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Nef Stimulates Human Immunodeficiency Virus Type 1 Proviral DNA Synthesis

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The Nef protein of human immunodeficiency virus type 1 (HIV-1) stimulates viral infectivity. The mechanism of this phenotype was investigated. Viruses containing disrupted *nef* genes were 4 to 40 times less infectious than wild-type HIV-1 in a single-round infection. The Nef-mediated stimulation of HIV-1 infectivity was dependent on the association of Nef with the plasma membrane and could be observed when Nef was provided in *trans* in the virus producer but not target cells. The impaired infectiousness of *nef*-defective (ΔNef) virions was observed whether or not CD4 was present in either of these cells. Furthermore, it was independent of the mode of viral entry, since it was not rescued by pseudotyping Env$^-$ HIV-1 virions with the amphotropic murine leukemia virus envelope glycoproteins. As predicted from this result, wild-type and ΔNef virions entered cells with equal efficiencies. However, despite their normal content in viral genomic RNA and reverse transcriptase activity, ΔNef viruses were limited in their ability to perform reverse transcription once internalized in several cell types, including peripheral blood lymphocytes. Since Nef does not appear to be abundant in virions, these results suggest that Nef acts in producer cells to allow the generation of particles fully competent for completing steps that follow entry, leading to efficient reverse transcription of the HIV-1 genome. Using a *trans* complementation assay, we found that Nef proteins from a number of primary HIV-1 isolates as well as, to a milder degree, those from HIV-2$_{ST}$ and SIV$_{MAC239}$ could enhance the infectivity of ΔNef HIV-1. This indicates that the Nef-mediated stimulation of proviral DNA synthesis is highly conserved and likely plays an important role in vivo.

The genomes of lentiviruses, including human immunodeficiency virus type 1 (HIV-1), are characterized by the presence of a number of accessory genes in addition to the prototypic *gag*, *pol*, and *env* reading frames encountered in all retroviruses. The *nef* gene is found only in primate lentiviruses. In HIV-1, it encodes a 206-amino-acid-long cytoplasmic protein that associates with the plasma membrane through an N-terminal myristate (10). Nef is translated from a fully spliced mRNA produced in abundance early in the course of infection (20, 22). *nef* belongs to the class of so-called nonessential genes of HIV-1, because it is dispensable for viral growth in a number of in vitro systems. However, Nef is highly conserved, indicating that it must fulfill a crucial function in vivo. Initial experiments had suggested that Nef repressed viral replication by inhibiting transcription from the proviral long terminal repeat (LTR); hence the acronym Nef, for negative factor (1, 23, 27). Nevertheless, these results were not subsequently confirmed (7, 9, 15, 21, 25, 34, 35). Instead, studies in rhesus monkeys infected with *nef*-deleted strains of the simian immunodeficiency virus (SIV) have demonstrated that Nef is crucial for high-titer replication of SIV in vivo and for disease induction (19). The mechanism of this phenomenon is still imperfectly understood, but recent studies have revealed that Nef exerts unambiguous effects both on the host cell and on viral replication itself.

Nef triggers the rapid endocytosis of CD4 from the surface of HIV-1-infected cells (2, 30). This downregulation significantly precedes Env-mediated trapping of the receptor in the endoplasmic reticulum and renders cells relatively resistant to HIV infection by reducing the efficiency of virus entry (2, 4). Whether this benefits the virus by blocking lethal multiple superinfections during an acute infection remains to be defined. Alternatively but not exclusively, Nef-mediated CD4 downmodulation could prevent virus aggregation on the surface of some cells during budding, analogous to the role played by neuraminidase for influenza virus. Finally, it could participate in altering T-cell activation pathways through the release of the p56^{lck} tyrosine kinase from the CD4 cytoplasmic domain (2, 30).

In addition, Nef seems to directly promote HIV-1 replication by enhancing the infectivity of virions (7, 25, 34). This observation was first suggested by experiments comparing the replication rates of *nef*-positive and *nef*-defective HIV-1 clones in activated primary blood lymphocytes (PBL) (9). Subsequently, it was demonstrated that the defective phenotype of *nef*-mutated viruses could be accentuated if PBL were infected while resting and activated afterwards (25, 34). A positive effect of Nef on HIV-1 growth was also detected in terminally differentiated macrophages (25) as well as in human T-lymphoid CEM (7) and Jurkat (42) cells. Although the Nef effect was most marked at low multiplicities of infection (7, 25, 34), it was revealed in single-cycle infection assays (25). Finally, Nef-mediated CD4 downregulation in virus producer cells did not contribute to the stimulation of HIV replication, since *nef*-competent viruses released by transfected COS cells were also more infectious than their *nef*-defective counterparts (25).

In the present work, we have examined the molecular basis for the Nef-mediated enhancement of HIV-1 infectivity. We observe that viruses produced from proviral DNAs mutated in *nef* are 4 to 40 times less infectious than the wild type in a single-round infection system. The Nef effect is dependent on the association of this protein with the plasma membrane and

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is determined at the stage of virus particle formation. The reduced infectivity of ΔNef HIV-1 can be complemented in *trans* by expressing *nef* in virus producer cells in a dose-dependent manner. In contrast, it cannot be rescued by Nef in the virus target cell, indicating that it takes place entirely during the early steps of infection, before the virus genome is transcribed. The Nef-mediated enhancement of HIV-1 infectivity is observed whether or not CD4 is present in either virus producer or target cells. It is independent of the HIV-1 envelope glycoproteins, as *env*-defective viruses pseudotyped by the amphotropic murine leukemia virus (MuLV) envelope do exhibit a Nef-promoted infectiousness. In agreement with this result, virus uptake assays indicate that ΔNef virions enter cells normally. However, once inside the cell, these viruses are restricted in their ability to reverse transcribe their genome. Since *nef*-defective virions contain normal amounts of genomic RNA and exhibit wild-type levels of reverse transcriptase activity, these results suggest that Nef might act by altering the processing of the internalized virus core, making it more competent for viral DNA synthesis. A number of primary *nef* alleles isolated from HIV-1-infected individuals were found to enhance virus infectivity, as were, to a lesser extent, the *nef* genes of HIV-2_{ST} and SIV_{MAC239}. This indicates that the stimulation of HIV-1 reverse transcription is a highly conserved function of Nef and is likely to play an important role in vivo.

