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Peripheral Blood Mononuclear Cells Produce Normal Amounts of Defective Vif⁻ Human Immunodeficiency Virus Type 1 Particles Which Are Restricted for the Preretrotranscription Steps

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Previous studies have demonstrated the absence of viral replication of Vif⁻ mutants in stimulated primary blood mononuclear cells (PBMC). Human immunodeficiency virus type 1 strain NDK Vif⁻ mutants were propagated on the semipermissive CEM cell line, and the viral stock obtained was compared with the wild-type virus during a single cycle in PBMC. The Vif⁻ virus was able to enter PBMC with the same efficiency as the wild type, as demonstrated by quantification of the strong-stop cDNA, and retrotranscription was observed for both viruses within 4 h postinfection. Using a PCR assay with an *Alu*-long terminal repeat pair of primers, we detected integration for both the wild-type and Vif⁻ viruses. We then used qualitative and quantitative reverse transcription-mediated PCR techniques to study the steady-state level of intracellular and extracellular viral RNAs. All mRNA species were detected in PBMC infected with the wild-type virus or with the Vif⁻ virus 36 h postinfection. Furthermore, quantification of viral RNA released from infected cells demonstrated similar levels of virus produced after a unique cycle of replication. However, the Vif⁻ virus obtained after one replication cycle in PBMC was unable to initiate retrotranscription in permissive target cells. These data strongly suggest that the failure to infect target cells is due to a defect in the formation of the viral particle in PBMC.

Human immunodeficiency virus type 1 (HIV-1) has several regulatory genes, *tat*, *rev*, *vpr*, *vif*, *nef*, and *vpu* (32). The *vif* gene of HIV-1 has so far been considered an accessory gene. It encodes a 23-kDa protein which is absent from virions but present in infected cells (17). Recent observations have indicated that HIV-1 Vif is located in the cytoplasm both in a cytosolic form and associated with the cytoplasmic side of cellular membranes (12). This protein is encoded by all lentivirus genomes except that of equine infectious anemia virus (19) and is antigenic, since antibodies against Vif are detected in the sera of infected individuals (1, 17). The first functional studies concerning HIV-1 *vif* indicated that this gene was required for efficient virus transmission, since Vif⁻ mutants were described to be 10^2 to 10^3 less infectious than wild-type virus in a cell-free infection assay (29). Cell-to-cell transmission has also been reported to require *vif* activity (8). More recent studies have demonstrated that the requirement for *vif* is cell type dependent (7, 10, 22, 26, 31); although most $CD4^+$ continuous cell lines are permissive for Vif⁻ mutants, others, including H9 cells and peripheral blood mononuclear cells (PBMC), do not allow the replication of these mutants. Furthermore, certain cell lines are semipermissive for Vif⁻ virus replication and have been used to grow Vif⁻ viral stocks. In vivo experiments using Vif⁻ simian immunodeficiency viruses have shown a total absence of infection of the animals inoculated with such mutants (5), demonstrating a crucial role for *vif* in vivo.

The mechanism of *vif* action is still unclear; the fact that the Vif protein is encoded by a single spliced 5-kb Rev-dependent mRNA (25) suggests that *vif* acts at the late steps of the virus life cycle. Some reports have suggested that the role of *vif* could be either a qualitative (13) or a quantitative (22) modulation of the envelope glycoproteins. Other studies have indicated that Vif⁻ mutants are able to enter permissive cells (4) but are unable to integrate into the cell genome (26, 31) either because of a block in the reverse transcription or because of nonefficient nucleocapsid internalization. However, all these studies have investigated the role of *vif* in continuous cell lines and not in primary cells; the latter system not only is described as the most restrictive system for the replication of *vif* mutants but also is a more relevant physiological model.

In this report, in which the replicative cycle of a HIV-1 Vif⁻ virus in PBMC is examined, we show that Vif is active at a late stage in viral replication, subsequent to integration. When Vif⁻ and wild-type virus are produced in semipermissive CEM cells, no quantitative difference can be observed during the first replication cycle in PBMC up to and including virus production. However, the resulting Vif⁻ virus progeny is strongly restricted for the preretrotranscription steps, suggesting a severe defect in the construction of the viral particle in the absence of *vif*.

MATERIALS AND METHODS

Viral molecular clones and viral stocks. The wild-type and Vif⁻ HIV-1_{NDK} molecular clones have been described elsewhere (14, 28) and were used throughout this study. The Vif⁻ HIV-1_{NDK} molecular clone was obtained by deleting 240 bp in the *vif* open reading frame (from nucleotides [nt] 5113 to 5353). Viral

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stocks were obtained after transfection of these clones into CEM cells (9), obtained from Robin Weiss's laboratory, by using the DEAE-dextran transfection method (6). This cell line has been reported to be semipermissive for Vif$^-$ virus replication (10, 15, 26, 31). Titers of the stocks were determined by measuring reverse transcriptase (RT) activity (20) and by assaying the fusogenic properties on C8166 cells (23). This cell line was also used to determine the 50% tissue culture infective dose ($TCID_{50}$) of viral stocks. All viral stocks were treated with 20 μg of DNase per ml in the presence of 10 mM $MgCl_2$ for 30 min at 30°C. PBMC viral supernatants were assayed for the detection of p24 by the means of a HIV-1 p24 core profile enzyme-linked immunosorbent assay kit (Dupont).

