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The *nef* Gene of SIVmac239 Is Necessary for Efficient Growth in H9 Cells

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Abstract
AIDS viruses require an intact functional *nef* gene in order to inducedisease. The nonpathogenic molecular cloned virus SIVmac239*nef*-deletion encodes a truncated *nef* gene. This attenuated reading frame is expressed both in vitro and in a virus-infected animal in vivo. Encoding the first 58 amino acids of Nef, the reading frame retained its ability to down-modulate CD4 from the surface of T cells. CD4-down-modulated stable cell lines expressing full-length and truncated *nef* genes were significantly less infected by SIV. SIVmac239*nef*-open and SIVmac*nef*-deletion encoding a truncated *nef* clearly differed in replication kinetics in H9 cells and H9-derived cell lines. SIVmac239*nef*-deletion replication was delayed in H9.

nef, an acronym for negative factor, was named based on its ability to moderately down-regulate transcription from the long terminal repeat (LTR) [1, 31]. Although essential for pathogenesis in vivo [19, 36], *nef* seems non-essential in vitro [23, 42]. The *nef* gene is conserved in all the primate lentiviruses including all SIVs, HIV-1 and HIV-2 [28]. Its product modulates the induction of NFκβ and Sp1 [29, 30]. *nef* plays a role in facilitating the replication of virus in quiescent cells [27, 41], which may be accomplished through interaction with cellular kinases [14, 37, 39]. Guy et al. [17] and later Garcia et al. [15] showed that HIV-1 *nef* can down-modulate CD4 from the surface of lymphoid cells. The CD4 molecule is an integral membrane protein involved in the T-cell activation pathway [40], responding to the presentation of antigen and also serving as a receptor for primate lentiviruses [11]. Skowronski et al. [40] propose that *nef* interferes with cellular signaling by preventing appropriate and facilitating inappropriate T-cell activation.

Down-regulation of CD4 on the cell surface does not result from a change in CD4 mRNA or protein synthesis, since steady-state levels of the protein and mRNA are maintained [16]. The down-modulation is species-independent: HIV-1 *nef* can down-modulate rhesus CD4, SIV-*nef* can down-modulate human CD4 [7, 16 and this report] and also down-modulate mouse CD4 [Salkowitz and Kestler, unpubl.]. Presumably, *nef* induces rapid CD4 endocytosis and lysosomal degradation of the internalized protein [2, 34]. Benson et al. [7] postulated that *nef* facilitates assembly of retroviral particles by preventing intracellular CD4-gp 160 interaction early in infection, when gp 160 and CD4 are present in similar concentrations. HIV-1 *nef* genes obtained from primary patient samples down-modulate CD4 [5]. However, not all HIV-

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nef alleles are competent in down-modulating CD4 [25]. In this report, we examine the ability of SIV*nef* alleles with very different pathogenic potentials to down-modulate CD4 surface expression.

Given the in vivo effect of *nef,* a role for this gene in determinating the efficiency of virus production is likely. Nef is required for efficient SIV and HIV replication in quiescent lymphocytes [14, 27, 41]. The interaction of *nef* with cellular kinases such as Hck may mitigate that effect [37, 39]. We recently characterized the virus from a long-term AIDS nonprogressor and found it mutant in a determinant of *nef* necessary for Hck interaction. Additionally, virus encoding this allele was growth attenuated in the quiescent cell assay. *nef* from the subject's virus was normal in its ability to down-modulate CD4 [Salkowitz et al., unpubl.]. An in vitro replication assay appears to correlate with attenuated virus. Virus from long-term AIDS nonprogressors often cannot replicate in the H9 cell line [6, 32]. Premkumar et al. [32] have recently shown that *nef* was necessary for efficient H9 virus replication. HIV containing a full-length *nef* from a long-term AIDS nonprogressor has no ability to replicate in H9 cells, yet was competent in the ability to down-modulate CD4 [Salkowitz et al., unpubl.].

Materials and Methods

Cell Lines and Culture Conditions

CEMx174 [38], H9, CEM-SS and their derivatives were maintained in RPMI-1640 (Whittaker) containing 10% defined fetal bovine serum (FBS, HyClone), 20 μ*M L*-glutamine (Whittaker) and 100 U/ml penicillin-streptomycin (Whittaker). Where indicated, genticin sulfate (G418) was added to 750 U/ml (active weight, Gibco/BRL). Cell lines were obtained from the AIDS Reference and Reagent program, except for the CEMx174 cells used in figure 4 and 6 provided by Y.-T. Huang of Case Western Reserve University.

NIH3T3 (ATCC) cells were maintained in DMEM (Whittaker) supplemented with 10% FBS, 20 μ*M L*-glutamine and 100 U/ml penicillin-streptomycin. DMEM without methionine and dialysed FBS were obtained from Gibco/BRL. Cells were grown at 37°C in 5% CO_2.

SIVmac239*nef*-open and SIVmac239*nef*-deletion were transfected into CEMx174 as described previously [19]. Virus replication was measured by the reverse transcriptase (RT) assay [3]. Five million CEMx174, H9 or H9-derived cell lines were infected following incubation with 100,000 cpm RT units of viral supernatant. Cells were split 2:1 twice per week. Two milliliters supernatant were assayed for RT activity, at indicated times.

Acute Virus Infection

The acute virus infection assay was performed as previously described, with minor modifications [2]. Twenty micrograms SIVmac239*nef*-open, SIVmac239*nef*-deletion and SIVmac239*nef*-KO molecular cloned virus were transfected into COS-1 cells using DEAE-dextran for 6 h [3, 10, 19]. After 48 h, CEMx174 cells were overlayed onto the COS-1 transfectants or mock COS-1 control cells. The co-culture was continued for 24 h. Overlayed cells were removed by aspiration and washed in RPMI-1640 complete medium. Samples were removed every 24 h for CD4 determination by FACS as described below and virus determination by SIVp27 antigen ELISA provided by Cellular Products (Buffalo, N.Y., USA).

Construction of Plasmids

Two oligonucleotides, *onefHin*dA 5′-TATAAGCTTACCTACAATATGGGTGGAGCTATTTCC-3′ (sense) and *onefHin*dB 5′-CATAAGCTTGTGGAAAGTCCCTGCTGTTTCAGCGAG-3′ (antisense) that circumscribe the SIVmac239*nef* gene [33] were used to amplify the *nef* genes of SIVmac239*nef*-open and SIVmac239*nef*-deletion. Five femtomoles of the template DNA were subjected to 30 cycles of amplification using AmpliTaq (Perkin Elmer Cetus). The cycles consisted of the following parameters: DNA denaturation, 1 min at 94°C; annealing, 2 min at 48°C, and extension, 3 min at 72°C. The extension was automatically increased 5 s at each cycle. The PCR DNA was phosphorylated with T4 DNA kinase (USB). The ends of the DNA were repaired with T4 DNA polymerase (Gibco/BRL) and subsequently cloned into *Hpa*I-cleaved pLXSN [26]. The nucleotide sequences of the entire *nef* gene and adjoining sequences were confirmed for each subclone. SIVmac239*nef*-KO was constructed as described by Chakrabarti et al. [8].

