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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

ABDALLAH HARMACHE,[1] MICHÈLE BOUYAC,[1] GILLES AUDOLY,[2] CORINNE HIEBLOT,[1] PAOLA PEVERI,[3]† ROBERT VIGNE,[1] AND MARIE SUZAN[1]*

Institut National de la Santé et de la Recherche Médicale (INSERM) U372, 13276 Marseille Cedex 09,[1] and INSERM U271, 69424 Lyon Cedex 03,[2] France, and Institute of Veterinary Virology, CH-3001 Berne, Switzerland[3]

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Complex retrovirus genomes contain a variable number of accessory genes, among which is the *vif* gene. We investigated in vitro the role of the *vif* gene of caprine arthritis encephalitis virus (CAEV) by studying the phenotype of five *vif* mutants after infection of primary goat synovial membrane (GSM) cells and blood-derived monocytes/macrophages. Any deletion introduced into the *vif* gene resulted in slow and low viral replication and production of virions with an infectious titer lower than that of wild-type viral particles. The wild-type phenotype could be restored by the *trans* expression of the *vif* gene in a complementation assay. Quantitative PCR and reverse transcription-PCR analyses were performed in order to determine which stage of the replicative cycle was impaired by the *vif* deletion. Our results demonstrated that CAEV Vif did not act at the level of reverse transcription or transcription but rather at the late stage of virus formation and/or release, as lower amounts of virus were produced after a single replicative cycle. The *vif*-deleted CAEV produced after 24 h of infection was still able to infect GSM cells, indicating that the *vif* gene is not essential for virus infectivity but is required for efficient virus production.

Caprine arthritis encephalitis virus (CAEV) is a retrovirus that naturally infects goats and induces chronic inflammatory and degenerative disease of the joints, mammary glands, and central nervous system (3, 20). CAEV is closely related to visna virus, which infects sheep, and exhibits more distant homology with other animal and human lentiviruses. However, the genetic organization and the gene regulation of CAEV expression are characteristics of the complex retroviruses of which human immunodeficiency virus (HIV) is the prototype (4). Both the CAEV and visna virus genomes contain three genes coding for the structural proteins Gag, Pol, and Env, two genes encoding the regulatory proteins Tat and Rev, and an additional gene previously named *Q* (24, 28, 32), which will here be referred to as *vif* because of the increasing evidence of homology with the *vif* gene of other lentiviruses (23). The transcription pattern of CAEV is complex and temporally regulated (14), as it is for other complex lentiviruses such as visna virus (35) and HIV (17). All of these *vif* genes are transcribed late during the viral cycle and are expressed as a singly spliced large mRNA (30, 35). Other similarities are noticeable at the protein level. The Vif proteins of HIV and visna virus are expressed and are immunogenic during in vivo infection, as revealed by the detection of anti-Vif antibodies in the sera of HIV-infected patients or visna virus-infected sheep (1, 15, 18). Vif proteins are present in the cytoplasm of infected cells and are not associated with cell-free virions (1, 11, 19).

The function of the Vif protein has been mostly studied with *vif*-deleted (*vif*−) HIV-1 viruses and remained to be clearly defined. Vif has been described as necessary for virus infectivity and cell-free or cell-to-cell transmission (6, 34). This phenotype is dependent on host cells from which *vif*− viruses are derived (6, 27, 31, 36), and Vif is essential for infection of peripheral blood mononuclear cells (PBMC) (5, 7). The stage at which the defect in the replication cycle of *vif*− viruses occurs is now under investigation. Some reports have shown that in nonpermissive cells, the replication of *vif*− HIV-1 is blocked after virus entry, at the DNA synthesis level (33, 36), whereas other authors suggest that the defect occurs at a later stage of replication/maturation (2). Sakai et al. (27) demonstrated that Vif modulates the gp120 content of newly synthesized virions, although not at the intracellular level, suggesting a role for Vif at a posttranslational level. Other reports proposed that Vif could compensate for cellular factors required for the production of infectious virus particles, i.e., Vif enhances viral infectivity during virus production (7, 36).

This is the first report describing the phenotypic analysis of a *vif*− CAEV infection of primary goat synovial membrane (GSM) cells and blood-derived monocytes/macrophages as well as a quantitative study of the different stages of the replicative cycle. We show that CAEV Vif is essential for rapid and efficient virus replication. Quantitative PCR and reverse transcription (RT)-PCR analyses demonstrate that the defect in *vif*− CAEV replication during a single cell cycle must occur at the late stage of virus formation and/or production, since there is no defect in either provirus synthesis or RNA transcription. The *vif*− CAEV produced after one replicative cycle could infect GSM cells, but *vif*− virus production after this second replication cycle was even more decreased than that of the wild-type (wt) virus. These results suggest a crucial role for CAEV Vif in virus formation and/or release.

MATERIALS AND METHODS

Molecular clone and construction of mutants. The infectious molecular clone was obtained after ligation of the 9-kb and 0.5-kb *Hin*dIII clones of CAEV (24,

* Corresponding author. Mailing address: INSERM U372, BP 178, 13276 Marseille cedex 09, France. Phone: (33) 91 82 75 82. Fax: (33) 91 82 60 61.

† Present address: Institute of Animal Breeding, CH-3012 Berne, Switzerland.

