INTRODUCTION

The human immunodeficiency virus (HIV) Nef protein is conserved in most HIV and simian immunodeficiency virus (SIV) isolates. Although the nef gene is not necessary for virus replication in vitro cell culture systems, its protein appears to be essential for causing disease and maintaining virus load in vivo (Arora et al., 2002; Kestler et al., 1991; Kirchhoff et al., 1995). Although many biochemical properties of the Nef protein have been reported, major biological effects on the host cells that have been reproducibly observed in vitro, and without much controversy, are the down-regulation of cell surface CD4 and MHC class I molecules (Arora et al., 2002; Collins et al., 1998; Garcia & Miller, 1991; Guy et al., 1987; Schwartz et al., 1996). It has been thought that these properties of Nef might be involved in the prevention of superinfection and evasion of the immune system by the virus (Arora et al., 2002; Benson et al., 1993; Geyer et al., 2001; Lama et al., 1999; Parnes & Seong, 1994; Ross et al., 1999).

Several regions of the Nef protein have been reported to be involved in down-regulation of CD4 and MHC class I proteins (Geyer et al., 2001; Geyer & Peterlin, 2001; Piguet et al., 1999). A myristoylation signal (Geyer et al., 1999), amino acid 57–58 region (Grzesiek et al., 1996), the dileucine (LL) motif (Bresnahan et al., 1998; Craig et al., 1998; Greenberg et al., 1998a) and the EE155 diacidic sequence (Benichou et al., 1994; Janvier et al., 2001; Piguet et al., 1999) were reported to be necessary for CD4 down-regulation, while for MHC class I the proline rich (PxxP) motif (Greenberg et al., 1998b; Mangasarian et al., 1999) and an acidic amino acid stretch around the amino acid 65 region (Piguet et al., 2000) were identified as key motifs. However, the roles of some regions are controversial. For example, the EE155 region has been reported to be involved in CD4 down-regulation by interacting with β-COP protein, but contradictory observations have also been made (Janvier et al., 2001; Piguet et al., 1999). Most of this work on the biochemical and genetic characterization of Nef protein have employed nef sequences isolated from standard laboratory strains such as HIV-1 NL4-3 and SF2. These strains have been maintained for a long period in transformed cell
lines. However, it is now clear that the nef gene is not even necessary for virus replication in the in vitro cell culture system and thus such nef sequences might not represent the nef sequence present in vivo. Based on this rationale, our group and others have been isolating and characterizing nef sequences directly from infected individuals at various clinical stages. It was quite clear from these studies that variation in Nef amino acid sequences is significantly high among primary isolates, ranging up to 30% even within the same subtype, forcing us to revisit some of the previous work done on the laboratory nef sequences.

We previously reported that two primary nef sequences, KS2 (subtype B) and K306 (subtype D), significantly differ in their pattern of CD4 down-regulation from the NL4-3 nef sequence (Yoon et al., 2001). For example, KS2 Nef could efficiently down-regulate MHC class I, but not CD4. It was reasoned that such differential effects could be used to localize the regions or motifs important for the biochemical effects of Nef. In this report, we continued the analysis of KS2 Nef and found the EE region around amino acid position 155 to be crucial for CD4 down-regulation. During this analysis, it was also determined that the effects of Nef are more prominent during the early stage of nef expression and also in primary T cells.