Derivation of Cell Lines and FACS Analysis

Stable cell lines were constructed by transfection of the appropriate plasmid into CEMx174, H9 or CEM-SS [19]. Transfection was accomplished by lipofection, retroviral gene transfer or electroporation. Electroporation was performed by removing 2×10^7 cells from culture, washing in RPMI-1640 containing 20% FBS on ice and resuspending in 0.5 ml RPMI-1640 with 20% FBS. Plasmid DNA was linearized by cleaving 10 μg pLXSN or its derivatives with *Pvu*I. The digested DNA was added to cells and exposed to 150 V, 975 μF as described [3] using a BTX 600 Electrocell Manipulator. Retroviral gene transfer was accomplished by electroporation of plasmid DNA into PAC317 cells and selection in media containing G418 sulfate for 3 weeks. Supernatant containing retroviral particles was collected and added to indicated cells. Lipofections were performed using 2 μg supercoiled plasmid as described by the supplier (Gibco/BRL). Cells were allowed to recover for 48 h. The cells were then placed in RPMI containing 750 μg/ml G418 sulfate (Gibco/BRL) for 3 weeks. G418 sulfate was removed and cells were grown for 1 week in the absence of selection. In some experiments, cells were grown for an additional 3 weeks without selection or were returned to G418 sulfate selection media for 3 weeks. One million cells were stained with phycoerythrin-conjugated antihuman CD4, FITC-conjugated antihuman CD8 and peridinin-chlorophyll-protein-conjugated antihuman CD3 (Becton-Dickinson), and the relative fluorescence was determined. Cells in some experiments were obtained by sorting those cells transfected with pLXSN-239*nef*-1 for low CD4 surface expression. FACS analysis and FACS sorting were performed using a Becton-Dickinson FACScan and FACStar Plus. Data were analyzed using the Lysis II software.

Immunoprecipitation

NIH3T3 cells (10^7) were grown to confluency and inoculated at a MOI of 5 with a recombinant vaccinia virus construct containing the

SIVmac239*nef*-open gene [19]. At 6 h after infection, cells were incubated for 30 min in DMEM without methionine containing 10% dialysed FBS (Gibco/BRL). Cells were labeled following addition of 1 mCi ^{35}S-methionine (1,217 Ci/m*M*, New England Nuclear) for 4 h. The cells were scraped from the flask, washed twice in cold PBS and resuspended in 10 ml Harlow buffer (50 m*M* HEPES, pH 7.0, 250 m*M* NaCl, 0.1% NP40 [18] containing the following protease inhibitors: 1 m*M* PMSF, 5 μ*M* leupeptin, 5 μ*M* pepstatin, 0.5 μ*M* aprotinin, 0.5 μ*M* EDTA (all obtained from Boehringer Mannheim). Cells were incubated on ice for 30 min with vigorous vortexing every 3 min. The cell extract was centrifuged at 20,000 *g* for 15 min at 4°C. Approximately 2 × 10^6 cpm from the supernatant fraction was diluted to 300 μl in Harlow buffer + inhibitors, then was preabsorbed to 15 μl protein G plus/protein A agarose (Oncogene Science) and incubated at 4°C for 1 h while rotating slowly. The nonspecific protein-A-bound material was removed by centrifugation for 15 min at 13,600 *g* at 4°C, 5 μl serum was added to each precleared lysate and incubated for 150 min at 4°C. Fresh protein G plus/protein A agarose was added, and each sample was incubated for 60 min at 4°C. The protein G plus/protein A agarose complex was pelleted, washed 4 times in Harlow buffer + inhibitor, resuspended in 15 μl SDS-PAGE sample buffer (2% SDS, 10% glycerol, 1.2 *M* β-mercaptoethanol, 60 m*M* Tris, pH 6.8, 0.001% bromphenol blue) and heated to 95°C for 2 min. Supernatants were loaded onto a 12% SDS polyacrylamide gel [21]. The gel was fixed for 1 h in 5% methanol and 7.5% acetic acid, rinsed and dried. The dried gel was exposed to Kodak XAR 5 film for 22 days. The prestained protein markers (Gibco/BRL) electrophorese with the following apparent molecular weights in daltons: 215,000 – myosin; 105,100 – phosphorylase B; 69,800 – bovine serum albumin; 43,300 – ovalbumin; 28,300 – carbonic anhydrase; 18,100 – β-lactoglobulin, and 15,400 – lysozyme. Antisera from SIV-infected macaques [19] were provided by Dr. Ron Desrosiers.

Immunofluorescence

Cell lines were tested for *nef* expression by immunofluorescent staining of fixed cells. Cells were fixed with 50% methanol and 50% acetone for 10 min at –20°C to access total protein expression. The cells were then incubated with the rhesus monkey sera at 1:50 dilution from animals infected with either SIVmac239*nef*-open (fig. 1) or SIVmac239*nef*-deletion (fig. 5) for 1 h at room temperature in a humidified chamber. Cells were washed 3 times with PBS-Tween (PBS with 0.01% Tween-20) and then incubated with a 1:1,000 dilution of a rabbit anti-rhesus-monkey antibody conjugated with FITC (ACS, Westbury, N.Y., USA) for 1 h at room temperature in a dark humidified chamber. Cells were washed as above and examined at a magnification of ×200 on a Nikon microscope equipped for epifluorescence. Digital images were captured using a computer imaging system (Oncor Image Systems, Inc.).

Immuno Dot Blot

Purified SIV Nef (1 μg) and cell lysate from SIVmac239*nef*-open-infected cells (6,000 cpm RT units) were spotted onto nitrocellulose and allowed to air dry. The membrane was treated for 2 h with PBS-Tween-20 and 5% dried nonfat milk. Monkey sera were diluted 1:1,000 in PBS-Tween 1% dried milk with or without purified soluble Nef protein competitor and incubated for 2 h at 4°C. The blots containing *nef* and SIV were incubated with the monkey sera for 1 h at room temperature, then washed 3 times in PBS-Tween, and horseradish-peroxidase-conjugated rabbit anti-rhesus-monkey serum (ACS) was added 1:3,000 in PBS-Tween, 1% milk for 1 h at room temperature. Finally, the blot was washed 3 times in PBS-Tween and the ECL Western blot kit (Amersham) was used to illuminate positive spots.

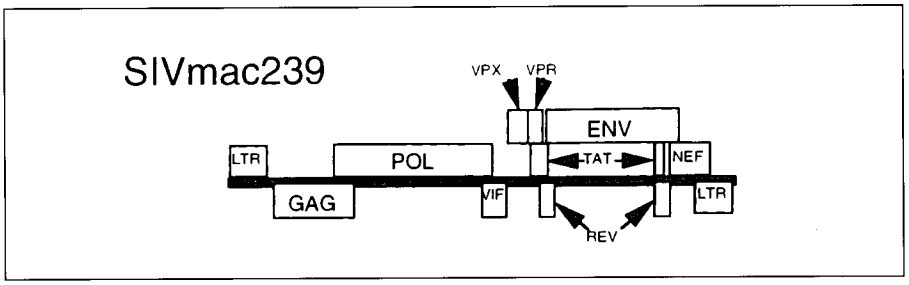
Detection of Truncated nef *by Western Blot*

Escherichia coli BL21 (DE3) cells were transformed with plasmids and induced with 0.5 m*M* IPTG. Induced cells lysed with radioimmunoprecipitation assay buffer [8] and 50 μg lysate were electrophoresed on a 10% SDS polyacrylamide gel. The gel was electroblotted onto nitrocellulose using a BioRad Mini Trans-Blot cell under conditions described by BioRad. The membrane was blocked by incubating with 5% nonfat dried milk in PBS containing 0.05% Tween 20 (PBS-T; BioRad) for 2 h at room temperature and then washed with PBS-T. A mixture containing 1% nonfat dried milk in PBS-T and a 1:3,000 dilution of rabbit antisera raised against Nef was added to the blot and incubated for 1 h at room temperature. The blot was rinsed with PBS-T twice, washed once with PBS-T for 15 min and washed 4 times in PBS-T for 5 min. The signal was generated by reacting the conjugated blot with the primary antibody, with a 1:10,000 dilution of goat antirabbit antibody conjugated with horseradish peroxidase and 1% nonfat dried milk in PBS-T for 1 h, at room temperature. The blot was rinsed with PBS-T twice, washed once with PBS-T for 15 min and washed 4 times in PBS-T for 5 min. Visualization was accomplished with the ECL reagent kit (Amersham).

