JOURNAL OF VIROLOGY, May 1997, p. 3641–3651 Vol. 71, No. 5
0022-538X/97/$04.00+0
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The *nef* Gene Products of Both Simian and Human Immunodeficiency Viruses Enhance Virus Infectivity and Are Functionally Interchangeable

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Received 21 August 1996/Accepted 16 January 1997

Adult rhesus macaques infected with *nef*-defective simian immunodeficiency virus (SIV) exhibit extremely low levels of steady-state virus replication, do not succumb to immunodeficiency disease, and are protected from experimental challenge with pathogenic isolates of SIV. Similarly, rare humans found to be infected with *nef*-defective human immunodeficiency virus type 1 (HIV-1) variants display exceptionally low viral burdens and do not show evidence of disease progression after many years of infection. HIV-1 Nef induces the rapid endocytosis and lysosomal degradation of cell surface CD4 and enhances virus infectivity in primary human T cells and macrophages. Although expression of SIV Nef also leads to down-modulation of cell surface CD4 levels, no evidence for SIV Nef-induced enhancement of virus infectivity was observed in earlier studies. Thus, it remains unclear whether fundamental differences exist between the activities of HIV-1 and SIV Nef. To establish more clearly whether the SIV and HIV-1 *nef* gene products are functionally analogous, we compared the replication kinetics and infectivity of variants of SIVmac239 that either do (SIV*nef*+) or do not (SIVΔ*nef*) encode intact *nef* gene products. SIV*nef*+ replicates more rapidly than *nef*-defective viruses in both human and rhesus peripheral blood mononuclear cells (PBMCs). As previously described for HIV-1 Nef, SIV Nef also enhances virus infectivity within each cycle of virus replication. As a strategy for evaluating the in vivo contribution of HIV-1 *nef* alleles and long terminal repeat regulatory sequences to the pathogenesis of immunodeficiency disease, we constructed SIV-HIV chimeras in which the *nef* coding and U3 regulatory regions of SIVmac239 were replaced by the corresponding regions from HIV-1/R73 (SIVR7*nef*+). SIVR7*nef*+ displays enhanced infectivity and accelerated replication kinetics in primary human and rhesus PBMC infections compared to its *nef*-defective counterpart. Converse chimeras, containing SIV Nef in an HIV-1 background (R7SIV*nef*+) also exhibit greater infectivity than matched *nef*-defective viruses (R7SIVΔ*nef*). These data indicate that SIV Nef, like that of HIV-1, does enhance virus replication in primary cells in tissue culture and that HIV-1 and SIV Nef are functionally interchangeable in the context of both HIV-1 and SIV.

Simian immunodeficiency virus (SIV) infection of rhesus macaques (*Macaca mulatta*) provides an excellent animal model for the study of infection and disease induction by the immunosupressive primate lentiviruses. An important aspect of this model system is its utility in the experimental evaluation of the in vivo contributions of individual gene products to virus replication and pathogenesis. A notable example of the value of this approach was provided by experimental infections of macaques with a *nef* deletion mutant of SIV which demonstrated the requirement of Nef for the attainment of high steady-state levels of virus replication in vivo and the consequent induction of AIDS (26). In macaques infected with SIV *nef* deletion mutants, CD4 counts remain normal and immunodeficiency does not develop. The importance of Nef in the pathogenesis of human immunodeficiency virus type 1 (HIV-1) disease was first revealed when an individual with hemophilia who has remained healthy despite prolonged HIV-1 infection (for more than 10 years) was found to be infected with a virus possessing deletions in the *nef* coding sequence and the U3 region of the long terminal repeat (LTR) (29). This individual showed an exceptionally low level of ongoing HIV-1 replication and viral load and stably high $CD4^+$ T-cell counts. More recently, an unusual cohort of HIV-1-infected blood transfusion recipients was described whose members, along with the blood donor that represented the common source of their infections, were all found to harbor *nef*-defective viruses (13). These individuals showed no evidence of progression to AIDS after 10 to 14 years of infection and, like rhesus macaques infected with *nef*-defective mutants of SIV, have maintained normal CD4 counts and very low viral loads. This experiment of nature suggests that the favorable clinical course seen in humans following infection with *nef*-defective HIV-1 variants, like that of macaques infected with *nef*-defective SIV variants, results from the functional debility of the infecting virus rather than any unique host factors.

The cellular and molecular functions that underlie the contribution of Nef to the establishment of high levels of virus replication in vivo and the induction of disease in rhesus macaques and humans have not been elucidated. Furthermore, it is not known how the various functions that have been ascribed to Nef in tissue culture model systems and in vitro studies contribute to the facilitation of virus replication in vivo. Nef is a myristoylated, membrane-associated protein of 25 to 27 kDa in HIV-1 and 32 kDa in HIV-2 and SIV and is one of the earliest viral gene products expressed in newly infected target cells (11, 27, 30, 36, 41). Both HIV-1 and SIV Nef have been demonstrated to induce the accelerated endocytosis and lyso-

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somal degradation of cell surface CD4 (1, 3, 5, 16, 17, 40). Independent of its role in CD4 down-regulation, HIV-1 Nef has also been shown to enhance virus replication in primary peripheral blood mononuclear cells (PBMCs) and macrophages (9, 14, 34, 35, 48). Although the mechanism of HIV-1 Nef-induced enhancement of virus infectivity has not been elucidated, it is known that the positive effect of Nef expression is exerted within producer cells during virus particle formation yet manifest only after the virus enters target cells (10, 35). Following entry into target cells, HIV-1 particles produced by Nef-expressing cells direct the synthesis of higher levels of proviral DNA than those produced in the absence of Nef, indicating that Nef acts, either directly or indirectly, to facilitate important early events in the virus life cycle (2, 10, 37, 46). The regions of the HIV-1 Nef protein that mediate CD4 down-modulation and infectivity enhancement have been defined by mutational analyses and shown to be distinct (19, 42). Interestingly, the regions of the HIV-1 Nef protein involved in the conduct of both of these functions are conserved between HIV-1 and SIV (19, 39, 43, 45, 47). In addition, both HIV-1 and SIV Nef have been found to physically interact with cellular serine kinases (18, 44, 45) and a subset of members of the Src family of tyrosine kinases that have been implicated in the enhancement of virus infectivity by HIV-1 Nef (43–45). However, despite the significant biologic and genetic similarity that exists between HIV-1 and SIV Nefs, no evidence for a replicative advantage of SIV Nef^{+} viruses was found in earlier studies of virus infections in culture (6, 26, 49).

HIV-1 Nef's ability to accelerate viral replication in tissue culture infections, together with the low viral burdens observed in animals and humans infected with *nef*-defective viruses, suggest that Nef's effect on disease pathogenesis is a consequence of the ability of this gene product to facilitate high levels of viral replication in an infected host. Surprisingly, however, the profoundly reduced in vivo viral load and lack of disease observed in rhesus macaques infected with *nef*-defective SIV did not correlate with reduced replication kinetics in cell culture infections of rhesus PBMCs (26, 49). It is plausible that differences in the *nef* coding sequences of HIV-1 and SIV affect the ability of each virus's Nef product to interact with cellular or viral constituents (39, 43, 47). Such differences could confer distinct biological functions to each virus or unique behaviors within specific host target cells or tissues. We therefore sought to examine, using assays that have been successfully applied to the study of HIV-1 Nef function, the potential influence that SIV Nef may exert on the efficiency of SIV or HIV-1 replication. In addition, we studied whether HIV-1 Nef could augment the infectivity of SIV. Our results indicate that, in contrast to earlier reports, SIV Nef does confer a positive growth advantage to SIV in both human and macaque primary cell cultures. We also demonstrate that SIV Nef, like HIV-1 Nef, enhances the infectivity of progeny virions within each cycle of virus replication and that HIV-1 and SIV Nef are functionally interchangeable within the context of intact, replicating HIV-1 and SIV recombinants.

MATERIALS AND METHODS

Construction of recombinant proviral vectors. (i) SIV constructs. The genome of SIVmac239 has been subcloned in two segments, p239SpSp5′ and p239SpE3′ (25) (kindly provided by Ron Desrosiers, New England Primate Research Center); the latter contains the full-length *nef* sequence with a premature translation termination signal at codon 93 (39). A *nef* open variant, in which the premature translation stop codon was repaired by site-directed mutagenesis, p239SpE3′/*nef*open (26), was also provided by Ron Desrosiers. An additional *nef*-minus mutant, p239Δ*nef*, was constructed by site-directed mutagenesis of p239SpE3′ to introduce an additional premature stop codon and to mutate the translation initiator methionine codon of *nef*, thus preventing the initiation of transcription. The additional premature translation stop was introduced by PCR amplification of p239SpE3′, using primers 1 (5′-tggagatctgcgacagagactctagcg-3′) and 2 (5′-atgcgaattctattgagtaccgagttgaccagg-3′). Primer 1 spans a region of *nef* that contains a unique *Bgl*II site (underlined) and mutates the leucine residue at position 21 to TAG while preserving the leucine codon of the overlapping *env* reading frame. Primer 2 is situated 3′ of the TATA box in U5 and includes an *Eco*RI site (underlined) at its 5′ terminus. The *Bgl*II-to-*Eco*RI fragment of p239SpE3′ (which contains flanking cellular DNA) was replaced with the amplified *Bgl*II-to-*Eco*RI fragment to create p239TAG. To eliminate the translation initiation codon, we used primers 3 (5′-gaactgtatcgattggaatt-3′) and 4 (5′-cgcagatctccagacggcctggaccgcctcatggaaatagctccacccgtatt-3′) to amplify a *Cla*I-to-*Bgl*II cassette. Primer 3 spans a unique *Cla*I site in the *env* region (underlined), while primer 4 spans the unique *Bgl*II site and the initiation codon which is mutated to ACG (underlined). This segment replaced the *Cla*I-to-*Bgl*II fragment of p239TAG to create p239Δ*nef*.

