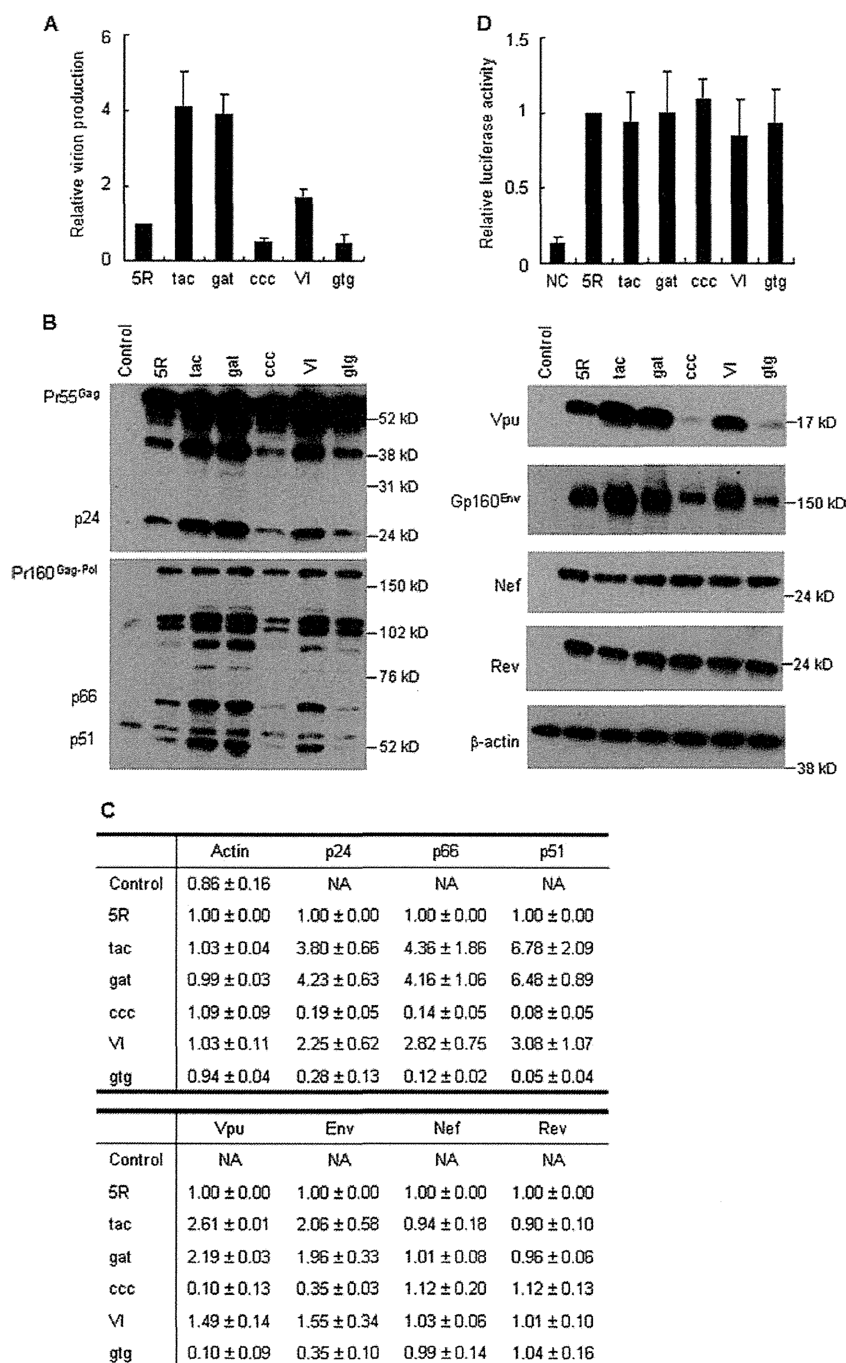


| | Actin | p24 | p66 | p51 |
|---|---|---|---|---|
| Control | 0.86 ± 0.16 | NA | NA | NA |
| 5R | 1.00 ± 0.00 | 1.00 ± 0.00 | 1.00 ± 0.00 | 1.00 ± 0.00 |
| tac | 1.03 ± 0.04 | 3.80 ± 0.66 | 4.36 ± 1.86 | 6.78 ± 2.09 |
| gat | 0.99 ± 0.03 | 4.23 ± 0.63 | 4.16 ± 1.06 | 6.48 ± 0.89 |
| ccc | 1.09 ± 0.09 | 0.19 ± 0.05 | 0.14 ± 0.05 | 0.08 ± 0.05 |
| VI | 1.03 ± 0.11 | 2.25 ± 0.62 | 2.82 ± 0.75 | 3.08 ± 1.07 |
| gtg | 0.94 ± 0.04 | 0.28 ± 0.13 | 0.12 ± 0.02 | 0.05 ± 0.04 |

| | Vpu | Env | Nef | Rev |
|---|---|---|---|---|
| Control | NA | NA | NA | NA |
| 5R | 1.00 ± 0.00 | 1.00 ± 0.00 | 1.00 ± 0.00 | 1.00 ± 0.00 |
| tac | 2.61 ± 0.01 | 2.06 ± 0.58 | 0.94 ± 0.18 | 0.90 ± 0.10 |
| gat | 2.19 ± 0.03 | 1.96 ± 0.33 | 1.01 ± 0.08 | 0.96 ± 0.06 |
| ccc | 0.10 ± 0.13 | 0.35 ± 0.03 | 1.12 ± 0.20 | 1.12 ± 0.13 |
| VI | 1.49 ± 0.14 | 1.55 ± 0.34 | 1.03 ± 0.06 | 1.01 ± 0.10 |
| gtg | 0.10 ± 0.09 | 0.35 ± 0.10 | 0.99 ± 0.14 | 1.04 ± 0.16 |

**FIG 8** Effect of single-nucleotide changes on virion production and intracellular expression levels of viral proteins. (A) Virion production in transfected cells. 293T cells were transfected with the indicated proviral clones, and on day 1 posttransfection, virion production was monitored by the amount of Gag-p24 in the culture supernatants. The amount of p24 relative to that produced by 5R is presented. Mean values ± SD from three independent experiments are shown. (B) Expression of various viral proteins in transfected cells. 293T cells were transfected with the indicated proviral clones, and on day 1 posttransfection, cell lysates were prepared for Western blotting using anti-Gag-p24, anti-RT, anti-Vpu, anti-Env-gp160, anti-Nef, anti-Rev, and anti-β-actin antibodies. The migration positions of mass standards are indicated on the right. Representative data from two independent transfection experiments are shown. Control, pUC19; tac, Y226tac; gat, D229gat; ccc, P233ccc; VI, V234I; gtg, V234gtg. (C) Quantitative analysis of the Western blot. Signal intensities of viral proteins were quantitated, and the intensities relative to those for 5R proteins are shown. Mean values ± SD obtained from the two independent transfection experiments in panel B are indicated. NA, not applicable. (D) Analysis of Tat activity. 293T cells were cotransfected with the indicated proviral clones and an LTR-driven luciferase reporter clone, and on day 1 posttransfection, cell lysates were prepared for luciferase assays. Luciferase activity relative to that exhibited by 5R is presented. Mean values ± SD from three independent experiments are shown. NC, negative control (basal luciferase activity of the LTR-driven luciferase reporter clone in the absence of proviral clones).

FIG 9 Effect of single-nucleotide changes on viral mRNA expression. (A) Nucleotide and amino acid sequences in the 3′ region of the *pol* gene. Nucleotides 4882 to 4942 correspond to the NL4-3 sequence (35) (GenBank accession no. AF324493). Black and gray bold letters in the nucleotide sequence show the sites at which the single-nucleotide substitution promoted or decreased viral replication efficiency, respectively. Bold letters in the amino acid sequence represent adaptive mutation sites. The SA1prox (this study), SA1 site (3, 4), ESE-Vif (27), ESE-M1 (28), and SLSA1 region (45) are as indicated. Numbers and underlines show the positions of amino acids in IN and their codons, respectively. (B) Schematic presentation of the 5R genome organization and various HIV-1 mRNA species. The genome structure of 5R is shown as in Fig. 1. Gray bars represent the regions used as probes for Northern blot analyses. The *Pol*4889-4923 region is also indicated.

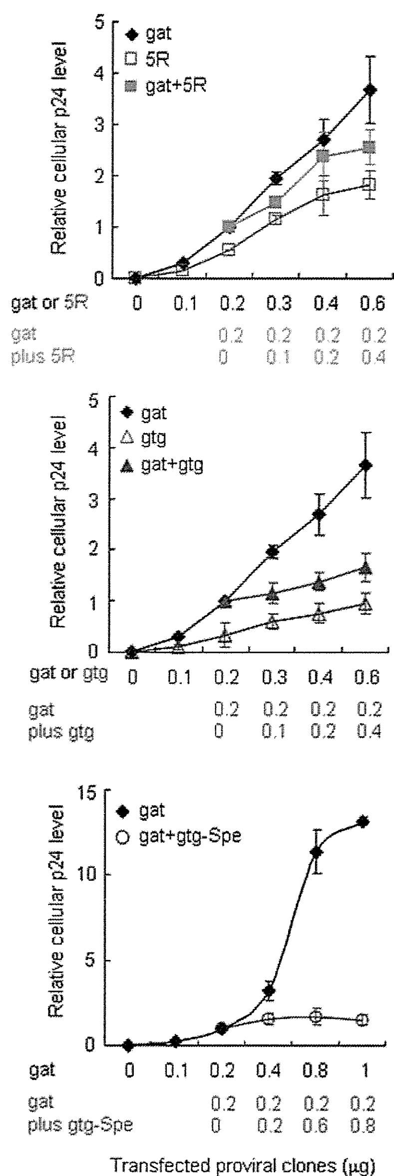


**FIG 10** Expression level of Gag-p24 in singly or doubly transfected cells. 293T cells were transfected with proviral clones as indicated, and on day 1 posttransfection, Gag-p24 expression level in cell lysates was determined. The amount of p24 relative to that produced upon transfection with 0.2 μg of the D229gat (gat) clone is presented. Mean values ± SD from three independent experiments are shown. gat, D229gat; gtg, V234gtg; gtg-Spe, a frameshift mutant of V234gtg.

