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Mutational Analysis of Human Immunodeficiency Virus Type 1 (HIV-1) Accessory Genes: Requirement of a Site in the *nef* Gene for HIV-1 Replication in Activated CD4$^+$ T Cells In Vitro and In Vivo

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Human immunodeficiency virus type 1 (HIV-1) accessory genes including *nef*, *vif*, and *vpr* are important factors that determine the replication and pathogenesis of HIV-1. The state of activation is also important for the replication of HIV-1. We evaluated the properties of *nef*-, *vif*-, and *vpr*-minus macrophage-tropic HIV-1$_{JR\text{-}CSF}$ in primary CD4$^+$ Th1- or Th2-like cell cultures which had been activated through CD3 molecules in the presence of interleukin-2 (IL-2) and IL-12 (Th1-like culture) or IL-4 (Th2-like culture), respectively. In activated Th1- or Th2-like cultures, replication of *nef*-minus HIV-1$_{JR\text{-}CSF}$ was markedly lower than that of wild-type HIV-1. Subsequent analysis by site-directed mutagenesis showed that (i) the presence of an acidic amino acid-rich domain (amino acid residues 72 to 75) in the Nef protein was critical for the enhancement of viral DNA synthesis, resulting in increased virus growth rate, and (ii) prolines that form part of Src homology 3 binding domain were not essential for viral replication. We also confirmed the importance of sites by using an HIV-1-infected animal model, the hu-PBL-SCID mouse system, representing HIV-1 replication and pathogenesis in activated CD4$^+$ T cells in vivo. These results indicate that Nef accelerates viral replication in activated CD4$^+$ T cells.

The genomes of human immunodeficiency virus type 1 (HIV-1) contain accessory genes such as *nef*, *vif*, and *vpr* that may be associated with the pathogenesis of HIV-1 infection (36). Nef is crucial for maintaining a high-titer viral replication of simian immunodeficiency virus (SIV) in SIV-inoculated monkeys (28). Analysis of the (SCID)-hu-Thy/Liv mouse, a mouse strain with severe combined immunodeficiency (SCID) that is transplanted with human thymus tissue and is susceptible to HIV-1 infection, showed that Nef of HIV-1 plays a critical role in high-level viral replication and CD4 depletion in this animal (3, 26). On the other hand, HIV-1 with truncated *nef* present in some patients with long-term nonprogressive HIV-1 infection may contribute to the low-level virus replication and nonpathogenicity (13, 29).

The functional role of the regulatory genes in HIV-1 replication is, however, poorly understood. Whereas Nef is not required for virus replication in transformed T cell lines and phytohemagglutinin (PHA)-activated peripheral blood mononuclear cells (PBMC) (4, 9, 17), it seems important for optimal replication in T cells during cellular induction from a quiescent to an activated state (37, 61). Several laboratories have recently reported that Nef enhances the infectivity of the progeny virions (9, 18, 37, 38) and increases viral DNA synthesis in infected cells (2, 10, 55). In addition, Nef associates with several cellular proteins including human hematopoietic cell kinase (Hck) (50), lymphocyte-specific protein tyrosine kinase (Lck) (11), mitogen-activated protein kinase (19, 20), protein kinase C-theta (59), and p21-activated kinase (41) and modulates the cellular activation state (19, 20, 33, 56). Although several biological functions of Nef have been reported, the relationship between these functions and the effect on viral replication, especially in activated T cells, remains unclear.

The biological properties of other accessory genes, *vif* and *vpr*, have also been reported. Vif protein is necessary for proper virion formation (6) and reverse transcription (12, 60, 66), and it modulates the postpenetration stability of the viral nucleoprotein complex (58). However, these effects of Vif are highly dependent on the cell type (15). Vif is essential in some cells (H9 and PBMC) but not in others (HeLa and SupT-1). Vpr protein has many biological functions, including induction of growth arrest (5, 22, 27) and prevention of the establishment of chronic infection (47, 49), and it enhances the preintegration of HIV-1 for effective nuclear transport in nondividing cells (23).

The biological features of HIV-1 strains vary in tropisms. T-cell-line-tropic HIV-1 strains, which were used conventionally, dominate in some patients who suffer from immunodeficiency syndromes. In contrast, macrophage-tropic HIV-1 strains capable of infecting both CD4$^+$ T cells and macrophages dominate in the acute phase of infection (71) and persistently replicate throughout the entire course of HIV-1 infection (54). Previous studies have shown that the presence of an activated state in HIV-1-infected cells is essential for the development of infection (63) and that the persistence of a macrophage-tropic HIV-1 strain is critical for HIV-1 replication at high levels throughout the entire clinical course (24, 45, 46, 67). Moreover, HIV-1 resides and replicates mainly in

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lymphoid tissue (14, 44), in which $CD4^+$ T cells and macrophages are activated in response to various antigens.

Thus, the effects of accessory genes on HIV-1 replication varies according to the state of activation of the infected cells and probably according to the viral strain used. Therefore, in an attempt to investigate the function of accessory genes in a simple model, we established a primary $CD4^+$ T-cell culture model of HIV-1 replication and evaluated the properties of accessory genes by using mutant viruses, lacking *vif*, *nef*, or *vpr*, that were derived from a macrophage-tropic HIV-1 strain. Our results showed that Vif was essential for HIV-1 infection in macrophages and activated T cells, while Nef was essential only for activated T cells. In addition, our results showed that an acidic amino acid-rich domain of Nef enhances the viral growth rate in primary activated $CD4^+$ T cells and in the hu-PBL-SCID mouse system.

MATERIALS AND METHODS

$CD4^+$ Th1- or Th2-like cell cultures. $CD4^+$ Th1- or Th2-like bulk cell cultures were prepared as described previously (64). Briefly, $CD4^+$ T cells were purified from nonadherent PBMC with Dynabeads M450 (Dynal, Oslo, Norway) coated with monoclonal antibodies to CD4. These $CD4^+$ T cells were stimulated in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), interleukin-2 (IL-2) (100 U/ml; Shionogi Pharmaceutical Inc., Osaka), and either IL-12 (10 ng/ml; R&D, Minneapolis, Minn.) or IL-4 (10 ng/ml; R&D) with immobilized OKT-3 anti-human CD3 monoclonal antibody. These cells were further stimulated twice during the next 8 days (on days 3 and 6). The Th type of bulk T cells was confirmed by assaying the culture supernatants for gamma interferon (IFN-γ) and IL-5; the IL-12-pretreated $CD4^+$ cells produced large amounts of IFN-γ (10 to 100 ng/ml) and small amounts of IL-5 (<20 pg/ml), while IL-4-pretreated $CD4^+$ T cells produced lower levels of IFN-γ (<8 ng/ml) but higher levels of IL-5 (>500 pg/ml). These results indicated that IL-12- and IL-4-pretreated $CD4^+$ T-cell cultures were of the Th1-like and Th2-like phenotype, respectively.

Monocyte-derived macrophage (MDM) culture. Monocytes were enriched from PBMC of healthy HIV-1-seronegative donors through Ficoll-Hypaque treatment by adherence at 37°C for 24 h to a plastic dish coated with human AB serum in Iscove's modified Dulbecco's medium supplemented with 20% FCS. One day later, nonadherent cells were removed, and the adherent cells were cultured for 2 days in Iscove's modified Dulbecco's medium supplemented with 15% FCS and 5% giant cell tumor supernatant (IGEN, Rockville, Md.). Adherent macrophages were treated with 0.5 mM EDTA in phosphate-buffered saline (PBS) for 10 min to remove the cells from the plates, seeded into 24-well plates at a density of 2.5×10^5 cells/well, and used for the infection experiment.

