The replicative impairment of Vif mutants of human immunodeficiency virus type 1 correlates with an overall defect in viral DNA synthesis

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The Vif protein of human immunodeficiency virus type 1 (HIV-1) is essential for the infectivity of virions produced by non-permissive cells. The primary replicative defect of Vif particles involves either synthesis or stability of viral DNA, but the mechanism of this defect is unknown. Here, we report the results of a detailed analysis of HIV-1 DNA synthesis by isogenic Vif mutants produced by different chronically infected H9 clones, which exhibit different degrees of impairment in their replicative capacity. We found that the degree of impairment of DNA synthesis by the mutant particles always correlated with the degree of their loss of infectivity. This impairment appears to be global, with a defect increasing along with synthesis of longer viral DNA species. We conclude that the primary replicative defect of Vif virus involves the capacity of the reverse transcription complex of HIV-1 to efficiently elongate viral DNA, resulting in an inability to produce full-length viral DNA genomes.

The human immunodeficiency virus type 1 (HIV-1) genome encodes several nonstructural proteins which have regulatory or auxiliary functions. The Vif protein is well-conserved in HIV-1 strains and is also found in most other lentiviruses (Chakrabarti et al., 1987; Guyader et al., 1987; Oberste & Gonda, 1992; Sonigo et al., 1985; Wain-Hobson et al., 1985). The HIV-1 Vif protein is expressed in the cytoplasm of infected cells (Gonçalves et al., 1994; Karczewski & Strebel, 1996), and has also been detected in association with the nucleoid of viral particles (Liu et al., 1995) although the specificity of Vif incorporation is not certain (Camaur & Trono, 1996). The magnitude of the replicative defect that characterizes Vif-defective (Vif−) mutants is dependent on the cell type that is producing the virus. In particular, Vif is absolutely required for virus growth in peripheral blood lymphocytes, macrophages and H9 cells (Gabuzda et al., 1992; Sakai et al., 1993; Von Schwedler et al., 1993). The nature of this replicative defect is not well-characterized. It has been reported as: (i) a defect in the internalization of the viral core in target cells (Sova & Volsky, 1993); (ii) a global viral DNA synthesis defect; (iii) a specific defect in the completion of reverse transcription (Von Schwedler et al., 1993); and (iv) a defect in viral DNA stability (Simon & Malim, 1996). Vif− mutant virions also possess an abnormally assembled viral core (Borman et al., 1995; Bouyac et al., 1997; Höglund et al., 1994), implying that the Vif protein could be an integral component of the core or could assist proper assembly or maturation of the particle.

This study was designed to further elucidate the consequences of the absence of Vif on HIV-1 DNA synthesis, using isogenic Vif− mutants produced by H9-derived cell clones. In a previous study (Bouyac et al., 1997), these clones were found to produce virions with striking differences in the degree of their replicative impairment in spite of the fact that H9 cells have been previously described as fully restrictive cells (Borman et al., 1995; Gabuzda et al., 1992; Sakai et al., 1993). The infectivity of these virions was titrated on P4 indicator cells using equal amounts of particles, as measured by p24 ELISA. In three independent experiments (Fig. 1A), Vif− virus from clone 1-8 was always the most affected. On average, its infectivity was 1% of wild-type (WT), while Vif− viruses from clone 4-5 and from population H9 Vif− had an infectivity of 1.8% and 3.8% of WT, respectively. Interestingly, although H9 cells have consistently been described as fully restrictive
regarding replication of Vif– HIV-1 mutants, we found that virus from clone 3-8 had an infectivity of 30% of WT HIV-1NDK.

The efficiency of viral DNA synthesis by Vif– virus was first evaluated by direct Southern hybridization. To ensure a high m.o.i., virus-producing cells were cocultivated with MT4 cells at a ratio of 1:4. Newly synthesized unintegrated viral DNA was extracted by the Hirt procedure (Hirt, 1967), digested by BamHI and subjected to Southern blot analysis with a specific probe representing the U3/Env region of pNL4-3. Unintegrated viral DNA was absent from both WT and Vif– (clone 1-8) cultures at the start of the coculture (Fig. 1B; time 0 of coculture), indicating that donor H9 cells were not the site of detectable viral DNA synthesis. In WT cocultures, linear DNA could be detected as early as 6 h after initiation of coculture. At 12 h, the amount of linear molecules reached a peak, while smaller amounts of circular DNA were visible. At 24 h, there was a notable decline in the amount of linear DNA, along with a strong accumulation of circles: a sign of nuclear import of viral DNA. In contrast with the active synthesis and processing of viral DNA in WT cocultures, no viral DNA could be detected in cells infected with Vif– virus as late as 24 h after infection. These results indicate that Vif– virus from clone 1-8 is unable to effect synthesis of detectable amounts of full-length viral DNA in newly infected cells.

