The Importance of *nef* in the Induction of Human Immunodeficiency Virus Type 1 Replication from Primary Quiescent CD4 Lymphocytes

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Summary

The viral regulatory gene, *nef*, is unique to the human immunodeficiency viruses (HIV) and their related primate lentiviruses. Expression of the *nef* gene has been shown to be essential to the maintenance of high levels of virus replication and the development of pathogenesis in the animal model of simian immunodeficiency virus (SIV) infection. In contrast to this in vivo model, the use of standard T cell culture systems to study *nef* function in vitro has produced a spectrum of contradictory results, and has failed to demonstrate a significant positive influence of *nef* on viral life cycle. We have developed a cell model to study regulation of HIV-1 replication that we believe reflects more accurately virus–cell interactions as they occur in vivo. Our experimental system used acute virus infection of purified, quiescent CD4 lymphocytes and subsequent induction of viral replication through T cell activation. With this cell model, NL4-3 virus clones with open and mutated *nef* reading frames were compared for replication competence. The clones with *nef* mutations showed reproducible and significant reductions in both rates of growth and maximal titers achieved. The degree of reduced replication was dependent on initial virus inoculum and the timing of T cell activation. The influence of *nef* was highly significant for induction of virus replication from a latent state within resting CD4 cells. Its effect was less apparent for virus infection of fully proliferating CD4 cells. This study demonstrates that *nef* confers a positive growth advantage to HIV-1 that becomes readily discernable in the primary cell setting of virus induction through T cell activation. The experimental cell model, which we describe here, provides not only a means to study *nef* function in vitro, but also provides important clues to the function of *nef* in HIV infection in vivo.

The viral regulatory gene, *nef*, is unique to HIV types 1 and 2, and the related primate lentiviruses. Although its mechanism of action is not understood, *nef* gene function has been shown to be essential for the maintenance of high levels of virus replication and the development of disease pathogenesis in the in vivo model of simian immunodeficiency virus (SIVmac)[1] infection of the macaque monkey (1). In HIV-1 infection of humans, there appears to be selective pressure for retention of highly conserved sequences of *nef* and an open reading frame (ORF) (2, 3). In contrast to these data implying an important role for *nef* during in vivo infection, the use of standard cell culture systems to study *nef* function in vitro has produced a wide spectrum of contradictory results. Several studies have described modest to severe repression of LTR driven transcription by *nef* expression (4–7), whereas other reports have demonstrated a lack of effect on rates of viral transcription or replication (8, 9). More recent studies have shown small to moderate positive effects of *nef* on HIV replication in PBMC cultures that were selective for certain allelic sequences of *nef* and that were revealed in a specific low growth, viral genetic background (10, 11). The only point of consensus drawn from in vitro studies has been the finding that *nef* gene function is not required for HIV replication in cell culture (4, 5, 12, 13).

Because productive replication of HIV-1 is dependent on T cell activation and proliferation (14, 15), all of the above mentioned in vitro experimental systems have utilized CD4 T cell lines of malignant phenotype or fully proliferating cultures of PBL to study components of virus infection and replication. The vast majority of T lymphocytes in the natural in vivo setting, however, are quiescent nondividing cells with

[1] *Abbreviations used in this paper:* ORF, open reading frame; SIVmac, simian immunodeficiency virus; $TCID_{50}$, 50% tissue culture infectious dose.

only a very minor proportion undergoing cell division and clonal expansion at any given time (16). Study of HIV infection and control of virus replication in continuous cultures of proliferating CD4 cells may not represent the most relevant in vitro model. In fact, data from several different studies indicate that HIV found in CD4 lymphocytes of the peripheral circulation and lymph nodes is maintained predominantly in a nonproductive viral state either as unintegrated proviral DNA (17) or in stages of blocked RNA transcription (18–20). In addition, recent work to elucidate a function for the HIV-1 *nef* gene indicates that *nef* has the ability to perturb normal T cell functions. Several research groups have reported that expression of NEF protein in T cells causes a wide range of disturbance including downregulation of CD4 cell membrane expression (21, 22), suppression of IL-2 gene transcription (23), and inhibition of nuclear factor (NF)-κB induction (24). All of these affected events occur in the process of normal T cell stimulation. Potential interactions with regulatory components of HIV replication might be lost or minimized in a cellular setting of abnormal, maximal, and continued proliferation.

In an attempt to reflect more accurately virus–cell interactions as they occur in vivo, we have developed a cell model to study the regulation of HIV-1 replication using acute virus infection of purified, quiescent primary CD4 lymphocytes to establish a nonproductive infection, and subsequent induction of productive viral replication through mitogen activation of the T cell proliferation cascade. To determine the influence of *nef* on HIV-1 replication, we have constructed isogenic *nef*-mutated clones from the NL4-3 parental clone which contains ORFs for all the known genes of HIV-1, including *nef* (25). Using this experimental model, we report a significant positive effect of *nef* on HIV-1 replication that is demonstrated by 10–500-fold differences in replication, and that is apparent even with high viral inoculum. Our results show that *nef* has a major influence on the ability of HIV-1 to achieve maximal levels of replication in primary CD4 cells during cellular induction from a nonproductive viral state to a fully productive replication cycle.

