

Human Immunodeficiency Virus Type 1 Vif Is Efficiently Packaged into Virions during Productive but Not Chronic Infection

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Packaging of the human immunodeficiency virus type 1 Vif protein into virus particles is mediated through an interaction with viral genomic RNA and results in the association of Vif with the nucleoprotein complex. Despite the specificity of this process, calculations of the amount of Vif packaged have produced vastly different results. Here, we compared the efficiency of packaging of Vif into virions derived from acutely and chronically infected H9 cells. We found that Vif was efficiently packaged into virions from acutely infected cells (60 to 100 copies per virion), while packaging into virions from chronically infected H9 cells was near the limit of detection (four to six copies of Vif per virion). Superinfection by an exogenous Vif-defective virus did not rescue packaging of endogenous Vif expressed in the chronically infected culture. In contrast, exogenous Vif expressed by superinfection of wild-type virus was readily packaged (30 to 40 copies per virion). Biochemical analyses suggest that the differences in the relative packaging efficiencies were not due to gross differences in the steady-state distribution of Vif in chronically or acutely infected cells but are likely due to differences in the relative rates of de novo synthesis of Vif. Despite its low packaging efficiency, endogenously expressed Vif was sufficient to direct the production of viruses with almost wild-type infectivity. The results from our study provide novel insights into the biochemical properties of Vif and offer an explanation for the reported differences regarding Vif packaging.

The human immunodeficiency virus type 1 (HIV-1) accessory protein Vif plays an important role in regulating virus infectivity (20, 54). The lack of a functional Vif protein results in the production of virions with reduced or abolished infectivity (20, 33, 54). This effect of Vif on virus infectivity is producer cell-dependent and can vary by several orders of magnitude (2, 5, 6, 19, 20, 23, 33, 42, 54, 55). Virus replication in nonpermissive cell types, such as primary T cells and macrophages as well as a small number of T-cell lines, including H9, is strictly dependent on Vif, while Vif-defective viruses can efficiently replicate in permissive hosts, such as Jurkat cells. Results from heterokaryon analyses, which involved the fusion of restrictive cell types with permissive cell types, suggested the presence of an inhibitory factor in restrictive cell types (35, 47). Such a cellular factor, CEM15, was recently identified and was found to be expressed in nonpermissive cell types but not in permissive cell types (44). Interestingly, CEM15 is packaged into virions, though the significance of this finding remains to be investigated.

Vif is a 23-kDa basic protein that is expressed late during infection in a Rev-dependent manner (43). Immunocytochemical analyses revealed a largely cytoplasmic localization of Vif (24, 30, 46). In particular, confocal microscopy revealed that a significant amount of Vif associates with the intermediate filament network in virus-producing cells (30), causing severe alterations of the

intermediate filament structure (27, 30); however, the domain(s) in Vif responsible for this association as well as its functional significance remain to be determined. Recent reports also suggested that Vif associates with viral genomic RNA in vivo and in vitro, and deletions in the N-terminal and central regions were found to affect the ability of Vif to bind to poly(G)-conjugated agarose beads in vitro (16, 58). Aside from its affinity to RNA and intermediate filaments, Vif was reported to associate with cellular membranes through a mechanism involving a basic C-terminal domain in Vif (24, 26, 52). This same domain also was reported to be responsible for the interaction of Vif with the Gag precursor Pr55^{gag} (7) and was found to influence multimerization of Vif in vitro and in vivo (56).

Despite the critical role of Vif in regulating virus infectivity, its mechanism of action has thus far remained obscure. It is generally accepted that Vif-deficient viruses can attach to and penetrate host cells but are blocked at a postpenetration step early in the infection cycle (3, 13, 15, 40, 48, 55). Yet comparison of virion morphology or protein composition between wild-type and Vif-defective virions has thus far been inconclusive (6, 8, 22, 28, 38, 45). Several reports have suggested that Vif affects the stability of the viral nucleoprotein complex (28, 39, 48). In particular, nucleocapsid protein (NC) and reverse transcriptase were found to be less stably associated with viral cores in the absence of Vif (39). Nevertheless, it is still unclear whether virus association of Vif is functionally relevant. Numerous reports have observed packaging of Vif into virions; however, estimates on the amounts of Vif packaged have varied significantly, ranging from less than 1 molecule to as much as 100 molecules of Vif per virion (10, 17, 22, 34).

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We have recently reported that Vif packaging is specific and dependent on its interaction with viral genomic RNA and possibly NC (31). Virus-associated Vif is stably associated with the nucleoprotein complex (30, 31, 34). Moreover, we found that virus-associated Vif is proteolytically cleaved by the viral protease at a conserved site near the N terminus (32). Proteolytic processing of Vif by the HIV protease was restricted to virus-associated Vif. Mutations at or near the processing site had distinct effects on Vif processing and revealed an intriguing parallel with Vif function (32). These results demonstrated that virus-associated Vif is a substrate for the viral protease and suggested that packaging and processing of Vif are functionally relevant. In addition, proteolytic processing of Vif could, at least in part, explain the reported difficulties associated with the detection of Vif in virus preparations.

In the present study we have further investigated the mechanism of Vif packaging in an effort to more satisfactorily explain the conflicting results reported in the literature for the amounts of Vif packaged. A review of the published literature revealed interesting parallels between the source of virus used in individual studies and the efficiency of Vif packaging. We found that in general, studies employing virus from chronically infected cells or from stable cell lines reported lower amounts of virus-associated Vif (10, 17, 49) than studies using virus from productively infected cells (34, 49). To directly compare the packaging efficiency of Vif under conditions of productive and chronic infection in an internally controlled system, we made use of H9 cell lines that were chronically infected with either wild-type HXB2 or a Vif-defective variant. Wild type-infected H9 cells contained significant levels of Vif. Yet virtually no Vif was detectable in cell-free virus preparations. Surprisingly, superinfection of the chronically infected cells with Vif-defective NL4-3 virus did not rescue Vif packaging despite the fact that virus production was significantly enhanced. In contrast, when chronically infected cells were superinfected with wild-type NL4-3, de novo-synthesized Vif expressed from NL4-3 virus was efficiently packaged. Comparison of the steady-state subcellular distribution of Vif in chronically or acutely infected cells did not reveal any substantial differences that could account for the differences in the relative packaging efficiencies. However, the rate of de novo Vif synthesis was significantly higher in acutely infected cells, suggesting that newly synthesized Vif was more packaging competent than preexisting Vif. Analysis of virus infectivity in a single-cycle assay revealed that virus produced from cells chronically infected with a Vif-expressing virus was infectious even in the absence of exogenous Vif, suggesting that endogenously expressed Vif is biologically active and that packaging of large amounts of Vif is not required for viral infectivity. Our results provide novel insights into the biochemical properties of Vif and offer an explanation for the reported differences regarding Vif packaging.

MATERIALS AND METHODS

Plasmids. The full-length molecular clone pNL4-3 (1) was used for the production of wild-type infectious virus. Plasmid pHCMV-G contains the vesicular stomatitis virus glycoprotein G (VSV-G) gene expressed from the immediate early gene promoter of human cytomegalovirus (57) and was used for the production of VSV-G pseudotypes. For transient expression of Vif, the subgenomic expression vector pNL-A1 (54) was employed. This plasmid expresses all HIV-1

proteins except for *gag* and *pol* products. Construction of HXB2NEO, which carries defective *vpr*, *vpu*, *env*, and *nef* genes, has been described elsewhere (17). HXB2NEO carries the neomycin phosphotransferase gene (*neo^R*) cloned into the *nef* region of the genome. Construction of a Vif-defective variant, HXB2VifΔNEO, was described previously (16).

Antisera. Serum from an HIV-positive patient (APS) was used to detect HIV-1-specific proteins. The serum does not recognize Vif or Nef and reacts only poorly with gp120 in immunoblot assays. A polyclonal, monospecific antiserum to Vif was raised in rabbits against *Escherichia coli*-derived fusion proteins (30) and was used for all immunoprecipitation and some immunoblotting analyses. Monoclonal antibodies to Vif (MAb #319) and p24 Gag (MAb #24-3) were used for some of the immunoblot analyses and for the quantitation of Vif in virus preparations and were obtained through the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program (21, 22, 46, 50).

Tissue culture and transfections. HeLa cells were propagated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS). Uninfected H9 cell lines were maintained in complete RPMI 1640 medium supplemented with 10% FBS. H9 cells chronically infected with HXB2NEO or HXB2VifΔNEO (16) were grown in complete RPMI 1640 medium supplemented with 10% FBS and G418 (1 mg/ml). For stimulation with phorbol myristate acetate (PMA) or tumor necrosis factor alpha (TNF- α), cells were treated with PMA (10^{-8} M) or TNF- α (10 ng/ml) for 48 h. LuSIV cells are derived from CEMx174 cells and contain a luciferase indicator gene under the control of the SIVmac239 long terminal repeat (41). These cells were obtained through the NIH AIDS Research and Reference Reagent Program and were maintained in complete RPMI 1640 medium supplemented with 10% FBS and hygromycin B (300 μ g/ml).

