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Supplementary Information for

Defective HIV-1 proviruses produce viral proteins

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Supplementary Information Text

Supplemental Materials and Methods

Simultaneous isolation of HIV-1 DNA and HIV-1 RNA and cDNA synthesis

Total HIV-1 DNA and cell-associated HIV-1 RNA were simultaneously isolated from the same populations of $CD4^+$ T cells using the AllPrep DNA/RNA Mini Kit (Qiagen) following the protocol (including an on-column DNase digestion step) provided by the manufacturer. cDNA was synthesized from cellular RNA with the Superscript IV First-Strand cDNA Synthesis kit (Thermo Fisher Scientific) as previously described [1] using an anchored oligo$(dT)_{20}$ primer that consists of a string of 20 deoxythymidylic acid resides followed by five nucleotides (TGAAG) complementary to the R region of the HIV-1 3' LTR (Primer 5T25) [2].

Amplification of near full-length HIV-1 DNA and unspliced HIV-1 RNA

Genomic DNA and cDNA were subjected to limiting dilution prior to amplification as previously described [5]. Briefly, the PCR was performed with KAPA HiFi HotStart (KAPA Biosystems) with the initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 98 °C for 20 s, annealing at 65 °C for 15 s, and extension at 72 °C for 6 min, with the final extension at 72 °C for 6 min. PCR reactions were performed in volumes of 25 µL in 0.2 mL PCR microtubes. The final concentrations of both primers were 400 nM. Amplification of a near full-length HIV-DNA was conducted by 5'LTR-to-3'LTR PCR utilizing the following primer pairs: DNA F1 (HXB2 coordinates: 623-649): 5'-AAATCTCTAGCAGTGGCGCCCGAACAG-3' and DNA R1 (9662-9686): 5'-TGAGGGATCTCTAGTTACCAGAGTC-3' were used for the first round PCR; and DNA F2 (682-705): 5'-TCTCTCGACGCAGGACTCGGCTTG-3' and DNA R2 (9603-9632): 5'-GCACTCAAGGCAAGCTTTATTGAGGCTTA-3' were used for the second round PCR. For amplification of a near full-length unspliced HIV-RNA, RNA F1 (HXB2 coordinates: 760-777): 5'-TTTTGACTAGCGGAGGCT-3'

and RNA R1 (9603-9632): 5'-GCACTCAAGGCAAGCTTTATTGAGGCTTA-3' were used for the first round PCR; and RNA F2 (796-816): 5'-GCGAGAGCGTCAGTATTAAGC-3' and RNA R2' (9438-9458): 5'-GGAAAGTCCCCAGCGGAAAGT-3' were used for the second round PCR. The PCR was performed to achieve 30% or fewer positive nested PCR reactions, indicating a greater than 80% probability of containing a single amplified provirus [3]. The PCR products were purified with AMPure XP beads (Beckman Coulter) prior to sequencing.

Sequencing and Sequence analyses

Single molecules were sequenced as near-full-length amplicons directly from PCR products using the 3500xL Genetic Analyzer (Applied Biosystems by Thermo Fisher Scientific) with a BigDye Terminator v3.1 Cycle Sequencing Kit. During post-sequencing analyses of data, samples that contained more than one template (evidenced by having heterogeneous peaks on sequencing chromatograms or having degenerate sequences) were discarded and excluded from the further analyses. Direct sequencing results were analyzed with the HIVAlign program (www.hiv.lanl.gov/content/sequence/VIRALIGN/viralign.html)[4] in order to map the regions that the input sequences touched relative to the reference sequence HXB2 of HIV-1. Hypermutants were detected using the HyperMut program (www.hiv.lanl.gov/content/sequence/HYPERMUT/hypermut.html). The Highlighter program (www.hiv.lanl.gov/content/sequence/HIGHLIGHT/highlighter_top.html), AliView (v1.19-beta-3)[5] and the Sequencher program (v5.4, Gene Codes Corporation) were used to generate sequence alignments. The ORF finder program (www.ncbi.nlm.nih.gov/orffinder) was used to detect presence of valid open reading frames. The nucleotide sequences of HIV-1 isolates derived from this study have been submitted to GenBank. The authors declare no competing financial interests.

