ORIGINAL

Morphological study on biologically distinct *vpx/vpr* mutants of HIV-2

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Abstract : We have previously shown that human immunodeficiency virus type 2 (HIV-2) without functional *vpx* and *vpr* genes is severely defective for viral growth in lymphocytic cells, and suggested that the virions produced in the absence of Vpx and Vpr are critically damaged. To examine the nature of replication-defect for the *vpx/vpr* double mutant, we quantitatively and morphologically studied the virions produced in cells transfected or infected with wild type clone, single (*vpx* and *vpr* mutants) or the double mutant. While no significant difference in virion production was found for various virus clones in transfected cells, a major growth retardation in infected cells was readily observed for the *vpx* and *vpx/vpr* mutants. In particular, no viral growth was detected for the double mutant. By contrast to the very distinct growth characteristics of the three mutant clones, no appreciable difference in virion morphology was noted. These results indicated that Vpx and Vpr of HIV-2 may cooperatively contribute to virion infectivity without affecting virion morphogenesis. J. Med. Invest. 53 : 271-276, August, 2006

Keywords **:** *HIV-2, accessory proteins, Vpx, Vpr*

INTRODUCTION

All human and simian immunodeficiency viruses (HIVs and SIVs) isolated so far contain a unique set of accessory genes in their genomes. HIV type 2 (HIV-2) and some of SIVs, such as SIVmac isolated from rhesus monkeys, carry a *vpx* gene in addition to *vpr* (1). Both *vpx* and *vpr* are required for SIVmac to grow optimally in lymphocytic cells (2), and cause AIDS efficiently in monkeys (3, 4). The *vpx* and *vpr* encode small proteins of approximately 100 amino acids which are specifically incorporated into viral particles (5, 6).

We have previously demonstrated that, in lymphocytic cells, the replication of single *vpx* mutant, but not *vpr*, was impaired, and that of a *vpx-vpr* double mutant was more severely damaged (2). Defective replication sites of the *vpx* single and *vpx-vpr* double mutants were shown to be mapped, respectively, to the nuclear import of viral genome and to both of the nuclear import and virus assembly/release steps. While the mutational effect of *vpr* was rather small, the replication efficiency in one cycle of the *vpx* mutant relative to that of wild-type (wt) virus was estimated to be 10%. Without the *vpx* and *vpr*, the virus replication was negligible. These results have raised a possibility that Vpx and Vpr play an important role(s) for the release and maturation of fully-infectious viral particles. In this report, we have examined the

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level of progeny virion production in transfected and infected cells, and the virion morphology in those cultures by extensive electron microscopic observation.

MATERIALS AND METHODS

Cells

A monolayer cell line 293T (7) was maintained in Eagles's minimal essential medium containing 10% heat-inactivated fetal bovine serum as previously described (8). A lymphocytic cell line HSC-F (9, 10) was maintained in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum as previously described (8).

Transfection

293T cells were transfected by the calcium-phosphate co-precipitation method as previously reported (8).

Infection

HSC-F cells were infected with cell-free viruses prepared from transfected 293T cells as previously described (11).

Reverse transcriptase (RT) assay

RT assay using ^{32}P-dTTP has been previously described (12).

DNA constructs

An infectious DNA clone of HIV-2 designated pGL-AN has been previously described (2, 13, 14). Proviral mutant clones of pGL-AN designated pGL-St (*vpx* frame-shift mutant, ΔVpx), pGL-Ec (*vpr* frame-shift mutant, ΔVpr), and pGL-St/Ec (*vpx-vpr* double mutant, ΔVpx/ΔVpr) have also been previously described (2, 13, 14).

Electron microscopy (EM)

For transfected 293T cells, fixation and embedding were performed according to the method described previously (15, 16). Infected HSC-F cells were centrifuged at 250 x g and the resultant cell pellets were then washed with phosphate buffered saline before fixation. During the fixation with glutaraldehyde, the pellets were centrifuged again at 1,200×g to make the cells more compactly packed. Thereafter, the samples were treated according to the original procedure. The cells embedded in LUVEAK-812 (Nakalai Tesque, Inc., Kyoto, Japan) were cut into ultrathin sections using a REICHERT-JUNG ULTRACUT E ultramicrotome, doubly stained with uranyl acetate and lead citrate, and examined under a JEOL JEM-1200EX II transmission electron microscope.

RESULTS AND DISCUSSION

Characteristics of virions produced in transfected cells

The human 293T cell line has been frequently and widely used to prepare stocks of various HIV/SIVs because of its high susceptibility to transfection. Furthermore, 293T cells are CD4-negative, and the late phase of virus replication can be easily assessed by monitoring RT production in the culture supernatants. Various proviral clones designated pGL-AN (wt), pGL-St (ΔVpx), pGL-Ec (ΔVpr) and pGL-St/Ec (ΔVpx/ΔVpr) were transfected into 293T cells, and virus production was determined on day 2 post-transfection. As shown in Fig.1, all the clones tested here gave similar results upon transfection. Mutations in *vpx*, *vpr* or both did not affect significantly the ability of the virus to release progeny virions as monitored by RT assay. The mutant virions produced in transfected 293T cells were functionally normal since they all exhibited similar infectivity to M8166 cells (data not shown), which are permissive for any *vpx* mutants (2, 13). As shown in Fig. 2, the virions

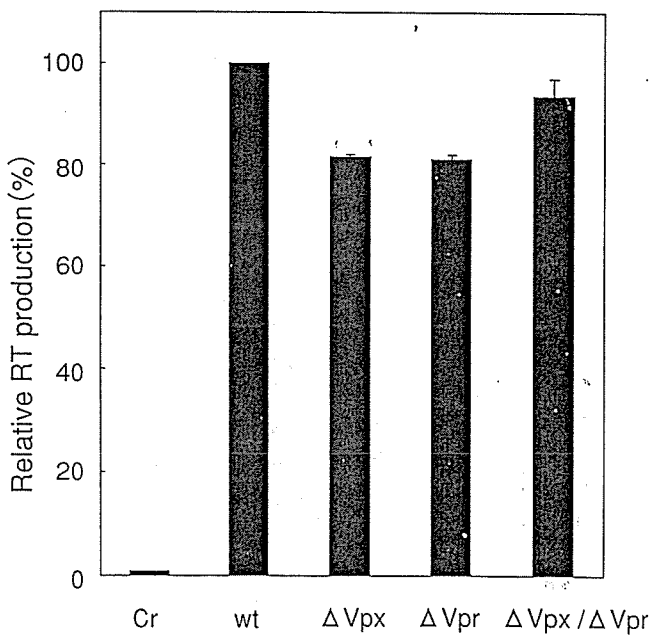

Fig. 1 Progeny virion production in 293T cells transfected with Vpx/Vpr mutant clones. Cells were transfected with various proviral clones indicated, and on day 2 post-transfection, virus production was determined by RT assay. RT production relative to that of wt clone is shown.

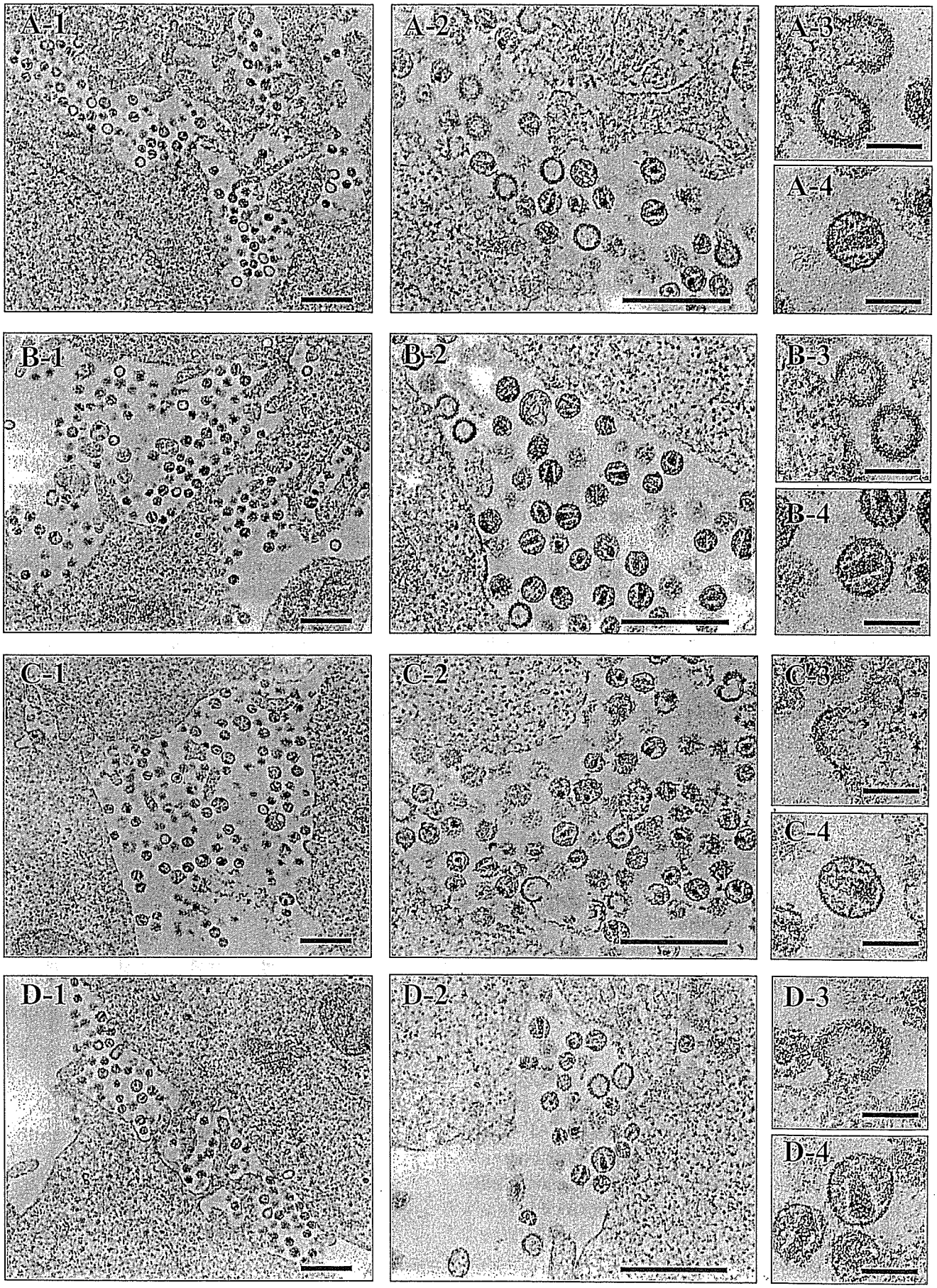

Fig. 2 EM analysis of Vpx/Vpr mutant virions produced in transfected 293T cells. Cells on day 2 post-transfection in Fig.1 were subjected to EM analysis as described in MATERIALS AND METHODS. Samples:A-1 to A-4, wt; B-1 to B-4, Δ Vpx;C-1 to C-4, Δ Vpr; D-1 to D-4, Δ Vpx/Δ Vpr. Bars : 500nm for A-1 & A-2 to D-1 & D-2 ; 100nm for A-3 & A-4 to D-3 & D-4.