For transfection of HeLa cells, cells were grown in 25-cm² flasks to about 80% confluency. Cells were transfected using LipofectAMINE PLUS (Invitrogen Corp., Carlsbad Calif.) following the manufacturer's recommendations. A total of 4 to 5 μ g of plasmid DNA per 25-cm² flask was used. Cells were harvested 24 to 48 h posttransfection. For electroporation of uninfected or chronically infected H9 cells, 5×10^6 cells were washed once in OptiMEM (Invitrogen Corp.) and suspended in 300 μ l of RPMI supplemented with 10 mM D-glucose and 0.1 mM dithiothreitol. Plasmid DNAs (a total of 20 μ g per transfection) were combined with the cells. The mixture was then transferred to a sterile electroporation cuvette (Bio-Rad Gene Pulser cuvette, 0.4 cm) and pulsed with a Bio-Rad Gene Pulser II (975 μ F, 0.2 kV). Electroporated cells were subsequently transferred to tissue culture flasks containing 5 ml of RPMI-10% FBS and G418 (1 mg/ml) and cultured for 24 to 48 h.

Preparation of virus stocks. Virus stocks were prepared by transfecting HeLa cells with appropriate plasmid DNAs (5 μ g/25-cm² flask) using LipofectAMINE PLUS. For the production of virus stocks pseudotyped with the vesicular stomatitis virus glycoprotein G (VSV-G), HIV plasmids were cotransfected with pHCMV-G (4 μ g of viral plasmid plus 1 μ g of pHCMV-G per 25-cm² flask). Virus-containing supernatants were harvested 48 h after transfection. Cellular debris was removed by centrifugation (3 min, 3,000 \times g), and clarified supernatants were filtered (0.45 μ m) to remove residual cellular contaminants.

Cell fractionation, immunoblotting, and immunoprecipitation. For cell fractionation, cells were resuspended in phosphate-buffered saline (PBS) and lysed by two cycles of freezing and thawing (3 min each at -70° C and 37° C, respectively). Insoluble material was pelleted for 3 min at 15,000 \times g, and the supernatant (fraction 1) was collected. The pellet was extracted with CHAPS buffer (50 mM Tris-HCl [pH 8.0], 5 mM EDTA, 100 mM NaCl, and 0.5% [vol/vol] 3-[(3-cholamidopropyl)-diethylammonio]-1-propanesulfonate] containing 0.2% deoxycholate, incubated on ice for 5 min, and pelleted as before. Detergent-soluble material present in the supernatant (fraction 2) was collected. Proteins present in the detergent-resistant pellet fraction (fraction 3) were solubilized by boiling in sample buffer (4% sodium dodecyl sulfate, 125 mM Tris-HCl [pH 6.8], 10% 2-mercaptoethanol, 10% glycerol, and 0.002% bromphenol blue) for 10 min at 95° C. Prior to immunoprecipitation, all fractions were adjusted to equal volumes, ionic strengths, and detergent concentrations. Cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); proteins were transferred to polyvinylidene difluoride (PVDF) membranes and reacted with appropriate antibodies as described in the text. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences, Piscataway, N.J.) and visualized by enhanced chemiluminescence (ECL) (Amersham Biosciences). For immunoprecipitation, cell lysates were precleared on protein A-Sepharose beads (Sigma-Aldrich Inc., St. Louis Mo.) followed by immunoprecipitation with appropriate antisera as indicated in the text. Proteins were solubilized by boiling in sample buffer and separated by SDS-PAGE. Radioactive bands were visualized by fluorography, and quantitation was performed using a Fuji BAS 2000 Bio-Image analyzer. To analyze total intracellular levels of Vif, whole-cell extracts were

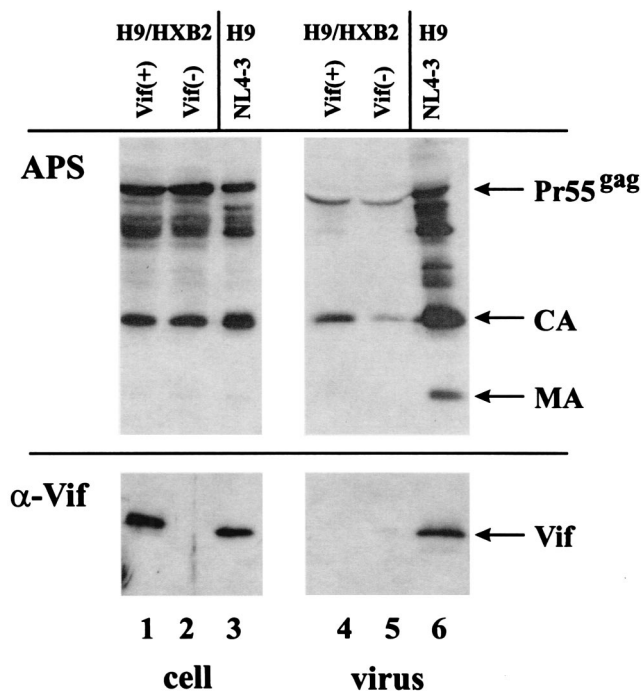


FIG. 1. Packaging of Vif into virions from chronically or productively infected H9 cells. For the analysis of productively infected cells, H9 cells (5×10^6) were infected with a concentrated stock of NL4-3 virus. Virus replication was monitored by reverse transcriptase assay, and virus-containing supernatant and infected cells were harvested near peak virus production (day 5). For the analysis of virus from chronically infected H9 cells, H9/HXB2NEO and H9/HXB2VifΔNEO cells were cultured in parallel with the acutely infected cells. Virus from 10 ml of chronically or productively infected cultures was concentrated by ultracentrifugation, suspended in 250 μ l of sample buffer, and immediately heated to 95°C for 5 min. Cells (10^7 each) from chronically or productively infected cultures were washed once in PBS, suspended in 500 μ l of PBS, and mixed with 500 μ l of sample buffer. Cell lysates were boiled and occasionally vortexed to shear cellular DNA until the proteins were completely solubilized. Cell lysates (40 μ l of chronically infected samples; 15 μ l of acutely infected sample) and viral pellets (60 μ l of chronically infected samples; 23 μ l of acutely infected sample) were separated by SDS-13% PAGE. Proteins were transferred to PVDF membranes and subjected to immunoblotting using an HIV-positive patient serum (APS) or a Vif-specific polyclonal antibody (α -Vif). Proteins are identified on the right.

prepared in essence as described above, except that the contents of the three fractions were pooled.

Quantitation of virus-associated Vif. For the quantitative assessment of virus-associated Vif, we used virus preparations from productively infected H9 cells (same virus stock as in Fig. 1, lane 6), H9 cells electroporated with NL4-3 plasmid DNA, or chronically infected H9/HXB2Neo cells electroporated with Vif-expressing NL4-3 or its Vif-defective variant NL4-3Vif(-) (same virus stocks as used for Fig. 6A, lanes 7 and 8). For standardization, recombinant p55 Gag (strain HIV-1 LAV; obtained through the NIH Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases) and recombinant Vif (32) were used. Recombinant proteins were assessed by gel electrophoresis and staining with Coomassie brilliant blue R250 for their purity and integrity, and protein concentrations were verified using the Bio-Rad protein assay according to the manufacturer's standard assay protocol (Bio-Rad Laboratories, Hercules, Calif.). Recombinant proteins (5 to 100 ng) were subjected to SDS-13% PAGE in parallel with concentrated virus samples. Proteins were transferred to PVDF membranes and reacted with either a Vif-specific monoclonal antibody (MAb #319) or a p24 Gag-specific monoclonal antibody (MAb #24-3; obtained through the NIH Research and Reference Reagent Program). Proteins were visualized by ECL and quantified by densito-

metric scanning of the films. The number of Vif molecules per virion was calculated based on the predicted molecular weights for Vif (22.70 kDa), CA (25.60 kDa), and Pr55^{gag} (55.80 kDa) and assuming 2,000 molecules of Gag per virion (4, 12).

