

Hairpin-induced tRNA-mediated (HITME) recombination in HIV-1

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ABSTRACT

Recombination due to template switching during reverse transcription is a major source of genetic variability in retroviruses. In the present study we forced a recombination event in human immunodeficiency virus type 1 (HIV-1) by electroporation of T cells with DNA from a molecular HIV-1 clone that has a 300 bp long hairpin structure in the Nef gene (HIV-1hNef). HIV-1hNef does not replicate, but replication-competent escape variants emerged in four independent cultures. The major part of the hairpin was deleted in all escape viruses. In three cases, the hairpin deletion was linked to patch insertion of tRNA^{asp}, tRNA^{glu} or tRNA^{trp} sequences. The tRNAs were inserted in the viral genome in the anti-sense orientation, indicating that tRNA-mediated recombination occurred during minus-strand DNA synthesis. We here propose a mechanistic model for this hairpin-induced tRNA-mediated (HITME) recombination. The transient role of the cellular tRNA molecule as enhancer of retroviral recombination is illustrated by the eventual removal of inserted tRNA sequences by a subsequent recombination/deletion event.

INTRODUCTION

The HIV-1 RNA genome is packaged into virus particles as a dimer. After virus entry into a host cell, the genomic RNA is reverse transcribed into dsDNA. This process is mediated by the virion-associated enzyme reverse transcriptase (RT) and a cellular tRNA is used as a primer that binds to a complementary sequence in the viral genome, referred to as the primer-binding site (PBS) (1). Although HIV-1 particles contain a subset of cellular tRNAs, only tRNA^{lys3} is found in tight association with the viral RNA (vRNA) due to base pairing with the PBS (2,3).

During reverse transcription, the nascent DNA has to dissociate from the 5' end of the RNA template and reanneal to a repeat (R) sequence at the 3' end of the vRNA (4). A second strand transfer is needed for the completion of reverse transcription. The RT therefore possesses a relatively low affinity for its template RNA and a low processivity (5). RT frequently undergoes intra- or intermolecular template switching, which will result in sequence deletion or duplication when executed imprecisely at non-homologous (without sequence identity) donor and acceptor sites. Intermolecular template switching can also result in homologous recombination when the RT switches template at homologous donor and acceptor sites (6,7).

The original forced-copy choice model of recombination proposed that stalling of RT at certain sequence or structure motifs may increase the probability of template switching (8). A more recently proposed dynamic-copy choice model of recombination states that the steady state between the rates of DNA polymerization during minus-strand synthesis and RNA degradation determines the frequency of RT template switching (9). Analysis of the requirements for RT template switching *in vitro* and *in vivo* has revealed that sequence similarity at the donor and acceptor sites greatly facilitates recombination (10–14). Deletion studies in HIV-1, spleen necrosis virus and murine leukemia virus have shown that the size of the direct repeats and the distance between them influence the rate of template switching (7,12,15–19). We and others have shown that RNA templates with hairpin structures could favour RT stalling and template switching (14,19–22). For instance, Beerens *et al.* stabilized a hairpin structure in the untranslated leader of the HIV-1 genome. Virus replication defects were imposed by hairpins with a perfect stem of 14 or more base pairs, reflecting a local thermodynamic stability of $\Delta G = -42.3$ kcal/mol (20).

To study RT-mediated template switching, we forced a recombination event by introducing an extended 300 bp long hairpin in the HIV-1 Nef gene (1hNef). This unnatural hairpin is extremely stable ($\Delta G = -648.2$ kcal/mol) and therefore induces a severe replication defect. We subsequently selected several replication-competent escape viruses with

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deletions in the lhNef hairpin, some of which had acquired tRNA sequences. Cellular tRNAs may be preferred recombination partners due to their accessible CCA 3' end. We propose a novel hairpin-induced tRNA-mediated (HITME) recombination mechanism to explain non-homologous recombination in HIV-1.

MATERIALS AND METHODS

DNA constructs and proviral DNA analysis

The full-length molecular HIV-1 clone LAI (23) was used to produce wild-type virus. DNA of LAI was subjected to PCR with primers BamHI-JvdV174 (CTAGTGGATCCTTAGCACTTATCTGGGACG, +8067 to +8091) and JvdV175 (CCAGATAACTTATAGCAAAATCCTTTCCAAGCCC, +8388 to +8363), yielding a 330 bp fragment corresponding with the 3' end of the env gene, and primers XhoI-JvdV176 (CCCCTCGAGTGTCTGCTTGTGCCTGGCTAGAAGCACAAAGAGG, +8544 to +8576) and JvdV177 (GCTATAAGTTATCTGGCTCAACTGGTACTAGCTTGTAGC, +8844 to +8813), yielding a 300 bp fragment corresponding to the central part of the Nef gene. Nucleotide numbers refer to the position on the genomic HIV-1 RNA transcript, with +1 being the capped G residue. HIV-1 sequences are underlined and restriction sites are in italics. Both DNA fragments were mixed and subjected to a fusion PCR with primers BamHI-JvdV174 and XhoI-JvdV176, yielding a 630 bp fragment. Because of the partial sequence complementarity between primers JvdV175 and JvdV177, it was possible to invert the Nef sequences (+8544 to +8844) in the fusion PCR. The BamHI-JvdV174/XhoI-JvdV176 PCR product was digested with XhoI and BamHI and cloned into the corresponding sites of Blue-3' LTR (24), resulting in 3' LTR-lhNef. The BamHI-BglII fragment of 3' LTR-lhNef was cloned into the corresponding sites of LAI, yielding HIV-long-hairpin-Nef (HIV-lhNef). HIV-lhNef has a 93 nt deletion in the 5' region of Nef and a 300 bp inverted repeat within the Nef gene (Figure 1).

For cellular DNA isolation, cells were pelleted for 4 min at 4000 r.p.m. and solubilized in 150 μ l lysis buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5% Tween-20] and 200 μ g/ml proteinase K (Roche) at 56°C for 1 h and at 95°C for 10 min. Proviral DNA sequences were PCR amplified from 5 μ l cellular lysate using the 5' Env primer tTA1-AD (+8269 to +8289) and 3' U5 primer CN1 (+9283 to +9253) (Figure 1). PCR amplification was performed in a 50 μ l reaction containing 1 \times PCR amplification buffer (Invitrogen), 0.5 mM MgCl₂, 25 pmol of each primer, 0.2 mM dNTPs and 2.5 U of AmpliTaq DNA polymerase (Perkin Elmer Applied Biosystem). The PCR program was as follows: 95°C for 5 min, 30 cycles of 30 s at 94°C, 30 s at 55°C, 30 s at 72°C and a final extension for 7 min at 72°C. The PCR products were separated on a 1% agarose gel, stained with EtBr and compared to a standard DNA size marker (Eurogentec). PCR products were excised from gel, purified with the QIAquick gel extraction kit (Qiagen) and cloned into pCR2.1 TOPO vector (Invitrogen) or sequenced directly with the same primer pair using the BigDye Terminator v1.1 Cycle Sequencing Kit (Perkin Elmer Applied Biosystem). HIV-lhNef could not be amplified

with this PCR due to the extended inverted repeats that may form a cruciform DNA structure.

To create molecular clones of HIV-lhNef escape variants, tTA1/CN1 PCR fragments were digested with XhoI and BamHI and cloned into the corresponding sites of the plasmid Blue-3' LTR. The XhoI-BglII fragments of these plasmids were used to replace the wild-type sequence in LAI, resulting in the molecular clones AS44, AS19, AS15, AS11 and AS8.

The RNA structures formed by HIV-lhNef and the AS escape variants was predicted with the Mfold program (25) at <http://www.bioinfo.rpi.edu/applications/mfold>.

Cells, DNA transfection and virus infection

The human T cell line SupT1 was cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% FCS (Hybond), penicillin (100 U/ml) and streptomycin (100 μ g/ml) at 37°C and 5% CO₂. SupT1 cells (2.5×10^6) were electroporated with different amounts of DNA from HIV (LAI clone), HIV-lhNef, or the AS molecular clones as described previously (26). Cells were cultured in 25 cm² flasks and split 1 to 10 twice a week. To select escape virus variants, cultures were maintained for up to 73 days. When HIV-induced cytopathic effects were observed, virus replication was maintained by passage of the cell-free culture supernatant onto uninfected SupT1 cells.

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats by standard Ficoll-Hypaque density centrifugation, activated with phytohemagglutinin (3 μ g/ml) and cultured in complete RPMI medium with IL-2 (100 U/ml). Cells (5×10^6) were transfected by electroporation in 250 μ l RPMI with 20% FCS in 0.4 cm cuvettes at 250 V and 960 μ F, and 1×10^6 fresh cells and 5 ml complete RPMI with IL-2 were added afterwards. After 3–4 days half of the culture medium was replaced by fresh complete RPMI medium with IL-2.

Human embryonic kidney (HEK) 293T cells were grown as a monolayer in DMEM (Invitrogen) supplemented with 10% FCS, minimal essential medium nonessential amino acids, penicillin (100 U/ml) and streptomycin (100 μ g/ml) at 37°C and 5% CO₂. One day before transfection, cells were trypsinized, resuspended in DMEM and seeded in 24-well plates at a density of 1.5×10^5 cells per well. Cells were co-transfected with 500 ng proviral DNA with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

SupT1 cells were infected with an equal amount (5 ng CA-p24) of 293T-produced viruses. Virus was passaged at the peak of infection as witnessed by massive syncytia. At each passage, cell and supernatant samples were stored at -70°C. Virus spread was followed by CA-p24 enzyme-linked immunosorbent assay (ELISA) on the culture supernatant as described previously (27).