These mutations were identified by both viral adaptation experiments (32) and comparative analysis of numerous HIV-1/SIVcpz genomes (Table 2). These findings suggest that the nucleotide sequence in the SA1prox may be involved in the viral adaptation/evolution process. Recent RNA structure analysis has shown that the nucleotide sequence proximal to SA1 within the HIV-1 genome forms a stem-loop structure (45, 46). Moreover, Pollom et al. reported that this stem-loop structure, designated "SLSA1," was conserved between HIV-1 NL4-3 and SIVmac239, suggesting the virological importance of the SLSA1 structure (45). Interestingly, all single-nucleotide mutations analyzed in Fig. 8 and 9 were mapped onto the SLSA1 sequence (at positions 4901 to 4942 in Fig. 9A). The emergence of novel mutations within this region may be limited due to its effect on IN functions, SA1 functions, and the SLSA1 structure. This may explain the low frequency of single-nucleotide mutations that alter viral replication efficiencies among HIV-1 genomes (Table 2). Nevertheless, the presence of such single-nucleotide variations in SLSA1 represents the plasticity of viruses with the ability to adapt themselves under various constraints. Our results on the replication-altering mutations within SLSA1 may also be useful for analyzing changes in the RNA sequence/structure and their effects on viral replication. Because the SLSA1 structure is conserved between HIV-1 NL4-3 and SIVmac239, it is of interest to determine whether naturally occurring single-nucleotide synonymous mutations in this region affect the replication efficiency of SIVmac239 and its closely related primate lentiviruses.

The change in virion production/replication ability was reflected in the expression levels of viral late proteins (Gag, Gag-Pol, Vpu, and Env) but not in those of the early proteins (Nef and Rev) (Fig. 7 and 8). However, a direct positive correlation between the steady-state levels of viral mRNAs and their corresponding late proteins was not observed (Fig. 8 and 9). More viral RNAs and a large number of viral RNA species were synthesized in cells producing low expressors of viral late proteins (5R, P233ccc, and V234gtg) than in those producing high expressors (Y226tac, D229gat, and V234I). This may imply that the expression level of mRNAs directed by high expressors is necessary and sufficient for the efficient expression of viral proteins and optimal viral replication. We initially assumed that various kinds of viral transcripts by low expressors may hinder the efficient translation of viral late proteins. However, interference assays between variants (Fig. 10) showed that this prediction may not be accurate. Moreover, no significant difference in the Tat activity of 5R and its variants was observed (Fig. 8D). Therefore, it is reasonable to assume that single-nucleotide changes in the SA1prox act in *cis* and may influence splicing, mRNA stability, mRNA transport, and/or translation efficiency from mRNAs.

The abundance of the *vif* transcript (species *1 in Fig. 9C and D) observed for 5R and low expressors (P233ccc and V234gtg) suggests enhanced splicing at the SA1 site. On one hand, a combination of two synonymous mutations in SLSA1 that changes its RNA structure was reported to affect splicing at the SA3 site downstream of SA1 but not that at the SA1 site, showing long-range RNA interactions and cross talk between splicing sites (45). This may explain variations in the expression levels of transcripts (the

RRE, Rev-responsive element; U, universal. (C and D) Steady-state expression levels of HIV-1 mRNA species. Total RNA was prepared from 293T cells transfected with the indicated proviral clones, and poly(A)$^+$ RNA was selected. After the DNase I treatment, samples were subjected to Northern blot analysis using the indicated probe. GAPDH was used as an internal standard. Three major species of viral mRNA (~9 kb, ~4 kb, and ~1.8 kb) are shown by arrowheads. The other extra bands *1 to *4 are indicated by arrows. RNA size markers (8 kb, 4 kb, and 2 kb) are on the left. Representative data from four independent experiments (C, left panel [5R and its mutants]) and from two independent experiments (C, right panel [NL4-3 and its mutants] and D) are shown. Control, pUC19; tac, Y226tac; gat, D229gat; ccc, P233ccc; VI, V234I; gtg, V234gtg.

*3, *4, and 1.8-kb species) among 5R/low expressors and high expressors. Since these transcripts do not contain the RRE region, it is clear that they are generated via splicing downstream of SA1. The abundance of these transcripts (*3, *4, and 1.8-kb species) for 5R and low expressors may also imply that the overall splicing efficiency of these clones is higher than that of high expressors. Efficient splicing at SA sites may compete with Rev function, and equilibrium between the strength of splicing acceptors and Rev function for the nuclear export of Rev-dependent mRNAs is important for virus replication (28). Thus, increased splicing for 5R and low expressors may obstruct the function of Rev, which results in a decrease in the Rev-dependent expression of late proteins from RRE-containing transcripts. On the other hand, while the amounts of 1.8-kb mRNAs of 5R and low expressors were larger than those of high expressors, the expression levels of viral early proteins were similar among 5R and its variants. A high concentration of Rev was previously shown to inhibit the translation from various RNAs (47). It is possible that the expression of viral early proteins may be regulated at an optimal level for viral replication. Alternatively, the translation efficiency of ~40 mRNA isoforms synthesized by alternative splicing events may vary due to differences in their noncoding sequences and/or structures. Viral mRNA species within 1.8-kb and 4-kb RNAs were shown to be altered by mutations that change splicing efficiency at SA1 or the structure of SLSA1 (27, 45, 48). Viral mRNA isoforms with a low translation efficiency, even if present in abundance, may not express a high level of their corresponding proteins.

*vif* mRNA expression is strongly influenced by splicing efficiency at the SA1 site. The regulation of splicing at SA1 is complicated and is determined by various elements, including three different exonic splicing enhancers (ESE-Vif and ESE-M1 [Fig. 9A] and ESE-M2 [nt 4956 to 4962 in NL4-3]), a suboptimal D2 splicing site (nt 4960 to 4970 in NL4-3), a GGGG silencer (nt 4968 to 4971 in NL4-3), and a G run ($G_{I2}$-1, nt 5034 to 5038 in NL4-3), which are located within the region from SA1 to just upstream of the *vif* start codon (nt 5041 in NL4-3) (27, 28, 48). The proviral clone 5R was constructed by introducing SIVmac239 *vif* into the downstream region of the *pol* open reading frame in the NL4-3 genome (Fig. 1 and 9B) (34). As a result, while the *pol* and *vif* genes of NL4-3 partially overlap, those of 5R do not. Since splicing efficiency is dependent on the sequence around the splice sites and their distance from the regulatory elements, the insertion of SIVmac239 *vif* into NL4-3 may have changed the splicing event at SA1. Indeed, 5R produced abundant amounts of the *vif* transcript (the *1 species in Fig. 9). The increase in *vif* mRNA was previously shown to decrease virion production, and the proportion between unspliced and spliced mRNAs has been suggested to be important for virion production (27). In agreement with this finding, we found that the virion production level from 293T cells transfected with 5R was lower than that from cells transfected with NL4-3 (data not shown). The decrease in *vif* transcript (*1 species) expression for high expressors may have caused the increase in virion production.

The splicing balance of viral mRNAs has been suggested to have biologically significant effects on viral replication (4, 9–11). Accumulating evidence has shown that HIV-1 gene expression processes, composed of transcription, poly(A) tailing, splicing, mRNA export, and subsequent translation, are mutually affected and coupled, even though these processes are biochemically distinguished (1, 2, 49). In addition, various elements within the HIV-1 genome and a number of virus/host factors have been shown to be involved in HIV-1 gene expression (3, 4, 9–11, 25–30, 50–55). The virological importance of the nucleotide sequence in the SA1prox is evident from the increase or decrease in viral replication caused by naturally occurring single-nucleotide changes. Further studies are needed to elucidate the molecular mechanism underlying the modulation of overall HIV-1 gene expression generated by single-nucleotide changes in the SA1prox.

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We declare that no competing interests exist.

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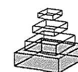

# Role of poly-proline motif in HIV-2 Vpx expression

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Human and simian immunodeficiency viruses (HIV and SIVs) contain several auxiliary genes not found in other retroviruses. These genes are thought to be functionally important for optimal viral replication and persistence in infected individuals. Primate lentiviruses can be classified by the composition of these accessory genes. While viruses of the HIV type1 (HIV-1) group have *vif*, *vpr*, *vpu*, and *nef* genes, those of the HIV-2 group carry *vif*, *vpx*, *vpr*, and *nef* genes (Fujita et al., 2010). Vpx protein encoded by the *vpx* gene is unique to non-HIV-1 viruses, and is essential for viral replication in macrophages in contrast to its structural paralog Vpr (Fujita et al., 2010). The most outstanding sequence feature to distinguish Vpx from Vpr is the presence of poly-proline motif (PPM) at its C-terminal region. We have recently shown, by *in vitro* and *in vivo* assay systems, that the PPM in HIV-2 Vpx is critical for its efficient translation (Miyake et al., 2014).

Although PPM consisting of seven consecutive prolines has been demonstrated to be required for efficient HIV-2 Vpx translation, thereby acquiring viral infectivity in macrophages, the effects of PPM mutations on the degradation of Vpx in cells was not formally analyzed as yet (Fujita et al., 2008; Miyake et al., 2014). Therefore, in this study, we asked whether the PPM plays a role in keeping away from proteasomal and/or lysosomal degradation (**Figure 1**). In order to assess this, we used various expression plasmids for HIV-2 Vpx (pEF-Fvpx series) described in a previous study (Miyake et al., 2014): wild-type (WT) plasmid has the *vpx* gene derived from HIV-2 GL-AN clone (Kawamura et al., 1994); mutants 103/4A and 106/4A have four consecutive alanine-substitutions at the site of P103-P106 and P106-P109, respectively, and have been shown to express a low/minimum level of mutant Vpx proteins in cells (**Figure 1A**); a negative control is a frame-shift mutant pEF-FxSt that lacks Vpx expression (ΔVpx).

