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Functional Characterization of Human Immunodeficiency Virus Type 1 *nef* Genes in Patients with Divergent Rates of Disease Progression

NELSON L. MICHAEL,[1]* GEORGE CHANG,[2] LISA A. D'ARCY,[2] CONRAD J. TSENG,[2] DEBORAH L. BIRX,[1] AND HAYNES W. SHEPPARD[3]

Division of Retrovirology, Walter Reed Army Institute of Research,[1] and Henry M. Jackson Foundation,[2] Rockville, Maryland, 20850, and California State Department of Health Services, Berkeley, California 94704[3]

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We have studied the sequence and function of the human immunodeficiency virus type 1 (HIV-1) *nef* genes from nine patients with highly divergent rates of disease progression enrolled in a longitudinal study of HIV disease. Over an average of 7.8 years of follow-up, three patients had net positive changes in $CD4^+$ T-cell counts, three patients had net negative changes in $CD4^+$ T cells but did not develop AIDS, and three patients progressed to AIDS. The *nef* gene from each of these patients was amplified and cloned, and the sequence of 8 to 10 clones was determined. Only 2 of 88 (2.3%) *nef* genes recovered from these nine patients were grossly defective. Moreover, there was no relationship between the phylogeny of *nef* sequences and the corresponding rates of disease progression from these patients. Representative *nef* genes from all nine patients were tested for their abilities to downregulate cell surface CD4 in a transient-transfection assay. There was no correlation found between the functions of the *nef* genes from these patients and their corresponding rates of disease progression. We conclude that the *nef* gene is not a common mediator of the rate of HIV disease progression in natural infection.

Much recent attention has been devoted to the analysis of patients who display extremely slow rates of human immunodeficiency virus type 1 (HIV-1) disease progression (9, 18, 26, 27, 29, 31, 33, 44, 50, 53, 62). Despite a high rate of viral turnover in HIV-1 infection (13, 22, 57), several reports have now confirmed that, on average, slow progressors express relatively low steady-state levels of viral RNA compared with those of rapid progressors (16, 39, 48). Thus, a fundamental question in HIV-1 pathogenesis is whether these patients mount particularly effective antiviral immune responses, harbor relatively attenuated viral genotypes, or represent some combination of both of these mechanisms (52). The dissection of qualitatively superior immune responses to HIV-1 would provide in vivo modeling of desired immune responses for both immunotherapy and preventive vaccines, while the understanding of attenuated viral strains could provide a blueprint for the development of live, attenuated viral vaccines.

Intensive efforts have focused on the role of the *nef* gene in HIV-1 pathogenesis since Kestler and coworkers demonstrated that *nef*-defective simian immunodeficiency virus causes an attenuated disease course in primates (28). Multiple groups have demonstrated that the *nef* gene is capable of downregulating cell surface CD4 molecules (2, 4, 6, 8, 15, 20, 25, 36, 54). The mechanism of Nef-mediated cell surface CD4 downregulation involves the active sequestration of cell surface CD4 molecules in lysosomes, where they are subsequently degraded (1, 46, 51). The *nef* gene has also been shown to directly influence the propagation of HIV-1 by its positive effect on the infectivities of progeny virions (12, 41, 42, 47). Finally, there is evidence that Nef both inhibits and stimulates the normal activation pathways of T cells (5, 19, 54). With these functions considered together, the *nef* gene would seem to be an attractive modulator of disease progression in HIV-1-infected patients.

A recent report described extensive and inactivating deletions in the *nef* gene from a single long-term nonprogressor from whom samples were repeatedly taken over time (29). However, another study of 10 long-term survivors found only 8.9% of *nef* genes to be grossly defective by sequence analysis (24). We have studied the viral sequences from a long-term survivor of HIV-1 infection who lacks cultivable virus and have found evidence for defective *vif*, *vpr*, and *vpu* genes but also wild-type *nef* alleles by both DNA sequence and functional analyses (37). We now report the results of DNA sequence and functional analyses of *nef* genes recovered from nine patients from the San Francisco Men's Health Study (SFMHS) representing divergent rates of disease progression. No correlation between the structure or function of the *nef* genes from these patients and their corresponding rates of disease progression was found. We also studied CD4 cDNA sequence heterogeneity for a subset of these patients and found no correlation with either rate of disease progression or *nef* function.

MATERIALS AND METHODS

Description of cohort. Study subjects were selected from the SFMHS, a population-based cohort of single men recruited in 1984 from the 19 census tracts of San Francisco, California, with the highest incidence rates of AIDS at that time (30). The men were between the ages of 25 and 54 years at study entry and have been monitored at 6-month intervals for up to 114 months. Of the 1,034 men enrolled, 400 were HIV-1 seropositive at cohort entry and an additional 48 have seroconverted during follow-up. Of these 448 seropositive men, 276 (62%) have met the 1993 Centers for Disease Control and Prevention expanded case definition for AIDS (CD4 count, $<200/\mu l$) (10) and 234 (52%) have died of AIDS-related illnesses.

Patient selection criteria. The entire cohort was stratified according to the least-squares fit (slope) of the patients' cumulative CD4 counts into 10 deciles as described previously (53). Five patients were randomly selected from the decile group with the least-negative CD4 slope and four patients were randomly selected from the decile group with the most-negative CD4 slope for *nef* analysis. Once the individual patients were selected from the two divergent decile groups, patients were then further sorted into three final groups on the basis of the slopes of their individual $CD4^+$ T-cell counts (Table 1). The slow progressor group, patients SP-1, SP-2, and SP-3, were all from the decile group with the least-

* Corresponding author. Mailing address: Division of Retrovirology, Walter Reed Army Institute of Research, 13 Taft Ct., Suite 200, Rockville, MD 20850. Phone: (301) 762-0089. Fax: (301) 762-4177. Electronic mail address: nmichael@hiv.hjf.org.

TABLE 1. Patient characteristics

Patient	CD4 count (cells/μl) at:		Study period (yr)	DNA sampling (yr)	CD4 slope (cells/μl · yr)
	Study entry	Follow-up			
SP-1	523	518	8.0	8.0	11.76
SP-2	1,043	910	8.0	8.0	13.80
SP-3	1,067	635	8.0	8.0	1.75
IP-1	1,005	240	7.0	5.0	−67.61
IP-2	728	220	8.0	8.0	−21.85
IP-3	587	383	8.0	8.0	−40.89
RP-1	819	182	8.0	6.0	−97.47
RP-2	832	20	7.0	5.0	−135.31
RP-3	840	125	8.0	7.0	−84.16

negative CD4 slope. They showed positive CD4 slopes over the 8-year follow-up period, with final CD4 counts all greater than 500 cells per μl. The rapid progressor group, patients RP-1, RP-2, and RP-3, were all from the decile group with the most-negative CD4 slope. They all showed highly negative CD4 slopes and progressed to AIDS (CD4 count, <200/μl). The intermediate progressor group, patients IP-1, IP-2, and IP-3, were drawn from both decile groups. They showed negative CD4 slopes but failed to progress to AIDS during the follow-up period (CD4 count, >200/μl). (See Fig. 1 for the CD4 counts for each patient over the follow-up period.)

