Generation of HIV-1 derivatives that productively infect macaque monkey lymphoid cells

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The narrow host range of human immunodeficiency virus type 1 (HIV-1) is caused in part by innate cellular factors such as apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G) and TRIM5α, which restrict virus replication in monkey cells. Variant HIV-1 molecular clones containing both a 21-nucleotide simian immunodeficiency virus (SIV) Gag CA element, corresponding to the HIV-1 cyclophilin A-binding site, and the entire SIV *vif* gene were constructed. Long-term passage in a cynomolgus monkey lymphoid cell line resulted in the acquisition of two nonsynonymous changes in *env*, which conferred improved replication properties. A proviral molecular clone, derived from infected cells and designated NL-DT5R, was used to generate virus stocks capable of establishing spreading infections in the cynomolgus monkey T cell line and CD8-depleted peripheral blood mononuclear cells from five of five pig-tailed macaques and one of three rhesus monkeys. NL-DT5R, which genetically is >93% HIV-1, provides the opportunity, not possible with currently available SIV/HIV chimeric viruses, to analyze the function of multiple HIV-1 genes in a broad range of nonhuman primate species.

APOBEC3 | host range | monkey model | TRIM5α | cyclophilin A

The narrow host range of human immunodeficiency virus type 1 (HIV-1) has been a major impediment for developing tractable animal models for studies of viral pathogenesis and vaccine development. Because simian immunodeficiency virus (SIV) has a genomic organization similar to that of HIV-1 and some SIV strains cause disease in Asian macaques, SIV/HIV chimeric viruses (SHIVs) were generated to assess the role of some HIV-1-encoded proteins in nonhuman primates (1–3). The commonly used SHIVs contain the HIV-1 *tat*, *rev*, *vpu*, and *env* genes inserted into an SIVmac239 genetic backbone; efforts to extend the incorporated HIV-1 gene segment to include *pol* and *gag* sequences have resulted in viruses unable to replicate in monkey cells (ref. 1; unpublished data). Although SHIVs have proven useful in characterizing the immune responses to primate lentiviruses (4, 5), and specifically, the role of antibodies directed against the HIV-1 envelope glycoprotein (6, 7), the absence of the other HIV-1 structural proteins has restricted analyses of their function *in vivo*.

It is now appreciated that many mammalian species encode factors conferring resistance to retroviral infections. Some, such as the apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3) family of cytidine deaminases, modify minus strand viral DNA during reverse transcription, resulting in either its degradation or its integration into host chromosomal DNA as a hypermutated provirus (8–10). The retroviral inhibitory effect of APOBEC3G results from its packaging into progeny virions during particle assembly (11–12). The deleterious activities of APOBEC3G are countered by lentiviral Vif proteins, which prevent the encapsidation of APOBEC3G into nascent virions (13–16). The sensitivity of APOBEC3G from different animal species to the Vif proteins expressed by different viruses varies widely. For example, although HIV-1 Vif can potently suppress human APOBEC3G, it is not effective against rhesus monkey (RhM) APOBEC3G, explaining in part the restriction of HIV-1 replication in macaque cells (11).

Another recently described restriction factor, TRIM5α, targets incoming viral capsids, and it blocks retroviral replication in a species-specific manner (17–19). For example, TRIM5α from RhMs potently suppresses HIV-1 but not SIV infectivity in monkey cells (19). Although its mechanism of action is still unclear, TRIM5α restriction is thought to affect virus uncoating, thereby blocking subsequent steps in the replication cycle (20). Cyclophilin A (CypA), which binds to a proline-rich loop on the surface of the HIV-1 capsid (CA) protein, augments HIV-1 infection in human cells and inhibits its replication in monkey cells (21, 22). Recent reports suggest that by binding to HIV-1, CypA may modulate the conformation of the virion core, rendering it sensitive to TRIM5α restriction in simian cells (23, 24).

In this work, we have generated HIV-1 derivatives, which carry only the SIVmac239 *vif* gene and a short 7-aa segment from SIV *gag* corresponding to the HIV-1 CypA-binding loop. Molecularly cloned viruses bearing these two SIV regions are able to establish spreading infections in a cynomolgus monkey (CyM) T cell line and CD8-depleted PBMCs from pig-tailed macaques (PtMs) and RhMs. These results indicate that the incorporation of two SIV gene segments into the HIV-1 genome can effectively counter two known species-specific restriction factors that block virus replication in monkey cells. They raise the possibility of generating HIV-1 derivatives, containing all of its structural proteins and capable of infecting macaque monkeys.

Results

Construction and Characterization of HIV-1 Molecular Clones Containing CA and Vif Sequences from SIVmac239. Three proviral DNA constructs were generated to counteract the restriction of HIV-1 replication in macaque monkey cells. In the first, the entire 214-aa Vif ORF from SIVmac239 was amplified by PCR and

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Abbreviations: AGM, African green monkey; APOBEC3G, apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G; CA, capsid; CyM, cynomolgus monkey; CypA, cyclophilin A; PBMC, peripheral blood mononuclear cell; p.i., postinfection; PtM, pig-tailed monkey; RhM, rhesus monkey; RT, reverse transcriptase; SHIV, SIV/HIV chimeric virus; SIV, simian immunodeficiency virus; SIVmac, simian immunodeficiency virus isolated from rhesus macaques; VSV-G, vesicular stomatitis virus type G.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AB266485, AB266486, AB226487, and AB266488 for pNL-DT5R and APOBEC3Gs from AGM, human, and CyM, respectively).

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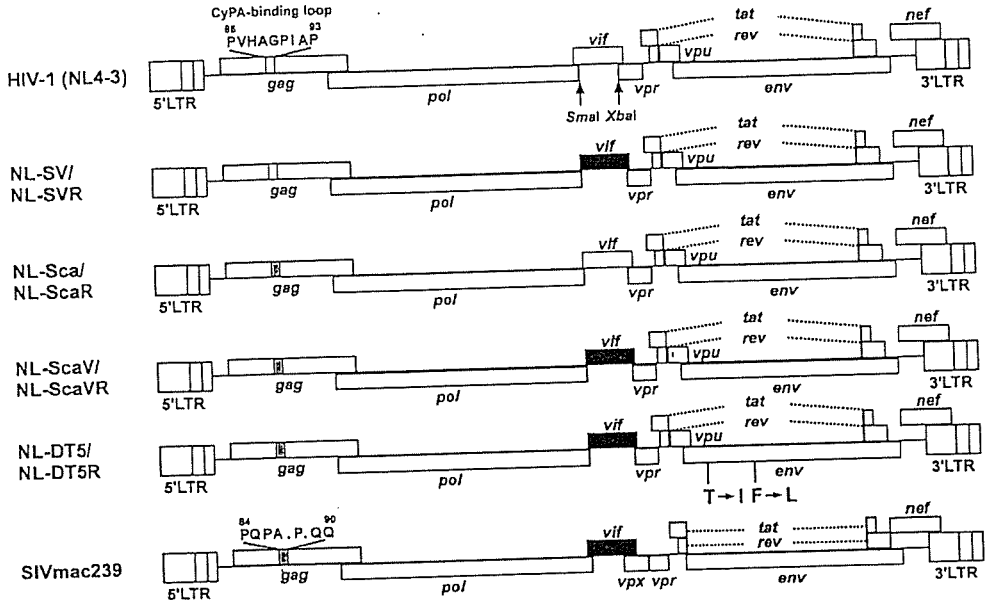

Fig. 1. Structure of chimeric clones between HIV-1 NL4-3 and SIVmac239 in this study. Eight chimeric proviral clones shown here were generated from a pNL4-3 derived vector pNL-SX (25) as described in *Materials and Methods*. Each chimeric clone has the entire *vif* (black area) and/or partial *gag* (gray area; analog of HIV-1 CypA-binding loop) of SIVmac239 as shown. For insertion of *vif* into the clones, the SmaI–XbaI site in pNL-SX was used. The two amino acid changes in *env* unique to pNL-DT5 and pNL-DT5R are indicated.

inserted into SmaI–XbaI-digested pNL-SX, a pNL4-3-derived vector, previously used for functional analyses of HIV-1 *vif* genes (25). This SIV Vif-encoding construct was designated NL-SV (Fig. 1). Because of the reported association of CypA with HIV-1 sensitivity to TRIM5α during infections of cells from Old World monkeys (21, 22), the 9-aa CypA-binding loop in NL4-3 was converted to the 7-residue SIVmac239 CA analog by site-directed mutagenesis of the pNL-SX vector carrying the HIV-1 *vif* gene (26). This construct was designated NL-Sca (Fig. 1). A final clone, containing both SIV elements, was generated by inserting SIV *vif* into NL-Sca and designated NL-ScaV (Fig. 1).

Expression of the lentiviral genes present in the three newly derived cloned proviruses was assessed by immunoblot analyses of lysates prepared from transfected 293T cells. The production of Gag, Pol, Env, Vpu, and Nef proteins directed by all three constructs was comparable with that observed with the parental pNL4-3; levels of Vpr expression, however, were markedly reduced (data not shown). The latter was subsequently shown to be caused by the presence of the TCT trinucleotide, introduced into the pNL-SX vector to generate the XbaI cloning site (25). When the TCT was removed by site-specific mutagenesis, Vpr expression was restored to wild-type levels in cells transfected by all three constructs (data not shown). The "Xba site-repaired" clones were designated NL-SVR, NL-ScaR, and NL-ScaVR, respectively, as indicated in Fig. 1.