Various expression plasmids were transfected into human 293T cells (Lebkowski et al., 1985) as described before (Adachi et al., 1986), and the amounts of WT and mutant Vpx proteins produced in cells in the absence or presence of a proteasome inhibitor MG-132 (Fujita et al., 2004; McCulley and Ratner, 2012) were comparatively examined by Western blotting (Miyake et al., 2014). A drastic reduction in Vpx expression was observed for mutants 103/4A and 106/4A, 106/4A in particular, both in the absence

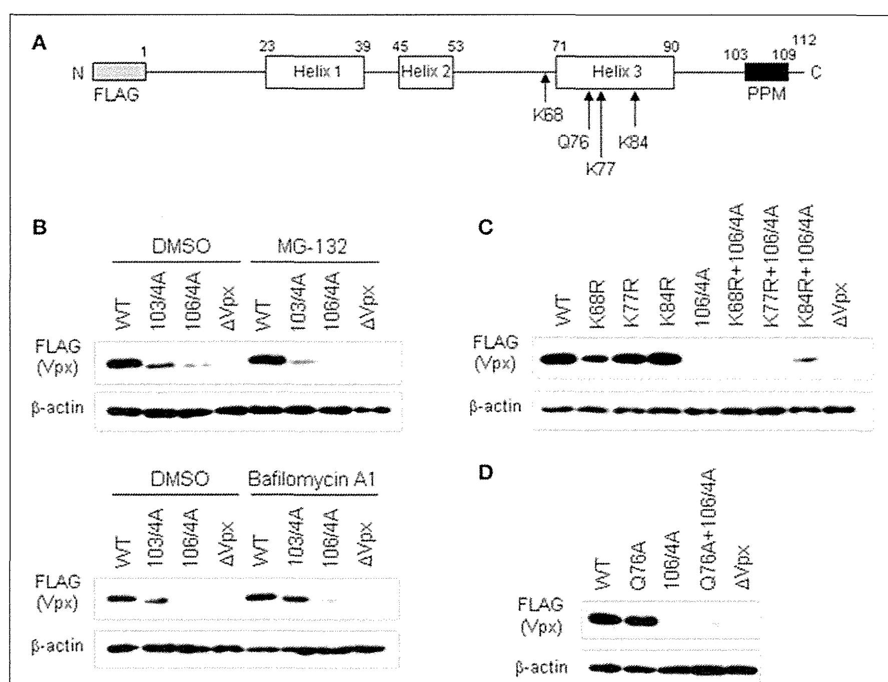


**FIGURE 1 | Steady-state levels of various Vpx-PPM mutants in cells as monitored by Western blotting. (A)** Structure of the FLAG-tagged HIV-2 GL-AN Vpx construct. Numerals above the schema represent amino acid numbers of the Vpx protein. Positions of lysine and glutamine residues mutated are indicated. **(B)** Expression of Vpx-PPM mutants in the presence of a proteasome inhibitor MG-132 or a lysosome inhibitor Bafilomycin A1. **(C)** Expression of lysine-mutants with or without 106/4A mutation. **(D)** Expression of Q76A mutants with or without 106/4A mutation. For **(B)** to **(D)** experiments, 293T cells were transfected with the plasmids indicated, and harvested for Western blotting 24 h later. To examine lysosomal and proteasomal degradation processes **(B)**, 100 nM of BafilomycinA1 (Yoshimori et al., 1991) and 7.5 μM of MG-132 (McCulley and Ratner, 2012) were added at 5 and 16 h post-transfection, respectively. WT, pEF-Fvpx; ΔVpx, pEF-FxSt.

and presence of MG-132 (**Figure 1B**). These results showed that neither of these mutants could be rescued with MG-132, suggesting no involvement of the PPM in the proteasome-mediated degradation. Similarly, a lysosome inhibitor Bafilomycin A1 (Yoshimori et al., 1991) did not affect much the level of 103/4A and 106/4A in transfected 293T cells, although a small increase was observed for both mutants (**Figure 1B**). These results suggested that the low expression level of these PPM mutants may not be attributable to the lysosomal degradation.

Proteasomal degradation is generally triggered by the polyubiquitin modification of lysine residues in a protein. There are three lysines in the Vpx of HIV-2 GL-AN clone (Khamsri et al., 2006) (**Figure 1A**). We generated several clones carrying mutations in these residues. Furthermore, we focused on the 76th glutamine residue (**Figure 1A**). This amino acid has been reported to interact with DCAF1 for formation of Cullin4-based E3 ubiquitin ligase complex to degrade an anti-HIV restriction factor SAMHD1 (Hrecka et al., 2011; Laguette et al., 2011) by proteasome (Le Rouzic et al., 2007; Srivastava et al., 2008). Mutants K68R, K77R, K84R, and Q76A with or without the 106/4A mutation were constructed as described previously (Miyake et al., 2014) (**Figure 1A**), and examined for their expression in transfected cells (**Figures 1C,D**). As shown in **Figure 1C**, only one clone with K84R and 106/4A mutations showed a slight enhancement in agreement with a previous report (Srivastava et al., 2008). Moreover, no significant effect was observed for a mutant carrying Q76A and 106/4A mutations (**Figure 1D**). These results also suggested that PPM may not be associated with the proteasome-mediated degradation.

In total, proteasomal or lysosomal degradation does not account for the extremely low expression level of Vpx exhibited by the PPM mutants. This is consistent with our previous conclusion that PPM is critical for efficient translation of Vpx (Miyake et al., 2014). Molecular mechanism by which PPM enhances Vpx translation to a remarkable extent needs to be determined.

## ACKNOWLEDGMENTS

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# Poly-proline motif in HIV-2 Vpx is critical for its efficient translation

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Human immunodeficiency virus type 2 (HIV-2) carries an accessory protein Vpx that is important for viral replication in natural target cells. In its C-terminal region, there is a highly conserved poly-proline motif (PPM) consisting of seven consecutive prolines, encoded in a poly-pyrimidine tract. We have previously shown that PPM is critical for Vpx expression and viral infectivity. To elucidate the molecular basis underlying this observation, we analysed the expression of Vpx proteins with various PPM mutations by *in vivo* and *in vitro* systems. We found that the number and position of consecutive prolines in PPM are important for Vpx expression, and demonstrated that PPM is essential for efficient Vpx translation. Furthermore, mutational analysis to synonymously disrupt the poly-pyrimidine tract suggested that the context of PPM amino acid sequences is required for efficient translation of Vpx. We similarly analysed HIV-1 and HIV-2 Vpr proteins structurally related to HIV-2 Vpx. Expression level of the two Vpr proteins lacking PPM was shown to be much lower relative to that of Vpx, and not meaningfully enhanced by introduction of PPM at the C terminus. Finally, we examined the Vpx of simian immunodeficiency virus from rhesus monkeys (SIVmac), which also has seven consecutive prolines, for PPM-dependent expression. A multi-substitution mutation in the PPM markedly reduced the expression level of SIVmac Vpx. Taken together, it can be concluded that the notable PPM sequence enhances the expression of Vpx proteins from viruses of the HIV-2/SIVmac group at the translational level.

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## INTRODUCTION

Primate immunodeficiency viruses carry a set of accessory proteins necessary for their optimal growth in host individuals (Blanco-Melo *et al.*, 2012; Harris *et al.*, 2012; Malim & Bieniasz, 2012; Zheng *et al.*, 2012). Extensive virological and molecular biological studies carried out so far have revealed that these auxiliary proteins profit the viruses mostly by antagonizing cellular antiviral restriction factors (Blanco-Melo *et al.*, 2012; Harris *et al.*, 2012; Malim & Bieniasz, 2012; Zheng *et al.*, 2012). One such viral protein, Vpx, is highly conserved among viruses of the human immunodeficiency virus type 2 (HIV-2) group, and plays a critical role in viral replication in different cell types (Fujita *et al.*, 2010). Vpx produced in cells is subsequently incorporated into progeny virions through a specific interaction of the putative third α-helix region (Jin *et al.*, 2001; Park & Sodroski, 1995) with the p6 domain of Gag (Accola *et al.*, 1999; Pancio & Ratner, 1998). The packaged Vpx then confers optimal infectivity on the virions in specific target cells such as macrophages and primary T-cells. Recently, it has been demonstrated that Vpx induces proteasomal degradation of host factors SAMHD1 (Hrecka *et al.*, 2011; Laguette *et al.*, 2011) and APOBEC3A (Berger *et al.*, 2011), relieving the restriction of virus infection.

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Vpx presumably has three major α-helices and unstructured amino/carboxy termini like its paralogue Vpr (Khamsri *et al.*, 2006; Mahnke *et al.*, 2006), another accessory protein known to be abundantly virion-associated. Despite this similarity, there is a notable poly-proline motif (PPM) near the C terminus of Vpx (seven consecutive prolines in the Vpx proteins of HIV-2; simian immunodeficiency virus from rhesus monkeys, SIVmac; and SIV from sooty mangabey monkeys, SIVsmm), which is not present in Vpr. We have previously generated a series of proviral HIV-2 mutant clones and performed systemic virological studies on Vpx using primary macrophage cultures and a T-lymphocyte cell line as infection targets (Fujita *et al.*, 2008a, b, 2010; Ueno *et al.*, 2003). Although all 19 point mutants, with mutations scattered throughout the *vpx* gene, produced virions containing Vpx at a comparable level to a WT clone upon transfection, many of them were found to be defective for virus growth in macrophages and/or T-cells. The defective replication step of these mutants was shown to be in the early phase (before/during viral DNA synthesis and/or its nuclear import) by extensive virological and molecular analyses (Fujita *et al.*, 2008a, b, 2010; Ueno *et al.*, 2003). In contrast to above, the other two multi-substitution mutants of the proline stretch designated 103/4A and 106/4A (Fig. 1a) failed to express Vpx upon transfection and produced progeny virions without detectable Vpx (Fujita *et al.*, 2008a, b). Consistently, the two mutant viruses were growth-defective both in macrophages and T-cells (Fujita *et al.*, 2008b). In particular, the 106/4A mutant virus behaved exactly like a ΔVpx virus in infection experiments (Table 1). Although severely impeded, the 103/4A mutant virus was still infectious for macrophages and T-cells (Table 1). Because the expression of 103/4A and 106/4A Vpx proteins was below the detection level of the system used (Table 1), the reason for the different growth abilities of the two viruses remained to be determined. Notably, it has been shown that a PPM-deletion mutant, if expressed to some extent, retains Vpx functionality in single-round infection experiments (Goujon *et al.*, 2008; Gramberg *et al.*, 2010).

In this study, we have focused on the role of the PPM in Vpx expression and analysed the underlying molecular basis. Expression plasmids of HIV-2 Vpx with the PPM mutations were constructed for quantitative comparison and utilized for protein expression analysis using various cellular and *in vitro* cell-free translation systems. Our results demonstrated that the PPM in HIV-2 Vpx is critical for its efficient expression in the eukaryotic as well as prokaryotic translation machineries. In addition, we found that this effect is determined by the context of PPM amino acid sequences, but not the nucleotide sequences. These data support the notion that the PPM plays an important role in enhancing the translational level of HIV-2 Vpx in infected cells, thereby conferring optimal replication ability on the virus in target cells.

## RESULTS

### PPM in Vpx is critical for its efficient expression in cells

We have previously shown that the expression of Vpx in PPM mutants carrying P103/4A or P106/4A is at an undetectable level both in cells and in progeny virions produced from transiently transfected cells (Table 1). However, while the 106/4A mutant virus exhibited a ΔVpx growth-like phenotype in lymphocytic HSC-F cells and no viral growth in macrophages, the 103/4A mutant virus grew better in both cell types than the ΔVpx virus (Table 1). These results led us to assume that the expression plasmid, pME18Neo-Fvpx, used in the study (Fujita *et al.*, 2008b) was unable to efficiently express the protein. Therefore, we have constructed a new expression plasmid based on pEF1/*myc*-HisA (pEF-Fvpx in Fig. 1b), and compared its ability with the old version (pME18Neo-Fvpx in Fig. 1b). As clearly observed in Fig. 1(b), pEF-Fvpx was much more efficient at producing Vpx than pME18Neo-Fvpx upon transfection.

