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The *vpx* Gene of Simian Immunodeficiency Virus Facilitates Efficient Viral Replication in Fresh Lymphocytes and Macrophages

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***vpx* is a unique open reading frame found in simian immunodeficiency virus (SIV) and human immunodeficiency virus type 2 (HIV-2) but not in HIV-1. It encodes a 12- to 16-kDa virion-associated protein. Although *vpx* is dispensable for viral replication in several established human lymphocyte cell lines, there is no consensus regarding whether this gene is required for efficient viral replication in freshly isolated lymphocytes. We report here that the *vpx* mutant of SIV_{mac} exhibits different degrees of impairment from wild-type SIV_{mac} in freshly isolated lymphocytes. This defect is more pronounced in macrophages from the same donors. Our findings suggest that *vpx* is required for efficient viral replication in fresh lymphocytes and macrophages.**

Simian immunodeficiency viruses (SIVs) have been isolated from rhesus macaques (SIV_{mac}) (3, 14), sooty mangabeys (SIV_{sm}) (7, 17), African green monkeys (SIV_{agm}) (18), mandrills (SIV_{mnd}) (22), and chimpanzees (19). They are closely related to human immunodeficiency virus types 1 (HIV-1) and 2 (HIV-2). The close relationship among SIV_{mac}, SIV_{sm}, and some isolates of HIV-2 (12) suggests a common origin for this group of primate lentiviruses. The genomic organization of this group of viruses is quite similar to that of HIV-1 and other SIVs. Nevertheless, a unique gene, *vpx*, is present in this group of viruses but not in other primate lentiviruses. Originally, the *vpx* gene was also identified in the genome of SIV_{agm} (6). However, it has recently been suggested that the *vpx* gene in SIV_{agm} should be classified as a *vpr* gene on the basis of amino acid homology (21). The *vpx* gene products of HIV-2, SIV_{mac}, and SIV_{sm} are associated with mature virions (5, 11, 15, 23) and bind to nucleic acids in vitro (11).

Previously we reported that the *vpx* gene of SIV_{mac} appears to be dispensable for virus replication in an established human T-lymphoid cell line (Hut-78) (23). Similar results have also been observed with HIV-2 (8, 13, 20). However, conflicting results concerning the question of whether the *vpx* gene is required for efficient replication of HIV-2 in fresh human peripheral blood lymphocytes (PBLs) have been obtained (8, 13). One study demonstrated that HIV-2 *vpx* mutants have a severe defect in replication in PBLs (8), while another study showed that *vpx* mutants and wild-type viruses replicate equally well in PBLs (13). In this study, we explored the question of whether the *vpx* gene of SIV_{mac} is required for efficient replication in fresh PBLs and macrophages.

The *vpx* mutant of SIV_{mac} used in this study was described previously (23). An *Xba*I linker which contains an in-frame stop codon was inserted into the *Stu*I site. This restriction site is 60 nucleotides downstream from the *vpx* gene initiator codon. Both the wild-type (pBK-28) (16) and the *vpx* mutant (pBK-x) proviral DNA were transfected into Hut-78 cells. Virus stocks were prepared with supernatants from transfected cells and adjusted according to the level of reverse transcriptase (RT) activity and the amount of virus antigens (enzyme-linked immunosorbent assay [ELISA] kit; Abbott, North Chicago, Ill.). Wild-type and mutant viruses with equivalent RT activity were inoculated into CEMX174 and MT-4 cells, which are sensitive to SIV_{mac} infection. Virus production was monitored by RT activity in the supernatant of infected cells. As shown in Fig. 1, both the wild-type and the *vpx* mutant viruses are infectious and produce viruses, as judged by RT activity in the supernatant. The kinetics of replication for both viruses are relatively equal in CEMX174 and MT-4 cells (Fig. 1), although the *vpx* mutant appears to release slightly (two- to threefold) less virus to the supernatant of infected CEMX174 cells than the wild type (Fig. 1a). This confirmed our previous findings that the *vpx* gene is dispensable for SIV_{mac} replication in established human cell lines (23). Similar findings have been reported for HIV-2 *vpx* mutants (8, 13, 20).