in the cultures were then examined for their morphology by EM. No significant difference of virion morphology was seen among wt and the mutants (A-1 & A-2 to D-1 & D-2). Budding of virions from cells (A-3 to D-3 at a high magnification), immature virions (B-3 at a high magnification), and mature virions with a cone-shaped core (A-4 to D-4) were similary observed.

Characteristics of virions produced in infected cells

We recently reported that a monkey lymphocytic cell line designated HSC-F behaved exactly like primary human lymphocytes for mutant viruses of HIV-2 (2). We were interested in comparing the morphology of the mutants produced in infected HSC-F cells. Cell-free virus samples were prepared from 293T cells transfected with various clones as above, and an equal amount as determined by RT assay was inoculated into HSC-F cells. As shown in Fig. 3, whereas ΔVpr grew equally well with wt virus, ΔVpx displayed a very retarded growth pattern. The double mutant ΔVpx/ΔVpr did not grow significantly during the observation period. The cultures on day 11 post-infection were then subjected to extensive EM analysis. As shown in Fig. 4, the number of virions produced in each culture was significantly different. Cells infected with wt virus or ΔVpr generated a large number of virions (A-1 & A-2 and C-1 & C-2) but those with ΔVpx yielded a relatively small number of progenies (B-1 & B-2). Progeny virions of ΔVpx /ΔVpr were seen only rarely (D-1 & D-2). These observations were in good agreement with the results in Fig. 3. In contrast to the production level of progeny virions, no remarkable difference was noticed for virion morphology among wt and mutant viruses. Immature (A-3 to D-3 at a high magnification) and mature virions (A-4 to D-4 at a high magnification) were seen in all cultures examined.

Conclusion

Our results described here indicated that Vpx and Vpr of HIV-2 do not affect virion morphogenesis appreciably both in permissive (293T) and non-permissive (HSC-F) cells for ΔVpx. In the absence of Vpx and Vpr, the virus almost lost its infectivity (Fig. 3) but still retained the intact structure as a virion (Fig. 4). We have recently demonstrated, by homology modeling, that HIV-2 Vpx and Vpr are structurally very similar (1). This may account for the cooperative function of Vpx and Vpr. The biological and molecular basis needs to be experimentally determined.

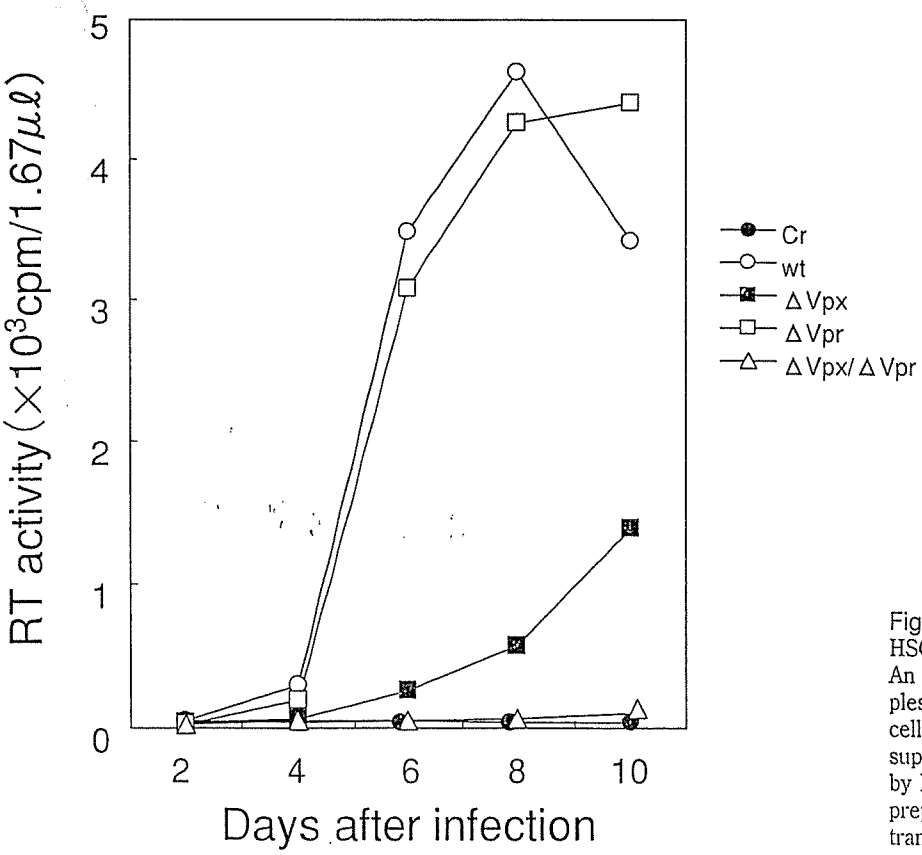

Fig. 3 Growth kinetics in lymphocytic HSC-F cells of Vpx/Vpr mutant viruses. An equal amount of cell-free virus samples indicated was inoculated into HSC-F cells, and virus production in the culture supernatants was monitored at intervals by RT assay. Input cell-free viruses were prepared from 293T cells on day 2 post-transfection.

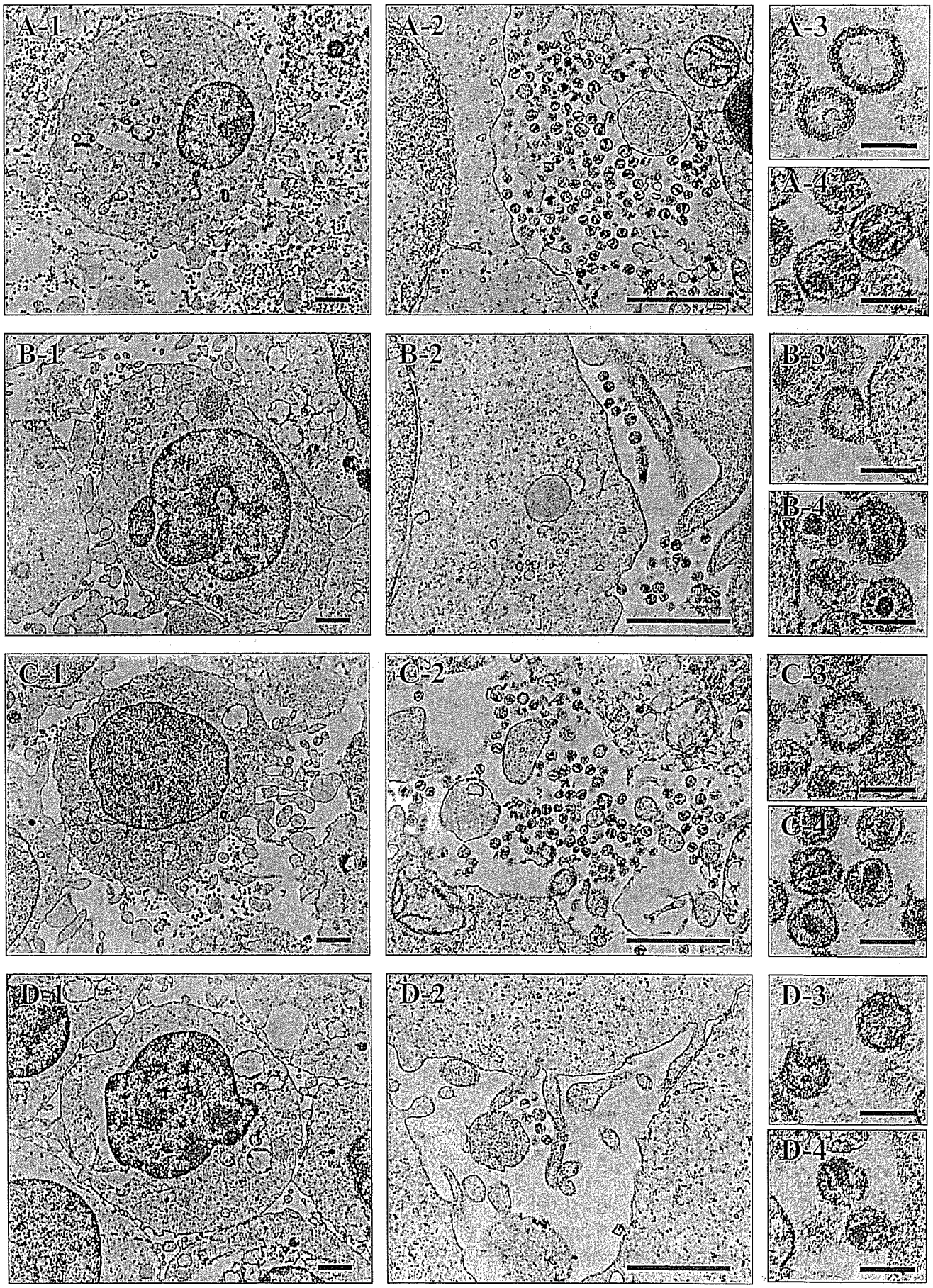

Fig. 4 EM analysis of Vpx/Vpr mutant virions produced in infected HSC-F cells. Cells on day 11 post-infection in Fig.3 were subjected to EM analysis as described in MATERIALS AND METHODS. Samples : A-1 to A-4, wt ; B-1 to B-4, Δ Vpx ; C-1 to C-4, Δ Vpr ; D-1 to D-4, ΔVpx/ΔVpr. Bars : 1μm for A-1 & A-2 to D-1 & D-2 ; 100nm for A-3 & A-4 to D-3 & D-4.