DNA synthesis by Vif– viruses from the different H9-derived cell clones was next examined using a PCR assay. Viral DNA could be detected as early as 6 h after initiation of coculture, the amount of linear molecules reached a peak, while smaller amounts of circular DNA were visible. At 24 h, there was a notable decline in the amount of linear DNA, along with a strong accumulation of circles: a sign of nuclear import of viral DNA. In contrast with the active synthesis and processing of viral DNA in WT cocultures, no viral DNA could be detected in cells infected with Vif– virus as late as 24 h after infection. These results indicate that Vif– virus from clone 1-8 is unable to effect synthesis of detectable amounts of full-length viral DNA in newly infected cells.

Target H9 cells were infected with equal amounts of DNase I-treated particles, trypsinized after a 2 h infection period and further cultured at 37 °C. At different times post-infection, cells were lysed and subjected to PCR amplification. To ensure that comparable amounts of cells were analysed, β-actin was amplified in parallel reactions. Elongation of viral DNA was followed over time by amplification of selected segments of the HIV genome with the specific primer pairs R/U5, U3/U5 and U3/gag (Courcoul et al., 1995). Amplification of the circle junction in circular species of viral DNA carrying two copies of the LTR was carried out with the R/U3′ primer pair (U3′ antisense, 5′ CAGGGAAGTAGCCTTGTGTGTGG 3′). These amplification products were hybridized to the LTR3 probe (Courcoul et al., 1995). As shown in Fig. 2(A), the difference in viral DNA synthesis between the 1-8, 4-5, 3-8 and WT virions was clearly perceptible with the R/U5 primers, reflecting the degree of replicative impairment observed in P4 cells. With the other primer pairs, the viral DNA synthesis defect increased progressively as the corresponding PCR products were representative of longer viral DNA species. Only the 3-8 virus was able to carry out synthesis of DNA detectable by the U3/U5 pair. Furthermore, the amount of DNA amplified with the U3/gag pair was clearly less than with WT virus. Finally, amplification of the circle junction in 2-LTR circles, which are generated after full completion of both strands of viral DNA, occurred with WT only.

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Fig. 2. For legend see page 1948.
synthesis by Vif− mutants is markedly reduced compared to WT virus. However, they do not exclude the possibility that this reduction could be related to a defect in particle entry into target cells. To exclude this, the amount of cell extract used in each PCR reaction was adjusted so that the amounts of R/U5 present in each PCR reaction were identical in the WT and the Vif− reactions as measured by PCR amplification using the R/U5 primers. These experiments were done with WT virus and Vif− virus produced by an uncloned population of H9 cells, whose infectivity was 3-8% that of WT virus (Fig. 1A). As shown in Fig. 2(B), the amounts of U3/U5 DNA were perceptibly smaller in the Vif− virus than in WT virus at 4 h post-infection and again were clearly reduced with the U3/gag pair. Finally, primers amplifying 2-LTR circle junctions yielded no signal in the Vif− virus. These results reveal that even when adjusted to the amounts of early DNA species in WT-infected cells, an overall defect in viral DNA synthesis can be observed in cells infected by Vif− virus.

Next, we examined the efficiency of reverse transcription (RT) in isolated WT and Vif− viral particles. In exogenous RT reactions, where polymerization by RT from lysed virions uses an exogenous homopolymeric template:primer, we found comparable RT activity in WT and Vif− virus (data not shown). In endogenous RT reactions, DNA is synthesized within the detergent-permeabilized virion using the particle-associated genomic viral RNA as a template. Unpurified WT and Vif− viral particles from infected H9 cells were concentrated on Macrosep centrifugal concentrators (Pall Filtron) and adjusted to 60 ng of HIV-1 p24 per reaction. The reaction mixtures contained dNTPs (final concentration 100 mM each) and different concentrations of NP40 in TME (500 mM Tris–HCl pH 8.1, 30 mM MgCl2, 30 mM EGTA). A control for contamination was done for each virus by incubation with NP40 and no dNTPs. After a 2 h reaction, the endogenously synthesized viral DNA was analysed by PCR with the U3/U5 primer pair, representing early DNA product, the PPT2/4947 primer pair (PPT2 sense, 5′ GCCGGCGCTTTGTTGGGCA-GGG 3′; 4947 antisense, 5′ GTTCTTTTCAAACCTGGAT-CTCTGCTGTCCCTGTA 3′), detecting DNA species of intermediate length, and the U3/gag pair, detecting late DNA products. The PCR products were analysed for their specificity with the LTR3 probe. The PPT3 probe (sense, 5′ CATAAT-AGCAACAGACATAAAAC 3′) was used to detect intermediate products. As shown in Fig. 3, there was a clear correlation between the impairment in Vif mutant infectivity and the efficiency of viral DNA synthesis in isolated particles. Early reverse transcripts were detectable in all viruses, although the amounts of such species were dramatically reduced in the 1-8 Vif− virus. Intermediate DNA products were not detectable in 1-8, and were markedly reduced in H9 Vif− and 3-8 viruses compared with WT, particularly at the suboptimal 0-01% NP40. Finally, the late products were undetectable in the 1-8 virus, poorly detectable in virions from the uncloned H9 population but still readily detectable in the 3-8 virus. Reaction mixtures lacking detergent produced only very small amounts of viral DNA and reactions lacking nucleotides yielded no viral DNA, ruling out the presence of exogenously synthesized viral DNA or of significant amounts of viral DNA synthesized prior to the reaction. Interestingly, there seemed to be an important difference in the requirement for non-ionic detergents in the 1-8 virus compared to the other viruses. U3/U5 DNA synthesis was maximal at 0-5% NP40 in the 1-8 Vif− viruses, while the optimal concentration of detergent was always 0-025% for the other viruses. These results confirm those obtained in newly infected cells and reveal an intrinsic defect in DNA synthesis within the Vif− viral particle.

