Human immunodeficiency virus 1 (HIV-1) virion infectivity factor (Vif) is part of reverse transcription complexes and acts as an accessory factor for reverse transcription

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Abstract

Virion infectivity factor (Vif) facilitates HIV infection by counteracting APOBEC3G late in replication in virus-producer cells. Here, we show that early after infection of new target cells Vif is part of the HIV reverse transcription machinery and acts as an accessory factor for reverse transcription. Vif protein was present in gradient fractions containing reverse transcription complexes ( RTCs ), and anti-Vif antibody immunoprecipitated HIV reverse transcription products from these gradient fractions. To investigate a role for Vif in RTCs independent of APOBEC3G, we created an intracellular environment that would restrict reverse transcription by pre-treating permissive target cells with 5-Fluoro-2-deoxyuridine, a thymidylate synthetase inhibitor, prior to infection with virus from permissive cells. Infectivity assays and quantitation of reverse transcription products demonstrated that replication of HIV lacking Vif was inhibited to a greater degree than wild type, without concurrent mutation of reverse transcription products, suggesting compromised reverse transcription in the absence of Vif.

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Introduction

The human immunodeficiency virus (HIV) virion infectivity factor (Vif) is essential for efficient viral replication in natural target cells (Gabuzda et al., 1992; Sova et al., 1995; von Schwedler et al., 1993). A major function of Vif is to prevent the action of APOBEC3G, a cellular inhibitor of HIV replication (Sheehy et al., 2002), or other APOBEC3 family members such as APOBEC3F (Zheng et al., 2004). APOBEC3 enzymes are cytosine deaminases that, in the absence of Vif, deaminate C to U residues in newly synthesised HIV minus strand DNA, leading to G–A mutation in the HIV proviral DNA (Cullen, 2006; Harris et al., 2003; Lecossier et al., 2003; Mangeat et al., 2003; Zhang et al., 2003). In viral producer cells, Vif induces degradation of and inhibits virion incorporation of APOBEC3G, thus preventing its inhibitory actions on HIV replication in subsequent target cells (Kao et al., 2003b; Mariani et al., 2003; Marin et al., 2003; Sheehy et al., 2003; Yu et al., 2003).

However, broader biological roles of Vif in HIV infection remain to be fully explored. Whilst some reports describe that virions produced in the absence of Vif have normal viral protein and RNA content (Bouyac et al., 1997; Fouchier et al., 1996; Gaddis et al., 2003; Ochsenbauer et al., 1997; von Schwedler et al., 1993), others describe abnormal virion morphology and less stable virion cores (Borman et al., 1995; Ohagen and Gabuzda, 2000; Sakai et al., 1993). Virus particles from Vif-deficient HIV enter target cells normally but are defective in production or accumulation of reverse transcription products (Courcoul et al., 1995; Nascimbeni et al., 1998; Simon and Malim, 1996; Sova and Volsky, 1993; von Schwedler et al., 1993). This well described observation may relate to the actions of APOBEC3G that in the absence of Vif can induce mutation in reverse transcription products (Harris et al., 2003; Lecossier...
deoxyuridine (FdUrd), a pyrimidine analogue that alters cellular virus in target cells that had been treated with 5-Flouro, 2-HIV was compromised to a greater degree than wild type (WT) permissive cells, reverse transcription by Vif-deficient (RTCs). In the absence of APOBEC3G or other factors from non-products in RTCs and show that Vif protein co-sediments with solution that Vif is physically associated with reverse transcription process. We demonstrate by co-immunoprecipita-

tion products. Early and late reverse transcription products were co-precipitated by an anti-IN but not p24 antibody, consistent with reports that RTCs contain IN but not p24 under these conditions (Bukrinsky et al., 1993; Karageorgos et al., 1993) (data not shown). In a similar way we attempted to immunoprecipitate newly synthesised reverse transcription products using a Vif antibody following WT and ΔVif infections. However, these experiments required initiation of infection using cell-free virus and the results were inconclusive due to the lower levels of reverse transcription products present in infection with cell-free virus. To examine whether Vif was associated with other known RTC components we analysed whether Vif antibody could co-precipitate RT activity. Cells were lysed and immunoprecipitated, as above with pre-immune sera, Vif and IN polyclonal antibody. The immunoprecipitates were assayed for their ability to catalyse RT-PCR of an exogenous RNA substrate (in vitro transcribed dengue virus (DV) RNA) and compared with RT-PCR reactions performed with AMV RT, the initial HIV infected cell lysate or no RT enzyme control. Both Vif and IN antibodies but not pre-immune sera successfully immunoprecipitated RT-PCR activity (Fig. 1B). Thus, in newly infected cells Vif was associated with both newly synthesised reverse transcription products and RT enzyme activity, two essential components of an active RTC.

