JOURNAL OF VIROLOGY, Mar. 1993, p. 1676–1680
0022-538X/93/031676-05$02.00/0

Vol. 67, No. 3

Disulfide Bond Formation in the Human Immunodeficiency Virus Type 1 Nef Protein

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Received 6 October 1992/Accepted 24 November 1992

Substitution of alanine for cysteine residues of the human immunodeficiency virus type 1 LAI (BRU) and ELI Nef proteins was used to determine pairing of the cysteine residues present in each protein. The results show that under nonreducing conditions, alternative pairing of the cysteines occurs. The preferred pairing of cysteine residues of the LAI and ELI proteins differs. In the experimental system used, viruses carrying the ELI *nef* allele are found to express Nef proteins which accelerate virus replication. Mutation in critical cysteine residues of the protein reduce the rate of virus replication. In the same system, viruses harboring the LAI *nef* allele fail to replicate. These observations raise the possibility that differences in the observed biological activity of *nef* alleles may be attributed, at least in part, to differences in the secondary structure of the proteins.

The *nef* gene of the primate lentiviruses, the human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2), and the simian immunodeficiency viruses is located at the 3′ end of the viral genome (3, 4, 14). *nef* from a macaque strain of simian immunodeficiency virus, SIV_{239}, is required for prolific replication and for induction of immunosuppressive disorders in monkeys (7).

The role of *nef* in replication of virus in culture has been difficult to define. In some experiments *nef* is reported to accelerate virus replication (8, 16). *nef* is also reported to retard virus replication in other experiments (9, 15). Differences in virus strains as well as in host cells used in these experiments may account for the apparent discrepancy. The Nef proteins of different strains of HIV-1 also appear to be heterogeneous in size on sodium dodecyl sulfate (SDS)-polyacrylamide gels. In most experiments the Nef protein is observed to migrate as a doublet on SDS-polyacrylamide gels following immunoprecipitation from infected cell lysates (5, 10, 18).

It is notable that Nef proteins from different strains of HIV-1 contain three cysteine residues capable of forming disulfide bonds. The positions of two of the cysteine residues are highly conserved among diverse HIV-1 isolates, whereas the location of the other cysteine varies. In this study formation of disulfide bonds between cysteine residues was analyzed by substitution of individual cysteine for alanine residues. Nef proteins derived from two strains, LAI (formerly BRU) (17) and ELI (2), were used. The LAI and ELI *nef* alleles were selected for this study as they represent extreme variants of HIV-1 isolates. Forty-four of the 207 amino acids differ between the two proteins (2). These two *nef* alleles have also been previously used for studies of *nef* function (1, 8, 16).

In the initial experiments, infection of the $CD4^+$ human T-cell line Jurkat was initiated by transfection with full-length infectious viral DNA. The recombinant HIV-1 DNA constructs used are shown in Fig. 1. In the HxB-ELI 1 recombinant provirus all sequences 5′ to the *Bam*HI site (8478) located in *env* are specified by the HxB2 clone of HIV-1. Sequences between the *Bam*HI site and the *Sac*I (9575) site in the 3′ long terminal repeat derived from the ELI isolate. Sequences 3′ to the *Sac*I site are specified by HxB2. The HxB-LAI provirus is isogenic to HxB-ELI 1 except for sequences between the *Sma*I (8800) and the *Sac*I (9575) sites which are derived from the LAI isolate of HIV-1. The *Sma*I site was created previously 5′ to the *nef* initiation codon by oligonucleotide-directed mutagenesis, as described previously (19). Jurkat cells (5×10^7) were incubated with 50 μg of proviral DNA carrying the wild-type and mutant *nef* genes in 0.25 mg of DEAE-dextran per ml at 37°C for 60 min. The cells were then washed with RPMI 1640, cultured for 48 h, and labeled for 3 h in the presence of 100 μCi of [^{35}S]methionine per ml. The cells were lysed in radioimmunoprecipitation assay buffer, and Nef proteins were immunoprecipitated with a rabbit polyclonal Nef-specific antiserum raised against the LAI Nef protein, made in *Escherichia coli* (18). The proteins were analyzed on SDS–18% polyacrylamide gels before or after treatment with reducing agents.

The autoradiographs of Fig. 2 and 3 confirm previous reports that Nef proteins migrate as a doublet (5, 10, 18). Two LAI Nef proteins of approximately 24 and 21 kDa (Fig. 2A, lane 2) and two ELI Nef proteins of 24 and 23 kDa (Fig. 3, lane 2) were immunoprecipitated from the labeled cellular extracts when the proteins were not reduced prior to loading onto polyacrylamide gels.

