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.

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 z-helices and unstructured amino/carboxy termini like its parologue 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 polyproline 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 impaired, 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. 1b, 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.
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 pEF1/6tag on the Vpx expression. 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 pEF1/6tag on the Vpx expression.

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 in both the eukaryotic and prokaryotic systems.

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

<table>
<thead>
<tr>
<th>Clone</th>
<th>Mutation*</th>
<th>Vpx expression†</th>
<th>Viral replication‡</th>
<th>Cells</th>
<th>Virions</th>
<th>HSC-F</th>
<th>MDM</th>
</tr>
</thead>
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<tr>
<td>GL-AN</td>
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<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>GL-St</td>
<td>ΔVpx</td>
<td>UD</td>
<td>UD</td>
<td>ΔVpx</td>
<td>UD</td>
<td>UD</td>
<td>UD</td>
</tr>
<tr>
<td>GL-xP103A</td>
<td>P103A</td>
<td>WT</td>
<td>ND</td>
<td>WT</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>GL-x103/4A</td>
<td>P103/4A</td>
<td>UD</td>
<td>M</td>
<td>M</td>
<td>M</td>
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<td>M</td>
</tr>
<tr>
<td>GL-x106/4A</td>
<td>P106/4A</td>
<td>UD</td>
<td>ΔVpx</td>
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<tr>
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<td>ND</td>
<td>WT</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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</table>

*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.

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 (Khamers 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 proteins.
expression plasmids/tagged proviral clones (Goujon et al., 2008; Gramberg et al., 2010; Khamisi 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 Vpx 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 SIVmm2 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 relative 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. 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.](image-url)
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

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**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.
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,
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. 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-Pvpx 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 pEF/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 C-terminal 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 XbaI) 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’-GCCAGGAAAACAGTGAGA-3’ and 5’-GCTTGGTGACATCCCTTTGGT-3’ for measurement of WT and mutant Vpx mRNAs; 5’-CTAGAGGGCCCCTCGAACAACA-3’ and 5’-GCTGCAACTAGAGAGCACACA-3’ for simultaneous measurement of Vpx and Vpr mRNAs. For normalization, a primer set specific for the human GAPDH gene (5’-CACACCATGAGAAGGCGT-3’ and 5’-GCTGATGACCTTGAGGCGTGT-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 SVvysk 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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