Minor Contribution of Chimeric Host-HIV Readthrough Transcripts to the Level of HIV Cell-Associated gag RNA

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Minor Contribution of Host-HIV Readthrough Transcripts to the Level of HIV Cell-associated *gag* RNA

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Abstract

Cell-associated HIV unspliced RNA is an important marker of the viral reservoir. HIV *gag* RNA-specific assays are frequently used to monitor the reservoir activation. Because HIV preferentially integrates into actively transcribed genes, some of the transcripts detected by these assays may not represent genuine HIV RNA but chimeric host-HIV readthrough transcripts. Here we demonstrate that in HIV-infected patients on suppressive cART, such host-derived transcripts do not significantly contribute to the HIV *gag* RNA level.

Cell-associated (CA) HIV unspliced RNA is an important marker of the viral reservoir and the response to combination antiretroviral therapy (cART) (1). Recently, there has been considerable interest in the utilization of CA HIV RNA as a surrogate marker of virus activation by latency-reversing agents (LRA) (2), and it has been used as a main output measure in several clinical trials aimed at reduction of the HIV reservoir (3-6). Primers specific for the HIV *gag* region are frequently used in PCR-based assays that quantify unspliced RNA (7,8). However, because HIV integrates preferentially within actively transcribed host genes (Fig. 1A) (9), it has been suggested that some of the transcripts detected by the *gag*-specific assays may not represent genuine HIV RNA but rather chimeric host-HIV readthrough transcripts that are transcribed from host promoters (10). In this case, an effect of LRA measured by induction of *gag* RNA transcription could represent activation of a host gene, instead of HIV latency reversal. Therefore, to properly interpret the results of the *gag* assays, it is necessary to determine the relative contribution of such readthrough transcripts to the total HIV *gag* RNA signal in cART-treated patients.

We developed a sensitive nested real-time PCR assay that amplifies the 5' long terminal repeat (LTR)-encoded U3 – packaging signal region (U3-Psi) of HIV-1. As the forward primers are

48 located 5' of the HIV LTR transcription start site, this assay specifically detects host-HIV
49 readthrough transcripts but not genuine HIV-1 unspliced RNA (Fig. 1B). The assay has a linear
50 range of five orders of magnitude and the sensitivity of four copies per reaction (Fig. 2). For this
51 study, we used peripheral blood mononuclear cells (PBMC) of 48 cART-treated patients visiting the
52 HIV outpatient clinic of the Academic Medical Center in 2011-2013 and participating in the Co-
53 morBidity in Relation to AIDS (COBRA) cohort, whose plasma viremia had been undetectable (<40
54 copies/ml) for a median of 7 years prior to the time of sampling. The median $CD4^+$ T-cell count was
55 675.5 cells/mm^3. Total DNA and total RNA were isolated from the patient PBMC using the Boom
56 isolation method (11) and CA HIV DNA and RNA were separately quantified using both the U3-Psi
57 assay and the seminested real-time PCR assay specific for the HIV *gag* region (Fig. 1B) (7,8).
58 Cellular RNA was treated with DNase (DNA-free™ kit, Ambion®) to remove DNA that could
59 interfere with the quantitation, and reverse transcribed using random primers and Superscript III
60 reverse transcriptase (both Invitrogen). As HIV integrates in a random orientation with regard to the
61 host genes, we used random primers to allow detection of readthrough RNA transcribed in both
62 directions, from both upstream and downstream host promoters (Fig. 1A). Same-volume aliquots of
63 the same DNA or cDNA preparations were used as input for U3-Psi and *gag* assays. HIV DNA and
64 RNA were normalized to the cellular inputs as described previously (12).