We next wished to test whether reverse transcription products could also be co-immunoprecipitated with Vif after gradient fractionation of RTCs. Our laboratory has previously established and validated a system for gradient isolation of HIV RTCs (Carr et al., 2006; Karageorgos et al., 1993). Lysates from infected cells were harvested at 6 h pi and fractionated on sucrose velocity gradients and the fractions were analysed for three known RTC properties: (i) the presence of newly synthesised reverse transcription products, (ii) the presence of endogenous RT activity, and (iii) velocity sedimentation rate. Fractions were also subjected to immunoprecipitation using pre-immune sera or HIV Vif antibody. Before immunoprecipitation, in vitro endogenous RT activity peaked in fr 4–5 (Fig. 2A), whilst ssDNA was more broadly distributed and peaked in fr 5–6 (1.08–1.09 g/ml sucrose) consistent with the sedimentation rate of HIV RTCs (Carr et al., 2006; Karageorgos et al., 1993). ssDNA that was specifically precipitable by Vif antibody was more sharply confined to frs 5 and 6 only (Fig. 2B). Similarly, U5-gag DNA, a marker of later reverse transcription, was distributed broadly across the gradient, peaking in fr 5–6 and was specifically precipitated by Vif antibody from fr 6 only. The lack of precipitation of U5-gag DNA from fr 5 by Vif antibody may be due to loss or inaccessibility of Vif protein in the later U5-gag containing RTC structures in fr 5. Together these results indicate that structures sedimenting at a rate characteristic for RTCs and containing both reverse transcription products and RT enzyme activity also contain HIV Vif protein. To confirm the association between Vif protein and RTCs further, RTCs

Results

HIV Vif co-precipitates with reverse transcription products in RTCs

Infection was initiated using a highly productive one-step synchronous cell-to-cell infection model, involving mixing of infected donor cells (H3B; a clone of H9 cells persistently infected with HTLV-IIIB in which there is minimal extrachromosomal HIV DNA or active reverse transcription) with uninfected recipient Hut-78 cells. This results in a burst of new HIV reverse transcription and DNA synthesis (Li and Burrell, 1992). Following infection cells were lysed, immunoprecipitated with Vif or control antibodies and DNA was extracted from precipitated complexes and analysed for HIV reverse transcription products. Early and late reverse transcription products were precipitated by a rabbit polyclonal Vif antibody but not with the pre-immune sera control (Fig. 1A). A monoclonal Vif antibody also co-precipitated U5/gag DNA at 6 h pi confirming that HIV reverse transcription products specifically co-precipitate with Vif. The quantitative difference in immunoprecipitation using different antibodies and at different time points post infection (pi) may reflect different antibody binding ability or accessibility of the Vif protein in the RTC. In separate experiments to validate the immunoprecipitation technique, HIV reverse transcription products were co-precipitated by an anti-IN but not p24 antibody, consistent with reports that RTCs contain IN but not p24 under these conditions (Bukrinsky et al., 1993; Karageorgos et al., 1993) (data not shown). In a similar way we attempted to immunoprecipitate newly synthesised reverse transcription products using a Vif antibody following WT and ΔVif infections. However, these experiments required initiation of infection using cell-free virus and the results were inconclusive due to the lower levels of reverse transcription products present in infection with cell-free virus. To examine whether Vif was associated with other known RTC components we analysed whether Vif antibody could co-precipitate RT activity. Cells were lysed and immunoprecipitated, as above with pre-immune sera, Vif and IN polyclonal antibody. The immunoprecipitates were assayed for their ability to catalyse RT-PCR of an exogenous RNA substrate (in vitro transcribed dengue virus (DV) RNA) and compared with RT-PCR reactions performed with AMV RT, the initial HIV infected cell lysate or no RT enzyme control. Both Vif and IN antibodies but not pre-immune sera successfully immunoprecipitated RT-PCR activity (Fig. 1B). Thus, in newly infected cells Vif was associated with both newly synthesised reverse transcription products and RT enzyme activity, two essential components of an active RTC.

