Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 5549–5553, June 1993
Medical Sciences

CD4 down-regulation by *nef* alleles isolated from human immunodeficiency virus type 1-infected individuals

ROBERTO MARIANI AND JACEK SKOWRONSKI*

Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724

Communicated by Maxine F. Singer, February 24, 1993

ABSTRACT **PCR was used to clone isolates of the human immunodeficiency virus type 1 (HIV-1) *nef* gene directly from peripheral blood leukocytes of HIV-1-infected individuals. A transient expression system with human CEM T cells was used to assess the effect of *nef* on CD4 antigen expression on the cell surface. We show that CD4 down-regulation is a frequent property of primary HIV-1 *nef* alleles. Mutations in conserved amino acid motifs of Nef disrupted CD4 down-regulation. Our observations strongly suggest that CD4 down-regulation reflects a conserved function of *nef*, which is selected *in vivo* in human HIV-1 infection. Methodology described here provides quantitative assays to establish whether alterations in *nef* correlate with the dynamics of disease progression in human AIDS.**

nef genes of human immunodeficiency virus type 1 and type 2 (HIV-1 and HIV-2) and simian immunodeficiency virus encode related small cytoplasmic proteins (1, 2). Data from *in vivo* experiments in rhesus monkeys indicate that *nef* is an important pathogenic determinant of these viruses and that *nef* is essential for high viral load and disease induction in this primate model for human AIDS (3).

In contrast to the data from *in vivo* studies, *nef* is not required for viral replication in primary T cells nor established T-cell lines under the commonly used *in vitro* conditions (3–5), and *nef* open reading frames (ORFs) from viral isolates that have been propagated *in vitro* frequently encode prematurely terminated and, hence, most likely nonfunctional Nef proteins (6, 7). So far no consistent function has been ascribed to *nef* through the *in vitro* studies. The initial reports of a negative effect of *nef* on viral replication (4, 5, 8) and expression (9, 10) were either not confirmed (11, 12) or contradicted (13) by more recent studies. A block to interleukin 2 gene induction was observed in stable cell lines expressing some, but not other, HIV-1 *nef* alleles (14). Also, it was reported that *nef* from the HIV-1 SF2 isolate down-modulates CD4 antigen on the cell surface (15), but these observations were not confirmed by other studies (16, 17). We have reported previously that Nef protein from the HIV-1 NL43 isolate, but not from the HIV-1 HxB3 isolate, down-regulated expression of CD4 antigen in human and murine T cells (18). HIV-1 NL43 Nef had a dramatic effect on development and activation of $CD4^+$ T cells when expressed in transgenic mice, and this correlated with CD4 down-regulation on thymic T cells (18).

The apparently contrasting results from *in vitro* experiments frequently reflect genetic variation among commonly studied *nef* alleles (13, 14, 18), and this variation may result from the absence of selection for *nef* function upon *in vitro* propagation of cloned viruses. Therefore, in an attempt to circumvent limitations resulting from lack of genetic selection for *nef* under the *in vitro* conditions, we isolated a large number of HIV-1 *nef* alleles directly from two patients infected with HIV-1. In this report we present results of the analysis of CD4 down-regulation by these primary HIV-1 *nef* alleles.

MATERIALS AND METHODS

PCR Amplification of Primary HIV-1 *nef* Isolates. DNA isolated from peripheral blood leukocytes of asymptomatic, HIV-1-positive patients SK1 and SK4 (W. Phares, P. Baron, J. Gold, and W. Herr, personal communication) and Nef-1 (GCCGAATTCTAGCAGTAGCTGAGGGGACAGATAG) and HU3-3 (AGGCAAGCTTTATTGAGG) primers, which hybridize to HIV-1 sequences; positions 8410–8935 and 375–352 in the North American consensus sequence (1), respectively, were provided by W. Phares and W. Herr (Cold Spring Harbor Laboratory). PCR (19) was done with denaturation at 94°C (40 sec), hybridizing at 62°C (40 sec), and extension at 72°C (3 min) for 25 cycles in the conditions recommended by the manufacturer (Boehringer Mannheim). Subsequently, amplification reactions were diluted 200-fold with the reaction buffer and the second round of PCR with NEF5EX and NEF3-Mlu (18) primers, which introduce the *Xba* I and *Mlu* I cloning sites flanking *nef* ORF, was done for another 25 cycles, as described above. Amplified 0.7-kb fragments that compose the Nef ORF were isolated by PAGE, digested with *Xba* I and *Mlu* I, and subcloned into *Xba* I- and *Mlu* I-cut CD3 TEX expression vector (18).