MATERIALS AND METHODS

DNA constructions. The HIV-1 HXB2 proviral constructs R7, NL, ΔN, ΔNX, and R7 NefG$_2$A and the *nef* expression plasmid CMX-Nef1 have been described (2). Envelope-defective versions of R7 and ΔN (ΔE and ΔEΔN, respectively) were constructed as previously described (38). R7-BaL contains a modified HXB2 provirus carrying the envelope gene of BaL (39), a macrophage-tropic strain of HIV-1 (16). Plasmids CMV-LA and SVHIV-2_{ST}nef were gifts of A. Panet and J. Kaminchik (18). Plasmid CMV-HIV-2_{ST}nef was made by replacing the *Pvu*I-*Hin*dIII fragment of CMV-LA, which expresses HIV-1_{LAI} *nef* from the cytomegalovirus (CMV) promoter, with the corresponding fragment of SVHIV-2_{ST}nef. Plasmid CMX-NefSIV$_{MAC239}$ was constructed by ligating a 956-bp *Pst*I-*Hpa*II fragment of p239Spe3′-nef open, a plasmid carrying the 3′ region of SIV_{MAC239}, into CMX opened with *Sma*I and *Pst*I. Primary alleles of *nef* (24), obtained from J. Skowronski, were subcloned from the CD3 Tex vectors by cutting with *Xba*I, end-filling with the Klenow fragment of DNA polymerase I, cleaving with *Bam*HI, and ligating the resulting DNA piece into CMX cut with *Sma*I and *Bam*HI. Plasmid SV-A-MLV-*env*, expressing the amphotropic MuLV *env* gene, was obtained from Ned Landau (28).

Cell lines and transfections. P4-2 and Z-24 cells were provided by F. Clavel (5, 6). P4-2, Z-24, and 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. The 293 cells were transfected by the calcium phosphate method as described before (3). Two days later, virus-containing supernatants were harvested, filtered through a 0.45-μm cellulose acetate syringe filter, and assayed for p24 content with the DuPont capture enzyme-linked immunosorbent assay (ELISA) kit. Virus stocks were stored at −75°C until needed.

nef-Expressing P4-2 cells were produced by infection with supernatant from the murine amphotropic packaging line CRIP (8), which had been previously transduced with LNef1.SHD, a retrovirus vector expressing HIV-1 *nef*. Clones were selected in histidine-free medium containing 1 mg of histidinol (Sigma) per ml and evaluated for *nef* expression by Western immunoblotting with rabbit antiserum produced against recombinant Nef protein and for surface CD4 levels by fluorescence-activated cell sorting (FACS) analysis, as previously described (2).

Peripheral blood mononuclear cells were isolated by banding of whole blood from seronegative donors on Ficoll-Paque (Pharmacia LKB). Monocytes were removed by overnight adherence to plastic. The resulting PBL were removed and activated by treatment with 3 μg of phytohemagglutinin (PHA) per ml for 48 h, after which the PHA was removed and the cells were cultured in RPMI 1640 containing 10% fetal bovine serum and 10 U of recombinant human interleukin-2 (Bethesda Research Laboratories) per ml. Cultures were split as needed and fed fresh interleukin-2 every 3 days.

Infections. For routine assays of HIV-1 infectivity, 40,000 P4-2 cells were plated in 12-mm wells and infected 1 day later with 0.25 ml of virus supernatant (diluted to 2 to 4 ng of p24 per ml) containing 20 μg of DEAE-dextran per ml. Two hours later, 2 ml of complete medium was added. Cells were fixed and stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) at 2 days postinfection, and the number of blue foci was counted under the light microscope.

PCR analysis of proviral DNA synthesis. For PCR analyses of proviral DNA synthesis in P4-2 cells, 300,000 cells were plated in 35-mm wells and infected with 10 or 50 ng of p24-containing supernatant (in 1 ml) which had been treated for 30 min at 37°C with 20 μg of DNase I (Boehringer Mannheim) per ml in the presence of 10 mM $MgCl_2$. Cells were harvested at each time point by washing once with phosphate-buffered saline (PBS), treating with trypsin to detach cells from the plate and eliminate bound virus, and washing two more times first with complete medium and then with PBS. One-week-old PBL (1.6×10^7) were infected in 8 ml with 400 ng of p24-containing virus supernatants. Viral inocula were removed by washing cells 3 h after infection. At the indicated times, aliquots of the cultures were removed, pelleted, and lysed.

Cell pellets were lysed in 100 μl of PCR lysis buffer as described before (36, 40). PCR was performed in 50-μl reaction mixes with the following primer pair to detect early products of reverse transcription: LTR-5 (5′GGCTAACTAGG GAACCCACTGCTT), corresponding to nucleotides 496 to 516 of HIV-1 HXB2 (as numbered by Ratner et al.) (29), and LTR-6 (5′CTGCTAGAGGATTTTC CACACTGAC), complementary to nucleotides 612 to 635. Primer pair LTR-5 and 5NC-2 (5′CCGAGTCCTGCGTCGAGAGAGC), the latter being complementary to nucleotides 677 to 698 of HIV-1, were used to detect viral DNA produced after the second template switch of reverse transcription. Primer pair β-globin-1 (5′TCTACCCTTGGACCCAGAGG), corresponding to nucleotides 274 to 293 of the human β-globin gene, and β-globin-2 (5′CTGAAGTTCTCAG GATCCACG), complementary to nucleotides 465 to 485, were used to evaluate the relative amounts of DNA in cell lysates. Reactions (50 μl) were performed for 30 cycles in a Perkin-Elmer 9600 thermal cycler with the following protocol: 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C. The products were analyzed by electrophoresis on 1.2% agarose gels and Southern blotting, as previously described (36, 40).

Virus uptake assays. Assays of virus entry were performed by infecting P4-2 cells at 4 or 37°C as described above and washing the cell monolayers twice with PBS^- 2 h after infection. After washing, cells were either lysed directly in 0.5 ml of 0.5% Triton X-100 or trypsinized and washed prior to resuspension of the cell pellet in viral lysis buffer. The p24 antigen content of the lysates was determined by ELISA.

Slot blot analysis of virion RNA. Viral RNA was purified from DNase I-treated viral supernatants with TriReagent (Molecular Research Center, Inc.). After treatment with RQ1 DNase (Promega), the RNA was vacuum-blotted onto nitrocellulose and probed as previously described (37).

Reverse transcriptase assays. Exogenous reverse transcriptase assays were performed by a modification of the procedure of Goff et al. (14). Briefly, 10 μl of virion supernatant was added to 20 μl of 50 mM Tris-HCl (pH 7.9)–75 mM KCl–2 mM dithiothreitol (DTT)–5 mM $MgCl_2$–25 μg of poly(A) oligo(dT_{12-18}) (Pharmacia)–0.05% Nonidet P-40–50 μCi of ^{3}H-TTP per ml. Reaction mixes were incubated at 37°C for 2 h, and the entire volume was spotted on 2.3-cm-diameter DE81 paper circles (Whatman). The disks were washed three times for 5 min in 2× SSC (1× is 0.15 M NaCl, 15 mM sodium citrate) and once in 95% ethanol and then air-dried. Tritium incorporation was determined by liquid scintillation counting. The values obtained were all within the linear portion of the assay and ranged between 2,000 and 10,000 cpm, compared with background levels of 100 to 200 cpm of ^{3}H.

