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Human Immunodeficiency Virus Type 1 Vif Does Not Influence Expression or Virion Incorporation of *gag*-, *pol*-, and *env*-Encoded Proteins

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The Vif protein of human immunodeficiency virus type 1 is required for productive replication in peripheral blood lymphocytes and a limited number of immortalized T-lymphoid lines (nonpermissive cells). In contrast, Vif is fully dispensable for virus replication in other T-cell lines (permissive cells). Because the infection phenotype of released virions is determined by producer cells and by the presence of Vif in those cells, we have analyzed the protein contents of purified viral particles in an attempt to define compositional differences that could explain the infection phenotype. Surprisingly, we were unable to discern any Vif- or cell-type-dependent quantitative or qualitative difference in the Gag, Pol, and Env proteins of virions or virus-producing cells that correlates with virus infectivity. We were, however, able to demonstrate that Vif itself is present in virions and, using semiquantitative Western blotting (immunoblotting), that there is an average of 30 to 80 molecules of Vif incorporated into each virion. Importantly, parallel analyses of total lysates of the producer cells revealed that the cell-associated expression levels of Vif are close to those of the Gag proteins. Given the dramatically higher abundance of Vif in cells than in virions, we speculate that Vif exerts its principal activity during the processes of virus assembly and budding and that this function could be of a structural-conformational nature.

It has been established that the virally encoded *vif* gene is a critical positively acting modulator of lentivirus infections (11, 13, 15, 24, 25, 27, 28). In particular, the *vif* gene of the prototypic lentivirus human immunodeficiency virus type 1 (HIV-1) is maintained in virus populations in vivo (19, 30) and is required for productive replication in cultured primary cells as well as certain immortalized T-cell lines (5, 9, 10, 12, 20, 23, 26, 29). However, in spite of Vif's profound influence on virus infectivity, we currently understand very little regarding its mechanism of action.

There have been a number of reports describing the nature of the defect(s) in *vif*-deficient (Δ*vif*) HIV-1 infections. A pair of early reports proposed that Vif modulates the postpenetration processivity of the virion reverse transcriptase (RT) such that Δ*vif* viruses are defective in the ability to synthesize significant levels of HIV-1 DNA (26, 29). In contrast, two later analyses indicated that Δ*vif* viruses fail to synthesize any viral DNA following exposure to susceptible cells (5, 9); it was therefore suggested that these viruses might be impaired at the stage(s) of receptor binding at the cell surface, membrane fusion, or uncoating. Most recently, we have found that Δ*vif* infections can proceed to the second-strand transfer of reverse transcription as efficiently as wild-type infections but that these DNAs are not stably maintained in the absence of Vif and are eventually cleared by the challenged cells (23).

Although these various findings might appear to be incompatible with each other, we have previously hypothesized that the target cells used for these experiments could result in the same fundamental defect being manifested in different ways. In certain cells, penetrating Δ*vif* nucleocapsids might be relatively stable and would support the synthesis of significant levels of viral DNA, in other cells they might be extremely unstable and may disassemble prior to any reverse transcription, and in yet others they may display an intermediate phenotype. Several groups have established that a genotypically Δ*vif* virus can be complemented, (i.e., rendered infectious) by the expression of Vif in *trans* in cells that are producing virus (4, 5, 9, 12, 29). This finding therefore suggests that Vif influences virus assembly and/or maturation in a manner that results in the acquisition of the infectious phenotype by the released virus particles. We have speculated that this phenotype ultimately reveals itself as nucleocapsid stability following penetration into a susceptible cell (23).

Given that Vif functions to impart infectivity on HIV-1 particles, it seems reasonable to expect that Vif might influence the composition and/or conformation of virions or virion components. With respect to the possible effect(s) of Vif on virion structure, two groups have used electron microscopy to show that Δ*vif* particles frequently display an immature morphology whereby the cores appear less electron dense, nonhomogeneously packed, and positioned away from the center of the virions (5, 16). Correlating these ultrastructural abnormalities of *vif*-deficient virions with the relevant compositional differences (if any) between Δ*vif* and wild-type particles and, ultimately, with the modulation of infectivity constitutes a major challenge for understanding Vif function. Although a number of groups have addressed this issue, the data that have been documented to date have been somewhat inconsistent and contradictory. For example, it has been suggested that Vif alters virion composition by enhancing the proteolytic processing of $p55^{Gag}$ (5, 21), increasing the incorporation of $gp160/120^{Env}$ (5, 20), and catalyzing a truncation within the carboxy-terminal 15 amino acids of $gp41^{Env}$ (14). In contrast, others have been unable to discern any differences between the viral protein content of wild-type and Δ*vif* virions (29). Until recently, it also remained unclear whether Vif itself was incorporated into viral particles. In this regard some conformity now

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exists, and it has been reported that there are between 7 and 100 molecules of Vif per HIV-1 particle (5, 8, 17, 18).