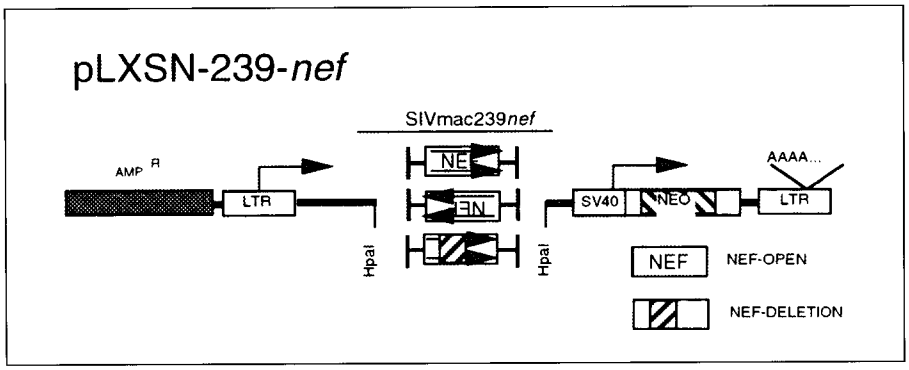
Results

The *nef* gene of SIVmac239*nef*-open and SIVmac239*nef*-deletion [19] were subcloned into the mammalian expression vector pLXSN shown in figure 1 and used to derive stable CEMx174, H9 and CEM-SS cell lines. The products of the *nef*-open gene and the truncated *nef*-deletion gene are expressed in stable cell lines (fig. 1C). H9 cells, H9 pLXSN, H9 *nef*-open cells, and H9 *nef*-deletion cells were tested for *nef* expression by immunofluorescence using antisera from a rhesus monkey infected with SIV. All cells containing the *nef*-open or the *nef*-deletion allele in the correct orientations were fluorescent positive (fig. 1C). The parent strain, H9 (fig. 1C), the vector cell line, H9-LXSN (fig. 1C), the *nef* antisense (as) cell line and H9 *nef*-as (not shown) were negative. The fields shown by phase contrast microscopy on the left are the same as shown by immunofluorescent microscopy on the right.

The *nef*-open gene from the pathogenic molecular clone SIVmac239*nef*-open was found to down-modulate human CD4 from the cell surface of two different cell lines (fig. 2A, D), while *nef* in the antisense orientation had no effect (fig. 2A, C, D). The cell lines CEMx174/pLXSN*nef*-1 (fig. 2A) and CEMx174/pLXSN *nef*-2 (fig. 2B) were independently derived *nef*-open sense



A



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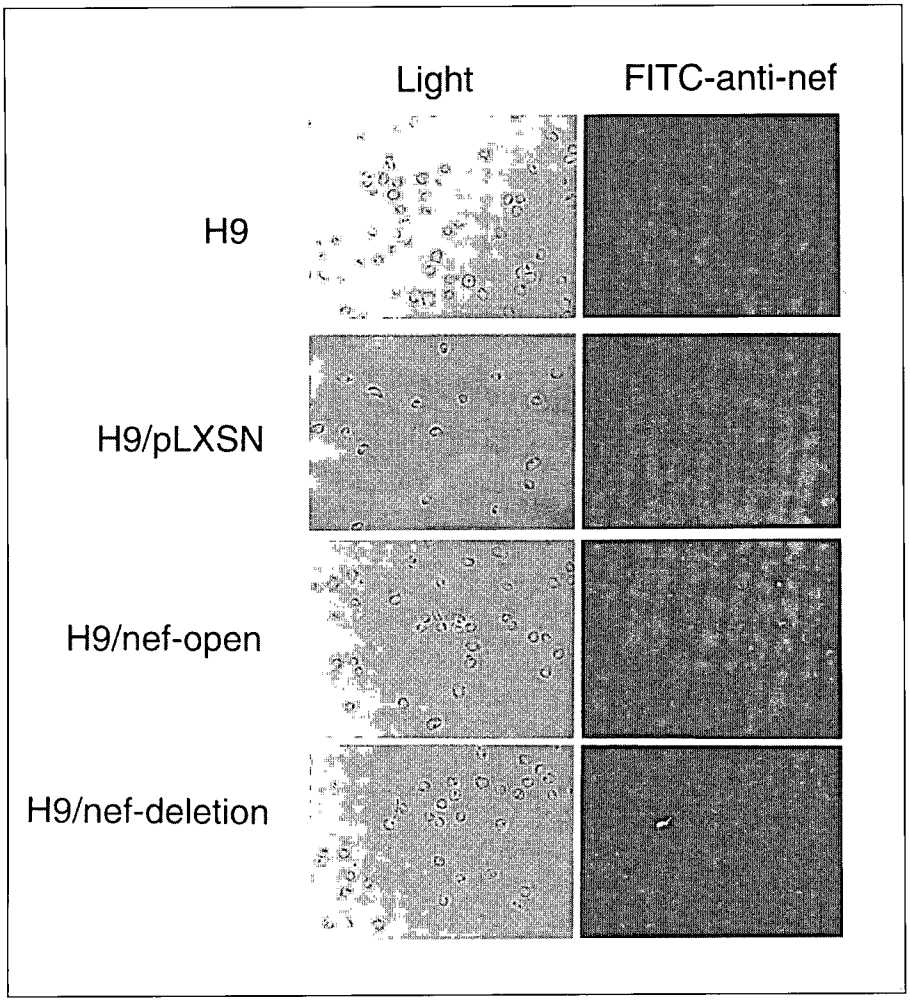
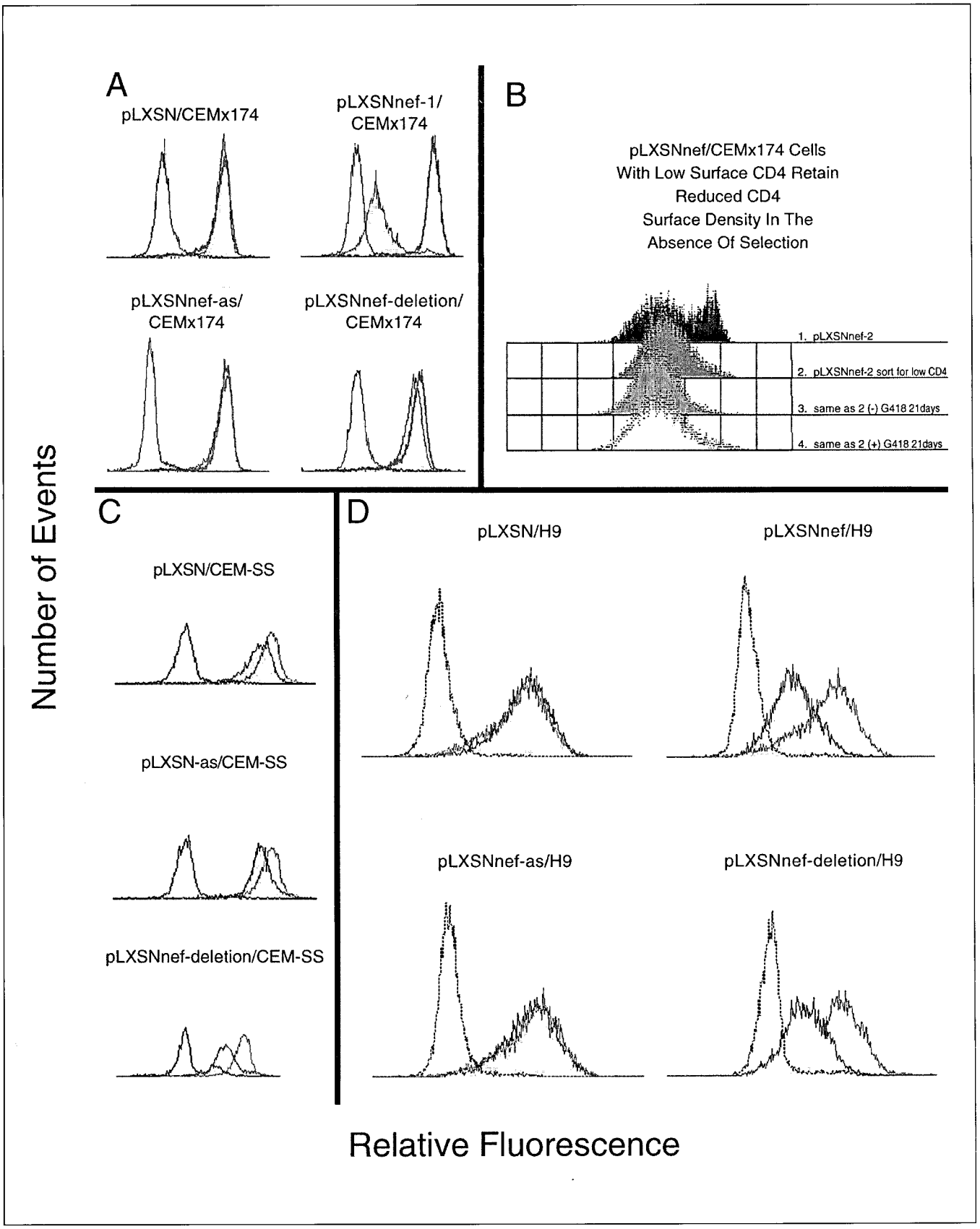