(ii) SIV-HIV *nef* chimeras. The *nef* coding sequence and U3 regulatory region, containing the NF-κB and SP1 binding sites of SIV, was replaced by the corresponding region of the HIV/R73 provirus (13) by overlap extension PCR (21) to create p239R7*nef*+ as follows. The *nef*-U3 region of HIV/R73 was amplified by using primers 5 (5′-cttgagctcactctcttgtaag**atgggtggcaagtggtcaaaaagt**-3′), and 6 (5′-cagagcgaaatgcagtgatatttat**atgcaggatctgagggct**-3′), while the SIV R/U5 region of p239SpE3′ was amplified by using primers 7 and 8. Primer 5 contains the 5′ end of HIV-1 *nef*, including the initiation codon (in boldface) and the 3′ end of SIV *env*, including a unique *Sac*I site (underlined); primer 6 contains the 3′ end of HIV-1 U3 (in boldface) and the 5′ end of SIV R. Primer 7 (5′-**acgccctcagatcctgcatataa**atatcactgcatttcgctctg-3′) overlaps and is complementary to primer 6 (HIV-1 sequences in boldface), while primer 8 contains the 3′ end of SIV U5 followed by an *Eco*RI site. The two PCR products were combined in a third PCR with primers 5 and 8 to create a chimeric DNA consisting of the entire HIV-1 *nef* coding sequence and U3 region flanked on the 5′ end by the SIV *env* sequence and on the 3′ end by the SIV R and U5 regions. This PCR product was digested with *Sac*I and *Eco*RI and then used to replace the corresponding *Sac*I-*Eco*RI fragment of the SIV mutant p239Δ*nef*; since the wild-type SIV *nef* translation begins prior to the end of the *env* region, disruption of the SIV *nef* initiator codon was required to permit optimal HIV-1 *nef* expression. As described above, the initiation codon of SIV *nef* was mutated in p239Δ*nef*. A *nef*-deleted version of p239R7*nef*+, p239R7Δ*nef*, was created by deletion of a portion of the 5′ region of HIV-1 *nef*, from ATG to the unique *Xho*I site of HIV-1. The *Cla*I-*Xho*I fragment of p239R7*nef*+ was replaced with a *Cla*I-to-*Xho*I-digested PCR product amplified from p239R7*nef*+ by using primers 3 and 9. As described above, primer 3 spans the unique *Cla*I site in SIV *env*; primer 9 (5′-cgtaatctcgagtctcacaagagagagtgagctcaagc-3′) contains the 3′-most region of SIV *env*, followed by an *Xho*I site (underlined).

(iii) HIV-SIV chimeras. The *nef* coding sequence of SIV was used to replace the corresponding sequence of HIV-1 to create chimeric constructs converse to those described above. The *nef* reading frames of p239SpE3′/*nef*open and p239Δ*nef* were amplified by PCR using primers 10 and 11 (Fig. 1). Primer 10 (5′-cgatcaacgcgtacaatatgggtggagct-3′) spans the translation initiation codon of SIV *nef* and contains an *Mlu*I site (underlined). For amplification of p239Δ*nef*, the initiator ATG of primer 10 was changed to ACG to retain the mutated initiation codon. Primer 11 (5′-gtccctgctgtttcagcgagtttc-3′) contains the 3′ end of the SIV *nef* sequence. The PCR products were cloned into the TA PCR cloning vector (Invitrogen, San Diego, Calif.). The resulting *Mlu*I-to-*Eco*RV fragment was then subcloned into an HIV-1 HXB cassette, pC3/Mlu, that contains the *Bam*HI-*Xba*I fragment of HXB, from which a portion of the *nef* gene has been deleted and replaced with a *Mlu*I site (15). The resulting *Bam*HI-to-*Xba*I fragments containing intact or defective SIV *nef* were then cloned into the HIV-1 molecular clone R73 (15).

Cell lines and primary cells. 293T cells (provided by Warren Pear, Massachusetts Institute of Technology) were maintained in Dulbecco's modified Eagle medium with 10% heat-inactivated fetal bovine serum (FBS) (38). CEMx174 cells (National Institutes of Health AIDS Research and Reference Reagents Program) were maintained in RPMI with 10% heat-inactivated FBS. PBMCs were purified by Ficoll-Plaque (Pharmacia-LKB, Piscataway, N.J.) density gradient centrifugation of buffy coats. Normal human blood was obtained from commercial human donors; rhesus macaque blood was obtained from a pool of normal animals maintained as blood donors (California Regional Primate Research Center, Davis, Calif.). Following purification, PBMCs were grown in RPMI with 6% pooled human AB serum (Gemini Bioproducts, Calabasas, Calif.) to avoid adventitious activation of T cells by FBS.

Viral stocks. For generation of SIV stocks, 10 μg of p239SpSp5′ and 10 μg of the respective 3′ constructs were digested with *Sph*I, purified by phenol-chloroform extraction, and ligated with T4 DNA ligase. The ligation mixtures were digested with *Eco*RI then transfected by calcium phosphate transfection into 293T cells (38). Twelve hours after transfection, medium was changed and 293T cells were cocultivated with CEMx174 cells. After 3 to 5 days of cocultivation, supernatants were harvested from the cocultivated cells. These supernatants were used to generate high-titer viral stocks by incubating cells at a density of 10×10^6 per ml in neat supernatant for 2 h at 37°C. Cells were then resuspended in RPMI with 10% FBS and maintained at concentration of 5×10^5/ml. The total supernatant was harvested daily, and cells were resuspended in fresh medium. Supernatants were cleared and stored at −80°C, and an aliquot was tested for

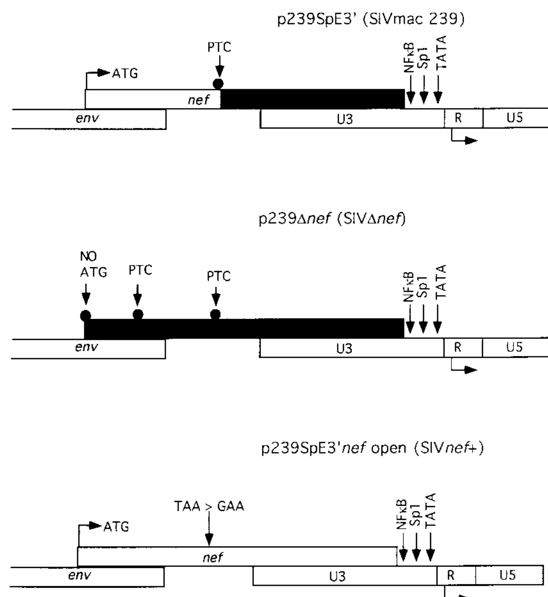

FIG. 1. Construction of SIV recombinant. The 3′ end of the sequence from the end of *env* through the *nef* coding sequence and the 3′ LTR is depicted for each construct. The wild-type molecular clone p239SpE3′ and derivative p239SpE3′*nef*open have been described elsewhere (26). The former contains a premature translation termination signal (PTC) in the *nef* sequence at codon position 93, repaired by Kestler et al. (26) to create p239SpE3′*nef*open. The *nef*-defective mutant p239Δ*nef* was created by mutating the initiation codon, while preserving the coding sequence of the overlapping envelope gene, and introducing a second premature stop codon. p239SpE3′, p239SpE3′*nef*open and p239Δ*nef* were used to generate the viruses SIV239, SIV*nef*+, and SIVΔ*nef*, respectively.

SIV $p27^{gag}$ concentration by enzyme-linked immunosorbent assay (ELISA; Coulter Corporation, Miami, Fla.).

Immunoblotting. Viral supernatants generated from CEMx174 infections were centrifuged at 1500 × *g* for 90 min at 4°C to pellet virus. Viral pellets were lysed in 50 to 100 μl of Nonidet P-40 lysis buffer and stored at −20°C. Then 10 to 50% of the viral lysate was diluted in sodium dodecyl sulfate (SDS) sample buffer, electrophoresed along with prestained SDS-polyacrylamide gel electrophoresis low-range standards (Bio-Rad, Hercules, Calif.) on a 10% polyacrylamide gel containing 0.1% SDS, and then transferred onto nitrocellulose (Bio-Rad). Blots were blocked overnight in 5% nonfat dry milk and 0.5% Tween in phosphate-buffered saline (PBS). The blot was then incubated with polyclonal HIV-1 BH10 Nef antiserum (National Institutes of Health AIDS Research and Reference Reagents Program) diluted 1/3,000 in PBS with 0.1% Tween 20 and 0.1% bovine serum albumin. The secondary antibody was horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin G (Amersham Life Sciences, Cleveland, Ohio) diluted 1/3,000 in PBS with 0.1% Tween 20. Following each antibody incubation, the blot was washed extensively in PBS with 1% Tween 20. Immunoreactive protein was visualized by using the ECL detection system (Amersham).

Nucleotide sequence determination. The SIV *nef* gene was amplified from virion RNA present in viral stocks by reverse transcription-PCR. PCR products were purified on spin columns (Qiagen, Chatsworth, Calif.) and directly sequenced by using a PRISM Ready Reaction Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, Calif.). Sequencing reactions were purified on Centristep spin columns (Princeton Separations), and the purified reactions run on an Applied Biosystems automated sequencer.

Virus infections. Infections were performed in 24-well plates by incubating 2×10^6 to 4×10^6 cells overnight in 1 to 2 ml of viral supernatant normalized to 10 ng of $p27^{gag}$ per ml. CEMx174 cells were infected in RPMI supplemented with 10% FBS; virus was removed by washing the cells in PBS. Cells were resuspended in FBS supplemented RPMI; supernatants were collected every 2 to 3 days and stored at −20°C until testing for virus by ELISA (Coulter). While supernatant was harvested, one-half to one-third of the medium was replaced with fresh medium, and excess cells were removed if necessary to prevent overcrowding. PMBCs were either infected directly after isolation (unstimulated infections) or cultured for 2 days in RPMI with 6% human AB serum and lectin; the cells were then washed in PBS to remove the lectin and resuspended in RPMI with 6% AB serum and recombinant interleukin-2 (rIL-2; Gibco, Grand Island, N.Y.) prior to infection (stimulated infections). Human PBMCs were lectin stimulated in 4 μg of phytohemagglutinin (PHA-P; Sigma, St. Louis, Mo.) per ml and grown in 10 U of rIL-2 per ml, while rhesus PBMCs were stimulated with 5 μg of concanavalin A (Sigma) per ml and maintained in 40 U rIL-2 per ml. The morning after infection, PBMCs were washed in PBS and resuspended in RPMI with 6% human AB serum and rIL-2, if cells had been previously lectin stimulated. For unstimulated infections, PBMCs were cultured after overnight incubation with virus in medium supplemented with the appropriate lectin and rIL-2 as described above; 48 h later, lectin was washed off and cells were returned to RPMI with 10% AB serum and rIL-2. Supernatant was harvested every 2 days and stored at −20°C until the $p27^{gag}$ concentration was determined by ELISA. As supernatants were collected, one-third of the volume of medium was replaced with fresh RPMI with AB serum and rIL-2.

