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The Human Immunodeficiency Virus Type 1 Vif Protein Modulates the Postpenetration Stability of Viral Nucleoprotein Complexes

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The *vif* gene of human immunodeficiency virus type 1 is absolutely required for productive infection of primary cells derived from human blood and certain immortalized T lymphoid cells, for example, H9. Cells with this restrictive phenotype are termed nonpermissive, whereas cell lines in which *vif*-deficient virus can replicate efficiently are known as permissive. In this paper, we describe experiments in which virus stocks derived from single-cycle infections of strictly nonpermissive H9 cells were used to determine the fate of *vif*-deficient infections. By PCR-based approaches, it was found that Vif has no significant impact on the biosynthetic capability of the virion reverse transcriptase in infected C8166 T cells. Specifically, the initial appearance of all DNA species up to and including initiated second (plus) strands as well as the early accumulation of these replicative intermediates is equivalent for wild-type and *vif*-deficient infections. However, whereas these viral DNAs are stably maintained in wild-type infections and can proceed to establish proviruses, they are largely degraded by the later time points of *vif*-deficient infections and, as a result, are prevented from forming proviruses. Subcellular fractionation analyses indicated that the majority of viral DNA is localized to the nucleus within 2 h of infection and that the turnover of reverse transcripts that occurs in these *vif*-deficient infections presumably takes place in the nucleus. Given that the ultimate infection phenotype of the virions is determined during virus production, we propose that Vif is required for an aspect of virus assembly and/or maturation that endows penetrating viral nucleoprotein cores with the ability to mature into functional preintegration complexes that can proceed to provirus establishment. In contrast, viruses that are produced in the absence of Vif give rise to nucleoprotein complexes that disassemble prematurely in challenged cells and fail to protect their RNA/DNA contents from nucleolytic destruction.

Full infectivity of the retrovirus human immunodeficiency virus type 1 (HIV-1), unlike that of its long-studied oncogenic murine and avian counterparts, is not conferred by the *gag*, *pol*, and *env* genes alone. In particular, three of the six additional and/or auxiliary genes of HIV-1, *vif*, *vpr*, and *nef*, appear to modulate virion infectivity. There remains considerable uncertainty regarding the mechanism(s) by which each of these genes influences the infection process and whether any of them have overlapping functions. The ~15-kDa Vpr protein is present at relatively high levels in HIV-1 virions (8, 35, 40, 52) and has been shown to be required for efficient replication in nondividing cells (3, 10, 25). Further analyses have demonstrated not only that Vpr is retained as a component of the preintegration complex but also that the import of this complex into the nucleus is inefficient for *vpr*-deficient viruses (25). In contrast to Vpr, the ~27-kDa Nef protein has not (to date) been identified as a virion component; moreover, Nef seems to act at an earlier step of infection than Vpr by allowing efficient reverse transcription to occur once entry has taken place (2, 6). Importantly, this effect is observed for viruses produced in CD4-negative cells (2, 22, 37) and is, therefore, independent from the well-established ability of Nef to down-modulate the surface expression of CD4 (1, 20).

Although the ~23-kDa Vif protein was recognized as a modulator of infectivity at the time of its initial identification in 1986 (46), its function(s) during HIV-1 infection remains ill defined and controversial. One of the major reasons for this uncertainty has likely stemmed from the observation that Vif is required for efficient HIV-1 replication only in certain cells. While the underlying basis for this effect is still an enigma, it is generally agreed that *vif*-deficient (Δ*vif*) viruses are unable to replicate in primary lymphocytes and macrophages as well as in a limited number of T-cell lines (collectively termed nonpermissive cells) but are fully replication competent in other T-cell lines such as Sup-T1 and C8166 (termed permissive cells) (4, 13, 15–17, 28, 42, 45, 47, 51). Importantly, and unlike *vpr* and *nef*, equivalents of the HIV-1 *vif* gene have been identified not only in HIV-2 (36) and the simian immunodeficiency viruses (SIVs) (21, 45) but also in all but one (equine infectious anemia virus) of the distantly related lentiviruses of nonprimate hosts (24, 39, 43, 49). It, therefore, seems likely that Vif plays a fundamental and conserved role in the replication of lentiviruses in vivo.

Attempts to define the nature of the defect(s) in Δ*vif* viruses have yielded an array of conflicting results. With respect to the viral particles themselves, electron microscopy analyses have shown that Vif may be required for the complete maturation of newly budded virions (4, 26). Specifically, it was noted that an absence of Vif resulted in a failure of the virion cores to adopt the characteristic cone-like structure of fully matured HIV-1 particles. It is unknown how this morphologic abnormality may be related to Vif's reported ability to enhance the proteolytic processing of $p55^{Gag}$ (4, 44), increase the incorporation of $gp160/120^{Env}$ into virions (42), and catalyze a truncation within the carboxy-terminal 15 amino acids of $gp41^{Env}$ (23) or even to its own incorporation into particles (4, 33). Importantly, the ultimate infection phenotype of HIV-1/Δ*vif* has also remained

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unresolved. Earlier studies suggested that Δ*vif* viruses are able to penetrate susceptible cells efficiently but are unable to undergo processive reverse transcription (47, 51); in agreement with this, the synthesis and accumulation of reverse transcripts, in addition to provirus formation, were found to be diminished in Δ*vif* infections. In contrast, two more recent reports have proposed that Δ*vif* viruses are incapable of giving rise to detectable reverse transcription following virus challenge (4, 11). These observations suggest that the block in Δ*vif* infections occurs earlier in the infection process—perhaps at receptor engagement, nucleocapsid penetration, uncoating, or the initiation of reverse transcription itself.

In this paper, we present a detailed analysis of the effect of Vif on HIV-1 infection using *vif*-deficient and *trans*-complemented viruses derived from nonpermissive H9 cells. Using permissive C8166 T cells as targets, we report that reverse transcription (and by inference, viral entry) can proceed unimpaired to the point of the second-strand transfer in the absence of Vif. However, the reverse transcripts synthesized in HIV-1/Δ*vif* infections are not maintained for prolonged periods and are degraded by the later time points of infection. Because no such turnover of viral DNA is observed in wild-type infections, proviruses are formed and a further round of virus expression and production, therefore, takes place. On the basis of these findings and the reported effects of Vif on the ultrastructure of virion cores, we propose that the nucleoprotein (preintegration) complex in which reverse transcription occurs may, in the case of HIV-1/Δ*vif*, disassemble inappropriately in infected cells, resulting in the subsequent clearance of viral nucleic acids by host cell degradative mechanisms.

MATERIALS AND METHODS

Molecular clones. The wild-type and *vif*-deficient HIV-1 expression vectors pIIIB and pIIIB/Δ*vif* have been described elsewhere (45). The retrovirus vectors that confer resistance to G418 and carry the wild-type *vif* genes of HIV-1 and SIV_{mac} or the defective *vif* gene of pIIIB/Δ*vif* are termed LN-M/hVif, LN-M/sVif, and LN-M/ΔhVif, respectively, and have been described previously (45). The murine sarcoma virus-based retroviral vector GVL3CATs harbors the bacterial chloramphenicol acetyltransferase (*CAT*) gene under the transcriptional control of nucleotides −524 to +80 of the HIV-1 long terminal repeat (LTR) and confers resistance to G418 (14). The SV-A-MLV-*env* and SV-Ψ$^-$-*env*$^-$-MLV vectors were used for retroviral transduction; they express the amphotropic murine leukemia virus Env and Moloney murine leukemia virus Gag and Pol proteins, respectively (32).

Cells and cell line derivation. The parental human T-cell lines H9, CEM-SS, C8166, and Jurkat were maintained at 0.1×10^6 to 2×10^6 cells per ml in RPMI 1640 medium supplemented with 10% fetal bovine serum and 50 μg of gentamicin sulfate per ml (complete medium). Human peripheral blood lymphocytes (PBLs) were obtained by venipuncture, purified over lymphocyte separation medium according to the instructions of the manufacturer (Organon Teknika Corp., Durham, N.C.), and cultured in complete medium supplemented with 5 μg of phytohemagglutinin (Sigma Chemical Co., St. Louis, Mo.) per ml for 72 h; the cells were then washed once with complete medium and were maintained thereafter in medium containing 20 U of recombinant interleukin-2 (Immunotech S.A., Westbrook, Maine) at 1×10^6 to 3×10^6 cells per ml.

