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Biological characterization of human immunodeficiency virus type 1 and type 2 mutants in human peripheral blood mononuclear cells

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Summary. Mutants of human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2), which have been shown to be infectious in established cell lines, were tested for ability to replicate and induce syncytium formation in human peripheral blood mononuclear cells (PBMC). The *vpu* mutant of HIV-1 showed depressed kinetics of replication in an established T cell line, as reported previously, but in PBMC, its replication was similar to that of the wild type virus. The *vpx* gene of HIV-2 was required for efficient virus propagation in PBMC, but not in an established T cell line, as previously reported. However, the growth rates of the *vpx* mutant in PBMC preparations from two individuals were different. The results of experiments on infection of PBMC with the *vif* and *vpr* mutants of HIV-1 and HIV-2 were essentially consistent with previous results of infection of established T cell lines. No negative effect of the *nef* gene products of HIV-1 and HIV-2 was observed. The abilities of the wild type virus and the mutants of HIV-1 to induce syncytium formation in both PBMC and established cell lines were similar. In contrast, neither the wild type nor any of the mutants of HIV-2 induced syncytium formation in PBMC. These results suggest that the functions of some genes can be detected only in mixed populations or primary cells such as PBMC. Studies on the roles of these genes in PBMC may provide a better understanding of their functions in vivo.

Introduction

Human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) are known to be the causative agents of acquired immune deficiency syndrome (AIDS). In addition to the structural genes, *gag*, *pol* and *env*, the genomes of these viruses

have at least six regulatory genes. Two trans-activator genes, *tat* and *rev*, are indispensable for virus replication [7, 10, 23, 28], but the other regulatory genes, named *vif, vpr, vpu, vpx* and *nef*, are not always essential for infectivity. Effects of mutations in these non-essential genes on virus replication in $CD4^+$ T cell lines have been reported [2, 3, 5, 6, 8, 9, 17, 19–22, 24–26, 29, 30]. A recent report has shown that *vpx* mutants of HIV-2 can propagate normally in T cell and monocytic lines, but are unable to establish productive infection in primary lymphocytes [14]. The behavior of mutants in primary cell cultures, therefore, may reflect the functions of HIV genes in vivo more precisely than those in established cell lines.

Here, we have examined phenotypes of the gene-specific infectious mutants of HIV-1 (*vif, vpr, vpu* and *nef* mutants) [2] and HIV-2 (*vif, vpx, vpr* and *nef* mutants) [22] in human primary blood mononuclear cells (PBMC), and demonstrate that some of the mutants display different growth potentials in PBMC from those in established cell lines.

Materials and methods

Cell culture

Human PBMC were separated from blood samples obtained from several individuals on Ficoll-Hypaque gradients, stimulated with 5 μg/ml Concanavalin A and cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum for one day. The culture medium was then replaced by the same medium containing 3% recombinant human IL-2. The $CD4^+$ human T cell lines, A3.01 [11] and Molt3 (kindly provided by Dr. A. Adachi, Kyoto University), were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum. A human colon carcinoma cell line, SW480 (ATCC CCL228), was maintained in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum.