$TCID_{50}$ determination. The 50% tissue culture infective doses ($TCID_{50}$s) of viral stocks were determined on CEMx174 cells and on PHA-P-stimulated human PBMCs (24). Sextuplicate infections of 10^5 PBMCs or 2×10^4 CEM-X174 cells were performed in a 96-well plate with fourfold serial dilutions of virus from 10 ng of p27 per ml to 0.1525 pg/ml. Infected cells were maintained in RPMI supplemented with 10% FBS and 10 U of rIL-2 per ml for PBMCs. Every 3 days, 50% of the medium was exchanged for fresh medium, and cells were examined for evidence of cytopathicity and split if necessary. Infection of CEMx174 cells was scored by cytopathic effect, as these cells develop large balloon-like syncytia upon infection with SIV. PBMC infections were scored by $p27^{gag}$ ELISA; positive wells were those in which 100 μl of supernatant was 2 standard deviations higher than for residual virus, determined by p27 of 100 μl of supernatant from the well inoculated with virus in the absence of cells, which received fresh medium every 3 days.

Single-cell infectivity assay. To assess SIV infectivity, we used a single-cell infectivity assay modeled on the MAGI cell assay originally described by Kimpton et al. (28). As the HeLa-β-gal cell line used in the MAGI assay is not permissive for SIV infection, an analogous cell line has been developed by Chackerian et al. (8), using CMMT cells (a rhesus macaque mammary tumor cell line) to permit analysis of SIV infectivity. These cells, referred to as sMAGI cells, express human CD4 and a construct that expresses β-galactosidase (β-Gal) under the control of the HIV-1 LTR. The presence of HIV-1 or SIV Tat activates expression from the HIV-1 LTR, and β-Gal expression can subsequently be detected by incubation with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). We therefore used these cells to analyze viral infectivity in a single round of infection. Cells were maintained in Dulbecco's modified Eagle medium supplemented with 0.2 mg of G418 (GibcoBRL) and 48 μg of hygromycin (Calbiochem) per ml. The day before infection 2.5×10^4 cells were plated into 24-well plates; 24 h later, selective medium was removed and cells were infected by addition of 400 μl of viral supernatant to each well. All infections were performed in triplicate with serial twofold dilutions of viral supernatants normalized by $p27^{gag}$ antigen. The morning after, infection fresh medium containing zidovudine at a concentration of 10 mM was added to prevent viral spread. Forty-eight hours postinfection, medium was removed and cells were fixed for 5 min in 0.2% glutaraldehyde and 1% formaldehyde in PBS. Wells were washed twice with PBS, and 200 μl of the substrate (0.4 mg of X-Gal per ml, 4 mM potassium ferrocyanide, 4 mM potassium ferrocyanide, 2 mM $MgCl_2$) was added. After incubation of the plates for 50 min at 37°C, the substrate solution was removed and replaced with PBS. Foci of blue cells were counted in a light microscope. Occasionally, small numbers of blue cells were observed in wells that were not infected. Multiple uninfected wells were counted on each plate, and the number of background cells was subtracted from the total number of blue foci observed in each infected well.

To assess the infectivity of wild-type HIV-1 and recombinants derived from it, the previously described (28, 34) MAGI cell assay was used. The day before infection, 7×10^3 cells were plated into 96-well plates, and 24 h later, cells were infected by addition of 100 μl of viral supernatant to each well. All infections were performed in triplicate with serial twofold dilutions of viral supernatants normalized by $p27^{gag}$. Blocking of subsequent rounds of replication and visualization of β-Gal expression were performed as described above.

RESULTS

SIV stocks. Viral stocks were generated by transfection of plasmid DNAs containing the 5′ half of the SIVmac239 genome (p239SpSp5′) after ligation in vitro with the various plasmid clones of the 3′ portion of the various viral constructs with intact or altered *nef* genes (Fig. 1). Thus, the virus named SIV*nef*+ was derived from ligation of p239SpSp5′ with p239*nef*open; SIV239 (SIVmac239) was obtained by ligation with p239SpE3, and SIVΔ*nef* was derived from ligation with p239Δ*nef*. Transfected cells were cocultivated with CEMx174 cells to obtain high-titer viral stocks. To assess whether alterations had occurred during virus transfection or passage, the *nef* sequences in each preparation of viral stocks was subjected to reverse transcription-PCR and direct DNA sequencing. On

occasion, prolonged culture of SIV239 in CEMx174 cells resulted in repair of the premature stop codon in a proportion of viruses. Only viral stocks that did not harbor detectable levels of reversion in the *nef* reading frame were used in the experiments described. The replication competence of these viruses was assessed in CEMx174 cells by infection with inocula normalized to 10 ng of p27^{gag} antigen per ml and the subsequent monitoring of viral growth kinetics. All three viruses replicated well in this transformed cell line (Fig. 2A), induced syncytia, and were highly cytopathic, killing the majority of cells in the culture by 14 days postinfection. Typically, there was very little difference between the growth of *nef*$^+$ and *nef*-defective viruses (Fig. 2A). The observed similarity between replication kinetics of SIV*nef*$^+$ and SIVΔ*nef* in transformed CD4$^+$ cell lines mirrors that seen for HIV-1 *nef*$^+$ and *nef*-defective isolates. Thus, any potential contribution of Nef to enhancing SIV replication may not be sensitively revealed in immortalized cell lines in culture.

SIV*nef*+ displays a replication advantage over SIV239 and SIVΔ*nef* in PBMC infections. As HIV-1 Nef has previously been shown to enhance viral replication in primary human PBMCs, we compared the replication kinetics of SIV*nef*+, SIV239 (which contains a premature stop codon in the *nef* reading frame), and SIVΔ*nef* in lectin-stimulated human and rhesus macaque PBMCs. In contrast to CEMx174 cell infection, SIV*nef*+ displayed a distinct positive growth advantage in comparison with SIVΔ*nef* and SIV239 in activated human PBMC infections. A representative infection of human PBMCs is shown in Fig. 2B. The SIV*nef*+-infected human PBMCs produced detectable levels of p27^{gag} antigen earlier, and peak virus production attained higher titers than *nef*-defective viruses. The observation that the SIV*nef*+ and SIVΔ*nef* used to initiate these infections replicate with equivalent kinetics in immortalized cell lines provides a useful control for the integrity of the viral genes other than *nef* and for the equivalence of the virus inocula used to initiate the infections. Infections of rhesus PBMCs with SIV*nef*+ and SIVΔ*nef* also demonstrated reproducibly that Nef enhances SIV replication in primary cell cultures (Fig. 2C and 6B). However, the magnitude of the difference between SIV*nef*+ and SIV239 or SIVΔ*nef*, in terms of the maximum viral titer measured over the course of the experiment and the time of onset of viral replication, differed between individual experiments and was often less dramatic in rhesus than in human PBMCs. These discrepancies may reflect differences in the efficiency with which rhesus and human cells grow under tissue culture conditions optimized for the growth of human PBMCs. Specifically, we found that the life span of rhesus PBMCs and the amount of virus produced increased dramatically when cultures were stimulated with concanavalin A (as opposed to PHA) and maintained in substantially greater (fourfold higher) concentrations of IL-2 than are normally used to maintain human PBMCs (data not shown). In general, we observed that when T-cell viability and proliferation were higher, virus production in infected cultures was greater and the difference in levels of virus replication between SIV*nef*+ and SIVΔ*nef* viruses was more pronounced, even when the same macques served as the PBMC donor. Nevertheless, infections of activated, primary human and rhesus T cells show consistently that SIV Nef, like HIV-1 Nef, facilitates virus replication in nontransformed cells in culture.

HIV-1 infection of resting PBMCs has been reported to reveal a greater difference between *nef*$^+$ and *nef*-defective virus than is observed in stimulated PBMCs (34, 48). We therefore infected both resting human and rhesus PBMCs directly after isolation and subsequently activated the virus-exposed cells by lectin stimulation (Fig. 3). In these experiments, there was a delay in the initial detection of virus compared with infections of stimulated cells. In unstimulated human PBMCs, there was an even greater difference between SIV*nef*+ and SIVΔ*nef* infections than was observed in stimulated PBMCs. Indeed, in some experiments, the SIVΔ*nef* failed to replicate to detectable levels (Fig. 3A). Infections using rhesus macaque

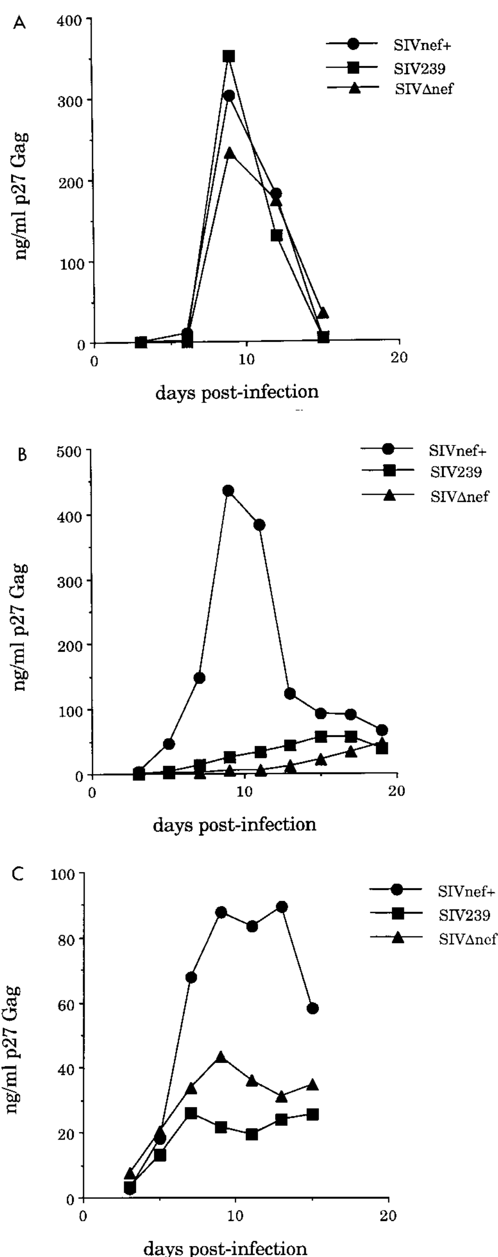

FIG. 2. SIV replication in activated primary PBMCs is accelerated in the presence of Nef. (A) Viruses generated from SIV*nef*+ and the *nef*-defective molecular clones SIV239 and SIVΔ*nef* replicate equally well in the immortalized cell line CEMx174. Cells were infected with equivalent virus inocula (normalized to 10 ng of p27^{gag} per ml) of the indicated viruses, and the accumulated p27 concentration in the supernatant was assessed by SIV Gag antigen capture ELISA. Human (B) or rhesus (C) PBMCs were isolated and lectin stimulated for 48 h prior to infection with SIV*nef*+, SIV239, or SIVΔ*nef* normalized to 10 ng of p27^{gag} per ml. The virus was washed off the cells 12 h postinfection, and the cells were grown in IL-2-supplemented medium. Every two days one-third of the medium was exchanged for fresh IL-2-containing medium and the supernatant was assayed for p27^{gag}.

