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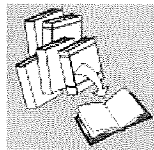
REVIEW



Multifaceted activity of HIV Vpr/Vpx proteins: the current view of their virological functions

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SUMMARY

Primate immunodeficiency viruses encode viral proteins that are uniquely auxiliary to their growth in host cells. Of these accessory proteins, those designated Vpr and Vpx are least well understood with respect to their functions in the viral replication cycle. Moreover, their assigned roles based on the results in published studies remain controversial. This review summarises current knowledge on human immunodeficiency virus (HIV) Vpr/Vpx proteins, and discusses their functional activities during the viral life cycle in macrophages and T lymphocytes, the two major target cells of HIV infection. Copyright © 2010 John Wiley & Sons, Ltd.

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INTRODUCTION

HIV type 1 (HIV-1) causes the acquired immunodeficiency syndrome (AIDS) pandemic, while type 2 (HIV-2) is found within the confines of West Africa and is believed to be less pathogenic for humans [1,2]. This difference may be due to the accessory proteins they encode, although experimental data supporting this possibility are not yet available. Both viruses have Vif, Vpr and Nef, but Vpu and Vpx are unique to HIV-1 and HIV-2, respectively (Figure 1). Extensive studies on these proteins have revealed that Vif and Vpu act as antagonist of cellular anti-viral factors. Vif degrades APOBEC proteins present in natural target cells, and plays a critical role in viral replication in those cells [3]. Vpu down-modulates tetherin in cells, making it important for virus release from cells, for virus spread among cells and for viral pathogenicity [4,5]. In addition, an enormous number of publications in a wide variety of research fields have clearly indicated that Nef acts and functions against acquired immunity in hosts and is crucial in AIDS pathogenesis [5,6].

In contrast with these three accessory proteins, the functional roles of HIV-1 Vpr and HIV-2 Vpr/Vpx in the viral life cycle are still unclear and controversial. First of all, Vpr proteins of HIV-1 and HIV-2 have been frequently reported and found to be unnecessary or dispensable for viral replication in any cell type, including monocyte-derived macrophages (MDMs) and primary T lymphocytes (see below). Although HIV-1 Vpr has been shown to have multiple functions (nuclear import of viral DNA, cell cycle arrest, regulation of apoptosis, transactivation of long terminal repeat (LTR) etc.) during the virus replication process [7], its dispensability has cast some doubt on the essential role of Vpr in viral growth, at least in cultured cells. Furthermore, whether the observed cytopathogenic activities of HIV-1 and HIV-2 Vpr proteins are directly related to viral pathogenesis also remains to be determined. HIV-2 Vpx, another viral protein of the HIV Vpr/Vpx lineage, is essential for virus growth in MDMs and has long been thought to be required for nuclear import of viral DNA [8] until very recently, when we and others have demonstrated that it is critical to the reverse

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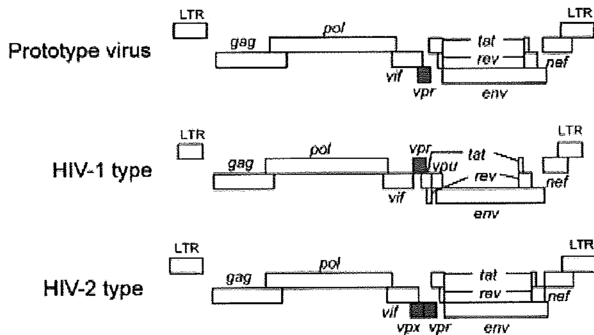

Figure 1. Proviral genome structure of primate immunodeficiency viruses. Genetic organisation of the three major viruses is schematically shown. *Vpr* and *vpx* genes are highlighted. SIVcpz (Chimpanzee), SIVgor (Gorilla), SIVgsn (Greater spot-nosed monkey), SIVmus (Mustached monkey) and SIVmon (Mona monkey) are categorised as the HIV-1 type. SIVsmm (Sooty mangabey), SIVmac (Rhesus monkey), SIVmnd-2 (Mandrill), SIVrcm (Red-capped mangabey) and SIVdrl (Drill) belong to the HIV-2 type. Many other SIVs are classified as the prototype

transcription step [9,10]. Consistently, it is also reported that Vpx is required for infection of monocyte-derived dendritic cells and that Vpx promotes accumulation of full-length viral DNA [11].