65 As expected, both U3-Psi and *gag* assays detected HIV DNA in >90% of the patients (44/48
66 and 46/48, respectively) with no significant quantitative bias between the assays (0.13±0.50 $\log_{10}$;
67 P>0.05 for comparison of the difference to 0) (Fig. 3), and a highly significant correlation between
68 the two measurements was observed (P=0.001), demonstrating the functionality of the U3-Psi assay.
69 However, a major difference in detectability of HIV RNA was observed. HIV *gag* RNA was
70 detected in 44/48 of these patients (92%) with a median copy number of 590 (interquartile range,
71 217-1194) copies/µg total RNA. However, the detectability of readthrough RNA was only 40%

(19/48 patients) (Fig. 4A). In these 19 patients where the readthrough RNA was detected, its median copy number was 49 (41-122) copies/µg total RNA (P=0.0001 for the paired comparison with the HIV *gag* RNA) (Figs. 4B, 4C). This represented only 8.3% (2.4%-11.2%) of the HIV *gag* RNA (Fig. 4D). Notably, this is a large overestimation and the real readthrough/*gag* RNA ratio is much lower, as patients with undetectable readthrough RNA (60% of all patients) were excluded from this calculation. No significant correlation was observed between HIV *gag* RNA and the readthrough RNA (P=0.64).

Although the existence of host-HIV readthrough transcripts has been demonstrated previously (9,13), this is the first quantitative comparison of these transcripts with HIV *gag* RNA in cells from HIV-infected patients. Our results compellingly show that in PBMC of HIV-infected patients on suppressive cART, the contribution of host-derived transcripts to the RNA measured by HIV *gag* assays is very small. The host-HIV readthrough RNA transcribed in the same direction as HIV (sense) is most probably polyadenylated at the HIV 5' LTR, whereas HIV has evolved a number of strategies to suppress polyadenylation of its nascent RNA transcript (14,15). However, polyadenylation cannot be the only explanation of the scarcity of host-HIV readthrough transcripts that we found, as the readthrough RNA transcribed in the antisense direction is not expected to be polyadenylated at the HIV LTRs. Rather, as introns represent the absolute majority of HIV integration sites within genes (9), the low abundance of host-HIV readthrough transcripts compared to genuine HIV RNA might reflect a combination of the short half-lives of pre-mRNA and intronic RNA in a cell (16) and the relative strength of the HIV LTR promoter.

A limitation of this study is that we only quantified HIV RNA in total PBMC. It is possible that the HIV readthrough/*gag* RNA ratio is different in resting $CD4^+$ T-cells. However, although the HIV transcription level is lower in resting than in activated $CD4^+$ cells (17), host cell transcription is also expected to be lower due to the absence of nuclear forms of key transcription factors (e.g.,

NFκB and NFAT) in resting cells (18). In addition, to monitor the efficacy of LRA clinical trials, HIV *gag* RNA is usually quantified in total $CD4^+$ cells or PBMC (3,4,6). Therefore, our report is relevant for the interpretation of the outcome of such trials.

In summary, we observed only a minor contribution of host-HIV readthrough transcripts to the level of HIV *gag* RNA. The vast majority of HIV *gag* RNA transcripts in cART-treated patients represent genuine HIV unspliced RNA.

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References

1. **Pasternak AO, Lukashov VV, Berkhout B**. 2013. Cell-associated HIV RNA: a dynamic biomarker of viral persistence. Retrovirology **10**:41.

2. **Bruner KM, Hosmane NN, Siliciano RF.** 2015. Towards an HIV-1 cure: measuring the latent reservoir. Trends Microbiol **23**:192-203.

3. **Elliott JH, Wightman F, Solomon A, Ghneim K, Ahlers J, Cameron MJ, Smith MZ, Spelman T, McMahon J, Velayudham P, Brown G, Roney J, Watson J, Prince MH, Hoy JF, Chomont N, Fromentin R, Procopio FA, Zeidan J, Palmer S, Odevall L, Johnstone RW, Martin BP, Sinclair E, Deeks SG, Hazuda DJ, Cameron PU, Sekaly RP, Lewin SR.** 2014. Activation of HIV transcription with short-course vorinostat in HIV-infected patients on suppressive antiretroviral therapy. PLoS Pathog **10**:e1004473.

4. **Rasmussen TA, Tolstrup M, Brinkmann CR, Olesen R, Erikstrup C, Solomon A, Winckelmann A, Palmer S, Dinarello C, Buzon M, Lichterfeld M, Lewin SR, Østergaard L, Søgaard OS.** 2014. Panobinostat, a histone deacetylase inhibitor, for latent-virus reactivation in HIV-infected patients on suppressive antiretroviral therapy: a phase 1/2, single group, clinical trial. The Lancet HIV **1**:e13-e21.