Analysis of Primary HIV-1 *nef* Isolates. The nucleotide sequence of NA and NB clones was determined, as described (20). Oligonucleotide-directed mutagenesis was done with the NA7 *nef*, subcloned into the pBS vector (Stratagene), as described (21). All mutations were verified by DNA sequence analysis. Electroporation, flow cytometry analysis of CEM E5 cells (22), and immunoblot analysis of Nef expression were done as described (18).

RESULTS

Cloning of Primary HIV-1 *nef* Isolates. The *nef* gene was isolated from DNA isolated from peripheral blood leukocytes of two asymptomatic HIV-1-infected patients, SK1 and SK4, by two rounds of PCR (see *Materials and Methods* for further details). Amplified fragments were cloned directly into the CD3β T-cell-specific expression vector, and clones containing *nef* inserts were identified by PCR. The two groups of clones derived from SK1 and SK4 patients are further referred to as CD3 NA (18 clones) and CD3 NB (3 clones), respectively. The NA and NB clones were used in two types of assays. First, to identify functional Nef ORFs, individual clones were transiently expressed in CEM T cells, and protein extracts were analyzed by immunoblotting. Subsequently, all clones that directed detectable expression of Nef protein were analyzed for CD4 down-regulation.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: ORF, open reading frame; HIV-1, human immunodeficiency virus type 1.

*To whom reprint requests should be addressed.

Identification of Functional HIV-1 *nef* ORFs. To identify CD3 NA and NB clones that direct synthesis of the 27-kDa Nef polypeptide individual CD3 NA and NB clones were introduced into human CD4+ CEM T cells, and protein extracts prepared from transfected cells were analyzed by immunoblotting with a rabbit anti-Nef serum (18). Altogether, 15 out of 18 NA, and 3 out of 3 NB clones tested directed readily detectable expression of Nef proteins, and a representative result from this analysis is shown in Fig. 1. Clones NA1, -3, -4, -5, -6, -8, and -14 directed expression of Nef at levels comparable to that of the NL43 and HxB3 proteins, which were used as standards. In contrast, clones NA11, -15, and -17 directed significantly lower expression, suggesting lower stability of proteins encoded by these *nef* alleles. The electrophoretic mobility of most of the NA Nef proteins was indistinguishable from that of the HIV-1 NL43 Nef, except for NA4 Nef, which migrated slightly faster. The observed variation in Nef expression and electrophoretic mobility suggested heterogeneity within the NA population of *nef* alleles.

CD4 Down-regulation Is a Frequent Property of Primary HIV-1 *nef* Isolates. A transient assay in human CD4+ CEM T cells (18) was used to assess the effect of primary *nef* alleles on CD4 antigen expression on the cell surface. As illustrated in Fig. 2*A*, transfection with the CD3–LacZ reporter construct, but not with a control CD3 TEX plasmid, resulted in a high level of β-galactosidase activity in a large fraction of electroporated cells (β-galactosidase-positive cells; compare *1* and *2*), and β-galactosidase expression had no effect on CD4 expression on the surface of β-galactosidase-positive cells (compare the right and left upper quadrants in *2*). As shown before (18), transfection with the HIV-1 NL43 *nef*, but not with HxB3 *nef*, caused an ≈50-fold decrease in CD4 staining (*4* and *5*, respectively).

Results of the analysis of 6 of the 14 CD3 NA clones tested are shown in Fig. 2*A*, *6–10*, and a compilation of analyses done with *nef* isolates from the SK1 and SK4 patients is shown in Fig. 2*B*. Expression of Nef from all clones derived from SK1 effected a decrease in CD4 antigen expression on the surface of CEM T cells, although to different extents. Interestingly, *nef* alleles from several of these clones, including NA3, -7, -10, -13, -14, -18, -11, and -15, were more potent in CD4 down-regulation than was NL43 *nef*. The NA1, -5, -6, -8, and NB1 alleles were as potent as the NL43 Nef. In contrast, the NA4, NA17, NB5, and NB9 alleles were exceptional in that they had little effect on surface CD4 expression.