In this report, we present an analysis of Vif's influence on the expression patterns of HIV-1-encoded proteins in cells and in viral particles. As sources of viruses, we have used cells that are restrictive to Δ*vif* virus replication (nonpermissive cells) as well as cells that allow wild-type and Δ*vif* virus to replicate equivalently (permissive cells). Furthermore, to rule out potential artifacts that could be attributed to experimental strategy, we have derived viruses not only from cells transiently transfected with provirus expression vectors but also from cells subjected to single-cycle infections with high-titer virus stocks. Throughout all of these analyses, the sole compositional difference that we could detect between wild-type and *vif*-deficient HIV-1 was that Vif itself was present in wild-type virions. We have therefore concluded that the ~100-fold effect that Vif can have on viral infectivity is unlikely to be due to differences in the posttranslational processing or virion incorporation of proteins derived from the viral *gag*, *pol*, or *env* gene.

MATERIALS AND METHODS

Molecular clones, cells, and cell lines. The wild-type and Δ*vif* HIV-1 provirus expression vectors, pIIIB and pIIIB/Δ*vif*, have been described elsewhere (24). The human CD4-positive T-cell lines H9, Jurkat, and CEM-SS were cultured as described previously (23, 24); these lines have previously been shown to be nonpermissive, semipermissive, and fully permissive, respectively, to the replication of pIIIB/Δ*vif*-derived virus (23, 24). The transduced derivative lines Jurkat/sVif, H9/hVif, and H9/ΔhVif express the intact *vif* gene of simian immunodeficiency virus from macaques, the intact *vif* gene of HIV-1, and the defective *vif* gene of pIIIB/Δ*vif*, respectively, and were maintained in G418-containing medium (23). Human peripheral blood lymphocytes (PBLs) were obtained by venipuncture, purified using Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden), stimulated with 5 μg of phytohemagglutinin per ml for 72 h, and maintained in medium containing 20 U of recombinant interleukin-2 per ml as described previously (23).

Virus production. In experiments in which transient transfections were used to generate virus, 10×10^6 T cells (PBLs, H9 cells, or CEM-SS cells) were electroporated with 15 μg of pIIIB or pIIIB/Δ*vif*, using a Bio-Rad gene pulser (Bio-Rad Laboratories, Hercules, Calif.). At 24 h postelectroporation, the cells were harvested by centrifugation at $500 \times g$ for 5 min at room temperature. The cell pellets were examined for viral protein expression (see below), and the bulk of the supernatants was used for the purification of viral particles on continuous sucrose gradients. Importantly, an aliquot of every supernatant was used for the determination of viral infectivity (see below).

In other experiments, T cells that had been infected with either wild-type or Δ*vif* HIV-1 were used as sources of virus. To express HIV-1/Δ*vif* from H9 cells (a line that does not support a spreading replication of HIV-1/Δ*vif*), a previously described coculture strategy that delivers a relatively high titer of Δ*vif* virus to these cells was used (23). Briefly, an acute infection of HIV-1/Δ*vif* was established in Jurkat/sVif cells. H9/hVif or H9/ΔhVif cells were then challenged continuously for 48 h by coculture except that the cell populations were kept separate by the utilization of Transwell culture dishes (0.4-μm pore size). The H9 cells were then harvested, treated with trypsin to eliminate the input inocula, and used as the producers of phenotypically wild-type (H9/hVif) or *vif*-deficient (H9/ΔhVif) viruses. When Jurkat or CEM-SS cells were used as virus producers, infections by pIIIB- or pIIIB/Δ*vif*-derived virus were allowed to proceed until maximum levels of virus expression were attained. In the case of Δ*vif* infections of Jurkat cells, a line that we have shown to be semipermissive for the replication of this virus (24), an inoculum that was ~100-fold greater than for the wild-type infection had to be used for similar levels of virus production to be attained by ~10 days postchallenge. The cultures were treated as described above to obtain cell pellets and purified viruses.

Quantitation of virion production and infectivity. For all methods of virus expression, viral particles from culture supernatants were quantitated by an enzyme-linked immunosorbent assay for soluble $p24^{Gag}$. The relative infectivity of each virus preparation was determined by using the previously described C8166/HIV-*CAT* T-cell line (23). Specifically, 5×10^5 cells were challenged with equal $p24^{Gag}$ values (0.5 to 1.0 ng/ml) for each pair of viruses (namely, wild type and *vif* deficient). At 24 h, the cells were harvested, lysates were prepared, and the induction of chloramphenicol acetyltransferase (CAT) activity was determined relative to that in a mock-infected culture.

Purification of virions on continuous sucrose gradients. At a time when virion production was at or close to its maximum, the cells were harvested as described above, an aliquot of the supernatant was reserved for the determination of infectivity, and the remainder was used for the purification of virus particles. First, the cleared supernatant was passed through a 0.45-μm-pore-size filter and layered onto a 0.5-ml 60% (wt/vol) sucrose (in phosphate-buffered saline [PBS])–2.0 ml 20% (wt/vol) sucrose (in PBS) step gradient and centrifuged at $150,000 \times g$ for 2 h at 4°C, using an SW41 rotor (Beckman Instruments Inc., Fullerton, Calif.). Virus particles were recovered from the interface, adjusted to 20% (wt/vol) sucrose (in PBS), and loaded onto the top of a 20 to 60% (wt/vol) 12-ml continuous sucrose gradient (in PBS). Centrifugation was at $75,000 \times g$ for 1.5 h at 4°C in a Beckman SW41 rotor. Fractions of 0.5 ml were harvested from the top of the gradient, and the peak of virus production was identified by $p24^{Gag}$ content or RT activity. Finally, the two or three peak fractions were pooled, and virus particles were pelleted by centrifugation in a Beckman SW41 rotor at $150,000 \times g$ for 2 h at 4°C.