**METHODS**

**Plasmid construction.** MLV (murine leukemia virus)-based retroviral vectors, MIG–nef/KS2 and MIG–nef/NL4-3, expressing the Nef and GFP proteins simultaneously as a bicistronic message driven from the MLV LTR, were constructed from HIV-1 KS2 and NL4-3, respectively. Both Nef proteins were tagged with the bacterial FLAG protein. Various C-terminally FLAG-tagged nef constructs were also cloned into the MIN5 retroviral expression vector (Lee et al., 2004), which expresses the nef gene and the bacterial neo sequence as a bicistronic message, for expression in mammalian cells. Mutations in the nef coding sequence were generated by PCR-mediated site-directed mutagenesis following the protocols of the manufacturer. Site-directed mutagenesis was performed on plasmid MIN5-nef/NL4-3 and MIN5-nef/KS2, respectively, which contains the full-length nef gene, utilizing a QuickChange Site-Directed Mutagenesis kit (Stratagene). The mutagenic primers were as follows: NL4-3K primer was 5'-TAGTACAGTTGGCCAGACAGCTGAGGAGGAC-3' (mutated nucleotides are in bold); NL4-3KE+ primer was 5'-TAGTACAGTTGGCCAGACAGACTGTA- GGAGGAGGAC-3'; KS2N primer was 5'-ACCTACCTGAGCCAGCATAAGGT-GGGCCAGACACTGTA- GGAGGAGGAC-3'; KS2NE primer was 5'-ACCA GTTGGACCCAGATAAGGTAGCCAATAAAAGGGGAGG-3'; and KS2NE/Q primer was 5'-TGACCCAGATAAGGTACACAGGG- CCAAATAAAGGGG-3'. All mutations and all constructs were verified by DNA sequencing. To amplify provirus nef sequences, a nested PCR amplification method was employed with sets of primers; the outer primers were 5'-AGACAGGGCTTGGGAAGG-3' and 5'-GGGATCTTGTTGTTACAG-3', and the inner primers were 5'-ATGGGTGGCAAGTGGTCAAA-3' and 5'-TCAGCAGTTCTTGTTGTTACAG-3'. Amino acid sequences aligned using Clustal W and Boxshade 3.21 programs. Some nef sequences described in this study are available at the Los Alamos HIV Sequence Database and have been deposited in GenBank.

**Transfections and transductions.** Retroviral constructs were transfected to 293T cells by a three-plasmid transfection method (Soneoka et al., 1995) using the calcium phosphate method. Cell culture supernatant was taken 2 days after transfection and filtered through a 0.45-µm-pore size filter. 5 × 10⁶ human T lymphoid lines, Jurkat and CEM-SS cells, were incubated with virus supernatant in the presence of 8 µg polybrene ml⁻¹ for 48 h at a 37°C incubator. The virus supernatants were then replaced with fresh medium containing 1 µg polybrene ml⁻¹. Following selection in G418, Nef was detected by Western blot analysis. All transductions were tested and found positive for Nef expression. For cells transiently expressing Nef, the constructs were transfected to 293T cells by a three-plasmid transfection method using FuGene6 (Roche). Culture supernatants were taken 2 days post-transfection and transduced three times at 12 h intervals by centrifugation. The virus supernatant was concentrated 100-fold by centrifugation at 29000 r.p.m. in a Beckman SW-40 rotor for 2 h at 20°C.

**Purification of CD4⁺ T cells from PBMCs.** CD4⁺ T cells were purified from the peripheral blood mononuclear cells (PBMCs) of healthy individuals. PBMCs were separated by using a Ficoll–Paque Plus (Pharmacia Biotech) density gradient and then the CD4⁺ T cells were purified by panning, as described previously (Wysocki & Sato, 1978). Cells were stimulated with 13 µg phytohaemagglutinin ml⁻¹ (Sigma) and cultured with 25 ng IL-2 ml⁻¹ (Endogen) in RPMI complete medium for 8 days. The percentage of CD4⁺ T cells in the purified cells was determined by flow cytometry.

**Antibodies.** Rabbit anti-Nef serum was obtained from the NIH AIDS Research and Reference Reagent Program. Polyclonal GFP antisera was purchased from Invitrogen. Mouse monoclonal anti-FLAG M2 antibody, anti-rabbit IgG-peroxidase conjugate, anti-mouse IgG–R-phcoerythrin-conjugated antibody and anti-mouse IgG1 antibody were purchased from Sigma. Anti-mouse IgG–horseradish peroxidase conjugate was purchased from Pierce and R-phcoerythrin-conjugated mouse anti-human monoclonal CD4 antibody or FITC-conjugated anti-human MHC class I antibody from BD PharMingen.