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Original article

Construction of *gag*-chimeric viruses between HIV-1 and SIVmac that are capable of productive multi-cycle infection

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Abstract

Forty-nine recombinant viral clones between human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus from the rhesus monkey (SIVmac), which carry chimeric *gag* (capsid/p2 region) genes in the background of the HIV-1 genome, were constructed to establish an HIV-1/monkey infection model system for human AIDS. Upon transfection, all the recombinants generated progeny virions at a level comparable to the parental HIV-1 clone and no major abnormalities were found in the virions, as examined by Western blot analysis. In infection experiments, 18 recombinants grew in human lymphocytic cells and six of these clones propagated as well as the parental virus, as monitored by virion associated-reverse transcriptase production. By contrast, none of the recombinants grew at a detectable level in monkey lymphocytic cells. The defective replication site(s) in human cells for non-infectious recombinants was mapped to the step before and/or during reverse transcription. Our results described here showed that HIV-1 type chimeric viruses between HIV-1 and SIVmac, which are capable of spreading productive infection, are readily constructed throughout the capsid/p2 region. In addition, it is suggested that there may be a viral determinant(s), other than Gag, responsible for the species-specific tropism of HIV-1 and which is associated with viral DNA synthesis.

Keywords: HIV-1; SIVmac; Gag; Capsid/p2; Chimeric virus

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) has been shown to have a much narrower host range than simian immunodeficiency viruses (SIVs), such as SIVmac [1]. This species-specific tropism of HIV-1 (tropism for humans and chimpanzees) has hindered the development of effective model systems for basic AIDS study. Early works have demonstrated that the non-*env* sequence is critical for the species tropism [2,3]. While SIVmac grows well both in human and simian lymphocytes, HIV-1 does not replicate in the latter cells, and the major viral determinant(s) for this restriction is most likely to be the Gag capsid (CA)-p2 region of HIV-1 [2,4–7]. Furthermore, mutations in *gag* can affect the cellular tropism of HIV-1. Some *gag* mutant viruses, with a postentry early defect in some human lymphocytic cells, were shown to grow well in others [8–11]. On the basis of these studies, it is quite likely that the early function of Gag, i.e., uncoating and/or reverse transcription, is involved in the restriction of HIV-1 growth in monkey cells. By extensive genetic and molecular analyses, recent studies have clearly indicated that Gag-CA is associated with the postentry early replication block of HIV-1 in monkey cells [12–14].

To develop a new and effective model of HIV-1 infection in practically useful non-human primates, recombinant viruses between HIV-1 and SIVmac in an HIV-1 background are critically required. In this report, various sequences in the SIVmac CA-spacer domain were inserted into the corresponding regions of HIV-1 to generate HIV-1-based *gag*-chimeric viruses. Forty-nine recombinants thus constructed were examined for their ability to grow in human and simian lymphocytic cell

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Table 1
SIVmac amino acid sequences inserted into HIV-1 capsid-p2 region

Recombinants	Amino acid sequences of MA239 inserted
CS2/15	(2)VQQIGGNYVHLPL(15)
CS2/15–86/122	(2)VQQIGGNYVHLPL(15) (86)PAPQQGQLREPSGSDIAGTTSSVDEQIQWM YRQQNPI(122)
CS2/15–110/ 112–119/122	(2)VQQIGGNYVHLPL(15) (110)EQI(112) (119)YRQQN(122)
CS2/15–110/122	(2)VQQIGGNYVHLPL(15) (110)EQIQWMYRQQNPI(122)
CS5/15	(5)IGGNYVHLPLS(15)
CS9/15	(9)YVHLPLS(15)
CS13/15	(13)PLS(15)
CS26/27	(26)IE(27)
CS26/100	(26)IEEKKFGAEVVPGFQALSEGCTPYDINQMLNC VGDHQAAMQIIRDIINEEAADWDLQHPQPAPQQ GQLREPSGSD(100)
CS26/149	(26)IEEKKFGAEVVPGFQALSEGCTPYDINQML NCVGDHQAAMQIIRDIINEEAADWDLQHPQ PAPQQ GQLREPSGSDIAGTTSSVDEQIQW MYRQQNPIPV GNIYRRWIQLGLQKC VRMYNPTNIL(149)
CS31/34	(31)FGAE(34)
CS37/47	(37)PGFQALSEGCT(47)
CS39/47	(39)FQALSEGCT(47)
CS47	(47)T(47)
CS47/52	(47)TPYDIN(52)
CS47/54	(47)TPYDINQM(54)
CS58/61	(58)VGDH(61)
CS68/72	(68)IRDII(72)
CS70/72	(70)DII(72)
CS79/100	(79)WDLQHPQPAPQQGQLREPSGSD(100)
CS86/93	(86)PAPQQGQL(93)
CS86/100	(86)PAPQQGQLREPSGSD(100)
CS86/112–119/122	(86)PAPQQGQLREPSGSDIAGTTSSVDEQI(112) (119)YRQQN(122)
CS86/122	(86)PAPQQGQLREPSGSDIAGTTSSVDEQIQWMYR QQNPI(122)
CS110/112–119/122	(110)EQI(112) (119)YRQQN(122)
CS110/122	(110)EQIQWMYRQQNPI(122)
CS110/149	(110)EQIQWMYRQQNPIPVGNIYRRWIQLGLQKCV RMYNPTNIL(149)
CS119/122	(119)YRQQN(122)
CS128/131	(128)YRRW(131)
CS135	(135)G(135)
CS139/141	(139)CVR(141)
CS146/149	(146)TNIL(149)
CS153/154	(153)QG(154)
CS153/215	(153)QGPKEPFQSYVDRFYKSLRAEQTDAA VKNWMTQTLLIQNANPDCKL VLKGLGVNPTLEEMLTA(215)
CS162/163	(162)YV(163)
CS171	(171)R(171)
CS177/180	(177)AAVK(180)
CS187	(187)L(187)
CS191	(191)N(191)
CS200/201	(200)LK(201)
CS204	(204)G(204)
CS207/209	(207)PTL(209)
CS215	(215)A(215)
CS226	(226)A(226)
CS230/231	(230)AE(231)
CS235/240	(235)EALAPV(240)
CS235/245	(235)LKEALAPVPI(245)
CS235/245-f	(235)LKEALAPVPIPFAA(245)
CS238/240	(238)APV(240)

Amino acid sequences in CA-p2 of NL432 (HIV-1) were replaced with those of MA239 (SIVmac) as shown. The first and last amino acid nos. of NL432 sequences replaced are indicated in parentheses. GenBank accession nos. for pNL432 and pMA239 are AF324493 and M33262, respectively. For schematic representation of the recombinants, see Fig. 1.

lines. We show here that 18 recombinant viruses are growth-competent in human but not at all in simian cells. Characterization of these viruses may contribute to the design of a new HIV-1 that optimally escapes the early replication block in monkey cells.

2. Materials and methods

2.1. Plasmids

The full-length infectious molecular clones of HIV-1, HIV-2 and SIVmac, designated pNL432 [15], pGL-AN [16] and pMA239 [2], respectively, have been described previously. An *env*-minus mutant clone of pNL432, pNL-Kp, has also been described [17]. Various *gag*-chimeric clones (Fig.1 and Table 1) were constructed from pNL432 by the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA), as previously described [18,19]. Chimeric clones, designated pNL-CS26/100, pNL-CS110/149, pNL-CS26/149 and pNL-CS86/93, in this report were previously referred to as pNL-SC1, pNL-SC2, pNL-SC3 and pNL-CAi2, respectively [20]. The GenBank accession nos. for pNL432 and pMA239 are AF324493 and M33262, respectively.

2.2. Cells, transfection and infection

A human kidney cell line, 293T [21], was cultured in Eagle's minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum. Human and simian lymphocytic cell lines, M8166 [2] and HSC-F [22], respectively, were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum. For transfection of the 293T cells, the calcium-phosphate coprecipitation method was used as previously described [15]. Infection of M8166 and HSC-F cells to monitor viral growth kinetics was performed essentially as previously described [23].

2.3. Reverse transcriptase (RT) assay

RT assay using ^{32}P-dTTP was carried out as previously described [24].