Results from immunoblot analysis (see Fig. 1) indicated some degree of correlation between the steady-state level of Nef protein expression and CD4 down-regulation. For example, all Nef alleles that effected CD4 down-regulation also directed detectable expression of Nef proteins in transiently transfected CEM T cells. The relatively weak CD4 down-regulation by NA17 Nef and NB5 and NB9 alleles correlated with a relatively low steady-state-level expression of corresponding Nef proteins in CEM T cells. Only the NA11 Nef was exceptional in that it had a potent effect on CD4 in spite of a relatively low steady-state expression. Thus, the relatively weak CD4 down-regulation may, in many cases, reflect decreased stability of the corresponding Nef proteins. In contrast, the weak NA4 allele is clearly exceptional because the steady-state-level expression of NA4 Nef was comparable to that of NL43 Nef protein.

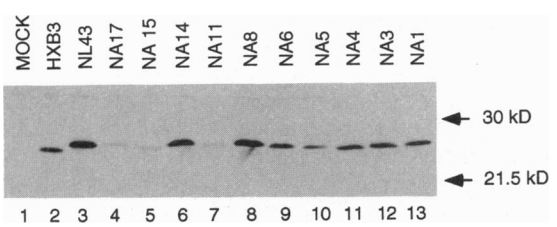

FIG. 1. Immunoblot analysis of Nef proteins from SK1 *nef* alleles. One hundred-microgram aliquots of protein extracts prepared from CEM T cells electroporated with the indicated expression vectors were separated by SDS/PAGE and immunoblotted with anti-Nef serum. CEM cells transfected with CD3 HxB3 and NL43 vectors, which direct expression of the HIV-1 HxB3 and N143 Nef proteins, were used as a positive control. CEM cells electroporated with CD3 TEX, which contains HIV-1 *tat* cDNA, provided a negative control (MOCK). Molecular size of proteins revealed by this analysis was estimated by using molecular size standards (kD, kDa; Amersham).

Mutations in Conserved Nef Motifs Disrupt CD4 Down-regulation. To characterize HIV-1 *nef* alleles further, the nucleotide sequence of 14 *nef* isolates from patient SK1 and of 3 *nef* isolates from patient SK4 was determined. The predicted amino acid sequences of respective Nef proteins from SK1 segregated into two closely related families (NA groups I and II), for which consensus sequences are shown in Fig. 3. Within each of these two groups the amino acid variation between Nef proteins was <1% (0.9% and 0.3% within group I and II proteins, respectively). In contrast, each of the group I Nef sequences differed from those of group II at nine diagnostic positions (4.4% divergence). A total of 19 amino acid substitutions was found among SK1 clones (Fig. 3 and Table 1). The consensus amino acid sequence derived from SK4 *nef* isolates differed from that of SK1 at 35 positions (17% divergence). Seventeen amino acid differences were found between NB clones (8.3% divergence, see Fig. 3 and Table 1). Table 1 shows that most amino acid variation involved conservative substitutions and had a marginal or nondetectable effect on CD4 down-regulation. Analysis of sequences of the defective NA17 and NA4 proteins revealed two candidate loss-of-function mutations in NA17 (Val-148 → Asp and Glu-155 → Val) and one in NA4 (Asp-36 → Gly). The comparison of NL43 Nef with the defective HxB3 Nef identified five additional candidate disabling mutations (Val-33 → Ala, Glu-65 → Lys, Cys-142 → Arg, Val-153 → Leu, and Phe-191 → Leu, Fig. 3).

To identify amino acid residues associated with loss of CD4 down-regulation by NA4, NA17, and HxB3 Nefs, mutations specific to these partially defective genes were transferred onto the background of the highly active NA7 *nef* allele. In an additional experiment the conserved Gly-2 was replaced with alanine. This mutation disrupts the N-terminal myristoylation site conserved in Nef proteins of both human and simian viruses (1, 23). The effect of these mutations on CD4 down-regulation was quantitated and correlated with the steady-state expression level of mutant Nef proteins in a dose–response experiment.