Plasmid construction. The *nef*-, *vif*-, and *vpr*-minus $HIV\text{-}1_{JR\text{-}CSF}$ (31) infectious plasmid DNAs were generated by a frameshift at the *Xho*I (nucleotide 8914 of $HIV\text{-}1_{JR\text{-}CSF}$), *Nco*I (nucleotide 5309 of $HIV\text{-}1_{JR\text{-}CSF}$), or *Afl*II (nucleotide 5646 of $HIV\text{-}1_{JR\text{-}CSF}$) site. The *nef*-minus $HIV\text{-}1_{NL4\text{-}3}$ (1) infectious plasmid DNA, which has also a frameshift at *Xho*I (nucleotide 8944 of $HIV\text{-}1_{NL4\text{-}3}$), was generously provided by A. Adachi (Tokushima University). The *nef* mutants of $HIV\text{-}1_{JR\text{-}CSF}$ infectious plasmid DNA with mutations in the highly conserved core region of Nef were generated by site-directed mutagenesis by a two-stage PCR procedure (34). First, PCR was performed with an outer-sense primer versus an inner-antisense primer and with an inner-sense primer versus an outer-antisense primer. The inner-sense and inner-antisense primers were complementary for 24 bp at the 3′ end. The inner-sense primer contained an oligonucleotide sequence for site-directed mutagenesis. After purification of both PCR products, a second PCR was performed with the outer-sense primer versus the outer-antisense primer, with a mixture of the two PCR products as a template. The second PCR product contained the full-length *nef* sequence. It was cloned into pGEM-T (Promega, Madison, Wis.), and the mutated bases were confirmed by sequencing. The *Xho*I (nucleotide 8914 of $HIV\text{-}1_{JR\text{-}CSF}$)-*Eco*RV (nucleotide 9137 of $HIV\text{-}1_{JR\text{-}CSF}$) fragment containing the *nef* mutation (M1 to M5 [see Fig. 5]) was substituted into the wild-type $HIV\text{-}1_{JR\text{-}CSF}$. Oligonucleotides used for PCR-based site-directed mutagenesis included outer-sense (5′-CGCGGATCCTCCATGGGCTATAAGATGGGTGGCAAG-3′), outer-antisense (5′-CGCGGATCCTCAGCAGTTCTTGAAGTACTC-3′), inner-sense for M1 (5′-GATGAGGAAGTGGGTTTTCCAGTCAGAGCTCAGGTAGCTTTAAGA-3′), inner-antisense for M1 (5′-GACTGGAAAACCCACTTCCTCATC-3′), inner-sense for M2 (5′-GGACTGGAAGGGCTAATTTACTCACAGTTATTACAAGATATC-3′), inner-antisense for M2 (5′-TGAGTAAATTAGCCCTTCCAGTCC-3′), inner-sense for M3 (5′-GATTGTGCCTGGCTAGAAGCATATCCTTTAAGACCAATGACT-3′), inner-sense for M4 (5′-GATTGTGCCTGGCTAGAAGCATATGCGGCTGCGGCAGTGGGTTTTCCA-3′), inner-sense for M5 (5′-GATTGTGCCTGGCTAGAAGCATATGTGGGTTTTCCAGTCAGACCTCAG-3′), and inner-antisense for M3, M4, and M5 (5′-ATATGCTTCTAGCCAGGCACAATC-3′).

Viruses. COS cells (10^7 cells) were transfected with 40 μg of HIV-1 proviral DNA by electroporation at 230 V and 950 μF with the Bio-Rad (Richmond, Calif.) gene pulser. The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. One day after transfection, the medium was replaced with fresh RPMI 1640 medium supplemented with 10% FCS. At 2 and 3 days after transfection, the viruses were recovered, filtered through a membrane (pore size, 0.22 μm), and assayed for HIV-1 $p24^{gag}$ content by enzyme-linked immunosorbent assay (ELISA) (Coulter, Hialeah, Fla.). Aliquots of the viral stocks were stored at −70°C until use.

The titer of each virus stock was determined by end-point titer determination of threefold limiting dilution in triplicate on PHA-activated PBMC from a single donor. Briefly, human PBMC were obtained from an HIV-1-seronegative normal donor by Ficoll-Hypaque sedimentation, activated for 3 days with 1 μg of PHA per ml in RPMI 1640 medium containing 10% FCS, and suspended at 5×10^5 cells/ml in RPMI 1640 medium containing 10% FCS and 100 U of IL-2 per ml. In the next step, 100 μl of the cell suspension was mixed with 100 μl of threefold serial dilutions of virus solutions in the wells of a flat-bottom microplate and incubated at 37°C. Four days after infection, the medium was replaced with fresh medium, and the mixture was incubated for another 3 days at 37°C. Seven days after infection, the levels of $p24^{gag}$ in the supernatants were measured by a $p24^{gag}$ ELISA (Coulter). Then the 50% tissue culture infective dose ($TCID_{50}$) was calculated by the Reed-Muench method (32).

Immunoblot analysis of Nef expression. Cytoplasmic extracts from COS cells transiently transfected with wild-type $HIV\text{-}1_{JR\text{-}CSF}$ or altered form of *nef* infectious plasmid DNA were prepared. At the same time, cells were cotransfected with murine leukemia virus long term repeat-driven luciferase expression plasmid to normalize the transfection efficiency. Aliquots which corresponded to identical levels of luciferase activity prepared from COS cells were denatured in reducing sample buffer, resolved on 12% polyacrylamide gels, and electroblotted onto polyvinylidene difluoride membranes (Immobilon; Millipore Corp.). The membranes were incubated for 1 h in a blocking solution containing TBS-T buffer (150 mM NaCl, 10 mM Tris-HCl [pH 8.0], 0.5% Tween 20) plus 1% bovine serum albumin and 5% nonfat dried milk (Carnation) and then exposed to anti-HIV-1 Nef mouse monoclonal antibody (Advanced Biotechnologies Inc.) in the blocking solution. The membranes were washed three times with TBS-T buffer, incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G antibodies, and developed by using the enhanced chemiluminescence detection system under the conditions recommended by the manufacturer (Amersham, Tokyo, Japan).

Electron microscopy of mutant viral particles. COS cells were transfected by electroporation with HIV-1 mutant plasmid DNA and were pelleted 2 days later, prefixed with cold 2% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) at 4°C for 1 h, and subsequently fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer at 4°C for 1 h. The fixed pellet was dehydrated through a series of diluted ethanol solutions and embedded in epoxy resin. Ultrathin sections were prepared and stained with uranyl acetate and lead citrate and examined under an electron microscope (Hitachi, H-300 and H-800).

Endogenous reverse transcription reaction. The procedure of endogenous reverse transcription has been described in detail by Masuda et al. (35). Briefly, DNase-treated virus stocks were prepared as described above. Aliquots of virus suspensions containing 20 pg of $p24^{gag}$ were incubated in 30 μl of endogenous reverse transcription reaction mixture (0.01% Triton X-100, 50 mM NaCl, 50 mM Tris-HCl [pH 8.0], 10 mM dithiothreitol, 5 mM $MgCl_2$, 100 μM each dATP, dCTP, dGTP, and dTTP). As a negative control, reactions without dTTP were performed in parallel. After 2 h of incubation at 37°C, the reaction was terminated by adding 60 μl of stop mix (proteinase K, 50 μg/ml; tRNA, 20 μg/ml; EDTA, 1.5 mM [pH 8.0]). After incubation at 60°C for 1 h, proteinase K was heat inactivated by boiling for 10 min. Finally, 5-μl aliquots of the reaction mixtures were subjected to PCR analysis.

Assays of viral entry. Anti-CD3 activated $CD4^+$ Th1-like cells (10^6) were exposed to the virus (100 ng of $p24^{gag}$ antigen) at 37°C for 1 h. The cells were washed three times with PBS and then incubated for 5 min at 37°C in PBS containing 0.25% trypsin and 0.53 mM EDTA. After trypsinization, the cells were washed three times with PBS and pelleted. The cell-associated $p24^{gag}$ antigen content was determined by ELISA (Coulter). As a negative control for the entry assay, cells were exposed to the virus in parallel experiments at 0°C and more than 97% of the noninternalized virus was removed by trypsin treatment. We could detect no $p24^{gag}$ in cell extracts infected by heat-inactivated (30 min at 65°C) HIV-1.

