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Growth Dominance of a Revertant Virus Generated during *in Vitro* Serial Passage of *nef* Frameshift Mutant of HIV-1[1]

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We prepared a series of *nef* mutant HIV-1 with a frameshift mutation at the *Xho* I site by up to 50 serial transfers into MT-4 cells. Here, we studied revertants. Immunofluorescence using an anti-Nef monoclonal antibody revealed that cells first became Nef antigen-positive at the 23rd passage. The percentage of Nef antigen-positive cells gradually increased and reached almost 100% by the 27th passage. The sequence of the provirus in the cells supported the generation of a revertant. This revertant mutated at the site immediately after the initially introduced frameshift mutation. This resulted in the substitution of only three amino acids and the insertion of two, which restored the proline-rich domain, a conserved region believed essential to viral replication, at the middle of Nef. Thus, the growth dominance of the revertant virus, compared with the original *nef* mutant, was directly demonstrated *in vitro* using serial passages consisting of mixed HIV-1 populations in a single cell. © 1996 Academic Press, Inc.

The *nef* gene of human immunodeficiency virus type 1 (HIV-1) is necessary for efficient viral replication in peripheral blood mononuclear cells and other cell lines (1, 2). In addition, the *nef* gene is essential for the pathogenicity of simian immunodeficiency virus in rhesus macaques by maintaining a high virus load *in vivo* (3), and for efficient replication in severe combined immunodeficiency mice (4). However, the precise mechanism for these observations is largely obscure.

Two major molecular functions of the *nef* gene product have been clarified. One is downregulation of the receptor, CD4 (5), which is also downregulated after HIV-1 infection by other viral gene products such as Env (6) and Vpu (7). However, the mechanism of CD4 downregulation by Nef is different from those by Env or Vpu (8, 9). Thus, CD4 downregulation by Nef prevents superinfection at the acute phase of infection. The other function is upregulation of viral infectivity (10). This phenomenon is believed to be due to the increased amount of viral DNA synthesis by viral reverse transcriptase (11, 12). Using several *nef* mutant viruses, the functional Nef domain for CD4 downregulation has been mapped to the N-terminal region, whereas the effect on viral replication is located in the extreme N-terminal region and the conserved PxxP motif in central region (10, 13).

In this study, we examined the appearance of revertants during serial passage of HIV-1 with a frameshift mutation at the 5′-prime side of the *nef* gene in the MT-4 cell line. Cells that reacted with antibodies to the Nef central region were first detected at the 23 passage and became predominant within 5 further passages. Nucleotide sequencing of the cellular *nef* revealed an insertion of two adenine residues at a site immediately after the initially introduced

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Abbreviations used are: HIV-1, human immunodeficiency virus type 1; wt, wild-type; IF, immunofluorescence.

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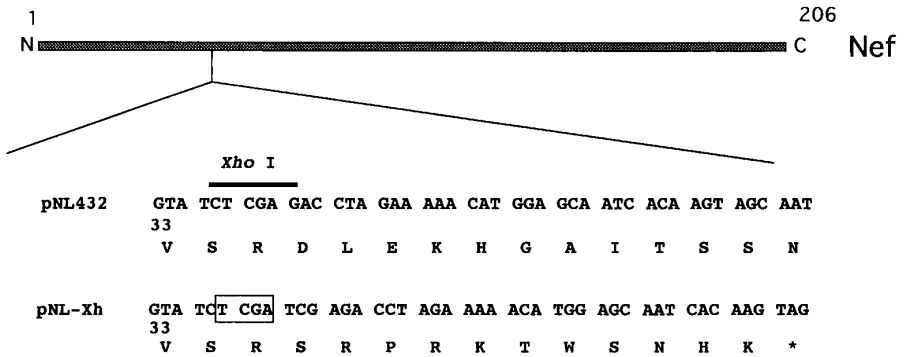

FIG. 1. The nucleotide and the amino acid sequences of *nef* in pNL432 (wt) and pNL-Xh (*nef* mutant). The thick bar on the nucleotide sequence shows the *Xho* I site in which frameshift was introduced in pNL-Xh, as shown by boxed nucleotides. ''*'' shows the termination codon (TAG) which would be on frame by the frameshift in pNL-Xh.

frameshift site. This insertion would recover Nef protein with the same amino acid residues as wild-type, except for only 5 amino acid residues.

