Vif is a RNA chaperone that could temporally regulate RNA dimerization and the early steps of HIV-1 reverse transcription

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ABSTRACT

HIV-1 Vif (viral infectivity factor) is associated with the assembly complexes and packaged at low level into the viral particles, and is essential for viral replication in non-permissive cells. Viral particles produced in the absence of Vif exhibit structural defects and are defective in the early steps of reverse transcription. Here, we show that Vif is able to anneal primer tRNA^{Lys3} to the viral RNA, to decrease pausing of reverse transcriptase during (–) strand strong-stop DNA synthesis, and to promote the first strand transfer. Vif also stimulates formation of loose HIV-1 genomic RNA dimers. These results indicate that Vif is a _bona fide_ RNA chaperone. We next studied the effects of Vif in the presence of HIV-1 NCp, which is a well-established RNA chaperone. Vif inhibits NCp-mediated formation of tight RNA dimers and hybridization of tRNA^{Lys3}, while it has little effects on NCp-mediated strand transfer and it collaborates with nucleocapsid (NC) to increase RT processivity. Thus, Vif might negatively regulate NC-assisted maturation of the RNA dimer and early steps of reverse transcription in the assembly complexes, but these inhibitory effects would be relieved after viral budding, thanks to the limited packaging of Vif in the virions.

INTRODUCTION

In addition to the structural _gag_, _pol_ and _env_ genes, the human immunodeficiency virus type 1 (HIV-1) genome contains several regulatory genes: _tat_, _rev_, _nef_, _vpr_, _vif_ and _vpu_. The HIV-1 Vif (viral infectivity factor) protein, first considered as an accessory factor, is a positive modulator of viral infectivity in several cell types. In particular, Vif is required for efficient HIV-1 replication in so called 'non-permissive' cells such as primary CD4 + T lymphocytes and macrophages and some T-cell lines (H9, HUT78), whereas HIV-1 lacking _vif_ (HIV-1Δ_vif_) can replicate in fibroblasts and most T-cell lines termed 'permissive' (1–6).

This cell type-dependent requirement for Vif can be explained by the expression of APOBEC3G and APOBEC3F, two cellular inhibitors of HIV-1 replication, in non-permissive cells (5,7). APOBEC3G/3F are members of a large family of cytidine deaminases (8). In the absence of Vif, APOBEC3G/3F associate with Gag and RNA during viral assembly and are packaged into virions (9–14). APOBEC3G/3F induce hypermutation of the HIV-1 genome by mediating deamination of cytidine to uracil on the newly synthesized (–) strand DNA during reverse transcription, thus leading to guanosine to adenosine transitions in the viral genome (9,15–17). Independently from their catalytic activity, APOBEC3G/3F factors also impair particle infectivity by affecting virion morphology and by destabilizing the reverse transcription complex (18–21). Vif counteracts the antiviral activity of APOBEC3G/3F by several mechanisms. Vif seems to directly impair packaging of APOBEC3G/3F by an unknown mechanism (10,22–24), induces degradation of APOBEC3G/3F through the ubiquitination-proteasome pathway (22,25–27), and negatively regulates APOBEC3G/3F translation (10,22,23).

Vif is part of a large cytoplasmic ribonucleoprotein (RNP) complex and it is now usually accepted that Vif is packaged into viral particles through interactions with the viral genomic RNA, co-packaged cellular RNAs and the nucleocapsid (NC) domain of Gag (14,28–33). Vif defective viruses produced from non-permissive cells display defects not only at early assembly events but also at post-entry steps of infection, resulting in a failure

to complete reverse transcription and integration (34–39). Moreover, viral particles produced in the absence of Vif show structural defects such as aberrant core morphology and reduced stability (37,40,41). In particular, NC and reverse transcriptase (RT) were found to be less stably associated with viral cores in the absence of Vif (40), explaining in part why Δvif virions are defective in the reverse transcription step (29,36,42,43). Initiation of reverse transcription is completely impaired in Δvif viruses, suggesting that Vif may serve as an auxiliary factor for HIV-1 RT (44) and allows formation of a functional RNP (30). It has also been shown that Vif is able to modulate viral protease (PR) activity and the proteolytic processing of the Gag precursor at the p2/NC site, leading to the possibility that virion incorporation of Vif could stabilize NC intermediates (45–47).

Considering that NCp7 (48–50) and Vif (44) are both involved in the reverse transcription process and in structural rearrangements of HIV-1 RNA (29), we were interested to know whether Vif could modulate RNA dimerization, a prerequisite for RNA packaging (51,52), and the early steps of reverse transcription either alone or in presence of various intermediate processing products of Gag (NCp7, NCp9 and NCp15). In the absence of NC proteins, we found that Vif possesses RNA chaperone activity, resembling but distinct from the chaperone properties of NCp proteins with respect to RNA dimerization, hybridization of tRNA^{lys3} to the PBS, initiation of reverse transcription by RT and the first strand transfer. Surprisingly, Vif inhibited the initiation phase of reverse transcription. At high Vif/NCp ratio (1/3), Vif also inhibited NCp-induced maturation of the RNA dimer and tRNA^{lys3} annealing, whereas both NCp and Vif contributed to increase the processivity of RT. Vif had modest effects on the NCp-induced strand transfer reaction. At low Vif/NCp ratio (1/30), Vif had very limited effects on reverse transcription. Considering that Gag maturation is a highly ordered process that can be modulated by Vif (47), and that Vif is a RNA chaperone influencing RNA dimerization, tRNA annealing, RT processivity, and the first strand transfer, our results suggest that during the assembly steps, Vif might be a negative temporal regulator of RNA dimerization and packaging, preventing premature initiation of reverse transcription, while promoting tRNA annealing, a process that could be affected by APOBEC3F/3G.

MATERIALS AND METHODS

RNA template and primers

The donor and acceptor RNAs, corresponding respectively to nucleotides 1–311 and 8607–9229 of HIV-1 genomic RNA (Mal isolate) were synthesized by *in vitro* transcription of plasmids pJCB and pFB1 using T7 RNA polymerase and purified as previously described (53). Plasmid containing a 3-nt substitution in the self-complementary sequence of the RNA dimerization initiation site (DIS) loop (pDIS-AAA) (54) was digested by RsaI prior to *in vitro* transcription to generate mutated RNA 1–311. RNA 1–615 used in the dimerization assays

has been obtained after digestion of plasmid pHIV_615 by PvuII (55). Prior to 5′-end labeling, 10 µg of tRNA^{Lys3} purified from beef liver (56) were denatured for 2 min at 90°C in 25 mM Tris–HCl pH 8, 0.1% SDS (wt/vol), 15% (vol/vol) formamide, cooled on ice and incubated with 1000 U of BAP (Fermentas) at 70°C for 1 h. After phenol/chloroform extraction and ethanol precipitation, the dephosphorylated tRNA^{lys3} was purified by denaturing polyacrylamide gel electrophoresis (PAGE). Dephosphorylated tRNA^{lys3} (450 ng) was incubated 5 min at 70°C, cooled at room temperature and radiolabeled using 15 U of phage T4 polynucleotide kinase (PNK, New England Biolabs) and 100 µCi of [γ-^{32}P] ATP (Amersham), for 30 min at 37°C in the buffer supplied with the enzyme in a 30 µl final volume. Labeled tRNA^{lys3} was then purified by denaturing PAGE. ODN, an 18-mer oligodeoxyribonucleotide complementary to the PBS was chemically synthesized and 5′-end labeled for 45 min at 37°C using 100 µCi of [γ-^{32}P] ATP and 10 U of PNK (New England Biolabs). Internal labeling of RNA 1–615 was achieved by addition of [α-^{32}P] ATP (Amersham) during transcription (57).