In this review, to define the present status of our virological understanding of HIV Vpr/Vpx proteins, we mainly focus on their biological functions. We summarise the most recent developments in the research field of Vpr/Vpx and discuss their functional roles in the HIV life cycle.

ORIGIN AND STRUCTURAL COMPARISON OF HIV VPR/VPX PROTEINS

Diverse primate species in Africa are naturally infected with various simian immunodeficiency viruses (SIVs). Interspecies transmission of viruses occurs among primate hosts. During infection and transmission, viral adaptation and recombination between different lineages of primate lentiviruses occurs, generating new viruses [5,12]. HIV-1 has been shown to originate from SIVcpz of chimpanzees [13] or SIVgor of gorillas [14] in central Africa by recent zoonotic transmission. Similarly, it was shown that HIV-2 came from SIVsmm of the sooty mangabey in West Africa [15]. Evolution of SIV/HIVs has been dynamic and complex, but their genomic organisation is classified into only three types (Figure 1) [5,12]. Viruses of HIV-1 and HIV-2 have *vpu* and *vpx*, respectively, in addition to the genes of the prototype virus. Importantly, all viruses including the prototype have *vpr*. Therefore, *vpr* of all SIV/HIVs could have a common origin. Viruses of the HIV-2 group have *vpr* and *vpx*, which encode structurally related Vpr and Vpx. A detailed phylogenetic analysis has suggested that SIVsmm acquired its *vpx* from *vpr* of African green monkey SIVagm by recombination [16], and not by gene duplication [17,18]. It is also suggested that the origin of SIVsmm *vpr* was *vpr* of SIVcpz [19]. The recombination theory based on these studies is quite reasonable, since Vpx and Vpr have distinct functions [8].