Fig. 4*A* shows that doses of 0.5 μg of the CD3 NA7 construct effected a decrease in CD4 expression and doses of 5 μg and higher saturated the response. In contrast, three of the tested mutations reduced down-regulation of surface CD4 by NA7 Nef (Fig. 4). First, mutation that disrupted the N-terminal myristoylation site in the NA7.1 Nef essentially abolished CD4 down-regulation at DNA doses below 5 μg, whereas higher doses of 15 and 50 μg effected a modest decrease in CD4 expression on the cell surface. As shown in Fig. 4*B*, immunoblot analysis of cytoplasmic extracts prepared from cells transfected with 15 μg and 50 μg of the wild-type CD3 NA7 or the CD3 NA7.1 constructs revealed that the steady-state expression level of mutant NA7.1 Nef was higher than that of NA7 Nef for both these doses (compare lanes 1 and 2 with 7 and 8, respectively). Thus, disruption of the conserved N-terminal myristoylation signal in Nef reduced CD4 down-regulation to a large extent. The second mutation, Asp-36 → Gly (NA7.3 Nef), originally found in the defective CD3 NA4 clone, decreased CD4

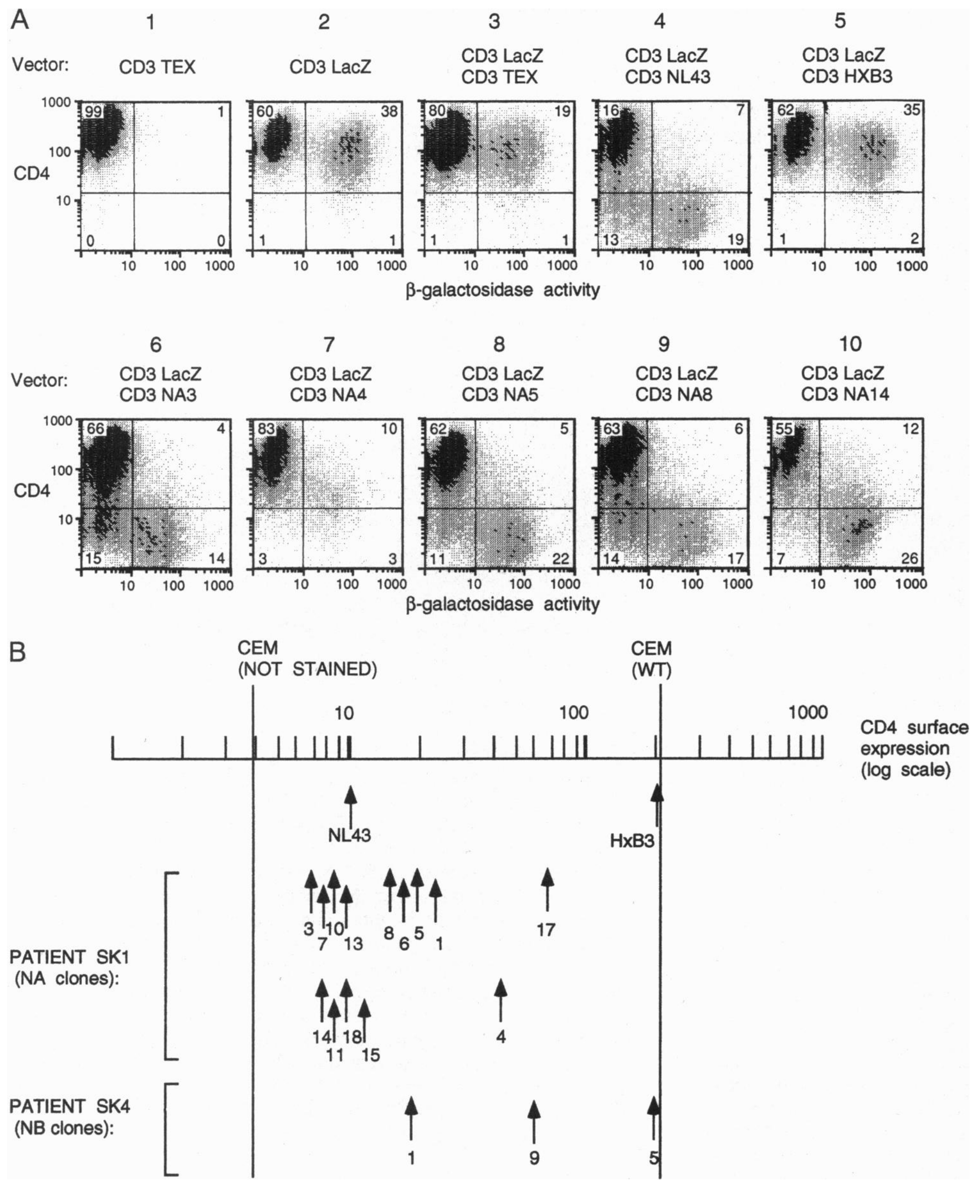

FIG. 2. Primary HIV-1 Nef isolates down-regulate CD4 in a transient assay. (*A*) CEM T cells were electroporated with control expression vectors containing HIV-1 *tat* cDNA (CD3 TEX), NL43 *nef* (CD3 NL43), HxB3 *nef* (CD3 HxB3), and the CD3 LacZ reporter plasmid (CD3 LacZ) (*Upper*), or with selected CD3 NA clones (*Lower*). β-Galactosidase activity and CD4 antigen expression on the cell surface were analyzed by flow cytometry and are shown on the abscissa and ordinate, respectively, in the logarithmic scale. Percentage fraction of cell populations expressing wild-type or reduced levels of CD4 and high or low levels of β-galactosidase activity are shown in the corners of each quadrant. (*B*) Compilation of results from flow cytometry analyses of *nef* alleles from patients SK1 and SK4. Each arrow represents an individual NA or NB *nef* clone, which is identified by a number below. Each arrow reflects the median level of CD4 expression on the surface of β-galactosidase-positive CEM cells transfected with the respective expression vector. Vertical lines represent the wild-type (WT)-level CD4 expression on CEM T cells and the autofluorescence of fluorescein digalactoside-loaded CEM cells that were not stained for CD4 (CEM not stained) and are provided as a reference. CD4 expression on CEM cells electroporated with the CD3 NL43 and CD3 HxB3 vectors is shown for comparison. Representative results from three independent experiments are shown.