Proteins

Wild-type HIV-1 Vif protein was expressed in *Escherichia coli* with an N-terminal 6-His fusion tag and purified as previously described (58). NCp7, NCp9 and NCp15 proteins from NL4.3 (55, 72 and 122 amino acids, respectively), expressed in *E. coli* and purified as described (59), were reconstituted with one equivalent of Zn^{2+} per zinc finger in milliQ H_2O (Millipore), aliquoted, layered with mineral oil and stored at −80°C (50). Wild-type and RNase H(–) E478Q HIV-1 RTs were expressed in *E. coli* with a N-terminal 6-His fusion tag and purified as previously described (60).

Heat annealing of tRNA^{Lys3} and ODN

All annealing reactions were performed using 1 pmol of RNA 1–311 and 0.3 pmol of 5′-end labeled ODN or tRNA^{Lys3}. Primer and template were denatured 2 min at 90°C then cooled on ice. After addition of 0.1 M NaCl and 6 µM $ZnCl_2$, samples containing ODN or tRNA^{Lys3} were incubated 20 min at 50 or 70°C, respectively, and cooled on ice.

Protein-mediated tRNA^{Lys3} annealing

Primer and template were first denatured separately by incubation 2 min at 90°C, ice-cooled and renatured 10 min at 37°C in 50 mM Tris–HCl pH 7.2, 50 mM NaCl, 6 µM $ZnCl_2$ and 5 mM $MgCl_2$ (buffer H). RNAs were then mixed together, incubated 10 min at 37°C, 5 min at room temperature and put on ice while adding Vif and/or NC proteins. In parallel, heat-annealed complexes were formed as described above and adjusted to 50 mM NaCl, 50 mM Tris–HCl pH 7.2 and 5 mM $MgCl_2$. Heat- and protein-mediated annealing reactions (10 µl) were incubated 20 min at 37°C, and split in two equal volumes, in order to monitor hybridization and to assay extension by wild-type HIV-1 RT. To monitor hybridization, samples were deproteinized with 1.3 mg/ml proteinase K

(Roche) in 8 mM NTPs, 1 mM spermidine, 1.3% SDS and 33 mM EDTA for 1 h at 37°C. Spermidine and NTPs help minimizing aggregation of nucleic acids by NC (61). Volume was then adjusted to 60 μl with buffer H and following phenol/chloroform extraction, 50 μl of RNA-containing aqueous phase supplemented with glycerol-containing loading buffer were analyzed by non-denaturing 6% PAGE. Electrophoresis was performed at 4°C in 0.5× Tris–Borate buffer supplemented with 0.1 mM MgCl$_2$.

Protein-mediated RNA dimerization

In a typical experiment, 100 nM of unlabeled HIV-1 1–615 RNA fragment were diluted in 10 μl of Milli-Q (Millipore) water with the corresponding labeled RNA (5000 c.p.m., 3–5 nM). Samples were denatured for 2 min. at 90°C, and snap-cooled on ice for 2 min. Dimerization was initiated by addition of Vif and/or NCp7 proteins in conditions disfavoring salt-induced RNA dimerization (50 mM sodium cacodylate pH 7.5, 50 mM NaCl, 0.1 mM MgCl$_2$). RNA samples were incubated 30 min at 37°C and deproteinized as above, then re-suspended in glycerol-containing loading buffer, split in two equal volumes and analyzed on a 0.8% agarose gel in native (Tris–Borate 0.5×, MgCl$_2$ 0.1 mM, run at 4°C) or denaturating (Tris–Borate-EDTA 1×, run at 20°C) electrophoresis conditions. Gels were fixed in 10% trichloroacetic acid for 10 min. and dried for 1 h under vacuum at room temperature. Radioactive bands corresponding to monomeric and dimeric species were visualized and quantified using a FLA 5000 (Fuji).

Synthesis of + 6 extension products with wild-type HIV-1 RT

To assay extension by HIV-1 RT, heat- or protein-annealed tRNA^{Lys3}/RNA 1–311 complexes were first treated with 1.5 mg/ml proteinase K in 1.5% SDS for 1 h at 37°C. After addition of 0.3 M sodium acetate, complexes were phenol/chloroform extracted and precipitated in ethanol. Following centrifugation and vacuum drying, nucleic acids pellets were mildly solubilized in buffer E1 (50 mM Tris–HCl pH 8.0, 50 mM NaCl, 1 mM DTT, 6 mM MgCl$_2$ and 1 mM spermidine). tRNA^{Lys3}/RNA 1–311 complexes were incubated 4 min at 37°C with 70 nM HIV-1 RT and reverse transcription was initiated by addition of dTTP, dGTP, dCTP (100 μM each) and 15 μM ddATP as a chain terminator. After 45 min at 37°C, polymerase activity was blocked by adding 20 mM EDTA, samples were phenol/chloroform extracted and precipitated in ethanol. Purified nucleic acids were re-suspended in urea-containing loading buffer and analyzed by 8% PAGE. Radioactive bands were visualized and quantified using a BioImager BAS 2000 (Fuji).

Synthesis of + 6 extension products in the presence of Vif

To test the influence of Vif on the initiation step of reverse transcription, heat-annealed tRNA^{Lys3}/RNA 1–311 complexes were incubated 20 min at 37°C in buffer H with increasing concentrations of Vif. Half of

the reaction medium was treated as described above to monitor hybridization, then 90 nM HIV-1 RT and 1 mM spermidine were added on the remaining half and after 4 min incubation at 37°C, reverse transcription was initiated by addition of dTTP, dGTP, dCTP (50 μM each) and 7.5 μM ddATP as a chain terminator. After 20 min at 37°C, polymerase activity was blocked by adding 33 mM EDTA, 1% SDS and 1 mg/ml proteinase K. After proteolysis for 1 h at 37°C, samples were phenol/chloroform extracted and precipitated with ethanol. Nucleic acids were re-suspended in urea-containing loading buffer and analyzed by 8% PAGE.

(–) Strong-stop DNA synthesis and strand transfer assays

For (–) strand strong-stop DNA (ssDNA) synthesis and strand transfer experiments, we used the 5′-end labeled ODN primer annealed to donor RNA 1–311. In both assays, NC and/or Vif proteins were incubated with 380 nM WT or E478Q HIV-1 RT for 4 min at 37°C in buffer E2 (0.1 M NaCl, 60 mM Tris–HCl pH 8.0, 6 mM MgCl$_2$, 50 μM dNTPs, 1 mM DTT). In strand transfer experiments, 2 pmol of acceptor RNA were denatured 2 min at 90°C, ice-cooled and re-natured at 37°C for 15 min in 0.1 M NaCl, 60 mM Tris–HCl pH 8.0, 6 mM MgCl$_2$. Acceptor RNA was then added to proteins and incubated 4 min at 37°C in buffer E2. Reverse transcription was initiated by addition of pre-incubated primer/template complexes and proceeded for 5–60 min at 37°C. Polymerization was blocked by addition of 10 mM EDTA, 1% SDS and 2 mg/ml proteinase K. Samples were treated as described above and analyzed by 6% PAGE.