To analyze whether *vpx* of SIV_{mac} is required for virus replication in fresh lymphocytes and macrophages, we isolated human PBLs from healthy donors by the Ficoll-Hypaque method. Macrophages were further separated by the adherent method. The PBLs were maintained in RPMI 1640 plus 20% fetal calf serum and 20 U of recombinant interleukin 2 (Boehringer Mannheim, Indianapolis, Ind.) per ml and stimulated with phytohemagglutinin P (Sigma, St. Louis, Mo.) for 48 h before infection. The macrophages were maintained in RPMI 1640 plus 10% fetal calf serum for 5 days before infection. The same virus stocks used for human cell line studies were used for these fresh PBL and macrophage studies. Five million fresh PBLs or macrophages were incubated with 5×10^5 cpm (RT activity) of wild-type or *vpx* mutant virus at 37°C for 12 h and washed two times with phosphate-buffered saline before they were resuspended in fresh complete medium. The supernatants of infected cells were monitored for virus production by RT assay and ELISA. Representative results generated from the cells of three different donors are shown in Fig. 2 and 3. In the first donor, the *vpx* mutant virus replicated much more slowly than the wild-type virus in PBLs. Only after 15 days postinfection could significant amounts of the *vpx* mutant virus be detected in the supernatant of infected cells (Fig. 2a). This phenomenon was not observed when PBLs from the other two healthy donors were used. Instead, the *vpx* mutant virus replicated with kinetics similar to those of the wild-type virus, but the production of *vpx* mutant virus was reduced

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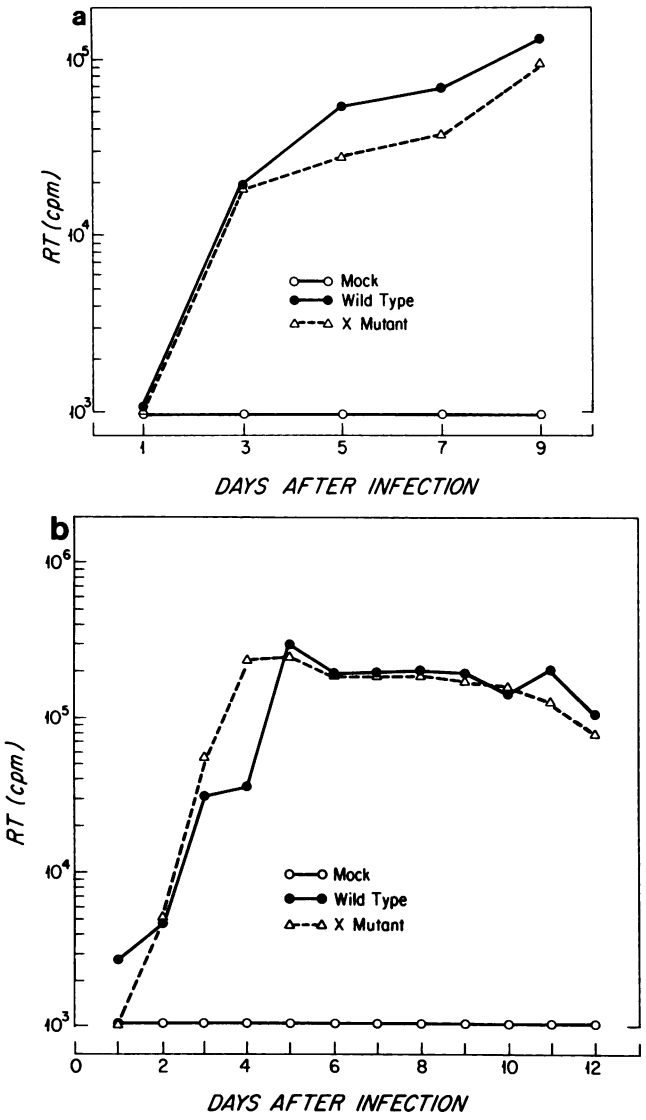

FIG. 1. Kinetic comparison of wild-type and *vpx* mutant (X Mutant) viruses in CEMX174 cells (a) and MT-4 cells (b) by RT assay as previously described (24). The RT values represent 0.1 ml of supernatant from infected cultures. Mock, mock-infected cells.