MATERIALS AND METHODS

Cells and viruses. The M10 clonal cell line (14), derived from the MT-4 cell line (15), was cultured in complete medium (RPMI-1640 medium supplemented with 10% fetal bovine serum) at 37°C in a CO_2 incubator. The inocula for wild-type (wt) or *nef* defective (Xh) HIV-1 were the conditioned media from SW480 cells transfected as described (16), with the pNL432 plasmid containing full-length HIV-1 DNA (17) and pNL-Xh (18), respectively. The pNL-Xh plasmid was constructed from pNL432 by inserting a frameshift mutation of four bases at the *Xho* I site, yielding Nef protein in pNL-Xh of only 35 amino acid residues (Fig. 1).

Infection with HIV-1. HIV-1 was serially passaged as described (19). Briefly, M10 cells were infected with wt and Xh viruses. After 3 to 4 days, HIV-1 in the conditioned media was serially passaged up to 50 times. Virus infection was achieved as follows. M10 cells were infected with wt or Xh virus at a multiplicity of infection of 0.01 for 1 h. After washing, the cells were adjusted to a density of 5×10^5/ml in complete media and cultured at 37°C. The cells were counted every 2 to 3 days by Trypan blue exclusion and adjusted to a density of 5×10^5 cells/ml in complete media. Viral spread was confirmed by the immunofluorescence (IF) test, as described (19).

Western blotting. Cells (1×10^7/ml) cultured for 3 to 4 days after acute HIV-1 infection were lysed in SDS sample buffer (New England Biolabs). Aliquots (10 μl) were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes (Millipore). Nef protein was detected using an anti-Nef monoclonal antibody (56D5) obtained from a BALB/c mouse immunized with recombinant Nef protein (20).

PCR amplification and nucleotide sequencing. Total cellular DNAs were extracted from acutely infected cells as described (19). Viral sequences were amplified by 30 cycles of 1 min at 94°C, 2 min at 60°C and 2 min at 72°C, using a PCR kit (Perkin Elmer Cetus) with primers NI5 (5′-TAGGGATCCAGATGGGTGGCAAGTGGTCA-3′ at nucleotides 8785 to 8804) and NT3 (5′-AGTCTGCAGAAGTCCCTTGTAGCAAGCTCG-3′ at nucleotides 9431 to 9411), as described (20). The underlined portions of the primers indicate the restriction sites of *Bam* HI and *Pst* I, respectively. The PCR products were directly cloned into the pCR II vector (Invitrogen). Nucleotides were sequenced by dideoxynucleotide chain termination using the Sequenase version 2.0 kit (USB).

RESULTS

Expression of Nef proteins reactive with anti-Nef antibody in Xh passaged virus-infected cells. The *nef* mutant virus (Fig. 1) causes a frameshift to generate a new termination codon at codon 47, resulting in Nef truncation (first 35 amino acids out of the 47 amino acids are identical to wt Nef protein). The Xh and wt viruses were serially passaged up to 50 times (named Xh-1 to -50 and wt-1 to -50, respectively) in M10 cells. Viral replication was measured by IF using an anti-Gag p24 monoclonal antibody (V107) (21). In addition, Nef protein expression was also measured by IF using the 56D5 antibody recognizing the central region of Nef. Epitope mapping of this 56D5 using several truncated recombinant Nef and Nef-related