DNA construct

The infectious molecular clones pNL-432 [1] and pGH-123 [22] were used as wild type (wt) DNAs of HIV-1 and HIV-2, respectively. The HIV-1 mutants designated as pNL-Nd (*vif* mutant), pNL-Af2 (*vpr* mutant), pNL-Ss (*vpu* mutant) and pNL-Xh (*nef* mutant) were derived from pNL-432 [2]. The HIV-2 mutants named pGH-Xb (*vif* mutant), pGH-St (*vpx* mutant), pGH-Ec (*vpr* mutant) and pGH-Ml (*nef* mutant) were derived from pGH-123 [22]. Table 1 shows the mutations in these DNA clones. Some biological properties of these mutants have been reported [2, 21, 22]. Chloramphenicol acetyl transferase (CAT) constructs under the control of viral LTRs were made by placing LTR fragments derived from HIVs/SIVs in front of the CAT gene of pHd-CAT [22; Sakuragi et al., submitted]. The details of the construction of the *nef* expression plasmid, pcD-SRα*nef*460, and the control plasmid, pcD-SRα*nef*ΔKpnI473 will be described elsewhere (Yokota et al., manuscript in prep.). Briefly, the Hinf I (nucleotides 8623) – Hind III (nucleotides 9606) fragment, containing the *nef* gene was excised from the pNL-432 DNA [1] and was repaired with Klenow fragment. After adding Bam HI linkers to both ends [18], the fragment was inserted into dephosphorylated Bgl II site of the pcDL-SRαBgl II456 [27; Takebe et al., unpubl.], generating the pcD-SRα*nef*473 plasmid, in which the *nef* gene is expressed under the control of the SRα promoter [27]. In the pcD-SRα*nef*ΔKpnI473, the Kpn I site (nucleotides 9005) in the *nef* gene was deleted by T4 DNA polymerase, creating a frame-shift mutation in the *nef* gene.

Table 1. HIV-1 and HIV-2 mutants used in this study

Plasmid	Region of mutation	Description of mutation[a]	Predicted size of mutated protein[b]
pNL-432	wild type (HIV-1)	none	
pNL-Nd	vif	2 bp insertion at Nde I (5122)	28 (192)
pNL-Af2	vpr	4 bp insertion at Afl II (5634)	26 (96)
pNL-Ss	vpu	8 bp linker insertion at Ssp I (6153)	32 (81)
pNL-Xh	nef	4 bp insertion at Xho I (8887)	35 (206)
pGH-123	wild type (HIV-2)	none	
pGH-Xb	vif	4 bp insertion at Xba I (5064)	67 (215)
pGH-St	vpx	4 bp insertion at Sty I (5518)	60 (112)
pGH-Ec	vpr	4 bp insertion at Eco RI (5756)	42 (120)
pGH-Ml	nef	4 bp insertion at Mlu I (8637)	34 (246)

[a] Nucleotide sequence data were from Los Alamos data bank (Los Alamos, NM, U.S.A.) for pNL-432 and from [16] for pGH-123. Values in parentheses are the first nucleotide no. of the enzyme recognition sites

[b] Values indicate no. of wt amino acid residues from N-terminus of mutated proteins. No. of amino acid residues of wt are also shown in parentheses. Data of mutants in HIV-1 were cited from [2]

DNA transfection

For transfection, uncleaved plasmid DNA was introduced into SW 480 cells by the calcium phosphate coprecipitation method [13, 31].

Infection

The growth kinetics of the mutants was determined in PBMC and $CD4^+$ T cell lines. Culture supernatants of transfected SW 480 cells were filtrated (0.45 μm pore size) and appropriate volumes of filtrates were added to 1×10^6 cells as previously described [11].

RT assays

Virion-associated reverse transcriptase (RT) activity of progeny virions was assayed as previously described [32].

Syncytium formation

Syncytium formation was monitored at intervals by microscopic examination.

CAT assays

CAT assays were carried out as previously described [12]. Equivalent amounts of cell lysates from transfected SW 480 cells were used for determination of CAT activity.

Results

Biological characterization of mutants

For assessment of the biological activities of HIV-1 and HIV-2 mutants in PBMC, equivalent amounts of cell-free viruses (5×10^4 cpm of RT activity)

prepared from transfected SW 480 cells were inoculated into PBMC and CD 4^+ T cell lines. Virus replication and syncytium formation were monitored by measuring RT production and by microscopic examination, respectively. Both of the wt viruses replicated well in PBMC, A 3.01 (HIV-1) or Molt 3 (HIV-2) cells.