5. **Archin NM, Liberty AL, Kashuba AD, Choudhary SK, Kuruc JD, Crooks AM, Parker DC, Anderson EM, Kearney MF, Strain MC, Richman DD, Hudgens MG, Bosch RJ, Coffin JM, Eron JJ, Hazuda DJ, Margolis DM**. 2012. Administration of vorinostat disrupts HIV-1 latency in patients on antiretroviral therapy. Nature **487**:482-485.

6. **Søgaard OS, Graversen ME, Leth S, Olesen R, Brinkmann CR, Nissen SK, Kjaer AS, Schleimann MH, Denton PW, Hey-Cunningham WJ, Koelsch KK, Pantaleo G, Krogsgaard K, Sommerfelt M, Fromentin R, Chomont N, Rasmussen TA, Østergaard L, Tolstrup M.** 2015. The Depsipeptide Romidepsin Reverses HIV-1 Latency In Vivo. PLoS Pathog **11**:e1005142.

7. **Pasternak AO, Adema KW, Bakker M, Jurriaans S, Berkhout B, Cornelissen M, Lukashov VV**. 2008. Highly sensitive methods based on seminested real-time reverse transcription-PCR for quantitation of human immunodeficiency virus type 1 unspliced and multiply spliced RNA and proviral DNA. J Clin Microbiol **46**:2206-2211.

8. **Kiselinova M, Pasternak AO, De Spiegelaere W, Vogelaers D, Berkhout B, Vandekerckhove L**. 2014. Comparison of droplet digital PCR and seminested real-time PCR for quantification of cell-associated HIV-1 RNA. PLoS One **9**:e85999.

9. **Han Y, Lassen K, Monie D, Sedaghat AR, Shimoji S, Liu X, Pierson TC, Margolick JB, Siliciano RF, Siliciano JD**. 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.

10. **Bullen CK, Laird GM, Durand CM, Siliciano JD, Siliciano RF**. 2014. New ex vivo approaches distinguish effective and ineffective single agents for reversing HIV-1 latency in vivo. Nat Med **20**:425-429.

11. **Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-van Dillen PM, van der Noordaa J.** 1990. Rapid and simple method for purification of nucleic acids. J Clin Microbiol **28**:495-503.

12. **Pasternak AO, Jurriaans S, Bakker M, Prins JM, Berkhout B, Lukashov VV.** 2009. Cellular levels of HIV unspliced RNA from patients on combination antiretroviral therapy with undetectable plasma viremia predict the therapy outcome. PLoS One **4**:e8490.

13. **Sherrill-Mix S, Ocwieja KE, Bushman FD.** 2015. Gene activity in primary T cells infected with HIV89.6: intron retention and induction of genomic repeats. Retrovirology **12**:79.

14. **Brown PH, Tiley LS, Cullen BR**. 1991. Efficient polyadenylation within the human immunodeficiency virus type 1 long terminal repeat requires flanking U3-specific sequences. J Virol **65**:3340-3343.

15. **Das AT, Klaver B, Berkhout B.** 1999. A hairpin structure in the R region of the human immunodeficiency virus type 1 RNA genome is instrumental in polyadenylation site selection. J Virol **73**:81-91.

16. **Clement JQ, Qian L, Kaplinsky N, Wilkinson MF**. 1999. The stability and fate of a spliced intron from vertebrate cells. RNA **5**:206-220.

17. **Kaiser P, Joos B, Niederost B, Weber R, Gunthard HF, Fischer M**. 2007. Productive human immunodeficiency virus type 1 infection in peripheral blood predominantly takes place in CD4/CD8 double-negative T lymphocytes. J Virol **81**:9693-9706.

