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REVIEW

Species barrier of HIV-1 and its jumping by virus engineering

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SUMMARY

Monkey infection models are absolutely necessary for studies of human immunodeficiency virus type 1 (HIV-1) pathogenesis and of developing drugs/vaccines against HIV-1. In addition, currently unknown roles of its accessory proteins for *in vivo* replication await elucidation by experimental approaches. Due to the fact that HIV-1 is tropic only for chimpanzees and humans, studies of this line have been impeded for a long time, although various investigations have been carried out utilising genetically related SIV and SIV/HIV chimeric virus (SHIV) as pathogens. Recent findings of anti-HIV-1 innate factors such as tripartite motif protein 5α (TRIM5α) and APOBEC3G/F prompted us to re-initiate an old and vital research project which would, as a result, confer the capability to overcome the species barrier on the HIV-1. We currently have obtained, by virus engineering through genetic manipulation and adaptation, some new and promising HIV-1 clones for *in vivo* studies in macaque monkeys as mentioned above. In this review, we summarise the past, present and future of HIV-1/SIV chimeric viruses with special reference to relevant basic HIV-1/SIV studies. Copyright © 2008 John Wiley & Sons, Ltd.

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INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) productively infects only humans and chimpanzees but not Old World monkeys, and is specifically pathogenic to humans causing AIDS and AIDS-associated diseases. This narrow host range of HIV-1 has compelled us to use SIV or SHIV, a chimera between HIV-1 and SIV, as input viruses for *in vivo* model studies in macaque monkeys. Although SIV isolated from rhesus monkeys (SIVmac) is similar to HIV-1 in its genome organisation and pathogenic potential, it is a genetically and virologically distinct virus from HIV-1 in a number of important points. The same is quite true for various SHIVs that are basically SIVmac derivatives carrying only a portion of HIV-1 genome sequence [1–6]. Of note, HIV-1 genome contains a unique set of accessory genes that are believed to play essential functional roles for virus persistence, spread and pathogenesis in natural target cells and/or in individuals by modulating and optimising viral replication.

It is now well appreciated that many mammalian species including primates encode factors conferring resistance to retroviral infections. In fact, human/simian tripartite motif protein 5α (TRIM5α) and apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G) (A3G) have been identified as such factors and demonstrated to have strong anti-HIV-1 activity. Importantly, the genomic regions of HIV-1 that are critical for the restriction of viral replication are also determined. Based on these studies, we could have designed a novel chimeric virus totally different from the pre-existing SHIVs, and actually created it *in vitro*. The resultant chimeric virus belongs to the HIV-1 group by standard scientific criteria and has been demonstrated to represent

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Abbreviations used
HIV-1, human immunodeficiency virus type 1; SHIV, SIV/HIV chimeric virus; TRIM5α, tripartite motif protein 5α; A3G, apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G); A3F, APOBEC3F; CA, capsid protein; N-MLV, N-tropic murine leukaemia virus; EIAV, equine infectious anaemia virus; CypA, cyclophilin A; CyM, cynomolgus monkey; GFP, green fluorescent protein

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Table 1. Primates and their immunodeficiency viruses

	Primate species	Virus designation	Virus grouping	Pathogenicity
Apes	Human	HIV-1	HIV-1/SIVcpz	+
		HIV-2	SIVmac/SIVsmm/HIV-2	+
	Chimpanzee	SIVcpz	HIV-1/SIVcpz	−
Old World monkeys	Macaque			
	Rhesus monkey	SIVmac	SIVmac/SIVsmm/HIV-2	+
	Cynomolgus monkey	SIVcyn		
	Pig-tailed monkey	SIVmne		
	Stamp tail monkey	SIVstm		
	Sooty mangabey	SIVsmm	SIVmac/SIVsmm/HIV-2	−
	African green monkey	SIVagm	SIVagm	−
	Mandrill	SIVmnd	SIVmnd-1 SIVmnd-2	−

Immunodeficiency viruses isolated from various primate species are classified by their genome organisation and/or nucleotide sequence homology (>90%). HIV-1 is a unique primate lentivirus. For details, see Reference [137]. (+) and (−) in the pathogenicity column indicate that the virus can induce AIDS in individuals, or not, respectively.

the first and prototype monkey-tropic HIV-1. However, the virus was found to grow less efficiently in macaque cells relative to the standard pathogenic clone SIVmac239. We are, therefore, now generating a second generation of monkey-tropic HIV-1s with positive results by virus engineering through recombinant DNA techiniques and virus adaptation in cells. In this review, we describe our basic research on chimeric viruses as well as the related virological topics.

VIROLOGICAL PROPERTIES OF SIVmac AND HIV-1

SIV was initially isolated from a rhesus monkey as an infectious agent to induce an AIDS-like disease [7,8]. Molecular clones of the virus (SIVmac) were then generated and shown to be capable of causing an AIDS-like disease in the monkeys [9–11]. Subsequently, species-specific SIVs were isolated from various monkeys including sooty mangabeys (SIVsmm) [12–14], African green monkeys (SIVagm) [15–17] and mandrill (SIVmnd) [18,19]. These SIVs establish asymptomatic chronic infections and do not develop any disease in their natural hosts (Table 1). It has been suggested that SIVmac emerged by a cross-species infection of the rhesus macaque with SIVsmm naturally found in sooty mangabeys [12,13,20]. SIVmac is similar to HIV-1 in genomic organisation (Figure 1) and in biology including pathogenicity *in vivo* [6]. Both viruses target CD4+ cells such as T-lymphocytes and macrophages, and use CCR5 as a co-receptor, resulting in the complete loss of CD4+ T-cells. They elicit persistent replication, chronic disease and eventually immunodeficiency. The infection of macaque monkeys with SIVmac is widely used as a model for HIV/AIDS to study disease progression and virus transmission.

However, some significant genetic and biological differences do exist between these two viruses. Both HIV-1 and SIVmac possess four accessory proteins, but HIV-1 can be distinguished from SIVmac by the presence and absence of Vpu and Vpx, respectively. Since it has been shown that the accessory proteins are, in many cases, dispensable for viral replication *in vitro*, the precise roles of these proteins for viral replication and pathogenesis need to be elucidated by *in vivo* study. In addition, SIVmac and HIV-1 show only 30–50% homology of the amino acid sequences, resulting in some functional differences of HIV-1 proteins and their SIVmac counterparts. First, SIVmac exhibits sensitivities to antiviral drugs that are not observed for HIV-1 [1–3]. Second, although simian and human AIDS are pathologically similar, the disease course of SIVmac is short relative to that of HIV-1 infection. SIVmac induces the immunodeficiency in individuals in 1–3 years versus

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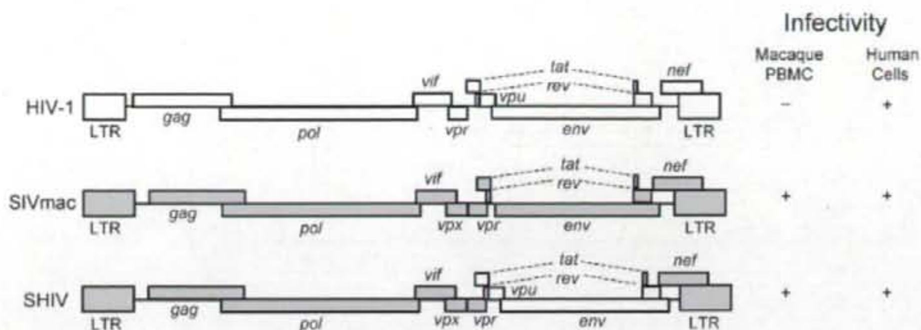