Figure 1 shows the growth kinetics of HIV-1 mutants in A 3.01 cells and PBMC. The mutants of the *vpu* and *nef* genes exhibited different growth patterns in the two cell types. Virus replication of the *vpu* mutant was clearly less efficient than that of wt virus in A 3.01 cells but both viruses replicated equally well in PBMC. In contrast, growth of the *nef* gene mutant was similar to that of the wt virus in A 3.01 cells, but was slightly slower than that of the wt virus in PBMC. The product of the *nef* gene of HIV-1 was detected by Western blotting in both A 3.01 cells and PBMC infected with wt virus, but not in cells infected with the *nef* mutant (data not shown). The *vif* mutant did not grow in either type of cells. The *vpr* mutant and the wt virus replicated similarly in both types of cells when infected at a relatively high input dose (Fig. 1), but 10-fold decrease in the input dose of the former delayed the virus growth markedly in A 3.01 cells and slightly in PBMC (Fig. 2 A and B).

Figure 3 shows the growth kinetics of HIV-2 mutants in Molt 3 cells and PBMC. The growth characteristics of the *vif* and *vpx* mutants in PBMC were

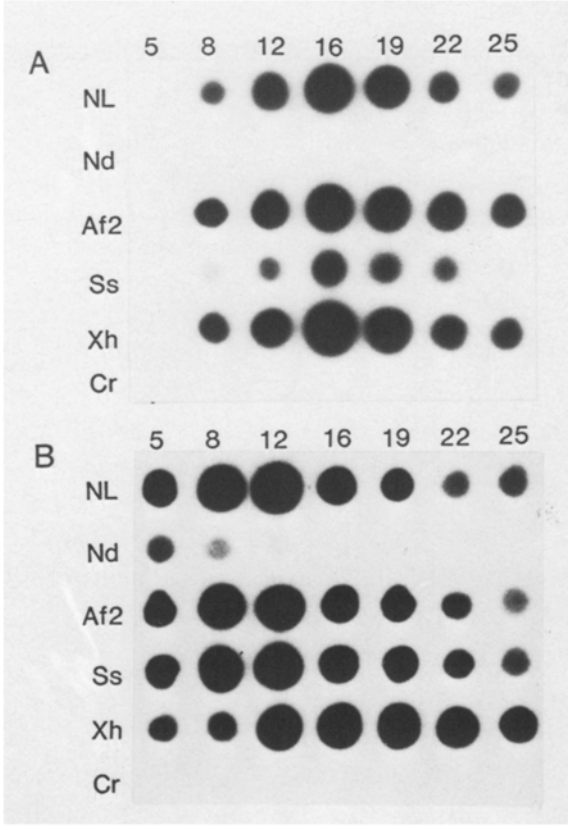

Fig. 1. Growth kinetics of various HIV-1 mutant clones in A 3.01 cells (**A**) and PBMC (**B**). 1×10^6 cells were simultaneously inoculated with an equivalent amount (5×10^6 cpm of RT activity) of cell-free virus from transfected SW 480 cells, and RT production was monitored at intervals with a negative control (mock infected; *Cr*). Numbers on the top indicate days after infection. *NL* Virus from the pNL-432 wild type clone of HIV-1; *Nd* pNL-Nd (*vif* mutant); *Af 2* pNL-Af 2 (*vpr* mutant); *Ss* pNL-Ss (*vpu* mutant); *Xh* pNL-Xh (*nef* mutant)