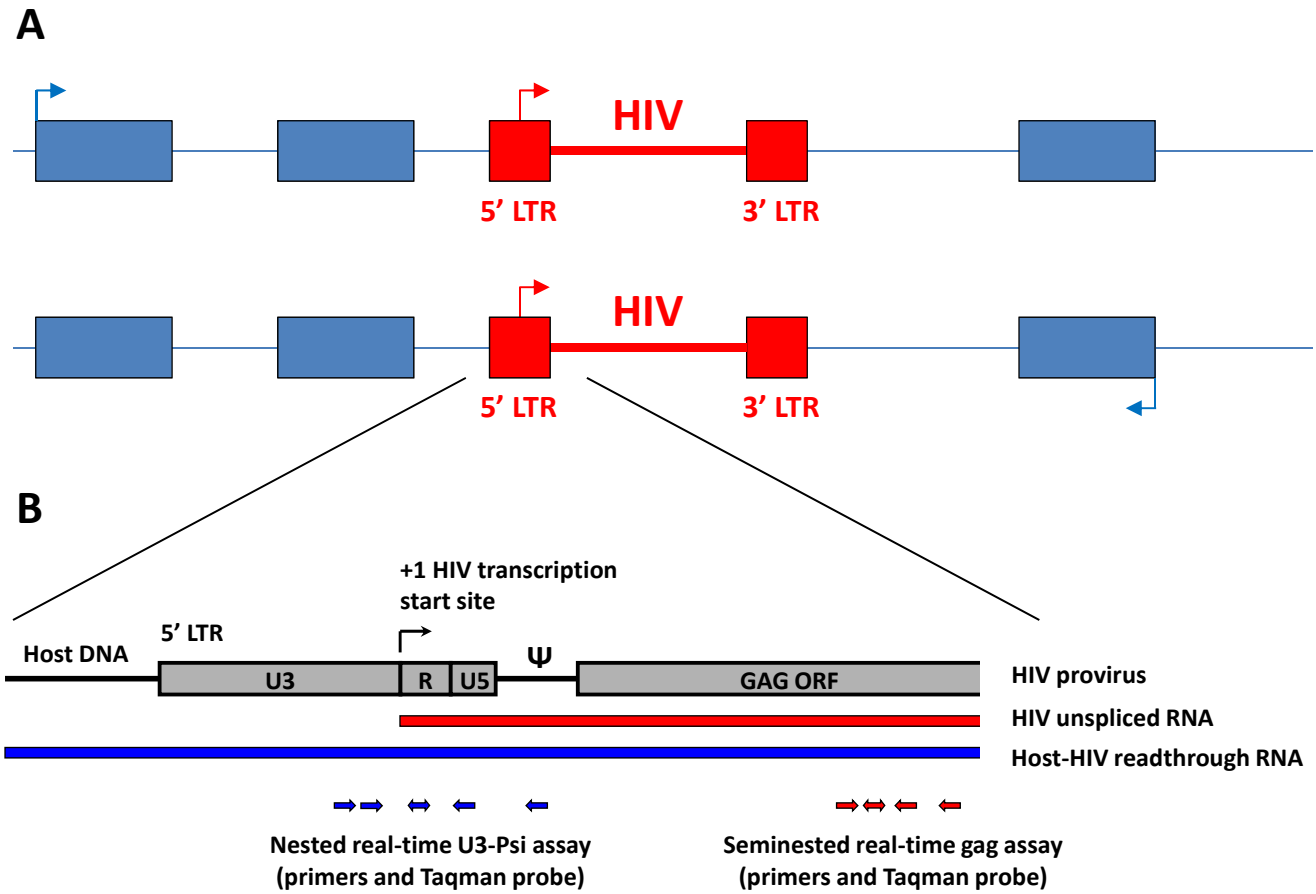
18. **Siliciano RF, Greene WC**. 2011. HIV latency. Cold Spring Harb Perspect Med **1**:a007096.

Figure Legends

Figure 1. (A) HIV proviruses integrate in intronic regions of transcriptionally active host genes, in the same (upper panel, sense) or opposite (lower panel, antisense) orientation with regard to local host gene transcription. (B) A close-up of the 5' HIV region, with a schematic representation of real-time PCR assays for detection of readthrough and *gag* RNA. LTR, long terminal repeat; ORF, open reading frame; Ψ, HIV packaging signal (Psi).