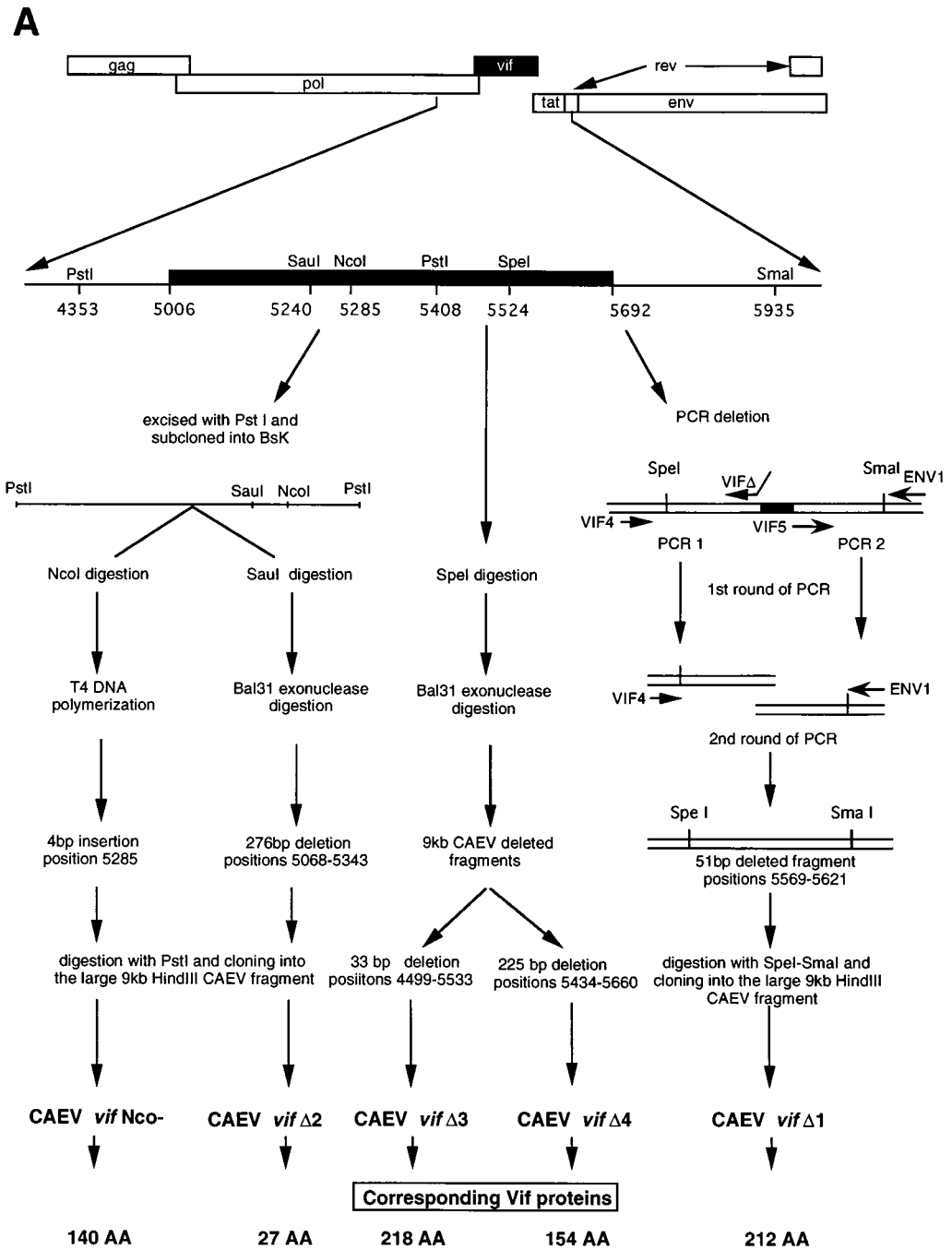


FIG. 1. (A) *vif* deletions introduced into the 9-kb *Hin*dIII clone of CAEV and (B) structure of the corresponding predicted proteins. (A) The *vif* gene spans nucleotides 5006 to 5692 (black box). The different strategies used to construct the mutants are described in Materials and Methods. *vif* Nco− resulted from a 4-bp insertion at the *Nco*I site (position 5285); *vif* Δ2, Δ3, and Δ4 were obtained by *Bal* 31 digestion from the *Sau*I (position 5240) or the *Spe*I (position 5524) site, respectively; *vif* Δ1 was a PCR deletion mutant. (B) The CAEV wt Vif protein sequence (CAEV Co [28]) is compared with the Vif sequences from visna virus isolates EV1 (29), SA OMVV (25), and K1514 (32). Below the sequences, an asterisk indicates AA identity and a dot indicates homologous AA residues. Underlined is the sequence deleted in the *vif* Δ2 mutant (AA 28 to 229). The shaded box (AA 95 to 140) indicates the sequence modified in the *vif* Nco− mutant, which ends at AA 140. The sequence in brackets (AA 83 to 94) indicates the region where reversions from *vif* Nco− infected cells were detected. Bars above the sequences indicate the deletions introduced by *vif* Δ1 (AA 189 to 205), Δ3 (AA 165 to 176), and Δ4 (AA 144 to 217). The open box (AA 170 to 175) indicates the motif conserved among all lentivirus isolates (23).

28). The overall strategy for mutant production is depicted in Fig. 1A. The 9-kb *Hin*dIII clone was digested with *Pst*I (positions 4353 to 5408) and cloned into a Bluescript vector (BsK). The *vif* Nco− mutant was obtained by digestion at the *Nco*I site (position 5285) and filling in with T4 DNA polymerase, resulting in a 4-bp insertion which removed the *Nco*I site and introduced a frameshift, creating a stop codon at position 5426. The *vif* Δ2 mutant was obtained by cleavage at the *Sau*I site (position 5240) followed by *Bal* 31 digestion. A 276-bp out-of-frame deletion was obtained (positions 5068 to 5343), which introduced a stop codon in position 5361. Cleavage of the 9-kb *Hin*dIII clone with *Spe*I (position 5524) and digestion with *Bal* 31 resulted in two mutants: *vif* Δ3, with a 33-bp in-frame deletion (positions 4499 to 5533), and *vif* Δ4, with a 225-bp in-frame deletion (positions 5434 to 5660). The *vif* Δ1 mutant was created by PCR to delete a 51-bp

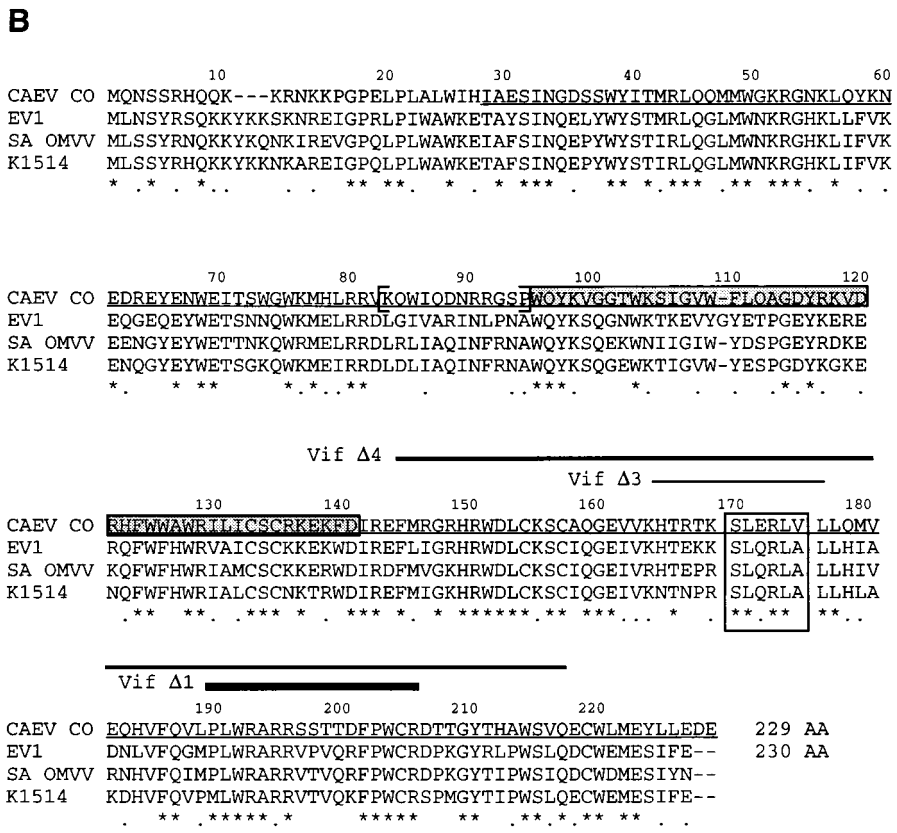


FIG. 1—*Continued.*

fragment (positions 5569 to 5621). In the first reaction, two independent PCRs were run with primers VIF4 and VIFΔ and primers VIF5 and ENV1, with VIFΔ and VIF5 having overlapping ends. In the second PCR, the amplified products were mixed and run with primers VIF4 and ENV1. The final amplified product was digested with *Spe*I and *Sma*I and replaced into the 9-kb *Hin*dIII clone.

Cells, transfection, and infection. Primary GSM cells were derived from carpal joints as previously described (21) and maintained in culture in Eagle's minimal essential medium (MEM) supplemented with 1% glutamine, penicillin, streptomycin, and 10% fetal calf serum. Monocytes/macrophages were derived from PBMC freshly isolated on Ficoll-Paque gradients (Pharmacia) as described before (22) and cultured for 10 to 12 days in Teflon bags in RPMI 1640 medium supplemented with 1% glutamine, penicillin, streptomycin, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 10^{-5} M β-mercaptoethanol, and 10% sheep serum.

Transfections of GSM cells were performed with 5 μg of proviral DNA per 50×10^4 cells with the Lipofectin reagent (Gibco BRL) as previously described (16). The medium was changed every 3 to 4 days, and the supernatants were harvested to measure the reverse transcriptase (RT) activity and virus titers.

Infections were carried out at a multiplicity of infection (MOI) of 0.01 to 0.04 for 2 h at 37°C on subconfluent GSM cells or on six-well plates (Falcon) containing PBMC-derived mononuclear cells or macrophages after 10 to 12 days of culture in Teflon bags. Infection of GSM cells was monitored by RT activity. The 50% tissue culture infectious dose ($TCID_{50}$) per milliliter was determined on supernatants from infected mononuclear cells or macrophages at days 3 and 7.

For PCR and RT-PCR analyses of the replicative cycle, GSM cells were infected under the same conditions except that viral supernatants were pretreated with 20 μg of DNase I (Boehringer) per ml and 10 mM $MgCl_2$ for 30 min at 30°C before being added to the cells. Heat-inactivated (30 min at 80°C) wt CAEV was used as a negative control. After 2 h at 37°C, the cells were washed twice with phosphate-buffered saline (PBS), incubated with 15 μg of trypsin type XIII (Sigma) per ml for 15 min at 4°C, washed again twice with PBS, and then cultured in MEM–10% fetal calf serum. At different times postinfection, cells were trypsinized and lysed for DNA or RNA extraction.