**Western blot analysis.** Transduced cells were washed with PBS and lysed with lysis buffer (500 mM NaCl, 10 mM Tris, 1 mM EDTA, 0.5% Triton X-100, 1 mM PMSE, 0.5 mM DTT) by freezing and thawing; lysates were clarified by centrifugation. Proteins were resolved by SDS-PAGE and transferred to a PVDF membrane (Amersham). After blocking with 5% skimmed milk in Tris-buffered saline (10 mM Tris pH7.5, 0.15 M NaCl), blots were probed with appropriate primary antibody and incubated with horseradish peroxidase-conjugated secondary antibody. Filters were washed and visualized by enhanced chemiluminescence (Pierce). The total levels of GFP, FLAG and Nef in MIG–nef were assessed by Western blot analysis with the corresponding antibodies. The blot was probed with an antibody against FLAG and then stripped and reprobed with an antibody specific for GFP. Equivalent amounts of proteins as used in previous blots were electrophoresed and then Western blotting was performed with Nef (Soneoka et al. For reprobing, blots were treated with Restore stripping solution (Pierce).

**Flow cytometry analysis.** Flow cytometric analysis of CD4 or MHC class I expression in cells transduced with retrovirus constructs expressing various Nef was performed on a Becton Dickinson FACSort, using CellQuest (BD) data acquisition and analysis software. Briefly, 1 × 10⁶ cells were incubated with saturating amounts of phcoerythrin-conjugated human anti-CD4 or anti-MHC class I monoclonal antibodies. Cells were then washed three times with PBS and resuspended in 500 µl PBS. CD4 or MHC class I expression levels were measured by FACS.
RESULTS AND DISCUSSION

Construction of retroviral vectors expressing Nef and GFP as a bicistronic message

The KS2 nef sequence was isolated directly from an infected individual who can be classified as a long-term non-progressor (LTNP). Its nucleotide and amino acid sequences differ from the nef gene of NL4-3 by 15% and 25%, respectively (Yoon et al., 2001). We previously reported that KS2 Nef down-regulates MHC class I as efficiently as NL4-3, but its ability to down-regulate CD4 is severely compromised (Yoon et al., 2001). These studies were done using transformed cell lines stably expressing the Nef protein. To be certain that KS2 Nef indeed has such an effect on CD4, we tested whether KS2 Nef could demonstrate the same effect in a transient manner as well as in the primary cells. Because these experiments would require analysis of the transient Nef protein, we constructed an MLV (murine leukaemia virus)-based retroviral vector, MIG–nef, that expresses the Nef and GFP protein simultaneously as a bicistronic message driven from the MLV LTR (Fig. 1A). This construct would allow us to analyse semi-quantitatively the effects of Nef on the cell surface molecules by measuring the fluorescence intensity of the GFP protein by FACS. MIG–nef/KS2 and MIG–nef/NL4-3 express the Nef protein from HIV-1 KS2 and NL4-3, respectively. For easy detection, both Nef proteins were tagged with the bacterial FLAG protein. The FLAG-tagged Nef proteins have previously been shown to have normal biological activities as measured by CD4 and MHC class I down-regulation (Nunn & Marsh, 1996; Stoddart et al., 2003). As a negative control, MIG was also used, which contained neither nef nor flag sequences. We first tested whether MIG–nef expressed the two proteins as expected. Retroviral vectors were prepared by the three-plasmid transfection method. Cell-free virus supernatants were used to transduce Jurkat cells by centrifugation, and transduced cells were analysed for production of GFP and Nef. The results from the Western blot analysis using antibodies against FLAG and Nef demonstrated that both Nef proteins derived from NL4-3 and KS2 were expressed at a similar level (Fig. 1B). GFP was also produced at a readily detectable level, as measured by Western blotting (Fig. 1B) as well as FACS (see below).