For endogenous reverse transcriptase assays, viruses were first pelleted by centrifugation at 14,000 × *g* in a microcentrifuge for 90 min at 4°C. The viral pellet was resuspended in 25 μl of endoRT buffer (167 mM Tris-HCl [pH 8.0], 25 mM NaCl, 2.5 mM magnesium acetate, 25 mM DTT) and kept on ice for 15 to 30 min. To an aliquot of 15 μl of the resuspended virus, 5 μl of 0.05% Nonidet P-40 and 5 μl of a solution of the four deoxynucleoside triphosphates (20 μM each), including 10 μCi of [^{3}H]TTP (Amersham; 50 Ci/mmol), were added, and the reaction mixes were incubated for 2 h at 41°C. The extent of the reaction was determined as described above. The values obtained in this assay ranged between 12,000 and 24,000 cpm of ^{3}H, with background values of 300 cpm.

RESULTS

Enhancement of HIV-1 infectivity requires Nef membrane association. To study the molecular basis for the Nef-mediated enhancement of HIV-1 infectivity, a single-round infection system was used. P4-2 cells are CD4-positive HeLa cells which contain the β-galactosidase (βGal) gene under control of the HIV-1 LTR; upon HIV infection, production of Tat induces βGal expression, which can be scored by X-Gal staining (6). Human fibroblast 293 cells were transfected with HIV-1 proviral DNA constructs expressing either wild-type *nef* (R7), a mutant encoding a nonmyristoylated protein (R7.Nef$_{G}$2$_{A}$), a variant with a translational frameshift at codon 31 (ΔN), or one in which the first 100 nucleotides of *nef* were deleted (ΔNX) (Fig. 1A). Virions purified from the supernatant of these cells

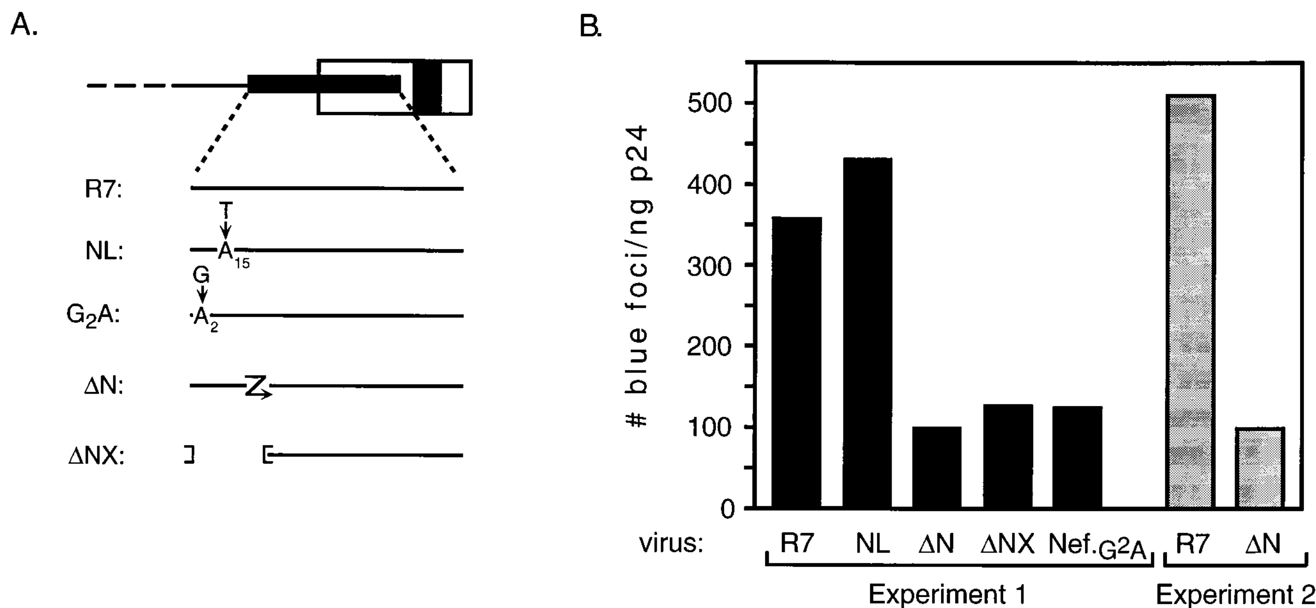

FIG. 1. Myristoylated Nef enhances HIV-1 infectivity. (A) Wild-type and Nef-mutated HIV-1 constructs used in this study. R7 is an HXB2 proviral clone containing a full-length *nef* coding region. NL is an identical construct carrying a point mutation changing Thr-15 to Ala. ΔN carries a frameshift affecting amino acid 34 of Nef. ΔNX contains a deletion of nucleotides 8796 to 8896 in R7, eliminating the first 100 bp of *nef*. R7 $Nef_{G}2_{A}$ contains a Gly-to-Ala change at position 2, resulting in the production of a nonmyristoylated Nef protein. (B) Nef enhances HIV-1 infectivity in a single-round infection assay. Viruses produced by transfecting 293 cells with proviral DNA constructs were harvested by filtration and assayed for p24 content by ELISA. Normalized amounts of p24 were used to infect P4-2 cells, and the number of blue foci was scored after staining with X-Gal 2 days later. The results shown are the mean of triplicate infections, which commonly gave variabilities within 10%. Two representative experiments are shown.

were used to inoculate P4-2 cells, and their infectivity was expressed as the number of infectious units per nanogram of p24 antigen (Fig. 1B). In these experiments, a 4- to 40-fold greater infectivity was consistently observed for viruses producing a wild-type Nef protein than for those expressing truncated or deleted versions of the gene. Although *nef*-mutated virus was always significantly less infectious, the magnitude of its defect varied from experiment to experiment (compare Fig. 1B and Fig. 6A). The reasons for this variability, also reported by others (25), are unknown; in particular, no correlation with the cell type producing the virus was found. The Nef enhancement of HIV infectivity was dependent upon myristoylation, indicating that the protein must associate with the membrane to exert its positive influence (Fig. 1B). Of note, Western blot analyses of Nef-expressing cells previously demonstrated that nonmyristoylated Nef is stable (2). This was confirmed by pulse-chase analyses with transfected 293 cells (data not shown).