RESULTS

Construction and testing of HIV-lhNef

We tried to induce non-homologous recombination in HIV-1 by insertion of a highly stable hairpin structure into the vRNA genome. For this reason, the HIV-1 molecular clone LAI was modified by adding an ~300 nt Nef sequence (+8844 to +8544) in antisense polarity upstream of the cognate sense Nef sequence (antisense Nef, asNef), but downstream of the

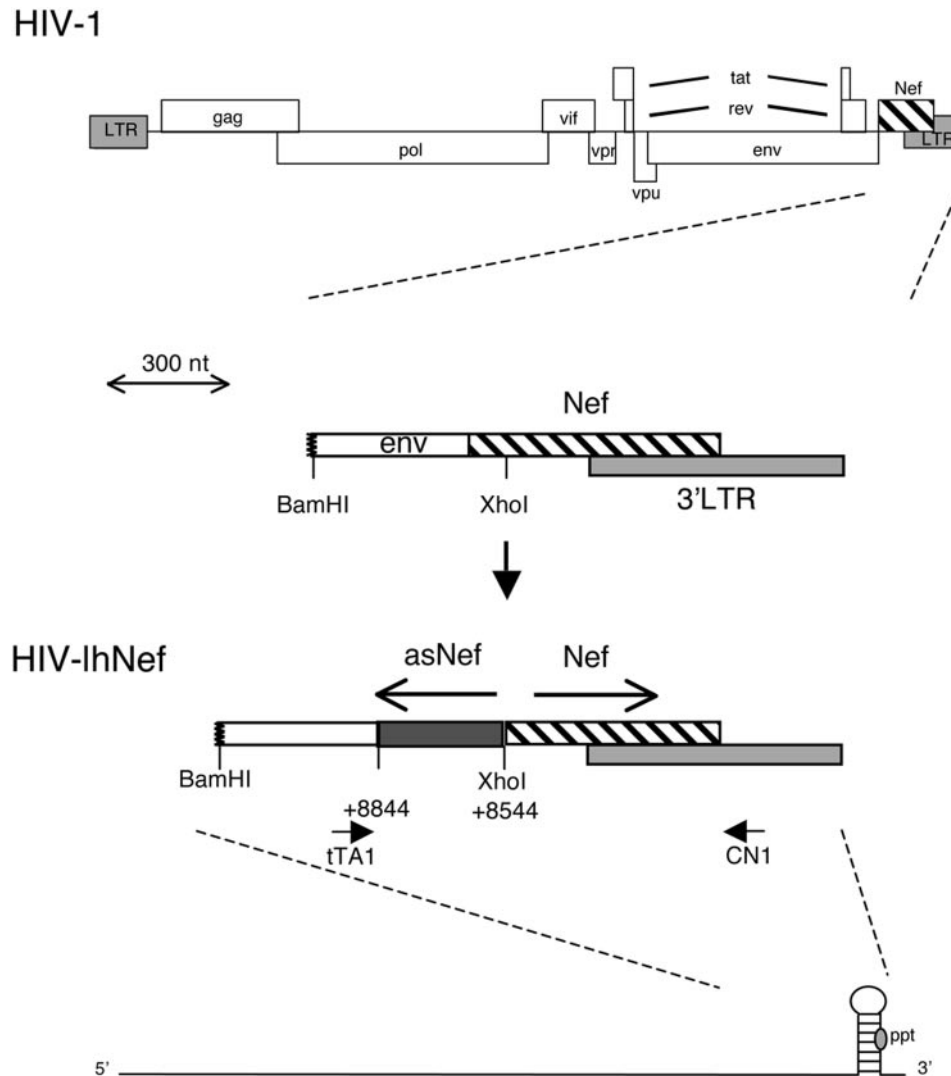


Figure 1. Design of the HIV-1lhNef construct. Schematic of the HIV-1 DNA genome with the insertion of the antisense asNef fragment (dark grey box, original Nef coordinates +8544 to +8844). Nucleotide numbers refer to the position on the genomic HIV-1 RNA transcript, with +1 being the capped G residue. The insert results in the formation of a 300 bp hairpin structure in the RNA genome of HIV-1lhNef. This hairpin encompasses the ppt. The 5' end of the Nef gene (hatched box) is deleted in the construction of HIV-1lhNef. The large arrows indicate the complementary asNef and Nef sequences, the small arrows the primers used to amplify the inverted repeat region.

Env gene (Figure 1). In this HIV-long-hairpin-Nef (HIV-1lhNef) variant, 93 nt from the 5' end of the Nef gene—including the AUG start codon—were deleted, thereby interrupting the Nef coding potential. The Nef protein, however, is not essential for virus replication *in vitro*. The hairpin structure in HIV-1lhNef is present in the full-length genomic RNA and in all spliced viral mRNAs. This was done to maximize the chance of inducing a severe replication defect of HIV-1lhNef.

To study the replication potential of HIV-1lhNef, proviral DNA (1–20 μg) was transfected into SupT1 T cells. As a control, cells were transfected with the wild-type HIV-1 (10 μg). SupT1 cells express the CD4 receptor and CXCR4 co-receptor and are fully susceptible for HIV-1 replication. Whereas the wild-type HIV-1 replicated efficiently, resulting in a high CA-p24 level and syncytia formation within 4 days after transfection, no virus replication was initially detected for HIV-1lhNef (Figure 2A). These results demonstrate that the

hairpin effectively interferes with one or multiple replication steps. However, after prolonged culturing, replication-competent variants could be selected in the cultures started with 5–20 μg DNA (cultures A–D), but not in culture E, which was started with 1 μg of DNA. Virus-induced syncytia were first observed at day 11 in cultures A and B (20 and 10 μg DNA) and at day 16 in cultures C and D (5 μg DNA). All HIV-1lhNef escape variants could be passaged to uninfected SupT1 cells and were cultured for up to 73 days.

Description of HIV-1lhNef escape variants

To determine the sequence of the HIV-1lhNef escape variants, proviral DNA from cell samples collected at different times after transfection was PCR amplified with primers flanking the lhNef region (Figure 1). The input HIV-1lhNef construct could not be amplified with this PCR due to the 300 bp long hairpin structure. The size of the expected PCR product (1213 nt) is

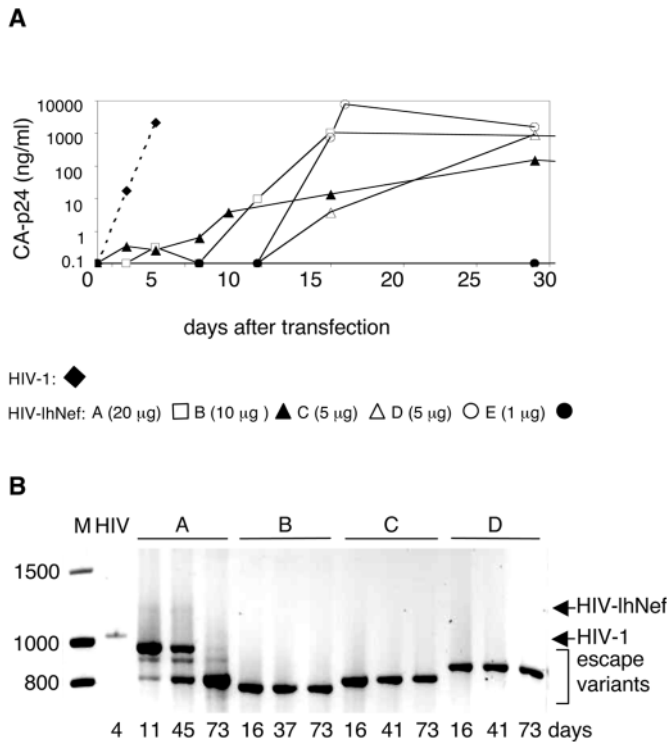


Figure 2. Evolution of the replication-impaired HIV-lhNef virus. (A) SupT1 cells were transfected with different amounts of HIV-lhNef (culture A, 20 μ g; B, 10 μ g; C, 5 μ g; D, 5 μ g; E, 1 μ g) and as a control 10 μ g of the wild-type HIV-1 molecular clone LAI. To select for replication-competent escape variants, cultures were maintained for 73 days. Virus spread was followed by CA-p24 ELISA of the culture supernatant. Virus-induced syncytia were first observed at day 11 in cultures A and B (20 and 10 μ g HIV-lhNef) and day 16 in cultures C and D (both 5 μ g). (B) Cell samples were taken at different time points for analysis of the proviral DNA by PCR across the hairpin insert with primers tTA1 and CN1 (see Figure 1). The amplification products were analysed by 1% agarose gel electrophoresis. The culture is indicated above and the day of harvest below the gel. M is a Smart DNA Ladder (Eurogentec). HIV-1 is the wild-type LAI control. HIV-lhNef could not be amplified with this PCR due to the long hairpin structure in its genome. Indicated is the position of the expected PCR product.

indicated in Figure 2B. The escape variants yielded a PCR product much shorter than that of the predicted HIV-lhNef product, indicating that large deletions occurred in the lhNef region. The four independent virus cultures yielded PCR products of different sizes, indicating the emergence of a distinct deletion mutant in each cell culture. In one culture, two replication-competent virus variants were present until 45 days post-transfection, of which the variant with the shorter PCR product predominated the culture at day 73 (Figure 2B, culture A).

