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High Frequency of Defective *nef* Alleles in a Long-Term Survivor with Nonprogressive Human Immunodeficiency Virus Type 1 Infection

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A large number of *nef* alleles were obtained from peripheral blood mononuclear cells (PBMC) of four long-term nonprogressing survivors of human immunodeficiency virus type 1 (HIV-1) infection and from five individuals with progressive HIV-1 infection. These primary *nef* alleles were characterized by DNA sequence analysis and for their ability to downregulate CD4 surface expression. Intact *nef* open reading frames that directed the expression of Nef protein were recovered from all of the individuals. Most of the Nef proteins derived from three of four individuals with nonprogressive infection and from all five individuals with progressive infection were functional as judged by their ability to induce a decrease in surface CD4 expression. In contrast, one individual with nonprogressive HIV-1 infection yielded an unusually high frequency of disrupted *nef* open reading frames and Nef proteins defective for CD4 downregulation. Approximately 70% of the *nef* clones obtained from the PBMC of this individual at eight time points over a 12-year period were disrupted or defective for CD4 downregulation. While functional Nef proteins were demonstrated early in the course of infection (1983), functional *nef* alleles have surprisingly not come to predominate over time in PBMC DNA in this individual.

Substantial variation in the rates of disease progression has been observed among individuals infected with human immunodeficiency virus type 1 (HIV-1). The median time to development of AIDS in most cohorts is approximately 10 years following initial exposure to the virus (30, 35). However, a small fraction of HIV-1-infected people remain clinically healthy and show no decline in $CD4^+$ T-cell counts even though they have been seropositive for 10 years or more (6, 16, 23, 37, 38). The mechanisms involved in the establishment of such long-term nonprogressive infection with HIV-1 are not clear. The immune response mounted by the host and the ability of the virus to replicate in the face of this response are two important factors that probably determine the course of the disease (7, 20, 22–24).

Defects in viral genes have been linked to nonprogressive infection. Rhesus monkeys experimentally inoculated with simian immunodeficiency virus (SIV) carrying deletions in the *nef* gene have low viral loads and normal $CD4^+$ T-cell counts and show no signs of disease progression (20). Deletion of *vpx* and *vpr* produces a similar outcome (13). These characteristics are very similar to those of individuals with long-term nonprogressive HIV-1 infection (7, 24, 32). An early study documented one long-term nonprogressor infected only with *nef*-deleted HIV-1 since 1983 (22). More recently, six Australian long-term nonprogressors were shown to be infected with *nef*-deleted HIV-1 from a single blood donor (9). These results demonstrate that viral defects can be responsible for, or at the very least contribute to, the absence of disease progression in SIV-infected rhesus monkeys and HIV-1-infected humans.

How intact *nef* provides a selective advantage for viral replication in vivo is not understood. *nef* is essential for high viral loads and progression to AIDS in SIV-infected adult rhesus monkeys and for efficient viral replication in human HIV-1-infected fetal thymic tissue (19, 20), but it is dispensable for viral replication under commonly used laboratory conditions (20, 25, 33). Evidence from HIV-1-infected SCID-hu mice implanted with human lymphoid tissue suggests that the positive effect of *nef* on viral replication in vivo does not require a functional immune response (19).

Two in vitro effects have been consistently observed with many *nef* alleles. One effect is to down-modulate the surface expression of CD4 antigen and to disrupt the association between CD4 and $p56^{lck}$ protein tyrosine kinase (1, 3, 12, 36). These events reflect accelerated endocytosis and lysosomal degradation of CD4 induced by the viral protein (1, 34). The other effect of Nef is to stimulate viral replication and/or to increase infectivity in peripheral blood mononuclear cells (PBMC) and in certain cell lines (8, 11, 29, 42). The positive effect of Nef on viral replication in vitro is likely to be independent of CD4 down-modulation (15). Since these effects of *nef* seem to be conserved in primary isolates of HIV-1 and/or SIV, they are likely to reflect the positive effects of *nef* for viral replication in vivo (2, 4, 5, 26).

In this study, we characterized *nef* genes from PBMC of a group of four individuals with long-term nonprogressive HIV-1 infection; the fifth individual from the same group was shown previously to contain only grossly defective forms of *nef* (22). As controls, *nef* genes from five individuals with progressive HIV-1 infection were also studied. Together, more than 110 *nef* alleles from the two groups of individuals were sequenced and/or assayed for CD4 downregulation. We found that the vast majority of clones from both groups contained intact *nef* open reading frames and most of the individuals yielded Nef proteins that could downregulate CD4 expression. In contrast, *nef* sequences encoding defective Nef proteins were recovered

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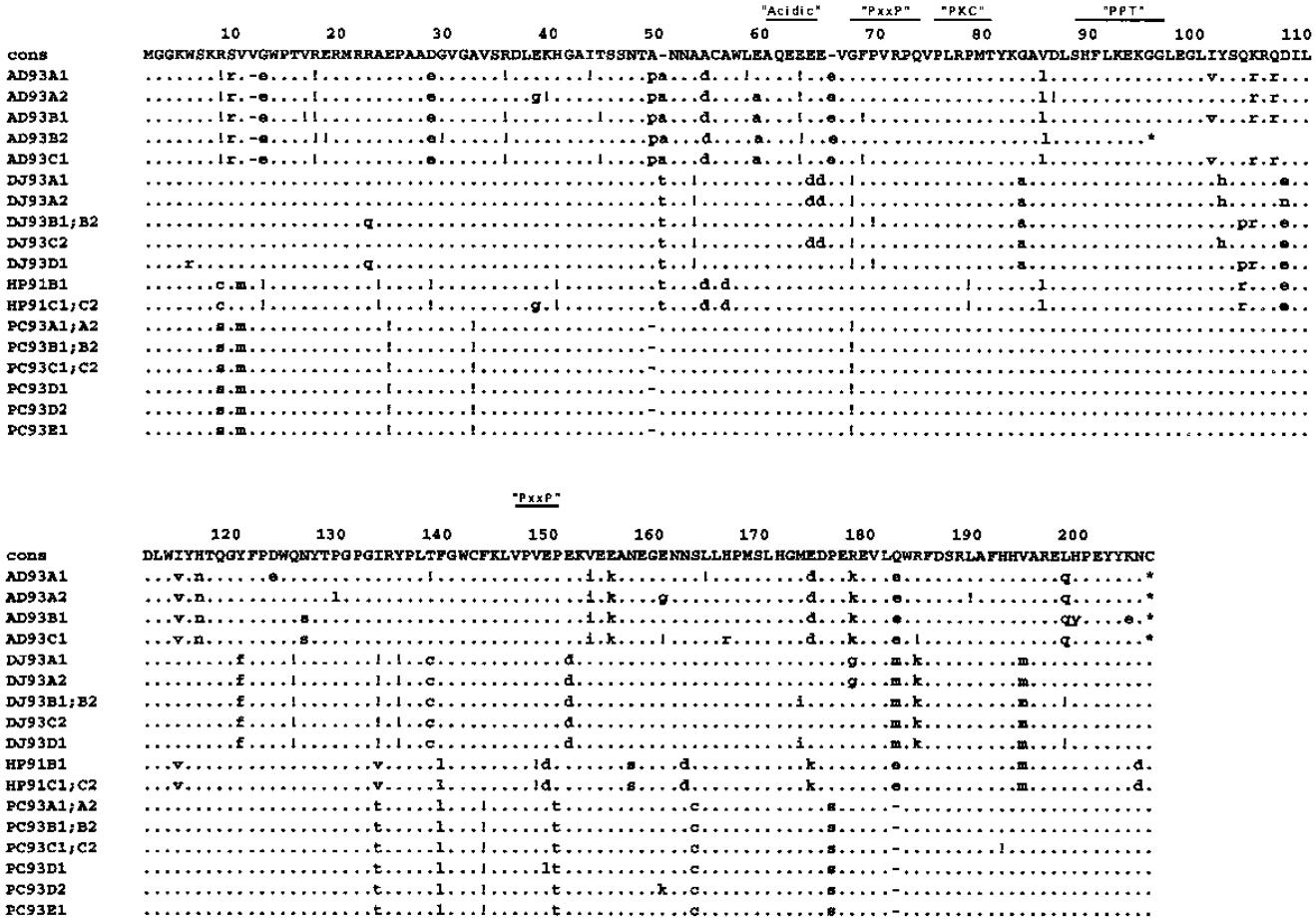