Western blot (immunoblot) analyses and antibodies. Whole cell pellets or purified virions were denatured in standard lysis buffers under reducing conditions. Viral proteins were resolved by one- or two-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). In the latter case, the proteins were subjected to nonequilibrium pH gradient electrophoresis (NEPHGE; pH range, 3.0 to 10) (2) followed by standard SDS-PAGE. Separated proteins were transferred to protean membrane (Schleicher & Schuell, Inc., Keene, N.H.) by electroelution and subjected to hybridization with a variety of monoclonal and polyclonal antibodies. Frequently, one filter was used for multiple hybridizations; in such cases, the filters were stripped of primary antibody by washing for 30 min at 50°C in 2% SDS–62.5 mM Tris-HCl (pH 6.8)–100 mM β-mercaptoethanol. The anti-$p24^{Gag}$ (capsid [CA]) and anti-$p17^{Gag}$ (matrix [MA]) monoclonal antibodies were purchased from DuPont, NEN, Inc. (Billerica, Mass.), the anti-RT monoclonal antibody was purchased from Intracel Corp. (Cambridge, Mass.), the anti-integrase (IN) monoclonal antibody was obtained from A. M. Skalka (3), the anti-$gp160/120^{Env}$ rabbit polyclonal antiserum was obtained from R. W. Doms (7), and the anti-Vif monoclonal antibody (monoclonal antibody 319) has been described previously (24). Bound antibodies were visualized by secondary hybridization with appropriate species-specific horseradish peroxidase-conjugated antibodies, enhanced chemiluminescence, and autoradiography.

Quantitation of Vif in HIV-1 virions. Cell lysates and sucrose gradient-purified virus particles were obtained from cultures of H9, Jurkat, and CEM-SS cells acutely infected with pIIIB-derived wild-type virus. Vif and $p24^{Gag}$ (CA) were overexpressed in *Escherichia coli* BL21(DE3)pLysS, each with an amino-terminal extension of Met-Arg-Gly-Ser-His_6-Ser, using the pET expression system (Novagen Inc., Madison, Wis.), and purified by Ni^{2+} chelate affinity chromatography in the presence of 6 M guanidine hydrochloride. The column eluates were dialyzed against 100 mM NaCl–50 mM Na_2HPO_4 (pH 5.0 for Vif; pH 10 for CA), and the final concentrations of protein were determined by the method of Bradford (6). Samples consisting of total cell lysates and purified virions were then resolved on SDS–12% polyacrylamide gels and subjected to Western analyses (as described above) alongside standard curves of the recombinant Vif and $p24^{Gag}$ proteins; monoclonal antibodies specific for Vif and $p24^{Gag}$ were used for the initial filter hybridizations. Following autoradiography, the quantities of Vif, $p55^{Gag}$, $p24^{Gag}$, and processing intermediates were determined for all samples by using a densitometer (model 300A; Molecular Dynamics, Sunnyvale, Calif.). From this information, viral and cellular ratios of Vif to Gag could be deduced and the average number of Vif molecules per virion could therefore be calculated.

RESULTS

As discussed earlier, Vif exerts an effect on HIV-1 infectivity only in certain cell types. In particular, it has been demonstrated that *vif*-deficient viruses fail to replicate in PBLs and in the H9 T-cell line (nonpermissive cells), replicate poorly and with substantially delayed kinetics in lines such as Jurkat and MT-4 (semipermissive cells), and replicate efficiently in the CEM-SS and C8166 T-cell lines (permissive cells) (5, 9–12, 20, 23, 24, 26, 29). Although the basis of this cell selectivity is unknown, if one were to believe that Vif exerts its effect on viral infectivity by modulating the composition of virions, then one might predict that such an effect could be overcome by permissive cells. For this reason, we have investigated the effect of Vif on HIV-1 protein expression, in terms of incorporation into virions as well as cell-associated expression patterns, in cells that do and do not support the robust replication of *vif*-deficient HIV-1.

Virus produced by transient transfection. For the first set of experiments, we used the transient transfection of provirus expression vectors as a means to introduce viral DNA into the cells destined to become virus producers. The rationale behind this procedure was that the short time span over which such

TABLE 1. Infectivity of HIV-1 derived by transfection

HIV-1 genotype	CAT activity[a] (U)	Fold induction[b]
PBL		
Donor 1		
Wild type	22,866	16
Δ*vif*	3,461	
Mock	2,146	
Donor 2		
Wild type	17,217	7
Δ*vif*	4,326	
Mock	2,117	
H9		
Expt 1		
Wild type	13,654	18
Δ*vif*	1,580	
Mock	866	
Expt 2		
Wild type	12,086	13
Δ*vif*	1,751	
Mock	905	
CEM-SS		
Expt 1		
Wild type	21,355	1.4
Δ*vif*	15,781	
Mock	866	
Expt 2		
Wild type	13,989	1.3
Δ*vif*	10,978	
Mock	905	

[a] With the C8166/HIV-*CAT* cell line used as an indicator, the accumulation of CAT at ~24 h postinfection serves as a sensitive measure of infection.