2.4. Western immunoblot analysis

Cell and virion lysates were prepared from transfected 293T cells as previously described [20,25,26], and were

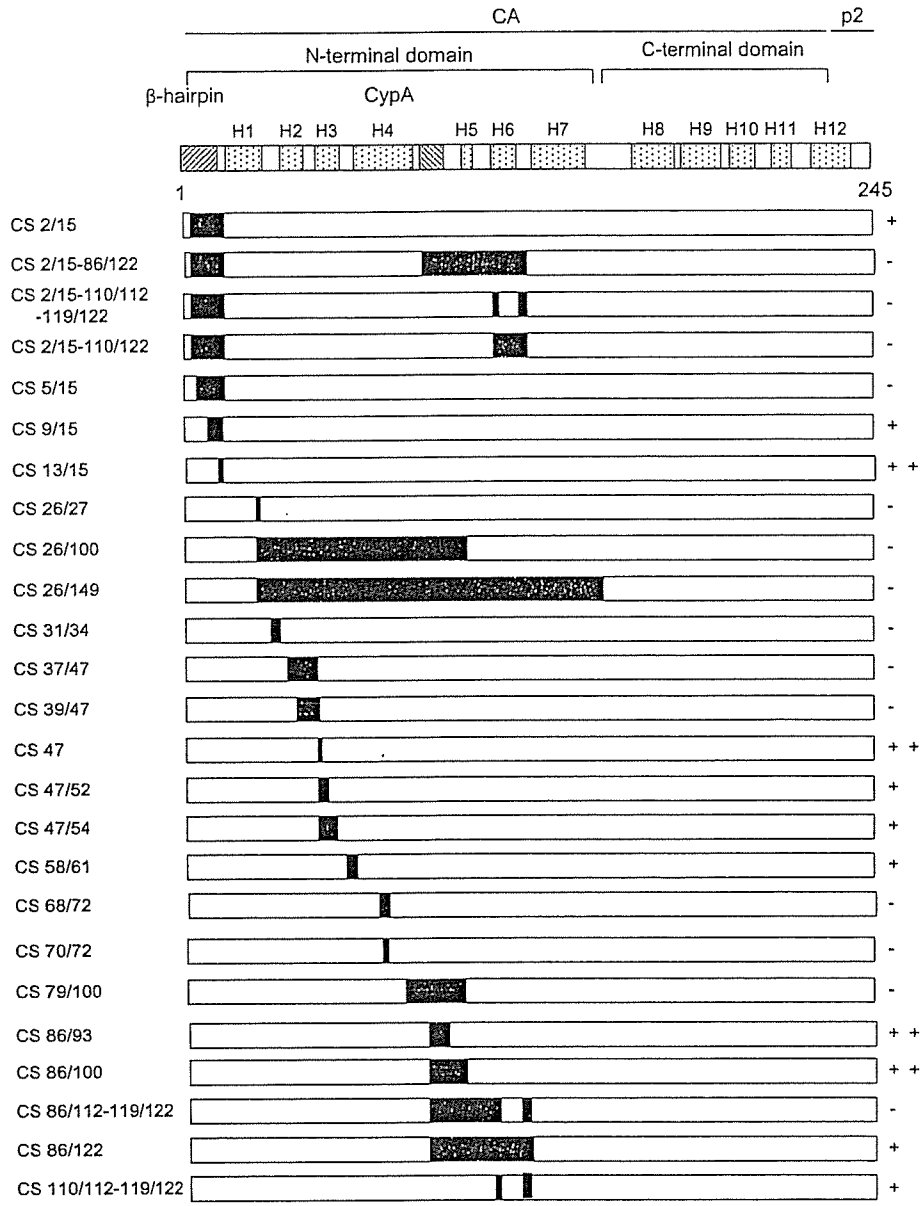

Fig. 1. *Gag*-chimeric viruses between HIV-1 and SIVmac used in this study. Location of SIVmac Gag sequence (MA239) inserted into HIV-1 Gag CA-p2 region (NL432) is indicated by black area. For the sequences inserted, see Table 1. Growth ability of viruses in M8166 cells is given as ++ (wt growth), + (retarded growth), and − (no growth) on the right. For examples of virus growth kinetics, see Fig. 2. Structural domains of HIV-1 Gag CA-p2 [29,30] are indicated at the top. H, α-helix; Cyp A, cyclophilin A-binding loop.

subjected to Western blot analysis with a human anti-HIV-1 antiserum as reported previously [19].

2.5. Polymerase chain reaction (PCR) analysis

M8166 cells were infected with an equal amount of cell-free virus samples from transfected 293T cells for 16 h in the presence of EGTA/DNase I [11,27]. On day 2 post-infection, cells were harvested for DNA extraction as previously described [27]. To monitor viral DNA synthesis in cells, DNA samples were PCR-amplified and analyzed essentially as previously described [27]. For the amplification of viral DNA, the early (R/U5) and late (U5/5′-non-coding region) primer pairs [27] were used. As a control for PCR, β-globin was amplified as previously described [16,28].

3. Results

3.1. Construction and biological characterization of gag-chimeric clones

We have recently shown that the transfer of a minute region of SIVmac CA to the corresponding region of HIV-1 could confer the cyclophilin A-independent replication potential of

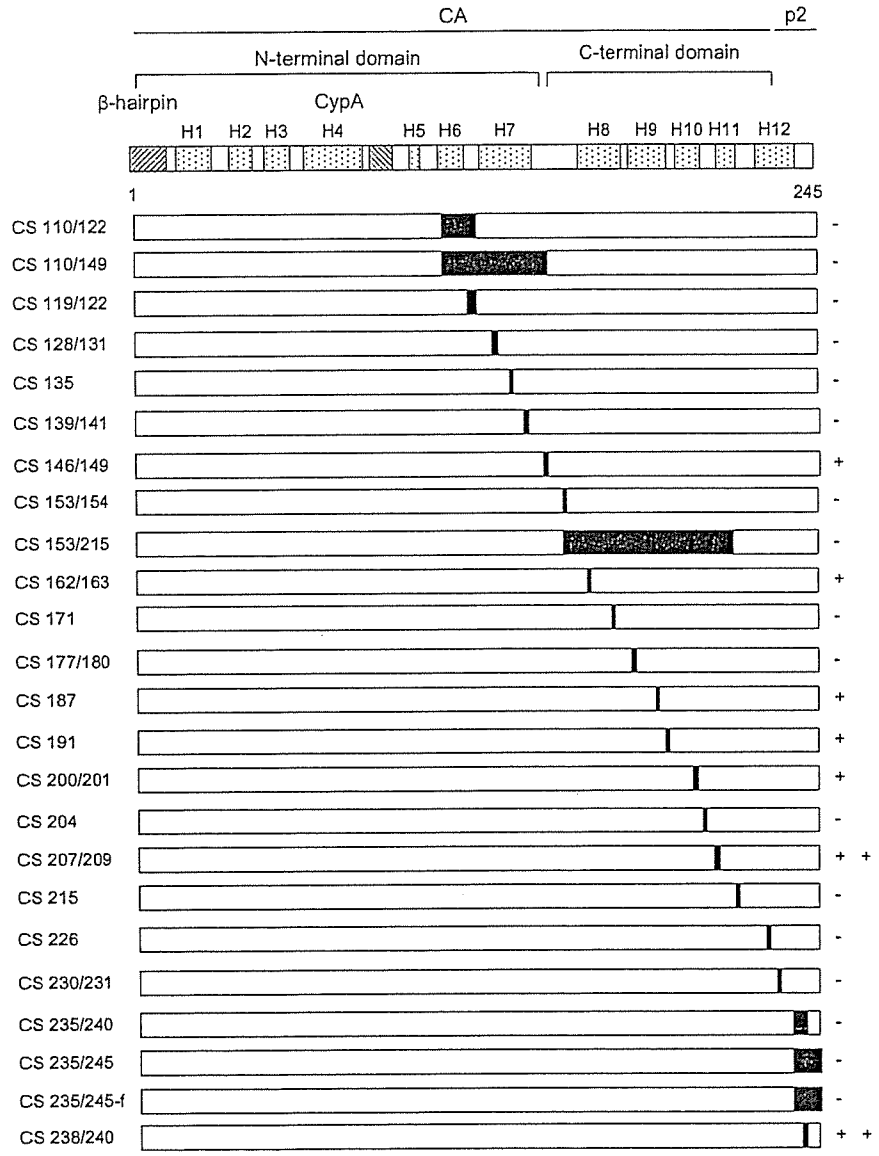

Fig. 1 (*continued*).

SIVmac on the virus [20]. However, the replication-competent virus, designated NL-CAi2, did not grow at all in monkey HSC-F cells [20]. These results prompted us to construct and characterize *gag* (CA-p2)-hybrid viruses more extensively. Structural analyses have already revealed the unique features of HIV-1 CA and provided a model for the intact protein [29,30]. Based on these findings, we introduced SIVmac *gag* sequences into all functionally important domains of HIV-1 CA-p2 as small or large insertions. As shown in Fig. 1, 49 recombinants in total were finally constructed.

Upon transfection into 293T cells, all the recombinants (Fig. 1) produced progeny virions at a level comparable to the wt clone (40–120%), as judged by RT production. These results indicated that the recombinants may have no major late replication defects in cells. We then asked whether these recombinants display multi-cycle infectivity in human and simian cells. All the viruses (5×10^6 RT units of each) prepared from transfected 293T cells were inoculated into human lymphocytic M8166 cells (1×10^6 cells), and their growth kinetics were determined. Representative growth properties in the cells of the recombinants are shown in Fig. 2, and all the data obtained from the infectivity assay are summarized in Fig. 1. Out of the 49 recombinants constructed, 18 (CS2/15, CS9/15, CS13/15, CS47, CS47/52, CS47/54, CS58/61, CS86/93, CS86/100, CS86/122, CS110/112-119/122, CS146/149, CS162/163, CS187, CS191, CS200/201, CS207/209 and CS238/240) were found to be infectious toward M8166 cells. Six of the 18 clones (CS13/15, CS47, CS86/93, CS86/100, CS207/209 and CS238/240) grew similarly well to the wt virus. These results showed that SIVmac *gag* sequences can be inserted into various parts of the corresponding HIV-1 CA-p2 region without abolishing the infectivity of the virus. However,

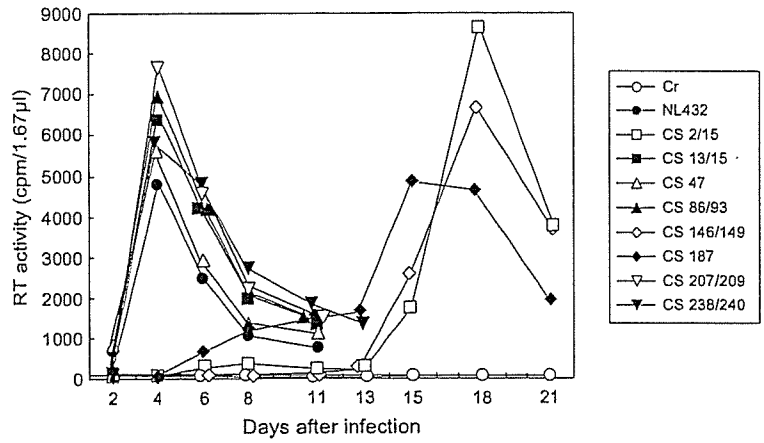

Fig. 2. Growth kinetics in M8166 cells of chimeric viruses. Cells were infected with cell-free viruses as described in the text, and virus replication was monitored at intervals by RT production in the culture supernatants. Input viruses were prepared from 293T cells transfected with 20 μg of the clones indicated on the right. Cr, pUC19.

almost all recombinants carrying an insertion in the α-helix grew poorly or not at all (Fig. 1). We then inoculated all the recombinants (1×10^7 RT units for each) into monkey lymphocytic HSC-F cells (1×10^7 cells) using SIVmac prepared from 293T cells transfected with pMA239 [2] as a positive control. No recombinants were found to be infectious for HSC-F cells (data not shown).

