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Human Immunodeficiency Virus Type 1 Vif⁻ Mutant Particles from Restrictive Cells: Role of Vif in Correct Particle Assembly and Infectivity

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Disruption of the *vif* gene of human immunodeficiency virus (HIV) type 1 affects virus infectivity to various degrees, depending on the T-cell line used. We have concentrated our studies on true phenotypic Vif⁻ mutant particles produced from CEMx174 or H9 cells. In a single round of infection, Vif⁻ virus is approximately 25 (from CEMx174 cells) to 100 (from H9 cells) times less infectious than wild-type virus produced from these cells or than the Vif⁻ mutant produced from HeLa cells. Vif⁻ virions recovered from restrictive cells, but not from permissive cells, are abnormal both in terms of morphology and viral protein content. Notably, they contain much reduced quantities of envelope proteins and altered quantities of Gag and Pol proteins. Although wild-type and Vif⁻ virions from restrictive cells contain similar quantities of viral RNA, no viral DNA synthesis was detectable after acute infection of target cells with phenotypically Vif⁻ virions. To examine the possible role of Vif in viral entry, attempts were made to rescue the Vif⁻ defect in H9 cells by pseudotyping Vif⁺ and Vif⁻ HIV particles with amphotropic murine leukemia virus envelope. Vif⁻ particles produced in the presence of HIV envelope could not be propagated when pseudotyped. In contrast, when only the murine leukemia virus envelope was present, significant propagation of Vif⁻ HIV particles could be detected. These results demonstrate that Vif is required for proper assembly of the viral particle and for efficient HIV Env-mediated infection of target cells.

The conservation of the *vif* gene sequence between different human immunodeficiency virus type 1 (HIV-1) isolates and the presence of open reading frames with similar coding potentials in many primate and nonprimate lentiviruses (4, 18, 24, 30, 37) suggest that the Vif protein has an important function. The *vif* open reading frame of HIV-1 encodes a protein of approximately 23 kDa (20). Vif is expressed from a singly spliced viral mRNA in a Rev-dependent manner (13, 28) late in the infectious cycle (21). Vif is apparently located in close proximity to the internal cell surface in infected cells (15). Höglund et al. recently demonstrated that Vif⁻ mutants of HIV-1 possess an abnormally assembled or matured viral core (19), suggesting that Vif functions late in the infectious cycle. However, no molecular basis was presented for this morphological defect.

The presence of Vif has been reported to increase the infectivity of viral particles by up to 1,000-fold (10, 22, 33). However, the role of the Vif protein in infection remains largely unknown. In vitro, mutation of the *vif* gene gives dramatically different effects according to the cell line used (11, 26). In restrictive cells (peripheral blood lymphocytes, macrophages, or H9 cells), Vif is essential for viral replication (3, 11, 26, 36). Conversely, no Vif⁻ defect is apparent when virus is produced in permissive cells (HeLa, COS, or M8166 cells) (11, 26). Furthermore, there exist a number of semipermissive (to different degrees) cell lines (SupT1 or some CEM cells) in which Vif⁻ mutants are able to replicate to intermediate levels (11, 26, 31, 36). It has been demonstrated that the Vif defect is conferred upon the virus by the producer cell (11, 36). Hence, a Vif defect can be rescued by coexpression of Vif in the producer cell but not by coexpression of Vif in the target cell (36). Thus, a phenotypically Vif⁻ virus produced in a restrictive cell is unable to replicate in any target cell, but a Vif⁻ virus produced in a totally permissive cell replicates with apparently wild-type (WT) efficiency in a single cycle in all cells. All recent reports are in agreement concerning the existence of permissive, semipermissive, and restrictive cell lines for HIV-1 Vif mutants. However, varied results have been obtained in different laboratories when similar cell lines are infected with Vif⁻ virus (3, 26, 31, 36), suggesting that apparently similar T-cell lines might differ greatly in terms of permissivity. This difficulty in defining which cell lines are truly restrictive is possibly one of the reasons why numerous different functions have been suggested for Vif. Aside from a possible role in particle assembly as described above, it has been proposed that Vif is required for the completion of reverse transcription (36), the incorporation of envelope proteins (26), a posttranslation modification of gp41 (17), or the efficient internalization of the viral core (31).

We have studied the effects of a *vif* deletion mutation on the replication of HIV-1 virions produced from truly restrictive cells, using a coculture procedure to produce large quantities of WT and Vif⁻ virus from such cells. Vif⁻ virions produced from restrictive cells have a greatly reduced infectivity and display an abnormal morphology and viral protein content. These abnormal virions were shown to be unable to initiate infection in target cells. The Vif⁻ defect could be partially rescued by pseudotyping with amphotropic murine leukemia virus (MuLV) envelope, but only when HIV-1 Env was absent. These results demonstrate that the block to Vif⁻ viral infection in restrictive cells results from an assembly or maturation de-

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fect and is first detected as a failure of mutant virus to initiate infection in new target cells.

MATERIALS AND METHODS

DNA constructions. The WT virus used in these experiments was pNL43 (1). This infectious provirus has previously been shown to express all known HIV-1 proteins (references 1, 2, 33, and 34 and references therein). The Vif⁻ mutant, which contains a deletion between the upstream *Nde*I and *Pfl*MI sites of pNL43, was generated by K. Strebel. Env⁻ derivatives of the WT and Vif⁻ plasmids were generated by deletion of the 1.3-kb *Kpn*I-*Bgl*II fragment in HIV-1 *env*. Plasmid pEGA, which contains the gibbon ape leukemia virus envelope under the control of the Friend MuLV promoter, was generated by J.-L. Battini. For the construction of the Vif expression vectors p205Vif and p205Vif.inv, the 1.6-kb *Bgl*II fragment of pNLA3 (33) containing the *vif* gene was inserted in each orientation behind an inducible metallothionein promoter in a modified p205 plasmid (35). The *vif* gene was in the sense and antisense orientations (with respect to the promoter) in plasmids p205Vif and p205Vif.inv, respectively.

Cells. HeLa cells were maintained as subconfluent monolayers in Dulbecco's modified minimal essential medium (DMEM) with 5% fetal calf serum. CEMx174, H9, SupT1, and MT4 T-lymphoid cell lines were maintained in RPMI 1640 supplemented with 10% fetal calf serum. H9-Ampho cells were generated by coculture of uninfected H9 cells with amphotropic MuLV (4070A strain)-infected NIH 3T3 cells for 3 months in RPMI 1640 with Polybrene (4 μg/ml). When H9 cells were deemed to be chronically infected (no syncytium formation without addition of fresh H9 cells, and high reverse transcriptase [RT] activity), they were removed from the coculture, selected for nonadherence, and cultured alone in RPMI 1640 with Polybrene. The P4 indicator cell line (HeLa CD4 LTR-LacZ) were cultured in DMEM with 5% fetal calf serum and 500 μg of G418 per ml as described previously (5). The P4Vif and P4Vif.inv cells lines were generated by transfection of P4 cells with p205Vif and p205Vif.inv, respectively. Transfected cells were selected in DMEM with 5% fetal calf serum and hygromycin (300 μg/ml). Expression of the *vif* gene in each orientation was achieved by the addition of Zn and Cd (100 and 1 μM, respectively) 36 h prior to infections.

Infections. WT and Vif⁻ viral stocks were produced from HeLa cells after transfection using a standard calcium phosphate protocol and were quantified for particle-associated HIV-1 p24 antigen by enzyme-linked immunosorbent assay (ELISA) (Dupont) of viral pellets produced by ultracentrifugation of filtered supernatants (see below). To determine the effect of Vif mutations on HIV replication in various T-cell lines, T cells were infected with filtered cell-free transfection supernatants containing equal quantities of WT and Vif⁻ viral particle-associated p24. At 2- or 3-day intervals, samples of cell-free supernatant from infections were assessed for virus content by p24 ELISA and stored frozen. For the analyses of specific infectivity and of viral protein or RNA content, aliquots from equivalent times after infection (in terms of virus production and the severity of infection of the bulk culture) were used. The P4 indicator cells were infected with various quantities of particle-associated HIV p24 in the presence of 20 μg of DEAE-dextran per ml for 24 h. Cells were then washed, fixed, and colored with 5-bromo-4-chloro-3-β-D-galactopyranoside (X-Gal) as described previously (5). Infectivity was scored as the number of blue cells per nanogram of infecting p24.