down-regulation by severalfold (Fig. 4*A*) despite similar steady-state expression of both mutant NA7.3 and wild-type NA7 Nef proteins (Fig. 4*B*, compare lanes 9 and 10 with lanes 1 and 2, respectively). Therefore, the NA7.3 Nef encodes stable, but partially inactive, protein. The third mutation was Cys-122 → Lys (NA7.17), found in HxB3 Nef. Fig. 4 shows that a high dose of 50 μg of the mutant CD3 NA7.17 only marginally affected CD4 expression. Results from immuno-

```
                                                                             70
MGGKWSKRSA GGWSAVRKRM EQAEPAADGV GAVSRDLEKY GAITSSNTAT NMADCAWLEA QEEEEVGFPV   cons
.......... .......... .......... .......... .......... .......... ..........   NA group I
.......... ...P...E.. K......... .........H .......... .......... ..K.......   NA group II
.......S.V V..P...E.. RR........ ........RH .........A T......... ..E.......   NB cons
.......S.V I..P...E.. RR........ .........H .........A ...A...... ..........   NL43
.......S.V V..P...E.. RR........ ..A......H .........A ...A...... ....K.....   HXB3

                                                                            140
RPQVPLRPMT YKAAVDISHF LKEKGGLEGL IHSQRRQDIL DLWIYHTQGF FPDWQNYTPG PGIRYPLTFG   cons
.......... .......... .......... .......... .......... .......... ..........   NA group I
.......... ......L... .......... .......... ...V...... .......... ..........   NA group II
.......... F.G.L.L... .......... .Y..EGRE.. .........Y .......... ..T.......   NB cons
T......... ......L... .......... .......... .........Y .......... ..V.......   NL43
T......... ......L... .......... .......... .........Y .......... ..........   HxB3

                                                                     206
WCFKLVPVEA EQVEEANTGE NNSLLHPMSL HGMEDAEREV LQWKFDSRLA FHHMARELHP EYYKDC       cons
.......... .......... .......... .......... .......... .......... ......       NA group I
.........P .......... .......... .......K.. .......... .......... ......       NA group II
.........Q .RI....K.. ..C....... ...-.S.G.. .E.R...S.. ...V...... ......       NB cons
..Y......P DK.....K.. .T....V... ...D.P.... .E.R...... ...V...... ..F.N.       NL43
.RY......P .KL....K.. .T....V... ...D.P.... .E.R...... L..V...... ..F.N.       HXB3
```

FIG. 3. Predicted amino acid sequences of NA Nef proteins. Consensus amino acid sequences for Nef proteins derived from SK1 (NA clones) and SK4 patient (NB clones) are shown in the upper line (cons). The consensus sequences for NA group I, group II, and NB Nef proteins are aligned below. Amino acid sequences of the HIV-1 NL43 and HxB3 Nef proteins are shown for comparison. Dots indicate sequence identity, and amino acid sequences are shown in the one-letter code. Group I consists of 11 clones (NA1, -3, -5, -6, -7, -8, -10, -11, -13, -15, and -17), and group II consists of 3 clones (NA4, -14, and -18). Amino acid variation within group I and group II clones is shown in Table 1.