In vitro infection. Anti-CD3 activated bulk $CD4^+$ Th1- or Th2-like cells induced for 8 days with IL-12 or IL-4 were collected and washed twice with PBS. The cells were exposed for 2 h at 37°C to 27 to 184 $TCID_{50}$ per 5×10^5 cells. After triplicate washing with the medium to remove residual free virus, the cells were cultured at 37°C in the presence of IL-2 (100 U/ml). MDM were exposed for 2 h at 37°C to 1,000 $TCID_{50}$ of wild-type, *nef*-minus and *vpr*-minus HIV-1 or 100 $TCID_{50}$ of *vif*-minus HIV-1. After triplicate washing with the medium, MDM were cultured in Iscove's modified Dulbecco's medium supplemented with 20% FCS. Virus production in culture supernatants of HIV-1-infected cells was monitored by an ELISA specific for the $p24^{gag}$ antigen (Coulter). The total number of anti-CD3 activated $CD4^+$ Th1- or Th2-like cells in each culture was counted simultaneously with a hemocytometer.

TABLE 1. Summary of the properties of mutant viruses

Virus	Amt of $p24^{gag}$ (ng/ml)[a]	Amt of endogenous RT[b] (copies/pg of $p24^{gag}$)[a]	No. of viral particles (per 30 cells)[a]	Entry in Th1-like culture (%)[a]	Viral DNA synthesis after 24-h infection in Th1-like culture (copies/ng of $p24^{gag}$)[a]	$TCID_{50}$/ng of $p24^{gag}$ in PHA-activated PBMC
Wild type	35.8 ± 1.4	2,680 ± 84	276 ± 43	0.081 ± 0.006	827 ± 77	140
nef minus	55.0 ± 1.7	2,860 ± 860	353 ± 31	0.084 ± 0.002	442 ± 136	81
vif minus	47.2 ± 1.7	3,700 ± 200	188 ± 62	0.096 ± 0.002	771 ± 101	27
vpr minus	22.8 ± 0.5	3,600 ± 100	180 ± 57	0.081 ± 0.004	818 ± 137	184

[a] Values are means ± standard deviations.
[b] RT, reverse transcriptase.

Measurement of DNA synthesis in HIV-1-infected $CD4^+$ T cells. To reduce residual plasmid DNA remaining after transfection, viral stocks were treated with 20 μg of DNase per ml in the presence of 10 mM $MgCl_2$ at room temperature for 30 min. As a negative control for PCR analysis, heat-inactivated viruses were prepared by incubation for 30 min at 65°C. Then 5×10^5 cells were infected with HIV-1 mutant strains at a dose of 10 ng of HIV-1 $p24^{gag}$ as described above. At 24 h after infection, the cells were washed in PBS, lysed in urea lysis buffer (4.7 M urea, 1.3% [wt/vol] sodium dodecyl sulfate, 0.23 M NaCl, 0.67 mM EDTA [pH 8.0], 6.7 mM Tris-HCl [pH 8.0], and then subjected to phenol-chloroform extraction and ethanol precipitation. Total nucleic acids resulting from this purification procedure were used for the PCR analysis.

HIV-1 DNA was quantitated as described before (70). The nucleotide sequences of the oligonucleotide primers used for HIV-1 DNA detection were M667 at 496 to 516 in the $HIV\text{-}1_{JR\text{-}CSF}$ (5′-GGCTAACTAGGGAACCCACTG-3′, sense) and M668 at 656 to 637 (5′-CGCGTCCCTGTTCGGGCGCC-3′, antisense). A pair of oligonucleotide primers were complementary to the first exon of the human β-globin gene at positions 14 to 33 (5′-CACAACTGTGTT CACTAGC-3′, sense) and 123 to 104 (5′-CAACTTCATCCACGTTCACC-3′, antisense). Prior to gene amplification, each sense primer was labeled with ^{32}P at the 5′ end. After gene amplification, the PCR products were subjected to polyacrylamide gel electrophoresis (8% polyacrylamide) and visualized by direct autoradiography of the dried gels. The densities of the positive bands were analyzed with a Bio-image-analyzer-BAS 2000 (Fuji Film, Tokyo, Japan). HIV-1 or β-globin DNA was quantitated during PCR amplification by analyzing a standard dilution curve of cloned $HIV\text{-}1_{JR\text{-}CSF}$ DNA or total cellular DNA, respectively. The amount of HIV-1 DNA was normalized to the amount of β-globin DNA in each sample.

In vivo HIV-1 infection. C.B-17-*scid*/*scid* mice were purchased from Clea Japan (Kawasaki, Japan) and maintained under specific-pathogen-free conditions after microbiological screening. Blood samples from these mice were checked by single radial immunodiffusion (Medical and Biological Laboratory, Nagoya, Japan) to detect serum immunoglobulin M. The mice were 6 weeks old