Figure 1. Genomic organisation and cellular tropism of HIV-1, SIVmac and a standard SHIV. White and grey boxes represent sequences of HIV-1 and SIVmac, respectively. (+) and (−) on the right indicate growth-competence and -incompetence, respectively

10 years on average for HIV-1 [6]. In particular, an accelerated disease progression is observed in some rhesus macaques inoculated with pathogenic strains of SIVmac and SIVsmm. In these cases, infected rhesus macaques fail to elicit immune responses and develop disease in less than 6 months [6,21,22]. This may be due to distinctive cellular and humoral immune responses that are SIV specific. Furthermore, the host cell tropism is quite different between HIV-1 and SIVmac. HIV-1 replicates in cells of humans and chimpanzees, but not in cells of Old World monkeys, such as the rhesus monkey and African green monkey, whereas SIVmac can establish productive infection in both human and monkey cells (Figure 1) [23–26]. Because of these differences between the two viruses, SIVmac macaque monkey models may restrict the utility for studies on the disease progression, the significance of HIV-1 accessory proteins for pathogenesis and the development of vaccine/drug specific for HIV-1.

GENERATION AND *IN VIVO* CHARCTERISATION OF SIVmac-DERIVED SHIV CLONES

In order to overcome the limitations of SIVmac-monkey models and to generate models that would more closely reflect HIV-1 infection and disease progression, various SHIVs have been designed and constructed. The first generation of SHIV contained HIV-1 *tat*, *rev*, *vpu* and *env* in the genetic backbone of SIVmac (Figure 1) [23,27]. This SHIV clone was shown to infect and induce immunologic responses in macaques, but did not cause AIDS-like disease. The pathogenic SHIV was obtained later by serial animal-to-animal passages of infected blood and bone marrow or by depletion of CD8+ T-cells in infected monkeys [28–31]. Macaques infected with SHIVs have been used to determine the effect of neutralising antibodies against, particularly, HIV-1 Env to prevent SHIV infection [32,33], for the evaluation of antiviral therapy and for the analysis of drug resistance emergence [2,34]. In contrast to the disease progressions observed for SIV in macaques (1–3 years) and HIV-1 in humans (10 years), SHIV cause a rapid, systemic complete loss of CD4+ T-lymphocytes within several weeks of inoculation in unvaccinated animals. Despite the rapid disease progression by SHIV, SHIV has been shown to be controlled easily by vaccines [35–37], whereas it is difficult to control SIV replication by vaccines [38–40]. It has been suggested that the rapid disease progression in SHIV-infected animals may be due to the difference in chemokine receptor usage by SHIVs and SIVs routinely employed in these experiments. SIVs predominantly use CCR5 as a coreceptor (R5-tropic), which is expressed on memory CD4+ T-lymphocytes. SIV infection induces massive loss of memory CD4+ T-lymphocytes, particularly in the gastrointestinal tract [41–43]. In contrast, SHIVs use the CXCR4 coreceptor (X4-tropic), which is expressed on naïve CD4+T-cells, for infection. X4-tropic SHIVs target naïve CD4+ T-cells and cause depletion of naïve CD4+ T-cells. The loss of naïve CD4+ T-cells results in the abrogation of any CD4+ T-cells renewal, causing a rapid and complete depletion of CD4+ T-cells in infected macaques [4,5]. In this regard,

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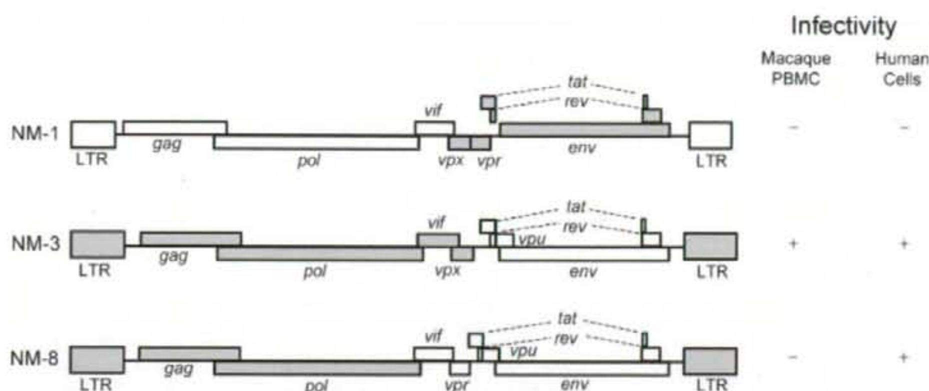

Figure 2. Genomic organisation and cellular tropism of SIVmac/HIV-1 chimeric viruses. White and grey boxes represent sequences from the parental clones NL4-3 (HIV-1) and MA239 (SIVmac), respectively. (+) and (−) on the right indicate growth-competence and -incompetence, respectively

R5-tropic pathogenic SHIV strains have been made by replacing the CXCR4-Env with CCR5-Env [44–46]. Because most primary isolates of SIVs and HIV-1 are R5-tropic, R5-ropic SHIV strains may be more relevant to HIV-1 infection and have been used for the evaluation of vaccine regimens [47,48] and for the studies of disease progression [49,50].

INNATE ANTI-HIV-1 FACTORS IN MONKEY CELLS

Early studies on viral determinants for species tropism

In order to search viral determinants that restrict the replication of HIV-1 in macaque PBMC, several SIVmac-HIV-1 chimeric viruses were constructed (Figure 2) [23,27]. Among them, only NM-3 exhibited infectivity to macaque PBMC. Genome comparison of NM-1 with NM-3 revealed that the determinant(s) of macaque cell tropism resides in the 5′-genomic region of SIVmac, since NM-1 containing the 3′-genomic region of SIVmac (*vpx, vpr, tat, rev* and *env*) did not grow in macaque PBMC, whereas NM-3 carrying the 5′-genomic region of SIVmac (LTR, *gag, pol, vif* and *vpx*) did. Worthy of note, NM-8 containing *vif* and *vpr* of HIV-1 instead of *vif* and *vpx* of SIVmac did not replicate in macaque PBMC. These results indicated that the central region of the SIVmac genome (*vif* and/or *vpx*) is important for species tropism.

In addition to the determinant(s) mentioned above, the Gag-CA sequence has been suggested to be important for the tropism by analysing a chimeric virus designated SIV/HIV-CA [51]. This virus is an SIV derivative containing a portion of the HIV-1 *gag* gene that encodes the CA-p2 region, and was found to grow in human but not at all in macaque PBMC.

Overall, early studies on chimeric SIV/HIV viruses showed that viral determinants for species tropism exist in both the CA-p2 domain and the central genomic region of SIVmac.

TRIM5α

As mentioned above, HIV-1 infects human and chimpanzee cells but not cells of Old World monkeys [23,24]. HIV-1 is able to enter these monkey cells, but appears to be blocked before and/or during the reverse transcription process [24–26,52,53]. Since this restriction can be somewhat overcome by infection at a high dose or by pre-treating target cells with high titres of restricted virus-like particle [25,26,54,55], it was postulated that a saturable factor exists in restricting target cells.