3.2. Biochemical characterization of gag-chimeric clones

Fourteen recombinants were selected and examined for their biochemical properties in cells. These included non-infectious (CS5/15, CS39/47, CS86/112-119/122, CS153/154, CS204 and CS235/245-f), poorly infectious (CS9/15 and CS146/149) and highly infectious (CS13/15, CS47, CS86/93, CS86/100, CS207/209 and CS238/240) clones for M8166 cells. The insertion sites of SIVmac *gag* sequences in these recombinants are located throughout the CA-p2 region of HIV-1 (Fig. 1).

First, the Gag expression in cells and the Gag profile in virions were confirmed. Because RT production in 293T cells transfected with the recombinants was fairly normal, no major defects were expected to be observed. 293T cells were transfected with various clones, and on day 2 post-transfection, cells were harvested for Western blot analysis. As shown in Fig. 3, no clear abnormality was seen for the recombinants tested, except CS235/245-f. Consistent with the insertion

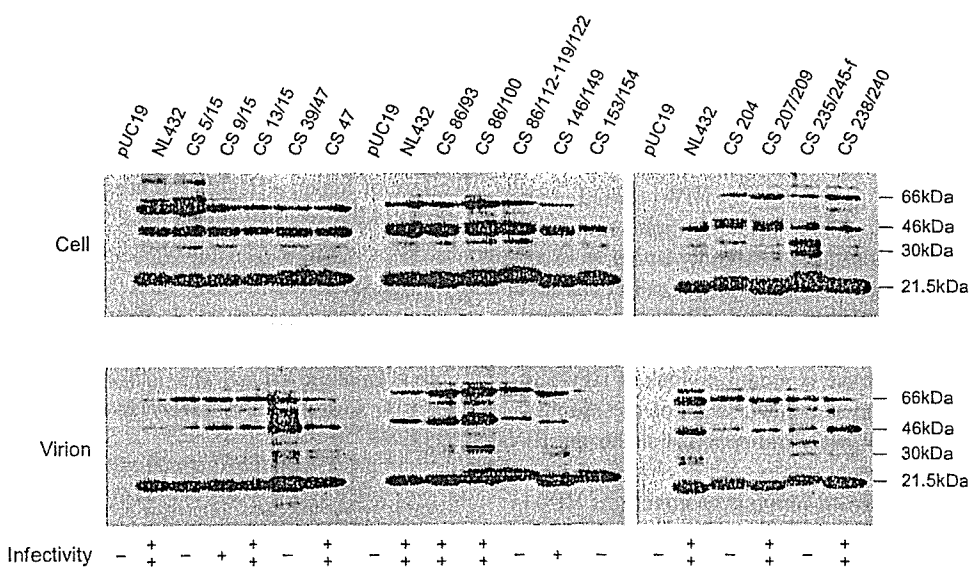

Fig. 3. Western blot analysis of chimeric viruses. Cell and virion lysates were prepared from 293T cells transfected with various clones (20 μg) indicated at the top, as described previously [20,25,26]. Each sample was then subjected to Western blot analysis using a human anti-HIV-1 antiserum as reported before [19]. Results obtained from three independent experiments are shown. The infectivity of viruses for M8166 cells is given as ++ (wt growth), + (retarded growth) and − (no growth) at the bottom. Protein size is shown on the right. Cell, lysates from transfected cells; virion, virion lysates.

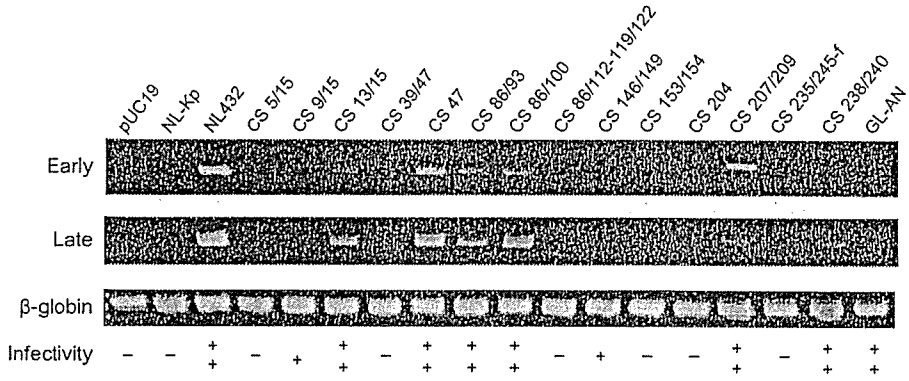

Fig. 4. PCR analysis of chimeric viruses. M8166 cells were infected with cell-free viruses, as described in the text. Input viruses were prepared from 293T cells transfected with 20 μg of the clones indicated at the top. DNA was extracted from infected cells on day 2 post-infection, and subjected to PCR analysis using the early and late primers as described previously [27]. To ascertain an approximate equality of DNA amount in each sample, β-globin gene was amplified by PCR [16,28]. The infectivity of viruses for M8166 cells is given as ++ (wt growth) + ,(retarded growth) and − (no growth) at the bottom.

that CS235/245-f carries (Fig. 1), it produced a considerable amount of Gag processing intermediates in cells, 33- and 34-kDa proteins, reported by us [31]. However, in the virions of CS235/245-f, the insertional effect was relatively small.

Second, the viral DNA synthesis in cells was monitored. M8166 cells were infected with the recombinants as above using an Env-minus HIV-1 mutant (NL-Kp) [17] and wt HIV-2 (GL-AN) [16] as negative controls. On day 2 post-infection, cells were harvested for PCR analysis. As shown in Fig. 4, parallel with the high infectivity in M8166 cells, the recombinants directed the synthesis of viral DNA. Viral specific DNA was readily detected only in cells infected with wt HIV-1 (NL432) or highly infectious recombinant viruses. We then monitored the synthesis of viral DNA in HSC-F cells infected with the same viruses as above. However, definite data, as evaluated by the PCR method here, were difficult to obtain probably due to the relatively low susceptibility of the cells to viruses.

4. Discussion

In this study, we have generated 18 HIV-1 based *gag*-chimeric viruses that are capable of productive and spreading infection in M8166 cells. These recombinant viruses, particularly the highly infectious ones, would be useful to construct an HIV-1 that is tropic for monkey cells to establish an animal infection model in the near future. They are also important as tools to analyze the basis for the replication block of HIV-1 in monkey cells. Understanding the mechanism of the species-specific tropism of HIV/SIV may add new insight to the research field of basic virology.

Although we have obtained a number of infectious HIV-1 carrying SIVmac *gag* sequences, none of them were able to grow in HSC-F cells, as monitored by virion-associated RT production. The defective site(s) in the cells of our recombinants is currently unclear. However, on the basis of the data presented here, it is quite possible that they have an early replication defect at the postentry step. Also, we have previously shown that the block for HIV-1 replication in monkey cells resides in the process of uncoating and/or reverse transcription [5]. Furthermore, the viral proteins responsible for the block appear to be Gag and an undetermined viral protein(s) [5–7]. Taken altogether, we may conclude that there is a viral factor, other than Gag CA, critical for the escape from the replication restriction and for viral DNA synthesis in monkey cells. We are now constructing a new series of chimeric viral clones to substantiate this hypothesis.

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Original article

Comparative study on the structure and cytopathogenic activity of HIV Vpr/Vpx proteins

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Abstract

The three-dimensional (3-D) structure of human immunodeficiency virus type 2 (HIV-2) Vpr/Vpx was predicted by homology modeling based on the NMR structure of human immunodeficiency virus type 1 (HIV-1) Vpr. The three proteins similarly have three major amphipathic α-helices. In contrast to HIV-1 Vpr, Vpr/Vpx of HIV-2 have a long N-terminal loop and clustered prolines in the second half of the C-terminal loop. HIV-2 Vpx uniquely contains a long region between the second and third major helices, and bears several glycines in the first half of the C-terminal loop. Instead of the glycines, there is a group of hydrophilic amino acids and arginines in the corresponding regions of the two Vprs. To compare the cytopathogenic potentials of HIV-1 Vpr and HIV-2 Vpr/Vpx, we examined the production of luciferase as a marker of cell damage. We further analyzed the characteristics of cells transduced with *vpr/vpx* genes driven by an inducible promoter. The results obtained clearly show that structurally similar, but distinct, HIV Vpr/Vpx proteins are detrimental to target cells.