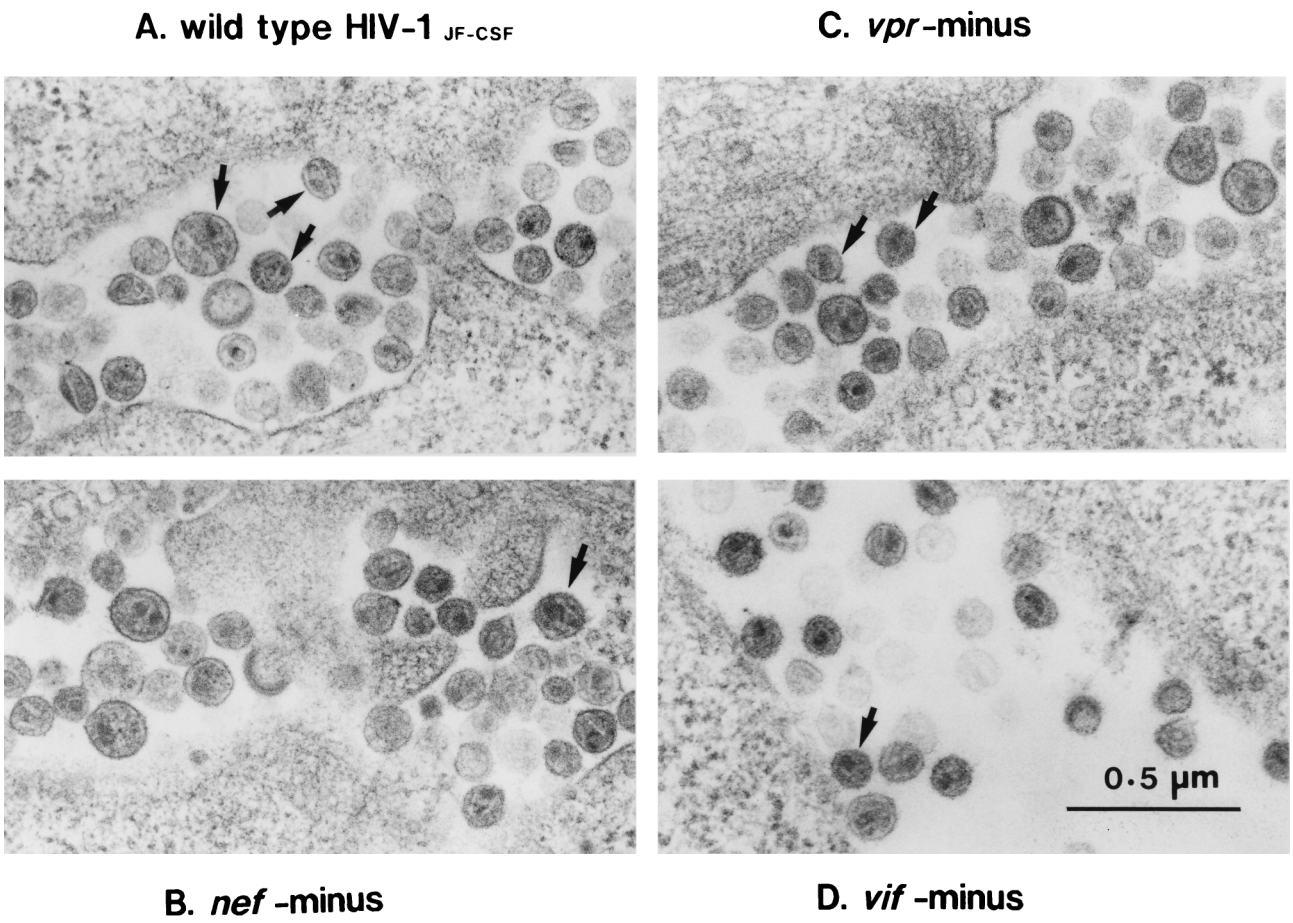

FIG. 1. Electron micrographs of thin sections of wild-type and mutant virions of $HIV\text{-}1_{JR\text{-}CSF}$ (A) Wild type; (B) *nef* minus; (C) *vpr* minus, (D) *vif* minus. Samples were prepared as described in Materials and Methods. Note the presence of typical mature particles containing a conical nucleoid with a dense broad end (arrow). The particles of wild-type HIV-1 and most of the particles of mutant HIV-1 had a mature morphology. The morphology was similar in these viruses.

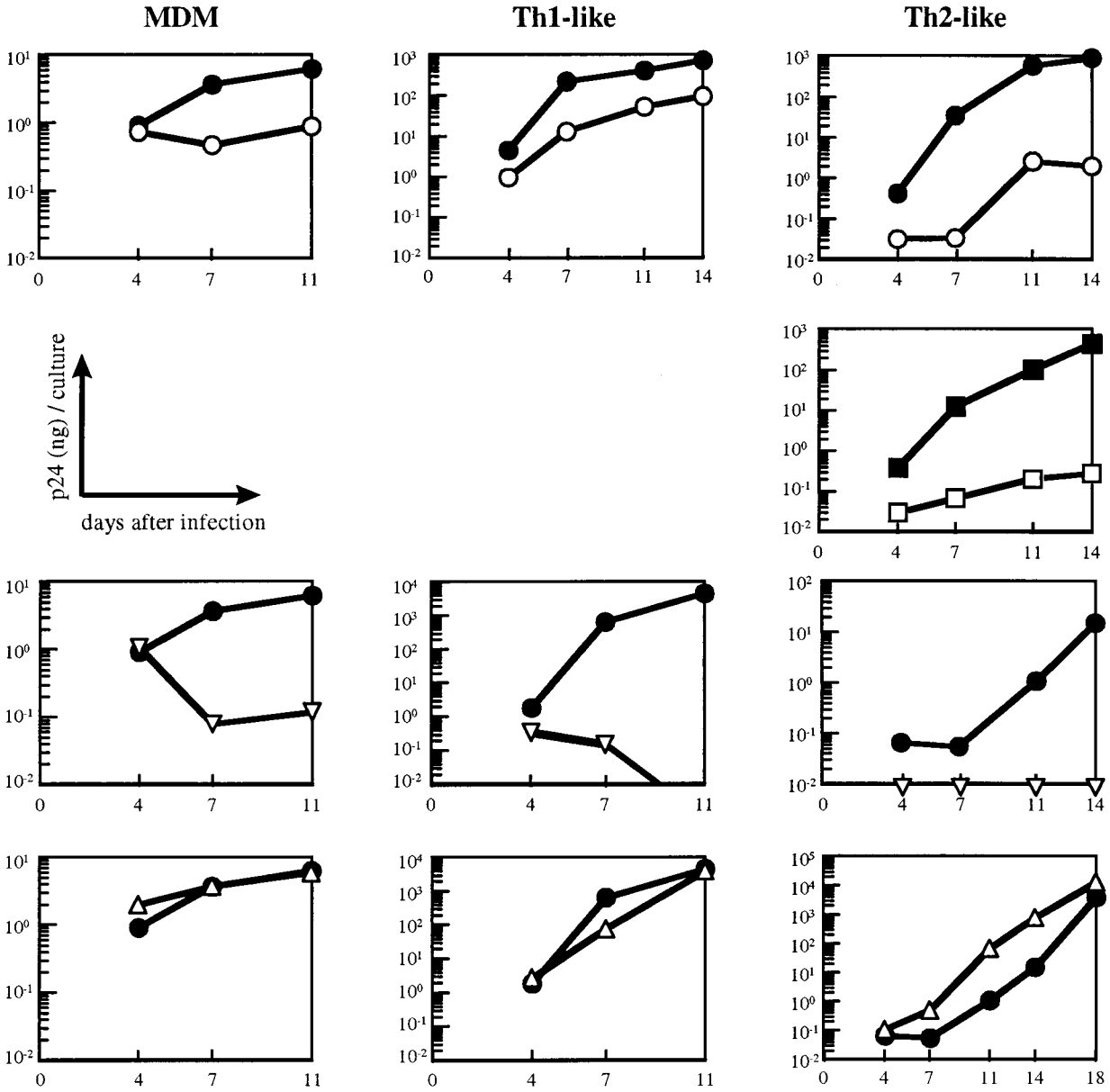

FIG. 2. Kinetics of *nef*-, *vif*-, and *vpr*-minus HIV-1_{JR-CSF} and *nef*-minus HIV-1_{NL4-3} in MDM and anti-CD3-activated $CD4^+$ Th1- and Th2-like cells. Anti-CD3-activated $CD4^+$ T cells (5×10^5 cells) were infected with 27 to 184 $TCID_{50}$, and MDM cells (5×10^5 cells) were infected with 1,000 $TCID_{50}$ of wild-type, *nef*-minus, and *vpr*-minus HIV-1 or 100 $TCID_{50}$ of *vif*-minus HIV-1. Experiments with HIV-1_{NL4-3} were performed only in Th2-like $CD4^+$ T cells because HIV-1_{NL4-3} did not replicate well in Th1-like $CD4^+$ T cells and MDM cultures. The results of one representative experiment with three different blood donors are shown. The range of HIV-1 production from culture supernatants in different donors varied from 3-fold (MDM) to 15-fold (Th1- or Th2-like cultures). Symbols: ●, wild-type HIV-1_{JR-CSF}; ○, *nef*-minus HIV-1_{JR-CSF}; ▽, *vif*-minus HIV-1_{JR-CSF}; △, *vpr*-minus HIV-1_{JR-CSF}; ■, wild-type HIV-1_{NL4-3}; □, *nef*-minus HIV-1_{NL4-3}.

at the time of cell transfer. The experimental protocol was approved by the ethics review committees of the participating institutions. PBMC were isolated from blood samples obtained from healthy HIV-1-seronegative donors by density gradient centrifugation. To deplete NK cells in mice, the animal was treated with anti-IL-2 receptor β-chain monoclonal antibody TMβ-1 (1.0 mg/animal) (32) 3 days prior to transfusion. Six days after intraperitoneal inoculation of 1×10^7 to 2×10^7 human PBMC in 0.5 ml of RPMI 1640 medium containing 10% FCS, 1,000 $TCID_{50}$ of viruses were injected intraperitoneally. The mice were sacrificed 4 or 8 days after infection, and human lymphocytes were recovered from peritoneal lavages. The collected cells were examined by flow cytometry and PCR analysis as described previously (32). Heparinized blood was collected by cardiocentesis, and mouse plasma was used for the $p24^{gag}$ ELISA (Coulter).