Transcomplementation assay. The *vif* gene of CAEV was cloned into the eucaryotic expression vector pRC-CMV (InvitroGen), which contains a neomycin resistance gene. Briefly, the *vif* gene was amplified with the VIF1 and VIF7 primers described below and subcloned into the *Sma*I site of the Bluescript vector. After sequence verification, a *Hin*dIII-*Xba*I fragment was excised and ligated to *Hin*dIII- and *Xba*I-digested pRC-CMV. GSM cells were transfected by lipofection with 10 μg of the pRC-CMV vector as a negative control (pRC) or 10 μg of this plasmid containing the CAEV *vif* gene (pRC vif). After selection with geneticin (Gibco BRL; 1 mg/ml) for neomycin resistance, the transfected cells were grown to confluency and then infected with wt or *vif*− CAEV supernatants containing the same RT activity (10,000 cpm per flask). Complementation was monitored by RT activity measurements in the cell-free supernatants.

RT activity and virus titration. Culture supernatants were clarified by centrifugation for 10 min at 500 × *g* and then stored at −80°C. The virion particles contained within 1 ml were pelleted by centrifugation for 5 min at 100,000 × *g* and lysed in 10 μl of 10 mM Tris-HCl (pH 7.8)–100 mM NaCl–1 mM EDTA–0.1% Triton X-100. This solution was mixed with 40 μl of 50 mM Tris-HCl (pH 7.8)–20 mM $MgCl_2$–20 mM KCl–2 mM dithiothreitol–0.5 μg of poly(A)-oligo(dT) (Pharmacia)–2.5 μCi of [^{3}H]dTTP (Amersham). The reaction mix was incubated for 1 h at 37°C, spotted onto DE81 paper, air dried, and washed three times for 5 min each in 5% anhydrous Na_2HPO_4, twice in distilled water, and once in 70% ethanol. Incorporated radioactivity was measured in a liquid scintillation counter (Packard Instruments).

Virus titers were determined on confluent monolayers of GSM cells in 24-well plates (Falcon). One milliliter of undiluted supernatant or 10-fold dilutions, up to 10^5, were inoculated in quadruplicate. The plates were incubated for 10 to 12 days; the cells were then fixed with 0.2% glutaraldehyde–2% formaldehyde and stained with 1% crystal violet (Sigma). Wells were scored for the presence of multinucleated syncytia, and the $TCID_{50}$ was determined as the highest dilution causing syncytium formation in two of the four wells.

DNA and RNA extraction for PCR and RT-PCR analyses. DNA extraction was performed on GSM cells at 2, 6, and 24 h postinfection with the nucleic acid extraction kit Isoquick (Microprobe) according to the manufacturer's protocol. Extracted DNA was dissolved in distilled water and quantified by optical density.

RNA was extracted from GSM cells at 24 h postinfection or from cell supernatants. Ten milliliters of supernatant was ultracentrifuged in a Beckman SW41 rotor for 45 min at 40,000 rpm. The pellet was dried and resuspended in 300 μl of RNAzol (Bioprobe), whereas pelleted cells were lysed in 500 μl of RNAzol. In both cases, RNA was double extracted according to the manufacturer's instructions and then dissolved in 10 μl of RNase-free distilled water (for virion-associated RNA) or quantified by optical density (for total cellular RNA).

PCR and RT-PCR analyses. For the RT reaction, total cellular RNA was reverse transcribed in a final volume of 20 μl with 10 U of avian myeloblastosis virus reverse transcriptase (Promega), 250 ng of random hexamer primers (Pharmacia), and 1 mM each of the four deoxynucleoside triphosphates (dNTPs). The

reaction was run on a Hybaid thermocycler as follows: denaturation for 3 min at 65°C, 30 s at 60°C, a ramp for 21 min to 39°C, followed by 25 min of extension at 39°C. Control RT reactions were performed under similar conditions, but the reverse transcriptase was omitted and in this case, no RT-PCR product was detected. Virion RNA was reverse transcribed as described above except that only 40 ng of the random hexamer primers was used.

PCR was carried out in a final volume of 25 μl of 1× PCR buffer (Promega), 200 μM each of the dNTPs, 1.5 mM $MgCl_2$, 6 ng of each primer per μl, 1.5 U of *Taq* polymerase (Promega), and various amounts of cellular DNA or RT reaction product, using the following conditions: a first denaturation step for 3 min at 94°C, followed by 35 amplification cycles of a 1-min denaturation step at 94°C, 1 min of annealing at a temperature determined for each primer pair, followed by 1 to 2 min of extension at 72°C (depending on the size of the expected PCR product), with a final 10 min of extension. For each temperature change, a scale of 1.5 s/°C was imposed to minimize the effect of the heterogeneity of the apparatus.

After amplification, each sample was run on a 1.5% agarose gel, transferred to a nylon membrane (Hybond N+; Amersham), and hybridized with a specific ^{32}P-labeled oligonucleotide probe. Hybridization was performed at 52°C overnight in 1 M NaCl–10 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 7.0]–0.1% sodium dodecyl sulfate (SDS)–5% dextran sulfate–0.2% Denhardt's reagent. Filters were washed once or twice in 2× SSC–0.1% SDS and once in 0.8× SSC–0.1% SDS at 52°C before being exposed to X-ray film (Kodak) at −70°C (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

Quantitative competitive RT-PCR. The competitor plasmids were kindly provided by A. P. Ravazolo, P. Turelli, and G. Quérat. The CAEV Δ*env* mutant was obtained by deletion of an *Aat*II fragment (positions 8398 to 8581) from the *env* gene. The CAEV Δ*pol* mutant was constructed by deleting 55 bp (positions 3824 to 3879) from the *pol* gene of the 9-kb *Hin*dIII CAEV clone. Amplification of these plasmids with the ENV2/ENV3 and POL S/POL A primers, respectively, led to shorter products than the RT-PCR product obtained from the RNA samples. The amount of viral cDNA in the RT reaction was first approximated by titration against a fivefold dilution series of the mutant *env* or *pol* fragment. This was followed by a more precise quantitation by titrating 2 μl of cDNA against a threefold dilution series spanning the first determination (26), using the conditions described above.

Primers and oligonucleotide probes. The exact sequences and positions of the primers used have been chosen according to the nucleotide sequence of CAEV (28) and are as follows: R (sense primer), 5′-GAGTTCTAGGAGAGTCCCTCC-3′ (1 to 21); U5 H (hybridization oligonucleotide), 5′-GTATTGCACAGATTAAGGGAC-3′ (145 to 125); U5 (antisense primer), 5′-CTGCGAGAGCCGCTCTGGTA-3′ (162 to 143); GAG (antisense primer), 5′-CAGGATTCTCGACCACCAAG-3′ (432 to 413); POL S (sense primer), 5′-GATAGGATAGGAGTGCATTG-3′ (3721 to 3740); POL H (hybridization oligonucleotide), 5′-TATTTCCGAAATATATTTGTC-3′ (3801 to 3781); POL A (antisense primer), 5′-TGAGTCTATGATTCCTCCT-3′ (4020 to 4002); POL B (sense primer), 5′-TCAGGAGAGTGGAAAGGACC-3′ (4902 to 4921); VIF1 (sense primer), 5′-CAGGATCCATGCAAAATTCATCCCGCC-3′ (*Bam*HI site [underlined] and 5006 to 5024); VIF2 (antisense primer), 5′-GGTAATTCTGGTCCAGGT-3′ (5067 to 5050); VIF3 (sense primer), 5′-TGCACCTAAGGAGAGTGA-3′ (5235 to 5252); VIF4 (sense primer), 5′-GTCTGGAAAGACTAGTAC-3′ (5514 to 5531); VIFΔ (antisense primer), 5′-CCCGTTGTGTGCAATACTTGAAACACATGC-3′ (5631 to 5621 [underlined], complementary to the underlined sequence in VIF5, and 5569 to 5551); VIF5 (sense primer), 5′-GACACAACGGGATACACGCA-3′ (5621 to 5640); VIF6 (antisense primer), 5′-ATGCGTGTATCCCGTTGTGT-3′ (5641 to 5622); VIF7 (antisense primer), 5′-CAGGATCCCTTTGAGGCAGTTCTTCAC-3′ (*Bam*HI site [underlined] and 5710 to 5692); REV (sense primer), 5′-GATACATGCGCTTAACTGGG-3′ (6028 to 6047); ENV1 (antisense primer), 5′-CCCAGTTAAGCGCATGTATC-3′ (6047 to 6028); ENV2 (sense primer), 5′-CATCAATACTGTATAACCTC-3′ (8184 to 8203); ENV H (hybridization oligonucleotide), 5′-GGACGAGCAGCCTCAGCAGG-3′ (8745 to 8726); ENV3 (antisense primer), 5′-AGATTCCCATCGTCAGCG-3′ (8766 to 8749); and U3 (sense primer), 5′-CCGCAAGTGCTGACAGATGT-3′ (8998 to 9017).

