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Conservation of an Intact Human Immunodeficiency Virus Type 1 *vif* Gene In Vitro and In Vivo

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Replication of *vif*-negative human immunodeficiency virus type 1 (HIV-1) is attenuated in certain cell lines and highly impaired in peripheral blood lymphocytes in vitro. To determine whether intact *vif* is positively selected during natural HIV-1 infection and to determine *vif* sequence variability, we employed PCR amplification, cloning, and sequencing to investigate the *vif* region of replicating virus in short-term-passage HIV-1 primary isolates from five asymptomatic individuals and from five persons with AIDS. A total of 46 *vif* clones were obtained and analyzed. Recombinant proviruses were constructed from selected *vif* clones from one patient and found to be fully infectious. We found that 38 of the 46 clones sequenced carried open *vif* reading frames and that there was a low degree of heterogeneity of *vif* genes within isolates from the same individual and among isolates from different donors. The cysteines previously found to be essential for *vif* protein function were conserved in all clones. A phylogenetic tree constructed from all available *vif* nucleotide sequences resulted in a virus grouping similar to those of *gag* and *env*. Direct sequencing of *vif* amplified by PCR from uncultured lymphocytes of 15 individuals at various stages of progression toward AIDS demonstrated *vif* open reading frames in 13 of 15 samples tested. There was no obvious correlation between disease status and the presence of an intact *vif* within this sample group at the time of sample procurement. The conservation of the *vif* open reading frame in vitro and in vivo and its limited variability following virus transmission in vitro are consistent with a role for *vif* in natural HIV-1 infection.

The human immunodeficiency virus type 1 (HIV-1) *vif* gene encodes a 23-kDa protein which facilitates HIV-1 infection (1, 8, 10, 28, 33–35, 38) and cytopathicity (16, 20, 28) in certain T-cell lines. The *vif* open reading frame is conserved among most lentiviruses (25), suggesting that *vif* is important for natural infection. This notion is further supported by reports that HIV-1 clones deficient in *vif* are impaired in replication in primary lymphocytes (1, 10, 31, 38) and macrophages (23, 38). If *vif* is absolutely required for HIV-1 infection in vivo, one can expect selection within the replication-competent quasispecies of an individual in favor of HIV-1 variants which carry intact *vif*. Until now, few analyses of *vif* sequences in HIV-1 isolates from a single donor have been published. Groenink et al. sequenced the *vif* region of four infectious molecular clones of HIV-1 from a culture of peripheral blood lymphocytes (PBL) from an AIDS patient and found that they were highly homologous and that they all encoded full-length proteins (11). We analyzed *vif* in two molecular clones of an HIV-1 isolate from an individual with lymphadenopathy (28). One of the two clones had an incapacitating 35-bp deletion in *vif*, but they were more than 98% homologous in this region (29). The analysis of *vif* in both studies was limited in that it evaluated only a small number of molecular clones of cultured HIV-1 isolates from a single donor.

In this work, we extended the analysis of the structure of *vif* to primary HIV-1 strains in short-term cultures and to HIV-1 sequences present in uncultured lymphocytes from HIV-1-positive individuals as a first step to determine the contribution of *vif* to natural infection. Our goal was to determine the prevalence of intact *vif* in vivo and during replication of primary HIV-1 in peripheral blood mononuclear cells (PBMC) and to determine the extent of *vif* sequence variability within the replicating virus population. To minimize the selection of minor HIV-1 variants potentially present in donor PBMC, we examined *vif* sequences in cells during short-term (less than 2 weeks) virus transmission from HIV-1-positive PBMC to uninfected PBMC. We used PCR amplification to produce viral DNA for analysis of the *vif* sequences. Certain *vif* clones were evaluated functionally by constructing and assaying the infectivity of recombinant viruses. To assess whether there are any marked features within *vif* which correlate with disease status, as has been suggested for the V3 region of *env* (9, 15, 22), we compared *vif* sequences from HIV-1-infected, asymptomatic individuals with those from persons with AIDS. To evaluate the relatedness of the 46 clones presented here and those previously published, we constructed a phylogenetic tree from all available *vif* sequences. Finally, we also amplified the *vif* gene by PCR directly from uncultivated PBMC from 15 asymptomatic and symptomatic persons with HIV-1 infection and analyzed the amplified DNA by direct sequencing. Our results

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TABLE 1. Characteristics of donors and primary virus isolates

Isolate no.	No. of clones analyzed	No. with intact open reading frames	Disease status at sample date[a]	% $CD4^+$ cells in PBMC	Infectivity or cytopathicity of viral isolates		
					PBMC (p24, ng/ml)	H9 (p24, ng/ml)	MT-2 (syncytium formation)[b]
1	4	4	AS	43	2.4	0.037	0
2	2	2	AS	27	2.4	0.027	0
3	7	2	AS	30	10.4	0.27	0
4	12	12	AS	35	7.6	0.23	0
5	2	2	AS	20	2.4	0.32	0
6	6	5	AI	14	171	259	4+
7	2	2	AI	18	218	178	4+
8	6	4	AI	NA	153	147	4+
9	6	3	AI	5	111	188	4+
10	2	2	AI	2	215	82	4+

[a] AS, asymptomatic; AI, AIDS; NA, not available.
[b] 4+, more than 10 syncytia per field.

indicate that the *vif* open reading frame in vivo and in vitro and *vif* sequences in culturable virus are generally highly conserved, as has been reported for the structural gene *gag*. Notably, however, the frequency of inactive *vif* genes in vivo and in HIV-1 isolates exceeded that of *gag* (19, 30).