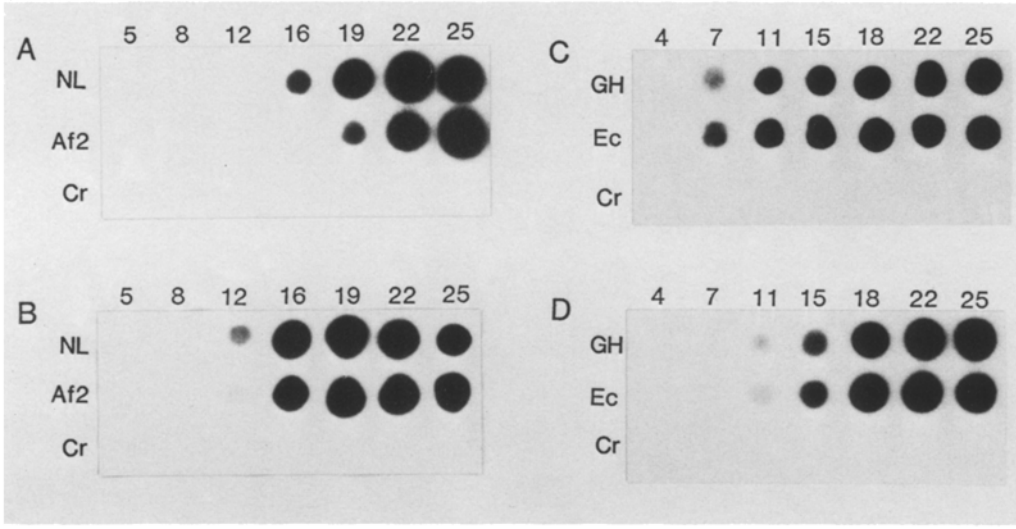

Fig. 2. Growth kinetics of wild type viruses and *vpr* mutants of HIV-1 (**A, C**) and HIV-2 (**B, D**) in A 3.01 cells (**A**), PBMC (**B, D**) and Molt 3 cells (**C**). 1×10^6 cells were infected with an equivalent amount (5×10^5 cpm of RT activity) of cell-free virus from transfected SW 480 cells, and RT production was monitored at intervals with a negative control (mock infected; *Cr*). Numbers on the top indicate days after infection. *NL* Virus from the pNL-432 wild type clone of HIV-1; *Af2* pNL-Af2 (*vpr* mutant of HIV-1); *GH* pGH-123 wild type clone of HIV-2; *Ec* pGH-Ec (*vpr* mutant of HIV-2)

different from those in Molt 3 cells. The *vpx* mutant replicated well in Molt 3 cells but very poorly in PBMC. To examine whether the retarded growth of the *vpx* mutant in PBMC was a general feature, we carried out a similar infection experiment by using PBMC samples prepared from another individual. As shown in Fig. 4, the *vpx* mutant propagated poorly but the reduction in its growth rate to one-third of that of the wt virus was not so remarkable as that shown in Fig. 3. The *vif* mutant grew poorly in Molt 3 cells, and showed no productive infection in PBMC. The kinetics of growth and the levels of virus production of the *vpr, nef* mutants and wt virus were similar in PBMC and Molt 3 cells. A 10-fold reduction in the input dose had no significant effect on the relative growth kinetics of the wt virus and the *vpr* mutant (Fig. 2 C and D).

Results of syncytium formation by the wt virus and the mutants are summarized in Table 2. The wt virus and all the mutants of HIV-1 except the *vif* mutant induced syncytium formation in both of the cell types. On the other hand, neither HIV-2 wt virus nor any of its mutants induced syncytium formation in PBMC, which is in sharp contrast to their effects on Molt 3 cells.

Effect of expression of HIV-1 nef on HIV/SIV LTRs

Earlier studies showed that mutations of the *nef* gene lead to more efficient viral replication [3, 19, 30] or transcription directed by the HIV-LTR [3, 30].