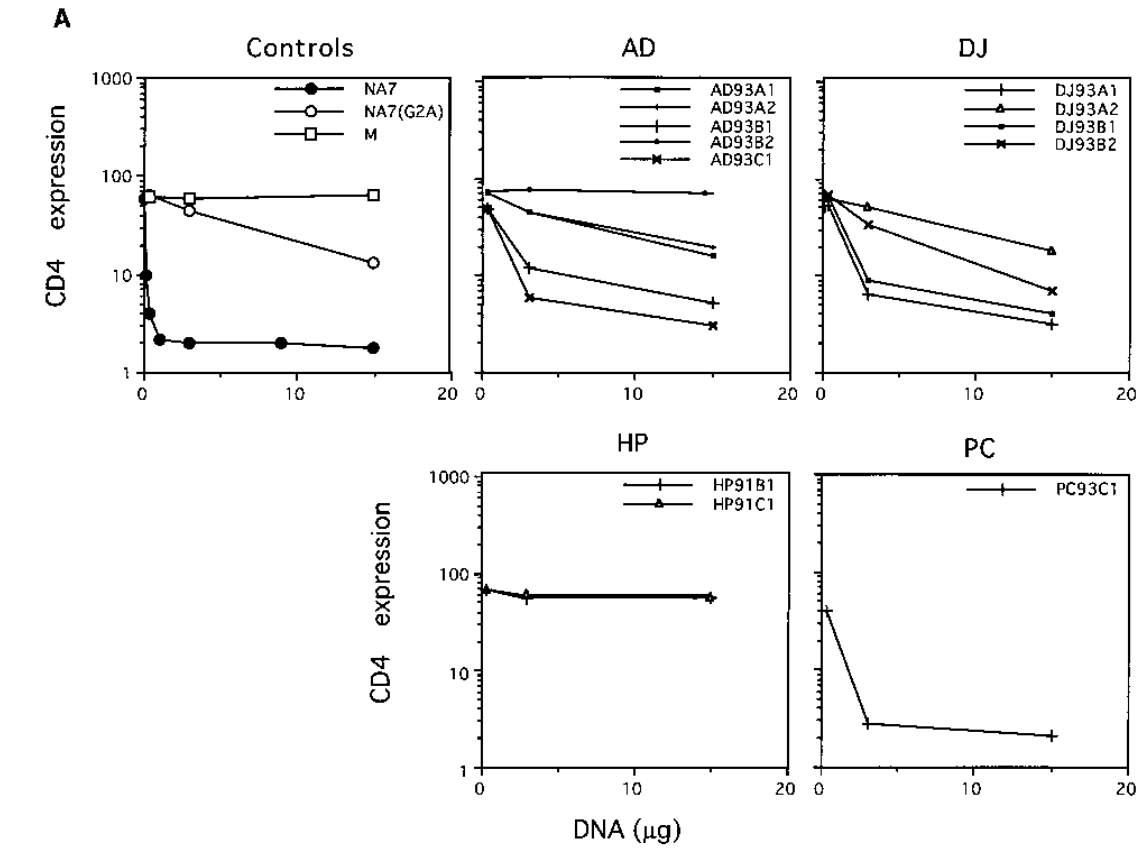
FIG. 1. Predicted amino acid sequences of Nef proteins from long-term nonprogressing survivors of HIV-1 infection. The consensus amino acid sequence for Nef proteins derived from nonprogressors AD, DJ, and PC is shown in the top line. The amino acid sequences predicted for individual alleles are aligned below. Dots indicate sequence identity, bars indicate gaps, asterisks indicate premature termination codons, slashes reflect frameshift mutations, and exclamation signs reflect synonymous changes. Amino acid sequences are shown in the one-letter code. The conserved amino acid sequence elements (39) are schematically shown above the consensus sequence. The origin of each sequence is identified by its name: the first two letters indicate the sampled individual, the following two digits indicate the year of sampling, and the subsequent letter and a digit identify the PCR of origin and the clone number, respectively. Five PCRs were performed for AD, DJ, and PC, each with 2.5 μg of DNA, and all were positive. Limiting-dilution analysis of the proviral copy number indicated that the cutoff concentration was about 0.6 μg for AD, 0.3 μg for DJ, and 0.05 μg for PC. Therefore, most PCR-derived clones from these individuals should represent different proviral sequences. PBMC from these nonprogressors were obtained in 1993. Of 10 PCRs performed with HP PBMC DNA from the 1991 time point, 3 were positive. Therefore, clones derived from the same PCR may reflect the same proviral genomes.

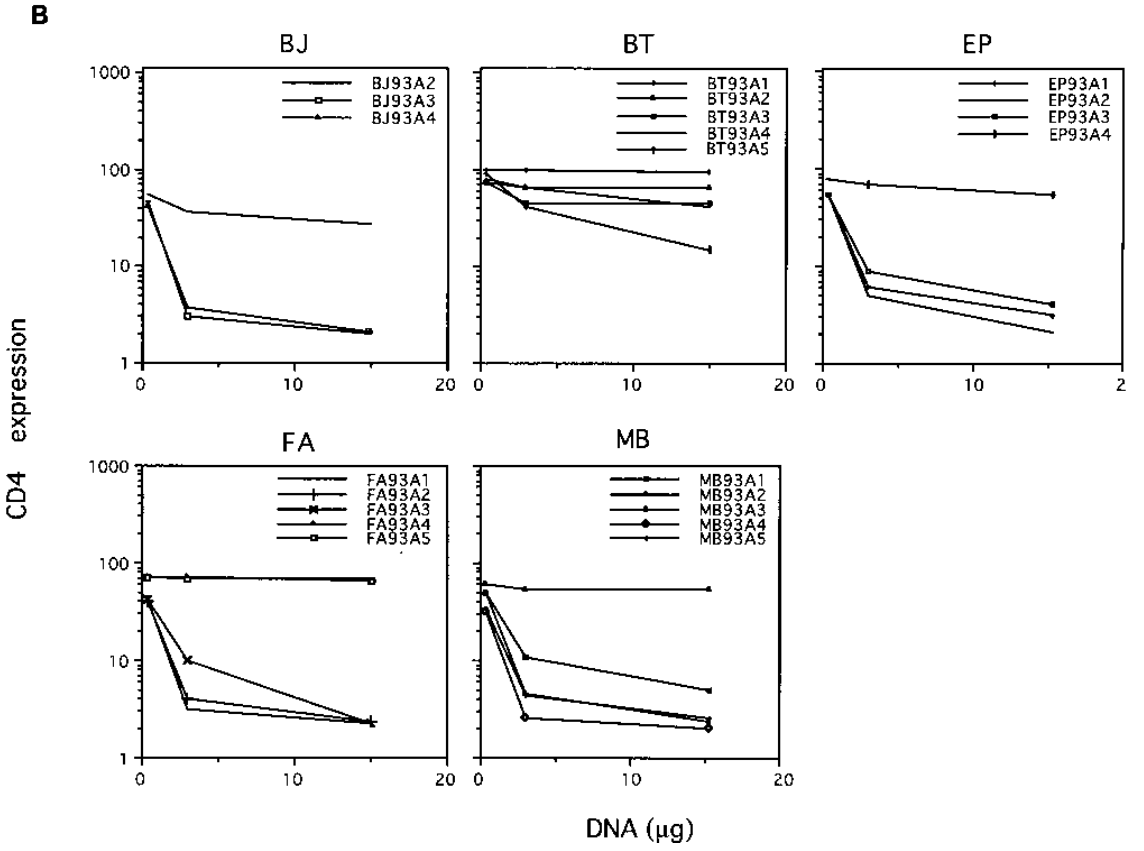
at an unusually high frequency from one of the individuals with nonprogressive HIV-1 infection. The observed persistence of defective forms of Nef over time appears to reflect effective control of HIV-1 infection rather than primary exposure to a *nef*-defective HIV-1 strain.

MATERIALS AND METHODS

Study participants. All study participants were monitored by the New England Area Comprehensive Hemophilia Center at the Medical Center of Central Massachusetts, Memorial Hospital, Worcester, Mass. Some of these individuals were described previously (16, 22). All participants have given informed consent. All of the individuals included in this report have severe hemophilia A and were infected with HIV-1 by infusions with contaminated factor VIII concentrates. All seroconverted prior to 1984 (see Table 1). The enumeration of lymphocyte subsets during 1991 and 1992 was used to classify disease progression for those monitored since 1984 or earlier. Study participants were considered to have long-term nonprogressive HIV-1 infection if they met the following criteria: (i) they have remained asymptomatic, (ii) they were never treated with antiretroviral agents, and (3) they have maintained absolute $CD4^+$ lymphocyte counts greater than 400/mm^3 with a CD4 percentage greater than 30%, or they have maintained absolute $CD4^+$ lymphocyte counts greater than 600/mm^3 irrespective of the CD4 percentage. This group comprised 7 members of the cohort of 119 who were surveyed. These individuals continue to meet these criteria in 1995. The clinical history of individual HP (the designations of patients are not their initials) was reported previously (16). Subjects were considered to have progressive infection if absolute $CD4^+$ lymphocyte counts declined to less than 200/mm^3 with a CD4 percentage less than 20% or if absolute CD4 lymphocyte counts fell below 100/mm^3 irrespectively of CD4 percentages (n = 72). Individuals who did not meet the criteria of either category were considered to have slowly progressive infection (n = 40).