HIV-1 Constructs Bearing the SIV *vif* Gene Are Able to Suppress the Inhibitory Effects of Simian APOBEC3Gs. It has been previously reported that RhM and African green monkey (AGM) APOBEC3Gs are resistant to HIV-1 *vif*, possibly explaining, in part, the restriction of HIV-1 replication in cells from Old World monkeys (11). To determine whether the simian APOBEC3Gs could block HIV-1 constructs carrying the SIVmac239 *vif* gene, VSV-G-pseudotyped viruses were generated in 293T cells in the presence of different APOBEC3Gs. For this experiment, species-specific APOBEC3G cDNAs were prepared from H9 (human), HSC-F (CyM) (27), and Vero (AGM) cells by RT-PCR and inserted into pcDNA3.1-FLAG, an expression vector containing an epitope tag, as described in *Materials and Methods*. Comparable levels of human, CyM, and AGM APOBEC3Gs were produced in transfected 293T cells, as monitored by immunoblotting by using anti-FLAG antibodies (Fig. 2*A*). The progeny virions generated in cells expressing human or the two monkey APOBEC3Gs were collected from culture supernatants at 48 h, and their infectivities were assayed in MAGI cells (Fig. 2*B*). Not unexpectedly, the replication of viruses (NL4-3 and NL-ScaR) bearing the HIV-1 *vif* gene and produced in cells expressing CyM and AGM APOBEC3Gs was potently suppressed. In contrast, the constructs (NL-SVR and NL-ScaVR) carrying the SIVmac239 *vif* gene were refractory to the effects of both CyM and AGM APOBEC3Gs. When the expression of the SIVmac *vif* gene in NL-ScaVR was abrogated by a frameshift mutation, the resulting virus (NL-ScaVR-dBgl) became sensitive to all three APOBEC3Gs, and its infectivity was markedly reduced. These results indicate that under the same experimental conditions in which simian APOBEC3Gs restrict wild-type HIV-1, the derivative clones expressing SIV Vif direct the production of virions able to replicate in MAGI cells.

HIV-1 Constructs Carrying a 7-Amino Acid SIV CA Element Exhibit Increased Replication in Simian Cells. The capacity of HIV proviruses bearing the SIV Gag analog of the HIV-1 CypA-binding loop to escape restriction in simian cells was assessed by using VSV-G-pseudotyped viruses (NL4-3, NL-ScaR, NL-SVR, and NL-ScaVR) in single-cycle replication assays by using human (293T), RhM (LLC-MKII), and owl monkey (OMK637) cell lines (Fig. 2*C*). Three-fold serial dilutions of each virus stock were added to the cultures, and the amounts of p24 Gag protein present in cell lysates 72 h postinfection (p.i.) was determined by ELISA. All four viruses expressed p24 with similar efficiencies in human cells. In contrast, the NL-ScaR and NL-ScaVR derivatives, both of which carry the SIV CA element, produced substantially more p24 Gag in monkey cells than the constructs (NL4-3 and NL-SVR) bearing the HIV-1 CypA-binding loop. This effect was particularly striking in owl monkey cells in which constructs carrying the SIV CA expressed 50-fold more viral protein. Not unexpectedly, the presence of SIV *vif* alone in NL-SVR did not result in increased p24 Gag production com-

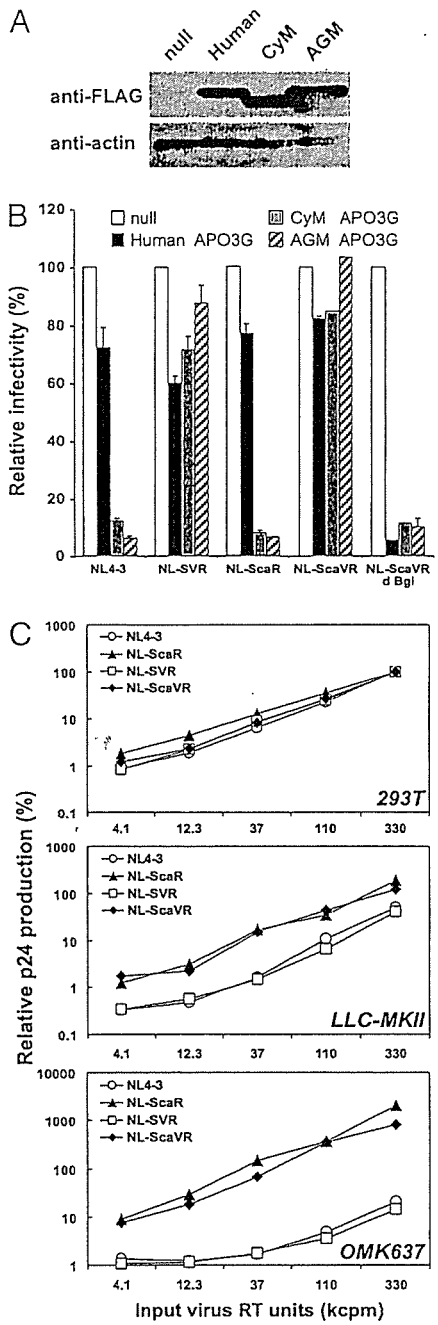

Fig. 2. Single-cycle replication properties of HIV-1 chimeric clones. (*A*) The expression of human, CyM, and AGM APOBEC3Gs containing the FLAG epitope was monitored in 293T cells by immunoblotting 48 h posttransfection. (*B*) VSV-G-pseudotyped viruses were prepared in 293T cells cotransfected with one of the three expression vectors for APOBEC3G (human, CyM, and AGM), and their infectivity was examined in MAGI cells. Infectivity relative to that of virus produced in the absence of APOBEC3G (null) is indicated. (*C*) Production of p24 Gag in human (293T), RhM (LLC-MKII), and owl monkey (OMK637) cells after infection with increasing amounts of the indicated VSV-G-pseudotyped viruses was measured on day 3 p.i. Expression levels of p24, relative to that generated by the virus sample with the highest RT activity in 293T cells, are indicated.

pared with wild-type HIV-1 in this single-cycle replication assay. The results shown in Fig. 2*C* indicate that the incorporation of a 21-nucleotide SIV *gag* gene element into HIV-1 proviral DNA is sufficient to suppress the endogenous restriction factors resident in RhM and owl monkey cells.

An HIV-1 Derivative Containing both SIV *vif* and the SIV CA Element Is Able to Establish Spreading Infections in a Monkey Lymphocyte Cell Line. Although single-cycle replication experiments using pseudotyped retroviral particles like those shown in Fig. 2 *B* and *C* can furnish valuable information about virus entry, uncoating, reverse transcription, integration, and the production of viral proteins, they provide no data about the functional properties of the progeny virions that are generated. The latter information accrues from spreading multicycle infections. Toward this end, NL-ScaR, NL-SVR, and NL-ScaVR virus stocks, prepared from transfected 293T cells and normalized for equivalent amounts of reverse transcriptase (RT) activity, were used to infect human (M8166) and cynomolgus (HSC-F) cells. HSC-F is a $CD4^+CXCR4^+CCR5^-$ CyM T cell line originally immortalized by *Herpesvirus saimiri* (27). Both cell types were also infected with similar amounts of the parental NL4-3 and SIVmac239, which served as controls. As shown in Fig. 3*A*, all of the viruses did, in fact, establish spreading infections in M8166 cells, although the three bearing the SIV *vif* gene (SIVmac239, NL-SVR, and NL-ScaVR) reached lower levels of peak RT activity compared with NL4-3 and NL-ScaR.

A completely different result was obtained during infections of the CyM cell line. As expected, SIVmac239 readily established a spreading infection, which peaked on day 6 p.i.; wild-type NL4-3 produced no measurable progeny virions (Fig. 3*B*). Of the three NL4-3 derivatives carrying SIV sequences, only NL-ScaVR exhibited any infectivity, which became detectable on day 15 p.i. The delayed appearance of NL-ScaVR progeny is reminiscent of previously described second-site revertants of HIV-1 mutants, which acquire changes during extended tissue culture passage that confer augmented replicative properties (28, 29). Therefore, to characterize more fully the late emerging virus, new and independent infections of HSC-F cells were initiated by using both NL-ScaV- and NL-ScaVR-derived virus preparations as inocula; in both cases, the production of progeny virions was again markedly delayed (data not shown). Culture supernatants from the NL-ScaV infection were collected on days 39 and 51 p.i., normalized for RT activity, and used as inocula for infections of fresh HSC-F cells. As shown in Fig. 3*C*, the viruses harvested on days 39 and 51 both exhibited accelerated replication kinetics compared with the original NL-ScaV virus, suggesting that long-term passage in HSC-F had resulted in the acquisition of genetic alterations.

Molecular Cloning and Characterization of an HIV-1 Derivative Able to Cause Spreading Infections in Macaque Primary Cells. The emergence of virus exhibiting an augmented replication phenotype prompted us to initiate the molecular cloning of cell-associated viral DNA collected from HSC-F cultures infected with the "sup 51" inoculum on day 18 p.i. Integrated proviruses were amplified from genomic DNA as two overlapping fragments by PCR, and virus stocks were prepared from 293T cells after transfection with reconstructed full-length clones. The replication properties of one of the infectious clones obtained (NL-DT5) is shown in Fig. 3*D*. Although production of progeny virus was delayed compared with that directed by SIVmac239, NL-DT5 still exhibited robust infection kinetics and released more particle-associated RT activity than the SIV control.