Cloning and sequencing of vif genes from H9 cells chronically infected with HXB2NEO. Genomic DNA from H9 cells chronically infected with HXB2NEO or HXB2VifΔNEO (16, 17) was prepared from 10×10^6 cells each as follows: cells were washed once in 10 ml of TSS (25 mM Tris [pH 7.4], 25 mM KCl, 250 mM sucrose) and then suspended in 2 ml of TSS buffer. Cells were allowed to swell on ice for 10 min. Then 2 ml of TSS buffer containing 0.4% NP-40 was added, and incubation was continued for 10 min on ice. Nuclei were harvested by centrifugation (10 min, 2,500 rpm, 4°C; Sorval HS4 rotor). Pelleted nuclei were washed once in TSS buffer, pelleted, and suspended in 0.7 ml of cold TSS. An equal volume of 2 \times proteinase K buffer (100 mM Tris [pH 7.5], 200 mM NaCl, 10 mM EDTA, 1% SDS) was added, and samples were treated with proteinase K (200 μ g/ml) for 5 h at 55°C. Samples were then extracted twice with phenol-chloroform, and DNA was precipitated with ethanol and finally suspended in 100 μ l of H₂O. vif sequences were amplified by nested PCR using 5' GATAATAG TGACATAAAAGTAG and 3' GCCCTAAGCCATGGAGCC external primers followed by a second amplification with 5' CACCGAATTCATGGAAACAG ATGGC and 3' ATAAGGATCCTAGTGCCATTCATTG internal primers. PCR products were purified and digested with *Eco*RI and *Bam*HI and cloned into the *Eco*RI and *Bam*HI sites of the in vitro transcription vector pSP65. Two independent clones were sequenced for each of the Vif⁺ or Vif⁻ variants.

Single-cycle infectivity assay. LuSIV cells (5×10^5) were infected in a 24-well plate with 500 μ l of unconcentrated virus supernatants. Cells were incubated for 3 h at 37°C, rocking the plate every hour. Following the 3-h infection, 1 to 2 ml of complete RPMI was added to each culture, and incubation was continued for 24 h at 37°C. Cells were then harvested, washed once with PBS, and lysed in 200 μ l of 1 \times reporter lysis buffer (Promega Corp., Madison, Wis.). To determine the luciferase activity in the lysates, 50 μ l of each lysate was combined with luciferase substrate (Promega) by automatic injection, and light emission was measured for 10 s at room temperature in a luminometer (Optocomp II; MGM Instruments, Hamden, Conn.).

RESULTS

Efficient packaging of Vif into virions from acutely but not chronically infected H9 cells. Previous studies on the packaging of Vif have produced a broad range of estimates for the amounts of Vif incorporated into viral particles, ranging from less than 1 to as much as 100 molecules per virion (10, 17, 34, 49). Our own recent experiments performed in acutely infected or single-cycle HIV-infected H9 cells or in transfected HeLa cells are consistent with the findings by Simon et al. (49) suggesting that packaging of Vif is dynamic and dependent on the intracellular expression levels (31). Indeed, variations in the intracellular expression levels were reflected in proportionate changes in the levels of virus-associated Vif (31; also unpublished data). In general, we found that approximately 10% of the total Vif protein synthesized during infection was packaged into virions from acutely infected cells. Based on these observations, it seemed possible that the observed variations in the levels of virus-associated Vif were caused—at least in part—by differences in the relative intracellular expression levels. In that regard we noted that, in general, viruses derived from stable cell lines or chronically infected cells contained lower levels of Vif than viruses derived from productively infected or transiently transfected cells (10, 17, 34, 49).

To investigate the impact of the experimental setup, i.e., productive versus chronic infection, on the packaging of Vif, we directly compared the relative packaging efficiency of Vif into virions derived from either productively or chronically infected H9 cells. H9 cells were productively infected using an NL4-3 virus stock that was produced in HeLa cells and was pseudotyped with VSV-G. H9/HXB2NEO and H9/HXB2VifΔNEO cells were cul-

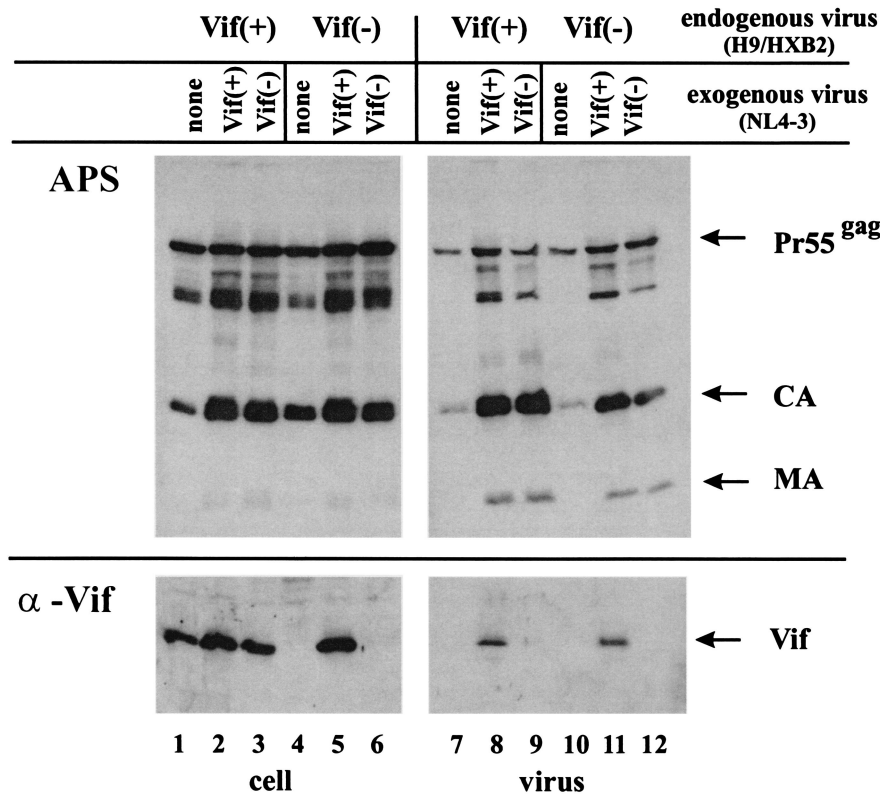


FIG. 2. Superinfection of chronically infected H9 cells does not rescue Vif packaging. H9 cells chronically infected with either HXB2NEO (lanes 1 to 3 and 7 to 9) or HXB2Vif Δ NEO (lanes 4 to 6 and 10 to 12) were superinfected with VSV-G-pseudotyped virus stocks of Vif-expressing NL4-3 (lanes 2, 5, 8, and 11) or Vif-defective NL4-3Vif(-) (lanes 3, 6, 9, and 12). Chronically infected H9 cells not subjected to superinfection were included as controls (lanes 1, 4, 7, and 10). Input virus was removed 5 h after infection. To minimize carryover of residual input virus, virus produced during the first 24 h after infection was discarded, and only virus produced between 24 and 48 h following infection was used for further analysis. Cell-free virus was concentrated by ultracentrifugation through 20% sucrose, and viral and cell lysates were subjected to immunoblotting using an HIV-positive patient serum (APS) or a Vif-specific polyclonal antiserum (α -Vif). Viral proteins are identified on the right.

tured in parallel and treated in exactly the same manner as the acutely infected cells. Virus production in the acutely infected culture was monitored by a reverse transcriptase assay. Virus-containing supernatants and cells were harvested near peak virus production, and whole-cell lysates and concentrated virus preparations were subjected to immunoblotting using a polyclonal Vif-specific antiserum or an HIV-positive human patient serum (Fig. 1). Sample loading was normalized for comparable levels of intracellular Gag proteins. Virus release from the acutely infected culture (lane 6) was significantly more efficient than that from chronically infected cultures (lanes 4 and 5), and substantial amounts of Vif were detectable in the virus preparation from the acutely infected H9 culture (lane 6, bottom panel). Interestingly, despite the presence of high levels of intracellular Vif, which were in fact comparable for acutely and chronically infected cells (lanes 1 and 3, bottom panels), Vif was virtually undetectable in the concentrated virus preparation from the chronically infected culture (lane 4, bottom panel). However, since the overall virus production from the productively infected culture was significantly higher than that from the chronically infected culture (compare lanes 4 and 5 with lane 6, top panels), it was possible that the inefficient export of Vif in the chronically infected culture was due to the overall low level of virus production from these cells.

Superinfection of chronically infected H9 cells does not rescue Vif packaging. To determine whether the low levels of virus production could account for the inefficient export of Vif from chronically infected cells, we attempted to boost virus production from the chronically infected cultures. A number of chronically infected T-cell lines were previously found to respond to mitogen treatment or treatment with cytokines, such as TNF- α , with increased virus production (9, 14). However, the stimulation of our cultures with neither TNF- α nor PMA treatment resulted in an increase in virus production from the chronically infected H9 cells (data not shown). We therefore attempted to increase virus production by superinfecting the chronically infected cultures with concentrated VSV-G-pseudotyped NL4-3 virus stocks. Please note that for the remainder of this report we will use the terms endogenous and exogenous Vif to discriminate between Vif expressed in the chronically infected culture and Vif produced by the superinfecting virus, respectively.