Isolation, propagation, and infection of PBMC. PBMC were obtained from a normal donor lymphopheresis sample which was aliquoted and stored in liquid nitrogen. After each thawing, PBMC were stimulated with 1/500 phytohemagglutinin (PHA) P (Difco) and cultivated in RPMI supplemented with 15% fetal calf serum and 10% interleukin-2 (Boehringer Mannheim). Three days after stimulation, the medium was replaced by a PHA-free medium supplemented with 15% fetal calf serum and 10% interleukin-2, and PBMC were allowed to grow for 2 days before being infected.

PBMC were infected with virus at a multiplicity of infection (MOI) of 1 $TCID_{50}$ per cell. Infection was usually performed for 1 h at 37°C in the presence of 20 μg of DEAE-dextran per ml, which was omitted when entry PCR was the purpose of the experiment. In each experiment, PBMC were also infected with heat-inactivated virus (30 min at 56°C) as a negative control (27). PBMC were then washed twice with serum-free RPMI and incubated with 15 μg of trypsin type XIII (Sigma) per ml at 4°C to remove any virus particles bound at the membrane surface. PBMC were again washed twice with serum-free RPMI before being grown in RPMI supplemented with 10% fetal calf serum and 10% interleukin-2.

Preparation of DNA for PCR amplification. Cells (10^6) were lysed by incubation in 1 ml of a buffer containing 20 mM Tris (pH 8.5), 100 mM KCl, 0.8% Nonidet P-40, and 0.1 mg of proteinase K per ml at 56°C for 3 h. DNA samples were heated at 95°C for 15 min. For each amplification reaction, 25 μl of the DNA sample was taken.

Preparation of RNA for RT-mediated PCR (RT-PCR) amplification. (i) Cellular RNAs. Cells (2.5×10^7) were lysed in 8 ml of 6 M guanidium isothiocyanate–0.5% sodium lauryl sarcosinate–5 mM sodium citrate–0.1 M β-mercaptoethanol at pH 7. This cell extract was centrifuged through a 5.7 M cesium chloride gradient at 30,000 rpm for 18 h in a Kontron TST41 rotor. The RNA pellet was resuspended in diethyl pyrocarbonate-treated water at a concentration of 1 μg/ml.

(ii) Viral RNA. Twelve milliliters of infected cell supernatant containing virus was concentrated by ultracentrifugation in a Beckman SW41 rotor for 90 min at 35,000 rpm. The viral pellet was lysed in 200 μl of RNA_{zol} (Bioprobe), and the RNA was then resuspended in a final volume of 10 μl in diethyl pyrocarbonate-treated water after purification as instructed by manufacturer (Bioprobe).

PCR assays, primers, and oligonucleotide probes. PCRs were performed as described previously (21). Briefly, 25 μl of DNA prepared as previously indicated was assayed in a final volume of 50 μl in the presence of 1.5 mM $MgCl_2$, 100 ng of each amplification primer, 0.2 mM each deoxynucleoside triphosphate, and 2.5 U of *Taq* polymerase (Promega). Samples were run on a Hybaid thermocycler for 30 cycles, with 30 s at 94°C, 60 s at an annealing temperature (determined as the lowest melting temperature of both primers empirically diminished by 5°C), and 2 min at 72°C. For RT-PCR, 2 μg of RNA was retrotranscribed in 20 μl with 10 U of avian myeloblastosis virus RT (Promega), using 0.3 μg of antisense primer in the buffer supplied with the enzyme source. Retrotranscription was carried out at 50°C for 20 min immediately after a linearization step of 3 min at 65°C. Following retrotranscription, 2 μl of the product was assayed by PCR using the commercial buffer (Promega) and the same conditions for primers and cycles as described above.

After amplification, each sample was run on an agarose gel, transferred to a nylon membrane (GeneScreen Plus), and hybridized with a ^{32}P-labeled specific oligonucleotide. Hybridization was performed at 58°C for 16 h in 10% dextran sulfate–1 M NaCl–1% sodium dodecyl sulfate (SDS)–25 mM Tris (pH 7.5)–100 μg of salmon sperm DNA per ml. Filters were washed twice for 30 min in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% SDS at 58°C before being exposed.

(i) Detection of the strong-stop cDNA (entry PCR). The method used has been described by others and ourselves (4, 11, 33). The primers and probe used were R (sense primer; 5′ggctaactagggaacccactgg3′, nt 495 to 516), U5 (antisense primer; 5′ctgctagagattttccacactgac3′, nt 635 to 612), and the long terminal repeat (LTR) oligonucleotide LTR3 (hybridization oligonucleotide probe; 5′gtgtgtgc ccgtctgttgtgtg3′, nt 556 to 567).

(ii) Detection of the proviral DNA (central region). The primers and probe used were Vif$^+$ (sense primer; 5′gggacagcagagatccaatttgg3′, nt 4903 to 4925), Vif$^-$ (antisense primer); 5′gtccattcattgtatggctccc3′, nt 5605 to 5584), and Vif_H (hybridization oligonucleotide probe; 5′ggactaactatatgtcctaatatgg3′, nt 5423 to 5372).

(iii) Detection of the proviral DNA (second jump region). The primers used were U3 (sense; 5′gattggcagaactacacaccag3′, nt 78 to 99) and *gag* (antisense; 5′gctctcgcacccatctctctcc3′, nt 803 to 781. The U5 oligonucleotide described above was used as the hybridization probe.

(iv) Detection of integrated proviral DNA (*Alu* PCR). The method used has been previously described (3). A first PCR round was performed with the *Alu* primer (5′gcctcccaaagtgctgggatta3′), which is representative of redundant *Alu* sequences disseminated along the human genome, and the *gag* primer. This reaction was then nested by a second PCR round using the same *Alu* primer and the U5 primer. The specificity of the product was assayed by Southern blotting, and hybridization was performed with the ^{32}P-labeled internal LTR3 primer.