Analysis of HIV-1 integration sites

Analysis of integration sites was performed by an inverse PCR method [6] with a slight modification using BclI for digestion of genomic DNA [7]. Primer pairs used for the inverse PCR were -576 (antisense, HXB2 coordinates: 576-596): 5'-GATCTCTAGTTACCAGAGTCA-3' and +2165 (sense, 2165-2187): 5'-CAGAAGAGAGCTTCAGGTTTGGG-3' in a first round reaction; and -558 (antisense, 558-579): 5'-GTCACACAACAGACGGGCACAC-3' and +2208 (sense, 2208-2229): 5'-TCAGAAGCAGGAGCCGATAGAC-3'. The inverse PCR was performed with the KAPA HiFi HotStart (KAPA Biosystems). Each round of PCR consisted of 30 cycles, with the initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 98 °C for 20 s, annealing at 65 °C for 15 s, and extension at 72 °C for 1 min 30 sec, with the final extension at 72 °C for 5 min. The amplicons captured the junction between the 5' end of the HIV-1 genome and the host cell DNA. The DNA was sequenced as described in the methods section for Sequencing and Sequence analyses. The human genomic sequence in each inverse PCR product was identified as a unique best-hit by BLAT search at the UCSC Bioinformatics Human Genome database (http://www.genome.ucsc.edu; Dec. 2013: GRCh38/hg38). The integration site for the defective provirus in the P36-5 clone was determined by virtue of sequencing the whole genome (BGI Americas Corp).

A

		Copy no. of HTLV-1	Genomic DNA (ng)
HTLV-1 transformed cell line	MT-4	15,449	6
↓	ED	420	6
↓	ALT1	26,672	6
↓	MT-2	16,986	6
↓	SLB1	39,889	6
HTLV-1 negative cell line	Hut-78	<3	6
↓	Jurkat	<3	6
Test sample	H9MN uncloned	<3	6
↓	H9MN FI subclone	<3	6
↓	H9MN FD subclone	<3	6
↓	H9MN SD subclone	<3	6
↓	H9MN neg for HIV-1	<3	6

B

pX-S, 5'-CGGATACCCAGTCTACGTGTT-3'
pX-AS, 5'-CAGTAGGGCGTGACGATGTA-3'
pX2 probe, 5'-6FAM CTGTGTACAAGGCGACTGGTGCC BHQ1-3'

Table S1. The status of the HTLV-1in the H9MN subclones tested in the present study was assessed by quantitative PCR. (A) Quantitative PCR analysis was performed using TaqMan mix (Thermo Fisher Scientific), according to the manufacturer's instructions. (B) The HTLV-1 primer pairs and the probe used in this quantification are shown [8]. No signal appearing after >45 PCR cycles was considered a negative result and shown as <3 in the table. The cell lines MT-4, ED, ALT1, MT-2 and SLB1 were used as positive control cells. HUT78 and Jurkat cell lines were used as negative controls (HTLV-1-negative transformed T-cell lines). The qPCR data confirmed that our HUT78 was HTLV-1 negative; and that the H9MN subclones used in the current study were also negative for HTLV-1.