Because of the apparent common origin of HIV Vpr/Vpx as described above, they show a remarkably similar three-dimensional (3-D) structure, as revealed by the homology modelling method. The three proteins (HIV-1 Vpr, HIV-2 Vpr and HIV-2 Vpx) have three major α-helices with amphipathic characteristics surrounded by N- and C-terminal loops [20]. This 3-D structure model was predicted from the nuclear magnetic resonance (NMR) structure of HIV-1 Vpr [21] on the basis of sequence alignment of HIV-1 Vpr and HIV-2 Vpr/Vpx (Figure 2). Sequence homologies of HIV-1 Vpr (89.6 strain, 96 amino acids) versus HIV-2 Vpr (GH-1 strain, 105 amino acids), HIV-1 Vpr versus HIV-2 Vpx (GH-1 strain, 112 amino acids) and HIV-2 Vpr versus HIV-2 Vpx were 44, 24 and 22%, respectively. A similar modelling analysis of HIV-2 Vpx was also performed, and gave essentially the same 3-D structure [22]. Although HIV Vpr/Vpx share a basic framework, there are unique structural features in the N-terminal loop, the region between major helices 2 and 3, and the C-terminal loop of HIV-2 Vpx [20].

```
                                              Helix 1              Helix 2
HIV-1 Vpr(NL4-3)  MEQAP———EDQGPQRE-PYNEWTLELLEELKSEAVRHFPRIWLHNLGQ———HIYETY 50
HIV-1 Vpr(89.6)   MEQAP———EDQGPQRE-PYNDWTLELLEELKNEAVRHFPRIWLHSLGQ———HIYETY 50
HIV-2 Vpr(GH-1)   MTEAPTEFPPEDGTPRRE-LGGDWVIRILGEIKEEALKHFDPRLLIALGN———YIHSRH 55
HIV-2 Vpx(GH-1)   MTDPRERVPPGNSGEETIGEAFEWLDRTIEALNREAVNHLPRELIFQVWQRSWRYWHDEQ 60
                  *                  *          **  *

                             Helix 3
HIV-1 Vpr(NL4-3)  GDTWAGVE-AIIRILQQLLFIHFRIGCRHSRIGVTRQ-RRARNGASRS——— 96
HIV-1 Vpr(89.6)   GDTWTGVE-ALIRILQQLLFIHFRIGCRHSRIGIIQQ-RRTRNGASKS——— 96
HIV-2 Vpr(GH-1)   GDTPEGAR-ELIRILQRALFVHLRAGCHRSRISQTRR-RTPFPAAPTPRGMY 106
HIV-2 Vpx(GH-1)   GMSPSYTKYRYLCLMQKAVFIHFKRGCTCLGGGHGPGGWRSGPPPPPPPGLV 112
                  *            *   * *   **
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Figure 2. Amino acid sequence alignment of HIV Vpr/Vpx proteins. Sequence alignment of HIV-1 Vpr and HIV-2 Vpr/Vpx was performed by the Clustal W programme version 2 [66]. Amino acids that constitute or are predicted to constitute α-helices are shaded as previously reported [20]. Identical amino acids and gaps are indicated by (*) and (-), respectively. GenBank accession numbers for NL4-3, 89.6 and GH-1 are AF324493, U39362 and M30895, respectively

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FUNCTIONAL ANALYSIS OF HIV VPR/VPX PROTEINS

Role of Vpr/Vpx for virus replication in cultured cells

The observations that Vpr is present at a high level in virions, that the pre-integration complex contains Vpr and that Vpr localises to the cell nucleus suggested that Vpr is involved in the nuclear import process of HIV-1 DNA [1]. Despite the proposed role for HIV-1 Vpr in nuclear import of viral DNA in non-dividing cells, deletion of Vpr has been widely reported to have only a modest effect on HIV-1 replication, even in MDMs [1]. It has also been reported by us and others that Vpr-deleted viruses of the HIV-2 group grow as well as wild-type virus in MDMs [23–25]. In sharp contrast to HIV-1 Vpr and HIV-2 Vpr, HIV-2/SIV Vpx is essential for viral replication in MDMs [8,9,23,24,26–31]. A typical example of these observations is shown in Figure 3A. Consistent with the growth property of viruses, HIV-2 Vpx, but not HIV-1 Vpr, is required for reverse transcription of viral RNA and/or nuclear import of viral DNA in MDMs (Figure 3B). However, a small number of reports have indicated that HIV-1 Vpr is essential for viral replication in MDMs [32,33], and that HIV-1 Vpr is required for the nuclear import of the viral genome [34,35].