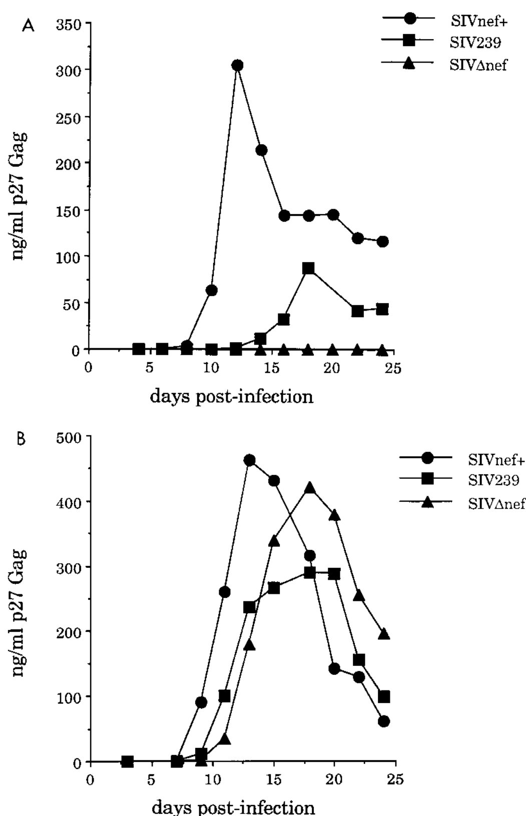

FIG. 3. Virus replication is accelerated in primary PBMCs infected with SIV while in a resting state. Unstimulated human (A) or rhesus (B) PBMCs were infected directly following isolation with the indicated viruses as described for Fig. 2. Following infection, cells were transferred to medium containing lectin and IL-2 and cultured as described for Fig. 2.

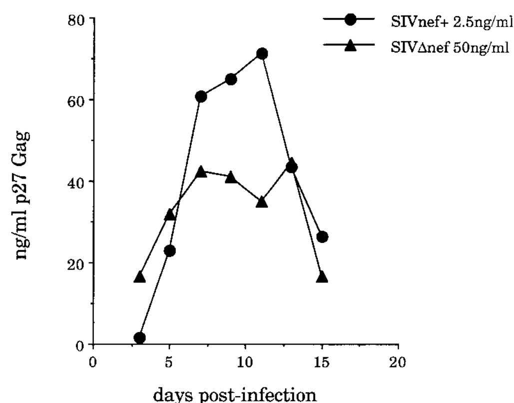

FIG. 4. Infection of stimulated rhesus PBMCs with a 20-fold excess of SIVΔ*nef* over SIV*nef*+ does not compensate for the accelerated replication of SIV*nef*+. Rhesus PBMCs were lectin stimulated for 48 h and then infected with the indicated amounts of each virus. Twelve hours postinfection, virus was washed off the cells and the cells were resuspended in IL-2-supplemented medium. Every 2 days, one-half of the medium was exchanged for fresh IL-2-supplemented medium and supernatants were tested for accumulated $p27^{gag}$.

PBMCs prepared from different animals yielded somewhat variable results. Cells from one animal, illustrated in Fig. 3B, showed earlier onset and peak of replication of SIV*nef*+ compared with SIVΔ*nef* and SIV239, although there was little difference in maximal viral titer achieved. Infection of PBMCs from other animals showed a more dramatic difference in both the onset of viral replication and maximal viral titers between SIV*nef*+ and the SIVΔ*nef* (see Fig. 6C). The reason why SIV*nef*+ and SIVΔ*nef* infections of resting rhesus T cells yielded more variable and sometime less dramatic differences than those seen in infections of resting human T cells is not known. However, we and others have noted that the presence of higher levels of activated cells in resting-cell cultures can diminish the magnitude of the difference observed between the replication kinetics of nef^{+} and *nef*-defective viruses (34, 48), and at least two factors may give rise to higher levels of activated cells in cultures of macaque T cells. First, we have observed variable levels of in vivo-activated $CD4^{+}$ ($CD25^{+}$ $CD69^{+}$) T cells in peripheral blood samples from different macaque donors (data not shown). Second, as human and not rhesus serum was used to maintain rhesus PBMCs in a resting state, these cultures may have contained more adventitiously activated T cells than are present in the corresponding cultures of human cells PBMCs. If rhesus T cells were more activated than human T cells upon infection of unstimulated cultures, the magnitude of the difference in replication kinetics observed between nef^{+} and *nef*-defective viruses may be reduced. In all, these data indicate that SIV Nef confers a replication advantage during virus infections of both human and rhesus macaque PBMCs and that this advantage is best revealed when the infection process is initiated when the target cells are in a resting state.

To determine whether the accelerated replication kinetics of SIV*nef*+ was due to differences in the infectivity of the initial inoculum alone, infections were performed with an excess quantity of SIVΔ*nef* (based on $p27^{gag}$ antigen content). Infection of stimulated rhesus PBMCs with a 20-fold excess of SIVΔ*nef* over SIV*nef*+ is shown in Fig. 4. At early time points after infection, virus production by SIVΔ*nef*-infected cells was greater than in SIV*nef*+ cells, possibly reflecting the higher numbers of cells initially infected by an excess of SIVΔ*nef*. However, at later time points, $p27^{gag}$ produced by SIV*nef*+-infected PBMCs exceeded that produced by PBMCs infected with a 20-fold excess of SIVΔ*nef*. Similar infections of human PBMCs gave analogous results (data not shown). Thus, increasing the amount of the SIVΔ*nef* by as much as 20-fold fails to compensate for the accelerated replication of SIV*nef*+. These data indicate that, as has been previously reported to HIV-1 Nef (34), SIV Nef-related differences in virus replication are not simply a reflection of infectivity differences of the initial virus inoculum but are likely compounded with each successive cycle of replication.

HIV-1 Nef can functionally replace SIV Nef upon infection of PBMCs. With a view to developing an in vivo system for the evaluation of the ability of specific HIV-1 *nef* alleles and LTR sequences to establish infection and induce disease in the macaque model, we have generated SIV-HIV chimeric constructs in which the *nef* coding and U3 regulatory regions of SIVmac239 are replaced by corresponding regions from HIV-1 (derived from the molecular clone HIV-1/R73 [15]) (Fig. 5A). The *nef* gene coding sequences of both SIV and HIV-1 extend well into the U3 region of the 3′ LTRs of their parental viruses. Given recent data indicating that the transcriptional regulatory motifs present in the U3 regions of SIV and HIV-1 may differ, we chose to exchange the entire U3 region (upstream of the TATA sequence) along with the *nef* genes when creating hybrid viruses. In this way, we sought to avoid potential artifacts that might arise by creation of hybrid U3 regions whose regulatory behavior might differ from that of the native transcriptional regulatory motifs (4a, 22). Further, to maximize expression of HIV-1 Nef, the in-frame translation initiation codons that are normally used in the expression of SIV Nef (that

FIG. 5. (A) Construction of SIV–HIV-1 chimeras. The 3′ end of the sequence from the end of *env* through the *nef* coding sequence and the 3′ LTR is depicted for each construct. HIV-1 *nef* was introduced into p239Δ*nef* to create p239R7*nef*+. In this construct, the *nef* initiation codon, which overlaps the SIV *env* sequence, is mutated to allow efficient initiation of the HIV-1 *nef* initiation codon which was inserted downstream of the SIV*nef* initiation codon, immediately following the termination of the SIV *env* coding sequence. The resident and introduced premature translation termination codons in the *nef* region of SIVmac239 (PTC) are shown. A *nef*-defective version of this construct, p239R7Δ*nef*, was created by deletion of the 5′ end of the HIV-1 *nef* gene from the initiation codon to the introduced *Xho*I site. (B) HIV-1 Nef is expressed by SIVR7*nef*+ and not by SIVR7Δ*nef*. Virus was pelleted from supernatants of CEMx174 cell infections, and an immunoblot analysis was performed with a rabbit anti-Nef polyclonal serum. Sizes are indicated in kilodaltons.

overlap the 3′ region of the SIV *env* open reading frame) were eliminated by site-directed mutagenesis. The *nef* gene present in the HIV-1/R73 proviral clone encodes a protein product known to both down-modulate cell surface CD4 and increase virus infectivity (1, 19, 34, 35). Chimeric SIV viral stocks that contained the HIV LTR and *nef* regions in place of the corresponding SIV regions were generated by ligation and transfection of p239SpSp5′ with p239R7*nef*+ to generate SIVR7*nef*+ or with p239R7Δ*nef* to generate SIVR7Δ*nef*. Following cocultivation of transfected cells with CEMx174 cells and generation of high-titer viral stocks, the presence or absence of HIV-1 Nef was assessed by immunoblotting of virus-containing supernatants. Pelleted virus supernatants contain detectable Nef protein that is either associated with virions or with membrane fragments (33a, 37, 51). As shown in Fig. 5B, the HIV-1 Nef-specific antibody detected a band of 27 kDa from SIVR7*nef*+-infected supernatants that is identical to the band obtained from HIV-1/R73 viral supernatant and was not present in SIVR7Δ*nef* or the parental SIV.