C

Fig. 1. *nef* constructs used in this study. **A** Map of *nef* genes derived from SIVmac239 [19]. SIVmac239*nef*-open and SIVmac239*nef*-deletion contain the same virus backbone and only differ in the *nef* gene. **B** nef of SIVmac239*nef*-open and SIVmac239*nef*-deletion were subcloned into pLXSN [26] by PCR amplification of the molecular cloned viruses [33]. **C** Expression of Nef in H9, H9/pLXSN, H9/*nef*-open and H9/*nef*-deletion. Left panels are cells observed by light microscopy. The same fields observed by fluorescent microscopy are shown on the right.


A
pLXSN/CEMx174
pLXSNnef-1/
CEMx174
pLXSNnef-as/
CEMx174
pLXSNnef-deletion/
CEMx174
B
pLXSNnef/CEMx174 Cells
With Low Surface CD4 Retain
Reduced CD4
Surface Density In The
Absence Of Selection
1. pLXSNnef-2
2. pLXSNnef-2 sort for low CD4
3. same as 2 (-) G418 21days
4. same as 2 (+) G418 21days
C
pLXSN/CEM-SS
pLXSN-as/CEM-SS
pLXSNnef-deletion/CEM-SS
D
pLXSN/H9
pLXSNnef/H9
pLXSNnef-as/H9
pLXSNnef-deletion/H9
Number of Events
Relative Fluorescence

orientation cell lines. Only the cell lines containing the vector maintained the parental CD4 surface density (fig. 2A, C, D). It was suggested that *nef* stable cell lines may be difficult to obtain by co-transfection [24]. We also obtained *neo*-resistant *nef* cell lines that fail to express *nef* and down-modulate CD4, even though *nef* is cloned in the sense orientation (data not shown). Therefore, we addressed the question: Is CD4 down-modulation as a consequence of *nef* expression a stable phenotype?

The *nef*-2-open stable cells in CEMx174 had two distinct populations expressing different amounts of CD4 on the surface: the down-modulated peak of cells and a population of cells appearing to have a normal level of CD4 on the surface. We examined whether the down-modulated phenotype might be unstable and, therefore, under selective pressure to revert to the parental density. Stable cells shown in figure 2B, curve 1, were sorted for low surface levels of CD4 and were re-examined for CD4 surface expression by FACScan (fig. 2B, curve 2). This relatively pure population of down-modulated cells was split into two fractions: one was subjected to G418 selection for an additional 3 weeks (fig. 2B, curve 4) and the second grown in the absence of G418 for 3 weeks (fig. 2B, curve 3). While there appeared to be a slight broadening of the curve in the down-modulated population grown without additional drug selection (fig. 2B, curve 3), the increase in CD4 surface density among some cells in the culture did not approach the normal density nor did it significantly differ from cells grown in the presence of G418. Thus, *nef*-mediated down-modulation of surface CD4 appears to be a stable phenotype. The presence of cells with parental CD4 surface density in stable cell lines occurred as a consequence of damage to the *nef* allele during the transfection procedure, but once the *nef* gene was established, it was stably expressed and was able to continuously down-modulate CD4. This result was corroborated by CEMx174/pLXSN*nef*-1 in which the entire population appears as a single peak down-modulated for CD4 (fig. 2A). That phenotype was also stably maintained over time (data not shown).

Fig. 2. Relative CD4 cell surface density of *nef* stable cell lines. Cells were stained with fluorescent anti-CD4 antibodies and subjected to FACS analyses. The control cells CEMx174, H9 and CEM-SS are shown by the open curve on the right in each panel. Experimental plots are shown by the shaded area. Unstained isotypic antibody control cells are shown by the curve on the left. **A** CEMx174-derived cell lines. **B** CEMx174/pLXSN*nef*-2 cells tested for stability of the reduced CD4 density phenotype. **C** CEM-SS-derived cell lines. **D** H9-derived cell lines.

Although the *nef*-deletion gene encodes only 58 amino acid residues of the 5′ end of *nef* (fig. 3), expression of this truncated protein was found to down-modulate CD4 from the surface of CEMx174 cells (fig. 2). The truncated *nef*-deletion allele is capable of inducing the down-modulation of CD4 to a similar extent in H9 cells (fig. 2D), and it is also able to induce the down-modulation in CEM-SS cells (fig. 2C). As shown in figure 2D, *nef*-open is capable of inducing down-modulation of CD4 in H9, while the vector pLXSN or the antisense are not. The H9 and CEM-SS cell lines have significant reductions in surface CD4 when the *nef*-deletion gene or the *nef*-open gene are expressed. No significant effect of *nef* was detected on two other lymphoid surface markers, CD8 and CD3, for all cell lines (data not shown).

The complete pathogenic potential of SIV requires the presence of an intact *nef* gene. SIVmac239*nef*-deletion does not cause AIDS [19]. Virus loads in animals infected with this virus are significantly reduced [19, 36] and are protected from superinfection with pathogenic virus [12]. SIVmac239*nef*-deletion, however, maintains a partial *nef*-open reading frame capable of producing a truncate *nef* protein consisting of the first 58 amino acids or 22% of the reading frame followed by 20 amino acids from an alternative reading frame (fig. 3). This truncated protein was able to cause a moderate decrease in the cell surface CD4 level in CEMx174 (fig. 2A). In contrast, the two *nef*-open CEMx174 cell lines had a more significant effect on surface CD4 (fig. 2A and B). Expression of *nef*-deletion was more pronounced on the CD4 cell surface density in H9 and CEM-SS cell lines. H9/*nef*-open, H9/*nef*-deletion and CEM-SS/*nef*-deletion stable cell lines were all CD4-down-modulated to similar extents. The presence of the *nef*-deletion allele in the H9/*nef*-deletion cell line was confirmed by Southern analysis [Salkowitz and Kestler, unpubl. results]. These results indicate that the determinant CD4-down-modulation maps at least partially to the N-terminal portion of the *nef* protein.