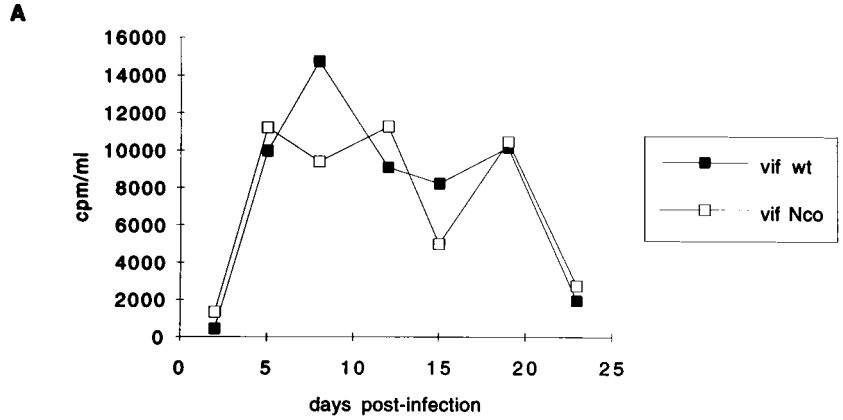
RESULTS

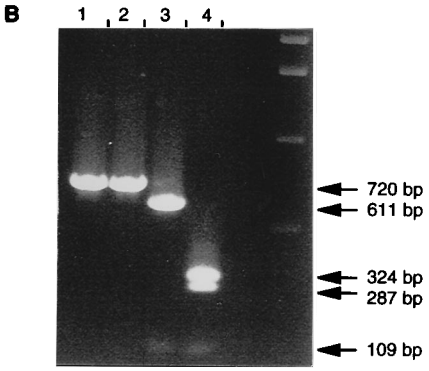
Molecular clones and mutants. Figure 1A summarizes the overall strategy developed to construct the different CAEV *vif* mutants used in this study. The *vif* Nco− mutant coded for a 140-amino-acid (AA) protein truncated at its COOH-terminal sequence (Fig. 1B). Moreover, the deduced amino acid sequence of the truncated protein was divergent after AA 95 (Fig. 2C, panel 2). All the other mutants obtained either by *Bal* 31 digestion (*vif* Δ3 and *vif* Δ4) or by PCR (*vif* Δ1) were deleted in domains located in the most conserved regions of the different ungulate lentiviral Vif proteins (Fig. 1B). Vif Δ1 was deleted from AA 189 to 205, Vif Δ3 was deleted from AA 165 to 176, and both deletions are contained within the Vif Δ4 mutant, deleted from AA 144 to 217. The deletion introduced into the Vif Δ3 mutant concerned the amino acid motif SLQXL (positions 170 to 175), described as being conserved among almost all the retroviral Vif proteins (23). The *vif* Δ2 mutant coded for a protein expressing only the first 27 NH_2-terminal amino acids, among which the last 6 were the result of the frameshift introduced by the deletion (Fig. 1B).

***vif* gene of CAEV is necessary for efficient viral replication.** The first mutant studied was *vif* Nco−, with a 4-bp insertion at the *Nco*I site (position 5285), which codes for a 140-AA protein instead of the 229 AA of the wt. Viral supernatants obtained after transfection of GSM cells with the wt or *vif* Nco− CAEV molecular clone were used to infect GSM monolayers, and infection was monitored by RT activity in the cell supernatants every 3 to 4 days. As shown in Fig. 2A, no obvious difference was observed between the replication of wt and *vif* Nco− CAEV. Total cellular DNA was extracted, and PCR amplification of the *vif* gene was performed with the VIF1/VIF7 primer pair, which produced a 720-bp fragment for both *vif* wt and *vif* Nco− (Fig. 2B, lanes 1 and 2). Digestion of the amplified products, which contain another *Nco*I site at position 5609, with *Nco*I generated fragments of the expected sizes and revealed that only one *Nco*I site (position 5609) was present in *vif* Nco− instead of the two observed in *vif* wt (Fig. 2B, lanes 3 and 4). This result demonstrated that the initial mutation introduced into the *vif* gene was still conserved but did not exclude any modification in other parts of the gene.

Amplified products from two independent transfection/infection experiments were sequenced, and the nucleotide sequences are shown in Fig. 2C (panel 1). In the first case, *vif* R1, a 4-bp deletion occurred (positions 5251 to 5254) upstream from the 4-bp insertion at the *Nco*I site (underlined). The deduced amino acid sequence (Fig. 2C, panel 2) revealed that an entire Vif protein identical to the wt one except for a stretch of 12 AA at positions 83 to 94 could be expressed. In the second case, *vif* R2, one base was deleted (position 5249) in the same region as for *vif* R1 and resulted in a 230-AA protein (one additional asparagine residue) with the same characteristics as Vif R1. It should be noted that the reversion took place in a region corresponding to a variable domain of the ungulate Vif proteins (Fig. 1B). In both cases, the same results were obtained by sequencing DNA prepared from GSM cells infected with supernatants taken at two different times after transfection. Moreover, we were able to detect the reverted sequences in DNA extracted from the transfected cells (not shown). These experiments suggested that the revertant mutations occurred during transfection and were contained within the viral inoculum used for subsequent infections, explaining why the initial virus production during infection was not affected by the initial *vif* mutation. These data imply that CAEV needs a functional *vif* gene for efficient replication. To test this hypothesis, we went on studying the effects of larger *vif* deletions on virus replication and phenotype.

***vif*− CAEV have slow and low replication rates.** The phenotypes of the four *vif* deletion mutants were established by infecting GSM cells with the same amount of RT activity from transfection supernatants (20,000 cpm per flask), corresponding to an MOI of 0.01 to 0.04. The RT activity curves obtained for *vif* Δ1, Δ2, Δ3, and Δ4 were superimposable and revealed slower and lower growth kinetics compared with that of the wt virus (Fig. 3A). Total cellular DNA was extracted on day 20 postinfection and subjected to PCR amplification with the VIF1/VIF7 primer pair. As shown in Fig. 3B, Southern blot hybridization of the amplified products with the ^{32}P-labeled VIF2 oligonucleotide revealed the expected band size in each case: 720 bp for the wt virus (lane 2), 670 bp for *vif* Δ1 (lane 4),





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          5245                                                    5300
1 vif wt  AGAGTGAAACAATGGATACAAGACAACAGGAGAGGAAGCCCATG    GCAGTACAAAGT
  vif Nco AGAGTGAAACAATGGATACAAGACAACAGGAGAGGAAGCCCATGCATGGCAGTACAAAGT
  vif R1  AGAGTGAA    TGGATACAAGACAACAGGAGAGGAAGCCCATGCATGGCAGTACAAAGT
  vif R2  AGAGTG AACAATGGATACAAGACAACAGGAGAGGAAGCCCATGCATGGCAGTACAAAGT

          80        90        100       110       120       130       140
2 Vif wt  RRV KQWIQDNRRGSPWQYKVGGTWKSIGVWFLQAGDYRKVDRHFWWAWRILICSCRKEKFD
  Vif Nco ... .....CMAVQSRRNMEKYRSVVP.SRRLQKG.QALLVGMEDTDMFLQ.RKV
  Vif R1  ... NGYKTTGEEAHA.....................................................
  Vif R2  ...NNGYKTTGEEAHA.....................................................