Sequencing of NL-DT5 DNA revealed that it had acquired four nucleotide changes, compared with NL-ScaV, during the 51-day passage in HSC-F cells. Two were nonsynonymous changes in *env* (nts 6633 and 7043), resulting in T110I (V1) and F247L (C2) substitutions in gp120. One of the other two was a synonymous change in the Pro coding sequence (nt 2300) and the other was a G to A substitution in the U3 region of the 3′ LTR. The functional significance of these changes is not presently known. Because NL-DT5 was derived from cells originally infected with NL-ScaV, the XbaI cloning site present upstream

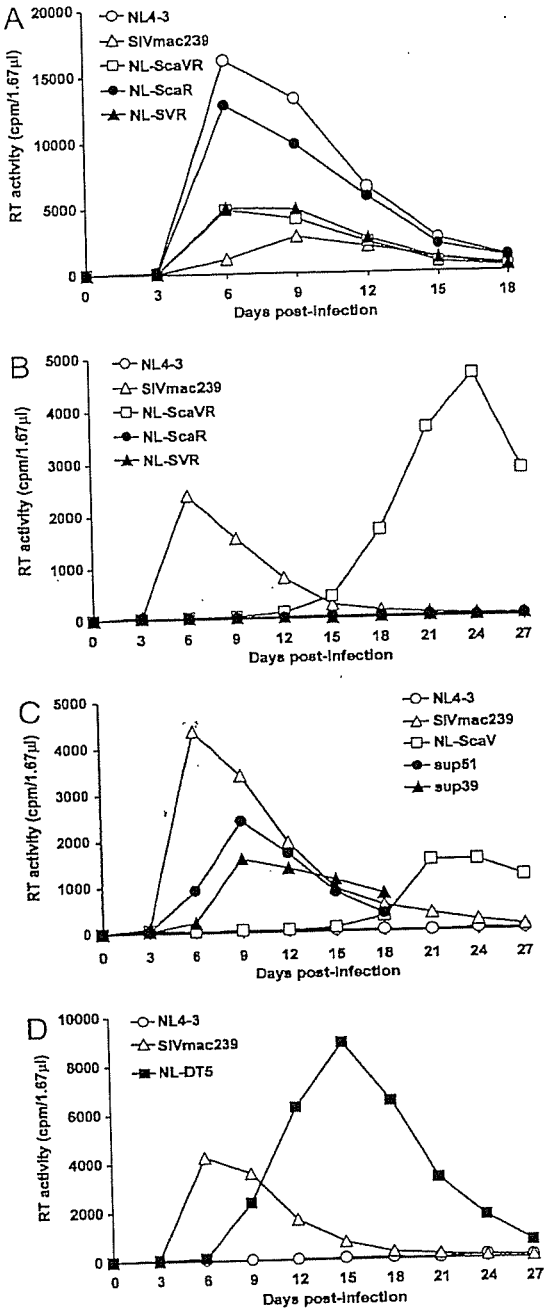

Fig. 3. Multicycle growth potential of various chimeric viruses in human and monkey lymphocyte cell lines. Virus samples were prepared from 293T cells transfected with the indicated proviral clones, and they were inoculated into human M8166 (*A*) or CyM HSC-F (*B*) cells. HIV-1 NL4-3 and SIVmac239 served as controls. (*C* and *D*) Growth properties of viruses generated in infected HSC-F cells. Culture supernatants from NL-ScaV-infected HSC-F cells collected on days 39 and 51 p.i. (sup39 and sup51 in *C*) and from 293T cells transfected with a molecular clone derived from sup51 (NL-DT5 in *D*) were inoculated into HSC-F cells. NL4-3, SIVmac239, and NL-ScaV from transfected 293T cells served as controls. Virus replication was monitored by RT activity released into the culture supernatants.

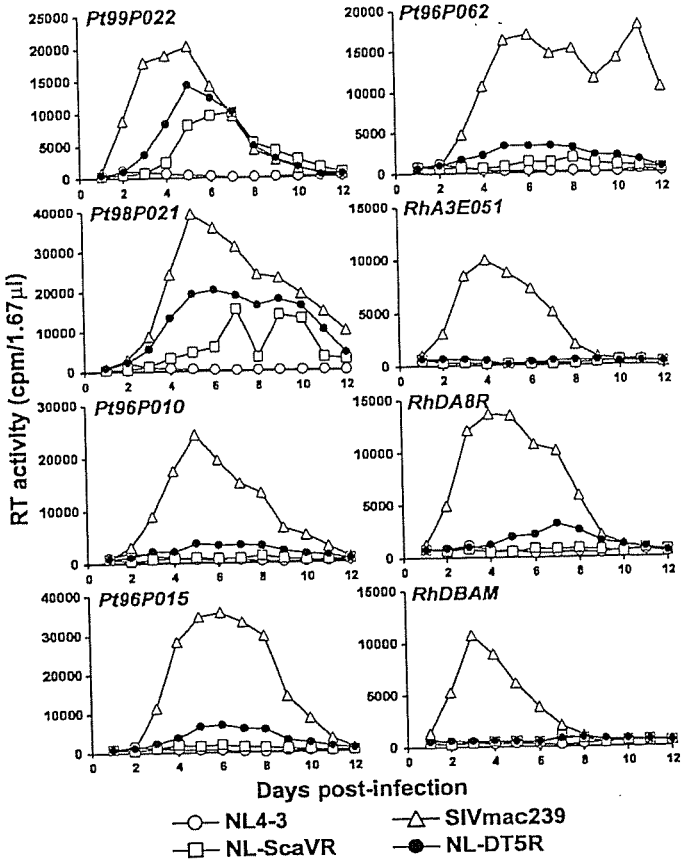

Fig. 4. Growth potential of the chimeric viruses in CD8-depleted PBMCs from PtM and RhM. Virus samples were prepared from 293T cells transfected with the proviral clones indicated at the bottom, and they were infected into CD8-depleted PBMCs by spinoculation (30). Virus replication was monitored daily by RT production in the culture supernatants. HIV-1 NL4-3 and SIVmac239 served as controls. Animal identifications are indicated at the top of each panel.

from *vpr* was repaired by deleting the TCT trinucleotide, as described earlier, and the resulting molecular clone was designated NL-DT5R.

Because the ultimate use of NL-DT5R would be as a virus inoculum in nonhuman primate studies, a more rigorous test of its infectivity would be replication in macaque PBMCs. In an initial experiment, unfractionated and ConA-activated PBMC from five PtM and three Indian origin RhM were infected with NL-DT5R, NL4-3, and SIVmac239 by spinoculation (30). Production of SIVmac239 progeny virions was initially detected on day 3 and peaked on day 6 p.i.; no replication of NL4-3 or NL-DT5R was observed during the 12-day course of this infection (data not shown). In contrast to these results, NL-DT5R was able to establish spreading infections in five of five PtM and one of three RhM PBMC preparations when $CD8^+$ T lymphocytes were removed with magnetic beads (Fig. 4). In cells from two of the PtMs (Pt99P022 and Pt98P021), the kinetics and amounts of virus produced were similar to those seen for SIVmac239. It should be noted that NL-DT5R exhibited augmented replication in primary macaque cells compared with NL-ScaVR, the original nontissue culture-passaged construct. As expected, no replication of NL4-3 was detected in the $CD8^+$ T cell-depleted primary monkey cells.

Discussion

Our results are consistent with and extend numerous previously published single-cycle virus replication experiments that have reported species-specific APOBEC3G and TRIM5α restriction of HIV-1 in monkey cells. In our work, the establishment of spreading HIV-1 infections in simian cells represents an important step in significantly increasing the host range of HIV-1. It was conferred by inserting a 21-nucleotide SIV Gag CA element and the entire SIV *vif* gene into the genetic backbone of the

pNL4-3 HIV-1 molecular clone, plus four additional nucleotide changes acquired during long-term passage in a CyM lymphoid cell line. The proportion of HIV-1 sequences in the molecularly cloned NL-DT5R derivative obtained (93%) is substantially greater than that present in currently available CXCR4 (X4) using SHIVs (28–30%). It may be possible to increase the HIV-1 content of NL-DT5R further by mutating the DRMR amino acid residues at positions 14–17 of HIV-1 Vif to SEMQ, which is similar to the analogous region of SIV Vif. Such a change in the *vif* gene has recently been reported to confer replication competence to HIV-1 constructs in the presence of RhM APOBEC3G (31). Construction of other NL-DT5R variants bearing CCR5 using *env* genes from a variety of HIV-1 clades is also a future goal of these studies.

In contrast to commonly used X4 SHIVs, which carry SIV *gag* and *pol* genes, the NL-DT5R variant provides the opportunity to assess nonnucleoside RT inhibitors and a full spectrum of protease inhibitors, which specifically target HIV-1-, not SIV-, encoded enzymes. HIV-1 variants like NL-DT5R may also permit analyses of the cellular responses directed against HIV-1 Gag proteins that are associated with immunologic control and escape, not possible with currently available X4-tropic SHIVs.