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were isolated by sucrose velocity sedimentation, which is influenced by shape, size and density and were then further purified on a sucrose equilibrium gradient, which separates according to density. We have previously shown that authentic RTCs (identified by the presence of RT activity, newly synthesised reverse transcription products and IN protein) band at 1.21–1.22 g/ml sucrose under these conditions (Carr et al., 2006). We took the RTC-containing fractions banding in this second gradient at 1.20–1.24 g/ml sucrose (fr 7–9) and containing reverse transcription products and performed

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immunoprecipitations. Both early (ssDNA) and late (U5-gag DNA) reverse transcription products in the major RTC fraction at 1.22 g/ml sucrose were co-precipitated by Vif antibody (Fig. 3) thereby confirming that the association between Vif and RTCs remains after more extensive purification of RTCs.

We next examined directly whether Vif protein could be detected in these immunoprecipitating fractions and if the sedimentation of Vif in these fractions was dependent on the presence of active reverse transcription and the presence of RTCs. The sedimentation of Vif protein alone without the presence of other HIV proteins was assessed by sucrose gradient fractionation and Western blot of a cell lysate from 293 cells transfected with a Vif expression vector (pCMV-Vif myc) (Feng et al., 2004). Vif protein was detected at the top of the gradient (fr 1) and in the sucrose cushion (fr10), but not within the gradient where RTCs are found (Fig. 4A). Next, to assess the velocity sedimentation of Vif protein in the presence of other HIV proteins but in the absence of active reverse transcription, chronically infected H3B cells were mixed with uninfected Hut-78, lysed immediately (0 h) and subjected to sucrose velocity gradient sedimentation and Western blot. At 0 h pi Vif was distributed at the top of the gradient (fr 2–5) and p24 overlapped with this with a slightly broader distribution (fr 1–5; Fig. 4B.2) whilst as previously described (Li and Burrell, 1992) no significant reverse transcription products were present (Fig. 4B.1). These findings are likely to represent Vif-containing HIV assembly complexes derived from the chronically infected H3B cells. When the cultures were incubated for 6 h after cell mixing before harvesting to allow the onset of reverse transcription, ssDNA and Vif proteins were present in frs 1–6 with a peak together in fr 3–4. U5/gag DNA was in a more discrete and more rapidly sedimenting structure in frs 4–6. In contrast to the results at 0 h, the distribution of p24 protein did not directly overlap with Vif but was maximal at the top of the gradient (Fig. 4C.1 and 2). These results suggest that (i) Vif protein and reverse transcription products can both be detected at a sedimentation velocity (1.08–1.09 g/ml sucrose) from which we have shown successful co-immunoprecipitation of reverse transcription products with Vif antibody and (ii) that Vif and p24 proteins are present in complexes prior to reverse transcription, and upon activation of reverse transcription, the majority of Vif remains in complexes but the majority of p24 is lost and sediments as free protein. These findings further demonstrate that Vif is an integral part of HIV RTCs.

**An additional function of Vif in HIV reverse transcription independent of APOBEC3G**

Earlier studies have demonstrated that Vif-deficient (∆vif) virus can replicate normally in permissive cells. However, the intracellular environment within HIV target cells is well known to influence the efficiency of viral replication. We thus aimed to assess the function of Vif in early HIV infection in a target cell environment that did not contain APOBEC3G but was constrained for normal viral replication for other reasons. This situation was produced by pre-treating permissive (i.e. APOBEC3G negative) target cells with a low concentration of the uridine analogue FdUrd. This agent inhibits thymidylate synthase resulting in the disruption of the normal cellular balance of dNTPs, with elevated levels of dUTP and decreased dTTP (Jackson, 1978). The concentration(s) of FdUrd used were in the nanomolar range which was non-cytotoxic over the duration of the experiment (data not shown) and was less than the micrometer concentration range generally employed to induce DNA mutation and cell death (Meyers et al., 2005)

The permissive indicator cell line HeLaCD4 LTR-βgal was pre-treated with FdUrd and infectivity assays were performed with WT and ∆Vif virus that had been produced from permissive CEM-SS cells and thus would not contain any APOBEC3G incorporated within virions. A dose dependent
inhibition of HIV infectivity by FdUrd was seen with more marked restriction of ΔVif than WT infection at all doses tested (Fig. 5A). Analysis at a single FdUrd concentration showed that infectivity of ΔVif was reproducibly and significantly inhibited approximately 40% further than that observed for WT HIV (Fig. 5A, B).