In a previous study using *nef* of the LAI strain, it was reported that a rapidly migrating form of the Nef protein arises from initiation at an internal methionine codon located at amino acid 20 (6). This report is curious, as under the conditions used, the Nef protein of the ELI strain also migrates as a doublet but lacks the corresponding methionine at amino acid 20. To examine the possibility that secondary structure rather than initiation at an internal methionine accounts for the rapid electrophoretic mobility of the 21-kDa LAI protein of LAI, the methionine codon at position 20 of this protein was altered to isoleucine by site-directed mutagenesis (13, 19). The substrate was a plasmid carrying the *Bam*HI (8478)-to-*Sac*I (9575) HIV-1 viral DNA fragment. Isoleucine was chosen as the ELI Nef protein specifies this amino acid at position 20. The electrophoretic mobilities of the parental and mutant proteins were compared by analysis on SDS-polyacrylamide gels using

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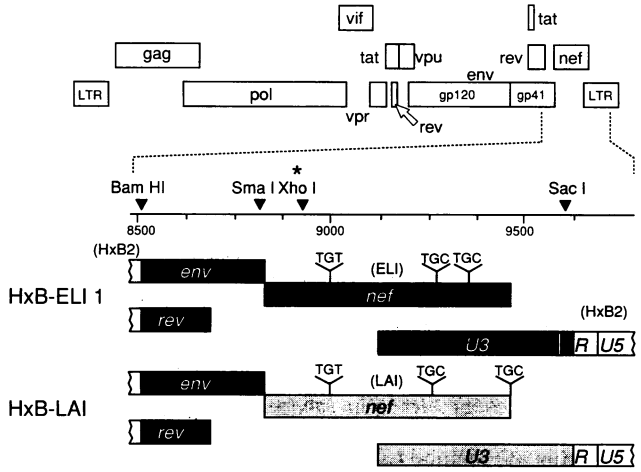

FIG. 1. Schematic representation of the HIV-1 genome. The HxB2 viral DNA fragment between the *Sma*I (8800) and the *Sac*I (9575) sites was replaced with the same sequences from either the LAI or the ELI strain. The three cysteine codons present in the LAI and ELI *nef* coding sequences are also depicted.

similar conditions. The results show that the methionine 20 mutant of the LAI Nef protein also migrates as a 24-kDa–21-kDa doublet (Fig. 2A, lane 3).

Formation of disulfide bonds between cysteine residues can alter the apparent molecular mass of a protein in SDS-polyacrylamide gels. To determine whether disulfide bonds might account for the distinct electrophoretic mobilities of the LAI and ELI Nef proteins, the proteins in the immunoprecipitates were treated with *N*-ethylmaleimide (NEM), a sulfhydryl-specific modifying agent, prior to analysis on SDS-polyacrylamide gels (12). Immunocomplexes were boiled in 20 μl of a solution containing 1.5% SDS with 20 mM dithiothreitol for 10 min. The supernatant containing reduced proteins was then treated with 10 μl of 0.2 M NEM dissolved in water for 1 h at 4°C. Laemmli buffer containing β-mercaptoethanol was added to the samples before loading onto the SDS-polyacrylamide gels.

The results show that both LAI and ELI Nef proteins treated with NEM migrate as single molecular mass species of 25 and 27 kDa, respectively (Fig. 2A, lane 7; Fig. 3, lane 6). These results indicate that both LAI 24-kDa–21-kDa and ELI 24-kDa–23-kDa Nef proteins contain disulfide bonds. The results also raise the possibility that the two forms of the Nef proteins present result from protein stabilized in two different configurations as a consequence of different pairwise formation of the three cysteine residues present in each protein. The difference in the apparent molecular masses of the fully reduced LAI and ELI Nef proteins can be attributed in part to residues at amino acid 54. Proteins that have an alanine residue at this position, such as the LAI Nef protein, have previously been shown to migrate with a rapid electrophoretic mobility, whereas proteins with an aspartic acid residue at amino acid 54, including the ELI Nef protein, migrate more slowly (10, 18).

The formation of specific pairs of disulfide bonds in the LAI and ELI Nef proteins was assessed by substitution of individual cysteine residues for alanine. The mutants were introduced into the viral DNA (Fig. 1) and expressed in Jurkat cells, and the electrophoretic mobilities of the proteins were determined by analysis on SDS-containing polyacrylamide gels before or after treatment with NEM.