H9 cells chronically infected with HXB2NEO (Fig. 2, Vif⁺) or HXB2Vif Δ NEO (Vif⁻) were superinfected at a high multiplicity of infection (>1) with Vif-encoding (Vif⁺) or Vif-defective (Vif⁻) virus stocks of NL4-3 produced in HeLa cells. Input virus was removed 5 h following infection. To minimize carryover of residual input virus, virus produced during the

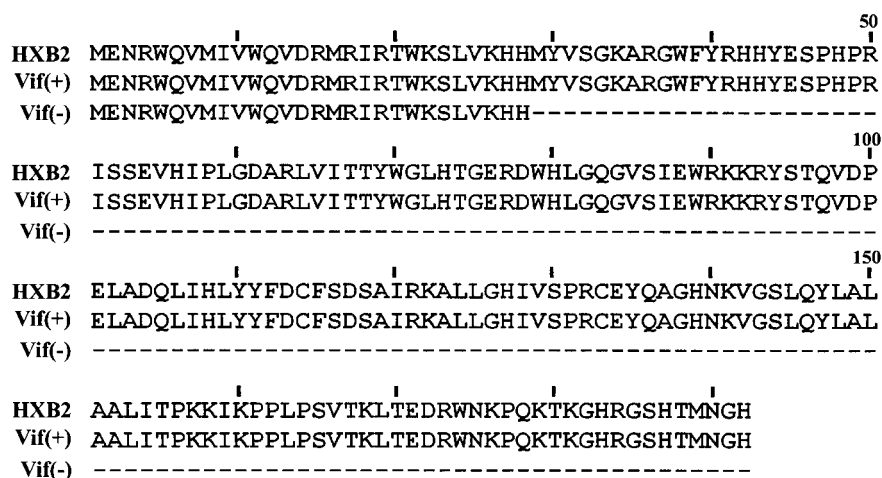


FIG. 3. Absence of mutations in the *vif* gene of multiply passaged chronically infected H9 cells. Genomic DNA from H9/HXB2NEO and H9/HXB2Vif Δ NEO cells was prepared from 10^7 cells and used for PCR amplification of the proviral *vif* genes. PCR products were purified and cloned into the *Eco*RI and *Bam*HI sites of the in vitro transcription vector pSP65. Two independent clones were sequenced for each of the Vif⁺ [Vif(+)] or Vif⁻ [Vif(-)] variants. The translated Vif sequence was aligned against the published HXB2 Vif sequence. No sequence variation was observed for any of the wild-type Vif sequences. As predicted, the Vif⁻ provirus carries a 68-bp out-of-frame deletion that results in a frameshift at residue 28 in Vif followed by six nonsense residues (not shown) and premature termination.

first 24 h after infection was discarded, and only virus produced between 24 and 48 h following infection was used for further analysis. Cell-free virions were concentrated by ultracentrifugation through 20% sucrose, and virus lysates and cell lysates were subjected to immunoblotting using an HIV-positive patient serum (Fig. 2, top panels) or a Vif-specific polyclonal antiserum (Fig. 2, bottom panels). Superinfection significantly increased virus production from both chronically infected cell lines as evidenced by the increased levels of extracellular viral proteins (compare lanes 7 and 10 with lanes 8 to 9 and 11 to 12, respectively). Superinfection by wild-type NL4-3 virus resulted in the production of virions that contained significant levels of Vif (lanes 8 and 11). Surprisingly, superinfection of Vif-expressing H9 cells with a Vif-defective NL4-3 virus stock did not rescue packaging of endogenous Vif despite the presence of large amounts of intracellular Vif (compare lanes 2 and 3) as well as high levels of virus production (lane 9, top panel). These results suggest that the Vif packaging defect observed in the chronically infected culture in Fig. 1 was not a simple consequence of low-level virus production.

Absence of mutations in the *vif* gene of multiply passaged chronically infected H9 cells. One possible reason for the inefficient packaging of endogenous Vif could be the gradual accumulation of mutations in *vif* during the initial selection or in the course of the continued passage of the chronically infected cell lines in the presence of G418. We addressed this possibility by PCR amplification and cloning of the endogenous *vif* gene using as template genomic DNA of the chronically infected H9 cells that had been in culture for an extended period of time. Details of the experiment are described in Materials and Methods. Two independent clones were sequenced for each of the wild-type and the Vif-defective proviruses. The result of this analysis is shown in Fig. 3 and reveals the absence of any differences in the amino acid sequence of the protein encoded by the endogenous *vif* gene when com-

pared to the parental HXB2 Vif sequence. In fact, no silent mutations were observed either (data not shown), suggesting that the proviruses in both chronically infected cell lines are stable throughout continued passage. As expected, the Vif-defective provirus was found to carry a 68-bp deletion (not shown), resulting in a frameshift at residue 28 in Vif followed by six nonspecific residues and premature termination (Fig. 3). From this analysis we conclude that the difference in packaging efficiencies observed for exogenous and endogenous Vif in Fig. 2 was not caused by mutations in the endogenous *vif* gene that could have potentially affected the packaging of the protein.

Subcellular distribution of Vif is similar in acutely and chronically infected H9 cells. We have previously studied the subcellular distribution of Vif in transiently transfected HeLa cells and found that Vif partitions with different cellular compartments, including a soluble cytosolic compartment as well as a detergent-resistant compartment that presumably represents cytoskeleton-associated Vif (30). It is possible that packaging of Vif requires its presence in a plasma membrane-proximal compartment. This is consistent with our previous observation that the Vif mutant Vif Δ G, which is for the most part insoluble and accumulates in a detergent-resistant cell fraction, is largely packaging incompetent (31). To address this issue, we compared the steady-state distribution of Vif in acutely and chronically infected H9 cells (Fig. 4). For the analysis of acutely infected cells, H9 cells were infected with wild-type NL4-3 as described for Fig. 1. Virus replication was monitored by reverse transcriptase assay (not shown), and cells were harvested near peak infection. Acutely infected H9 cells (Fig. 4, H9) and chronically infected H9 cells (Fig. 4, H9/HXB2NEO) were fractionated as described in Materials and Methods. Soluble (lanes a), detergent-soluble (lanes b), and detergent-insoluble (lanes c) fractions were analyzed by immunoblotting using an HIV-positive patient serum (APS), a Vpu-specific antibody, or a Vif-specific monoclonal antibody. As

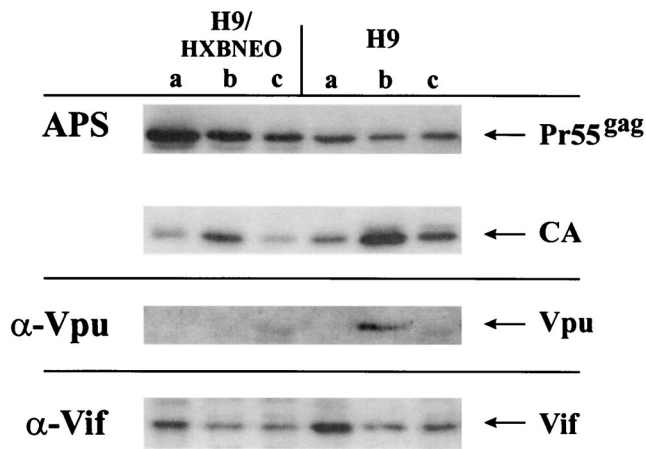


FIG. 4. Intracellular distribution of Vif in chronically and acutely infected H9 cells. Chronically (H9/HXB2NEO) or acutely (H9) infected cells were fractionated into soluble (a), detergent-soluble (b), and detergent-resistant (c) fractions as described in Materials and Methods. Cell lysates were separated by SDS-13% PAGE, transferred to PVDF membranes, and subjected to immunoblotting using an HIV-positive patient serum (APS) for detection of Pr55^{gag} and CA, a Vpu-specific polyclonal antibody (U2-3) for detection of Vpu (α -Vpu), or monoclonal antibody MAb #319 for detection of Vif (α -Vif). Proteins were visualized by ECL using appropriate horseradish peroxidase-conjugated secondary antibodies. A minor nonspecific protein cross-reacting with the Vpu antiserum is visible in fractions c of both cell types.

expected, the integral membrane protein Vpu was almost exclusively associated with the membrane fraction (fraction #2; H9, lane b), validating our fractionation procedure. No Vpu-specific signal was found in the chronically infected culture, since it carries a *vpu*-defective virus. Most of the cell-associated CA products associated with the membrane fraction as well, consistent with the notion that these products reflect budding virus. In contrast, the Pr55^{gag} precursor was more prominently associated with the soluble cytoplasmic fraction of the cells, although smaller amounts were recovered from the membrane and detergent-resistant fractions as well. Interestingly, approximately 50% of Vif partitioned with the soluble fraction (lanes a, bottom panel), with the remaining Vif protein equally distributed between the remaining two fractions. Thus, Vif showed an intracellular distribution very similar to that of Pr55^{gag}. Moreover, there was little or no difference in the relative distribution of Vif in chronically infected cells versus acutely infected cells. These results suggest that the inefficient packaging of Vif from chronically infected cells is not due to gross differences in the subcellular distribution of Vif in these cells.