Although the host range of the HIV-1 NL-DT5R derivative has expanded to include a monkey lymphoid cell line and CD8-depleted RhM and PtM PBMC, it still replicates less efficiently than SIV in simian cells. This observation undoubtedly reflects the high proportion (93%) of HIV-1 sequences present in the final construct, which have evolved for optimal replicative potential in human, not monkey cells. Additional changes will be required to achieve more robust infectivity for simian cells, which has already occurred to a limited extent with the acquisition of nucleotide substitutions after *in vitro* passaging. In addition to expected alterations in viral structural proteins, long-term passaging of NL-DT5R in monkeys may introduce changes in other HIV-1 sequences affecting analogous but subtly different SIV nonstructural proteins and cis-acting elements involved in processes such as transcriptional regulation and T cell activation pathways in monkey cells. Such alterations are likely to occur in the HIV-1 Nef protein, which is significantly smaller (205–210 aa long) than SIV Nef (260–265 aa long), and the HIV-1 LTR, which can be distinguished from its SIV analog by encoding: (*i*) different numbers/types of binding sites for transcriptional regulatory proteins and (*ii*) a single, not a double, stem-loop-bulge TAR element present at the 5′ termini of all viral transcripts (32). Although the replication properties of HIV-1 NL-DT5R in inoculated monkeys are presently unknown, they are very likely to be less robust than existing X4 SHIVs for the reasons noted above. Nonetheless, it is anticipated that extensive *in vivo* passaging of NL-DT5R will greatly augment its infectivity in macaque cells. In this regard, improved replicative and disease-inducing properties attended serial animal-to-animal transfers of first-generation nonpathogenic SHIVs, and they were associated with extensive sequence changes affecting multiple viral genes (33).

Materials and Methods

Construction of HIV-1 Proviral Clones. A pNL4-3-derived (34) vector, previously used for functional analyses of HIV-1 *vif* genes and designated pNL-SX (25), was the genetic backbone for the constructs shown in Fig. 1. In pNL-Sca, the 9-aa CypA-binding region of pNL-SX/NLVif (25) was replaced with the corresponding 7-residue segment from SIVmac239 CA by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). To construct pNL-SV, the entire SIVmac *vif* sequence was amplified by PCR by using pMA239 (1) as template with forward TCC*CCCGGG*ATGGAGGAGGAAAAGAGGTGG and reverse GC*TCTAGA*TCATGCCAGTATTCCCAAGACC primers containing embedded SmaI and XbaI sites, respectively. The reactions were heated at 95°C for 5 min for 1 cycle; 95°C for 1 min, 51°C for 1 min, and 72°C for 1.5 min for 10 cycles; 95°C for 1 min, 60°C for 1 min, and 72°C for 1.5 min for 25 cycles; and 72°C for 10 min for 1 cycle. The amplified product was subcloned into the SmaI–XbaI site of pNL-SX. To construct pNL-ScaV, the SmaI–XbaI fragment from pNL-SV was cloned into the equivalent sites of pNL-Sca. A negative-control clone, designated pNL-ScaV dBgl, contained a frameshift mutation at the BglII site of SIVmac239 *vif* in the pNL-ScaV.

Cell Culture. The 293T (human embryonic kidney), LLC-MKII (RhM kidney), Vero (AGM kidney), and OMK637 (owl monkey kidney) adherent cell lines were cultured in Eagle's MEM supplemented with 10% heat-inactivated FBS. A $CD4^+CXCR4^+CCR5^-$ CyM T cell line, HSC-F (27), was maintained in RPMI medium 1640 containing 10% FBS. RhM PBMCs were prepared and cultured as described previously (35). For PtM PBMC, a mixture of 95% Ficoll–Paque Plus (GE Healthcare, Piscataway, NJ) and 5% Dulbecco's modified PBS was used as a separation medium. To remove $CD8^+$ T cells from PBMCs, cells were stained with phycoerythrin (PE)-conjugated antihuman CD8 antibody (clone SK1; BD Bioscience, San Jose, CA), and then they were incubated with magnetic beads conjugated with anti-PE antibody (anti-PE MicroBeads; Miltenyi Biotec, Auburn, CA). Unstained cells were collected as the pass-through of a depletion column (LD Column, Miltenyi Biotec) according to the manufacturer's instructions.

Transfection, Infection, and RT Assays. Virus stocks were prepared by transfecting 293T cells with cloned HIV-1 NL4-3 derivatives by using either calcium phosphate coprecipitation (36) or Lipofectamine Plus (Invitrogen, Carlsbad, CA); 48 h later, culture supernatants were collected and stored at −80°C until use. Virion-associated RT activity was measured as described previously (28). HSC-F cells (1×10^7) were infected with equivalent amounts (1×10^7 RT units) of different virus preparations, and then they were monitored for RT activity in the culture supernatants. Macaque PBMCs (5×10^6) were infected with similar amounts (1×10^7 RT units) of the indicated viruses by spinoculation (30) for 1 h, and they were maintained for 12 days. The tissue culture medium was replaced daily.

Cloning of APOBEC3G Genes. Species-specific APOBEC3G cDNA was amplified from H9 (human), HSC-F (CyM), and Vero (AGM) cells by RT-PCR (described in *Supporting Methods*, which is published as supporting information on the PNAS web site) and cloned into pcDNA3.1-FLAG, an expression vector containing the FLAG tag sequence in pcDNA3.1 (Invitrogen). The expression levels of the three APOBEC3Gs in transfected 293T cells were monitored by immunoblotting using the anti-FLAG antibody.

Single-Cycle Replication Assays. The effects of the species-specific APOBEC3Gs on virus replication were evaluated by using VSV-G-pseudotyped HIV-1 stocks, prepared from 293T cells cotransfected with (*i*) individual *env*-deficient NL4-3 clones (NL4-3, NL-SVR, NL-ScaR, NL-ScaVR, and NL-ScaVR dBgl); (*ii*) pCMV-G (37), a VSV-G protein expression vector; and (*iii*) an individual species-specific APOBEC3G expression vector at a ratio of 8:1:1. The infectivity of the resultant viruses was determined by MAGI assay as described previously (38). To assess the effect of *gag* gene substitutions during single-cycle replication in cells from different primate species, VSV-G-pseudotyped viruses were prepared from 293T cells cotransfected with (*i*) individual *env*-deficient NL4-3 clones [NL4-3, NL-SVR, NL-ScaR, and NL-ScaVR] and (*ii*) pCMV-G, at a ratio of 9:1. Virus released into the medium and normalized for RT

activity was added directly or as 3-fold serial dilutions to 293T, LLC-MKII, and OMK637 cells, plated at a density of 5×10^4 cells per well in 24-well plates on the day before infection. On day 3 p.i., cells were lysed with CHAPS-based lysis buffer (28), and the amounts of intracellular p24 were determined by using the RETROtek p24 ELISA kit (ZeptoMetrix, Buffalo, NY). The total amount of protein in each sample was determined in parallel with the DC protein assay kit (Bio-Rad, Hercules, CA) to normalize for different cell-harvesting efficiencies.

Generation of pNL-DT5. HSC-F cells were infected NL-ScaV virus prepared from transfected 293T cells as described above. Half of the culture medium (5 ml) was replaced every 3 days, and the harvested supernatants were stored at −80°C. Fresh HSC-F cells (1×10^7) were added on days 27, 36, and 45 p.i., and the culture was maintained until 51 days p.i. The supernatants collected on days of 39 and 51 p.i. were filtered through a 0.45-μm filter and used to initiate a second round of infection (5×10^6 RT units of viruses added to 1×10^7 HSC-F cells). On day 18 p.i., cells infected with the day 51 supernatant (sup 51) were collected (Fig. 3*C*), and the integrated provirus was amplified from genomic DNA as two overlapping fragments by DNA PCR. The 5′ fragment extended from 5′ LTR to the Vpr-coding region, whereas the 3′ fragment spanned the Vif-coding region to the 3′ LTR. The 5′ fragment was amplified with the NL1–24Aat-5′ (AGTCA*GACGTC*TGGAAGGGCTAATTTGGTCCCAAA at nucleotide positions 1–24 in NL4-3) and NL5832–5855Bam-3′ (ATCGCGGATCCTCTAGTCTAGGATCTACTGGCTCC at 5832–5855) primer pairs, whereas the 3′ fragment was amplified with the NL5596–5619Xba-5′ (GCTAGTCTAGAAGCCATACAATGAATGGACACTAG at 5596–5619) and NL9686–9709Sph-3′ (ACATG*GCATGC*TGCTAGAGATTTTCCACACTGACT at positions 9686–9709) primer pairs. The reactions were heated at 95°C for 5 min for 1 cycle; 95°C for 0.5 min, 51°C for 0.5 min, and 72°C for 6 min for 10 cycles; 95°C for 0.5 min, 60°C for 0.5 min, and 72°C for 6 min for 25 cycles; and 72°C for 10 min for 1 cycle. The amplified 5′ and 3′ viral DNA segments were digested with AatII–EcoRI and EcoRI–SphI, respectively, and they were then cloned together into pUC19 digested with AatII–SphI. The resultant proviral clone was designated pNL-DT5.

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Effects of lysine to arginine mutations in HIV-1 Vif on its expression and viral infectivity

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Abstract. We previously demonstrated that the expression in cells of human immunodeficiency virus type 1 (HIV-1) Vif is maintained at low level by proteasome-degradation. We examined the contribution of 16 lysines present in Vif (NL432 clone), which is composed of 192 amino acids (aa), to its expression within cells and to viral infectivity for non-permissive cells. To this end, various lysine-arginine mutations were introduced into wild-type (wt) Vif, and the mutational effects were monitored by transfection experiments. When all the lysines were changed to arginines, the mutant Vif was expressed in cells at much higher level than wt and was much more stable. Both N-terminal (aa nos. 34 and 36) and C-terminal (aa nos. 179 and 181) lysines were found to be almost sufficient for wt property. Different from this observation, one of the lysines at aa nos. 22 and 26 was demonstrated to be essential for the virus to grow in non-permissive cells. Our results showed that there is no clear co-relationship between the expression level of HIV-1 Vif and viral infectivity.

