JOURNAL OF VIROLOGY, Jan. 1995, p. 93–100 Vol. 69, No. 1
0022-538X/95/$04.00+0
Copyright © 1995, American Society for Microbiology

Characterization of *nef* Sequences in Long-Term Survivors of Human Immunodeficiency Virus Type 1 Infection

YAOXING HUANG, LINQI ZHANG, AND DAVID D. HO*

Aaron Diamond AIDS Research Center, New York University School of Medicine, New York, New York 10016

Received 15 July 1994/Accepted 7 October 1994

Studies with the simian immunodeficiency virus have shown that *nef* deletion results in a low level of viremia and a lack of disease progression in monkeys. Given the similarity of this clinical profile to that observed in long-term survivors of human immunodeficiency virus type 1 (HIV-1) infection, we sought to examine the *nef* gene in 10 patients who are clinically healthy and immunologically normal despite 12 to 15 years of infection. PCR and DNA sequencing were used to determine *nef* sequences in peripheral blood mononuclear cells obtained from long-term survivors. We found that there is no gross deletion within *nef* in the cases studied; most *nef* sequences (91.1%) obtained from 10 subjects contained a full-length and intact open reading frame. In addition, at the protein level, there were no discernible differences between the Nef consensus sequences derived from long-term survivors and those from patients with AIDS. We therefore conclude that deletion of or gross sequence abnormality within *nef* is not likely to be a common explanation for the well-being of long-term survivors of HIV-1 infection. Moreover, phylogenetic analysis of *nef* sequences suggests that HIV-1 strains found in our study subjects do not have a common origin.

The *nef* gene of human immunodeficiency virus type 1 (HIV-1), HIV-2, and simian immunodeficiency viruses (SIV) is unique to primate lentiviruses, having no counterpart in other animal lentiviruses. Conservation of this coding region in HIV and SIV suggests a close evolutionary relationship between these primate lentiviruses as well as an important role for *nef* in viral infection and pathogenesis (8, 33, 39). The *nef* gene product of HIV-1 is encoded by an open reading frame located at the 3′ end of the genome, overlapping the U3 region of the 3′ long terminal repeat (LTR) (25, 33). Nef is expressed early in the viral replication cycle from multiply spliced mRNA transcripts (21, 37). Although Nef is primarily contained within the cytoplasm, it is also partly membrane associated due to myristylation of this 27-kDa protein at the N terminus (30, 47).

Considerable efforts have been made to understand the functional properties of Nef in viral replication and pathogenesis. Initial studies described *nef* as a negative regulatory factor, suppressing both viral replication and transcriptional activity of the LTR and thereby helping to maintain viral latency (1, 6, 25, 32). However, these results were not confirmed by subsequent investigations (15, 22). In fact, recent reports have demonstrated a positive effect of *nef* on the rate of HIV-1 replication in primary peripheral blood mononuclear cells (PBMC) (11, 29, 42, 48) as well as in certain T-cell lines (7). In addition, expression of *nef* in human T cells has been shown to down-regulate surface CD4 antigen expression and block interleukin-2 induction (13, 26, 27).

Substantial sequence polymorphism has been detected among *nef* genes from various cloned isolates of HIV and SIV (3, 10, 16, 31, 33). In most of the viruses studied, *nef* is intact, whereas in others, a premature stop codon is present. An analysis of *nef* sequence variation among isolates derived from patients with AIDS has been described recently (40). On the basis of this study, four stretches of residues have been identified that were highly conserved not only among the *nef* sequences derived from patients with AIDS but also among the *nef* sequences of HIV-2 and SIV. There appears to be a strong selective pressure in vivo for retention of these highly conserved sequences of *nef* as well as for an open reading frame.

The importance of *nef* expression in disease pathogenesis was convincingly demonstrated by experiments in which rhesus monkeys were infected with a SIV containing mutated *nef* sequences (20). The *nef* mutant containing a premature stop codon rapidly reverted to a full open reading frame in vivo and caused disease, whereas the *nef*-deleted virus replicated to only low levels and did not produce clinical disease or pathological changes. These findings suggested that *nef* can be considered a virulence factor in the pathogenesis of primate immunodeficiency virus infection, with deficiencies in *nef* causing viral attenuation.

The natural history and pathogenic process of HIV-1 infection in humans are complex and variable, depending on a multitude of viral and host factors and their interactions (19, 43–45). Host factors may result in a differential susceptibility to viral infection and its pathogenic effects, whereas HIV-1 variation may account for differential viral virulence and disease course. Although most HIV-1-infected persons develop AIDS-related symptoms within 10 years after seroconversion, there exists a small population of infected individuals, termed long-term survivors, who remain clinically healthy and immunologically normal for more than a decade (36a). Interestingly, the clinical characteristics of HIV-1 infection in these individuals are very similar to those observed in rhesus monkeys infected with *nef*-deleted SIV. Therefore, this similarity led us to determine whether these long-term survivors are infected with viruses that are attenuated because of defects in *nef*.

In the present study, we have directly isolated and analyzed *nef* alleles from clinical specimens from long-term survivors of HIV-1 infection by nested PCR and DNA sequencing. We have observed that there is no obvious deletion within *nef* in the 10 cases studied; the vast majority of *nef* sequences contained an intact *nef* open reading frame. In comparison with sequences published in the database, there is also no discernible difference between the *nef* consensus sequences

* Corresponding author. Mailing address: Aaron Diamond AIDS Research Center, New York University School of Medicine, 455 First Ave., New York, NY 10016. Phone: (212) 725-0018. Fax: (212) 725-1126.

TABLE 1. Characteristics of long-term survivors of HIV-1 infection

Subject	Sex	Risk factor	Duration of infection (yr)	CD4 cell count (no./mm^3)	HIV-1 DNA copies/ 10^6 PBMC	Isolation of HIV-1 from:	
						PBMC	Plasma
E	M	Homosexual	15	600–1,200	16	−	−
SF	M	IVDU[a]	≥12	500–700	20	−	−
LSS	M	Homosexual	≥12	560–740	21	−	−
RR	M	Homosexual	14	500–1,200	33	−	−
RP	M	IVDU	≥12	800–1,000	44	−	−
LM	F	Heterosexual	≥13	500–850	50	+[b]	−
BO	M	Homosexual	12	560–860	76	−	−
DH	M	Homosexual	15	500–700	94	+	−
D	M	Homosexual	≥14	600–1,100	296	+	−
CD	M	Homosexual	≥14	550–850	1,783	+	−

[a] IVDU, intravenous drug use.
[b] By CD8 depletion only.

obtained from patients with AIDS and those obtained from our long-term survivors.