Verification and quantification of strand transfer products

To distinguish genuine strand transfer products from self-priming products, bands containing nucleic acids longer than ssDNA were cut out of the dried gels, eluted at room temperature in 0.5 M ammonium acetate, 0.1 mM EDTA and 0.1% SDS and PCR-amplified with sense (corresponding to nucleotides 9038–9055) and antisense (ODN) primers. Percentage of full-length transfer product (FL) was calculated according to:

$$FL(\%) = \left(\frac{I_{FL}}{I_{FL} + I_{SS-FL}} \right) \cdot 100$$

where I_{FL} is the intensity of the band corresponding to full-length transfer product and I_{SS-FL} is the sum of the intensities of incomplete transfer products and ssDNA.

RESULTS

Vif induces formation of a functional tRNA^{Lys3}/template RNA complex

Δ*Vif* viruses exhibit defects in the early steps of reverse transcription (29,36,42,43) in cells expressing APOBEC3G/3F, and APOBEC3G has been recently
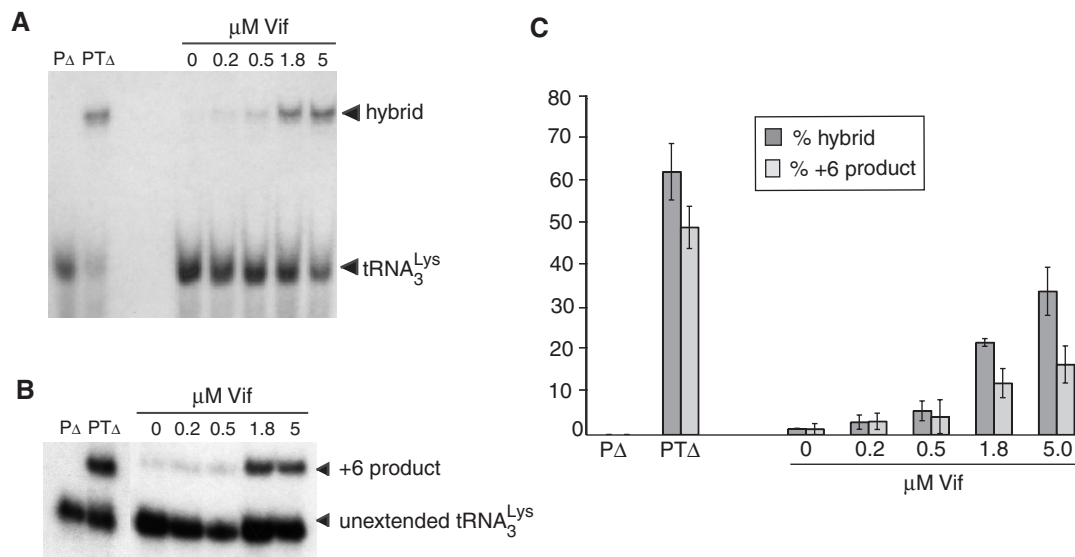
Figure 1. Vif-mediated annealing of tRNA^{Lys3}. (**A**) Bandshift assay showing heat- and Vif-mediated annealing of tRNA^{Lys3} to HIV-1 RNA 1–311. Free and annealed tRNA^{Lys3} are indicated by arrowheads. P$_\Delta$ and PT$_\Delta$ lanes correspond to heat-annealing reaction performed in the absence and presence of RNA 1–311, respectively. (**B**) After deproteinization of the complexes, tRNA^{Lys3} annealed in (A) was extended with HIV-1 RT using a mixture of dCTP, dGTP, dTTP and ddATP. (**C**) Quantification of the gels shown in (A) and (B). The errors bars correspond to the mean ± SD of three to six experiments.

shown to specifically inhibit tRNA^{Lys3}-primed DNA synthesis, possibly by inhibiting tRNA^{Lys3} annealing to the PBS, which occurs concomitantly to or immediately after budding (21). Gag precursor, mature NCp7, or maturation intermediates are thought to be the main players of this step thanks to their RNA chaperone activity (62). However, Vif is present in large amounts in the assembly complexes (Vif/Gag ratio ∼0.5) (32) and in stoichiometric amount with RT in the viral particles (∼100 molecules) (39) and could thus affect tRNA annealing. In favor of this hypothesis, Vif binds the 5′-end region of HIV-1 genomic RNA with high affinity (K_d ∼45 nM) and recognizes many secondary structures in the 5′-UTR, including the PBS region (33).

To test the influence of Vif on tRNA^{Lys3} placement onto the PBS, we performed annealing experiments using purified recombinant Vif, post-transcriptionally modified tRNA^{Lys3} and a RNA template spanning the first 311 nt of HIV-1 genomic RNA. Hybridization was monitored by native gel electrophoresis, and to ascertain specific and functional placement of tRNA^{Lys3} to the PBS, hybrids were tested for their ability to initiate reverse transcription.

In keeping with previous studies, annealing of tRNA^{Lys3} at 70°C in the absence of proteins proceeded with >80% efficiency (Figure 1A, lanes P$_\Delta$ and PT$_\Delta$) (61,63). Remarkably, we observed that Vif was able to significantly promote tRNA^{Lys3} annealing to the PBS at 37°C, in a dose-dependent manner. While no hybrid was observed at 37°C in the absence of Vif, ∼10% of tRNA^{Lys3} was hybridized in the presence of 0.5 μM Vif, and a maximum of 30% of tRNA^{Lys3} was annealed at 5 μM Vif (Figure 1A and C).

To check the functionality of the Vif-annealed tRNA^{Lys3}/RNA 1–311 complexes, we monitored

reverse transcription of the viral RNA from the annealed tRNA^{Lys3} by HIV-1 RT, after removal of Vif. Primer extension was performed using a mixture of dCTP, dTTP, dGTP and ddATP, which allowed addition of 6 nt to tRNA^{Lys3} (Figure 1B). We observed a good correlation between the percentage of hybrid and the amount of +6 extension products with both heat- and Vif-annealed complexes (Figure 1B and C), indicating that RT can recognize these complexes and initiate DNA synthesis. However, for both types of complexes, the amount of +6 extension product was systematically 20% lower than the amount of hybrid, suggesting a systematic loss of tRNA^{Lys3}/RNA 1–311 complexes during protein extraction and purification procedures. Taken together, these results clearly show that Vif is able to promote formation of a functional tRNA^{Lys3}/RNA complex.