Introduction

Vif is one of the human immunodeficiency virus type 1 (HIV-1) accessory proteins, and is conserved in all known primate immunodeficiency viruses (1). It is dispensable for the replication of HIV-1 in permissive cells like MT-4 (2) and M8166 (3), but is critical for the viral growth in non-permissive cells such as H9 (4) and peripheral blood mononuclear cells (5-9). Recent studies have shown that the non-permissive cells have a cytidine deaminase APOBEC3G carrying anti-viral activity, and that the Vif counteracts the virion incorporation of APOBEC3G (10-19). The precise molecular mechanism for this activity of Vif, however, remains to be elucidated.

We have recently demonstrated that the expression of HIV-1 Vif is controlled uniquely to be at low level among accessory proteins by proteasome degradation (20,21). Virological significance of this degradation can be explained by the fact that a high expression level of Vif inhibits viral infectivity through modulating proteolytic processing of the Gag precursor at the p2/nucleocapsid processing site (22). However, the experiments were done in a quite artificial system; expression of a large amount of Vif by pNL-A1 (23) and examination of infectivity by a single-round replication assay.

In this study, we investigated the relationship between the Vif expression level within cells and viral multi-cycle infectivity for the non-permissive cells. For this purpose, we introduced a wide variety of lysine to arginine mutations into wild-type (wt) Vif, because it is well known that proteins are poly-ubiquitinated at their lysine residues or N-terminus to become a marker recognized by the proteasome, and that the polyubiquitinated proteins are then degraded (24); the lysine and arginine have similar physicochemical characteristics. We identified lysines in Vif which are important for the wt expression level within cells and for the viral multi-cycle infectivity in non-permissive cells.

Materials and methods

Cells. A lymphocytic cell line H9 (4) was cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). A monolayer cell line 293T (25) was cultured in Eagle's minimal essential medium supplemented with 10% heat-inactivated FBS.

Transfection. For transfection of plasmid DNAs into adherent 293T cells, the calcium-phosphate coprecipitation technique (26) or the Lipofectamine Plus™ system (Invitrogen, Carlsbad, CA, USA) was used. For transfection of lymphocytic H9 cells, the electroporation method was used as previously described (26).

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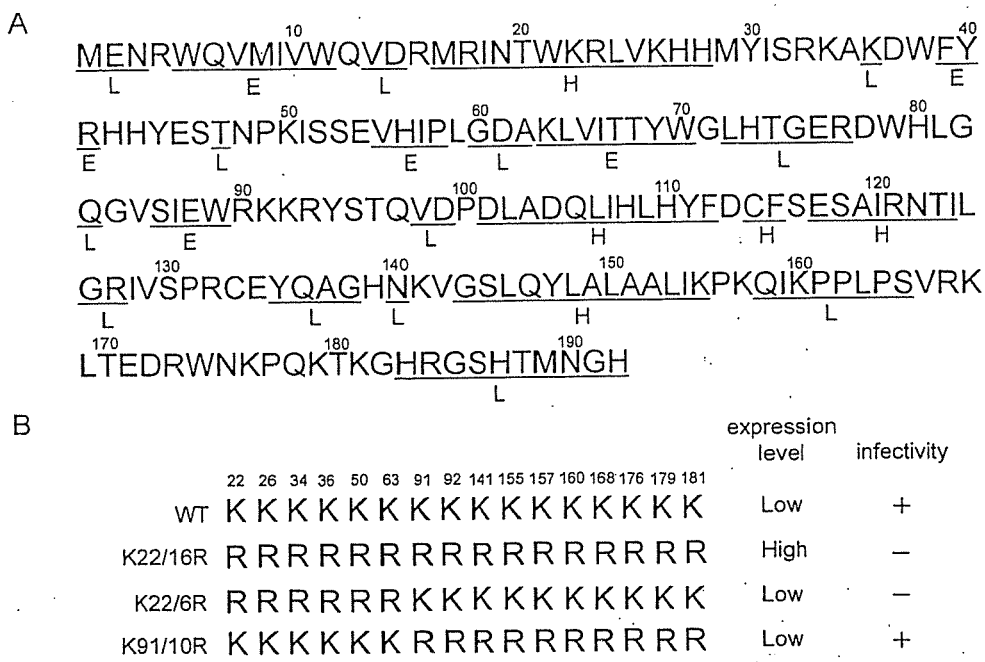

Figure 1. The lysine-arginine alterations in the first group of Vif mutants. Structure of the Vif of HIV-1 NL432 clone by the PredictProtein (http://www.predictprotein.org/) (A) and the alterations in this Vif (the first group mutants) (B) are indicated. L, E and H in (A) represent the loop, ß-strand and α-helix structures, respectively. Data on the expression level in cells of the mutated Vif proteins and the infectivity for H9 cells of the mutants are also shown in (B) (see Figure 2).

Reverse transcription (RT) assay. Virus production in the culture supernatants of transfected H9 cells was monitored by RT assay as previously described (27).

Western immunoblot analysis. Transfected 293T cells were collected and solubilized by dissolving in PBS-Laemmli's sample buffer (1:1) for SDS-PAGE as previously described (20). Samples resolved by the SDS-PAGE were then electrophoretically transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore, Bedford, MA, USA). The membranes were treated with anti-FLAG antibody (Ab) (ANTI-FLAG M2 Monoclonal Ab, Sigma-Aldrich, St. Louis, MO, USA) and visualized using the ECL plus Western blot detection system (Amersham Biosciences, Buckinghamshire, UK).

Pulse/chase analysis. Transfected 293T cells were pulse-labelled with ^{35}S, and chased as previously described (20). Cells harvested were lysed with the CHAPS/DOC buffer as described previously (20), and the cell lysates were precipitated with a Vif-specific polyclonal antibody Vif93 (28). Wt and mutant Vif proteins were identified by SDS-PAGE followed by fluorography as previously described (20).

DNA constructs for infection experiments. An infectious proviral clone of HIV-1 designated pNL432 (26) was used as wt clone for infection experiments. Appropriate fragments of pNL432 were subcloned into pBluescript SK(+) (Stratagene, La Jolla, CA, USA) and mutations were introduced. The alterations of lysines into arginines were performed by the QuikChange site-directed mutagenesis kit (Stratagene). The mutated fragments were cloned back to wt to construct pNL-K22/6R, pNL-K91/10R, pNL-K22/2R, pNL-K34/2R, pNL-K50R, pNL-K63R, pNL-K22R and pNL-K26R. To make pNL-K22/16R, appropriate fragments of pNL-K22/6R and pNL-K91/10R were used. Clone pNL-K34/14R was constructed from the pNL-K22/16R similarly as above. As a negative control, pNL-Nd (29) carrying a frame-shift mutation in *vif* was used.

DNA constructs for Western immunoblot and pulse/chase analyses. The pNL-A1S (21) was used to construct expression vectors for Western blot and pulse/chase analyses. To generate pNL-ASCF, a *Cla*I site and the FLAG sequence (in this order) were introduced just upstream of the stop codon of *vif* in pNL-A1S (21) by the QuikChange site-directed mutagenesis kit. The *vif* sequences of pNL432 and its mutants, pNL-K22/16R, pNL-K22/6R, pNL-K91/10R and pNL-K34/14R, were amplified by polymerase chain reaction (PCR) with *Sma*I at 5' and *Cla*I at 3' ends, respectively. The *Sma*I-*Cla*I fragment from the pNL-ASCF was replaced with these PCR-amplified sequences to construct pNL-ASCF-fWT, pNL-ASCF-fK22/16R, pNL-ASCF-fK22/6R, pNL-ASCF-fK91/10R and pNL-ASCF-fK34/14R. An appropriate fragment of pNL-ASCF-fK22/16R was subcloned into pBluescript SK(+), and mutations were introduced by the QuikChange site-directed mutagenesis kit. The mutated fragments were cloned back to pNL-ASCF-fK22/16R to construct pNL-ASCF-K22/2R/50/12R, pNL-ASCF-K22/6R/141/8R, pNL-ASCF-K22/9R/160/5R and pNL-ASCF-K22/14R. As an expression vector for luciferase, pGL3-Control Vector (Promega, Madison, WI, USA) was used.

Results

Expression and infectivity of various HIV-1 Vif mutants. We examined the importance of lysines present in Vif for its