MATERIALS AND METHODS

Study subjects. Ten long-term survivors were chosen on the basis of their asymptomatic status and normal CD4 cell counts despite 12 to 15 years of HIV-1 infection. Their clinical profiles have been presented elsewhere (5) and partially summarized in Table 1.

Samples. PBMC were isolated from each subject by centrifugation of blood through a Ficoll-Hypaque density gradient (Pharmacia). DNA was then extracted by standard methods (35) from purified PBMC. Sequential blood samples from one subject (D) were also obtained over a 5-year period, including June 1985, September 1988, and March 1990. Plasma samples from each individual were also cultured for infectious HIV-1 (5).

PCR amplification of viral *nef* sequence and cloning. Because HIV-1 proviral DNA is often present in low copy numbers, a nested-primer PCR amplification method was employed to amplify proviral sequences. Two sets of primers were designed, and the primer-binding sites flanking the HIV-1 *nef* gene were chosen to be as highly conserved as possible. The primer sequences are given, with their position in the HIV-1 clone HXB2 genome indicated in parentheses. The outer primers were 5′-GTAGCTGAAGGGACAGATAGGGTTAT-3′ (8687 to 8712) and 5′-GCACTCAAGGCAAGCTTTATTGAGGC-3′ (9631 to 9605); the inner primers were 5′-CGTCTAGAACATACCTAGAAGAATAAGACAGG-3′ (8748 to 8768) and 5′-CGGAATCCGTCCCCAGCGGAAAGTCCCTTGTA-3′ (9552 to 9429). The inner primers included restriction sites for *Xba*I and *Eco*RI (underlined). From each sample, 1 to 2 μg of template DNA was used in the first PCR. Briefly, PCR was performed by denaturation at 94°C (1 min), hybridization at 55°C (1 min), and extension at 72°C (1.5 min) for 30 cycles under conditions recommended by the manufacturer (Perkin Elmer). Subsequently, amplification reaction mixtures were diluted 20-fold with the reaction buffer, and the second round of PCR with inner primers was performed for another 30 cycles as described above. Amplified *nef* sequences were purified with the Magic PCR Preps DNA purification system (Promega), digested with *Xba*I and *Eco*RI, and subcloned into the M13 mp18 vector. To improve sampling, PCR products of each sample were generated from two independent amplifications prior to cloning.

Length analysis of PCR products. To investigate length variation in *nef*, PCR products from the second amplification were subjected to agarose gel electrophoresis. Migration distances were visualized by ethidium bromide staining of agarose gel. CEM cells persistently infected with the HIV-1 clone HXB2 were used as a reference size control.

Measurement of proviral load in PBMC. Proviral DNA was quantified by a limiting-dilution and nested-PCR method described previously (41). Oligonucleotides corresponding to the *env* gene of the HIV genome were used in the quantitation assay. Proviral DNA was initially diluted to an end point at which less than 25% of the subsequent PCR products were positive. The number of proviral copies was then estimated by $-\ln[F]$, where F is the fraction of negative reactions, assuming that the incidental appearance of a positive PCR product follows a Poisson distribution. Positive and negative controls were included in all quantitation assays performed.

Sequencing and sequence analysis. Cloned PCR products were sequenced by the Sequenase protocol (United States Biochemical Corp.). Nucleotide sequences were aligned with the Clustal V program, which was kindly provided by D. G. Higgins (17). Genetic distances between pairs of sequences were estimated with the DNADIST program implemented in the PHYLIP package (version 3.4) (12). Phylogenetic trees were constructed with several algorithms, including neighbor joining and parsimony. Similar phylogenetic relationships were also found in 1,000 bootstrap replicates of neighbor-joining trees (PHYLIP programs SEQBOOT and CONSENSE).

Nucleotide sequence accession numbers. The *nef* sequences described in this study have been deposited in GenBank; the accession numbers are U16863 to U16952.

RESULTS

Characteristics of the study population. Table 1 summarizes the characteristics of long-term survivors of HIV-1 infection, all of whom have been seropositive for 12 years or more. None has developed AIDS-related symptoms or $CD4^+$ T-cell decline over the course of infection. Details of their clinical characteristics are described by Cao et al. (5).

In addition, most subjects at the time of sample collection demonstrated a very low viral load. By a limiting-dilution PCR quantitation method, we were able to detect provirus in infected individuals down to a level of single molecule per reaction (41). The number of proviruses quantitated in this study population ranged from 16 to 296 copies per 10^6 PBMC except for the sample from subject CD, which yielded more provirus DNA copies (1,783/10^6 PBMC). The number of proviruses in subject D reflected the mean value of three sequential samples taken over a 5-year period. Independent determination of viral load was also carried out by quantitative culture (5). By the routine virus isolation method, samples from subjects DH, D, and CD were positive, whereas HIV-1 was recovered from PBMC of subject LM only after $CD8^+$ T-cell depletion. Extensive efforts to culture HIV-1 from the other subjects were unsuccessful, and no virus was isolated from plasma samples in this study population.

Study of the length polymorphism in the *nef* region of long-term survivors. DNA was extracted from PBMC obtained from 10 long-term survivors, and sequences spanning *nef* were amplified by PCR. CEM cells persistently infected with the HXB2 clone, which contains a full-length *nef*, were used as a reference size control. PCR products from the second round of amplification (approximately 750 bp long for the HXB2 control) were visualized by ethidium bromide staining after agarose gel electrophoresis (Fig. 1). All patient samples yielded positive results in PCR. The positive bands of samples from subjects CD, BO, and RR migrated slightly more slowly than that of the control HXB2 clone (Fig. 1). Nevertheless, in general, PCR products of *nef* amplified from our long-term survivors were of approximately the same length as that of the reference HXB2 control. Thus, no gross *nef* deletion was observed in this agarose gel mobility assay. In addition, intrasample length polymorphism was not detectable in any of the samples examined. No length variation was seen for subject