Vif has an inhibitory effect on the initiation of reverse transcription

A recent study showed that Vif stimulates HIV-1 RT activity by enhancing both the polymerization rate and RT binding to nucleic acids (44). Vif also stimulates DNA synthesis through abasic sites, a property that could play a role in counteracting APOBEC3G-mediated deamination of proviral DNA (16,64,65). These results suggest that Vif is a co-factor of HIV-1 RT, although these experiments were performed using an artificial primer–template complex (44). We previously showed that when using a RNA template corresponding to the 5′-end of the HIV-1 genomic RNA and tRNA^{Lys3} as primer, (−) strand ssDNA synthesis proceeds through two distinct steps. Initiation corresponds to the addition of the first 6 nt to tRNA^{Lys3}: during this step DNA synthesis is distributive, while the subsequent elongation is processive
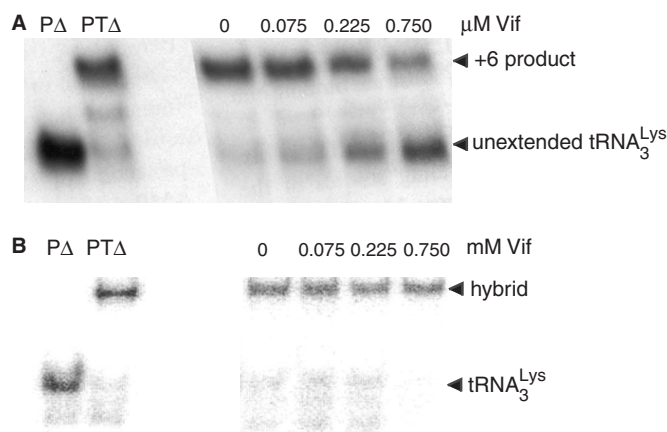
Figure 2. Effect of Vif on the initiation of reverse transcription. Heat-annealed tRNA^{Lys3}/RNA 1–311 complex (PT$_\Delta$) was incubated with HIV-1 RT and increasing concentrations of Vif. (**A**) Reverse transcription was initiated by addition of dTTP, dGTP, dCTP and ddATP. Polymerization was blocked with EDTA after 20 min. Following deproteinization, samples were analyzed by denaturing PAGE to visualize the initiation product (tRNA^{Lys3} extended by 6 nt). (**B**) Native PAGE control showing that Vif did not destabilize the heat-annealed complex.

(66,67). Conversely, priming DNA synthesis with an 18-mer DNA complementary to the PBS (ODN) allows DNA synthesis to start in the elongation mode (63).

To test the effect of Vif on the initiation of reverse transcription, heat-annealed tRNA^{Lys3}/RNA 1–311 complex was incubated with increasing concentrations of Vif and reverse transcription was initiated by adding RT and a mixture of dCTP, dTTP, dGTP and ddATP that allowed to complete the initiation phase of reverse transcription (see Experimental Procedures section) (Figure 2). Note that contrary to the +6 extension assay described above, Vif was present during DNA synthesis. Whereas increasing concentrations of Vif had no effect on the stability of heat-annealed hybrid (Figure 2B), we observed a dose-dependent decrease in +6 extension product correlated with an increase in unextended tRNA^{Lys3} primer (Figure 2A). We did not observe any reverse transcription product between these two forms, indicating that inhibition of reverse transcription takes place before the addition of the first deoxynucleotide. These data suggest that Vif decreased initiation of reverse transcription either by inhibiting the addition of the incoming nucleotide or by preventing binding of RT to the tRNA^{Lys3}/RNA 1–311:RT complex.

Vif stimulates (–) strand ssDNA synthesis

In order to test the effect of Vif on the elongation phase of reverse transcription, we primed ssDNA synthesis with an ODN annealed to the PBS of RNA 1–311. Whereas a low (0.2 μM) concentration of Vif had no significant effect on ssDNA synthesis, a 2 μM concentration of Vif increased ssDNA synthesis by ~2-fold after a 60-min reaction time (Figure 3). Analysis of the reverse transcription products by denaturing PAGE showed that the increase in DNA synthesis correlated with a decrease in RT
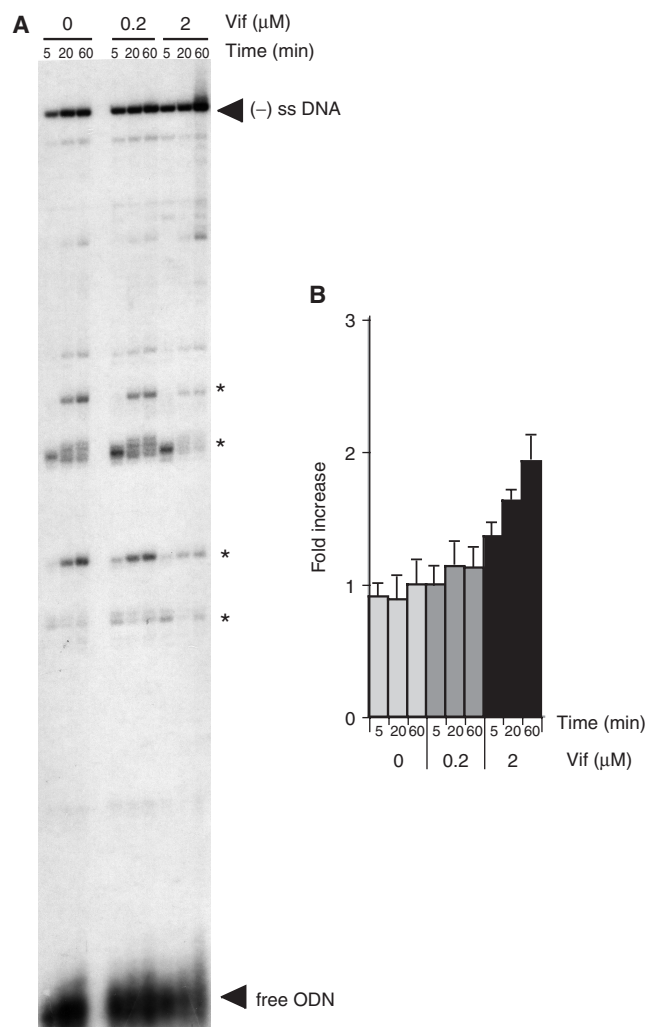

Figure 3. Effect of Vif on (–) strand strong-stop DNA synthesis. A DNA primer (ODN) heat-annealed to RNA 1–311 was extended by incubating with HIV-1 RT, Vif and dNTPs. Polymerization was for 5, 20 or 60 min and the samples were deproteinized before PAGE. (**A**) Denaturing gel showing the time course of labeled ODN extension in the presence of increasing concentrations of Vif. Minus strong-stop DNA and unextended ODN are indicated. Asterisks indicate RT pauses specifically reduced by the addition of Vif. (**B**) Quantification of (A), data is normalized to the ratio of ssDNA synthesized at 60 min in the absence of Vif. The errors bars correspond to the mean ± SD of three to six experiments.

pausing (Figure 3A, asterisks), suggesting that Vif-enhanced ssDNA synthesis by facilitating reverse transcription through stable secondary structures present in the RNA template. Indeed, secondary structures slow down RT, inducing dissociation of the enzyme and subsequently pauses (68,69). Thus, our data suggest that stimulation of ssDNA synthesis results from Vif–RNA interactions.