Flow cytometric analysis. A total of 0.3×10^6 to 1.0×10^6 cells were stained with antibodies specific for human cell surface markers (Dako Japan, Kyoto, Japan) for 30 min at 4°C and then washed three times. Fixed cells were analyzed within 24 h by flow cytometry (Coulter Epics Elite flow cytometer). The antibodies used for this analysis were phycoerythrin-conjugated anti-CD4, fluorescein isothiocyanate-conjugated anti-CD8, and phycoerythrin-conjugated anti-HLA class I.

RESULTS

Properties of *nef*-minus, *vif*-minus, and *vpr*-minus HIV-1. To evaluate the role of HIV-1 accessory genes in virus replication, we generated *nef*-minus, *vif*-minus, and *vpr*-minus HIV-1 by introducing frameshift mutation and examined a variety of their properties. We chose a macrophage-tropic HIV-1_{JR-CSF} strain because this isolate can effectively replicate in all systems by macrophages and activated $CD4^+$ Th1 and Th2 cells. Each

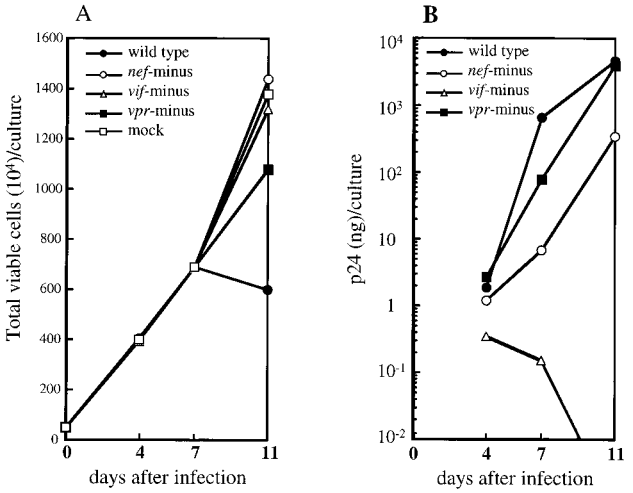

FIG. 3. Relationship between viral production and cell growth. (A) Total viable cells in each culture; (B) total HIV-1 $p24^{gag}$ production in culture supernatants.

mutant plasmid was transfected, and the virus was recovered from culture supernatants. The rate of $p24^{gag}$ release of *vpr*-minus HIV-1 was approximately 70% of that of the control virus, and that of *nef*-minus HIV-1 and *vif*-minus HIV-1 was approximately 150 and 130%, respectively, of that of the wild-type virus (Table 1). The level of endogenous reverse transcriptase RT activity of mutant HIV-1 particles was similar to that of the wild type. Although the morphology of each mutant particle was indistinguishable from the wild-type virus on electron microscopy, the number of viral particles per 30 cells varied (Fig. 1; Table 1). *nef* deletion increased the number of viral particles, whereas *vif* or *vpr* deletion resulted in a decrease in the number viral particles. We recently showed that macrophage-tropic HIV-1 isolates could efficiently replicate in both $CD4^+$ Th1-like cultures and $CD4^+$ Th2-like cultures and induce lytic infection particularly in $CD4^+$ Th1-like cells (64). Using this culture system, we examined the influence of Nef, Vif, and Vpr. Although we observed a similar efficiency of viral entry into primary $CD4^+$ Th1-like (Table 1) and Th2-like (data not shown) cultures, viral DNA synthesis in *nef*-minus virus in $CD4^+$ cultures was consistently approximately 50% of that observed in the wild type. Furthermore, the infectivity of *vif*-minus or *nef*-minus mutant virus in PHA-activated PBMC was about 20 and 60% of that of the wild type, respectively (Table 1).

Kinetics of *nef*-minus, *vif*-minus, and *vpr*-minus HIV-1 production in primary cultures. As shown in Fig. 2, replication of *nef*-minus virus in anti-CD3-activated $CD4^+$ Th1- and Th2-like cell cultures was 10 to 100 times less efficient, respectively, 14 days after infection, than that in the wild-type virus. In contrast, replication of *nef*-minus virus in PHA-activated PBMC was similar to that of the wild-type virus (data not shown), confirming the results of previous reports (37, 61). The effect of *nef* on virus replication in anti-CD3-activated $CD4^+$ Th1- or Th2-like cells was not specific to $HIV\text{-}1_{JR\text{-}CSF}$ isolate. *nef*-minus $HIV\text{-}1_{NL4\text{-}3}$, a prototype T-cell-line-tropic virus, also replicated less efficiently than the parental wild-type strain in $CD4^+$ Th2-like cell culture (Fig. 2). We could not evaluate the replication of $HIV\text{-}1_{NL4\text{-}3}$ in $CD4^+$ Th1-like cell culture because this isolate did not replicate sufficiently in Th1-like cells. In MDM cultures, *nef*-minus HIV-1 showed reduced virus production in one donor (Fig. 2), whereas a small change or no difference in virus production was observed in MDM cultures from two other donors (data not shown). Thus, the effect of *nef* on virus replication in MDM culture varied from one donor to another. Production of the *vif*-minus virus was always lower than that of the wild-type virus in all primary cultures including MDM and activated $CD4^+$ Th1- and Th2-like cell cultures, while *vpr*-minus virus replicated well in MDM and anti-CD3-activated $CD4^+$ Th1- and Th2-like cell cultures.

Since HIV-1 replication is highly dependent on cell proliferation (63), we counted the number of activated $CD4^+$ T cells infected with each mutant virus or mock virus to exclude a possible cytotoxic effect by mutations in *nef* or *vif*. As shown in Fig. 3A, the cell growth kinetics of $CD4^+$ Th1-like cell cultures infected with either *nef* or *vif* mutated virus were similar to those of mock-infected T cells. In contrast, reduced cell growth in wild-type virus-infected culture was apparent 1 week after infection, which could have resulted from high levels of HIV-1 replication (Fig. 3B). Thus, cytotoxicity paralleled HIV-1 production, and the reduced replication efficiency of *vif*-minus and *nef*-minus HIV-1 was not due to increased cell death in this culture system. Since no culture system is yet available to evaluate Nef function, especially of macrophage-tropic HIV-1 in activated T cells, our results indicate that the anti-CD3-activated $CD4^+$ Th1- or Th2-like cell culture system used in this study is particularly useful to investigate the effect of *nef* of HIV-1 on viral replication.

In the next step, we measured the exponential slope defining HIV-1 production for the wild-type and *nef*-minus HIV-1 in anti-CD3-activated Th1-like cell cultures before initiation of loss of T cells (Fig. 4). The ratio of wild-type HIV-1 to *nef*-minus HIV-1 growth rate was 2.53 ± 0.46 in two experiments with $CD4^+$ Th1-like cells from a single donor. Repeated experiments with two other donors showed similar results (data not shown). The exponential slope of HIV-1 production was probably multiplicity of infection independent, since a 100-fold increase in *nef*-minus HIV-1 load did not significantly change the value of the exponential slope of HIV-1 production (Fig. 4). These data suggest that *nef* is crucial for the high viral load in activated $CD4^+$ Th1 or Th2 cells.

Since macrophage-tropic HIV-1 vigorously replicates in $CD4^+$ Th1-like cells, the use of anti-CD3 activated $CD4^+$ Th1-like cell culture should be an adequate evaluation system

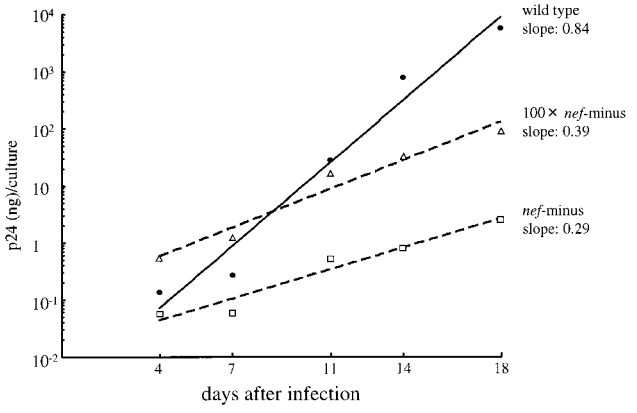

FIG. 4. Kinetics of *nef*-minus, 100-fold-increased (100× *nef*-minus), and wild-type $HIV\text{-}1_{JR\text{-}CSF}$ in anti-CD3-activated $CD4^+$ Th1-like cell culture. Anti-CD3 activated $CD4^+$ Th1-like cells (5×10^5 cells) were infected with 81 $TCID_{50}$ of *nef*-minus, 8,100 $TCID_{50}$ of *nef*-minus, or 140 $TCID_{50}$ of wild-type HIV-1. Lines represent the least-squares linear regression lines for data points between days 4 and 18.

