Table 1
S. pombe strains used in this study

Brief genotype	Genotype[b]	Description	Source
Wild type (WT)[a]	*wee1::HA$_3$-His$_6$-wee1$^+$*	Normal cell cycle; normal DNA damage and replication checkpoints	M. O'Connell
rad24	*wee1::HA$_3$-His$_6$-wee1$^+$* *ura4-D18 rad24::ura4$^+$*	No expression of Rad24 involved in cell cycle control and DNA damage checkpoint	Our lab
chk1	*wee1::HA$_3$-His$_6$-wee1$^+$* *ura4-D18 chk1::ura4$^+$*	No expression of Chk1, a transducer of DNA damage and replication checkpoints	Our lab
cds1	*wee1::HA$_3$-His$_6$-wee1$^+$* *ura4-D18 cds1::ura4$^+$*	No expression of Cds1, a transducer of DNA replication checkpoint	Our lab
chk1 cds1	*wee1::HA$_3$-His$_6$-wee1$^+$* *ura4-D18 chk1::ura4$^+$ cds1::ura4$^+$*	No expression of Chk1 or Cds1	Our lab

[a] The strain expresses Wee1 tagged with HA_3 and His_6 and is expediently designated as "wild type" in this study.
[b] The genetic background (*h$^-$ leu1–32*) common to all of the strains is omitted.

treated with RNase A (0.1 mg/ml) in 50 mM sodium citrate (pH 7.0) for 2 h, and stained with propidium iodide (10 μg/ml) for 1 h. The DNA content of the cells was then measured by a flow cytometer (FACSCalibur flow cytometry system; BD Biosciences).

2.4. Examination of yeast cell morphology

S. pombe cells in the mid-log growth phase were seeded in MM supplemented with or without thiamine at a density of 1×10^5/ml and grown at 30 °C for 36 h with vigorous shaking. Then, morphology of the cells was observed as described previously [8] under a phase-contrast microscope (Olympus) without fixation. Representative pictures were taken by using a CCD camera (KV-26B; Hitachi Electronics) and printed by a video copy processor (SCT-P67; Mitsubishi Electric Corp.).

2.5. Protein analysis

S. pombe cells in the mid-log growth phase were seeded in MM supplemented with or without thiamine at a density of 1×10^6/ml and grown at 30 °C for 16 h with vigorous shaking. Then, cell extracts were prepared by the glass beads method in urea buffer (8 M urea, 100 mM Na_2HPO_4, 10 mM Tris–HCl, pH 8.0) supplemented with phosphatase inhibitors I and II (Sigma). The samples were then electrophoresed on a NuPage Bis-Tris gel (Novex), and blotted to a polyvinylidene difluoride (PVDF) membrane. For immunological detection of HA-tagged Wee1 and HIV-1 Vpr, anti-HA monoclonal antibody 12CA5 (Roche Diagnostics) and anti-Vpr rabbit serum (NIH AIDS Research and Reference Reagent Program) were used, respectively. Human 14-3-3 proteins were detected by using antibodies specifically reacting with 14-3-3β (A-15; Santa Cruz Biotechnology) and 14-3-3ζ (C-16; Santa Cruz Biotechnology), or an antibody broadly reactive with all 14-3-3 isoforms (H-8; Santa Cruz Biotechnology). Binding of the antibodies was visualized by using peroxidase conjugated anti-mouse or rabbit IgG antibodies (Amersham Pharmacia Biotech) and a BM chemiluminescence blotting kit (Roche Diagnostics). As an internal control for the amount of loaded samples, α-tubulin was detected by reprobing the filters with an anti-α-tubulin monoclonal antibody (clone DM1A, Sigma).

For dephosphorylation of Wee1, cell extracts were added with Ni-NTA-agarose (Qiagen) for affinity isolation of Wee1 with a His_6 tag, and the beads were washed extensively in urea buffer. Then precipitates were washed three times in CIP buffer (50 mM Tris–HCl, 5 mM $MgCl_2$, pH 8.0) and treated with calf intestinal alkaline phosphatase (New England Biolabs) for 30 min at 37 °C and processed for gel electrophoresis and immunoblot analysis.

3. Results

3.1. Wee1 is phosphorylated and expressed at a higher level in the fission yeast cells expressing HIV-1 Vpr

It has previously been shown that expression of HIV-1 Vpr in the fission yeast *S. pombe* causes cell cycle G2 arrest manifested by elongated morphology (*cdc* phenotype) in a *wee1$^+$*-dependent manner [8]. In this study, strains carrying HA-*wee1$^+$* were used. Addition of a HA_3-His_6 tag to the N-terminus of Wee1 did not affect the susceptibility to Vpr-induced G2 arrest (Fig. 1A), and the *cdc* phenotype was observed (Fig. 1B). Immunoblot analysis with an antibody to the HA tag detected Wee1 as a protein of about 110 kDa (Fig. 1C, lanes 3–6, lower arrowhead) as described previously [13]. Interestingly, additional signal corresponding to a slightly larger molecular size was also detected for Vpr-expressing cells (Fig. 1C, lane 4, upper arrowhead). When the samples were treated with calf intestinal alkaline phosphatase, the upper band disappeared (Fig. 1D, lanes 4 and 6), indicating that the molecule with the larger size corresponded to a phosphorylated form of Wee1. It is also noteworthy that the total amount of Wee1 appeared to be increased in Vpr-expressing cells (Fig. 1C, lane 4 and Fig. 1D, lanes 2, 4, and 6).

