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DOI
10.1128/JVI.02597-15

Publication date
2016

Document Version
Accepted author manuscript

Published in
Journal of Virology

Citation for published version (APA):
https://doi.org/10.1128/JVI.02597-15

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Minor Contribution of Host-HIV Readthrough Transcripts to the Level of HIV

Cell-associated gag RNA

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Running title: Host-HIV readthrough transcripts and HIV gag RNA

Word count abstract: 74
Word count text: 1.000
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
located 5’ of the HIV LTR transcription start site, this assay specifically detects host-HIV readthrough transcripts but not genuine HIV-1 unspliced RNA (Fig. 1B). The assay has a linear range of five orders of magnitude and the sensitivity of four copies per reaction (Fig. 2). For this study, we used peripheral blood mononuclear cells (PBMC) of 48 cART-treated patients visiting the HIV outpatient clinic of the Academic Medical Center in 2011-2013 and participating in the Co-morBidity in Relation to AIDS (COBRA) cohort, whose plasma viremia had been undetectable (<40 copies/ml) for a median of 7 years prior to the time of sampling. The median CD4+ T-cell count was 675.5 cells/mm³. Total DNA and total RNA were isolated from the patient PBMC using the Boom isolation method (11) and CA HIV DNA and RNA were separately quantified using both the U3-Psi assay and the seminested real-time PCR assay specific for the HIV gag region (Fig. 1B) (7,8). Cellular RNA was treated with DNase (DNA-free™ kit, Ambion®) to remove DNA that could interfere with the quantitation, and reverse transcribed using random primers and Superscript III reverse transcriptase (both Invitrogen). As HIV integrates in a random orientation with regard to the host genes, we used random primers to allow detection of readthrough RNA transcribed in both directions, from both upstream and downstream host promoters (Fig. 1A). Same-volume aliquots of the same DNA or cDNA preparations were used as input for U3-Psi and gag assays. HIV DNA and RNA were normalized to the cellular inputs as described previously (12).

As expected, both U3-Psi and gag assays detected HIV DNA in >90% of the patients (44/48 and 46/48, respectively) with no significant quantitative bias between the assays (0.13±0.50 log₁₀; P>0.05 for comparison of the difference to 0) (Fig. 3), and a highly significant correlation between the two measurements was observed (P=0.001), demonstrating the functionality of the U3-Psi assay. However, a major difference in detectability of HIV RNA was observed. HIV gag RNA was detected in 44/48 of these patients (92%) with a median copy number of 590 (interquartile range, 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.

Funding information
The Co-morBidity in Relation to AIDS (COBRA) collaboration has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement no 305522. A.O.P. is financially supported by the Dutch AIDS Fonds (http://www.aidsfonds.nl/), grant nrs. 2011020 and 2012025. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements
We are grateful to the COBRA collaboration for making available the samples from COBRA study participants. The study was approved by the ethics commission of the Academic Medical Center, conducted in accordance with the ethical principles of the Declaration of Helsinki, and all patients provided written informed consent.

References


**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’-
CACAACAGACGGGACACAC-3’) primers (10) and probe (5’-(FAM)-GAGCTCTCTGGCTAACTAGGGAACCC-(TAMRA)-3’) in a total volume of 50 µl.

**Figure 3.** Bland-Altman plot of the *gag* and U3-Psi HIV DNA measurements. Horizontal lines indicate the average difference between the measurements, as well as average ± standard deviation.

**Figure 4.** Comparison of HIV-1 *gag* RNA and readthrough RNA: (A) in all patients, with undetectable values left-censored at the detection limits of corresponding assays shown by open circles, and (B) only in patients with detectable gag or readthrough RNA, (C) paired comparison in patients with detectable readthrough RNA, and (D) percentage of readthrough RNA in the HIV-1 *gag* RNA: only patients with detectable readthrough RNA are shown. Medians are shown in (A) and (B), and median and interquartile range is shown in (D).
A

B

Host DNA

5' LTR

U3 R US

Ψ

GAG ORF

HIV provirus

HIV unspliced RNA

Host-HIV readthrough RNA

+1 HIV transcription start site

Nested real-time U3-Psi assay (primers and Taqman probe)

Seminested real-time gag assay (primers and Taqman probe)
$R^2 = 0.991$
Average of \( \text{gag} \) and U3-\( \Psi \) DNA measurements, \( \log_{10} \) copies/10\(^6\) PBMC

Difference between \( \text{gag} \) and U3-\( \Psi \) DNA measurements, \( \log_{10} \)