[b] The Vif-mediated induction of infectivity was calculated by subtracting the mock value from the wild-type and Δ*vif* values and using the corrected Δ*vif* value as the denominator.

experiments are performed would likely eliminate (or at least minimize) any selective effects that virus gene expression and virion production could impose on producer cell populations. It is possible that selective pressures of this nature could have contributed to the disparate results obtained in previous analyses of Vif function.

To confirm that viruses generated by transient transfection do display the established Vif phenotype in terms of a cell-type-specific reduction of HIV-1 infectivity in the absence of Vif, cultures of PBLs (two donors), H9 cells, and CEM-SS cells were electroporated with the pIIIB and pIIIB/Δ*vif* expression vectors. At 24 h, the culture supernatants were tested for the presence of $p24^{Gag}$, and virus samples corresponding to ~1 ng of $p24^{Gag}$ per ml were used for the determination of viral infectivity, using the sensitive T-cell indicator line C8166/HIV-*CAT* (Table 1). According to this assay, a viral infection that proceeds to provirus establishment and expresses the viral Tat *trans* activator will stimulate transcription from the resident HIV-*CAT* expression cassette as well as from the newly formed provirus. Alternatively, infections that fail to establish a provirus also fail to induce CAT expression (23). Consistent with Vif being essential for productive HIV-1 replication in primary human cells, PBLs transfected with the Δ*vif* provirus gave rise to virions that were markedly less infectious than their wild-type counterparts. Importantly, H9 cells accurately recapitulated this phenotype (18- and 13-fold inductions of infectivity by Vif), whereas CEM-SS cells did not. This was to be expected as CEM-SS cells support the replication of HIV-1/Δ*vif* and H9 cells do not (23). Together, these data indicate that viruses derived by transient transfection represent a good system for studying the potential effects of Vif on virion composition.

Wild-type and *vif*-deficient viral particles from transiently transfected cultures of H9 cells (which yield considerably more virus than PBLs) and CEM-SS cells were purified on continuous sucrose gradients, pelleted by high-speed centrifugation, and subjected to Western blot analyses using a variety of antibodies specific for HIV-1-encoded proteins (Fig. 1A). In particular, antibodies that recognize $p24^{Gag}$ (CA), $p17^{Gag}$ (MA), $gp160/120^{Env}$, the 66- and 51-kDa subunits of RT, the 32-kDa IN protein, and the 23-kDa Vif protein were used. Inspection of these data reveals not only that an intact *vif* gene had no significant impact on the relative quantities of *gag*-, *pol*-, and *env*-encoded protein products present in virions but also that the nonpermissive versus permissive phenotype of the producer cell also had no effect. Because the peaks of virus were always present in the same gradient fractions (data not shown), we have further concluded that there were no significant variations in the particle densities of the viruses examined in these experiments. Of particular interest was the finding that the ratios of $p55^{Gag}$ to the mature CA and MA proteins were essentially the same in all of these virus preparations. These findings therefore conflict with those of others who have sug-

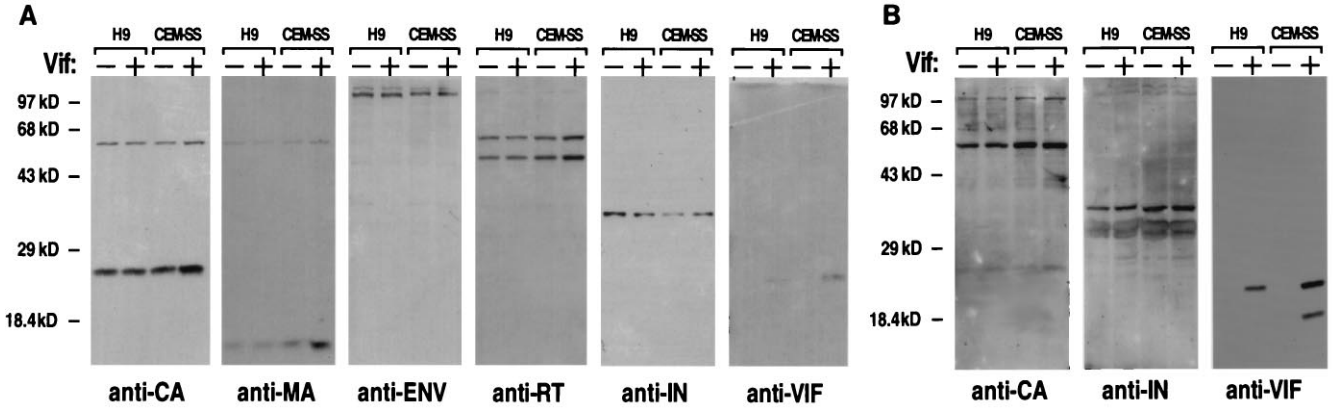

FIG. 1. Western analysis of HIV-1-encoded proteins in purified virions (A) and total cell lysates (B) following transient transfection. Cultures of H9 and CEM-SS cells were electroporated with the pIIIB (wild type) and pIIIB/Δ*vif* expression vectors and analyzed at 24 h. Virions corresponding to ~15 ng of $p24^{Gag}$ or lysates from ~5×10^5 cells were loaded in each lane. Antibodies that specifically recognize the indicated HIV-1 proteins were used for the initial filter hybridizations, and detection was accomplished with horseradish peroxidase-conjugated secondary antibodies, enhanced chemiluminescence, and autoradiography. The positions of prestained protein molecular mass standards are indicated to the left.

