. (1994). 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 *91*, 3862–3866.

Adhya, S., and Gottesman, M. (1982). Promoter occlusion: transcription through a promoter may inhibit its activity. Cell *29*, 939–944.

Alami, R., Greally, J.M., Tanimoto, K., Hwang, S., Feng, Y.Q., Engel, J.D., Fiering, S., and Bouhassira, E.E. (2000). {beta}-globin YAC transgenes exhibit uniform expression levels but position effect variegation in mice. Hum. Mol. Genet. *9*, 631–636.

Bohnlein, E., Lowenthal, J.W., Siekevitz, M., Ballard, D.W., Franza, B.R., and Greene, W.C. (1988). The same inducible nuclear proteins regulates mitogen activation of both the interleukin-2 receptor-alpha gene and type 1 HIV. Cell *53*, 827–836.

Callen, B.P., Shearwin, K.E., and Egan, J.B. (2004). Transcriptional interference between convergent promoters caused by elongation over the promoter. Mol. Cell *14*, 647–656.

Chun, T.-W., Finzi, D., Margolick, J.B., Chadwich, K., Schwartz, D., and Siliciano, R.F. (1995). In vivo fate of HIV-1-infected T cells: quantitative analysis of the transition to stable latency. Nat. Med. *1*, 1284–1290.

Chun, T.-W., Carruth, L., Finzi, D., Shen, X., Digiuseppe, J.A., Taylor, H., Hermankova, M., Chadwich, K., Margolick, J.B., Quinn, T.C., et al. (1997). Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. Nature *387*, 183–188.

Corbin, V., and Maniatis, T. (1989). Role of transcriptional interference in the Drosophila melanogaster Adh promoter switch. Nature *337*, 279–282.

Coull, J.J., Romerio, F., Sun, J.M., Volker, J.L., Galvin, K.M., Davie, J.R., Shi, Y., Hansen, U., and Margolis, D.M. (2000). The human factors YY1 and LSF repress the human immunodeficiency virus type 1 long terminal repeat via recruitment of histone deacetylase 1. J. Virol. *74*, 6790–6799.

du Chene, I., Basyuk, E., Lin, Y.L., Triboulet, R., Knezevich, A., Chable-Bessia, C., Mettling, C., Baillat, V., Reynes, J., Corbeau, P., et al. (2007). Suv39H1 and HP1gamma are responsible for chromatin-mediated HIV-1 transcriptional silencing and post-integration latency. EMBO J. *26*, 424–435.

Duh, E.J., Maury, W.J., Folks, T.M., Fauci, A.S., and Rabson, A.B. (1989). Tumor necrosis factor alpha activates human immunodeficiency virus type 1 through induction of nuclear factor binding to the NF-kappa B sites in the long terminal repeat. Proc. Natl. Acad. Sci. USA *86*, 6845–6849.

Dye, M.J., and Proudfoot, N.J. (2001). Multiple transcript cleavage precedes polymerase release in termination by RNA polymerase II. Cell *105*, 669–681.

Elledge, S.J., and Davis, R.W. (1989). Position and density effects on repression by stationary and mobile DNA-binding proteins. Genes Dev. *3*, 185–197.

Epshtein, V., Toulmé, F., Rahmouni, A.R., Borukhov, S., and Nudler, E. (2003). Transcription through the roadblocks: the role of RNA polymerase cooperation. EMBO J. *22*, 4719–4727.

Eszterhas, S.K., Bouhassira, E.E., Martin, D.I.K., and Fiering, S. (2002). Transcriptional interference by independently regulated genes occurs in any relative arrangement of the genes and is influenced by chromosomal integration position. Mol. Cell. Biol. *22*, 469–479.

Finzi, D., Blankson, J., Siliciano, J.D., Margolick, J.B., Chadwick, K., Pierson, T., Smith, K., Lisziewicz, J., Lori, F., Flexner, C., et al. (1999). Latent infection of $CD4^+$ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. Nat. Med. *5*, 512–517.

Francastel, C., Walters, M.C., Groudine, M., and Martin, D.I.K. (1999). A functional enhancer suppresses silencing of a transgene and prevents its localization close to centromeric heterochromatin. Cell *99*, 259–269.