Transient cocultures of transfected HeLa cells with T cells. To produce concentrated WT and Vif⁻ viral stocks from T-cell lines, uninfected T cells (approximately 1:1) were added to transfected HeLa cells 24 h posttransfection. After 24 h of coculture, T cells were removed, washed extensively, and cultured alone. Viral supernatants were collected 24 or 48 h later. All viral stocks were quantified for particle-associated p24 antigen by ELISA and for specific infectivity by using P4 indicator cells as described above.

Western blot (immunoblot) analysis of viral proteins. Viral particle-associated proteins were prepared from filtered cell-free supernatants by ultracentrifugation at 100,000 rpm (TL100; Beckman) for 10 min through a 10% (wt/vol) sucrose cushion. Viral pellets were resuspended in phosphate-buffered saline (PBS) and quantified for HIV p24 antigen by ELISA and for RT activity (38). For Western blot analysis, equal quantities of p24-containing virus (quantified by HIV p24 ELISA) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 12% gels. Transfer to Hybond C super (Amersham) was performed at 180 mA for 2 h, using a semidry blotting apparatus (Milliblot SDE; Millipore). Membranes were blocked in PBS with 5% (wt/vol) dried milk prior to exposure to serum from HIV-infected patients. For detection of nonstructural viral proteins in infected cell lysates, mono- or polyclonal antisera were used. Mouse anti-Vif antibodies were a gift of K. Strebel; monoclonal anti-gp120 antibodies [clone MN-A(F5)] were from Hybridolab, Pasteur; rabbit anti-Nef was a gift of O. Schwartz; and rat anti-Vpr was a gift of J.-L. Virelizier. Blots were developed by enhanced chemiluminescence (ECL Western blotting kit; Amersham) after incubation with the appropriate horseradish peroxidase-linked secondary antibody. Mock-infected cell lysates and supernatants were analyzed in parallel in all experiments.

For the detection of Vif protein in infected cell lysates and virions, pelleted virus produced from a known quantity of CEMx174 cells was analyzed alongside infected cell extracts by Western blotting. Blots were probed with monoclonal mouse anti-Vif antibodies and were developed by enhanced chemiluminescence.

Quantification of virion-associated viral RNA by RT-PCR. Equal quantities (by HIV p24 ELISA of ultracentrifuged virus) of WT or Vif⁻ virions produced at equivalent stages after infection of CEMx174 cells (see above) were analyzed for viral RNA content as follows. RNA was extracted from viral pellets as instructed for the RNA isolation kit (Stratagene), and extracted RNA was resuspended in a volume of water (in microliters) equal to the original concentration of virus used (in nanograms of p24). Different volumes of extracted RNA were reverse transcribed in 20-μl aliquots, using 10 U avian myeloblastosis virus RT (Promega) and 0.5 μg of antisense primer (oligonucleotide 1070) [CCGCT TAATACCG ACGCTCTCGCAC, nucleotides {nt} 818 to 793). After preheating of the reaction mixture to 65°C for 5 min, reverse transcription was carried out at 50°C for 25 min. Aliquots of 2 μl of the final reaction mixture were then added to PCR mixtures containing the antisense 1070 and sense Sac (TGAGC CTGGGAGCTCTCTGGCTAACTAG, nt 477 to 505) oligonucleotides. PCR was performed for 30 cycles of 45 s at 92°C, 45 at 52°C, and 1 min at 72°C. PCR products were phenol-chloroform extracted and analyzed by agarose gel electrophoresis. Detection of virus-specific PCR products was performed by hybridization of Southern-blotted DNAs with the ^{32}P-labeled internal NARS oligonucleotide (CTCTAGCAGTGGCGCCCGAACAGGG, nt 627 to 651). Negative controls were performed with equivalent quantities of ultracentrifuged supernatant from mock-infected cells and with viral RNA which had not been reverse transcribed prior to PCRs.

PCR assays for determining the efficiency of HIV DNA synthesis in newly infected cells. The method was essentially that described by others (12). Equal quantities of WT and Vif⁻ particles (in terms of particle-associated p24 antigen) purified at equivalent stages from infected CEMx174 cells were used to infect P4 cells. After 30 min on ice, cells were washed to remove unbound virus (time zero), and were incubated at 37°C. At various times after infection, cells were recovered by trypsinization and washed first in medium and then in PBS, and total cellular DNA was extracted as described previously (12). PCR amplification of viral DNA was performed either with the Sac and 1070 oligonucleotides described above or with Sac and U5AS (TAGAGATTTTCCACACTGACTAA, nt 633 to 609) oligonucleotides, with comparable quantities of WT- and Vif⁻-infected cell DNA. Hybridization of Southern blots after electrophoresis and membrane transfer of purified PCR products was performed with the radiolabeled internal NARS oligonucleotide in the case of the Sac-1070 primer pair. For PCR products generated with the Sac and U5AS primers, specificity of PCR products was assessed by restriction endonuclease mapping of purified PCR products.

Quantification of virus production in a single infectious cycle in restrictive cells. CEMx174 cells (6×10^6) were infected with WT or Vif⁻ virus produced from infections of MT4 cells. Infections were with 850 and 1,200 ng of particle-associated p24 for WT and Vif⁻ viruses, respectively, to ensure infection with equal quantities of infectious virus (as Vif⁻ virus stocks from MT4 cells showed approximately 70% of the infectivity of WT stocks from the same cells; see Results). Cells were incubated with virus for 2 h at 37°C and were then washed twice with PBS containing 0.1% trypsin (0 h postinfection). At 2 h postinfection, cells were rewashed twice with PBS-trypsin, divided into three equal volumes (cultures A, B, and C), and then cultured in RPMI 1640 as described above. Four hours postinfection, culture A of the WT- and Vif⁻-infected cells was centrifuged at low speed, and the cell-free supernatant was filtered and centrifuged as described above for the analysis of virion-associated proteins by Western blotting. Zidovudine (5 μM, final concentration) was added to cultures B and C at 10 and 30 h postinfection, respectively. Thus, the infections in culture B should be limited to a single cycle, whereas more than one cycle should occur in culture C. At 24 and 48 h postinfection, viral particles were harvested from cultures B and C, respectively. Equal cell equivalents of viral particle preparations from each culture were analyzed for HIV p24 antigen and by Western blotting (see above).

Electron microscopy of WT and Vif⁻ viral particles. For the analysis of virus particle morphology for WT and Vif⁻ virus preparations, infections were initiated in MT4, H9, or CEMx174 cells by coculture with transfected HeLa cells. Cocultured T cells were prepared for electron microscopy as described previously (25) 24 h after the end of coculture.

Pseudotyping experiments. For pseudotyping experiments, HeLa cells were transfected with WT, Vif⁻, Vif⁺ Env⁻, or Vif⁻ Env⁻ DNAs in the presence of plasmid pEGA. Coculture of transfected HeLa cells was performed as described above, using H9-Ampho cells as recipients. Infections initiated in H9-Ampho cells by coculture were normalized for HIV p24 antigen production, and spreading infections were monitored with the same assay. Equal numbers of uninfected H9 cells were added to the cultures at 3-day intervals. It should be noted that no infection was detectable in either the Vif⁺ Env⁻ or Vif⁻ Env⁻ cocultures if uninfected H9 cells were not added at regular intervals.