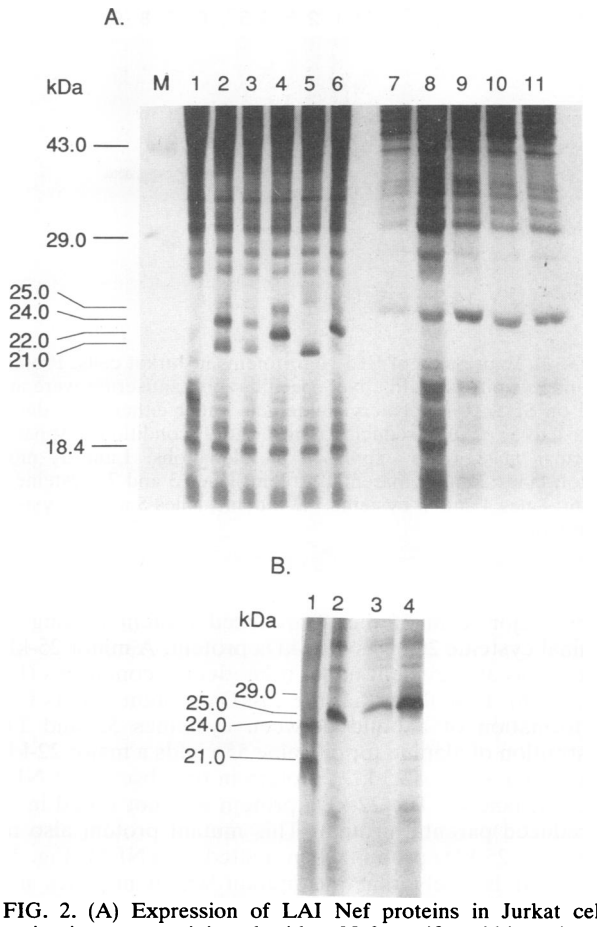

FIG. 2. (A) Expression of LAI Nef proteins in Jurkat cells. Proteins immunoprecipitated with a Nef-specific rabbit antiserum were analyzed on SDS–18% polyacrylamide gels under either nonreducing (lanes 1 to 6) or fully reducing (lanes 7 to 11) conditions. Apparent molecular masses are expressed in kilodaltons. Lane 1, mock control; lanes 2 and 7, wild-type protein; lanes 3 and 8, methionine 20 mutant; lanes 4 and 9, cysteine 55 mutant; lanes 5 and 10, cysteine 142 mutant; lanes 6 and 11, cysteine 206 mutant. (B) Expression of wild-type LAI Nef protein and cysteine 55-cysteine 206 double mutant protein in Jurkat cells under nonreducing conditions (lanes 1 and 2, respectively) or fully reducing conditions (lanes 3 and 4, respectively).

As described previously, the nondenatured LAI Nef protein migrated as a 24-kDa–21-kDa doublet (Fig. 2A, lane 2), whereas the reduced form of the protein migrated as a single 25-kDa protein (Fig. 2A, lane 7). Substitution of alanine for cysteine 142 eliminated the 24-kDa but not the 21-kDa protein observed under nonreducing conditions (Fig. 2A, lane 5). This substitution did not alter the electrophoretic mobility of the NEM-treated mutant protein (Fig. 2A, lane 10). This result indicates that the 21-kDa protein, which is one of the predominant forms of the parental protein, contains a disulfide bond between cysteines 55 and 206. This assignment is confirmed by the observation that substitution of alanine for cysteine 55 eliminates the 21-kDa protein under nonreducing conditions (Fig. 2A, lane 4). The 21-kDa form of the nonreduced protein was also eliminated by a mutation which creates a stop codon rather than a cysteine residue at the terminal residue (Fig. 2A, lane 6). The electrophoretic mobility of all three mutant proteins was unchanged when treated with NEM (Fig. 2A, lanes 9 to 11).