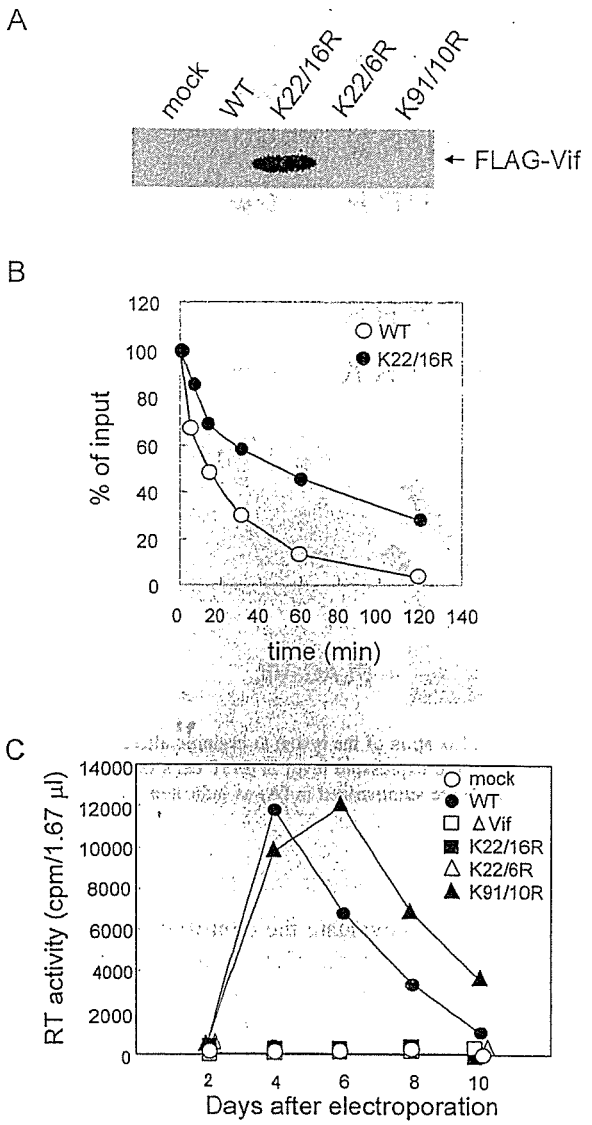

Figure 2. Characteristics of the first group of Vif mutants. (A) Expression level in cells of wt and mutant clones. Co-transfected 293T cells with 7.5 μg of the subgenomic viral clones derived from pNL-ASCF-fWT and 2.5 μg of an expression vector for luciferase, at 48-h post-transfection, were harvested for Western blot analysis and luciferase assay. The loading amount in each lane was normalized by the luciferase activity. mock, pUC19; WT, pNL-ASCF-fWT. (B) Degradation kinetics in 293T cells of the K22/16R mutant. Transfected 293T cells with 5 μg of the subgenomic clones, and at 24-h post-transfection, were collected, ^{35}S-labeled, and chased for ≤120 min for immunoprecipitation analysis. WT, pNL-ASCF-fWT. (C) Growth kinetics in non-permissive cells of wt and mutant clones. H9 cells were transfected with 10 μg of the full-length viral clones, and virus production in the culture supernatants was monitored by RT assay. mock, pUC19; WT, pNL432; ΔVif, a frame-shift mutant pNL-Nd.

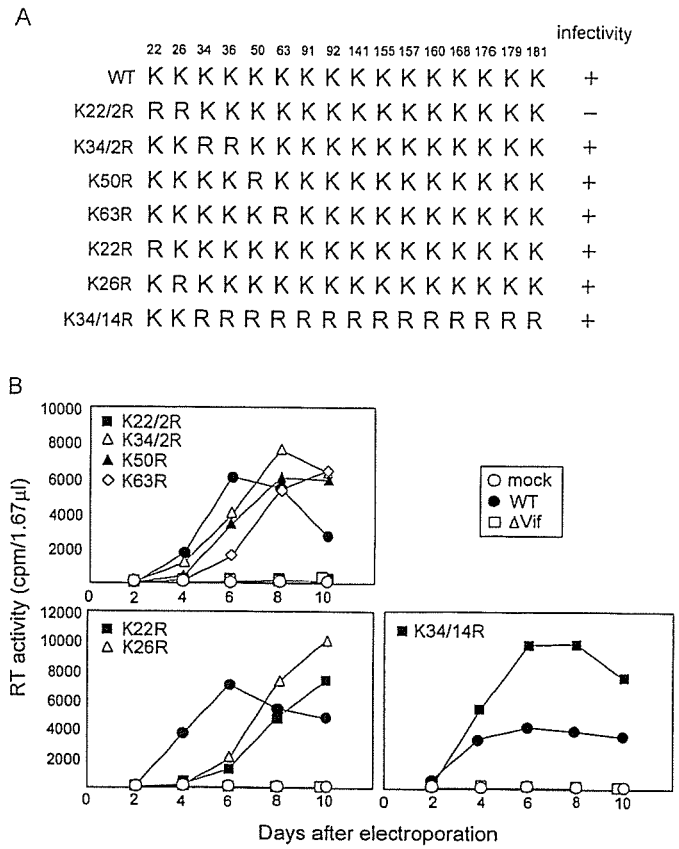

Figure 3. Identification of the lysine residue in Vif critical for viral infectivity. Location of the lysine to arginine alterations in the Vif of the mutants (A) and their growth kinetics in H9 cells (B) are shown. For determination of viral infectivity in H9 cells, cells were transfected with various full-length clones and monitored for virus production as above. Data in (B) are summarized in (A) as indicated. mock, pUC19; WT, pNL432; ΔVif, a frame-shift mutant pNL-Nd.

expression and viral infectivity. As the first group mutants for this study, clones K22/16, K22/6R and K91/10R, which carry all 16, N-terminal 6 and C-terminal 10 lysine-ariginine exchanges, respectively, were constructed and characterized (Fig. 1). For a quantitative comparison, their expression to a high level was achieved by the subgenomic-type clone (21) and analyzed by Western immunoblotting using anti-FLAG antibody. As shown in Fig. 2A, the mutant K22/16R produced its Vif at an extremely high level relative to those of wt, K22/6R and K91/10R. We determined the stability of the K22/16R Vif by the pulse/chase experiment as previously described (20). As shown in Fig. 2B, in a good agreement with the steady-state expression level, K22/16R was much more stable than wt. We next examined the infectivity of these mutants for non-permissive cells. H9 cells were electroporated with the full-length version of the mutant clones, and virus growth was monitored by RT assay (30). As shown in Fig. 2C, only the mutant K91/10R among the three mutants, which express a high or negligible level of Vif in cells (Fig. 2A), grew fairly well.

In total, our results described herein indicated that the lysines present in Vif were important for the stable expression of Vif, and that there is no clear negative or positive co-relationship between the expression level of Vif and infectivity of lysine-ariginine mutants.

Lysine residues in Vif important for viral infectivity. To determine the lysine residue in Vif crucial for viral infectivity, we constructed another set of proviral mutant clones. One or two lysines located at the N-terminal region of Vif were changed to the arginine residue (K22/2R, K34/2R, K50R and K63R in Fig. 3A), and the resultant clones were examined for their growth kinetics in H9 cells as above. As shown in Fig. 3B, only the K22/2R were not infectious. Therefore, we

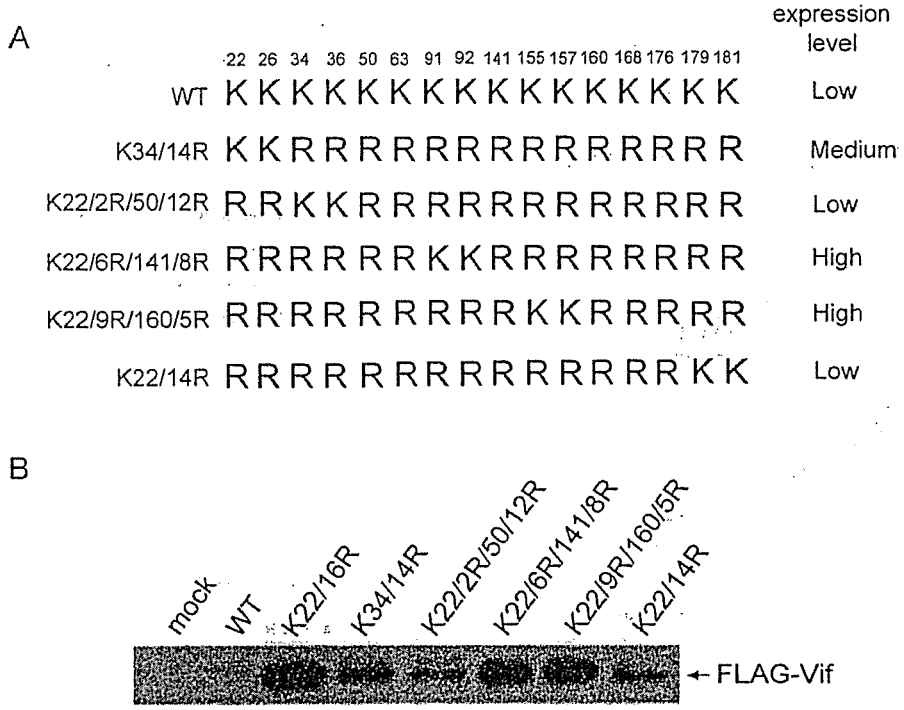

Figure 4. Identification of the lysine residue in Vif critical for the wt expression level within cells. Location of the lysine to arginine alterations in the Vif of the mutants (A) and their expression in the transfected 293T cells (B) are shown. For monitoring the expression level in 293T cells of the Vif, cells were transfected with various subgenomic clones and analyzed for their expression as above. Data in (B) are summarized in (A) as indicated. mock, pUC19; WT, pNL-ASCF-fWT.

constructed K22R, K26R and K34/14R (Fig. 3A) to determine whether one of the K^{22} and K^{26} or both are essential for viral infectivity, and whether the lysines other than K^{22} and K^{26} are critical for viral infectivity. The three additional mutants thus constructed were transfected into H9 cells, and their growth was examined. As shown in Fig. 3B, the three mutants grew to a comparable extent. All the mutants described above were confirmed to propagate in permissive M8166 cells (data not shown). Collectively, we concluded that either K^{22} or K^{26} of Vif is critical for the productive infection of HIV-1 NL432 in non-permissive cells.