Materials and Methods

Construction of Plasmid Clones with nef Gene Mutations. The HIV-1 plasmid clone pNL4-3, which contains ORFs for all the major viral genes (25), was used to derive *nef* gene mutants (Fig. 1). To construct the deletion mutation, Δ*nef*, the circular plasmid pNL4-3 (AIDS Research and Reference Reagent Program, National Institutes of Health, Bethesda, MD) which contains eight BglII cleavage sites was partially digested with diluted BglII such that 10–20% of the DNA was converted to full-length linear form. This DNA was completely digested at the unique EspI site and then treated with the large fragment of *Escherichia coli* DNA polymerase I and the four deoxynucleoside triphosphates. The resulting blunt-ended DNA was diluted to 100 ng/100 μl and treated with T4 DNA ligase to circularize the plasmid fragments. To linearize those DNA molecules which retained *nef* gene sequences between EspI and BglII, the circular DNA was digested with XhoI and used to transform *E. coli* DH5 (GIBCO BRL, Gaithersburg, MD). The desired plasmid DNA, in which the XhoI site was missing and the EspI site was fused to the BglII site at nucleotide position 9041, was identified by restriction enzyme mapping. A second *nef* mutant, *nef*/DS, was constructed using a PCR method (26) to introduce two point mutations resulting in stop signals at codons 37 and 61 in the NL4-3 *nef* reading frame. Three PCR amplifications were performed. Circular pNL4-3 was linearized with SalI digestion and used as template in the first two reactions. In the first amplification, the 5′ oligonucleotide primer contained sequence bases 8441-8466 (primer-1, GACAGATCCATTCGATTAGTGAACGG), and the 3′ primer sequence directed a G to A point mutation at position 8825 (primer-3, CATTCTTTCCCTTACAGCAGG<u>T</u>CATCC-AAT). Each primer (0.25 μg) was used in a DNA PCR of 100 μl containing 1.25 mM of each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, and 2.5 U Taq polymerase (Perkin Elmer Cetus, Los Angeles, CA). PCR was performed for 30 cycles, each of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. A second amplification was performed using a 5′ primer containing an A to T point mutation at position 8847 (primer-4, CCTGCT-GTAAGGGAAAGAATG<u>T</u>GACGAGCT) and a 3′ primer containing sequence bases 8966-8941 (primer-2, TGCTTCTAGCCA-GGCACAAGCAGCAT). The products from these two reactions were purified from a 3% agarose gel (NuSieve GTG; FMC Bioproducts, Rockland, ME) using a DNA purification system (Magic PCR Preps; Promega Corp., Madison, WI). The purified fragments were combined and used as template with primers 1 and 2 in the third PCR amplification. The resulting products were then digested with BamHI and XhoI for 3 h at 37°C. The digested DNA was then gel purified, ligated into BamHI/XhoI–cut, alkaline phosphatase–treated pNL4-3, and used to transform *E. coli* strain MC1061. Plasmid DNA was tested for the presence of the desired point mutations by screening with sequence-specific oligonucleotide probes. The presence of expected sequences in the mutant clones Δ*nef* and *nef*/DS was confirmed by DNA sequence analysis (Sequenase 2.0 kit, United States Biochemical Corp., Cleveland, OH). Plasmid preparations of pNL4-3, pΔ*nef*, and p*nef*/DS were grown in large scale and purified by banding on CsCl gradients.

Preparations of Infectious Virus Stocks. Stocks of infectious virus for clones NL4-3, Δ*nef*, and *nef*/DS were prepared by transfecting plasmid DNA into the CEM T lymphoblastoid cell line (27) with lipofectin (28). Virus preparations used in this study were derived from a single passage of each virus stock through the CEM line. A high titer stock of the LAI (LAV_{Bru}) strain of HIV-1 was prepared from infection of CEM cells. Titration of infectivity was performed by terminal dilution microassay with the MT-2 cell line (29), and the 50% tissue culture infectious dose ($TCID_{50}$) was calculated. Titration end points were determined by visible cytopathic effects, and confirmed by p24 antigen assay (Abbott Laboratories, North Chicago, IL).

Isolation of CD4 Lymphocytes from Peripheral Blood Samples. Peripheral blood was collected by standard venipuncture from healthy, HIV-seronegative donors. The mononuclear cell fraction was isolated by Ficoll-Hypaque density gradient and monocytes were removed from the cell preparation by ingestion of iron filings (30). The resulting lymphocyte-enriched preparation was processed for CD4 lymphocytes by negative selection with the panning method (31). Cell aliquots were incubated at 4°C for 30 min with a mixture of murine mAbs specific for three antigens: CD8 (OKT8; Ortho Diagnostic Systems, Raritan, NJ), CD16, and CD22 (Leu11b and Leu14; Becton Dickinson Immunocytometry Systems, San Jose, CA). The cells were washed with PBS, Dulbecco A and added to plastic petri dishes coated with goat antiserum to mouse Ig (IgG + IgM, Kirkegaard and Perry Laboratories, Gaithersburg, MD). After a 60-min incubation at 4°C, the nonadherent cells were re-

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moved, washed with PBS, and briefly reacted with diluted rabbit complement (Pel-Freeze Clinical Systems, Brown Deer, WI) at 37°C. The resulting cell preparations were at least 95% viable by trypan blue dye exclusion, and contained 90–98% $CD4^+$ lymphocytes as monitored by staining with FITC-conjugated mAb to CD4 (Leu3a+b, Becton Dickinson) and automated flow cytometry analysis on a cytofluorograf (50 H; Ortho Diagnostic Systems). To ensure that the separation procedure itself did not induce detectable cell activation, CD4 cells taken after separation were compared with the unfractionated mononuclear cells by direct-staining flow cytometry for membrane expression of antigens associated with cell activation: CD25, CD71, and DR (mAb anti-IL-2 receptor, antitransferrin receptor, anti-MHC II, respectively; Becton Dickinson).