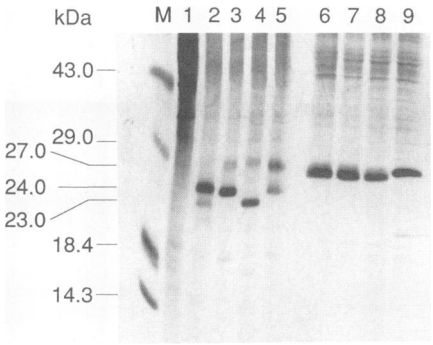

FIG. 3. Expression of ELI Nef proteins in Jurkat cells. Proteins immunoprecipitated with a Nef-specific rabbit antiserum were analyzed on SDS–18% polyacrylamide gels under either nonreducing (lanes 1 to 5) or fully reducing (lanes 6 to 9) conditions. Apparent molecular masses are expressed in kilodaltons. Lane 1, mock control; lanes 2 and 6, parental protein; lanes 3 and 7, cysteine 55 mutant; lanes 4 and 8, cysteine 143 mutant; lanes 5 and 9, cysteine 170 mutant.

The major form of the nonreduced protein lacking the terminal cysteine 206 was a 24-kDa protein. A minor 25-kDa protein was also evident under nonreducing conditions (Fig. 2A, lane 6). It is likely that the 24-kDa protein results from the formation of a bond between cysteines 55 and 142. Substitution of alanine for cysteine 55 yields a major 22-kDa protein and a minor 25-kDa protein in the absence of NEM (Fig. 2A, lane 4). The 22-kDa protein was not found in the nonreduced parental protein. This mutant protein also migrated as a 25-kDa protein when treated with NEM (Fig. 2A, lane 9). It is likely that the mutant which migrates as a 22-kDa protein contains a disulfide linkage between residues 142 and 206. Double mutation of cysteines 55 and 206 yields a single protein form migrating under nonreducing conditions with an apparent molecular mass of 25 kDa (Fig. 2B, lane 2). The electrophoretic mobility of this protein does not change after treatment with NEM (Fig. 2B, lane 4), indicating that no covalent cysteine linkages are present in this protein. This observation confirms that all forms of the LAI Nef protein that migrate under nonreducing conditions as 24-, 22-, or 21-kDa proteins contain disulfide bonds between cysteines 55, 142, and 206.

These results demonstrate that disulfide bonds can form between all three cysteine residues in the parental LAI Nef protein. When all these residues are present, two major forms of the protein are observed under nonreducing conditions. One form contains a covalent linkage between cysteines 55 and 206 and migrates with an apparent molecular mass of 21 kDa. The second form contains a covalent bond between cysteines 55 and 142 and migrates as a 24-kDa protein.

Similar experiments were done with the ELI protein which migrates under nonreducing conditions as a 24-kDa–23-kDa doublet (Fig. 3, lane 2). This protein has an apparent molecular mass of 27 kDa when fully reduced (Fig. 3, lane 6). Substitution of alanine for cysteine 55 yields a major 24-kDa protein and a minor 27-kDa protein under nonreducing conditions (Fig. 3, lane 3). Substitution of alanine for cysteine 143 results in a major 23-kDa form and a minor 27-kDa form (Fig. 3, lane 4). Substitution of alanine for cysteine 170 yields a mixture of the 24- and 27-kDa proteins (Fig. 3, lane 5). Previous studies showed that substitution of alanine for cysteine 170 results in a protein that is slightly less stable than the parental protein (18). All three mutant proteins migrate as a 27-kDa protein upon treatment with NEM (Fig. 3, lanes 7 to 9).

These results show that all three possible pairs of disulfide bonds can also form within the ELI Nef protein. The 23-kDa form of the nonreduced ELI Nef protein contains a disulfide bond between cysteines 55 and 170. Assignment of the disulfide linkages of the major 24-kDa protein observed under nonreducing conditions is less certain. Proteins that contain disulfide linkages between cysteines 55 and 143 as well as proteins that contain covalent bonds between cysteines 143 and 170 migrate as 24-kDa proteins under nonreducing conditions. The form of the protein in which cysteine 143 is linked to cysteine 170 appears to be preferred, as under nonreducing conditions little of the reduced 27-kDa protein is present either in the parental protein or in the mutant which is lacking cysteine 55 (Fig. 3, lanes 2 and 3). Formation of the bond between cysteines 55 and 143 appears to be less favored, as the mutant lacking cysteine 143 has a predominant 27-kDa protein form (Fig. 3, lane 5).