A

Name	Mutation	41 GAVSRDLEKHGAITSSNTAATNADCAWLEAYEDEEVGFPVRPQVPLRPMTYKAAIDLSHFLKEKGGLEGLIYSQKRQDIL 120
wild		GAVSRDLEKHGAITSSNTAATNADCAWLEAYEDEEVGFPVRPQVPLRPMTYKAAIDLSHFLKEKGGLEGLIYSQKRQDIL
M1	82A, 85A	••A••A••••••••••••••••••••••••••••••••••
M2	115LL	••LL••••
M3	deletion 72-84	••••••••••••••••••••••••••••••••-----------••••••••••••••••••••••••••••••••••
M4	deletion 72-75	••••••••••••••••••••••••••••••••----•••
M5	72AAAA	••••••••••••••••••••••••••••••••AAAA•••
nef-minus		•••••SRPGLTWSNHL

B

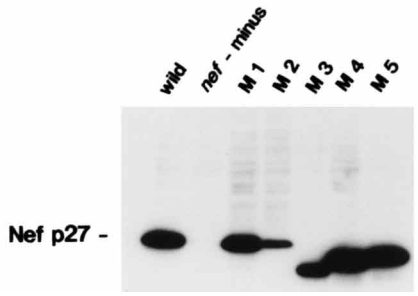

FIG. 5. Mutations of the central core region of Nef and immunoblot analysis. (A) Amino acid sequences of a set of mutant Nef are aligned with that of the central core region of wild-type HIV-1_{JR-CSF} Nef. Dots indicate amino acid identity with the wild-type protein, and letters identify amino acid substitutions in the single-letter code. The nomenclature of the mutant Nef and corresponding mutations are defined on the left. (B) Immunoblot analysis of Nef expression was performed with anti-Nef monoclonal antibody from transiently transfected COS cells by wild-type HIV-1_{JR-CSF} or an altered form of *nef* infectious provirus plasmid DNA. Wild-type and site-directed altered mutants (M1, M2, and M5) expressed 27-kDa Nef, and deletion mutants (M3 and M4) expressed smaller Nef. The Nef-minus mutant has no signal.

for HIV-1 production. Such a system can be used to examine the effects of Nef on HIV-1 replication as well as pathogenicity. In the next series of experiments, we examined Nef function in more detail with this culture system.

Functional domains of Nef that induce efficient HIV-1 production in activated CD4+ T cells. To determine the domains of Nef responsible for efficient HIV-1 replication in activated CD4+ Th1-like cells, we targeted three regions in the central core region of Nef for site-directed mutagenesis: a glutamic acid-rich segment (amino acids 72 to 75) (57), a proline-rich motif that can interact with the SH3 domain containing cellular proteins (amino acids 82 and 85) (50, 57), and conserved Lys/Arg-Arg residues (amino acids 115 and 116) essential for multiple function including the binding of Nef to cellular serine kinases, CD4 downregulation, and the effect on CD3 signaling in T cells (25, 52, 69). Figure 5 shows the sites of mutation introduced in Nef of HIV-1_{JR-CSF}. In the first step, we analyzed the steady-state levels of protein expression of each Nef mutant by immunoblotting. COS cells were transfected with each *nef*-mutated proviral DNA, and similar levels of $p24^{gag}$ release were observed (data not shown). Nef-specific antibody failed to detect signals from cell extracts in *nef*-minus HIV-1 DNA-transfected cells but could detect similar levels of mutated Nef expression (M1, M3, M4, and M5) and wild-type expression except for one mutant (M2). An approximately 10-fold reduction was observed in cells transfected with M2. In the next step, we compared the kinetics of HIV-1 production in cells infected by *nef*-mutant viruses with the kinetics in cells infected with the parental wild-type HIV-1_{JR-CSF} (Fig. 6). HIV-1 with point mutations in residues 115 and 116 (M2) and deletion of the acidic amino acid-rich domain (residues 72 to 84 [M3] or residues 72 to 75 [M4]) replicated two- or threefold less efficiently during 18 days of infection and showed less cytopathicity than did wild-type virus. However, changing the acidic amino acids (residues 72 to 75) to a neutral amino acid (M5) and mutation at the proline-rich motif (M1) resulted in no apparent change in HIV-1 production and cytopathic properties compared with the wild-type virus.

We also investigated the efficiency of viral DNA synthesis after 24 h of infection of anti-CD3-activated CD4+ Th1-like cells by each mutant virus (Fig. 7). The efficiency of viral DNA synthesis of both M3 and M4 was two- to fourfold lower than for the wild type, compared with the efficiency for complete *nef*-minus virus. M1 and M5, which grew in anti-CD3-activated CD4+ Th1-like cells as efficiently as the wild-type virus did, showed a rate of viral DNA synthesis in anti-CD3-activated CD4+ Th1-like cells similar to that of the wild type. Although the rate of M2 replication in anti-CD3-activated CD4+ Th1-like cells was always lower than that of the wild type, its rate of viral DNA synthesis in anti-CD3-activated CD4+ Th1-like cells was less impaired.

In vivo infection. To evaluate Nef function in vivo, we used a human/mouse chimera model consisting of SCID mice engrafted with human peripheral blood leukocytes (hu-PBL-SCID) (21, 31, 39, 40). Recent studies have shown that a human immune response and initial activation of CD4+ T cells occur in hu-PBL-SCID mice within the first 2 to 3 weeks following reconstitution (48, 65). Therefore, we injected HIV-1 into hu-PBL-SCID mice 6 days after reconstitution and analyzed the viral load and CD4+ T-cell depletion 4 and 8 days later. In this modified hu-PBL-SCID mice model, a severe CD4+ T-cell depletion was observed 8 days after infection with wild-type HIV-1_{JR-CSF} but not 4 days after infection (Table 2). We also examined the effects of four Nef mutants on CD4+

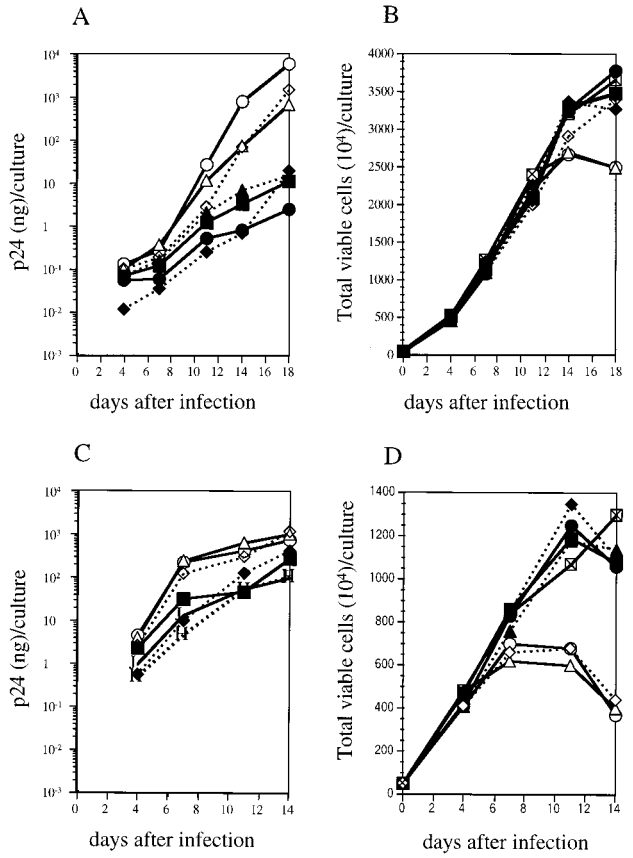

FIG. 6. HIV-1 production and cytopathicity on HIV-1_{JR-CSF}-infected or each *nef* mutant-infected Th1-like culture. Data are from two blood donors, donor M (A and B) and donor I (C and D). (A and C) Total production of HIV-1 $p24^{gag}$ into culture supernatants; (B and D) total viable cells in each culture. Symbols: ○, wild type; ●, *nef* minus; △, M1; ■, M2; ▲, M3; ◆, M4; ◇, M5; ⊠, mock.