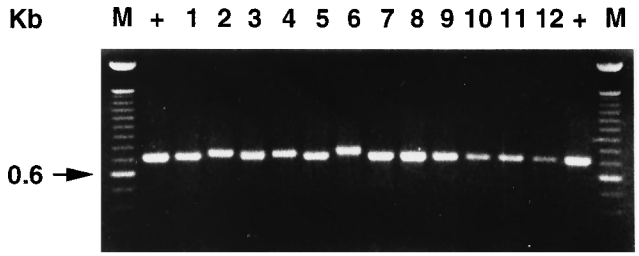

FIG. 1. Analysis of length polymorphism of the *nef* gene in the PBMC of long-term survivors. DNA fragments from the second PCR amplification were run on a 1.5% agarose gel and visualized by ethidium bromide staining. Lanes 1 to 9 represent the PCR products from samples from subjects DH, RR, LM, BO, LSS, CD, SF, RP, and E, respectively. Sequential samples obtained from subject D (D85, D88, and D90) are represented by lanes 10, 11, and 12, respectively. The positive control (lane +) consists of CEM cells persistently infected with the HIV-1 clone HXB2 (containing a full-length *nef*). Lane M contains a 100-bp ladder size marker (Gibco BRL).

D over a 5-year period. In multiple, independent PCR amplifications, these parameters remained consistent. All 10 samples were also subjected to sequence analysis (see below), the results of which supported the conclusions derived from agarose gel electrophoresis.

Nucleotide sequence variation in *nef*. In order to delineate the genetic features of *nef* in detail, a total of 90 full-length *nef* nucleotide sequences were obtained from 10 long-term survivors. Seventeen clones were derived from subject D (including sequential samples at three time points), 11 were derived from subject E, 13 each were derived from subjects SF and RP, and 6 each were derived from subjects BO, CD, DH, LM, LSS, and RR (sequences are not shown but have been deposited in GenBank). Consistent with observations from the agarose gel analysis, there were interpatient length variations, although intrasample length polymorphism was not observed in most of the subjects studied. Subject CD had the longest (648 bp) *nef* sequences, whereas subject LSS had the shortest (618 bp) *nef* sequences among the 10 subjects. The length variation is contributed mainly by the variation in a previously defined variable region near the 5′ end of *nef*. Nevertheless, all the length variations are in-frame changes and do not cause any frameshift or premature termination. Intrasample length polymorphism has been observed only in subject BO; in six sequenced clones, two were shorter by a stretch of six nucleotides, which resulted in the in-frame deletion of two amino acid residues within the variable region.

The degree of intrasample sequence diversity in long-term survivors varies from individual to individual. The upper panel of Table 2 shows the mean intrasample diversity of *nef* in long-term survivors, ranging from 0.04% in subject SF to 3.54% in subject DH. Furthermore, the intrasample sequence variation appears to be positively correlated with the number of distinct sequence variants observed. In other words, a greater genetic distance is associated with a greater number of sequence variants. For example, the genetic distance is higher in DH (3.54%) than in SF (0.04%); accordingly, the number of distinct sequence variants versus the total number of sequences obtained is greater in DH (5/6) than in SF (3/13). Similar correlations are seen for other subjects.

Temporal changes in genetic diversity within a single individual were observed for subject D. Five sequences were obtained from the sample collected in 1985, and six were obtained for each of the 1988 and 1990 samples. The intrasample diversity was 1.7% in 1985, 2.35% in 1988, and 1.53% in 1990 (Table 2). Interestingly, the intersample distance between 1985 and 1990 (2.4%) was less than that between 1985 and 1988 (2.75%), suggesting that the genetic distance over time is not necessarily additive.

TABLE 2. Comparison of Nef sequence variation between long-term survivors and patients with AIDS

Group (ref.)	No. of clones	Mean intrasample diversity (%)[a]
Long-term survivors		
BO	6	1.52
CD	6	1.83
D85	5	1.70
D88	6	2.35
D90	6	1.53
DH	6	3.54
E	11	1.12
LM	6	2.99
LSS	6	1.53
RP	13	0.06
RR	6	1.67
SF	13	0.04
		$\bar{x}$ = 1.65 (SD = 1.0)
Patients with AIDS (40)		
248	2	0.98
233	2	3.30
227	2	0.00
171	3	2.21
166	4	2.02
164	6	1.57
102	6	2.39
179	3	3.13
226	2	1.46
		$\bar{x}$ = 1.89 (SD = 1.0)

[a] SD, standard deviation.

The degree of intrasample genetic diversity obtained from long-term survivors was compared with those from patients with AIDS (Table 2). In long-term survivors, the mean genetic diversity was 1.65%, whereas in the patients with AIDS reported by Shugars et al. (40), the overall average was 1.89%. The differences in overall genetic distance between these two groups are statistically insignificant, suggesting that the degree of sequence variation in *nef* is not likely to be associated with the stage of HIV-1 infection.

Phylogenetic relationship of *nef* sequences. Several phylogenetic approaches have been applied to the analysis of *nef* nucleotide sequences (see Materials and Methods). Figure 2 depicts the neighbor-joining tree for *nef* sequences from 10 long-term survivors and from nine patients with AIDS studied by Shugars et al. (40). For clarity and ease of generating the tree, only those *nef* sequences representing the different variants of each sample from patients with AIDS and from long-term survivors are included in the phylogenetic analysis. Individual sequences are represented by their names at the end of each branch, which are drawn in accordance with their relative genetic distances.

The most obvious and consistent result from the phylogenetic analysis was that nucleotide sequences from long-term survivors interdigitated with those from patients with AIDS without apparent clustering among themselves (Fig. 2). The *nef* sequences from subject SF (SF17, SF18, and SF10), for instance, are more similar to a sequence from a patient with AIDS (248a) than to those from long-term survivors such as LM and CD. Furthermore, another feature of *nef* sequences from long-term survivors is their tight grouping for each sample. Although sequences obtained from subject D span 5