(v) Detection of *tat* mRNA. The primers and probe used were *tat1* (sense primer; 5′atggatccagtagatcctaatctag3′, nt 5820 to 5846), *tat2* (antisense primer); 5′gtcccctcgggactgggagg3′, nt 8364 to 8344), and tat_H (hybridization oligonucleotide probe; 5′gtctccgcttcttcctgccat3′, nt 5980 to 5960).

(vi) Detection of all other mRNAs. The R primer located in the leader sequence was used as the sense primer. The antisense primers were the following, corresponding to the mRNA for each region target: *nef* (5′cagggaagtagccttgtgtgtgg3′, nt 9123 to 9100) and *env* (5′ggtacacaggcatgtgtggccc3′, nt 6438 to 6417). For *vpr*, the Vif$^-$ primer was used since it is located immediately in 3′ of the splice site of the *vpr* open reading frame (25); for genomic RNA, the *gag* primer was used.

Specificity of the RT-PCR product was assayed by Southern blotting, and hybridization was done with the ^{32}P-labeled internal LTR3 primer.

Quantitative competitive RT-PCR. The competitor plasmid was constructed by deleting 50 bp from the unique *Bss*HII site (nt 711) of the HIV-1_{NDK} molecular clone, using BAL 31 nuclease. Amplification of this plasmid with the primers used to detect the genomic viral RNA (R and *gag*) therefore leads to a product slightly shorter than the RT-PCR product obtained from the RNA samples. cDNA samples (2 μl) were coamplified with serial dilutions of the competitor plasmid (in a range between 10 pg and 1 fg) in order to determine the 50% competing dilution in the same conditions as previously described.

RESULTS

Absence of replication of Vif$^-$ mutants in PBMC. We previously described the phenotype of Vif$^-$ virus propagated in CEM cells. We have shown that these viruses possess a normal infectivity in CEM cells, although the peak of RT activity after infection of these cells at a low MOI is lower in Vif$^-$ virus-infected cells than in wild-type virus-infected cells (4). When the amount of cell-free virus used to infect CEM cells is increased, the differential between the Vif$^-$ and wild-type viruses is no longer observed, indicating that these CEM cells are not very restrictive for Vif$^-$ mutants. Infection at a very high MOI can be achieved very easily, since HIV-1_{NDK} was used in these studies; this strain has been described as highly replicative (28), and highly infectious viral stocks (titers determined) in C8166 cells) can be obtained without requiring virus concentration. We took advantage of this point to examine whether we could produce a Vif$^-$ virus infection in primary cells by using a high MOI. PHA-stimulated PBMC (target cells) were infected, at 1 $TCID_{50}$ per cell, with Vif$^-$ or Vif$^+$ virus produced in CEM cells (source cells), and RT activity was monitored in the culture supernatant for 4 weeks postinfection. As shown in Fig. 1 and in accordance with previous observations (7, 31), the Vif$^-$ virus was not able to productively infect PBMC. Wild-type virus production can be observed from 7 days after infection, indicating that several rounds of virus replication are required before RT activity becomes detectable. To determine whether Vif$^-$ virus replication was blocked within the first replication cycle, infected PBMC were cocultivated 18 h postinfection with the indicator cell line C8166 (Table 1). This time point is earlier than that described in the literature as necessary to achieve a complete viral replicative cycle (16, 33). No syncytia were observed in the C8166 cells cocultivated with 10^6 Vif$^-$ virus-infected PBMC, while 10^2 wild-type virus-infected PBMC induced syncytium formation. This result suggests either that Vif$^-$ virus replication is blocked in PBMC during the first round of replication or that any Vif$^-$ virus produced from a first cycle is unable to infect new C8166 cells. We therefore decided to analyze the early steps of Vif$^-$ virus replication by PCR.

Vif$^-$ viruses propagated in CEM cells are normally retrotranscribed in PBMC. To determine the ability of Vif$^-$ viruses

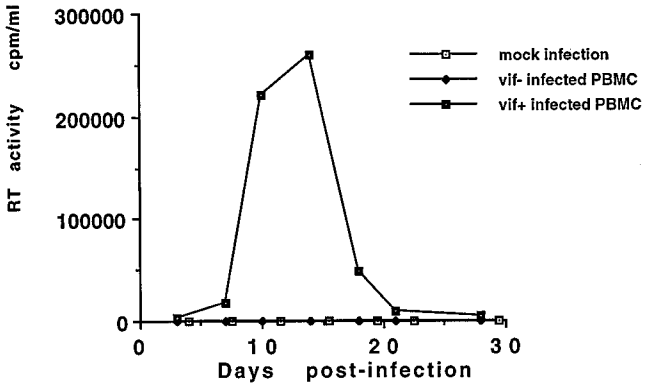

FIG. 1. Replication kinetics of HIV-1_{NDK} Vif^- and Vif^+ isolates in PBMC. PHA-stimulated PBMC (5×10^6) were infected with an MOI of 1 $TCID_{50}$ per cell. Following infection on day 0, supernatant RT activity was measured twice weekly and expressed as counts per minute per milliliter of cell-free supernatant. Mock infection was performed with culture medium.