Infection and Induction of Virus Replication from CD4 Lymphocytes. For infection, virus stocks were diluted in RPMI 1640 medium without serum and with 1 μg/ml polybrene to achieve equivalent infectivity titers among the specific virus clones used. Aliquots of 3–6 × 10^6 primary quiescent CD4 lymphocytes in 0.5–1.0 ml were infected with virus during 2–4 h of incubation at 37°C at multiplicities of infection (MOI) ranging from 0.5 to 0.005 $TCID_{50}$ per cell. After incubation, excess virus was removed by washing with PBS. Cell recovery was monitored by viable cell count with trypan blue dye exclusion. The infected cells were cultured in RPMI 1640 medium with 5% human AB serum (Gemini Bio-Products, Calabasas, CA) at 4–5 × 10^6 cells per 1 ml in 15-ml culture tubes (Corning Plastics, Corning, NY). At various times after infection, cell viability was determined; cells were distributed into round-bottom microtiter plates (Linbro Plastics; Flow Laboratories, McLean, VA) at 10^5 cells per 200 μl medium per well in triplicate; and 3 μg/ml PHA (Sigma Chemical Co., St. Louis, MO) plus 5 U/ml rIL-2 (DuPont, NEN Research Products, Boston, MA) were added to induce cell proliferation and virus replication.

Unstimulated, infected CD4 cells and uninfected cell controls were included for culture. For comparison, virus infection and replication in proliferating, prestimulated CD4 cells was determined in parallel in each experiment by infecting aliquots of primary CD4 lymphocytes that had been stimulated 3–4 d earlier with PHA plus rIL-2. In selected experiments, virus infection and replication was evaluated in a similar manner using the CEM T cell line, grown in RPMI medium with 10% FCS.

To minimize experimental variation due to individual cell donor differences, a given experimental design was repeated two to four times using primary cells from different donors. Each figure depicts the averaged, combined data obtained from two repeat experiments with different cell donors.

Quantitation of Virus Replication. Virus production was assessed by measuring soluble p24 antigen released into culture supernatants. Samples of 100 μl were taken from each microculture well at the indicated times, and stored at −70°C until completion of the experiment. Batch assays for p24 antigen quantitation were performed by the ELISA method (Abbott Laboratories). Supernatants from uninfected cell cultures were included as controls.

Each p24 value represents the average of triplicate culture wells. Initially, replicate sample aliquots were assayed individually for p24 content and a mean value was derived mathematically. In subsequent experiments, a different method was used to achieve an equivalent value; equal volumes from the replicate samples were mixed together and the combined sample was then assayed for average p24 content.

Determination of Cell Proliferation. To assess levels of cell activation and proliferation generated by mitogen stimulation, microcultures were pulsed with [^{3}H]thymidine (^{3}H-TdR, 2 μCi/well, Du Pont NEN Research Products) for 18–24 h on day 4 after the addition of PHA and rIL-2. Cell cultures were harvested onto glass fiber filters with a multiple automated sample harvester (PHD Cambridge Technology, Watertown, MA) and the amount of isotope incorporation was determined by liquid scintillation β-emission.

Results

HIV-1 Infection and Replication in Primary CD4 Lymphocytes. In an attempt to reflect more accurately virus–cell interactions as they occur in vivo, we chose to use an experimental culture system of primary T lymphocytes and virus infection initiated in a quiescent cell state. CD4 lymphocytes were purified with a negative selection technique from the peripheral blood of healthy, HIV-seronegative donors. The LAI strain (LAV_{BRU}; 32) and the NL4-3 clone (25) of HIV-1 were used to infect resting cells at a MOI of 0.5 $TCID_{50}$ per cell. At various times after infection, PHA mitogen plus recombinant IL-2 (rIL-2) were added to microcultures to induce lymphocyte proliferation and productive virus replication. At each time point tested, up to 10 d after infection, mitogen stimulation induced high levels of HIV-1 replication with 300–700-fold increases in soluble p24 antigen production (Fig. 2 *A*, *top*). Cells infected with either the LAI strain or the NL4-3 clone achieved peak levels of virus production 7–10 d after each mitogen induction that equaled or exceeded the levels of virus produced during parallel infections of fully proliferating CD4 lymphocytes, stimulated 3 d before infection (Fig. 2 *B*, *top*). These results were in general agreement with previously reported studies (33, 34) and showed that HIV-1 can readily infect quiescent CD4 cells, establish a stable nonproductive viral state, and be induced subsequently into productive replication through T cell activation. However, this in vitro model represented a unique departure from other experimental systems of primary T cells (15, 33, 34), because it utilized isolated CD4 lymphocytes and relatively high virus MOI to