Figure 2. Quantitation of the serially diluted plasmid pLAIΔRT, which is a molecular clone of HIV-1 harboring a deletion of the reverse transcriptase gene (7), with the U3-Psi nested real-time PCR assay. Pre-amplification (15 cycles) was performed with the forward (5'-AGTGGCGAGCCCTCAGATG-3') and reverse (5'-CAGCAAGCCGAGTCCT-3') primers in a volume of 25 µl. Two microliters of this PCR reaction were used as input for a nested real-time PCR performed with the forward (5'-CAGATGCTGCATATAAGCAGCTG-3') and reverse (5'-

190 CACAACAGACGGGCACACAC-3') primers (10) and probe (5'-(FAM)-
191 GAGCTCTCTGGCTAACTAGGGAACCC-(TAMRA)-3') in a total volume of 50 µl.
192
193 **Figure 3.** Bland-Altman plot of the *gag* and U3-Psi HIV DNA measurements. Horizontal lines
194 indicate the average difference between the measurements, as well as average ± standard deviation.
195
196 **Figure 4.** Comparison of HIV-1 *gag* RNA and readthrough RNA: (A) in all patients, with
197 undetectable values left-censored at the detection limits of corresponding assays shown by open
198 circles, and (B) only in patients with detectable gag or readthrough RNA, (C) paired comparison in
199 patients with detectable readthrough RNA, and (D) percentage of readthrough RNA in the HIV-1
200 *gag* RNA: only patients with detectable readthrough RNA are shown. Medians are shown in (A) and