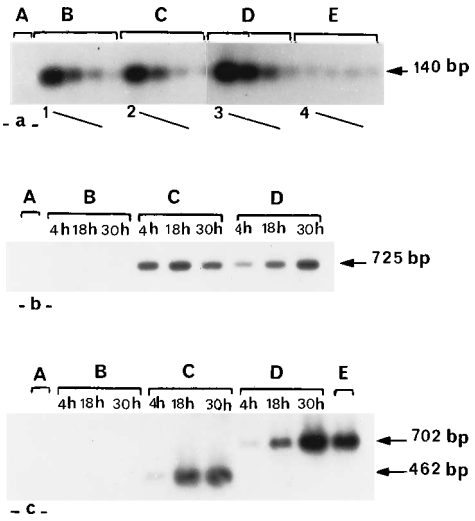

FIG. 2. Analysis of the reverse transcription step. (a) Amplification and titration of the early reverse-transcribed proviral DNA 4 h after infection of PBMC. Lane sets: A, culture medium; B, Vif^- virus; C, Vif^+ virus; E, heat-inactivated Vif^+ virus. Wild-type infection of CEM cells was used as positive control (lane sets D). Panels 1 to 4 show a threefold serial dilution of input DNA used for analysis. DNA corresponding to 10^5 cells was used as the highest input. Following amplification, products were electrophoresed on a 3% agarose gel and analyzed by Southern blotting followed by hybridization with the ^{32}P-labeled LTR3 oligonucleotide probe. (b) Detection of DNA proviral sequences at 4, 18, and 30 h after infection of PBMC. Lane sets: A, culture medium; B, heat-inactivated Vif^+ virus; C, Vif^- virus; D, Vif^+ virus. Amplification was performed with U3 and *gag* primers. Specificity of the PCR products was assayed by Southern blotting followed by hybridization with ^{32}P-labeled U5 oligonucleotide probe. (c) Detection of DNA proviral sequences at 4, 18, and 30 h after infection of PBMC. Lane sets: A, culture medium; B, heat-inactivated Vif^+ virus; C, Vif^- virus; D, Vif^+ virus. Wild-type infection of CEM cells was used as positive control (lane E). Amplification was performed with Vif^- and Vif^+ primers. Specificity of the PCR products was assayed by Southern blotting followed by hybridization with ^{32}P-labeled Vif_H oligonucleotide probe.

to enter PBMC, the strong-stop cDNA was detected by using entry PCR with the R and U5 primers (Fig. 2a). This previously described procedure (4, 11, 33) allows the analysis of the retrotranscribed viral DNA sequences a few hours postinfection, indicating that the entry process has occurred. Uninfected PBMC and PBMC infected with heat-inactivated virus were used as negative controls. Wild-type virus-infected CEM cells were used as a positive control. Following a high-MOI PBMC infection, low levels of strong-stop DNA were detected in the PBMC infected with the heat-inactivated virus (lanes E). This result is in accord with a previous demonstration (30) of the presence of partial reverse transcripts in virus particles. However, the amount of Vif^- (lanes B) and wild-type (lanes C) proviral DNA detected 4 h postinfection was significantly higher than that corresponding to the heat-inactivated virus. The quantification of the DNA target by serial dilution of the input DNA indicates similar levels of early retrotranscribed DNA products in both Vif^- and wild-type virus-infected PBMC. These results indicate that Vif^- viruses obtained after passage in these CEM cells are not defective in their ability to enter PBMC.

Since the initiation of reverse transcription was detected 4 h following infection, we then analyzed intermediate and completed products of reverse transcription. DNA was extracted from PBMC infected with the Vif^- and wild-type viruses at 4, 18, and 30 h postinfection and was assayed by PCR using two different primer couples. The U3-*gag* primer pair detects the second jump of the retrotranscriptase and can amplify only the completed double-stranded proviral DNA. The Vif^+/Vif^- pair of primers can amplify intermediate RT products and also verifies the absence of cross-contamination, since they allow amplification of both the wild-type and the truncated *vif* regions. The results in Fig. 2b show the detection of viral DNA sequences for both Vif^- and wild-type viruses. The detection of the second jump of the retrotranscriptase was observed regardless of the presence of *vif* in the viral genome when the U3-*gag* primer pair was used. Furthermore, these data verify the absence of wild-type contamination in the samples corresponding to the Vif^- virus infection, since the PCR product detected with the Vif^+ and Vif^- primers is 240 bp smaller than the product of the wild-type sample (Fig. 2c). Therefore, these data allowed us to conclude that retrotranscription occurs with both types of virus and leads to successfully completed proviral DNA molecules.

TABLE 1. Detection of syncytia after cocultivation of C8166 cells with wild-type virus-infected PBMC[a]

No. of PBMC	Syncytium formation
10^6	+
10^5	+
10^4	+
10^3	+
10^2	+
10	−

[a] Infected PBMC were diluted in culture medium and cocultivated 18 h postinfection with 10^5 C8166 cells in 200 μl of a 96-well plates; the presence of syncytia was observed 4 and 10 days after infection. In all cases, no syncytia were detected in Vif^- virus-infected PBMC.