Positive effect of Nef is determined at the stage of virus particle formation and is independent of the HIV envelope. Next, we asked whether the defect exhibited by *nef*-mutated HIV-1 could be complemented in *trans* by providing the viral protein in either virus producer or target cells. P4-2 infections revealed that the infectivity of ΔNef virions was very significantly improved when harvested from 293 cells cotransfected with a *nef* expression vector (Fig. 2). This effect, which correlated with the amount of Nef provided, indicated that Nef exerts at least part of its influence at the stage of viral particle formation. To ask whether Nef could also complement the infectivity of a *nef*-defective virus when expressed in the target cell, P4-2 cell clones stably expressing *nef* were generated by retroviral transduction. Nef expression was verified by Western blotting (data not shown), and its consequences were first evaluated by flow cytometric measurements of cell surface CD4 levels (Fig. 3A). This revealed that, as expected, Nef induced CD4 downregulation in P4-2 cells. Additional control experiments demonstrated that these cells could complement the infectivity of *nef*-defective virions when they were used as producer cells (data not shown). Because of the decreased CD4 levels at the surface of these cells, infections were performed with Env-defective HIV-1 virions pseudotyped with the amphotropic MuLV envelope. In the parental P4-2 cells, *nef*-competent HIV(ampho) pseudotypes were 10- to 40-fold more infectious than their *nef*-defective counterparts. This difference was fully preserved when the *nef*-expressing P4-2 cells served as targets (Fig. 3B). This demonstrates that the Nef-mediated enhancement of HIV-1 infectivity is not restricted to a mode of

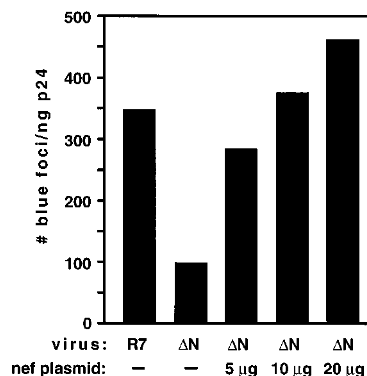

FIG. 2. Infectivity of *nef*-mutated virions can be complemented by coexpression of *nef* in the virus producer cell. Viruses were produced by cotransfection of 293 cells with ΔN proviral DNA and various amounts of CMX-Nef1, a CMV-based *nef* expression plasmid, and assayed by infection of P4-2 cells. The results shown are the average of triplicate infections and are representative of at least two independent experiments.

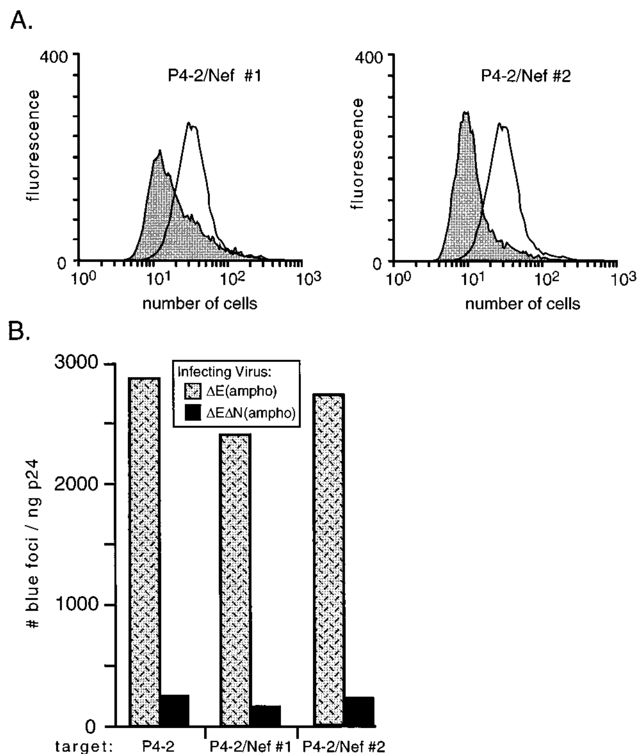

FIG. 3. *nef*-defective virions are not complemented by expression of *nef* in the target cell. Clones of P4-2 cells expressing *nef* were generated by infection with the retroviral vector LNef1.SHD packaged in CRIP cells. (A) FACS analysis comparing surface CD4 levels on *nef*-transduced P4-2 clones (shaded) and parental P4-2 cells (open). (B) Results of infectivity assays with wild-type and *nef*-pseudotyped virions to infect parental and *nef*-expressing P4-2 cells.

viral entry mediated by gp120 and CD4 and that it is entirely determined during the steps leading to viral particle formation.

Stimulation of HIV-1 infectivity by Nef in the absence of CD4 in both virus producer and target cells. Because the above-described experiments used viruses produced in fibroblasts, they confirmed an earlier report indicating that the effect of Nef on HIV-1 infectivity is not related to the presence of CD4 in cells releasing the virions (25). In agreement with these results, comparable phenotypes were observed with viruses harvested either from chronically infected CD4-positive T-cell lines or from fibroblasts cotransfected with a CD4 expression vector and the proviral plasmids (data not shown). To examine further whether the effect of Nef required the presence of CD4 in the virus target cell, the experiments were repeated with a CD4-negative version of the P4-2 cells, called Z-24 (5), as targets. Env-defective HIV-1 virions pseudotyped by the amphotropic MuLV envelope glycoproteins were used in this experiment to circumvent the absence of the normal HIV receptor at the surface of these cells. The magnitude of the Nef effect was as strong in the $CD4^-$ Z-24 cells as in the $CD4^+$ P4-2 cells (Fig. 4). The enhancement of HIV-1 infectivity by Nef is therefore completely independent of CD4 down-regulation by the viral protein.

Nef-defective virions enter cells as efficiently as the wild type. The data presented so far indicate that Nef manifests its effect on HIV-1 infectivity at a step of the virus life cycle following the production of virions but preceding the expression of the viral genome in target cells. In order to characterize further the nature of this effect, a p24 antigen uptake assay was used to evaluate the efficiency of viral entry (Fig. 5). In this

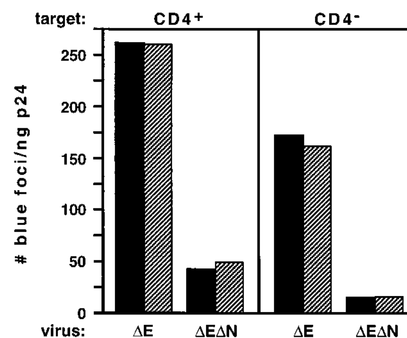

FIG. 4. Nef-dependent enhancement of HIV-1 infection is independent of the presence of CD4 in the target cell. Pseudotyped virions were produced by cotransfection of *env* or *env nef* mutant HIV-1 proviral constructs with SV-A-MLV-*env* into 293 cells, and supernatants were harvested and filtered through a 0.45-μm filter 48 h later. An aliquot of supernatant containing 1 ng of p24 antigen was used to infect P4-2 cells (Hela-CD4/LTR-lacZ). Cells were fixed and stained with X-Gal 2 days after infection, and the number of blue foci was counted. Bars represent separate infections with individual virus stocks.