To obtain the recombinant retrovirus stocks used for transduction, 35-mm monolayer cultures of the African green monkey kidney cell line COS were cotransfected with 2.5 μg each of SV-A-MLV-*env*, SV-Ψ$^-$-*env*$^-$-MLV, and, as appropriate, LN-M/hVif, LN-M/sVif, LN-M/ΔhVif, or GVL3CATs with DEAE-dextran and chloroquine. At 48 h, these transient stocks were used to challenge 10^6 T cells by cocultivation; after a further 24 h, the T cells were harvested and then maintained in complete medium supplemented with 1 mg of G418 (Gibco BRL Inc., Gaithersburg, Md.) per ml until resistant polyclonal cultures emerged. These lines were named in accordance with the parental T-cell line and Vif protein (or *CAT* gene) that they expressed.

Western analysis and monoclonal antibodies. Total lysates from $\sim 5 \times 10^5$ T cells were resolved in sodium dodecyl sulfate-14% polyacrylamide gels, and the proteins were transferred to nitrocellulose filters by electroelution. The HIV-1 Vif and host cell C1/C2 heterogeneous ribonucleoprotein particle proteins were detected by primary hybridization with either MAb319 (45) or 4F4 (41), respectively. Bound antibodies were visualized by secondary hybridization with a horseradish peroxidase-conjugated anti-mouse antibody raised in goats (FisherBiotech, Pittsburgh, Pa.), enhanced chemiluminescence (Amersham Corp., Arlington Heights, Ill.), and autoradiography.

Preparation of HIV-1 stocks. The inocula of wild-type HIV-1 and HIV-1/Δ*vif* that were used for the determination of host cell phenotypes (see Fig. 1) were derived from pIIIB and pIIIB/Δ*vif*, respectively, and were propagated in the permissive cell line C8166 as previously described (45). Additional stocks of wild-type HIV-1 were also generated from H9 and CEM-SS by the same approach.

Genotypically Δ*vif* viruses that were utilized for the detailed analysis of HIV-1 infection (see Fig. 4, 5, 6, 7, and 8) were generated by the coculture strategy represented in Fig. 2. Here, an acute infection of HIV-1/Δ*vif* was first established in the Jurkat/sVif cell line in complete medium. At a point when HIV-1/Δ*vif* production was high, these cells were transferred to the lower chambers of Transwell culture dishes (Corning Costar, Cambridge, Mass.). The cells from which virus was ultimately to be produced (these were already transduced with either LN-M/hVif or LN-M/ΔhVif) were then added to the upper chamber in complete medium. Following 48 h of coculture, the cells from the upper chamber were recovered, washed with phosphate-buffered saline (PBS), and treated with trypsin on 3 consecutive days to eliminate input virus. These HIV-1/Δ*vif*-infected cells were maintained for 24 h, and virus stocks were prepared from the culture supernatants by adjustment to 10 mM $MgCl_2$, passage through 0.45-μm-pore-size filters, treatment with 9,000 U of DNase I (U.S. Biochemicals, Cleveland, Ohio) per ml for 30 min at 37°C and storage at −70°C in aliquots. By exploiting this approach, HIV-1/Δ*vif* corresponding to ~2 ng of $p24^{Gag}$ per ml could be derived routinely from H9/ΔVif cells.

Analysis of HIV-1 replication by spreading infection. All T-cell lines were challenged with wild-type or Δ*vif*HIV-1 in 1 ml of complete medium containing 2×10^6 cells. At 24 h, the cells were washed and maintained at 0.1×10^6 to 2×10^6 cells per ml for the duration of the experiment. PBLs were infected in the same manner ~24 h after transfer to interleukin-2-containing medium; at 1 week postchallenge, these cultures were supplemented with freshly stimulated uninfected PBLs obtained from the same donor. Virus production and, hence, replication were monitored over time by determining the expression of soluble $p24^{Gag}$ in the culture medium by enzyme-linked immunosorbent assay (ELISA) (Dupont, NEN, Inc., Billerica, Mass.).

Quantitation of productive HIV-1 infection with C8166/HIV-*CAT* cells. A total of 0.5×10^6 C8166 T cells that had previously been stably transduced with the GVL3CATs retroviral vector were challenged with HIV-1 virus stocks corresponding to 1 ng of $p24^{Gag}$ in a final volume of 1 ml. The cells were washed at 1 h and were maintained in complete medium until 24 h postchallenge. Total cell lysates were prepared by resuspension of cell pellets in 100 mM Tris-HCl (pH 7.8)–0.5% Triton X-100, and the levels of CAT activity were determined by the diffusion method (38).

PCR analysis of HIV-1 reverse transcription. DNase I-treated stocks of HIV-1/Δ*vif* that contained 1.5 ng of soluble $p24^{Gag}$ and that had been derived from H9/ΔVif or H9/Vif cells were used to challenge 7.5×10^5 T cells in a volume of 1.5 ml on ice. Following a 2-h incubation on ice (during which virus binding but no postbinding events could occur), 5×10^4 cells were removed, washed once in PCR wash buffer (20 mM Tris-HCl [pH 8.4], 100 mM KCl), and lysed in 100 μl of PCR lysis buffer (wash buffer plus 0.5 mg of proteinase K [Boehringer Mannheim Corp., Indianapolis, Ind.], 0.1% Nonidet P-40) by heating at 60°C for 2 h and at 95°C for 15 min; these samples represented the zero time points. Complete medium prewarmed to 37°C was added to the remainder of the infection mixtures, and the cultures were transferred to a 37°C CO_2 incubator (postbinding events could then be initiated). At 50 min post-37°C transfer, the cells were washed twice with prewarmed PBS and were resuspended in complete medium. At that time (the 1-h time point) and at various times thereafter, aliquots of 5×10^4 cells were removed, washed, and lysed.

Following the collection of samples from all time points, 10 μl of 10-fold dilutions of the lysates was analyzed for the presence of various reverse transcription DNA products by using specific DNA primer pairs, standard PCR buffer (9), and *Taq* polymerase (Perkin-Elmer Corp., Norwalk, Conn.). The primers are named with reference to the region of HIV-1 DNA to which they hybridize and whether they themselves consist of plus (+) or minus (−) sense sequence; accordingly, the first (minus)-strand transfer, first-strand extension, and second (plus)-strand transfer reverse transcripts were detected with U3+/U5−, *gag*+/*gag*−, and U3+/*gag*− primer pairs, respectively. The sequences of these have been described previously (9), with the exception of the U3+ primer, which was 5′-GGC GCT ACA AGC TAG TAC CAG-3′. All HIV-1-specific PCRs utilized a program in which an initial denaturation of 2 min at 94°C was followed by 30 cycles of annealing for 1 min at 60°C, extension for 2 min at 72°C, and denaturing for 1 min at 94°C. To allow quantitative comparisons of viral DNA contents in the test reaction mixtures, each primer pair was used in parallel reaction mixtures containing no (negative control sample), 1, 10, 10^2, 10^3, and 10^4 ACH2 cells in a background of a 5×10^4 cells. In addition, the DNA contents of all samples from a given infection were also established as consistent by performing reactions with primers specific for the cellular β-globin gene (9). The products of all PCRs were electrophoresed through 1.5% agarose gels, transferred to hybond-N^+ membranes (Amersham Corp.), subjected to Southern hybridization by using random-primed radiolabeled DNA probes (Pharmacia Biotech Inc., Piscataway, N.J.), and visualized by autoradiography. The DNAs used for radiolabeling were obtained following PCR amplification of segments of pIIIB or a human β-globin expression vector with the relevant primer pairs.