                150       160       170       180
  Vif wt  IREFMRGRHRWDLCKSCAQGEVVKHTRTKSLERLVLLQMVEQHVF
  Vif Nco
  Vif R1  ..........................................
  Vif R2  ..........................................
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FIG. 2. Analysis of the *vif* Nco− mutant. (A) RT activity measurements in the supernatants from GSM cells infected with wt or *vif* Nco− CAEV. The medium was changed every 3 to 4 days, and RT activity was measured in 1 ml of supernatant concentrated 100-fold. (B) PCR analysis of DNA extracted from cells infected wt (lanes 2 and 4) or *vif* Nco− (lanes 1 and 3) virus. The amplified products (VIF1/VIF7 primers) were analyzed on an agarose gel, either undigested (lanes 1 and 2) or after *Nco*I digestion (lanes 3 and 4). The *vif* wt has two *Nco*I sites (positions 5285 and 5609) and generated three fragments of 324, 287, and 109 bp (lane 4). *vif* Nco− harbors only one *Nco*I site (position 5609) and generated two fragments, 611 and 109 bp (lane 3). (C) Panel 1. Nucleotide sequences from two revertants (*vif* R1 and *vif* R2) isolated from independent experiments, compared with the wt and *vif* Nco− sequences. The VIF1/VIF7 primers were used to amplify the *vif* gene to be sequenced, and the region (5243 to 5301) where mutational events resulted in a frameshift is shown. Underlined is the initial 4-bp insertion at the *Nco*I site. ACAA is the upstream sequence deleted in *vif* R1, while one A was deleted in *vif* R2. Panel 2. Deduced amino acid sequences of the Vif proteins encoded by the different *vif* mutants. Only the region (AA 80 to 185) where the different mutations introduced into the *vif* gene resulted in sequence modification is shown. Dots indicate identity with the wt sequence.

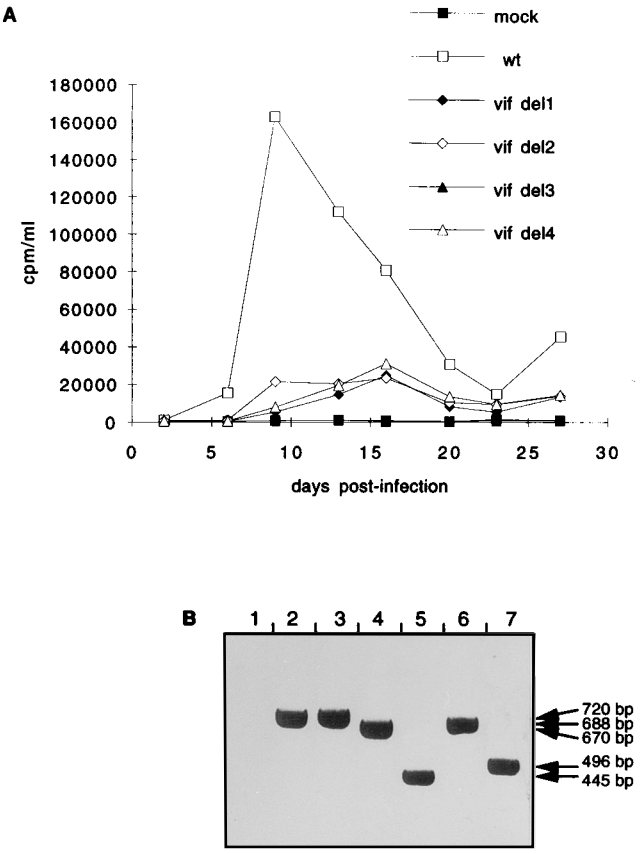

FIG. 3. (A) RT activity production by the *vif* Δ1, Δ2, Δ3, and Δ4 deletion mutants in infected GSM cells compared with that by wt CAEV. The medium was changed every 3 to 4 days, and RT activity was measured in 1 ml of supernatant concentrated 100-fold. (B) PCR analysis with the VIF1/VIF7 primers on cellular DNA extracted at day 20 postinfection from GSM cells either mock infected (lane 1) or infected with wt (lane 2), *vif* Nco (lane 3), *vif* Δ1 (lane 4), *vif* Δ2 (lane 5), *vif* Δ3 (lane 6), or *vif* Δ4 (lane 7). The Southern blot of the amplified products was hybridized with the ^{32}P-labeled VIF2 oligonucleotide probe.

445 bp for *vif* Δ2 (lane 5), 688 bp for *vif* Δ3 (lane 6), and 496 bp for *vif* Δ4 (lane 7). In lane 3 of Fig. 3A is shown the amplified product from *vif* Nco− infected cells in a parallel experiment, whereas no product could be amplified from mock-infected cells (lane 1). These experiments revealed that deletions introduced into any part of the 3′ half of the *vif* gene of CAEV, as in *vif* Δ1, Δ3, and Δ4, resulted in the same attenuated phenotype as in the largest deletion mutant, *vif* Δ2. This supports the importance of the 3′ moiety of the protein for function and confirms the need for a complete and functional *vif* gene in CAEV replication.

Virus titration was carried out on day 27 postinfection supernatants, and the $TCID_{50}$ appeared to be 10- to 100-fold lower for all the *vif*− viruses than for the wt virus (data not shown). Infection experiments were performed on primary undifferentiated mononuclear cells or on differentiated macrophages to determine the phenotype of *vif*− viruses on cells which are the natural targets of infection (8, 9, 22). For the remainder of this study, we tested only *vif* Δ2, as being representative of all the other *vif*− viruses, and the results are summarized in Table 1. It can be seen that the *vif*− virus did not grow on undifferentiated mononuclear cells and grew poorly on differentiated macrophages (0 and 10^2 $TCID_{50}$/ml in day 7 supernatants, respectively), compared with the wt virus,

TABLE 1. Titers of wt and *vif*− CAEV produced by infected-blood-derived mononuclear cells or macrophages[a]

Cells	$TCID_{50}$/ml			
	wt CAEV		*vif*− CAEV	
	Day 3	Day 7	Day 3	Day 7
Mononuclear	0	10^2	0	0
Macrophages	$10^{2.5}$	$10^{6.75}$	0	10^2

[a] Infections were done at an MOI of 0.01, and supernatants were taken on days 3 and 7 postinfection to determine the $TCID_{50}$ on GSM cells. Mononuclear cells were derived from heparin-treated blood and infected while undifferentiated at day 0. Macrophages were obtained and infected on day 12 after culture of mononuclear cells in Teflon bags.

which grew at a low level on mononuclear cells and at a high level on macrophages (10^2 and $10^{6.75}$ $TCID_{50}$/ml in day 7 supernatants, respectively). These results demonstrated that the slow-and-low phenotype observed on GSM cells can be reproduced on primary blood-derived macrophages, which can both be considered semipermissive cells for *vif*− CAEV replication.

Transcomplementation of *vif*− CAEV by *vif*. In order to assess whether the modified phenotype of the *vif*− CAEV was due to the deletion introduced into the *vif* gene, a transcomplementation assay was carried out by transfection of GSM cells with the pRC vif CAEV construct. Cells expressing the neomycin resistance gene contained in this vector were selected in the presence of geneticin and then infected with wt or *vif*− CAEV. As shown in Fig. 4A, the wt phenotype could be restored in cells transfected with pRC vif CAEV before infection with *vif*− virus, whereas cells transfected with the expression vector pRC alone before *vif*− virus infection produced a small amount of virions, characteristic of the *vif*− phenotype. The lower RT activity produced in Vif-expressing cells could be explained by the fact that the cells were not cloned and so had variable levels of expression of the *vif* gene. Virion-associated RNA was analyzed on day 21 postinfection, and RT-PCR was carried with the POL B/VIF6 primer pair, allowing only viral genomic DNA amplification.

The amplified products were analyzed by Southern blot hybridization with the ^{32}P-labeled VIF2 oligonucleotide probe; the pattern obtained is shown in Fig. 4B. Virions produced by cells infected with the wt virus contained the wt *vif* gene (740 bp) whether transfected with the vector alone (lane 2) or one expressing the *vif* gene (lane 5). RNA associated with the virions produced by the *vif*− virus-infected GSM cells revealed the same 464-bp amplified product, in the absence (lane 3) or in the presence (lane 6) of the *vif* gene product. No signal could be amplified from mock-infected cells whether transfected with pRC (lane 1) or pRC vif (lane 4).