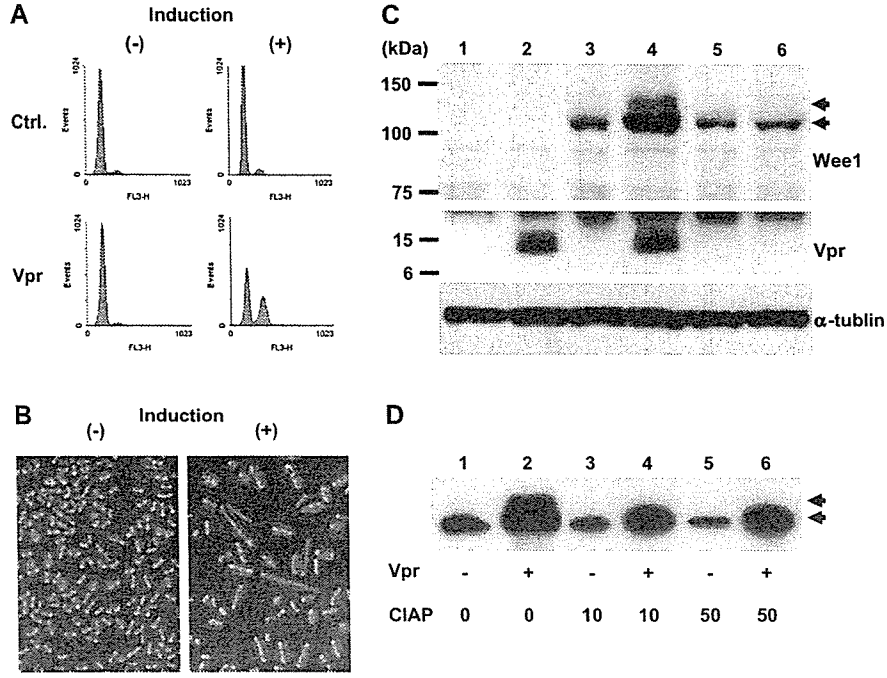

Fig. 1. HIV-1 Vpr induces the G2 cell cycle arrest in the fission yeast *S. pombe* associated with phosphorylation and increase of Wee1. (A) HA-*wee1*$^+$ *S. pombe* cells carrying pREP-1 (Ctrl.) or pREP1-vpr (Vpr) were grown in low nitrogen medium in the presence [Induction (−)] or absence [Induction (+)] of thiamine for 36 h, and the nuclear DNA content was measured for cell cycle analysis. (B) Photomicrographs of HA-*wee1*$^+$ *S. pombe* cells carrying pREP1-vpr grown under Vpr-inducing (+) and noninducing (−) conditions for 36 h. Original magnification, ×400. Elongated morphology (the *cdc* phenotype) of the cells indicates induction of the G2 cell cycle arrest. (C) Parental *wee1*$^+$ (lanes 1 and 2) and HA-*wee1*$^+$ (lanes 3–6) *S. pombe* cells carrying pREP1-vpr (lanes 1–4) or pREP-1 (lanes 5 and 6) were grown in the presence (lanes 1, 3, and 5) or absence (lanes 2, 4, and 6) of thiamine for 16 h. Extracts prepared from these cells were fractionated by gel electrophoresis (40 μg/lane) and transferred to a PVDF membrane. The membrane was probed with anti-HA monoclonal antibody (upper panel) or an anti-Vpr rabbit serum (lower panel). Positions of molecular size markers are shown on the left. Arrowheads on the right indicate positions of Wee1 and its high-molecular-weight form. Signals of an internal control, α-tubulin, indicate that the amounts of samples loaded in the lanes are comparable to each other. (D) Cells were grown under Vpr-inducing (+) or noninducing (−) conditions for 16 h, and the cell extracts were prepared. Then Wee1 with the HA_3-His_6 tag was precipitated on Ni-NTA agarose from the extracts (300 μg), treated with the indicated units of calf intestinal alkaline phosphatase (CIAP) and processed for immunoblot analysis with anti-HA monoclonal antibody. Lower and upper arrowheads on the right indicate positions of Wee1 and its phosphorylated form, respectively.

3.2. Increase in the phosphorylation and the amount of Wee1 is associated with induction of G2 cell cycle arrest by Vpr in WT S. pombe

To examine whether Vpr-induced G2 arrest and Wee1 phosphorylation are associated, *vpr* genes derived from HIV-1 clinical isolates were cloned in pREP-1, and their effects were compared. These clinically isolated genes encode Vpr with scattered amino acid substitutions compared with HIV-1_{NL4-3} Vpr (Fig. 2A). While Vpr from isolate #16 (Vpr16) failed to cause the cell cycle arrest in fission yeast, Vpr from isolate #17 (Vpr17) was able to induce G2 arrest manifested by *cdc* phenotype (Fig. 2B). The phosphorylated form of Wee1 was detected in the cells expressing HIV-1_{NL4-3} Vpr and Vpr17, but not Vpr16 (Fig. 2C, upper panel). The amount of non-phosphorylated Wee1 appeared to be the same for Vpr-, Vpr16-, and Vpr17-expressing cells. However, the total amount of Wee1 in Vpr- and Vpr17-expressing cells should be larger than in the non-expressing cells due to presence of the phosphorylated form in these cells. This was confirmed by comparison of the total amount of Wee1 after CIAP treatment (Fig. 2C, bottom panel). Therefore, the ability of Vpr to induce G2 arrest appeared to be associated with its ability to cause increase in the phosphorylation and the amount of Wee1.

3.3. Phosphorylation of Wee1 in Vpr-expressing cells requires neither Chk1 nor Cds1

Our previous studies showed that Vpr can cause G2 arrest in *chk1* and *cds1* mutants of *S. pombe* [8]. Consistently, Vpr induced G2 arrest in the *chk1* and *cds1* mutants with HA-*wee1*$^+$ background (Fig. 3A). A *chk1 cds1* double mutant was also susceptible to Vpr-induced G2 arrest (Fig. 3A). In response to Vpr expression, Wee1 was phosphorylated in these mutants at a level comparable to that in the strain with intact *chk1*$^+$ and *cds1*$^+$ (Fig. 3B). Therefore, neither Chk1 nor Cds1 was essential for Wee1 phosphorylation in Vpr-expressing *S. pombe*.

3.4. In rad24 mutant refractory to Vpr-induced G2 arrest, the level of Wee1 expression was lower than in WT in the absence and presence of Vpr

It was previously shown that *rad24* mutant of *S. pombe* was refractory to Vpr-induced G2 arrest, indicating that *rad24*$^+$ plays an essential role in G2 arrest induction by Vpr in *S. pombe* [8]. In order to study how *rad24*$^+$ contributes to Vpr-induced G2 arrest, *rad24* mutant of *S. pombe* carrying HA-*wee1*$^+$ was transformed with pREP1-vpr and effects of Vpr expression were examined. Consistent with the previous study