TABLE 2. Infectivity of HIV-1 derived by infection

HIV-1 genotype	CAT activity[a] (U)	Fold induction[b]
H9		
Wild type	9,457	130
Δ*vif*	218	
Mock	144	
Jurkat		
Expt 1		
Wild type	16,533	7.0
Δ*vif*	3,075	
Mock	815	
Expt 2		
Wild type	13,775	6.0
Δ*vif*	2,961	
Mock	808	
CEM-SS		
Expt 1		
Wild type	13,339	1.0
Δ*vif*	13,169	
Mock	744	
Expt 2		
Wild type	12,323	1.0
Δ*vif*	12,765	
Mock	381	

[a] Expression was determined ~24 h after challenge of the C8166/HIV-*CAT* cell line.
[b] The induction of infectivity by Vif was determined as for Table 1.

gested that the viral protease's ability to process the *gag* and *gag-pol* gene products is severely impeded in the absence of Vif in nonpermissive cells (5, 21). Thus, in our hands, there does not appear to be any quantitative difference in the Gag, Pol, or Env content of HIV-1 virions that correlates either with the induction of infectivity by Vif or with the phenotype of the producer cell. It was clear, however, that the Vif protein itself was present in the purified preparations of wild-type virus (Fig. 1A, anti-Vif; see below).

To address the possibility that Vif might modulate the patterns of viral protein expression in cells but not in viral particles, we also analyzed the cell pellets of the cultures that were used for virion isolation (Fig. 1B). Western blot analyses using the CA- and IN-specific antibodies revealed that neither Vif nor the producer cells had any discernible effect on the expression of $p55^{Gag}$ or IN. Subsequent analyses of the filters with MA-, RT-, and Env-specific antibodies yielded similar results (data not shown).

Virus produced by infection. We next wished to evaluate Vif's influence on virion composition and protein expression when the proviral DNA was present in T cells as a consequence of viral infection rather than transfection (as in Fig. 1). Wild-type and *vif*-deficient HIV-1 were obtained from acutely infected semipermissive Jurkat cells and fully permissive CEM-SS cells at a time when virus production was at its peak. Because HIV-1/Δ*vif* cannot productively infect nonpermissive H9 cells, an alternative strategy for generating virus in these cells was called for. As described previously (23), we used a culture acutely infected with Δ*vif* virus to deliver a moderate viral titer to H9 cells that harbored either an intact *vif* gene (H9/hVif) or a nonfunctional variant (H9/ΔhVif). Viruses harvested from such H9 cultures were therefore Vif complemented (namely, wild type) or *vif* deficient, respectively. The relative infectivities of all six virus types were determined in the C8166/HIV-*CAT* cell line as before (Table 2). As expected, Vif had the most dramatic impact on infectivity when the viruses were produced in H9 cells (in excess of 100-fold in this experiment). Viruses derived from Jurkat T cells also exhibited a substantial differential in infectivity depending on the presence of Vif (six- to sevenfold), whereas the two viruses from permissive CEM-SS cells displayed equivalent levels of infectivity.

To examine the protein contents of these virions, we initially focused our efforts on viruses propagated in Jurkat and CEM-SS cells. The reason for this was that significant quantities of wild-type and Δ*vif* virus were readily obtained from these cultures (namely, greater than 200 ng of $p24^{Gag}$ per ml at peak virus production). Sucrose gradient-purified virions and corresponding whole cell lysates were subjected to Western analysis using the same panel of HIV-specific antibodies as used for Fig. 2. In absolute agreement with our findings regarding viruses prepared by transient transfection, no differences in the Gag, Pol, or Env protein contents of these viruses that correlated either with Vif expression or the producer cell phenotype could be detected (Fig. 2A). Similarly, the patterns of expression in the cell lysates were comparable in all four infected cultures (Fig. 2B). However, and as before, we were able to find the Vif protein in the wild-type virions. One difference that we did reproducibly notice between virions derived from transfections and viruses obtained from acute infections was that the extent of $p55^{Gag}$ processing was reduced in viruses from transfected cells (compare the anti-CA panels in Fig. 1A and 2A). Importantly, however, this does not correlate with Vif's ability to induce infectivity, as viruses from transfected H9 cells display a greater Vif-mediated stimulation