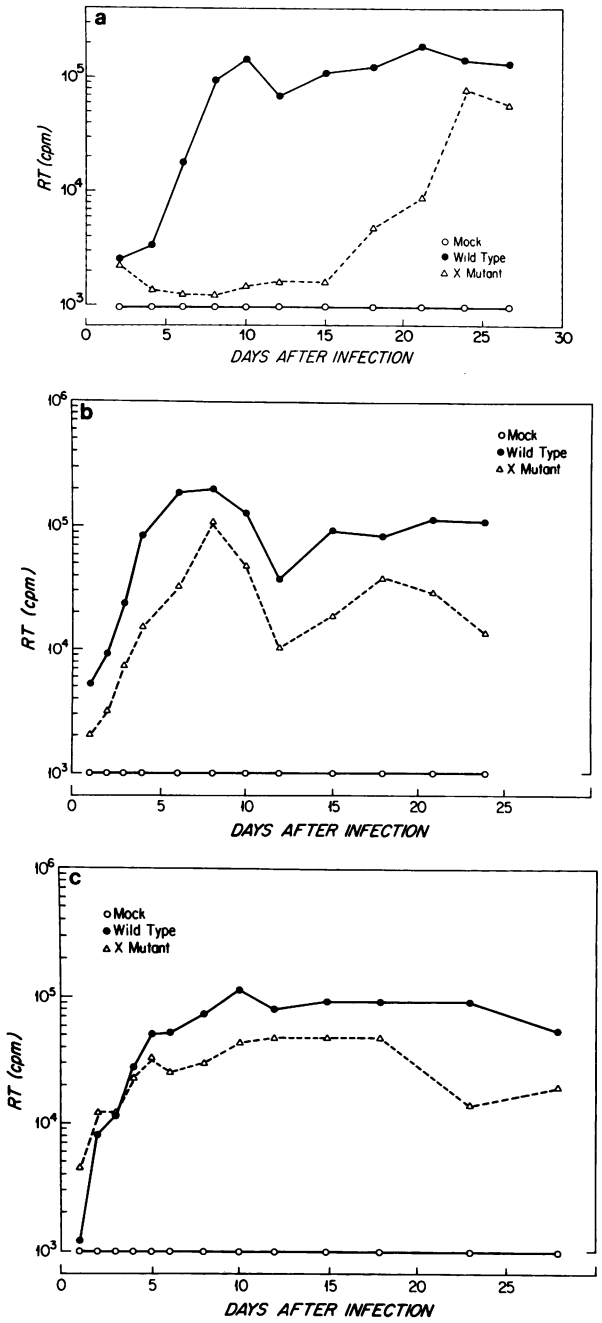

FIG. 2. Kinetic comparison of wild-type and *vpx* mutant (X Mutant) viruses in human PBLs. a, b, and c represent parallel studies using PBLs from three different donors. Mock, mock-infected PBLs.

two- to threefold at the peak level (Fig. 2b and c). These results indicate that the mutation in the *vpx* gene of SIV_{mac} has an effect on viral replication in fresh human lymphocytes. The variable degree of the defect appears to be donor dependent, since virus infection and preparation of the cells were performed under the same conditions. The effect of *vpx* mutation in HIV-2 has also been evaluated with human PBLs (8, 13). In one study, the *vpx* mutants of HIV-2 displayed a severe defect in PBLs (8). However, in another study it was demonstrated that the *vpx* mutants of HIV-2 had replication kinetics and a level of replication similar to those of the wild-type virus in PBLs (13). Therefore, our results are more compatible with the finding of the former study that the *vpx* gene facilitates efficient replication of this group of immunodeficiency viruses in fresh PBLs.