A breakthrough in determining this saturable restriction factor came from the identification of TRIM5α. The significance of TRIM5α for HIV-1 restriction was demonstrated by the inhibition of HIV-1 infection in human cells expressing rhesus

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TRIM5α and by the rescue of HIV-1 infectivity in TRIM5α-knocked out rhesus cells by small interfering RNA [56]. Subsequent studies have revealed that the block of retrovirus replication with TRIM5α was species-specific. Human TRIM5α inhibited N-tropic murine leukaemia virus (N-MLV) and equine infectious anaemia virus (EIAV) replication [57,58], and TRIM5α from various Old World monkey species suppressed HIV-1 infection but not SIV [56,59]. TRIM proteins contain a tripartite motif defined by the presence of a RING (really interesting new gene) domain that possesses ubiquitin ligase acitivity, B-box and coiled-coil domain [60,61]. Coiled-coil domain is involved in multimerization of TRIM5α proteins [62,63]. TRIM5α encodes the C-terminal B30.2 (SPRY) domain that is absent in other TRIM5 isoforms. This SPRY domain is responsible for the direct interaction with viral CA and for the species-specific restriction of retroviral infection [64–68]. TRIM5α-mediated restriction can occur rapidly after virus entry into target cells and impede the reverse transcription process. The interaction of trimeric TRIM5α and multimerised viral CA leads to abnormally accelerated uncoating of incoming HIV-1 cores [69,70]. It has been suggested that either the reduction or increase of HIV-1 core stability with the mutations in CA impairs the reverse transcription [71] and that rapid disassembly of incoming HIV-1 cores mediated by TRIM5α results in restriction of HIV-1 replication [69,70].

Proteasome inhibitors can relieve rhesus TRIM5α-imposed block to the reverse transcription, and rescue the viral cDNA accumulation but not the productive infection of HIV-1 due to the inhibition of nuclear translocation of viral cDNA [72,73]. These observations suggest that TRIM5α may restrict another distinct step(s) in HIV-1 infection [73,74]. In any case, the exact mechanism by which TRIM5α blocks retroviral infection remains to be elucidated.

Cyclophilin A (CypA)

It was reported that HIV-1 CA binds to the cytoplasmic protein CypA [75]. Subsequent studies have indicated that CypA is efficiently incorporated into virions via interaction with HIV-1 CA domain in producer cells [76,77]. CypA also interacts with incoming HIV-1 cores in target cells and this interaction enhances HIV-1 infectivity [78,79]. In simian cells, reversely, CypA decreases HIV-1 infectivity depending on the presence of TRIM5α. Both cyclosporine A and a small interfering RNA, by blocking the interaction between HIV-1 CA/CypA and by decreasing CypA expression level, respectively, rescue HIV-1 infectivity through reducing HIV-1 sensitivity to TRIM5α in simian cells [80–83]. In contrast to the effect of CypA on TRIM5α-mediated restriction in simian cells, the interaction of CypA with the incoming HIV-1 core protects from TRIM5α restriction in human cells and CypA is required for maximal infectivity. It has been shown in human cells that the decrease of TRIM5α expression has little effect on HIV-1 infectivity and that the decrease of HIV-1 infectivity by blocking CA-CypA interaction is independent of TRIM5α expression, suggesting the existence of an unknown antiviral factor(s) in human cells [82–84]. Putative models for the recognition and interaction of CypA with TRIM5α and CA in human and simian cells have been proposed [74,85].

APOBEC3G/F

HIV-1 Vif has been shown to be essential for HIV-1 infectivity in certain cell types including primary lymphocytes, monocyte-derived macrophages and some T-cell lines [86–88]. After extensive efforts by many researchers, human A3G was finally identified as a cellular target of HIV-1 Vif [89]. A3G is a member of a polynucleotide cytidine deaminase family that displays diverse functions [90,91]. Since the discovery of A3G, it has been shown that other APOBEC family members also exhibit antiviral properties [92–95]. Of these, APOBEC3F (A3F) has a similarly strong antiviral activity to that of A3G and is countered by Vif [92,96–100].

In the absence of Vif, human A3G and A3F are incorporated into HIV-1 virions. On the infection of new target cells, the virion-associated A3G and A3F deaminate cytidine in viral minus-strand DNA during reverse transcription, leading to either the G to A hypermutation of viral genome or degradation of the viral genome by cellular DNA repair enzymes [101–105]. Since the cytidine deaminase-inactivated A3G and A3F still retain antiviral activity [100,106], it is quite clear that A3G and A3F have deaminase-independent antiviral activity [100,106–110].

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HIV-1 Vif degrades A3G and A3F via the ubiquitin–proteasome pathway by recruiting an E3 ubiquitin ligase complex with Cullin 5, Elongin B and Elongin C [99,111–114]. Some investigators have reported that Vif inhibits A3G antiviral activity via mechanisms independent of the ubiquitin–proteasome pathway [105,115–117]. The ability of Vif to induce the degradation of A3G is species-specific, and this specificity is probably determined by Vif/A3G binding. HIV-1 Vif interacts with and degrades human A3G amd A3F but not A3Gs derived from the rhesus macaque and African green monkey. In contrast, SIVmac Vif is able to inactivate both human and simian A3Gs [118]. Very recently, binding sites of human A3G and A3F in HIV-1 Vif have been identified. It was demonstrated that the distinct regions of Vif are required for interaction with A3G and A3F [119–121; manuscript in preparation].

GENERATION OF MONKEY CELL-TROPIC HIV-1 DERIVATIVES

Although SHIVs have been used for studies of the immune responses to primate immunodeficiency viruses [122,123], of the protective role of antibodies directed against the HIV-1 Env [35,124], and of the disease progression [49,50], the SHIV/monkey system itself has many serious scientific issues as described earlier. After the discovery of two major host cell restriction factors, TRIM5α and A3G (Figure 3), we initiated a research project to construct HIV-1/SIVmac chimeric viruses of a new and distinct category. We designed a hybrid virus in the backbone of the HIV-1 genome that can infect and grow in macaque monkey cells. In other words, we wished to make HIV-1 derivatives (genetically containing 90% or more sequences from HIV-1) having minimum essential sequences of SIVmac. Towards this end, the potentially important sequences within the HIV-1 genome against TRIM5α and A3G, that is *gag* and *vif* genes, were mutated or replaced with the corresponding regions of SIVmac.

In our laboratory, numbers of *gag*-chimeric viruses between HIV-1 and SIVmac239 already have been constructed. Some of them lost the infectivity even in human cells, and none of them showed the infectivity in cynomolgus monkey (CyM) HSC-F cells [125,126]. HSC-F is a CyM T-cell line originally immortalised by *Herpesvirus saimiri* [127]. Together with the data reported in early studies, these results prompted us to substitute both the CypA-binding loop in Gag-CA and the Vif with the corresponding sequences of SIVmac239 to generate monkey cell-tropic HIV-1 derivatives. As described below, we thus constructed an HIV-1 derivative designated pNL-ScaVR, which carries only a short seven-aa segment of *gag* gene corresponding to the HIV-1 CypA-binding loop and the entire *vif* gene from SIVmac [128]. The nine-aa CypA-binding loop in NL4-3 Gag-CA (HIV-1) was converted to the seven-residue MA239 CA analogue (SIVmac) by site-directed mutagenesis of a pNL4-3-derived *vif*-expression vector pNL-SX carrying the HIV-1 *vif* gene [129]. The full 214-aa Vif ORF from MA239 was amplified by PCR and inserted into this clone to generate a proviral construct pNL-ScaV. When expression of Gag, Pol, Env, Vpr, Vpu and Nef of pNL-ScaV was examined by transfection analysis, the level of Vpr was markedly reduced. Since this Vpr-defect was subsequently found to be caused by one of the *vif*-cloning sites in pNL-ScaV, a Vpr-repaired proviral clone designated pNL-ScaVR was constructed [128].