Keywords: HIV-1; HIV-2; Vpr; Vpx; Homology modeling; Cytopathogenic activity

1. Introduction

All human and simian immunodeficiency viruses (HIVs and SIVs) isolated so far carry an accessory gene, *vpr*, in their genomes [1]. HIV type 2 (HIV-2) and SIVs isolated from rhesus (SIVmac) and sooty mangabey (SIVsm) monkeys constitute an independent sub-group (HIV-2 group) within primate immunodeficiency virus groups, and carry a *vpx* gene in addition to *vpr* [2]. Recently, SIVs from the mandrill (SIVmnd-2), red-capped mangabey monkey (SIVrcm) and drill (SIVdrl) have been reported to have both *vpr* and *vpx*, like viruses of the HIV-2 group [3–5]. A complete set of SIVmac *vpr* and *vpx* is required to cause AIDS efficiently in rhesus monkeys [6–8]. The *vpr* and *vpx* share considerable sequence similarity [2,5], and encode small proteins of approximately 100 amino acids. The three-dimensional (3-D) structure of HIV-1 Vpr determined by NMR is characterized by three major α-helices surrounded by N and C-terminal loops [9]. Structural analyses of Vpr/Vpx proteins other than HIV-1 Vpr, however, have not yet been carried out.

HIV-1 Vpr has been demonstrated to display cytopathogenic activities such as cell cycle arrest at the G_2 phase (G_2 arrest) [10–15] and apoptosis [15–17]. It was reported that the Vpr of HIV-2 group arrests cells at the G_2 phase [14,18–21], while Vpx does not [13,14,18,19,21]. The role of Vpr/Vpx of the HIV-2 group for apoptosis has not yet been well documented. While the virological significance of G_2 arrest and apoptosis induced by Vpr remains unclear, the ability to induce G_2 arrest is conserved among various primate immunodeficiency viruses [19,20,22]. The cytopathogenic potential of

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Vpr/Vpx, however, has not been compared yet under the same experimental conditions.

In this study, the 3-D structure and cytopathogenic activity of HIV-1 Vpr and HIV-2 Vpr/Vpx were compared. To analyze the framework of HIV-2 Vpr/Vpx, homology modeling based on the HIV-1 Vpr structure was performed. To evaluate their cytopathogenic activity, cells transiently or stably transfected with various expression vectors for HIV Vpr/Vpx were characterized biochemically and biologically. We demonstrate here that HIV-1 Vpr and HIV-2 Vpr/Vpx are structurally quite similar, but have distinct characteristics, and also that these proteins are detrimental to target cells.

2. Materials and methods

2.1. Homology modeling

Sequence alignment of HIV-1 Vpr and HIV-2 Vpr/Vpx was performed by the Clustal W program [23]. On the basis of this alignment, the 3-D structure of HIV-2 Vpr/Vpx was predicted from the NMR structure of HIV-1 Vpr (Protein Data Bank (PDB) code 1ESX) [9] by the MODELLER 6v2 program [24], and a diagram was generated by RASMOL software [25]. The amino acid sequence of HIV-1 Vpr and those of HIV-2 Vpr/Vpx are from HIV-1 P896 (GenBank accession no. U39362) [26] and HIV-2 GH-1 (GenBank accession no. M30895) [27] isolates, respectively. The sequences of *vpr/vpx* in HIV-2 GH-1 are identical with those in pGL-AN [28,29] used in this study.

2.2. Expression vectors

Vector pME18Neo-Fvpr was used to express HIV-1 Vpr with a FLAG tag at the N-terminus [30]. Expression vectors for HIV-2 Vpr and Vpx with FLAG tags designated pME18Neo-Fvpr2 and pME18Neo-Fvpx were constructed by replacement of the *vpr* of pME18Neo-Fvpr with *vpr* and *vpx*, respectively. *Vpr/vpx* were amplified by polymerase chain reaction (PCR) using pGL-AN [28,29] as a template. Vectors, a pGL3-Control Vector (Promega, Madison, WI, USA), pSG-Vif cFLAG [31], and pSG-Gag (p24) cFLAG [32] were used to express luciferase, HIV-1 Vif and HIV-1 Gag-p24, respectively. These vectors were transiently transfected into 293T cells [33] by the calcium phosphate coprecipitation method as previously described [34]. 293T cells were cultured in Eagle's minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) [33].

2.3. Full-length viral clones

For construction of full-length viral clones with tags at the 5′ site of *vpx/vpr* designated pGL-xFrH and pGL-xHrF, the *Xba* I-*Eco*R I fragment of pGL-AN [28,29] (nucleotides 5064-5756) was cloned into pUC19 to construct a subcloning vector pUC-GL(Xb-Ec). The FLAG and HA sequences were then introduced right after the ATG codon of *vpx/vpr* genes in pUC-GL(Xb-Ec) by a QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA), and the resultant DNA fragment was cloned back into pGL-AN [28,29] to construct pGL-xFrH and pGL-xHrF, as shown in the text. These clones were transiently transfected into 293T cells [33] by the calcium phosphate coprecipitation method as above [34].

2.4. Establishment of HeLa Tet-Off cell lines

Expression vectors based on pBI-EGFP under the control of an inducible and bidirectional promoter (Clontech, Palo Alto, CA, USA) were used to express EGFP and HIV Vpr/Vpx simultaneously. Vectors designated pBI-EGFP/Vpr, pBI-EGFP/Vpr2 and pBI-EGFP/Vpx to express HIV-1 Vpr, HIV-2 Vpr, and HIV-2 Vpx with a FLAG tag at N-terminus, respectively, were constructed by insertion of *vpr/vpx* and FLAG sequences into pBI-EGFP. HIV-1 *vpr* and HIV-2 *vpr/vpx* were amplified by PCR using pNL432 (GenBank accession no. AF324493) [34] and pGL-AN [28,29] as templates, respectively. Transient transfection into HeLa Tet-Off cells, cultured as described in the Tet Systems User Manual, was performed by the calcium phosphate coprecipitation method [34]. For establishment of Tet-Off/control and Tet-Off/Vpx, HeLa Tet-Off cells were transfected with pBI-EGFP or pBI-EGFP/Vpx as described above [34], and cultured in the selection medium described in the manual. These cell lines were maintained in Eagle's minimal essential medium supplemented with 10% heat-inactivated FBS in the presence of G418 (0.1 mg/ml), hygromycin B (0.1 mg/ml) and doxycycline (0.1 μg/ml). Induction of Vpr/Vpx and EGFP from these cells was achieved by removal of doxycycline from the culture medium.

2.5. Luciferase assay

Luciferase assays were performed with a Luciferase Assay System (Promega, Madison, WI, USA).

2.6. Western immunoblotting

Western immunoblotting was performed essentially as previously described [35]. Cell lysates for immunoblotting were prepared from 293T and HeLa Tet-Off cells transfected with various clones by CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate)/DOC (deoxycholate) [35] or Laemmli's sample [32] buffer, and resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrophoretically transferred to polyvinylidene fluoride membranes, and the membranes were treated with an ANTI-FLAG M2 Monoclonal Antibody (Ab) (Sigma–Aldrich, St. Louis, MO, USA), anti-EGFP Ab (Living Colors A.v. Peptide Ab, BD Biosciences, Palo Alto, CA, USA) or anti-HA Ab (Monoclonal Ab, HA.11, BAbCO, Berkeley, CA, USA). For visualization, ECL Plus Western Blotting Detection Reagents (Amersham Biosciences, Buckinghamshire, England) were used.

3. Results and discussion

3.1. Structure of HIV Vpr/Vpx

We made a structural comparison of HIV-1 Vpr and HIV-2 Vpr/Vpx by generating sequence alignment through the Clustal W [23] program. As shown in Fig. 1A, the three proteins have three major α-helices with amphipathic characteristics surrounded by N- and C-terminal loops.

Sequence homologies of HIV-1 Vpr vs. HIV-2 Vpr, HIV-1 Vpr vs. HIV-2 Vpx, and HIV-2 Vpr vs. HIV-2 Vpx are 44%, 24% and 22%, respectively. These figures show that sequence of HIV-2 Vpx is quite distinct from those of the other two. Sequence identities of HIV-2 Vpx with HIV-1 Vpr and HIV-2 Vpr are particularly low in a major helix 2 (nos. 47–55) and a region between major helices 2 and 3 (nos. 56–72). In this region (nos. 56–72), HIV-2 Vpx uniquely contains a short α-helix and a long loop. Tyrosines (nos. 68, 71 and 73) in and close to this region of Vpx have been reported to be critical for virion incorporation [36]. Furthermore, in the first half of the C-terminal loop (nos. 93–105), HIV-2 Vpx characteristically bears several glycines. Instead of the glycines, there are a group of hydrophilic amino acids and arginines in the corresponding regions of the two Vpr (nos. 95–104). The two arginines (nos. 100 and 101) of HIV-1 Vpr have been reported to be responsible for G_2 arrest [37].

Vpr/Vpx of HIV-2 have different characteristics from HIV-1 Vpr in N- and C-terminal loops. N-terminal loops of HIV-2 Vpr/Vpx are longer than that of HIV-1 Vpr, and there are two prolines in the region (nos. 8–14). In the second half of the C-terminal loop of HIV-2 Vpx, there are continuous seven prolines (nos. 106–112). HIV-2 Vpr similarly has several prolines (nos.102, 104, 107 and 109), while HIV-1 Vpr has no prolines in its C-terminal loops.