Table 1. Amino acid substitutions in Nef proteins from SK1 patient

SK1 clone	Xaa → Yaa substitution	CD4 down-regulation
NA group I		
NA3, -7, -10, -13	NA consensus I	++
NA1	Val-146 → Ala	++
NA5	Ser-34 → Pro	++
NA6	Val-146 → Ala	++
	Ser-169 → Asn	++
NA8	Gly-67 → Glu	++
NA11	Gly-11 → Arg	++
NA15	Thr-117 → Ala	++
	Pro-129 → Ser	++
NA17	Val-148 → Asp	++
	Glu-155 → Val	+/−
NA group II		
NA14, -18	NA consensus II	++
NA4	Asp-36 → Gly	+/−
NB		
NB1	Gln-71 → Trp	++
	Gly-106 → Arg	++
NB5	Ser-8 → Arg	++ (a)
	Ala-15 → Thr	++
	Thr-51 → Asn	++
	Glu-62 → Lys	ND
	Phe-81 → Tyr	++
	Gly-83 → Ala	++
	Tyr-102 → His	++
	Gln-104 → Leu	ND
	Arg-107 → Gln	++
	Thr-133 → Ile	++
	Gln-150 → Pro	++ (a)
	Arg-152 → Lys	++ (a)
	Cys-163 → Ser	++
	Ser-188 → Arg	++
	Val-194 → Met	++
NB9	NB consensus	+/−

Amino acid differences (Xaa → Yaa) between sequences predicted for Nef proteins encoded by individual NA alleles and the consensus sequences, presented in Fig. 3, are shown. To test the effect of individual amino acid substitutions on CD4 down-regulation, mutations were transferred onto the background NA7 *nef* and assayed as described for Fig. 2*B*. (a) Amino acid residues shared with NL43 Nef; ND, not determined.

blot analysis revealed a relatively low steady-state expression directed by 15 and 50 μg of mutant CD3 NA7.17, when compared with those directed by the wild-type CD3 NA7 construct (compare lanes 11 and 12 with 1–5, Fig. 4*B*). However, the steady-state level of NA7.17 Nef in cells transfected with 50 μg of this DNA was comparable to that directed by 5.0 μg of the wild-type CD3 NA7 construct (compare lane 11 and 3, Fig. 4*B*), which had a subsaturating effect on CD4 down-regulation (Fig. 4*A*). Therefore, the NA7.17 mutation has a 2-fold effect and affects both the stability and some function(s) of Nef protein required for CD4 down-regulation. The remaining four unique amino acid substitutions found in HxB3 Nef had no effect on CD4 down-regulation when tested on NA7 background (data not shown).

Finally, we analyzed two amino acid changes found in the defective NA17 Nef allele (Table 1). The Glu-155 → Val substitution had no detectable effect on either stability or CD4 down-regulation. In contrast, the Val-148 → Asp substitution resulted in a low steady-state expression of the mutant protein and partial loss of CD4 down-regulation (data not shown). Therefore, defective CD4 down-regulation by