T-cell killing in the hu-PBL-SCID mouse system. The mean percentages of human $CD4^+$ T cells based upon HLA^+ cells in the peritoneal cavity 8 days after infection are shown in Table 2. $CD4^+$ T-cell depletion was less pronounced in *nef*-minus virus-infected mice and only mild in M2 and M4 virus-infected mice. In contrast, depletion was severe in M1 virus-infected mice and wild-type virus-infected mice.

The amount of viral DNA in peritoneal cells from *nef*-minus-, M2-, and M4-infected mice was significantly smaller than in M1- or wild-type virus-infected mice 4 days after infection. The mean levels of HIV-1 DNA (per 10^5 human cells) in peritoneal cells from infected mice 4 days after infection are shown in Table 2. The levels of viral DNA in mice infected with *nef*-minus, M2, and M4 HIV-1 were about 70- to 700-fold lower than in mice infected with M1 or wild-type virus. In contrast, 8 days after infection, almost equal levels of HIV-1 DNA were found in peritoneal cells in all infected mice (Table 2). It seems that the level of viral DNA in mice after 8 days of infection reached a peak during the course of HIV-1 infection and no more permissive cells may reside in the chimera mice. The level of $p24^{gag}$ antigen in the plasma 4 days after infection showed a pattern similar to the level of DNA, and the level 8 days after infection was almost identical (data not shown). We concluded that the presence of residues 72 to 75 is important for the induction of HIV-1 replication and subsequent CD4 killing in vivo.

DISCUSSION

Our data clearly showed a crucial role for Nef and Vif of HIV-1 in activated T cells and demonstrated that Nef accelerated viral replication in activated T cells both in an in vitro primary culture system and in an in vivo system, hu-PBL-SCID mice. We found that residues 72 to 75 of Nef are important for viral replication in vitro and in vivo whereas the prolines that form part of the SH3 binding surface of Nef are not important for viral replication in activated T cells.

The process of HIV-1 replication in patients has been described as dynamic, involving continuous cycles of de novo virus infection (45) with an estimated average total HIV-1 production of 10.3×10^9 virions per day, a mean half-life of plasma virions of 0.24 day, a mean half-life of productively infected cells of 1.6 days, and an average HIV-1 generation time of 2.6 days. Furthermore, HIV-1 replicates mainly in the lymphoid organs (14, 44), where T cells are activated continuously by several antigens, and virus replication is enhanced after vaccination against influenza virus or IL-2 injection into HIV-1-infected individuals (30, 42, 62), suggesting the presence of a close relationship between T-cell activation and HIV-1 replication. Previous studies have demonstrated that *nef* of SIV or HIV significantly contributed to the high level of viral replication and pathogenesis in vivo (3, 26, 28) and that Nef played a positive role in the establishment of viral infection in quiescent T cells in vitro (37, 61). However, its role in primary activated T cells has not been demonstrated by in vitro conventional methods such as PHA-activated blast culture. Several investigators have observed the replication of *nef*-minus HIV-1 in PHA blast cultures at a rate similar to that of the wild type (61). To establish a suitable system of T-cell activation to study the function of Nef in activated T cells, we stimulated $CD4^+$ T cells with anti-CD3 in the presence of IL-2 in addition to either IL-12 or IL-4 and established an HIV-1 infection system with $CD4^+$ Th1- and Th2-like cell cultures. Our results showed a positive effect of Nef on HIV-1 replication in these cells. Using this culture system, we first examined the rate of virus growth. A high level of viral replication in in vitro culture may cause the loss of $CD4^+$ T cells followed by limitation of viral spreading. To exclude these possibilities, we ignored the peak levels of $p24^{gag}$ production and compared the growth rate of each mutant virus before the commencement of T-cell loss. By estimating the slope defining HIV-1 production, we were able to demonstrate that the growth-enhancing effects of Nef might be attained during a single cycle of viral replication. This finding is similar to those described by other investigators (37). It is conceivable that a reduction in the replication ability of Nef-mutated HIV-1 will result in a significant change after multiple rounds of infection, because a high rate of de novo infection and replication of HIV-1 in infected individuals may be crucial in the development of AIDS.

We also examined the effect of Nef in hu-PBL-SCID mice. Previous studies with SCID-hu-Thy/Liv mice showed that *nef*-minus HIV-1 replicates less efficiently and causes less depletion of $CD4^+$ T cells with a T-cell line-tropic HIV-1_{NL4-3} strain (3, 26). Although it was shown that a macrophage-tropic HIV-1 strain did not induce a severe cytokilling effect in the SCID-hu-Thy/Liv model, we were able to demonstrate augmentation of HIV-1 replication and severe $CD4^+$ T-cell depletion with Nef by using hu-PBL-SCID mice. Mosier and coworkers reported that macrophage-tropic HIV-1 induced more severe pathogenic changes than did T-cell line-tropic HIV-1 (40) and that *nef*-minus HIV-1 was less pathogenic in hu-PBL-SCID mice (21). It seems that the cell condition of transferred human T cells differs between SCID-hu Thy/Liv