A series of mutants based on pEF-Fvpx were then constructed (Fig. 1a, c, e) and examined for their expression. First, we monitored the expression level of the 103/4A and 106/4A mutants to see if there is a significant difference that can account for the distinct growth phenotype of viruses carrying these mutations (Table 1). As shown in Fig. 1(c), only a faint amount of Vpx was detected for the 106/4A mutant and a deletion mutant lacking the entire PPM-coding region (d7P). Although considerably reduced relative to the WT clone, the 103/4A mutant clearly generated more Vpx than the 106/4A mutant. This result correlated well with the growth potentials of the WT, 103/4A, and 106/4A viruses (Table 1). Next, we determined the effect of the number and position of the alanine substitutions in PPM on Vpx expression (Fig. 1d). A single substitution of proline with alanine did not cause major reductions except for P106A (approximately 50 % of the WT level). Double and triple alanine substitutions gave distinct results. While the P104/2A, P103/3A and P105/3A mutations did not have a significant effect, the expression level of P107/3A relative to that of WT markedly decreased (similar to the P103/4A level). The P106/2A and P108/2A mutants expressed Vpx at a slightly reduced level as observed for P106A. The results in Fig. 1(c, d) showed that the number and position of consecutive prolines in PPM are important for Vpx expression. In addition to the PPM mutations, we analysed the mutational effect of the glycine-rich domain (GRD), which is a presumably flexible region just upstream of PPM (Fig. 1a). In general, poly-proline sequences form a rigid structure whereas glycine repeats provide flexibility. Therefore, we speculated that the GRD may affect the ability of PPM to enhance Vpx expression. However, the introduction of alanine substitutions into the GRD showed no appreciable effects (Fig. 1e).

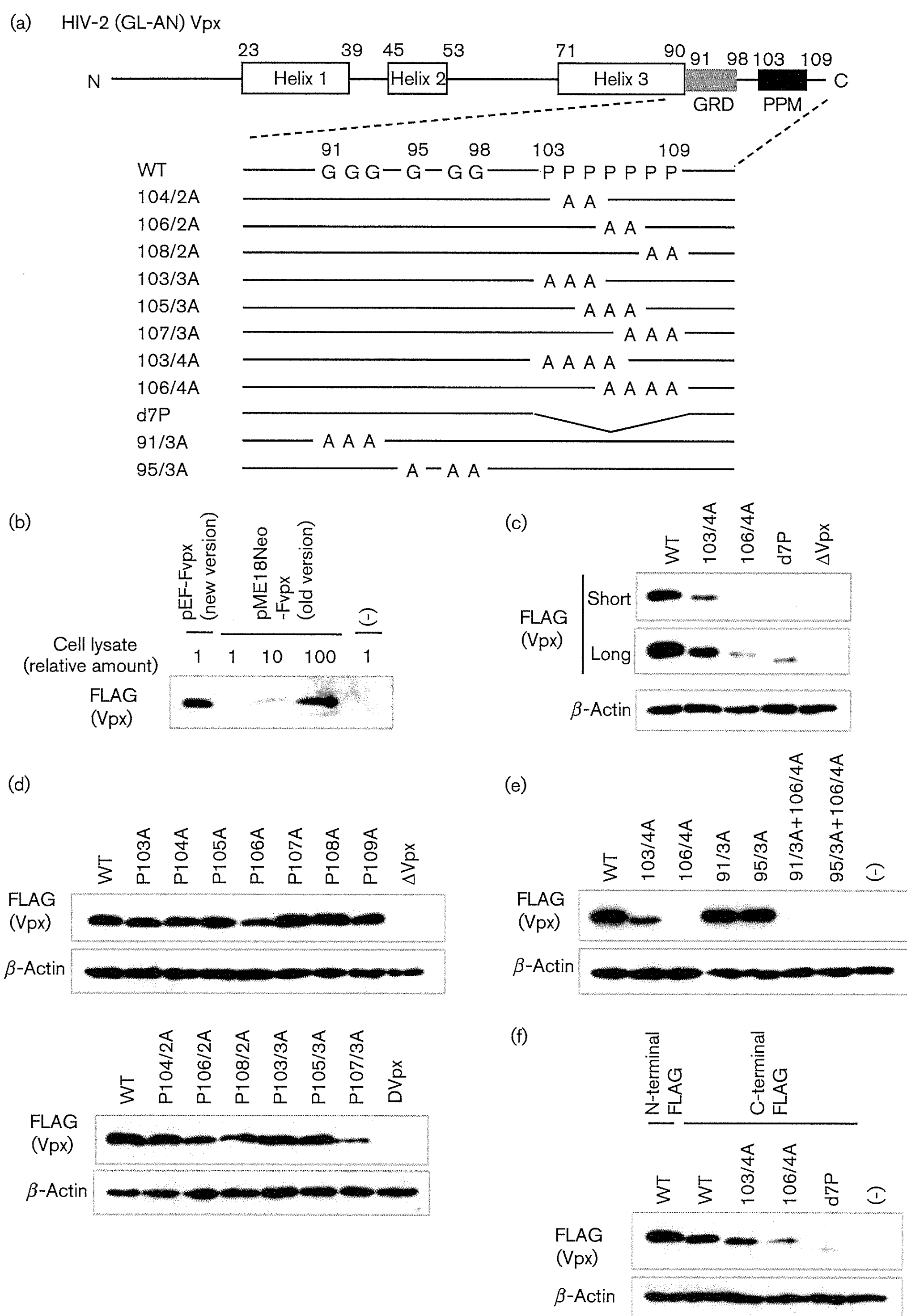


**Fig. 1.** Expression profiles of various Vpx–PPM mutants in transfected 293T cells. (a) A scheme of the domain structure and sequences of HIV-2 GL-AN Vpx (112 amino acids) and its mutants. Expression plasmids with N-terminal (pEF-Fvpx series)/C-terminal (pEF-vpxF series) FLAG were constructed in this study. Numbers indicate the positions of amino acid residues in the HIV-2 Vpx. GRD, glycine-rich domain; PPM, poly-proline motif. (b) Expression of Vpx from two expression plasmids designated pEF-Fvpx (this study) and pME18Neo-Fvpx (Fujita *et al.*, 2008a, b; Khamsri *et al.*, 2006). Relative amount of cell lysates used for Western blotting is indicated. (c) Expression of Vpx–PPM mutants carrying four successive alanine substitutions or a deletion. Short, short exposure; long, long exposure. (d) Expression of Vpx–PPM mutants carrying a single alanine substitution (upper) or two/three alanine substitutions (lower). (e) Expression of Vpx–GRD mutants with or without the 106/4A mutation. (f) Expression of Vpx–PPM mutants with a C-terminal FLAG tag. (-), pEF1/*myc*-HisA; WT, pEF-Fvpx or pEF-vpxF; ΔVpx, pEF-FxSt.

**Table 1.** Effect of mutations in HIV-2 GL-AN Vpx on its expression level and viral replication ability

Results obtained in 293T cells, lymphocytic HSC-F cells, and monocyte-derived macrophage (MDM) cultures (Fujita *et al.*, 2008a, b) are summarized.

| Clone | Mutation* | Vpx expression† | | Viral replication‡ | |
|---|---|---|---|---|---|
| | | Cells | Virions | HSC-F | MDM |
| GL-AN | None (WT) | WT | WT | WT | WT |
| GL-St | ΔVpx | UD | UD | ΔVpx | UD |
| GL-xP103A | P103A | WT | ND | WT | WT |
| GL-x103/4A | P103/4A | UD | UD | M | M |
| GL-x106/4A | P106/4A | UD | UD | ΔVpx | UD |
| GL-xP109A | P109A | WT | ND | WT | M |

*ΔVpx, a frame-shift mutation in the *vpx* gene (Kawamura *et al.*, 1994); see Fig. 1 for P103A, P103/4A, P106/4A and P109A mutations.
†WT, wt level expression; UD, undetectable; ND, not done. Vpx proteins in transfected 293T cells (cells) and in virions prepared from transfected 293T cells (virions) were monitored. Vpx in cells was examined by using proviral clones and/or FLAG-tagged Vpx-expression vectors.
‡WT, similar replication to wt virus; ΔVpx, similar replication to GL-St virus; UD, undetectable; M, medium replication phenotype between WT and GL-St viruses.

Although our data here on the 103/4A and 106/4A mutants were consistent with the viral growth properties (Table 1), we asked whether there is a positional effect of the FLAG tag on the Vpx expression. Expression plasmids with a C-terminal FLAG tag based on pEF1/*myc*-HisA (pEF-vpxF constructs: WT, 103/4A, 106/4A and d7P) were constructed, and their ability to express Vpx upon transfection was analysed. As shown in Fig. 1(f), the data obtained were quite similar to those in N-terminal FLAG-tagged vectors. However, the difference in the expression level between clones appeared to be smaller (Fig. 1c, f). This might result from the adjacent effect of the C-terminal FLAG tag on PPM. We used N-terminal tagged versions (pEF-Fvpx clones) thereafter.

## PPM facilitates translation of Vpx in a nucleotide sequence-independent manner

The results presented so far indicated that PPM is important for Vpx expression in cells. To further understand the mechanism underlying this observation, we compared the transcription and translation efficiencies of WT and PPM mutants (Fig. 2). We firstly measured mRNA levels in cells transfected with WT or three PPM mutants (103/4A, 106/4A and d7P). Total RNA was extracted from cells and relative *vpx* mRNA level was quantified by the real-time reverse-transcription-PCR (RT-PCR) method. As shown in Fig. 2(a), mutations in PPM did not significantly change the steady-state level of each mRNA in transfected cells. In agreement with this observation, the *in vitro* transcription assay gave similar results (Fig. 2a). However, when the Vpx proteins were synthesized by an *in vitro* transcription/translation system using rabbit reticulocyte lysates, the three PPM mutants were scarcely produced (Fig. 2b). In parallel with the data obtained in transfected cells, the amount of synthesized 103/4A was confirmed to be higher than that of 106/4A in independently repeated experiments (data not shown). Furthermore, we compared the translation efficiency of WT and 106/4A clones by an *in vitro* transcription/translation system using *Escherichia coli* S30 lysates. As seen in Fig. 2(b), the PPM mutation almost abrogated the translation of Vpx even in the bacterial system.