RESULTS

The effect of Vif mutations is producer cell type dependent. Given the large number of conflicting reports regarding proposed functions for the Vif protein of HIV-1, we initially

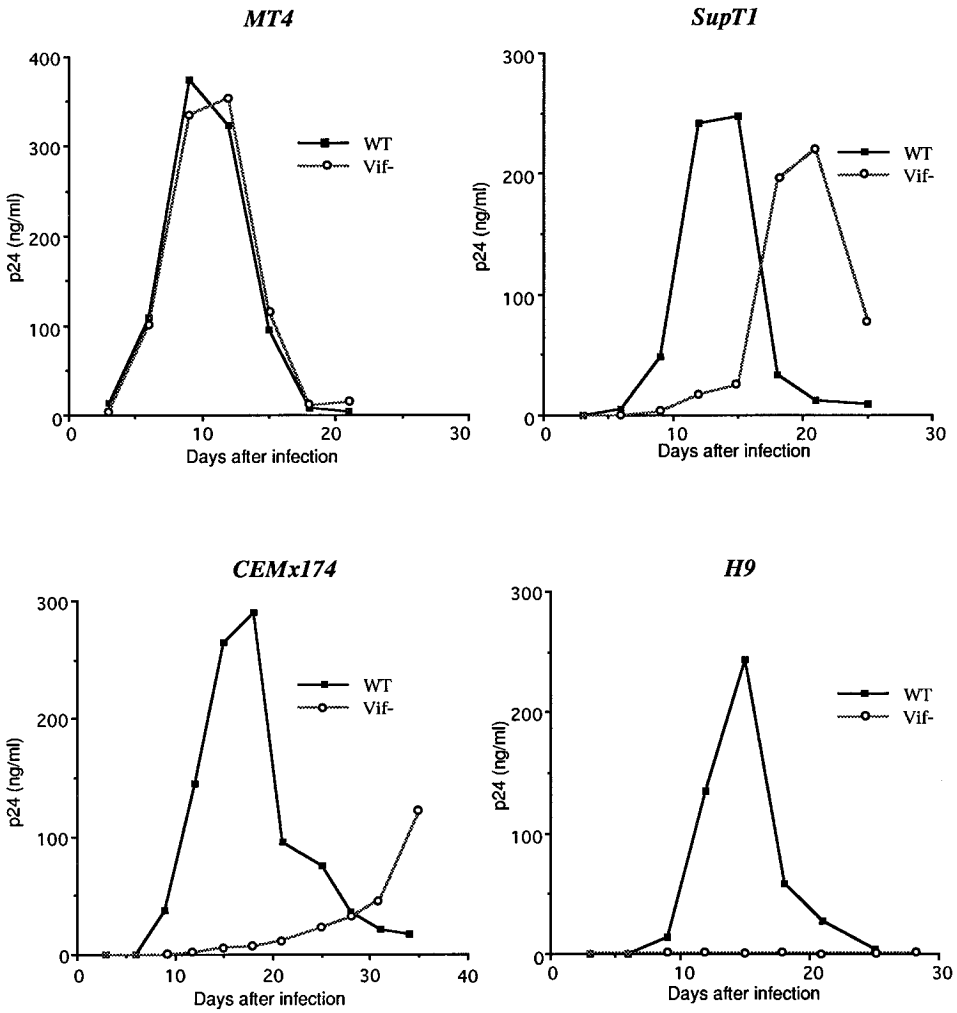

FIG. 1. The effect of Vif mutations is cell dependent. Continuous T-cell lines (as indicated) were infected with cell-free supernatants from HeLa cells transfected with WT and Vif$^-$ proviral clones. Infections were commenced with 20 ng of particle-associated p24 per 2×10^6 cells and were monitored for HIV p24 antigen production as described in Materials and Methods.

wished to analyze the effects of a Vif$^-$ deletion mutation in the various continuous T-cell lines available to us. The Vif$^-$ mutant used for these studies contains an extensive deletion in the *vif* open reading frame, thus reducing the possibility of primary-site reversion events during repeated passages. WT and Vif$^-$ viruses were generated by transfection of HeLa cells and were used to infect various transformed T-cell lines at relatively low virus input (usually 20 ng of particle-associated p24 per 2×10^6 cells). In agreement with previous reports (11, 26, 31, 36), T-cell lines could be classified into three categories according to the ability to support Vif$^-$ virus infection (Fig. 1). In permissive cells (MT4), Vif$^-$ virus replication was indistinguishable from that of WT virus in terms of peak p24 production and kinetics of infection. In cells which were semipermissive (SupT1 and CEMx174 cells), there was a marked delay in the emergence of detectable Vif$^-$ virus replication with respect to the WT virus. It should be noted that the delay in emergence of Vif$^-$ viral replication was much greater in CEMx174 cells than in SupT1 cells. Finally, in truly restrictive cells (peripheral blood lymphocytes [data not shown] and H9 cells [Fig. 1]), a peak of Vif$^-$ virus replication could not be detected even after several months in culture.

The delayed kinetics of virus emergence in the studies described above reflect an effect which is acting over many infectious cycles. To quantitate the severity of the Vif$^-$ defect, we studied virus produced during a single cycle of infection in T-cell lines. By using a system of transient coculture of various T-cell lines with transfected HeLa cells, it was possible to initiate a single massive round of infection even in totally restrictive T cells (see Materials and Methods; Fig. 2A). With WT virus, coculture leads to a spreading productive infection after several days, whereas Vif$^-$ virus infection of H9 cells is transient, again demonstrating the restrictive nature of H9 cells.

Virus produced from the cocultured T-cell lines was assayed for specific infectivity over a single infectious cycle in the P4 indicator cell line. Initially, to confirm the validity of this coculture approach, WT and Vif$^-$ viruses from transfected HeLa cells were compared for infectivity (Fig. 2B). Vif$^-$ particles from HeLa cells demonstrated approximately 80% of the infectivity of the WT preparation, confirming that Vif$^-$ virus from this source could be used to initiate infection efficiently. The results shown in Fig. 2B confirm previous reports which indicated that the Vif$^-$ phenotype is conferred by the producer