Lysine residues in Vif important for the expression level of Vif. To determine the lysine residue in Vif crucial for the Vif expression, we constructed a series of double lysine-arginine mutants other than the K34/14R, based on the subgenomic clone (21) (Fig. 4A). The mutants were then analyzed for their Vif expression by Western immunoblotting as described above. When the mutant K34/14R, which is infectious for H9 cells (Fig. 3B), was monitored for its Vif expression, it displayed a medium expression level between wt and the stable and non-infectious mutant K22/16R (Fig. 4B). This observation again indicated the absence of a detectable close relationship between viral infectivity and the Vif expression level. The data on the other mutants in Fig. 4B clearly showed that the K^{34} and K^{36} or the K^{179} and K^{181} are responsible for the low expression level of Vif. While the mutants K22/6R/141/8R and K22/9R/160/5R produced a similarly high expression level to that of the K22/16R, the mutants K22/2R/50/12R and K22/14R expressed a low level of Vif quite similar to the wt clone. We also constructed a complete set of single lysine-arginine mutants through one by one alterations to evaluate the contribution of each lysine to the Vif expression. All the single mutants constructed were found to produce Vif, upon transfection, at a level higher than that of the mutants K22/2R/50/12R and K22/14R (data not shown). These results strongly suggested that each lysine residue in Vif is less important than the combination of K^{34} and K^{36} or of K^{179} and K^{181} for the wt expression level of Vif.

Discussion

In this study, we showed by a mutational analysis that the lysines in HIV-1 Vif are important for its steady-state expression in transfected 293T cells, and that the two lysines in Vif (K^{34} and K^{36} or K^{179} and K^{181}) are nearly sufficient for the expression property of the Vif (Figs. 2 and 4). We also demonstrated that either K^{22} or K^{26} in Vif is critical for the replication of HIV-1 in H9 cells (Figs. 2 and 3). Thus, we did not find any clear co-relationship between the expression level in cells of Vif and viral infectivity for the non-permissive cells.

Together with the results previously published (20,22), it was reasonable to assume that the K^{34} and K^{36} or K^{179} and K^{181} are important to maintain the low appropriate expression level of Vif by the proteasome-degradation, and thereby enable the virus to grow in non-permissive cells. However, our data herein on the mutants demonstrating that either K^{22} or K^{26} is critical for viral infectivity do not support this prediction, and instead, the mechanism(s) and molecule(s) other than the proteasome-degradation and its associated factors which confer the infectivity on the virus should be considered. They would include 1) the structure of the lysine itself, 2) covalent modification(s) of the lysine and 3) interaction of the lysine

with some unknown factor(s). Further study is required to clarify the molecular basis for our observations on the Vif mutants.

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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

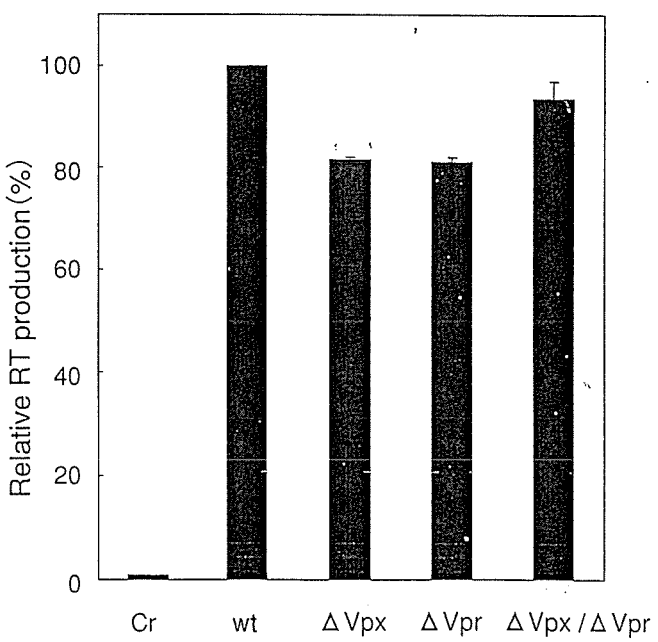

Fig. 1 Progeny virion production in 293T cells transfected with Vpx/Vpr mutant clones. Cells were transfected with various proviral clones indicated, and on day 2 post-transfection, virus production was determined by RT assay. RT production relative to that of wt clone is shown.

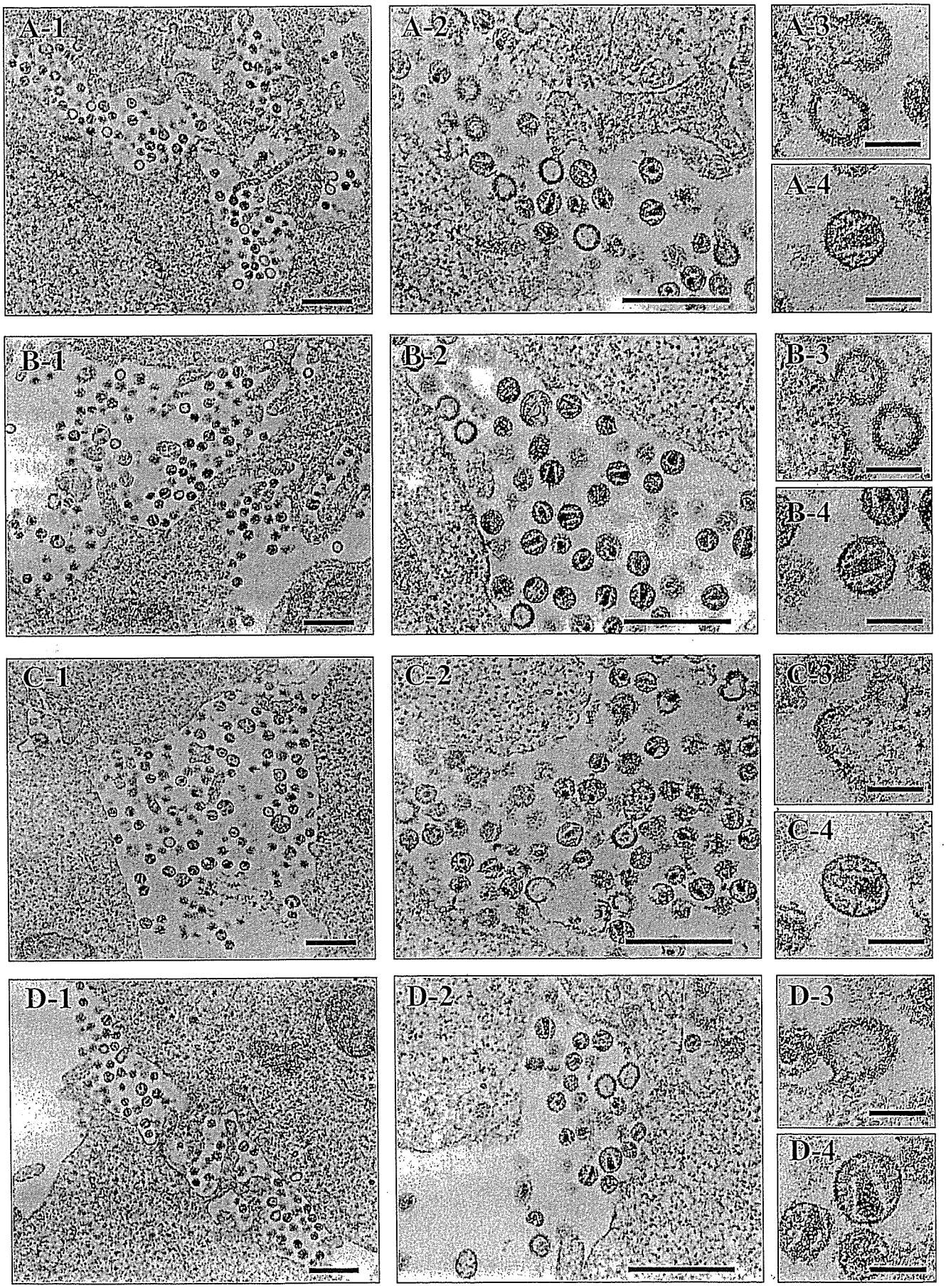

Fig. 2 EM analysis of Vpx/Vpr mutant virions produced in transfected 293T cells. Cells on day 2 post-transfection in Fig.1 were subjected to EM analysis as described in MATERIALS AND METHODS. Samples:A-1 to A-4, wt; B-1 to B-4, Δ Vpx;C-1 to C-4, Δ Vpr; D-1 to D-4, ΔVpx/ΔVpr. Bars : 500nm for A-1 & A-2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

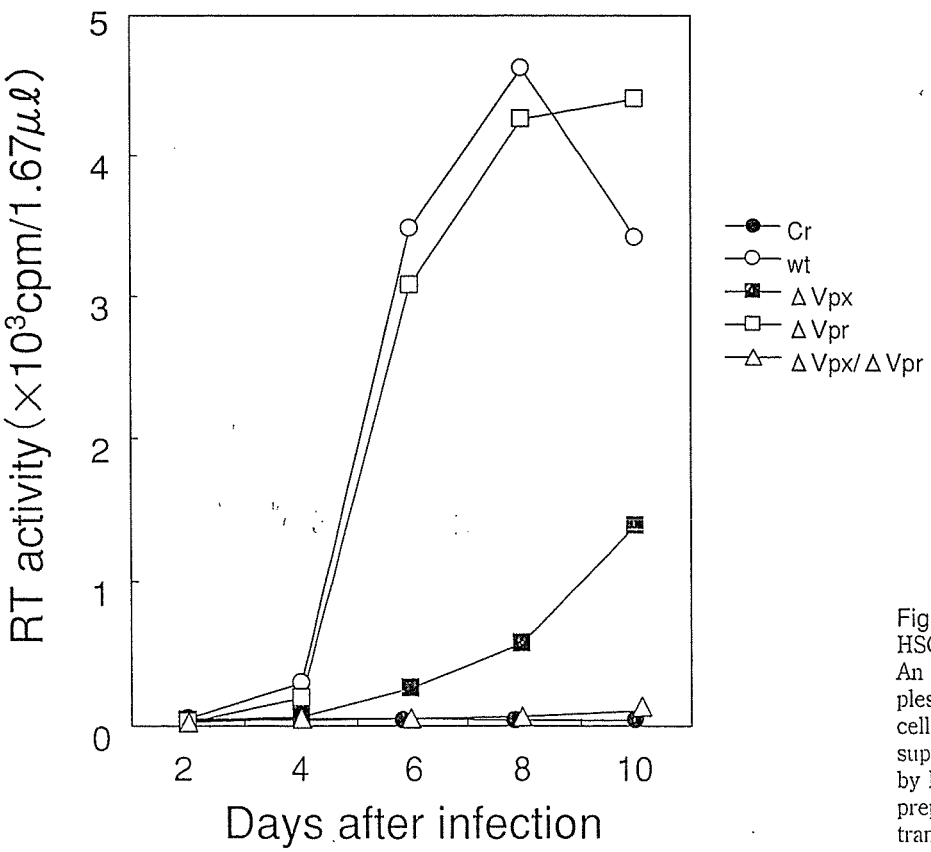

Fig. 3 Growth kinetics in lymphocytic HSC-F cells of Vpx/Vpr mutant viruses. An equal amount of cell-free virus samples indicated was inoculated into HSC-F cells, and virus production in the culture supernatants was monitored at intervals by RT assay. Input cell-free viruses were prepared from 293T cells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