MATERIALS AND METHODS

Patient populations and primary virus isolates. For primary virus isolation and PCR analysis, heparinized peripheral blood was obtained from 10 HIV-1-positive individuals enrolled in the Pittsburgh arm of the Multicenter AIDS Cohort Study (MACS) and the AIDS Clinical Trial Group (ACTG). None of the subjects was taking antiretroviral drugs. Mononuclear cells were obtained by Ficoll-Hypaque density centrifugation. At the time of sample procurement, the percentage of CD4-bearing cells in PBMC was determined by flow cytometry, and the clinical status of patients was established (Table 1). Cocultures were established by incubation of patient PBMC with lectin-stimulated PBMC from a negative donor as previously described (6). PBMC were harvested for PCR after 1 to 2 weeks of culture. The infectivity of primary HIV-1 isolates was evaluated in phytohemagglutinin (PHA)-stimulated PBMC and H9 cells as described previously (6). Briefly, 10^7 cells were incubated with 25 μg of DEAE-dextran per ml for 20 min at 37°C, washed, exposed to 10^5 cpm of reverse transcriptase (RT) units of virus in 1 ml for 1 h at 37°C, and cultured in RPMI 1640 containing 5% interleukin-2, conditioned medium, 20% fetal bovine serum (FBS), and antibiotics. The culture supernatant was tested for the content of HIV-1 p24 capsid antigen at day 8 after infection by the HIV-1 p24 capture kit (NEN/Dupont, Boston, Mass.). The ability of the primary isolates to induce syncytia was tested in MT-2 cells as described by O'Marro et al. (26). Briefly, 96-well flat-bottomed plates were seeded in triplicate with 4×10^4 MT-2 cells in 200 μl of RPMI 1640 medium containing 10% FBS. Following 2 to 4 h of incubation at 37°C, 100 μl of medium containing HIV-1 isolates (0.05×10^6 to 0.8×10^6 RT units per ml) were added to cell-coated wells. The plates were incubated for 3 to 5 days until extensive, characteristic cytopathology had developed in positive virus control wells containing syncytium-inducing HIV-1.

For *vif* analysis in uncultured PBMC, heparinized blood was obtained and PBMC were isolated as described above from three groups of HIV-1-positive individuals: gc1 and gc2 are nonprogressors enrolled in the Pittsburgh MACS, and i1 through i4, ib, and id are individuals with slowly progressing symptoms who met the criteria for diffuse infiltrative lymphocytosis syndrome, an immunogenetically determined host response to HIV-1 characterized by the development of persistent circulating CD8 lymphocytosis and infiltration of salivary glands and other viscera with CD8 T cells (13, 14). These individuals have low rates of opportunistic infections, maintain relatively well-preserved numbers of CD4-positive T cells for long periods of time, and carry lymphocyte-derived HIV-1 strains with narrow nucleotide divergence of the *env* V3 domain (14). gmk1 through gmk8 were recruited from participants in the St. Luke's-Roosevelt ACTG and represent various stages of progression toward AIDS as defined by CD4-positive T-cell depletion. Isolated PBMC were washed in phosphate-buffered saline (PBS) and frozen at −80°C until use.

Cell lines, viruses, and plasmids. Lymphoblastoid cell lines MT-2, H9, and SupT1 were obtained from the AIDS Research and Reference Reagent Program, National Institutes of Health, Bethesda, Md., and maintained in RPMI 1640 medium supplemented with 10% FBS. Human rhabdomyosarcoma RD cells were obtained from A. Srinivasan and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. The construction and characteristics of the recombinant viruses KS282 and KS242 have been described (29).

DNA preparation and PCR amplification. Cells from short-term cocultures or uncultured PBMC were lysed in buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.5% Nonidet P-40, 0.5% Tween 20, and 60 mg of proteinase K per ml at 60°C; the proteinase K was subsequently inactivated by heating the reaction mixture to 95°C. The HIV-1 *vif* and *env* region DNAs in cell lysates were amplified by nested PCR (12, 34). The outer *vif* region primers were VF4937 (5′-GGACCAGCAAAGCTCCTCTGGAAAGT-3′, 4937 to 4962) and VF5710 (5′-TGGCTTCCACTCCTGCCCAAGTAC-3′, 5710 to 5735); the inner *vif* region primers were VF52 and VF32 (34). The respective outer and inner *env* region DNA primers were E6940 (5′-CCGGATCCTACAAATGTCAGCACA GT-3′, 6940 to 6957) and E7730 (5′-GGGAATTCTCTTTGCCTTGGTGGG TG-3′, 7713 to 7730), BRU5, and BRU3 (39). The numbers indicate the positions of primers with the numbering of HXB-2 (24). PCR was performed essentially as described before (12, 34) with 0.6 mM each primer, 0.2 mM each of the four deoxyribonucleotide triphosphates, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, and 4 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). Both rounds of PCR consisted of 40 cycles, each cycle divided into a denaturation step (94°C, 1 min), annealing step (55°C, 1 min), and elongation step (72°C, 1 min), with the denaturation time lengthened to 5 min during first cycle and the elongation step lengthened to 10 min during the last cycle. The reaction mixture containing the amplified product from the first step was used as the template in the second round of PCR at a dilution of 1:50.