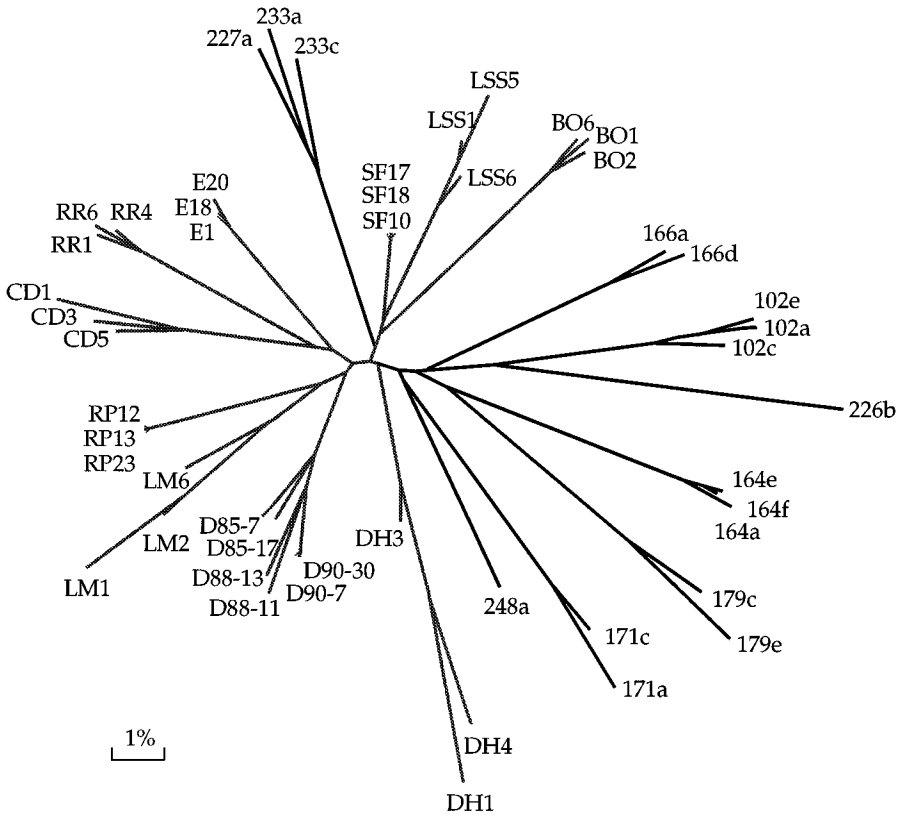

FIG. 2. Phylogenetic relationship of *nef* nucleotide sequences from patients with AIDS and long-term survivors. The phylogenetic tree was constructed by the neighbor-joining method (see Materials and Methods). Letter codes (e.g., BO) represent the sequences derived from the long-term survivors. Number codes (e.g., 248) represent sequence data from patients with AIDS obtained from Shugars et al. (40). All branch lengths are drawn in accordance with their relative genetic distances.

years, they still group tightly in the phylogenetic tree; similar observations can be made for the remaining subjects. The *nef* sequences are all specific to an individual, without any evidence for a common origin.

Features of deduced Nef amino acid sequences derived from long-term survivors. To investigate the features of amino acid sequences of Nef derived from long-term survivors of HIV-1 infection, 90 *nef* nucleotide sequences from 10 study subjects were translated to amino acid sequences (Fig. 3). Analysis of Nef protein sequences revealed that only eight were obviously defective, due to either the appearance of a premature stop codon (BO4, D85-17, DH1, E28, LSS4, RP23, and RR5) or the lack of an initiation codon (RP17). Thus, the overall defective rate was 8.9% in 90 *nef* alleles derived from long-term survivors, which approximated that found in blood or pathologic tissues of patients with AIDS (8 to 11%) (3, 40). In addition, the location of defects in *nef* appeared to be randomly distributed.

It has been noted that HIV-1 Nef sequences contain several putative functional motifs. We therefore investigated whether specific changes occurred in these motifs within Nef derived from long-term survivors. To this end, Nef amino acid sequences from each subject were optimally aligned, and a consensus Nef sequence was derived from this alignment. The consensus sequences derived from 10 long-term survivors were compared with consensus Nef sequences obtained by Shugars et al. (40) from patients with AIDS (Fig. 4). In most cases, sequences in previously defined functional regions are highly conserved, although a few changes have been observed in specific regions. For instance, four of six clones in patient LM were found to have a glycine (G)-to-serine (S) change in the N-terminal myristylation signal sequence (amino acids 1 to 7). Interestingly, changes from lysine (K) to glutamine (E) in the polypurine tract (PPT) were also observed in these clones. In patient RR, five of six clones have a glycine (G) insertion in the middle of a highly acidic charged region (amino acids 71 to 75), whereas in patient LSS amino acid residue changes from threonine (T) to serine (S) were observed in the potential protein kinase C phosphorylation region (amino acids 87 to 92) in four of six clones. A $(PxxP)_3$ repeat sequence that resembles a SH3-binding site (33a) (amino acids 79 to 88) is invariant. However, another well-conserved (PxxP) SH3-binding motif located between residues 157 and 160 was found to be replaced by (PxxQ) in subject RP. A predicted beta-turn motif (GPGI/V) at position 140 to 143 is highly conserved among Nef sequences obtained from patients with AIDS. In most Nef sequences derived from four long-term survivors (D, DH, LM, and RP), this motif has been replaced by GPGT, which may still form a beta-turn (46). The functional consequences, if any, of these observed changes in the *nef* sequences derived from long-term survivors are unknown.

Four stretches of Nef sequence that were highly conserved not only among the Nef sequences obtained directly from patients with AIDS but also among HIV-1, HIV-2, and SIV isolates have been identified as Nef-defining sequences (40). Multiple consensus sequence alignment allows comparison of this Nef-defining region with the Nef sequences derived from the long-term survivors. As can be seen in Fig. 4, sequences in blocks A (amino acids 71 to 98) and D (amino acids 189 to 200) are more variable than those in blocks B (amino acids 117 to