These results, together with a previous report regarding the effect of amino acid substitutions at residue 54 (10), account for the multiple forms of Nef protein seen in earlier studies. Under fully reducing conditions of the Nef proteins of LAI and ELI migrate as single homogeneous proteins. The differences in the apparent size of the fully reduced proteins, the ELI 27-kDa and LAI 25-kDa proteins, is not attributable to the length of the polypeptide chain (10). The Nef protein doublets observed in cells infected with either LAI or ELI viruses, as previously reported (5, 18), can now be attributed to alternative cysteine pairs formed between the three cysteine residues in both proteins. The faster-migrating species of the LAI protein, observed in our experiments, is not the result of initiation at an internal methionine as previously reported (6) but rather results from pairing of cysteines 55 and 142 to yield a 24-kDa protein and cysteines 55 and 206 to yield the 21-kDa protein. The multiple forms of the ELI Nef protein are also attributed to alternative pairing of cysteine bonds. In addition, no dimeric or other multimeric forms of the LAI and ELI Nef proteins are observed under nonreducing conditions.

The structural differences between the LAI and ELI proteins observed here raise the possibility that functional differences in these two proteins also exist. To evaluate the effect of wild-type and cysteine mutant ELI and LAI Nef proteins on virus replication, the $CD4^+$ T-cell line Jurkat and primary peripheral blood mononuclear cells (PBMCs) were infected with recombinant HIV-1 viruses carrying the various *nef* mutants. COS-1 cells were transfected with proviral DNA harboring the altered ELI and LAI *nef* genes (Fig. 1). These particular proviral DNA constructs were used as previous studies showed that the magnitude of the effect of *nef* upon virus replication depends on sequences in the virus itself that lie outside of *nef* (16). The HxB-ELI 1 recombinant virus was shown to replicate more rapidly in Jurkat cells in the presence of an active *nef* allele. Additionally, *nef*-defective viruses were created by a frameshift mutation at the *Xho*I (8900) site that prematurely truncates the protein at amino acid 35.

Sixty hours after transfection of COS-1 cells with 10 μg of proviral DNA, the supernatants were harvested, cleared, filtered through 0.2-μm-pore-size filters, and normalized for viral DNA polymerase activity, as described previously (11). Normalized virus (10^4 cpm) was used to infect 10^6 Jurkat cells and 5×10^6 primary PBMCs previously stimulated with 5 μg of phytohemagglutinin (Sigma) per ml and kept in

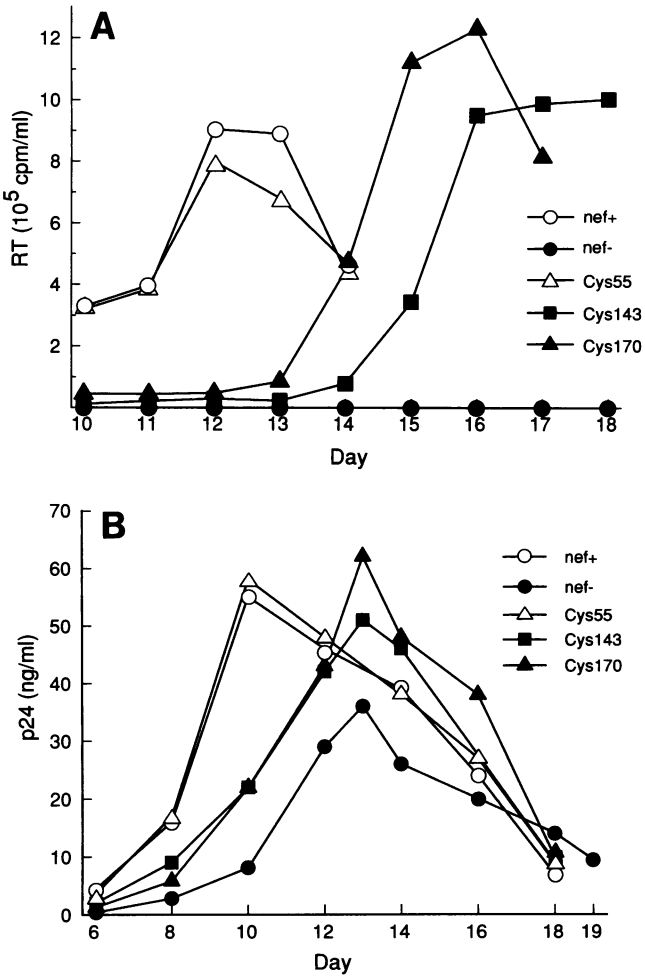

FIG. 4. (A) Replication of the ELI *nef* recombinant viruses in Jurkat T cells. Cells (10^6) were infected with 10^4 cpm of virus, as measured by reverse transcriptase activity, produced in COS-1 cells. Supernatants were changed and assayed for reverse transcriptase activity daily. (B) Replication of the ELI *nef* recombinant viruses in primary PBMCs. Primary PBMCs (5×10^6) were infected with 10^4 cpm of virus, as measured by reverse transcriptase activity in the supernatant of proviral DNA-transfected COS-1 cells. Supernatants from infected PBMCs were changed daily and assayed for p24 protein expression. Representative replication kinetics from repeat experiments are shown.

culture in the presence of 10 U of recombinant human interleukin 2 (Boehringer Mannheim) per ml. The medium was changed daily and assayed for either viral DNA polymerase activity or $p24^{gag}$ expression with a radioimmunoassay kit (NEN-Dupont).