Sequence analysis revealed that all replication-competent escape viruses had acquired deletions in the asNef sequence and the Nef coding region upstream of the polypurine tract (ppt). Thus, the hairpin structure had been removed through non-homologous recombination. A graphical presentation of all lhNef deletions is shown in Figure 3. We named the replication-competent escape virus variants according to the size of the remaining asNef sequence (AS), or more precisely the number of base pairs in the RNA hairpin that remains after recombination (Figure 3, e.g. AS19 is capable of forming a 19 bp long hairpin structure). The thermodynamic stability

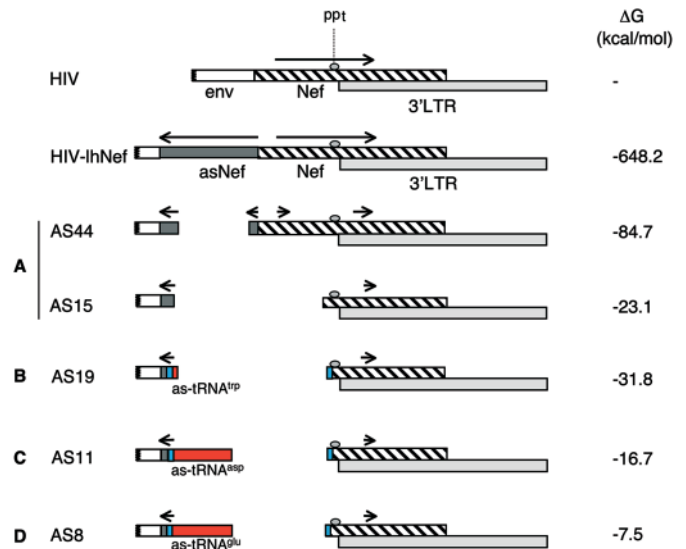


Figure 3. Deletions acquired in the HIV-lhNef escape variants. The name of the escape viruses reflects the number of base pairs in the remaining RNA hairpin. The ΔG column shows the thermodynamic stability score of the perfectly basepaired stem segment. Upstream Env gene sequences are in white; asNef is dark grey; Nef is hatched; 3' LTR is light grey. Arrows indicate the complementarity between Nef and asNef. The remaining asNef sequences in variant AS44 are capable of forming an additional 9 bp long stem structure in the central junction as indicated with additional arrows in the figure. These additional base pairs are not indicated in the name of AS44. Human tRNA sequences are inserted in antisense orientation at the recombination junctions in AS11, AS8 and AS19. The tRNA-inserts are marked in red, the blue flanks highlight sequence homology with the HIV-lhNef genome.

(ΔG) of the remaining perfectly base paired RNA stem is listed in Figure 3. These combined results suggest that a hairpin structure of -84.7 kcal/mol (AS44) is compatible with virus replication. However, this variant is outcompeted by variant AS15 with a larger deletion and consequently a less stable hairpin of -23.1 kcal/mol. All escape virus variants have intact ppt and 3' LTRs, which are essential *cis*-acting regulatory elements for virus replication. The deletions in all AS variants resulted in truncation of the Nef open reading frame, but Nef translation was already abrogated in HIV-lhNef.

The upstream and downstream recombination junctions of all HIV-lhNef escape viruses are depicted in Figure 4. The number within the deleted segments indicates the length of the deletion, which ranges from 237 nt in AS44 to 462 nt in AS11. Escape variant AS44 evolved further into AS15 and both are presented in the same alignment. The sequences were analysed for direct repeats at the junction sites that could potentially have been used for non-homologous recombination. In AS44 a CA sequence at the junction site (underlined in Figure 4) may have facilitated partial annealing of the nascent DNA strand to the acceptor site during minus-strand DNA synthesis, suggesting that deletion of lhNef sequences in this variant resulted from a non-homologous recombination event.

In AS11 and AS8, the hairpin deletion was linked to 63 and 65 nt patch insertions in the viral genome. A BLAST search of the inserts in the NCBI database showed nearly perfect homology to human tRNA^{asp} and tRNA^{glu} sequences, respectively. The tRNAs were inserted in the viral genome in the antisense orientation (red boxes in Figure 3). There were at least five

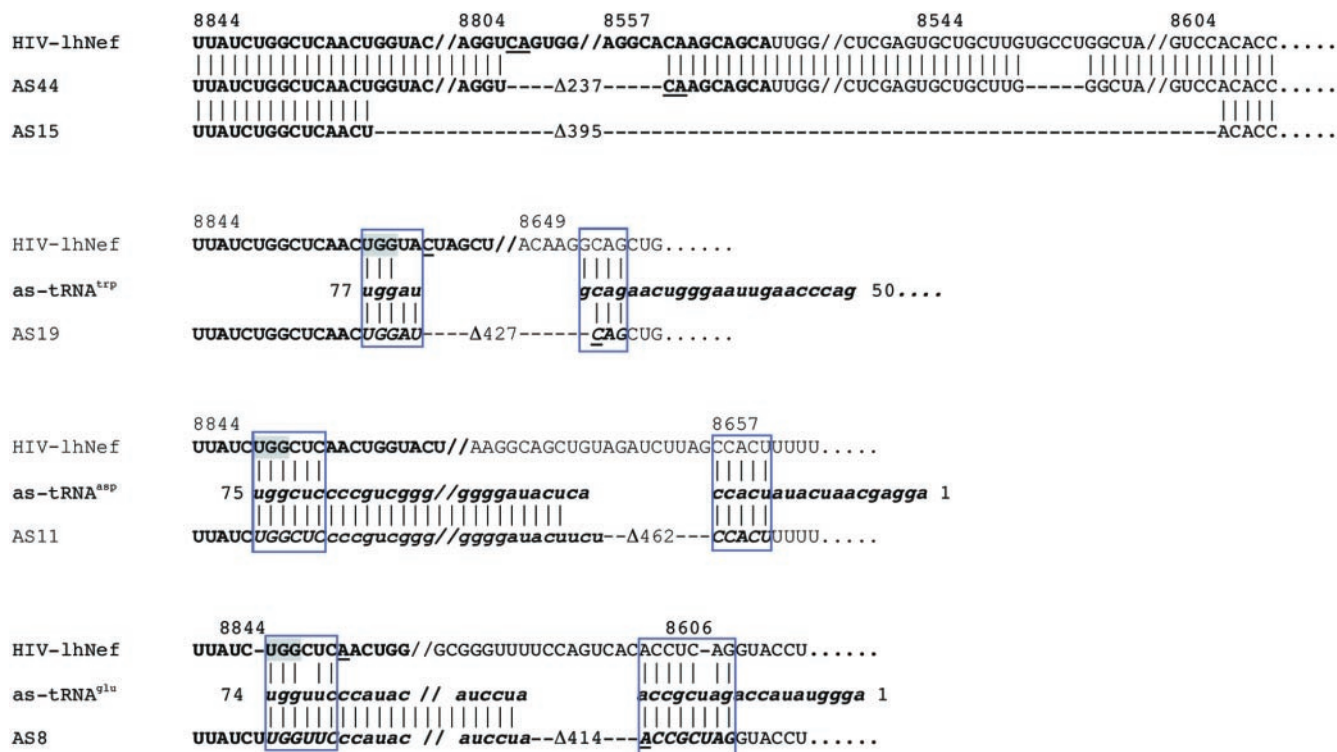


Figure 4. Alignment of recombination junctions between HIV-1hNef and AS escape variants. In all alignments, the upper sequence represents HIV-1hNef and the lower sequence the AS escape variant. Escape variant AS44 evolved further into AS15 and both are presented in the same alignment. The middle sequence in the AS11, AS8 and AS19 alignments is the antisense sequence of the tRNA that is inserted in the viral genome. Homologous sequences between the tRNA and HIV-1hNef are boxed in blue, the UGG sequences at recombination junctions are shaded in grey, repeated sequences at the recombination junctions of HIV-1hNef and the AS mutants are underlined. The vertical lines between the sequences indicate identity. Slashes represent sequences that are not shown. Deleted sequences are presented as dashes, asNef sequences are bold caps, Nef sequences are normal caps, tRNA sequences are bold lower-case italics. Nucleotide numbers above the upper sequence refer to the position on the genomic HIV-1 RNA transcript, with +1 being the capped G residue. Numbers in the middle sequence of the lower three panels refer to tRNA positions.

homologous nucleotides between the tRNA and the HIV genome, both at the 5' and 3' recombination junctions (blue boxes in Figures 3 and 4). A triple mismatch was apparent at the downstream HIV-1/tRNA^{asp} junction, the other three junctions were exact. Both tRNAs were apparently copied up to their 3'-CCA end (UGG sequence in Figure 4).

The AS19 evolution mechanism requires a closer inspection. At first glance, one might think that an RT-slippage event caused the deletion since the virus has not acquired a sequence insertion. However, we observed two special features at the recombination junction: a UGG motif (as observed in AS11 and AS8) and a 2 nt mismatch with both the donor and acceptor sequences. We therefore speculate that this could represent another tRNA-mediated recombination event. Inspection of the tRNA database indicated that tRNA^{trp} is the likely candidate primer in this scenario because it explains both the UGG sequence and the 2 nt mismatch. In fact, this tRNA primer does not just make a 5 nt match at the 5' recombination junction, but also a 4 nt match at the 3' junction (illustrated by blue boxes in Figures 3 and 4). The total match of 9 nt makes it very likely that tRNA^{trp} was used in the evolution event that created AS19. Thus, tRNA-mediated recombination occurred in three out of four evolution experiments.

The three tRNA sequences that were inserted in the HIV-1 RNA genome are presented in Figure 5. Marked in red is the region of the molecules that was actually inserted in the

antisense orientation in viral genome. The tRNA regions complementary to the viral RNA sequence are boxed in blue. Very similar regions of the tRNA^{asp} and tRNA^{glu} were inserted, starting in the D stem-loop region and running up to the extreme 3' end CCA of the mature tRNA species. A minimal tRNA^{trp} sequence was present in AS19, which was also extending to the extreme 3' CCA end.

Mechanistic models for tRNA-mediated recombination in HIV-1

The structure of the AS19, AS11 and AS8 escape viruses suggests a function of a tRNA molecule as recombination partner during reverse transcription. We propose two alternative models for this hairpin-induced tRNA-mediated (HITME) recombination, which are either based on (i) a second tRNA priming event during minus-strand DNA synthesis, or (ii) a template switch during plus-strand synthesis.