Amplification and cloning of HIV-1 *nef* alleles. PCR was performed with 2.5- to 5.0-μg (nonprogressors) or 0.5-μg (progressors) aliquots of DNA isolated from peripheral blood leukocytes as described previously (22). The initial two rounds of amplification were carried out with primers corresponding to nucleotides 8675 to 8698 (5′GCAGTAGCTGAGGGGACAGATAGG3′) and 9530 to 9507 (5′CCAGTACAGGCAAAAAGCAGCTGC3′) of NL4-3 (31). The first round involved 10 cycles of amplification with 5 pmol of each primer. In the second round, 35 additional cycles were performed with 50 pmol of each primer. Subsequently, another 35 PCR cycles were performed with 5 μl of the amplification products from the second PCR and with 50 pmol each of the second pair of nested primers pF3(Xba) (GCACATCTAGAGAAGAATAAGACAGG) and pF4(Mlu) (CCAGGACGCGTCTCCCTGGAAAGTCCC), containing *Xba*I and *Mlu*I restriction sites (underlined), respectively. PCR fragments were purified from agarose gels with the GeneClean Kit (Qiagen Inc., Chatsworth, Calif.) and subcloned into the CD3β expression vector (40). Clones were sequenced with the Sequenase version 2.0 DNA-sequencing kit (U.S. Biochemicals). Control experiments to estimate the PCR error rate resulted in 3 PCR mutations in 6,210 bases analyzed. Thus, the error rate was approximately 0.05%, or 0.3


A
Controls
AD
DJ
HP
PC
CD4 expression
DNA (μg)
NA7
NA7(G2A)
M
AD93A1
AD93A2
AD93B1
AD93B2
AD93C1
DJ93A1
DJ93A2
DJ93B1
DJ93B2
HP91B1
HP91C1
PC93C1


B
BJ
BT
EP
FA
MB
CD4 expression
DNA (μg)
BJ93A2
BJ93A3
BJ93A4
BT93A1
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BT93A3
BT93A4
BT93A5
EP93A1
EP93A2
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EP93A4
FA93A1
FA93A2
FA93A3
FA93A4
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MB93A1
MB93A2
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TABLE 1. Clinical characteristics of study participants

Patient code[a]	Age (yr) in 1995	Category of progression[b]	CD4 count in 1995[c]	Time to CD4 < 100/ml (yr) and yr[d]	Amt of viral RNA (copies/ml)[e]
AD	16	LTNP	865	NA[f]	732
DJ	21	LTNP	888	NA	338
HP	49	LTNP	1,175	NA	290[g]
PC	37	LTNP	1,137	NA	677
BT	37	SLOW PROG	550	NA	9,259
EP	38	SLOW PROG	84	>11 (1995)	21,232
BJ	41	PROG	58	>5 (1989)	ND[h]
FA	47	PROG	11	>8 (1992)	38,024
MB	36	PROG	7	>9 (1993)	70,111

[a] All patients suffered from severe hemophilia A and seroconverted prior to 1984. Patient codes are not their initials.
[b] LTNP, long-term nonprogressor; SLOW PROG, slow progressor; PROG, progressor.
[c] The absolute $CD4^+$ lymphocyte count in PBMC measured in 1995.
[d] Approximate time that elapsed between individuals first tested seropositive and when CD4 T-cell counts first declined to less than 100/μl, and the year of this decline are shown.
[e] The highest RNA PCR measurement from samples obtained during 1992 to 1994.
[f] NA, not applicable.
[g] Only one (1992) of four determinations performed between 1992 and 1995 was weakly positive.
[h] ND, not determined.

nucleotide substitution per *nef* allele. Oligonucleotide-directed mutagenesis was performed as described previously (26). The mutated *nef* sequences were verified by DNA sequencing. Standard precautions were taken to minimize the possibility of sample cross-contamination. In addition, collection of clinical samples, DNA isolation, PCR sample preparation, and analysis of PCR products were always performed in separate laboratory spaces and frequently in different institutions. Moreover, to facilitate detection of possible rare cross-contamination events, several PCRs without DNA template added were always included in each PCR amplification experiment.

Limiting-dilution analysis. For limiting-dilution analysis, 2.5 μg of PBMC DNA, isolated from an HIV-1-infected individual, was serially diluted into 2.5 μg of genomic DNA isolated from uninfected CEMx174 cells. Each dilution was done in triplicate. The genomic DNA samples were subjected to the nested PCR approach described above. A cutoff concentration is defined as the largest amount of PBMC DNA that did not result in amplification of *nef* sequences in at least one of the three PCR reactions.

CD4 downregulation assay. Dose-response analysis of the effect of *nef* on CD4 expression was performed with Jurkat T cells expressing high levels of CD4 (kindly provided by Dan Littman, The Skirball Institute, New York University Medical Center, New York, N.Y.) as described previously (26, 41), except that an Epics Elite flow cytometer (Coulter) was used. As described previously, to ensure reproducibility of transfection experiments, a reporter plasmid directing the expression of *Escherichia coli* LacZ was used in all transfections as an internal control (26). However, we found that expression of some *nef* alleles resulted in unusually low levels of reporter gene expression. In those cases, transfections were performed with several independently isolated DNA preparations to ensure that the results are representative.

Immunoblot analysis of Nef expression. Immunoblot analysis of Nef expression was performed with aliquots of extracts containing 40 μg of protein. Samples were denatured for 15 min at 70°C in reducing sample buffer, resolved on 15% polyacrylamide gels, and transferred to polyvinylidene difluoride membranes as described previously (36). Immunoblot analysis with rabbit serum raised against *E. coli*-expressed HIV-1 HxB3 Nef protein was performed as described previously (40), and the immunoblot was developed with the enhanced chemiluminescence detection system (Amersham).

Quantitative RNA PCR. Quantitative RNA PCR was performed by using the Amplicor HIV-1 monitor assay (Roche Diagnostic System Inc.) strictly as specified by the manufacturer. The plasma specimens used in these assays were from EDTA- or ACD-anticoagulated blood (Vacutainer; Becton Dickinson) processed within 6 h of phlebotomy and stored at −80°C.

Phylogenetic analysis of *nef* sequences. Nucleotide sequences isolated from individual HP were subjected to phylogenetic analyses by the maximum-parsimony method, as implemented by PAUP software (43, 44). A total of 31 *nef* sequences isolated from this individual, each derived from an independent PCR, were analyzed by the heuristic approach with the MULTIPARS option and the TBR swapping algorithm. The HIV-1 NA7 *nef* allele was used as an outgroup. In additional, the neighbour-joining method with distances calculated by the method of Jukes and Cantor, as implemented by MEGA software, was also used. The relative rates of nonsynonymous and synonymous mutations were analyzed by the one-parameter method as described previously (14). Statistical analysis of *nef* sequences was performed with the chi-square test and corrected by the method of Yates.

RESULTS

Characterization of HIV-1 *nef* alleles from long-term survivors. The cohort of individuals who participated in this study is described in detail in Materials and Methods. The clinical characteristics of these individuals are shown in Table 1. To assess the status of *nef* in individuals with nonprogressive HIV-1 infection, the nucleotide sequences of 20 *nef* alleles derived from PBMC collected in 1993 from individuals AD, DJ, and PC and of three alleles derived from PBMC collected from individual HP in 1991 were initially determined. A summary of characteristics of *nef* sequences analyzed in this study is shown in Table 2. As shown in Fig. 1, amino acid sequence alignments revealed a low level of variation among protein sequences derived from the same individual (0 to 4.5%) and greater variation between protein sequences derived from different individuals (10 to 16%). This extent of the observed intrapatient heterogeneity is similar to that found in the previous studies (0.04 to 3.54% [10, 17, 39]). Most of the variation, involving substitutions and insertions or deletions of single amino acid residues, was located outside the previously iden-

FIG. 2. Effect of *nef* from long-term nonprogressors on CD4 antigen expression. The effect of *nef* alleles isolated from HIV-1-infected individuals on CD4 expression on the cell surface was analyzed in dose-response experiments. Jurkat T cells were cotransfected with CD3 LacZ reporter (0.3 to 2 μg) and different amounts of CD3 vectors directing expression of control or primary HIV-1 *nef* alleles (0.1 to 15 μg), shown on the linear scale on the abscissa. CD4 expression on the cell surface was determined by flow-cytometric analysis 16 to 24 h later. The median level of CD4 on the surface of β-galactosidase-positive cells recorded on the logarithmic scale is shown on the ordinate. Each panel shows results from *nef* alleles isolated from a single individual. (A) in the Controls panel, NA7 is a strong HIV-1 *nef* allele, NA7(G2A) contains a single point mutation that disrupts the myristylation signal, and M is a control empty vector. Panels AD, DJ, HP, and PC show results for long-term survivors of nonprogressive HIV-1 infection. The NA7 and NA7(G2A) alleles were included as controls in all experiments (data not shown). (B) Panels BJ, BT, EP, FA, and MB show results from individuals with a progressive HIV-1 infection.