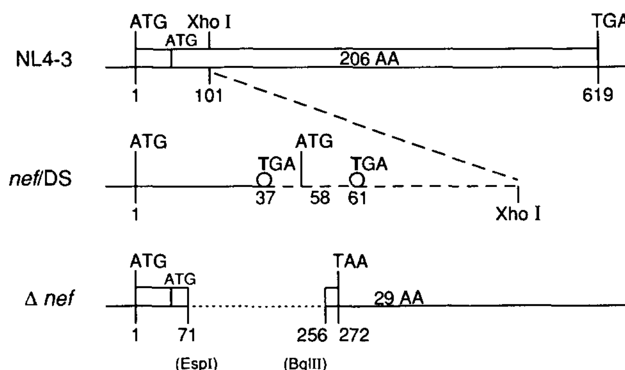

Figure 1. *Nef* mutations constructed from the NL4-3 clone. Schematic representation of the *nef* gene reading frame of NL4-3 and derived mutations. The translated sequences and the resulting AA length of the protein product are depicted by open boxes. Base 1 is the translation initiation codon which corresponds to sequence base 8787 for NL4-3 (46). Clone *nef*/DS is a PCR-derived mutant containing point mutations that create two premature termination codons, 3′ of each initiator ATG. Clone Δ*nef* is a deletion mutant that lacks 185 bases between the intragenic EspI and BglII restriction sites, resulting in a premature termination signal at nucleotide 272 with an expected product of 29AA. (See Materials and Methods.)

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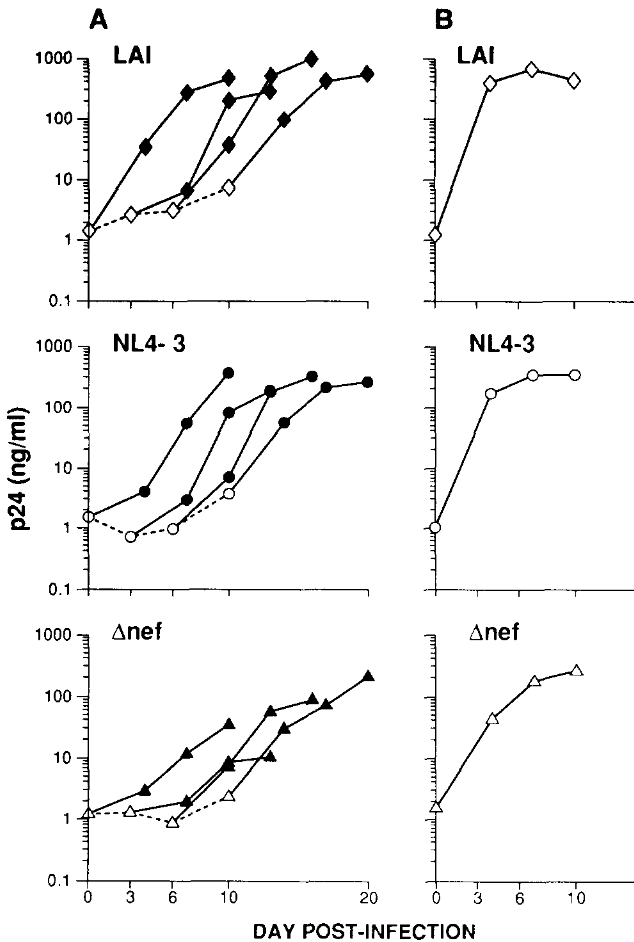

Figure 2. Isogenic *nef* deletion clone with reduced capacity to replicate. (*A*) Primary, quiescent CD4 cells were infected on day 0 with strain LAI (♦) or isogenic clones NL4-3 (●) and Δ*nef* (Δ) at MOI = 0.5 $TCID_{50}$/cell. PHA and rIL-2 were added on days 0, 3, 6, and 10 after injection to induce virus replication. Microculture supernatants were tested for p24 production on days 4, 7, and 10 after mitogen induction. (*B*) Virus infection and replication was determined in parallel cultures of proliferating primary CD4 cells, stimulated 3 d before infection with PHA plus rIL-2. Levels of p24 expressed as nanograms per milliliter on a $\log_{10}$ scale. Depicted are the combined, averaged results from two repeated experiments with different cell donors.

provide a means to evaluate HIV-1 replication early in the growth cycle and in the absence of potential factors contributed by other interacting cells and cytokines.