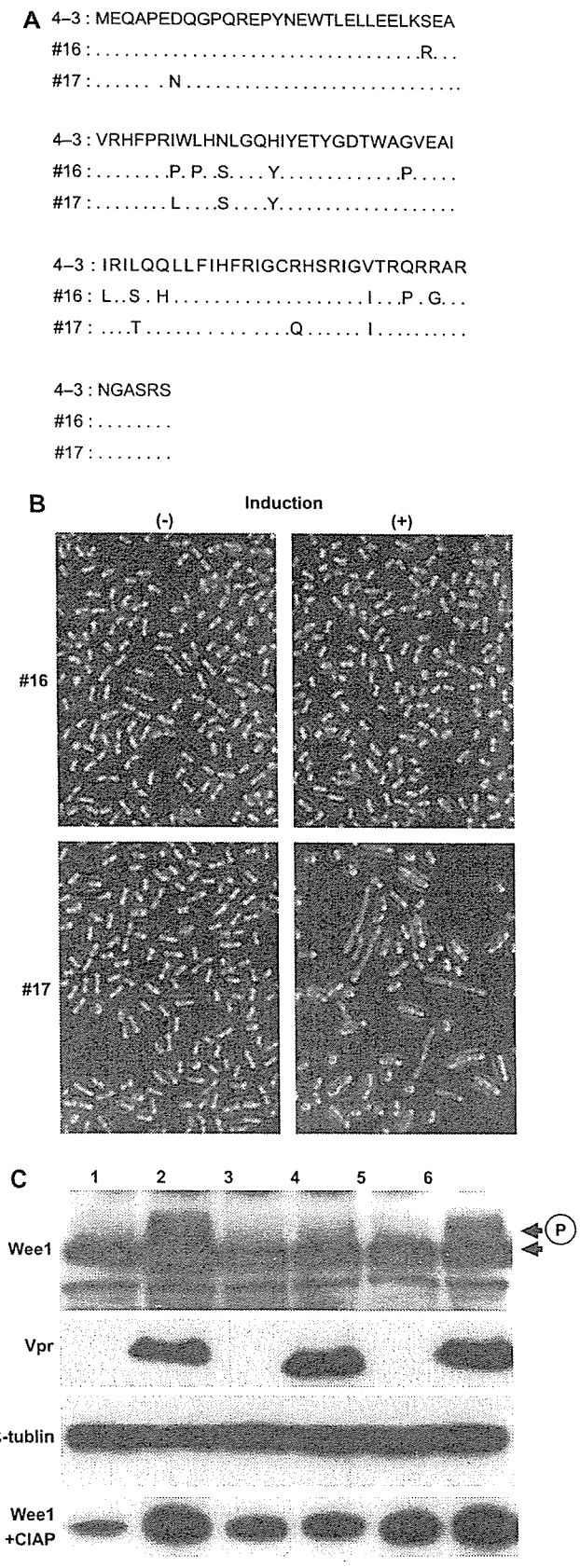

Fig. 2. Correlation between the effects of Vpr on Wee1 expression and induction of the G2 cell cycle arrest. (A) Comparison of the deduced amino acid sequences of Vpr derived from HIV-1_{NL4-3} and clinical isolates #16 and #17. (B) Photomicrographs of HA-*wee1*$^+$ *S. pombe* cells carrying pREP1-vpr16 and pREP1-vpr17, which had been grown under Vpr-inducing (+) and noninducing (−) conditions for 36 h. Original magnification, ×400. (C) HA-*wee1*$^+$ *S. pombe* cells carrying pREP1-Vpr (lanes 1 and 2), pREP1-vpr16 (lanes 3 and 4), or pREP1-vpr17 (lanes 5 and 6) were grown under Vpr-inducing (lanes 2, 4 and 6) or noninducing (lanes 1, 3 and 5) conditions for 16 h, and the cell extracts were prepared. For dephosphorylation reaction, Wee1 with the HA_3-His_6 tag was precipitated on Ni-NTA agarose from the extracts (300 μg) and treated with 10 units of CIAP. Then, immunoblot analysis was carried out as in Fig. 1C with anti-HA monoclonal antibody (upper and middle panel) and anti-Vpr rabbit serum (lower panel). Arrowheads on the right indicate positions of Wee1 and its phosphorylated form (P). Result of immunoblot analysis with an anti-α-tubulin antibody is shown as an internal control for the amounts of loaded samples.

[8], Vpr failed to induce G2 arrest in *rad24* mutant (Fig. 4A). The level of Wee1 expression in this mutant was lower than in WT in the absence of Vpr (Fig. 4B, lanes 1 and 3). When Vpr was expressed in *rad24* cells, the phosphorylated form of Wee1 was detected (Fig. 4B, lane 4). However, overall levels of Wee1 were clearly lower in *rad24* mutant than in WT in the presence of Vpr (Fig. 4B, lanes 2 and 4).

3.5. Human 14-3-3 complemented rad24 for susceptibility to Vpr-induced G2 arrest and increase of Wee1

Since Rad24 is a *S. pombe* counterpart of mammalian 14-3-3 proteins [15], we examined whether human 14-3-3 could complement *rad24* and confer susceptibility to Vpr-induced G2 arrest in *S. pombe*. *S. pombe* carrying *rad24* mutation was transformed with expression plasmid for human 14-3-3β, ε or ζ, or the control plasmid pAUR224. Expression of 14-3-3 in the transformants was demonstrated by immunoblot analysis (Fig. 5B). Transformants expressing 14-3-3β, ε and ζ were confirmed to have restored G2 checkpoint control in response to bleomycin-induced DNA-damage (data not shown). Those cells also responded to Vpr and manifested the *cdc* phenotype, whereas the cells transformed with the control plasmid did not (Fig. 5A). The level of Wee1 expression in the *rad24* cells transformed with the control plasmid was lower than in WT cells (Fig. 5B, lanes 1–4). However, *rad24* cells expressing human 14-3-3β, ε and ζ showed increase in the phosphorylation and amount of Wee1 upon Vpr expression at a level comparable to WT cells (Fig. 5B, lanes 5–10).

4. Discussion

Our previous studies showed that Wee1 is necessary for induction of G2 arrest by HIV-1 Vpr in the fission yeast *S. pombe* [8]. In this study, we demonstrated that Wee1 was phosphorylated and increased in Vpr-expressing *S. pombe*. The ability of Vpr molecules derived from clinical isolates to induce G2 arrest appeared to be correlated with their ability to cause increase in the phosphorylation and amount of Wee1. Increased amount of Wee1 in Vpr-expressing *S. pombe* is comparable to the previous observation that Vpr-induced G2 arrest in human cells was associated with higher levels of WEE1 due to delayed degradation [20]. Therefore, it is likely that

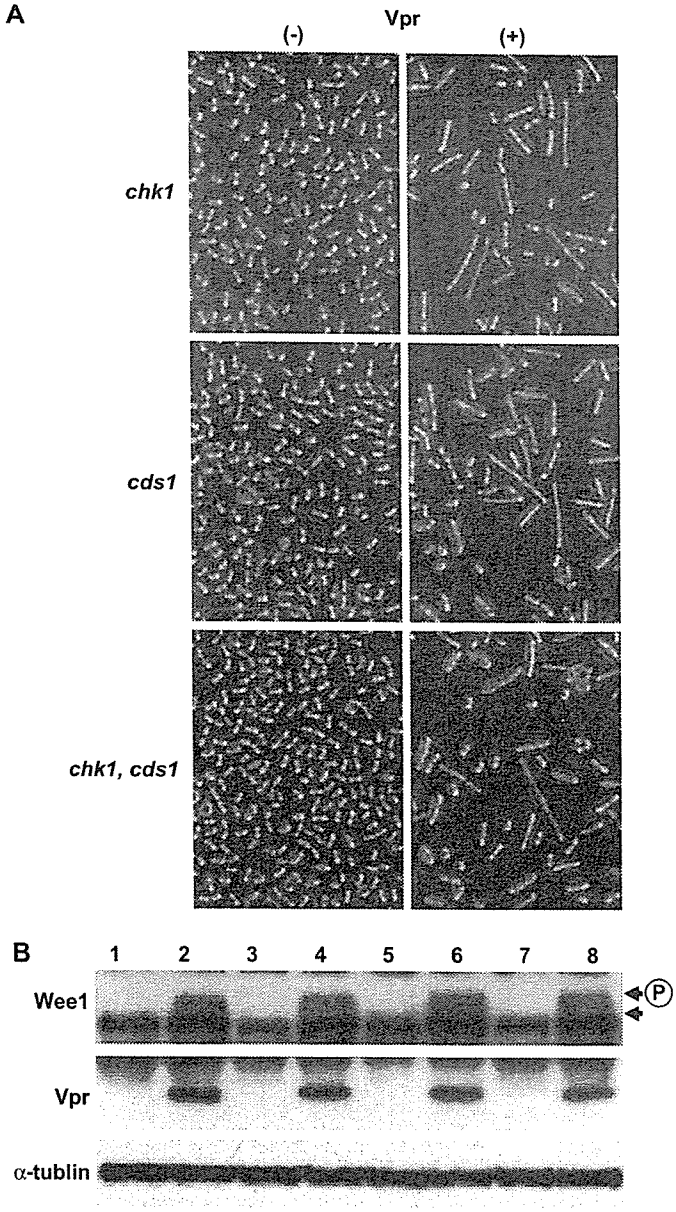

Fig. 3. Neither Chk1 nor Cds1 are required for Vpr-induced phosphorylation of Wee1. (A) Photomicrographs of HA-*wee1*$^+$ *S. pombe* cells with *chk1*, *cds1* or both mutations carrying pREP1-vpr, which had been grown under Vpr-inducing (+) and noninducing (−) conditions for 36 h. Original magnification, ×400. (B) HA-*wee1*$^+$ *S. pombe* cells with intact *chk1*$^+$ and *cds1*$^+$ (lanes 1 and 2), *chk1* mutation (lanes 3 and 4), *cds1* mutation (lanes 5 and 6), or both mutations (lanes 7 and 8) carrying pREP1-vpr were grown under Vpr-inducing (lanes 2, 4, 6, and 8) or noninducing (lanes 1, 3, 5, and 7) conditions for 16 h, and the cell extracts were prepared. Then, immunoblot analysis was carried out as in Fig. 1C with anti-HA monoclonal antibody (upper panel) and anti-Vpr rabbit serum (lower panel). Arrowheads on the right indicate positions of Wee1 and its phosphorylated form (P). Result of immunoblot analysis with an anti-α-tubulin antibody is shown as an internal control for the amounts of loaded samples.