We next assessed if the greater inhibition of Vif-deficient HIV infection in the presence of FdUrd was due to restriction of reverse transcription. Permissive CEM-SS cells were pre-treated with 50 nM FdUrd and infected with WT or ΔVif virus, as above. Extrachromosomal DNA was extracted at early time points pi and reverse transcription products quantitated. Consistent with the infectivity data, results showed a reduction in levels of first strand transfer reverse transcription products by FdUrd in ΔVif compared with WT HIV infection (Fig. 6B) with a significant and reproducible 40% greater inhibition of reverse transcription from ΔVif virus at 10 h pi (Fig. 6B).

The greater degree of inhibition of reverse transcription of Vif-deficient virus by FdUrd may be a consequence of greater frequency of FdUrd-induced mutation or reduced efficiency of the reverse transcription process itself. We investigated this by sequence analysis of the early reverse transcription products produced in the presence of FdUrd. The nef-LTR region (706 bp), representing products following first strand transfer reverse transcription products by FdUrd in ΔVif compared with WT HIV infection (Fig. 6A) with a significant and reproducible 40% greater inhibition of reverse transcription from ΔVif virus at 10 h pi (Fig. 6B).
within RTCs. Notably, Vif antibody immunoprecipitated a more sharply sedimenting major subset of RTCs, suggesting that RTC structures display heterogeneity in size, composition and possibly in function and that Vif may have a specific function in some RTCs only. Previous work in our laboratory was unable to detect Vif in RTCs by immunoprecipitation (Karageorgos et al., 1993). This earlier study examined RTC-containing fractions at a single sucrose density only, potentially omitting the RTC fraction of interest, whilst the study described here utilises different Vif antibodies and a more sensitive and quantitative PCR detection method.

The presence of Vif as part of RTCs in newly infected cells carries the prerequisite that Vif is present in virions. Although Vif can be non-specifically incorporated into murine leukaemia virus (MLV) virions and has not been detected in HIV virion preparations in some earlier studies (Camaur and Trono, 1996; Dettenhofer and Yu, 1999; Sova et al., 2001), other studies have identified Vif as part of HIV virions. The failure to detect Vif in virions reported in some instances may be due to intravirion proteolysis of Vif and/or lower levels of Vif virion incorporation from chronically infected cell lines (Camaur and Trono, 1996; Kao et al., 2003a; Karcewski and Strebel, 1996; Khan et al., 2001, 2002). Results of the present study support the view that Vif is part of the virion and has a functional requirement in the newly infected cell.

Although Vif-deficient virions are not grossly aberrant morphologically, there is evidence that they have altered physical and biochemical properties. Some studies have reported Vif-deficient virions to have altered core structure and reduced core stability (Borman et al., 1995; Bouyac et al., 1997; Ohagen and Gabuzda, 2000). Previous studies have also suggested that Vif-deficient virus has reduced ability for endogenous reverse transcription (Dettenhofer et al., 2000) although again this observation is not seen in all studies (Gaddis et al., 2003). Biochemical transcription studies using recombinant proteins have suggested that Vif facilitates RT–primer binding and RT activity in vitro (Cancio et al., 2004). Further recent data based on in vitro assays have shown that Vif may act as a chaperone for the early steps of reverse transcription by binding RNA, enhancing tRNA^lys^3 primer annealing and increasing the processivity of RT (Henriet et al., 2007). Thus, Vif within the RTC may directly promote reverse transcription or, in a similar manner to its role in core stability, may stabilise the RTC in the target cell, either by physically contributing to the tertiary structure required for efficient reverse transcription or by protecting reverse transcription products from host anti-viral defences such as APOBEC3G/F. However, such a role for Vif has previously been given little attention and its main documented role has been preventing the incorporation of APOBEC3 enzymes into virions and the subsequent anti-viral actions of APOBEC in newly infected target cells during reverse transcription. Virus produced from cells lacking APOBEC3G replicates normally in some cell lines, even in the absence of Vif, which led to the view that the defective phenotype of Vif-deficient virus is mediated by the action of APOBEC3G rather than the loss of other important accessory functions of Vif. However, these experiments measured HIV replication under optimally permissive in vitro replication conditions. Whether