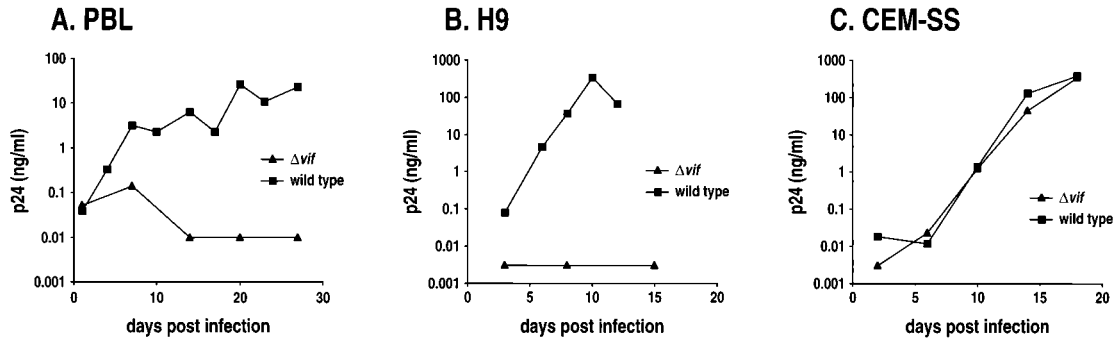

FIG. 1. Vif is essential for HIV-1 replication in PBLs and H9 T cells. Cultures containing 2×10^6 PBLs (A), H9 cells (B), or CEM-SS cells (C) were challenged with wild-type or Δ*vif* stocks of HIV-1 corresponding to 50, 10, or 10 ng of $p24^{Gag}$, respectively. The cultures were maintained in complete medium, and virus replication was monitored by ELISA for soluble $p24^{Gag}$ expression.

In some experiments, second-round infection (virus spread) was inhibited by the addition of 10 μg of the D47 neutralizing monoclonal antibody (5) per ml at 4, 8, and 24 h postinfection.

Subcellular fractionation. C8166 T cells were infected with DNase I-treated stocks of HIV-1/Δ*vif* as described above. At various times thereafter, 5×10^4 cells were removed, pelleted, and resuspended in 100 μl of ice-cold PCR wash buffer containing 0.1% Nonidet P-40. After a 5-min incubation on ice, the nuclei were pelleted by centrifugation at 16,000 × *g* for 5 s in a microcentrifuge, washed twice with PCR wash buffer, and lysed in 100 μl of PCR lysis buffer. The cytoplasmic samples, which correspond to the supernatants from the first centrifugation, were supplemented with 0.5 mg of proteinase K per ml. All lysates were then treated as described above, diluted 10-fold, and subjected to PCR analysis with primer pairs specific either for the first strand transfer of reverse transcription or for β-globin (nuclear) and cytochrome *b* (mitochondrial/cytoplasmic) DNA. The sequences of the cytochrome *b*+ and cytochrome *b*− primers were 5′-GGC GCC TGC CTG ATC CTC C-3′ and 5′-GGG GTT GGC TAG GGT ATA ATG G-3′, respectively. Following agarose gel electrophoresis, the reaction products were detected either by Southern hybridization (HIV-1 DNA) or by staining with ethidium bromide (β-globin and cytochrome *b* DNA).

RESULTS

Vif is essential for HIV-1 replication in primary human lymphocytes and the H9 T-cell line. It has been shown by a number of laboratories that an intact *vif* gene is essential for HIV-1 replication in primary cell cultures (11, 16, 17, 28, 44, 51). For experiments that utilize cultured T-lymphoid lines to be relevant for the analysis of Vif function, we considered it critical to identify cell lines that recapitulated this requirement for Vif. Stocks of wild-type and Δ*vif* HIV-1 were, therefore, prepared in permissive C8166 cells (45) and were used to challenge human PBLs as well as a number of T-cell lines. The respective extents of virus replication were monitored over time by determining the expression of soluble $p24^{Gag}$ in the culture medium. Representative replication profiles from some of these experiments are presented in Fig. 1. In agreement with the observations of others, we found that Vif was absolutely required for virus replication in PBLs (Fig. 1A). Importantly, H9 cells exhibited a similar phenotype in that the Δ*vif* virus always failed to replicate to any detectable degree (Fig. 1B). In contrast, the absence of Vif had either a negligible (for example, CEM-SS cells [Fig. 1C]) or a modest inhibitory influence on HIV-1 replication in the majority of cell lines tested. As defined above, PBLs and H9 cells are regarded as nonpermissive with respect to HIV-1/Δ*vif* replication, whereas cell lines such as CEM-SS are considered permissive. Given the experimental limitations of using primary cells, it is most likely that analyses performed with nonpermissive T-cell lines such as H9 will, therefore, be the most pertinent for understanding the role of Vif in the life cycle of HIV-1 in vivo.

Development of an expression system for HIV-1/Δ*vif* in nonpermissive H9 cells. There is a major technical obstacle that makes the experimental analysis of *vif*-deficient HIV-1 derived from nonpermissive cells problematic. Specifically, one cannot prepare virus stocks from a productive and spreading infection of nonpermissive cells, since the virus is, by definition, unable to replicate in such cells (Fig. 1). This situation can, therefore, be considered analogous to attempting to propagate a temperature-sensitive virus mutant at the nonpermissive temperature. However, and as will be shown below, Δ*vif* virus produced in permissive cells is capable of infecting nonpermissive cells and of giving rise to a single round of gene expression (11, 51). With this in mind, we were able to develop the strategy diagrammed in Fig. 2 for producing HIV-1/Δ*vif* from nonpermissive cells.

First, an acute infection of HIV-1/Δ*vif* was established in Jurkat cells transduced with the *vif* gene of SIV_{mac} (45). Although it was not essential to use Jurkat cells for this purpose, this was found to be advantageous, since high titers of virus could be obtained over sustained periods owing to the relatively noncytopathic character of these infections. The SIV_{mac} *vif* gene was chosen for *trans* complementation in these nonpermissive cells because of its limited (~30%) sequence identity with the HIV-1 gene; this, therefore, minimized the likelihood of repairing the *vif* defect of HIV-1/Δ*vif* by recombination. The infected Jurkat cells were then cocultivated with the cells that we wished to employ as the virus producers (for example, H9/ΔVif or H9/Vif) using Transwells. Importantly, the 0.45-μm-pore-size membrane of the Transwell's filters allowed virus

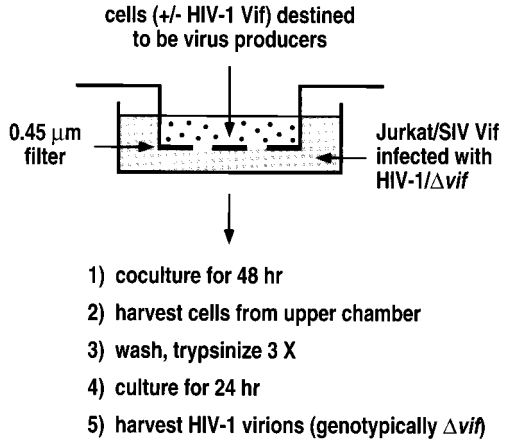

FIG. 2. Experimental strategy for the production of *vif*-deficient HIV-1 from nonpermissive cells. See text for details.