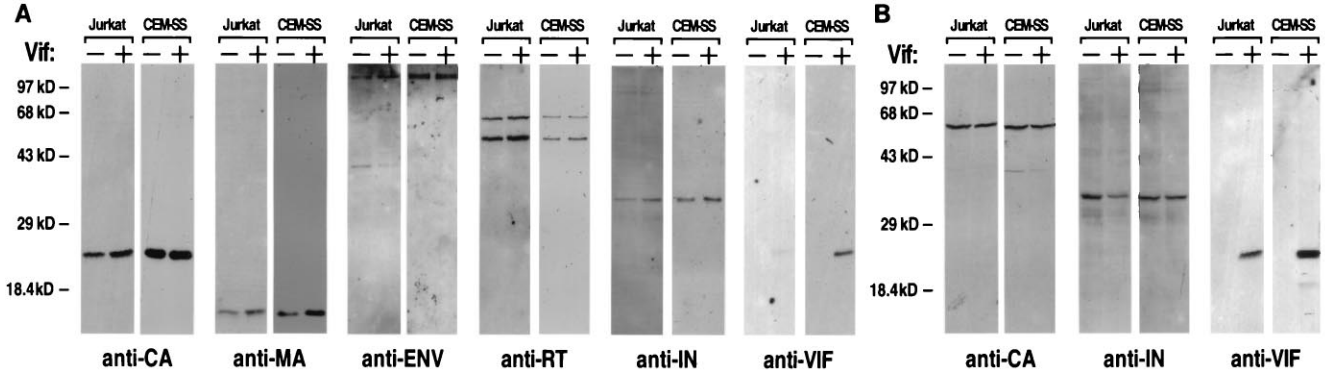

FIG. 2. Western analysis of HIV-1-encoded proteins in purified virions (A) and total cell lysates (B) from acutely infected T cells. Virus and cell samples were collected at the time of peak virus production. Virions corresponding to ~30 ng of $p24^{Gag}$ or lysates from ~5×10^5 cells were loaded in each lane. The detection of viral proteins was performed as for Fig. 1.

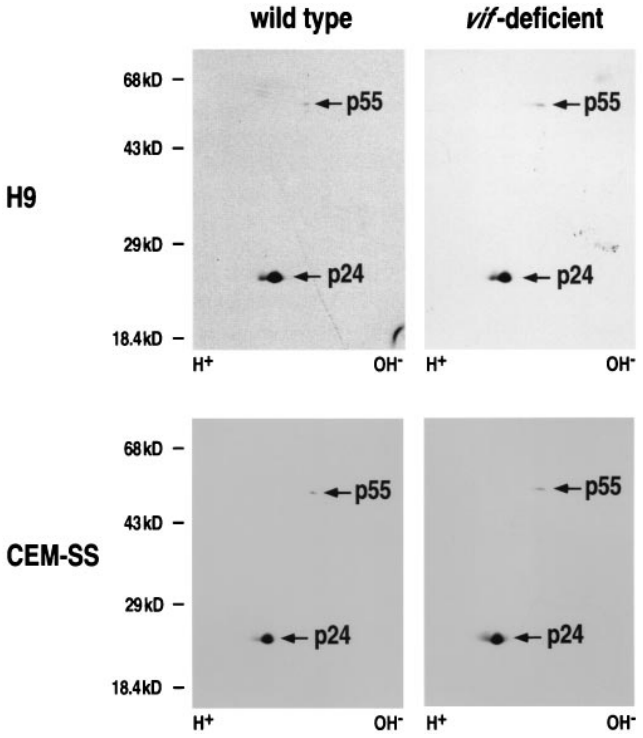

FIG. 3. Two-dimensional gel electrophoresis and Western analysis of $p55^{Gag}$ and $p24^{Gag}$ (CA) in wild-type and *vif*-deficient virions from H9 and CEM-SS T cells. Virions were purified from cultures of infected cells, and samples corresponding to ~5 ng of $p24^{Gag}$ were subjected to NEPHGE followed by SDS-PAGE. Initial hybridization was with a CA-specific monoclonal antibody, and visualization was accomplished as for Fig. 1.

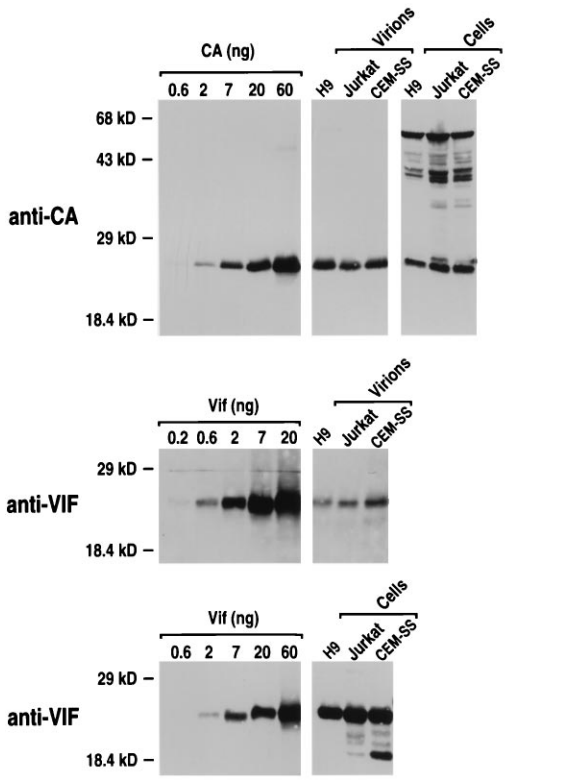

FIG. 4. Quantitation of Gag and Vif in purified HIV-1 virions and infected cell lysates. Virus preparations corresponding to 10 and 40 ng of $p24^{Gag}$ were used for the quantitation of incorporated Gag and Vif proteins, respectively. The test samples were subjected to Western analyses together with standard curves of recombinant proteins purified from *E. coli*. Initial hybridizations used CA- and Vif-specific monoclonal antibodies, and visualization was accomplished as for Fig. 1.

of infectivity than do viruses from infected Jurkat cells despite possessing significantly greater levels of unprocessed $p55^{Gag}$. To confirm that the virion isolation procedure itself did not adversely affect Vif's ability to regulate virus infectivity, C8166/HIV-*CAT* cells were challenged with gradient-purified wild-type and Δ*vif* viruses derived from Jurkat cells. Consistent with what was observed for culture supernatants (Table 2), Vif increased infectivity by sixfold in two separate experiments (data not shown).