The first model of second tRNA priming is shown in Figure 6. The second tRNA primer is marked in red and complementary nucleotides between this tRNA and the HIV-1hNef genome are highlighted by blue boxes. Reverse transcription is initiated by the tRNA^{lys3} primer that is annealed to the PBS near the 5' end of the vRNA genome. RT synthesizes the strong-stop minus-strand DNA (ss-DNA) by copying the 5' R-U5 region. Subsequently, the ss-DNA is

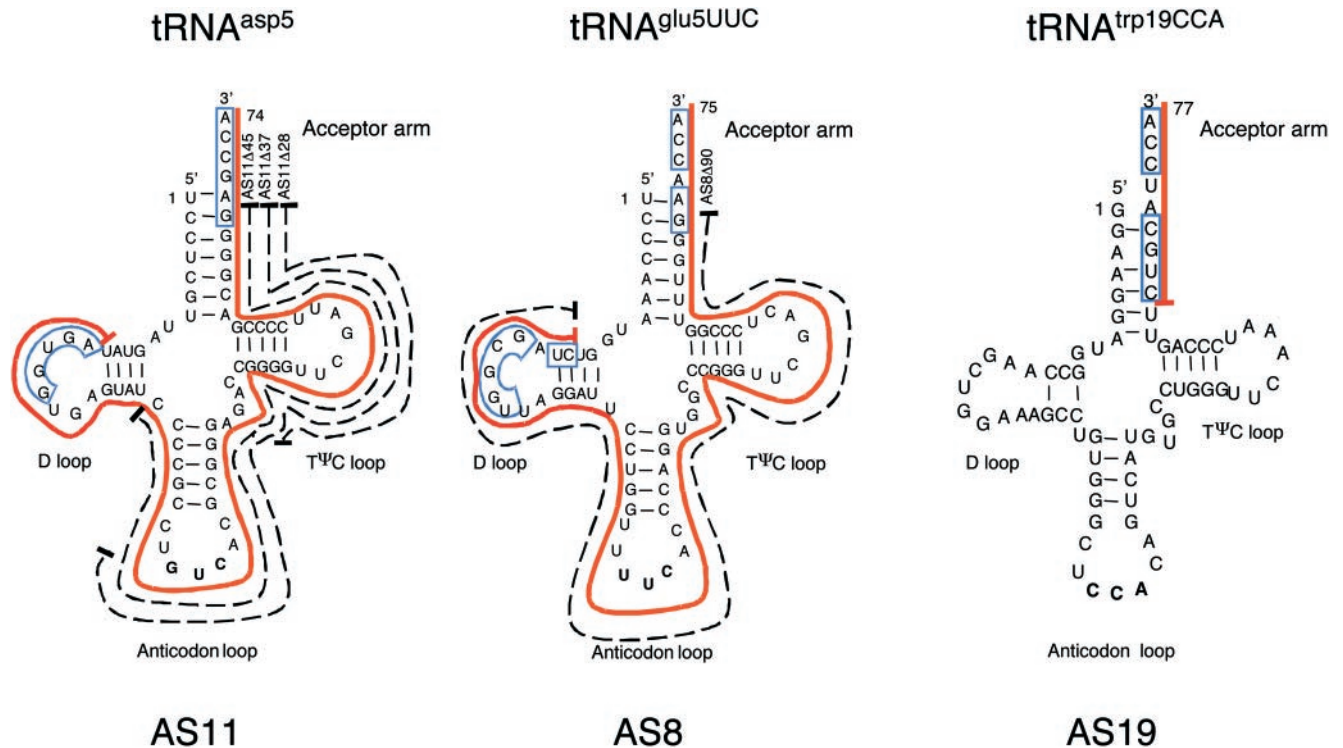


Figure 5. Cloverleaf structure of tRNA^{asp}, tRNA^{glu} and tRNA^{trp}. The human tRNA sequences were obtained from the tRNAscan-SE genomic tRNA database (<http://lowelab.ucsc.edu/GtRNAdb/credits-citation.html>): tRNA^{5-Asp} (GD0002843, chromosome 2), tRNA^{5-GluUUC} (GE0002900, chromosome 13) and tRNA^{19-TrpCCA} (Sc: 21.17, chromosome 11). The anticodon triplet is indicated in boldface. The red line indicates the sequence that is inserted in the HIV-1hNef escape variants. Homologous nucleotides between HIV-1hNef and the tRNA are boxed in blue. The dashed lines indicate the subsequent deletions in the tRNA insert observed during further evolution of the AS escape variants (see Figure 9 for details).

translocated to the homologous 3' R sequence (first strand transfer) and reverse transcription continues. The long hairpin in the vRNA template will cause pausing of the RT enzyme. Rescue of reverse transcription may be triggered by a tRNA that has some sequence complementarity with the vRNA, and that can act as a second primer for minus-strand DNA synthesis. We assume that the ppt sequence is copied by RT, even though this motif is part of the hairpin structure. The ppt of the vRNA primes synthesis of strong-stop plus-strand DNA (ss+DNA), which is translocated to the 5' end of the minus-strand cDNA in the second strand transfer. Upon continued plus-strand DNA synthesis the second tRNA primer is copied. Sequence complementarity between this DNA copy of the second tRNA primer and the minus-strand DNA facilitates a third strand transfer that is needed to complete reverse transcription. The non-natural priming and strand transfer events result in insertion of the tRNA sequence into the viral genome and deletion of the lhNef sequence. In support of this second tRNA priming model is the observation that the 5' end of the inserted sequence (5' UGG) is complementary to the 3' end of the tRNA primer (3'-CCA).

The second model involves a template switch during plus-strand synthesis (Figure 7). This model assumes that RT is able to copy the extended hairpin in the vRNA template during minus-strand synthesis, but the enzyme is paused by the hairpin during plus-strand DNA synthesis. A tRNA is subsequently used by RT as a transient template to bypass the hairpin, followed by a return to the vRNA template. Two regions of sequence complementarity between the tRNA

and the nascent viral DNA facilitate this process. Upon completion of reverse transcription, the proviral DNA is a hybrid between a plus-strand DNA with an inserted DNA copy of the tRNA, and a minus-strand cDNA containing the lhNef hairpin. Such proviral structures may be resolved by DNA repair and/or subsequent cell division. Our results strongly suggest that only the provirus without the lhNef structure is able to produce progeny.

Analysis of the relative fitness of the AS escape viruses and subsequent evolution

We made molecular clones of the HIV-1hNef escape variants to perform more detailed studies. HEK293T cells were transfected with proviral constructs encoding wild-type HIV-1, HIV-1hNef and the AS escape variants. HEK293T cells do not support HIV-1 replication, but produce virus particles upon DNA transfection. Virus production was measured by CA-p24 ELISA in the culture supernatant at 3 days post-transfection. Unlike the original HIV-1hNef, which demonstrated a severe CA-p24 production defect, all AS variants efficiently produced virus (Figure 8A).

The T cell line SupT1 and PBMCs were transfected with the DNA constructs to study the replication capacity of the AS mutants. CA-p24 production was measured to follow virus spread in the culture supernatant at several times post-transfection (Figure 8B). As expected, HIV-1hNef did not replicate in SupT1 and PBMCs. Escape variant AS44, with the intermediate length hairpin, replicated only marginally in