Effect of transient expression of Nef

The use of a retrovirus construct expressing GFP and Nef as a bicistronic message allowed us to study the effect of

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**Fig. 1.** Expression of FLAG, Nef and GFP from retroviral vectors expressing Nef and GFP. (A) Schematic representation of MLV-based retroviral vectors, MIG–nef/NL4-3 and MIG–nef/KS2, expressing respective Nef proteins together with GFP as a bicistronic message. The two genes are linked through the IRES (internal ribosomal entry sequence) from EMCV. Nef protein is tagged with the FLAG protein as indicated. The backbone of the retroviral vector is MT (Lee et al., 2004). (B) Immunoblot analysis of protein extracts prepared from 293T cells transfected with plasmids containing a retroviral vector sequence, MIG (lacking both nef and flag sequences), MIG–nef/NL4-3, or MIG–nef/KS2. Blots were probed with antibodies against FLAG (left panel), Nef (middle panel) or GFP (right panel). Identical amounts of proteins were used in all experiments.
Nef during early stage expression, for example the first 2 days after transfer of the nef gene to the target cells. Cell-free retroviral vectors were prepared again by the three-plasmid transfection method, and their titres were standardized. The T-lymphoid Jurkat cell line was transduced with identical amounts of three retroviral vectors, MIG, MIG–nef/KS2 or MIG–nef/NL4-3. Two days later, cells were analysed by a two-colour flow cytometric analysis using two channels, one for CD4 and the other for GFP (Fig. 2A). The magnitude of CD4 down-regulation by Nef was also evaluated by analysing the GFP-positive populations (Fig. 2B). Overall, transduction efficiency, as determined by the percentage GFP-positive population, was comparable between constructs: 51 %, 66 %, and 55 % for MIG, MIG–nef/NL4-3, and MIG–nef/KS2, respectively. In cells transduced with NL4-3, more than 95 % of the GFP + population was CD4 −, while almost 30 % of the cells transduced with MIG–nef/KS2 cells were still CD4 +. Similar results were obtained from three other independent experiments, which resulted in 91 ± 7.3 % and 62 ± 5.4 % of CD4 − cells in cells expressing NL4-3 Nef and KS2 Nef, respectively. This result indicated that the ability of KS2 Nef to down-regulate is significantly lower than that of NL4-3 Nef, consistent with the previous data (Yoon et al., 2001), and also that the effect of Nef is much more prominent when analysed during the early stage of Nef expression, distancing these two Nef proteins even further.

**Effect of KS2 Nef on primary T cells**

To test whether the primary KS2 nef sequence could also lack the ability to down-regulate CD4 in primary T cells, CD4 + cells were isolated from peripheral blood lymphocytes by a panning method using anti-CD4 antibody, and were cultured for 8 days in the presence of PHA and IL-2. The purity of the isolated CD4 + cell population was over 90 %, as measured by FACS using anti-CD4 antibody after a purification step (data not shown). CD4 + T cells were transduced with the same virus titre of either MIG–nef/KS2 or MIG–nef/NL4-3, and 2 days later, the magnitude of CD4 down-regulation was analysed using the GFP as a marker protein. When cultured for 8 days after CD4 + cell isolation, gene delivery efficiency was increased to 50 %, 32 % and 39 % for either MIG, MIG–nef/NL4-3 or MIG–nef/KS2, respectively (Fig. 3). However, it has to be noted that cells cultured for 8 days became very sensitive to the transduction process and were readily killed. From three independent experiments including the one shown in Fig. 3, it was found that 1 ± 0.8 % of total cells transduced with MIG–nef/KS2 showed significant down-regulation of

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**Fig. 2.** Effect of short-term expression of Nef on CD4 in Jurkat cells. One representative result from three independent flow cytometric analyses is shown. MIG is a negative control for CD4 down-regulation. The vertical axis shows phycoerythrin-conjugated anti-CD4 antibody, while the horizontal axis indicates GFP fluorescence (A). The percentage given in each quadrant indicates the relative percentage of cells. CD4 surface expression was also measured in the GFP-positive populations (B).
CD4, while 16±3.8% of cells expressing NL4-3 Nef displayed a decreased level of CD4. This result showed that the ability of KS2 Nef to down-regulate CD4 is significantly lower than NL4-3 Nef in primary T cells as well as in transformed cell lines.