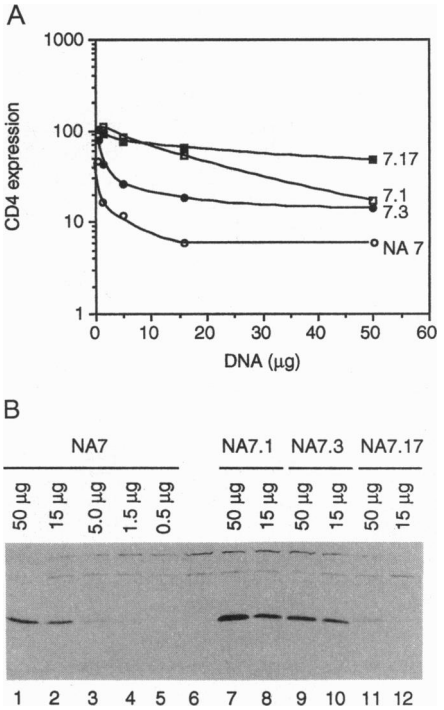

FIG. 4. Mutations in Nef that disrupt CD4 down-regulation. (*A*) The effect of selected mutations in NA7 Nef on CD4 down-regulation was analyzed in a dose–response experiment. CEM cells were coelectroporated with CD3 LacZ reporter (0.5 μg–5 μg) and different amounts of the wild-type (CD3 NA7) or mutant (CD3 NA7.1, 7.3, 7.17) expression vectors (0.5, 1.5, 5.0, 15, or 50 μg, shown on the abscissa). CD4 expression on the cell surface was determined by flow cytometric analysis 24 hr later. The median level of CD4 on the surface of β-galactose-positive cells recorded on the logarithmic scale is shown on the ordinate. (*B*) Immunoblot analysis of mutant Nef proteins. Steady-state expression of wild-type and mutant Nef proteins from the experiment shown in *A* was determined by immunoblot analysis of 25-μg aliquots of protein extracts prepared from CEM cells electroporated with the indicated amounts of CD3 NA7 construct (lanes 1–5), control CD3 TEX plasmid (lane 6), or mutant CD3 NA7.1 (lanes 7 and 8), CD3 NA7.3 (lanes 9 and 10), and CD3 NA7.17 (lanes 11 and 12).

NA17 Nef may result from decreased stability of the mutant protein.

DISCUSSION

In an attempt to circumvent limitations resulting from lack of genetic selection for *nef* under *in vitro* growth conditions, we isolated a large number of HIV-1 *nef* alleles directly from two asymptomatic patients infected with HIV-1. Immunoblot analyses determined that >80% of *nef* alleles isolated from patient SK1 directed synthesis of Nef proteins at levels comparable with that of the HIV-1 NL43 Nef. These observations are consistent with the previously observed requirement for *nef* in the natural setting of HIV infection (3) and strongly suggest that these *nef* alleles are functional. The low level of amino acid sequence divergence (4.3%) seen among 14 NA *nef* isolates was similar to that reported (24). Most amino acid replacements identified among Nef proteins from SK1 patients, except for those found in NA4 and NA17 Nef proteins, represented conservative amino acid substitutions. Thus, the observed divergence is likely to represent the preexisting heterogeneity of Nef *in vivo* in the SK1 patient rather than errors generated during PCR amplification *in vitro*. The two predominant families of NA Nef sequences observed are consistent with two major HIV-1 quasispecies

that evolved in the SK1 patient but may reflect two independent infection events.

Evidence from experiments presented here strongly suggests that CD4 down-regulation reflects a conserved, selected *in vivo* function of *nef*. (*i*) Approximately 80% of all *nef* alleles, including all those that directed detectable expression of Nef protein, down-regulated surface CD4 antigen upon transient expression in CEM T cells. The observation that the remaining 20% of SK1 *nef* alleles showed no effect on surface CD4 expression is not contradictory. A similar frequency of functionally defective alleles (30%) was observed before with the HIV-1 *tat* gene, which is essential for the viral life cycle (25). (*ii*) Although sequence variation observed among primary isolates of Nef proteins and the NL43 Nef amounted altogether to >40 amino acid substitutions, most reflected conservative changes with little or no detectable effect on CD4 down-regulation. (*iii*) CD4 down-regulation was disrupted by nonconservative replacements in amino acid motifs that are conserved among human and simian Nef proteins. Specifically, an intact N-terminal myristoylation site, which is invariantly found in both HIV-1 and HIV-2/simian immunodeficiency virus Nef proteins, was required for full Nef activity. Moreover, CD4 down-regulation is also disrupted by a mutation altering the conserved cysteine found in all HIV-1 Nefs in the central domain that is highly conserved between human and simian Nef proteins (1).

CD4 down-regulation by *nef* has been observed before in human and murine T cells stably expressing *nef* alleles derived from some (15, 18, 23), but not the other HIV-1 isolates that have been repeatedly passaged *in vitro* (18). Our observations indicate that CD4 down-regulation quantitatively depends on the level of Nef protein expression and that some *nef* alleles derived from the *in vitro* established viruses, or cloned directly from infected patients, encode defective and/or unstable proteins that are nonfunctional. These two variables may explain the lack of CD4 down-regulation by Nef reported by Gama Sosa *et al.* (16) and Schwartz *et al.* (17).

It is well established that dynamics of disease progression can vary greatly among HIV-infected people. Results from *in vivo* experiments in rhesus monkeys indicated that *nef* is essential for simian immunodeficiency virus pathogenesis (3) and *nef*-deficient simian immunodeficiency virus can protect infected animals upon subsequent superinfection with the wild-type virus (26). Methodology described here provides a quantitative assay to assess *nef* function in HIV-infected people and to establish whether alterations in *nef* are associated with differences in disease progression in human AIDS.