In Fig. 4C are shown the results obtained by RT-PCR analysis on virion-associated RNA. The primer pair used, VIF3/ENV1, allowed amplification only from wt RNA, since the VIF3 primer is located within the *vif* deletion. As can be seen, among GSM cells transfected with the pRC vif construct, only those infected with wt virus could produce an amplified signal of 813 bp (lane 2), while no amplification could be obtained from virions produced by *vif*− virus-infected cells (lane 3) or from mock-infected-cell supernatant (lane 1). These results indicated that restoration of the wt phenotype was due neither to contamination nor to recombination and demonstrated that the Vif protein synthesized from an expression vector could act in *trans* to complement a *vif*− CAEV. We then went on to

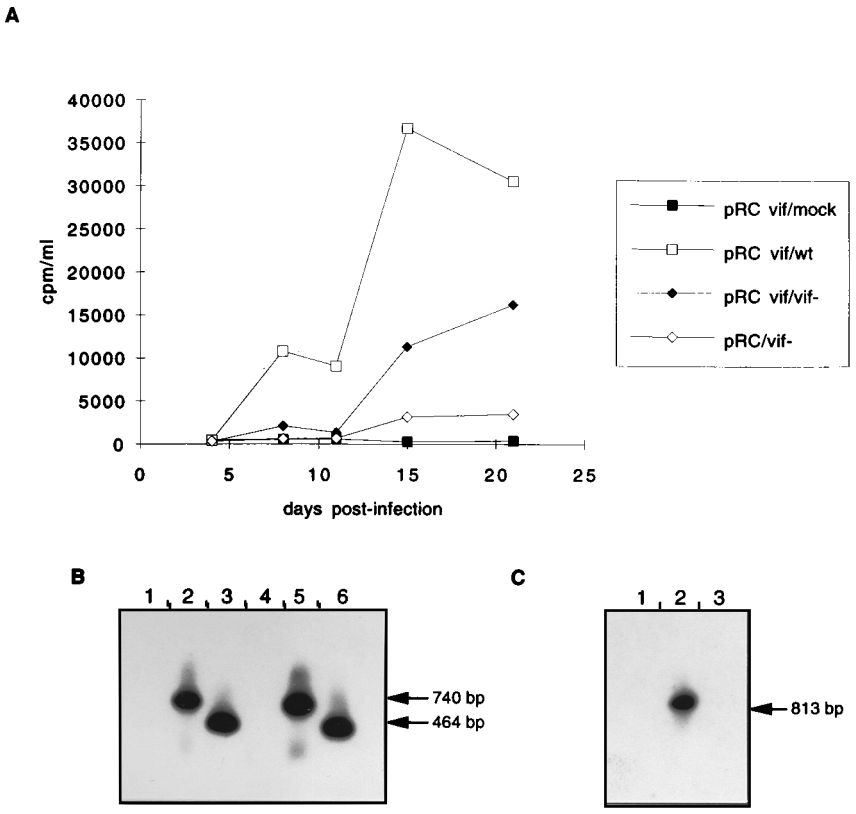

FIG. 4. Transcomplementation of *vif*− CAEV by the *vif* gene. (A) GSM cells were transfected with 10 μg of the expression vector alone (pRC) or one expressing the *vif* gene of CAEV (pRC vif), selected in the presence of 1 mg of geneticin per ml, and then infected with wt or *vif*− viral supernatants (10,000 cpm per flask). (B) PCR analysis with the POL B/VIF6 primers on cellular DNA extracted at day 21 postinfection from cells transfected with pRC (lanes 1 to 3) or pRC vif (lanes 4 to 6) and either mock infected (lanes 1 and 4) or infected with wt (lanes 2 and 5) or *vif*− (lanes 3 and 6) CAEV. The Southern blot of the amplified products was hybridized with the ^{32}P-labeled VIF2 oligonucleotide probe. (C) RT-PCR analysis of virion-associated RNA extracted from day 21 supernatant. Lane 1, pRC vif-transfected, mock-infected GSM cell supernatant; lane 2, pRC vif-transfected, wt CAEV-infected cell supernatant; lane 3, pRC vif-transfected, *vif*− CAEV-infected cell supernatant. Amplification was done with the VIF3/ENV1 primers, VIF3 being inside the *vif* deletion. The Southern blot of the amplified products was hybridized with the ^{32}P-labeled VIF6 oligonucleotide probe.

analyze a single replication cycle of the virus to determine which stage was modified by the *vif* deletion.

Reverse transcription is as efficient in wt CAEV- as in *vif*− CAEV-infected cells. GSM cells were infected with supernatants containing the same amount of RT activity (25,000 cpm per flask) of wt or *vif*− CAEV and of heat-inactivated wt virus as a negative control. The cells were lysed at 6 h postinfection, and DNA was extracted and analyzed for the levels of strong-stop DNA, which is synthesized early during reverse transcription. PCR amplification was performed with the R/U5 primers, and the amplified products were revealed by Southern blot hybridization with the ^{32}P-labeled U5H oligonucleotide probe (Fig. 5A). Serial threefold dilutions of input total DNA indicated similar levels of strong-stop DNA, as the penultimate dilution (1.23 ng) produced the same signal for the wt and *vif*− viruses. The complete double-stranded proviral DNA was then analyzed with the U3/GAG primers, and in each case, the last dilution of input DNA (1.03 ng) generated signals of the same intensity (Fig. 5B), indicating that the second jump of the RT occurred with the same efficiency in wt and *vif*− CAEVs.

To compare the relative amounts of proviral DNA in wt and *vif*− CAEV-infected cells, we used a quantitative competitive PCR assay, in which a constant amount of cellular DNA was coamplified with serial threefold dilutions of a *pol*-mutated internal standard containing a 55-bp deletion in the amplified fragment, allowing determination of the DNA amount in the extract. The analyses were carried out on DNA extracted at 2 and 6 h postinfection. Figure 5C shows that detection of proviral DNA was possible at 2 h postinfection, with the same amount of competitor DNA (0.04 pg) being the 50% competing dose. We could not determine whether this signal was due to DNA synthesis following infection or to DNA contained within the viral inoculum. Thus, the analysis was carried out at 6 h postinfection (Fig. 5D), and we determined that the 50% competing dose was 0.37 pg for the wt as well as the *vif*− genomic DNA. This result demonstrated a ninefold increase in DNA synthesis between 2 and 6 h postinfection, indicating active reverse transcription of viral RNA, but did not allow us to demonstrate any differences between the wt and *vif*− viruses at the reverse transcription stage.

***vif*− CAEV produces quantitatively less virion-associated RNA during a single replicative cycle.** Transcription was studied on cellular RNA extracted from 24-h wt or *vif*− CAEV-infected GSM cells. After a nonspecific RT reaction allowing the amplification of all the RNA species, specific primer pairs were chosen to amplify the *rev*, *env*, and genomic viral RNAs (Fig. 6). As shown in Fig. 6A, the REV/ENV3 primers amplified the spliced *rev* mRNA from serial threefold dilutions of the RT reaction. We obtained a signal of the same intensity for the fifth dilution of either wt or *vif*− *rev* RNA, suggesting no difference in the transcription of early multispliced mRNAs. An example of a late unspliced or singly spliced mRNA was the