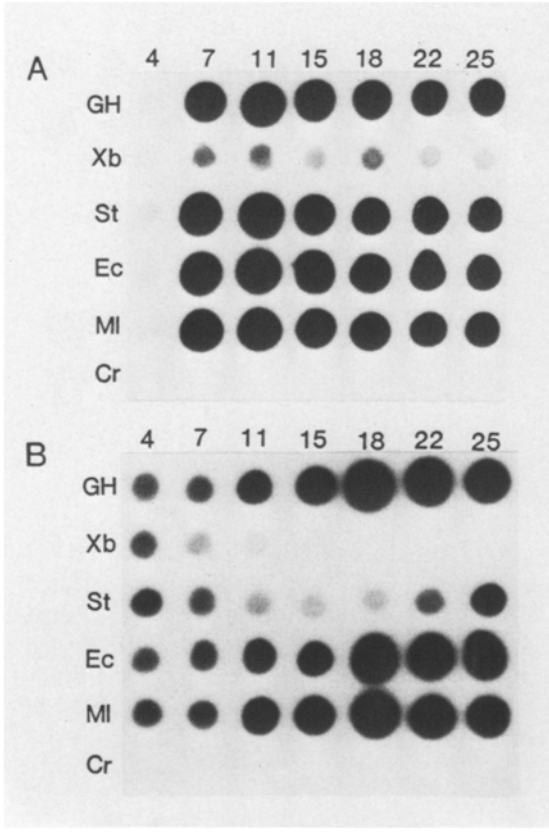

Fig. 3

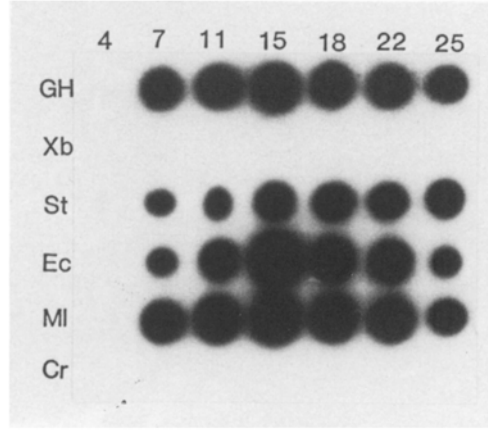

Fig. 4

Fig. 3. Growth kinetics of various HIV-2 mutant clones in Molt 3 cells (**A**) and PBMC (**B**). 1×10^6 cells were simultaneously inoculated with an equivalent amount (5×10^6 cpm of RT activity) of cell-free virus from transfected SW 480 cells, and RT production was monitored at intervals with a negative control (mock infected; *Cr*). Numbers on the top indicate days after infection. *GH* pGH-123 wild type clone of HIV-2; *Xb* pGH-Xb (*vif* mutant); *St* pGH-St (*vpx* mutant); *Ec* pGH-Ec (*vpr* mutant), *Ml* pGH-Ml (*nef* mutant)

Fig. 4. Growth kinetics of various HIV-2 mutant clones in another PBMC preparation. PBMC separated from a different individual (see Fig. 3) were inoculated with equivalent amounts (5×10^6 cpm of RT activity) of cell-free virus from transfected SW 480 cells, and RT production was monitored at intervals with a negative control (mock infected; *Cr*). Numbers on the top indicate days after infection. *GH* pGH-123 wild type clone of HIV-2; *Xb* pGH-Xb (*vif* mutant); *St* pGH-St (*vpx* mutant); *Ec* pGH-Ec (*vpr* mutant), *Ml* pGH-Ml (*nef* mutant)

However, the possible function of the *nef* gene is still controversial [4, 15, 17, 20]. In our infection experiments, the *nef* gene product showed no negative effect on virus replication. For further examination of the potential role of HIV-1 *nef* gene expression in transcription directed by the LTRs of various primate immunodeficiency viruses, cotransfection experiments were carried out. SW 480 cells were transfected with a series of LTR-CAT constructs and a

Table 2. Characterization of HIV-1 and HIV-2 mutants

Mutants	Mutated gene	Virus growth in		Syncytium formation in	
		cell line[a]	PBMC	cell line[a]	PBMC
pNL-432 (HIV-1)	None	+	+	+	+
pNL-Nd	vif	−	−	−	−
pNL-Af2	vpr	+[b]	+	+	+
pNL-Ss	vpu	+[c]	+	+	+
pNL-Xh	nef	+	+[b]	+	+
pGH-123 (HIV-2)	None	+	+	+	−
pGH-Xb	vif	+[c]	−	−	−
pGH-St	vpx	+	+[b]	+	−
pGH-Ec	vpr	+	+	+	−
pGH-Ml	nef	+	+	+	−