Vif^- proviruses are able to integrate the cellular genome of PBMC. The presence of an integrated proviral DNA in the cellular genome of Vif^-- and Vif^+-infected PBMC was assayed by nested *Alu* PCR at 24 h postinfection. This assay specifically amplifies integrated viral DNA sequences in the human genome, since the sense primer matches the repeated human *Alu* sequences whereas the antisense primer matches the proviral genome. As shown in Fig. 3, analysis of PCR products by Southern blotting followed by hybridization with the specific LTR3 probe indicates the presence of multiple integration events occurring with the Vif^- (lane C) as well as for the wild-type virus (lane D). No obvious difference in the kinetics of integration of either virus was noted when several time

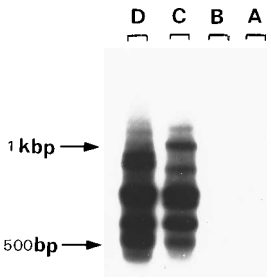

FIG. 3. Amplification of integrated proviral sequences in PBMC 18 h after infection. Lanes: A, culture medium; B, heat-inactivated Vif$^+$ virus; C, Vif$^-$ virus; D, Vif$^+$ virus. Nested PCR was performed by using a first amplification with *gag* and *Alu* primers followed by a second amplification with R and *Alu* primers. PCR products were electrophoresed on a 1.2% agarose gel and analyzed by Southern blotting followed by hybridization with the ^{32}P-labeled LTR3 oligonucleotide probe.

points after infection were assayed; the integration seems to appear in both cases 18 h postinfection (data not shown). To further investigate this point, the *Alu* PCR products obtained from the cells infected for 18 h with the Vif$^-$ or the Vif$^+$ virus were cloned into an M13 vector and sequenced by using a specific LTR primer (*nef* primer; see Materials and Methods). The junction sequences of several clones were determined. These sequences, indicated in Table 2, clearly demonstrate the occurrence of integration events in PBMC infected with the Vif$^-$ or the Vif$^+$ virus. No specific cellular integration sequences could be identified, since each M13 clone exhibits a distinct cellular sequence immediately 5′ of the common viral sequence corresponding to the viral LTR. Our data show definitively that integration events occur during the first round of replication in PBMC infected either with the Vif mutant virus or with the wild-type virus.

Vif$^-$ proviruses are transcribed in PBMC. We next attempted to determine whether Vif$^-$ viruses were normally transcribed and if complete viral mRNA expression could be detected after a unique round of replication. RT-PCR was used to detect the various species of viral mRNA in PBMC 36 h after infection with the Vif$^-$ or wild-type virus. We were confident that at this time point we would detect viral RNA resulting from a single replication cycle, since previous studies have indicated that the earliest detectable RNA molecules are seen between 24 and 48 h postinfection (33). Furthermore, zidovudine (AZT; 5 μg/ml) was added in the cultures 9 h after infection in order to limit infection to a single cycle. We chose to study the expression of *nef* and *tat* mRNAs since they represent the earliest transcripts which are Rev independent (24). We also chose to study the expression of some Rev-dependent transcripts, including those expressing *vpr* and *env*, and the genomic *gag-pol* mRNA. To allow differentiation of PCR products amplified from a cDNA from those amplified from DNA remaining in the RNA preparation, we chose primers matching distinct exons of the RNA species (Fig. 4a). In the case of *tat* mRNA, the sense and antisense primers were complementary to the first and second coding exons, respectively (2). In the case of *nef*, *vpr*, and *env* mRNAs, the sense primer was complementary to the common leader sequence and the antisense primer was located within the specific exon of each gene. Furthermore, each RNA sample was tested by PCR for the absence of any contaminating DNA material, this precaution being even more crucial when the presence of the unspliced *gag-pol* mRNA was analyzed.

Figure 4b shows the results of hybridization of several RT-PCR products with specific oligonucleotide probes. The data demonstrate the presence of *tat*, *nef*, *vpr*, and *env* mRNAs in PBMC infected either with the Vif$^-$ or the wild-type virus. The multiple-band pattern observed for the *env* mRNA is probably due to the existence of cryptic splice sites, which results in the detection of several transcripts. Unspliced *gag-pol* mRNA was detected in both Vif$^-$- and Vif$^+$-infected PBMC by using RT-PCR with the *gag*-R primer pair (Fig. 4c). These data thus suggest that all RNAs, genomic and subgenomic, from the wild-type and from the Vif$^-$ mutant are correctly transcribed regardless of the presence of *vif*.

To compare the relative amounts of genomic RNA in Vif$^-$ virus-infected PBMC and wild-type virus-infected PBMC, quantitative competitive RT-PCR was performed by means of a competitor plasmid introduced in the PCR. The results shown in Fig. 4c indicate that amounts of competitor plasmid lower than 8.5 fg are unable to compete for the amplification of the viral genomic RNA from either Vif$^-$ or wild-type virus-infected PBMC (sets B and C, lanes 4). The 50% competing dose is estimated at 1.03 pg for both types of RNA (compare sets B and C, lanes 2). No competition can be detected with the PBMC infected with heat-inactivated virus (set A). These experiments indicate no significant difference in the quantity of intracellular genomic RNA in PBMC infected with the Vif$^-$ or wild-type virus.

Vif$^-$ virus production is quantitatively normal. Quantification of virion production at the end of a first round of replication (about 36 h postinfection [16, 33]) is difficult to address with conventional techniques such as the measurement of RT activity (Fig. 1) or p24 antigen. This study is even more difficult when one is dealing with bulk populations of PBMC which include noninfectable CD4$^-$ cell types as well as noninfectable CD4$^+$ cells. Therefore, viral RNA harvested from the infected PBMC cell-free supernatant was assayed by quantitative competitive RT-PCR. Kinetics experiments were performed to verify that signals detected 36 h after infection were able to detect de novo-synthesized viral particles. AZT was added 9 h after infection in order to study the viral production of a single replication cycle. The results indicated in Fig. 5a demonstrate

TABLE 2. Junction sequences of integrated proviral DNA in the cellular genome of PBMC 18 h postinfection[a]