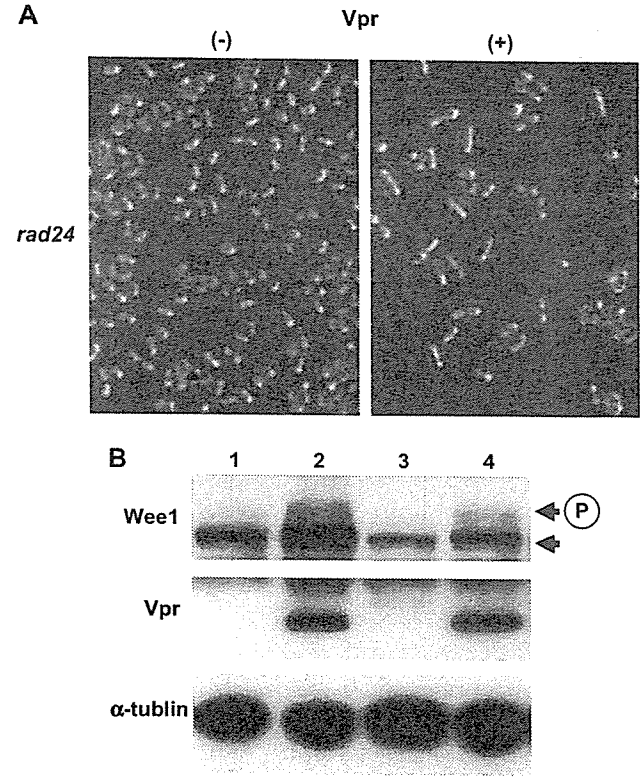

Fig. 4. In *rad24* mutant of *S. pombe* refractory to Vpr-induced G2 arrest, Wee1 was phosphorylated in the presence of Vpr, but its expression level was lower than in WT *S. pombe*. (A) Photomicrographs of HA-*wee1*$^+$ *S. pombe* cells with *rad24* mutation carrying pREP1-vpr, which had been grown under Vpr-inducing (+) and noninducing (−) conditions for 36 h. Original magnification, ×400. (B) HA-*wee1*$^+$ *S. pombe* cells without (lanes 1 and 2) or with (lanes 3 and 4) *rad24* mutation carrying pREP1-vpr were grown under Vpr-noninducing (lanes 1 and 3) or inducing (lanes 2 and 4) conditions for 16 h, and the cell extracts were prepared. Then, immunoblot analysis was carried out as in Fig. 1C with anti-HA monoclonal antibody (upper panel) and anti-Vpr rabbit serum (lower panel). Arrowheads on the right indicate positions of Wee1 and its phosphorylated form (P). Result of immunoblot analysis with an anti-α-tubulin antibody is shown as an internal control for the amounts of loaded samples.

upregulation of Wee1 plays a crucial role in Vpr-induced G2 arrest in both human and fission yeast.

Increase in the phosphorylation and amount of Wee1 observed in Vpr-expressing *S. pombe* is reminiscent of the Wee1 expression profile in the cells subjected to UV-induced DNA damage [13]. In *S. pombe*, Chk1 kinase, which plays an essential role in DNA damage checkpoint control [12], is genetically required for Wee1 phosphorylation in UV-irradiated cells [13]. Therefore, it is strongly suggested that Wee1 is upregulated through Chk1-mediated phosphorylation in response to DNA-damage. Another kinase, Cds1, has also been shown to phosphorylate Wee1 in vitro and be involved in the DNA replication checkpoint [14]. However, our present study revealed that neither Chk1 nor Cds1 was essential for Wee1 phosphorylation in Vpr-expressing *S. pombe*. In a previous study using human HeLa cells, a CHK1 inhibitor UCN-01 and siRNA-mediated knockdown of CHK1 relieved the cells from the Vpr-induced G2 arrest [21]. Thus, CHK1 appears to play a significant role in Vpr-induced G2 arrest in HeLa cells. Our present study does not exclude the possibility that Chk1 or Cds1 might play some role in Vpr-induced Wee1 phosphorylation in *S. pombe* as well. However, it was clearly demonstrated that other kinase could also phosphorylate Wee1 in Vpr-expressing *S. pombe* in the absence of Chk1 and Cds1.