Influence of the nef Gene on Virus Replication. Earlier studies from our laboratory and elsewhere have demonstrated that *nef* gene transcription is an early and predominant event in HIV-1 infection of proliferating T cells and monocyte-derived macrophages (35–37). Some of our initial studies with LAI infection of quiescent CD4 lymphocytes also indicated that *nef* gene transcription was the earliest detectable event after induction of virus replication (38, and Spina, C. A., manuscript in preparation). To investigate the impact of *nef* gene expression on HIV-1 infection of primary CD4 lymphocytes, a *nef* deletion clone was constructed from the pNL4-3 plasmid (Fig. 1). This mutant, Δ*nef*, contained a 185 base deletion within the unique *nef* reading frame that did not extend 5′ into *env* or 3′ into the polypurine tract of the LTR. The replication competence of the Δ*nef* mutant was compared with that of the isogenic NL4-3 parental clone by infection of resting-state CD4 cells (MOI = 0.5) and subsequent mitogen stimulation of virus replication, as described above. The Δ*nef* mutant showed severely reduced capacity to replicate (10–20-fold) after mitogen induction of the infected primary cells, especially at days 0 and 3 after infection (Fig. 2 *A*, *bottom*). When mitogen stimulation was applied at later time points, 6–10 d after infection, higher peak levels of Δ*nef* virus production were eventually attained. But, the growth kinetics of the *nef*-mutated clone still lagged behind that of the parental NL4-3 clone, and p24 antigen levels remained approximately threefold below those of NL4-3 until 10 d after mitogen induction. In contrast with the significant growth disadvantage seen when T cell activation was initiated after infection, the Δ*nef* mutant was capable of replicating to high titers, rapidly matching p24 antigen levels achieved by NL4-3, when virus infection occurred in cultures of prestimulated, proliferating CD4 lymphocytes (Fig. 2 *B*, *bottom*). Even in this cellular setting advantageous to virus replication, the *nef*-mutated clone demonstrated a reproducible two to threefold growth disadvantage at the earliest time point after infection.

The first series of experiments to test the influence of *nef* on virus replication were done with a relatively high virus inoculum of 0.5 $TCID_{50}$ per cell. Additional experiments were done comparing NL4-3 and Δ*nef* replication kinetics at three different MOI, spanning a 100-fold range from 0.005 to 0.5 $TCID_{50}$ per cell. Based on the results from earlier experiments, three time points of 0, 4, and 8 d after infection were chosen for PHA mitogen induction of virus replication. The NL4-3 clone replicated to high titers, and reached similar peak levels of p24 production at each MOI and all post-infection time points tested (Fig. 3 *A*). In contrast, the replication capacity of the Δ*nef* mutant was dependent on high virus dose and the timing of T cell activation after infection (Fig. 3 *A*). At the lowest MOI of 0.005, induced replication of Δ*nef* at 0 and 4 d after infection was 10–100-fold less than the isogenic NL4-3 clone. If infection of the resting CD4 cell cultures was allowed to proceed for 8 d before the induction of T cell stimulation, the *nef*-mutated clone was able to attain peak levels of virus production more similar to NL4-3, but still showed 3–10-fold reduction in the amounts of virus produced during the early cycles of replication. In comparison, infection of prestimulated CD4 lymphocyte cultures revealed less of a *nef* gene influence and minimized the effect of virus dose (Fig. 3 *B*). Taken together, the results from these experiments demonstrated a consistently positive influence of the NL4-3 *nef* gene on HIV-1 replication in primary CD4 lymphocytes. The effect of *nef* was significantly greater, and dose related, for virus replication induced from infected, resting CD4 cells in comparison with virus replication initiated in proliferating cultures of CD4 cells. The data indicate that *nef* gene function may confer a selective growth advantage to virus that infects quiescent CD4 cells and is maintained in a latent proviral form for some time before cellular activation, which induces productive viral replication.

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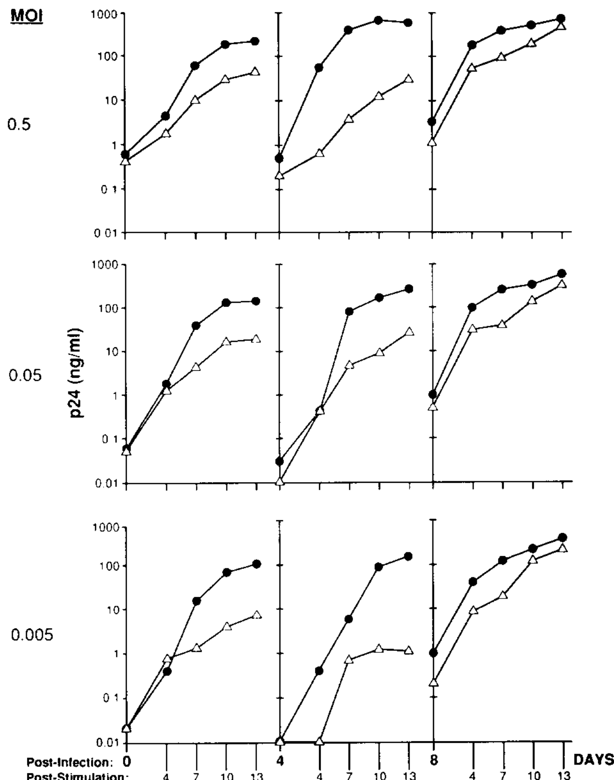