Cloning and sequencing. The reaction mixtures containing the amplified products from the second PCR step were used directly in a ligation reaction with the pCR vector (TA cloning kit; Invitrogen, San Diego, Calif.). Two to 10 clones from each patient were sequenced by the chain termination method in the Applied Biosystems automatic sequenator at the Core Facility of the Columbia Comprehensive Cancer Center; a 688-bp fragment encompassing the open reading frame of *vif* was sequenced in both directions from outer M13 forward and M13 reverse primers and inner VF5282 and VF5411 (34) primers. In the case of patients 4, 6, 8, and 9, PCR products were generated and cloned from two independent PCRs. The 307-bp fragment containing the *env* V3 area was sequenced in both directions from outer M13 forward and M13 reverse primers.

Direct sequencing of PCR products. Amplified *vif* DNA was gel purified and sequenced along both strands by the dideoxy chain termination method as described above. The sequencing primers were VF5001 (5′-AAGTAGTGCC AAGAAG-3′, 5001 to 5016), VF5282 (34), VF5323 (5′-CAGGGGTCTACTTG TGTGC-3′, 5323 to 5340), and VF32 (34). Some nucleotide residue positions were ambiguous, presenting as overlapping peaks in a chromatogram. In such cases, the larger peak was considered the prevalent nucleotide at that position and thus served for creating a consensus sequence.

Sequence analysis. Nucleotide and protein sequence analysis was computer assisted, using the ALIGN and PROTYLZE programs (S&E Software, State Line, Pa.), and the published HIV-1 sequences were obtained with the aid of the BLAST electronic mail server at the National Center for Biotechnology Information (NCBI, Bethesda, Md.) (3) and the local Gopher client program from the combined GenBank, EMBL, PIR, and Swiss-Prot data libraries. The consensus sequence of *vif* from each donor and a reference sequence of all subtype B *vif* sequences were constructed. The protein sequences of clones from each individual were aligned, and a consensus *vif* sequence for each donor was constructed by hand. The 10 consensus sequences from our donors were aligned with published *vif* sequences of HIV-1 strains of the B subtype, and the subtype B consensus sequence was constructed by hand to serve as an overall reference. The PROSITE library (5) was searched by the FASTA method (27) for functional motifs in the *vif* protein with the aid of the GenQuest sequence comparison e-mail server at Oak Ridge National Laboratory (Oak Ridge, Tenn.). Distance matrix and phylogenetic analyses were performed with neighbor-joining algorithms (32) in the TREECON package (37) and by using maximum-parsimony algorithms in the Phylogenetic Analysis Using Parsimony (PAUP) package (36).

Generation of recombinant HIV-1 clones with *vif* region amplified by PCR. DNA present in patient 9 (Table 1) PBMC lysates was amplified by nested PCR, generating the 1.1-kb fragment encompassing *vif*. The respective outer and inner primer pairs were VF4579 (5′-TGGCAGCAATTTCACCGG-3′, 4579 to 4586) and VF5971 (5′-TCTCCGCTTCTTCCTGCC-3′, 5971 to 5988) and VF4632 (5′-TCAAGCAGGAATTTGGAATTCCCTAC-3′, 4632 to 4657) and VF5732 (5′-GCAGTTGTTGCAGAATTCTTATTATGGC-3′, 5732 to 5759). The inner set of primers contained *Eco*RI restriction endonuclease sites, which facilitated cloning of the amplified 1.1-kb fragment into a cassette of the viral genome generated by *Eco*RI digestion of molecularly cloned proviral DNA from KS242 (29). The orientation of the insert was evaluated by cleavage with the unique restriction enzyme set. Recombinant plasmids were amplified in bacterial culture, and supercoiled plasmid DNA was column purified (Qiagen plasmid kit; Qiagen, Hilden, Germany). The viability of viral recombinants was assayed by transfection of plasmid DNA into RD cells by the calcium phosphate precipitation method (4); virus released into the culture medium was standardized by p24 core antigen levels and used for infection of SupT1 and MT-2 cells at 0.5 pg of p24 per cell, which in our hands equals a multiplicity of infection of 0.5 (29). The replication of viral recombinants was monitored by measuring expression of HIV-1 antigens by an indirect immunofluorescence assay with sera from AIDS

TABLE 2. Biological activity of recombinant HIV-1 clones carrying *vif* genes amplified by PCR from patient PBMC[a]

HIV-1 clone	HIV-1 p24 core antigen (ng/ml of culture supernatant)				
	RD cell cultures, 48 h after transfection	MT-2 cell cultures, on day after infection:			
		3	6	9	14
KS242	105.8	0.87	547.0	284.0	ND[b]
KS282	118.4	0.11	0.94	3.72	1.66
9.1	73.7	0.10	450.6	366.2	ND
9.21	90.5	1.41	149.9	360.0	ND

[a] HIV-1 DNA transfections, viral infection, and evaluation of virus expression were performed as described in Materials and Methods. Clones 9.1 and 9.21 are chimeric infectious molecular clones of HIV-1 containing a PCR-amplified *vif* region DNA from isolate 9.
[b] ND, not determined.

patients as a first antibody and by release of p24 core antigen into the culture medium.