Then, we asked whether the effect of PPM on Vpx translation is linked to the unique secondary structure and/or poly-pyrimidine tract of mRNA around the PPM-coding region (Fig. 3a). At first, mutant plasmids carrying a stop codon just upstream of PPM (G102St and +103St) were constructed (Fig. 3a), and the expression of these mutant proteins was examined in transfected cells as well as in the cell-free system using rabbit reticulocyte lysates. The truncated mutants, G102St and +103St, migrated faster than WT Vpx and were expressed at a much lower level (Fig. 3b). This was also observed in the cell-free system (Fig. 3b). These results suggested that the amino acid sequences of PPM, but not the context of the RNA sequence, are essential for efficient translation of Vpx. Moreover, we constructed various clones with synonymous mutations (106/3ccg, 106/3cca, 105ccg, 106ccg, 107ccg and 104,106ccg) that potentially disrupt the poly-pyrimidine tract (Fig. 3a), and examined their expression levels in transfected cells and in the cell-free system. As shown in Fig. 3(c), the synonymous mutants were expressed as efficiently as WT Vpx. These data also indicated that the role of Vpx PPM is primarily determined by the context of the amino acid sequences, but not by that of nucleotide sequences. Taken together (Figs 2 and 3), our findings showed that the consecutive proline residues of PPM play an essential role in efficient translation of HIV-2 Vpx both in the eukaryotic and prokaryotic systems.

## PPM of HIV-2 Vpx does not have a major effect on the expression level of HIV Vpr proteins

Vpx shares many properties with Vpr including virion-association, putative three-dimensional structure, and biological activities (Fujita *et al.*, 2010). However, no PPM is present in HIV-1 and HIV-2 Vpr proteins (Khamsri *et al.*, 2006). In addition, the stoichiometry of Vpx in the virion is much higher than that of Vpr (Singh *et al.*, 2000). Approximately 4000 Vpx are estimated to be packaged in one virion, while only 14–18 HIV-1 Vpr are encapsidated. In accordance with this observation, it has been previously reported that the expression level of HIV Vpr proteins in cells is low relative to that of HIV-2 Vpx as monitored by tagged

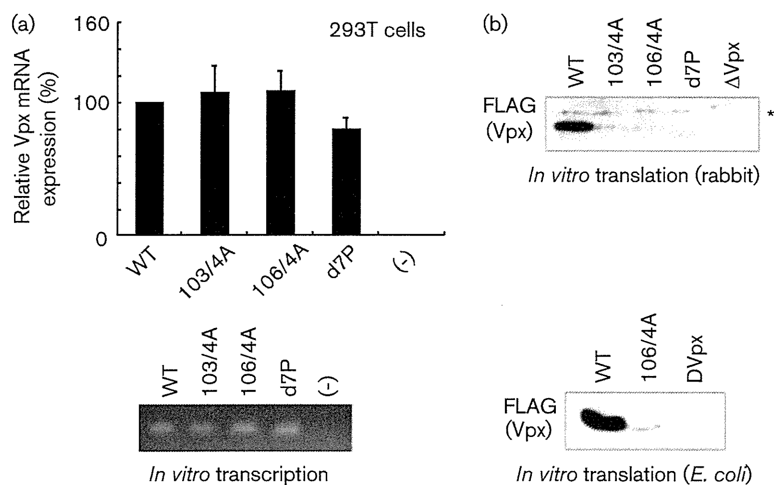


**Fig. 2.** Effect of PPM mutations on the expression of Vpx mRNA and protein. (a) Upper: relative amounts of Vpx mRNAs in transfected 293T cells. Total RNA was extracted from cells transfected with the expression plasmids indicated at 24 h post-transfection, and subjected to quantitative real-time RT-PCR analysis. Relative copy numbers are shown. (-), pEF1/*myc*-HisA. Lower: amounts of *in vitro* transcribed mRNAs for Vpx. (-), pEF1/*myc*-HisA. (b) Expression of Vpx–PPM mutants by an *in vitro* transcription/translation system using rabbit reticulocyte lysates (upper) or *E. coli* S30 lysates (lower). *, Non-specific bands; WT, pEF-Fvpx or pET-Fvpx; ΔVpx, pEF-FxSt or pET-FxSt.

expression plasmids/tagged proviral clones (Goujon *et al.*, 2008; Gramberg *et al.*, 2010; Khamsri *et al.*, 2006). Based on these results, we speculated that the addition of the HIV-2 Vpx PPM onto HIV-1/HIV-2 Vpr might enhance their expression.

We firstly compared expression levels of mRNA and protein for Vpx and Vpr. The mRNA levels for HIV-1 and HIV-2 Vpr proteins relative to that for HIV-2 Vpx in transiently transfected cells were measured by quantitative RT-PCR. As shown in Fig. 4(a), both Vpr mRNAs, HIV-1 Vpr in particular, were expressed to a lesser extent relative to Vpx mRNA. However, no major difference was noticed for Vpx and Vpr RNAs synthesized *in vitro* (Fig. 4a), probably due to T7 RNA polymerase in the reaction. When the protein expression levels were compared, more drastic results were obtained. HIV-1 and HIV-2 Vpr proteins were scarcely detectable in transfected cells and in the cell-free system (Fig. 4b), in contrast to Vpx. These results suggested that both transcription and translation processes are inefficient for Vpr expression. We then tested whether the PPM augments expression levels of HIV-1 and HIV-2 Vpr proteins by addition of the C-terminal flexible region of HIV-2 Vpx containing the PPM (Vpr1/Vpx and Vpr2/Vpx in Fig. 4c). In transfected cells, both Vpr1/Vpx and Vpr2/Vpx exhibited slightly higher expression relative to parental Vpr1 and Vpr2 clones, respectively (Fig. 4c). However, their expression levels obtained by adding the PPM were still much lower than that of Vpx. In addition, the *in vitro* transcription/translation analysis by rabbit reticulocyte lysates also gave little effect of the substitution with C-terminal flexible region on the translation efficiency (data not shown). These results showed that the addition of the Vpx PPM does not cause a major effect on the expression level of HIV Vpr proteins *in vivo* and *in vitro*.

## SIVmac Vpx has PPM consisting of a hepta-proline stretch and its expression is PPM-dependent

For detailed analysis of Vpx and PPM-containing Vpr proteins, we generated a phylogenetic tree of various Vpx/Vpr proteins using SIVsyk (SIV from Sykes' monkeys) Vpr (without PPM) as a reference (Fig. 5). The Vpr of SIV from African green monkeys (SIVagm) has been suggested as an origin of Vpx (Sharp *et al.*, 1996). Notably, the Vpr of SIVagm clone GRI1677 has a PPM composed of five consecutive prolines, and its expression level is markedly reduced as a result of PPM-deletion (data not shown). The PPM (four consecutive prolines) of SIVmnd2 Vpx is located at a relatively similar position (106th to 109th proline) to our Vpx clone (HIV2 GL-AN in Fig. 5). Substitution mutations in this region (P106/4A) almost abolished Vpx expression (Fig. 1). Among various Vpr/Vpx proteins in Fig. 5, other than HIV-2 Vpx, Vpx proteins of SIV from drills (SIVdrl), SIVsmm and SIVmac have seven consecutive prolines.

Based on the results summarized above, we asked whether the P106/4A mutation in the Vpx–PPM of SIVmac gives an effect similar to that observed for HIV-2 Vpx (Fig. 1). As shown in Fig. 6(a), the sequence homology between the two proteins is quite high, the N-terminal half in particular, and the PPM is conserved as described above. Unexpectedly, the amount of SIVmac Vpx produced upon transfection was found to be significantly lower relative to that of HIV-2 Vpx (Fig. 6b). However, as clearly observed, the PPM mutant protein of SIVmac Vpx (106/4A) was expressed at a very reduced level relative to WT Vpx (Fig. 6b), indicating the presence of PPM-dependent regulation. We were interested in mapping the determinant(s) responsible for the different expression levels seen for HIV-2 and SIVmac Vpx proteins. Three chimeric expression plasmids were constructed, and monitored for their expression upon transfection (Fig. 6c). Since the three chimeric constructs expressed Vpx at a similarly low level to the WT SIVmac clone, the putative helix 1 in Vpx was considered to be the determinant. We substituted four amino acids in HIV-2 Vpx helix 1 with corresponding residues in the helix 1 of SIVmac Vpx (Fig. 6a, c). Expectedly, as is clear in Fig. 6(c), the mutant with the four substitutions (GL-D26N/I29V/A31E/L32I) and the WT SIVmac clone produced Vpx at a similarly low level upon