DNA amplification and nucleotide sequencing of *nef* genes. DNA was prepared from cryopreserved peripheral blood mononuclear cells from the time points indicated in Table 1 by the IsoQuick technique (Molecular Probes). A nested PCR strategy was employed for the isolation of a 0.74-kb fragment (HXB2 positions [45] 8696-9438) containing the entire *nef* gene by using the following primers: Nef-outer 1, CGCTTGAGAGACTTAMTCTTGAYT; Nef-outer 2, AGGATCTGAGGGCTCGCCACT; Nef-inner 1, GRGACAGATAG GRTTATAGAA; and Nef-inner 2, GTCCCTTGTAGCAAGCTCGAT; where R = G + A, Y = C + T, and M = A + C. These amplicons were molecularly cloned with the TA cloning vector pCR-II (Invitrogen, Inc.). Plasmid DNA from multiple clones was prepared with a rapid column purification technique (Qiagen, Inc.) and then subjected to cycle sequencing with fluorescence-labeled oligonucleotide primer and dideoxynucleotide terminator approaches with an automated DNA sequencer (model 373A; Applied Biosystems, Inc., Foster City, Calif.). Nucleotide sequences were compiled and translated into amino acids with the EditSeq version 3.75 software package and aligned with the MegAlign version 1.05 software package (DNAStar, Madison, Wis.). For cladistic analysis, DNA sequences corresponding to the *nef* coding region were aligned and gap stripped with GeneWorks version 2.2 software (Intelligenetics, Inc.). These sequences were then used to compute a distance matrix and a neighbor-joining tree with the DNADIST, NEIGHBOR, and DRAWTREE programs contained in the Phylip version 3.45 package.

Amplification and nucleotide sequencing of CD4 cDNA. Total cellular RNA (1.0 μg), prepared by the RNAzol B method (Tel-Test, Inc.), was converted to cDNA with reverse transcriptase (SuperScript II; Gibco) and the primer CD4-dn. Following heat inactivation of the reverse transcriptase reaction, a nested PCR strategy was performed to isolate a 1.44-kb DNA fragment containing amino acid residues −15 through 433 (35) of the CD4 coding sequences in which the first residue of the mature CD4 protein is residue +1. These amplicons were molecularly cloned and sequenced by the same approach used for the *nef* genes. The primers used for the reverse transcriptase PCR strategy are given with the 5′ member of each pair listed first: (outer pair) CD4-up and CD4-dn, CCTCG GCAAGGCCACAATGAACCG and CCTAACCCAGGAGAAACAATGG CA, respectively; (inner pair) CD4-1 and CD4-2, TT CTGGTGCTGCAACTGG CGCTCCT and GTGGGATCTGCTACATTCATCTGGT, respectively. This strategy was validated by using RNA provided from the plasmid pT4B (a full-length CD4 cDNA clone).

Nef-mediated cell surface CD4 downregulation assay. The Nef-mediated cell surface CD4 downregulation assay is a modification of a previously published approach (36, 54). *nef* alleles were cloned into pCD3Tex (36) and then cotransfected by electroporation into A3.01 cells (a human neoplastic T-cell line) along with a β-galactosidase expression plasmid (pCMVβgal) (43). All subclone constructions were confirmed by nucleotide sequencing prior to transfection. Plasmid DNA concentrations were kept uniform with the addition of pSKCAT (40) as filler DNA. Plasmid DNAs were mixed with 1.5×10^7 log-phase A3.01 cells and then electroporated in disposable 0.4-cm-gap cuvettes with a Gene Pulser (Bio-Rad, Inc.) essentially as described previously except that the voltage was 200 V (38). After 40 h, the cells were harvested, hypotonically loaded with fluorescein di-β-D-galactopyranoside (Molecular Probes, Inc.), a fluorescein-releasing β-galactosidase substrate stained with a phycoerythrin-labeled, anti-CD4 monoclonal antibody, and then analyzed on a FACScan analytical flow cytometer with Lysis II software (Becton Dickinson, Inc.). Only those cells expressing fluorescein were gated for CD4 phycoerythrin fluorescence. This ensured that only those cells transduced with DNA were assessed for cell surface CD4 expression. The median channel fluorescence for 50,000 cells was computed for each experiment. Controls included cells transfected with filler DNA alone or pCMVβgal alone and known wild-type and defective *nef* alleles cotransfected with pCMVβgal.

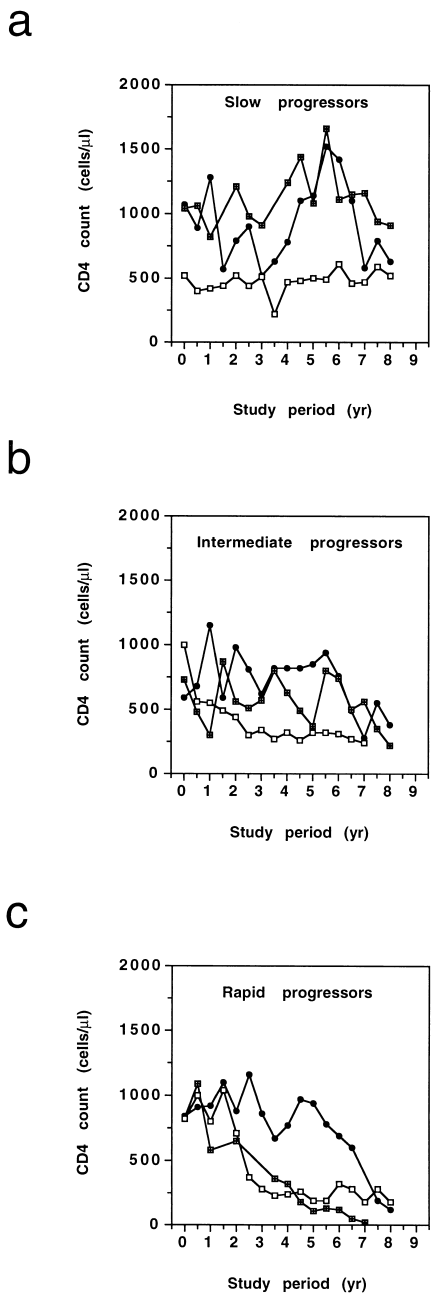


FIG. 1. Cohort peripheral blood CD4+ T-cell counts. The CD4 count is shown for each patient at 6-month intervals over the entire study period. Symbols for each group: □, patient 1; ⊞, patient 2; ●, patient three.