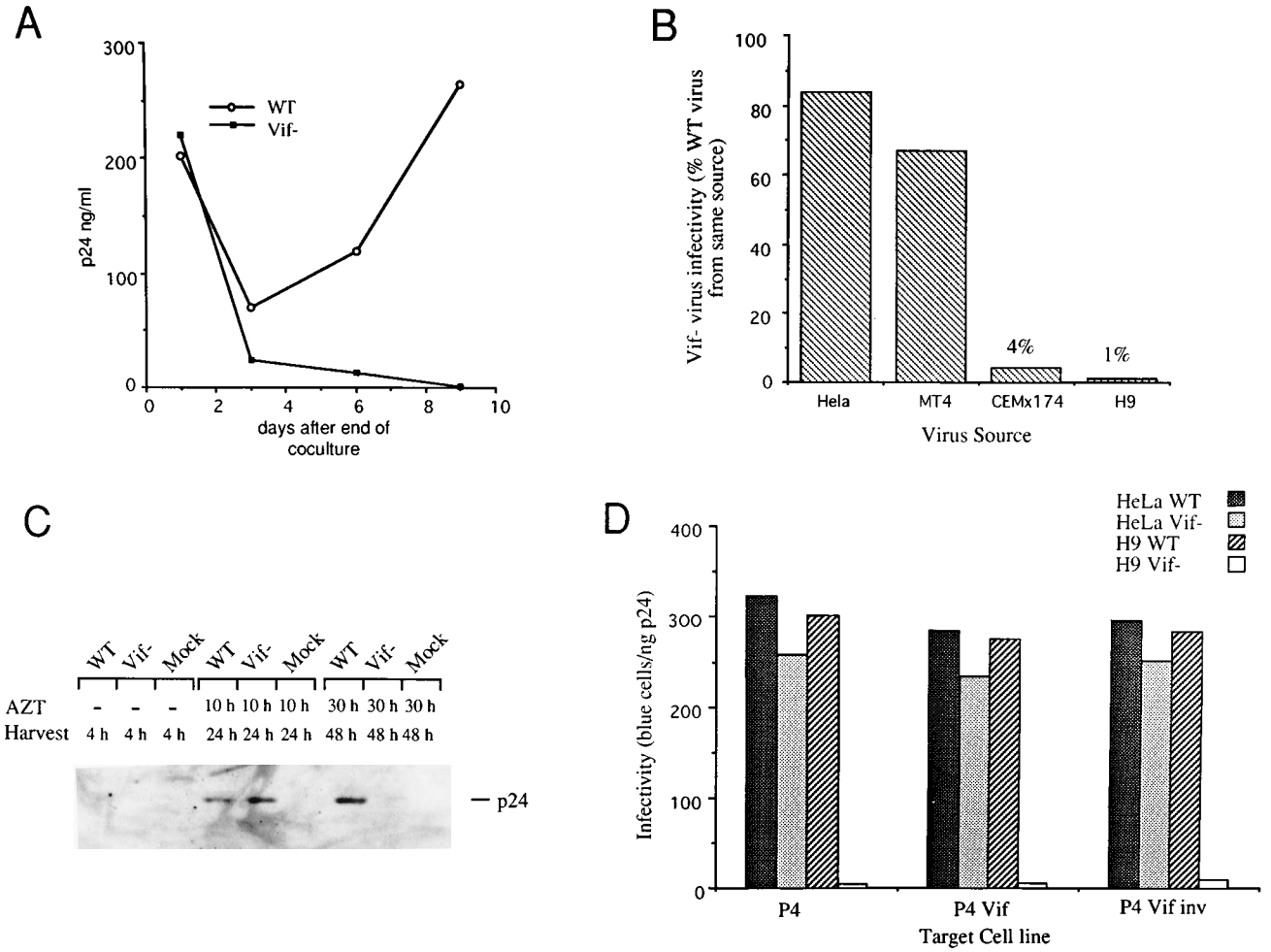

FIG. 2. The Vif defect is conferred by the producer cell. (A) Transient coculture of H9 cells with transfected HeLa cells. Subconfluent HeLa cells monolayers were transfected with WT or Vif⁻ proviral clones and were cocultured 24 h after transfection with uninfected H9 cells as described in Materials and Methods. After 24 h of coculture, H9 cells were removed, washed, and cultured alone. HIV p24 antigen in cell-free supernatants of the transfected HeLa cells and the H9 cells after coculture was quantified by p24 ELISA. For the experiment described, the transfected HeLa cells produced 160 (WT) and 195 (Vif⁻) ng/ml of particle-associated p24 in the 24 h prior to coculture. (B) WT and Vif⁻ viral stocks were produced after transfection of HeLa cells with WT and Vif⁻ provirus clones or after transient coculture of transfected HeLa cells with various T-cell lines (see Materials and Methods; panel A). Particle-associated HIV p24 was quantified for each viral stock, and p24 equivalents were used to infect P4 indicator cells. Twenty-four hours after infection, cells were fixed and stained with X-Gal. Infectivity was scored as blue cells per nanogram of p24. The graph shows relative infectivity of Vif⁻ viral stocks compared with the infectivity of WT virus produced in the same experiment from the same cell source. (C) Similar quantities of WT and Vif⁻ viral particles are produced after a single infectious cycle in CEMx174 cells. Quantification of virus production after a single infectious cycle was performed exactly as described in Materials and Methods. A typical autoradiograph of a Western blot analysis is shown. Time (hours after infection) of addition of zidovudine and harvesting of viral particles are given above each lane. The position of the p24 protein is shown. Mock-infected cell supernatants were analyzed in parallel. For the data shown, p24 antigen concentrations (as detected by ELISA of ultracentrifuged samples prior to electrophoresis) were less than 0.1 ng/ml at 4 h, 3.3 (WT) and 3.5 (Vif⁻) ng/ml at 24 h, and 7.8 (WT) and 1.6 (Vif⁻) ng/ml at 48 h. (D) The WT and Vif⁻ viral stocks produced from HeLa cells and H9 cells (as for the experiments described in panel A) were used to infect P4, P4Vif (expressing the *vif* gene in the sense orientation), or P4Vif.inv (expressing the *vif* gene in the antisense orientation) cells. Infectivity was scored as blue cells per nanogram of p24 as described for panel B. Similar values for infectivity were achieved whether the expression of *vif* in the target cell was induced with Zn and Cd (D) or not (data not shown).

cell (11, 26, 36). A relatively minor defect was seen with the mutant virus produced in either HeLa cells or MT4 cells (Fig. 2B), and an intermediate defect was observed with SupT1 cells (data not shown; relative infectivity approximately 40% of the WT level). However, Vif⁻ virus from CEMx174 and H9 cells showed a severe replicative defect in the single-round assay (25- and 100-fold less infectious, respectively, than the WT virus from the corresponding cells).

The greatly reduced infectious titer obtained with H9 and CEMx174 cells confirmed that in our coculture system, T-cell virus was not significantly contaminated with HeLa cell virus. Nevertheless, we found that similar quantities of WT and Vif⁻ viruses (in terms of p24 antigen released into the supernatants) could be produced from H9 cells in the 48 h following coculture, indicating that massive first-round infection of the H9 cells had occurred. To further examine the question of single-cycle production of Vif⁻ virus from restrictive cells, CEMx174 cells were infected with large, equivalent titers of WT and Vif⁻ viruses from permissive MT4 cells. By adding inhibitory concentrations of zidovudine at various times after infection, the CEMx174 cell infections could be limited to one or more than one infectious cycle. The results presented in Fig. 2C demonstrate that similar quantities of Vif⁻ and WT virions, in terms of particle-associated p24 (detected either by ELISA or Western blotting), were released from CEMx174 cells during a single infectious cycle. The reduction in recoverable particle-

associated p24 in the Vif$^-$ cultures between 24 and 48 h postinfection was reproducible in several independent experiments and probably reflects degradation of the noninfectious Vif$^-$ particles released after the first cycle of infection. This reduction again demonstrates that Vif$^-$ virions produced from CEMx174 cells are unable to efficiently initiate subsequent cycles of infection. The severe replicative defect which we obtained with Vif$^-$ virus from CEMx174 cells stresses the importance of independently controlling the relative permissivity of all cell lines used for the study of Vif$^-$ mutants. Clearly, the CEMx174 cell line, which is a somatic cell hybrid between a B- and a T-cell line (27), is more restrictive than other CEM cell lines (3, 31) in terms of the propagation of Vif$^-$ viruses. For these studies, only Vif$^-$ viruses produced from H9 or CEMx174 cells were considered to be sufficiently phenotypically Vif$^-$ to allow further analysis.

Next, the ability to complement the Vif$^-$ defect in the target cell was examined. Phenotypic Vif$^-$ virus and WT virus (both produced from H9 cells 24 h after transient coculture) were used to infect P4 cells expressing the *vif* gene in either the sense or antisense orientation. After 24 h of infection, cells were fixed and colored with X-Gal as described in Materials and Methods. As shown in Fig. 2D, the ability of both WT and Vif$^-$ viruses to complete a single cycle infection (up to and including the expression of Tat) was unchanged by the presence of Vif protein in the target cell (compare H9 WT and H9 Vif$^-$ columns for the three P4 cell lines; Fig. 2D). A reduction in single-cycle infectivity of the order of 100-fold was observed for the Vif$^-$ mutant even in P4 cells expressing the *vif* gene. That these cells were indeed synthesizing Vif was confirmed by Western blot analysis of cell extracts with and without Zn-Cd induction (data not shown). Thus, in agreement with previous reports, rescue of the Vif$^-$ defect is not possible by expression of the *vif* gene in the target cell.

Phenotypic Vif$^-$ virus particles have an abnormal protein composition and virion morphology. The Vif$^-$ phenotype conferred by H9 or CEMx174 cells apparently does not reflect a reduction in the level of viral production. As stated above, approximately equal quantities of total and particle-associated p24 were found for the WT and Vif$^-$ viruses after a single infectious cycle in restrictive cells (Fig. 2C). Thus, it seems that normal quantities of virus are released after a single cycle of Vif$^-$ mutant infection in restrictive cells, but that the virions released are noninfectious. The composition of such phenotypic Vif$^-$ particles was thus compared with that of the WT particles produced from the same type of cells. For these experiments, WT and Vif$^-$ viruses from H9, CEMx174, and MT4 cells were used. Infections were initiated in CEMx174 and MT4 cells by electroporation with either the WT or Vif$^-$ proviral clone and in H9 cells by transient coculture with transfected HeLa cells. Samples of filtered cell-free supernatant were assayed for HIV p24 antigen at 3-day intervals throughout CEMx174 and MT4 cell infections and were stored frozen. This was necessary to allow the analysis of samples from equivalent stages in the WT and Vif$^-$ infections (in terms of both virus production and cytopathic effects detectable in the infected cultures), given that Vif$^-$ virus replication is much delayed relative to that of WT virus, at least in CEMx174 cell infections. For H9 cell cocultures, WT and Vif$^-$ viruses were collected 24 h after the end of coculture.