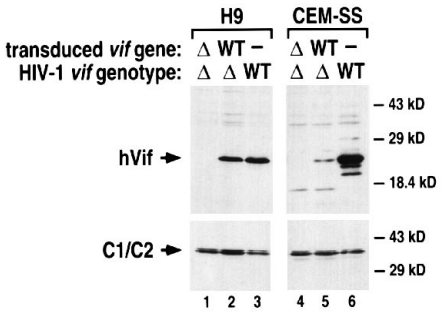

FIG. 3. Expression of Vif during the preparation of virus stocks; Western analysis of total cell lysates of H9 (lanes 1 to 3) and CEM-SS (lanes 4 to 6) cultures infected with HIV/Δ*vif* (lanes 1, 2, 4, and 5) or wild-type virus (lanes 3 and 6). The cultures were previously transduced with LN-M/hVif (lanes 1 and 4) or LN-M/ΔhVif (lanes 2 and 5) or were untransduced (lanes 3 and 6). Vif and the host cell C1/C2 proteins, the latter serving as a loading control, were visualized following initial hybridization with MAb319 and 4F4, respectively. The positions of prestained protein molecular mass standards (GIBCO BRL, Inc.) are indicated to the right.

to pass freely between the two cultures in the absence of any cell mixing. The culture destined to become the producer (upper chamber) was, therefore, exposed to a high titer of fresh virus for a prolonged time. After a series of trypsinization and washing steps (and DNase I treatments as appropriate), stocks of the genotypically *vif*-deficient HIV-1 were prepared from the cells of the upper chamber. The analyses of HIV-1/Δ*vif* stocks derived by this approach are described below.

HIV-1/Δ*vif* infectivity can be rescued by Vif expression in producer but not target cells. To evaluate the infection phenotypes of HIV-1/Δ*vif* derived from nonpermissive and permissive cells either in the presence or in the absence of Vif, virus stocks were obtained from H9 and CEM-SS cells transduced with LN-M/hVif or LN-M/ΔhVif by using the Transwell coculture system. Western analysis of whole-cell lysates prepared at the time of virus harvest confirmed that the Vif protein was present only in cultures that had been transduced with LN-M/hVif (Fig. 3; compare lanes 1, 2, 4, and 5 with the positive controls in lanes 3 and 6). Virus supernatants corresponding to 1 ng of $p24^{Gag}$ from each of these four stocks were then used to challenge the same four cell lines. The ability of a given virus-cell combination to support a productive and spreading infection during a 2- to 4-week period was scored by the induction of $p24^{Gag}$ expression in the culture medium and the appearance of cytopathic effects (Fig. 4).

A number of conclusions regarding the mode of action of Vif can be drawn from these data. First, a Δ*vif* virus expressed by a nonpermissive cell cannot be complemented (in other words, rescued) either by the expression of Vif in the cell being challenged or by the permissive nature of that target cell. This is inferred from the inability of HIV-1/Δ*vif* derived from a culture of H9/ΔVif to initiate a productive infection in any cell type (Fig. 4, column 1). Second, a Δ*vif* virus that is expressed by a nonpermissive cell can be rescued by the presence of Vif in that cell (Fig. 4, compare columns 1 and 2). Although such a virus is now infectious, a spreading infection that proceeds beyond the first round ensues only when the target culture is either permissive or expresses Vif (Fig. 4, compare rows 1, 2, and 4 with row 3). Third, HIV-1/Δ*vif* produced in permissive cells is phenotypically equivalent to virus derived from Vif-expressing nonpermissive cells regardless of whether Vif is present (Fig. 4, columns 2, 3, and 4). Taken together, these patterns of complementation imply that Vif functions at a stage of virus production (for example, assembly or maturation) that endows the released virions with the ability to undergo a productive infection following exposure to susceptible cells. These conclusions are not only consistent with those of others (4, 11, 16, 51) but also serve to validate our coculture strategy as a method for producing HIV-1 virions that carry a Δ*vif* genotype from permissive and nonpermissive cells.

In the following sections, we describe experiments in which the nature of the defect in Δ*vif* virus infections was evaluated. Although these data examine events that take place in the target cells, as opposed to the producer cells (in which the fate of the infection is actually determined), it was anticipated that defining the stage of infection beyond which a Δ*vif* virus fails to proceed would provide insight as to the possible role of Vif during virion production.

Vif is required for HIV-1 provirus establishment. As a first step toward defining the defect(s) in Δ*vif* infections, the influence of Vif on the formation of integrated double-stranded viral DNA, here termed the provirus, was determined. Since provirus establishment is required for significant HIV-1 gene expression (12, 31), we used the synthesis of one of the early gene products, the transcription *trans* activator Tat, as a surrogate marker for provirus formation. C8166 T cells were, therefore, stably transduced with a retroviral vector that harbored the *CAT* gene under the transcriptional regulation of the HIV-1 LTR (14). Productive infection of these C8166/HIV-*CAT* cells with wild-type HIV-1 results in the rapid accumulation of Tat and an ensuing efficient transcriptional stimulation not only of the newly formed provirus but also of the resident LTR-*CAT* cassette. Accordingly, the induction of CAT expression serves as a quantitative indicator of viral infection. This assay is, therefore, conceptually similar to the frequently used MAGI assay (30), except that we have used T cells rather than HeLa CD4 cells as the target of infection and CAT instead of β-galactosidase as the reporter gene.

C8166/HIV-*CAT* cells were challenged with normalized quantities of the four Transwell-derived stocks of HIV-1/Δ*vif* described above as well as with equivalent $p24^{Gag}$ levels of wild-type virus obtained from acute infections of parental H9 or CEM-SS cells. Following an incubation of 24 h, the level of CAT activity present in each culture was determined (Fig. 5). As expected, and in agreement with the lack of an effect of Vif on replication in permissive cells, all three virus stocks produced in CEM-SS cells exhibited similar infectivities. Importantly, and in sharp contrast, a *vif*-deficient virus expressed in nonpermissive H9 cells in the absence of Vif gave rise to a level of CAT activity that was ~50-fold less than that observed for the corresponding Vif-complemented virus and less than 2-fold

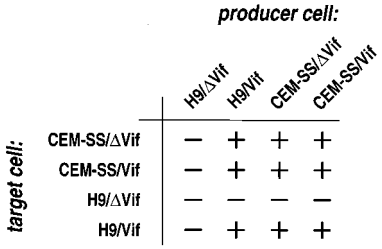

FIG. 4. Long-term growth phenotypes and complementation of *vif*-deficient HIV-1 propagated in nonpermissive and permissive T cells. Cells (2×10^6) were challenged with genotypically Δ*vif* viruses that were derived by Transwell coculturing and that corresponded to 1 ng of $p24^{Gag}$. The cultures were maintained in complete medium for 2 to 4 weeks, and virus replication was evaluated by ELISA for $p24^{Gag}$ accumulation and the appearance of cytopathic effects. +, marked cytopathic effect and >200 ng of $p24^{Gag}$ per ml; –, no cytopathic effect and <5 ng of $p24^{Gag}$ per ml.

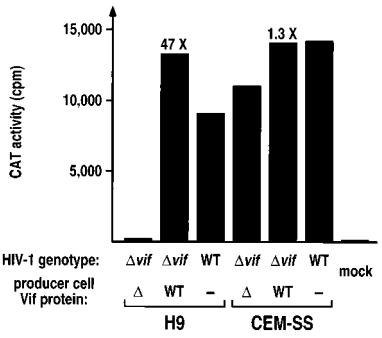

FIG. 5. Vif is required for HIV-1 provirus formation. C8166/HIV-*CAT* cells (0.5×10^6) were challenged with 1 ng of $p24^{Gag}$ of the indicated virus stocks. At 24 h, total cell lysates were prepared and the levels of CAT activity were determined. WT, wild type.

above that noted for the mock infection. As would be predicted, complementation in *trans*, even in these nonpermissive cells, was able to restore infectivity to a level that was comparable to that of the wild-type challenge. From these data, we concluded that HIV-1/Δ*vif* is severely attenuated in its ability to form proviruses following virus challenge. It was formally possible that proviruses that were transcriptionally inert and, therefore, unable to express Tat were, in fact, established in the infection with Δ*vif* virus from H9 cells. However, on the basis of the experiments that follow and the finding that equal levels of virus protein synthesis are initiated following the transfection of wild-type and Δ*vif* provirus expression plasmids into T cells (data not shown), this possibility was ruled out.