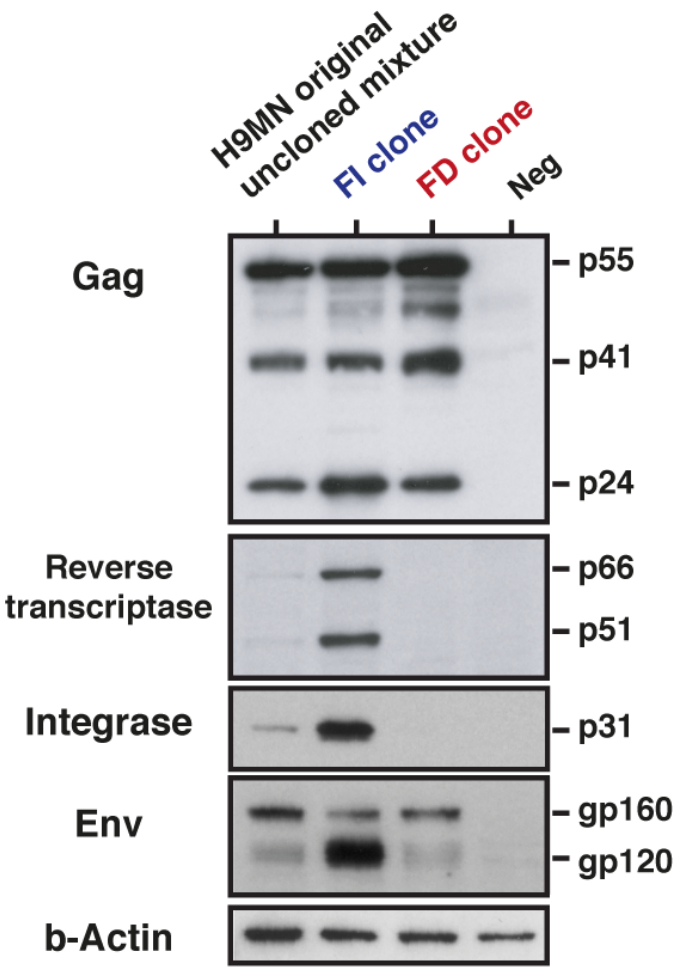
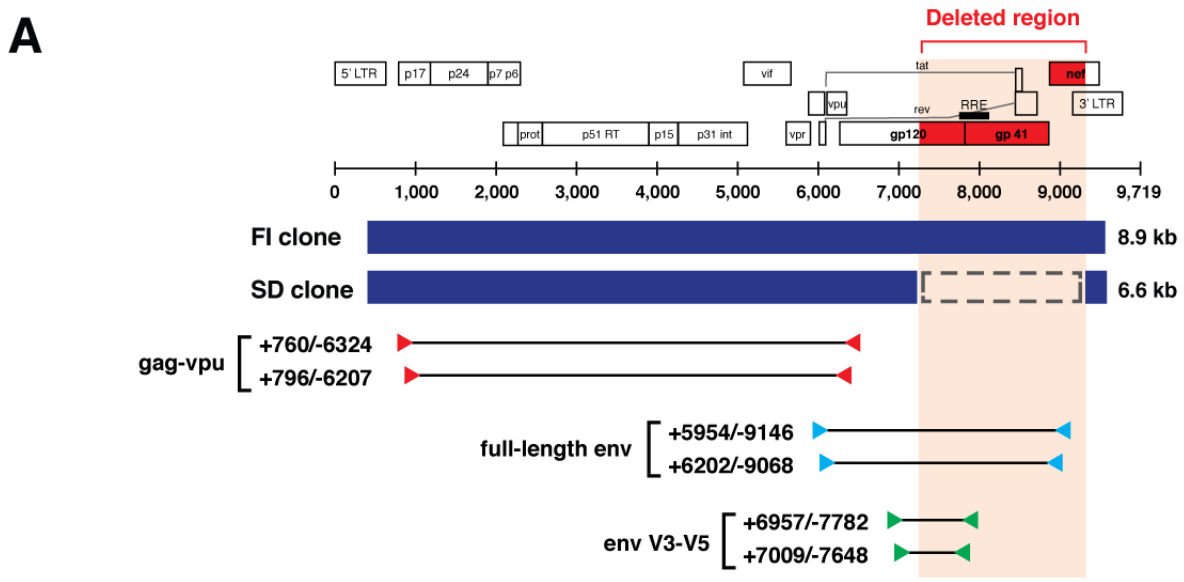