Previous studies have failed to detect major differences in viral protein content between WT and Vif$^-$ virions (11, 36). Here we found that, in agreement with a previous report (26), Vif$^-$ virions from restrictive cells apparently possess much reduced quantities of envelope glycoproteins gp120 and gp41. WT levels of Env proteins were not detected even in preparations of freshly budded Vif$^-$ particles (data not shown). However, Env was apparently expressed to WT levels at the surface of Vif$^-$ mutant-infected CEMx174 cells (as analyzed by FACScan analysis with anti-gp120 antibodies or by Western analysis of infected cell lysates; Fig. 3B). This finding suggests that the lack of Env on Vif$^-$ particles might reflect a problem of incor-

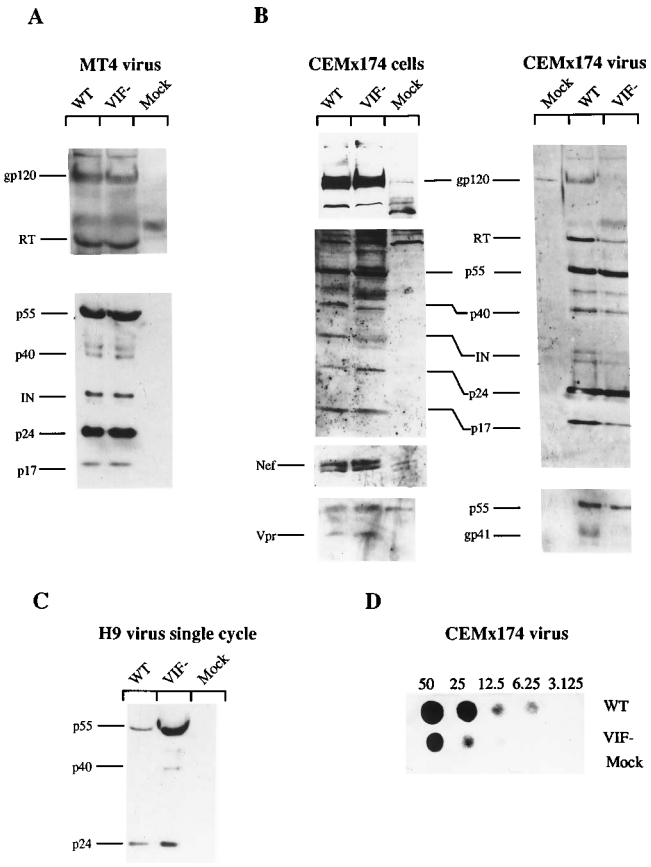

FIG. 3. Vif$^-$ particles from restrictive cells are abnormal in terms of viral protein content. (A to C) Western blot analyses of WT and Vif$^-$ virion-associated proteins. Viral particles were produced from either MT4 (A), CEMx174 (B, right), or H9 (C) cells. Equal quantities of particle-associated p24 antigen, as assessed by ELISA (A and B), or samples purified from equal volumes of supernatant and containing approximately equal RT activity (C) were subjected to Western blot analysis after electrophoresis on 15% polyacrylamide gels as described in Materials and Methods. Mock-infected supernatants were analyzed in parallel. Virus was produced after electroporation of MT4 or CEMx174 cells with WT and Vif$^-$ proviral clones (A and B) or after transient coculture of H9 cells (C). The positions of virus-specific proteins are given alongside each panel. The upper half of panel A was exposed for twice as long as the lower half to facilitate detection of gp120. (B, left) Western blots of WT-, Vif$^-$-, and mock-infected CEMx174 cell extracts were probed with antibodies against gp120 (top) or vpr or nef (bottom) or with HIV-infected patient sera (middle). (B, right) A second HIV-infected patient serum which shows reactivity against gp41 was used to probe the Western blot of WT and Vif$^-$ virus preparations from CEMx174 cells (bottom). (C) Approximately 6 and 11 ng of particle-associated p24 (as detected by p24 ELISA) were loaded for WT and Vif$^-$ virus particle preparations, respectively. With the quantities of virus used, only Gag proteins were detectable by Western blot. (D) Exogenous RT assay of WT and Vif$^-$ viral particles produced from CEMx174 cells. Virus particles were prepared from cell-free supernatants of CEMx174 cells infected with either WT or Vif$^-$ virus (infections initiated by electroporation) and were analyzed for HIV p24 antigen by ELISA. Doubling dilutions of particle-associated viral proteins (quantity given above each set of two spots as nanograms of p24 analyzed) were subjected to an exogenous RT assay as described in Materials and Methods. After transfer of reactions to filter paper and exposure to photographic film, incorporated radioactivity was determined by scintillation counting. Values for incorporation of radioactivity were 26,500 cpm for WT and 8,280 cpm for Vif$^-$ at the highest concentration of virus assayed.

poration rather than of envelope stability. Of further interest is the finding that Vif⁻ particles have abnormal ratios of the Gag and Pol proteins. For equal quantities of particle-associated p24 antigen (as measured by ELISA), Vif⁻ particle preparations contained approximately 30% more p55 than WT preparations and only 25, 40, and 60%, respectively, of the WT quantities of integrase, Gag matrix p17, and mature p24 (as quantified by densitometry of different exposures of Fig. 3B). These Vif⁻ particle preparations also contained approximately fourfold less detectable RT protein and demonstrated four times less RT activity (Fig. 3D). A similar reduction in particle-associated RT activity was observed when viral stocks produced from H9 cells were used, but no difference was detectable between WT and Vif⁻ preparations from HeLa cells (data not shown). It should also be noted that no differences in the relative amounts of Gag and Pol proteins could be seen in cell lysates from infected CEMx174 cells (Fig. 3B). Thus, it seems that the abnormalities associated with cell-free preparations of Vif⁻ particles from these cells are due to aberrant virus assembly or protein incorporation and not due to reduced synthesis of the proteins in the infected cell.

Vif⁻ virions apparently have an abnormal protein composition, and hence neither p24 antigen nor RT activity seems an accurate means to standardize particle numbers. As described above, when equal quantities of p24 (by ELISA) are used to standardize gel loading, WT lanes contain globally more viral structural proteins. If RT activity is used to standardize protein loading, the converse is true. Expectedly, when loading is standardized to equal RT activity, Vif⁻ lanes contained four- to fivefold more uncleaved p55 and approximately twofold more p17/p24 protein than WT lanes (Fig. 3C). However, a dramatic reduction in Env proteins was still observed with such Vif⁻ preparations (data not shown). It should also be noted that the difference in viral protein profiles evident in Fig. 3 between the WT and Vif⁻ virions from CEMx174 cells was obtained with virion preparations harvested at equivalent stages of infection (in terms of severity of infection of the cell population) and also with viruses harvested 24 h after the end of a transient coculture experiment (data not shown), demonstrating that the aberrant Vif⁻ viral protein profile does not reflect contamination of viral pellets with different amounts of cellular debris or differences in culture age. Thus, it seems that there is a real and pronounced redistribution of both Gag/Pol proteins and Env with WT and Vif⁻ particles produced from restrictive cells. It should also be noted that no such Env- or Gag/Pol-related differences were detectable between WT and Vif⁻ particles produced in permissive MT4 cells (Fig. 3A).

Given the dramatically altered proportions of several viral proteins in phenotypically Vif⁻ viral particles as described above, we wished to ensure that the deletion in the *vif* gene of the provirus used to generate Vif⁻ viruses had not affected the expression of other viral proteins. In particular, it could be postulated that the deletion affected splicing of mRNAs encoding Vpr or Env. To rule out the possibility that our Vif⁻ virus was phenotypically multiply defective, we analyzed the quantities of other viral proteins expressed in WT and Vif⁻ virus-infected CEMx174 cells, since the WT (pNL43) provirus has previously been shown to possess all functional HIV-1 genes (references 1, 2, 33, and 34 and references therein). That the Env (and thus Vpu)-related mRNAs were efficiently spliced had already been shown by the indistinguishable levels of gp120 protein detected in WT and Vif⁻ virus-infected cells (Fig. 3B). Comparable quantities of the Nef and Vpr proteins were also detectable in WT and Vif⁻ virus-infected CEMx174 cell lysates, confirming that the expression of these proteins was also unaffected by the deletion in the *vif* gene. Thus, it can reasonably be assumed that the multiple defects seen in phenotypically Vif⁻ viral particles at the level of viral protein content result from the absence solely of Vif protein and not of some other accessory protein.

