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-lhNef). HIV-lhNef 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 tRNAasp, tRNAGlu or tRNAtrp sequences. The tRNAs were inserted in the viral genome in the antisense 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 tRNAlys 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 (lhNef). 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...
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 (CTAGTGGATCCCTACGCACTTATAGCAGAAGGCC, +8067 to +8091) and JvdV175 (CCAGATAAACCTATAGGACCG, +8388 to +8363), yielding a 330 bp fragment corresponding with the 3' end of the env gene, and primers XhoI-JvdV176 (CCCGCTCGAGTGCTCTGTGGCCTTGCTTCGGTACGGC, +8544 to +8576) and JvdV177 (GCTTATGGATGGCCTCAACTGGTACCAGTGGAG, +8844 to +8813), yielding a 330 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 capping 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 µl lysis buffer [10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 0.5% Tween-20] and 200 µg/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 µl 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 µl reaction containing 1 × 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 µg/ml) at 37°C and 5% CO2. SupT1 cells (2.5 × 106) 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 µg/ml) and cultured in complete RPMI medium with IL-2 (10 U/ml). Cells (5 × 106) were transfected by electroporation in 250 µl RPMI with 20% FCS in 0.4 cm cuvettes at 250 V and 960 µF, and 1 × 106 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 µg/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 × 103 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
Env gene (Figure 1). In this HIV-long-hairpin-Nef (HIV-lhNef) 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-lhNef 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-lhNef.

To study the replication potential of HIV-lhNef, 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-lhNef (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-lhNef escape variants could be passaged to uninfected SupT1 cells and were cultured for up to 73 days.

Description of HIV-lhNef escape variants

To determine the sequence of the HIV-lhNef 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-lhNef 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
cells were transfected with different amounts of HIV-lhNef (culture A, 20 μg; B, 10 μg; C, 5 μg; D, 5 μg; E, 1 μg) and as a control 10 μg 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 μg HIV-lhNef) and day 16 in cultures C and D (both 5 μg). (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 lysed by 1% agarose gel electrophoresis. The culture is indicated above and the number of base pairs in the remaining RNA hairpin that remains after recombination (Figure 3, e.g. AS19 is capable of forming a 9 bp long stem structure in the central 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 (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^as^ 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

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**Figure 2.** Evolution of the replication-impaired HIV-lhNef virus. (A) SupT1 cells were transfected with different amounts of HIV-lhNef (culture A, 20 μg; B, 10 μg; C, 5 μg; D, 5 μg; E, 1 μg) and as a control 10 μg 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 μg HIV-lhNef) and day 16 in cultures C and D (both 5 μg). (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 lysed by 1% agarose gel electrophoresis. The culture is indicated above and the number of base pairs in the remaining RNA hairpin that remains after recombination (Figure 3, e.g. AS19 is capable of forming a 9 bp long stem structure in the central 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).

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The AS19 evolution mechanism requires a closer inspection. At first glance, one might think that an RT-slipage 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 AS19 evolution mechanism requires a closer inspection. At first glance, one might think that an RT-slipage 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 tRNAasp 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-1 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

| 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 tripal mismatch was apparent at the downstream HIV-1/tRNAasp 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-slipage 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 tRNAasp 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. |
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 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-lhNef escape variants to perform more detailed studies. HEK293T cells were transfected with proviral constructs encoding wild-type HIV-1, HIV-lhNef 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-lhNef, 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-lhNef did not replicate in SupT1 and PBMCs. Escape variant AS44, with the intermediate length hairpin, replicated only marginally in...
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,
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 (AS44 D10 and AS8 D90). 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 AG values in

**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.
The AS44Δ10 reduces the hairpin stability from $-84.7$ to $-60.7$ kcal/mol, and AS15Δ8 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 tRNAasp insert (Figure 9C and illustrated in Figure 5). Mutant AS11Δ28 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 at several days post-transfection.

**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 leukemia 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-IhNef 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 AG 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-IhNef escape viruses and deletion variants asNef sequences are bold caps, tRNA sequences are lower-case italics. Homologous sequences between the tRNA and HIV-IhNef 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 tRNAs and tRNA precursors, 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 tRNAlys3 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 tRNAlys3 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 3's12 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 incidently act as recombination partner, which is driven by limited sequence complementarity.

It is interesting that RT was able to copy nearly the complete tRNA ap and tRNA 16u 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 (aminocylated) 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 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.

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