Effects of NC intermediates and Vif on viral RNA dimerization, tRNA^{Lys3} annealing and (–) ssDNA synthesis

Mature NCp7 is a well-characterized co-factor of HIV-1 RT (70), and at least some of the Vif-binding sites in the 5′ region of viral RNA are also NCp-binding
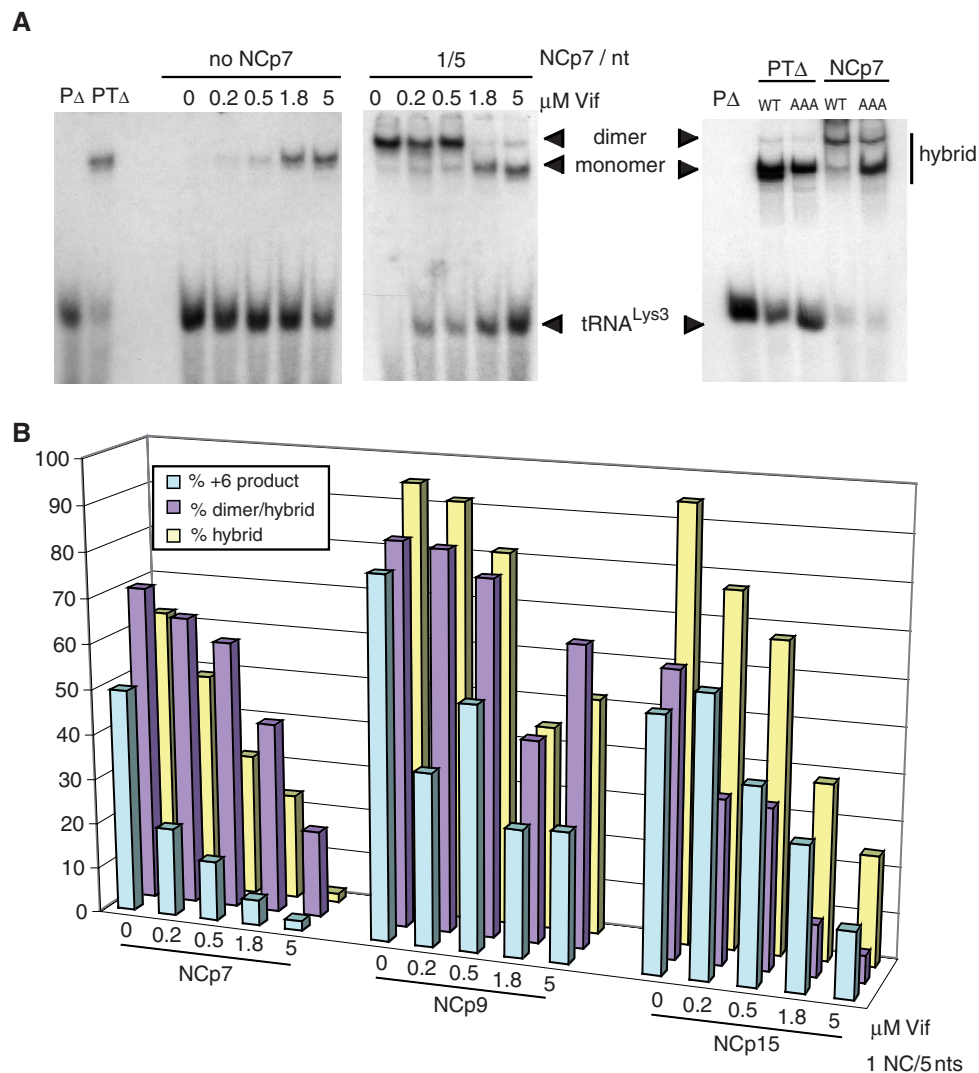
Figure 4. Effect of Vif on NCp-mediated tRNA^{Lys3} annealing and RNA dimerization. (**A**) Bandshift assay showing heat- (PT$_\Delta$) and NCp-mediated annealing of tRNA^{Lys3} to HIV-1 RNA 1–311 in presence of increasing concentration of Vif. Free and annealed tRNA^{Lys3} on monomeric or dimeric 1–311 RNAs are indicated. P$_\Delta$ and PT$_\Delta$ lanes correspond to heat-annealing reaction performed in the absence and presence of RNA 1–311, respectively. (**B**) Quantification of the data: blue bars, % of +6 product; purple bars, % of tRNA^{Lys3} annealed to dimeric RNA/tRNA^{Lys3} annealed to monomeric + dimeric RNA; yellow bars, % of tRNA^{Lys3} annealed to monomeric + dimeric RNA.

sites (62). While the effects of NC on tRNA^{Lys3} annealing and ssDNA synthesis have been well documented (62), the combined effects of Vif and NC on these steps have never been studied. Furthermore, as Vif has been shown to modulate processing of the Gag precursor (47), we also studied the effects of NCp9 and NCp15, together with Vif, on these steps. Primer tRNA^{Lys3} annealing and extension experiments were performed as described above, except that NC proteins were present at a concentration corresponding to complete coverage of the RNA template (1 NCp/5 nt) (71) (Figure 4). In the absence of Vif, this saturating concentration of NCp allowed very efficient hybridization of tRNA^{Lys3} to the PBS, with a maximum of 90% hybrids with NCp9 and NCp15 and a slightly reduced efficiency with NCp7 (70% hybrids) (Figure 4B, yellow bars), in agreement with previous studies (61), Unexpectedly, increasing Vif concentrations had an

inhibitory effect on the formation of tRNA^{Lys3}/vRNA hybrids induced by NCp (Figure 4A and B, yellow bars), with a corresponding decrease in +6 product formation (Figure 4B, blue bars).

Moreover, while the tRNA^{Lys3}/vRNA complex formed by heating or in the presence of Vif migrated as a single band, two tRNA^{Lys3}/vRNA complexes could be observed in the presence of NC proteins, with the main one migrating slower than the complex formed in the presence of Vif (Figure 4A). These two bands correspond to tRNA^{Lys3} annealed to monomeric and dimeric forms of HIV-1 RNA. Indeed, when using a fragment of HIV-1 genomic RNA with point mutations (AAA) in the dimerization initiation site (DIS), which is crucial for RNA dimerization (51,52), the monomeric form of the hybrid became largely predominant, even in the presence of NCp7 (Figure 4A, right panel). Interestingly, increasing

Vif concentrations progressively inhibited NCp7-induced RNA dimerization, and inhibition was almost complete at the highest Vif concentrations (Figure 4A, left panel). Vif had similar effects when tRNA^{Lys3} annealing and HIV-1 RNA dimerization were induced by NCp15, i.e. Vif efficiently inhibited both processes (Figure 4B right panel). However, tRNA^{Lys3} annealing and especially HIV-1 RNA dimerization promoted by NCp9 were more resistant to inhibition by Vif (Figure 4B, central panel).