[a] A 3.01 cells (HIV-1) and Molt 3 cells (HIV-2) were used as target cell lines for infection
[b] Delayed kinetics of virus growth
[c] Reduction of virus growth

plasmid expressing either the HIV-1 *nef* gene (pcD-SRα*nef*460) or the mutated *nef* gene (pcD-SRα*nef*ΔKpnI473), and the CAT expression was monitored. As shown in Fig. 5, pcD-SRα*nef*460 did not repress CAT expression of any of the HIV/SIV LTR-CAT constructs when compared to that by pcD-SRα*nef*ΔKpnI473. Essentially the same results were obtained in repeated experiments.

Discussion

We have examined in this study the effects of mutations in five regulatory genes of HIV (*vif, vpr, vpu* and *nef* of HIV-1, and *vif, vpx, vpr* and *nef* of HIV-2) on virus replication and syncytium formation in PBMC and established T cell lines. These genes are known to be dispensable for virus replication in established T cell lines [2, 3, 5, 6, 8, 9, 17, 19–22, 24–26, 29, 30]. Recently, Guyader et al. reported that *vpx* mutants of HIV-2 replicate normally in T and promonocytic cell lines, but not in peripheral blood lymphocytes (PBL) [14], suggesting that the *vpx* gene product has an important role in vivo. We examined whether the mutants of these regulatory genes exhibit distinct characteristics when inoculated into primary cells, and summarized the results in Table 2.

The *vpu* gene is unique to the HIV-1 genome. Our results (Fig. 1 A) and previous data [2, 26, 29] showed that the *vpu* gene is necessary for efficient virus replication in established T cell lines. Unexpectedly, the *vpu* mutant and the wt virus showed similar growth kinetics in PBMC (Fig. 1 B). This observation suggests that the requirement of the *vpu* product for virus replication may differ depending on cell origins. Further investigations will elucidate the function of the *vpu* gene in vivo.