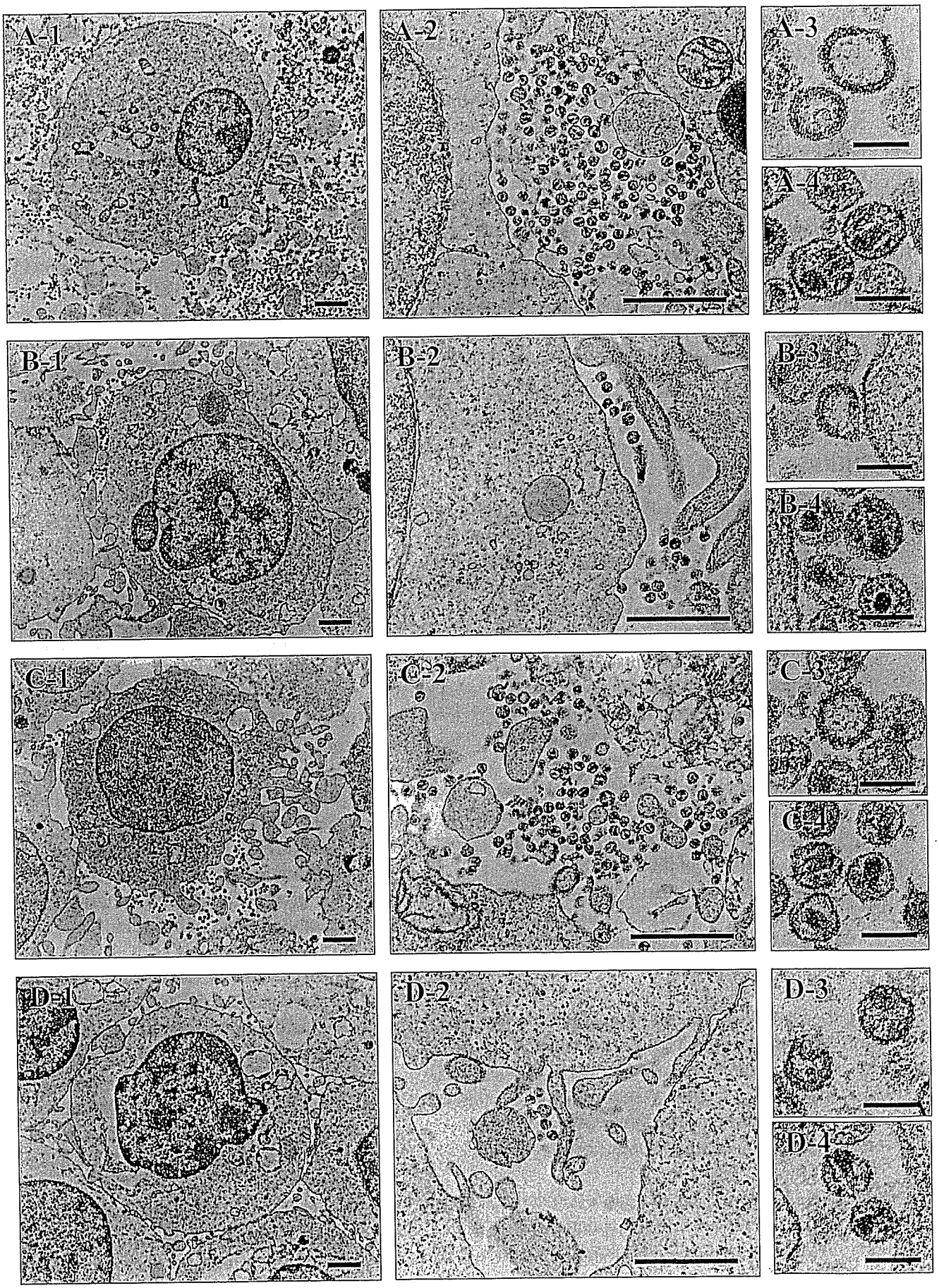

Fig. 4 EM analysis of Vpx/Vpr mutant virions produced in infected HSC-F cells. Cells on day 11 post-infection in Fig.3 were subjected to EM analysis as described in MATERIALS AND METHODS. Samples: A-1 to A-4, wt; B-1 to B-4, Δ Vpx; C-1 to C-4, Δ Vpr; D-1 to D-4, ΔVpx/ΔVpr. Bars: 1μm for A-1 & A-2 to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

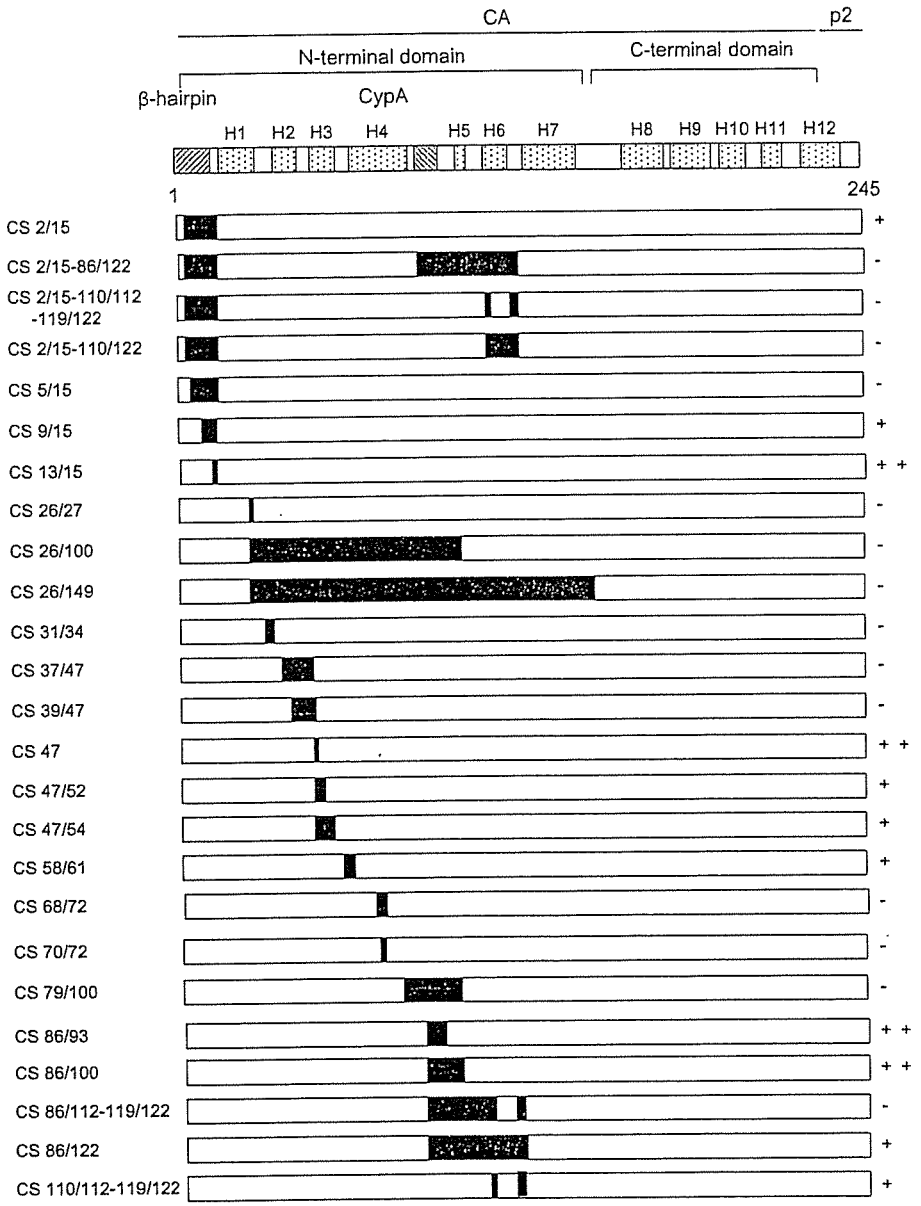

Fig. 1. *Gag*-chimeric viruses between HIV-1 and SIVmac used in this study. Location of SIVmac Gag sequence (MA239) inserted into HIV-1 Gag CA-p2 region (NL432) is indicated by black area. For the sequences inserted, see Table 1. Growth ability of viruses in M8166 cells is given as ++ (wt growth), + (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