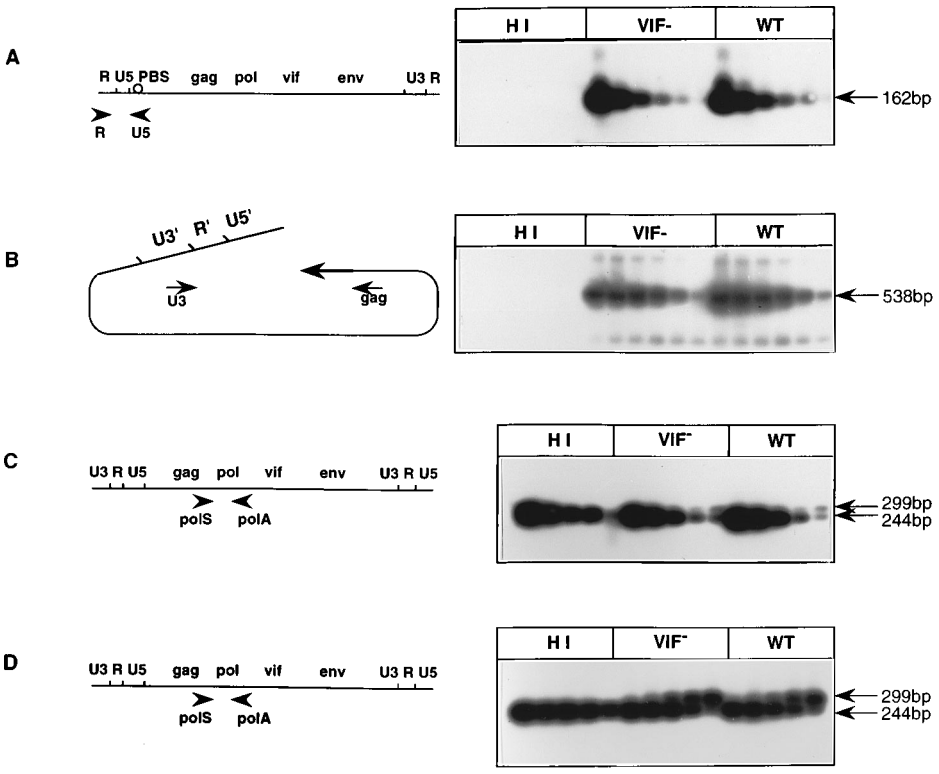

FIG. 5. PCR analysis of the reverse transcription steps during one replicative cycle of heat-inactivated wt (HI), wt, or *vif*− CAEV. Cellular DNA was extracted at 2 h (C) or 6 h (A, B, and D) postinfection. (A) Amplification with the R/U5 primers and semiquantitative analysis of serial threefold dilutions of DNA, starting with 100 ng. (B) Amplification with the U3/GAG primers of serial threefold dilution of DNA, starting with 250 ng. In both cases, Southern blots of the amplified products were hybridized with the ^{32}P-labeled U5H oligonucleotide probe. Cellular DNAs (100 ng) extracted at 2 h (C) or 6 h (D) postinfection were coamplified with the POL S/POL A primers in the presence of threefold dilutions of the Δ*pol* competitor plasmid, starting with 3.3 pg (C) or 10 pg (D). Both Southern blots were hybridized with the ^{32}P-labeled POL H oligonucleotide probe.

env mRNA (Fig. 6C). The relative amount of *env* mRNA was estimated by using a quantitative competitive PCR assay with the ENV2/ENV3 primers. Constant volumes of the RT reaction mix were coamplified with serial threefold dilutions of a competitor DNA containing a 183-bp deletion in the *env* gene. The 50% competing dose was 1 pg in each case, and this allowed us to conclude that the same amount of *env* mRNA was produced at 24 h postinfection in the wt and *vif*− CAEV-infected cells. The same approach was used to quantitate the genomic mRNA using the POL S/POL A primer pair (Fig. 6B); the 50% competing dose was 0.37 pg for the wt as well as for the *vif*− virus. All these results suggested that, at the intracellular level, viral mRNAs were transcribed with the same efficiency during one replicative cycle of wt or *vif*− CAEV, which appeared to be complete within 24 h instead of 72 h, as previously described for visna virus (35) and CAEV (14).

Viral supernatants were collected at 24 h postinfection, and virion-associated RNA was extracted and subjected to quantitative competitive RT-PCR with the POL S/POL A primers to quantify virus production. As shown in Fig. 6D, the 50% competing dose was 0.1 pg for the *vif*− virus, compared with 0.3 pg for the wt virus. Since these experiments demonstrated a significant quantitative difference in the relative amount of virion-associated RNA, it could be assumed that the defect introduced by the *vif* deletion was located in the very late stages of the replication cycle, most probably at the moment of virus formation and/or release.

Quantitative defect in *vif*− CAEV production is amplified after a second replicative cycle. The wt and *vif*− viral particles produced in the supernatant after the first replication cycle (24 h) were used to infect GSM cells, to analyze the consequences of the *vif* deletion on a second replicative cycle. Cellular DNA was extracted at 6 h postinfection and subjected to PCR amplification to detect the strong-stop cDNA and completion of the double-stranded DNA. Semiquantitative analysis of the amplified products revealed that for the early reverse-transcribed product (Fig. 7A), the lowest amounts of DNA producing a detectable signal were 12.3 ng of wt DNA and 37 ng of *vif*− DNA. The same threefold difference was also observed when analyzing the late reverse-transcribed product (Fig. 7B), in which the last dilution giving rise to an amplified signal corresponded to 30.8 ng for the wt DNA and 92.5 ng for the *vif*− DNA. Since this difference reflected the input quantitative difference, these results indicated that the *vif*− viruses produced after one replication cycle were not modified in their infectious capacity and that reverse transcription continued normally.

Cellular DNA was prepared at 24 h postinfection and subjected to quantitative competitive PCR amplification of the viral genomic DNA with the POL S/POL A primers as described above. Figure 7C shows that 50% competition was obtained with 0.1 pg of competitor DNA in the case of the wt virus (third dilution), compared with 0.03 pg for the *vif*− virus (fourth dilution), suggesting that transcription during the second cycle was equally efficient in both cases. Finally, virions produced at 24 h postinfection were concentrated, and associated RNAs were quantified in the same way (Fig. 7D). This experiment revealed a ninefold decrease in *vif*− virus production; the 50% competing dose was 11 fg for the wt virus (third

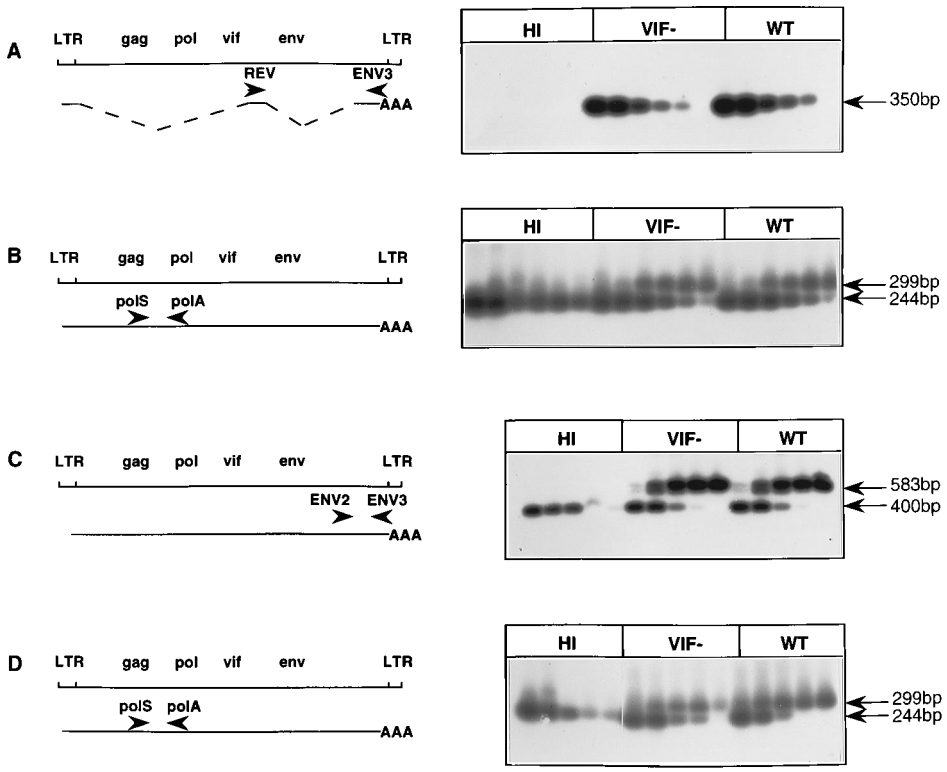

FIG. 6. RT-PCR analysis of the transcription steps during one replicative cycle of heat-inactivated wt (HI), wt, or *vif*− CAEV. Cellular RNAs (A, B, and C) or RNA associated with released virions (D) was extracted at 24 h postinfection. LTR, long terminal repeat. (A) Amplification of *rev* mRNA with the REV/ENV3 primers from threefold dilutions of the RT reaction mix, starting with 5 μl. (B and D) PCRs were performed with the POL S/POL A primers on mixtures containing a constant amount of 5 μl of the RT reaction mix and serial threefold dilutions of the Δ*pol* competitor plasmid, starting with 10 pg (B) or 1 pg (D). Both Southern blots were hybridized with the ^{32}P-labeled POL H oligonucleotide probe. (C) PCR was performed with the ENV2/ENV3 primers on a mixture of 5 μl of the RT reaction mix and threefold dilution of the Δ*env* competitor plasmid, starting with 3 pg. The Southern blot was hybridized with the ^{32}P-labeled ENV H oligonucleotide probe.

dilution) and 1.2 fg for the *vif*− virus (fifth dilution). Therefore, analysis of a second replication cycle gave the same finding as the first, namely, no difference in the reverse transcription or transcription events but impaired virus production, which is more pronounced in the second replication cycle, confirming the role of the *vif* gene product in the very late steps of viral replication. An increase in this defect during the time course of an infection could account for the slow and low replication of *vif*− mutants that we observed in GSM cells (Fig. 3) and primary macrophages (Table 1).