These results prompted us to analyze more precisely the capacity of Vif to modulate HIV-1 RNA dimerization in the absence of tRNA^{Lys3} and in absence or in presence of NCp7 (Figure 5). Indeed, HIV-1 RNA can form two different kinds of dimers *in vitro*, termed loose and tight dimers (51). Loose dimers are formed first and correspond to kissing complexes interacting by the DIS loop (57,72,73). Formation of tight dimers depends on the presence of the sequences 3′ to the major splice donor site, the incubation temperature and the presence of NCp. Although several authors have proposed that tight dimers could correspond to extended duplexes, this has not been demonstrated conclusively on large RNA fragments, and alternatively the kissing complexes could be stabilized by tertiary interactions that remain to be identified (51). Using an RNA fragment encompassing the first 615 nt of HIV-1 genomic RNA, we analyzed dimerization using two different electrophoresis conditions: (i) native electrophoresis in Tris–Borate magnesium (TBM) buffer at 4°C, under which both loose and tight dimers are stable (Figure 5A) and (ii) semi-denaturing electrophoresis in Tris–Borate-EDTA (TBE) buffer at room temperature, under which only the tight dimers survive (Figure 5B) (72). In the absence of NCp, Vif stimulated RNA dimerization in a concentration dependant manner, and a dimerization yield of 30% was observed at 5 μM Vif (Figure 5A). This Vif–induced RNA dimer is a loose dimer, as it dissociated during electrophoresis under semi-denaturing conditions (Figure 5B) (57,72,73). In the absence of Vif, a saturating NCp concentration induced >90% RNA dimerization, and the NCp-induced dimer was predominantly the tight dimer (compare Figure 5A and B), in keeping with previous studies (72,74). However, increasing Vif concentration progressively decreased the dimerization yield from >90% to ~50% (Figure 5A), and the RNA dimer remaining at the highest Vif concentration was exclusively the loose dimer (compare Figure 5A and B). Thus, Vif was able to promote formation of the loose dimer, and to inhibit formation of the tight dimer by NCp, indicating that, as in the tRNA^{Lys3} annealing experiments, the effect of Vif was dominant over NCp. Taken together, these results suggest that the dominant effect of Vif over NC proteins is due to Vif/NC interactions, rather than to Vif–RNA interactions.

Next, we compared the efficiency of ssDNA synthesis in the presence of NCp15, NCp9 and NCp7 (Figure 6). At a concentration of 1 NCp/5 nt, a ~4.5-fold increase of ssDNA was observed with NCp9 after 60 min of reaction, which correlated with a strong decrease in the intensity of RT pauses (Figure 6, middle panel, black triangles). Comparatively, NCp7 and NCp15 had
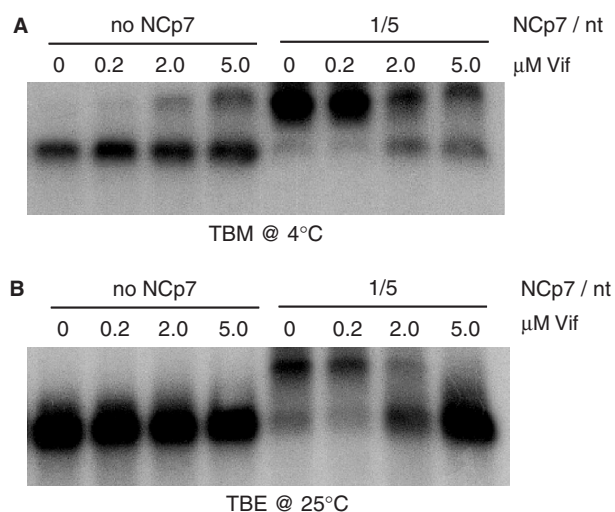

Figure 5. Vif and NC-mediated HIV-1 RNA dimerization *in vitro*. HIV-1 RNA 1-615 (100 nM) was allowed to dimerize *in vitro* with Vif (0, 0.2, 2.0 and 5.0 μM) in absence or in presence of NCp7 protein (saturating concentration, 6 μM). After removal of Vif and NCp7, RNA was loaded on an agarose gel and run in (**A**) native (Tris–Borate-Magnesium, TBM, at 4°C) and (**B**) semi-denaturing (Tris–Borate-EDTA, TBE, at 25°C) electrophoresis conditions.

a smaller stimulatory effect (~2.5 and ~3-fold, respectively) and less influence on RT pausing (Figure 6).

Given the stimulatory effect of Vif on ssDNA synthesis (Figure 3B), we tested whether Vif could act synergistically with NC to increase the yield of ssDNA. Compared with DNA synthesis performed with NC or Vif alone, reactions performed in the presence of both Vif and NC showed no significant synergy. Addition of 2 μM Vif to reactions containing saturating amounts of NC significantly delayed ssDNA synthesis, and similar yields of ssDNA were observed only at the last time point (60 min) (Figure 6). However, RT pauses specifically induced by either NCp7 or NCp15 (Figure 6, left and right panels, asterisks) were strongly diminished in the presence of 2 μM Vif. Note that NCp7 and NCp15 generated different pausing patterns, suggesting that these two proteins preferentially bind to different RNA motifs in the R region, but that Vif had similar effects in these two reactions. These results suggest that both NC and Vif contributed to increase the processivity of RT and that NC–RNA interactions are affected by Vif binding to RNA.

Stimulation of the first strand transfer by Vif and NC intermediates

The presence of repeated (R) sequences at both ends of retroviral genomes allows transfer of the neo-synthesized ssDNA from the 5′ to the 3′-terminal region of the viral RNA (75). It has been shown that the first strand transfer is strongly enhanced by NCp9 and NCp7 *in vitro* (62,76,77), and interactions between NC and the TAR loop of the viral RNA or its complement cTAR on the ssDNA play an important role in this process (49,78). As we recently showed that the TAR apical loop is a high affinity Vif-binding site (33) (Bernacchi *et al.*, in press for publication), we tested the influence of Vif on the first
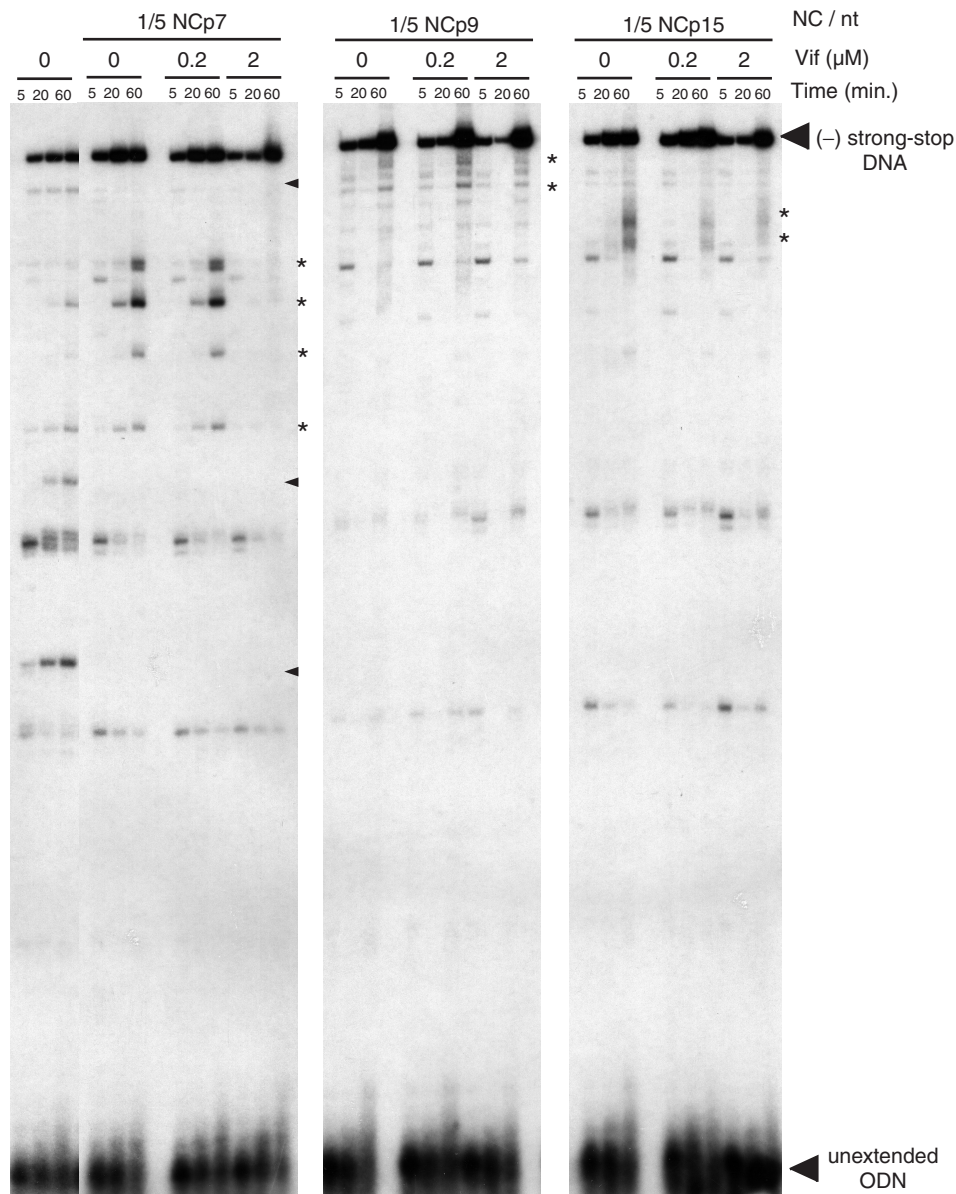
Figure 6. Minus strong-stop DNA synthesis in the presence of Vif and NC proteins. ODN extension products were analyzed by denaturing PAGE. Asterisks indicate RT pauses specifically induced by the different NC proteins and suppressed by high concentration of Vif proteins. Black triangles indicate RT pauses specifically suppressed by high concentration of NC proteins. The NC coverage of 1NCp/5 nt corresponds to a concentration of 6 μM of NCp.