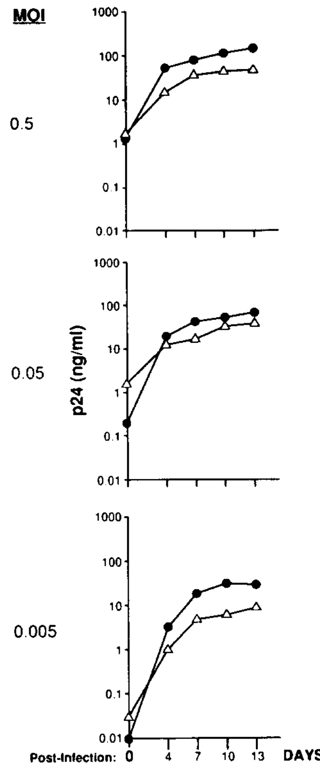

Figure 3. Contribution of virus dose on *nef* effects. (*A*) Resting CD4 cells were infected on day 0 with isogenic clones NL4-3 (●) and Δ*nef* (Δ) at three different MOI of 0.5, 0.05, and 0.005 $TCID_{50}$/cell. On days 0, 4, and 8 after infection, PHA plus rIL-2 were added and supernatant samples were assayed for p24 production on days 4, 7, 10, and 13 after mitogen induction. (*B*) Virus infection and replication was also determined in proliferating primary CD4 cells, stimulated 4 d earlier with PHA plus rIL-2. Levels of p24 are plotted on a $\log_{10}$ scale. Depicted are the averaged results from two repeated experiments with different cell donors.

Comparison of nef Mutants. A plasmid clone with point mutations in the NL4-3 *nef* ORF was constructed to contain two sequential stop codons within the NH_2 terminus (Fig. 1). This mutant, *nef*/DS, which contained minor base alterations from the parental genomic sequence, was used in further experiments to confirm results obtained with the deletion mutant Δ*nef*. As before, virus infection (MOI = 0.1) was initiated in resting CD4 cells, and productive virus replication was induced with mitogen stimulation at 0, 4, and 8 d after infection. The pattern of replication for the *nef*/DS mutant clone was similar to that of the Δ*nef* clone, but showed even greater reductions in growth capacity (Fig. 4 *A*). At all time points of mitogen induction, the *nef*/DS mutant produced p24 levels that were 20–500-fold less than those of the parental NL4-3 clone. The Δ*nef* mutant replicated 5–20-fold below the rate of NL4-3 at 0–4 d after infection, and 3–7-fold below, at 8 d after infection. The reason for these observed differences in replication levels between the two *nef* mutant clones is unclear. Although the Δ*nef* construct is predicted to express a greatly truncated, but myristoylated, fragment of the NEF protein, which might retain some function, we have not been able to detect such a protein product in these experiments. Analysis of infected cell lysates using Western blot with a NEF-specific rabbit antibody showed that the 27-kD product of *nef* could be detected after 4–7 d of mitogen stimulation in cells infected with NL4-3, but not in cells infected with Δ*nef* or *nef*/DS (data not shown). When virus replication was compared in proliferating cell cultures of prestimulated CD4 lymphocytes (Fig. 4 *B*) and the CEM T cell line (Fig. 4 *C*), the *nef*-mutated clones demonstrated better replication rates which were similar to those of NL4-3. However, the *nef*/DS mutant continued to show a relative disadvantage in replication in comparison with both NL4-3 and Δ*nef* clones. The degree of clonal difference seen with the CEM cell line (Fig. 4 *C*) was greatly minimized with the addition of several repeat experiments, and the relative growth pattern resembled more closely that seen with prestimulated CD4 lymphocytes (Fig. 4 *B*). These experiments confirmed that *nef* confers a positive viral growth advantage that is greatly magnified in primary T cells and during events of virus transition from a nonproductive state to a fully productive state.

Capacity of Infected CD4 Cells to Respond to T Cell Stimulation. Because our experimental model uses T cell stimulation of primary CD4 cells to induce productive HIV-1 replication, the level of virus replication should be directly related to the ability of the infected cells to respond to this stimulus and proliferate. The concentration of PHA mitogen used in this study was determined by prior titration to induce optimal proliferation in primary cultures of isolated CD4 lymphocytes, but it was not known if similar levels of cell activation would be achieved in the infected cell populations. To examine whether the observed growth deficiency of the *nef*-mutated clones was associated with an abnormal T cell response to mitogen or increased cytopathology, cell proliferation was monitored by measuring DNA synthesis with uptake of

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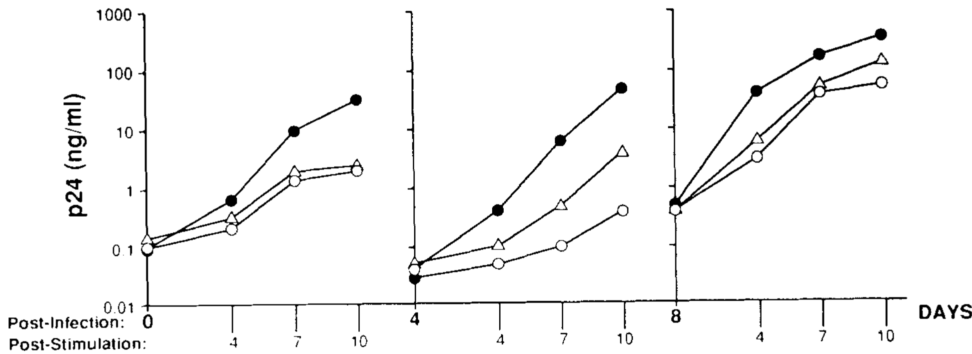