assay, P4-2 cells were incubated with normalized amounts of purified virions either at 4°C, a temperature permissive for virus binding but not internalization, or at 37°C, a setting which allows both processes. After 2 h, unbound viruses were removed by extensive washing. Cells were then either lysed directly or first treated with trypsin to remove virus simply attached to the cell surface but not internalized. Trypsin treatment removed approximately 90% of the p24 antigen associated with the cells at 4°C, indicating that noninternalized virus could be efficiently eliminated by this procedure. Upon warming to 37°C, there was a significant increase in cell-associated p24 antigen. The two wild-type (R7 and NL) and two *nef*-defective (ΔN and ΔNX) viruses tested induced similar amounts of p24 antigen uptake. This was an accurate reflection of viral entry, since control experiments with both a macrophage-tropic virus, which cannot infect P4-2 cells (unpublished), and an Env-defective virus yielded values that were threefold lower (Fig. 4 and data not shown). Thus, the reduced infectivity of *nef*-defective virions is not due to inefficient viral entry.

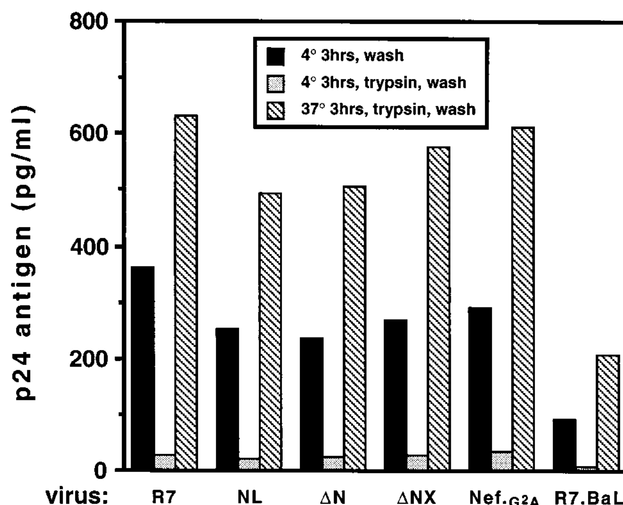

FIG. 5. *nef*-defective virions enter target cells as efficiently as the wild type. P4-2 cells (250,000) were inoculated with virions from 293 transfections (10 ng of p24), and the cultures were kept at 4 or 37°C. After 3 h, the cells were washed and lysed with 0.5% Triton X-100 or first trypsinized, washed, and then lysed. Lysates were analyzed for p24 content by ELISA.

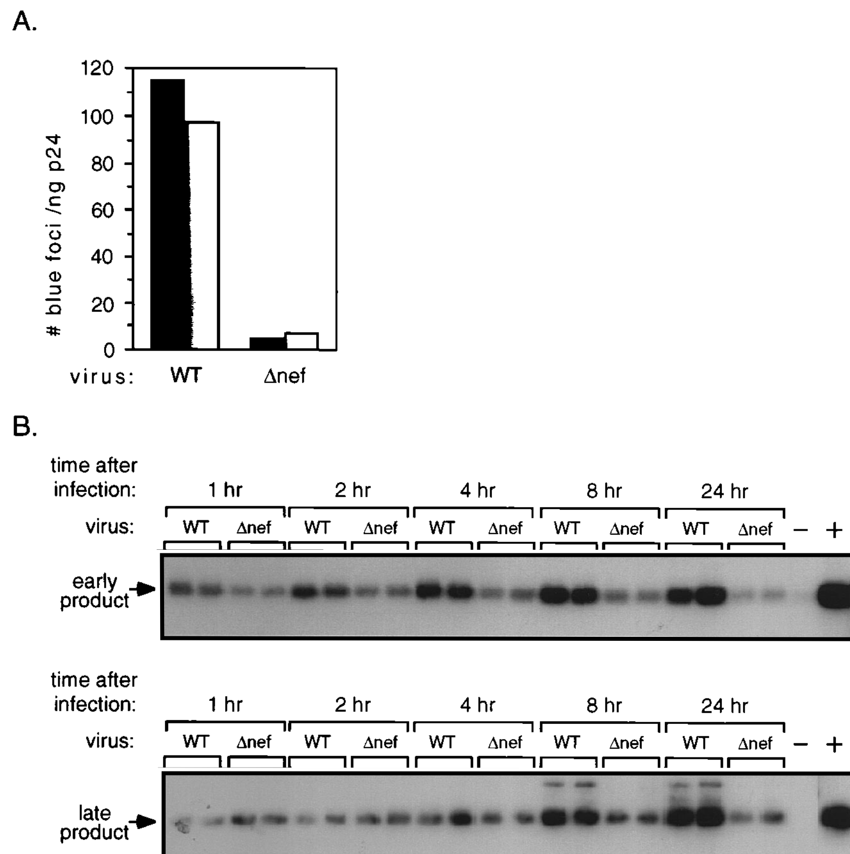

FIG. 6. ΔNef virions exhibit defective reverse transcription in P4-2 target cells. (A and B) Targets were $CD4^+$ HeLa cells. P4-2 cells (250,000) were inoculated in duplicate with 50 ng of p24-containing supernatant which had been treated with DNase I to eliminate carryover plasmid DNA. At the indicated times, the cells were harvested by trypsinization and washed, and lysates were prepared for PCR analysis. (A) Prior to lysis, a fraction of the cells were replated and stained with X-Gal 2 days later for an internal control for the ΔNef phenotype in this experiment. (B) PCR assays were performed for 30 cycles with primer pairs specific for minus-strand strong-stop DNA (top) or for transcripts generated after the second template switch (bottom). Products were analyzed by Southern blotting. The higher-molecular-weight bands generated by the primer pair specific for late products, in wild-type-infected cells, probably represent amplified two-LTR circular DNA.

This is consistent with the observed maintenance of the ΔNef infectivity phenotype when pseudotyped viruses were used.