Ganesh, L., Burstein, E., Guha-Niyogi, A., Louder, M.K., Mascola, J.R., Klomp, L.W.J., Wijmenga, C., Duckett, C.S., and Nabel, G.J. (2003). The gene product Murr1 restricts HIV-1 replication in resting $CD4^+$ lymphocytes. Nature *426*, 853–857.

Greger, I.H., Demarchi, F., Giacca, M., and Proudfoot, N.J. (1998). Transcriptional interference perturbs the binding of Sp1 to the HIV-1 promoter. Nucleic Acids Res. *26*, 1294–1301.

Guenther, M.G., Levine, S.S., Boyer, L.A., Jaenisch, R., and Young, R.A. (2007). A chromatin landmark and transcription initiation at most promoters in human cells. Cell *130*, 77–88.

Gulick, R.M., Mellors, J.W., Havlir, D., Eron, J.J., Gonzalez, C., McMahon, D., Richman, D.D., Valentine, F.T., Jonas, L., Meibohm, A., et al. (1997). Treatment with indinavir, zidovudine, and lamivudine in adults with human immunodeficiency virus infection and prior antiretroviral therapy. N. Engl. J. Med. *337*, 734–739.

Hammer, S.M., Squires, K.E., Hughes, M.D., Grimes, J.M., Demeter, L.M., Currier, J.S., Eron, J.J., Feinberg, J.E., Balfour, H.H., Deyton, L.R., et al, and The AIDS Clinical Trials Group (1997). A controlled trial of two nucleoside analogues plus indinavir in persons with human immunodeficiency virus infection and CD4 cell counts of 200 per cubic millimeter or less. N. Engl. J. Med. *337*, 725–733.

Han, Y., Lassen, K., Monie, D., Sedaghat, A.R., Shimoji, S., Liu, X., Pierson, T.C., Margolick, J.B., Siliciano, R.F., and Siliciano, J.D. (2004). 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. *78*, 6122–6133.

He, G., and Margolis, D.M. (2002). Counterregulation of chromatin deacetylation and histone deacetylase occupancy at the integrated promoter of human immunodeficiency virus type 1 (HIV-1) by the HIV-1 repressor YY1 and HIV-1 activator Tat. Mol. Cell. Biol. *22*, 2965–2973.

Hermankova, M., Siliciano, J.D., Zhou, Y., Monie, D., Chadwick, K., Margolick, J.B., Quinn, T.C., and Siliciano, R.F. (2003). Analysis of human immunodeficiency virus type 1 gene expression in latently infected resting $CD4^+$ T lymphocytes in vivo. J. Virol. *77*, 7383–7392.

Herrmann, C.H., and Rice, A.P. (1995). Lentivirus Tat proteins specifically associate with a cellular protein kinase, TAK, that hyperphosphorylates the carboxyl-terminal domain of the large subunit of RNA polymerase II: candidate for a Tat cofactor. J. Virol. *69*, 1612–1620.

Ho, C.K., and Shuman, S. (1999). Distinct roles for CTD Ser-2 and Ser-5 phosphorylation in the recruitment and allosteric activation of mammalian mRNA capping enzyme. Mol. Cell *3*, 405–411.

Hongay, C.F., Grisafi, P.L., Galitski, T., and Fink, G.R. (2006). Antisense transcription controls cell fate in Saccharomyces cerevisiae. Cell *127*, 735–745.

Imamura, T., Yamamoto, S., Ohgane, J., Hattori, N., Tanaka, S., and Shiota, K. (2004). Non-coding RNA directed DNA demethylation of Sphk1 CpG island. Biochem. Biophys. Res. Commun. *322*, 593–600.

International Human Genome Sequencing Consortium (2001). Initial sequencing and analysis of the human genome. Nature *409*, 860–921.

Eggermont, J., and Proudfoot, N.J. (1993). Poly(A) signals and transcriptional pause sites combine to prevent interference between RNA polymerase II promoters. EMBO J. *12*, 2539–2548.

Jaworski, D.M., Beem-Miller, M., Lluri, G., and Barrantes-Reynolds, R. (2007). Potential regulatory relationship between the nested gene DDC8 and its host gene tissue inhibitor of metalloproteinase-2. Physiol. Genomics *28*, 168–178.

Jiang, G., Espeseth, A., Hazuda, D.J., and Margolis, D.M. (2007). c-Myc and Sp1 contribute to proviral latency by recruiting histone deacetylase 1 to the human immunodeficiency virus type 1 promoter. J. Virol. *81*, 10914–10923.