The effect of *nef* on CD4 surface density can be tested in a viral infection even though other genes of primate lentiviruses perform the same function. The acute infection assay developed in the Trono laboratory allows one to observe *nef*-mediated CD4 down-modulation in the absence of an envelope-caused down-modulation [2]. The assay relies on the fact that *nef* is expressed early in the virus life cycle and the envelope is expressed late. Additionally, *nef* down-modulates CD4 in a posttranslational mechanism, and Env control of CD4 surface density occurs through the inhibition of de novo production of CD4. Since surface CD4 has a long half-life, the effect of

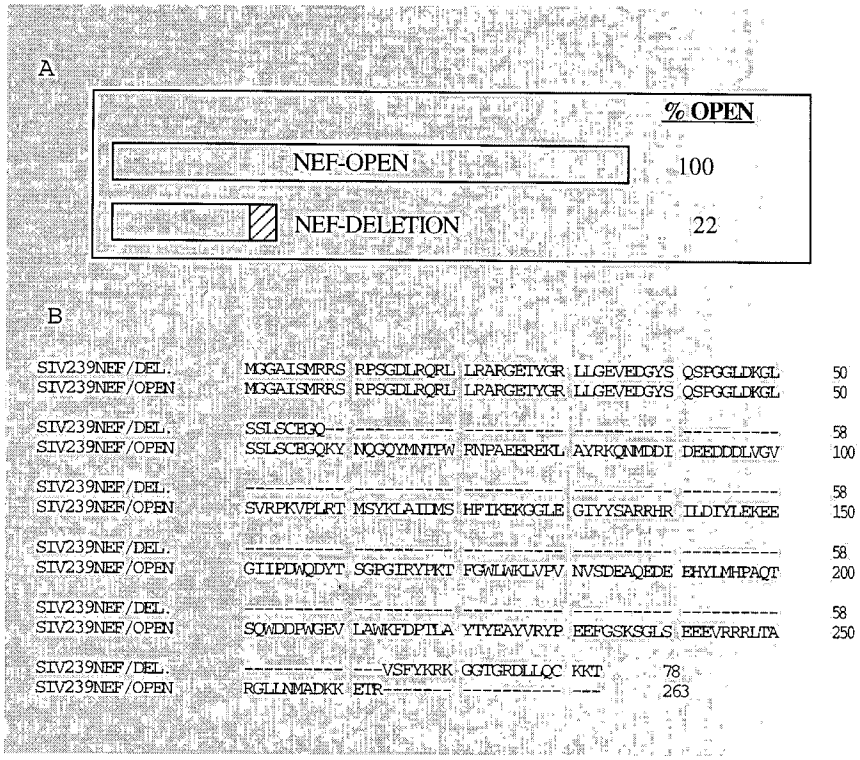
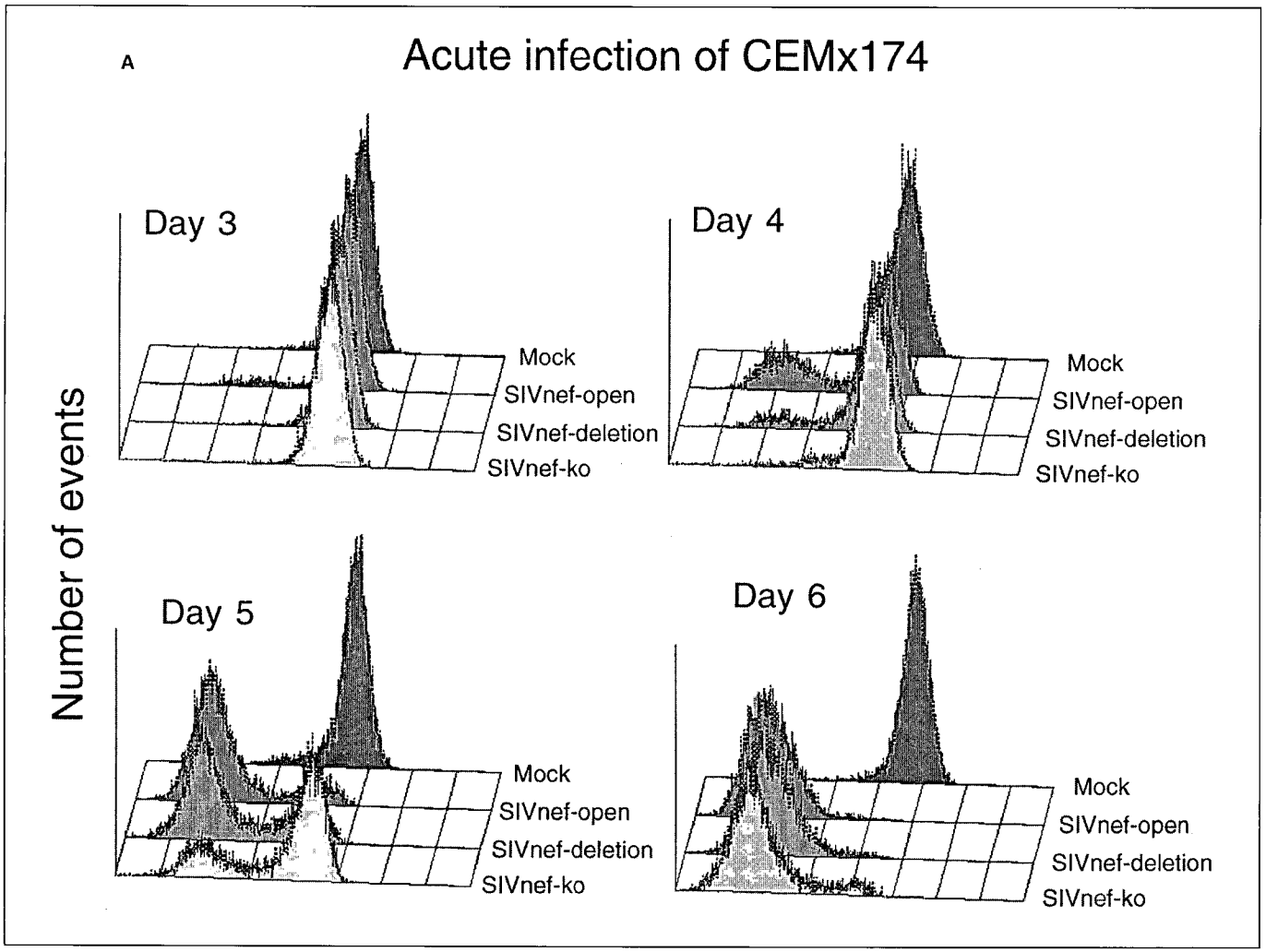


Fig. 3. Nonpathogenic SIVmac239*nef*-deletion encodes a truncated polypeptide. **A** Schematic diagram of *nef*-open versus *nef*-deletion. Both alleles share a common amino terminus. The *nef*-deletion reading frame shifts at the site of deletion of 182 base pairs resulting in the addition of 20 amino acids from an alternative reading frame (shown by the striped box). **B** The amino acid alignment of *nef*-open and *nef*-deletion. The amino acid abbreviations are those recommended by the IUPAC-IUB biochemical nomenclature commission.

nef can be detected by synchronously infecting large numbers of cells and measuring the density of CD4 early in the course of infection, before Env has a chance to act. COS-1 cells were transfected with 20 μg of infectious molecular cloned SIVmac239*nef*-open, SIVmac239*nef*-deletion or SIVmac239*nef*-KO (a virus not capable of expressing the *nef* sequence) [8]. Overlaying of CEMx174 cells on COS transfectants results in infection at a high multiplicity. The CD4 surface density for uninfected, SIVmac239*nef*-open-infected, SIVmac239*nef*-deletion-infected and SIVmac239*nef*-KO-infected CEMx174 was identical for the first 3 days after inoculation (fig. 4 and not shown). However, by day 4 the cells infected with the two viruses encoding either the N-terminal portion of Nef or the complete *nef* gene showed accumulation of cells with reduced surface CD4, while SIV*nef*-KO retained the parental density. At day 5 the two viruses encoding the *nef* sequence are almost entirely down-modulated and SIV*nef*-KO was beginning to show a down-modulated population. At day 6 all virus-infected cells were completely down-modulated (fig. 4A, C). Replication of these viruses was nearly identical, as shown by p27 antigen capture (fig. 4B).