Nef-mutated HIV-1 is defective in viral DNA synthesis in spite of normal levels of genomic RNA and reverse transcriptase activity. Because ΔNef and wild-type virions could enter target cells equally well, the rate and extent of reverse transcription performed by these viruses were examined in a single round of infection. P4-2 cells were infected with normalized amounts of purified *nef*-positive and *nef*-defective HIV-1(ampho) pseudotypes and harvested at various times after infection. The use of pseudotypes devoid of endogenous envelope prevented the spread of the infection beyond the first round, facilitating the interpretation of the results. Viral DNA synthesis was then analyzed by PCR with primers specific for the various intermediates and products of reverse transcription as previously described (36, 40). The accepted model of retroviral reverse transcription includes a succession of steps, beginning with the synthesis of DNA complementary to the U5 and R regions of the viral RNA (minus-stand strong-stop DNA), continuing after a first template switch with the elongation of this minus strand, with the final double-stranded product being generated after a second template switch. A first primer pair was thus selected to amplify the earliest DNA synthesized, the minus-strand strong-stop DNA, whereas a second pair was designed to detect the latest reverse transcripts, generated after the second jump. To measure in parallel the infectivity, an aliquot of the P4-2 cells was maintained in culture and stained with X-Gal 2 days later. In the experiment shown here, the ΔNef pseudotyped virus exhibited a 20-fold reduced titer relative to the *nef*-competent virus (Fig. 6A). The PCR analysis revealed that this decreased infectivity correlated with lower levels of viral DNA synthesis in cells infected with the *nef* mutant (Fig. 6B). Both primer pairs used in these procedures showed similar differences between the two types of viruses, indicating that Nef does not affect strand transfer, but instead augments the efficiency of reverse transcription.

These results were obtained in $CD4^+$ HeLa cells. To ask whether Nef also enhances HIV-1 reverse transcription in cells more relevant in vivo, PHA-activated PBL were used as targets. In these cells, the ΔNef virus was also markedly impaired for proviral DNA synthesis (Fig. 7). In addition, a similar phenotype was observed when CEM T-lymphoid cells were infected (not illustrated). In a series of independent experiments, the magnitude of the defect in DNA synthesis exhibited by ΔNef viruses closely paralleled the magnitude of their infectivity phenotype.

The reduced efficiency of viral DNA synthesis by the *nef*-defective virus suggested that it might contain decreased levels of genomic RNA or reverse transcriptase activity. However, slot blot analysis of purified virions revealed that Nef

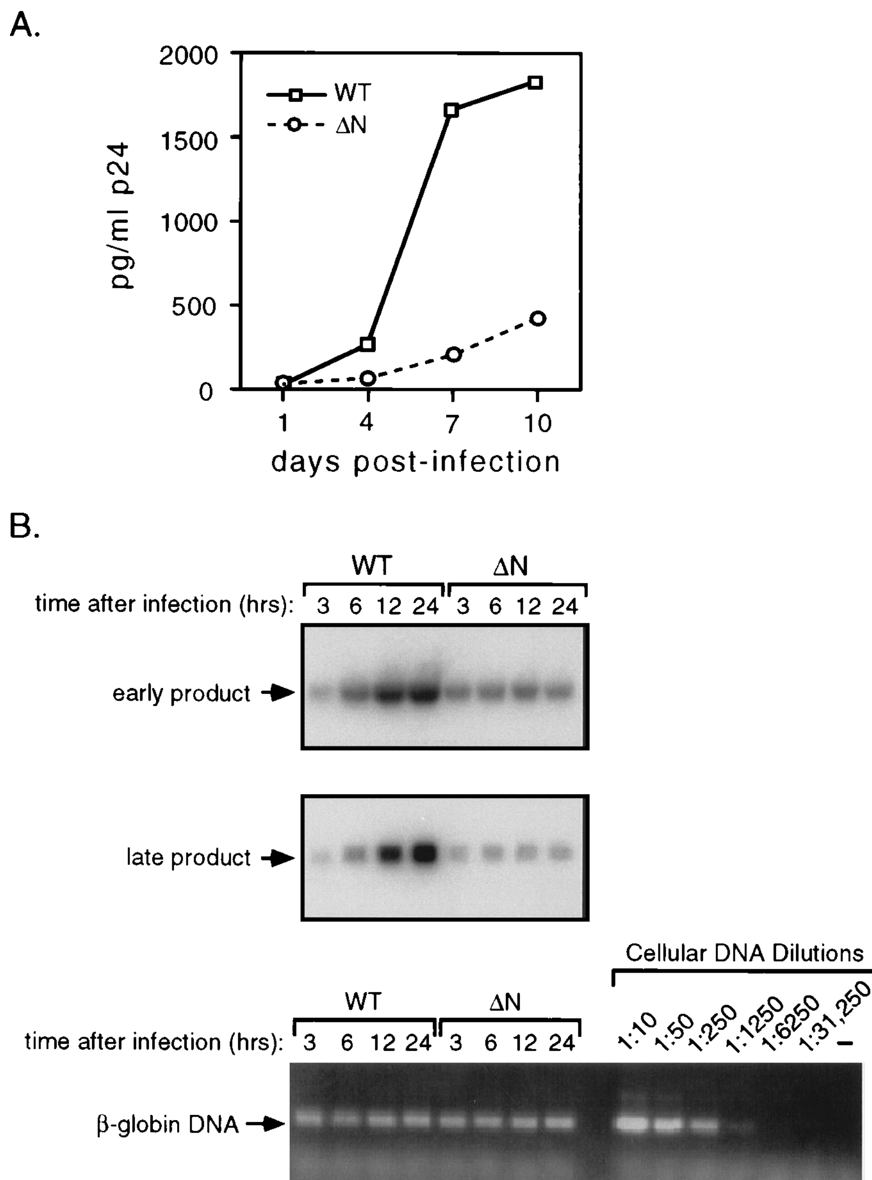

FIG. 7. Defective reverse transcription by ΔNef virions is also revealed in activated PBL. (A) One-week-old PHA-activated PBL were infected with equal amounts of wild-type and *nef*-defective viruses. Viral growth was monitored by p24 ELISA. Solid line, growth of R7; dashed line, growth of ΔN. (B) Upper two panels: PCR analysis of reverse-transcribed HIV-1 DNA was performed on total DNA from cell samples taken at the indicated time points, as described for Fig. 6B. Lower panel: PCR amplification of β-globin sequences with 1:250 dilutions of DNA samples for which results are shown in the upper two panels.

had no effect on the amounts of particle-associated genomic RNA (Fig. 8). Similarly, wild-type and *nef*-defective viruses contained comparable amounts of reverse transcriptase activity, whether it was measured with a polyribonucleotide (exo-RT assay) or the viral RNA itself (endo-RT assay) as the template (Fig. 9).

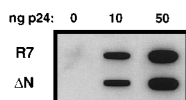

FIG. 8. *nef*-defective virions contain normal amounts of RNA. Supernatants from transfected 293 cells containing the indicated amounts of p24 antigen were treated with DNase I, and RNA was extracted with TriReagent. The RNA was again treated with DNase I and slot blotted onto nitrocellulose. RNA was detected by hybridization to a random-primed DNA probe generated from a restriction fragment containing the entire R7 provirus.

Taken together, these results indicate that Nef acts at the stage of viral particle formation to enhance the steps which follow HIV-1 internalization, facilitating the efficient completion of reverse transcription.