The SIVmac sequences in pNL-ScaVR were functionally active and counteracted effectively against the inhibitory factors in monkey cells as monitored by single-cycle replication assays for measuring *gag* and *vif* activities. As expected, the virus derived from pNL-ScaVR productively infected the CyM HSC-F cells. However, its growth kinetics were very much delayed relative to those of SIVmac from pMA239. Since it has been reported that HIV-1 acquires changes during extended tissue culture passage that confer augmented replicative properties [130,131], a viral adaptation experiment by long-term culture of infected HSC-F cells was performed. We successfully obtained viruses with enhanced growth abilities and finally have constructed by recombinant DNA techniques including PCR an infectious molecular clone (two biologically significant mutations in *env* gene) from them. It was designated pNL-DT5R [128] and used subsequently as a prototype clone (Figure 4). The NL-DT5R virus certainly grows in CD8-depleted PBMC prepared from pig-tailed and rhesus macaques and also in pig-tailed monkeys (see below), albeit less efficiently than SIVmac239. Another proviral clone designated stHIV-1, which contains the

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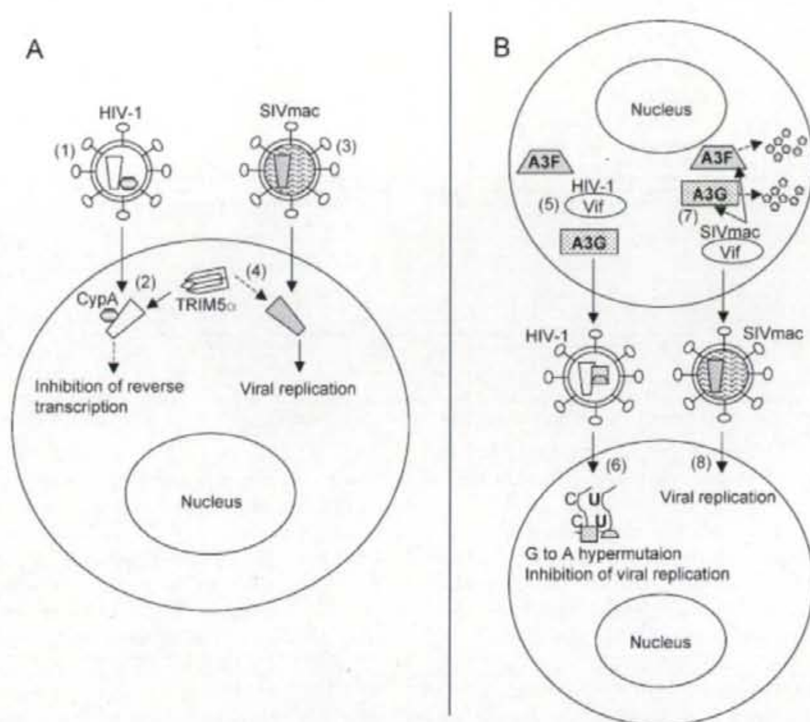

Figure 3. Schematic representation of species-specific restriction of HIV-1 replication by CypA/TRIM5α and by A3G/F in macaque monkey cells. Early HIV-1 replication steps affected by CypA/TRIM5α (A) and by A3G/F (B) are highlighted. (1) CypA is incorporated into HIV-1 virion. (2) After entry into cells, macaque TRIM5α interacts with CypA-CA core and promotes accelerated uncoating, resulting in the inhibition of reverse transcription. (3) No CypA is present in SIVmac virion. (4) In macaque cells, TRIM5α is unable to target SIVmac CA core and viral replication occurs efficiently. (5) HIV-1 Vif is unable to interact with and degrade macaque A3G/F, and both proteins are incorporated into HIV-1 virion. (6) In target cells, the virion-associated A3G/F deaminate cytidines in viral minus-strand DNA during reverse transcription, leading to the inhibition of viral replication by either G to A hypermutation in viral genome or the degradation of viral genome by cellular DNA repair enzyme. (7) SIVmac Vif inactivates macaque A3G/F in infected cells. (8) There is no carry-over of A3G/F, and SIVmac replication occurs normally in target cells

entire SIVmac CA and Vif coding sequences, was similarly constructed by others (Figure 4) and the virus was shown to be growth-competent in rhesus macaque PBMC [132]. Whether stHIV-1 can grow in monkeys is not yet reported.

To establish an ideal monkey model system for HIV-1/AIDS study, generation of HIV-1 derivatives that grow similarly well with SIVmac239, are pathogenic for macaque monkeys as well, and are R5-tropic, if possible, is essential. We already have started to modify the genome of NL-DT5R to further improve its growth potential in monkey cells. Extensive attempts to obtain *gag* and/or *vif* variants of NL-DT5R with an accelerated replication ability were unsuccessful (manuscripts in preparation; our unpublished data). Some of them were constructed based on the published reports [132,133]. Parallel attempts to construct R5-tropic viruses by *env*-substitution resulted in two full-length molecular clones designated pNL-DT5R5-1 and pNL-DT5R5-2 infectious for CD8-depleted PBMC of pig-tailed monkeys (our unpublished results). NL-DT5R5-1 was also infectious for HSC-F cells but less efficiently than the parental NL-DT5R as for PBMC. Clearly, biologically different from NL-DT5R, NL-DT5R5-1

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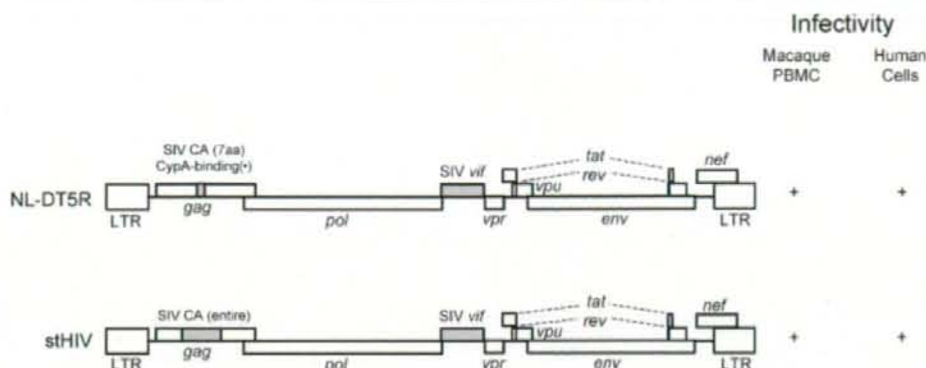

Figure 4. Genomic organisation and cellular tropism of HIV-1/SIVmac chimeric viruses of a novel class. White and grey boxes represent sequences from the parental clones NL4-3 (HIV-1) and MA239 (SIVmac), respectively. (+) on the right indicates growth-competence

rapidly induced severe cytopathic effects in HSC-F cells.