In this study, we demonstrate that the extent of the infectivity defect of Vif− viruses produced by different H9-derived cell clones correlates with a generalized defect in viral DNA synthesis both in newly infected cells and in isolated viral particles. The different cell clones studied produce isogenic Vif− viruses with different infectivities always following the same order: WT virus > clone 3-8 H9 Vif− (an uncloned population of H9 cells) > clone 4-5 > clone 1-8. The efficiency of viral DNA synthesis in cells newly infected by those different viruses followed exactly the same order. The strict correlation observed here between the infectivity and the DNA synthesis defects is demonstrative of the primary role of impaired DNA synthesis in the loss of infectivity of Vif− particles. Furthermore, this correlation could explain why results obtained in different laboratories using Vif− mutants produced by different cell systems yielded apparently conflicting interpretations on the role of Vif in HIV-1 DNA synthesis (Borman et al., 1995; Courcoul et al., 1995; Simon & Malim, 1996; Sova & Volsky, 1993; Von Schwedler et al., 1993). Similar to DNA synthesis in newly infected cells, the...
Role of Vif in HIV-1 DNA synthesis

Fig. 3. DNA synthesis by isolated virions. The endogenous reverse transcription reaction was performed with unpurified concentrated virion-rich supernatant as virus source. Equal amounts of WT and Vif\(^{-}\) viruses were used for reactions, which were done in the presence of increasing amounts of NP40 as indicated above each lane. Controls for contamination were done with 0.025% NP40 and no dNTPs. Incubation was at 41 °C for 2 h and the reaction products were analysed by PCR and hybridization with oligonucleotide probes. 1-8, 4-5, H9 Vif\(^{-}\), 3-8, Vif\(^{-}\) viruses; WT, wild-type virus; C, contamination control; Controls '+' and '−', positive and negative controls respectively.

efficiency of viral DNA synthesis in isolated particles from the different cell clones also correlated well with the infectivity of the corresponding viruses. These results are in agreement with those of Gonçalves et al. (1996) and further emphasize that the prime replicative defect of Vif\(^{-}\) particles relates to an inability to efficiently effect synthesis of full-length viral DNA.

Taken together, our results favour a model in which the absence of Vif from restrictive cells results in the production of viral particles that are intrinsically defective for viral DNA synthesis. Whether the Vif protein directly participates in the reverse transcription process or is required during assembly or maturation of the particle for proper conformation of the reverse transcription nucleoprotein complex remains speculative. Reverse transcription of all retroviral genomes occurs within an organized nucleoprotein structure derived from the particle nucleoid, which provides optimal conditions for the reverse transcription process itself. Any alteration in the precise arrangement of the different components of the complex is likely to affect reverse transcription and later stages of the virus replication cycle. It has been shown by several groups that the structure of the core of Vif\(^{-}\) particles is abnormal (Borman et al., 1995; Bouyac et al., 1997; Höglund et al., 1994). We have observed here that the concentration of nonionic detergent required for optimal endogenous reverse transcription is different for virus from clone 1-8, which displays maximal replicative and DNA synthesis defects, than for the other viruses. Although it may be too early to speculate on the molecular basis of this finding, it suggests that the DNA synthesis defect that characterizes Vif\(^{-}\) viral particles is related to a structural abnormality that could affect the sensitivity to detergent of the nucleoprotein complex that ensure optimal viral DNA synthesis. Whether this abnormality is the direct consequence of the absence of Vif from the particle or is due to an indirect effect of the absence of Vif from the site of synthesis or assembly of the components of the particle is unclear. Nonetheless, we believe that the replicative defect that is
characteristic of Vif− particles is a consequence of an abnormally assembled reverse transcription nucleoprotein complex, which in turn leads to a generalized defect in viral DNA synthesis.

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