While Vpx is completely unnecessary or dispensable for viral replication in established cell lines and primary lymphocytes from peripheral blood mononuclear cells [23], it has been reported to be critical for nuclear import of viral DNA in non-dividing cells such as MDMs [8,29], and thereby essential for virus replication in those cells. Biochemical properties of Vpx supported this notion [29,30]. However, the conclusions of the two articles [8,29] could not be generalised because materials used in each study varied, and only non-quantitative polymerase chain reaction (PCR) analyses were performed. We therefore re-evaluated the defective early stage of a series of HIV-2 *vpx* mutants in human MDMs by the real-time PCR analysis [9]. We clearly demonstrated that Vpx is critical for reverse transcription of the viral RNA genome in MDMs. Our results are consistent with those independently obtained by others [10]. Of note, recent studies have demonstrated that an anti-viral factor(s) exists in MDMs, and that Vpx overcomes the restriction imposed by the factor [36–38].

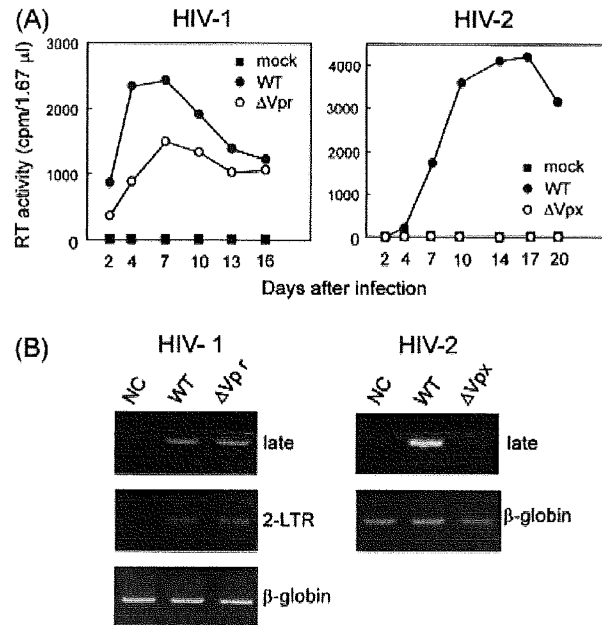

Figure 3. Comparative characterisation of *vpr*-minus HIV-1 and *vpx*-minus HIV-2 mutants. (A) Growth kinetics of the mutants in human MDMs. Wild-type virus clones used were pNL-8KB (a newly constructed clone; pNL4-3 carrying an *Stu* I-*BsaB* I fragment from p89.6 *env* gene) and pGL-AN [9,23] for HIV-1 and HIV-2, respectively. Mutant clones used were pNL-8KB-Ec (a newly constructed clone; pNL-8KB carrying a frame-shift mutation at *EcoR* I site in its *vpr* gene) and pGL-St [9,23]. MDMs were isolated from peripheral blood mononuclear cells by adhesion to the plastic and cultured in RPMI-1640 medium supplemented with 10% heat-inactivated human serum AB as previously described [9,23]. Virus samples (WT, wild-type virus; (Vpr, *vpr*-minus mutant NL-8KB-Ec; (Vpx, *vpx*-minus mutant GL-St) were prepared from transfected 293T cells, and equal reverse transcriptase (RT) units of viruses were inoculated into MDMs. Virus replication was monitored at intervals by RT production in the culture supernatants. As demonstrated by us and others [23–25], Vpr-deleted viruses of the HIV-2 group grow as well as wild-type virus in MDMs. (B) Semi-quantitative estimation of viral DNA synthesis in human MDMs. Mutant clones used were *env*-minus pNL-Kp (HIV-1) [67], *env/vpr*-minus pNL(Vpr-Kp (a newly constructed HIV-1 clone; pNL-Kp carrying an *Spe* I-*Sal* I fragment of pNLLuc(Bg((Vpr) [35]), *env*-minus pGL-Ns (HIV-2) [23] and *env/vpx*-minus pGL-Ns/St (HIV-2) [23]. Input pseudotype viruses (NC, negative control; WT, *env*-minus mutant pseudotyped with vesicular stomatitis virus [VSV]-G protein [G]; (Vpr, *env/vpr*-minus mutant pseudotyped with VSV-G; (Vpx, *env/vpx*-minus mutant pseudotyped with VSV-G) were prepared from transfected 293T cells, and equal RT units of viruses were inoculated into MDMs. On day 2 post-infection, DNA was extracted from cells, and samples were subjected to PCR analysis [23]. Late, late reverse transcription products; 2-LTR, two-LTR circles in the cell nucleus