Nucleotide sequence accession numbers. The sequences described here have been deposited at GenBank under accession no. U26060 through U26147.

RESULTS

Sequence analysis of the *nef* gene reveals a low rate of inactivating mutations. Nine patients that represented a spec-

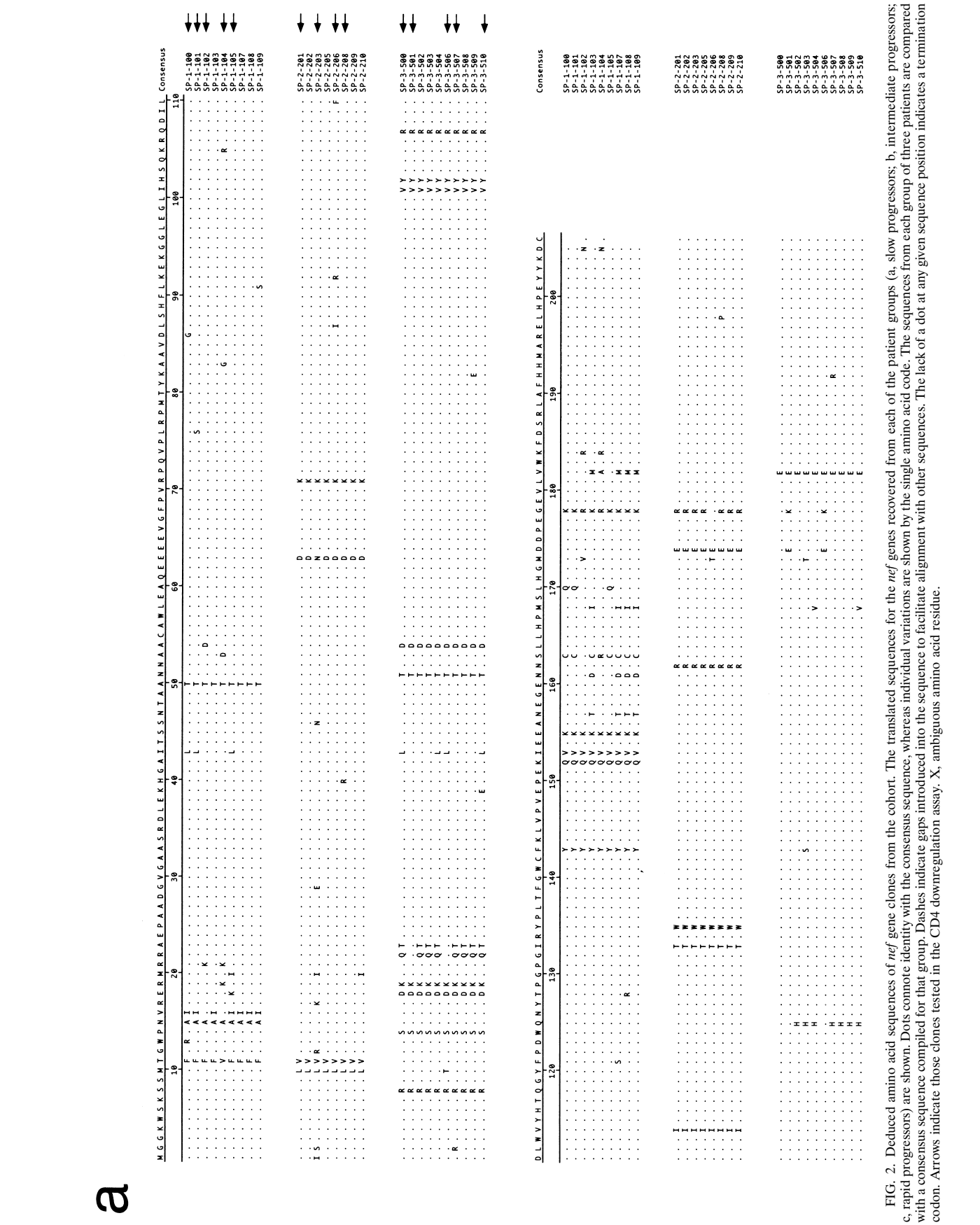


FIG. 2. Deduced amino acid sequences of *nef* gene clones from the cohort. The translated sequences for the *nef* genes recovered from each of the patient groups (a, slow progressors; b, intermediate progressors; c, rapid progressors) are shown. Dots connote identity with the consensus sequence, whereas individual variations are shown by the single amino acid code. The sequences from each group of three patients are compared with a consensus sequence compiled for that group. Dashes indicate gaps introduced into the sequence to facilitate alignment with other sequences. The lack of a dot at any given sequence position indicates a termination codon. Arrows indicate those clones tested in the CD4 downregulation assay. X, ambiguous amino acid residue.