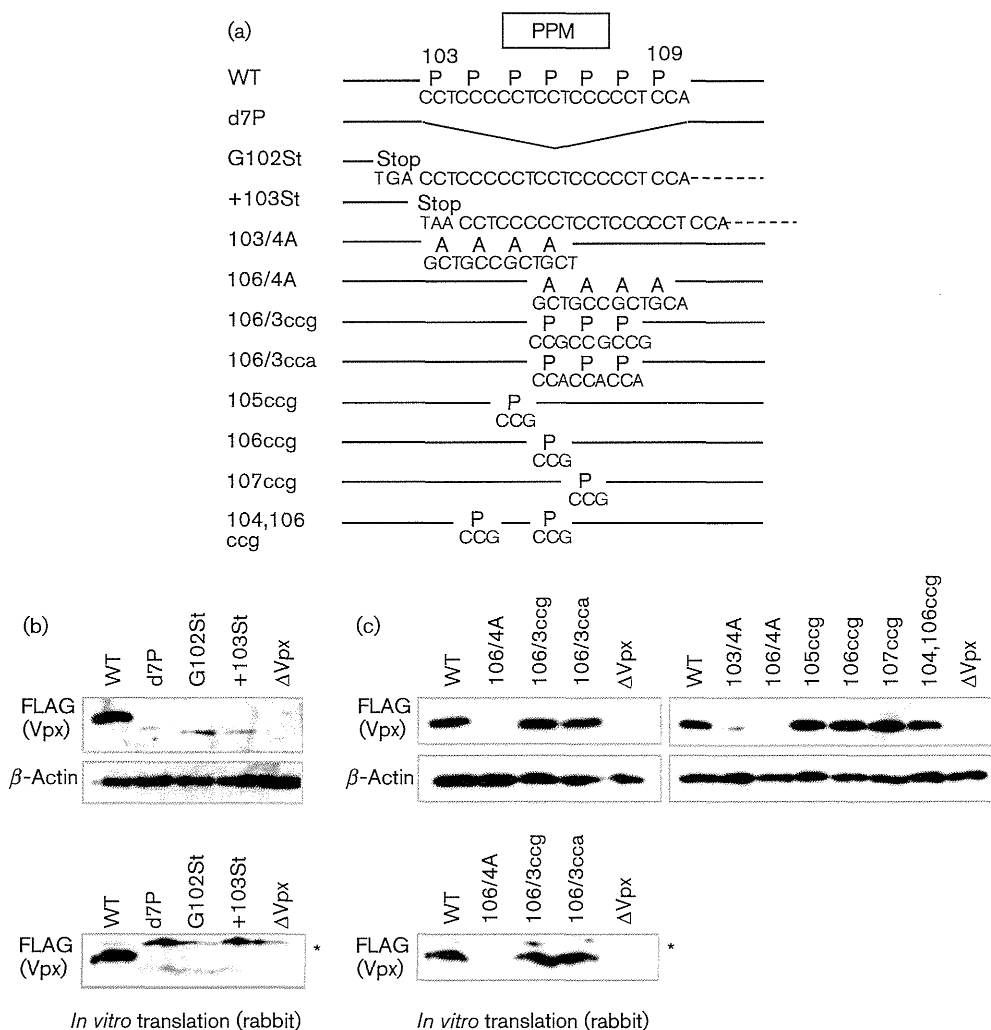


**Fig. 3.** Effect of poly-pyrimidine tract mutations on Vpx expression. (a) Sequences of the PPM region of WT and mutant clones. Red letters indicate nucleotides changed by mutagenesis. (b, c) Expression profiles of various Vpx–PPM mutants. Samples were prepared from transfected 293T cells (upper) or by an *in vitro* transcription/translation system using rabbit reticulocyte lysates (lower). *, Non-specific bands; WT, pEF-Fvpx; ΔVpx, pEF-FxSt.

transfection. Our results described above showed that the PPM-function itself, i.e., enhancing the Vpx expression level, is maintained in the HIV-2/SIVmac group.

## DISCUSSION

One of the most prominent features for Vpx proteins of the HIV-2/SIVsmm/SIVmac group is a highly conserved PPM consisting of a hepta-proline stretch in the C-terminal region (Fig. 5). Our previous studies showed that the PPM in HIV-2 Vpx is required for Vpx expression in cells and virions (Fujita *et al.*, 2008a, b). To gain mechanistic insights into the PPM-dependent Vpx expression, we performed a systemic mutational analysis. We found that each proline residue in PPM is not equally important for Vpx expression, but that the number and position of consecutive proline residues are critical (Fig. 1). Our data showed that at least four consecutive prolines are needed to impose a clear PPM-dependency on Vpx expression. Three (or perhaps two) consecutive prolines were effective if located at the C-terminal half of PPM. Quantitative real-time RT-PCR and *in vitro* transcription/translation assays revealed that the PPM is essential for efficient translation of Vpx in both the eukaryotic and prokaryotic systems (Fig. 2). Moreover, we showed that the stretch of PPM amino acid sequence, but not the nucleotide context, is required for enhancing translation (Fig. 3).

Our data on the expression level of Vpx–PPM mutants in cells (Fig. 1) were well correlated with the ability of mutant viruses to grow in primary macrophages and lymphocytic HSC-F cells (Table 1). While mutant viruses with ability to produce Vpx at a normal level (P103A and P109A) grew comparably

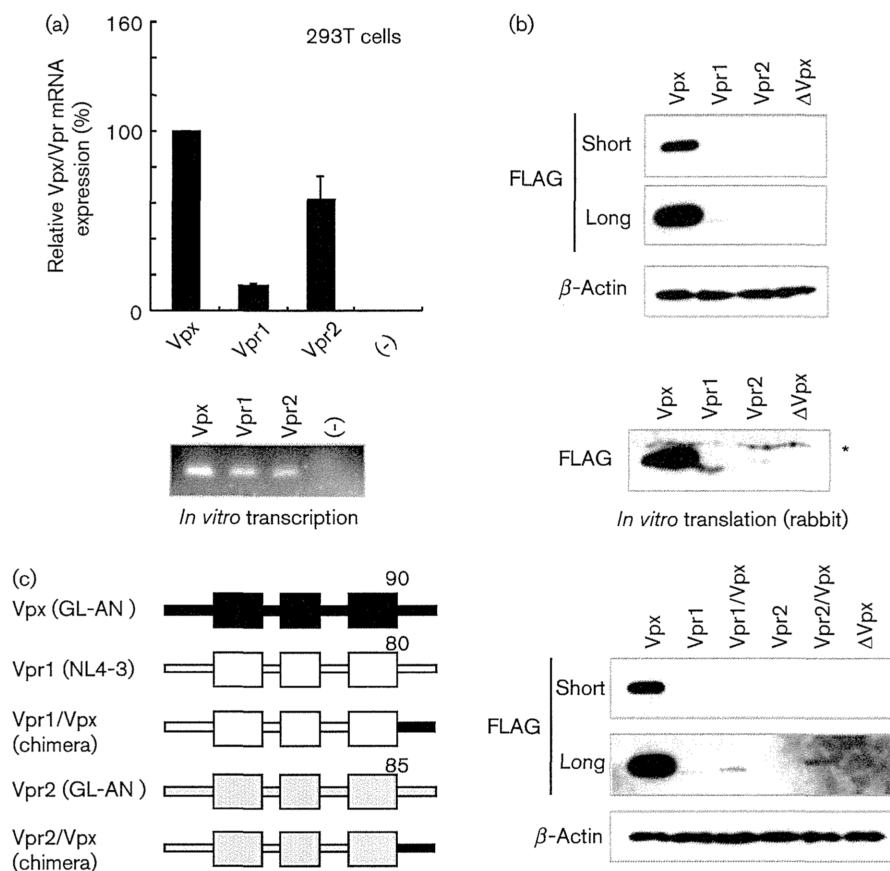


**Fig. 4.** Expression profiles of HIV Vpx/Vpr mRNAs and proteins. (a) Upper: expression level of Vpx/Vpr mRNAs in transfected 293T cells. Cells were transfected with the expression plasmids for HIV-2 Vpx (Vpx), HIV-1 Vpr (Vpr1) or HIV-2 Vpr (Vpr2). Total RNA was extracted at 24 h post-transfection, and subjected to quantitative real-time RT-PCR analysis with a primer set for the 3′ untranslated region of pEF1/*myc*-HisA-based vectors. Relative copy numbers are shown. (-), pEF1/*myc*-HisA. Lower: *in vitro* transcription by T7 RNA polymerase. Linearized plasmids were used as DNA templates for this assay. (-), pEF1/*myc*-HisA. (b) Upper: expression of Vpx and HIV-1/HIV-2 Vpr proteins in transfected 293T cells. Lower: expression of Vpx and HIV-1/HIV-2 Vpr proteins by an *in vitro* transcription/translation system using rabbit reticulocyte lysates. *, Non-specific bands. (c) Left: schematic structure of chimeric proteins between HIV-2 Vpx (black), HIV-1 Vpr (white), and HIV-2 Vpr (grey) proteins. Numbers indicate amino acid positions at the end of the putative third $\alpha$-helix. Right: expression of Vpx/Vpr chimeric proteins in transfected 293T cells. Short, Short exposure; long, long exposure; ΔVpx, pEF-FxSt.

with parental WT virus in both cell types, a mutant (103/4A), which expresses a small amount of Vpx, grew very poorly in those cells (Fig. 1, Table 1). A mutant (106/4A), which expresses a negligible amount of Vpx, was unable to grow in macrophages and grew similarly poorly to the ΔVpx mutant virus in HSC-F cells (Fig. 1, Table 1). These results suggested that the PPM is critical for Vpx expression but not for its activity. Functionality, i.e. the potential to confer infectivity on virions, of a PPM-deletion mutant and of a Vpx/Vpr chimeric clone lacking the PPM support this conclusion (Goujon *et al.*, 2008; Gramberg *et al.*, 2010).

Very recently, it has been reported that translation elongation factor P (EF-P) is linked to the adjustment of translational efficiency for poly-proline-containing proteins in the bacterial system (Doerfel *et al.*, 2013; Ude *et al.*, 2013). During the translation, poly-proline stretch sequences tend to induce ribosome stalling, which is likely to be rescued by the EF-P (Doerfel *et al.*, 2013; Ude *et al.*, 2013). It has been reported that eIF5A, like its orthologue EF-P in the bacterial system, promotes translation of PPM-containing proteins in the yeast system (Gutierrez *et al.*, 2013). These results demonstrate the suppressive effect of poly-proline sequences on translation in cells. In contrast, our present study showed that the PPM of HIV-2 Vpx contributes to the enhancement of Vpx translation (Figs 2 and 3) and that the translational enhancement of Vpx occurs in both prokaryotic and eukaryotic machineries. How can we rationalize such opposite effects of poly-proline sequences on translation? At this moment, we do not have the answer but might assume that the HIV-2 PPM could hijack the functions of EF-P and/or eIF5A, which are the factors that stimulate the peptidyltransferase activity of the ribosome. Otherwise,