As an even more rigorous test of Vif's potential ability to influence $p55^{Gag}$ processing, we also wished to examine the composition of virions produced from single-cycle infections of nonpermissive H9 cells. Rather than using one-dimensional gel systems, we turned to two-dimensional gel electrophoresis for these experiments (Fig. 3). By taking this approach, we could not only evaluate lower quantities of viral material (an unavoidable consequence of the single-step coculture strategy of virus production in H9 cells) but also obtain information regarding potential posttranslational modifications. Purified wild-type and Δ*vif* virions from H9 and CEM-SS cells corresponding to ~5 ng of $p24^{Gag}$ were therefore subjected to isoelectric focusing by NEPHGE and resolution according to molecular mass on SDS–12% polyacrylamide gels. Following transfer to membranes, the $p55^{Gag}$ and CA proteins were detected by hybridization with a CA-specific monoclonal antibody. As can be visualized, all four panels appear to be equivalent; even the small quantity of $p55^{Gag}$ that was detected was apparently the same in each of the virus preparations. Moreover, even though the CA signals (namely, those at ~24 kDa) are consistent with there being isoforms with at least two different isoelectric points, this experiment failed to reveal any Vif-dependent variation in the ratios between these isoforms among these virus samples. Subsequent hybridization of these filters with a pool of all the HIV-specific antibodies used in the assays described above also failed to reveal any differences in the Gag, Pol, or Env contents of these viruses (data not shown).

Quantitation of Vif in virions and infected cell lysates. Using both of our methodologies for the production of HIV-1, we were able to detect Vif in wild-type particles (Fig. 1 and 2). Although these findings are consistent with those of others (5, 17, 18), there remains some discrepancy regarding the average number of Vif molecules that are present per virion. Specifically, copy numbers ranging from 7 to as high as 100 have been recorded (8, 18). As a result, there has been considerable debate concerning the specificity of Vif incorporation and whether incorporation constitutes the difference between wild-type and Δ*vif* virions that renders viruses from nonpermissive cells infectious. Importantly, however, the efficiency of Vif incorporation relative to another virion component, for example, Gag, has yet to be addressed. We therefore decided to calculate the relative abundance of both Vif and Gag in HIV-1 virions as well as in corresponding producer cells.

To accomplish this, purified virions and whole cell lysates were derived from cultures of H9, Jurkat, and CEM-SS cells acutely infected with wild-type HIV-1 and then subjected to semiquantitative Western analyses using monoclonal antibodies specific for Vif and CA (Fig. 4). For each analysis, the

TABLE 3. Quantitation of Gag and Vif in HIV-1 virions and infected cells

Sample	Vif[a] (ng/sample)	Gag[b] (ng/sample)	Vif/Gag ratio[c]	Molecules of Vif/virion[d]
H9				
Virions	0.54	14	1:89	31
Cells	17	21	1:1.1	NA
Jurkat				
Virions	0.67	8.9	1:47	58
Cells	31	47	1:1.3	NA
CEM-SS				
Virions	1.2	12	1:35	78
Cells	37	35	1:0.8	NA

[a] The total amount of protein reactive with the Vif-specific monoclonal antibody was determined for each sample, using the dilution series of recombinant protein as standard curves (Fig. 4), the calculated molecular masses of the native and recombinant proteins, and densitometry.

[b] The total amount of protein (including $p24^{Gag}$, $p55^{Gag}$, and the various processing intermediates) reactive with the CA-specific monoclonal antibody was determined for each sample, using the dilution series of recombinant protein as the standard curve (Fig. 4). Note that the quantities of virions used for the analyses of Gag were only 25% of those used for Vif.

[c] Molar ratios were calculated by using the calculated molecular masses of Vif and CA.

[d] Based on the assumption that there are 2,750 CA monomers per retroviral particle (1). NA, not applicable.

amount of Vif or Gag in a given sample was determined by reference to a standard curve generated by using recombinant protein purified from bacteria. From these values, the ratios of Vif to Gag for each viral and cell sample as well as the average number of Vif molecules per virion were calculated (Table 3). In agreement with our earlier results (Fig. 1 and 2), Vif could readily be detected in preparations of wild-type virus. Moreover, the extents of Vif incorporation were similar for the three producer cell lines, ranging from 31 to 78 molecules per virion (Table 3). The unexpected result, however, was that the cell-associated expression levels of Vif were so high (Fig. 4, lower panels). In fact, the levels of expression of Gag (p55, CA, and all processing intermediates) and Vif in total cell lysates from these infected cultures appear, on a molar basis, to be approximately equivalent (Table 3). Given this level of abundance of Vif and the relatively low amounts that are detected in viral particles compared with Gag, it is tempting to speculate that Vif's incorporation into virions might be governed by stochastic events rather than by specific packaging.