On the basis of our results and observations described above, we decided to modify the genomes of NL-DT5R and NL-DT5R5-1 by viral adaptation within HSC-F cells (Figure 5). Virus stocks prepared from transfected 293T cells were inoculated into HSC-F cells, and the cultures were maintained until progeny virus production was undetectable. Fresh HSC-F cells were then added to the cultures (on day 45 post-infection) and they were monitored for virus production. Viruses soon emerged in both co-cultures and grew to a higher level relative to that of viruses in early infection days. In order to ascertain the adapted nature of the emerged viruses, culture supernatants, collected from HSC-F cells infected with either NL-DT5R or NL-DT5R5-1 on day 57 (Figure 5) and normalised by RT activity, were inoculated into HSC-F cells (Figure 6). As is clear, the late

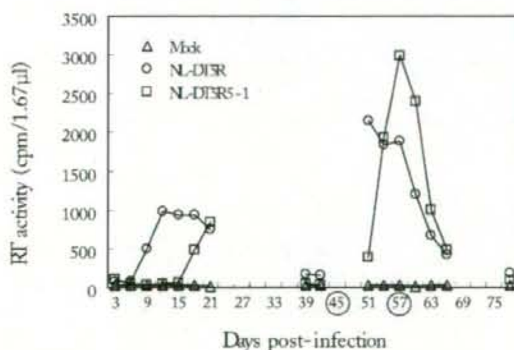

Figure 5. Growth properties of NL-DT5R (X4-tropic) and NL-DT5R5-1 (R5-tropic) viruses during a long-term passage in HSC-F cells. Virus samples were prepared from 293T cells transfected with the indicated proviral clones (Mock, pUC19), and equal RT units of viruses were inoculated into CyM HSC-F cells. Virus replication was monitored by RT production in the culture supernatants. On day 45 post-infection, fresh uninfected HSC-F cells were added to the cultures. On day 57 post-infection, cell-free viruses were prepared from the cultures, and used for monitoring their growth properties (see Figure 6)

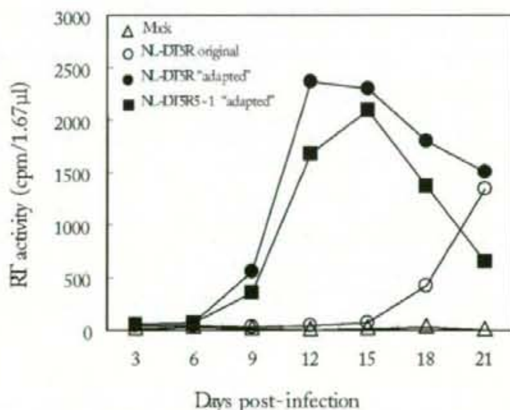

Figure 6. Growth kinetics of viruses generated during a long-term passage in HSC-F cells. Culture supernatants from NL-DT5R- or NL-DT5R5-1-infected HSC-F cells (adapted) were collected on day 57 post-infection (Figure 5), and equivalent RT units were inoculated into HSC-F cells. Virus replication was monitored by RT production in the culture supernatants. For controls, samples prepared from 293T cells transfected with NL-DT5R (original) or pUC19 (Mock) were used

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emerging viruses did in fact exhibit the adapted phenotype, that is, more robust viral replication and more extensive viral cytopathic effects than the original parental non-HSC-F-passage viruses. We have obtained a number of molecular clones from these adapted viruses by the method described above, and confirmed that viruses derived from them reproduce a similar biological property or even show a more enhanced phenotype in infected HSC-F cells (our unpublished results).

INFECTIONS OF MONKEYS WITH THE PROTOTYPE MONKEY CELL-TROPIC HIV-1 NL-DT5R

As a first monkey infection experiment of monkey cell-tropic HIV-1, NL-DT5R was inoculated into four pig-tailed macaques with/without anti-CD8 antibody treatment [134]. All the monkeys showed signs for viral productive and persistent infection. Plasma viral loads as monitored by viral RNA were elevated, reached a peak and gradually declined to an undetectable level by 5 to 11 weeks post-inoculation. Viral DNA in PBMC similarly persisted for 45 weeks post-infection. Virus in the infected animals could be transferred to another pig-tailed monkey. The NL-DT5R challenge into pig-tailed macaques also induced anti-HIV-1 antibody directed against HIV-1 encoded p17, p24, gp41, gp120 and gp160. However, the numbers of circulating CD4+ T-lymphocytes did not change appreciably during the observation period. Depletion of CD8+ cells had a small but significant effect on most of the above-described outcomes. In total, although NL-DT5R induced plasma viraemia and anti-HIV-1 antibodies in pig-tailed macaques, no significant depletion of CD4+ T-cells and no evidence of clinical disease were observed. Based on these results, other sets of monkey experiments are now taking off. Infection of CyMs, rhesus monkeys and pig-tailed monkeys by NL-DT5R and its improved versions may be a milestone for the establishment of monkey models for HIV-1/AIDS study.

CONCLUSIONS AND FUTURE DIRECTIONS

Recently discovered and extensively studied host cell restriction factors against HIV-1 have given us a rationale for generation of monkey-tropic HIV-1 to establish a pivotal monkey system for both basic and clinical studies. These factors have explained, at least in part, the narrow host range of HIV-1, and a number of new HIV-1s that are able to infect monkey cells have been generated by us and others [128,132; our unpublished data]. The genome of monkey-tropic HIV-1s basically contains both a 21-nucleotide SIVmac239 Gag-CA element, corresponding to the HIV-1 CypA-binding site, and the entire SIVmac 239 *vif* gene (Figure 4). While the prototype monkey-tropic HIV-1 designated NL-DT5R clearly established spreading infections in a CyM T-cell line, CD8-depleted PBMCs from pig-tailed and rhesus macaques, it did not cause AIDS-like symptoms at all in pig-tailed monkeys. Because the virus grows more poorly than the SIVmac239 virus both in cultured simian cells and in monkeys, improvement of viral replication by the modification of the viral genome is required for developing tractable monkey models for HIV-1/AIDS study. Our extensive attempts to obtain such variant viruses through genetic manipulation of *gag* and *vif* genes have so far been unsuccessful. In sharp contrast, viral adaptation in cells to speed up the growth rate appeared to occur quite efficiently (Figures 5 and 6), and infectious molecular clones with adapted viral phenotypes tropic either for X4 or R5 cells were obtained (our unpublished results). Sequencing the genome of adapted viruses and subsequent functional analysis may reveal the presence of an otherwise undiscovered genetic region(s) responsible for the species tropism of HIV-1. We have been convinced that the above-mentioned new viral clones would give us a good chance to develop HIV-1/AIDS-nonhuman primate models. According to recent reports [135,136], incorporation of green fluorescent protein (GFP) into HIV-1 clones enables one to visualise the virus itself. We are interested in generating 'visible viruses', since they are eminently useful to determine the movement of viruses in monkeys that would be involved in or associated with disease progression.