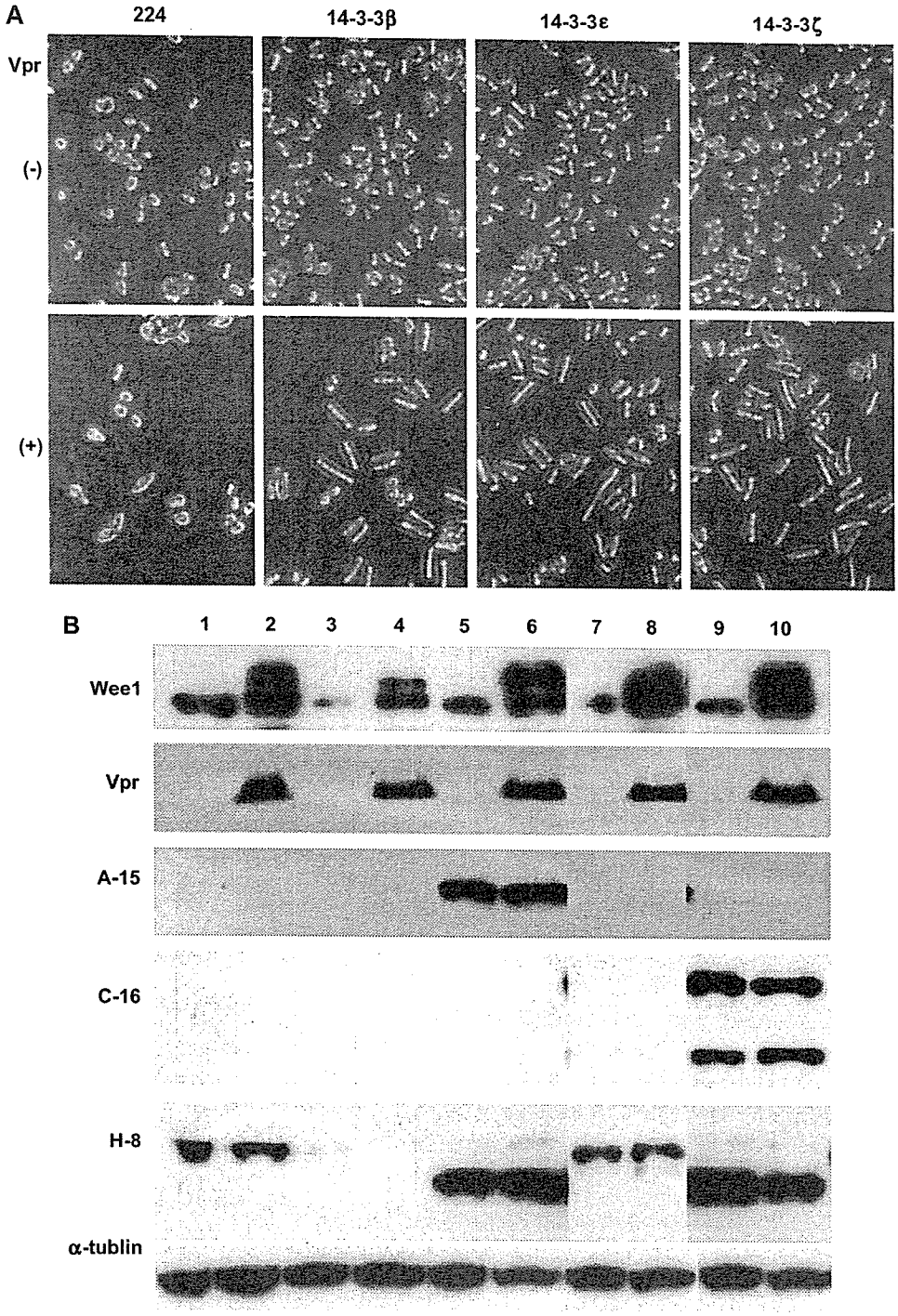

Fig. 5. (A) Photomicrographs of HA-*wee1*$^+$ *S. pombe* cells with *rad24* mutation carrying both pREP1-vpr and pAUR224, pAUR-14-3-3β, ε or ζ, which had been grown under Vpr-inducing (+) and noninducing (−) conditions for 36 h. Original magnification, ×400. (B) HA-*wee1*$^+$ *S. pombe* cells with intact *rad24*$^+$ (lanes 1 and 2) or *rad24* mutation (lanes 3–10) carrying both pREP1-vpr and pAUR224 (lanes 3 and 4), pAUR-14-3-3β (lanes 5 and 6), ε (lanes 7 and 8), or ζ (lanes 9 and 10) were grown under Vpr-inducing (lanes 2, 4, 6, 8 and 10) or noninducing (lanes 1, 3, 5, 7 and 9) conditions for 16 h, and the cell extracts were prepared. Then, immunoblot analysis was carried out as in Fig. 1C with anti-HA monoclonal antibody (Wee1), anti-Vpr rabbit serum and antibodies A-15, C-16, and H-8, which react with 14-3-3β, ζ, and various isoforms of 14-3-3 including ε, respectively. The lower band detected by C-16 likely correspond to a truncated from of 14-3-3ζ generated by proteolysis. Result of immunoblot analysis with an anti-α-tubulin antibody is shown as an internal control for the amounts of loaded samples.

Therefore, although the mechanisms for Vpr-induced G2 arrest and the physiological G2 checkpoint may share a common pathway, their mechanisms do not appear to be identical to each other in *S. pombe*. What might be the kinase responsible for Wee1 phosphorylation in the Vpr-expressing *chk1 cds1* mutant? Nim1/Cdr1 and Cdr2 have been shown to phosphorylate Wee1 in vitro [11,22]. However, it is unlikely that these kinases are involved because they are negative regulators of Wee1 [11,22]. In fact, Vpr-induced G2 arrest was observed in *nim1* and *cdr2* mutants ([8] and our unpublished data).

With no putative kinase domain, Vpr by itself is unlikely able to phosphorylate Wee1, although it is possible that Wee1 autophosphorylates itself in the presence of Vpr. Another more plausible explanation would be that Vpr can exploit or activate a novel kinase capable of phosphorylating Wee1. Studies are in progress to identify the molecules responsible for Wee1 phosphorylation in Vpr-expressing *S. pombe*.

It was shown that *rad24* mutant of *S. pombe* was refractory to Vpr-induced G2 arrest [8]. Our present study demonstrated that the amount of Wee1 in *rad24* mutant was considerably smaller than in WT even in the presence of Vpr, suggesting that the degree of Vpr-induced Wee1 upregulation might be insufficient for causing G2 arrest. Rad24 is a *S. pombe* ortholog of human 14-3-3, and among 7 isoforms, amino acid sequence of 14-3-3ε is relatively similar to that of Rad24. Therefore, it is reasonable that 14-3-3ε could complement *rad24* mutation, conferring susceptibility to Vpr-induced upregulation of Wee1 and G2 arrest. Phylogenetic analysis has indicated that 14-3-3 β and ζ are comparably close to each other, while they are rather distantly related to 14-3-3ε and Rad24 [23]. On the other hand, it has been demonstrated in some vertebrate cells that 14-3-3β, ε, and ζ bind to and stabilize phosphorylated Wee1 [24–26]. Therefore, it is possible that despite the difference in their primary structures, 14-3-3β, ε and ζ share common functional properties, which could contribute to the complementation of *rad24* mutation in *S. pombe* observed in this study. Further studies are in progress to examine whether these 14-3-3 isoforms are involved in Vpr-induced G2 arrest in human cells.

Recent studies have suggested that alteration of Cdc25 activity might be involved in Vpr-induced G2 arrest in *S. pombe* and human cells [27,28]. It has also been suggested that 14-3-3η and σ might be involved in Vpr-induced G2 arrest in human cells through its effects on CDC25 activity [29]. However, since relative activities of Wee1 and Cdc25 are thought to play a crucial role in controlling G2/M transition of the cell cycle, reduction of Cdc25 activity alone may not be sufficient for Vpr-induced G2 arrest, as well as the physiological checkpoint control. Our present study provides a novel insight that Chk1/Cds1-independent phosphorylation and Rad24/14-3-3-dependent upregulation of Wee1 might also contribute to Vpr-induced G2 arrest. Since *S. pombe* shares the cell cycle regulatory mechanisms with higher eukaryotes, additional studies using this model organism may provide useful insights into the mechanism for Vpr-induced G2 arrest in mammalian cells. It has been shown that HIV1-Vpr could exert pleiotropic effects on *S. pombe* cell cycle regulation in addition to the G2 arrest [30]. Therefore, HIV-1 Vpr used in the fission yeast model may serve as a useful molecular tool for investigating mechanisms for eukaryotic cell cycle regulation as well.