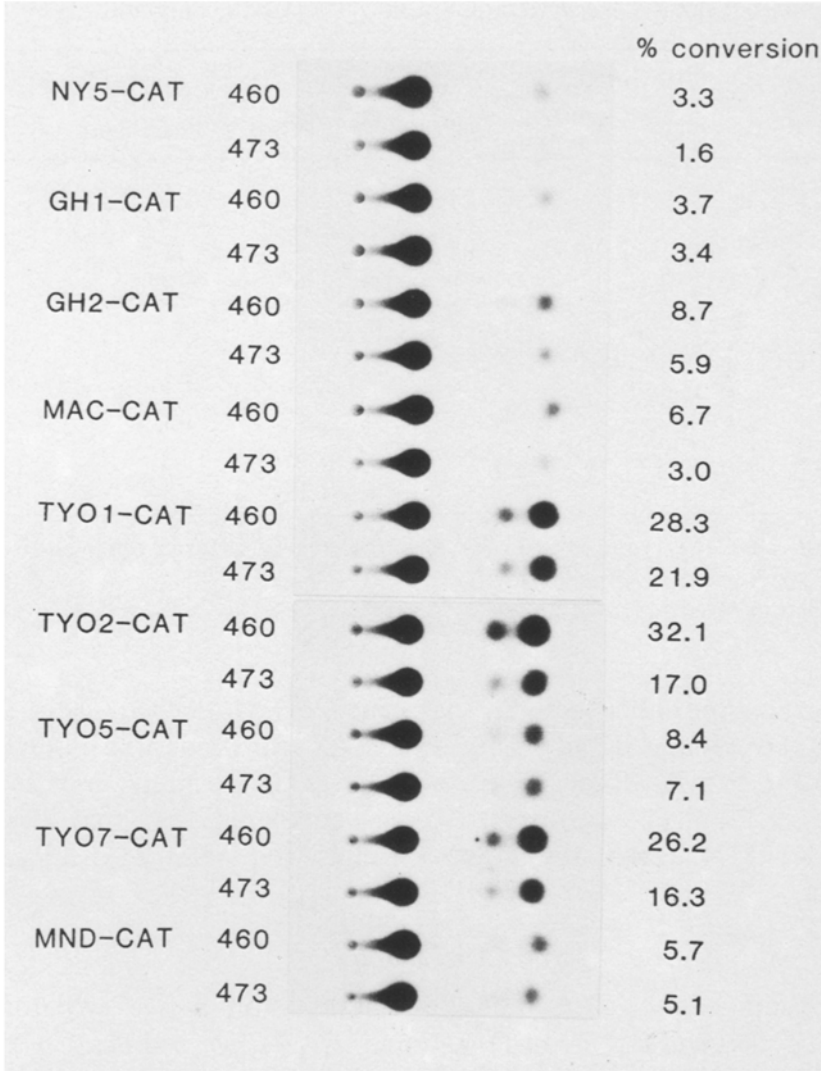

Fig. 5. Effect of *nef* gene expression on various LTRs. 10 μg of expression vector of the *nef* gene from the pNL-432 HIV-1 clone pcD-SR*α*-*nef*460 (460) or its mutant pcD-SR*α*-*nef*ΔKpnI473 (473) were cotransfected into SW 480 cells with 10 μg of each of HIV/SIV LTR-CAT constructs, NY 5-CAT (HIV-1), GH 1-CAT (HIV-2), GH 2-CAT (HIV-2), MAC-CAT (SIV_{MAC}), TYO 1-CAT to TYO 7-CAT (SIV_{AGM}) and MND-CAT (SIV_{MND}). Details for these constructs are described elsewhere (J. Sakuragi et al., submitted). CAT activity in the cell lysates was determined at 48 h post-transfection. The percent conversion of chloramphenicol to its acetylated forms is indicated on the right

The *vpx* gene is conserved in all primate lentiviruses except HIV-1. This gene of HIV-2 was reported to be necessary for virus replication in human PBL but not in established cell lines [14]. Our results were almost consistent with the reported findings. However, we found that the phenotype of the *vpx* mutant varied significantly in PBMC preparations from different individuals (Fig. 4).

These results also indicate the importance of the character of target cells for infectivity of mutant viruses.

The *vif* genes of HIV-1 and HIV-2 have been demonstrated to be required for efficient replication in established cell lines, but the effect of mutation in the *vif* gene varies depending on the cells used [2, 9, 22, 24, 25]. In our experiments, mutants of the *vif* genes of neither HIV-1 nor HIV-2 replicated in PBMC, suggesting that *vif* gene product is indispensable for virus replication in vivo.

There are reports that the *vpr* product of HIV-1 increases the rate of replication and accelerates the cytopathic effect of the virus in established T cell lines [6, 21]. In the case of HIV-2, however, *vpr* mutants exerted no effect on the growth kinetics [8, 22]. The results of our experiments in PBMC were essentially consistent with these previous reports.

Earlier studies showed that *nef* mutants multiply faster than the wt virus in established T cell lines [3, 19, 30]. However, in the present investigation, we obtained no evidence that *nef* gene product exerts negative influences on virus growth (Fig. 1 and 3). The *nef* gene product has also been reported to reduce HIV-1 LTR-mediated transcription [3, 20], but more recent data indicated the absence of the negative influence of the *nef* gene [4, 15, 17]. Cheng-Mayer et al. found that the *nef* products from several isolates had different effects on HIV replication in vitro [5]. Moreover, we found that the *nef* protein of HIV-1 NL-432 did not repress CAT activity directed by LTRs derived from HIV-1, HIV-2, SIV_{MAC}, SIV_{MND} and SIV_{AGM}, which are representatives of major primate immunodeficiency viruses (Fig. 5). This finding is consistent with the results of our infection experiments. More biochemical functional analyses are required to re-evaluate the function of the *nef* gene.

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