Vif is required for the stable accumulation of HIV-1 reverse transcripts. Having demonstrated that our coculture strategy yields virus stocks from nonpermissive T cells that are ~50-fold less infectious when Vif is absent, we wanted to determine the step of infection beyond which these Δ*vif* viruses are unable to proceed. Importantly, and as discussed above, this issue still awaits resolution. Highly permissive C8166 T cells were, therefore, challenged with Transwell-derived DNase I-treated stocks of HIV-1/Δ*vif* produced in H9/ΔVif and H9/Vif cells. At various times after the temperature of the infection was raised to 37°C, total cell lysates were prepared and evaluated for the presence of reverse transcripts by using semiquantitative PCR and Southern hybridization (Fig. 6). Each pair of primers was designed in accordance with the standard model for reverse transcription in which first (minus)-strand DNA synthesis commences at the 3′ boundary of the LTR (specifically the U5) sequences located at the 5′ terminus of the RNA and continues following its transfer to the 3′ terminus of the RNA. Second (plus)-strand DNA synthesis, which utilizes the minus-strand DNA as a template, is initiated at the 5′ boundary of the U3 sequences that ultimately become part of the proviral 3′ LTR and then continues into *gag* once its transfer to the extending end of the minus strand has taken place (7, 27). The U3+/U5− and *gag*+/*gag*− primer pairs, therefore, detect early first-strand transfer and extended first-strand DNAs, respectively, whereas the U3+/*gag*− primers are specific for the early second-strand transfer DNA. Of note, the first-strand transfer DNA that is detected by U3+/U5− is distinct from the initial pretransfer species, namely the strong-stop DNA, which has itself been found to be relatively abundant within cell-free virions (34, 50). In fact, the absence of a signal in any of the zero time point samples of this experiment demonstrates that all of the HIV-1 DNAs that were detected were the products of newly initiated reverse transcription.

Inspection of the analyses for first-strand transfer DNA (Fig. 6, upper panels) shows that substantial reverse transcription had taken place during the 1st h of infection for both Δ*vif* and Vif-complemented viruses and that peak accumulation of this replicative intermediate had occurred by 4 h postinfection. However, whereas the levels of this DNA were sustained throughout the infection with Vif-complemented (phenotypically wild-type) virus, they had diminished significantly by the later time points of the Δ*vif* infection (24- and 48-h samples). Strikingly similar results were obtained in the analyses of the first-strand extension DNA (Fig. 6, second set of panels) as well as of the second-strand transfer DNA (third set of panels). In particular, not only was each DNA species initially detected

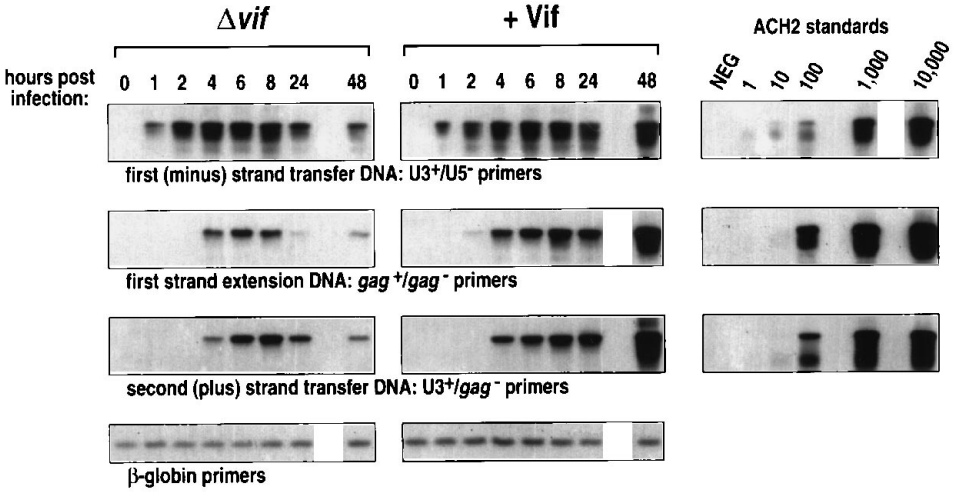

FIG. 6. Vif is required for the accumulation of reverse transcripts in HIV-1-infected T cells. C8166 cells were challenged with DNase I-treated stocks of HIV-1/Δ*vif* derived from nonpermissive H9 cells in the absence (left-hand panels) and presence (central panels) of Vif. At various times thereafter, total cell lysates were prepared from aliquots of 5×10^4 cells and were subjected to PCR analysis with primer pairs specific for the products of reverse transcription (upper three rows of panels) or β-globin (bottom panels). A dilution series of ACH2 cells was also subjected to amplification with each pair of HIV-1-specific primers (right-hand panels); these reactions served to confirm that the analyses were performed within the semiquantitative range. All reaction products were visualized by Southern hybridization and then by autoradiography. NEG, negative control.

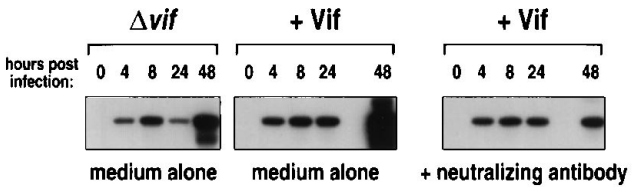

FIG. 7. Analysis of Vif's influence on HIV-1 reverse transcription in the absence of second-round infections. C8166 cells were challenged with DNase I-treated stocks of Δ*vif* virus derived from H9 cells in the absence (left-hand panel) and presence (center and right-hand panels) of Vif as described in the legend to Fig. 6. At 4 h postinfection, virus spread was inhibited in one of the Vif-complemented challenges by the addition of the neutralizing antibody D47 (right-hand panel). At various times during the infection, total cell lysates were prepared from 5×10^4 cells, analyzed by PCR with primers specific for first-strand extension DNA, and visualized by Southern hybridization and then by autoradiography.

at approximately 4 h postinfection for both viruses (although in this particular experiment, a trace amount of the extended first strand could be visualized in the wild-type infection at 2 h), but the levels of each were also markedly reduced by the later time points of the Δ*vif* but not the wild-type infection. Importantly, these dramatic differences in the abilities of Δ*vif* and wild-type HIV-1 infections to give rise to the sustained expression of reverse transcripts, seen most clearly here in the 24-h samples analyzed with the U3+/*gag*− and U3+/U5− primers, are consistent with the ~50-fold differential in infectivity already noted for this pair of viruses (Fig. 5).

The clear amplification in signal observed for all primer pairs with the Vif-complemented virus between 24 and 48 h postinfection is due to reverse transcription that occurred during second-round infections (see below). This was to be expected, since the C8166 target cells used for this experiment are, like CEM-SS cells (Fig. 4), known to be permissive for the replication of a genotypically Δ*vif* virus once a provirus has been established (45). In addition, and again as would be predicted, similar PCR-based analyses carried out with CEM-SS cells as virus producers demonstrated that Vif had no effect on the accumulation of reverse transcripts synthesized by HIV-1 derived from permissive cells (data not shown).

Although these data suggest that the diminution of HIV-1 reverse transcripts that was seen by the later times of infection was largely restricted to the Δ*vif* virus, it was formally possible that a similar turnover occurred in the Vif-complemented infection but that this was masked by the synthesis of reverse transcripts during second-round infections. Indeed, it has been noted previously that the levels of HIV-1 DNA do decline late in infections of T cells prior to the onset of second-round events (9, 29). To address this issue directly, we monitored the accumulation of reverse transcripts in wild-type infections of T cells in the absence of viral spread (Fig. 7). To accomplish this, C8166 cells were challenged with stocks of HIV-1/Δ*vif* derived from H9 cells as before; in this case, however, two Vif-complemented challenges were initiated and second-round infections were inhibited in one of them by the addition of the potent neutralizing antibody D47 to the culture medium (Fig. 7, right-hand panel). Cell lysates were then prepared at various times and were analyzed for the presence of viral DNA by PCR. As expected, the addition of D47 was extremely effective at preventing virus spread in a wild-type challenge (compare the 48-h samples in the center and right-hand panels). Most importantly, whereas the levels of reverse transcripts were maintained at a comparatively constant level in the presence of Vif and in the absence of second-round infections (right-hand panel), these HIV-1 DNAs diminished significantly by 24 h postinfection in cells challenged with the Δ*vif* virus (Fig. 6 and left-hand panel of Fig. 7).