Acknowledgments

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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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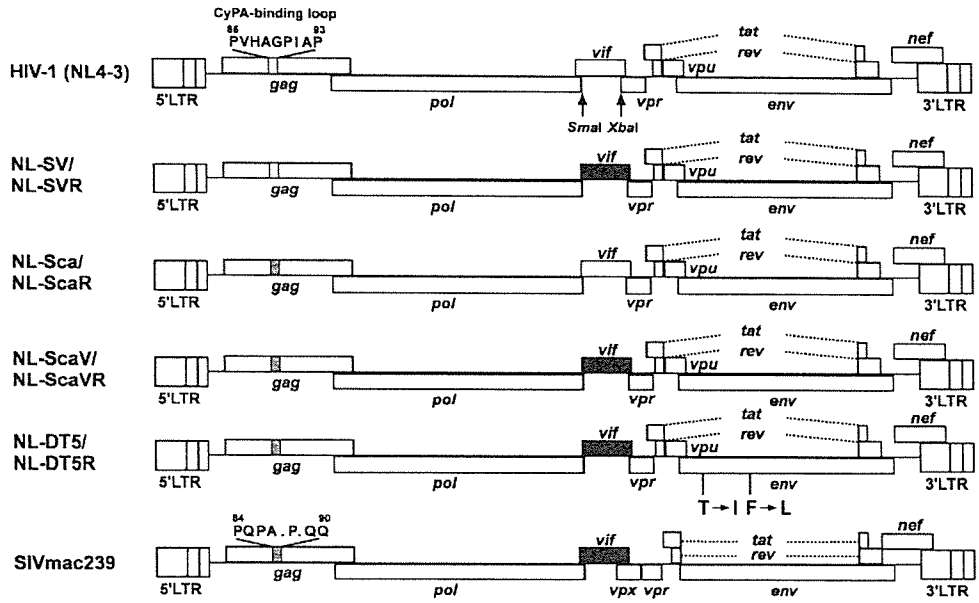

Fig. 1. Structure of chimeric clones between HIV-1 NL4-3 and SIVmac239 in this study. Eight chimeric proviral clones shown here were generated from a pNL4-3 derived vector pNL-SX (25) as described in *Materials and Methods*. Each chimeric clone has the entire *vif* (black area) and/or partial *gag* (gray 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-

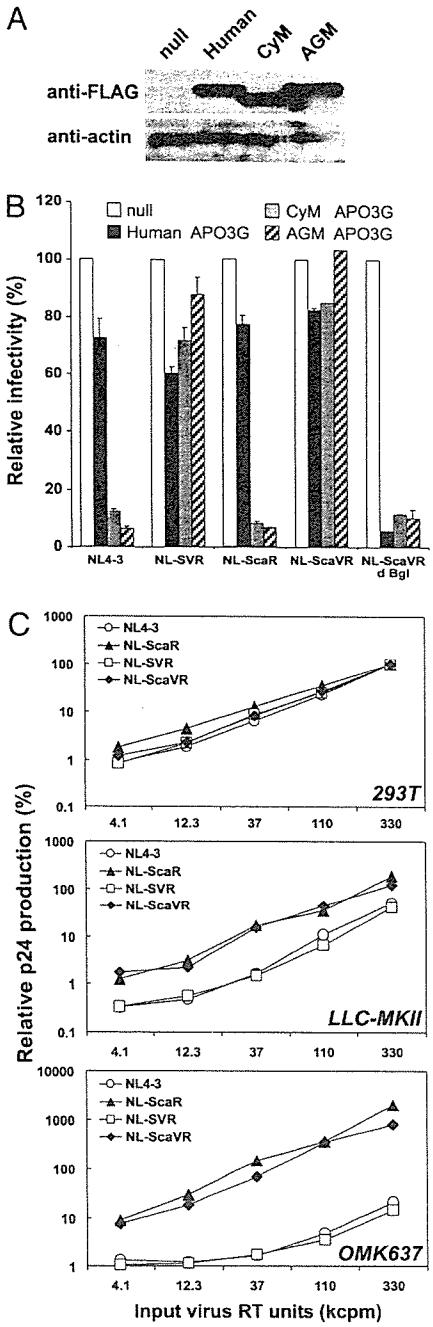

Fig. 2. Single-cycle replication properties of HIV-1 chimeric clones. (*A*) The expression of human, CyM, and AGM APOBEC3Gs containing the FLAG epitope was monitored in 293T cells by immunoblotting 48 h posttransfection. (*B*) VSV-G-pseudotyped viruses were prepared in 293T cells cotransfected with one 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

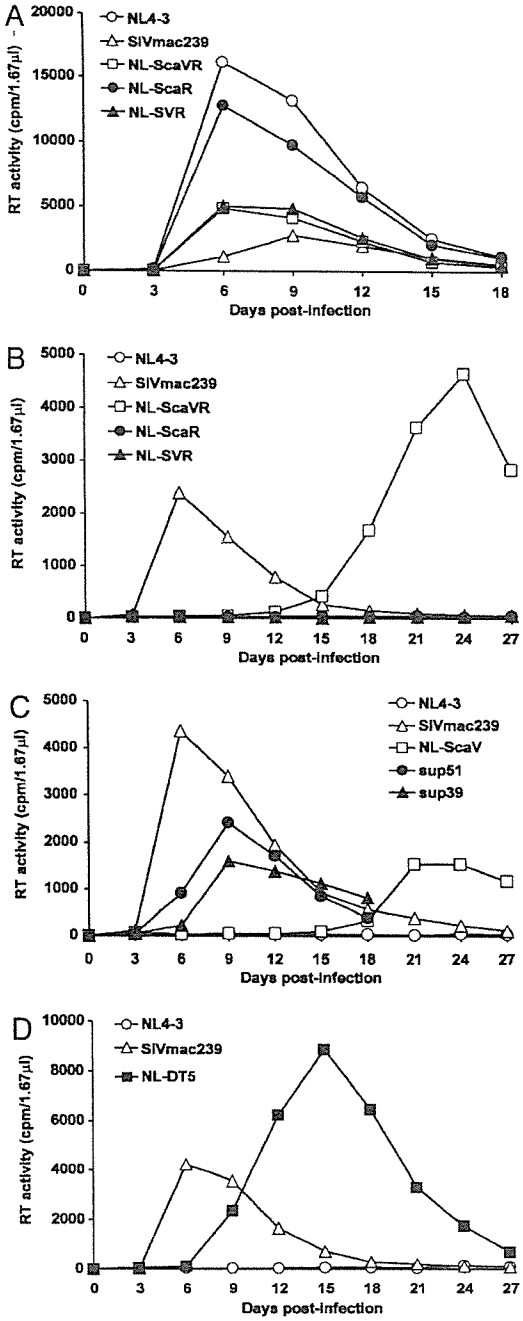

Fig. 3. Multicycle growth potential of various chimeric viruses in human and monkey lymphocyte cell lines. Virus samples were prepared from 293T cells transfected with the indicated proviral clones, and they were inoculated into human M8166 (*A*) or CyM HSC-F (*B*) cells. HIV-1 NL4-3 and SIVmac239 served 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.