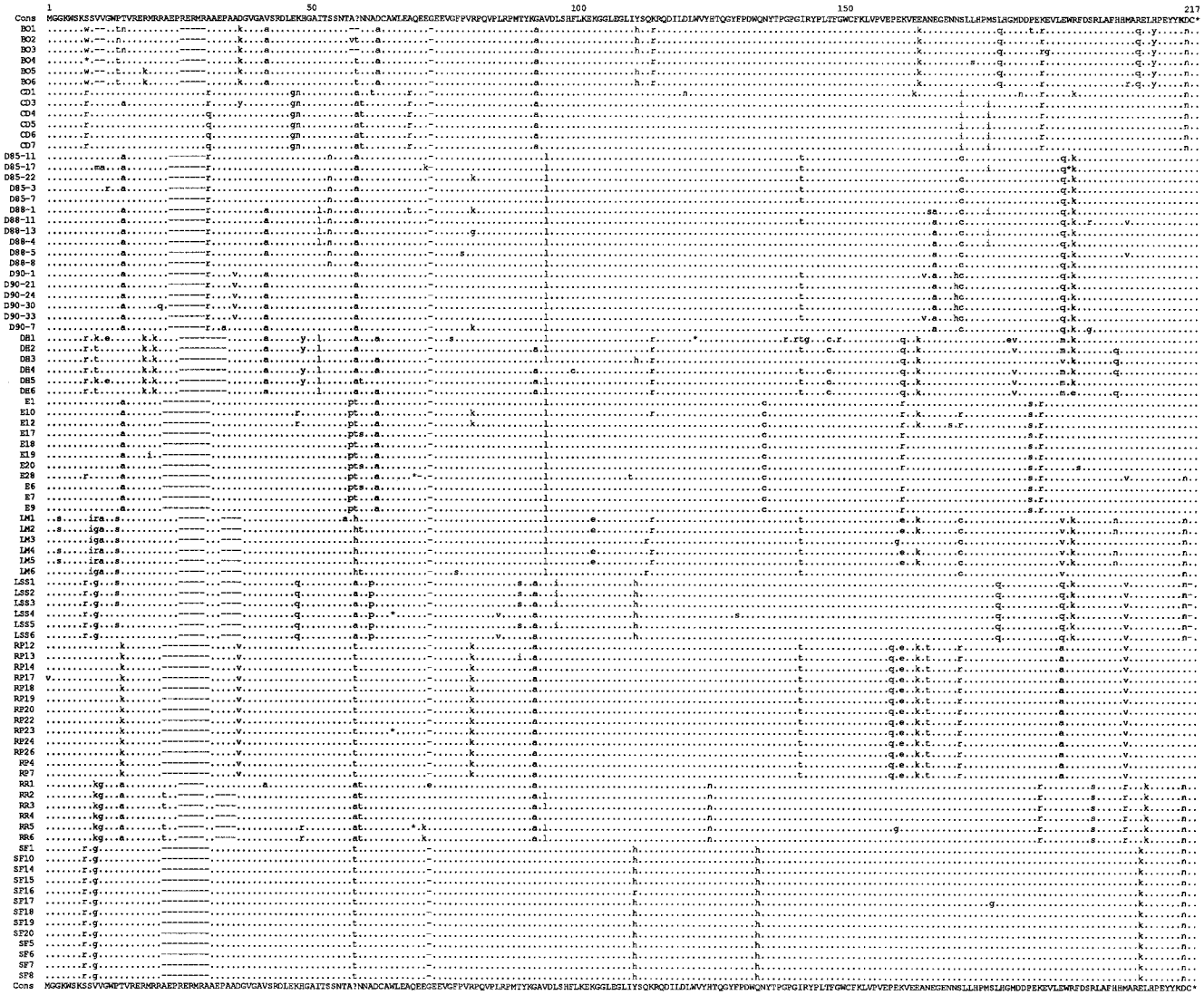

FIG. 3. Alignment of the Nef protein sequences derived from long-term survivors. Ninety clones from 10 subjects were sequenced. The deduced amino acid sequences were aligned and are shown in the single-letter code. The consensus (Cons) sequences derived from the individual clones were placed on the top and bottom of the figure. Gaps (−) were introduced to maximize homology. ·, identical protein sequences; *, position of premature termination.

124) and C (amino acids 139 to 158). Of these four blocks, block B is the most conserved among long-term survivors and patients with AIDS. A similar observation has also been made by Shugars et al. (40). The overall conservation in these four stretches in the long-term survivors suggests that their *nef* alleles are likely to be functionally equivalent.

DISCUSSION

Substantial variation in the rate of disease progression has been observed among patients infected with HIV-1. In most cases, the infected individual has an extended asymptomatic period prior to the development of AIDS, whereas some become immunosuppressed and develop opportunistic complications rapidly (2, 28). It has become evident recently from several cohort studies (4, 36a) that a small proportion (approximately 5 to 8%) of infected individuals has remained clinically healthy and without evidence of immunodeficiency despite prolonged infection. Although the precise definition of long-term survivors of HIV-1 infection has varied in the literature, these persons generally have been infected for over a decade while lacking clinical symptoms and maintaining normal and stable $CD4^+$ T-cell counts (14, 23, 24, 38).

Multiple factors, including host, viral, and environmental factors, are believed to be involved in disease progression induced by HIV-1 infection. The clinical sequela of each infection, therefore, is dependent on the fine balance of these multifactorial interactions. Although it has been suggested that intrinsic host properties such as the HLA type of an infected individual may confer a differential susceptibility to HIV-1 infection and its pathogenic effects (19, 45), our results to date suggest that $CD4^+$ T cells from long-term survivors are not inherently more resistant to HIV-1 infection in vitro (5). Characterization of host immune responses in the San Francisco Clinic Cohort has revealed substantially higher levels of HIV-1-specific antibodies and $CD8^+$ T cells in long-term survivors (24). Our group has recently found strong HIV-1-neutralizing antibodies and virus-suppressive $CD8^+$ T-cell