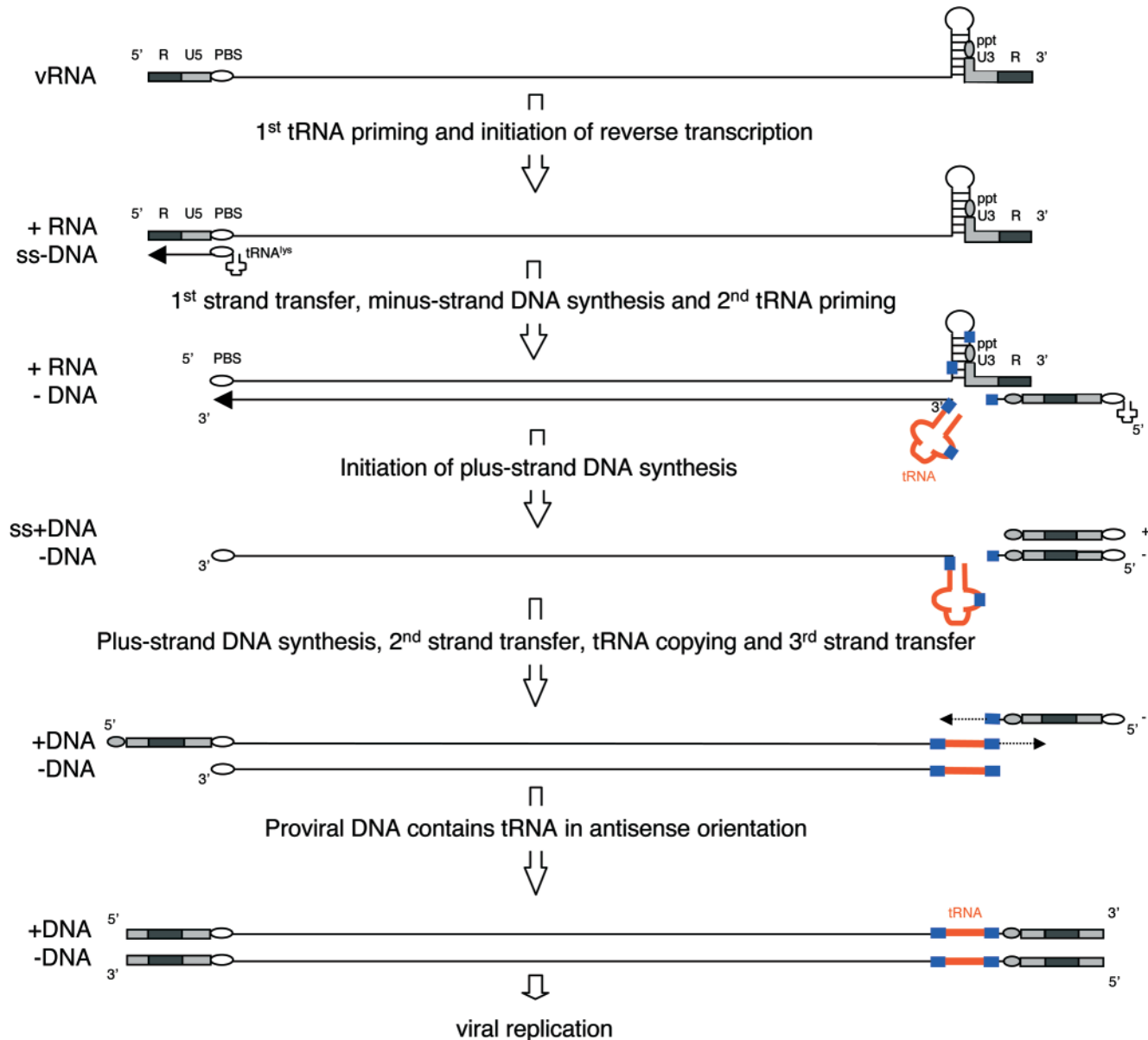


Figure 6. Mechanistic model for HITME recombination by a second tRNA priming event during minus-strand DNA synthesis. Reverse transcription is initiated by the tRNA^{lys3} primer that is annealed to the PBS. Strong-stop minus-strand DNA (ss-DNA) is synthesized by RT by copying the 5' R region. The ss-DNA is translocated to the homologous 3' R sequence (first strand transfer), and reverse transcription continues. The extended lhNef hairpin will cause pausing of the RT enzyme. A spurious tRNA acts as a second primer for minus-strand DNA synthesis. The ppt primes the synthesis of strong-stop plus-strand DNA (ss+DNA), which is translocated to the 5' end of the minus-strand cDNA (second strand transfer). The minus-strand cDNA and the 2nd tRNA primer are copied during continued plus-strand DNA synthesis. Sequence complementarity between plus-strand copy of the 2nd tRNA primer and the minus-strand DNA facilitates a third strand transfer that is needed to complete reverse transcription. The non-natural priming and strand transfer events result in insertion of the tRNA sequence into the viral genome and deletion of the lhNef sequence. The tRNA sequence is in red and complementary nucleotides between tRNA and HIV-lhNef are boxed in blue.

both cell types. All other AS variants replicated at least as efficiently as wild-type HIV in SupT1 cells. In contrast, all these AS mutants replicated less efficiently than wild-type HIV-1 in PBMC. This phenotypic difference can be explained by the Nef-minus genotype of these viral strains. It has been reported that the accessory Nef gene has no impact on HIV-1 replication fitness in T cell lines, but Nef contributes to virus replication in primary cells (28,29).

The fitness of the five AS variants was determined in a direct competition experiment in SupT1 cells (Figure 9). SupT1 cells were infected with an equimolar mixture of the five variants

(AS44, AS19, AS15, AS11 and AS8) that were produced in HEK293T cells, and virus was passaged at the peak of infection. Cellular DNA was extracted at different times post-infection and the proviral Nef region was PCR amplified (see Figure 1). Since this PCR product will differ in size for each virus variant, all variants can be detected by subsequent agarose gel electrophoresis. All five variants were indeed detected in the culture at day 12 (Figure 9A). A single PCR product was observed at day 16, indicating that one AS variant dominated the viral population. Shorter PCR products were observed at later times. The PCR products from day 5,

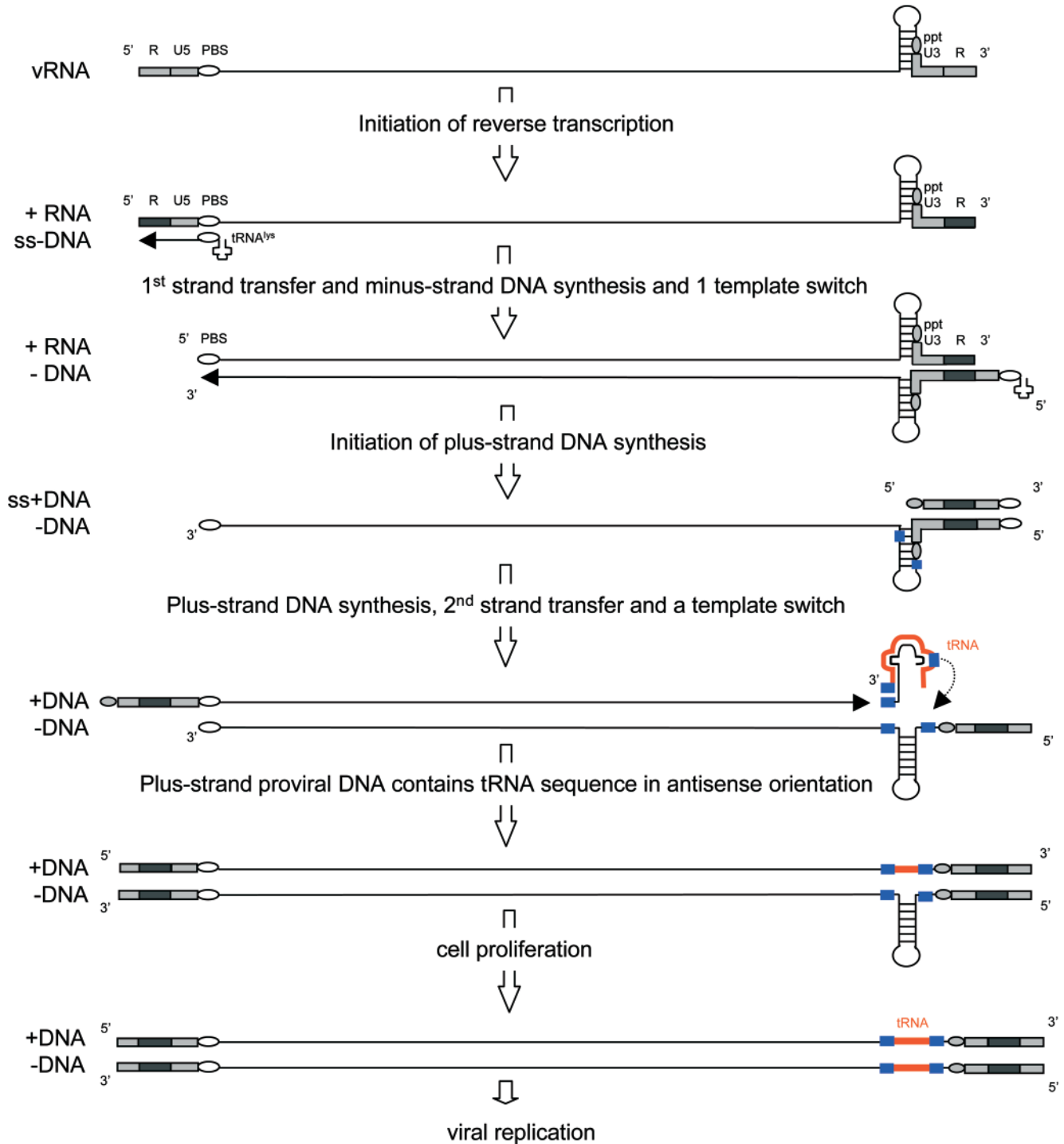


Figure 7. HITME recombination mechanism by a template switch during plus-strand synthesis. Reverse transcription is initiated as described in Figure 6. In this model, we assume that RT is able to copy the complete lhNef during minus-strand cDNA synthesis, but the enzyme is paused by the long hairpin during plus-strand DNA synthesis. A tRNA is subsequently used by RT as template. The complementarity between the tRNA and the viral DNA facilitates this process. Upon completion of reverse transcription, the proviral DNA is a hybrid between a plus-strand DNA with an incorporated tRNA sequence, and a minus-strand cDNA containing lhNef. The hybrid provirus structure will be resolved by DNA repair and/or subsequent cell division.

22 and 33 were cloned, and multiple clones were sequenced (Figure 9B). The day 5 sample consists of several input viruses (AS19, AS11, AS8), but also variants thereof (AS44Δ10 and AS8Δ90). Sequence details of these new variants are shown in Figure 9C. Subsequent samples also indicated deletion of

sequences for the AS15 and AS11 variants. These results indicate that virus replication can be further improved by additional removal of tRNA or Nef sequences. Improved replication is likely due to further truncation of the inserted secondary structures, as illustrated by the ΔG values in