Since both *nef*-open and the truncated *nef*-deletion can cause CD4 down-modulation, we tried to determine whether the *nef* deletion allele is expressed in vivo in virus-infected animals. Infection of man with HIV and monkeys with SIV generates a humoral response to *nef* [4, 19]. SIVmac239*nef*-open and SIVmac239*nef*-deletion having an open and truncated *nef* gene were tested for expression in rhesus monkeys. Recently, a group of AIDS nonprogressors who were shown to harbor *nef*-deleted HIV had antibody responses to Nef [13, pers. commun. Dale McPhee]. This response was due to the strong *rev*-independent expression of the *nef* transcript occurring early after infection [35]. The presence of *nef* antibodies indicates that *nef* or a portion of *nef* was expressed. Animals who received SIVmac239*nef*-deletion, SIVmac239*nef*-stop and SIVmac239*nef*-open were tested for antibodies to the *nef*-open gene. An animal which received the *nef*-deletion virus had antibodies to *nef* (fig. 5), indicating that the truncated protein was expressed in this animal. The monkey serum was examined in three assays. Immunoprecipitation of a vaccinia virus expressing the full-length Nef protein with preimmune serum (fig. 5, lane 1) showed no *nef* precipitate; however, in sera from all 3 animals infected with SIVmac239*nef*-stop, SIVmac239*nef*-deletion and SIVmac239*nef*-open (lanes 2–4), we saw a precipitate migrating with the molecular weight of *nef*. In figure 5B, immunofluorescence shows that an animal infected with SIVmac239*nef*-deletion had made



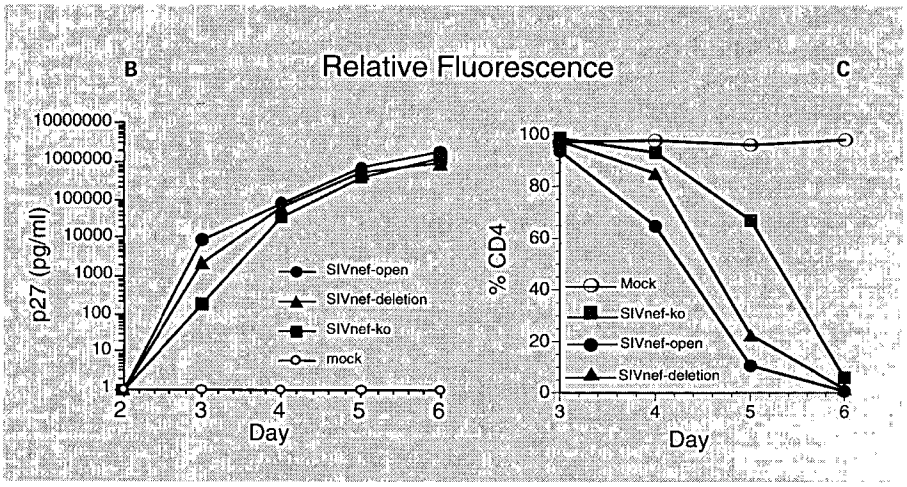


Fig. 4. Acute infection fo CEMx174. **A** FACS CD4 analysis of acutely infected CEMx174 cells at various times after injection. **B** Replication of indicated virus in CEMx174-infected cells as measured by antigen capture over time after infection. **C** Percent CD4 down-modulation as a function of time after infection.