b

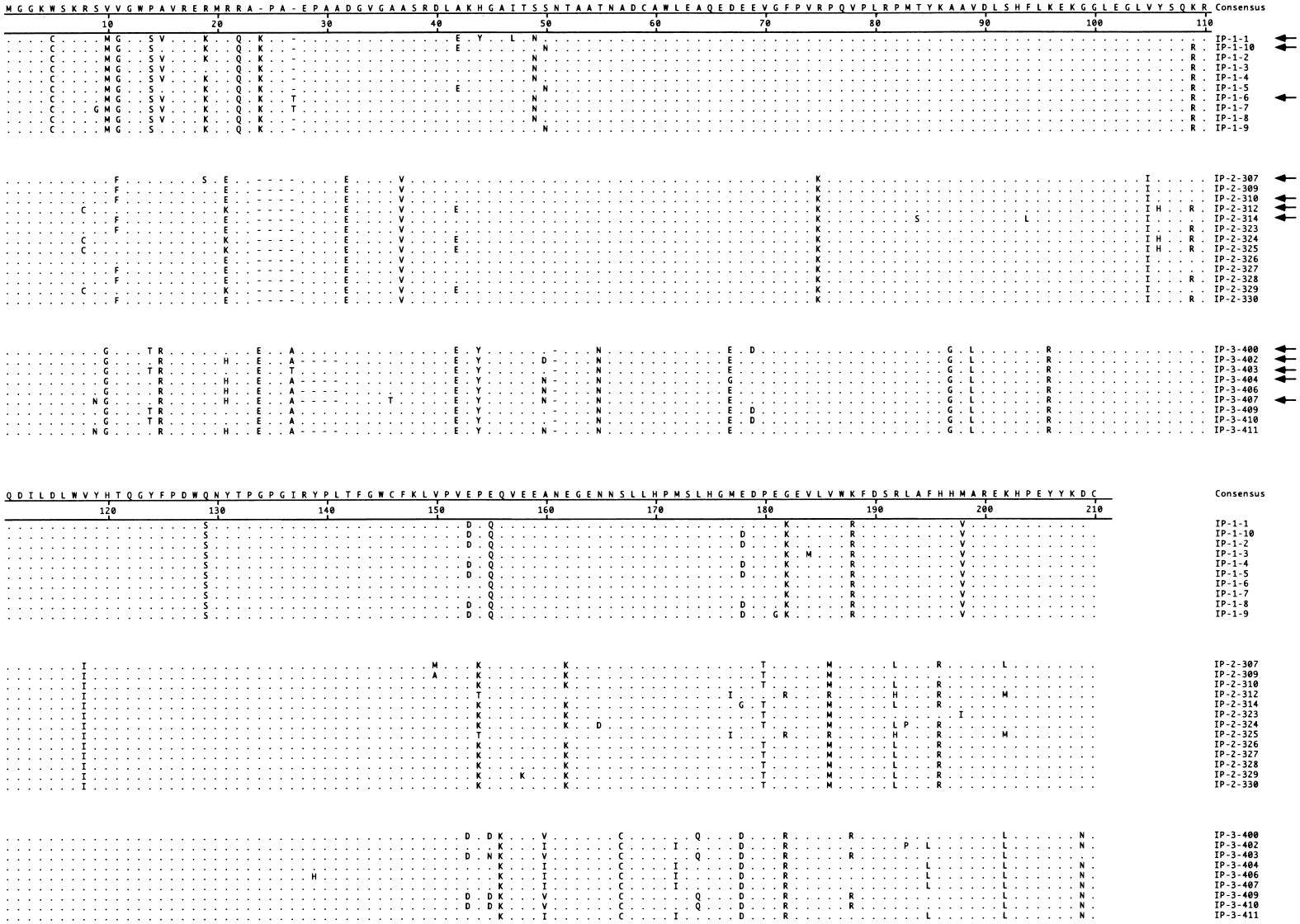


FIG. 2—*Continued.*

C

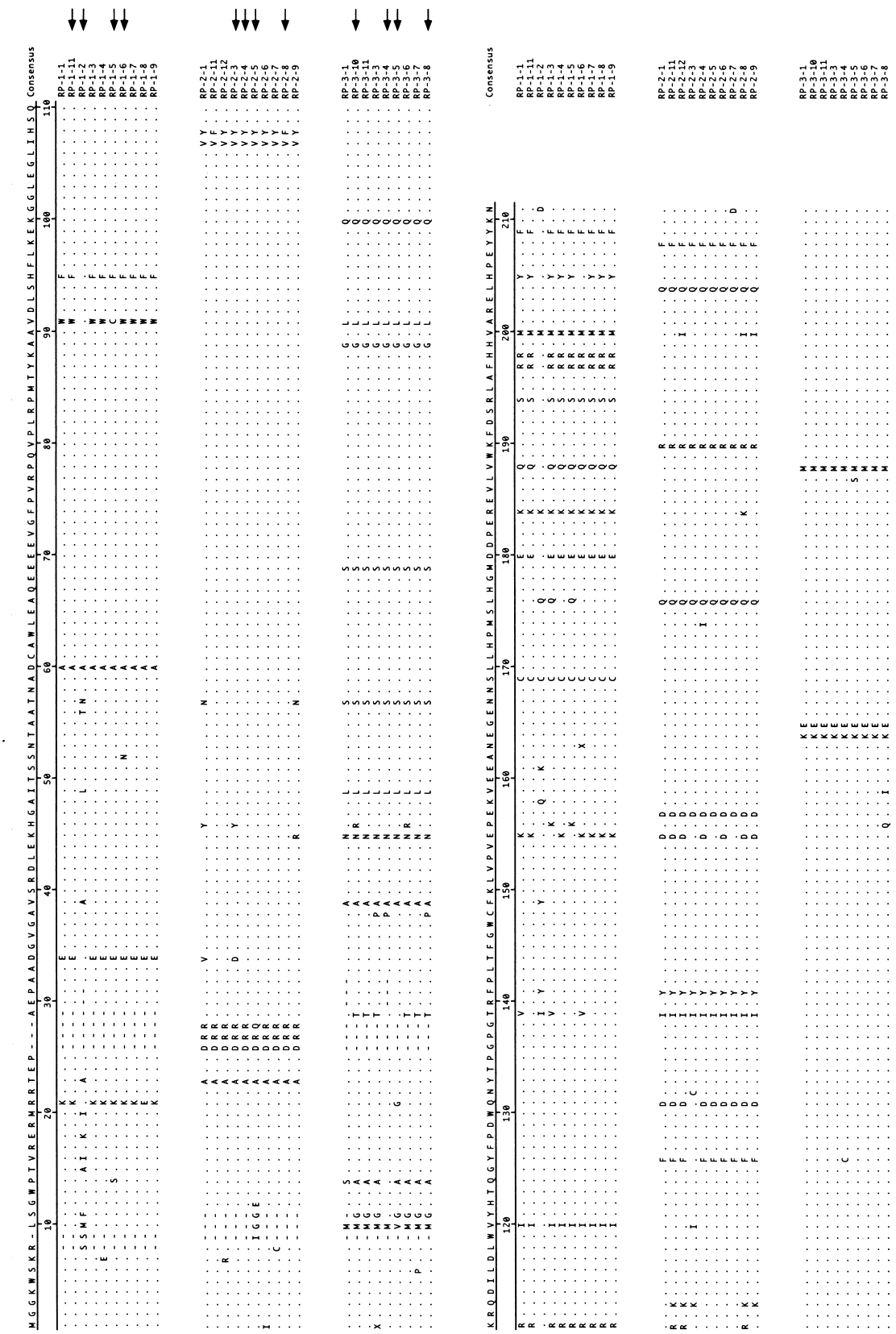


FIG. 2–*Continued.*

TABLE 2. Functional statuses of patient *nef* clones

Patient group	Patient	No. of clones				
		Sequenced	Analyzed[a]	Functional	Partially functional	Defective[b]
Slow progressors	SP-1	9	5	4	0	1
	SP-2	8	7	4	1	2
	SP-3	10	5	3	0	2
Group total (%)		27	17	11 (64.7)	1 (5.9)	5 (29.4)
Intermediate progressors	IP-1	10	4	1	2	1
	IP-2	13	5	3	2	0
	IP-3	9	7	6	0	1
Group total (%)		32	16	10 (62.5)	4 (25.0)	2 (12.5)
Rapid progressors	RP-1	10	6	2	4	0
	RP-2	10	5	4	0	1
	RP-3	9	5	2	0	3
Group total (%)		29	16	8 (50.0)	4 (25.0)	4 (25.0)
Cohort total (%)		88	49	29 (59.2)	9 (18.4)	11 (22.4)

[a] Includes nontested clones identical in sequence to tested clones.
[b] Includes one grossly defective clone (50349-6) that was not directly tested.