Needless to say, monkey infection models provide powerful tools for the elucidation of the unknown role of HIV-1 accessory proteins in the HIV-1 life cycle and pathogenesis, and more importantly, for the development of vaccines and drugs for the prevention and treatment. In a different point of view, we are also interested in analysing, by determining the alterations of viral genomes, the difference of responses against HIV-1 infection among individuals and/or

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species. HIV-1 may variably mutate and evolve during the course of spreading persistent infection. The information from these studies would offer new avenues for clinical features as well as basic research in HIV-1.

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Vpx Is Critical for Reverse Transcription of the Human Immunodeficiency Virus Type 2 Genome in Macrophages[▽]

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The abilities of wild-type and *vpx*-defective human immunodeficiency virus type 2 (HIV-2) clones to synthesize viral DNA in human monocyte-derived macrophages (MDMs) and lymphocytic cells were comparatively and quantitatively evaluated. While the *vpx*-defective mutant directed the synthesis of viral DNA comparably to the wild-type virus and normally in lymphocytic cells, no appreciable viral DNA was detected in MDMs infected with the mutant. To substantiate this finding and to determine whether there is some specific region(s) in Vpx crucial for viral DNA synthesis in MDMs, we generated a series of site-specific point mutants of *vpx* and examined their phenotypes. The resultant five mutants, with no infectivity for MDMs, showed, without exception, the same defect as the *vpx*-defective mutant. Our results here clearly demonstrated that the entire Vpx protein is critical for reverse transcription of the HIV-2 genome in human MDMs.

Viruses of the human immunodeficiency virus type 2 (HIV-2) group carry a *vpx* gene that encodes virion-associated Vpx protein. Vpx is an accessory viral protein and is completely unnecessary and dispensable for virus replication in established cell lines and primary lymphocyte cells prepared from peripheral blood mononuclear cells (10, 21, 25). However, in human monocyte-derived macrophages (MDMs), the *vpx*-defective viruses do not grow at all (6, 20, 21, 25). Because Vpx is specifically incorporated into virions by association with Gag-p6 protein in significant quantities (1, 11, 12, 24), it has been believed that Vpx has a specific and early functional role at the Env-independent postentry replication step. In fact, there have been some articles directly addressing the early function of Vpx in the life cycle of HIV-2. Worthy of note, one report has shown that Vpx is dispensable for reverse transcription of the viral RNA genome but important for nuclear import of the viral preintegration complex in MDMs (6). But in that study (6), a unique simian immunodeficiency virus (SIV) isolated from the sooty mangabey (SIV_{SM}PBj1.9), which causes an acute fatal disease in pig-tailed monkeys (5), was used to determine the defect of *vpx* mutants in simian MDMs. Another paper has described results similar to those mentioned above, obtained for a U937 cell line growth arrested by mimosine treatment (20). Furthermore, in both studies (6, 20), the conclusions were based on the data obtained from rather qualitative PCR analysis. Therefore, quite surprisingly, virtually no studies focusing on the functional role of HIV-2 Vpx in human MDMs with clear and convincing data have been published yet. In this study, we have performed an extensive mutational functional analysis by quantitative assays of HIV-2 Vpx in human MDMs. We demonstrate here, in contrast to the previously published conclusions, that Vpx is critical for reverse transcription of the HIV-2 genome in human MDMs.

We first evaluated the extent of viral DNA synthesis by a *vpx*-defective mutant at the postentry step in human MDMs by using HSC-F cells (3, 4) as a cell control (21). The mutant used was derived from a well-characterized and widely distributed molecular clone (13, 14, 21) for easy scientific comparison. Infection of human MDMs by virus samples from 293T cells (17) transfected with proviral clones was very much inefficient and gave ambiguous data. To obtain reproducible quantitative results, we conducted the assay as follows. Virus samples (pseudotype viruses) were prepared from 293T cells cotransfected with an expression vector of the vesicular stomatitis virus G protein (pCMV-G) (23) and an *env*-defective proviral clone (pGL-Ns) (21) for the wild type (WT) or an *env*- and *vpx*-defective clone (pGL-Ns/St) (21) for the *vpx* mutant and inoculated into HSC-F and MDM cells. On day 2 postinfection, DNAs were prepared from these infected cells and subjected to real-time PCR analysis using appropriate primer pairs to detect the late reverse transcription product (U5/5′-end noncoding region) in the cytoplasm and the two-long terminal repeat (two-LTR) circle in the cell nucleus. As is clear in Fig. 1, a major replication defect in HSC-F cells of the *vpx*-defective mutant was noticed at the nuclear import process of viral DNA, in good agreement with our previous report (21). By contrast, the mutant was unable to synthesize viral DNA in MDMs, as judged by the absence of the late reverse transcription product, indicating that Vpx is crucial for reverse transcription of the viral RNA genome in a cell type-dependent manner. The same experiments were repeated, using MDMs from different individuals, with perfectly reproducible outcomes. These results prompted us to do a systemic mutational analysis of HIV-2 Vpx in MDMs to dissect its function in the virus replication cycle.

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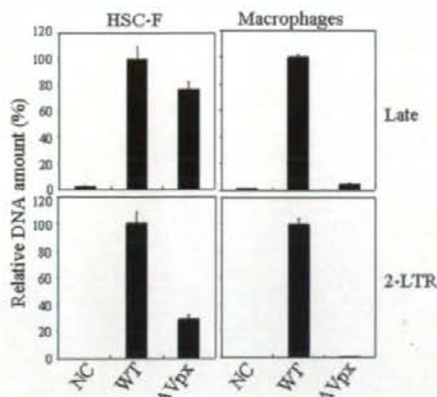

FIG. 1. Quantitative estimation of viral DNA synthesis in HSC-F and human MDM cells infected with the *vpx*-defective mutant. Cell-free virus samples (pseudotype viruses) were prepared from 293T cells cotransfected with 10 μg of pCMV-G and 10 μg of pGL-Ns (WT) or pGL-Ns/St (ΔVpx). For a negative control (NC), pGL-Ns (10 μg) and pUC19 (10 μg) were used for cotransfection. HSC-F cells (1×10^7) and confluent human MDMs, which had been prepared from peripheral blood mononuclear cells and cultured in each well of six-well tissue culture plates as previously described (7, 21) and were 95 to 97% CD68 positive and completely negative for CD3, were infected with equal amounts of these cell-free viruses (4×10^7 reverse transcriptase [RT] units [22] and 1.4 μg of Gag-p27, as determined by enzyme immunoassays of SIV Gag-p27 [Coulter, Miami, FL], for HSC-F and MDM cells, respectively) in the presence of DNase I (40 μg/ml), $MgCl_2$ (10 mM), EGTA (2 mM), and DEAE-dextran (5 μg/ml), as previously described (16). On day 2 postinfection, DNA was extracted from the infected cells and subjected to real-time PCR analysis using TaqMan probes (Applied Biosystems, Foster City, CA) to detect the late reverse transcription product (U5/5′-end noncoding region) in the cytoplasm and the two-LTR circle in the cell nucleus. The β-globin gene was amplified for normalization (19). The primers and probes used were as follows: for the late reverse transcription product (U5/5′-end noncoding region), 5′-TCCGCTTTGGGAATCCAA-3′ (forward primer), 5′-GGGCTTCTCAGTCCCTTTCAA-3′ (reverse primer), and 5′-FAM (6-carboxyfluorescein)-AAAATCCCTAGCAG GTTGGCGCCC-TAMRA (6-carboxytetramethylrhodamine)-3′ (probe); and for the two-LTR circular product (U5/U3 region), 5′-TCGCCGCCT GGTCATT-3′ (forward primer), 5′-CCCTACTGTAAAACATCCCA TCCA-3′ (reverse primer), and 5′-FAM-ACCCTGGTCTGTTAGGA CCCTTCCGC-TAMRA-3′ (probe). The reaction mixtures were heated at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