Virus	Sequence					
VIF$^-$ a	attgagctaa	atggggcaga	gtcaaaatcc	tgggttctTG	GAAGGGCTAA	TTCACTCCCA
VIF$^-$ b	ggtgtgagcc	acgtgcccgg	cccattctgt	taactgttTG	GAAGGGCTAA	TTCACTCCCA
VIF$^-$ c	taattaattg	atttttattt	caatagcttt	gggggtacTG	GAAGGGCTAA	TTCACTCCCA
VIF$^+$ a	tattcaagca	ctgctagaga	ttttccacac	tgacgaagTG	GAAGGGCTAA	TTCACTCCCA
VIF$^+$ b	gtgctgcatt	tggaggatgc	tgagcaacca	cttcatatTG	GAAGGGCTAA	TTCACTCCCA

[a] PBMC were infected either with Vif$^-$ or wild-type virus and cultivated for 18 h before sampling. *Alu* PCR products were cloned in M13mp18, and recombinants plaques were chosen randomly and sequenced by using a specific primer matching the viral LTR sequence. The first three clones were generated from the Vif$^-$-infected PBMC DNA; the two last clones were generated from the wild-type-infected PBMC DNA. Uppercase letters correspond to the viral sequences; lowercase letters to the cellular sequences.

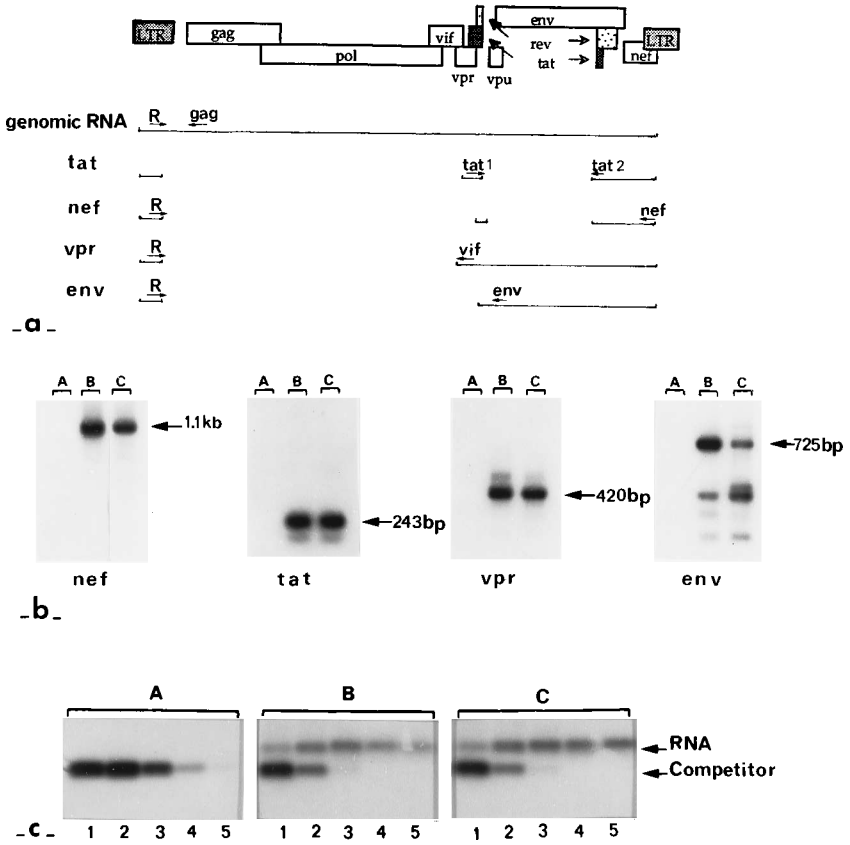

FIG. 4. Detection by RT-PCR of mRNA transcripts in PBMC 36 h after infection. Lanes: A, heat-inactivated Vif$^+$ virus; B, Vif$^-$ virus; C, Vif$^+$ virus. (a) Primers used for amplification; (b) qualitative amplification of spliced mRNA (the product of the expected size is indicated by the arrow); (c) quantitative amplification of the viral genomic mRNA. Sampling was performed 36 h after infection; AZT (5 μg/ml) was added in the culture 9 h after infection. The competitor doses added to the reactions in lanes 1 to 5 were 11.4 pg, 1.03 pg, 93 fg, 8.5 fg, and 0.8 fg, respectively. Specificity of the PCR products was assayed by Southern blotting and hybridization with a specific ^{32}P-labeled oligonucleotide probe.

a dramatic increase of the RT-PCR signals 36 h postinfection, corresponding to viral production. Weak signals can be observed at earlier time points; they might correspond to some initial viral inoculum which was still bound to the cellular membrane. However, the quantitative difference between the remaining virus 8 h postinfection and the newly produced virus 36 h postinfection was estimated to be increased of at least 12-fold, as measured by the quantitative competitive RT-PCR technique (data not shown).

Therefore, we decided to compare the amounts of viral RNA in Vif$^-$ and wild-type virus-infected supernatant PBMC 36 h postinfection. The results shown in Fig. 5b indicated that the addition of less than 1.2 fg of plasmid competitor in the PCR is unable to compete with the viral cDNA (lanes B9 and C9). Once again, the same dose of competitor plasmid was required for 50% competition of both the wild-type and Vif$^-$ PCRs. Indeed, 3.5 fg of competitor was required to inhibit 50% of the amplification of both the Vif$^-$ supernatant genetic material (lane B8) and the wild-type supernatant (lane C8). This result suggests that the first replication cycle in PBMC of Vif$^-$ viruses is quantitatively normal.