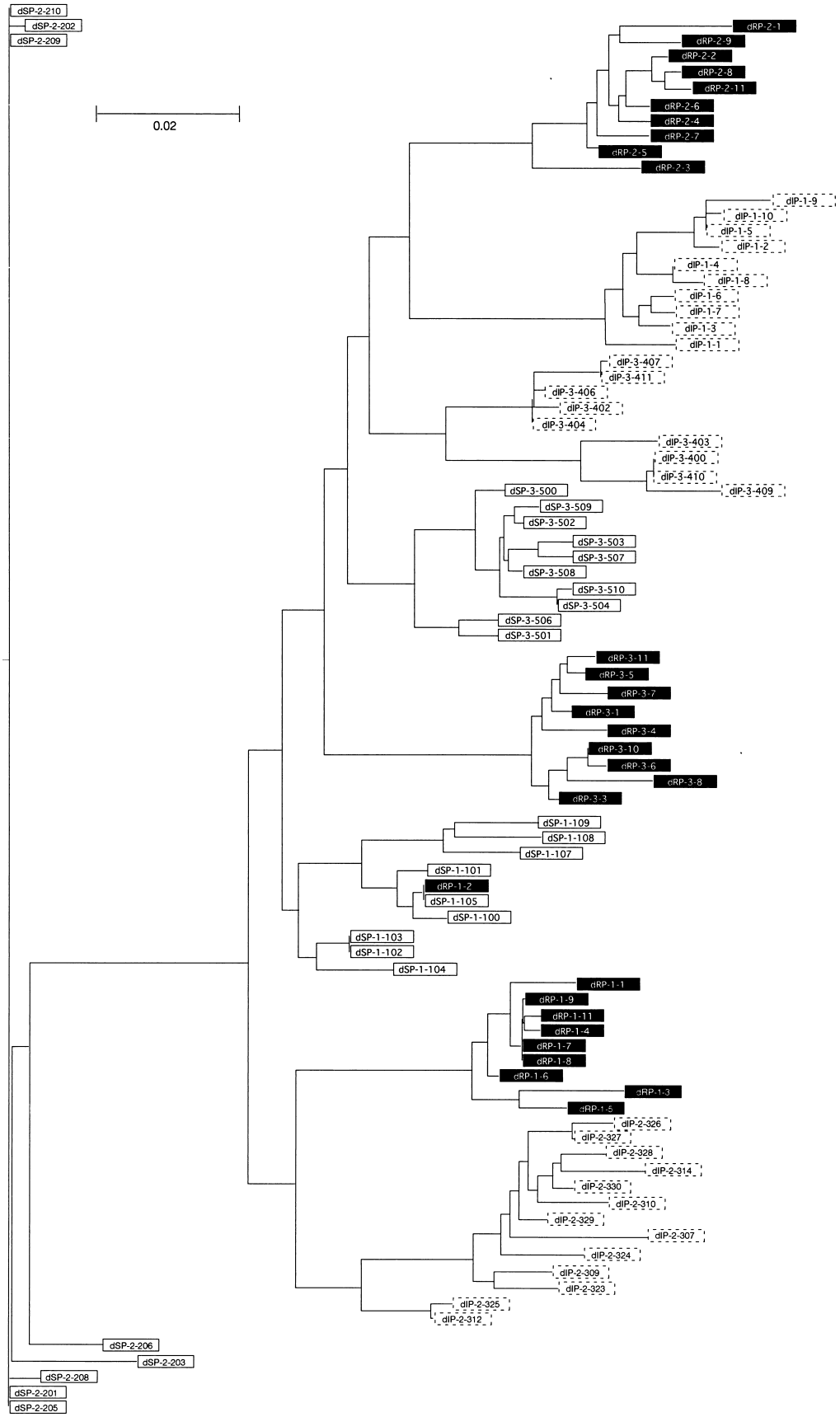
trum of HIV-1 disease progression were selected from the SFMHS cohort. Three patients were slow progressors, three patients were intermediate progressors, and three patients were rapid progressors (see Materials and Methods for patient selection criteria; CD4 counts are shown in Fig. 1). DNA fragments encompassing the entire *nef* gene from each of these patients were amplified, cloned, and sequenced. The deduced amino acid sequences from multiple clones for each patient are shown in Fig. 2. By inspection, each group of clones for each individual patient appears to be distinctive. The sequence data for the three slow progressors are given in Fig. 2a. Only a single grossly defective clone, from patient SP-2, out of 27 *nef* sequences from these three patients was observed. This clone, SP-2-203, contains an M-to-I mutation at position 1 (M_1I mutation) at the initiation codon. The next in-frame methionine residue does not occur until position 79. None of the 32 *nef* sequences recovered from the three intermediate progressors were grossly defective (Fig. 2b). For the 29 sequences from the three rapid progressor patients (Fig. 2c), one grossly defective clone was identified from patient RP-2. Clone RP-2-6 contained an M_1I mutation at the initiation codon, although another in-frame methionine was present at position 20. Overall, two grossly defective *nef* sequences were recovered from 88 clones (2.3%) from nine patients.

We next asked if rates of disease progression correlated with cladistic associations between *nef* coding sequences. Figure 3 shows a neighbor-joining tree constructed from all of the DNA sequences from the nine patients studied. All sequences were gap stripped prior to compilation of distances and neighbor-joining phenogram development with the DNADIST and NEIGHBOR or DRAWTREE applications, respectively, from the Phylip package. With the exception of a single sequence, all sequences sorted by patient and not by subcohort. Moreover, there was no discernible cladistic association of patients belonging to any given subcohort. One *nef* sequence of ten recovered from the rapid progressor RP-1 (RP-1-2) sorted closely with clone SP-1-105 from the slow progressor SP-1. Although this association may be biologically valid, it is also possible that this sorting represents a laboratory artifact (cross-contamination or mislabeling).