Jordan, A., Defechereux, P., and Verdin, E. (2001). The site of HIV-1 integration in the human genome determines basal transcriptional activity and response to Tat transactivation. EMBO J. *20*, 1726–1738.

Jordan, A., Bisgrove, D., and Verdin, E. (2003). HIV reproducibly establishes a latent infection after acute infection of T cells in vitro. EMBO J. *22*, 1868–1877.

Kao, S.Y., Calman, A.F., Luciw, P.A., and Peterlin, B.M. (1987). Anti-termination of transcription within the long terminal repeat of HIV-1 by tat gene product. Nature *330*, 489–493.

Lassen, K., Han, Y., Zhou, Y., Siliciano, J., and Siliciano, R.F. (2004a). The multifactorial nature of HIV-1 latency. Trends Mol. Med. *10*, 525–531.

Lassen, K.G., Bailey, J.R., and Siliciano, R.F. (2004b). Analysis of human immunodeficiency virus type 1 transcriptional elongation in resting $CD4^+$ T cells in vivo. J. Virol. *78*, 9105–9114.

Leupin, O., Attanasio, C., Marguerat, S., Tapernoux, M., Antonarakis, S.E., and Conrad, B. (2005). Transcriptional activation by bidirectional RNA polymerase II elongation over a silent promoter. EMBO Rep. *6*, 956–960.

Lewinski, M.K., Bisgrove, D., Shinn, P., Chen, H., Hoffmann, C., Hannenhalli, S., Verdin, E., Berry, C.C., Ecker, J.R., and Bushman, F.D. (2005). Genome-wide Analysis of chromosomal features repressing human immunodeficiency virus transcription. J. Virol. *79*, 6610–6619.

Liu, H., Dow, E.C., Arora, R., Kimata, J.T., Bull, L.M., Arduino, R.C., and Rice, A.P. (2006). Integration of human immunodeficiency virus type 1 in untreated infection occurs preferentially within genes. J. Virol. *80*, 7765–7768.

Lobe, C.G., Koop, K.E., Kreppner, W., Lomeli, H., Gertsenstein, M., and Nagy, A. (1999). Z/AP, a double reporter for Cre-mediated recombination. Dev. Biol. *208*, 281–292.

Marban, C., Suzanne, S., Dequiedt, F., de Walque, S., Redel, L., Van Lint, C., Aunis, D., and Rohr, O. (2007). Recruitment of chromatin-modifying enzymes by CTIP2 promotes HIV-1 transcriptional silencing. EMBO J. *26*, 412–423.

Martianov, I., Ramadass, A., Serra Barros, A., Chow, N., and Akoulitchev, A. (2007). Repression of the human dihydrofolate reductase gene by a non-coding interfering transcript. Nature *445*, 666–670.

Mazo, A., Hodgson, J.W., Petruk, S., Sedkov, Y., and Brock, H.W. (2007). Transcriptional interference: an unexpected layer of complexity in gene regulation. J. Cell Sci. *120*, 2755–2761.

Mitchell, R.S., Beitzel, B.F., Schroder, A.R.W., Shinn, P., Chen, H., Berry, C.C., Ecker, J.R., and Bushman, F.D. (2004). Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences. PLoS Biol. *2*, E234.

Nabel, G., and Baltimore, D. (1987). An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature *326*, 711–713.

Perelson, A.S., Essunger, P., Cao, Y., Vesanen, M., Hurley, A., Saksela, K., Markowitz, M., and Ho, D.D. (1997). Decay characteristics of HIV-1-infected compartments during combination therapy. Nature *387*, 188–191.

Petruk, S., Sedkov, Y., Riley, K.M., Hodgson, J., Schweisguth, F., Hirose, S., Jaynes, J.B., Brock, H.W., and Mazo, A. (2006). Transcription of bxd noncoding RNAs promoted by trithorax represses Ubx in cis by transcriptional interference. Cell *127*, 1209–1221.

Pierson, T., Hoffman, T.L., Blankson, J., Finzi, D., Chadwick, K., Margolick, J.B., Buck, C., Siliciano, J.D., Doms, R.W., and Siliciano, R.F. (2000). Characterization of chemokine receptor utilization of viruses in the latent reservoir for human immunodeficiency virus type 1. J. Virol. *74*, 7824–7833.