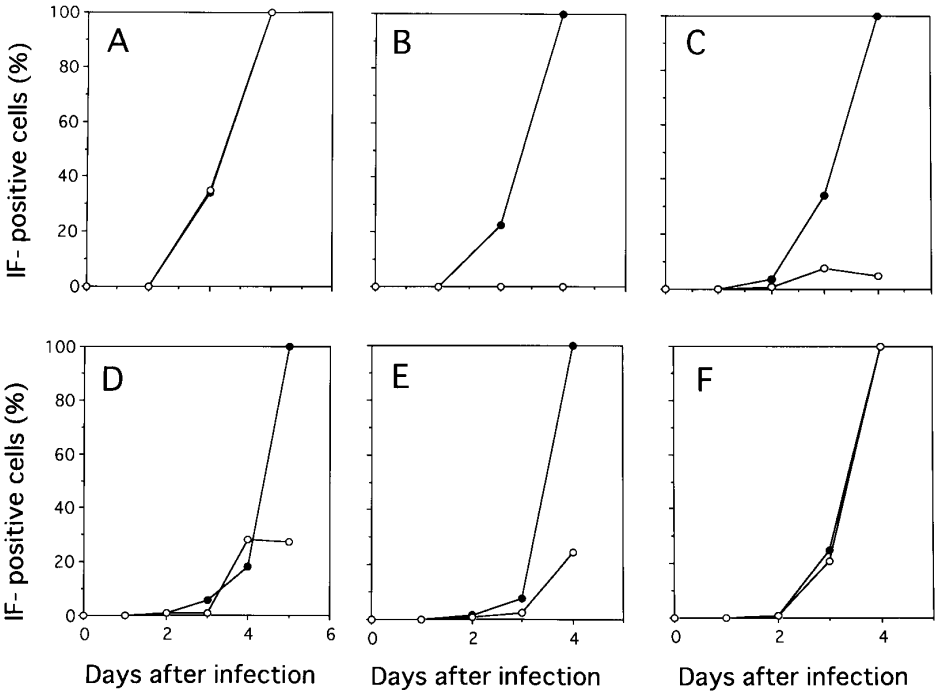

FIG. 2. Kinetics of the appearance of Nef antigen-positive cells after infection with passaged Xh virus. M10 cells were infected with wt-23 (A), Xh-17 (B), Xh-23 (C), Xh-25 (D), Xh-26 (E), and Xh-27 (F) at the same multiplicity of infection, 0.01. On every 2 days after infection, the cells were smeared and fixed in cold acetone. The IF test was performed using anti-Gag (V107, ●) or anti-Nef (56D5, ○) monoclonal antibody. The results are shown by the percentages of the positive cells.

synthetic peptides as described (22) revealed the region of amino acid residues 117 to 128 (not shown).

Figure 2 shows the populations of either Gag or Nef antigen-positive cells among those infected with wt or Xh virus. The populations of Gag-positive cells infected with wt-23 were similar to those of Nef-positive cells at every time point after the infection (Fig. 2A), while in cells infected with Xh-17 virus (Fig. 2B) or at a lower passage number (not shown), no Nef-positive cells were detected even when almost all the cells expressed Gag antigen. Nef-positive cells were first detected in the cells infected with Xh-23 (Fig. 2C). Thereafter, the IF-positive cells gradually increased with passage (Fig. 2D and E) and accounted for almost 100% of the cells infected with Xh-27 (Fig. 2F). The kinetics of the appearance of Nef-positive cells were similar to those of Gag-positive cells among those infected with Xh-27, indicating that almost all the cells expressed both Gag and Nef antigens. Almost 100% of the infected cells were Nef-positive between 27 up to 50 passage (not shown). We examined the expression of Nef protein in acutely infected cells by Western blotting (Fig. 3). Total cellular proteins extracted from acutely infected cells were separated by SDS-PAGE, then Western-blotted with 56D5. The antibody reacted with an apparently single 27 kDa protein in wt-23-infected cells (Fig. 3, lane 1), but not in those infected with Xh-17 and -23 (Fig. 3, lanes 2 and 3). A faint band was first detected in Xh-25-infected cells (Fig. 3, lane 4). The amount of 56D5-reactive protein increased in infected cells with advanced passages of Xh (Fig. 3, lanes 4 to 6), being at a similar level in Xh-27- and wt-infected cells (Fig. 3, lane 6 versus lane 1). The molecular weights of the proteins reactive with 56D5 in cells infected with Xh and wt viruses were