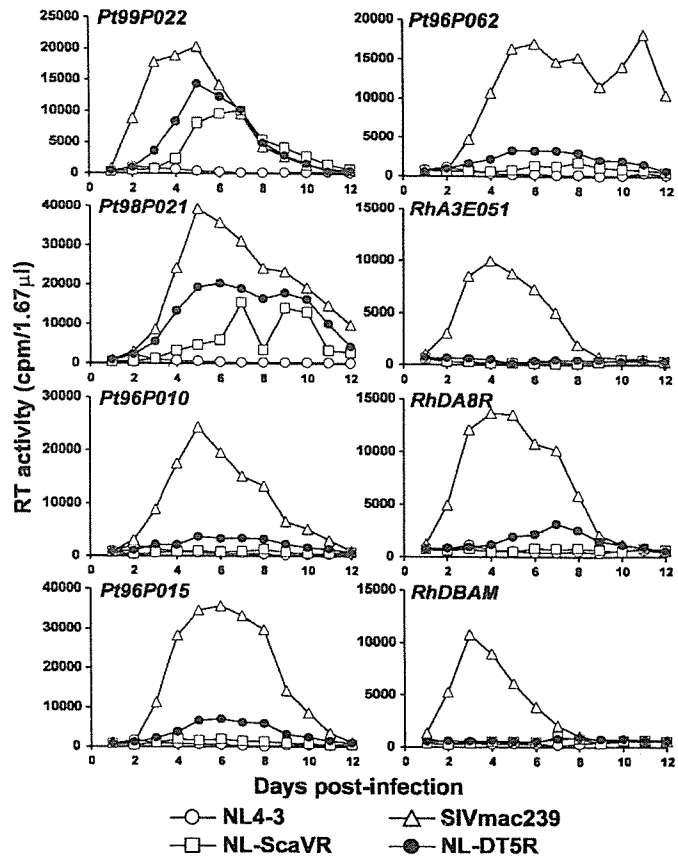

Fig. 4. Growth potential of the chimeric viruses in CD8-depleted PBMCs from PtM and RhM. Virus samples were prepared from 293T cells transfected with the proviral clones indicated at the bottom, and they were infected into CD8-depleted PBMCs by spinoculation (30). Virus replication was monitored daily 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′ (GCTAGTCTAGAAGCCATA-CAATGAATGGACACTAG at 5596–5619) and NL9686–9709Sph-3′ (ACATG*GCATGC*TGCTAGAGATTTTCCA-CACTGACT 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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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

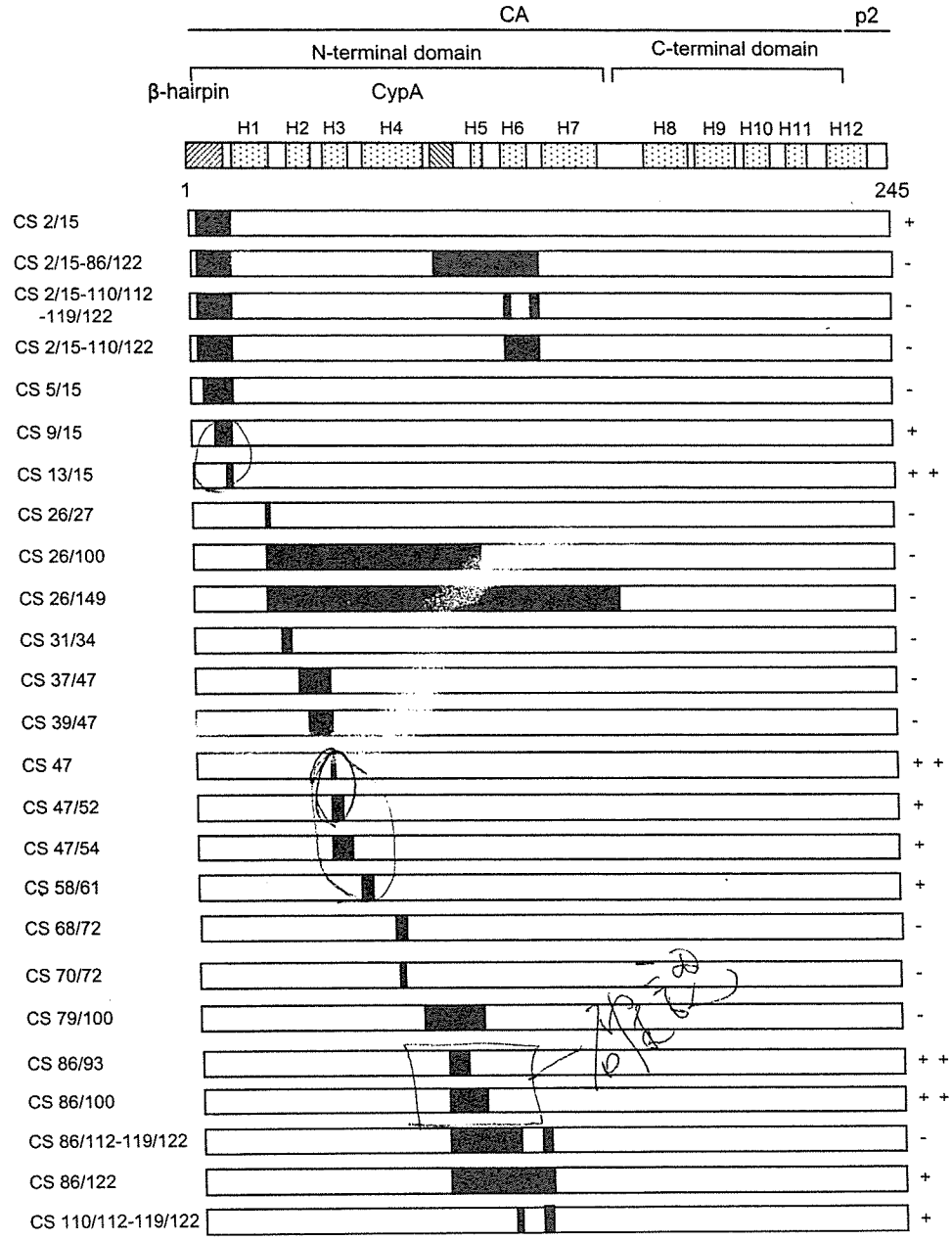

Fig. 1. *Gag*-chimeric viruses between HIV-1 and SIVmac used in this study. Location of SIVmac Gag sequence (MA239) inserted into HIV-1 Gag CA-p2 region (NL432) is indicated by black area. For the sequences inserted, see Table 1. Growth ability of viruses in M8166 cells is given as ++ (wt growth), +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