DISCUSSION

In this report, we demonstrate that the HIV-1 Vif protein has no detectable impact, either qualitatively or quantitatively, on the *gag*-, *pol*-, or *env*-encoded contents of viral particles that correlates with its ability to stimulate virus infectivity (Fig. 1 to 3). We also report that the cell-associated expression patterns of Gag, Pol, and Env do not appear to be influenced by the presence of Vif. Of particular importance to these studies was our analysis of viruses derived not only by transient transfection (Fig. 1) but also by infection (Fig. 2 and 3). In addition, we used cells that are nonpermissive (H9 and Jurkat) as well as permissive (CEM-SS) to efficient HIV-1/Δ*vif* replication as sources of virus. It was our hope that by using this variety of expression strategies and producer cell types, we might be able to address some of the discrepancies that currently exist concerning Vif's effect(s) on virion composition.

Our findings confirm and extend those of von Schwedler et al. (29) but stand in contrast to those of others who have reported that Vif enhances the processing of $p55^{Gag}$ (5, 21) and increases the incorporation of $gp160/120^{Env}$ into virions (5, 20). At this time, we cannot offer reasonable hypotheses to explain the differences between these results and our own. However, the fact that we used more than one experimental approach to address this issue leads us to believe that Vif does not affect the expression, processing, or virion incorporation of the Gag, Pol, or Env protein in a manner that can explain its capacity to modulate infectivity. One difference between our experimental systems and those of other groups who have described Vif-dependent differences in $p55^{Gag}$ processing is that virus production was equivalent in our Vif-expressing and *vif*-deficient samples at the time of analysis. This contrasts with the studies of others, in which the levels of virus gene expression were substantially diminished in the Δ*vif* samples (21), though why this might affect the activity of the viral protease is open to speculation. In some of our experiments, we were able to observe a slight diminution of $p55^{Gag}$ processing in Δ*vif* infections (data not shown). However, these experiments were very unsatisfactory as the extents of infection were difficult to normalize between the wild-type and Δ*vif* samples. Not only had virus production progressed well past its peak, but the cultures also displayed marked cytopathic effects; it is also conceivable that this could have resulted in the release of some immature particles into the culture medium. Importantly, it is unlikely that this latter finding has any relevance to Vif function since it was equally apparent in permissive and semipermissive cells.

The sole difference between wild-type and *vif*-deficient virus that we could detect was that the Vif protein itself was present in wild-type virions (Fig. 1, 2, and 4). This conclusion is consistent with those of others (5, 8, 17, 18), and our calculation that there are approximately 30 to 80 molecules of Vif per virion (Table 3) is in good agreement with those of Liu et al. (18) and Camaur and Trono (8), who have reported that there are 60 to 100 and 7 to 20 molecules of Vif per virion, respectively. In the latter case, the number of Gag molecules per virion was assumed to be 1,500 rather than 2,750; use of this copy number would have raised the abundance of Vif to 13 to 37 molecules per virion. A novel and unexpected finding was, however, that Vif is expressed at such high levels in the HIV-1-infected cells themselves (Table 3). Indeed, our calculations reveal that the cell-associated concentrations of Vif are approximately equivalent to those of the Gag protein. In light of this level of abundance and the observation that Vif and Gag colocalize in these infected T cell cultures (22), it is perhaps not surprising that a certain amount of Vif becomes incorporated into budding virions. Thus, it is possible that Vif packaging is an unavoidable consequence of expression level together with subcellular localization and may not be driven by a specific packaging process. This hypothesis would be consistent with the recent finding that HIV-1 Vif can also be incorporated into the budding virions of a retrovirus, murine leukemia virus, that does not carry a *vif* gene (8). Ultimate resolution of this issue will presumably require the identification of Vif mutants in which virion incorporation and biological activity no longer cosegregate.

Our current understanding of Vif function is that its expression in virus-producing cells confers released virions with the ability to complete DNA synthesis and establish proviruses during the next round of infection. Moreover, recent data have suggested that this may be accomplished via a Vif-mediated stabilization of penetrating viral nucleocapsids. To achieve this end result, Vif could conceivably function in producer cells, cell-free virions, postentry viral structures, or some combination of these three. The abundance of Vif in producer cells is suggestive of a structural role, perhaps in determining the

conformation of Gag monomers as they assemble into viral structures, as opposed to an enzymatic function. Notably, this would be consistent with the abnormalities in virus core ultrastructure that have been described for *vif*-deficient viruses derived from nonpermissive cells (5, 16). It is also possible that Vif could exert a structural-conformational effect on virions or penetrated nucleocapsids; however, the relatively low levels of Vif in virions compared with producer cells might argue against this conjecture.

In summary, the only compositional difference between wild-type and *vif*-deficient HIV-1 particles that we have been able to detect is that Vif itself is present in wild-type virions. If, as we have speculated, Vif subserves a structural role during virus assembly that has conformational consequences for Gag and released virions, then the identification of relevant interacting partners (viral as well as cellular) will likely be important for elucidating its precise function.

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