Positive effect of Nef on virus infectivity is observed with primary alleles of HIV-1 *nef* as well as with *nef* from HIV-2 and SIV. With a convenient *trans* complementation system in hand, the conservation of the Nef effect on virus infectivity was examined. First, a number of alleles originally isolated by PCR from peripheral blood leukocytes of HIV-1-infected individuals were tested. Those had been previously used by others to study Nef-mediated CD4 downregulation (24). A large fraction of these alleles were found to increase HIV-1 infectivity in a

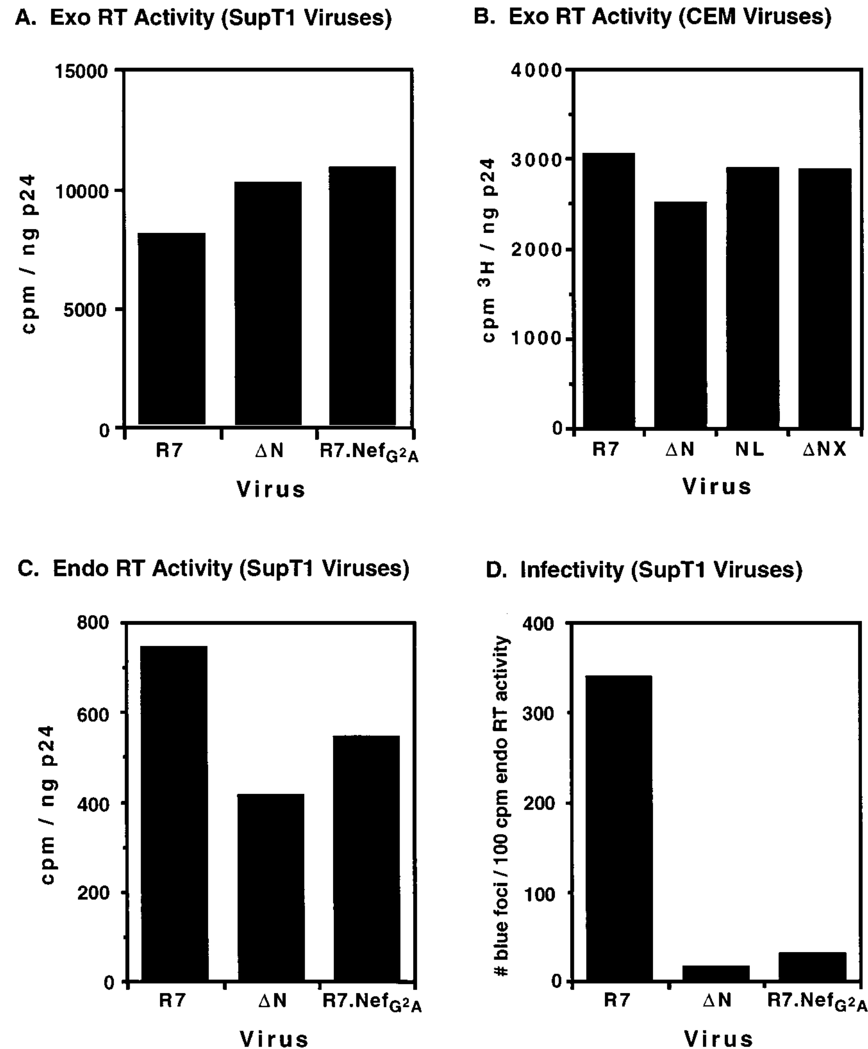

FIG. 9. Infectivity defect in *nef*-mutated HIV-1 is not due to a decreased level of reverse transcriptase (RT) activity. (A and B) Exogenous RT assays of virions produced from two different human T-cell lines fail to reveal a significant difference between wild-type and *nef*-mutated virions. The values shown are those obtained after normalization of the supernatants for p24 concentrations. The virions assayed were produced from SupT1 cells (A) and CEM cells (B). (C) Representative data from endogenous RT assays of the same supernatants shown in panel A. (D) Infectivity of SupT1 viruses is shown as a function of their endogenous RT activity. The 39-fold infectivity difference between the wild-type and *nef*-defective virions when expressed as a function of p24 antigen content (not illustrated) is not accounted for by the slight difference in RT activity between these samples.

single-round assay (Fig. 10A). This is as expected if a strong selective pressure is exerted in vivo to conserve this function of *nef.*

Consistent with this view, Nef proteins from HIV-2_{ST} and SIV_{MAC239} could increase the infectivity of *nef*-defective HIV-1, albeit to a lesser degree than Nef from HIV-1 (Fig. 10B). In a representative experiment, whereas HIV-1 Nef stimulated the infectiousness of a ΔNef virus by approximately 5-fold, Nef from SIV_{MAC239} and HIV-2_{ST} induced 2.5- and 2-fold effects, respectively. Such interstrain complementation suggests that elements necessary for this function of Nef are common to the three primate lentiviruses. Moreover, since SIV_{MAC239} is the molecular clone which was used to demonstrate the importance of Nef for AIDS pathogenesis in rhesus monkey (19), this result implies further that the stimulation of virus infectivity by Nef might play an important role in vivo.

DISCUSSION

Several recent studies have indicated that Nef promotes HIV-1 replication (7, 9, 25, 34, 35). The present work reveals that Nef appears to exert this positive influence by stimulating proviral DNA synthesis. Of note, this result is in agreement with that of another study showing that Nef enhances HIV-1 reverse transcription (33). Whether Nef also promotes steps that follow reverse transcription but precede viral gene expression, such as nuclear transport and integration, cannot be inferred from our analyses. However, other investigators found that the levels of circular and linear viral DNA were equally decreased in cells infected with *nef*-defective virus compared with the wild type, indicating that Nef does not affect viral nuclear import (33). Furthermore, the magnitude of the effect of Nef on reverse transcription in target cells closely paralleled that of its effect on viral infectivity, as scored in a single-round infection assay. Thus, it does not appear to be necessary to

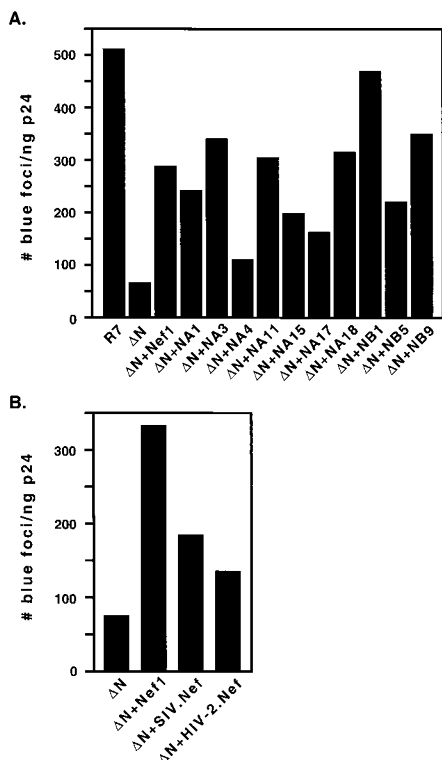

FIG. 10. Many primary alleles of *nef* as well as *nef* from SIV_{MAC239} and HIV-2 can complement the infectivity of ΔNef HIV-1. (A) PCR clones of *nef* alleles from two patients were transferred into the pCMX expression construct and cotransfected with ΔN HIV-1 proviral DNA into 293 cells. After normalizing for p24 content, viral supernatants were assayed on P4-2 cells. (B) SIV_{MAC239} and $HIV\text{-}2_{ST}$ *nef* alleles were assayed similarly for complementation of ΔN HIV-1 infectivity. The results shown are representative of three assays.

invoke a role for Nef following reverse transcription in order to explain the decreased infectivity of *nef*-defective virions.