TABLE 2. Characteristics of *nef* sequences from patients with different courses of HIV-1 infection

Category of progression[a]	Patient code[b]	No. of *nef* sequences				
		Sequenced	Grossly defective	Tested	Functional	Nonfunctional
LTNP	AD	5	1	5	4	1[c]
	DJ	6	0	4	4	0
	HP	59	19[d]	12	5	7[e]
	PC	9	0	7	7	0
SLOW PROG	BT	8	0	5	3	2
	EP	4	0	4	3	1
PROG	BJ	5	0	3	2	1
	FA	9	2	5	3	2[f]
	MB	7	0	5	4	1

[a] See Table 1, footnote *b*.
[b] See Table 1, footnote *a*.
[c] Includes the grossly defective AD93B2 allele.
[d] The frequency of grossly defective *nef* sequences is significantly higher for HP (19 of 59) than that for the other nonprogressors (1 of 20 [$P < 0.04$]), for the progressors (2 of 33 [$P < 0.01$]), or for both groups combined (3 of 53 [$P < 0.001$]).
[e] Only data from those alleles that were tested directly are shown. Includes the grossly defective HP89B1, HP93A1, and HP93C1 alleles.
[f] Includes two grossly defective *nef* sequences.

tified conserved regions of the protein (31, 39). For each of the individuals, the vast majority of *nef* alleles encoded complete Nef proteins (22 clones). Only one of six *nef* clones from AD (AD93B2) had a frameshift mutation that predicted a truncated protein. No gross defects in the remaining *nef* sequences from these four individuals with long-term nonprogressive infection were detected by nucleotide sequence analysis.

Ability of Nef from long-term nonprogressors to downregulate CD4 expression. To assess the functional status of Nef proteins from individuals with long-term nonprogressive HIV-1 infection, we assayed their ability to downregulate CD4 surface expression. The relative strength of selected *nef* alleles from AD, DJ, PC, and HP was quantitated in dose-response experiments in human $CD4^+$ Jurkat T cells by using a transient-transfection assay (26, 41). As shown in Fig. 2A, panel Controls, a strong HIV-1 NA7 allele and a weak NA7(G2A) allele with a single amino acid substitution disrupting the N-terminal myristoylation signal were used as standards (26). The NA7 *nef* resulted in an approximately 50-fold decrease in surface CD4 expression, and this effect was saturated with approximately 1 μg of the expression construct (Fig. 2A, Controls). In contrast, the weak myristoylation-defective NA7(G2A) *nef* did not saturate even at the highest dose of 15 μg. Nevertheless, it still functioned for CD4 downregulation when overexpressed.

Dose-response curves obtained with *nef* alleles from the four nonprogressors are shown in Fig. 2A. Interestingly, different individuals displayed different characteristics of the dose-response profiles of the *nef* alleles. For example, a relatively large degree of intrapatient variation was observed with *nef* alleles derived from AD and DJ but not with those derived from PC and HP. Thus, dose-response curves obtained with clones AD93B1 and AD93C1 were very similar to that obtained with the strong NA7 allele. In contrast, clones AD93A1 and AD93A2 were considerably weaker and clone AD93B2, which encodes a severely truncated Nef protein, did not show any detectable activity in the assay.

A very uniform population of *nef* was found in individuals PC and HP. Seven of nine alleles derived from PC were predicted to encode identical proteins which were all highly active in the CD4 downregulation assay (Fig. 2A; only results from the PC93C1 allele are shown). In contrast, none of the *nef* alleles amplified from the 1991 HP sample had a detectable effect on CD4 antigen expression on the cell surface (Fig. 2A).

To assess the normal range of variability in the strength of *nef* alleles, additional *nef* sequences from five individuals with progressive HIV-1 infection were characterized. As shown in Fig. 2B, the majority of *nef* alleles recovered from the control individuals were active in the CD4 downregulation assay, albeit several inactive clones were also found. A relatively high level of intrapatient variability in the strength of *nef* alleles was observed for each of the control subjects, indicating that this variability is not uniquely associated with nonprogressive HIV-1 infection. In contrast to the findings from individual HP, however, *nef* sequences capable of downregulating CD4 expression predominated in all subjects with progressive HIV-1 infection (Table 2). Thus, the status of *nef* in individual HP was more closely examined.

High frequency of defective *nef* sequences in HP. To further explore the status of *nef* genes in HP, DNA was obtained from PBMC collected from this individual at seven additional time points spanning a 12-year period from 1983 to 1995. Multiple PCRs were performed with 5 μg of PBMC DNA each, and between two and seven independently amplified *nef* sequences were obtained for each of the time points. Alignment of amino acid sequences predicted by *nef* sequences derived from HP over a 12-year period is shown in Fig. 3.

A significant fraction of HP *nef* alleles were grossly defective. Of 59 sequences, 19 carried point mutations or internal deletions that disrupted the protein-coding sequence of *nef*. Ten *nef* alleles derived from 1983, 1993, and 1995 time points shared a mutation that inactivated the initiator methionine (Fig. 3, HP83B, HP93A, HP93B, HP93C, and HP95A). Frameshift mutations disrupted open reading frames in five additional alleles (Fig. 3, HP83D, HP83F2, and HP95C). Polypeptides specified by the remaining four alleles suffered deletion of 4 or 10 amino acids in conserved parts of the Nef protein and are therefore likely to be defective (Fig. 3, HP89B and HP95F). In agreement with these observations, neither the 27-kDa Nef protein nor smaller peptides reacting with the anti-Nef antibody were detected in cytoplasmic extracts prepared from cells transfected with the prematurely terminated HP83D1 or with the initiation codon-deficient HP93 clones (Fig. 4A, lanes 7, 16 and 17). This 32% frequency of grossly defective *nef* open reading frames in HP (19 of 59) is significantly higher than that found for other nonprogressors (1 of 20; mean value, 5% [$P < 0.04$]) and progressors (2 of 33; mean value, 6.1% [$P < 0.01$]) characterized in this study and higher than that found in other studies (mean values, 2.3 to 11% [17, 28, 39]).

The remaining 40 *nef* alleles were predicted to encode full-length Nef protein. Twenty-two *nef* alleles from this group shared characteristic amino acid substitutions at positions 56 and/or 174 of Nef protein. As shown in the example of HP83F1, HP91B1, and HP91C1, substitutions at positions 56 and/or 174 correlated with the loss of downregulation of surface CD4 expression by Nef, even though they did not interfere with the expression of the Nef protein (Fig. 4A, lanes 8, 14, and 15; Fig. 4B and 2A, panels 1983 and 1991, respectively). Subsequent experiments demonstrated that each of these substitutions was sufficient individually to disrupt the ability of Nef to downregulate surface CD4 expression (see below). Thus, an additional 37% of HP *nef* sequences specified functionally defective proteins.