Next, the phenotypically Vif⁻ and WT particles produced from either CEMx174 or MT4 cells were examined by transmission electron microscopy as described previously (25). WT viral particles from either cell type exhibited the characteristic morphology of mature HIV-1 particles (14), with an electron-dense, conical core when sectioned vertically, the wide end of this core being precisely centered in the viral particle in horizontal sections (Fig. 4A and B; quantified in Table 1). Vif⁻ virus particle preparations from MT4 cells also demonstrated a normal proportion of particles with this characteristic mature morphology (data not shown). Although such particles were also apparent in the Vif⁻ virus preparation from CEMx174 cells, the majority of Vif⁻ virions possessed an abnormally condensed core. In a significant proportion of Vif⁻ virions studied, the electron-dense material appeared mostly displaced in the wide end of the core, and when viewed in horizontal section, this core was situated toward the side of the virion (Fig. 4C and D). Furthermore, numerous particles with either no visible core (Fig. 4E) or with the electron-dense material apparently located outside the core (Fig. 4F) were seen. Similar particle abnormalities have recently been reported by Höglund et al. (19) for preparations of Vif⁻ particles produced from apparently restrictive CEM cells.

Thus, the data from Western analyses and electron microscopic studies of phenotypically Vif⁻ virions strongly suggest that Vif protein is required in restrictive cells at the stage of particle assembly or maturation.

Phenotypic Vif⁻ viruses are unable to synthesize viral DNA in new target cells. We next tested the ability of phenotypically Vif⁻ viruses to initiate and complete viral DNA synthesis in target cells. To ensure that any observed differences were not due to abnormal quantities of viral RNA in Vif⁻ particles, RNA content was assayed by RT-PCR of RNA purified from equivalent quantities of pelleted WT and Vif⁻ viruses produced from CEMx174 cells. It is clear from the Southern blot analysis of the products of RT-PCR (Fig. 5A) that WT and Vif⁻ particles from restrictive cells contain similar amounts of viral RNA. Thus, viruses from these same stocks were used to infect P4 target cells. At various times after infection, cells were recovered by trypsinization (which serves also to remove virus bound to the cell surface which has not been internalized), washed several times, and lysed for PCR analysis as described in Materials and Methods. Viral DNA synthesis in infected cells was assessed by using two different sets of PCR primers. Effectively, the Sac-U5AS primer pair detects strong-stop viral DNA and thus will detect virtually all early reverse transcription events. Conversely, the Sac-1070 primer pair detects only the products of late reverse transcription. Specific viral DNA synthesis was easily detected with both primer pairs after infection with WT virus (Fig. 5B and C). Similar results were obtained with Vif⁻ virus produced from nonrestrictive MT4 cells (data not shown). Conversely, no DNA synthesis was detected with either primer pair after infection with phenotypically Vif⁻ virus (Fig. 5B and C).

The failure to detect even strong-stop DNA with this Vif⁻ virus effectively demonstrates that the replicative block in Vif⁻ virus infection is either prior to or extremely early in the initiation of reverse transcription. However, we have already demonstrated here that phenotypically Vif⁻ virions possess less RT and less Env proteins (Fig. 3). Thus, the failure to detect early viral DNA synthesis in the experiments described above could result from an inability of virions to efficiently

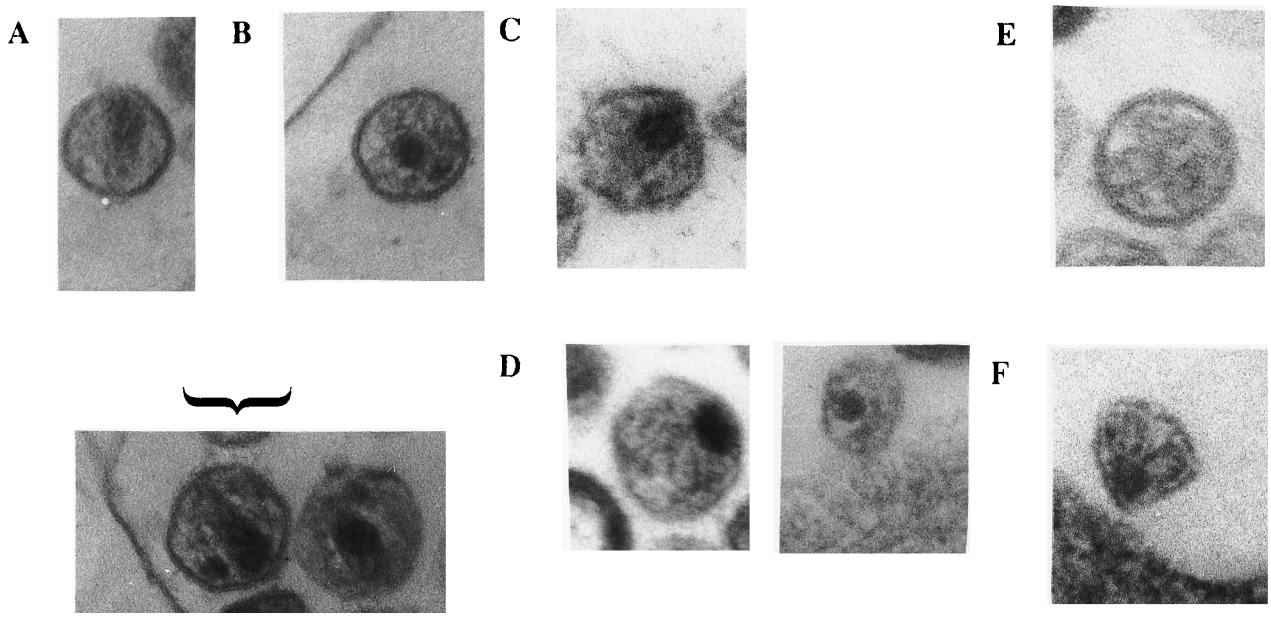

FIG. 4. A subtle morphological defect is associated with Vif$^-$ particles produced from CEMx174 cells. Three independent populations of CEMx174 cells infected with WT or Vif$^-$ virus by transient coculture were prepared for transmission electron microscopy as described in Materials and Methods. Shown are photographs of mature WT virions in vertical and horizontal section (A and B, respectively) and a selection of Vif$^-$ virions showing different aberrant morphologies (C, E, and F in vertical section; D in horizontal section). The virions shown in panels D (right-hand side) and F were photographed at 66% of the magnification of the virions shown in the other panels. The various viral particle morphologies seen with WT and Vif$^-$ particles produced from CEMx174 cells were quantified. The cumulative percentages of three independent experiments are shown in Table 1. For WT and Vif$^-$ viruses, 100 and 170 particles, respectively, were examined at magnifications equal to or exceeding ×25,000.

enter cells (due to the Env-related defect), from the reduced quantities of virion-associated RT protein, or from a combination of both of these defects.

The Vif$^-$ defect can be partially rescued by pseudotyping with amphotropic envelope. If one of the primary blocks to infection by phenotypic Vif$^-$ virions is related to the abnormal quantities of Env proteins detected with Vif$^-$ virions, it should be possible to overcome this defect by bypassing the normal HIV-1 Env-dependent entry pathway. It has previously been shown that cells coinfected with HIV-1 and MuLV produce HIV particles with envelopes from both viruses (23). Such pseudotyped viruses have an extended host range and may infect cells lacking CD4 (32). However, it has been suggested that the Vif$^-$ defect cannot be overcome by pseudotyping, at least when both the HIV and MuLV envelopes are present in the producer cell (36). Thus, we attempted to produce pure pseudotyped HIV WT and Vif$^-$ particles, using H9 cells which were chronically infected with amphotropic MuLV as a source of amphotropic envelope (H9-Ampho cells; Materials and Methods). HeLa cells were transfected with WT and Vif$^-$ DNAs with either an intact or interrupted *env* open reading frame together with an expression vector for gibbon ape leukemia virus Env. These cotransfected HeLa cells were cocultured with H9-Ampho cells in order to initiate an infection in these restrictive cells. Infections were assessed by ELISA measurement of p24 antigen production.