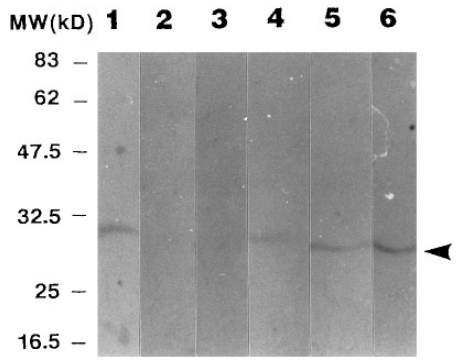

FIG. 3. Western blot analysis of Nef protein in cells infected with passaged Xh virus. The cells incubated for 3 or 4 days after infection with wt-23 (lane 1), Xh-17 (lane 2), Xh-23 (lane 3), Xh-25 (lane 4), Xh-26 (lane 5) and Xh-27 (lane 6) were lysed in SDS-PAGE sample buffer. The aliquots were separated in SDS-PAGE. The Nef proteins in these cells were analyzed by Western blotting using anti-Nef monoclonal antibody, 56D5. The arrowhead shows the position of wt Nef protein.

similar (Fig. 3, lane 1 versus lanes 4 to 6). These results were essentially consistent with the IF observations.

Genetic analysis of Xh passaged virus. The 56D5 reactive Nef protein that appeared during serial passage of *nef* frameshift mutant was not due to contamination with wt virus during serial passage of Xh virus, since every passage of Xh conserved the frameshift mutation originally introduced at *Xho* I site (not shown). This finding, together with the IF observation using 56D5, indicated that revertant Nef expression in Xh passage-infected cells could be due to a mutation(s) at downstream of the *Xho* I site of *nef* gene.

To define the induced mutation in Xh virus, we amplified and cloned the *nef* genes in the Xh-29-infected cells, which mostly expressed 56D5-reactive Nef protein (not shown). The sequence results revealed the insertion of two adenines in a cluster of five starting from nucleotide 8903 in 5 of 7 clones examined (as shown by the result of 29-1 clone as a representative in Fig. 4A and 4B). This insertion recovered the truncation of the Nef protein in Xh virus with a frameshift at the *Xho* I site (Fig. 1 and 4B), which produced Nef protein with the identical amino acid sequence to that of wt Nef protein, except for 5 amino acid residues at 36 to 41 (Fig. 4C). Thus, the translated protein should be of a size similar to that of wt Nef protein, as shown in Fig. 3. There were no other mutations such as substitution, insertion or deletion that affected Nef expression (Fig. 4B). Consequently, the insertion of two adenines seemed to be the major mutation that brought about the expression of the revertant Nef protein.

DISCUSSION

The *nef* gene is essential for the progression of disease in simian immunodeficiency virus-infected monkeys, by maintaining a high virus load *in vivo* (3). The population in monkeys infected with the virus carrying a premature termination codon in the *nef* gene shifts to *nef*$^+$ by back mutation at the termination codon, leading to disease progression. In this report, we showed that a similar back mutation, with the concomitant acquisition of a growth advantage, occurred *in vitro* using serial passages of HIV-1 with frameshift mutation at *nef.*

During serial passages of *nef* mutant, a mutation occurred at downstream of the *Xho* I site where a frameshift was originally introduced. This second mutation leading to the reading frame of intact Nef protein, was first detected in cells infected with Xh-23 (Fig. 2). Thereafter, the expression of revertant Nef protein increased by infection with advanced passages, finally accounting for almost 100% of Xh-27-infected cells (Fig. 2). These results indicated that the