Fig. S1. Expression of HIV-1 proteins in three distinct H9MN single-cell clones determined by western blot: FI, a subclone containing an 8.9 kb full-length intact provirus; FD, a subclone containing an 8.9 kb full-length defective provirus with a 1bp frame-shift lethal mutation in the RT (reverse transcriptase) gene; Neg, a subclone negative for HIV-DNA. A mouse monoclonal antibody for HIV-1 p24 (clone: 39/5.4A, Abcam, ab9071), a rabbit polyclonal antibody for HIV-1 RT (Abcam, ab63911), a mouse monoclonal antibody for HIV-1 Integrase (clone: IN-2, Abcam, ab66645), and a goat polyclonal antibody for HIV-1 Env (Abcam, ab21179) were used. After detection of the target proteins, the membranes were stripped and reprobed with rabbit anti-beta-Actin antibody (Abcam, ab1801). Complete abolishment of the RT and integrase expression in FD clone was observed in the FD clone, as predicted from the DNA and RNA sequences from this clone. Of note, expression of the Gag and Env proteins was clearly demonstrated in the FD clone.



B

Name	HXB2 coordinates	Sequence (5' -> 3')
+760	760-777	TTTTGACTAGCGGAGGCT
-6324	6324-6352	GGTACCCCATAATAGACTGTGACCCACAA
+796	796-816	GCGAGAGCGTCAGTATTAAGC
-6207	6207-6231	CTCTCATTGCCACTGTCTTCTGCTC
+5954	5954-5982	GGCTTAGGCATCTCCTATGGCAGGAAGAA
-9146	9146-9171	CTGCCAATCAGGGAAGTAGCCTTGTGT
+6202	6202-6228	AGAAAGAGCAGAAGACAGTGGCAATGA
-9068	9068-9096	TAGCCCTTCCAGTCCCCCCTTTTCTTTTA
+6957	6958-6977	TACAATGTACACATGGAATT
-7782	7782-7811	AGTGCTTCCTGCTGCTCCCAAGAACCCAAG
+7009	7010-7029	TGGCAGTCTAGCAGAAGAAG
-7648	7648-7668	CACTTCTCCAATTGTCCCTCA

C

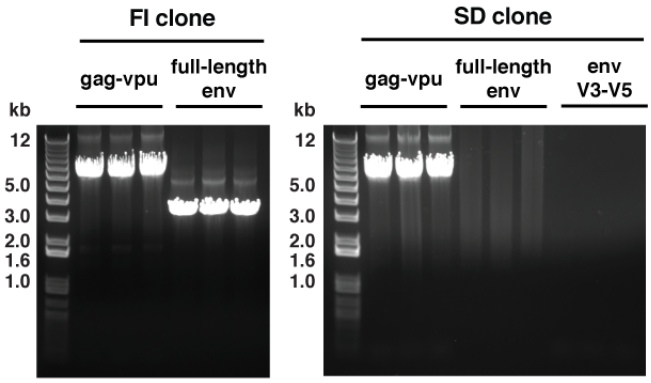


Fig. S2. To rule out the possibility of viral protein production from contaminant full-length proviruses in putative T-cell clones harboring truncated "defective" proviruses with large internal deletions, the absence of full-length proviruses was confirmed by a nested PCR that specifically amplified the env region of HIV-1 genome. (A) Locations of primer pairs used for the nested PCR are shown. (B) Sequences of HIV-1 primers. (C), Agarose gel pictures depicting sizes of HIV-DNA PCR fragments generated for the FI (full-length intact provirus) and SD (short defective provirus). The HIV-DNA sequence in the SD clone contained a large internal deletion (approximately 2.3 kb) affecting the gp41 and *nef* coding regions. The failure of the env PCR primer pairs to amplify HIV-DNA confirmed the absence of contaminant full-length proviruses.