Superinfection increases the rate of de novo synthesis of Vif.

The results from the previous experiments suggest that the inefficient packaging of Vif from chronically infected cells is not due to differences in the steady-state level of Vif in the virus-producing cell or in its subcellular distribution. We therefore speculated that differences in the rate of de novo Vif synthesis may account for the observed phenomenon. To test this possibility we compared the rate of Vif synthesis in chronically infected H9 cells with or without prior superinfection. Superinfection was done as described for Fig. 2 using a con-

centrated, VSV-G-pseudotyped stock of NL4-3 virus. One day following superinfection, cells were metabolically labeled for up to 30 min as indicated in Fig. 5. Whole-cell lysates were prepared and subjected to immunoprecipitation with an HIV-positive patient-derived serum (Fig. 5A, top panel) or a Vif-specific polyclonal antiserum (Fig. 5A, lower panel). Protein bands corresponding to Pr55^{gag} or Vif were quantified by densitometric scanning and plotted as a function of time (Fig. 5B). Background noise, which increased over time for each sample, was subtracted individually from each sample. In chronically infected cells, the rate of Vif synthesis was very low and near the limit of detection (Fig. 5B). The rate of synthesis calculated for Vif from chronically infected cells in Fig. 5B may, in fact, be an overestimation due to the poor signal/noise ratio. The synthesis of viral Gag proteins in chronically infected cells was low as well but nevertheless readily detectable (Fig. 5B, Pr55^{gag} chronic). As expected, superinfection significantly increased the rate of synthesis for both Vif and the viral Gag proteins (Fig. 5B) and thus provides an increased pool of newly synthesized Vif.

Rescue of viral infectivity by endogenous Vif. To determine how much the observed differences in the relative packaging efficiencies of endogenous and exogenous Vif affect viral infectivity, we measured the infectivity of viruses produced in chronically infected H9 cells using a single-cycle infectivity assay. To avoid possible background problems stemming from residual input virus, chronically infected H9 cells were directly electroporated with wild-type NL4-3 or its Vif-defective variant, NL4-3vif(-). To increase the sensitivity of the infectivity assay, viruses were pseudotyped with VSV-G. As reported previously, pseudotyping HIV-1 with VSV-G does not bypass the requirement for Vif in restrictive cells but allows for more efficient entry of viruses into target cells independent of their CD4 expression status (3). H9/HXB2NEO or H9/HXB2Vif Δ NEO cells were electroporated with a combination of VSV-G (5 μ g) and either NL4-3 or NL4-3Vif(-) (15 μ g) plasmid DNAs as described in Materials and Methods. Cells and virus-containing supernatants were harvested 24 h after electroporation. Virus from 50% of the culture supernatant was concentrated by ultracentrifugation. Whole-cell and virus lysates were prepared as described for Fig. 2. Proteins were separated by SDS-13% PAGE and subjected to immunoblotting using an HIV-positive patient-derived serum or the Vif-specific monoclonal antibody MAb #319 (Fig. 6A). As can be seen in Fig. 6A, samples derived from cells electroporated with NL4-3 or NL4-3Vif(-) were very similar within each cell type even though intracellular and extracellular amounts of viral proteins were slightly higher in samples from H9/HXB2NEO cells (compare lanes 1 and 2 with lanes 3 and 4, and compare lanes 5 and 6 with lanes 7 and 8). Consistent with the results from Fig. 2, significant amounts of endogenous Vif (lane 4, bottom panel) were detected, with its levels approaching those of exogenous Vif (lane 1, bottom panel). Virus-associated Vif was readily detectable in virus preparations from cells expressing exogenous Vif (lanes 5 and 7, bottom panel), while endogenous Vif was very low, yet still detectable, in cell-free virus (lane 8, bottom panel). These results are consistent with the results from Fig. 2 and confirm our

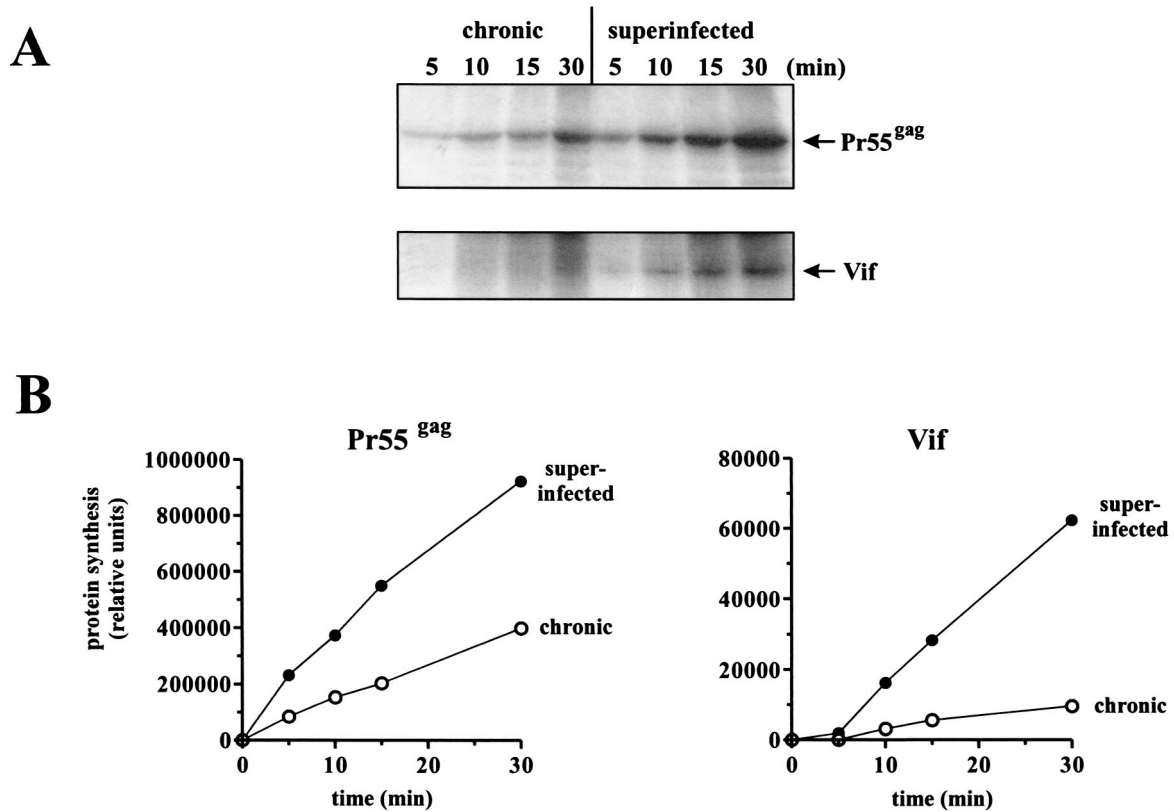


FIG. 5. Superinfection increases the rate of de novo synthesis of Vif. (A) Chronically infected H9/HXB2NEO cells (10×10^6) were infected with concentrated VSV-G-pseudotyped NL4-3 virus. H9/HXB2NEO cells (10×10^6) not subjected to superinfection were used in parallel. Both cultures were maintained in the presence of G418 throughout the experiment. Twenty-four hours after infection, both cultures were metabolically labeled with [³⁵S]methionine (2 mCi/ml; ICN Biomedical Inc., Costa Mesa, Calif.) for 5, 10, 15, or 30 min. Labeling reactions were terminated by removing the isotope and storing cells on dry ice. Whole-cell lysates were preadsorbed on protein A-Sepharose to minimize nonspecific binding. Cell lysates were then immunoprecipitated with an HIV-positive patient-derived serum (25% of lysate) (Pr55^{gag}) or a Vif-specific polyclonal antibody (75% of lysate) (Vif) followed by SDS-13% PAGE. Proteins were visualized by fluorography. A 2-day exposure is shown for Pr55^{gag}, and an 8-day exposure is shown for Vif. (B) Pr55^{gag}-specific and Vif-specific bands were quantified as described for Fig. 4 and plotted as a function of time.

observation that endogenous Vif is inefficiently packaged but not completely absent from cell-free virus.