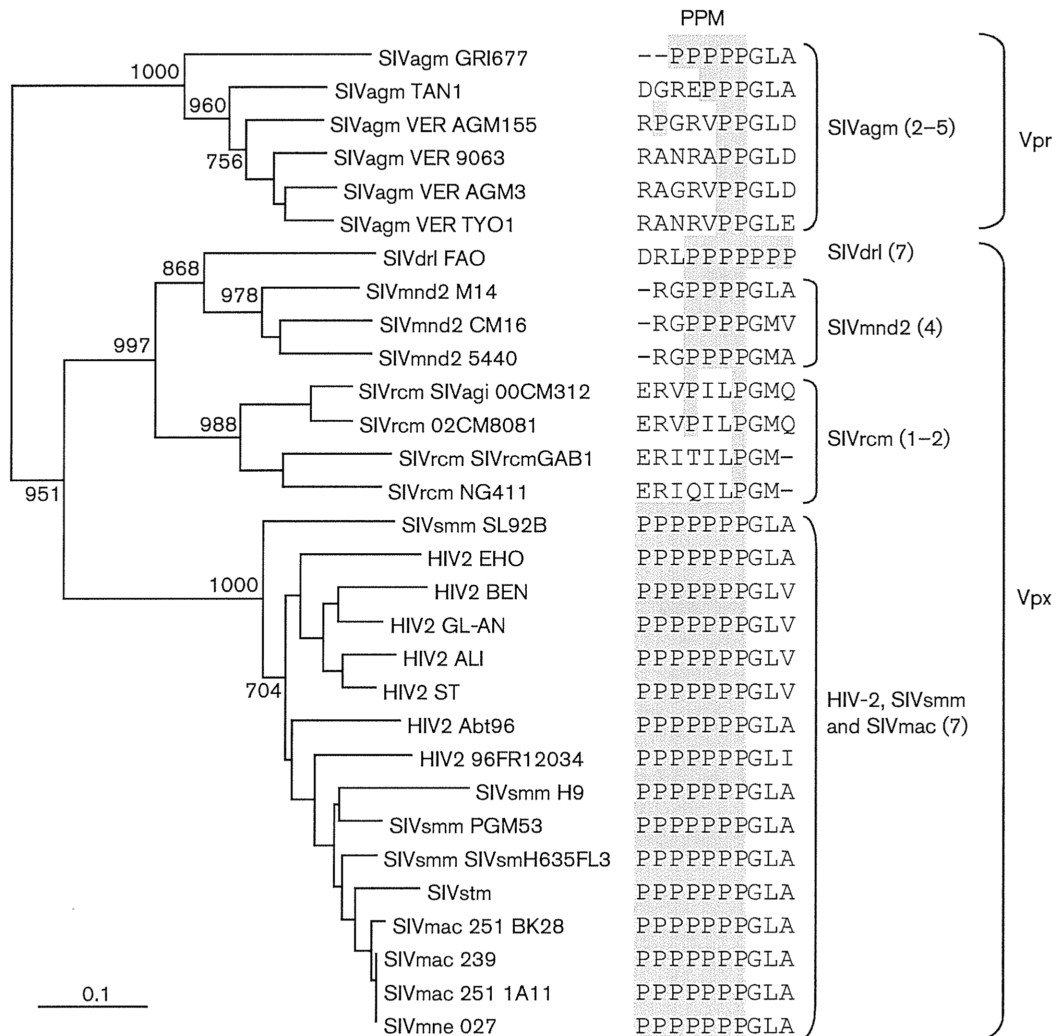


**Fig. 5.** Phylogenetic analysis of various Vpx and Vpr proteins. Phylogenetic tree was generated by the neighbour-joining method. The genetic distance corresponding to the lengths of branches is shown by the scale bar. Branches were calculated from 1000 bootstrap replicates, and the bootstrap values are labelled on the major branches. Amino acid sequences of the PPM region and numbers of proline residues in parentheses are shown on the right.

an additional unidentified factor(s) important for PPM-mediated protein expression may exist in cells. Moreover, of note, PPM alone did not enhance the synthesis of Vpr proteins in our present study (Fig. 4). Consistently, Vpr engineered to have the C-terminal flexible region of Vpx exhibited inefficient expression in cells, indicating that the PPM alone is insufficient for promoting protein translation (Gramberg *et al.*, 2010). There may be a region(s) and/or amino acids in Vpx other than the PPM sequence important for PPM-dependent efficient translation of Vpx. The putative helix 1 in Vpx was shown to be important for fixing its expression level in cells, but the effect of helix 1 appeared to be independent of the PPM-regulation (Fig. 6). Further study is required to elucidate the molecular mechanism for the PPM-dependent translation enhancement of Vpx.

The PPM sequence is found in a large number of prokaryotic and eukaryotic proteins (UniProt Knowledgebase, http://www.uniprot.org). As expected, a wide range of human DNA and RNA viruses encode PPM (seven or more consecutive prolines)-containing proteins. Examples include adenoviruses, herpesviruses and hepatitis viruses. However, whether these PPMs are responsible for efficient expression of the PPM-containing proteins is as yet undetermined. Extensive studies on these proteins remain to be performed to have a general picture of PPM-mediated protein expression. This paper is the first report, to the best of our knowledge, that describes and demonstrates the PPM-dependent efficient translation of animal virus proteins. Furthermore, we have shown the minimal requirements constituting a 'functional PPM' as described above (Figs 1 and 4). In HIV/SIVs, PPM sequence is also intriguing from an evolutional point of view

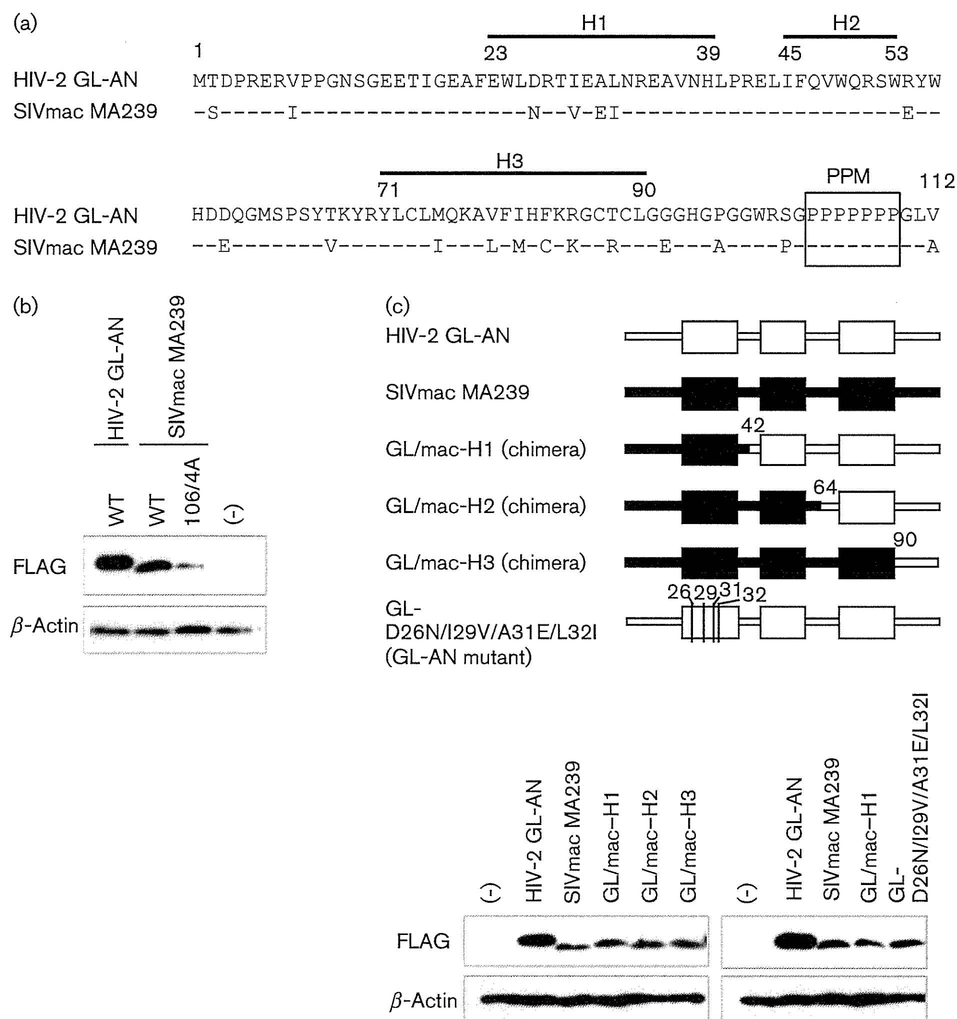


**Fig. 6.** Expression profiles of various SIVmac and HIV-2 Vpx proteins. (a) Sequence alignment of HIV-2 GL-AN (Kawamura *et al.*, 1994) and SIVmac MA239 (Shibata *et al.*, 1991) Vpx proteins. Numbers indicate the positions of amino acid residues in Vpx. Different amino acids in the putative first α-helix (H1) of HIV-2 GL-AN and SIVmac MA239 Vpx proteins are shaded. (b) Expression of an SIVmac Vpx–PPM mutant carrying four successive alanine substitutions at amino acids 106–109 (P106/4A) in transfected 293T cells. (c) Upper: schematic structure of chimeric proteins between HIV-2 Vpx (white) and SIVmac Vpx (black) proteins. A clone with four mutations relative to HIV-2 GL-AN Vpx is also shown. Numbers indicate the positions of amino acid residues. Lower: expression of HIV-2/SIVmac chimeric Vpx proteins and an HIV-2 mutant Vpx protein in transfected 293T cells. (-), pEF1/*myc*-HisA.

(Fig. 5). HIV-1/HIV-2 Vpr proteins without a PPM were found to be expressed at an extremely low level relative to HIV-2 Vpx (Fig. 4). While the PPM-dependent enhancement was also true for the expression of SIVmac Vpx (Fig. 6) and SIVagm Vpr (our unpublished data), some SIV Vpx/Vpr proteins lack a typical PPM sequence (three, four or more consecutive prolines) (Fig. 5). Although many of the Vpx/Vpr proteins in Fig. 5 are unanalysed to date for the function and expression, it is not unreasonable to assume that each virus in Fig. 5, during its persistent infection in its natural host, has acquired appropriate Vpx and/or Vpr for optimal viral replication and maybe for viral persistence/spread. It would be, therefore, of interest to perform functional and virological studies on the Vpx/Vpr proteins with/without PPM derived from a variety of HIV/SIVs. Studies in this direction are in progress in our laboratory.

## METHODS

**Plasmids.** Expression plasmids for HIV-2 Vpx (GL-AN clone) (Kawamura *et al.*, 1994) with an N-terminal FLAG tag designated pME18Neo-Fvpx and its mutant derivatives have been previously described (Fujita *et al.*, 2008a, b; Khamsri *et al.*, 2006). New plasmids for various Vpx proteins with an N-terminal FLAG, pEF-F series, were constructed by introduction of an appropriate *vpx* gene fragment into pEF1/*myc*-HisA (Life Technologies). The resultant plasmids for WT and its frame-shift mutant (ΔVpx) were designated pEF-Fvpx and pEF-FxSt, respectively. Expression plasmids for HIV-1 Vpr (NL4-3 clone) (Adachi *et al.*, 1986) and HIV-2 Vpr (GL-AN),

designated pEF-Fvpr1 and pEF-Fvpr2, respectively, were constructed by replacement of the *vpx* gene in pEF-Fvpx with each *vpr* gene. Expression plasmids for Vpx/Vpr chimeras were generated by PCR-based mutagenesis using pEF-Fvpx, pEF-Fvpr1 and pEF-Fvpr2. To construct an expression plasmid for HIV-2 Vpx with a C-terminal FLAG tag, the *gag* gene of pSG-Gag cFLAG (Anraku *et al.*, 2010) was swapped with the *vpx* gene of pEF-Fvpx, and both the *vpx*-cFLAG portion and Kozak consensus sequence at the 5′ untranslated region were inserted into pEF1/*myc*-HisA (Life Technologies). The resultant plasmid was designated pEF-vpxF and used for expression of HIV-2 Vpx with a C-terminal FLAG tag. Various mutant clones were constructed from pEF-vpxF by PCR-based mutagenesis. An expression plasmid for SIVmac Vpx with an N-terminal FLAG tag was constructed by replacement of the *vpx* gene in pEF-Fvpx with SIVmac *vpx* gene (MA239 clone) (Shibata *et al.*, 1991), and designated pEF-Fvpx-SIVmac. Expression plasmids for an SIVmac Vpx mutant and HIV-2/SIVmac Vpx chimeras were generated by PCR-based mutagenesis using pEF-Fvpx and pEF-Fvpx-SIVmac. For *in vitro* transcription/translation analysis by *E. coli* S30 lysates, each *vpx* gene was inserted into pET-21b(+) (Novagen) to express Vpx with a FLAG tag at the N terminus (designated pET-Fvpx, pET-Fx106/4A, and pET-FxSt).