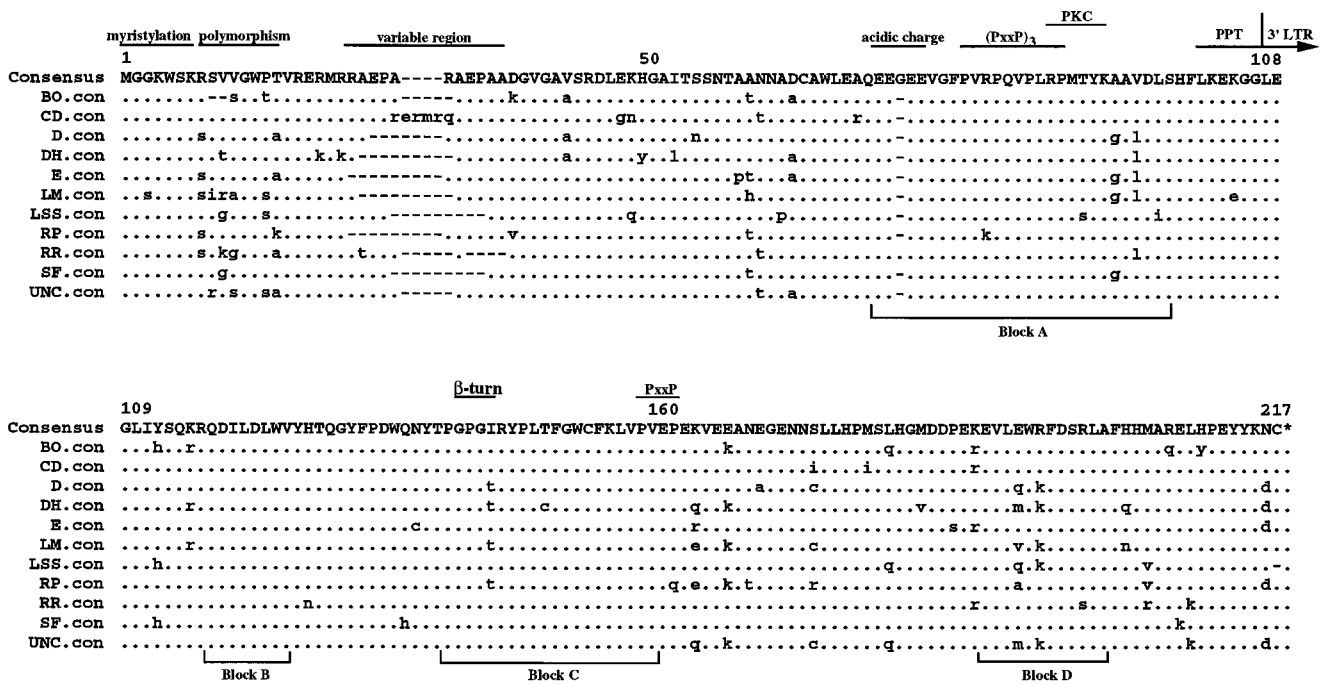

FIG. 4. Multiple consensus sequence alignment and comparison of Nef protein sequences derived from patients with AIDS and long-term survivors. UNC.Con. denotes the consensus Nef amino acid sequence obtained from patients with AIDS (40). Ten consensus sequences derived from long-term survivors are represented as BO.con, CD.con, D.con, DH.con, E.con, LM.con, LSS.con, RP.con, RR.con, and SF.Con. The top consensus sequence represents an overall predicted consensus sequence derived from this alignment. Gaps (−) were introduced to maximize alignment; · indicates amino acid identity. The location of predicted motifs of the myristylation signal, sequence polymorphism, variable region, acidic charged residues, (PxxP) repeat sequence, putative phosphorylation site (PKC), polypurine tract (PPT) 5′ border of the 3′ LTR, and beta-turn are shown above the consensus Nef protein sequence, while Nef-defining sequences, blocks A, B, C, and D, are bracketed underneath the aligned consensus sequences.

activity in our 10 subjects (5). These findings, collectively, suggest that some long-term survivors do well due in part to their vigorous immune responses.

There is ample evidence to stress the importance of viral load and phenotype in influencing disease progression to AIDS (9, 18, 34, 36, 41, 43). Two studies, in particular, strongly support the notion that viral characteristics play a critical role in long-term nonprogression. First, an astute epidemiological observation made by Learmont et al. (23) showed that six transfusion recipients of blood from an HIV-1-infected individual have remained clinically well and immunologically stable despite a decade of infection. Likewise, the blood donor has remained healthy. The existence of this cluster of long-term survivors raises the possibility that an attenuated strain of HIV-1 may have been transmitted. Second, a set of elegant experiments carried out by Kestler et al. (20) showed that monkeys experimentally inoculated with *nef*-deleted SIV exhibited no sign of disease and maintained a low viral burden along with normal $CD4^+$ T-cell counts. These characteristics are very similar to what we have seen in long-term survivors of HIV-1 infection, thereby prompting us to investigate the possibility that defects in *nef* may be responsible for the well-being of our long-term survivors.

We used nested PCR to amplify *nef* from each of the long-term survivors. Length polymorphism of the PCR product was seen; however, no gross deletion was found (Fig. 1). For the 10 subjects whose *nef* sequences were determined, all contained a full-length *nef*, although slight length variation was again noted. In examining the deduced amino acid sequences (Fig. 3), eight clones (8.9%) were found to be obviously defective due to either the presence of a premature stop codon or the lack of an initiation codon. Nevertheless, the frequency of gross *nef* defect was no different from the 8 to 11% found in patients with AIDS (3, 40). Although *nef*-truncated HIV-1 has been found by Desrosiers (11a) in a long-term survivor, our results would suggest that *nef* truncation or deletion is not likely to be a common explanation for the clinical stability of these subjects.

Several highly conserved motifs with putative functions have been identified in Nef. In comparing the amino acid sequences of Nef in our subjects, few changes were found in these regions. Therefore, we conclude that there are no major differences between Nef sequences derived from long-term survivors and those from patients with AIDS. It is possible that most HIV-1 *nef* alleles in long-term survivors are functionally intact. Indeed, preliminary biological functional analysis of *nef* alleles obtained from patients D, RP, and SF support this conclusion (unpublished results).

We have phylogenetically analyzed a total of 120 *nef* sequences, 30 of which were obtained from patients with AIDS (40) and 90 of which were from the current study. The analysis showed that, as expected, sequences from each long-term survivor were more similar to each other than to those from another subject or to those from a patient with AIDS. More importantly, the *nef* sequences derived from different long-term survivors were not clustered together phylogenetically; instead, they were randomly distributed among *nef* sequences obtained from patients with AIDS (Fig. 2). This finding suggests that HIV-1 strains found in long-term survivors do not have a common origin, as the epidemiological data had already indicated.

In sum, our results demonstrate that most *nef* alleles derived from long-term survivors in the study population are full length with an open reading frame. At the protein level, there is no

discernible difference in domains of putative functional importance within Nef between patients with AIDS and long-term survivors. Moreover, our phylogenetic analysis of *nef* sequences shows that HIV-1 strains in our long-term survivors are not linked epidemiologically and that they do not have a common origin.

ACKNOWLEDGMENTS

We thank Y. Cao for technical assistance; C. Cheng-Mayer, R. A. Koup, N. R. Landau, and J. P. Moore for critical reading of the manuscript; and W. Chen for preparation of the figures.

This work was supported by NIH grants AI24030, AI25541, AI27742, AI32427, AI27665, AI27742, and AI45218; the Ernst Jung Foundation; and the Aaron Diamond Foundation. Y.H. was supported by NIH training grant AI07180, awarded to the Department of Microbiology, New York University School of Medicine.