**Localization of the region responsible for CD4 down-regulation**

There are several regions in the Nef protein that are known to be involved in CD4 down-regulation. They are a myristoylation signal, amino acid 57–58 region, the dileucine motif and the EE155 region. However, for the EE155 a myristoylation signal, amino acid 57–58 region, the dileucine motif and the EE155 region, there have been contradictory reports (Janvier et al., 1999). In one study, the EE155 region was reported to be involved in CD4 down-regulation and to have a major region interacting with β-COP (Piguet et al., 1999). In another study, the same region was shown to have nothing to do with CD4 down-regulation and β-COP (Janvier et al., 2001). Ironically, the comparative analysis of amino sequences of both nef sequences indicated that the most significant difference between KS2 and NL4-3 Nefs is the lack of the EE155 region in the former, while all other major ‘regulatory’ regions are present in both sequences (Fig. 4A). The amino acid sequence around the EE155 region of NL4-3 is EPDKVEANK, while for KS2 it consists of only five amino acids, GPD-----TV, i.e. completely omitting EE155. To test whether the lack of the EE155 region in KS2 Nef is involved in its decreased ability to down-regulate CD4, the equivalent region, consisting of ten or five amino acids, was swapped in a precise manner between NL4-3 and KS2 nefs, resulting in NL4-3K and KS2N. In these mutant constructs, the nef sequences are identical to the parental sequences except that each contains the other nef sequence around the EE155 region. As controls, EE155 sequences were either added to the NL4-3K nef or deleted from the KS2N nef, resulting in NL4-3KE+ and KS2NE−, respectively. We also generated the mutant KS2NE/Q, in which EE was replaced with QQ (Fig. 4B). All these mutants were inserted into retroviral vector MIN5, which expresses the bacterial neo sequence and the nef gene as a bicistronic message (Fig. 4B). In these experiments, the drug-resistance gene was used because G418 selection allows us to obtain more than 95% of transduced cells and thus analyse the effect(s) of Nef in a more quantitative manner.

Cell-free retroviral vectors were prepared from 293T cells again by a three-plasmid transfection method, and their titres were standardized to an m.o.i. of 0.1. Jurkat cells were transduced followed by G418 selection, and a drug-resistant population was obtained. In order to test whether these mutant nefs indeed expressed their respective proteins, total protein extracts were prepared from each population followed by Western blotting analysis using antibody against FLAG. As shown in Fig. 5(A), all chimeric proteins were expressed at comparable levels.

Next, cells were analysed by FACS using an antibody against CD4 (Fig. 5B, C). Consistent with the previous result, the magnitude of CD4 down-regulation by KS2 Nef was significantly lower than that by NL4-3. When the EE155 region of NL4-3 nef was replaced with the equivalent region from KS2 nef, the ability of this mutant NL4-3 to down-regulate CD4 was severely weakened. The exact opposite effect was observed in KS2N. When EPDKVEANK of NL4-3 was introduced into KS2 nef after deletion of the original five amino acids (GPD-----TV), KS2N was able to down-regulate CD4 as efficiently as NL4-3. These data clearly indicated that the EE155 region is important for the down-regulation of CD4. This conclusion was confirmed by properties shown by NL4-3KE+, KS2NE− and KS2NE/Q, where two Es were added, deleted or replaced, respectively. In these constructs, the ability to down-regulate CD4 was recovered as for the original parental sequence, NL4-3 and KS2. Similar observations were made using another T lymphoid line, CEM-SS (Fig. 5B, C). It is interesting to note that the magnitude of Nef CD4 down-regulation is generally lower in Jurkat than in CEM-SS cells. Jurkat is a more mature T cell line