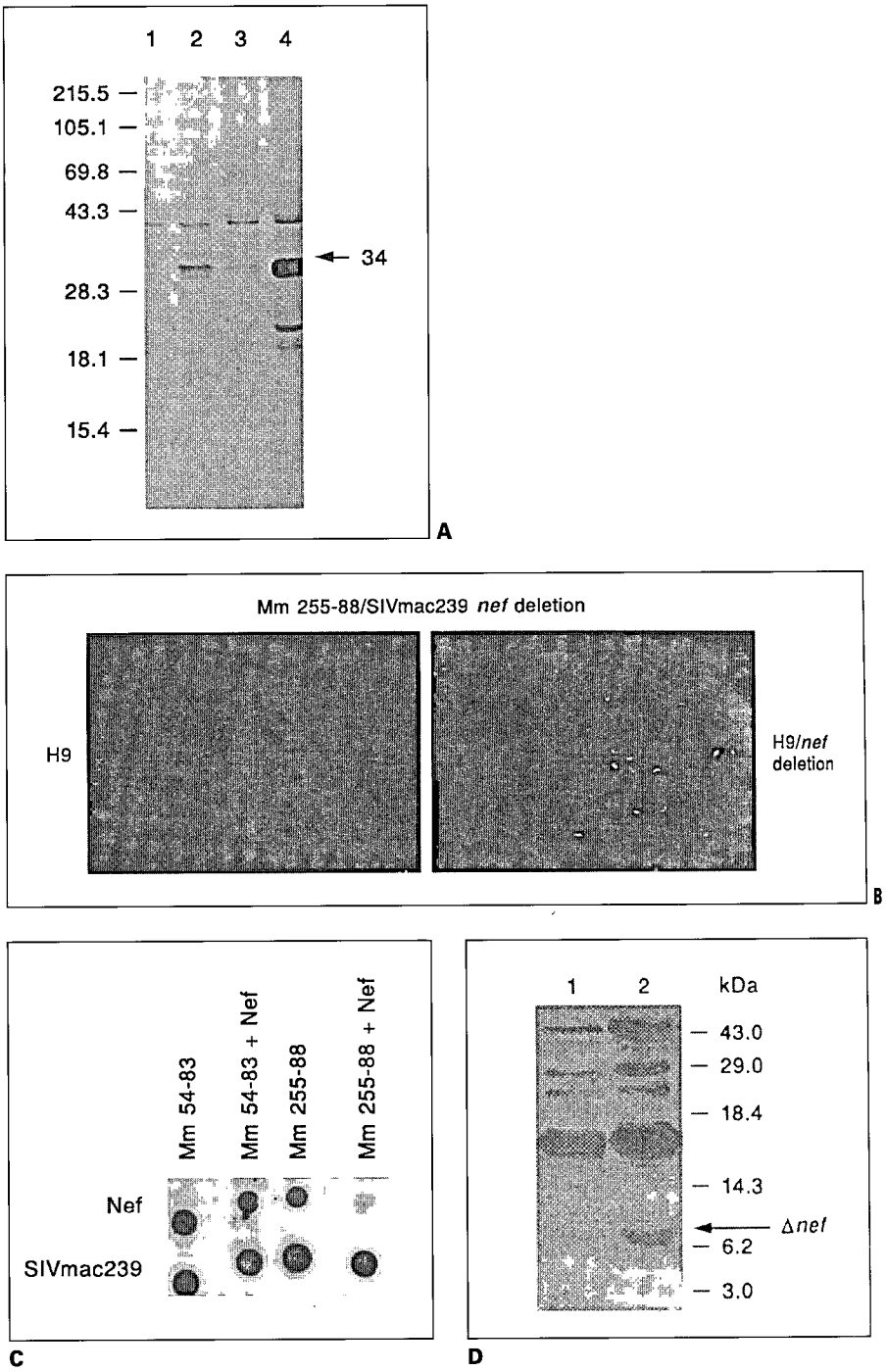


Fig. 5. The truncated *nef*-deletion allele is expressed in vivo. **A** Immunoprecipitation of *nef* by sera from experimentally infected monkeys. Lane 1: immunoprecipitation of radiolabeled *nef*-open with sera from *Macaca mulatta* (Mm) 54-83 before inoculation; lane 2: immunoprecipitation of radiolabeled *nef*-open with sera from Mm 54-83 8 weeks after this animal had been inoculated with SIVmac239*nef*-stop; lane 3: immunoprecipitation of radiolabeled *nef*-open with sera from Mm 255-88 1 year after inoculation with SIVmac239*nef*-deletion; lane 4: immunoprecipitation of radiolabeled *nef*-open with sera from Mm 358-88 1 year after inoculation with SIVmac239*nef*-open. Molecular weight expressed in kilodaltons. **B** Immunofluorescence of H9 and H9/*nef*-deletion cell lines using antisera from an animal experimentally infected with SIVmac239*nef*-deletion. **C** *nef* immunoblot with monkey sera. *nef* and SIV immobilized on nitrocellulose were used to test for antibodies to *nef*. Serum from Mm 54-83 1 year after infection with SIVmac239 was tested for the ability to respond to Nef protein. Lane 2 shows the signal generated when serum is preincubated with free soluble *nef*. Serum from Mm 255-88 1 year after infection with SIVmac239 *nef*-deletion also has antibodies to *nef*, which can be blocked by preincubation with free soluble *nef*. **D** Western blot of bacterially produced *nef* deletion. Lane 1: B121 (DE3)pET3a bacterial lysate; lane 2: B121 (DE3)pET3-a-*nef*-deletion bacterial lysate.

antibodies to truncated *nef*-deletion expressed on H9 cells (right), but not to any other antigen present in the parent H9 cell line (left). Figure 5 demonstrates by immunoblot that the antisera from Mm 54-83, as animal infected with SIVmac239 encoding a wild-type *nef*, can be prevented from binding to *nef* immobilized on the filter, by preincubating with soluble *nef* (reduction in spot intensity). Likewise, antisera from Mm 255-88, an animal infected with SIVmac239*nef*-deletion, were prevented from binding to immobilized *nef* by preincubation with soluble *nef*. The addition of *nef* in the preincubation step did not produce a signal reduction in the SIV control spots. This demonstrates that the antibody behavior observed in figure 5A and B is due to *nef*-specific antibodies present in

both SIVmac239*nef*-open- and SIVmac239*nef*-deletion-infected animals. Mm 255-88, the animal infected with SIV*nef*-deletion in figure 5, was the only animal tested with quantifiable antibody titers to *nef*. Other animals given *nef*-deletion virus did not have quantifiable antibodies to *nef* by immunoblot, immunoprecipitation or immunofluorescence [Salkowitz and Kestler, unpubl. results]. Perhaps the presence of *nef* antibodies in Mm 255-88 is related to the fact that the virus load in this animal was higher than in other animals infected with SIVmac239*nef*-deletion, as measured by *gag* PCR [Kestler and Desrosiers, unpubl. results]. The partial reversion of virus load has not affected the health of this animal, nor has it affected the ability of SIVmac239*nef*-deletion to serve as a vaccine [12, 20]. Perhaps other animals infected with SIVmac239*nef*-deletion do not maintain detectable titers of *nef* antibodies, because persistent virus expression is very low.

The truncated SIVmac239 Nef-deletion protein encodes the first 58 amino acids of *nef* followed by 20 amino acids in another reading frame. The predicted molecular weight of such a polypeptide is 8.5 kD. We tested whether this polypeptide could be expressed by cloning it into pET3a, transforming *E. coli* and performing a Western blot analysis on the bacterial lysate, using anti-SIV*nef* antibody. A 7.3-kD polypeptide recognized by Nef antibodies was detected in lysates transfected with pET3a-*nef*-deletion (fig. 5D).

The down-modulation of CD4 could produce a barrier to superinfection, since CD4 is the receptor for SIV and HIV. We tested the replication of virus in H9 cell lines expressing *nef*-open and *nef*-deletion. Figure 6E and F shows an inhibition of SIV growth in H9 cell lines that express both truncated and full-length *nef*. The replication of SIV in H9 is dependent on the presence of *nef*. SIV*nef*-open and SIV*nef*-deletion show no difference in their replication kinetics in CEMx174 (fig. 6A) but show a clear difference in H9 (fig. 6B). We also tested replication of SIV in stable cell lines encoding the antisense *nef* (H9/*nef*-as; fig. 6C) and those encoding just the vector (H9/pLXSN; fig. 6D). In all 3 cases, virus encoding a full-length *nef* appeared in the culture approximately 5 days earlier than the virus encoding a truncated *nef*.

Discussion

The defect introduced to construct SIVmax239*nef*-deletion did not totally eliminate the *nef* reading frame [19]. The nonpathogenic molecular cloned virus SIVmac239*nef*-deletion encodes a truncated *nef* that is expressed in vivo and in vitro. We showed that the protein produced by this truncated reading frame retains a biological function: the ability to down-modulate CD4. This result was confirmed using two different assays employing a variety of cell types. Cells having a reduced CD4 on their surface due to *nef* did not revert to the parental CD4 density, which suggests that once established, *nef* cell lines are genetically stable.

The CEMx174/pLXSN*nef*-2 cells appear to have two distinct CD4 density populations, while CEMx174 has only one density of CD4 on the surface (fig. 2A and B). We showed that cells with low CD4 surface density maintained that density in the presence or absence of additional selection. The difference between CEMx174/pLXSN*nef*-2 and CEMx174/pLXSN*nef*-1 is unknown and perplexing, since both cells were created using the same plasmid. Possibly the difference is related to the method of transfection. CEMx174/pLXSN*nef*-2 was derived by electroporation of pLXSN*nef* into CEMx174 and CEMx174/pLXSN*nef*-1 by lipofection of pLXSN*nef* into the PAC317 packaging cell line followed by retroviral gene transfection of CEMx174. Electroporation of the plasmid may have induced damage to a number of *nef* copies in that cell line. More studies are necessary to determine if damage to *nef* in the electroporation process is, indeed, occurring.

The truncated allele of *nef* encoded by SIVmac239*nef*-deletion is expressed in vivo as demonstrated by the fact that the SIVmac239*nef*-deletion-infected animal produced a humoral response to *nef*. Binding of Nef antibodies from the serum of this SIVmac239*nef*-deletion-infected animal to immobilized Nef protein can be blocked by adding soluble Nef protein to the reaction indicating that the antiserum was, in fact, recognizing Nef.

A humoral response to *nef* was not observed in other animals infected with SIVmac239*nef*-deletion, but even with SIVmac239*nef*-open-infected animals the titers of antibodies to Nef can be quite low or absent [Kestler and Desrosiers, unpubl.]. Direct visualization of truncated Nef by Western blot in an infected or stable cell is technically very difficult. Figure 5 demonstrates expression of truncated Nef by immunofluorescence. Additionally, the truncated *nef* gene encodes a 7.3-kD protein recognized by anti-Nef antibodies when overexpressed in a bacterial system. Expression of the truncated *nef* reading frame in a virus context was also observed when *nef*-deletion was fused to a herpes virus thymidine kinase gene [8].