Several of the remaining 18 alleles encoded Nef proteins capable of downregulating surface CD4 expression. Eleven *nef*

TABLE 3. Characteristics of nucleotide substitutions in *nef* sequences from HP

HP *nef* sequence[a]	No. of clones analyzed (PCRs)[b]	Mutation[c]				
		No. of mutations (no. of positions)[d]	S:N	K_s	K_n	K_s/K_n
Clade A	10 (5)	37 (36)	6:31	0.038	0.073	0.5
Clade B	13 (7)	16 (15)	6:10	0.033	0.023	1.4
Non-A/non-B	26 (13)	25 (24)	6:19	0.027	0.045	0.6

[a] The following sequences were used for analysis: clade A, HP83B1, HP83B2, HP93A1, HP93A2, HP93B1, HP93B2, HP93C1, HP93C2, HP95A1, and HP95A2; clade B, HP91A1, HP91A2, HP91B1, HP91C1, HP91C2, HP94E1, HP94E2, HP94F1, HP94F2, HP95E1, HP95E3, HP95F1, and HP95F2; non-A/non-B group comprises all nef sequences that do not have the loss-of-function mutations M1I, A56D, and/or E174K: HP83A1, HP83A2, HP83C1, HP83C2, HP83D1, HP83D2, HP83E2, HP87A1, HP87A2, HP89A1, HP89A2, HP89C1, HP89C2, HP90A1, HP90A2, HP90B2, HP94A1, HP94A2, HP94B1, HP94B2, HP95B1, HP95B2, HP95C1, HP95C2, HP95D1, and HP95D2.

[b] In most cases, two sequences from each PCR were used, except for HP91B in clade B, where HP91B1 was the only available sequence, and HP83E in the non-A/non-B group, where HP83E1 allele has a A56D mutation.

[c] The total number of nonsynonymous (N) and synonymous (S) differences found among *nef* sequences from each group, and the estimates of nonsynonymous (K_n) and synonymous (K_s) substitutions per site (14) and of the ratio between the two values (K_n/K_d) are shown.

[d] Two distinct differences were found at position 1 of codon 29 in clade A sequences, at position 1 of codon 19 in clade B sequences, and at position 1 of codon 176 in non-A/non-B sequences.

sequences, obtained from seven independent PCRs performed with DNA from 1983, 1989, and 1994 time points, specified polypeptides which were identical to the predicted consensus HP Nef (Fig. 3). As shown with the HP83A2 and HP89A1 alleles, these HP Nef proteins were expressed well when placed under the control of the CD3 promoter (Fig. 4A, lanes 5 and 10). Their ability to downregulate surface CD4 expression was readily detectable and was intermediate between those of the strong NA7 and weak NA7(G2A) proteins (Fig. 4B, panels 1983 and 1989). Four additional *nef* sequences were tested. While all four directed detectable expression of the Nef polypeptide, only three had a measurable effect on CD4 expression in the transient-transfection assay (Fig. 4, HP83C1, HP87A1, HP90A1, and HP90B2).

Thus, although Nef proteins that can downregulate CD4 expression were recovered from HP through the observation period, approximately 70% of *nef* sequences from this individual specified defective *nef* open reading frames or directed expression of Nef proteins that were defective for downregulation of CD4 expression.

Loss-of-function mutations in 1991 and 1993 HP *nef* alleles. To further understand the population of *nef* alleles in HP, we analyzed which mutations were responsible for loss of function in 1991 and 1993 *nef* alleles. To map the region of HP91 Nef that contains the inactivating mutation(s), a chimeric protein combining the N-terminal part of the inactive HP91B1 with the C-terminal segment from the active NA7 was constructed (Fig. 5A, mutants 1, 2, and 3). This HP91-NA7 chimera had only a very weak effect on surface CD4 expression (Fig. 5A, mutant 3). This indicated a defect in the N-terminal portion of HP91 protein.

To identify the amino acid residue(s) underlying the defect in HP91-NA7, amino acid differences found in the defective protein were replaced with those found in NA7 Nef (Fig. 3, compare sequences HP91 and NA7). As shown in Fig. 5A, substitution of alanine for aspartic acid 56 in the background of HP91-NA7 restored full activity of the chimera (HP91[D56A]-NA7 [Fig. 5A, mutant 4]). Moreover, the reciprocal A56D mutation in the NA7 background disrupted CD4 downregulation by this protein (NA7[A56D] [Fig. 5A, mutant 6]). The residual effect of NA7[A56D] Nef was very similar to that found in the HP91-NA7 chimera (Fig. 5A, compare mutants 3 and 6). Thus, the D56 substitution appeared to be the only loss-of-function substitution in the N-terminal segment of HP91 Nef. The D56A substitution, however, failed to rescue the parental HP91 molecule (HP91[D56A] [Fig. 5A, mutant 5]). Therefore, additional mutations residing in the C-terminal portion of the HP91B1 Nef were likely to contribute to the defect.

A similar approach was used to map a defect in the C-terminal region of HP91B1 Nef. Substituting lysine for glutamic acid 174 in the background of NA7 Nef disrupted CD4 downregulation by this NA7[E174K] protein (Fig. 5B, mutant 8). Reverting K174E alone in the HP91B1 was not sufficient to restore function, but a double-point mutation reverting both D56 and K174 resulted in a readily detectable gain of function of this allele (HP91[K174E] and HP91[D56A,K174E] [Fig. 5B, mutants 9 and 10]). These observations identified K174 as a second loss-of-function substitution in HP91 alleles.

The HP93A1 and HP93C1 alleles which lacked the initiator methionines were also analyzed (Fig. 3). Restoring the consensus initiation codon in HP93 proteins was not sufficient to restore their activity (Fig. 5C, compare mutants 13 and 14 with 11 and 12). Since no Nef protein was detected by immunoblot analysis of cytoplasmic extracts prepared from Jurkat T cells transiently transfected with the reverted HP93A1[I1M] and HP93C1 alleles (Fig. 5D), we suspect that some of the additional mutations present in HP93 alleles have a destabilizing effect on Nef polypeptides.

Phylogenetic analysis of HP *nef*. Thirty-one HP *nef* nucleotide sequences, each derived from a separate PCR, were subjected to phylogenetic analyses with two different phylogenetic algorithms: the maximum-parsimony approach and the neighbour-joining method (43, 44). Both approaches resulted in similar trees and revealed that three major classes of *nef* sequences coexisted in individual HP through the 12-year observation period (Fig. 6). As shown in Fig. 6, two of these classes correspond to the phylogenetic lineages designated clades 1 and 2. Both these clades comprised exclusively defective *nef* sequences. Sequences clustered in clade 1 contained the characteristic inactivating M1I substitution and were unable to direct the expression of Nef polypeptide (Fig. 3 and 6). *nef* sequences clustered in clade 2 carried the A56D and E174K mutations that disrupted the ability of Nef to downregulate CD4 expression.

The third class of *nef* sequences comprised those that could not be classified to the phylogenetic lineages defined by clades 1 and 2. One subset of these sequences carried the A56D and/or E174K mutation characteristic of clade 2 sequences (Fig. 3 and 6, HP83F1, HP83G1, HP87B1, and HP89B). It is possible that these sequences have a close phylogenetic relationship to the clade 2 lineage, but they possessed to few