**Transfection.** Human 293T cells (Lebkowski *et al.*, 1985) were maintained in MEM medium containing 10 % heat-inactivated FBS and used for transfection experiments. For transfection, 2.5 μg of each expression plasmid DNA was introduced into 293T cells by the calcium-phosphate coprecipitation method (Adachi *et al.*, 1986). Cells were harvested at 24 h post-transfection for Western blot and RT-PCR analyses.

**Western blotting.** Western blot analysis was performed as described previously (Fujita *et al.*, 2008a, b). Cells were lysed in buffer composed of 10 mM Tris/HCl (pH 7.5), 10 mM NaCl, 1 % NP-40 and 1 % protease inhibitor cocktail (Sigma). Lysates were centrifuged for 5 min at 12 000 r.p.m. at 4 °C and the supernatants were used as samples after normalization of total protein amounts by a DC protein assay (Bio-Rad). Samples were separated on 12.5 or 15 % SDS-PAGE and transferred onto PVDF membranes (Immobilon-P; Millipore). The membranes were probed with anti-FLAG M2 antibody (Sigma) or anti-β-actin AC-15 antibody (Sigma), and with HRP-conjugated secondary antibody. Immunoreactive proteins were visualized by chemiluminescence using ECL Plus Western blotting detection reagents (GE Healthcare Bio-Sciences). Experiments were repeated at least three times, and the representative results are shown.

***In vitro* RNA transcription.** *In vitro* transcription was conducted by T7 RNA polymerase (New England Biolabs) using linearized plasmid DNAs (cut with *Xba*I) as templates. Transcribed RNA was quenched by EDTA and denatured by incubating at 65 °C for 15 min in MOPS, 50 % formamide and 12 % formaldehyde as indicated in the manufacturer's instructions. Denatured RNA was then mixed with ethidium bromide, separated by 1.5 % agarose gel containing MOPS and 18 % formaldehyde, and visualized by ethidium bromide staining. Experiments were repeated three times, and the representative results are shown.

***In vitro* transcription/translation.** A TNT T7 Quick Coupled Transcription/Translation System using rabbit reticulocyte lysates and a S30 T7 High-Yield Protein Expression System (Promega) were used to monitor the Vpx/Vpr expression in eukaryotic and prokaryotic cell-free systems, respectively. *In vitro* reactions were conducted according to the manufacturer's instructions. *In vitro* translated proteins were analysed by Western blotting. Experiments were repeated at least three times, and the representative results are shown.

**Quantitative real-time RT-PCR.** 293T cells were transfected with various expression plasmids, and harvested 24 h later. Levels of Vpx/Vpr mRNA in transfected cells were determined by quantitative real-time RT-PCR. After washes with PBS, total RNA was extracted with an RNeasy Plus Mini kit (Qiagen), and cDNA was synthesized using SuperScript III (Invitrogen) using oligo(dT) as a primer. PCR was performed with an ABI7500 (Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems). Primer sets used were: 5′-GCCAGGAAACAGTGGAGA-3′ and 5′-GCTTGGTGACATCCCTTGGT-3′ for measurement of WT and mutant Vpx mRNAs; 5′-CTAGAGGGCCCTTCGAACAA-3′ and 5′-GCTGGCAACTAGAAGGCACA-3′ for simultaneous measurement of Vpx and Vpr mRNAs. For normalization, a primer set specific for the human *GAPDH* gene (5′-CACCACCATGGAGAAGGCTG-3′ and 5′-GCTGATGATCTTGAGGCTGTTGT-3′) was used. Values were calculated by the manufacturer's software. Standard curves were generated by amplifications of serially diluted cDNA samples. Experiments were repeated three times, and the mean values with standard deviations are presented.

**Phylogenetic analysis.** Phylogenetic analysis was performed for Vpx proteins of HIV-2/SIVs and Vpr proteins of the SIVagm group. PPM-minus SIVsyk Vpr was used as a reference. These amino acid sequences were obtained from the HIV sequence database at Los Alamos National Laboratory (http://www.hiv.lanl.gov) and aligned by the CLUSTAL_X 2.0.11 program (Jeanmougin *et al.*, 1998; Thompson *et al.*, 1997). Phylogenetic tree was generated by the neighbour-joining method using the CLUSTAL_X 2.0.11 program. The branch significance was analysed by bootstrap with 1000 replicates. The tree was visualized by the TreeView 1.6.6 program (Page, 1996) and the reference was manually removed.

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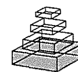

# Growth potentials of CCR5-tropic/CXCR4-tropic HIV-1mt clones in macaque cells

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Human immunodeficiency virus type 1 (HIV-1) is strictly adapted to humans, and cause AIDS only in humans. Consequently, no experimental animals susceptible to HIV-1 and suitable for the AIDS model study are available to date (Nomaguchi et al., 2008, 2011). To overcome this issue, viruses genetically related to HIV-1 have been challenged into macaque monkeys to mimic the natural HIV-1 infection. Viruses used for these experiments are simian immunodeficiency viruses (SIVs), SIVs chimeric with parts of HIV-1 sequences (SHIVs), and macaque tropic HIV-1 derivatives carrying a small portion of SIV genome (HIV-1mt clones). Because viruses of the SHIV and HIV-1mt groups carry HIV-1 genes/sequences, their scientific/medical significance and impact are evident. Although some SHIVs indeed induce AIDS in macaques, accumulating evidences have demonstrated that the genuine CCR5-tropism of input viruses is prerequisite for superimposing the experimental outcome on the natural disease progression in humans (Feinberg and Moore, 2002; Margolis and Shattock, 2006). Therefore, a number of CCR5-tropic SHIVs currently have been generated and utilized for *in vivo* macaque experiments (Hsu et al., 2003; Humbert et al., 2008; Nishimura et al., 2010; Fujita et al., 2012).

Recently, prototype HIV-1mt clones, CXCR4-tropic NL-DT5R, and dual-tropic (CXCR4- and CCR5-tropic) stHIV-1, have been generated by us (Kamada et al., 2006) and others (Hatziioannou et al., 2006), respectively. We selected three distinct Env sequences and made three proviral constructs in the backbone of the NL-DT5R genome to obtain CCR5-tropic/dual-tropic viruses (**Figure 1A**), based on the published results (Hsu et al., 2003; Hatziioannou et al., 2006; Matsuda et al., 2010; Nishimura et al., 2010). Of the three clones constructed, while NL-DT562 grew in a cynomolgus macaeque cell line HSC-F (Akari et al., 1996; Fujita et al., 2003), the other two viruses designated NL-DT589 and NL-DT5AD did not (Doi et al., 2010; our unpublished observations). The replication efficiency in HSC-F cells of NL-DT562 was much lower than that of the parental virus NL-DT5R (Doi et al., 2010). When examined in $CD8^+$ cell-depleted pig-tailed macaque peripheral blood mononuclear cells (PBMCs), NL-DT5AD was found to be replication-competent in addition to NL-DT562 (Igarashi and Adachi, unpublished results). However, NL-DT5AD grew more poorly than NL-DT562, and NL-DT562 itself propagated much more inefficiently again than NL-DT5R in these PBMCs. Of note, NL-DT562 was confirmed to use CCR5 for cell entry (our unpublished data). To improve the replication ability of NL-DT562, we extensively modified its genome by adaptation to macaque cells and also by *in vitro* mutagenesis (Nomaguchi et al., 2008, 2011, 2013a,b; Nomaguchi et al., submitted). As a result, the same mutations were introduced into the corresponding genomic regions of NL-DT5R and NL-DT562 encoding Gag-capsid, Pol-integrase, and Vpu-transmembrane domain. Numerous growth-enhancing adaptive mutations were found to separately occur in the Env of NL-DT562, but only one in the Env of NL-DT5R (Nomaguchi et al., 2013b). Since the enhancement of virus growth by these mutations is strictly Env sequence-dependent (Nomaguchi et al., 2013b), only a single best mutation for viral replication was introduced into the *env* gene of each clone. As shown in **Figure 1B**, the final version of CCR5-tropic virus currently constructed (MN5/LSDQgtu in **Figure 1A**) surely grew extremely better than NL-DT562 in a rhesus macaque cell line M1.3S (Doi et al., 2011), but more poorly relative to MN4/LSDQgtu (Nomaguchi et al., submitted) (**Figure 1A**), a CXCR4-tropic virus derived from NL-DT5R (a virus corresponding to MN5/LSDQgtu). Taken all together, we are unable yet to have a CCR5-tropic HIV-1mt clone that grows better or equally well in macaque cells relative to CXCR4-tropic MN4/LSDQgtu. Virological and molecular basis for this negative result is presently unknown, but it is certain that the Env sequence is important for viral growth potentials. Extensive search for appropriate Env sequences to confer CCR5-tropism and high replication-ability on HIV-1mt clones is required for our final purpose, i.e., the generation of proviral clones virologically similar to viruses of the SIVmac group that are pathogenic for macaques. In this regard, it is tempting to use "intracellular homologous recombination" as a measure to readily generate recombinant HIV-1 clones (Fujita et al., 2012).

Despite the every effort of researchers, so far, no appreciable disease was induced in pig-tailed and cynomolgus macaques infected with various HIV-1mt clones (Igarashi et al., 2007; Hatziioannou et al., 2009; Saito et al., 2011, 2013; Thippeshappa et al., 2011). Although the rhesus macaque is thought to be the best macaque species for infection experiments of this kind from various virological and primatological points of view, no attempts to infect it with HIV-1mt clones have been made to date, probably due to its highly resistant nature to the viruses. Common characteristics of the non-morbifical infections as described above are low viral loads relative to those in pathogenetic infections with SIV/SHIV/HIV-1 and no apparent viral set points in the course of infection. Without initial burst of viruses in hosts to guarantee viral amount and diversity enough for persistent infection, viruses may not survive in individuals/populations. Further improvement