Sequence identities of HIV-2 Vpr/Vpx with HIV-1 Vpr are enough to build an appropriate homology model for HIV-

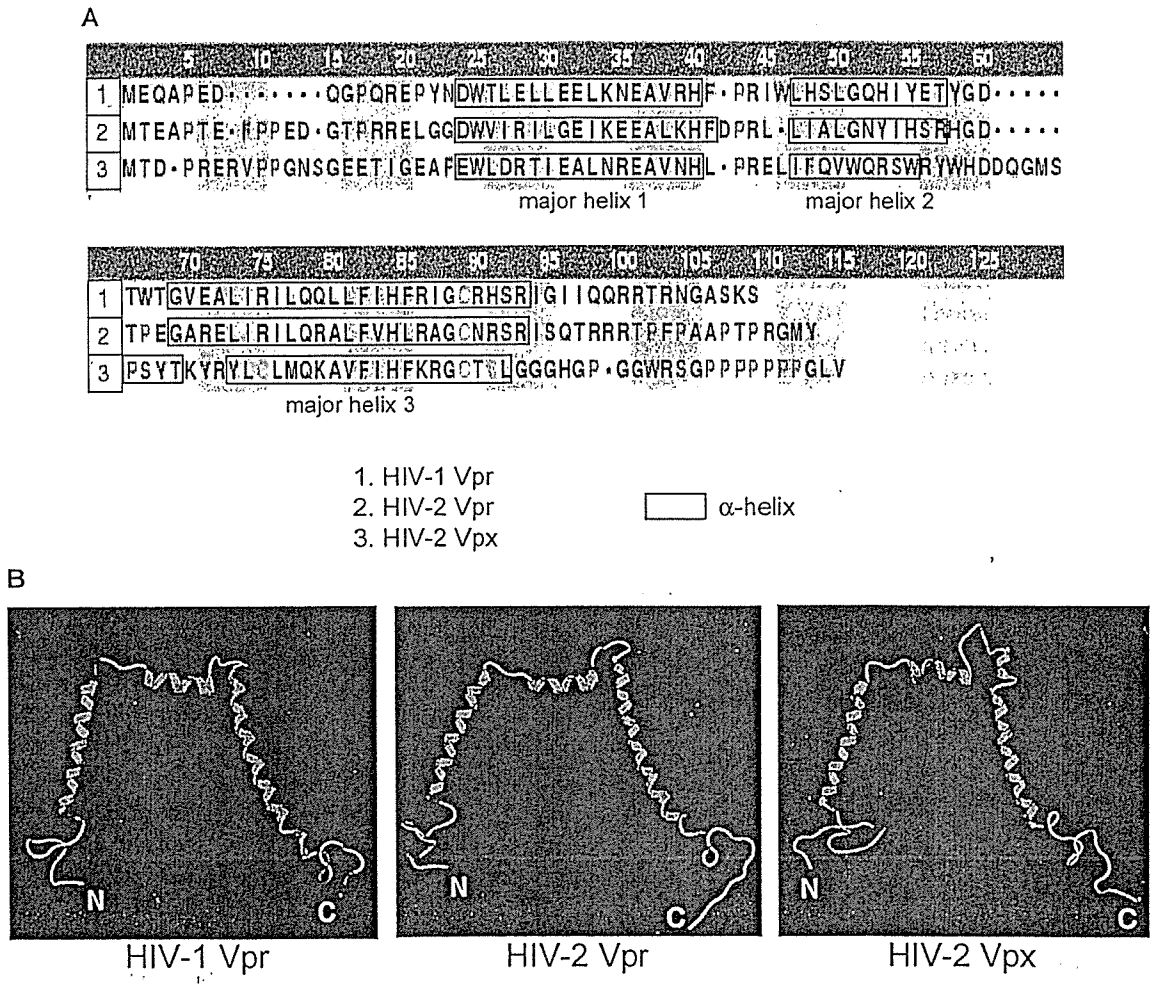

Fig. 1. Structure of HIV Vpr/Vpx proteins. (A) Sequence alignment of Vpr/Vpx. The sequence alignment, was performed, by the Clustal W program [23]. Colored codes indicate amino acids with distinct characteristics as follows: red, acidic; blue, basic; pink, hydrophilic; green, hydrophobic; light green, aromatic; yellow, cysteines; orange, prolines. Amino acids that constitute α-helices in HIV-1 Vpr as determined by NMR [9], or are predicted to constitute α-helices in HIV-2 Vpr/Vpx, are boxed. Amino acid sequence of HIV-1 Vpr and those of HIV-2 Vpr/Vpx are from HIV-1 P896 (GenBank accession no. U39362) and HIV-2 GH-1 (GenBank accession no. M30895) isolates, respectively. (B) Ribbon diagram of Vpr/Vpx. On the basis of the NMR structure of HIV-1 Vpr (PDB code 1ESX) [9], the 3-D structure of HIV-2 Vpr/Vpx was predicted by homology modeling from the sequence alignment shown in (A). Colored parts of the diagram indicate unique structures as follows: red, α-helices; blue, tight turn (3_{10} helices); green, other turn; light blue, others. N and C indicate N- and C-termini of the proteins, respectively.

2 Vpr/Vpx based on the NMR structure of HIV-1 Vpr [9]. 3-D structures of HIV-2 Vpr/Vpx were thus predicted by the MODELLER 6v2 program [24], and their diagrams were generated by RASMOL software [25]. As is clear in Fig. 1B, the three proteins share a basic framework, but 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. The different shape of Vpx in these regions may be associated with its conspicuous activity, e.g. lack of ability to induce G_2 arrest [13,14,18,19,21].

3.2. Cytopathogenic activity of HIV Vpr/Vpx

HIV Vpr has been shown to have cytopathogenic activities such as induction of G_2 arrest and apoptosis [10–21], while there have been no reports to demonstrate Vpx having this activity [13,14,18,19,21]. The cytopathogenic potential of Vpx was assessed by transfection experiments using the two Vprs as controls. We first monitored the production of marker protein luciferase upon co-expression. 293T cells were transiently co-transfected with expression vectors of luciferase and Vpr/Vpx, and cell lysates were analyzed for luciferase activity. As shown in Fig. 2A, when HIV-1 Vif and Gag-p24 were co-expressed, production of luciferase was not significantly affected. In contrast, when HIV Vpr/Vpx were co-expressed, production of luciferase was markedly decreased. To normalize the detrimental activity of the Vpr/Vpx observed here, the expression level of HIV Vpr/Vpx proteins in the cell lysates was determined by Western blot analysis. As shown in Fig. 2B, the expression level of HIV-2 Vpr was much lower than that of HIV-1 Vpr and HIV-2 Vpx. Taken together, the cytopathogenic activity of HIV-2 Vpr was much higher than those of HIV-1 Vpr and HIV-2 Vpx.

To evaluate more definitively the detrimental effects of Vpr/Vpx on cells, we next established various HeLa-Tet Off cell lines, which carry an inducible promoter for expression of the two proteins. Expression vectors of Vpr/Vpx designated pBI-EGFP/Vpr, pBI-EGFP/Vpr2 and pBI-EGFP/Vpx were constructed, and transfected into HeLa-Tet Off cells. The cells were then incubated in the absence (induction +) or presence (induction –) of doxycycline, and cell lysates were prepared. As shown in Fig. 3A, while expression of marker proteins EGFP, HIV-1 Vpr, and HIV-2 Vpx was readily observed upon induction, HIV-2 Vpr was not detected. Stable HeLa-Tet Off cell lines carrying pBI-EGFP (Tet-Off/control) and pBI-EGFP/Vpx (Tet-Off/Vpx) were then established. Cell lines harboring HIV-1 *vpr* and HIV-2 *vpr* genes were found to be unstable and difficult to maintain. A tiny amount of cytotoxic Vpr could cause the death of target cells. As expected, expression of HIV-2 Vpx in Tet-Off/Vpx cells was induced by removal of doxycycline (Fig. 3B). Using these cell lines, the effect of Vpx on cells was monitored by determining cell numbers. As shown in Fig. 3C, Tet-Off/Vpx cells did not grow substantially after induction of Vpx. In sharp contrast, Tet-Off/control cells and Tet-Off/Vpx cells without induction grew

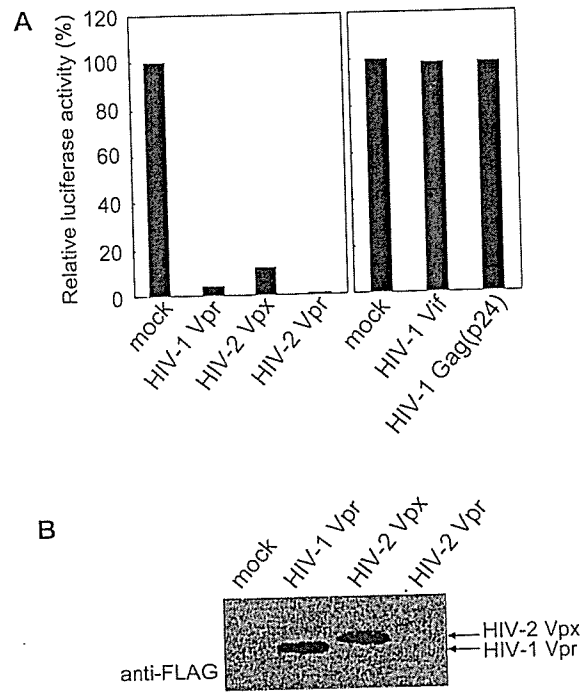

Fig. 2. Expression of HIV Vpr/Vpx in 293T cells. (A) Effects in cells of Vpr/Vpx on luciferase production. 293T cells were co-transfected with the expression vector for luciferase (pGL3-Control Vector) (2.5 μg), and one of the expression vectors for Vpr/Vpx (pME18Neo-Fvpr [30], pME18Neo-Fvpx and pME18Neo-Fvpr2) (7.5 μg) (left panel) or of those for Vif/Gag-p24 (pSG-Vif cFLAG [31] and pSG-Gag (p24) cFLAG [32]) (7.5 μg) (right panel). At 48 h post-transfection, cells were harvested for luciferase assays (A) and for Western blotting analysis (B). Luciferase activity relative to that of mock (pUC19 in the left panel and pSG5 in the right panel)-transfected cells is shown. (B) Expression level of Vpr/Vpx as monitored by Western blot analysis. Cell lysates were analyzed by Western immunoblotting using anti-FLAG Ab (anti-FLAG M2 monoclonal Ab). Loading amount in each lane was normalized by the luciferase activity shown in (A). Mock, pUC19.

fairly well. These results clearly demonstrated for the first time, the cytostatic effect of HIV-2 Vpx. We were interested in the extremely low expression level of HIV-2 Vpr observed in Fig. 2B and Fig. 3A. Expression of Vpr in the context of the full-length HIV-2 genome was, therefore, examined. For this purpose, FLAG and HA tags were introduced into the 5′-ends of *vpx* and *vpr* genes of pGL-AN in different combinations (Fig. 4A). The resultant clones were transfected into 293T cells, and cell lysates were analyzed by Western blotting using anti-FLAG and anti-HA Abs. As shown in Fig. 4B, Vpx was easily detected in cells transfected with pGL-xFrH and pGL-xHrF. In contrast, expression of Vpr was not observed at all in the transfected cells. Proteasome degradation may account for the extremely low expression level of HIV-2 Vpr observed here, as reported for SIVmac [38].