201 (B), and median and interquartile range is shown in (D).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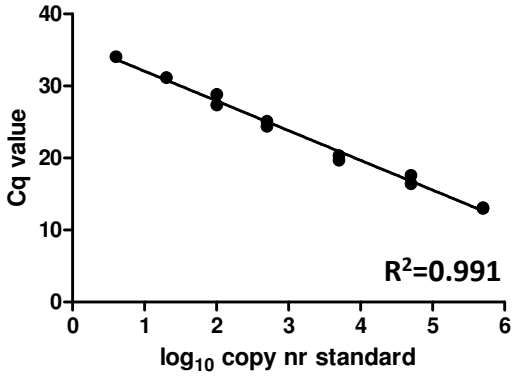
HIV
5’ LTR
3’ LTR
HIV
5’ LTR
3’ LTR
B
+1 HIV transcription
start site
5’ LTR
Host DNA
U3
R
U5
Ψ
GAG ORF
HIV provirus
HIV unspliced RNA
Host-HIV readthrough RNA
Nested real-time U3-Psi assay
(primers and Taqman probe)
Seminested real-time gag assay
(primers and Taqman probe)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Cq value
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2
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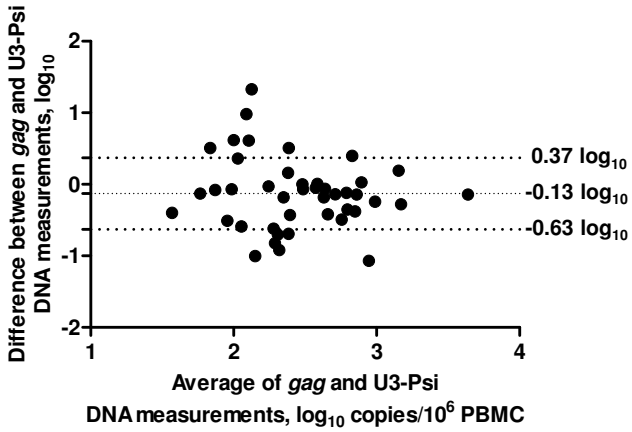
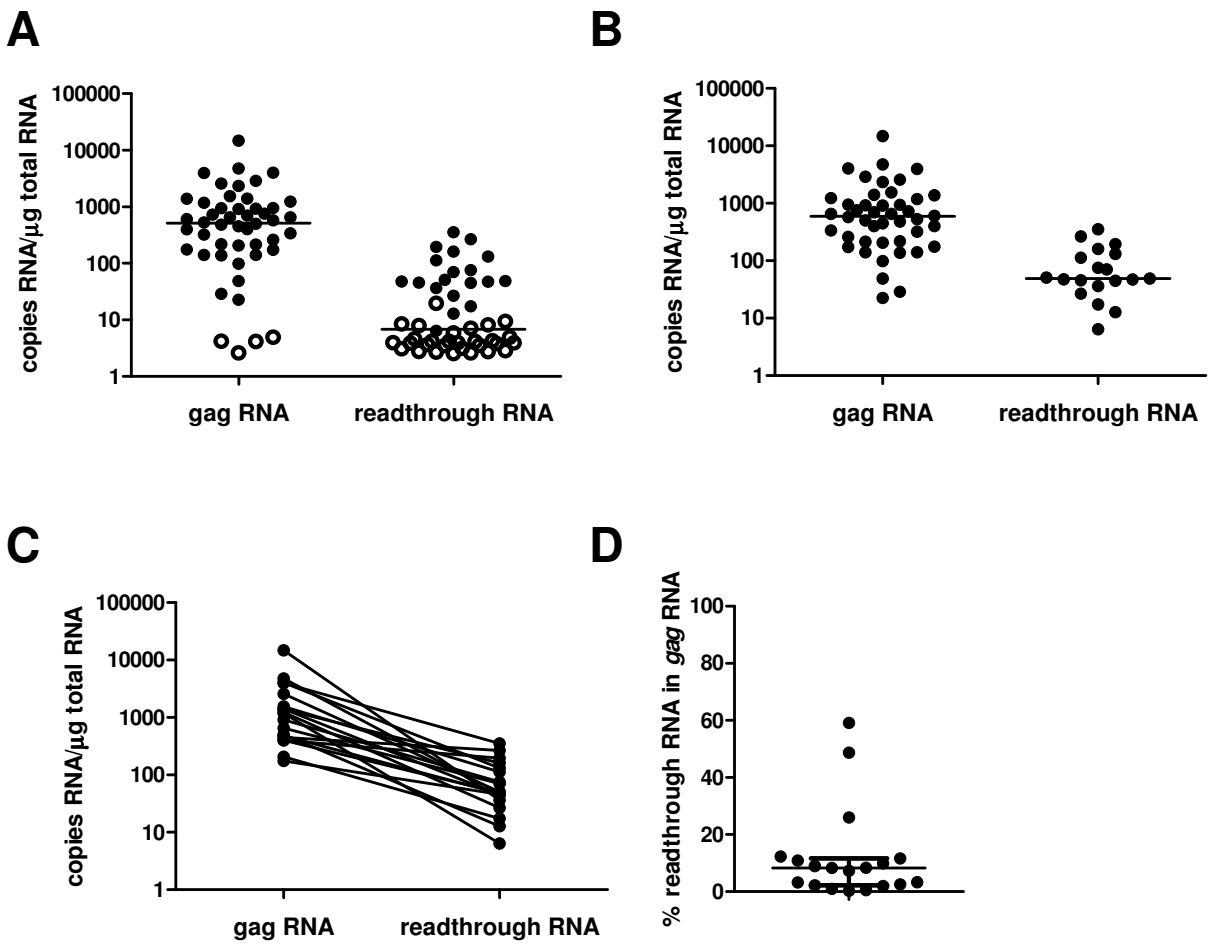
log10 copy nr standard
R2=0.991


Difference between gag and U3-Psi DNA measurements, log10
2
1
0
-1
-2
1
2
3
4
0.37 log10
-0.13 log10
-0.63 log10
Average of gag and U3-Psi DNA measurements, log10 copies/10^6 PBMC


A
copies RNA/µg total RNA
100000
10000
1000
100
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1
gag RNA
readthrough RNA
B
copies RNA/µg total RNA
100000
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1000
100
10
1
gag RNA
readthrough RNA
C
copies RNA/µg total RNA
100000
10000
1000
100
10
1
gag RNA
readthrough RNA
D
% readthrough RNA in gag RNA
100
80
60
40
20
0