RESULTS

Characteristics of patient population and *vif* sequence data obtained. HIV-1 isolates from 10 HIV-1-infected individuals in the Pittsburgh area were selected for virological studies in vitro. The criteria for inclusion in this study were the disease status of the subjects and the phenotype of the virus isolates from these subjects. Five donors who were asymptomatic and whose virus isolates were non-syncytium inducing and did not replicate in T cell lines (9) were chosen. The other five donors were AIDS patients; their HIV-1 isolates were capable of replication in T-cell lines and syncytium inducing (9). The donor characteristics and their viral phenotypes are summarized in Table 1. This study used 1- to 2-week cocultures of PBMC of these donors as the source of viral DNA both to evaluate replicating virus and to minimize selection of minor viral variants in vitro.

PCR amplification of the 688-bp area encompassing the entire *vif* open reading frame and parts of the *pol* and *vpr* genes was conducted on PBMC extracts. Amplified fragments were cloned, and the nucleotide sequences of 46 individual clones were determined. Forty-five of 46 sequences were of the same length; the sequence of one of the clones from patient 9 had a deletion of two nucleotides. The numbers of clones sequenced for each patient and statistics about the integrity of the *vif* gene sequences are given in Table 1. The results show that 38 of the 46 clones sequenced carried *vif* open reading frames. Of the remaining clones, two lacked start codons, five had stop codons that resulted in premature termination, and one carried a frameshift mutation. These results demonstrate that the majority of the *vif* clones (83%) amplified from short-term primary cultures of viruses from 10 HIV-1-positive individuals carried *vif* genes with intact open reading frames.

To study the functionality of a representative *vif* derived from primary virus cultures, *vif* clones from patient 9 were exchanged with the corresponding *vif* region in KS242, a prototype HIV-1 (28, 29). KS242 served as a positive control, and *vif*-negative KS282, which carries an incapacitating 35-bp deletion in *vif* (29), represented the *vif*-negative phenotype. Control and recombinant virus were used for infection of SupT1 and MT-2 cells; the former cells are known to be permissive for infection with *vif*-deficient HIV-1, whereas the latter are nonpermissive (10, 34). Table 2 summarizes the infections; only the *vif*-deletion mutant virus KS282 displayed a *vif*-negative infection in MT-2 cells. The recombinant viruses and wild-type virus displayed a similar cytotoxic and highly productive course of infection in SupT1 and MT-2 cells. We conclude that the *vif* isolated by PCR amplification from patient 9 is fully functional.

Analysis of *vif* protein sequences. *vif* protein sequences were first analyzed to identify any features common to sequences of primary HIV-1 isolates from either AIDS patients or asymptomatic persons which would distinguish them from sequences from the other group. The amino acid sequences were deduced from the nucleotide sequences, and the 46 *vif* sequences presented here and all the available *vif* sequences, including those from HIV-1 strains of the A, B, and D subtypes, were aligned in reference to the subtype B consensus sequence (Fig. 1). We found no obvious pattern in *vif* limited to viruses from either asymptomatic individuals or persons with AIDS. Prematurely terminated clones were found in isolates from both an asymptomatic patient and two AIDS patients. One clone each from patients 6 and 9 lacked the accepted initiation codon; however, the presence of an alternative start site 21 bp downstream may indicate a variable amino terminus in *vif* protein.

We also made two analyses of *vif* protein for sequences required for function. In one we examined all *vif* proteins for the presence of cysteines 114 and 133, which we have shown to be critical for *vif* function in HIV-1 infection (21). Both cysteines were conserved in every *vif* protein translated, indicating a strong selection for the cysteine residues in *vif* in functional virus (Fig. 1). The other analysis involved a global search for functional motifs with the PROSITE library to analyze the *vif* sequence of KS242, which we have demonstrated to be active (29). Inspection of the KS242 *vif* protein sequence revealed no prominent functional domains, including zinc fingers, nuclear localization signals, or nucleic acid-binding regions. However, 10 potential phosphorylation sites were identified in the functional protein sequence, some of which appear to be conserved over most of the sequences analyzed (Fig. 1). More sequence data are required to map any conserved or variable regions of the *vif* gene and to better identify functional regions.