Functional analysis of the *nef* gene reveals no correlation with disease progression. The inspection of gene sequences alone cannot fully predict their functions. Many groups have reported the ability of the *nef* gene to downregulate cell surface CD4 (2, 4, 6, 8, 15, 20, 25, 36, 54). Recent data suggest that the mechanism of Nef-mediated cell surface CD4 downregulation involves the active sequestration of cell surface CD4 molecules in lysosomes, where they are subsequently degraded (1, 46, 51). Therefore, we tested the abilities of representative *nef* genes from each of these patients to downregulate cell surface CD4 using a well-established transient-transfection assay (36, 54).

Representative *nef* genes (Fig. 2) from each of the nine patients studied were subcloned into a eukaryotic expression vector and cotransfected with a β-galactosidase expression plasmid into A3.01 cells. Cell surface CD4 density was determined 40 h posttransfection with a fluorescence-labeled CD4 monoclonal antibody from only that population of cells that demonstrated β-galactosidase activity. The results of these experiments are shown in Fig. 4. For each series of patient *nef* genes tested, positive (NL4-3) and negative (HXB3) control *nef* expression vectors were simultaneously tested for comparison.

The data for the slow progressors are given in Fig. 4a to c. In each case, the NL4-3 positive control *nef* expression vector mediates dose-dependent cell surface CD4 downregulation whereas little or no effect was seen with the HXB3 negative control *nef* expression vector. Patient SP-1 (Fig. 4a) showed four fully functional clones (similar to NL4-3) of the five tested. Clone SP-1-102 was intermediate in function compared with the positive and negative control genes. This clone differed from its functional counterparts at numerous positions. In contrast, only clone SP-2-201 of the four clones tested from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dSP-2-208
dSP-2-201
dSP-2-205

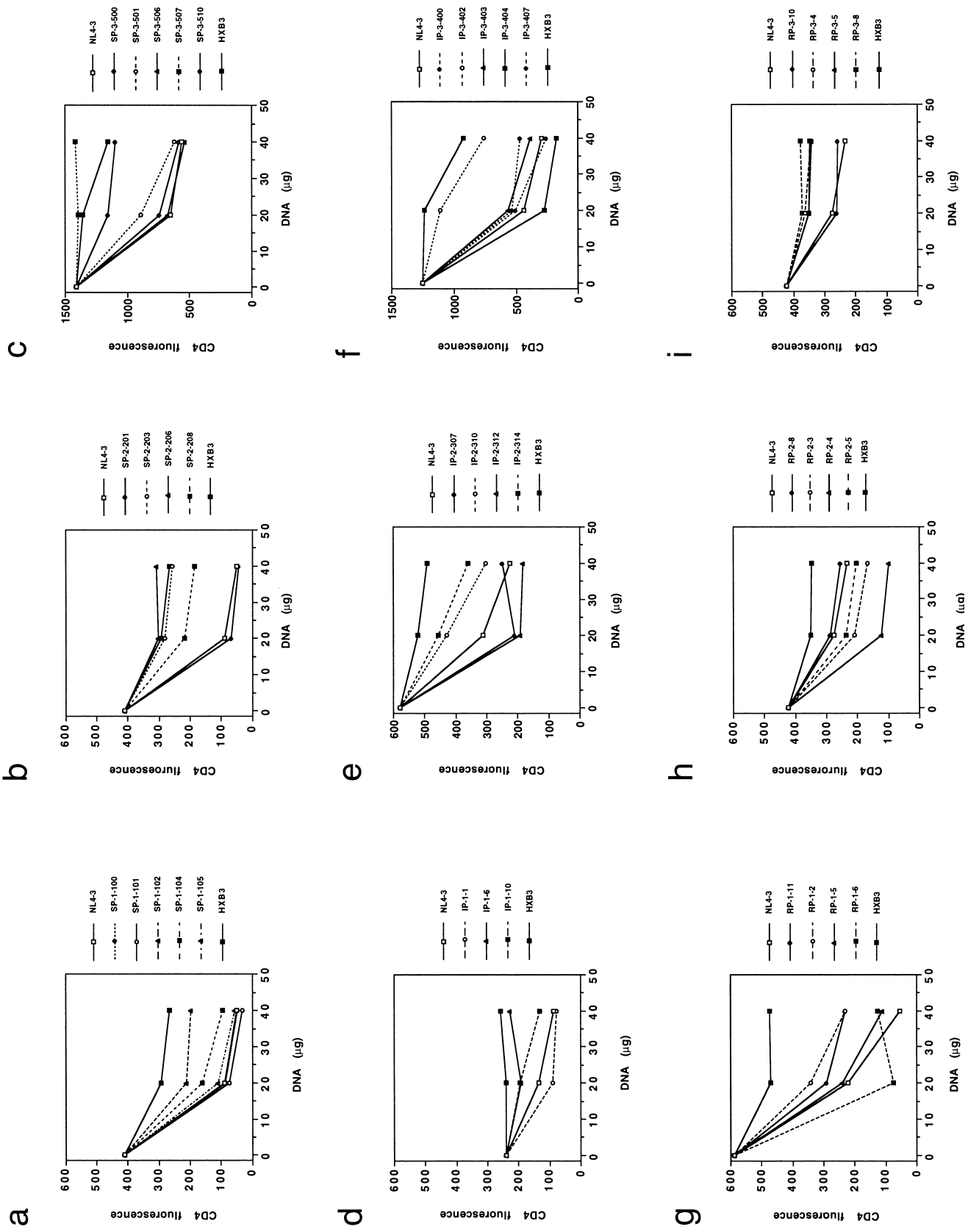
slow progressor SP-2 was fully functional (Fig. 4b) although this clone was identical to three other clones from this patient that were not tested. Clone SP-2-203, which was previously identified as grossly defective because of an M_1I mutation (Fig. 2a), was shown by functional analysis to be completely defective (similar to HXB3). Clone SP-2-206 was also completely defective, whereas clone SP-2-208 was partially functional. The sequence of clone SP-2-208 differs from that of the fully functional clone SP-2-201 by both an $H_{40}R$ and an $L_{198}P$ substitution (Fig. 2a). The third slow progressor, patient SP-3, showed three fully functional *nef* clones (clones SP-3-500, SP-3-501, and SP-3-506) of five tested (Fig. 4c). Clones SP-3-507 and SP-3-510 were both functionally defective. Although clone SP-3-507 possessed a mutation of the glycine residue at position 2, known to be essential to posttranslational myristoylation and function of the *nef* gene (36, 61), clone SP-3-510 differed from the wild-type gene SP-3-500 by both a $K_{39}E$ and an $M_{168}V$ substitution (Fig. 2a). All of the clones from the slow progressors showed conservation of the three PXXP motifs at positions 69 to 72, 75 to 78, and 147 to 150 (Fig. 2a). These proline repeats have been implicated as SH3 binding domains that mediate protein-protein interactions of the Nef protein with the Src family of nonreceptor tyrosine kinases (47). Further, the PXXP repeats have been shown to be important for Nef-mediated enhancement of virion infectivity but not CD4 downregulation (47). The functional analyses of the *nef* genes from the slow progressors are summarized in Table 2. Of the three slow progressor patients, 17 *nef* genes from a total of 27 genes recovered were functionally assessed for their abilities to downregulate cell surface CD4. Of these genes, 11 (64.7%) were fully functional, 1 (5.9%) was partially functional, and 5 (29.4%) were completely nonfunctional.