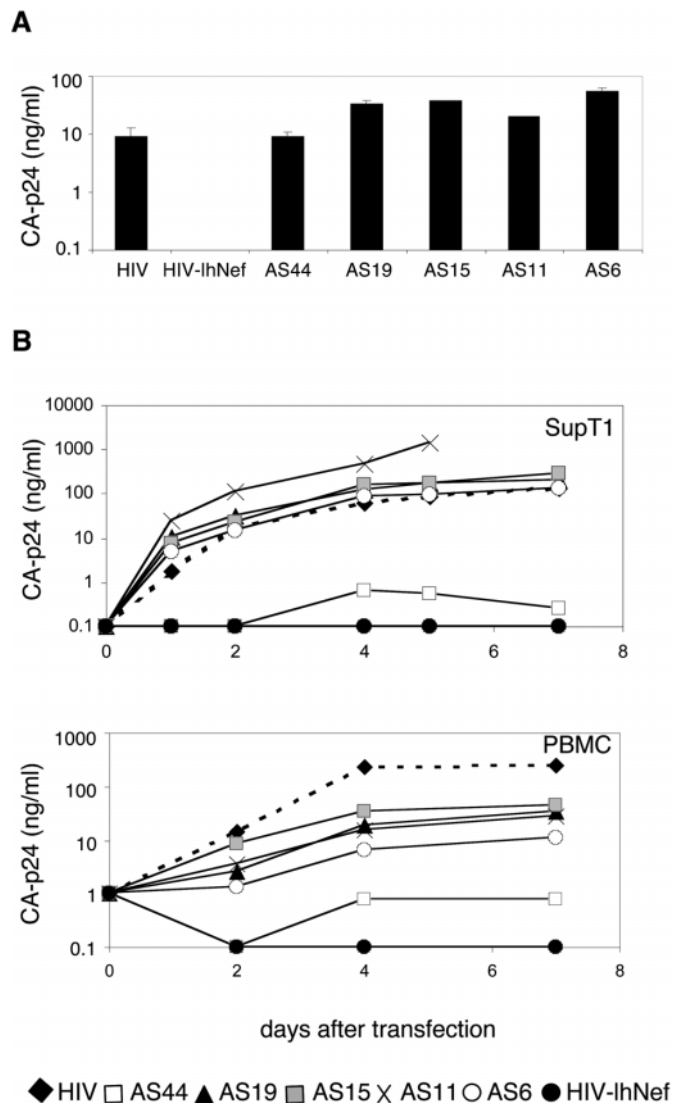


Figure 8. Virus production and replication of HIV-1 and the escape variants. (A) Virus production in HEK293T cells. Cells were transfected with the proviral constructs encoding wild-type HIV-1, HIV-lhNef and the AS escape mutants. Virus production was measured by CA-p24 ELISA in the culture supernatant at three days post-transfection. (B) Virus replication in SupT1 and PBMC cells. Cells were transfected with proviral DNA constructs and CA-p24 production was measured in the culture supernatant at several days post-transfection.

Figure 9B. The AS44 Δ 10 reduces the hairpin stability from -84.7 to -60.7 kcal/mol, and AS15 Δ 8 from -23.1 to -6.0 kcal/mol.

The escape variants with a large tRNA insert showed an interesting evolution path. AS11 acquired additional deletions of 28, 37 and 45 nt within the tRNA^{asp} insert (Figure 9C and illustrated in Figure 5). Mutant AS11 Δ 28 dominated the virus population in the last sample (13 out of 19 clonal sequences) taken at day 33. This result is consistent with the replication experiment in SupT1 cells, in which AS11 showed the highest level of replication (see Figure 8B). AS11 harbours the largest deletion in the Nef gene (Figure 3). The secondary structure has a ΔG value of -16.7 kcal/mol that is comparable to the stability of natural secondary structures in the HIV-1 RNA

genome, which never consist of more than 11 consecutive base pairs.

A variant of AS8 with a nearly complete deletion of tRNA^{glu} and 33 flanking Nef nucleotides was repeatedly observed in the co-culture, although it never became dominant in the viral quasispecies. The tRNA deletion is illustrated in Figure 5. Partial sequence complementarity may have facilitated some of the tRNA deletions (underlined in Figure 9C, e.g. C in AS8 Δ 90). The instability of the tRNA-containing variants AS11 and AS8 indicates that the cellular tRNA was only transiently beneficial as a partner in the recombinatorial rescue of HIV-lhNef.

DISCUSSION

Generation of genetic variation in HIV-1 is the result of mutation and recombination (30). In this study, we forced recombination in HIV-1 by creating an excessively stable 300 bp lhNef hairpin. Many steps of the HIV-1 replication cycle could in theory be affected by the lhNef insertion, e.g. RNA splicing, RNA export out of the nucleus or mRNA translation. In addition, the genomic RNA may also face problems with packaging in virus particles or reverse transcription and the long hairpin may also induce an antiviral response through RNA interference (RNAi) or interferon pathways. Indeed, we found that HIV-lhNef exhibits a severe gene expression and replication defect. After prolonged culturing in T cells however, replicating variants emerged through recombination events that lead to the nearly complete removal of the lhNef hairpin structure. Previous studies have suggested that homologous retroviral recombination occurs two to three orders of magnitude more frequently than non-homologous recombination (31,32). However, only non-homologous recombination can remove the hairpin structure in the HIV-lhNef genome. HIV-lhNef escape variants emerged with limited nucleotide complementarity at the cross-over sites. Three escape variants acquired an insertion corresponding to a human tRNA sequence. We propose that this occurred via a particular mechanism referred to as HITME recombination. Two possible mechanistic models are proposed in which this recombination is either mediated by (i) a second tRNA priming event during minus-strand DNA synthesis, or by (ii) a template switch during plus-strand DNA synthesis. The second model seems less likely because it assumes that RT can copy the complete lhNef structure during minus-strand cDNA synthesis. Alternatively, one could propose a recombination event at the DNA level (33), but the involvement of a tRNA-recombination partner rules out this possibility.

It has previously been suggested that RNA secondary structure presents a hot spot for non-homologous RT-mediated recombination in Moloney murine leukaemia virus- and HIV-based vectors or viruses (22,34–36). For instance, Beerens *et al.* (21) demonstrated *in vitro* that the viral RT enzyme halted near the base of an extended hairpin, and this triggered the selection of a deletion that truncates the structure upon virus replication. The results presented in this study are consistent with this idea since all deletions were observed within the hairpin structure, and we did not observe a consensus pattern of sequence homology at the recombination junctions. On the other hand, the selection of replication-competent escape viruses will strongly bias for deletion of hairpin sequences.

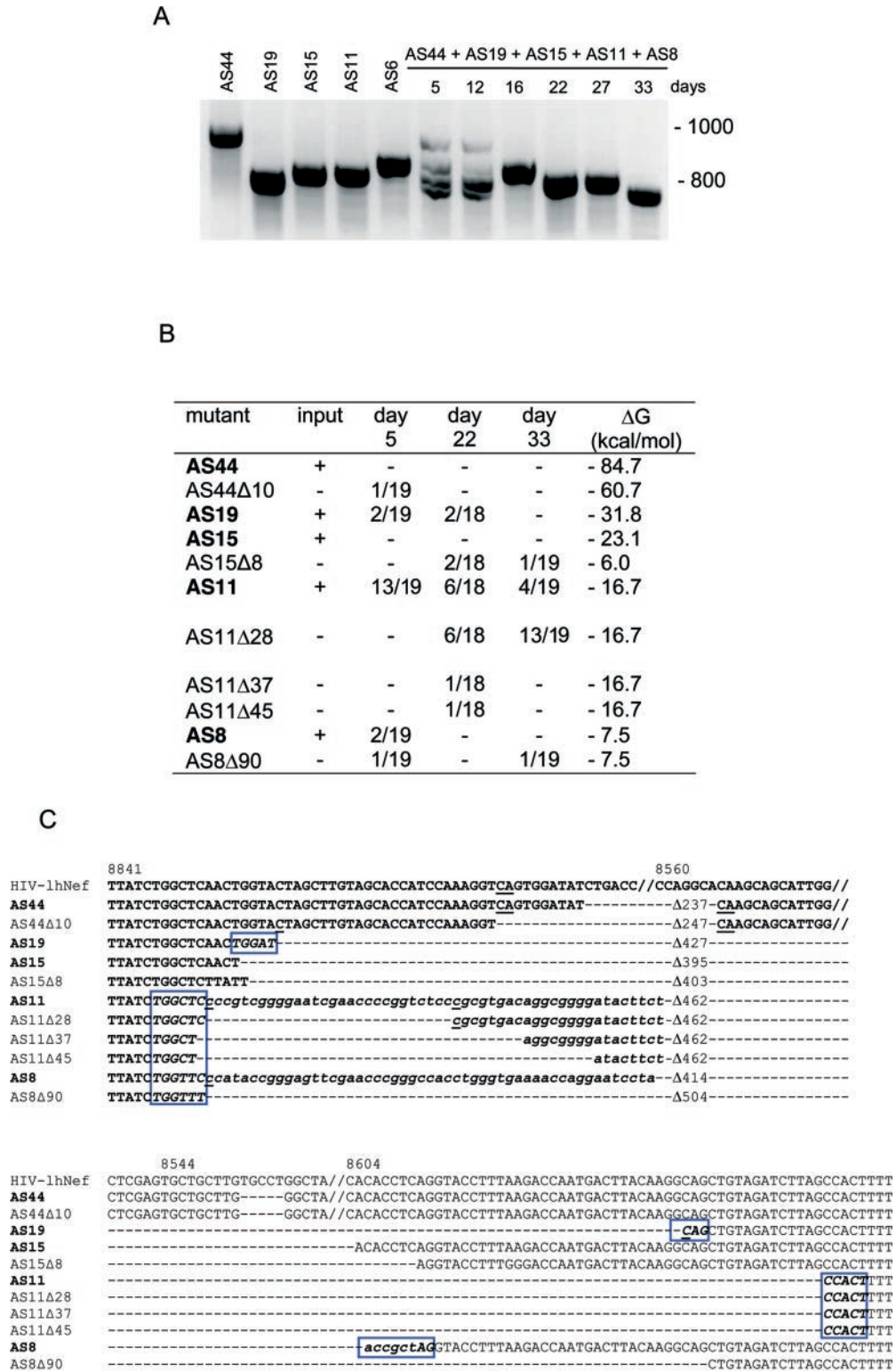


Figure 9. Comparison of the replication capacity of the HIV-1hNef escape viruses and continued evolution. (A) SupT1 cells were infected with an equimolar mixture of the five AS escape variants (5 ng CA-p24 of AS44, AS19, AS15, AS11 and AS8) that were produced in HEK293T cells. Virus was passaged at the peak of infection. Cellular DNA was extracted at days 5, 12, 16, 22, 27 and 33 post-infection and proviral DNA around the hairpin insert was PCR amplified with primers tTA1 and CN1 (see Figure 1). On the right are indicated the Smart DNA Ladder sizes (bp). (B) The PCR products from samples obtained at day 5, 22 and 33 were cloned in the pCR2.1 vector, and 18 or 19 clones were sequenced per sample. The composition of the virus mixture is indicated by the number of clones. The input virus names are in boldface. Newly evolved sequence variants are shown in (C). The free energy ΔG value is a thermodynamic stability score of the perfect duplex structure, e.g. 44 bp for AS44 and 11 bp for AS11. (C) Sequence alignment of HIV-1hNef escape viruses and deletion variants asNef sequences are bold caps, tRNA sequences are lower-case italics. Homologous sequences between the tRNA and HIV-1hNef at the recombination junctions are boxed in blue. Repeated sequences at the recombination junctions are underlined.