To determine the extent of *vif* variability in the replicating virus pool, we also analyzed nucleotide sequence variation. The pairwise difference between sequences was calculated as the number of mismatched nucleotides between each pair divided by the length of the sequence, multiplied by 100 (percent value). For the *vif* area, the intra-individual isolate differences ranged from 0 to 2.5% and interindividual isolate differences ranged from 1.3 to 8.7% within subtype B and from 10 to 14% when compared among different subtypes. The average of these pairwise differences between clones from a single donor, the average of differences between clones from each pair of donors, and the distances between the sequences presented here and those of *vif* clones from representatives of HIV-1 subtypes A, B, and D were also calculated. The results indicate that sequence variability between *vif* clones obtained from viral cultures in vitro is on the same order as that of *gag* (22, 30). As may be expected from the origin of our clones, the *vif* sequences are more closely related to the representative subtype B sequence than to representative sequences of subtype A or D.

Phylogenetic tree analysis based on *vif* sequences. We next tested whether the differences in *vif* sequences among primary isolates and their quasispecies permit isolate grouping and HIV-1 subtype designation. Phylogenetic trees were constructed by using neighbor-joining algorithms (32, 37) for alignment of 579 unambiguously alignable base pairs in the *vif* open reading frame of the 46 clones presented here and of 18 previously published *vif* sequences (Fig. 1). Bootstrap analysis of 10,000 resamplings confirmed the stability of the major branches and of the patient-specific clusters. This data set was

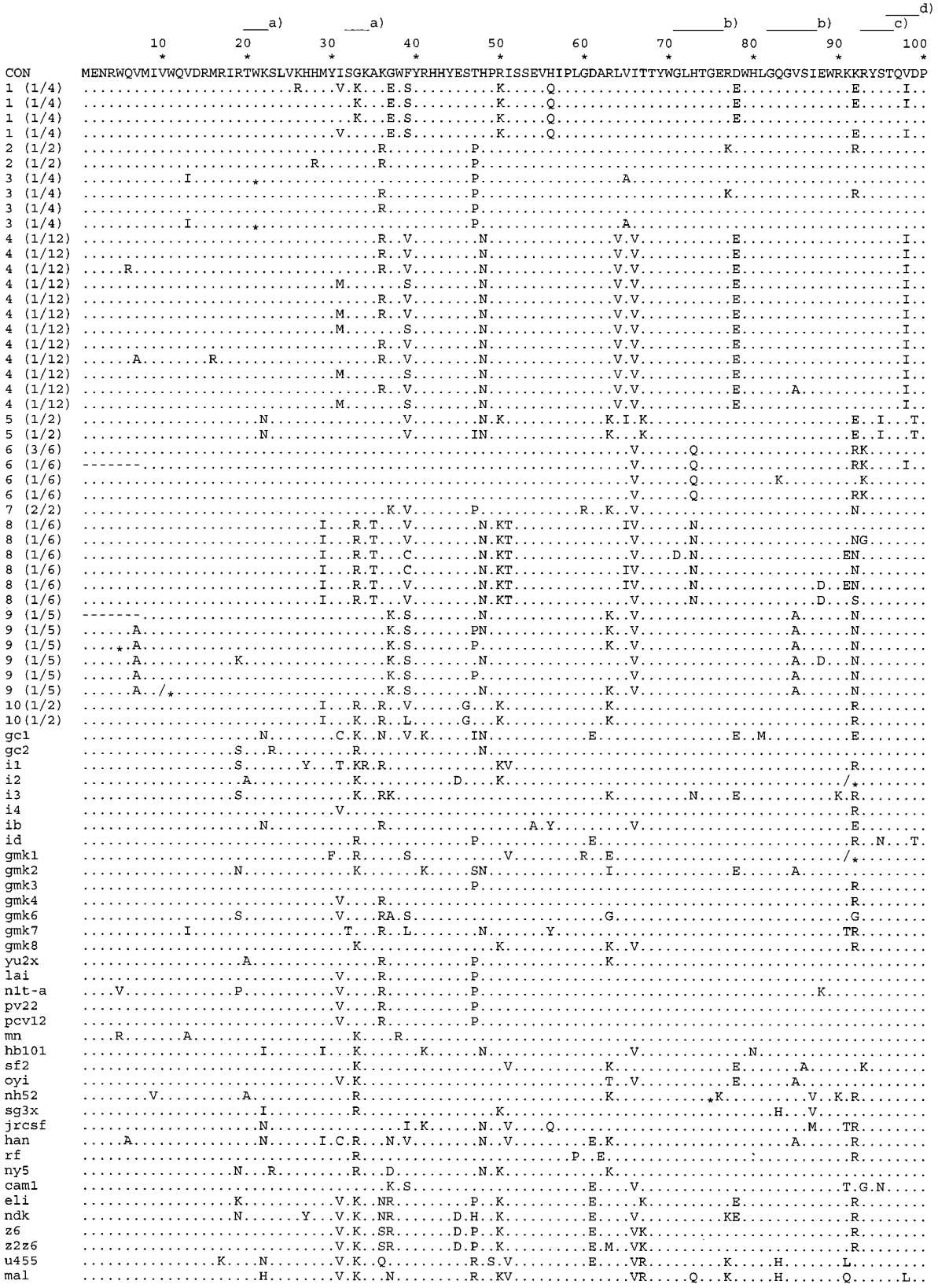

FIG. 1. Alignment of deduced amino acid sequences of *vif* protein. The nucleotide sequences of clones studied here and published elsewhere were translated and aligned relative to a reference sequence representing the subtype B consensus sequence in *vif*. The bars above the alignment indicate motifs identified by search of the PROSITE library: (a) protein kinase C phosphorylation site, (b) N-myristoylation site, (c) cyclic AMP- and cyclic GMP-dependent protein kinase phosphorylation site, and (d) casein kinase II phosphorylation site. •, identity with the consensus sequence; —, a gap; *, a stop codon or a frameshift. For some clones from patients 3, 8, and 9, as well as the strains nh52, and z2z6, the amino acid sequence following the stop codon or frameshift is supplied as if this change did not occur to illustrate the sequence alignment.