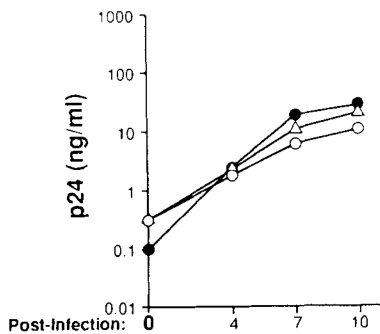

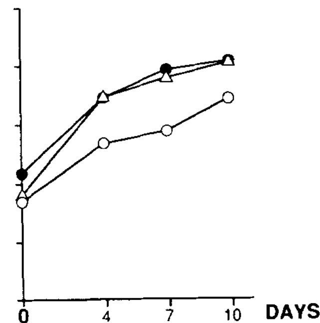

Figure 4. Comparison of *nef* mutants containing deletion or point mutations. (*A*) Resting CD4 cells were infected on day 0 with isogenic clones NL4-3 (●), Δ*nef* (Δ), and *nef*/DS (○) at MOI of 0.1 $TCID_{50}$/cell. On days 0, 4, and 8 after infection, PHA plus rIL-2 were added and supernatant samples were tested for p24 production on the days indicated. Virus infection and replication were compared in (*B*) proliferating primary CD4 cells, stimulated 4 d earlier, and (*C*) the CEM line. Depicted are the averaged results from two repeated experiments with different cell donors.

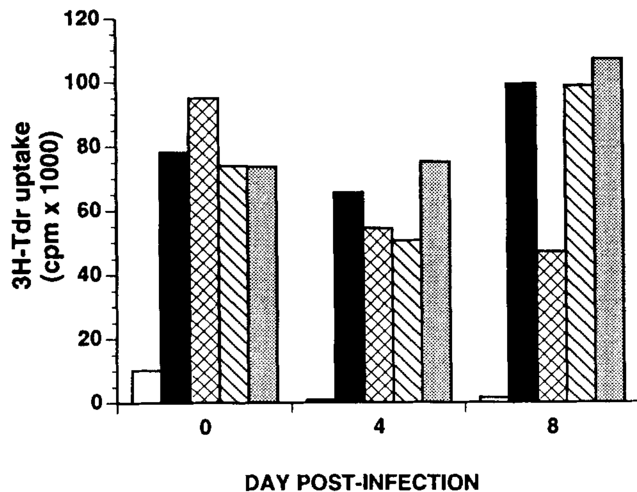

Figure 5. Proliferation capacity of infected primary CD4 cells. Microcultures of HIV-infected and uninfected cells, taken from the experiment described in Fig. 4, were assayed for levels of DNA synthesis by [^{3}H]TdR uptake. Cells were stimulated with PHA plus rIL-2 on days 0, 4, and 8 after infection. Cell proliferation was measured on day 4 after mitogen stimulation, and expressed as cpm (× 1,000). Each value represents the mean of triplicate culture wells. The graph depicts the averaged results from two repeated experiments. (□) Unstimulated CC; (■) stimulated CC; (▧) stimulated/NL4-3; (▨) stimulated/Δ*nef*; and (▤) stimulated/*nef*-DS.

[^{3}H]thymidine. In the experiment described above, comparing replication of the Δ*nef* and *nef*/DS mutants with NL4-3, the infected cell microcultures were analyzed for cell proliferation after each cycle of mitogen induction on days 0, 4, and 8 after infection. 4 d after the addition of PHA and rIL-2, the microwell cultures were pulse labeled for 18–24 h. The peak levels of DNA synthesis in CD4 cells infected with the *nef*-mutated clones were similar to those of the uninfected cell control at all three time points examined (Fig. 5). In addition, the induced proliferation of cells infected with NL4-3 was not different from the levels of proliferation seen in cells infected with the mutant clones, except at day 8 after infection. At day 8, cells infected with NL4-3 exhibited approximately half the level of DNA synthesis detected in cells infected with either *nef* mutant or the uninfected cell control. This comparative decrease in cell proliferation was probably due to the cytopathology associated with high levels of parental NL4-3 replication. Results from these experiments demonstrated that the reduced capacity of the *nef* mutant clones to replicate in primary CD4 lymphocytes was not associated with an inhibition of induced cell proliferation, or with a reduced ability of these infected cells to achieve peak levels of DNA synthesis.

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Discussion

We have developed an in vitro cell model to study the regulation of HIV-1 replication based on our current understanding of the natural in vivo life cycle of the CD4 lymphocyte, the major target of HIV-1 infection. We have infected primary CD4 lymphocytes in a quiescent state, and induced productive virus replication by activation of the T cell response at various and extended time periods after infection. Data generated by this experimental approach were compared to data obtained from parallel infections of fully proliferating CD4 cells. Our premise has been bolstered by a series of both recent and prior reports which indicate that HIV-1 infection in the majority of CD4 lymphocytes, in both the peripheral circulation (17, 18) and in lymph nodes (19, 20), is maintained in a nonproductive, or semiblocked proviral state. Using our in vitro cell model to study the influence of the HIV-1 *nef* gene on viral replication, we have found that *nef* confers a positive growth advantage to HIV that is highly significant for viral replication induced by T cell activation, but much less so for virus infection of proliferating cells.