The ability of the chimeric SIVR7*nef*+ and SIVR7Δ*nef* constructs to replicate was determined by infection of CEMx174 cells. In these cells, the chimeric viruses replicate as well as the parental SIV isolates, and they efficiently induce syncytium formation and cell death. As previously seen with the parental SIV Nef$^+$ isolate, no evidence for an accelerated replication rate of the Nef$^+$ chimeric virus was observed in this immortalized T-cell line (Fig. 6A). The replication kinetics of the chimeric viruses in primary rhesus PBMCs were compared to those SIV*nef*+ and SIVΔ*nef*, using the methods described above. As illustrated in Fig. 6B, SIVR7*nef*+ demonstrated accelerated replication kinetics compared with SIVR7Δ*nef* upon infection of stimulated rhesus PBMCs. Furthermore, the replication kinetics of the chimeric viruses closely paralleled the respective parental viruses SIV*nef*+ and SIVΔ*nef*. Upon infection of resting rhesus macaque PBMCs prior to lectin stimulation, *nef*$^+$ viruses again showed increased replication kinetics with respect to the *nef*-defective viruses (Fig. 6C). A comparison of the replication kinetics of SIVR7*nef*+ and SIVR7Δ*nef* was also performed in unstimulated human PBMCs (Fig. 6D). As observed in previous infections with parental SIV, the effect of Nef on replication kinetics was more pronounced in human than in rhesus PBMCs. Thus, under parallel infection conditions, SIV Nef and HIV Nef are functionally equivalent in their abilities to enhance the replication of SIV in tissue culture infections.

SIV Nef and HIV-1 Nef enhance the infectivity of SIV. We next sought to determine whether the accelerated replication resulting from Nef expression might be due to enhancement of SIV infectivity by Nef, as has previously been described for HIV-1 (9, 34). Therefore, we used two types of analyses to compare infectivity of *nef*$^+$ and *nef*-defective viruses. First, a limiting dilution titration ($TCID_{50}$ analysis) was performed on CEMx174 cells and on activated PBMCs to compare the infectivities of virus preparations normalized for p27 antigen content. Second, the recently developed sMAGI cell line (8) was used to assess infectivity in a single round of infection. The HeLa cell-based MAGI cell line has been previously used to compare the infectivities of *nef*$^+$ and *nef*-defective HIV-1 in a single round of infection (34, 35). These cells are stably transfected with CD4 and the HIV LTR-directed β-Gal expression vector; upon infection and production of Tat, LTR-driven RNA transcription is transactivated and β-Gal is expressed. The MAGI cell line cannot be infected with SIV; however, an analogous cell line, termed sMAGI cells, that is permissive for SIV infection has recently been developed (8). The sMAGI cell line is derived from a rhesus macaque mammary tumor cell line that, like the HeLa-based MAGI indicator cell line, was transfected with human CD4 and a Tat-dependent HIV LTR–β-Gal expression vector (8, 28).

CEMx174 cells and activated PBMCs were infected with limiting dilutions of virus and then assessed for the presence or absence of infection. $TCID_{50}$s were calculated by the method of Reed and Muench (24). As shown in Table 1, $TCID_{50}$s were reduced 8- to 11-fold upon infection of CEMx174 cells for SIV239 and SIVΔ*nef* compared with SIV*nef*+. For activated PBMC infections, a 16-fold decrease in the $TCID_{50}$ was obtained for both *nef*-defective viruses compared with SIV*nef*+. Similar differences in $TCID_{50}$s were obtained for the chimeric viruses. A sixfold reduction was observed for the SIVR7Δ*nef* infection of CEMx174 cells compared with SIVR7*nef*+, and an eightfold difference was observed between these two viruses upon infection of activated human PBMCs. These limiting-dilution titrations demonstrate that when normalized for $p27^{gag}$, SIVs with an intact *nef* reading frames, derived from either HIV-1 or SIV, are more infectious than viruses deficient in *nef*. The magnitude of difference in virus infectivity between SIV*nef*+ and SIVΔ*nef* is, when measured by $TCID_{50}$ analyses, equivalent to that seen in identical analyses of HIV-1 *nef*+ and *nef*-defective viruses (34).

To elucidate the stage of the virus life cycle where SIV and HIV-1 Nefs act to augment SIV infectivity, sMAGI cells were

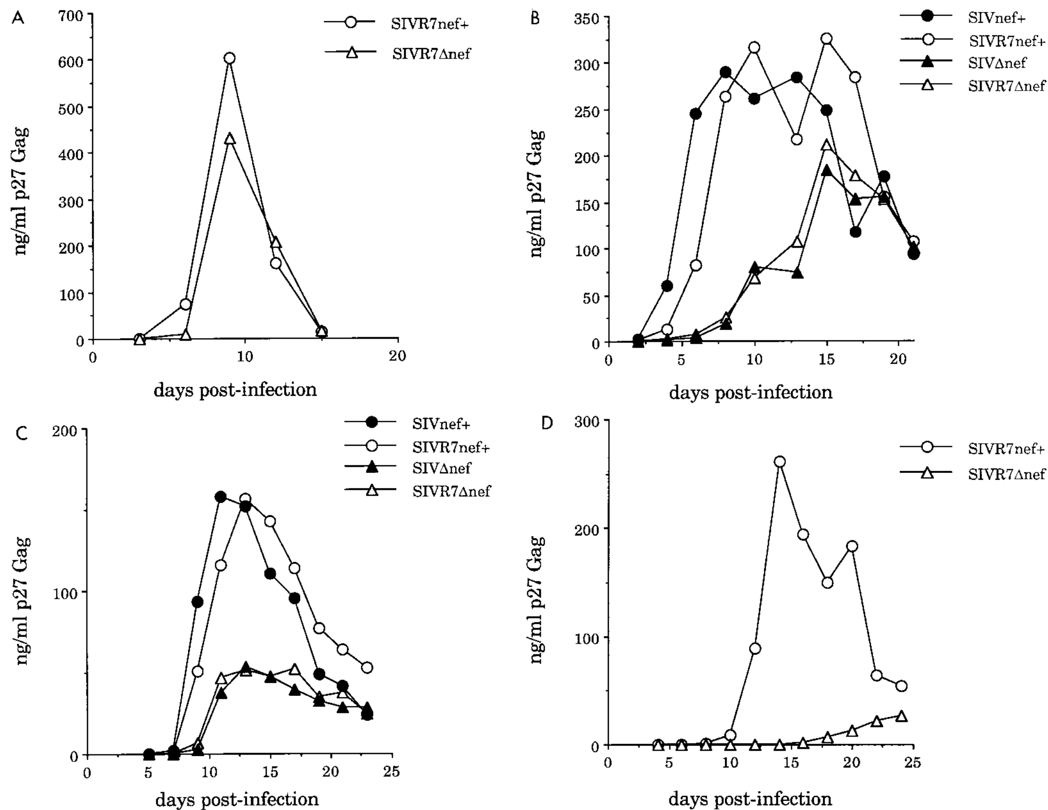

FIG. 6. HIV-1 Nef accelerates SIV viral replication in both human and rhesus PBMCs. (A) Viruses made from both constructs (SIVR7*nef*+ and SIVR7Δ*nef*) replicate equally well in the immortalized cell line CEMx174. Cells were infected overnight with the indicated viruses, normalized to 10 ng of $p27^{gag}$ per ml. Every 3 days, one-half of the tissue culture medium was exchanged for fresh medium and the accumulated $p27^{gag}$ in the supernatant was measured. (B) Rhesus PBMCs were isolated and lectin stimulated for 48 h and then infected with the SIV parental and chimeric isolates normalized to 10 ng of p27 per ml. The virus was washed off the cells 12 h postinfection, and the cells were grown in IL-2-supplemented medium. Every 2 days, one-third of the medium was exchanged for fresh IL-2-containing medium and the supernatant was assayed for $p27^{gag}$. Unstimulated rhesus (C) or human (D) PBMCs were infected directly following isolation with the indicated viruses normalized to 10 ng of $p27^{gag}$ per ml. Twelve hours later, the cells were washed and transferred to medium containing lectin and IL-2 and cultured as described above.

infected with SIV*nef*+, SIVΔ*nef*, SIVR7*nef*+, and SIVR7Δ*nef*. Approximately 12 to 16 h following infection, zidovudine was added to infected cultures to prevent secondary cycles of virus infection. SIV*nef*+ infected three to five times more sMAGI cells than SIVΔ*nef* (Fig. 7A), and SIVR7*nef*+ infected three to eight times more cells than SIVR7Δ*nef* (Fig. 7B). These results indicate that early events, culminating in Tat production by infected cells, are enhanced by both HIV-1 and SIV Nef. Although the relative differences between the *nef*$^+$ and *nef*-defective versions of both sets of viruses were equivalent, SIV-*nef*+ productively infected three to four times more cells than the chimera, SIVR7*nef*+. It is plausible that the hybrid LTR of SIVR7*nef*+ is less efficient than the parental SIV LTR region in activating viral RNA transcription in the sMAGI cells.

TABLE 1. Limiting-dilution infection of CEMx174 cells and activated human PBMCs demonstrates a reduction in infectivity of *nef*-defective viruses

Virus	$TCID_{50}$ at 10 ng of p27/ml (fold reduction compared with SIV*nef*+ or SIVR7Δ*nef*)	
	CEMx174 cells	Activated human PBMCs
SIV*nef*+	4.09×10^3	1.69×10^3
SIVΔ*nef*	3.84×10^2 (11)	1.05×10^2 (16)
SIV239	4.95×10^2 (8)	1.05×10^2 (16)
SIVR7*nef*+	2.92×10^3	3.55×10^2
SIVR7Δ*nef*	5.13×10^2 (6)	4.17×10 (8.5)

SIV Nef enhances the infectivity of HIV-1. We have demonstrated that SIV and HIV-1 Nef proteins are functionally interchangeable in the context of SIV. To determine if SIV Nef could replace HIV-1 Nef in the context of HIV-1, we generated further chimeric constructs in which the *nef* coding sequence of HIV-1 was replaced with the corresponding SIV sequence (Fig. 8). The *nef* coding sequences of SIV*nef*+ and SIVΔ*nef* were used to generate R7SIV*nef*+ and R7SIVΔ*nef*. The relative infectivities of these chimeric viruses were compared to the infectivity of the parental HIV-1 *nef*$^+$ and *nef*-defective viruses (R73 and R73mlu) in the MAGI assay. As shown in Fig. 9, R7SIV*nef*+ was more infectious than R7SIVΔ*nef* and R7mlu, although SIV Nef did not fully restore the level of infectivity to that of the parental HIV-1 *nef*$^+$ virus R73. It is possible that the failure to fully complement virus infectivity in this context may derive, at least in part, from the creation of a hybrid SIV–HIV-1 LTR U3 region that separates interacting transcriptional regulatory motifs that differ between SIV and HIV-1 (4a, 22).