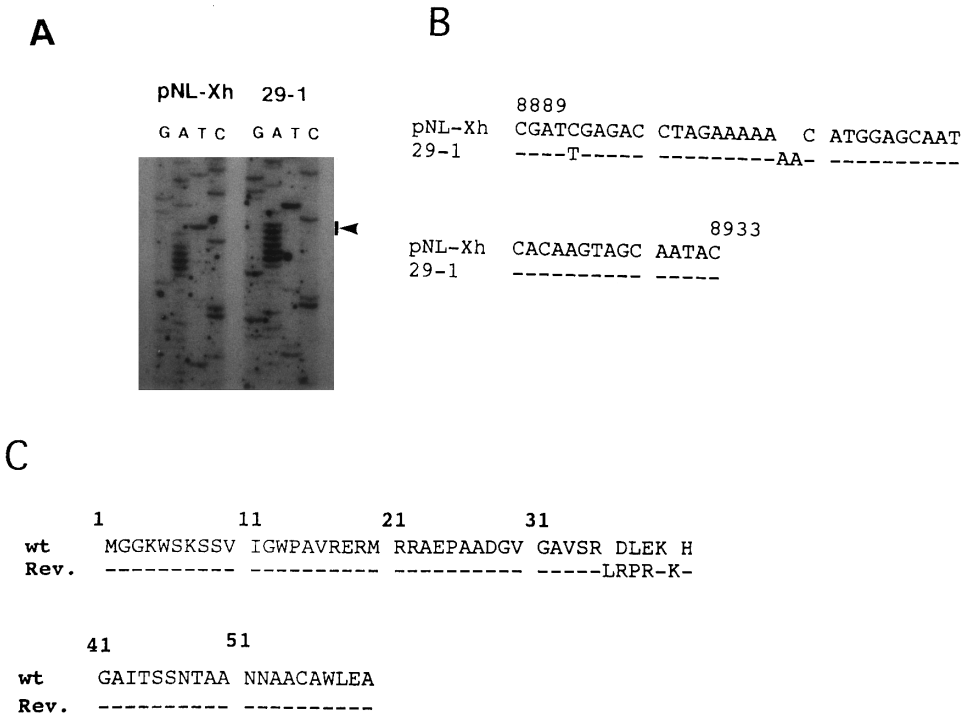

FIG. 4. Nucleotide sequences of *nef* gene in proviral DNA from passaged Xh virus-infected cells. A, An *nef* DNA clone (29-1) was obtained from provirus DNA in Xh-29-infected cells. The sequence result of 29-1 with two adenine insertion which detected immediately at downstream of the initially introduced frameshift site (as indicated by arrowhead) is comparatively shown with that of original pNL-Xh. B, The nucleotide sequence around the initially introduced frameshift site in 29-1 was compared with that in wt. ''-'' indicates the same nucleotide as in pNL-Xh. C, Deduced amino acid sequence at N-terminal region of the revertant (Rev.) is comparatively shown with that of wt. ''-'' indicates the same amino acid in wt.

nef revertant had a significant advantage in viral replication compared with the original *nef* mutant. This finding is consistent with several recent studies, which showed by comparison of growth rates between wild-type and *nef* mutant viruses, that Nef is required for efficient viral replication in primary T lymphocytes (1, 2). Our culture system may be a useful model for understanding whether the human situation is similar to that in monkeys carrying a high virus load of nef^+ compared with nef^- (3). Mutational analyses have shown that the membrane-targeting sequences at the extreme N-terminal region and a conserved PxxP repeat in the central region of Nef protein are important for efficient virus replication (13, 23). These results are also consistent with the growth dominance of the *nef* revertant isolated here, since revertant Nef had the same nucleotide and amino acid sequences in both motifs as in wt (Fig. 4).

Although IF showed that almost 100% of the infected cells expressed 56D5-reactive Nef antigen, the virus population containing the insertion at *nef* was relatively lower (5 of 7 DNA clones examined) (Fig. 2). This discrepancy might be due to the fact that multicopies of HIV-1 genome are present in a single cell (24, 25). Because Nef protein functions for the downregulation of the major viral receptor CD4 from the cell membrane, and prevents subsequent superinfection (26), we speculate that superinfection would frequently occur in infected cells lacking Nef expression. Consequently, the phenotype of the cells carrying both the revertant and original frameshift mutant might be dominated by the former in terms of viral replication rate.

The site generating a revertant of Xh corresponds to the *nef* variable region identified in primary isolates (27). Duplications in the region were also detected in infected rhesus monkeys (28) or patients (29-31), with a possible link to pathogenicity (29). It is notable that the back mutation can be caused solely by selection for HIV-1 growth dominance with the wild-type *nef* structure at the single cell level, without *in vivo* effects such as immune responses.

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