Vif does not influence the nuclear import of HIV-1 nucleoprotein complexes. The above data suggest that the infection phenotype of HIV-1/Δ*vif* is not that reverse transcription fails to occur (4, 11) or that it is inherently nonprocessive (47, 51). Instead, these observations indicate that reverse transcription can occur normally in Δ*vif* infections but that the DNA products are not stably maintained in the challenged cell and are, therefore, degraded. There are a number of defined events that take place during a productive HIV-1 infection that occur subsequent to the second-strand transfer and that could conceivably impact on, or be affected by, DNA stability. Thus, for a *vif*-deficient infection (i) reverse transcription could be blocked at an even later stage than the second-strand transfer, (ii) trimming of the 3′ termini of the full-length linear DNA strands may not take place, (iii) the viral nucleoprotein complex might not be able to enter the nucleus, or (iv) the integration reaction itself could be inhibited. Unfortunately, the first two possibilities could not be addressed, since no quantitative assay that detects full-length linear plus-strand DNA or recessed 3′ ends at the level of sensitivity required for our Δ*vif* virus stocks is currently available. Moreover, it seemed unlikely that an inability to undergo integration would result in the inappropriate degradation of DNA, since reverse transcripts have been shown to be stably maintained in cells challenged with integrase-deficient HIV-1 (48). However, we were in a position to examine the possibility that Vif affects the import of nucleoprotein complexes into the nucleus (Fig. 8).

C8166 cultures were challenged with the two stocks of H9-derived HIV-1/Δ*vif*, and the cells were fractionated into cytoplasmic and nuclear samples at various times. All fractions were then analyzed by PCR for the presence of either HIV-1 DNA or, in the same reactions, cytochrome *b* DNA (a cytoplasmic gene) and β-globin DNA (a nuclear gene). Inspection of the viral DNA analyses (Fig. 8, upper panels) revealed that greater than 90% of newly synthesized reverse transcripts were localized to the nucleus (right-hand panels) by the 2-h time point in both the Δ*vif* and wild-type infections. This conclusion is valid, even though the signals for cytochrome *b* DNA were similar in the nuclear and cytoplasmic samples (lower panels), since the levels of HIV-1 DNA detected in the fractions that were free of nuclear DNA and, therefore, contained only cytochrome *b* DNA (left-hand panels) were always markedly lower than those in the β-globin DNA-containing counterparts. Importantly, our finding that the bulk of HIV-1 DNA can be found in the nucleus (or at least associated with the nucleus) within 2 h of infection is consistent with recent observations showing that protein components of HIV-1 preintegration complexes accumulate in the nucleus by 4 h postinfection (18, 19). Moreover, our data also suggest that Vif does not influence the rapid nuclear import of actively reverse transcribing viral nucleoprotein complexes and that the degradation of HIV-1 DNA that is seen in these Δ*vif* infections of C8166 cells likely occurs in the nucleus.

DISCUSSION

Although it has been firmly established that the HIV-1 Vif protein is essential for virus replication in primary cells (11, 16, 17, 28, 44, 51) (Fig. 1), the definition of the defect in *vif*-deficient virus infections has remained elusive and controversial. Here, we have used virus preparations derived from single-cycle infections of nonpermissive cells to analyze the infection phenotype of HIV-1/Δ*vif*. Surprisingly, we found that Δ*vif* virus is able to penetrate susceptible C8166 T cells and

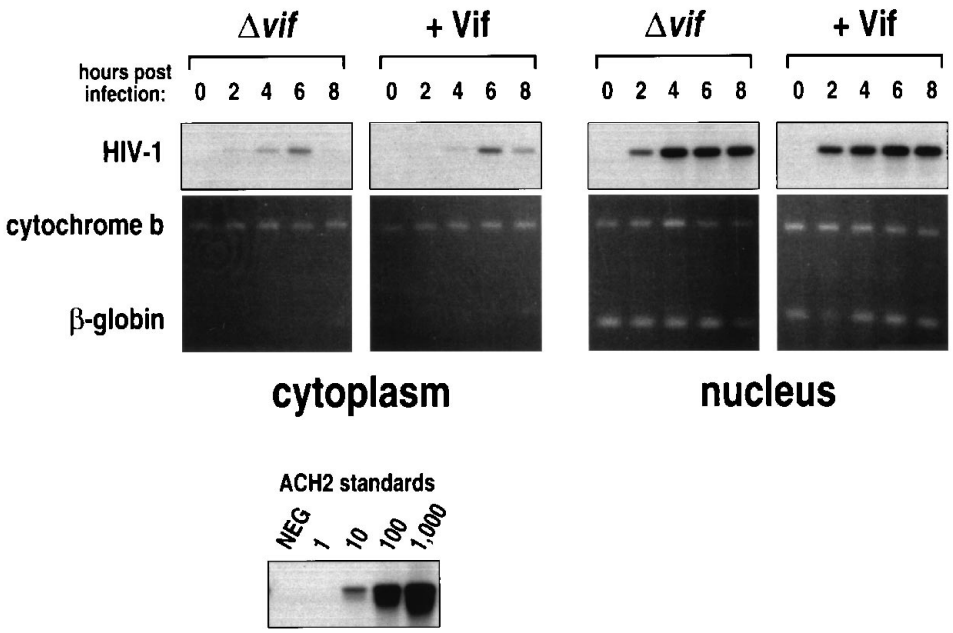

FIG. 8. Vif does not affect the nuclear import of HIV-1 nucleoprotein complexes. C8166 cells were challenged with DNase I-treated stocks of Δ*vif* virus derived from H9 cells in the absence and presence of Vif as described in the legend to Fig. 6. At various times thereafter, 5×10^4 cells were fractionated into cytoplasmic (left-hand set of panels) and nuclear (right-hand set of panels) samples and analyzed by PCR with primers specific for first-strand transfer DNA (top panels) or the host cell cytochrome *b* and β-globin genes (bottom panels). ACH2 controls were employed for the analysis of HIV-1 DNA as described in the legend to Fig. 6. The virus-specific reaction products were visualized by Southern hybridization and autoradiography, whereas the cytochrome *b* and β-globin-specific products were analyzed by ethidium bromide staining. NEG, negative control.

initiate reverse transcription in a manner that is indistinguishable from that of wild-type HIV-1 (Fig. 6). In particular, neither the kinetics with which the first-strand transfer, first-strand extension, and second-strand transfer DNAs appear nor the levels to which these reverse transcription intermediates initially accumulate are substantially influenced by Vif in these cells. However, whereas these newly synthesized reverse transcripts are stably maintained following challenge with virus expressed in the presence of Vif, the vast majority of them are degraded by the later time points of Δ*vif* infections. In agreement with the observed turnover of viral DNA in Δ*vif* infections, provirus formation was noted to be ~50-fold-less efficient for Δ*vif* viruses expressed in nonpermissive cells (Fig. 5). Thus, Vif does not appear to influence the ability of penetrated HIV-1 nucleocapsids to undergo DNA synthesis by directly affecting the enzymatic activity of reverse transcriptase itself. Rather, our data imply that Vif is required for the protection of those reverse transcripts from degradation within virally infected cells.