mutations and 1 clone with 4/706 G–A substitutions, showing very few DNA sequence substitutions whilst WT + 50 nM FdUrd showed no mutations (0/1412 bp, data not shown). Similarly, sequencing of 6 clones from ΔVif + 5 nM FdUrd, which still has an inhibitory effect on ΔVif replication (Fig. 5A), showed no sequence mutations. Thus ΔVif infection + FdUrd which is associated with restricted reverse transcription in the absence of APOBEC3G showed a distinctly lower mutation frequency (4/8472 or 0.05%) compared to the rate (2.4%) we previously observed for ΔVif infection of non-permissive Hut-78 cells that is associated with the presence of APOBEC3G (Carr et al., 2006). Since sequencing of multiple independent clones failed to identify hypermutation of cytoplasmic reverse transcription products from ΔVif virus in the presence of FdUrd, this suggests that the reduction of reverse transcription and infectivity in this situation was directly due to reduced efficiency rather than fidelity of the reverse transcription process.

Discussion

In this study we have shown that the accessory protein Vif is part of the HIV replication machinery during early reverse transcription and can act as an accessory factor to promote reverse transcription and viral infectivity.

We identified RTCs in this study based on sucrose velocity gradient sedimentation, the presence of RT activity and newly synthesised HIV reverse transcription products and also by the presence of IN protein as shown in previous experiments from our laboratory (Carr et al., 2006; Karageorgos et al., 1993). We observed co-sedimentation of Vif protein with RTCs and co-precipitation of HIV reverse transcription products using a Vif antibody, from either infected cell lysates or gradient fractions containing RTCs indicating a true physical association of Vif within RTCs. Notably, Vif antibody immunoprecipitated a more sharply sedimenting major subset of RTCs, suggesting that RTC structures display heterogeneity in size, composition and possibly in function and that Vif may have a specific function in some RTCs only. Previous work in our laboratory was unable to detect Vif in RTCs by immunoprecipitation (Karageorgos et al., 1993). This earlier study examined RTC-containing fractions at a single sucrose density only, potentially omitting the RTC fraction of interest, whilst the study described here utilises different Vif antibodies and a more sensitive and quantitative PCR detection method.

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virus lacking Vif can replicate equally well in cells where the intracellular environment is not as conducive to viral replication, i.e. situations where any accessory functions could become important, has not been investigated. We have mimicked this situation by treating cells with a pyrimidin analogue that inhibits pyrimidine biosynthesis and disrupts the balance of cellular dNTP levels. Under these conditions we found that infectivity and reverse transcription of Vif-deficient virus were impaired to a greater extent than that of WT HIV. The effect was not associated with increased mutation of reverse transcription products that could result from increased dNTP misincorporation or reduced DNA repair in the absence of Vif. Thus the greater impairment in reverse transcription of Vif-deficient virus compared to WT virus in the presence of FdUrd appears to be due to reduced efficiency rather than loss of fidelity of reverse transcription in the absence of Vif.

These results show for the first time a biological difference in intracellular reverse transcription of Vif-deficient compared with WT virus in permissive target cells, consistent with the previously observed stimulation by Vif of reverse transcription in vitro. When combined with our data showing Vif is part of RTCs, these results show both appropriate localisation and a functional role for Vif in reverse transcription. Thus, Vif can act in two ways to ensure efficient HIV infection of target cells (1) by preventing the actions of anti-viral APOBEC enzymes and (2) by promoting the reverse transcription process per se. This latter activity may be particularly relevant in cell types or biological situations that are important in HIV pathogenesis in vivo but are not modelled in conventional laboratory HIV culture systems.