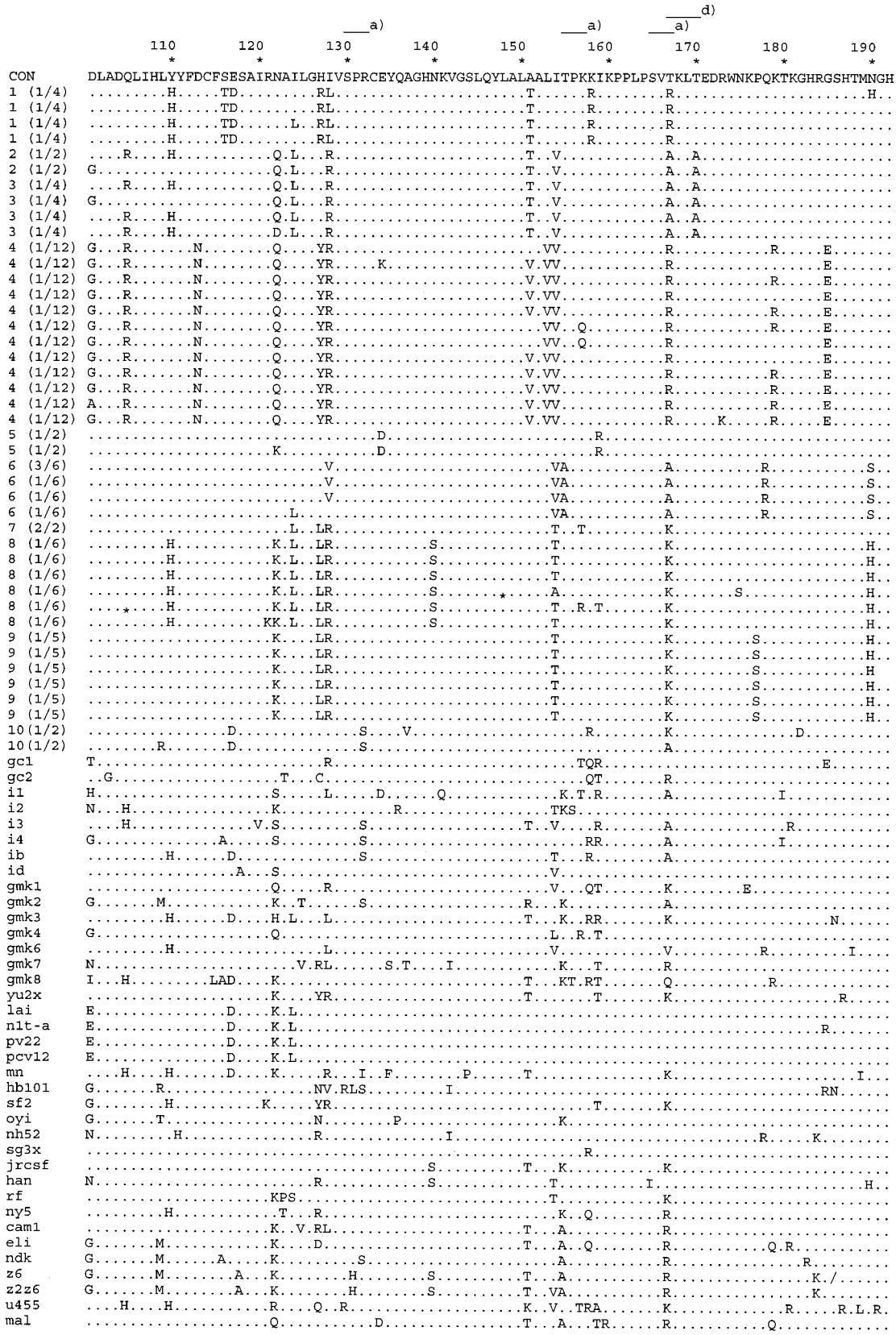

FIG. 1.—*Continued.*

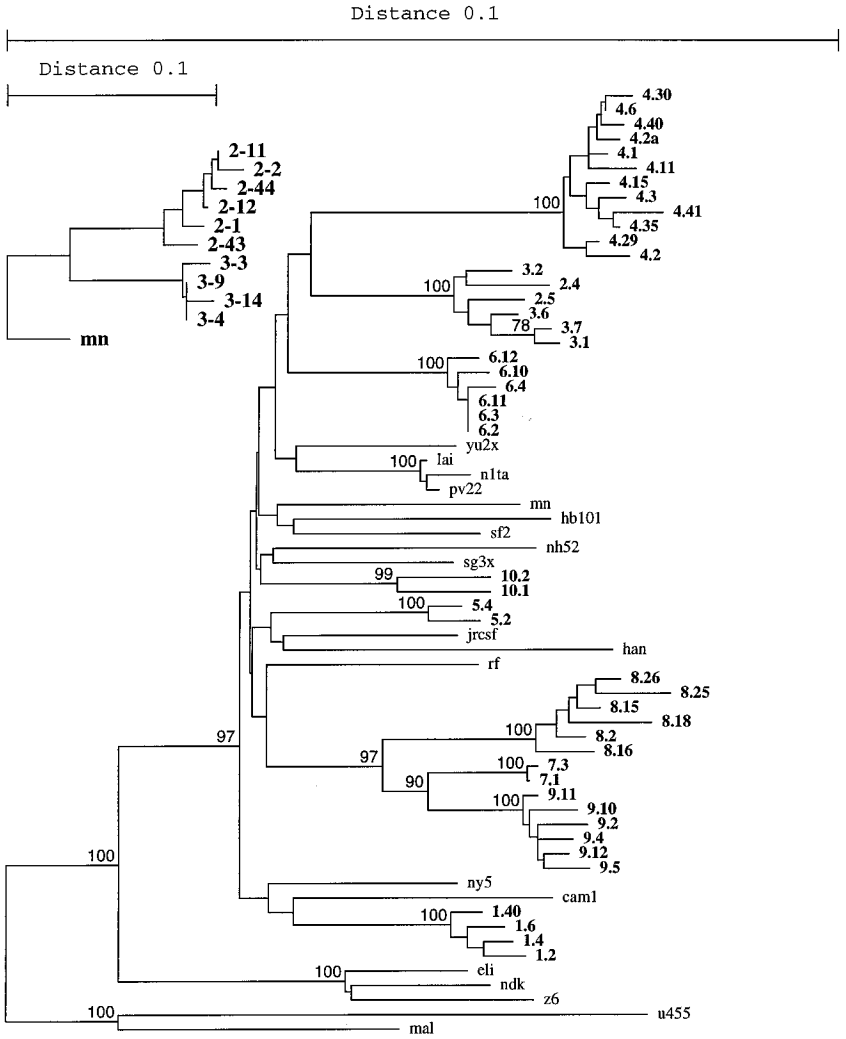

FIG. 2. Phylogenetic tree based upon all available *vif* nucleotide sequences. The tree was constructed as described in Materials and Methods and in references 29 and 34. Numbers at nodes indicate the frequency, as a percentage, with which the cluster descending from that node was found by bootstrapping on 10,000 resamplings. Numbers are indicated only for groups found in greater than 90% of the bootstrap trees. The inset contains a neighbor-joining phylogenetic tree based upon 307 bp in the V3 region of *env* from 10 sequences from patients 2 and 3.

also used in a maximum-parsimony analysis (36), resulting in the same overall tree topology (data not shown). Three clusters of HIV-1 strains can be discerned on the basis of their *vif* sequences (Fig. 2): (i) a cluster containing Ugandan U455 and Zairian MAL isolates (subtype A), (ii) a cluster containing Zairian ELI, Z6, and NDK isolates (subtype D), and (iii) a cluster of mostly North American and European isolates (subtype B). Therefore, the *vif* sequences group similarly to the *env* or *gag* sequences, which formed the basis of the HIV-1 subtype designation (19, 24).

It is interesting that clones of *vif* sequences from each individual were clearly clustered, separate from other patient sequences or published *vif* sequences, except the clones from patient 2 and patient 3. Clone 2-4 and clone 3-2 were more similar to each other than to the other clones from the same donor. Similarly, clone 2-5 clusters with clones 3-1, 3-6, and 3-7. In detailed comparison of aligned nucleotide sequences, however, some residues of clone 2-4 were identical with those of clone 3-1 and different from those of 3-2, and at other residues, the clones from patient 2 differed from the clones from patient 3. To determine whether the viruses from patients 2 and 3 are related, we amplified, cloned, and sequenced clones from *env* encompassing the V3 loop, because this has been the region of choice in defining relationships among HIV-1 isolates (17). As an outlier, we also analyzed the V3 region from patient 8. Six clones from patient 2, four clones from patient 3, and four clones from patient 8 were examined. Nucleotide sequences from the V3 area of the clones from patients 2 and 3 were used for construction of a neighbor-joining phylogenetic tree (inset in Fig. 2). The alignment of translated sequences with HIV-1 MN as a reference is presented in Fig. 3. These data show that the isolates from patients 2 and 3 are clearly different in the V3 region of *env*.