We thank B. Stillman and J. D. Watson for their interest and support of these studies. We also thank W. Phares, P. Baron, J. Gold, and W. Herr for providing DNA samples from SK1 and SK4 patients and for PCR primers; L. Usher and S. Salghetti for technical assistance; and P. Burfeit for assistance in flow cytometry analysis. We acknowledge W. Herr for critical reading of the manuscript. This work was supported by a grant from Johnson & Johnson and by Cold Spring Harbor Laboratory funds.

1. Myers, G., Berzofsky, J. A., Rabson, A. B. & Smith, T. E. (1990) *Human Retroviruses and AIDS* (Los Alamos Natl. Lab., Los Alamos, NM).
2. Franchini, G., Robert-Guroff, M., Ghrayeb, J., Chang, N. T. & Wong-Staal, F. (1986) *Virology* **155**, 593–599.
3. Kestler, H. W., Ringler, D. J., Mori, K., Panicali, D. L., Sehgal, P. K., Daniel, M. D. & Desrosiers, R. C. (1991) *Cell* **65**, 651–663.
4. Terwilliger, E., Sodroski, J. G., Rosen, C. A. & Haseltine, W. A. (1986) *J. Virol.* **60**, 754–760.
5. Luciw, P. A., Cheng-Meyer, C. & Levy, J. A. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 1434–1438.
6. Ratner, L., Starcich, B., Josephs, S. F., Hahn, B. H., Reddy, E. P., Livak, K. J., Petteway, S. R., Jr., Pearson, M. L., Haseltine, W. A., Arya, S. K. & Wong-Staal, F. (1985) *Nucleic Acids Res.* **13**, 8219–8229.
7. Regier, D. A. & Derosiers, R. C. (1990) *AIDS Res. Hum. Retroviruses* **6**, 1221–1231.
8. Cheng-Meyer, C., Ianello, P., Shaw, K., Luciw, P. A. & Levy, J. A. (1989) *Science* **246**, 1629–1632.
9. Ahmad, N. & Venkatesan, S. (1988) *Science* **241**, 1481–1485.
10. Niederman, T. M. J., Thielan, B. J. & Ratner, L. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 1128–1132.
11. Hammes, S. R., Dixon, E. P., Malim, M. H., Cullen, B. R. & Greene, W. C. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 9549–9553.
12. Kim, S., Ikeuchi, R., Byrn, R., Groopman, J. & Baltimore, D. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 9544–9548.
13. Terwilliger, E., Langhoff, E., Gabuzda, D., Zazopoulos, E. & Haseltine, W. A. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 10971–10975.
14. Luria, S., Chambers, I. & Berg, P. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 5326–5330.
15. Garcia, V. & Miller, A. D. (1991) *Nature (London)* **350**, 508–511.
16. Gama Sosa, M. A., DeGasperi, R., Kim, Y.-S., Fazely, F., Sharma, P. & Ruprecht, R. M. (1991) *AIDS Res. Hum. Retroviruses* **7**, 859.
17. Schwartz, O., Arenzana-Seisdedos, F., Heard, J.-M. & Danos, O. (1992) *AIDS Res. Hum. Retroviruses* **8**, 545–551.
18. Skowronski, J., Parks, D. & Mariani, R. (1993) *EMBO J.* **12**, 703–713.
19. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988) *Science* **239**, 487–494.
20. Hattori, M. & Sakaki, Y. (1986) *Anal. Biochem.* **152**, 232–238.
21. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 488–492.
22. Scheppler, J. A., Nicholson, J. K. A., Swan, D. C., Ahmed-Ansari, A. & McDougal, J. S. (1989) *J. Immunol.* **143**, 2858–2865.
23. Guy, B., Kieny, M. P., Riviere, Y., Le Peuch, C., Dott, K., Girard, M., Montagnier, L. & Lecocq, J. P. (1987) *Nature (London)* **330**, 266–269.
24. Delassus, S., Cheinier, R. & Wain-Hobson, S. (1991) *J. Virol.* **65**, 225–231.
25. Meyerhans, A., Cheynier, R., Albert, J., Seth, M., Kwok, S., Sninsky, J., Morfeldt-Manson, L., Asjo, B. & Wain Hobson, S. (1989) *Cell* **58**, 901–910.
26. Daniel, M. D., Kirchhoff, F., Czajak, S. C., Sehgal, P. K. & Desrosiers, R. C. (1992) *Science* **258**, 1938–1941.