The recombination junctions recovered from separate evolution experiments differ, which suggests that multiple recombination events can trigger the hairpin deletion.

The usage of a tRNA primer for reverse transcription is a hallmark of the retrovirus family, and an initial tRNA-recombination event may have been involved in the acquisition of a PBS motif in the retroviral genome during the early days of evolution. It could even be hypothesized that spurious tRNA-recombination could add a second PBS element to the retroviral genome, but it is questionable whether such an additional motif will be helpful during reverse transcription. However, we know of such additional priming events for plus-strand synthesis, e.g. the central-ppt.

An assortment of host cell RNAs, such as rRNAs and tRNAs, are encapsidated in retroviral virions, although not as efficiently as full-length vRNA (37–40). We report three escape viruses in which a tRNA sequence is incorporated in the viral genome. A very similar event has previously been described for the cognate tRNA^{lys3} primer, which was copied in the HIV-1 genome in an unusual transduction event (41). Several other studies have reported inserts of the cognate tRNA primer or non-cognate tRNA sequences in the minus-strand orientation, which may all have been initiated by a tRNA priming event (31,41–43). In one of these studies, an aberrant tRNA^{met(i)} primer was incorporated that apparently lacked the 3'-CCA end (43), which may indicate packaging of immature, not fully processed tRNAs in virion particles. Carrasco *et al.* (43) described a tRNA insert in the plus-orientation, which would involve two extra template switches during minus-strand synthesis: onto the tRNA, and back onto the vRNA. The use of a diversity of tRNAs as recombination partner indicates that HIV-1 RT can use these molecules as primer. This variety in recombination primer usage contrasts with the exclusive usage of tRNA^{lys3} to initiate HIV-1 reverse transcription at the PBS site. In fact, several attempts to switch the HIV-1 primer usage by PBS-mutation failed (1,44), which is likely due to the presence of additional motifs in the viral genome that pair with the primer tRNA, including the primer-activation signal (45). We recently succeeded to make an HIV-1 variant that stably uses the non-self tRNA^{lys1,2} primer by mutation of both the PBS and PAS motifs (46), but we did not succeed to make more radical tRNA switches. These results underscore the high specificity of tRNA priming during initiation of reverse transcription, whereas multiple tRNAs, even the low abundant ones, can incidentally act as recombination partner, which is driven by limited sequence complementarity.

It is interesting that RT was able to copy nearly the complete tRNA^{asp} and tRNA^{glu} molecules, including a number of modified bases present in the mature tRNAs. Combined with the results of other studies (41–43), we suggest that RT is surprisingly versatile at copying modified bases in RNA. The alternative explanation is that an unmodified precursor form of the tRNA acted as the primer, which we consider unlikely given the presence of the 3' terminal CCA modification that is added post-transcriptionally and that unmodified tRNAs are rapidly degraded (47). Weiner and Maizels (48) postulated the CCA genome tag hypothesis based on the usage of tRNA primers by retroelements and tRNA-like structures with (aminoacylated) CCA ends in many RNA viruses. The finding that tRNA has been repeatedly borrowed and adapted in evolution for a role

in virus replication is likely due to its unique priming abilities through the extended CCA end.

tRNA may be particularly suited as recombination partner for retroviruses because it is packaged in virions and because it has an extended and single-stranded CCA 3' end that can initiate an aberrant priming event. In fact, tRNA-mediated recombination may occur much more frequently than noticed. First, it may be difficult to recognize such an event when only a minimal tRNA cassette is inserted as described for AS19. Second, in case a large tRNA segment is incorporated, our results with AS11 and AS8 indicate that spontaneous deletions in the tRNA insertions occur upon prolonged virus replication. This genetic instability of the replication-competent variants AS11 and AS8 may suggest that, upon tRNA-mediated recombinatorial rescue of HIV-lhNef, the emerged variants further optimize the acquired secondary structure in their Nef coding domain. Inspection of genomic viral RNAs revealed that those of most plant and mammalian viruses causing persistent infections are highly structured, and these secondary RNA structures may render the viral RNAs less sensitive to innate antiviral responses in cells (49). HIV-1 RNA is also highly structured and additional secondary RNA structures are selected when an antiviral RNAi response was evoked, rendering HIV-1 RNA less susceptible for degradation by the RNAi machinery (50,51). On the other hand, the eventual removal of a large segment of the tRNA-inserts demonstrates that the inserts generally do not contribute to virus replication fitness, and reinforces the idea that the tRNAs were instrumental only transiently as recombination partner.

Retroviruses are known for their ability to transduce host cellular sequences (52,53). Most notably, the acutely transforming retroviruses harbour oncogenes that were acquired via transduction of cellular counterparts termed proto-oncogenes. The most prevalent mechanism of transduction by retroviruses proposes that the provirus integrates next to the sequence that will be transduced (5). Read-through transcription will result in an extended transcript that fuses viral and cellular sequences. Intramolecular recombination during reverse transcription can result in the incorporation of the cellular sequence into the progeny (6). An intermolecular mechanism has also been proposed by random co-packaging of a cellular RNA into a virion, followed by recombination during reverse transcription (54). The transduction of tRNA sequences may present a special case, since the proposed HITME mechanisms are exclusively intermolecular with a co-packaged tRNA molecule. This may be triggered by the accumulation of small tRNA molecules in retroviral particles, where up to 30% of the total RNA is tRNA (55). As transduction of unknown sequences is observed quite frequently in the evolution of drug-resistant HIV-1 variants (56,57), tRNA-mediated recombination events may occur during virus evolution in a clinical setting.