strand transfer. For that purpose RNA 1–311 was used as the donor template, and RNA 8607–9229 as the acceptor RNA, as previously described (53).

During ssDNA synthesis, as the RNA template is being degraded by RNase H, the cTAR sequence in ssDNA can fold into a hairpin structure that is able to 'self-prime' reverse transcription, yielding abortive products that are longer than ssDNA. First, we used an RNase H(–) HIV-1 RT that is unable to give rise to strand transfer and self-priming products to unambiguously identify ssDNA (Figure 7A, first lane). Longer products were observed with wild-type RT in the presence of acceptor RNA but in the absence of Vif or NC proteins. These were self-priming products, since PCR amplification of the corresponding

gel-eluted nucleic acid bands did not yield any products when using primers specific to strand-transfer products (see Experimental Procedures section, data not shown). On the other hand, strand transfer reactions performed with wild-type RT and increasing Vif concentrations yield a faint but reproducible product near the top of the gel, identified as full-length strand transfer product by PCR (Figure 7A). Quantification showed that ∼5% of the ssDNA was converted into full-length transfer product in the presence of 2 μM Vif. Thus, Vif can stimulate the strand transfer reaction, albeit with a limited efficiency.

In the absence of Vif and in the presence of a saturating concentration of NC proteins, strand transfer proceeded with varying efficiency, depending on the protein: the
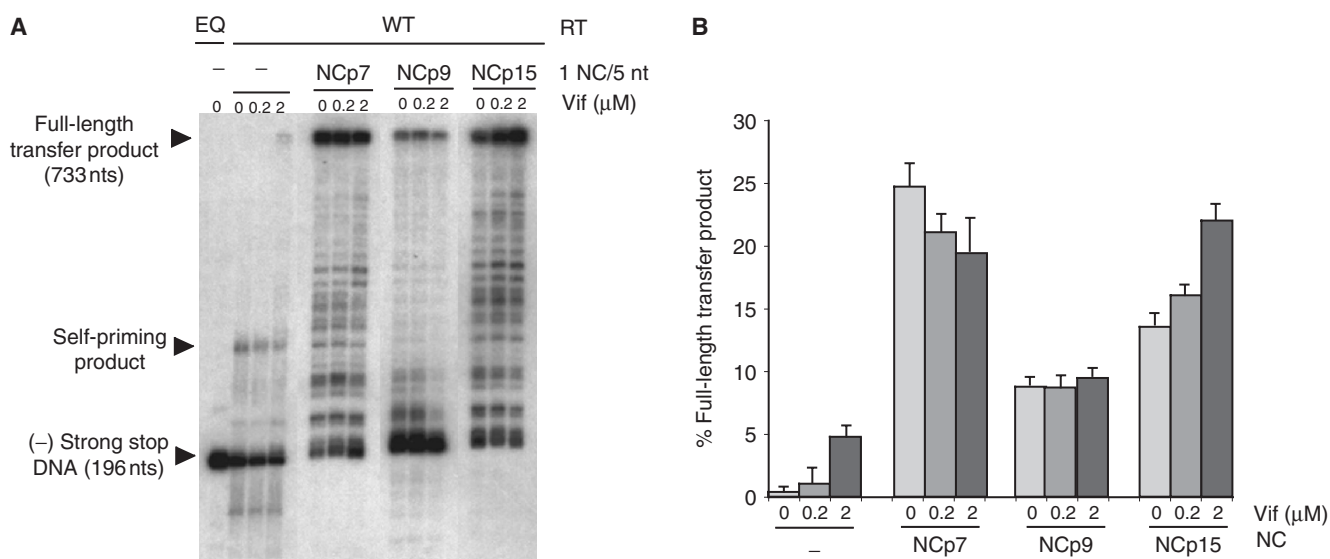
Figure 7. Effects of Vif and NC proteins on the first strand transfer. (**A**) PAGE analysis of transfer products. ODN/RNA 1–311 complexes were incubated with RT, dNTPs, acceptor RNA, Vif and/or NC proteins (6 µM). A control using RNase H(–) E478Q HIV-1 RT is shown in first lane. Minus strong-stop DNA, self-priming products and full-length transfer product are indicated. (**B**) Quantification of the data strand transfer efficiency was calculated as described in the Materials and Methods section. The errors bars correspond to the mean ± SD of three experiments.

highest efficiency was obtained with NCp7, giving rise to ∼25% of full-length transfer product, while NCp15 and NCp9 were less potent (∼14% and ∼8% of full-length product, respectively) (Figure 7). This result clearly shows that mature NCp7 promotes strand transfer more efficiently than NC maturation intermediates, probably due to the different nucleic acids chaperone and aggregating activities of these proteins (59). Adding increasing concentrations of Vif to a saturating concentration of NC led to a dose-dependent modulation of NCp7 and NCp15 activity, while no effect was observed on NCp9. Although Vif moderately decreased the amount of full-length transfer product in NCp7-mediated transfer reaction, Vif showed an opposite effect in presence of NCp15, where addition of 2 µM of Vif reproducibly increased the yield of full-length transfer product by ∼40%. Taken together, these results show that Vif alone slightly promote the first strand transfer reaction and that this protein is able to specifically modulate the activity of NCp7 and NCp15 in this process.