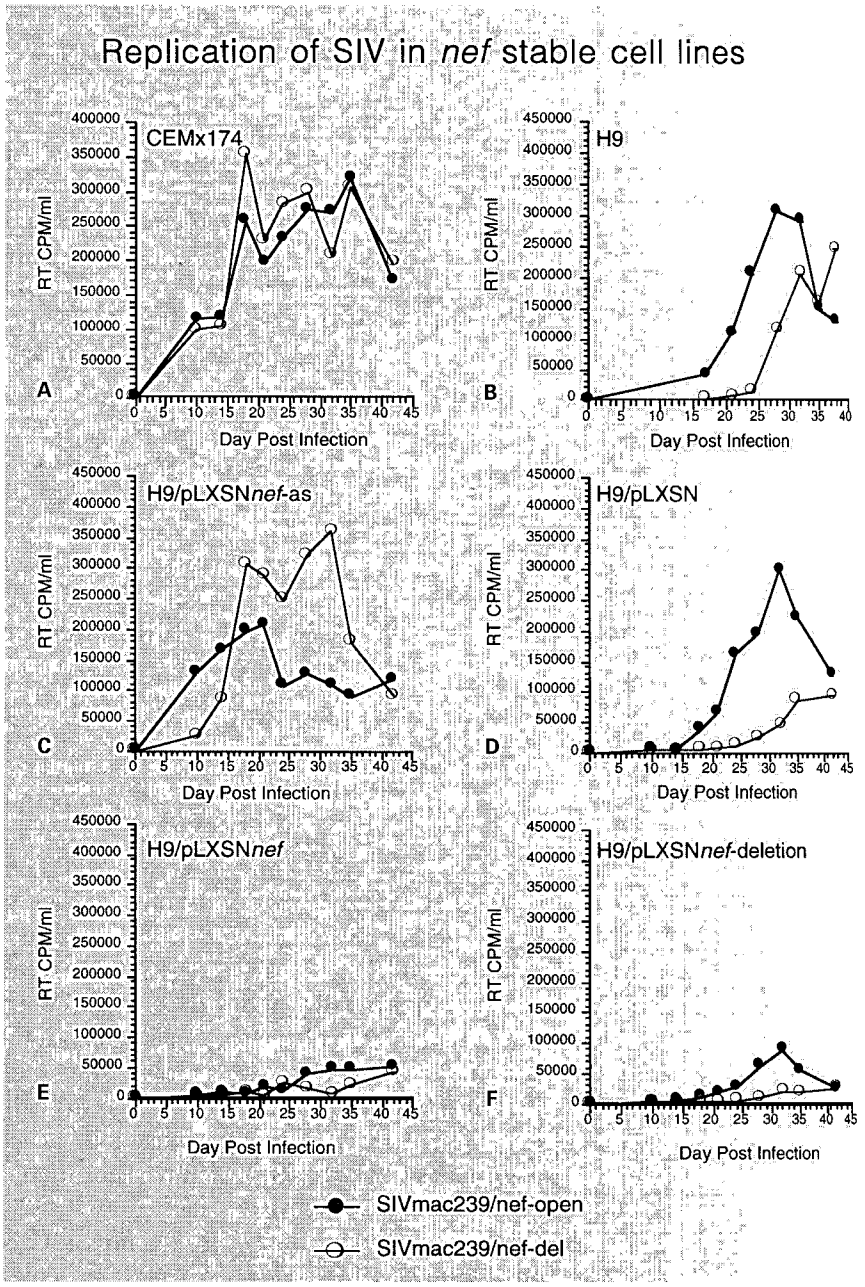


Fig. 6. Replication of SIVmac239*nef*-open and SIVmac239*nef*-deletion in *nef* stable cell lines. Viral growth kinetics of SIVmac239*nef*-open and SIVmac239*nef*-deletion in six different cell lines. SIV replication: CEMx174 (**A**), H9 (**B**), H9/pLXSN*nef*-as (antisense, **C**), H9/pLXSN (vector, **D**), H9/pLXSN*nef* (**E**), H9/pLXSN*nef*-deletion (**F**). An equal inoculum (100,000 cpm RT units) was used to infect an equal number of cells (5×10^6).

Our results suggest that ability or inability to down-modulate CD4 by *nef*-deletion is not the reason for the absence of pathogenicity of virus encoding the truncated *nef*-deletion allele, since both *nef*-deletion and *nef*-open possess this capacity, and since both alleles are expressed in a natural infection. However, the difference in pathogenic potential is possibly due to a quantitative defect in the ability to down-modulate CD4. The pathogenicity of a virus encoding a full-length *nef* mutant in determinants necessary to down-modulate CD4 has not been evaluated.

The ability of HIV*nef* to down-modulate CD4 was genetically separated from its ability to promote viral growth in primary and H9 cells [32, 37, Salkowitz et al., unpubl.]. While down-modulation of CD4 was the most consistently observed phenotype of *nef*, the value of the phenotype to the virus life cycle was not established, and the role of this property in determining pathogenic potential is questionable.

Given the effect of *nef* on the virus load in vivo, it is likely that the product of this gene plays a role in facilitat-

ing the replication of virus. Since the humoral and cellular responses to both viruses appear to be similar [19], it is unlikely that Nef plays a role in the decay of virus. Nef was required for efficient SIV and HIV replication in quiescent primary lymphocytes [14, 27, 41]. The interaction of *nef* with cellular kinases such as Hck may mitigate that effect [37, 39]. We have recently characterized the *nef* gene from virus from a long-term AIDS nonprogressor and found that while the gene was fully open, *nef* was mutant in a determinant of *nef* necessary for interaction with Src family kinases. Additionally, virus encoding this allele was growth attenuated in the quiescent cell assay [Ma et al., unpubl.]. Nef from the virus of this individual was normal in its ability to down-modulate CD4 [Salkowitz and Kestler, unpubl.].

Efficient replication of AIDS viruses in the H9 cell line is also dependent on *nef*. Viruses from long-term AIDS nonprogressors are often unable to replicate in the H9 cell line [9, 32]. *nef* has recently been shown by Premkumar et al. [32] to be necessary for efficient H9 virus replication. A full-length *nef* from a long-term AIDS nonprogressor caused virus to lose its ability to replicate in H9 cells, yet was competent in the ability to down-modulate CD4 [Salkowitz et al., unpubl.]. This study has shown that *nef* of SIV is also required for efficient replication in H9. H9 and H9 stable cell lines containing *nef*, in the antisense orientation, the retroviral vector or the open or truncated forms of *nef* were better in supporting the replication of SIVmac239 encoding a full-length open *nef* gene than SIVmac239*nef*-deletion encoding a truncated *nef* gene. However, Nef-stable cell lines encoding a fully open SIV-*nef* or a truncated *nef* were severely deficient in their ability to support efficient replication of SIV. The block in replication is due, at least in part, to the reduced concentration of CD4 on their cell surface.

Ability of *nef* to play a role in determining the rate and efficiency of replication is likely to be the primary (pathogenic) function of this in vivo essential gene product. Here we showed a defect in the replication of virus encoding a truncated *nef*. The efficient replication of AIDS viruses in quiescent primary cells [14, 27, 41] and in H9 depends on an open *nef* gene. Why quiescent primary cells and H9 cells are indicative of *nef* function and other tumor cell lines or stimulated primary cells are not is unknown. The block appears to be at a step in the virus life cycle, after entry. When molecular cloned SIVmac239*nef*-deletion was transfected directly into H9, we were unable to recover virus [Salkowitz et al., unpubl.]. A possible explanation is that H9 and quiescent lymphocytes may utilize a signaling pathway in which a cellular factor docks with Nef protein to signal increased cell growth. Other transformed cells and stimulated lymphocytes may use alternative signaling pathways that are *nef* insensitive. Examples of Nef interactions with factors involved in the cell growth control apparatus do exist. Recently, the crystal structure of Nef complexed to Src family proteins has been solved [22]. That structure employed only the C-terminal portion of the Nef protein. Nef can also interact with a serine kinase. This association appears to be required to obtain the full pathogenic potential of the virus [Sawai and Luciw, pers. commun.]. The residues necessary for both interactions are missing on the truncated version of *nef* used in this study.

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