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REFERENCES

- Das, A.T., Klaver, B. and Berkhout, B. (1995) Reduced replication of human immunodeficiency virus type 1 mutants that use reverse transcription primers other than the natural tRNA(3Lys). *J. Virol.*, **69**, 3090–3097.
- Jiang, M., Mak, J., Wainberg, M.A., Parniak, M.A., Cohen, E. and Kleiman, L. (1992) Variable tRNA content in HIV-1III_B. *Biochem. Biophys. Res. Commun.*, **185**, 1005–1015.
- Mak, J., Jiang, M., Wainberg, M.A., Hammarskjöld, M.L., Rekosh, D. and Kleiman, L. (1994) Role of Pr160gag-pol in mediating the selective incorporation of tRNA(Lys) into human immunodeficiency virus type 1 particles. *J. Virol.*, **68**, 2065–2072.
- Telesnitsky, A. and Goff, S.P. (1997) Reverse transcriptase and the generation of retroviral DNA. In Coffin, J.M., Hughes, S.H. and Varmus, H.E. (eds), *Retroviruses*. Cold Spring Harbor Laboratory Press, NY, pp. 121–160.
- Temin, H.M. (1993) Retrovirus variation and reverse transcription: abnormal strand transfers result in retrovirus genetic variation. *Proc. Natl Acad. Sci. USA*, **90**, 6900–6903.
- Hu, W.-S. and Temin, H.M. (1990) Retroviral recombination and reverse transcription. *Science*, **250**, 1277–1283.
- Galetto, R., Giacconi, V., Veron, M. and Negroni, M. (2005) Dissection of a circumscribed recombination hot spot in HIV-1 after a single infectious cycle. *J. Biol. Chem.*, **281**, 2711–2720.
- Coffin, J.M. (1990) Retroviridae and their replication. In Fields, B.N. and Knipe, D.M. (eds), *Virology*. Raven Press, N.Y., pp. 1437–1500.
- Hwang, C.K., Svarovskaia, E.S. and Pathak, V.K. (2001) Dynamic copy choice: steady state between murine leukemia virus polymerase and polymerase-dependent RNase H activity determines frequency of *in vivo* template switching. *Proc. Natl Acad. Sci. USA*, **98**, 12209–12214.
- Pathak, V.K. and Temin, H.M. (1992) 5-Azacytidine and RNA secondary structure increase the retrovirus mutation rate. *J. Virol.*, **66**, 3093–3100.
- Jetst, A.E., Yu, H., Klarmann, G.J., Ron, Y., Preston, B.D. and Dougherty, J.P. (2000) High rate of recombination throughout the human immunodeficiency virus type 1 genome. *J. Virol.*, **74**, 1234–1240.
- Pfeiffer, J.K. and Telesnitsky, A. (2001) Effects of limiting homology at the site of intermolecular recombinogenic template switching during Moloney murine leukemia virus replication. *J. Virol.*, **75**, 11263–11274.
- An, W. and Telesnitsky, A. (2002) Effects of varying sequence similarity on the frequency of repeat deletion during reverse transcription of a human immunodeficiency virus type 1 vector. *J. Virol.*, **76**, 7897–7902.
- Derebail, S.S. and DeStefano, J.J. (2004) Mechanistic analysis of pause site-dependent and -independent recombinogenic strand transfer from structurally diverse regions of the HIV genome. *J. Biol. Chem.*, **279**, 47446–47454.
- Klaver, B. and Berkhout, B. (1994) Premature strand transfer by the HIV-1 reverse transcriptase during strong-stop DNA synthesis. *Nucleic Acids Res.*, **22**, 137–144.
- Berkhout, B., van Wamel, J. and Klaver, B. (1995) Requirements for DNA strand transfer during reverse transcription in mutant HIV-1 virions. *J. Mol. Biol.*, **252**, 59–69.
- van Wamel, J.L.B. and Berkhout, B. (1998) The first strand transfer during HIV-1 reverse transcription can occur either intramolecularly or intermolecularly. *Virology*, **244**, 245–251.
- Svarovskaia, E.S., Delviks, K.A., Hwang, C.K. and Pathak, V.K. (2000) Structural determinants of murine leukemia virus reverse transcriptase that affect the frequency of template switching. *J. Virol.*, **74**, 7171–7178.
- Berkhout, B., Vastenhout, N.L., Klasens, B.I. and Huthoff, H. (2001) Structural features in the HIV-1 repeat region facilitate strand transfer during reverse transcription. *RNA*, **7**, 1097–1114.
- Klasens, B.I.F., Huthoff, H.T., Das, A.T., Jeeninga, R.E. and Berkhout, B. (1999) The effect of template RNA structure on elongation by HIV-1 reverse transcriptase. *Biochim. Biophys. Acta*, **1444**, 355–370.
- Beerens, N., Groot, F. and Berkhout, B. (2000) Stabilization of the U5-leader stem in the HIV-1 RNA genome affects initiation and elongation of reverse transcription. *Nucleic Acids Res.*, **28**, 4130–4137.
- Moumen, A., Polomack, L., Unge, T., Veron, M., Buc, H. and Negroni, M. (2003) Evidence for a mechanism of recombination during reverse transcription dependent on the structure of the acceptor RNA. *J. Biol. Chem.*, **278**, 15973–15982.
- Peden, K., Emerman, M. and Montagnier, L. (1991) Changes in growth properties on passage in tissue culture of viruses derived from infectious molecular clones of HIV-1LAI, HIV-1MAL, and HIV-1ELI. *Virology*, **185**, 661–672.
- Klaver, B. and Berkhout, B. (1994) Comparison of 5' and 3' long terminal repeat promoter function in human immunodeficiency virus. *J. Virol.*, **68**, 3830–3840.
- Zuker, M. (2003) Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res.*, **31**, 3406–3415.
- Das, A.T., Klaver, B. and 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.
- Jeeninga, R.E., Hoogenkamp, M., Armand-Ugon, M., de Baar, M., Verhoef, K. and Berkhout, B. (2000) Functional differences between the long terminal repeat transcriptional promoters of HIV-1 subtypes A through G. *J. Virol.*, **74**, 3740–3751.
- de Ronde, A., Klaver, B., Keulen, W., Smit, L. and Goudsmit, J. (1992) Natural HIV-1 NEF accelerates virus replication in primary human lymphocytes. *Virology*, **188**, 391–395.
- Deacon, N.J., Tsykin, A., Solomon, A., Smith, K., Ludford-Menting, M., Hooker, D.J., McPhee, D.A., Greenway, A.L., Ellett, A., Chatfield, C. *et al.* (1995) Genomic structure of an attenuated quasi species of HIV-1 from blood transfusion donor and recipients. *Science*, **270**, 988–991.
- Galetto, R. and Negroni, M. (2005) Mechanistic features of recombination in HIV. *AIDS Rev.*, **7**, 92–102.
- Zhang, J. and Temin, H.M. (1993) Rate and mechanism of nonhomologous recombination during a single cycle of retroviral replication. *Science*, **259**, 234–238.
- An, W. and Telesnitsky, A. (2004) Human immunodeficiency virus type 1 transductive recombination can occur frequently and in proportion to polyadenylation signal readthrough. *J. Virol.*, **78**, 3419–3428.
- Schwartz, J.R., Duesberg, S. and Duesberg, P.H. (1995) DNA recombination is sufficient for retroviral transduction. *Proc. Natl Acad. Sci. USA*, **92**, 2460–2464.
- Moumen, A., Polomack, L., Roques, B., Buc, H. and Negroni, M. (2001) The HIV-1 repeated sequence R as a robust hot-spot for copy-choice recombination. *Nucleic Acids Res.*, **29**, 3814–3821.
- Duch, M., Carrasco, M.L., Jepsen, T., Aagaard, L. and Pedersen, F.S. (2004) An RNA secondary structure bias for non-homologous reverse transcriptase-mediated deletions *in vivo*. *Nucleic Acids Res.*, **32**, 2039–2048.
- Lanciault, C. and Champoux, J.J. (2006) Pausing during reverse transcription increases the rate of retroviral recombination. *J. Virol.*, **80**, 2483–2494.
- Anderson, D.J., Stone, J., Lum, R. and Linial, M.L. (1995) The packaging phenotype of the SE21Q1b provirus is related to high proviral expression and not *trans*-acting factors. *J. Virol.*, **69**, 7319–7323.
- Berkowitz, R., Fisher, J. and Goff, S.P. (1996) RNA packaging. *Curr. Top. Microbiol. Immunol.*, **214**, 177–218.
- Cimarelli, A., Sandin, S., Høglund, S. and Luban, J. (2000) Basic residues in human immunodeficiency virus type 1 nucleocapsid promote virion assembly via interaction with RNA. *J. Virol.*, **74**, 3046–3057.
- Onafuwa-Nuga, A.A., King, S.R. and Telesnitsky, A. (2005) Nonrandom packaging of host RNAs in moloney murine leukemia virus. *J. Virol.*, **79**, 13528–13537.
- Sun, G., O'Neil, P.K., Yu, H., Ron, Y., Preston, B.D. and Dougherty, J.P. (2001) Transduction of cellular sequence by a human immunodeficiency virus type 1-derived vector. *J. Virol.*, **75**, 11902–11906.
- Colicelli, J. and Goff, S.P. (1986) Structure of a cloned circular retroviral DNA containing a tRNA sequence between the terminal repeats. *J. Virol.*, **57**, 674–677.
- Carrasco, M.L., Duch, M. and Pedersen, F.S. (2004) Strand transfer to the 5' part of a tRNA as a mechanism for retrovirus patch-repair recombination *in vivo*. *J. Gen. Virol.*, **85**, 1965–1969.
- Zhang, Z., Kang, S.-M., LeBlanc, A., Hajduk, S.L. and Morrow, C.D. (1996) Nucleotide sequences within the U5 region of the viral RNA genome are the major determinants for an human immunodeficiency

- virus type 1 to maintain a primer binding site complementary to tRNA^{His}. *Virology*, **226**, 306–317.
45. Beerens, N., Groot, F. and Berkhout, B. (2001) Initiation of HIV-1 reverse transcription is regulated by a primer activation signal. *J. Biol. Chem.*, **276**, 31247–31256.
 46. Abbink, T.E.M., Beerens, N. and Berkhout, B. (2004) Forced selection of a human immunodeficiency virus type 1 variant that uses a non-self tRNA primer for reverse transcription: involvement of viral RNA sequences and the reverse transcriptase enzyme. *J. Virol.*, **78**, 10706–10714.
 47. Alexandrov, A., Chernyakov, I., Gu, W., Hiley, S.L., Hughes, T.R., Grayhack, E.J. and Phizicky, E.M. (2006) Rapid tRNA decay can result from lack of nonessential modifications. *Mol. Cell*, **21**, 87–96.
 48. Weiner, A.M. and Maizels, N. (1987) tRNA-like structures tag the 3' ends of genomic RNA molecules for replication: implications for the origin of protein synthesis. *Proc. Natl Acad. Sci. USA*, **84**, 7383–7387.
 49. Simmonds, P., Tuplin, A. and Evans, D.J. (2004) Detection of genome-scale ordered RNA structure (GORS) in genomes of positive-stranded RNA viruses: implications for virus evolution and host persistence. *RNA*, **10**, 1337–1351.
 50. Das, A.T., Brummelkamp, T.R., Westerhout, E.M., Vink, M., Madiredjo, M., Bernards, R. and Berkhout, B. (2004) Human immunodeficiency virus type 1 escapes from RNA interference-mediated inhibition. *J. Virol.*, **78**, 2601–2605.
 51. Westerhout, E.M., Ooms, M., Vink, M., Das, A.T. and Berkhout, B. (2005) HIV-1 can escape from RNA interference by evolving an alternative structure in its RNA genome. *Nucleic Acids Res.*, **33**, 796–804.
 52. Stehelin, D., Varmus, H.E., Bishop, J.M. and Vogt, P.K. (1976) DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA. *Nature*, **260**, 170–173.
 53. Rosenberg, N. and Jolicoeur, P. (1997) Retroviral pathogenesis. In Coffin, J.M., Hughes, S.H. and Varmus, H.E. (eds), *Retroviruses*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 475–586.
 54. Stuhlmann, H., Dieckmann, M. and Berg, P. (1990) Transduction of cellular neo mRNA by retrovirus-mediated recombination. *J. Virol.*, **64**, 5783–5796.
 55. Peters, G.G. and Hu, J. (1980) Reverse transcriptase as the major determinant for selective packaging of tRNA's into avian sarcoma virus particles. *J. Virol.*, **36**, 692–700.
 56. Lobato, R.L., Kim, E.Y., Kagan, R.M. and Merigan, T.C. (2002) Genotypic and phenotypic analysis of a novel 15-base insertion occurring between codons 69 and 70 of HIV type 1 reverse transcriptase. *AIDS Res. Hum. Retroviruses*, **18**, 733–736.
 57. van der Hoek, L., Back, N., Jebbink, M.F., de Ronde, A., Bakker, M., Jurriaans, S., Reiss, P., Parkin, N. and Berkhout, B. (2005) Increased multinucleoside drug resistance and decreased replicative capacity of a human immunodeficiency virus type 1 variant with an 8-amino-acid insert in the reverse transcriptase. *J. Virol.*, **79**, 3536–3543.