As shown in Fig. 2, 19 point mutations were introduced into scattered regions of WT *vpx* of an infectious HIV-2 molecular clone designated pGL-AN (13) by using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). To determine the target amino acids for mutation, amino acids that are well conserved among various HIV-2 isolates were carefully selected. In addition, since the 5′ region of *vpx* encoding amino acids 1 to 58 of Vpx was overlapping with *vif*, care was taken not to change the amino acids of WT Vif. The mutants thus constructed were introduced into 293T cells, and all the mutants generated progeny virions at a normal level, with MAGI infectivity (15) comparable to that of the WT virus, as expected (data not shown). Various virus clones were then inoculated into human MDMs, and their growth properties were determined. As shown in Fig. 2, out of 19 mutants, 9 grew more poorly than the WT virus and 5 did not grow at all in MDMs. These results were confirmed in repeated experiments, using MDMs from different individuals. The mutations causing the noninfectious mutants (E15G, W24L, H39L, W49L, and Q76A) were not clustered, suggesting that there may be no specific regions or domains important for virus growth in MDMs.

There was a possibility that the damaged or noninfectious nature of the 14 mutants (Fig. 2) is due to the lack of incorporation of mutant Vpx proteins into virions. Initial attempts to detect the expression of Vpx in transfected 293T cells by Western immunoblotting were mostly unsuccessful, probably because the monoclonal and polyclonal antibodies against Vpx used for detection were insensitive. We therefore constructed a Vpx expression vector with a FLAG tag for the five noninfectious mutants and monitored the transfected 293T cells for mutant Vpx by anti-FLAG antibody. A Vpx protein level comparable to that for the WT clone was detected for each mutant (Fig. 3A). We then determined whether the mutant Vpx proteins were actually detectable in the progeny virions. We previously reported that HIV-1 virion-associated viral proteins can be examined after partial purification and concentration of the virions by ultracentrifugation (8). We applied the same method to monitor Vpx in HIV-2 virions. As controls for this experiment, we newly constructed two Gag-p6 site-specific mutants (designated p6/3AS and p6/2A) from pGL-AN, which have mutated amino acid sequences in Gag-p6 critical for the incorporation of Vpx into virions (1) (Fig. 3B). These two mutants were transfected into 293T cells, and 3 days later, virion samples for Western blot analysis were prepared as described above. As is clear in Fig. 3B, the incorporation of Vpx into virions was not detected at all for the two control mutants, as expected, indicating that the procedure used for HIV-1 can be applicable for the preparation of HIV-2 virions. Mutant virions prepared from transfected 293T cells by this method were then monitored for Vpx. As shown in Fig. 3C, the virions of the P4L (WT growth properties), P10L (intermediate growth properties [between those for WT and noninfectious viruses]), and E15G (noninfectious virus) mutants (Fig. 2) contained Vpx, like those of the WT virus. The presence of Vpx in virions of all the other mutants shown in Fig. 2 was also verified by this Western blot analysis. The percentages of specific virion incorporation of Vpx (Vpx/Gag-p27 ratio, as quantified by immunoblotting [Fig. 3C]) of the five noninfectious mutants (E15G, W24L, H39L, W49L, and Q76A) relative to the WT level were 195 ± 16, 193 ± 14, 28 ± 6, 56 ± 6, and 103 ± 19, respectively. Of note, mutant N33S, having a low level of virion-incorporated Vpx in this assay (14 ± 6), was still somewhat infectious for MDMs (Fig. 2).

We finally evaluated the abilities of the five point mutants noninfectious for MDMs (E15G, W24L, H39L, W49L, and Q76A) to synthesize viral DNA in infected human MDMs. MDMs were infected with the mutants (pseudotype viruses) as described above, and the infected cells were similarly analyzed by real-time PCR using two sets of primer pairs. As shown in Fig. 4, the reverse transcription processes at early and late phases of the five mutants were critically impaired, generating no significant quantities of viral DNA in the cell nucleus. The Q76A mutant appeared less attenuated for reverse transcrip-

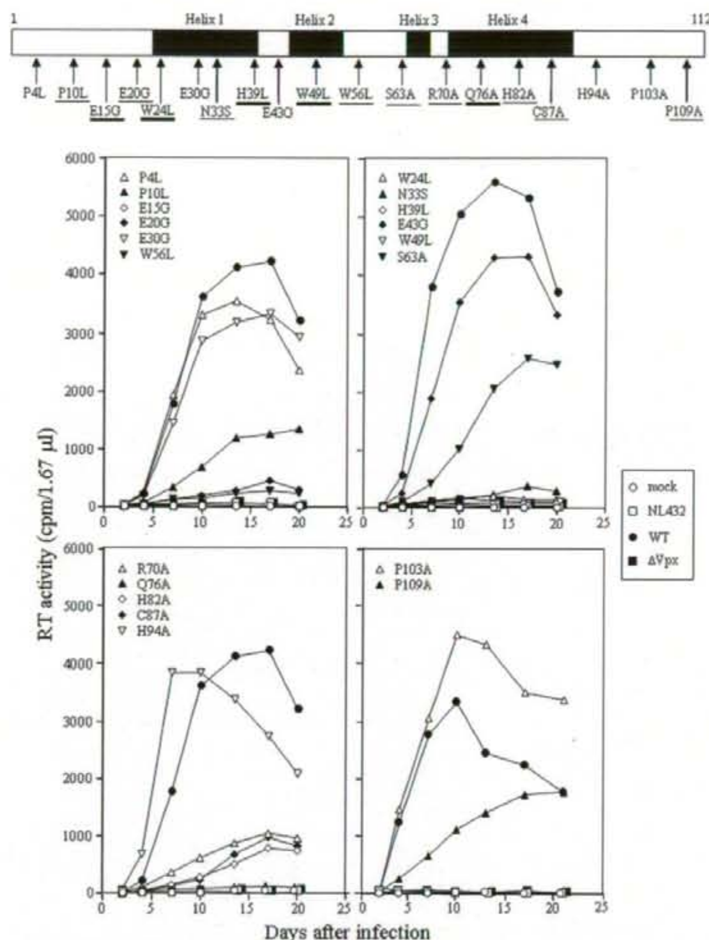

FIG. 2. Growth kinetics in human MDMs of various *vpx* point mutants. Confluent MDMs in each well of 24-well tissue culture plates prepared as described in the legend to Fig. 1 were infected with equivalent numbers of RT units of cell-free viruses (6×10^5) in the presence of DEAE-dextran (5 μg/ml), and viral replication was monitored at intervals by determining RT production in the culture supernatants (22). Input viruses were prepared from 293T cells transfected with 20 μg of pGL-AN, its *vpx* mutants, or the HIV-1 infectious clone pNL432 (2) as a negative control. At the top, the locations of the point mutations in pGL-AN Vpx, consisting of 112 amino acids (GenBank accession no. M30895) with four predicted helices (14), and the standard designations of the *vpx* mutants are indicated. The noninfectious and growth-defective mutants are indicated by bold and thin underlines, respectively. Mock, pUC19; WT, pGL-AN (13); ΔVpx, pGL-St (13).

tion than the other four mutants. The experiment for Fig. 4 was repeated extensively, with reproducible results.