Figure 4A shows that the parental HxB-ELI 1 virus replicates actively, as evaluated by the level of viral DNA polymerase activity in the supernatant fluid of infected Jurkat cells, whereas its isogenic *nef*-defective counterpart fails to replicate. The peak levels of viral p24 protein expression in the supernatant of primary PBMCs infected with the *nef*-defective virus are delayed by 3 days (Fig. 4B). Mutation of cysteine 55 to alanine has no effect upon virus growth. The rate of replication of viruses harboring this change is similar to that of the parental virus in both Jurkat cells and primary PBMCs (Fig. 4B). However, alterations in cysteines 143 and 170 result in a reduction of the relative rate of virus replication in both cell types. The peak viral DNA polymerase activity in the supernatant of Jurkat cells is delayed by 4 to 6 days (Fig. 4A). The peak of viral p24 core protein expression in the supernatant of infected primary PBMCs is delayed by 3 days (Fig. 4B). The effect of these two mutations on virus replication is not as severe as the effect of the premature truncation of the ELI Nef protein. It is possible that changes in the cysteine residues of the ELI Nef protein reduce but do not eliminate *nef* function. It is also possible that the *nef* compromised phenotype observed upon replication of the virus carrying the mutation in cysteine 143 is in part due to the relative instability of this altered Nef protein (18).

Similarly, HxB-LAI virus, produced by transfection of COS-1 cells with proviral DNA, was used to infect Jurkat cells and primary PBMCs. In repeat experiments, all viruses harboring the wild-type and the cysteine-altered LAI *nef* genes failed to replicate in both Jurkat cells and primary PBMCs. No viral DNA polymerase activity or $p24^{gag}$ protein expression was detectable in the culture fluid of infected cells, and no syncytia could be observed for up to 1 month after infection. COS-1 cells transfected with the HxB-LAI constructs display normal virus protein expression, including Nef protein expression, as evaluated by immunoprecipitation using patient serum (data not shown), and abundant virus production, as measured by viral polymerase activity in the supernatant fluid. In addition, in the same genetic context, various *nef* alleles are shown to accelerate virus replication in both Jurkat cells and primary PBMCs (unpublished observations).

In previous studies, viruses carrying the LAI *nef* allele replicated more slowly in A3.01 T lymphocytes (1) and slightly faster in CEM-SS cells (8) than did their isogenic *nef*-defective counterparts. In this study, the failure of viruses carrying the LAI *nef* allele to replicate may be attributed to differences in the genetic context of the viruses used. It was previously shown that proviral sequences beyond the boundaries of the *nef* open reading frame influence the magnitude of the effect of *nef* on virus replication (16). The recombinant virus used here requires an active *nef* allele for efficient replication in cells (16, 18). The replication of viruses carrying the LAI *nef* allele observed in the above-mentioned studies may be due to a lack of requirement of *nef* for active replication in cells. Additionally, differences in the host cells may contribute to the apparent discrepancy.

It is possible that active Nef proteins contain a disulfide link between cysteines in the center of the protein corresponding to the cysteine residues at amino acids 143 and 170. The lack of replication of viruses harboring the LAI *nef* allele might be attributed to an improper folding of the protein due to the formation of disulfide bonds between amino acid residues 55 and 206 and residues 55 and 142, having as a consequence the generation of a nonfunctional protein. The possibility that also other defects are present in the LAI Nef protein sequence cannot be excluded. Additionally, *cis*-acting sequences present in the LAI *nef* coding region that affect long terminal repeat function may exert a negative effect upon virus replication.

We thank Jan Welch for manuscript preparation.

E.Z. is supported by the National Foundation for Cancer Research. This work was supported by grants U01 AI24845 from the National Institutes of Health National Cooperative Drug Develop-

ment Program, P30 CA06516 from the Cancer Center, and P30 AI28691 from the Center for AIDS Research.

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