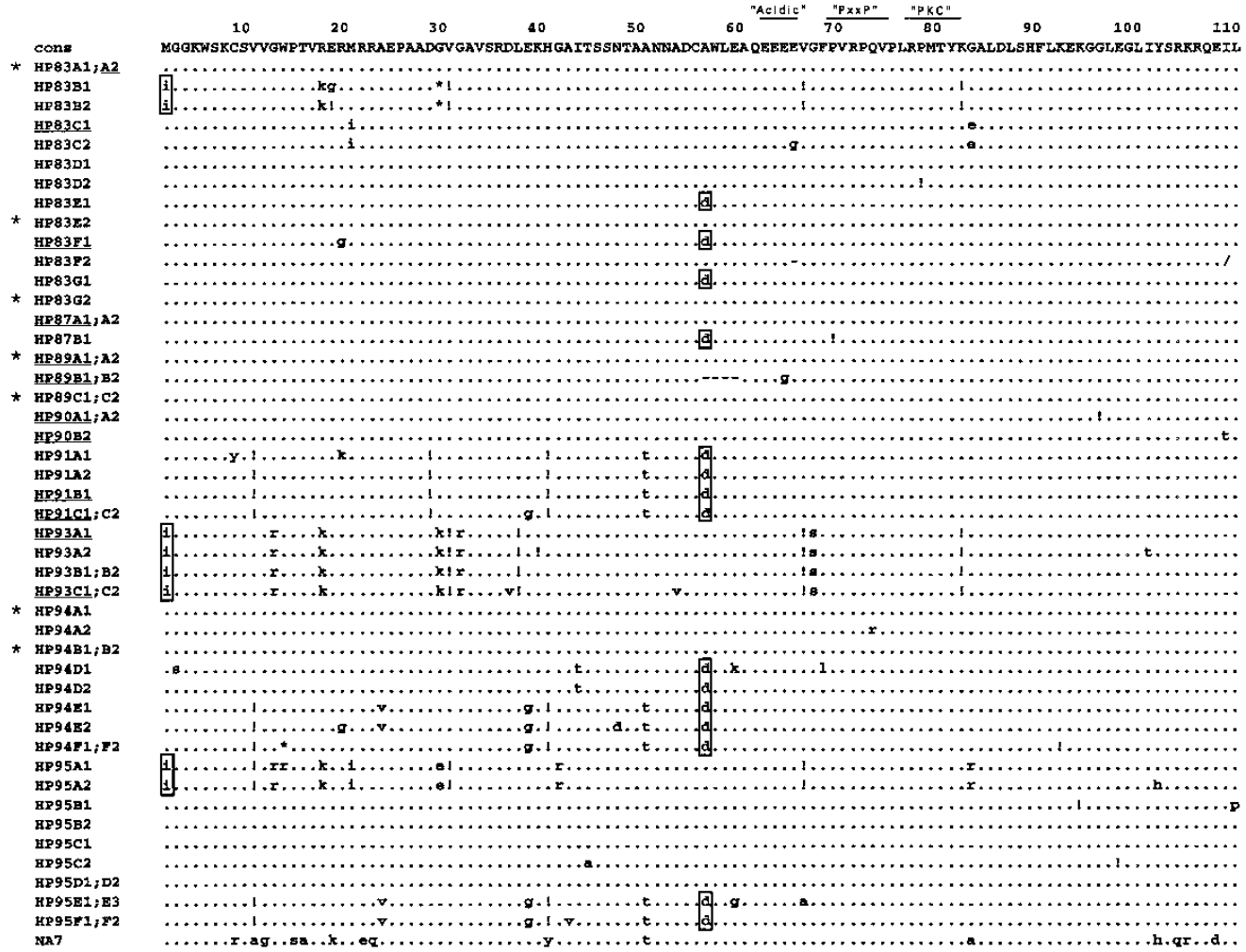

FIG. 3. Predicted amino acid sequences of HP Nef proteins. The consensus amino acid sequence for Nef proteins derived from HP over a 12-year period, defined by the predominant amino acid residues at each position, is shown in the top line. The conserved amino acid sequence elements (39) are schematically shown above the consensus sequence. The individual Nef amino acid sequences are aligned below. The amino acid sequence of NA7 Nef that was used as a positive control in functional studies is shown for comparison in the bottom line. The loss-of-function mutations identified in the 1991 and 1993 alleles are boxed. The origin of individual sequences is indicated as described in the legend to Fig. 1. For each time point, only a fraction (5 to 70%) of PCRs resulted in amplification of *nef* sequences. The high incidence of nucleotide sequence identity found with *nef* sequences derived from the same PCR and a lower incidence with those from independent PCRs indicate that most but not all amplifications resulted from single proviral copies. Asterisks on the left side of the panel indicate amino acid sequences identical to the predicted consensus HP Nef. Exclamation signs reflect synonymous changes. Alleles selected for functional analysis are underlined.

phylogenetically informative sites to prove this relationship. Alternatively, some of these sequences may have evolved independently. A second subset included the HP83A, HP83E2, HP89A, HP89C, HP94A, and HP94B *nef* alleles. Nucleotide sequences of these alleles were identical to each other. As shown in Fig. 6, their location on the phylogenetic tree implied that they are identical, or closely related, to an ancestral HP *nef* sequence. The frequent nucleotide sequence identity among *nef* alleles from this group may reflect persistence of these proviral sequences in PBMC (archival viral DNA). As shown with the HP83A2 Nef, proteins encoded by these alleles were functional for CD4 downregulation (Fig. 3 and 4B).

The relative frequencies of synonymous and nonsynonymous differences found among sequences from clade 1, from clade 2, and among those that did not contain changes diagnostic for clade 1 or clade 2 sequences are shown in Table 3 (14). With clade 1 (or clade 2), only sequences that were downstream of nodes with bootstrap values higher that 90 were used for these calculations. Clade 1 sequences were characterized by a relatively high frequency of nonsynonymous differences (K_s/K_n = 0.52 [Table 3]). This is consistent with relatively low, if any, selective pressure to maintain the Nef coding region of these grossly defective clade 1 alleles. The relative frequency of nonsynonymous differences among clade 2 sequences was relatively low (K_s/K_n = 1.4 [Table 3]). This is in contrast to the findings from the two remaining groups of HP *nef* sequences and suggests that a selective pressure existed to maintain the amino acid sequence of these Nef proteins defective for CD4 downregulation. Thus, it appears that the major classes of HP *nef* sequences were subject to different selective pressures.

DISCUSSION

nef sequences from PBMC of four individuals with long-term nonprogressive HIV-1 infection and from five control individuals were characterized in this study. All individuals

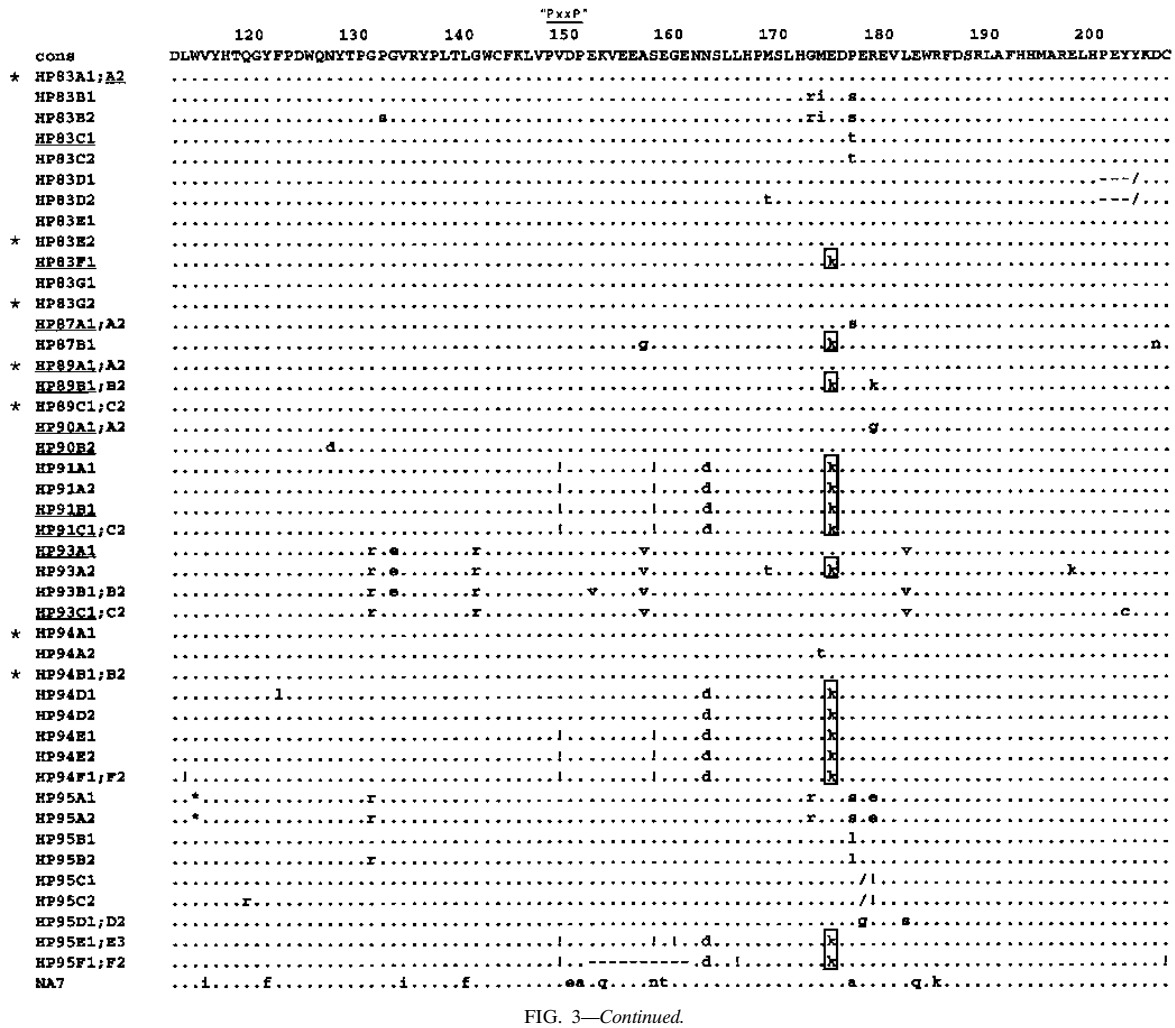

FIG. 3—*Continued.*

studied here were hemophiliacs infected with HIV-1 by infusion of contaminated factor VIII concentrates. Results from phylogenetic analysis of *nef* sequences isolated from these individuals suggest that each infection may have resulted from a single, rather homogeneous viral strain (data not shown). This is in agreement with previous evidence that not every individual who received infusion of contaminated factor VIII became infected.