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It has been generally accepted that HIV-1 Vpr has little influence on viral growth in proliferating primary T cells and T-cell lines [1]. Nonetheless, a few articles have shown that the *vpr*-minus mutant displays a severe growth defect relative to wild-type virus in primary T cells [39,40]. On the other hand, HIV-2/SIV Vpr/Vpx were required for efficient viral replication in a herpesvirus saimiri-immortalised simian T-cell line designated HSC-F and in proliferating peripheral blood lymphocytes and mononuclear cells [23,25]. In HSC-F cells, it was found that HIV-2 Vpx enhanced nuclear import of the viral genome [9,23], and that HIV-2 Vpr promoted viral replication at a late phase [23]. Therefore, HIV-2 Vpx apparently acts at a different step in viral replication in MDMs (reverse transcription of the RNA genome) and in T lymphocytes (nuclear import of the DNA genome). However, we have noticed that HIV-2 Vpx may also affect the nuclear import process of the viral genome in MDMs. As shown in Figure 4, when barely replication-competent *vpx*-mutant viruses designated E20G, N33S and W56L [9] were assessed for their ability to accomplish the early replication stage, they all appeared to be somewhat defective at both the reverse transcription and nuclear import steps.

A summary of mutational studies on HIV Vpr/Vpx described above is presented in Table 1.

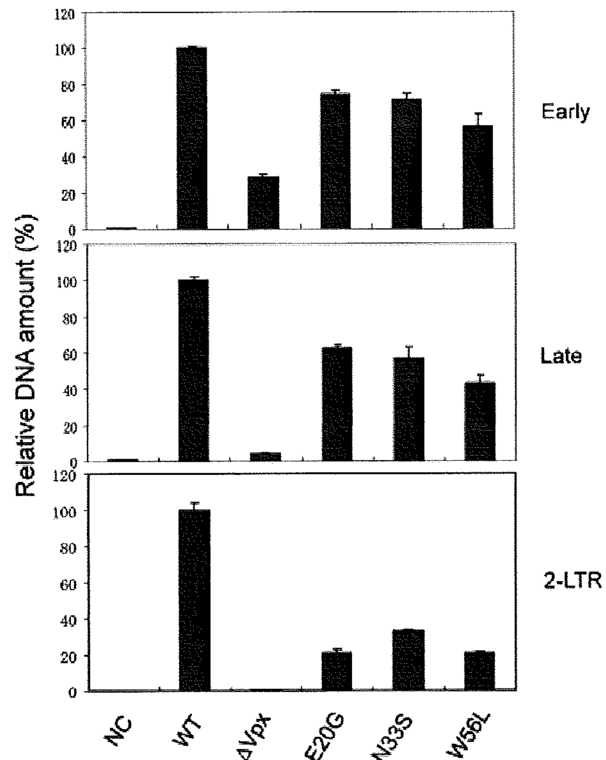

Figure 4. Quantitative estimation of viral DNA synthesis in human MDMs infected with *vpx* point mutants of HIV-2. Proviral clones of HIV-2 described in the legend to Figure 3 and those of *vpx* point mutants [9] were used. Human MDMs were infected with pseudotype viruses as described in the legend to Figure 3B [9], and on day 2 post-infection, DNA samples were subjected to real-time PCR analysis [9]. Amount of viral DNA synthesised in MDMs infected with a mutant (*env*-minus/*vpx*-point mutant pseudotyped with VSV-G) relative to that by WT (*env*-minus mutant pseudotyped with VSV-G) is shown. NC, negative control; (Vpx, *env*/*vpx*-minus mutant pseudotyped with VSV-G; early, early reverse transcription products; late, late reverse transcription products; 2-LTR, two-LTR circles in the cell nucleus. E20G, N33S and W56L are the site-directed *vpx* point mutants of HIV-2 which barely grow in human MDMs [9]

Cytopathogenic activity of HIV Vpr/Vpx

It is well established that HIV-1 Vpr is a cytostatic protein which arrests cells in the G_2 phase of the cell cycle [41–45]. It has also been reported that HIV-2/SIV Vpr arrests cells at the G_2 phase [8,46–48], while Vpx does not [8,43–45,47]. The mechanism for G_2 arrest has been clarified through extensive studies [49]. Of particular note, HIV-1 Vpr is demonstrated to manipulate the Cul4-DDB1-DCAF1 E3 ligase to induce ATR-dependent cell cycle arrest in G_2 via proteasomal degradation of an unknown cellular target [50–56]. Similarly, SIV Vpx is reported to act as an adaptor in the Cul4-DDB1-DCAF1 E3 ligase complex to counteract macrophage restriction by proteasomal degradation of an unknown target [10,36]. The virological significance of G_2 arrest by Vpr is presently unclear, although this property is well conserved among HIV/SIVs. It has been shown that viral gene expression is optimal at the G_2 phase of the cell cycle [57].