The *nef* gene of NL4-3 was used as the functional ORF prototype to derive mutated proviral sequences. Other investigators have demonstrated that expression of this particular NL4-3 *nef* gene is associated with disruption of normal T cell functions, causing decreased cell membrane expression of the CD4 molecule (21), suppression of IL-2 gene transcription (23), inhibition of NF-κB induction (24), and abnormal depletion of thymic T cells in transgenic mice (39). For our studies of virus replication, isogenic *nef* mutants were constructed from NL4-3 to contain either a deletion or point mutation. By using purified, primary CD4 lymphocytes as targets for infection and relatively high virus inoculum, we were able to examine early growth cycle events and compare kinetics of replication, as well as, peak levels of virus production. The virus clones with *nef* mutations displayed reproducible and significant reductions in both growth rate and maximal titers achieved. The magnitude of these reductions was dependent on initial virus inoculum and the timing of T cell activation.

We do not understand fully the observed phenomenon of relative enhancement of virus replication when mitogen induction is applied to infected, quiescent CD4 cells after an extended time (8 d). This phenomenon is very consistent, however, and has been found with several different parental clones of HIV-1 and additional, nonisogenic mutants of *nef* (data not shown). We do not believe that it is related to an overt change in the resting state of the isolated CD4 cells over time in culture. Both uninfected and infected cultures of resting cells have been monitored for signs of T cell activation over 8–10 d in culture. No changes have been detected in basal levels of DNA synthesis, shifts in cell cycle from G_0/G_1 to G_2/S, or expression of membrane markers of IL-2 receptor, transferrin receptor, MHC class II, and CD45RO (data not shown). Rather than a change in cell state, a subtle change in the predominant state of the virus has been observed. Data from another series of experiments employing PCR analysis suggest that full-length proviral DNA is detected and accumulates slowly after 5–6 d of infection in primary cultures of resting CD4 lymphocytes (40, and Spina, C. A., manuscript in preparation). The implications of these findings for our study reported here are unclear, but may relate to the stability of the proviral form and/or the complicated dynamics of induced virus replication in primary CD4 cells. After mitogen stimulation, two parallel series of events occur: the progression of the HIV-1 life cycle to transcription and translation of regulatory genes, and the progressive induction of T cell regulatory factors required to move the cell into DNA synthesis and mitosis. The initiation and elongation of viral RNA transcripts (41, 42), as well as the switch from regulatory to structural gene transcription (43–45), is highly dependent on interactions between viral regulatory proteins and induced cellular factors. It is likely that the protein product of *nef* also interacts directly or indirectly with cellular factors, and this interaction would be dependent on the presence and abundance of both *nef* and cellular products during the sequence of events following T cell activation.

Our data indicate that *nef* has a predominant, selective effect on HIV-1 replication that is relevant to the biological setting of primary T cells undergoing transition from a resting to an activated state. When virus infection occurs in prestimulated, highly proliferating cells, the effect of *nef* is greatly diminished. This suggests that the presence and/or abundance of putative cellular factors, found in a state of maximal proliferation, reduces the requirement for *nef* in the virus life cycle. Other studies, which have shown a modest, positive influence of *nef* on virus replication in cultures of proliferating PBMC and some T cell lines, have used low virus inoculum and *nef* genes from primary clinical isolates (10) and the Eli clone (11) expressed in the genetic background of HXB2, a low growth HIV-1 phenotype that contains additional gene defects in *vpr* and *vpu* (46). Because it has been shown that the *vpu* gene product also interacts to downregulate CD4 expression and allow efficient virus production, it has been unclear whether the effects of *nef* on HIV replication in vitro could be revealed only in the particular genetic context of defective *vpu* function. Our study has used the high growth virus phenotype of NL4-3, which has ORF for all major genes, and derived isogenic *nef* mutants in experiments with a range of high to moderate MOI. Our results indeed confirm that *nef* exerts a positive growth influence on HIV-1 replication, but also demonstrate a significantly greater impact of *nef* that is selective for T cell induction of virus replication from a nonproductive state into a productive cycle. The experimental cell model that we describe here provides not only a means to study *nef* function in vitro, but also provides important clues to the function of *nef* in HIV infection in vivo.

It has been difficult to reconcile the divergent findings for the influence of the *nef* gene between in vitro culture studies and the in vivo study of SIV infection of the macaque (1). Minimal to no effects have been found for *nef* in HIV-1 infection of T cell cultures, whereas *nef* has been shown to be essential for high rates of virus replication and associated disease pathogenesis in the SIV animal model. Our study demon-

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strates a positive contribution of *nef* to the HIV-1 life cycle in primary CD4 cells that is consistent with findings in the animal model of SIV infection. We propose that *nef* confers a significant growth advantage to HIV-1 in vivo by providing a maximal virus burst from infected CD4 cells early after induction by T cell activation, thus ensuring high virus production from an infected cell before it is recognized and removed by immune competent cells. We hypothesize that in the presence of a functional immune system, *nef* is required for the rapid spread and seeding of virus in host cells during initial infection and for the sustained spread of virus within the CD4 lymphocyte population, especially during earlier phases of HIV-1 disease.

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