3.3. Perspectives relating to this study

We used the NMR structure of HIV-1 Vpr analyzed in H_2O/trifluoroetanol as a template [9] in the homology modeling here. When acetonitrile was used instead of trifluoroethanol, folding of three α-helices around a hydrophobic core,

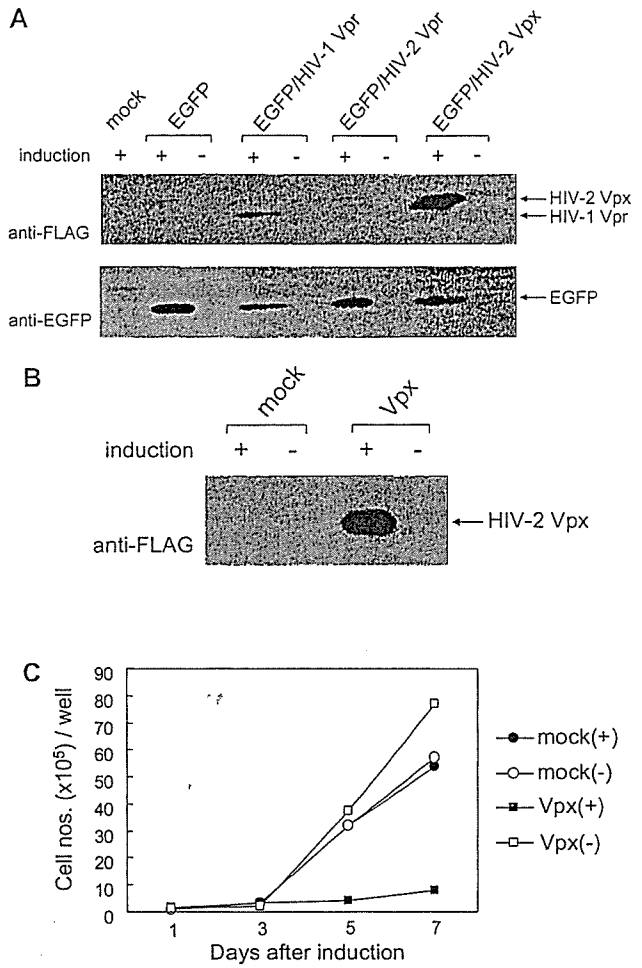

Fig. 3. Expression of HIV Vpr/Vpx in HeLa Tet-Off cells. (A) Transient expression of Vpr/Vpx in HeLa Tet-Off cells. Cells were transfected with various expression vectors (pBI-EGFP, pBI-EGFP/Vpr, pBI-EGFP/Vpr2 and pBI-EGFP/Vpx) (15 μg), and incubated for 48 h with (+) or without (–) induction. Cell lysates were then prepared and analyzed by Western immunoblotting using anti-FLAG Ab (ANTI-FLAG M2 Monoclonal Ab, upper) and anti-EGFP Ab (Living Colors A.v. Peptide Ab, lower). Induction of Vpr/Vpx and EGFP was done by removal of doxycycline from the culture medium. Mock, pUC19. (B) Expression of Vpx in HeLa Tet-Off cells stably carrying pBI-EGFP/Vpx (Tet-Off/Vpx). Cells were incubated for 5 days with (+) or without (–) induction, and lysates were prepared for Western immunoblotting using anti-FLAG Ab (ANTI-FLAG M2 Monoclonal Ab). Each lane contained 20 μg of total protein. As a control, HeLa Tet-Off cells carrying pBI-EGFP (Tet-Off/control) were used. (C) Growth of Tet-Off/control and Tet-Off/Vpx cells. Cells were cultured for 7 days with (+) or without (–) induction, and nos. of cells were determined at intervals. Variable cell counts and days after induction are plotted.

which is more likely to be formed in physiological conditions, was observed [39]. Homology modeling based on this structure should be carried out.

Fletcher et al. suggested that two major functions carried out by HIV-1 Vpr, G_2 arrest and nuclear import of viral reverse transcription complexes, are borne by Vpr and Vpx of SIVsm, respectively [18]. In this study, however, we found that Vpx has cytopathogenic activity, the same as Vpr. We need to clarify the structural basis for this activity.

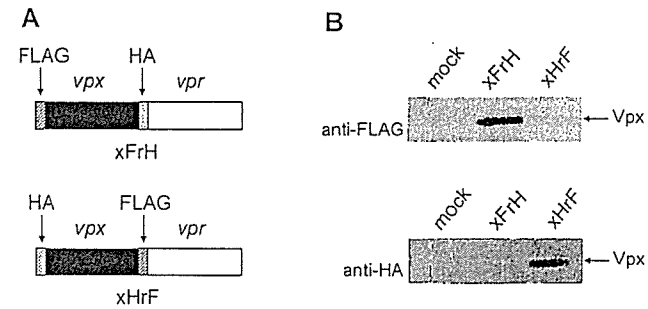

Fig. 4. Expression of Vpr/Vpx in the context of the full-length HIV-2 genome. (A) Location of tags in pGL-xFrH and pGL-xHrF. FLAG and HA tags are placed in the *vpx/vpr* region as indicated. (B) Expression level in cells of Vpx/Vpr. 293T cells were co-transfected with one of the full-length viral clones (pGL-xFrH and pGL-xHrF) (7.5 μg) and the expression vector for luciferase (pGL3-Control Vector) (2.5 μg). At 48 h post-transfection, cells were harvested for luciferase assays and Western blot analysis using anti-FLAG Ab (ANTI-FLAG M2 Monoclonal Ab, upper) and anti-HA Ab (Monoclonal Ab, HA.11, lower). The loading amount in each lane was normalized by luciferase activity. Mock, pUC19.

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Immunopathology and Infectious Diseases

Role of Neuronal Interferon-γ in the Development of Myelopathy in Rats Infected with Human T-Cell Leukemia Virus Type 1

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Human T-cell leukemia virus type 1 (HTLV-1) is the causative agent of not only adult T-cell leukemia but also HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Among the rat strains infected with HTLV-1, chronic progressive myelopathy, named HAM rat disease, occurs exclusively in WKAH rats. In the present study, we found that HTLV-1 infection induces interferon (IFN)-γ production in the spinal cords of HAM-resistant strains but not in those of WKAH rats. Neurons were the major cells that produced IFN-γ in HTLV-1-infected, HAM-resistant strains. Administration of IFN-γ suppressed expression of *pX*, the gene critically involved in the onset of HAM rat disease, in an HTLV-1-immortalized rat T-cell line, indicating that IFN-γ protects against the development of HAM rat disease. The inability of WKAH spinal cord neurons to produce IFN-γ after infection appeared to stem from defects in signaling through the interleukin (IL)-12 receptor. Specifically, WKAH-derived spinal cord cells were unable to up-regulate the *IL-12 receptor β2* gene in response to IL-12 stimulation. We suggest that the failure of spinal cord neurons to produce IFN-γ through the IL-12 pathway is involved in the development of HAM rat disease. *(Am J Pathol 2006, 169:189–199; DOI: 10.2353/ajpath.2006.051225)*

Human T-cell leukemia virus type 1 (HTLV-1) is the causative agent of adult T-cell leukemia (ATL)[1,2] and so-called HTLV-1-associated diseases such as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP),[3,4] HTLV-1 uveitis (HU),[5] HTLV-1-associated arthropathy (HAAP),[6] T-cell alveolitis,[7] Sjögren's syndrome,[8] polymyositis,[9] and infective dermatitis.[10] Only a small proportion (<5%) of HTLV-1-infected individuals develop ATL or HTLV-1-associated diseases, whereas more than 95% of carriers remain asymptomatic for life.[11] Little is known about the factors that govern susceptibility to diseases caused by HTLV-1.

We previously established a rat model of HAM/TSP in which chronic progressive myelopathy with paraparesis of lower limbs occurred in WKAH rats 15 to 22 months after HTLV-1 infection.[12] Although the provirus was detected in the systemic organs of all HTLV-1-infected strains examined, myelopathy, hereafter referred to as HAM rat disease, occurred exclusively in WKAH rats. Histopathological alterations were limited to the thoracic spinal cord in HAM rat disease. The most crucial finding was apoptotic cell death of oligodendrocytes in the anterior and lateral funiculi of the upper thoracic cord, which became manifest 7 months after inoculation with HTLV-1.[13] Subsequently, demyelination occurred with infiltration of activated macrophages, and at the end stage of the disease, proliferation of astrocytes was observed in the affected region.[14] Interestingly, lymphocytic infiltration into the spinal cord, which is characteristic of human HAM/TSP,[15] was absent throughout the disease process in the HAM rat model. Although the significance of this finding is not clear, lymphocytic infiltration in human HAM/TSP may represents a cellular response to tissue damage.

The HTLV-1 provirus, which predominantly localizes in microglia and macrophages, becomes detectable in the spinal cord of both HAM-resistant and -susceptible rats 3 months after infection.[16] Selective expression of the

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