Our finding that Δ*vif* infections of C8166 T cells abort following the second-strand transfer of reverse transcription differs from earlier reports in which Δ*vif* viruses gave rise to undetectable (4, 11) or reduced (47, 51) levels of reverse transcription. Although it is difficult to provide rational explanations for all of these differences, there are a few points that are worth noting. First, many more time points were examined in our PCR-based analyses of infections (Fig. 6) than in those of others. It is, therefore, plausible that transient accumulations of reverse transcripts in Δ*vif* virus infections could have been overlooked in some cases. Second, the T cells that we utilized as virus producers are absolutely nonpermissive to the replication of Δ*vif* viruses. Notably, some of these other groups derived their virus stocks in cells that are not truly nonpermissive, in that they do allow restricted levels of HIV-1/Δ*vif* replication. As would be expected, such preparations of Δ*vif* virus are able to maintain levels of reverse transcripts in challenged cells that are less than that for wild-type virus but nevertheless significant (data not shown); a failure to detect the transient peak in viral DNA production could, therefore, give the appearance of reduced reverse transcriptase processivity in the absence of Vif. Third, a major point of difference between our studies and those of others is the choice of target cell. Here, we have used C8166 T cells because we have found them to be highly infectable by HIV-1 and, therefore, exquisitely sensitive to infection by low titers of virus (data not shown). In contrast, other groups have used less-susceptible cells such as H9, MT-2, and PBLs (11, 47, 51). We speculate that cells of this genre might provide a more hostile environment for incoming Δ*vif* nucleocapsids such that the protection they afford to viral RNA and DNA may be much shorter lived than what we have observed for C8166 cells. As a result, reverse transcription could be arrested at very early time points or, even more extremely, could fail to take place. Thus, the defect(s) in Δ*vif* infections could, in the former case, appear to be at the level of reverse transcription processivity (47, 51) or, in the latter case, appear to be at viral entry or uncoating (11). Fourth, our observation that the progression of viral DNA synthesis during the early stages of HIV-1 infection is equivalent for Δ*vif* and wild-type viruses implies that Vif does not influence viral entry. This conclusion is entirely consistent with the finding that Vif has no effect on the efficiency of HIV-1 nucleocapsid uptake into CD4-positive cells (51).

The most important result of the analyses described here is that Vif expression does not modulate the biosynthetic potential of virion reverse transcriptase per se. Instead, it appears that Vif is required for the sustained accumulation of reverse transcripts in cells challenged with HIV-1 and that its absence culminates in the degradation of these DNAs. The key issue

concerning Vif function is, therefore, the elucidation of the mechanism by which the presence of this protein in virus-producing cells endows the expressed virions with the ability to synthesize and maintain viral DNA in infected cells and, as a result, to proceed to provirus formation. Interestingly, abnormalities in the ultrastructure of *vif*-deficient virus cores have been observed by electron microscopy (4, 26). Thus, it is tempting to speculate that the nucleoprotein complexes that are formed following the penetration of Δ*vif* viruses are also structurally altered; such differences might then translate into complex instability, susceptibility to nuclease attack, and the eventual clearance of reverse transcripts from the interior of the infected cell.

Although qualitative and quantitative alterations in the protein composition of Δ*vif* viruses have been described elsewhere (4, 42, 44), no consensus view as to the relevant difference(s) has emerged. Moreover, it also remains to be established whether the presence of Vif itself within virions (4, 33) is required for the acquisition of the infectious phenotype. Gaining greater insight in these difficult areas will clearly be critical to understanding the essential role that Vif plays in the replication of HIV-1 in vivo.

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REFERENCES

1. **Aiken, C., J. Konner, N. R. Landau, M. E. Lenburg, and D. Trono.** 1994. Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain. Cell **76:**853–864.
2. **Aiken, C., and D. Trono.** 1995. Nef stimulates human immunodeficiency virus type 1 proviral DNA synthesis. J. Virol. **69:**5048–5056.
3. **Balliet, J. W., D. L. Kolson, G. Eiger, F. M. Kim, K. A. McGann, A. Srinivasan, and R. Collman.** 1994. Distinct effects in primary macrophages and lymphocytes of the human immunodeficiency virus type 1 accessory genes *vpr*, *vpu*, and *nef*: mutational analysis of a primary HIV-1 isolate. Virology **200:**623–631.
4. **Borman, A. M., C. Quillent, P. Charneau, C. Dauguet, and F. Clavel.** 1995. Human immunodeficiency virus type 1 Vif$^-$ mutant particles from restrictive cells: role of Vif in correct particle assembly and infectivity. J. Virol. **69:**2058–2067.
5. **Broder, C. C., P. L. Earl, D. Long, S. T. Abedon, B. Moss, and R. W. Doms.** 1994. Antigenic implications of human immunodeficiency virus type 1 envelope quaternary structure: oligomer-specific and -sensitive monoclonal antibodies. Proc. Natl. Acad. Sci. USA **91:**11699–11703.
6. **Chowers, M. Y., M. W. Pandori, C. A. Spina, D. D. Richman, and J. C. Guatelli.** 1995. The growth advantage conferred by HIV-1 *nef* is determined at the level of viral DNA formation and is independent of CD4 downregulation. Virology **212:**451–457.
7. **Coffin, J. M.** 1990. Retroviridae and their replication, p. 1437–1500. *In* B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsch, J. L. Melnick, T. P. Monath, and B. Roizman (ed.), Virology. Raven Press, New York.
8. **Cohen, E. A., G. Dehni, J. G. Sodroski, and W. A. Haseltine.** 1990. Human immunodeficiency virus *vpr* product is a virion-associated regulatory protein. J. Virol. **64:**3097–3099.
9. **Collin, M., and S. Gorden.** 1994. The kinetics of human immunodeficiency virus reverse transcription are slower in primary human macrophages than in a lymphoid cell line. Virology **200:**114–120.
10. **Connor, R. I., B. K. Chen, S. Choe, and N. R. Landau.** 1995. Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes. Virology **206:**935–944.
11. **Courcoul, M., C. Patience, F. Rey, D. Blanc, A. Harmache, J. Sire, R. Vigne, and B. Spire.** 1995. Peripheral blood mononuclear cells produce normal amounts of defective Vif$^-$ human immunodeficiency virus type 1 particle which are restricted for the preretrotranscription steps. J. Virol. **69:**2068–2074.
12. **Engelman, A., G. Englund, J. M. Orenstein, M. A. Martin, and R. Craigie.** 1995. Multiple effects of mutations in human immunodeficiency virus type 1 integrase on viral replication. J. Virol. **69:**2729–2736.
13. **Fan, L., and K. Peden.** 1992. Cell-free transmission of Vif mutants of HIV-1. Virology **190:**19–29.
14. **Felber, B. K., and G. N. Pavlakis.** 1988. A quantitative bioassay for HIV-1 based on trans-activation. Science **239:**184–187.
15. **Fisher, A. G., B. Ensoli, L. Ivanoff, M. Chamberlain, S. Petteway, L. Ratner, R. C. Gallo, and F. Wong-Staal.** 1987. The *sor* gene of HIV-1 is required for efficient virus transmission in vitro. Science **237:**888–893.
16. **Gabuzda, D. H., K. Lawrence, E. Langhoff, E. Terwilliger, T. Dorfman, W. A. Haseltine, and J. Sodroski.** 1992. Role of *vif* in replication of human immunodeficiency virus type 1 in CD4$^+$ T lymphocytes. J. Virol. **66:**6489–6495.
17. **Gabuzda, D. H., H. Li, K. Lawrence, B. S. Vasir, K. Crawford, and E. Langhoff.** 1994. Essential role of vif in establishing productive HIV-1 infection in peripheral blood T lymphocytes and monocyte/macrophages. J. Acquired Immune Defic. Syndr. **7:**908–915.
18. **Gallay, P., S. Swingler, C. Aiken, and D. Trono.** 1995. HIV-1 infection of nondividing cells: C-terminal tyrosine phosphorylation of the viral matrix protein is a key regulator. Cell **80:**379–388.
19. **Gallay, P., S. Swingler, J. Song, F. Bushman, and D. Trono.** 1995. HIV nuclear import is governed by the phosphotyrosine-mediated binding of matrix to the core domain of integrase. Cell **83:**569–576.
20. **Garcia, J. V., and A. D. Miller.** 1991. Serine phosphorylation-independent downregulation of cell-surface CD4 by *nef*. Nature (London) **350:**508–511.
21. **Gibbs, J. S., D. A. Regier, and R. C. Desrosiers.** 1994. Construction and *in vitro* properties of SIV_{mac} mutants with deletions in "nonessential" genes. AIDS Res. Hum. Retroviruses **10:**607–616.
22. **Goldsmith, M. A., M. T. Warmerdam, R. E. Atchison, M. D. Miller, and W. C. Greene.** 1995. Dissociation of the CD4 downregulation and viral infectivity enhancement functions of human immunodeficiency virus type 1 Nef. J. Virol. **69:**4112–4121.
23. **Guy, B., M. Geist, K. Dott, D. Spehner, M.-P. Kieny, and J.-P. Lecocq.** 1991. A specific inhibitor of cysteine proteases impairs a Vif-dependent modification of human immunodeficiency virus type 1 Env protein. J. Virol. **65:**1325–1331.
24. **Harmache, A., M. Bouyac, G. Audoly, C. Hieblot, P. Peveri, R. Vigne, and M. Suzan.** 1995. The *vif* gene is essential for efficient replication of caprine arthritis encephalitis virus in goat synovial membrane cells and affects the late steps of the virus replication cycle. J. Virol. **69:**3247–3257.
25. **Heinzinger, N. K., M. I. Bukrinsky, S. A. Haggerty, A. M. Ragland, V. Kewalramani, M.-A. Lee, H. E. Gendelman, L. Ratner, M. Stevenson, and M. Emerman.** 1994. The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. Proc. Natl. Acad. Sci. USA **91:**7311–7315.
26. **Höglund, S., Å. Öhagen, K. Lawrence, and D. Gabuzda.** 1994. Role of *vif* during packing of the core of HIV-1. Virology **201:**349–355.
27. **Katz, R. A., and A. M. Skalka.** 1994. The retroviral enzymes. Annu. Rev. Biochem. **63:**133–173.
28. **Kawamura, M., T. Ishizaki, A. Ishoto, T. Shioda, T. Kitamura, and A. Adachi.** 1994. Growth ability of human immunodeficiency virus type 1 auxiliary gene mutants in primary blood macrophage cultures. J. Gen. Virol. **75:**2427–2431.
29. **Kim, S., R. Byrn, J. Groopman, and D. Baltimore.** 1989. Temporal aspects of DNA and RNA synthesis during human immunodeficiency virus infection: evidence for differential gene expression. J. Virol. **63:**3708–3713.
30. **Kimpton, J., and M. Emerman.** 1992. Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated β-galactosidase gene. J. Virol. **66:**2232–2239.
31. **LaFemina, R. L., C. L. Schneider, H. L. Robbins, P. L. Callahan, K. LeGrow, E. Roth, W. A. Schleif, and E. A. Emini.** 1992. Requirement of active human immunodeficiency virus type 1 integrase enzyme for productive infection of human T-lymphoid cells. J. Virol. **66:**7414–7419.
32. **Landau, N. R., and D. R. Littman.** 1992. Packaging system for rapid production of murine leukemia virus vectors with variable tropism. J. Virol. **66:**5110–5113.
33. **Liu, H., X. Wu, M. Newman, G. M. Shaw, B. H. Hahn, and J. C. Kappes.** 1995. The Vif protein of human and simian immunodeficiency viruses is packaged into virions and associates with viral core structures. J. Virol. **69:**7630–7638.
34. **Lori, R., F. di Marzo Veronese, A. L. De Vico, P. Lusso, Jr., M. S. Reitz, and R. C. Gallo.** 1992. Viral DNA carried by human immunodeficiency virus type 1 virions. J. Virol. **66:**5067–5074.
35. **Lu, Y.-L., P. Spearman, and L. Ratner.** 1993. Human immunodeficiency virus type 1 viral protein R localization in infected cells and virions. J. Virol. **67:**6542–6550.
36. **Michaels, F. H., N. Hattori, R. C. Gallo, and G. Franchini.** 1994. The human immunodeficiency virus type 1 (HIV-1) vif protein is located in the cytoplasm of infected cells and its effect on viral replication is equivalent in HIV-2. AIDS Res. Hum. Retroviruses **9:**1025–1030.
37. **Miller, M. D., M. T. Warmerdam, I. Gaston, W. C. Greene, and M. B.**