It is also well known that HIV-1 Vpr readily induces apoptosis [58,59], and this mechanism has been extensively studied [49]. On the contrary, apoptosis due to HIV-2/SIV Vpr/Vpx has not yet been well documented. We and others have shown that Vpx is detrimental to cells via an unknown mechanism [10,20]. More importantly, although a causal role of Vpr in the induction of apoptosis is evident, no reports directly studying its virological significance have been published to the best of our knowledge. Apoptosis due to Vpr may not contribute to promoting viral replication, but may be

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Table 1. Requirement of HIV Vpr/Vpx proteins for viral replication in target cells

	Cells		
	Established lines	**MDMs**	**Primary or immortalized T lymphocytes**
HIV-1 Vpr	Unnecessary[a]	Dispensable[b]/unknown Essential[c]/NI[d]	Unnecessary Dispensable/NI
HIV-2 Vpr	Unnecessary	Unnecessary	Dispensable/unknown
HIV-2 Vpx	Unnecessary	Essential/RT[e], NI	Dispensable/NI

Requirement of Vpr/Vpx proteins for HIV replication, based on replication properties of mutant viruses reported to date, and proposed mechanism for the phenotype are indicated. For details, see text.
[a]Unnecessary; mutant viruses without Vpr or Vpx replicate in the cells as well as wild-type virus.
[b]Dispensable; mutant viruses without Vpr or Vpx replicate in the cells but poorly relative to wild-type virus.
[c]Essential; mutant viruses without Vpr or Vpx do not replicate at all in the cells.
[d]NI; nuclear import of viral DNA genome.
[e]RT; reverse transcription of viral RNA genome.

important in AIDS pathogenesis, as may be true for the cell cycle arrest by Vpr.

In vivo study of Vpr/Vpx

Because HIV-1 is tropic only for chimpanzees and humans, no substantial animal studies towards understanding the functional role of HIV-1 Vpr have been performed [60]. Instead, rhesus monkeys infected with SIVmac (HIV-2 group) have been extensively analysed. These studies indicated that Vpr/Vpx are not major contributors to viral replication in experimentally infected animals [2]. Moreover, many of the animals infected with the *vpr*-minus or *vpx*-minus virus have progressed to AIDS [2]. However, deletion of both *vpr* and *vpx* markedly attenuated the virus in animals by an unknown mechanism [61]. On the other hand, in pig-tailed monkeys, SIV Vpx is important for viral dissemination and pathogenesis [62].

CONCLUSIONS