REFERENCES

1. **Ahmad, N., and S. Venkatesan.** 1988. Nef protein of HIV-1 is a transcriptional repressor of HIV-1 LTR. Science **241:**1481–1485.
2. **Bacchetti, P., and A. R. Moss.** 1989. Incubation period of AIDS in San Francisco. Nature (London) **338:**251–253.
3. **Blumberg, B. M., L. G. Epstein, Y. Saito, D. Chen, L. R. Sharer, and R. Anand.** 1992. Human immunodeficiency virus type 1 *nef* quasispecies in pathological tissue. J. Virol. **66:**5256–5264.
4. **Buchbinder, S. P., D. Mann, L. Louie, F. Katz, and S. D. Holmberg.** 1993. Healthy long-term positives (HLPs): genetic cofactors for delayed HIV disease progression, p. 46. *In*: IXth International Conference on AIDS.
5. **Cao, Y., L. Qing, L. Zhang, J. Safrit, and D. D. Ho.** Virological and immunological characterization of long-term survivors of human immunodeficiency virus type 1 infection. Submitted for publication.
6. **Cheng-Mayer, C., P. Iannello, K. Shaw, P. A. Luciw, and J. A. Levy.** 1989. Differential effects of nef on HIV replication: implications of viral pathogenesis in the host. Science **246:**1629–1632.
7. **Chowers, M. Y., C. A. Spina, T. Jesse Kwoh, N. J. S. Fitch, D. D. Richman, and J. C. Guatelli.** 1994. Optimal infectivity in vitro of human immunodeficiency virus type 1 requires an intact *nef* gene. J. Virol. **68:**2906–2914.
8. **Colombini, S., S. K. Arya, M. S. Reitz, L. Jagodzinski, B. Beaver, and F. Wong-Staal.** 1989. Structure of simian immunodeficiency virus regulatory genes. Proc. Natl. Acad. Sci. USA **86:**4813–4817.
9. **Connor, R. I., H. Mohri, Y. Cao, and D. D. Ho.** 1993. Increased viral burden and cytopathicity correlate temporally with CD4+ T-lymphocyte decline and clinical progression in human immunodeficiency virus type 1-infected individuals. J. Virol. **67:**1772–1777.
10. **Delassus, S., R. Cheynier, and S. Wain-Hobson.** 1991. Evolution of human immunodeficiency virus type 1 *nef* and long terminal repeat sequences over 4 years in vivo and in vitro. J. Virol. **65:**225–231.
11. **deRonde, A., B. Klaver, W. Keulen, L. Smit, and J. Goudsmit.** 1992. Natural nef accelerates virus replication in primary human lymphocytes. Virology **188:**391–395.

11a. **Desrosiers, R.** Personal communication.

12. **Felsenstein, J.** 1988. Phylogenies from molecular sequences: inference and reliability. Annu. Rev. Genet. **22:**521–565.
13. **Garcia, J. V., and A. D. Miller.** 1991. Serine phosphorylation-dependent downregulation of cell-surface CD4 by nef. Nature (London) **350:**508–511.
14. **Greenough, T. C., M. Somasundaran, D. B. Brettler, R. M. Hesselton, A. Alimenti, F. Kirchhoff, D. Panicali, and J. L. Sullivan.** 1994. Normal immune function and inability to isolate virus in culture in an individual with long-term human immunodeficiency virus type 1 infection. AIDS Res. Hum. Retroviruses **10:**395–403.
15. **Hammes, S. R., E. P. Dixon, M. H. Malim, B. R. Cullen, and W. C. Greene.** 1989. Nef protein of human immunodeficiency virus type 1: evidence against its role as a transcriptional inhibitor. Proc. Natl. Acad. Sci. USA **86:**9549–9553.
16. **Harris, M., S. Hislop, P. Pastilinacos, and J. C. Neil.** 1992. In vivo derived nef gene products are heterogeneous and lack detectable nucleotide binding activity. AIDS Res. Hum. Retroviruses **5:**537–542.
17. **Higgins, D. G., and P. M. Sharp.** 1989. Fast and sensitive multiple sequence alignments on a microcomputer. Comput. Appl. Biosci. **5:**151–153.
18. **Ho, D. D., T. Moudgil, and M. Alam.** 1989. Quantitation of human immunodeficiency virus type 1 in blood of infected persons. N. Engl. J. Med. **321:**1621–1625.
19. **Kaslow, R. D., R. Duquesnoy, M. Van Raden, L. Kingsley, M. Marrari, H. Friedman, S. Su, A. J. Saah, R. Detels, J. Phair, and C. Rinaldo.** 1990. A1, Cw7, B8, DR3 HLA antigen combination associated with rapid decline of T-helper lymphocytes in HIV-1 infection. Lancet **335:**927–930.
20. **Kestler, H. W., D. J. Ringler, K. Mori, D. L. Panicali, P. K. Sehgal, M. D. Daniel, and R. D. Desrosiers.** 1991. Importance of the nef gene for maintenance of high virus loads and/or development of AIDS. Cell **65:**651–662.
21. **Kim, S. Y., R. Byrn, J. Groopman, and D. Baltimore.** 1989. Temporal aspects of DNA and RNA synthesis during human immunodeficiency virus infection: evidence for differential gene expression. J. Virol. **63:**3708–3717.
22. **Kim, S. Y., K. Ikeuchi, R. Byrn, J. Groopman, and D. Baltimore.** 1989. Lack of a negative influence on viral growth by the nef gene of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA **86:**9544–9548.
23. **Learmont, J., B. Tindall, L. Evans, A. Cunningham, P. Cunningham, J. Wells, R. Penny, J. Kaldor, and D. A. Cooper.** 1992. Long-term symptom-less HIV-1 infection in recipients of blood products from a single donor. Lancet **340:**863–867.
24. **Lifson, A. R., S. P. Buchbinder, H. W. Sheppard, A. C. Mawle, J. C. Wilber, M. Stanley, C. E. Hart, N. A. Hessol, and S. D. Holmberg.** 1991. Long-term human immunodeficiency virus infection in asymptomatic homosexual and bisexual men with normal $CD4^+$ lymphocyte counts: immunologic and virologic characteristics. J. Infect. Dis. **163:**959–965.
25. **Luciw, P. A., C. Cheng-Mayer, and J. L. Levy.** 1987. Mutational analysis of the human immunodeficiency virus: the orf-B region down-regulates virus replication. Proc. Natl. Acad. Sci. USA **84:**1434–1438.
26. **Luria, S., I. Chambers, and P. Berg.** 1991. Expression of the type 1 human immunodeficiency virus Nef protein in T cells prevents antigen receptor-mediated induction of interleukin 2 mRNA. Proc. Natl. Acad. Sci. USA **88:**5326–5330.
27. **Mariani, R., and J. Skowronski.** 1993. CD4 down-regulation by nef alleles isolated from human immunodeficiency virus type 1-infected individuals. Proc. Natl. Acad. Sci. USA **90:**5549–5553.
28. **McLean, K. A., D. A. Holmes, and B. A. Evans.** 1990. Rapid clinical and laboratory progression of HIV-1 infection. AIDS **4:**369–371.
29. **Miller, M. D., M. T. Warmerdam, I. Gaston, W. C. Greene, and M. B. Feinberg.** 1994. The human immunodeficiency virus-1 nef gene product: a positive factor for viral infection and replication in primary lymphocytes and macrophages. J. Exp. Med. **179:**101–113.
30. **Murit, K. G., P. S. Brown, L. Ratner, and J. V. Garcia.** 1993. Highly localized tracks of human immunodeficiency virus type 1 Nef in the nucleus of cells of human $CD4^+$ T-cell line. Proc. Natl. Acad. Sci. USA **90:**11895–11899.
31. **Myers, G., J. A. Berzofsky, B. Korber, R. F. Smith, and G. N. Pavlakis.** 1992. Human retroviruses and AIDS 1992, vol. 2, p. 142–143. Los Alamos National Laboratory, Los Alamos, N. Mex.
32. **Niederman, T. M., B. J. Thielan, and L. Ratner.** 1989. Human immunodeficiency virus type 1 negative factor is a transcriptional silencer. Proc. Natl. Acad. Sci. USA **86:**1128–1132.
33. **Ratner, L., B. Starcich, S. F. Josephs, B. H. Hahn, E. P. Reddy, K. J. Livak, S. R. J. Petteway, M. L. Pearson, W. A. Haseltine, S. K. Arya, and F. Wong-Staal.** 1985. Polymorphism of the 3′ open reading frame of the virus associated with acquired immune deficiency syndrome, human T-lymphotropic type III. Nucleic Acids Res. **13:**8219–8229.