Based on the results described above, we concluded that Vpx is crucially required for reverse transcription of the HIV-2 RNA genome in human MDMs. We also claim here, by our mutational analysis, that a specific region or domain(s) in Vpx may not be responsible for the Vpx activity shown in this report; rather, the entire structure of Vpx is important. Of the five mutations that completely abrogate viral infectivity in MDMs, H39L and W49L might affect the stability of mutant proteins and give the phenotype shown in Fig. 2 and 4. However, this was quite unlikely, because the mutant proteins were stably expressed in cells by a FLAG tag expression vector (Fig. 3). Furthermore, the N33S mutant, which contains a smaller amount of Vpx in virions than the H39L and W49L mutants, still retained viral infectivity. In any case, our main conclusion, that the overall structure of Vpx is crucial for reverse transcription of the HIV-2 genome in human MDMs, is unchanged. Whether inactive or defective mutants other than the five noninfectious mutants shown in Fig. 2 display the defect in nuclear import of viral DNA is another intriguing question to address, and this needs to be determined. Determination of the subcellular localizations of these mutant Vpx proteins could explain their biological differences, if there are any.

Our results described in this report are quite distinct from those previously published (6, 20) but not inconsistent. Clearly,

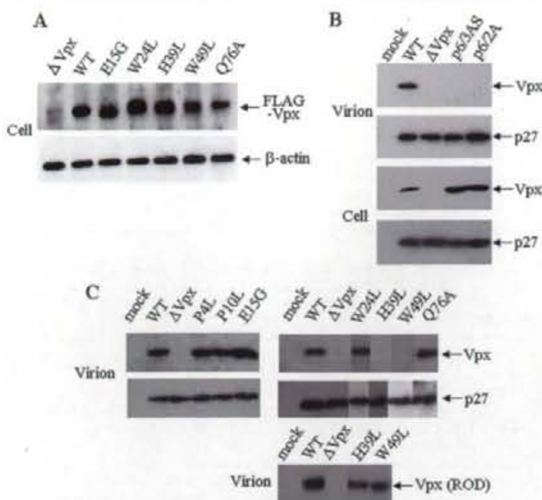

FIG. 3. Immunoblot analysis of mutant Vpx proteins in cells and virions. 293T cells were transfected with 10 μg of an expression vector of WT Vpx designated pME18Neo-Fvpx (14) and its mutants or 20 μg of WT proviral clone pGL-AN (13, 21) and its mutants, as previously described (2), and on day 2 or 3 posttransfection, cell or virion lysates were prepared as previously described (8, 22). The lysates normalized by β-actin or RT activity were then analyzed by Western immunoblotting (7, 8, 14) with the HIV-2 Vpx monoclonal antibody 6D2.6 (Vpx) (NIH AIDS Research and References Reagent Program, catalog no. 2710), an HIV-2 ROD Vpx polyclonal antibody (ROD) (catalog no. 2609), and an antiserum to SIV-p27 (p27) (NIBSC Centralised Facility for AIDS Reagent, repository reference no. ARP414). Commercially available monoclonal antibodies were used for detection of FLAG-Vpx (ANTI-FLAG M2; Sigma-Aldrich, St. Louis, MO) and β-actin (anti-β-actin clone AC-15; Sigma-Aldrich). The results obtained for the mutant Vpx proteins in cells, for the Vpx-defective-virion mutants as predicted by their Gag-p6 amino acid sequences (p6/3AS and p6/2A), and for the mutant Vpx proteins in virions are shown in panels A, B, and C, respectively. Gag-p6 mutants designated p6/3AS and p6/2A have A^{17}, S^{19}, A^{22}, and A^{23} (instead of D^{17}, A^{19}, L^{22}, and L^{23}, respectively) and A^{22} and A^{23} (instead of L^{22} and L^{23}, respectively) in the Gag-p6 amino acid sequence. Because the monoclonal antibody 6D2.6 did not react with the H39L and W49L mutant proteins, the polyclonal antibody was used to detect them as shown in panel C. Mock, pUC19.

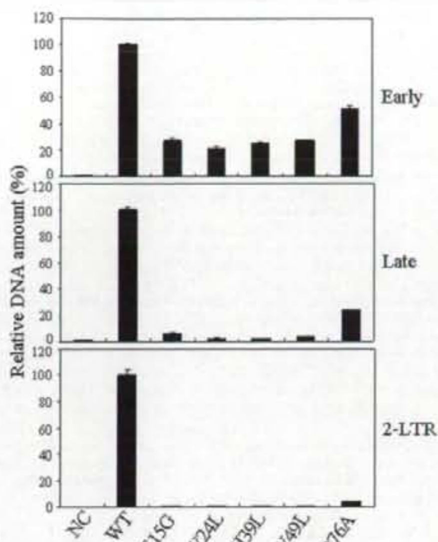

FIG. 4. Quantitative estimation of viral DNA synthesis in human MDMs infected with *vpx* point mutants. Pseudotype viruses were prepared by transfection and inoculated into human MDMs as described in the legend to Fig. 1. The procedures for real-time PCR analysis for Fig. 1 were also used, but the early reverse transcription product (R/U5 region) was additionally monitored here. The primers and probe for the early reverse transcription product (R/U5 region) were as follows: 5′-CAAGTTAAGTGTGTGTTCCCATCTCT-3′ (forward primer), 5′-CCAGGGTCTTGTTATTCAGATGAA-3′ (reverse primer), and 5′-FAM-CTAGTCGCCGCCTGGTCATTCGG-TAMRA-3′ (probe).

we and they have used different experimental systems, including different methods for infection, virus clones, cell types, and methods for analysis of viral DNAs. Therefore, the data obtained could be different. Interestingly, one report has described reproducible reductions in the abundances of reverse transcription products in MDMs infected with *vpx*-defective mutants (6). In agreement with this and our results here, it has recently been demonstrated that Vpx of SIV_{MAC} of the HIV-2 lineage plays an essential role for the reverse transcription process in human dendritic cells (9).

Determination of the molecular basis underlying the macrophage-specific requirement of Vpx for reverse transcription of the viral genome is virologically very important. In this regard, two recently published articles are quite provocative. Goujon et al. reported that Vpx may counteract a restriction factor present in human dendritic cells to escape the proteasome-mediated degradation pathway (9). Le Rouzic et al. showed that Vpx binds to DCAF1/VprBP, an adaptor molecule of the ubiquitin ligase complex (18). These findings have raised the possibility that there is a proteasome-dependent factor(s) in a certain cell type that suppresses reverse transcription. It is not unreasonable to assume that HIV-2 Vpx antagonizes such a factor, thus efficiently promoting viral replication. In addition, it has been well established that innate antiretroviral factors, such as TRIM5α and APOBEC3G/F, target the step of viral DNA synthesis. The association of HIV-2 Vpx with the reverse transcription process of the viral RNA genome needs to be biochemically proved to clarify the early events of HIV-2 replication precisely.

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