Materials and methods

Cells and plasmids

Hut-78 and CEM-SS cells were obtained from the NIH AIDS Research and Reference Reagent Program (NIH AR&RRP). H3B cells are a laboratory clone of H9 cells persistently infected with HTLV-IIIB, as described previously (Li and Burrell, 1992). pNL4.3 was obtaind from the NIH AR&RRP. pNL4.3 Δvif was generated by Ndel/PflMI digestion of the SpeI/EcoRI fragment of pNL4.3, end filling and re-ligation. The SpeI/EcoRI fragment containing Δvif was sub-cloned back into pNL4.3 to generate a full-length infectious clone, similar to previous Vif mutants (Karczewski and Strebel, 1996; Khan et al., 2001). This mutation generates a frame shift at amino acid 28 of Vif and creates a stop codon at amino acid 40 resulting in a severely truncated Vif protein but does not affect the neighbouring Vpr open reading frame.

Virus stocks

NL4.3 or Vif-defective (Δvif) NL4.3 virus was derived by transfection of 293 cells and amplification of virus supernatant in CEM-SS. Virus stocks were DNase I treated and filtered (0.22 μM) to remove contaminating DNA and cells. TCID50 was determined by titrating virus on CEM-SS cells, p24 content of virus stocks was determined by ELISA (Roche).

Cell–cell HIV infection

For cell-to-cell transmission of HIV, virus infected donor cells (H3B) and uninfected recipient cells (Hut-78) were co-cultured at a ratio of 1:4 (donor:recipient) at a density of 2×10^6 cell/ml, as previously described, achieving a highly synchronous one-step infection (Li and Burrell, 1992).

Cell lysis and gradient fractionation of lysates

At 6 h pi cells were harvested, trypsinised, washed and lysed for 15 min at room temperature in lysis buffer A (10 mM Tris, pH 7.4, 150 mM KCl, 5 mM MgCl2, 1 mM DTT, 20 μg/ml Aprotinin (LBA)) containing 0.1% Triton X-100, as previously described (Carr et al., 2006; Karageorgos et al., 1993). Nuclei were pelleted (2500×g, 5 min), supernatants clarified by centrifugation (10,000×g, 10 min) and the resultant cytoplasmic extracts were adjusted to 8% (w/v) sucrose, 0.5% (v/v) Triton X-100 and stored at −80 °C before analysis. Cell lysates (1–5% gradient volume) were fractionated on a 10 ml linear 15–30% sucrose velocity gradient with 60% sucrose cushion in LBA containing 0.5% Triton X-100 at 4 °C. Sucrose velocity gradients were centrifuged in a SW40 rotor (Beckman) at 35,000 rpm for 50 min. One milliliter fractions were collected from the top of the gradient and sucrose density determined by refractive index. Sucrose equilibrium gradient sedimentation was performed on a 10 ml linear 10–70% sucrose gradient and centrifuged at 28,000 rpm for 18 h.

Real time PCR for HIV reverse transcription products

Gradient fractions or immunoprecipitates were treated with proteinase K and extracted with phenol chloroform, and the nucleic acid was precipitated with ethanol for subsequent real time PCR analysis. Samples were analysed by real time PCR for viral strong stop (ss), 1st strand transfer (ST) or U5-Gag DNA. Reactions utilised primers pairs CTAACAGAGAACCTAGTCG and CTGCTAGGATTTCTCCAC (ss DNA), GAGCCCTCAGATCCTGCATA and TCCCTAGTTAGCCAAGGAGACG (1st ST DNA) and GGTAAGTAGTACCTCAG and AGAGCTCTCTGTGTTTCCCT (U5-Gag DNA). Reactions were performed using Syber green PCR mix (Qiagen) and amplification by real time PCR cycling with a Rotor-gene 3000 (Corbett Research). Viral DNA copy numbers were determined from genomic DNA standards derived from a mix of the H3B, ACH2 and 8E5 chronically HIV-infected cell lines and ranged from 3000 to 30 copies (Vandegraaff et al., 2001).