As can be seen from the results presented in Fig. 6A, no productive viral infection could be detected with Vif$^-$ particles produced in the presence of the HIV envelope (Vif$^-$ Env$^+$) or with either Vif$^+$ Env$^-$ or Vif$^-$ Env$^-$ when the amphotropic Env expression vector was omitted from the transfection mixes (data not shown). However, with amphotropic Env in the absence of HIV Env (Vif$^-$ Env$^-$; Fig. 6B), replication of the Vif$^-$ HIV particles could be detected in H9-Ampho cells. It should

TABLE 1. Quantification of results shown in Fig. 4

CEM cell infected with:	%					
	Immature	Mature				
		Homogeneous dense cone-shaped core[a]	Round dense central core[b]	Dense material in base of cone[c]	Displaced core[d]	No defined core, empty core[e] dense material outside core[f]
WT	14	34	29	1	11	11
Vif$^-$	7.5	9	19.5	12	23	29

[a] Characteristic mature electron-dense cone-shaped core in vertical sections (Fig. 4A).
[b] Central electron-dense circular core seen in horizontal sections of mature virions (Fig. 4B).
[c] Well-defined cone-shaped core with electron-dense material confined to the wide end (Fig. 4C).
[d] Circular central core is displaced to the inner surface of the viral membrane (Fig. 4D).
[e] Defined core in horizontal or vertical sections which apparently lacks dense matter (Fig. 4E).
[f] Dense material is found outside the defined core structure (Fig. 4F).

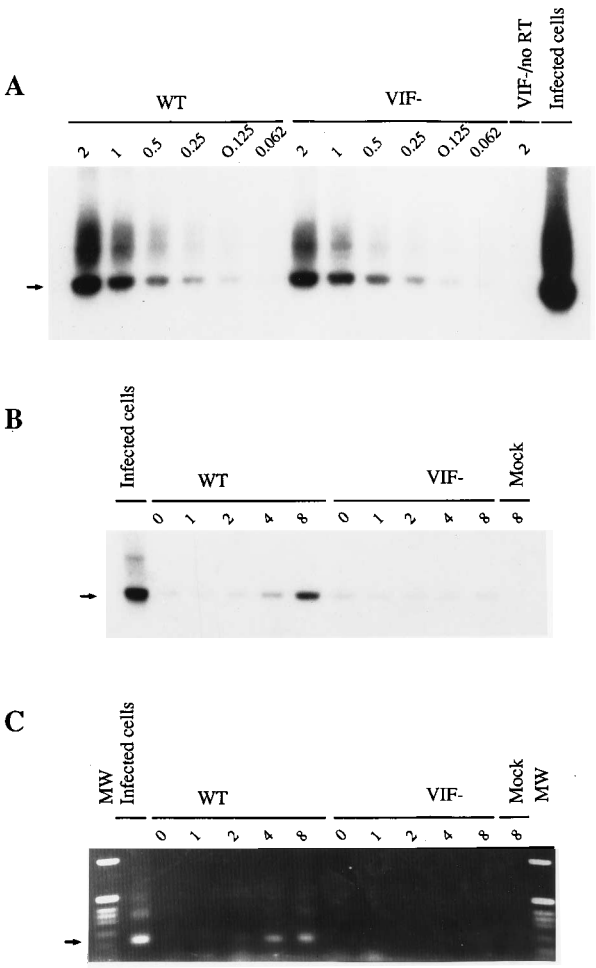

FIG. 5. Viral DNA synthesis is not detectable in cells infected with phenotypic Vif⁻ virus preparations. (A) RT-PCR analysis of the viral RNA present in cell-free preparations of WT and phenotypically Vif⁻ virions (produced from CEMx174 cells) was performed exactly as described in Materials and Methods, using RNA purified from the quantities of virus (in nanograms of particle-associated p24) given above the lanes. Positive and negative controls were performed by PCR (without RT) with WT-infected CEMx174 cell lysate (infected cells) and with RNA from 2 ng (particle-associated p24) of Vif⁻ virus, respectively. (B and C) PCR analysis of proviral DNA synthesis after infection of P4 cells with WT and phenotypically Vif⁻ virus from CEMx174 cells. Infection of P4 cells and extraction of total cell DNA were performed as described in Materials and Methods. DNA for PCR analysis was extracted from equal numbers of WT and Vif⁻ virus-infected cells at the time (in hours) given above each lane. Primer pairs were Sac-1070 (B) and Sac-U5AS (C). Specificity of PCR products was verified by Southern blotting and hybridization using the internal NARS oligonucleotide (B); PCR products synthesized with the Sac-U5AS primers were visualized by ethidium bromide staining of agarose gels (C), and specificity was verified by restriction endonuclease mapping of PCR products (not shown). Negative and positive control PCR reactions were performed with mock-infected cells (mock) and chronically infected CEMx174 cell DNA (infected cells), respectively. In all cases, the position of the authentic RT-PCR or PCR product (342 nt for the Sac-1070 primers pair and 157 nt for the Sac-U5AS primer pair) is indicated by an arrow at the left.

be noted that in independent experiments, the rescue of Vif⁻ mutant infectivity was always partial, and the levels of replication seen with the Vif⁻ Env⁻ double mutant never reached those seen with the Vif⁺ Env⁻ positive control. In addition, no rescue was detectable if noninfected H9 cells were not regularly added to the H9-Ampho cultures. Nevertheless, it appears that the replicative defect that is characteristic of Vif⁻ HIV particles in H9 cells can be partially overcome when the requirement for HIV envelope is bypassed. In this system, the presence of HIV envelope in the absence of Vif protein has an apparent negative *trans*-dominant effect on rescue of the Vif⁻ defect by MuLV envelope.

DISCUSSION

We have examined the role of Vif in HIV-1 infectivity by using a variety of human cell systems. In agreement with previous reports, we found that the effect of deletion of the *vif* gene on replication of HIV-1 was dependent on the type of cells in which the particles were produced (11, 26). Study of the nature of the Vif⁻ defect was greatly facilitated by the ability to produce large quantities of Vif⁻ virions, in a single cycle of infection, from cells which are totally restrictive for Vif⁻ mutants. When produced from such restrictive cells (H9 or CEMx174 cells), Vif⁻ viral particles were found to be noninfectious and to exhibit an abnormal protein content. These replication-defective Vif⁻ virions possess less envelope proteins and contain altered proportions of Gag and Pol proteins. Not surprisingly, these Vif⁻ virions were shown to be incapable of DNA synthesis in target cells. However, partial rescue of the Vif⁻ defect was possible in pseudotyping experiments under conditions in which infection was independent of HIV Env protein.

The Vif protein has been detected in the cytoplasm, close to the membrane of virus-producing cells (15). Such a cellular localization of Vif and the fact that it is synthesized late in the infectious cycle in a Rev-dependent manner have led to suggestions that Vif operates during virus assembly, release, or maturation (3, 26). Indeed, Höglund et al. recently demonstrated that Vif⁻ mutants of HIV-1 possess an abnormally assembled or matured viral core (19). Similar core abnormalities were also detectable with our preparations of Vif⁻ virus from restrictive cells. Furthermore, the quantities of Gag and Pol proteins in cell-free preparations of Vif⁻ particles were found to be abnormal. Reproducibly, Vif⁻ particle preparations were found to contain more Gag precursor ($p55^{gag}$) per unit of mature p24 than preparations of WT virus. Other abnormalities included a reduction in the amounts of Pol Proteins and, more strikingly, a marked decrease in the amount of particle-associated envelope proteins. These data are further evidence that Vif is required in restrictive cells for the proper incorporation and assembly of the HIV structural proteins into the viral particle. It should be noted that we have not attempted to conduct a precise quantitation of the amounts of viral proteins per particle, since available particle quantitation methods rely either on p24 ELISA or on RT activity, each of which seems to be directly affected by the Vif⁻ phenotype. However, much reduced quantities of envelope were consistently observed with the Vif⁻ mutant even when comparisons included clearly more Vif⁻ particles than WT particles. It is not certain whether the Gag- and Env-related defects observed for phenotypically Vif⁻ particles are separate consequences of the lack of Vif in the producer cell or, instead, whether Env proteins are poorly incorporated or retained on Vif⁻ particles because of the abnormally assembled viral core. Since the Gag MA protein has been shown to be essential for envelope incorporation into particles (8, 39), it is conceivable that an aberrantly organized or folded $p55^{gag}$ precursor could hinder the MA-Env interaction at the infected cell surface.