Infectivity of the viruses produced from the H9/HXB2NEO and H9/HXB2VifΔNEO cells was determined by infection of LuSIV cells (41) with equivalent amounts (500 μ l) of unconcentrated culture supernatants as described in Materials and Methods. Luciferase activity in the infected cultures was determined 24 h after infection, normalized for input virus, and plotted as relative light units (Fig. 6B). Infectivity of Vif-encoding wild-type virus (exogenous Vif⁺) produced from the two chronically infected cell lines was each defined as 100 percent. As expected, the infectivity of viruses produced from cells lacking both endogenous and exogenous Vif (Fig. 6B, lane 2) was significantly less than that of virus expressing exogenous Vif (Fig. 6B, lane 1). Interestingly, expression of endogenous Vif appeared to be sufficient to provide almost full infectivity (80% of wild-type NL4-3) to the Vif-defective NL4-3 variant (Fig. 6B, compare lanes 3 and 4). These results suggest that endogenous Vif, although inefficiently packaged into prog-

eny virions, is biologically active and sufficient to rescue viral infectivity.

DISCUSSION

While the main biological activity of Vif, i.e., the regulation of viral infectivity, is undisputed, the biochemical mechanism of Vif function remains under investigation. Vif has been found at multiple sites intracellularly, including the plasma membrane (24, 26, 46), the cytoplasm (24, 30, 36, 51, 58), the cytoskeleton (27, 30), and the nucleus (11, 29), as well as in purified virus preparations (10, 17, 22, 30, 34). This heterogeneous distribution of Vif makes it difficult, if not impossible, to predict a relevant site(s) of action. Phenotypically, Vif defects have been correlated with an altered stability of the viral nucleoprotein complex (18, 28, 39, 48) and with an inability of the virus to complete reverse transcription following entry into target cells (15, 25, 37, 48, 53, 55). However, aside from the presence or absence of Vif in virus preparations, no consistent differences have thus far been observed that could explain the

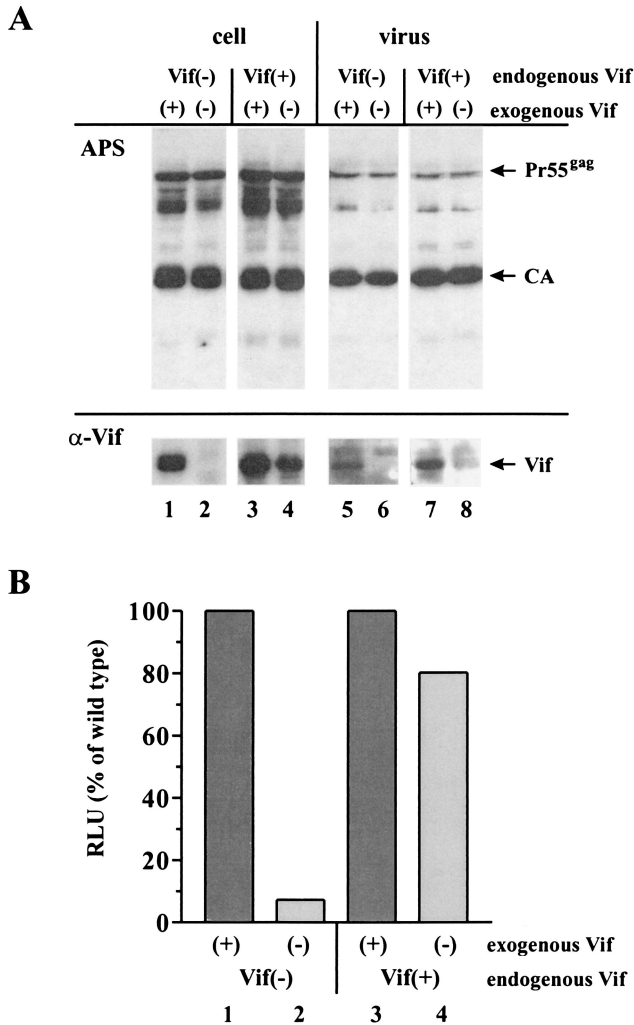


FIG. 6. Rescue of viral infectivity by endogenous Vif. (A) H9/HXB2NEO [Vif(+)] and H9/HXB2Vif Δ NEO [Vif(-)] cells (5×10^6 each) were electroporated with a combination of pHCMV-G (5 μ g) and 15 μ g of either pNL4-3 [(+)] or pNL4-3 Vif(-) [(-)] plasmid DNAs. Cells and virus-containing supernatants were harvested 24 h after electroporation. Virus from 50% of the supernatant was concentrated by ultracentrifugation through 20% sucrose and suspended in 200 μ l of sample buffer. Cells were washed once with PBS, and whole-cell lysates were prepared by suspending cells in 150 μ l of PBS plus 150 μ l of sample buffer. Viral and cell lysates were boiled (95°C, 15 min), and 30 μ l of cell lysates and 90 μ l of viral lysates were subjected to SDS-13% PAGE. Proteins were transferred to PVDF membranes and subjected to immunoblotting using MAb #319 (α -Vif). The same blot was subsequently reacted with an HIV-positive patient serum (APS). Proteins are identified on the right. (B) Unconcentrated virus-containing supernatants (500 μ l each) from panel A were used to infect LuSIV indicator cells. Input virus was quantified both by reverse transcriptase assay and p24 ELISA, and relative infectivity of the viruses was determined by quantifying the virus-induced luciferase activity as described in Materials and Methods. RLU, relative light units.

noted lack of infectivity associated with the absence of a functional *vif* gene (22, 31, 45). Yet, the concept that Vif may be a functional component of HIV virions has faced skepticism because of the low abundance of Vif in virus preparations and the high variability in the reported packaging efficiencies.

Our analyses of the intracellular distribution of Vif in

TABLE 1. Effect of virus source on Vif packaging

Virus source	No. of Vif molecules per virion	Reference
Chronically infected H9 cells	<1	Dettenhofer and Yu (17)
Chronically infected Molt IIIB cells	7–20	Camaur and Trono (10)
Stable H9 cell line	4–8	Simon et al. (49)
Chronically infected H9 cells ^a	4–6	This report
Productively infected T cells	30–80	Fouchier et al. (22)
Productively infected H9 cells	28	Simon et al. (49)
Productively infected SupT1 cells	60–100	Liu et al. (34)
Productively infected H9 cells ^b	60–100	This report
Transiently transfected H9 cells ^c	30–60	This report
Chronically infected H9 cells ^d (after superinfection)	30–40	This report

^a Same virus stock as used in Fig. 2, lane 7.

^b Same virus stock as used in Fig. 1, lane 6.

^c Virus produced by electroporation of H9 cells with pNL4-3 plasmid DNA as described in Materials and Methods.

^d Same virus stock as used in Fig. 2, lane 8.

acutely and chronically infected H9 cells are largely consistent with the above-mentioned heterogeneous distribution of Vif as they confirm the presence of Vif in various cellular compartments. However, the lack of obvious differences in the subcellular distribution of Vif in chronically infected cells versus acutely infected cells suggests that factors other than subcellular distribution are responsible for the observed differences in the packaging efficiencies for Vif from these cells. In fact, the only notable difference observed in our analysis was the relative rate of de novo Vif synthesis, which was near the limit of detection in chronically infected cells but was significantly elevated following superinfection. It is thus likely that virus-associated Vif is derived from the pool of newly synthesized Vif.

It is currently not clear why de novo-synthesized Vif should be more packaging competent than preexisting Vif. However, it is possible that after synthesis Vif gradually associates with cellular factors such as CEM15 (44) or vimentin (27, 30), thereby preventing packaging of Vif into virus particles. Indeed, the intracellular distribution of vimentin closely mimics that of Vif in our fractionation studies, and even low levels of intracellular Vif were found to have profound effects on the structure of intermediate filaments in H9 cells, suggesting a close association of Vif and vimentin in these cells (unpublished data). To what extent this phenomenon is linked to the regulation of viral infectivity by Vif or represents a secondary independent activity remains to be resolved. At any rate, the association of Vif with vimentin structures in combination with a low rate of de novo synthesis could explain the inefficient packaging of Vif into viruses produced from chronically infected cells.