DISCUSSION

We have demonstrated that SIV Nef accelerates SIV replication in human and rhesus primary peripheral blood T cells. As previously reported for HIV-1 Nef, the ability of SIV Nef to

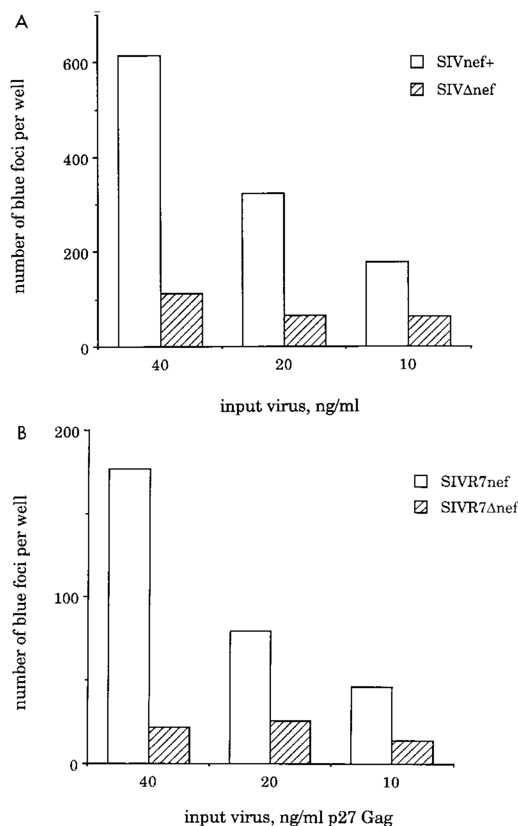

FIG. 7. SIV and HIV-1 Nef enhance the infectivity of SIV. A single-cycle infectivity assay was performed by infecting β-Gal indicator cells, sMAGI cells with parental (A) or chimeric (B) SIV. Forty-eight hours postinfection, cells were incubated with X-Gal and infected cells, expressing Tat, were counted.

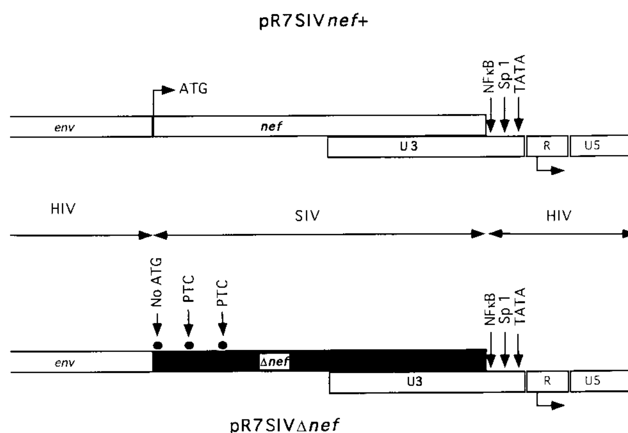

FIG. 8. Schematic diagram of recombinant HIV chimeras. R7SIV*nef*+ was constructed by replacement of the HIV-1 *nef* gene (and a portion of the HIV-1 LTR U3 region) with an intact SIV*nef* gene (and a portion of the SIV LTR U3 region) obtained from SIVmac239/*nef* open. R7SIVΔ*nef* contains the mutant SIV *nef* gene (and adjacent SIV LTR U3 sequences) obtained from the SIVΔ*nef* virus that is unable to express Nef by virtue of the absence of an appropriate translation initiation codon, the insertion of a premature translation termination codon, and the presence of the original premature termination codon found in SIVmac239. Premature translation termination codons are designated PTC.

accelerate the kinetics of virus replication in culture was correlated with a greater infectivity (as measured by determination of $TCID_{50}$s) of virus particles produced by Nef-expressing cells. The analyses of single cycles of SIV replication by using the sMAGI indicator cell line has established that SIV Nef, like HIV-1 Nef, acts early in the virus life cycle to enhance the efficiency of virus replication. Furthermore, the HIV-1 *nef* gene, when included in a chimeric SIV, confers upon the recombinant virus a growth phenotype in primary T cells and infectivity, as measured by $TCID_{50}$ analyses and sMAGI cell infections, that are virtually indistinguishable from those of parental SIV*nef*+. In experiments where SIV *nef* was substituted for the native *nef* gene in HIV-1, SIV Nef was able to augment the replication of the recombinant virus in primary T cells. SIV Nef's ability to enhance the infectivity of HIV-1 is also mediated at early stages of the virus life cycle, as assessed by studies of single cycles of virus infection using the MAGI cell line. These results support the proposal that SIV and HIV-1 Nef serve similar, if not identical, roles in the virus life cycle. Our finding that SIV Nef enhances the infectivity of HIV-1 is consistent with the recent results of Aiken and Trono, who demonstrated that Nef from both HIV-2 and SIV, supplied in *trans*, could enhance the infectivity of *nef*-defective HIV-1, albeit to a lesser extent than HIV-1 Nef (2).

Although our findings are in agreement with previous reports for HIV-1 Nef, they are in conflict with earlier reports on the role of Nef in SIV replication in culture where no difference in the replication of nef^+ and *nef*-defective SIV was detected in rhesus PBMCs (26, 49) or where Nef-mediated inhibition of SIV replication was described (6). Until more sensitive and reproducible assay systems were defined, numerous conflicting results concerning the impact of Nef on HIV-1 replication were also presented, which confounded the delineation of the role of Nef in the virus life cycle (11). However, the ability of HIV-1 Nef to enhance virus infectivity is now widely appreciated, and significant progress is being made in defining the molecular mechanism of this effect (2, 10, 19, 35, 37, 42, 44, 46, 51). Using cell culture systems modeled on those that have been successfully used to study HIV-1 Nef function (9, 34, 35, 48), we have found that SIV Nef also acts reproducibly to enhance virus infectivity. Kestler et al. studied the identical SIV isolate and *nef* allele used in our studies, and their inability to detect Nef-induced enhancement of virus infectivity cannot derive from genetic differences in viral se-

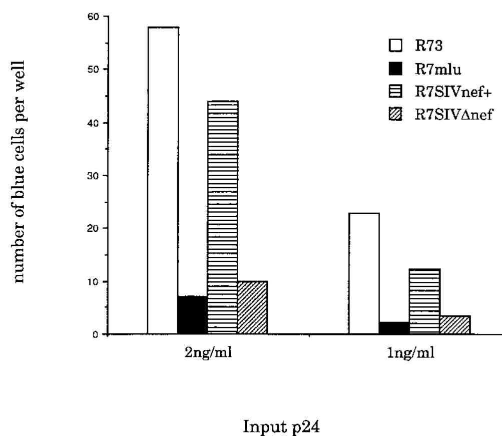

FIG. 9. SIV Nef enhances the infectivity of HIV-1. A single-cycle infectivity assay was performed by infecting β-Gal indicator cells (MAGI cells) with wild-type, nef^+ HIV-1 (R73), *nef*-defective HIV-1 (R7Mlu), an HIV-1 chimera that includes an intact SIV *nef* gene (R7SIV*nef*+), and a HIV-1 chimera that includes a defective SIV *nef* gene (R7SIVΔ*nef*). Infections were carried out at two concentrations of virus, 2 and 1 ng/ml. Forty-eight hours postinfection, numbers of blue (Tat-expressing) cells were enumerated.

quences (26). The reason for our discrepant results is not clear; however, differences in the amount of virus used as inocula, or in the processing or culture of the target cells used for virus infection, between the current and previous studies may have contributed. The ability of Nef to accelerate HIV-1 and SIV replication is less pronounced at higher multiplicities of infection or when, in the course of resting-cell infections, higher levels of activated $CD4^+$ T cells are present at the time of initiation of virus infections of target cell cultures (references 9, 34, and 48 and this work). In reports of SIV Nef function other than that of Kestler et al., it is unclear whether the SIV *nef* alleles studied were actually competent for either of the functions established for HIV-1 Nef, infectivity enhancement, or CD4 down-modulation. Unger et al. found no difference between the replication kinetics of the nonpathogenic SIVmac1A11 and a *nef* deletion mutant of this virus (49). However, the 1A11 isolate is severely attenuated in vivo, and it is not known which regions of the 1A11 genome are responsible for its attenuation (31, 32, 49). Indeed, it is plausible that the *nef* gene of 1A11 is potentially functionally compromised compared with the SIVmac239open *nef* allele. Binninger et al. studied the replication of SIV variants harboring insertion and deletion mutations in *nef* and reported that Nef expression led to impaired virus replication in culture (6). This observation is directly contradicted by our results. The study by Binninger et al. used infections of immortalized human T-cell lines exclusively, and it is known that this culture system does not sensitively reveal the ability of Nef to enhance virus replication (14, 34, 48). In addition, the SIV *nef* mutant that showed the fastest replication kinetics in this study, and which represented the primary focus of the work, was also deleted in the viral sequences encoding the carboxy-terminal 12 amino acids of the Env protein and is thus not a simple Nef mutant virus (6).

The apparent conservation of function between HIV-1 and SIV Nef in tissue culture infections suggests that they may also exert analogous effects in vivo, with HIV-1 Nef, like SIV Nef, acting to facilitate high level virus replication. The demonstration that SIV Nef and HIV-1 Nef are functionally interchangeable within infectious recombinant viruses argues that the ability of Nef to enhance virus infectivity is an evolutionarily conserved, and likely ancient, property of primate lentiviruses. These observations further suggest that key features of the virus constituent(s) that represents the target of Nef action must also be conserved between HIV-1 and SIV. Although the molecular bases by which Nef mediates the down-modulation of cell surface CD4 expression and the enhancement of virus infectivity have not been fully elucidated, domains important for both of these Nef functions are found to be conserved when the HIV-1 and SIV *nef* coding sequences are compared (19, 39, 41, 44, 47). In particular, regions conferring the ability to associate with certain cellular kinases are present in both HIV-1 and SIV *nef*. HIV-1 and SIV Nef specifically associate with and activate a cellular serine kinase, recently identified as a member of the p21-activated kinase (PAK) family of kinases, in infected cells (18, 44, 45). Nef association with this cellular serine kinase is dependent on a central highly conserved region found in all primate lentivirus *nef* alleles analyzed to date (18, 44, 45). While delineation of the precise function(s) conferred by Nef association with, and activation of, PAK will require further study, the importance of this association has been recently highlighted through experimental infections of rhesus macaques with SIV variants that either can or cannot bind PAK (44). Macaques infected with SIV variants harboring mutations in the di-arginine motif necessary for PAK association replicate poorly shortly after inoculation and are rapidly overgrown by viral revertants with restored ability to bind PAK (44). At the cellular and molecular levels, recent studies of HIV-1 suggest that the relevant cellular serine kinase may recruited into assembling virus particles by virtue of a specific association with Nef (7). At some point during or after virion assembly, the Nef-associated serine kinase may phosphorylate a component of the HIV-1 nucleoprotein complex (e.g., the matrix [gagMA] protein) in such a way that the efficiency of early stages of the viral life cycle is increased. Serine phosphorylation of gagMA found within nascent virions and within viral preintegration complexes has been suggested to facilitate dissociation of preintegration complexes from viral and cellular membranes, thereby freeing the complexes to traffic to the nucleus in an unimpeded manner (7). In addition to Nef association with serine kinases within infected cells, conserved SH3-like binding domains that confer the ability to bind certain members of the Src family of tyrosine kinases with high affinity (in particular Hck and to a lesser extent Lyn) in vitro are present in the *nef* coding sequence of HIV-1 (42). While these SH3 domains are dispensable for CD4 down-regulation, it is reported that the replicative potential of HIV-1 is diminished when they are ablated (42). The coding sequences of SIV and HIV-2 *nef* also contain SH3-like domains (37, 40), although they possess only three of the four proline repeats present in HIV-1 Nef. However, the two central prolines thought to be involved in infectivity enhancement by HIV-1 Nef are conserved in both SIV and HIV-2 *nef* coding sequences, as is the ability to bind to the SH3 domain with high affinity (42). The mechanism by which Src family kinases contribute to enhanced infectivity of HIV-1 has yet to be defined. Given the emerging importance of Nef association with cellular serine and tyrosine kinases in the enhancement of HIV-1 infectivity, and the demonstration that SIV Nef binds to the same cellular kinases as HIV-1 Nef, it might be expected that Nef-induced infectivity enhancement would be also be conserved between these primate lentiviruses. Our results indicate that this is property of Nef is, indeed, shared between HIV-1 and SIV.