The vast majority of *nef* sequences from three of the four nonprogressors encoded full-length Nef proteins (19 of 20 sequences), and most of these sequences were functional for CD4 downregulation (13 of 14 that were tested). Similar frequencies of intact *nef* open reading frames and functional Nef proteins were found in the five control individuals with progressive HIV-1 infection, and these in turn are similar to the frequencies reported previously in other progressors and nonprogressors (17, 22, 28, 39). Thus, while consistent deletions in the *nef* gene appear to be responsible for some remarkable cases of nonprogression (9, 22), defects in *nef* do not appear to be responsible for most other cases of nonprogression (17, 18, 28).

Our analyses suggest that different factors may be responsible for the lack of progression among the four subjects studied here. For example, two observations differentiated PC from HP and all other individuals. First, *nef* alleles recovered from PC were unusually active in downregulating surface CD4 expression (Fig. 2A, panel PC). The frequency of nucleotide substitutions of 0.3 per clone found in *nef* sequences from PC was exceptionally low compared with that found for all other individuals, regardless of the clinical status of HIV-1 infection (Fig. 1). The limited variation observed in *nef* sequences from PC is likely to be representative of the actual sequences present in PBMC, since clones from five independent PCRs were analyzed. Although it is difficult at present to reconcile these observations, together they suggest that nonprogressive infection in PC may have a fundamentally different basis from those in the other four nonprogressors in this cohort.

Results with *nef* genes from individual HP are in sharp contrast with those from other nonprogressors and from the control subjects. Many of the Nef proteins recovered from this individual early in the infection (1983) were functional as judged by two criteria: their ability to downregulate cell surface CD4 expression (Fig. 4B, HP83A1) and their ability to enhance the infectivity of *nef*-deleted HIV-1 in a single-round infection assay (21, 25a, 29). Surprisingly, however, an exceptionally large proportion of *nef* sequences isolated from this individual at various time points over a 12-year observation period were nonfunctional or encoded defective proteins (42 of a total of 59 sequences analyzed). A similar finding of an unusually high frequency of defective accessory genes was recently reported

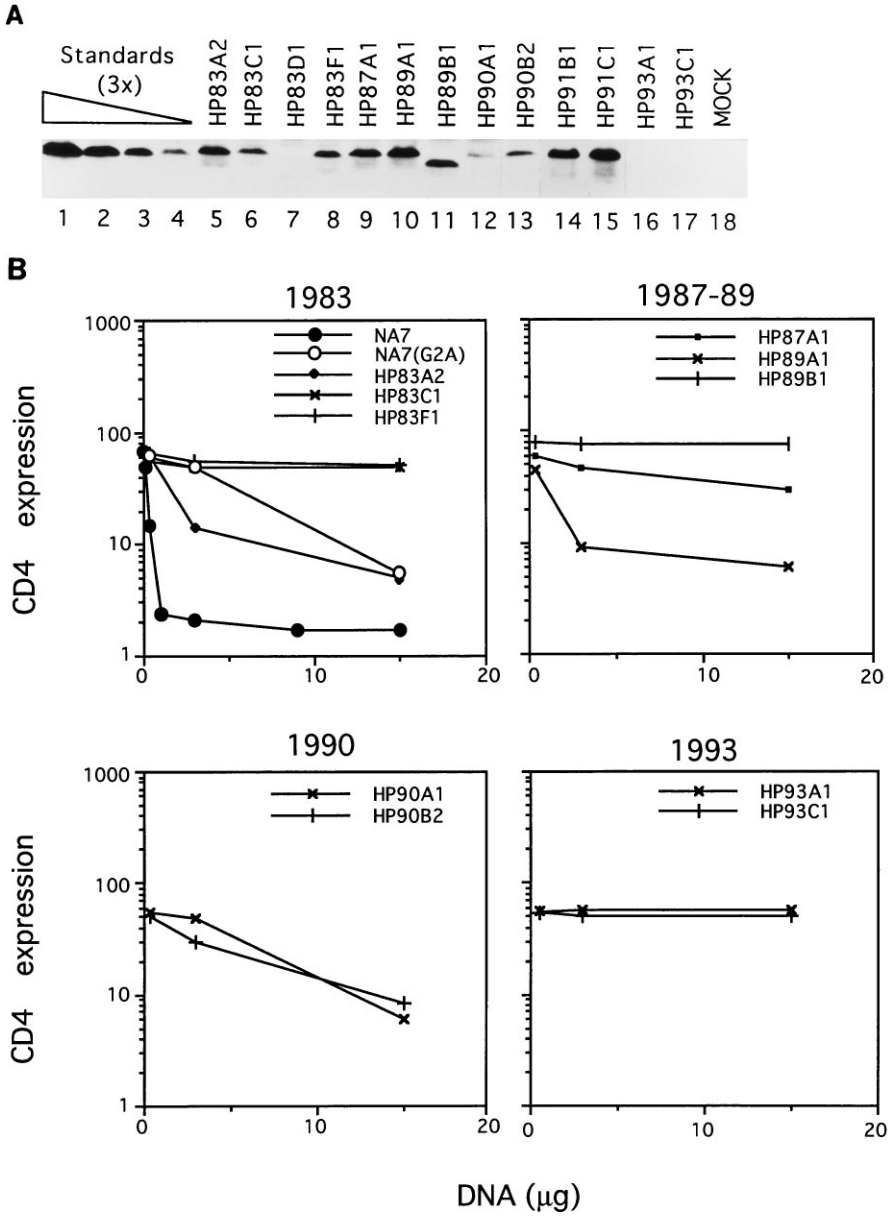

FIG. 4. Effect of HP Nef proteins on CD4 expression. (A) Immunoblot analysis of Nef proteins from HP. The steady-state expression of HP Nef proteins from the experiment shown in panel B was determined from immunoblot analysis of 40-μg aliquots of protein extracts prepared from Jurkat cells transfected with 3 μg of CD3β expression vector containing various HP *nef* sequences (lanes 5 to 17). Extracts from cells transfected with 9, 3, 1, and 0.3 μg of CD3 NA7 plasmid were used as a reference for the proportionality of the assay (lanes 1 to 4, respectively). Cells transfected with a control empty vector were used as a negative control (MOCK). (B) A dose-response analysis of *nef* alleles from HP PBMC samples collected between 1983 and 1993 was performed as described in the legend to Fig. 2.

for another individual with long-term nonprogressive HIV-1 infection from whom HIV-1 could not be isolated (27).

The lack of selection for functional *nef* alleles in PBMC DNA of HP is unusual, surprising, and different from the usual course of SIV and HIV infection. Functional forms of *nef* are quickly and efficiently selected in rhesus monkeys infected experimentally with SIV that is defective in *nef* (20). Drug-resistant mutants evolve quickly and efficiently in HIV-1-infected people treated with antiviral drugs (45). Why functional *nef* alleles were not selected over time in HP PBMC is not known but can be speculated upon given the existing data. Since this individual maintains high antibody and cytotoxic T-lymphocyte levels, he appears to remain persistently infected. However, since the viral loads in this patient are so extremely low, we have no way of knowing the extent to which the sequences analyzed represent actively replicating virus versus archival or defective genomes. In any event, the data indicate an extraordinary level of effective control of HIV-1 infection in this individual. Factors that could conceivably be contributing to this effective control include (i) a defect elsewhere in the genome that severely limits viral replication, (ii) an unusually effective immune response that severely limits viral replication, (iii) immune system selective pressure on Nef itself, and (iv) some combination of these and/or other factors. Continued study of these and other long-term nonprogressing survivors should shed light on the different underlying causes of non-