The virus particles produced from Vif$^-$-infected PBMC are defective for the preretrotranscription steps. Having found no quantitative difference between Vif$^-$ and wild-type virus pro-

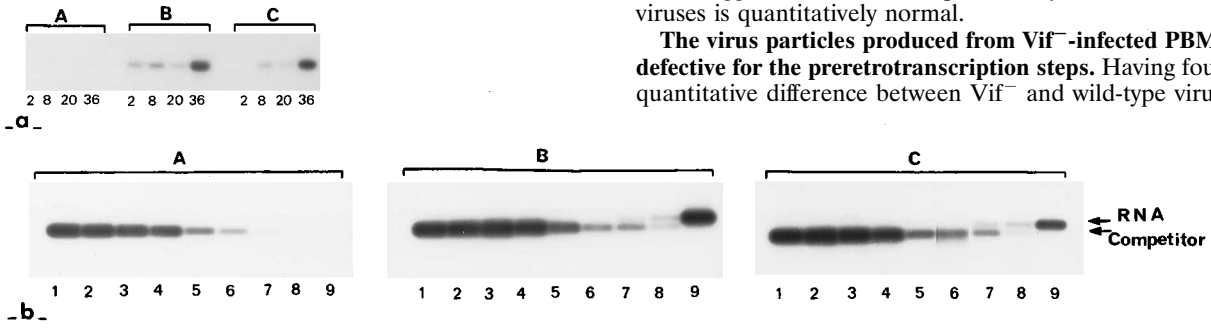

FIG. 5. Amplification of the viral genomic mRNA in PBMC cell-free supernatant. Lane sets: A, heat-inactivated Vif$^+$ virus; B, Vif$^-$ virus; C, Vif$^+$ virus. (a) Kinetic analysis of viral production. Three milliliters of supernatant was harvested at 2, 8, 20, and 36 h postinfection. AZT (5 μg/ml) was added in the culture 9 h after infection. Viral pellets were assayed for RT-PCR by using the R-*gag* primer pair. (b) Quantitative amplification of the viral RNA. Sampling was performed 36 h after infection; AZT (5 μg/ml) was added in the culture 9 h after infection. The competitor doses added to the reactions in lanes 1 to 9 were 8.4 pg, 2.76 pg, 907 fg, 298 fg, 98 fg, 32 fg, 10.6 fg, 3.5 fg, and 1.2 fg, respectively.

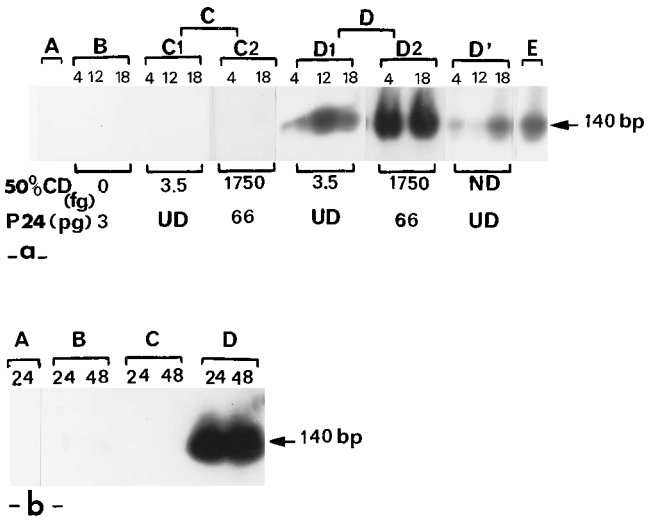

FIG. 6. Determination of the ability of the Vif$^-$ and Vif$^+$ viruses obtained after a single round of replication in PBMC (36 h postinfection) to reinfect PBMC by cell-free infection (a) or HeLa-CD4 cells (b) by cocultivation. (a) PBMC were infected with 5 ml of 36-h-postinfection supernatants produced from PBMC infected with culture medium (A) heat-inactivated Vif$^+$ virus (B), Vif$^-$ virus (C), Vif$^+$ virus (D) or a 1/50 dilution of D1 (D′). C1 and C2 and D1 and D2 represent two distinct experiments. Wild-type infection of CEM cells was used as a positive control (E). Determination of the 50% competing dose (50%CD) and of the p24 amount contained in the infecting supernatants is indicated below each panel. ND, not done; UD, undetectable. Entry PCR was performed as previously indicated at 4, 12, and 18 h postinfection. (b) HeLa-CD4 cells (2×10^5) were cocultivated with 10^6 PBMC infected 36 h previously with culture medium (A), heat-inactivated Vif$^+$ virus (B), Vif$^-$ virus (C), or Vif$^+$ virus (D). Entry PCR was performed as previously indicated at 24 and 48 h postcocultivation.

duction, we then examined whether the Vif$^-$ virions were qualitatively normal. Cell-free supernatant, harvested 36 h postinfection from PBMC infected with either Vif$^-$ or wild-type virus, were used to infect normal PBMC. A 50-fold dilution of the wild-type supernatant was assayed as a control of the sensitivity of the system. A first set of experiments was performed with supernatants obtained in the same conditions as described above except that addition of AZT was omitted (Fig. 6a, panels C1 and D1). Since no p24 was detectable in the 36-h-postinfection PBMC supernatants, a second set of experiments was done with 36-h-postinfection PBMC supernatants containing higher amounts of viruses (panels C2 and D2), as standardized by quantitative competitive RT-PCR and by p24 determination. This stock was obtained in the same conditions except that the incubation time between the infecting virus and the target PBMC was increased to 2.5 h.