![Fig. 3. Effects of Nef expression in primary CD4+ T cells. One representative result from three independent flow cytometric analyses is shown. Cells were cultured for 8 days in the presence of PHA and IL-2 after isolation of CD4+ T cells. The vertical axis shows phycoerythrin-conjugated anti-CD4 antibody, while the horizontal axis indicates GFP fluorescence. The percentage shown in each quadrant indicates the relative percentage of cells.](http://vir.sgmjournals.org)
Fig. 4. Mutational analysis of Nef. (A) Amino acid sequence alignment of Nef from KS2 and NL4-3. Black and scarlet characters represent shared or different amino acids between the two Nef sequences, respectively. The amino acid sequences around the EE155 region are indicated in blue. Four important domains of Nef are shown as shaded boxes (Geyer & Peterlin, 2001). Cellular proteins known to bind to Nef are indicated (Geyer et al., 2001; Greenberg et al., 1998a).

(B) Schematic representation of the various nef mutants. The mutants were generated by PCR-mediated site-directed mutagenesis and cloned into a retroviral vector, MIN5, which contains the respective nef and bacterial neo sequences.
than CEM-SS in the context of T cell receptor expression, which presumably results in the difference in cellular factors expressed in these two lines.

We also measured levels of MHC class I in these transduced T cell lines to test whether the EE155 region was also involved in MHC class I down-regulation. For NL4-3 Nef, the relative MHC class I down-regulation in Jurkat cells was approximately 36.4 ± 13.07 % and 23 ± 6.43 % for Nefs containing and lacking the EE155 region, respectively (Fig. 5B, C), while it was 30 ± 1.16 % and 23 ± 4.69 % for KS2 Nef. Similar results were obtained in CEM-SS. These data indicated that the EE155 region did not exert any significant influence on MHC class I down-regulation, consistent with the previous result showing that KS2 Nef down-regulates MHC class I as efficiently as NL4-3 (Yoon et al., 2001).

Analysis of clinical samples from KS2 and other pathologically defined individuals

Because KS2 is defined as an LTNP, we compared the nef sequence and basic clinical data of KS2 with those of eight other individuals whose pathological status has been followed by the National Institute of Health in Korea, and
whose nef sequences were determined also (Table 1). One of the noticeable observations made from KS2 was that this individual maintained an unusually high number of CD8\(^+\) T cells in peripheral blood over the 6-year period, ranging from 1252 to 2000 cells mm\(^{-3}\). Because CD4\(^+\) T cells were maintained at around 540 cells, the individual showed a

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Fig. 5. Effect of mutant Nef proteins on CD4 and MHC class I down-regulation. (A) Expression of mutant Nef proteins. Immunoblot analysis was carried out using protein extracts prepared from CEM-SS cells stably transduced with the respective retroviral vectors. The blot was probed with antibody against FLAG. The equivalent of \(5 \times 10^5\) cells was loaded per lane. (B) Flow cytometric analysis of T cell lines expressing various Nefs. Jurkat (top panel) and CEM-SS (bottom panel) cells were transduced with the respective retroviral vectors followed by selection with G418. Drug-resistant cells were obtained and the expression of Nef proteins was confirmed as shown in Fig. 5(A) followed by flow cytometric analysis. The surface expression of CD4 and MHC class I was measured by flow cytometry and the results are shown as indicated. MIN5 lacking the nef sequence was used as a control. (C) Quantitative analysis of the flow cytometric plots. The percentage down-regulation of CD4 and MHC class I was calculated from three independent flow cytometric analyses. The percentage relative CD4 down-regulation was calculated as \(100 - 100 \times (\% \text{CD4}^+ \text{cells in the experimental vector-containing cell line divided by } \% \text{CD4}^+ \text{cells in a cell line transduced with the control vector (MIN5) lacking Nef})\). Experimental vectors include those for the wild-type and mutant nefs. The percentage relative MHC class I down-regulation was calculated in the same manner. NL4-3 Nef derivatives are indicated as an open bar and KS2 Nef derivatives are indicated as a hatched bar.
relatively lower ratio of CD4/CD8, ranging from 0.24 to 0.47. This is in contrast to other LTNPs whose CD4/CD8 ratio was generally higher than 0.5 at almost all time-points, but similar to that of the three RPs (rapid progressors) compared in this study. Since the last observation made in March 2003, KS2 showed no sign of any significant change in these numbers, and KS2 is still not undergoing any drug treatment. The significance of this finding remains unclear at this time.