The Nef effect is determined at the stage of virus particle formation, as the infectivity of ΔNef virus could be enhanced by providing the protein in virus producer but not target cells. Furthermore, the phenotype of *nef*-defective virions was maintained in HIV-1(ampho) pseudotypes, indicating that the presence of the HIV-1 envelope glycoproteins is not required for Nef activity. As predicted from this result, and confirming recently published data (26), Nef did not affect viral entry. Instead, ΔNef viruses exhibited a dramatic reduction in the efficiency of reverse transcription after they were internalized.

Immediately after infection, the amounts of viral DNA detectable in cells infected with wild-type and ΔNef viruses were roughly comparable, whereas a marked difference was noted at later time points (Fig. 6 and 7). This correlates the presence of equal amounts of partial reverse transcripts in wild-type and *nef*-mutated virions (not illustrated) and suggests that Nef stimulates viral DNA synthesis only after the virus enters target cells. At that stage, the synthesis of both minus-strand strong-stop DNA and full-length double-stranded DNA was impaired in the absence of Nef (Fig. 6 and 7). This indicates that Nef does not act on a step such as strand transfer, but rather augments the effectiveness of DNA synthesis itself. The mechanism of this effect remains obscure. Nef does not influence the efficiency of viral RNA packaging or the activity of virion-associated reverse transcriptase, whether the latter is tested through an exogenous or an endogenous reaction. Nef could thus play its role by influencing the processing of the internalized virus, perhaps facilitating its uncoating or its release from the plasma membrane, or the penetration of elements essential for reverse transcription, such as cations or nucleotides, into the core. Alternatively, Nef could increase the stability of the virus reverse transcription complex in the target cell.

It is interesting that the Nef-mediated enhancement of HIV-1 infectivity was best revealed by infecting resting T cells prior to stimulation (25, 34). On the basis of the present results, it is tempting to speculate that it is due to the fact that reverse transcription is already an inefficient process in quiescent T lymphocytes (41). This relative inefficiency may serve to amplify effects which are less pronounced in actively dividing cells. However, an additional effect of Nef on the activation of resting T cells cannot be excluded.

It is unclear why the significant effect of Nef on HIV-1 infectivity, as revealed in a single round of infection, does not translate into a more spectacular phenotype when growth curves of *nef*-defective virus are performed in T-lymphoid cells. However, the propagation of a virus within a cell culture involves parameters that are not all reflected in a single-round infection with cell-free virus. These parameters include cell-to-cell transmission of the virus, the increasing multiplicity of infection as the virus spreads in the culture, and possibly factors released by infected cells.

Does Nef act directly or indirectly? The infectivity of a virus expressing a nonmyristoylated form of the protein is as impaired as that of a *nef*-deleted virus. Nef must therefore associate with the plasma membrane to exert its effect. As HIV-1 assembly also takes place at the plasma membrane, this raises the possibility that some Nef might be incorporated into virions. Even though Nef was not detected in previous studies analyzing the composition of HIV-1 particles (12, 13), it might be present in amounts much lower than those of structural proteins such as Gag and Env. Alternatively, Nef might act in the virus producer cell to influence reverse transcription in an indirect manner, for instance, by modifying another viral or cellular protein involved in the early steps of the virus life cycle.

The results described here are reminiscent of those previously reported for Vif; like Nef, Vif acts at the stage of viral particle formation to increase the efficiency of HIV-1 proviral DNA synthesis (40). Nevertheless, several differences can be noted. First, the Vif requirement is strictly cell type specific (11, 40). For instance, *vif*-defective viruses produced from transfected fibroblasts or from a number of T-lymphoid cell lines exhibit a wild-type phenotype. In contrast, the Nef effect is more general and is visible with virus released by cells that do not reveal Vif action, such as COS, 293, Jurkat, and Sup-T1 cells (25, 34, 35, 42; this study). Second, when measured in the appropriate setting, the influence of Vif is far greater than that of Nef. ΔVif virions produced from PBL and primary macrophages are completely defective (11, 40), whereas *nef*-deleted viruses are able to replicate in these cells, albeit with a reduced efficiency compared with the wild type (25, 34; this study). This allows us to conclude that Nef does not act through Vif. Recently, Nef has been reported to bind to a serine-specific protein kinase present in many cell types (32). It is conceivable that the recruitment of this kinase, and perhaps its activation, are involved in the Nef-dependent enhancement of proviral DNA synthesis. Still, the target of this enzyme remains to be identified.

We find that the positive effect of Nef on HIV-1 reverse transcription is independent of the presence of CD4. This had been suggested by Miller et al., who showed that the infectivity of COS-produced HIV-1 is increased by Nef (25). Here, we demonstrate in addition that Nef also manifests its action when CD4 is absent from the virus target cells. These data still do not allow us to conclude formally that downregulation of CD4 and

stimulation of proviral DNA synthesis are distinct functions of Nef. However, recent studies indicate that a *nef* allele mutated in a conserved (PxxP) repeat is still competent for CD4 downmodulation yet fails to enhance HIV-1 replication in PBL infected prior to stimulation (31). It will be interesting to ask whether this latter phenotype corresponds to a defect in reverse transcription.

Nef proteins from a number of primary alleles of Nef as well as, to a lesser degree, from HIV-2_{ST} and SIV_{MAC239} can enhance the infectivity of a *nef*-deleted HIV-1 clone. This indicates that this function of Nef, like CD4 downregulation, is highly conserved and maintained by a strong selective pressure in vivo. In the future, *nef* mutants impaired in CD4 downregulation but not proviral DNA synthesis, and vice versa, should be tested in the SIV-rhesus monkey (19) and HIV/SCID-hu (17) model systems. This will allow the evaluation of the respective contributions of these two effects of Nef to AIDS pathogenesis.

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