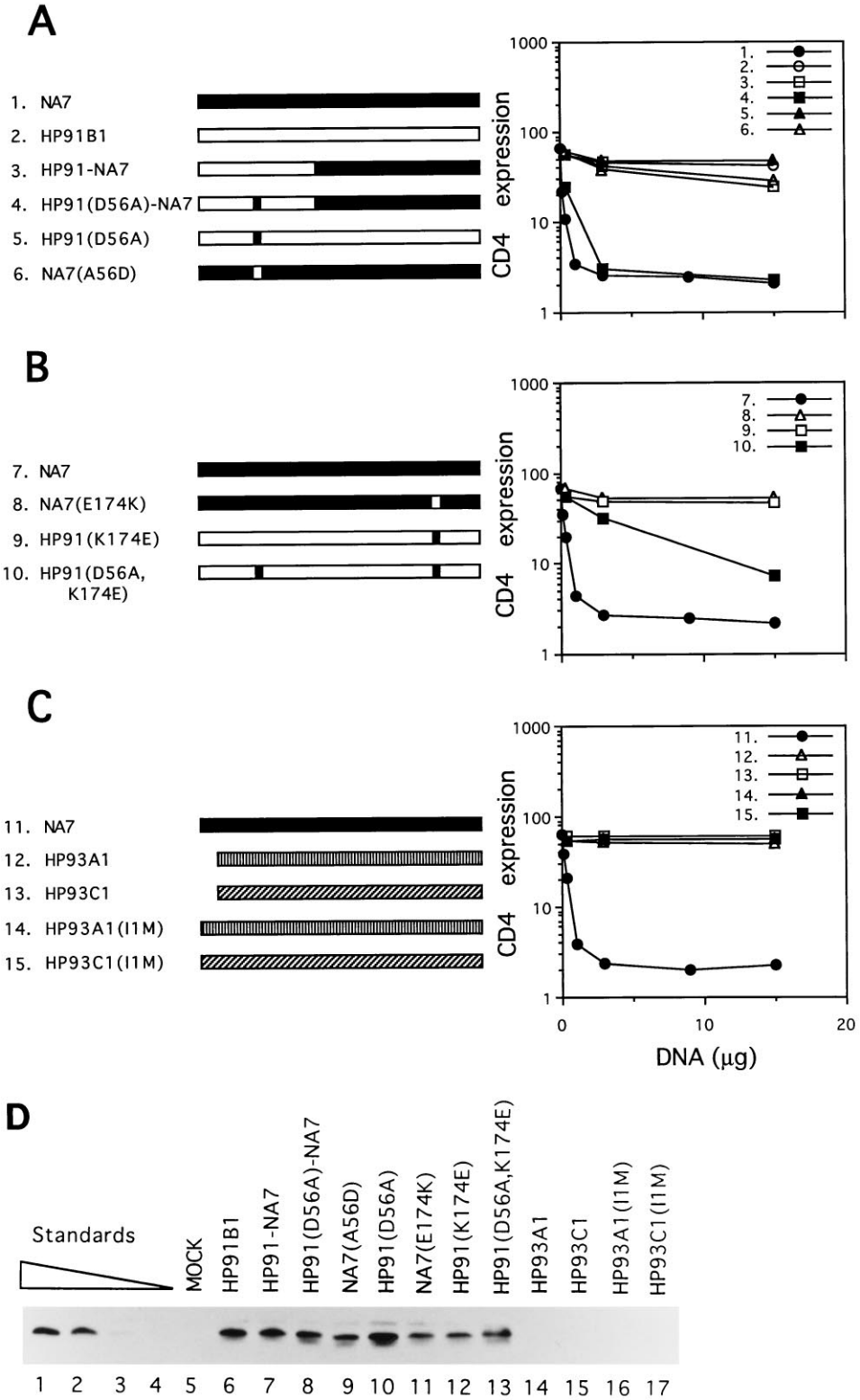

FIG. 5. Mapping loss-of-function mutations in 1991 and 1993 HP *nef* alleles. Mapping the debilitation mutations in the N-terminal (A) and C-terminal (B) segments of HP91B1 protein and in HP93A1 and HP93C1 proteins (C). In each panel, the structures of chimeric and mutant Nef proteins are shown on the left, and the effects of mutant Nef proteins on CD4 expression on the cell surface are shown on the right. Sequences derived from NA7 are represented by solid rectangles, sequences derived from HP91B1 are indicated by open rectangles, and those from HP93A1 and HP93C1 are indicated by striped bars. In the HP91-NA7 chimera, amino acids 1 to 74 are from HP91B1 Nef and amino acids 75 to 206 are from NA7 Nef. The location and origin of amino acid substitutions introduced into chimeras and parental proteins are schematically shown by vertical bars. (D) Immunoblot analysis of mutant Nef proteins was performed as described in the legend to Fig. 4A.

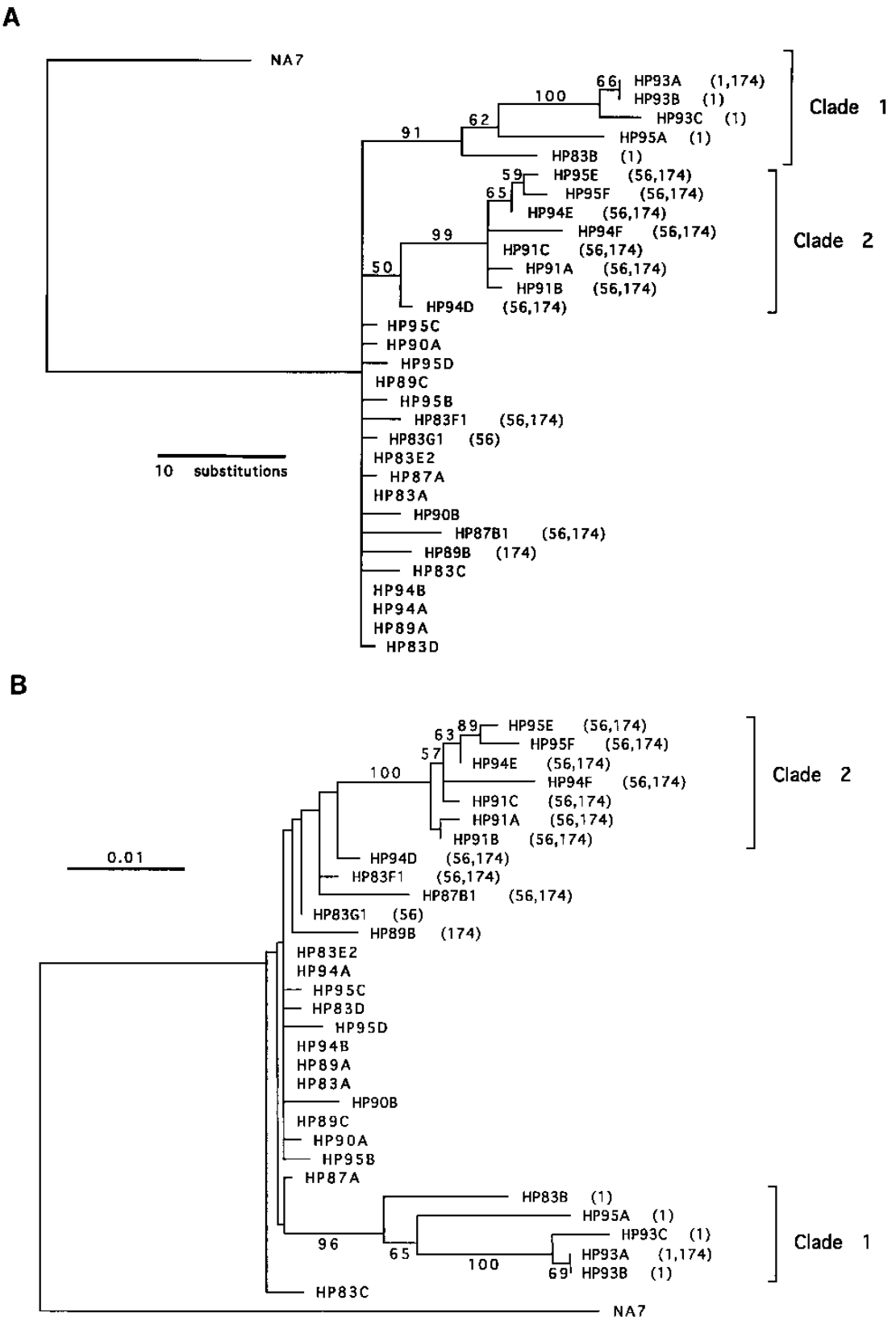

FIG. 6. Phylogenetic relationship of *nef* sequences isolated from individual HP over a 12-year period. For each of the PCRs performed with HP DNA samples, one *nef* sequence was selected for phylogenetic analysis. (A) Trees were estimated by the maximum parsimony-approach with PAUP software (43, 44). Branch lengths are expressed as the number of nucleotide substitutions along with the number of unambiguous substitutions (absolute distance minus homoplasy). (B) Trees were estimated by the neighbor-joining method, with distances calculated by the method of Jukes and Cantor with MEGA software. The number of bootstrap replications (of a total of 100) in support of the adjacent node is shown. The HIV-1 NA7 *nef* allele (26) was used as an outgroup. The distribution of debilitating mutations M1I, A56D, and E174K among the analyzed sequences is shown in brackets on the right.

progression and on the mechanisms by which HIV-1 infection can be controlled.

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