It appears that the EE155 region does not have any strong correlation with pathological status. Samples from four out of six LTNPs contained the intact EE155 region, samples from individuals KS2 and KU8 did not contain this region. All three RPs had the intact EE155 region. The nef sequences obtained during longitudinal analysis of infected individuals in Korea were also examined. No significant changes were observed, such as the appearance or disappearance of the EE155 region over time on the pathological status (Table 1, second column from the left). All these analyses indicate that the EE155 region is well conserved, but there is no close correlation between the presence of the EE155 region and the progress of the disease.

### Analysis of nef sequences available from the public database

We checked the 481 nef sequences available from the Los Alamos HIV Sequence Database. Seven nef sequences (approx. 1.5% of the total) lack the EE155 region, while

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<td>1131</td>
<td>0.16</td>
</tr>
<tr>
<td>EE (8)</td>
<td>2/98</td>
<td>136</td>
<td>836</td>
<td>0.16</td>
<td></td>
</tr>
</tbody>
</table>

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*Numbers in parentheses show the year of first detection.
†Numbers in parentheses indicate the number of DNA clones analysed during the study.
§One out of nine clones analysed contains no EE155 region.
$Not available.
||One out of eight clones isolated has the QE replacement.

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HIV-1 Nef and CD4 down-regulation
one of the two Es was replaced with other amino acids in 88 nef sequences (approx. 18·3%). Among 34 nef sequences isolated from 21 LTNP available from the PubMed database, only one sequence did not have the EE155 region, while no replacement was found in other sequences. Five nef sequences from five RPs were also available; one had the replaced EE155 region, while all the others contained the two glutamic acids.

We found the EE155 region crucial for CD4 down-regulation by Nef protein. Thus far two regions, the LL165 motif and the EE155 region, have been reported to play a role(s) in CD4 down-regulation. However, the involvement of the latter region has been controversial (Janvier et al., 2001; Pigu et al., 1999). Our data are more consistent with the findings of Pigu et al., showing that the EE155 region is needed for CD4 down-regulation. The actual mechanism of how the EE155 interacts with CD4 molecules remains to be elucidated, and various biochemical experiments are in progress.

There have been a few reports showing correlation between the presence of particular Nef regions and disease progression (Mariani & Skowronski, 1993; Asamitsu et al., 1999). However, data on the possible relationship between the EE155 region in particular, CD4 down-regulation and AIDS pathogenesis is extremely limited. One report discusses the possible role of the EE155 region in CD4 down-regulation (Mariani & Skowronski, 1993), while the other report indicates no role (Asamitsu et al., 1999). However, the number of individuals used in these studies was only one or two, numbers too low to derive any conclusion. In our study, we analysed eight individuals, whose status of disease progression was identified, as well as 34 Nef sequences from 21 LTNP available from the database. Although the profile of CD4+ and CD8+ T cells in the individual KS2 is intriguing, we did not discover any significant correlation between the progress of disease and presence of two glutamic acids. Based on the bulk of previous reports, it is likely that there are multiple factors involved in Nef-driven CD4 down-regulation, and thus it may not be possible to come to any acceptable conclusion about AIDS pathogenesis and the EE155 region alone. However, the EE155 region was very well conserved and present in the majority of nef sequences, indicating a crucial role in Nef protein function. Biological and clinical data are interesting enough to warrant further studies.

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