The data for the intermediate progressors are given in Fig. 4d to f. Patient IP-1 (Fig. 4d) showed one fully functional clone (IP-1-1), two partially functional clones (IP-1-10 and its identical counterpart IP-1-5), and one defective clone (IP-1-6). Although these clones differ from one another at multiple positions, the completely defective clone IP-1-6 has a threonine insertion at position 27 compared with the functional and partially functional clones (Fig. 2b). In the absence of systematic reversion mutation analysis, however, the link between these structural observations and functionality remains unclear. Three clones from patient IP-2 were functional (clones IP-2-307 and IP-2-312 were directly tested, and clone IP-2-325 is identical to clone IP-2-312), and two clones (IP-2-310 and IP-2-314) were partially functional (Fig. 4e). The only consistent difference in amino acid sequences between the functional and partially functional clones is the presence at position 202 of a hydrophobic residue (leucine or methionine) in the former and the charged residue lysine in the latter (Fig. 2b). Although all of the clones recovered from this patient had conservation of the first two PXXP motifs (positions 73 to 76 and 79 to 82), the third repeat (position 151-154) was mutated to PXXK/T (Fig. 2b). Given that PXXP motif mutations do not effect Nef-mediated CD4 downregulation (47), the degree of functional impairment of the *nef* genes from patient IP-2 may be underestimated. However, Saksela and coworkers have shown that the proline residue in the fourth position of the third PXXP motif has less functional significance than the proline residue in the first position of the repeat (47). The next intermediate progressor, patient IP-3, showed six fully functional *nef* genes and a single defective gene (Fig. 4f). Clones IP-3-400, IP-3-403, IP-3-404, and IP-3-407 were determined to be functional experimentally; clones IP-3-409 and IP-3-410 were identical to clone IP-3-400. The single defective clone, IP-3-402, differed from the functional clones by both an $N/S_{50}D$ and an $L_{193}P$ substitution (Fig. 2b). Overall, 16 *nef* genes from a total of 32 genes recovered from the intermediate progressor patients were functionally assessed (Table 2). Of these genes, 10 (62.5%) were fully functional, 4 (25.0%) were partially functional, and 2 (12.5%) were completely nonfunctional.

The data for the rapid progressors are given in Fig. 4g to i. Patient RP-1 had two fully functional clones, RP-1-5 and RP-1-6, and four partially functional clones (Fig. 4g). Two of the latter clones, RP-1-2 and RP-1-11, were directly tested, and clones RP-1-7 and RP-1-9 were identical in sequence to clone RP-1-11. However, given that clones RP-1-5 (functional) and RP-1-3 (not tested) had a $P_{156}K$ mutation in the third PXXP repeat (Fig. 2c), it is possible that the functions of the *nef* genes from this patient were underestimated. In contrast, all four of the tested *nef* genes from patient RP-2 were functional (Fig. 4h). Clone RP-2-6 was previously identified as grossly defective, given the M_1I mutation that abolished the initiating methionine residue. The third rapid progressor, patient RP-3, had two functional clones and three defective clones (Fig. 4i). One of the functional clones, RP-3-10, was directly tested, while the other, RP-3-6, was identical in sequence to RP-3-10. Each of the three functionally defective clones, RP-3-4, RP-3-5, and RP-3-8, differed from the wild-type clones at multiple positions (Fig. 2c). The defective clone RP-3-8 also contained a $P_{156}Q$ mutation in the third PXXP repeat. Thus, for the three rapid progressor patients, 16 *nef* genes from a total of 29 genes recovered were functionally assessed (Table 2). Of these genes, 8 (50.0%) were fully functional, 4 (25.0%) were partially functional, and 4 (25.0%) were completely nonfunctional. For the nine patients studied, 49 *nef* genes of 88 genes recovered were functionally analyzed. Of these genes, 29 (59.2%) were functional, 9 (18.4%) were partially functional, and 11 (22.4%) were completely defective. These proportions were not fundamentally different for any given progression group. Thus, these data do not support a correlation between the function of the *nef* genes from these patients and their corresponding rates of disease progression. We conclude that the *nef* gene is not a common mediator of the rate of HIV disease progression in natural infection.

CD4 gene structure is not a modulator of disease progression. Given that the CD4 molecule is the viral receptor (14, 34) and that Nef is known to interact (either indirectly or, possibly, directly) with residues in the cytoplasmic tail of CD4 (1, 3, 49), we reasoned that variation in *nef* gene product function may also be a function of CD4 sequence variation. The functional consequences of *nef* sequence variation could theoretically be compensated for by changes in the CD4 coding sequences. If so, then the use of the A3.01 cell CD4 downregulation assay would be insensitive to putative CD4 sequence heterogeneity. Another HIV-1 protein, Vpu, has also been implicated in CD4

FIG. 3. Phylogenetic relationship between *nef* genes from slow, intermediate, and rapid progressors. A neighbor-joining phenogram computed on the basis of DNA distance data from gap-stripped *nef* coding sequences is shown. Horizontal distance between nodes is directly proportional to sequence variance. Sequence variance between individual clones is given by the sum of all horizontal distances connecting them. Vertical distance between nodes is significant only for optimal visualization of sequences and has no bearing on sequence variance. The bar indicates 0.02 distance units (2% variance). Individual clones are identified by patient group as follows: open boxes, slow progressors; dashed boxes, intermediate progressors; solid black boxes, rapid progressors. All clone identifiers are preceded by the letter d to indicate their composition as DNA sequences.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SP-2-206
SP-2-208
SP-3-500
SP-3-501
SP-3-506
SP-3-507
SP-3-510
IP-1-1
IP-1-6
IP-1-10
IP-2-307
IP-2-310
IP-2-312
IP-2-314
IP-3-400
IP-3-402
IP-3-403
IP-3-404
IP-3-407
RP-1-11
RP-1-2
RP-1-5
RP-1-6
RP-2-8
RP-2-3
RP-2-4
RP-2-5
RP-3-10
RP-3-4
RP-3-5
RP-3-8