DISCUSSION

Several reports demonstrated that deletion of the *vif* gene affected reverse transcription during the entry phase of the viral life cycle (39,40,42,44) and prevented endogenous reverse transcription (29,36,43), suggesting that Vif could interact with virion components involved in the regulation of reverse transcription such as RT (44), NC (31,32), tRNA^{Lys3} (29,79) and genomic RNA (28–30,33). However, the molecular mechanisms of Vif function in reverse transcription have remained unclear. Since we recently showed that Vif is an RNA-binding protein that preferentially binds to the 5′ terminal region of HIV-1 genomic RNA, including the

PBS, we decided to examine for the first time the *in vitro* contribution of Vif to the initial steps of reverse transcription, either alone or in combination with NC proteins at different maturation stages. In addition, since Vif is present in the HIV-1 assembly complexes (32), we studied the effect of Vif on RNA dimerization, as this step is a prerequisite to efficient HIV-1 RNA packaging (51). In order to analyze the intrinsic properties of Vif, we first studied this protein in the absence of other viral proteins. Then, to evaluate the potential biological significance of our results, we studied the effects of Vif in the presence of NC proteins.

In the absence of NC proteins, Vif enhances several early steps of the reverse transcription process. First, Vif is able to anneal tRNA^{Lys3} to a PBS-containing RNA fragment quite efficiently (Figure 1A), even though Vif is less efficient than NCp7, NCp9 and NCp15 in this respect (Figure 4B). In addition, the resulting primer–template complex is fully functional, since HIV-1 RT was able to initiate reverse transcription of these complexes (Figure 1B). The annealing activity of Vif might be important for HIV-1 replication in non-permissive cells, since it was recently shown that inhibition of tRNA^{lys3}-primed reverse transcription of ∆*Vif* viruses by APOBEC3G and 3F accounts for an important part of its antiviral effect, independently of its deaminase activity (21,80).

Second, Vif significantly decreases pausing of RT and enhances ssDNA synthesis (Figure 3). RT pausing most often occurs when RT is blocked by stable secondary structures present in the RNA template (68,69). Indeed, we previously showed that Vif binds to several secondary structure motifs in the R and U5′ regions of the genomic RNA (33), and our present results suggest that Vif is able to destabilize these structures, allowing a better processivity of HIV-1 RT. The effect of Vif on RT

pausing might also be the result of an affinity increase of RT for the primer–template complex in the presence of Vif, as observed by Cancio *et al.* (44) using poly(rA)/oligo(dT). Even though the exact mechanism by which Vif increases RT processivity remains to be established, this activity is analogous to the previously described effect of NCp7 on RT pausing (62,68).

We also reproducibly observed that Vif stimulates the first strand transfer, albeit with a reduced efficiency compared to NC proteins: Vif was 2-fold less efficient than NCp9, 3-fold less efficient than NCp15 and 5-fold less efficient than NCp7 (Figure 7). Nevertheless, Vif shares with NC proteins the ability to anneal tRNA^{Lys3} to the PBS, decrease RT pausing, and promote strand transfer. These properties, especially the first and the third one, are characteristic of the RNA chaperone activity of NC proteins (59). In addition, we found that Vif, like NCp (74), promotes dimerization of HIV-1 RNA, even though the former induces formation of loose dimers, while the latter favors tight dimers. Therefore, our results show that Vif is an authentic RNA chaperone (81).

These results prompted us to look for potential synergic effects between Vif and various Gag processing products such as NCp15, NCp9 and NCp7. Processing of the Gag precursor takes place in a sequential manner during maturation of HIV-1 viral particles due to differences in cleavage site efficiency (82). Cleavage starts at the p2/NC junction, resulting in the production of MA-CA-p2 and NCp15 intermediates products. Secondary cleavage releases the mature MA protein as well as the NCp9 (NCp7-p1) intermediate after removal of p6 (83). Tertiary cleavage produces mature CA and NCp7 proteins. Mature viral particles are not only composed of fully processed Gag and Gag-Pol products, but also contain residual amounts of Gag precursors and processing intermediates (47). Moreover, Vif modulates the HIV-1 protease activity, leading to the possibility that virion incorporation of Vif could stabilize NC intermediates (45–47). A number of molecular rearrangements occur in the RNP during or just after budding, including tRNA^{Lys3} hybridization (70), RNA dimer maturation (84) and initiation of reverse transcription (70).

In the tRNA^{Lys3} annealing reactions, Vif clearly has a dominant effect over NC proteins (Figure 4). Indeed, increasing concentrations of Vif significantly inhibited NC-induced tRNA^{lys3} annealing (Figure 4B) and RNA dimerization (Figures 4A and 5). This inhibitory effect of NC-mediated functions might explain why excessive Vif expression is detrimental to viral infectivity (47). Importantly, the highest Vif concentration used in this study corresponds to a Vif/NCp ratio of 1/3, which is close to the 1/2 Vif/Gag ratio in the assembly complexes (32,39). Thus, Vif might initiate tRNA^{lys3} annealing in the assembly complexes, and since it is mostly excluded from the virions (the Vif/NCp ratio in virions is between 1/20 and 1/40), its inhibitory effect would be relieved after assembly of the virions, and NC proteins could complete tRNA^{Lys3} annealing. In this context, inhibition of the initiation of reverse transcription by Vif (Figure 2) might prevent premature initiation of DNA synthesis in the assembly complexes of the producing cell.

Interestingly, identical effects have been observed with the Tat protein (85,86). Similarly, inhibition of the NC-induced viral RNA dimerization by Vif (Figure 5) might temporally regulate RNA packaging and prevent premature maturation of the loose kissing loop complex into a more thermostable tight dimer. Thus, our results suggest that Vif is a negative regulator of several NCp-associated functions in the assembly complexes, and that the NC domain (either in Gag or its maturation product) becomes fully active in the viral particles, from which Vif is mostly excluded. Inhibition of the NCp-mediated tRNA^{lys3} annealing and RNA dimerization, as well as inhibition of the initiation of reverse transcription by Vif might allow temporal fine-tuning of these steps.

During ssDNA synthesis, both Vif and NC proteins decreases RT pausing. However, NC proteins also appear to induce pausing at sites that were specific for each NC species (Figure 6). These pauses could result from increased RNase H activity of RT in the presence of NC proteins (62). No additional pauses were observed with Vif; on the contrary Vif suppressed the NC-induced pauses. Thus, Vif act in cooperation with NC proteins to increase RT processivity and favor synthesis of long DNA products.

The effect of Vif on NC-induced strand transfer is limited, but, unexpectedly, Vif has either a positive, null or negative effect depending on the maturation stage of NC proteins (Figure 7). Significantly, NCp9 is the most efficient NC species in ssDNA synthesis, whereas NCp7 has the most efficient strand transfer activity, suggesting that maturation of the NC intermediates might regulate the early steps of HIV-1 reverse transcription.

Thus far, it was unclear whether Vif has a direct role in reverse transcription. Our data strongly suggest that Vif does play a role during the early phase of this process in coordination with other components of the viral core such as Gag and its maturation products and during the dimerization of genomic RNA. Taken together, our data lead to the possibility that Vif might be a temporal regulator during viral assembly: (i) by interacting with genomic RNA and NC-derived products, Vif may prevent RNA dimerization/packaging and premature initiation of reverse transcription; (ii) however, still in conjunction with Gag precursors, Vif could promote the placement of tRNA^{Lys3} on to the PBS, stabilizing NC intermediates to increase, at the right time, the efficiency of the early steps of reverse transcription. Obviously, a detailed temporal analysis of the effects of Vif on HIV-1 replication will be required to test our hypothesis.

ACKNOWLEDGEMENTS

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