Conservation of *vif* open reading frame in vivo. To determine whether the observed preponderance of functional *vif* genes in primary HIV-1 isolates reflects the frequency of functional *vif* in vivo, we evaluated the status of *vif* sequences present in uncultured PBMC from HIV-1-infected individuals. PBMC were obtained from 15 persons at various stages of HIV-1-related disease at the time of sample procurement,

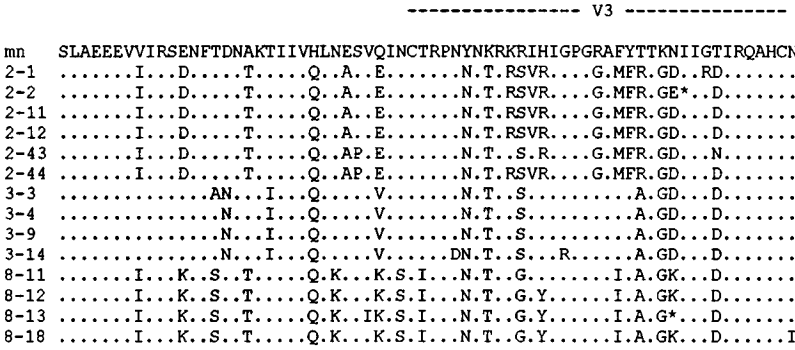

FIG. 3. Alignment of the deduced amino acid sequence of the V3 region of *env*. The V3 region of the clones from patients 2, 3, and 8 is aligned relative to that of HIV-1 MN (24) as described in the legend to Fig. 1.

including two nonprogressors (gc1 and gc2), six slow progressors (i1, i2, i3, i4, ib, and id), and eight AIDS patients (gmk1 through gmk8). The *vif* region DNA was amplified by PCR and sequenced by direct sequencing, and consensus sequences from individual samples were translated and aligned relative to a reference sequence of the subtype B consensus sequence in *vif* (Fig. 1). Thirteen of 15 patient PBMC samples contained intact *vif* open reading frames. Two of the samples, i2 and gmk1, contained frameshift mutations involving the addition of alanine in position 277 of *vif* and thus likely causing premature termination of translation (Fig. 1). All the patient samples analyzed contained conserved cysteine residues in positions 114 and 133, confirming the critical role of these residues for *vif* function (21). Thus, within the cohort of 15 patients tested, the frequency of intact *vif* in uncultured PBMC, as indicated by the presence of undisturbed *vif* open reading frames, was 87%. This is similar to the 83% frequency of intact *vif* found in short-term cultures (Fig. 1). It should be noted, however, that at the time of printing, patient i2 had progressed to full-blown AIDS and patient gmk1 had CD4-positive cell counts of less than 100. The conservation of the intact *vif* open reading frame in vivo reported here accords well with the data of Wieland et al., who found an intact *vif* in about 90% of 61 patient samples analyzed (40). We conclude that the frequency of an intact *vif* gene in short-term cultures closely resembles that in PBMC in vivo.

DISCUSSION

We performed structural and functional analysis on the auxiliary gene *vif* in vivo and in short-term cultures of primary HIV-1 isolates in vitro. A total of 83% of the DNA clones obtained from in vitro cultures contained undisturbed *vif* open reading frames. The cysteines required for function in *vif* (21) were uniformly conserved among all the genes described here, as well as those previously published. Similarly, intact *vif* was present in uncultured PBMC in the majority (87%) of the 15 HIV-1-positive individuals tested, in no obvious correlation to their disease status at the time of sampling. The significance of finding inactive *vif* open reading frames in uncultured PBMC from patient i2, who subsequently progressed to AIDS, and in patient gmk1, who maintains low CD4 cell levels (see above), needs to be explored in a larger patient population. These results are consistent with the recently published analysis of *vif* sequences in vivo (40), and together, the results strongly support the notion that *vif* plays a critical role in natural HIV-1 infection (1, 10, 21, 31, 34, 38).

It is of interest that our findings do not provide evidence for strong selection for the intact *vif* open reading frame during virus transmission. The frequency of inactive *vif* genes in transmitted viral isolates was actually slightly higher than that in uncultured PBMC from different groups of patients analyzed in this work (Fig. 1) and elsewhere (40). Seventeen percent of the *vif* genes in transmitted viruses were rendered inactive by premature termination or by the absence of initiation of transcription. This is a greater frequency of inactivating mutations than that observed for *gag* (1.5%) (19, 30) or for *nef* (3.3%) (27). It is unlikely that these mutations arose from errors in amplification or sequencing, because the overall mutation frequency among the clones sequenced was not high. Duplicate sequencing of a single clone yielded identical results (not shown), and the mutation rate of the *Taq* polymerase used (1.1×10^{-4}) (12) is much lower than required to account for the observed mutations. One possibility is that these inactivating *vif* mutations resulted from errors of reverse transcription during virus transmission. Alternatively, natural *vif* mutants may have been transmitted from PBMC at a low rate during primary culture. This would be consistent with recent studies in which we have shown that inactivating *vif* mutations impair but do not abolish viral DNA synthesis and progeny virus production in PBL in vitro (31). Together, these results raise the possibility that HIV-1 variants carrying inactive *vif* mutations persist in vivo. In our limited survey, the presence of putative *vif* mutants was not associated with a particular clinical status for an HIV-1 carrier. However, the persistence of such variants, if confirmed, may indicate a role in the natural history of HIV-1 infection.

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