To directly compare our results with the published literature, we performed a quantitative analysis of the amounts of Vif packaged under various conditions. Indeed, our data are quite consistent with the published literature (Table 1) and demonstrate that virus preparations from productively infected

(or transfected) H9 cells contain higher levels of Vif (30 to 100 molecules per virion) than virus preparations from chronically infected cells (4 to 6 molecules of Vif per virion), even when virus output was boosted from the chronically infected cells through exogenous virus. It is interesting that these differences in packaging efficiency were not paralleled by corresponding changes in viral infectivity. This suggests that either Vif functions intracellularly or only small amounts of virus-associated Vif are sufficient to yield infectious virus. There are valid arguments to support either view. An intracellular function of Vif would be consistent with the noted cell type dependence of Vif, which presumably is due to the presence of an inhibitory factor in nonpermissive cells that must be neutralized by Vif to allow the assembly of infectious viruses (35, 47). If the neutralization of a cellular inhibitor were the only function of Vif, one would predict that Vif increases viral infectivity in a dose-dependent manner, reaching a plateau once Vif is present at saturating levels. However, preliminary data suggest that high levels of Vif can actually inhibit viral infectivity, suggesting that the activity of Vif goes beyond a mere inactivation of a putative cellular inhibitor (unpublished data). A virus-associated activity of Vif, on the other hand, would be consistent with its noted effect on the stability of nucleoprotein complexes in particular, since Vif itself is part of this complex (30, 31, 34). Our limited understanding of the biochemical activity of Vif does not currently allow us to speculate on the minimal number of Vif molecules that would be required for a presumed function within the viral nucleoprotein complex. Yet a putative role of Vif in stabilizing components of the viral reverse transcription complex could be accomplished with levels of Vif comparable to those of reverse transcriptase or integrase. Also, it should be pointed out that the amounts of Vif present in virus derived from chronically infected cells may actually be higher than the four to six molecules calculated in our study. By quantitating only full-length Vif, our calculations did not take into account the fact that a portion of Vif is proteolytically processed by the viral protease (32). Finally, it is conceivable that Vif is not evenly distributed among all virus particles but enriched in infectious virions, which represent only a small percentage of the whole virus population. Such a selective distribution of Vif is plausible because of the direct correlation of Vif with the infectivity of HIV particles and could be accomplished through selective packaging of Vif in association with viral genomic RNA.

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REFERENCES

1. Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin. 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. *J. Virol.* **59**:284–291.
2. Akari, H., J. Sakuragi, Y. Takebe, K. Tomonaga, M. Kawamura, M. Fuka-

- sawa, T. Miura, T. Shinjo, and M. Hayami. 1992. Biological characterization of human immunodeficiency virus type 1 and type 2 mutants in human peripheral blood mononuclear cells. *Arch. Virol.* **123**:157–167.
3. Akari, H., T. Uchiyama, T. Fukumori, S. Iida, A. H. Koyama, and A. Adachi. 1999. Pseudotyping human immunodeficiency virus type 1 by vesicular stomatitis virus G protein does not reduce the cell-dependent requirement of vif for optimal infectivity: functional difference between Vif and Nef. *J. Gen. Virol.* **80**:2945–2949.
4. Bess, J. W., Jr., P. J. Powell, H. J. Issaq, L. J. Schumack, M. K. Grimes, L. E. Henderson, and L. O. Arthur. 1992. Tightly bound zinc in human immunodeficiency virus type 1, human T-cell leukemia virus type I, and other retroviruses. *J. Virol.* **66**:840–847.
5. Blanc, D., C. Patience, T. F. Schulz, R. Weiss, and B. Spire. 1993. Transcomplementation of VIF– HIV-1 mutants in CEM cells suggests that VIF affects late steps of the viral life cycle. *Virology* **193**:186–192.
6. Borman, A. M., C. Quillent, P. Charneau, C. Daugey, and F. Clavel. 1995. Human immunodeficiency virus type 1 Vif– mutant particles from restrictive cells: role of Vif in correct particle assembly and infectivity. *J. Virol.* **69**:2058–2067.
7. Bouyac, M., M. Courcoul, G. Bertoia, Y. Baudat, D. Gabuzda, D. Blanc, N. Chazal, P. Boulanger, J. Sire, R. Vigne, and B. Spire. 1997. Human immunodeficiency virus type 1 Vif protein binds to the Pr55_{Gag} precursor. *J. Virol.* **71**:9358–9365.
8. Bouyac, M., F. Rey, M. Nascimbeni, M. Courcoul, J. Sire, D. Blanc, F. Clavel, R. Vigne, and B. Spire. 1997. Phenotypically Vif– human immunodeficiency virus type 1 is produced by chronically infected restrictive cells. *J. Virol.* **71**:2473–2477.
9. Butera, S. T., B. D. Roberts, L. Lam, T. Hodge, and T. M. Folks. 1994. Human immunodeficiency virus type 1 RNA expression by four chronically infected cell lines indicates multiple mechanisms of latency. *J. Virol.* **68**:2726–2730.
10. Camaur, D., and D. Trono. 1996. Characterization of human immunodeficiency virus type 1 Vif particle incorporation. *J. Virol.* **70**:6106–6111.
11. Chatterji, U., C. K. Grant, and J. H. Elder. 2000. Feline immunodeficiency virus Vif localizes to the nucleus. *J. Virol.* **74**:2533–2540.
12. Chertova, E., J. W. Bess, Jr., B. J. Crise, R. C. Sowder II, T. M. Schaden, J. M. Hilburn, J. A. Hoxie, R. E. Benveniste, J. D. Lifson, L. E. Henderson, and L. O. Arthur. 2002. Envelope glycoprotein incorporation, not shedding of surface envelope glycoprotein (gp120/SU), is the primary determinant of SU content of purified human immunodeficiency virus type 1 and simian immunodeficiency virus. *J. Virol.* **76**:5315–5325.
13. Chowdhury, I. H., W. Chao, M. J. Potash, P. Sova, H. E. Gendelman, and D. J. Volsky. 1996. *vif*-negative human immunodeficiency virus type 1 persistently replicates in primary macrophages, producing attenuated progeny virus. *J. Virol.* **70**:5336–5345.
14. Clouse, K. A., D. Powell, I. Washington, G. Poli, K. Strebel, W. Farrar, P. Barstad, J. Kovacs, A. S. Fauci, and T. M. Folks. 1989. Monokine regulation of human immunodeficiency virus-1 expression in a chronically infected human T cell clone. *J. Immunol.* **142**:431–438.
15. Courcoul, M., C. Patience, F. Rey, D. Blanc, A. Harmache, J. Sire, R. Vigne, and B. Spire. 1995. Peripheral blood mononuclear cells produce normal amounts of defective Vif– human immunodeficiency virus type 1 particles which are restricted for the preretrotranscription steps. *J. Virol.* **69**:2068–2074.
16. Dettenhofer, M., S. Cen, B. A. Carlson, L. Kleiman, and X. F. Yu. 2000. Association of human immunodeficiency virus type 1 Vif with RNA and its role in reverse transcription. *J. Virol.* **74**:8938–8945.
17. Dettenhofer, M., and X. F. Yu. 1999. Highly purified human immunodeficiency virus type 1 reveals a virtual absence of Vif in virions. *J. Virol.* **73**:1460–1467.
18. Dornadula, G., S. Yang, R. J. Pomerantz, and H. Zhang. 2000. Partial rescue of the Vif-negative phenotype of mutant human immunodeficiency virus type 1 strains from nonpermissive cells by intravirion reverse transcription. *J. Virol.* **74**:2594–2602.
19. Fan, L., and K. Peden. 1992. Cell-free transmission of Vif mutants of HIV-1. *Virology* **190**:19–29.
20. Fisher, A. G., B. Ensoli, L. Ivanoff, M. Chamberlain, S. Petteway, L. Ratner, R. C. Gallo, and F. Wong-Staal. 1987. The *sov* gene of HIV-1 is required for efficient virus transmission in vitro. *Science* **237**:888–893.
21. Fouchier, R. A., B. E. Meyer, J. H. Simon, U. Fischer, and M. H. Malim. 1997. HIV-1 infection of non-dividing cells: evidence that the amino-terminal basic region of the viral matrix protein is important for Gag processing but not for post-entry nuclear import. *EMBO J.* **16**:4531–4539.
22. Fouchier, R. A., J. H. Simon, A. B. Jaffe, and M. H. Malim. 1996. Human immunodeficiency virus type 1 Vif does not influence expression or virion incorporation of *gag*-, *pol*-, and *env*-encoded proteins. *J. Virol.* **70**:8263–8269.
23. Gabuzda, D. H., K. Lawrence, E. Langhoff, E. Terwilliger, T. Dorfman, W. A. Haseltine, and J. Sodroski. 1992. Role of *vif* in replication of human immunodeficiency virus type 1 in CD4⁺ T lymphocytes. *J. Virol.* **66**:6489–6495.
24. Goncalves, J., P. Jallepalli, and D. H. Gabuzda. 1994. Subcellular localization of the Vif protein of human immunodeficiency virus type 1. *J. Virol.* **68**:704–712.