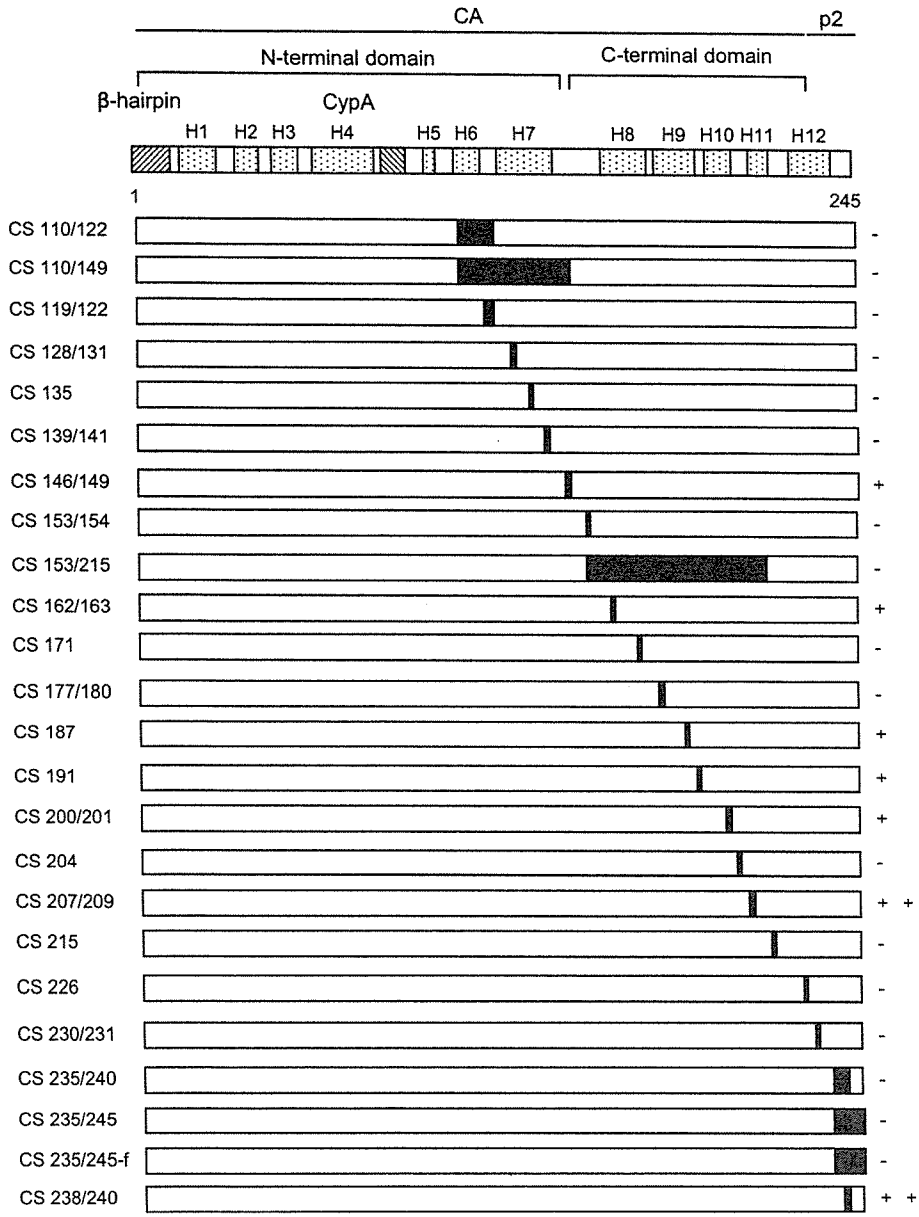

Fig. 1 (*continued*).

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,

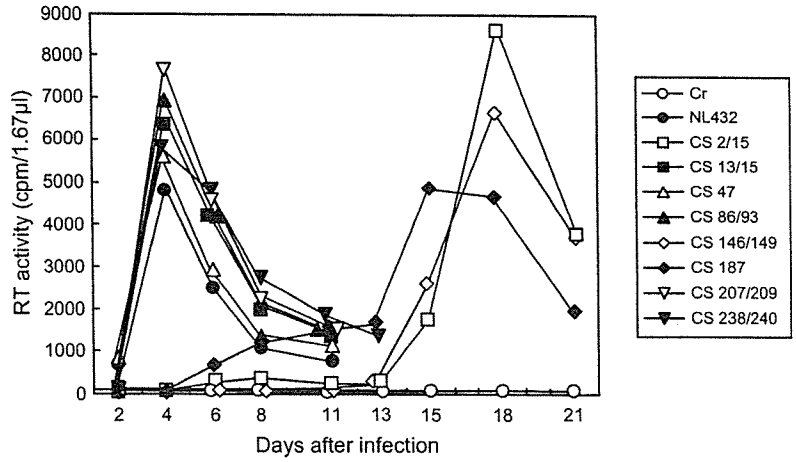

Fig. 2. Growth kinetics in M8166 cells of chimeric viruses. Cells were infected with cell-free viruses as described in the text, and virus replication was monitored at intervals by RT production in the culture supernatants. Input viruses were prepared from 293T cells transfected with 20 μg of the clones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

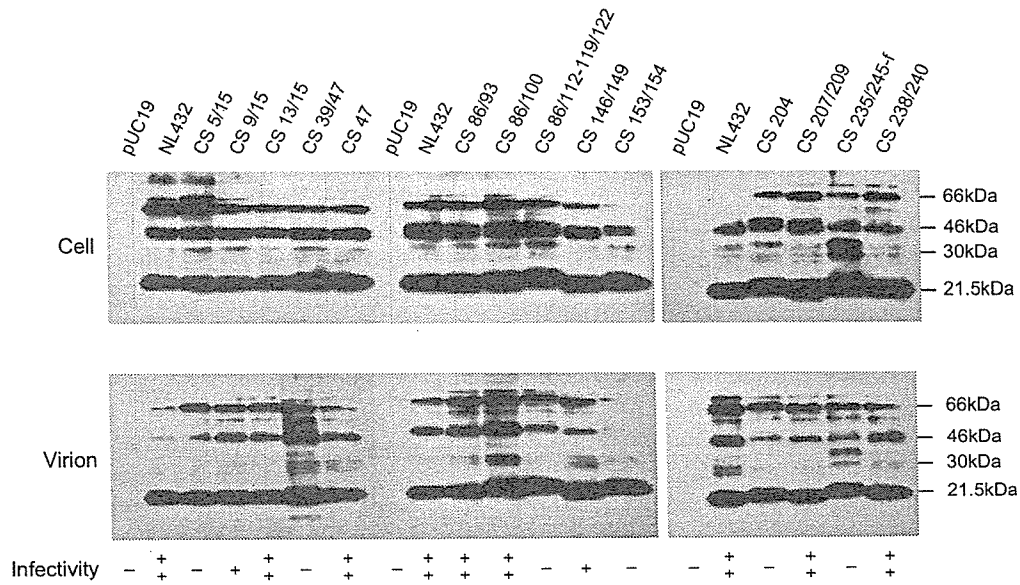

Fig. 3. Western blot analysis of chimeric viruses. Cell and virion lysates were prepared from 293T cells transfected with various clones (20 μg) indicated at the top, as described previously [20,25,26]. Each sample was then subjected to Western blot analysis using a human anti-HIV-1 antiserum as reported 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.

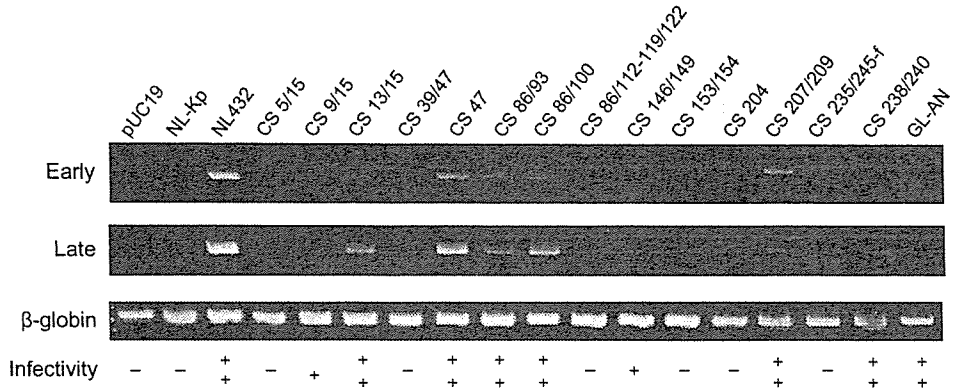

Fig. 4. PCR analysis of chimeric viruses. M8166 cells were infected with cell-free viruses, as described in the text. Input viruses were prepared from 293T cells transfected with 20 μg of the clones indicated at the top. DNA was extracted from infected cells on day 2 post-infection, and subjected to 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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