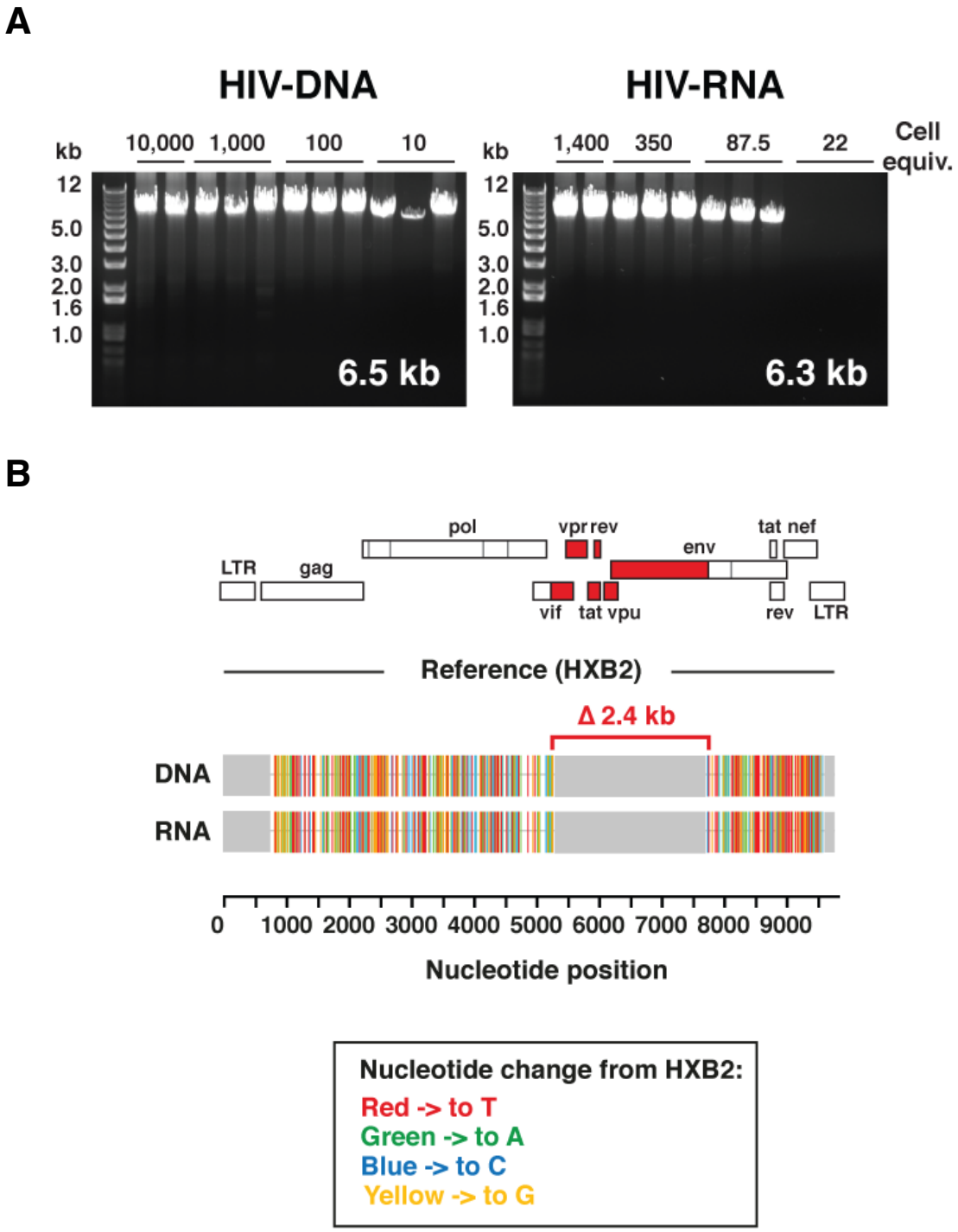


Fig. S3. (A) Agarose gel pictures depicting the sizes of HIV-DNA (6.5 kb) and HIV-RNA (6.3 kb) PCR fragments generated from the P36-5 clone using 5'LTR-to-3'LTR PCR. (B) HIV-RNA sequences corresponded precisely to the HIV-DNA sequences for the P36-5 clone (same as Fig. 4A)

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