Immunoprecipitation (IP) of HIV reverse transcription products and RT activity

Total cell lysates or sucrose gradient fractions from 1×10^6 cell equivalents were divided and incubated with rabbit pre-immune sera (negative control), Vif antisera (rabbit anti-sera raised in-house against recombinant Vif or Vif mAb TG002, #2746, AR&RP) or IN antisera (mix of rabbit antisera #756, 757, 758, AR&RP) rotating for 3 h at 4 °C. Equilibrated protein
A sepharose (1/20th the volume) was then added and the lysate incubated for a further 1 h at 4 °C. Protein antibody complexes were precipitated by centrifugation and washed five times in cold LBA containing 0.5% Triton X-100, with vortexing in between each wash. For analysis of HIV reverse transcription products, DNA associated with the precipitated proteins was extracted by proteinase K digestion, phenol chloroform extraction and ethanol precipitation and analysed for HIV DNA by real time PCR. For analysis of HIV RT activity, immunoprecipitated proteins were resuspended in AMV reaction buffer between each wash. For analysis of HIV reverse transcription, immunoprecipitated proteins were resuspended in AMV reverse transcriptase buffer (Roche) and 1/10th the immunoprecipitate utilised in a reverse transcriptase reaction with 20 ng of in vitro transcribed dengue virus type 2 (DV) positive strand capsid RNA and primer Tag-3.2 (Wati et al., 2007). Positive controls were performed with 1 U of AMV reverse transcriptase (Roche) and 5 μl of the unprecipitated HIV infected cell lysate. Negative control reactions were also performed with no RT enzyme. Tag primed-DV cDNA was then subjected to 40 cycles of real time PCR amplification as described previously (Wati et al., 2007) and equivalent copy number DV RNA determined as a quantitative measure of the amount of input RT enzyme activity.

**RT assay**

Endogenous RT assays were performed by methyl-3H-TTP incorporation as described previously (Karageorgos et al., 1993).

**Western blot analysis**

Total protein from sucrose gradient fractions was precipitated at 4 °C by addition of 1/10th volume 100% (w/v) trichloroacetic acid (TCA, Sigma Corp). The precipitated protein was resuspended in 62.5 mM Tris, 2% SDS, 5% glycerol, pH 6.8 and 0.07% β-mercaptoethanol and subjected to denaturing SDS–PAGE in 25 mM Tris, 192 mM glycine, 0.1% SDS with a 4% stacking and 12.5% separating gel. Separated proteins were transferred to a nitrocellulose membrane, blocked in Tris buffered saline containing 0.1% (v/v) Tween-20 (TBS-T) and 5% skim milk powder and then probed with rabbit anti-Vif antibody (#2221; NIH AIDS Research and Reference Reagent Program, AR&RP, 1/6000) or rabbit anti-p24 antibody (#6458, NIH AR&RP, 1/500). Membranes were then incubated with anti-rabbit IgG–HRP secondary antibody conjugates, and the proteins were visualised by chemiluminescence (GE Healthsciences).

**Infectivity assays**

Infectivity assays were performed as previously described (Feng et al., 2004; Kimpton and Emerman, 1992). Briefly, HeLaCD4LTR-β-gal cells were plated in 24 well plates at 1.5 × 10⁴/well. Twenty four hours later supernatant was removed and cells were left untreated or pre-treated with 5-Flouro, 2-deoxyuridine (FdUrd, Sigma) at the indicated concentration for 2 h. Medium was removed and cells were infected with 100 ng p24 in a volume of 100 μl cell culture medium±FdUrd for 2 h. Medium was removed, 300 μl fresh medium with half the original concentration of FdUrd added and cells cultured. At 48 h post infection medium was removed, cells fixed and stained for β-gal and blue cells enumerated under light microscopy.

**Analysis of early HIV reverse transcription**

CEM-SS cells were left untreated or pre-treated with 50 nM FdUrd for 2 h prior to infection. Cells were inoculated with 20 ng p24/10⁶ cells of WT or ΔVif virus by centrifugal enhancement, as above. At 2 h pi (immediately after centrifugal enhancement procedure) and 10 h pi, 2 × 10⁶ cells were harvested and extrachromosomal DNA extracted according to the Hirt method (Hirt, 1967; Vandegraaff et al., 2001). Early reverse transcription products were quantitated by real time PCR.

**Cloning and sequencing of early reverse transcription products**

Extrachromosomal DNA from cells taken at 10 h post infection was amplified by PCR using Platinum Taq high fidelity DNA polymerase (Invitrogen) and primer pair CACA-CACAAGGCTACTTCCC and GCCCTACTCCCCAGTC-CGGCCAGG. The PCR product was cloned by TA cloning (TOPO cloning kit, Invitrogen) and sequenced using universal and reverse sequencing primers (Big Dye Terminator, Applied Biosystems).

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**References**


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