FIG. 4. Nef-mediated cell surface CD4 downregulation assay. The complete coding sequences for representative *nef* genes from each of the nine patients studied and from the molecular clones NL4-3 and HXB3 were cloned into an expression vector containing the CD3 promoter. These plasmids were then cotransfected in increasing amounts with a fixed amount of the β-galactosidase expression vector pCMVβgal into A3.01 cells. The cells were loaded at 40 h posttransfection with a fluoresceinated β-galactosidase substrate and were subsequently stained with a phycoerythrin-labeled anti-CD4 monoclonal antibody. Only those cells positive for fluorescein were gated on to ensure that cell surface CD4 expression was measured only in that subpopulation of cells that was transduced with plasmid DNA. Cell surface CD4 expression for each transfection was determined as median channel phycoerythrin fluorescence. Clone identifiers are given to the right of each figure. NL4-3, a positive control Nef expression plasmid. HXB3, a negative control Nef expression plasmid. a to c, slow progressors; d to f, intermediate progressors; g to i, rapid progressors.

degradation, albeit in the endoplasmic reticulum, via residues in the CD4 cytoplasmic tail (7, 11, 32, 56, 59, 60). Although there have been limited reports of CD4 sequence polymorphisms in humans and other mammals (17, 21, 23, 55, 58), we are aware of no published reports of CD4 sequence variation for patients with divergent rates of disease progression.

We amplified, cloned, and sequenced a 1.44-kb cDNA fragment containing residues −15 through 433 (35) of the CD4 coding sequences from RNA extracted from the peripheral blood mononuclear cells of two seronegative volunteers (one of Russian/Polish ancestry and one of Korean ancestry), two slow progressors (SP-1 and SP-2), and two intermediate progressors (IP-1 and IP-2). Although this was a very limited data set, we found no evidence of CD4 cDNA sequence polymorphism in either the two seronegative volunteers or the four HIV-1-infected patients (data not shown). Thus, CD4 cDNA sequence heterogeneity did not correlate with either rates of disease progression or *nef* function in these patients.

DISCUSSION

We have analyzed the HIV-1 *nef* genes from nine patients drawn from a large, natural-history cohort and have found that the structure and function of *nef* does not correlate with disease progression. A previous study by Huang and coworkers of *nef* gene sequences from 10 long-term survivors found only 8.9% of all clones to be grossly defective (24). This is in good agreement with our observed rate of 1 grossly defective clone out of 27 sequenced clones (3.7%) from three slow progressors in the SFMHS. Further, the overall rate of grossly defective clones from patients representing the full spectrum of HIV-1 disease progression was 2.3% (2 of 88 clones). Cladistic analysis by Huang and coworkers of *nef* sequences from long-term survivors showed no association with *nef* sequences from historical controls with those from late-stage disease (24). We have now shown that *nef* sequences from patients with divergent rates of disease progression drawn from the same longitudinal cohort also have no cladistic association.

Despite these initial observations, the low rate of recovery of grossly defective clones in both studies casts serious doubt on the sensitivities of purely structural analyses to predict the dysfunction of viral genes which may be associated with disease progression. Further, the analysis of patients representing only one extreme of HIV-1 disease progression may bias conclusions regarding virologic associations with disease progression. Thus, we designed the present study to analyze the functional statuses of *nef* genes from patients representing the entire spectrum of disease progression. The rates of recovery of defective and partially functional *nef* clones, 22.4 and 18.4%, respectively, were much higher by CD4 downregulation assay than the rate of recovery of grossly defective clones by sequence analysis alone (2.3%). Despite this increase in sensitivity, the proportions of functional, partially functional, and defective clones did not appreciably differ among the three progression subgroups. Interestingly, only 59.2% of the 49 *nef* clones tested from the nine patients studied were fully functional. These data speak to the wide range of functional genotypes previously reported from peripheral blood mononuclear cell proviral DNA populations (38). Additionally, we have shown that sequence heterogeneity in the viral receptor CD4 is apparently not involved in the modulation of disease progression. This observation further validates the use of a common cell line as the assay system for the fitness of individual *nef* genes to mediate CD4 downregulation.

We were unable to precisely discern the molecular lesion for every defective or partially functional *nef* gene in this study

because of the frequent recovery of defective clones with multiple amino acid changes compared with the recovery of wild-type clones. Unlike in systematic mutation studies of a single molecular clone, the intent of this work was not a mechanistic dissection of the molecular basis of *nef* function for each patient. Similarly, we did not assess the stability of the Nef proteins produced from each molecular clone to discriminate between those clones defective by virtue of rapid protein turnover and those defective by virtue of stable but less functional protein. The intent of these studies was to establish if there was a link between *nef* gene function and the rate of disease progression in natural infection prior to establishing the precise molecular constraints governing *nef* gene function.

Several laboratories have demonstrated the positive role of *nef* in determining the infectivities of progeny virions (12, 41, 42, 47). Given reports of *nef* mutations in the PXXP repeats that affect infectivity enhancement but not CD4 downregulation (47), our study may have underestimated the degree of *nef* functional impairment by testing *nef* genes only for their ability to downregulate cell surface CD4. However, since the only PXXP mutations were seen for a single intermediate progressor and two rapid progressors, this putative bias would only strengthen our conclusion that *nef* is not a common mediator of reduced rates of HIV-1 disease progression in natural infections. However, the report of a single HIV-1-positive long-term nonprogressor with serial proviral DNA amplifications yielding only grossly defective *nef* genes (29) clearly indicates that such patients do exist in nature, albeit rarely. The recovery of some proportion of functional *nef* genes in the majority of patients with HIV-1 disease speaks to the importance of this gene in natural infection. Further, the observation in this study of rapid progressors with a mixture of functional and defective *nef* genes argues strongly for the validity of deletion mutations in the design of live, attenuated viral vaccines.

We have recently studied an HIV-1-infected long-term survivor lacking cultivable virus and found functionally wild-type long terminal repeats and *nef* genes but also a concentration of inactivating mutations in *vif*, *vpr*, and *vpu* (37). A larger number of such patients are under study to learn if defective accessory genes are a more common molecular correlate of disease progression in natural infections than are defective *nef* genes. We envision that the results of these studies will provide for a more precise understanding of the virologic basis of long-term survival, with a view to the rational design of candidate live, attenuated vaccines for the prevention of HIV disease.

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