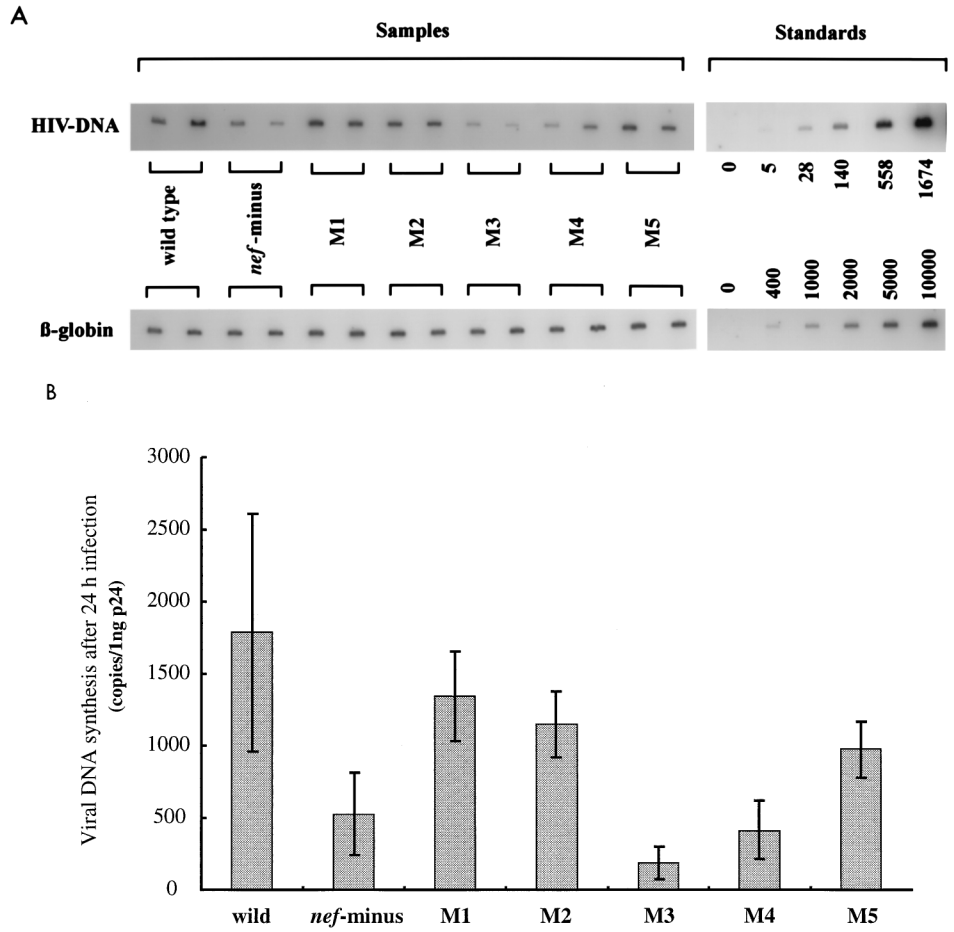

FIG. 7. PCR analysis of human β-globin DNA and HIV-1 DNA in cells infected with wild-type or *nef* mutant viruses at 24 h after infection. (A) Detection of human β-globin DNA and HIV-1 DNA. "Standards" indicates the number of proviral DNA or human β-globin DNA per each reaction. (B) Quantitation of HIV-1 DNA in infected cells at 24 h after infection with *nef* mutated viruses. Specific signals of human β-globin DNA and HIV-1 DNA were measured from panel A and calculated from a standard curve of serially diluted HIV DNA or human DNA. The level of HIV-1 DNA synthesis after entry was expressed per nanogram of input $p24^{gag}$ antigen. Each bar represents the mean ± standard deviation (n = 2) from a representative experiment.

and hu-PBL-SCID mice. At 14 days after engrafting human PBMC into SCID mice, all the human T cells in the peritoneal cavity became CD45RO positive memory T-cell phenotype and responded to anti-CD3 stimulation when the affected cells were observed under in vitro conditions (64a). On the other hand, 50% of recovered T cells from the thymus tissue in SCID-hu-Thy/Liv expressed CD45RO molecules and the rest were CD45RA-positive naive T cells. These results suggest that the cells susceptible to HIV-1 infection in our hu-PBL-SCID mice system are fully activated memory T cells. Therefore, the present results confirm that Nef is also necessary for viral replication in vivo in activated T cells.

Based on our finding that anti-CD3-stimulated $CD4^+$ T cells were useful for examination of the effect of Nef, we also investigated the effect of Vif and Vpr on viral replication by using the same in vitro system. Consistent with previous results obtained with the SCID-hu-Thy/Liv system (3) or by the conventional in vitro method (15), our results suggested that Vif is

TABLE 2. Results for the hu-PBL-SCID-mice infected with HIV-1[a]

Virus	Day 4		Day 8	
	HIV-1 DNA/10^5 human cells	% CD4-positive cells	HIV-1 DNA/10^5 human cells	% CD4-positive cells
Mock	ND[b]	36.5 ± 6.1	ND	34.6 ± 11.4
Wild type	3,877 ± 2,135	35.3 ± 11.9	127,000 ± 25,000	1.7 ± 0.9
nef minus	5.3 ± 2.6	29.2 ± 3.2	179,000 ± 114,000	27.7 ± 10.4
M1	1,463 ± 1,300	35.4 ± 0.9	73,700 ± 27,400	1.2 ± 0.4
M2	54 ± 5	32.7 ± 4.6	192,000 ± 49,200	4.1 ± 1.1
M4	32 ± 12	35.3 ± 5.2	217,000 ± 125,000	28.4 ± 18.7

[a] Results of three representative experiment for one blood donor are shown. Values are means ± standard deviations.
[b] ND, not detected.

essential in all primary T-cell cultures. In contrast, we observed no significant difference in the replication of *vpr*-minus HIV-1 in the activated $CD4^+$ T-cell culture system compared with that of wild-type virus. These results do not necessarily exclude a potential role for Vpr in HIV-1-mediated pathogenesis of infected individuals, although Vpr may have no direct effect on virus production in activated T cells in vivo.

Several studies have shown that Nef enhances virus infectivity, as determined by the efficiency of viral DNA synthesis rather than the level of binding or entry into target cells (2, 10, 55). Our study also confirmed these observations. Goldsmith et al. (18) demonstrated that deletion of 10 amino acids, corresponding to the M3 mutant in our present study and including a part of the proline-rich domain, abrogated infectivity. While M1 had little effect on viral DNA synthesis in the present study, only the 4-amino-acid-deleted mutant (M4) showed reduced viral DNA synthesis in infected cells. Thus, these amino acids must play an important role in Nef-induced HIV-1 augmentation. Although we cannot explain how Nef enhances viral DNA synthesis and the lack of effect of the site-directed mutant (M5) on viral DNA synthesis, future studies should clarify these issues. Reduced infectivity of *nef*-minus HIV can be complemented by expression of Nef on virus producer cells (2, 38, 43). Nef is incorporated into the virion (8, 43, 68) and is cleaved specifically by the viral protease (8, 68). The specific cleavage site of Nef is contiguous with the acidic domain (16, 53). Cyclophilin A-deficient HIV-1 also shows an impaired viral life cycle at the initiation of viral DNA synthesis (7). Hence, we assumed that the acidic domain of Nef may be important for Nef incorporation into the virion or for proteolytic processing of Nef by the viral protease and that cleaved Nef itself could act at the stage of virus uncoating as a molecular chaperone that stabilizes the viral RNA-reverse transcriptase complex. Alternatively, other unknown cellular factors, e.g., a member of the chaperonin group, could associate with Nef and may be incorporated into virion together with Nef.

Several groups of investigators have shown that enhancement of infectivity and viral growth are both dependent on an intact proline-rich motif (18, 50, 69), findings different from those of the present study. These differences may be due to different experimental procedures used in the two studies. In the above studies, the HeLa-CD4-LTR/β-galactosidase cell system was used for the infectivity assay, and Goldsmith et al. (18) measured the expressed β-galactosidase by ELISA. Therefore, the intact proline-rich motif may be required for HIV-1 infection in HeLa-CD4 cells. Other results reported by Saksela et al. (50) showed that the proline-rich motif should be significant for the induction of virus replication from a latent state within resting T cells. Therefore, the phenomenon that the proline-rich motif in Nef may not be necessary for virus replication may be characteristic in activated normal T cells.

Although previous reports suggested that the Nef protein is associated with a cellular serine kinase through the conserved Lys/Arg-Arg residues (residues 115 and 116) (19, 51, 52, 69), the functional importance of this protein with regard to viral replication has remained elusive. Recently, mutation in residues 115 and 116 was shown to disrupt several independent functions of Nef including CD4 downregulation and the effect of Nef on signal transduction in T cells, in addition to disrupting the association of Nef with serine kinase activity (25). These phenomena seem to be explained by the stability of Nef, because the Nef mutant with mutation in this 115 and 116 residues was unstable (25) (Fig. 5). The reduced viral replication of M2 in vitro and in vivo in the present study may also result from the instability of mutant Nef. Unstable expression of the Nef mutant (M2) might also influence the results of the long-term experiment. The rate of viral DNA synthesis in our in vitro T-cell culture was less impaired, although the rate of viral production was lower than in the wild type. The rate of viral DNA synthesis was measured at 24 h postinfection, whereas viral production was monitored for 18 days after infection.

In summary, our study demonstrated the significant role of the *nef* gene in HIV-1 replication and its role in the pathogenesis of primary $CD4^+$ T cells stimulated through the T-cell receptor in the presence of IL-12 or IL-4. We also identified the critical domain of Nef in the expression of its effects. These data will be helpful for the development of both specific therapeutic agents targeting Nef and safe live attenuated vaccines.

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