Prescott, E.M., and Proudfoot, N.J. (2002). Transcriptional collision between convergent genes in budding yeast. Proc. Natl. Acad. Sci. USA *99*, 8796–8801.

Proudfoot, N.J., Furger, A., and Dye, M.J. (2002). Integrating mRNA processing with transcription. Cell *108*, 501–512.

Reiser, J., Lai, Z., Zhang, X.Y., and Brady, R.O. (2000). Development of multigene and regulated lentivirus vectors. J. Virol. *74*, 10589–10599.

Sabl, J.F., and Henikoff, S. (1996). Copy number and orientation determine the susceptibility of a gene to silencing by nearby heterochromatin in Drosophila. Genetics *142*, 447–458.

Schroder, A.R.W., Shinn, P., Chen, H., Berry, C., Ecker, J.R., and Bushman, F. (2002). HIV-1 integration in the human genome favors active genes and local hotspots. Cell *110*, 521–529.

Schuettengruber, B., Chourrout, D., Vervoort, M., Leblanc, B., and Cavalli, G. (2007). Genome regulation by polycomb and trithorax proteins. Cell *128*, 735–745.

Shearwin, K.E., Callen, B.P., and Egan, J.B. (2005). Transcriptional interference - a crash course. Trends Genet. *21*, 339–345.

Siliciano, J.D., Kajdas, J., Finzi, D., Quinn, T.C., Chadwick, K., Margolick, J.B., Kovacs, C., Gange, S.J., and Siliciano, R.F. (2003). Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting $CD4^+$ T cells. Nat. Med. *9*, 727–728.

Smit, A.F. (1999). Interspersed repeats and other mementos of transposable elements in Mamm. Genomes. Curr. Opin. Genet. Dev. *9*, 657–663.

Strathdee, D. (2006). Expression of transgenes targeted to the Gt(ROSA)26Sor locus is orientation dependent. PLoS ONE *1*, e4.

Swingler, S., Morris, A., and Easton, A. (1994). Tumor necrosis factor alpha and interleukin-1 beta induce specific subunits of NFκB to bind the HIV-1 enhancer: characterization of transcription factors controlling human immunodeficiency virus type 1 gene expression in neural cells. Biochem. Biophys. Res. Commun. *203*, 623–630.

Teixeira, A., Tahiri-Alaoui, A., West, S., Thomas, B., Ramadass, A., Martianov, I., Dye, M., James, W., Proudfoot, N.J., and Akoulitchev, A. (2004). Autocatalytic RNA cleavage in the human [beta]-globin pre-mRNA promotes transcription termination. Nature *432*, 526–530.

Tong-Starksen, S.E., Luciw, P.A., and Peterlin, B.M. (1987). Human immunodeficiency virus long terminal repeat responds to T-cell activation signals. Proc. Natl. Acad. Sci. USA *86*, 6845–6849.

Tyagi, M.K.J. (2007). CBF-1 promotes transcriptional silencing during the establishment of HIV-1 latency. EMBO J. *26*, 4985–4995.

Verdin, E., Paras, P., Jr., and Van Lint, C. (1993). Chromatin disruption in the promoter of human immunodeficiency virus type 1 during transcriptional activation. EMBO J. *12*, 3249–3259.

Ward, D.F., and Murray, N.E. (1979). Convergent transcription in bacteriophage [lambda]: Interference with gene expression. J. Mol. Biol. *133*, 249–266.

West, S., Gromak, N., and Proudfoot, N.J. (2004). Human 5[prime] [rarr] 3[prime] exonuclease Xrn2 promotes transcription termination at co-transcriptional cleavage sites. Nature *432*, 522–525.

Williams, S.A., Chen, L.F., Kwon, H., Ruiz-Jarabo, C.M., and Verdin, E. (2006). NF-kappaB p50 promotes HIV latency through HDAC recruitment and repression of transcriptional initiation. EMBO J. *25*, 139–149.

Winslow, B.J., Pomerantz, R.J., Bagasra, O., and Trono, D. (1993). HIV-1 latency due to the site of proviral integration. Virology *196*, 849–854.

Yahata, K., Maeshima, K., Sone, T., Ando, T., Okabe, M., Imamoto, N., and Imamoto, F. (2007). cHS4 insulator-mediated alleviation of promoter interference during cell-based expression of tandemly associated transgenes. J. Mol. Biol. *374*, 580–590.