The magnitude of the effect of Nef, although not dramatic in culture, may be sufficient to tip the balance in vivo between virus replication and host immune containment in favor of the virus. Maximization of virus infectivity enhancement by Nef may be particularly important in in vivo settings where the availability of susceptible target cells to support virus replication is limiting, or yet to be defined handicaps to virus spread within intact lymphoid organs that preclude efficient in situ transmission of Nef-defective viruses may exist. Even in circumstances where no countervailing antiviral immune response is present, as is the case in SCID/Hu mice, Nef is necessary for high-level virus replication and efficient CD4 T-cell depletion (23). However, in experimental infections of neonatal rhesus macaques born to SIV-uninfected mothers where large numbers of susceptible T cells are available for infection, host immune responses are immature, and no maternal antiviral antibodies are present; even multiply deleted SIV variants (e.g., SIVΔ3 lacking intact *nef*, *vpr*, and LTR sequences) can induce immunodeficiency when infection is initiated with a sufficiently high virus inoculum (4, 52). This observation indicates that Nef is not necessary for pathogenic consequences of virus infection per se but functions primarily to enhance replication of viruses whose cytopathic properties derive from expression of other viral gene products (e.g., Env). Whatever the precise mechanism of Nef-induced facilitation of virus replication in vivo may prove to be, it is likely that when virus replication is less efficient, as in the case of *nef*-defective virus, effective host immune control may be more readily accomplished (13, 26, 29). As rates of virus replication are linked

to rates of CD4$^+$ T-cell destruction and disease progression, the ability of *nef*-defective HIV-1 or SIV variants to induce disease in the immunocompetent host is consequently abrogated (20, 33, 43, 50).

The ability of macaques infected with *nef*-defective SIV to resist experimental challenge with fully pathogenic SIV isolates has provided a rationale for and potential approach to the derivation of *nef*-defective HIV-1 variants as candidate live-attenuated AIDS vaccines in humans (12). However, concerns about the safety of attenuated HIV-1 vaccines have been raised (4). Thus, for both theoretical and practical reasons, it is important to demonstrate the functional equivalence of the SIV and HIV-1 genes that are deleted in candidate vaccines and to elucidate the molecular basis through which specific viral gene products contribute to disease induction. Unfortunately, in contrast to many other live-attenuated vaccines now in widespread use in humans, empiric approaches to development of a live-attenuated HIV-1 vaccine are not feasible, and no acceptable animal model system that permits the evaluation of HIV-1 infection, disease induction, and vaccine efficacy has been identified. That Nef is required for high-level HIV-1 and SIV replication in vivo also raises the possibility that anti-HIV-1 Nef therapeutics reduce virus replication and thereby delay disease progression in HIV-1-infected people. Although the recent definition of tissue culture assays to evaluate Nef functions should aid in the identification of candidate Nef antagonists, the evaluation of the therapeutic efficacy of anti-Nef agents will greatly benefit from the derivation of an in vivo test system based on a well-characterized animal model of lentivirus-induced AIDS.

In hopes of facilitating the experimental evaluation of HIV-1 Nef function in vivo, we generated chimeric SIVs where the corresponding regions of the viral genomes have been replaced with the HIV-1 LTR and *nef* coding sequences. In the construction of these chimeric viruses, care was taken to maximize HIV-1 Nef expression by eliminating, through site-directed mutagenesis, all of the upstream translation initiation codons normally used for the expression of SIV Nef (39). Further, along with the HIV-1 *nef* gene, we chose to replace all of the SIV U3 region upstream of the TATA sequence with the corresponding sequences of the HIV-1 U3 region. By exchanging both the *nef* coding sequences and the transcriptional regulatory elements of the LTR U3 regions, unintended effects resulting the creation of hybrid transcriptional regulatory motifs that function less well or in different ways than the native regulatory elements may be minimized (4a, 22). The SIV–HIV-1 Nef chimeras described in this study replicate in tissue culture infections with similar kinetics to those of the parental SIV and may provide a novel approach to evaluate the contribution of HIV-1 Nef and LTR sequences to the level and consequences of virus replication in vivo. Should HIV-1 *nef* complement the replicative debility of *nef*-defective SIV as effectively in vivo as it does in tissue culture, direct comparison of the contribution of SIV and HIV-1 Nefs to disease pathogenesis will be possible. Chimeric SIVs that express HIV-1 Nef should also provide a valuable approach to test the ability of potential therapeutic strategies to interfere with HIV-1 Nef function in vivo. Given the accumulating information concerning the structure and function of HIV-1 Nef, the characterization in vivo of chimeric viruses harboring mutations that abolish specific Nef functions, alone and in combination, should help illuminate the complex biology of this multifunctional viral gene product.

ACKNOWLEDGMENTS

We thank R. Desrosiers for generously providing p239SpSp5′, p239SpE3′, p239*nef*open, and the CEMx174 cell line; W. Pear for his gift of the 293T cell line; B. Chackerian and J. Overbaugh for providing the sMAGI cell line; R. Andino for helpful advice; and M. Miller and K. Page for advice, helpful discussions, and review of the manuscript. The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health: HeLa-CD4-LTR-β-gal from M. Emerman and the monoclonal antibody to recombinant HIV-1 NEF (NF2-B2).

REFERENCES

1. **Aiken, C., J. Konner, N. R. Landau, M. E. Lenburg, and D. Trono.** 1994. Nef induces CD4 endocytosis: requirement for a critical motif in the membrane-proximal CD4 cytoplasmic domain. Cell **76:**853–864.
2. **Aiken, C. and D. Trono.** 1995. Nef stimulates human immunodeficiency virus type 1 proviral DNA synthesis. J. Virol. **69:**5048–5056.
3. **Anderson, S., D. C. Shugars, R. Swanstrom, and J. V. Garcia.** 1993. Nef from primary isolates of human immunodeficiency virus type 1 suppresses surface CD4 expression in human and mouse T cells. J. Virol. **67:**4923–4931.
4. **Baba, T. W., Y. S. Jeong, R. Bronson, M. F. Greene, and R. M. Ruprecht.** 1995. Pathogenicity of live, attenuated SIV after mucosal infection of neonatal macaques. Science **267:**1820–1825.

4a. **Barbosa, P., and M. Feinberg.** Unpublished data.

5. **Benson, R. E., A. Sanfridson, J. S. Ottinger, C. Doyle, and B. R. Cullen.** 1993. Downregulation of cell-surface CD4 expression by simian immunodeficiency virus Nef prevents viral superinfection. J. Exp. Med. **177:**1561–1566.
6. **Binninger, D., J. Ennen, D. Bonn, S. G. Norley, and R. Kurth.** 1991. Mutational analysis of the simian immunodeficiency virus SIVmac *nef* gene. J. Virol. **65:**5237–5243.
7. **Bukrinskaya, A. G., A. Ghorpade, N. K. Heinzinger, T. E. Smithgall, R. E. Lewis, and M. Stevenson.** 1996. Phosphorylation-dependent human immunodeficiency virus type 1 infection and nuclear targeting of viral DNA. Proc. Natl. Acad. Sci. USA **93:**367–371.
8. **Chackerian, B., N. L. Haigwood, and J. Overbaugh.** 1995. Characterization of a CD4-expressing macaque cell line that can detect virus after a single replication cycle and can be infected by diverse simian immunodeficiency virus isolates. Virology **213:**386–394.
9. **Chowers, M. Y., C. A. Spina, T. J. Kwoh, N. J. S. Fitch, D. D. Richman, and J. C. Guatelli.** 1994. Optimal infectivity in vitro of HIV-1 requires an intact *nef* gene. J. Virol. **68:**2906–2914.
10. **Chowers, M. Y., M. W. Pandori, C. A. Spina, D. D. Richman, and J. C. Guatelli.** 1995. The growth advantage conferred by HIV-1 *nef* is determined at the level of viral DNA formation and is independent of CD4 downregulation. Virology **212:**451–457.
11. **Cullen, B. R.** 1994. The role of Nef in the replication cycle of the human and simian immunodeficiency viruses. Virology **205:**1–6.
12. **Daniel, M. D., F. Kirchhoff, S. C. Czajak, P. K. Sehgal, and R. C. Desrosiers.** 1992. Protective effects of a live attenuated SIV vaccine with a deletion in the *nef* gene. Science **258:**1938–1941.
13. **Deacon, N. J., A. Tsykin, A. Solomon, K. Smith, M. Ludford-Menting, D. J. Hooker, D. A. McPhee, A. L. Greenway, A. Ellett, C. Chatfield, V. A. Lawson, S. Crowe, A. Maerz, S. Sonza, J. Learmont, J. S. Sullivan, A. Cunningham, D. Dwyer, D. Dowton, and J. Mills.** 1995. Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. Science **270:**988–991.
14. **de Ronde, A., B. Klaver, W. Keulen, L. Smit, and J. Goudsmit.** 1992. Natural HIV-1 Nef accelerates virus replication in primary human lymphocytes. Virology **188:**391–395.
15. **Feinberg, M. B., D. Baltimore, and A. D. Frankel.** 1991. The role of Tat in the human immunodeficiency virus life cycle indicates a primary effect on transcriptional elongation. Proc. Natl. Acad. Sci. USA **88:**4045–4049.
16. **Foster, J. L., S. J. Anderson, A. L. Frazier, and J. V. Garcia.** 1994. Specific suppression of human CD4 expression by Nef from the pathogenic simian immunodeficiency virus SIVmac239open. Virology **201:**373–379.
17. **Garcia, J. V., and A. D. Miller.** 1991. Serine phosphorylation-independent downregulation of cell-surface CD4 by *nef*. Nature **350:**508–511.
18. **Garcia, J. V., and J. L. Foster.** 1996. Structural and functional correlates between HIV-1 and SIV Nef isolates. Virology **226:**161–166.
19. **Goldsmith, M. A., M. T. Warmerdam, R. E. Atchison, M. D. Miller, and W. C. Greene.** 1995. Dissociation of the CD4 downregulation and viral infectivity enhancement functions of human immunodeficiency virus type 1 Nef. J. Virol. **69:**4112–4121.
20. **Ho, D. D., A. U. Neumann, A. S. Perelson, W. Chen, J. M. Leonard, and M. Markowitz.** 1995. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature **373:**123–126.
21. **Horton, R. M.** 1995. PCR-mediated recombination and mutagenesis. SOEing together tailor-made genes. Mol. Biotechnol. **3:**93–99.
22. **Ilyinskii, P. O., and R. C. Desrosiers.** 1996. Efficient transcription and rep-