In this study, we have observed that phenotypically Vif⁻ virions possess less Env protein and that the infectivity defect associated with such virions can be partially rescued in pseudotyping experiments using the amphotropic MuLV Env. This partial rescue was possible only in the absence of HIV

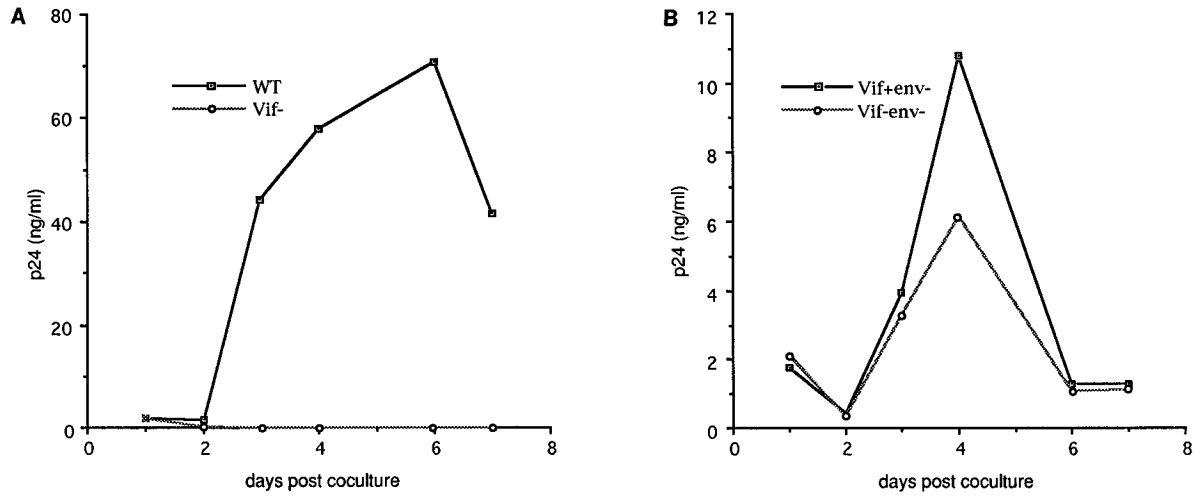

FIG. 6. The Vif⁻ defect can be partially rescued by pseudotyping with amphotropic envelope. Pseudotype rescue experiments were performed exactly as described in Materials and Methods. HeLa cells were transfected with the WT or Vif⁻ proviral clone (A) or the HIV Env⁻ derivative of the WT or Vif⁻ clone in the presence of an expression vector for gibbon ape leukemia virus envelope (B). Transfected HeLa cells were transiently cocultured with H9-Ampho cells, and the amount of HIV p24 antigen produced from H9-Ampho cells after coculture was standardized and quantified at 1- or 2-day intervals as described in Materials and Methods. The graphs show p24 antigen in the cell-free supernatants of H9-Ampho cell cultures at various times after coculture.

Env from the infected cells. Effectively, these data demonstrate that one of the primary blocks to Vif⁻ virus infection is due to inefficient HIV Env-mediated infection of target cells. Whether pseudotyping overcomes a problem with virus binding, fusion, or entry into target cells is uncertain at the present time. In contrast, a previous study of the early stages of the infectious cycle of Vif⁻ virions concluded that Vif was required for completion of reverse transcription in the infected cell (36). This conclusion was based on the inefficient proviral DNA synthesis observed in infections using Vif⁻ virions and on the failure to rescue the Vif⁻ defect in pseudotyped virions which had been produced in the presence of HIV Env. However, given that we have observed here that phenotypically Vif⁻ virions possess less RT protein and are apparently incapable of proviral DNA synthesis in the target cell, it is possible that inefficient reverse transcription contributes to the proportion of the Vif⁻ defect which was not rescuable by pseudotyping. In addition, it is believed that reverse transcription is at least initiated, if not completed, within an organized viral capsid structure (6), a structure which we and others (19) have shown to be abnormally assembled in Vif⁻ virions.

In this study, no differences in particle morphology, protein content, or efficiency of proviral DNA synthesis were detectable between WT and Vif⁻ viruses produced from permissive cells or semipermissive cells (even though Vif⁻ virus preparations from the semipermissive SupT1 line demonstrated only about 40% of WT virus infectivity; data not shown). Likewise, the severities of the Vif⁻ defect were quantitatively similar in terms of viral protein profiles and virion morphology with Vif⁻ virions from either CEMx174 or H9 cells, even though Vif⁻ virus from H9 cells was reproducibly four times less infectious than its CEMx174 counterpart. These discrepancies, which are presumably due to the relative insensitivity of the available assay systems, emphasize the importance of quantifying the permissivity of all cell lines used for the generation of Vif⁻ virions and of studying phenotypically Vif⁻ virus produced from truly restrictive cells. It seems likely that certain of the disparate functions suggested for the Vif protein (17, 31, 36) (see the introduction) are a consequence of using cells which are not truly restrictive cells for the production of Vif⁻ virus. In the light of our results, which show that the abnormal viral protein content is detectable only with preparations of severely defective Vif⁻ virus, use of semipermissive cells for Vif⁻ virus generation probably explains why the majority of previous studies of Vif⁻ virions failed to detect differences in viral protein content (11, 36). Even though an Env defect has previously been observed with Vif⁻ mutants produced from the semipermissive A3.01 cell line (26), the more subtle Gag/Pol protein defect was not detected with the partially defective virions produced from such cells.

Previously, two hypotheses have been proposed to explain the permissive/restrictive nature of different T-cell lines with respect to Vif⁻ mutants. It has been postulated either that permissive cells possess a protein capable of complementing Vif function or conversely that restrictive cells possess a protein(s) with a deleterious effect on HIV replication in the absence of Vif. In view of the dramatically different effects (in terms of severity) of Vif mutation in the various T-cell lines, and since primary cultures of peripheral blood mononuclear cells (9) are totally restrictive to Vif⁻ virus, it will be particularly interesting to define the feature(s) which confer permissivity or restrictivity to T cells. To date, we have been unable to transfer a Vif-like protein from permissive cells to restrictive producer cells using a heterokaryon approach, although the existence of a "toxic" protein in restrictive cells, which requires neutralization by Vif, has not yet been examined.

Finally, it has recently been reported that the Vif protein is present in cell-free preparations of HIV particles (2a). In this report, we have confirmed that extremely small quantities of Vif protein are indeed detectable by Western analysis of cell-free preparations of WT HIV-1 particles (Fig. 7). Unfortunately, it is difficult to investigate whether the Vif protein is specifically encapsidated (albeit at low levels) into virions, or whether its presence results from nonspecific encapsidation of Vif protein present in the infected cell at the site of particle assembly. To test this, it would be necessary to attempt detection of encapsidation of a cellular protein of approximately the same size and charge as Vif and with a cellular localization similar to that of Vif. However, it remains possible that particle-associated Vif protein is physiologically significant for HIV

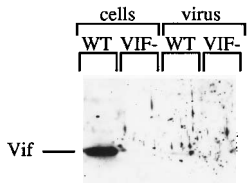

FIG. 7. Vif protein is present in cell-free preparations of HIV-1 particles. Western blots for the detection of Vif protein were performed exactly as described in Materials and Methods. The analysis was performed on lysates from 10^5 WT- or Vif⁻-infected CEMx174 cells and cell-free virus (approximately 180 ng of particle-associated p24 antigen) purified from the culture medium of 3×10^6 of the same WT- or Vif⁻-infected CEMx174 cell cultures. Thus, in terms of cell equivalents, the virus analyzed had been produced from 30 times more cells than the cell extracts which were loaded in parallel.

infection of restrictive cells and that the absence of Vif from viral particles rather than from the infected cell is responsible for certain of the defects observed in this study with phenotypically Vif⁻ virions. Further studies will be needed to clarify these issues.

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