33a. **Saksela, K.** Personal communication.

34. **Saksela, K., C. Stevens, P. Rubinstein, and D. Baltimore.** 1994. Human immunodeficiency virus type 1 mRNA expression in peripheral blood cells predicts disease progression independently of the numbers of $CD4^+$ lymphocytes. Proc. Natl. Acad. Sci. USA **91:**1104–1108.
35. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
36. **Schnittman, S. M., J. J. Greenhouse, M. C. Psallidopoulos, M. Baseler, N. P. Salzman, A. S. Fauci, and H. C. Lane.** 1990. Increased viral burden in $CD4^+$ T cell from patients with human immunodeficiency virus (HIV) infection reflects rapidly progressive immunosuppression and clinical disease. Ann. Intern. Med. **113:**438–443.

36a. **Schrager, L. K., J. M. Young, M. G. Fowler, B. J. Mathieson, and S. H. Vermund.** 1994. Long-term survivors of HIV-1 infection: definition and research challenges. AIDS **8**(Suppl. 1)**:**S95–S108.

37. **Schwartz, S., B. K. Felber, D. M. Benko, E. M. Fenyo, and G. N. Pavlakis.** 1990. Cloning and functional analysis of multiply spliced mRNA species of human immunodeficiency virus type 1. J. Virol. **64:**2519–2529.
38. **Sheppard, H. W., W. Lang, M. S. Ascher, E. Vittinghoff, and W. Winkelstein.** 1993. The characterization of non-progressor: long-term HIV-1 infection with stable $CD4^+$ T-cell levels. AIDS **7:**1159–1166.
39. **Shibata, R., T. Miura, M. Hayami, K. Ogawa, H. Sakai, T. Kiyomasu, A. Ishimoto, and A. Adachi.** 1990. Mutational analysis of the human immunodeficiency virus type 2 (HIV-2) genome in relation to HIV-1 and simian immunodeficiency virus SIV_{AGM}. J. Virol. **64:**742–747.
40. **Shugars, D. C., M. S. Smith, D. H. Glueck, P. V. Nantermet, F. Seillier-Moiseiwitsch, and R. Swanstrom.** 1993. Analysis of human immunodeficiency virus type 1 *nef* gene sequences present in vivo. J. Virol. **67:**4639–4650. (Author's correction, **68:**5335.)
41. **Simmonds, P., P. Balfe, L. F. Peutherer, C. A. Ludlam, J. O. Bishop, and A. J. Leigh Brown.** 1990. Human immunodeficiency virus-infected individuals contain provirus in small numbers of peripheral mononuclear cells and at low copy numbers. J. Virol. **64:**864–872.
42. **Spina, C. A., T. Jesse Kwoh, M. Y. Chowers, J. C. Guatelli, and D. D.**

Richman. 1994. The importance of nef in the induction of human immunodeficiency virus type 1 replication from primary quiescent CD4 lymphocytes. J. Exp. Med. **179:**115–123.
43. **Tersmette, M., R. A. Gruters, and F. De Wolf.** 1989. Evidence for a role of virulent human immunodeficiency virus (HIV) variants in the pathogenesis of acquired immunodeficiency syndrome: studies on sequential HIV isolates. J. Virol. **63:**2118–2125.
44. **Weiss, R. A.** 1993. How does HIV cause AIDS? Science **260:**1273–1279.
45. **Williams, L. M., and M. W. Cloyd.** 1991. Polymorphic human gene(s) determines differential susceptibility of CD4 lymphocytes to infection by certain HIV-1 isolates. Virology **184:**723–728.
46. **Wilmot, C. M., and J. M. Thornton.** 1988. Analysis and prediction of the different types of β-turn in proteins. J. Mol. Biol. **203:**221–232.
47. **Yu, G., and R. L. Felsted.** 1992. Effect of myristoylation on p27 nef subcellular distribution and suppression of HIV-LTR transcription. Virology **187:**46–55.
48. **Zazopoulos, E., and W. A. Haseltine.** 1993. Effect of nef alleles on replication of human immunodeficiency virus type 1. Virology **194:**20–27.