Figure 5 summarises the various activities of HIV Vpr/Vpx reported to date. Major consensus conclusions regarding the function of HIV Vpr and Vpx proteins are as follows: (1) Vpr proteins of HIV-1 and HIV-2 arrest cells in the G_2 phase of the cell cycle [8,41–48]. (2) HIV-1 Vpr induces apoptosis [58,59]. (3) Vpx is essential for viral replication in macrophages and is important for viral replication in T-cells [8,9,23–31]. Additional important functions of HIV Vpr/Vpx so far proposed include the promotion of full-length viral DNA accumulation (HIV-2 Vpx) [9–11], nuclear import of viral preintegration complex (HIV-1 Vpr and HIV-2 Vpx) [1,8,23,29,34,35], transcriptional transactivation of viral and cellular promoters (HIV-1 Vpr and HIV-2 Vpr) [63–65] and cytopathogenic activity by an unknown mechanism (HIV-2 Vpx) [10,20]. Generally, mutational effects of HIV Vpr on viral replication are smaller than those of HIV-2 Vpx, and therefore, care should be taken to interpret the results of experiments. In this regard, it is interesting to note that Vpr proteins of HIV-1 and HIV-2 strongly affect cell physiology, and are detrimental to cells.

Of the multiple functions of HIV Vpr/Vpx so far reported, those clearly and directly associated with viral replication are still subject to debate. Are HIV-1 Vpr and HIV-2 Vpx indispensable, or important to a certain extent for nuclear import of the viral DNA in non-dividing cells? Does HIV-2 Vpx have bi-functional roles in the early phase of viral replication (reverse transcription and nuclear import) in non-dividing cells? If so, what is the mechanism? Recent observations that Vpx, but not HIV-1 Vpr, efficiently counteracts a cellular restriction factor in non-dividing macrophages have shed some light on the issue. Identification of the anti-viral factor in macrophages is in urgent need of clarification.

HIV-1 Vpr evidently displays cytopathogenic potential by inducing G_2 arrest and apoptosis. It

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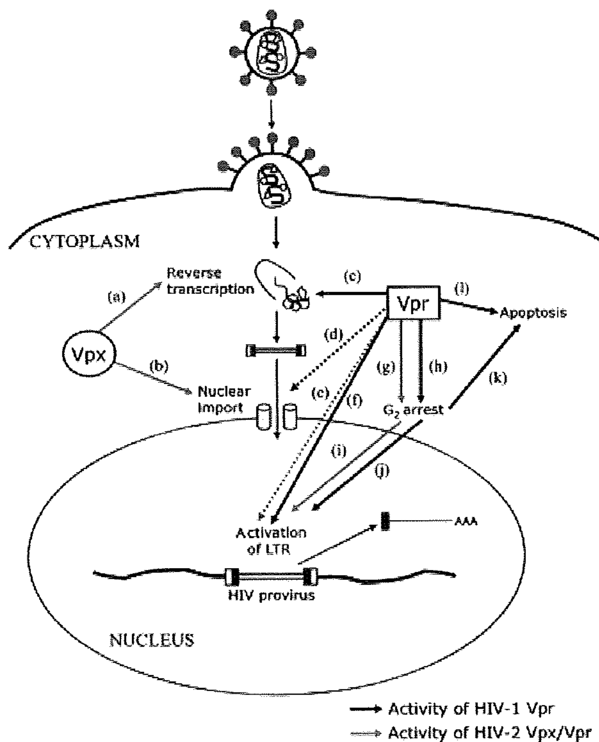

Figure 5. Function of HIV Vpr/Vpx in target cells. Various activities of HIV Vpr/Vpx in macrophages and T-lymphocytes reported to date are schematically shown. Broken lines indicate that the activity is still subject to debate. Whether all the activities in this figure have positive effects on HIV replication is currently unclear. Because HIV virions contain a large amount of Vpr/Vpx, HIV Vpr/Vpx are believed to play functional roles at the early infection phase. (a) Promotion of reverse transcription of the viral RNA in macrophages [9,10]. (b) Promotion of nuclear import of the viral DNA in T-cells (and in macrophages?) [23]. (c) Increase of fidelity of reverse transcription [68,69]. (d) Promotion of nuclear import of the viral DNA in macrophages (?) [1,34,35]. (e) Trans-activation of proviral LTR (?) [65]. (f) Trans-activation of proviral LTR [63,64]. (g) and (h) Induction of G_2 arrest [8,41–48]. (i) and (j) Up-regulation of transcription via G_2 arrest [70–73]. (k) Induction of apoptosis via G_2 arrest [49,74]. (l) Induction of apoptosis [58,59]

is currently unclear whether this viral ability contributes to enhance viral replication in target cells, and in individuals to some degree. However, it is interesting to study the role of HIV-1 Vpr in the pathophysiology of AIDS. Appropriate animal models to approach this issue are presently unavailable. In this regard, our attempt to generate monkey-tropic and -pathogenic HIV-1 is important. The resultant monkey infection model would provide a powerful tool to elucidate the unknown role of HIV-1 Vpr.

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