Feinberg. 1994. The human immunodeficiency virus-1 *nef* gene product: a positive factor for viral infection and replication in primary lymphocytes and macrophages. J. Exp. Med. **179:**101–113.
38. **Neumann, J. R., C. A. Morency, and K. O. Russian.** 1987. A novel rapid assay for chloramphenicol acetyltransferase gene expression. BioTechniques **5:** 444–447.
39. **Oberste, M. S., and M. A. Gonda.** 1992. Conservation of amino-acid sequence motifs in lentivirus Vif proteins. Virus Genes **6:**95–102.
40. **Paxton, W., R. I. Connor, and N. R. Landau.** 1993. Incorporation of Vpr into human immunodeficiency virus type 1 virions: requirement for the p6 region of *gag* and mutational analysis. J. Virol. **67:**7229–7237.
41. **Piñol-Roma, S., and G. Dreyfuss.** 1991. Transcription-dependent and transcription-independent nuclear transport of hnRNP proteins. Science **253:** 312–314.
42. **Sakai, H., R. Shibata, J.-I. Sakuragi, S. Sakuragi, M. Kawamura, and A. Adachi.** 1993. Cell-dependent requirement of human immunodeficiency virus type 1 Vif protein for maturation of virus particles. J. Virol. **67:**1663–1666.
43. **Shacklett, B. L., and P. A. Luciw.** 1994. Analysis of the *VIF* gene of feline immunodeficiency virus. Virology **204:**860–867.
44. **Simm, M., M. Shahabuddin, W. Chao, J. S. Allan, and D. J. Volsky.** 1995. Aberrant Gag protein composition of a human immunodeficiency virus type 1 *vif* mutant produced in primary lymphocytes. J. Virol. **69:**4582–4586.
45. **Simon, J. H. M., T. E. Southerling, J. C. Peterson, B. E. Meyer, and M. H. Malim.** 1995. Complementation of *vif*-defective human immunodeficiency virus type 1 by primate, but not nonprimate, lentivirus *vif* genes. J. Virol. **69:** 4166–4172.
46. **Sodroski, J., W. C. Goh, C. Rosen, A. Tartar, D. Portetelle, A. Burny, and W. Haseltine.** 1986. Replicative and cytopathic potential of HTLV-III/LAV with *sor* gene deletions. Science **231:**1549–1553.
47. **Sova, P., and D. J. Volsky.** 1993. Efficiency of viral DNA synthesis during infection of permissive and nonpermissive cells with *vif*-negative human immunodeficiency virus type 1. J. Virol. **67:**6322–6326.
48. **Stevenson, M., T. L. Stanwick, M. P. Dempsey, and C. A. Lamonica.** 1990. HIV-1 replication is controlled at the level of T cell activation and proviral integration. EMBO J. **9:**1551–1560.
49. **Tomonaga, K., J. Norimine, Y.-S. Shin, M. Fukasawa, T. Miyazawa, A. Adachi, T. Toyosaki, Y. Kawaguchi, C. Kai, and T. Mikami.** 1992. Identification of a feline immunodeficiency virus gene which is essential for cell-free virus infectivity. J. Virol. **66:**6181–6185.
50. **Trono, D.** 1992. Partial reverse transcripts in virions from human immunodeficiency and murine leukemia viruses. J. Virol. **66:**4893–4900.
51. **von Schwedler, U., J. Song, C. Aiken, and D. Trono.** 1993. *vif* is crucial for human immunodeficiency virus type 1 proviral DNA synthesis in infected cells. J. Virol. **67:**4945–4955.
52. **Yuan, X., Z. Matsuda, M. Matsuda, M. Essex, and T.-H. Lee.** 1990. Human immunodeficiency virus *vpr* gene encodes a virion-associated protein. AIDS Res. Hum. Retroviruses **6:**1265–1271.