DISCUSSION

This paper describes the phenotype of several *vif* deletion mutants of CAEV which appeared to have a slow and low replication profile on GSM cells compared with the wt virus. The four *vif* mutants were obtained by deletion of different conserved domains among the Vif proteins of ungulate (*vif* Δ1 and Δ4) or all (*vif* Δ3) lentiviruses (Fig. 1B), and they all exhibited the same phenotype as the largest deletion mutant, *vif* Δ2, suggesting the importance of these domains in protein structure and function. This conclusion was also reached with the *vif* Nco− mutant, in which the initial mutation, while being conserved, was compensated for by a second mutational event, resulting in restoration of the wt *vif* phenotype. These events took place in a region upstream from the mutation site, which is one of the most variable regions in the ungulate Vif proteins (Fig. 1B). Furthermore, a complementation experiment demonstrated that the wt *vif* phenotype could be restored by expression in *trans* of the *vif* gene (Fig. 4). This suggested the necessity for CAEV to have a functional *vif* gene in order to replicate efficiently.

We previously reported (1) that the *vif* gene of visna virus was expressed late in the replication cycle and that the Vif protein was localized in the cytoplasm of infected cells but was not associated with the viral particles. On the basis of the close relationship between visna virus and CAEV, one could assume that these features are also valid for Vif of CAEV. The Vif protein of HIV-1 also shares these characteristics (11, 18, 19), and one report described the existence of HIV-1 Vif as a soluble cytosolic form and as a membrane-associated form in infected cells (11). These authors demonstrated that the C-terminal part of the Vif protein was important in both membrane association and function and that the hydrophobic region (residues 142 to 154) containing the highly conserved motif SLQXL was required for complementation of Vif function in *trans*. Our results demonstrated the importance of this domain in CAEV Vif function, as the *vif* Δ3 mutant, coding for a protein which is deleted in this domain, has the same impaired phenotype as the largest deletion mutant, *vif* Δ2. This domain is conserved in 34 of 38 lentivirus Vif proteins, and it is also found in nonviral proteins associated with cell membranes (23). It is most likely that at least part of the function of the Vif proteins is conserved among all the lentiviruses as a result of the conservation of this short domain in spite of little or no global sequence similarity. However, the precise function of Vif remains to be definitively established in primate and nonprimate lentiviruses.

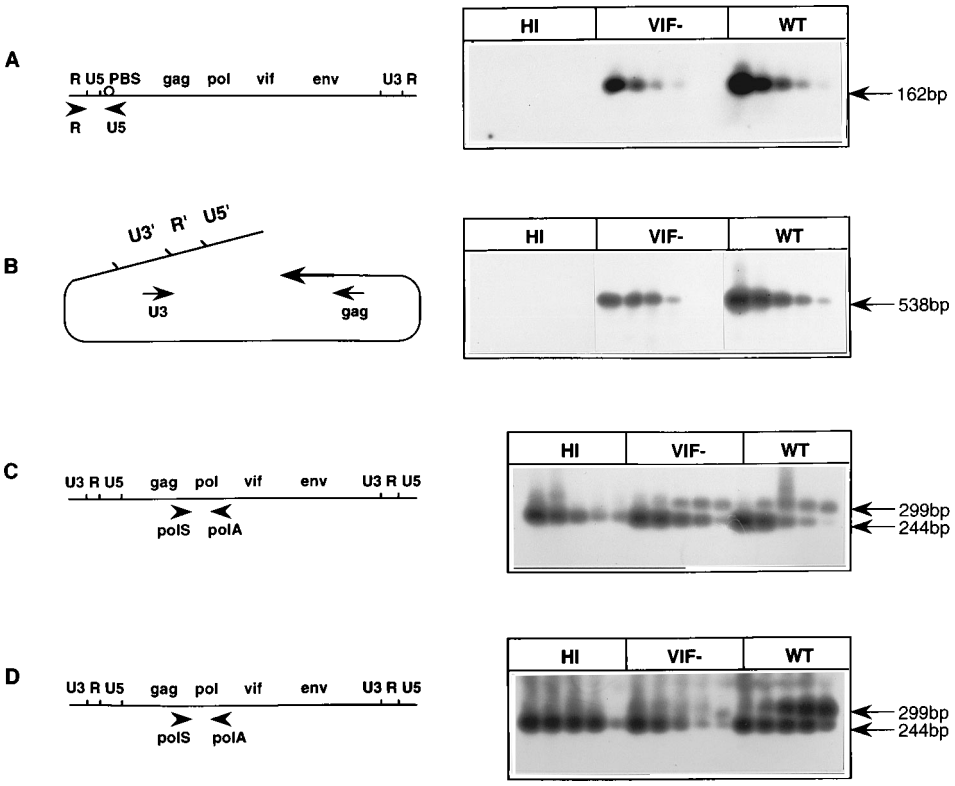

FIG. 7. PCR and RT-PCR analyses of a second replication cycle of heat-inactivated wt (HI), wt, or *vif*− CAEV produced at 24 h postinfection. Cellular DNA was extracted at 6 h (A and B) or 24 h (C) postinfection. Virion-associated RNA was prepared from cell supernatant at 24 h postinfection (D). Primers and ^{32}P-labeled oligonucleotide probes were as described in the legend to Fig. 5. For semiquantitative PCR analyses, the highest inputs of DNA were 1 μg (A) and 2.5 μg (B) which were then threefold diluted. For quantitative competitive RT-PCR analyses, 5 μl of the RT reaction mix was coamplified with a threefold dilution of the Δ*pol* competitor plasmid, starting with 0.1 pg (C and D).

We investigated which stage of the replicative cycle was affected by the *vif* deletion during replication in primary GSM cells. Quantitative analyses of different steps of the reverse transcription and transcription process through two consecutive replication cycles revealed no detectable differences between the wt and *vif*− viruses, indicating that the *vif* gene product might act at a posttranscriptional level. However, the extracellular production of *vif*− virus showed a threefold decrease compared with that of the wt virus; this difference was more marked after the second replication cycle. This quantitative difference argues for a role of Vif in particle formation and/or release. Our work did not address the question of the protein content and morphological structure of the released *vif*− virions, as recently reported for *vif*− HIV-1 (13). However, our experiments demonstrated that the *vif*− viral particles produced at 24 h postinfection were still able to enter GSM cells, suggesting no major effect of Vif on the virion structure and therefore on the infectivity of the virus particles. These results are in agreement with the conclusions drawn by Blanc et al. (2) and Fan and Peden (5) from studies of *vif*− mutants of several HIV-1 isolates grown on permissive cell lines. These authors and others (7, 10, 19) showed that *vif*− HIV-1 and HIV-2 are unable to productively infect activated fresh PBMC. Our results established that primary GSM cells or primary macrophages could be considered semipermissive for *vif*− CAEV replication. It thus appeared that, in contrast to studies on *vif*− primate lentiviruses, we did not have fully restrictive cell lines or primary cells with which to study the function of the *vif* gene of CAEV. This could be a potential reason to explain the difference between our results and those concerning the HIV *vif* gene, but we cannot exclude the possibility that the *vif* genes of primate and nonprimate lentiviruses might have diverged in their function(s).

The ungulate lentiviruses are known to latently infect blood monocytic cells (8, 9, 22) and dendritic cells (12). Our experiments showed that the *vif*− CAEV did not grow on undifferentiated monocytes and grew poorly on differentiated macrophages compared with the wt virus. This would suggest an important role for Vif during CAEV infection in vivo. Infection of goats with the infectious molecular clone of CAEV resulted in the establishment of persistent infection and pathogenesis characterized by the induction of arthritic lesions in the joints a few weeks after inoculation (34a). Experiments in vivo are in progress to study the effect of the *vif* deletion on virus replication, persistence, and disease progression in this animal model.

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