To detect the infectivity of the viral particles, entry PCR was then performed at 4, 12, and 18 h after reinfection. The results shown in Fig. 6a indicate that the Vif$^-$ virions were unable to infect PBMC even when high MOIs were used (panel C2), whereas wild-type particles did infect PBMC. The Vif$^-$ virus was also unable to infect cells by coculture; Fig. 6b compares Vif$^-$ and Vif$^+$ virus entry into HeLa-CD4$^+$ cells after cocultivation with infected PBMC 36 h postinfection. No strong-stop DNA was detected in the cells cocultivated for 24 and 48 h with the Vif$^-$-infected PBMC, indicating a total absence of infectivity of the viral particles. Altogether, these data strongly argue for a critical role of the *vif* gene product in the formation of a competent viral particle for the preretrotranscription steps.

DISCUSSION

The purpose of this study was to investigate which step(s) of the replication cycle was limited during the replication of a Vif$^-$ virus produced in a semipermissive cell line and propagated in a primary cell system such as PBMC. No differences were observed at the level of virus entry, RNA retrotranscription, or DNA integration when single replication cycles of Vif$^-$ and wild-type virus produced from CEM cells (source cells) were compared. Furthermore, our data give a clear-cut demonstration that such virions cannot be propagated on PBMC (target cells) because of a defect in the late steps of the virus life cycle posttranscriptionally. Our results show that this defect does not lead to a quantitative difference in the amount of virus produced but rather leads to a qualitative difference: the same amount of Vif$^-$ virus is produced in a single cycle of replication in the PBMC restrictive system (source cells). However, the Vif$^-$ virus produced is totally noninfectious in a second round of infection and is blocked at the level of the preretrotranscription steps even in a cell-to-cell infection system. This main qualitative difference argues strongly for Vif being required at the level of the particle formation in restrictive cells. Very recent electronic microscopy studies have shown that Vif$^-$ virus particles are released from semipermissive cells but exhibit abnormal core structures, suggesting a defect in the final stages of packing of the viral nucleoprotein core (15). Western blot (immunoblot) analysis of noninfectious Vif$^-$ particles from restrictive cells demonstrates aberrant protein profiles which also gave rise to similar morphological defects (4a). The precise consequence of the absence of Vif on the replication cycle limiting step in our system remains to be determined; Vif$^-$ virions released from PBMC can be restricted either at the level of the attachment on the CD4 molecule, during the fusion and penetration, or during the decapsidation step. Pseudotyping studies could be done in this primary cell model to differentiate receptor-mediated steps. Previous studies (31) have shown that HIV-1 Vif$^-$ (murine leukemia virus) pseudotypes produced in semipermissive cells are blocked in H9 cells after the entry step per se, as judged by monitoring the synthesis of the retrotranscribed products by PCR. However, the role of the steps between entry and retrotranscription, i.e., penetration and uncoating, remains to be determined.

Other reports (26, 31) described a noncompletion of the retrotranscription and a lack of integration of Vif$^-$ proviruses in the target cells. However, the authors were able to detect partially reverse transcription products. In these systems, the Vif$^-$ viruses were propagated in CEM cells (source) and assayed in H9 cells (target), which do not allow replication of Vif$^-$ viruses. These cells are considered the closest cellular model to primary cells, though they remain slightly permissive for viral replication of Vif$^-$ mutants (8). However, the main difference between the system used in these studies and our model might result from the CEM cell strain which was used to propagate Vif$^-$ viruses (source cell). Von Schwedler et al. (31) and Sova and Volsky (26) used CEM-SS cells, while classical CEM cells were used in our study. It is possible that the CEM-SS cells are less permissive than classical CEM cells for the replication of Vif$^-$ mutants, and thus mutants propagated on such cells are already abnormal. Early retrotranscription products might be detected with such mutants, but it is likely that quantification with a competitive quantitative PCR assay would reveal a significant difference. The absence of completed reverse transcription described in these previous reports might reflect the consequence of a partial defect of the very early replicative steps of the particles produced from the

CEM-SS cells. In our system, Vif$^-$ mutants replicate efficiently in the CEM producer cell lines, though some differences in the level of replication were observed (4). Formation of the viral particles is therefore probably normal, allowing the analysis of the role of *vif* during the late steps of virus replication.

The fact that the Vif protein has been localized to the cytoplasmic side of the cellular membranes (12) supports the results presented in the present study: *vif* acts at the level of particle formation. This observation raises some additional questions; for example, on which modification and on which viral or cellular target protein does Vif act? It has been proposed that the Vif protein has a protease activity and cleaves the C-terminal end of the gp41 glycoprotein (13). However, alteration of this gp41 region does not modify the requirement for *vif* (10, 18). Nevertheless, it cannot be excluded that a putative protease activity of Vif on a different target could be associated with its biological role. One report of a study using A301 cells, which are a semipermissive for the replication of Vif mutants, suggested that *vif* could play a role in the incorporation of envelope proteins in the virion (22). We could not analyze the protein patterns in a fully restrictive cell system such as PBMC since it is totally restrictive for the growth of Vif mutants. However, it will be interesting to study protein expression within a single round of replication. The development of new technologies such as immuno-PCR (34), which can detect proteins through a PCR system combined with monoclonal antibodies, might help to define the role of *vif* in the late steps of the virus replication cycle. Determination of the precise action of Vif protein will be crucial since it will help in the design of therapeutic agents directed against one of the indispensable regulatory proteins of HIV.

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