lication of simian immunodeficiency virus in the absence of NF-κB and Sp1 binding elements. J. Virol. **70:**3118–3126.
23. **Jamieson, B. D., G. M. Aldrovandi, V. Planelles, J. B. M. Jowett, L. Gao, L. M. Bloch, I. S. Y. Chen, and J. A. Zack.** Requirement of human immunodeficiency virus type 1 *nef* for in vivo replication and pathogenicity. J. Virol. **68:**3478–3485.
24. **Johnson, V. A., and R. E. Byington.** 1990. Quantitative assays for virus infectivity, p. 71–86. *In* A. Aldovini, A. and B. D. Walker (ed.), Techniques in HIV research. Stockton Press, New York, N.Y.
25. **Kestler, H., T. Kodama, D. Ringler, M. Marthas, N. Pedersen, A. Lackner, D. Regier, P. Sehgal, M. Daniel, N. King, and R. Desrosiers.** 1990. Induction of AIDS in rhesus monkeys by molecularly cloned simian immunodeficiency virus. Science **248:**1109–1112.
26. **Kestler, H., D. J. Ringler, K. Mori, D. L. Panicali, P. K. Sehgal, M. D. Daniel, and R. C. Desrosiers.** 1991. Importance of the *nef* gene for maintenance of high virus loads and for development of AIDS. Cell **65:**651–662.
27. **Kim, S., R. Byrn, J. Groopman, and D. Baltimore.** 1989. Temporal aspects of DNA and RNA synthesis during human immunodeficiency virus infection: evidence for differential gene expression. J. Virol. **63:**3708–3713.
28. **Kimpton, J. and M. Emerman.** 1992. Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated β-galactosidase gene. J. Virol. **66:** 2232–2239.
29. **Kirchhoff, F., T. C. Greenough, D. B. Brettler, J. L. Sullivan, and R. C. Desrosiers.** 1995. Absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection. N. Engl. J. Med. **332:**228–232.
30. **Klotman, M. E., S. Kim, A. Buchbinder, A. DeRossi, D. Baltimore, and F. Wong-Staal.** 1991. Kinetics of expression of multiply spliced RNA in early human immunodeficiency virus type 1 infection of lymphocytes and monocytes. Proc. Natl. Acad. Sci. USA **88:**5011–5015.
31. **Lohman, B. L., M. B. McChesney, C. J. Miller, E. McGowan, S. M. Joye, K. K. A. Van Rompay, E. Reay, L. Antipa, N. C. Pedersen, and M. L. Marthas.** 1994. A partially attenuated simian immunodeficiency virus induces host immunity that correlates with resistance to pathogenic virus challenge. J. Virol. **68:**7021–7029.
32. **Marthas, M. L., R. A. Ramos, B. L. Lohman, K. K. A. Van Rompay, R. E. Unger, C. J. Miller, B. Banapour, N. C. Pedersen, and P. A. Luciw.** 1993. Viral determinants of simian immunodeficiency virus (SIV) virulence in rhesus macaques assessed by using attenuated and pathogenic molecular clones of SIVmac. J. Virol. **67:**6047–6055.
33. **Mellors, J. W., C. R. Rinaldo, P. Gupta, R. M. White, J. Todd, and L. A. Kingsley.** 1996. Prognosis in HIV-1 infection predicted by quantity of virus in plasma. Science **272:**1167–1170.
33a. **Miller, M. D., and M. B. Feinberg.** Unpublished data.
34. **Miller, M. D., M. T. Warmerdam, I. Gaston, W. C. Greene, and M. B. Feinberg.** 1994. The HIV-1 *nef* gene product: a positive factor for viral infection and replication in primary lymphocytes and macrophages. J. Exp. Med. **179:**101–113.
35. **Miller, M. D., M. T. Warmerdam, K. A. Page, M. B. Feinberg, and W. C. Greene.** 1995. Expression of the human immunodeficiency virus type 1 (HIV-1) *nef* gene during HIV-1 production increases progeny particle infectivity independently of gp160 or viral entry. J. Virol. **69:**579–584.
36. **Munis, J. R., R. S. Kornbluth, J. C. Guatelli, and D. D. Richman.** 1992. Ordered appearance of human immunodeficiency virus type 1 nucleic acids following high multiplicity infection of macrophages. J. Gen. Virol. **73:**1899–1901.
37. **Pandori, M. W., N. J. S. Fitch, H. M. Craig, D. D. Richman, C. A. Spina, and J. C. Guatelli.** 1996. Producer-cell modification of human immunodeficiency virus type 1: Nef is a virion protein. J. Virol. **70:**4283–4290.
38. **Pear, W. S., G. P. Nolan, M. L. Scott, and D. Baltimore.** 1993. Production of high-titer helper-free retroviruses by transient transfection. Proc. Natl. Acad. Sci. USA **90:**8392–8396.
39. **Regier, D. A., and R. C. Desrosiers.** 1990. The complete nucleotide sequence of a pathogenic molecular clone of simian immunodeficiency virus. Aids Res. Hum. Retroviruses **6:**1221–1231.
40. **Rhee, S. S., and J. W. Marsh.** 1994. Human immunodeficiency virus type 1 Nef-induced down-modulation of CD4 is due to rapid internalization and degradation of surface CD4. J. Virol. **68:**5156–5163.
41. **Robert-Guroff, M., M. Popovic, S. Gartner, P. Markaham, R. C. Gallo, and M. S. Reitz.** 1990. Structure and expression of *tat*-, *rev*-, and *nef*-specific transcripts of human immunodeficiency virus type 1 in infected lymphocytes and macrophages. J. Virol. **64:**3391–3398.
42. **Saksela, K., G. Cheng, and D. Baltimore.** 1995. Proline-rich (PxxP) motifs in HIV-1 Nef bind to SH3 domains of a subset of Src kinases and are required for the enhanced growth of Nef+ viruses but not for down-regulation of CD4. EMBO J. **69:**484–491.
43. **Saksela, K., C. Stevens, R. Rubinstein, and D. Baltimore.** 1994. Human immunodeficiency virus type 1 mRNA expression in peripheral blood cells predicts disease progression independently of the number of CD4+ lymphocytes. Proc. Natl. Acad. Sci. USA **91:**1104–1108.
44. **Sawai, E. T., A. Baur, H. Struble, B. M. Peterlin, J. A. Levy, and C. Cheng-Mayer.** 1994. Human immunodeficiency virus type 1 Nef associates with a cellular serine kinase in T lymphocytes. Proc. Natl. Acad. Sci. USA **91:**1539–1543.
45. **Sawai, E. T., A. S. Baur, B. M. Peterlin, J. A. Levy, and M. C. Cheng.** 1995. A conserved domain and membrane targeting of Nef from HIV and SIV are required for association with a cellular serine kinase activity. J. Biol. Chem. **270:**15307–15314.
46. **Schwartz, O., V. Marechal, O. Danos, and J. M. Heard.** 1995. Human immunodeficiency virus type 1 Nef increases the efficiency of reverse transcription in the infected cell. J. Virol. **69:**93–100.
47. **Shugars, D. C., M. S. Smith, D. H. Glueck, P. V. Nantermet, F. Seillier-Moiseiwitsch, and R. Swanstrom.** 1993. Analysis of human immunodeficiency virus type 1 *nef* gene sequences present in vivo. J. Virol. **67:**4639–4650.
48. **Spina, C. A., T. J. Kwoh, M. Y. Chowers, J. C. Guatelli, and D. D. Richman.** 1994. The importance of Nef in the induction of HIV-1 replication from primary quiescent CD4 lymphocytes. J. Exp. Med. **179:**115–123.
49. **Unger, R. E., M. L. Marthas, L. E. Pratt, P. A. Padrid, and P. A. Luciw.** 1992. The *nef* gene of simian immunodeficiency virus SIVmac1A11. J. Virol. **66:** 5432–5442.
50. **Wei, X., S. K. Ghosh, M. E. Taylor, V. A. Johnson, E. A. Emini, P. Deutsch, J. D. Lifson, S. Bonhoeffer, M. A. Nowak, B. H. Hahn, M. S. Saag, and G. M. Shaw.** 1995. Viral dynamics in human immunodeficiency virus type 1 infection. Nature **373:**117–122.
51. **Welker, R., H. Kottler, H. R. Kalbitzer, and H. G. Krausslich.** 1996. Human immunodeficiency virus type 1 Nef protein is incorporated into virus particles and specifically cleaved by the viral proteinase. Virology **219:**228–236.
52. **Wyand, M. S., K. D. Manson, A. A. Lackner, and R. C. Desrosiers.** 1997. Resistance of neonatal monkeys to live attenuated vaccine strains of simian immunodeficiency virus. Nat. Med. **3:**30–36.