25. **Goncalves, J., Y. Korin, J. Zack, and D. Gabuzda.** 1996. Role of Vif in human immunodeficiency virus type 1 reverse transcription. *J. Virol.* **70**: 8701–8709.
26. **Goncalves, J., B. Shi, X. Yang, and D. Gabuzda.** 1995. Biological activity of human immunodeficiency virus type 1 Vif requires membrane targeting by C-terminal basic domains. *J. Virol.* **69**:7196–7204.
27. **Henzler, T., A. Harmache, H. Herrmann, H. Spring, M. Suzan, G. Audoly, T. Panek, and V. Bosch.** 2001. Fully functional, naturally occurring and C-terminally truncated variant human immunodeficiency virus (HIV) Vif does not bind to HIV Gag but influences intermediate filament structure. *J. Gen. Virol.* **82**:561–573.
28. **Hoglund, S., A. Ohagen, K. Lawrence, and D. Gabuzda.** 1994. Role of vif during packing of the core of HIV-1. *Virology* **201**:349–355.
29. **Huvent, I., S. S. Hong, C. Fournier, B. Gay, J. Tournier, C. Carriere, M. Courcoul, R. Vigne, B. Spire, and P. Boulanger.** 1998. Interaction and cocapsidation of human immunodeficiency virus type 1 Gag and Vif recombinant proteins. *J. Gen. Virol.* **79**:1069–1081.
30. **Karczewski, M. K., and K. Strebel.** 1996. Cytoskeleton association and virion incorporation of the human immunodeficiency virus type 1 Vif protein. *J. Virol.* **70**:494–507.
31. **Khan, M. A., C. Aberham, S. Kao, H. Akari, R. Gorelick, S. Bour, and K. Strebel.** 2001. Human immunodeficiency virus type 1 Vif protein is packaged into the nucleoprotein complex through an interaction with viral genomic RNA. *J. Virol.* **75**:7252–7265.
32. **Khan, M. A., H. Akari, S. Kao, C. Aberham, D. Davis, A. Buckler-White, and K. Strebel.** 2002. Intravirion processing of human immunodeficiency virus type 1 Vif protein by the viral protease may be correlated with Vif function. *J. Virol.* **76**:9112–9123.
33. **Kishi, M., Y. Nishino, M. Sumiya, K. Ohki, T. Kimura, T. Goto, M. Nakai, M. Kakinuma, and K. Ikuta.** 1992. Cells surviving infection by human immunodeficiency virus type 1: vif or vpu mutants produce non-infectious or markedly less cytopathic viruses. *J. Gen. Virol.* **73**:77–87.
34. **Liu, H., X. Wu, M. Newman, G. M. Shaw, B. H. Hahn, and J. C. Kappes.** 1995. The Vif protein of human and simian immunodeficiency viruses is packaged into virions and associates with viral core structures. *J. Virol.* **69**:7630–7638.
35. **Madani, N., and D. Kabat.** 1998. An endogenous inhibitor of human immunodeficiency virus in human lymphocytes is overcome by the viral Vif protein. *J. Virol.* **72**:10251–10255.
36. **Michaels, F. H., N. Hattori, R. C. Gallo, and G. Franchini.** 1993. The human immunodeficiency virus type 1 (HIV-1) vif protein is located in the cytoplasm of infected cells and its effect on viral replication is equivalent in HIV-2. *AIDS Res. Hum. Retrovir.* **9**:1025–1030.
37. **Nascimbeni, M., M. Bouyac, F. Rey, B. Spire, and F. Clavel.** 1998. The replicative impairment of Vif⁻ mutants of human immunodeficiency virus type 1 correlates with an overall defect in viral DNA synthesis. *J. Gen. Virol.* **79**:1945–1950.
38. **Ochsenbauer, C., T. Wilk, and V. Bosch.** 1997. Analysis of vif-defective human immunodeficiency virus type 1 (HIV-1) virions synthesized in 'non-permissive' T lymphoid cells stably infected with selectable HIV-1. *J. Gen. Virol.* **78**:627–635.
39. **Ohagen, A., and D. Gabuzda.** 2000. Role of Vif in stability of the human immunodeficiency virus type 1 core. *J. Virol.* **74**:11055–11066.
40. **Reddy, T. R., G. Kraus, O. Yamada, D. J. Looney, M. Suhasini, and F. Wong-Staal.** 1995. Comparative analyses of human immunodeficiency virus type 1 (HIV-1) and HIV-2 Vif mutants. *J. Virol.* **69**:3549–3553.
41. **Roos, J. W., M. F. Maughan, Z. Liao, J. E. Hildreth, and J. E. Clements.** 2000. LuSIV cells: a reporter cell line for the detection and quantitation of a single cycle of HIV and SIV replication. *Virology* **273**:307–315.
42. **Sakai, H., R. Shibata, J. Sakuragi, S. Sakuragi, M. Kawamura, and A. Adachi.** 1993. Cell-dependent requirement of human immunodeficiency virus type 1 Vif protein for maturation of virus particles. *J. Virol.* **67**:1663–1666.
43. **Schwartz, S., B. K. Felber, and G. N. Pavlakis.** 1991. Expression of human immunodeficiency virus type 1 vif and vpr mRNAs is Rev-dependent and regulated by splicing. *Virology* **183**:677–686.
44. **Sheehy, A. M., N. C. Gaddis, J. D. Choi, and M. H. Malim.** 2002. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. *Nature* **418**:646–650.
45. **Simm, M., M. Shahabuddin, W. Chao, J. S. Allan, and D. J. Volsky.** 1995. Aberrant Gag protein composition of a human immunodeficiency virus type 1 vif mutant produced in primary lymphocytes. *J. Virol.* **69**:4582–4586.
46. **Simon, J. H., R. A. Fouchier, T. E. Southerling, C. B. Guerra, C. K. Grant, and M. H. Malim.** 1997. The Vif and Gag proteins of human immunodeficiency virus type 1 colocalize in infected human T cells. *J. Virol.* **71**:5259–5267.
47. **Simon, J. H., N. C. Gaddis, R. A. Fouchier, and M. H. Malim.** 1998. Evidence for a newly discovered cellular anti-HIV-1 phenotype. *Nat. Med.* **4**:1397–1400.
48. **Simon, J. H., and M. H. Malim.** 1996. The human immunodeficiency virus type 1 Vif protein modulates the postpenetration stability of viral nucleoprotein complexes. *J. Virol.* **70**:5297–5305.
49. **Simon, J. H., D. L. Miller, R. A. Fouchier, and M. H. Malim.** 1998. Virion incorporation of human immunodeficiency virus type-1 Vif is determined by intracellular expression level and may not be necessary for function. *Virology* **248**:182–187.
50. **Simon, J. H., T. E. Southerling, J. C. Peterson, B. E. Meyer, and M. H. Malim.** 1995. Complementation of vif-defective human immunodeficiency virus type 1 by primate, but not nonprimate, lentivirus vif genes. *J. Virol.* **69**:4166–4172.
51. **Simon, J. H. M., E. A. Carpenter, R. A. M. Fouchier, and M. H. Malim.** 1999. Vif and the p55^{Gag} polyprotein of human immunodeficiency virus type 1 are present in colocalizing membrane-free cytoplasmic complexes. *J. Virol.* **73**: 2667–2674.
52. **Sova, P., W. Chao, and D. J. Volsky.** 1997. The redox state of cysteines in human immunodeficiency virus type 1 Vif in infected cells and in virions. *Biochem. Biophys. Res. Commun.* **240**:257–260.
53. **Sova, P., and D. J. Volsky.** 1993. Efficiency of viral DNA synthesis during infection of permissive and nonpermissive cells with vif-negative human immunodeficiency virus type 1. *J. Virol.* **67**:6322–6326.
54. **Strebel, K., D. Daugherty, K. Clouse, D. Cohen, T. Folks, and M. A. Martin.** 1987. The HIV 'A' (sor) gene product is essential for virus infectivity. *Nature* **328**:728–730.
55. **von Schwedler, U., J. Song, C. Aiken, and D. Trono.** 1993. Vif is crucial for human immunodeficiency virus type 1 proviral DNA synthesis in infected cells. *J. Virol.* **67**:4945–4955.
56. **Yang, S., Y. Sun, and H. Zhang.** 2001. The multimerization of human immunodeficiency virus type I Vif protein: a requirement for Vif function in the viral life cycle. *J. Biol. Chem.* **276**:4889–4893.
57. **Yee, J. K., A. Miyanojara, P. LaPorte, K. Bouic, J. C. Burns, and T. Friedmann.** 1994. A general method for the generation of high-titer, pantropic retroviral vectors: highly efficient infection of primary hepatocytes. *Proc. Natl. Acad. Sci. USA* **91**:9564–9568.
58. **Zhang, H., R. J. Pomerantz, G. Dornadula, and Y. Sun.** 2000. Human immunodeficiency virus type 1 Vif protein is an integral component of an mRNP complex of viral RNA and could be involved in the viral RNA folding and packaging process. *J. Virol.* **74**:8252–8261.