The replication potential of the *vpx* mutant virus in macrophages was also analyzed. Adherent macrophages isolated from the donors of the PBLs used in the experiment whose results are shown in Fig. 2 were infected with equal amounts of wild-type or *vpx* mutant viruses. Virus production was monitored by RT activity in the culture supernatants. Significant differences between the wild type and the *vpx* mutant can be seen with the macrophages (Fig. 3). In the macrophages from the first donor, no significant level of viral replication by the *vpx* mutant was detected during the entire 27 days of follow-up monitoring. The RT activity in the

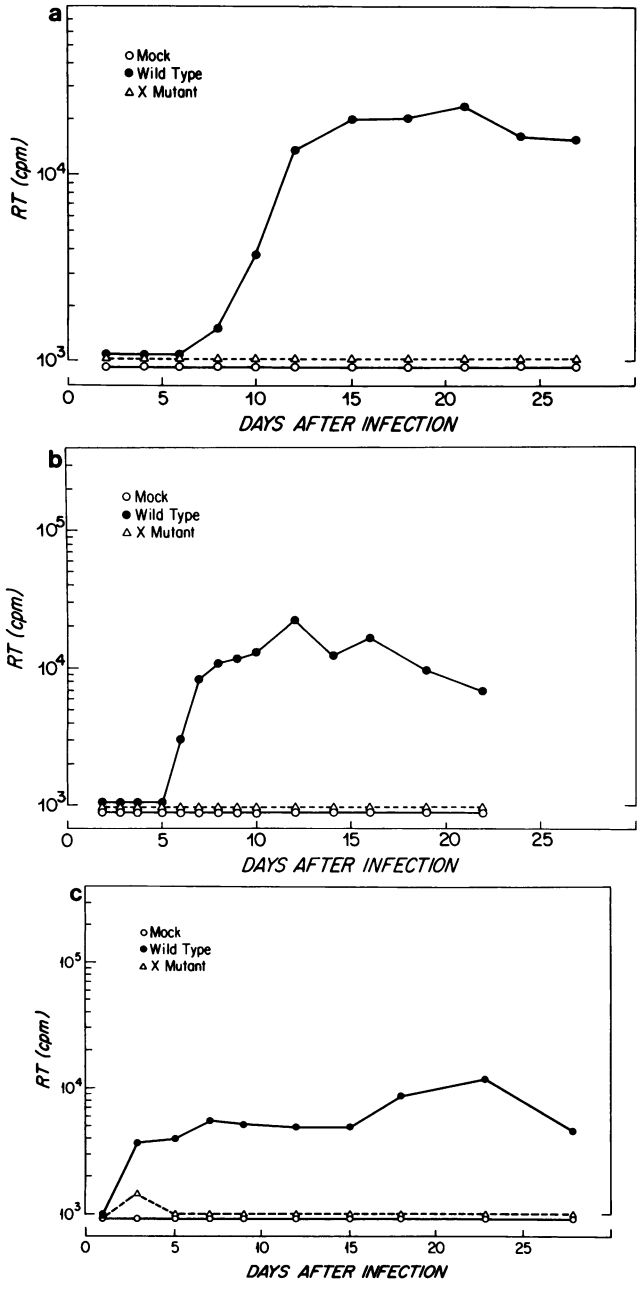

FIG. 3. Kinetic comparison of wild-type and *vpx* mutant (X Mutant) viruses in enriched human macrophages. a, b, and c represent parallel studies using adherent macrophages from the donors mentioned in the legend to Fig. 2. Mock, mock-infected macrophages.

supernatant of the macrophages infected by the *vpx* mutant was only a few times higher than that in the mock-infected culture. In contrast, the RT activity of wild-type virus-infected macrophages was more than 50-fold higher than that in the control culture (Fig. 3a). In the cases of the other two donors, although the *vpx* mutant showed only two- to threefold less virus production in PBLs than the wild-type virus (Fig. 2b and c), the replication of the *vpx* mutant in macrophages never reached a level comparable to that seen in the wild-type-infected macrophages (Fig. 3b and c). These

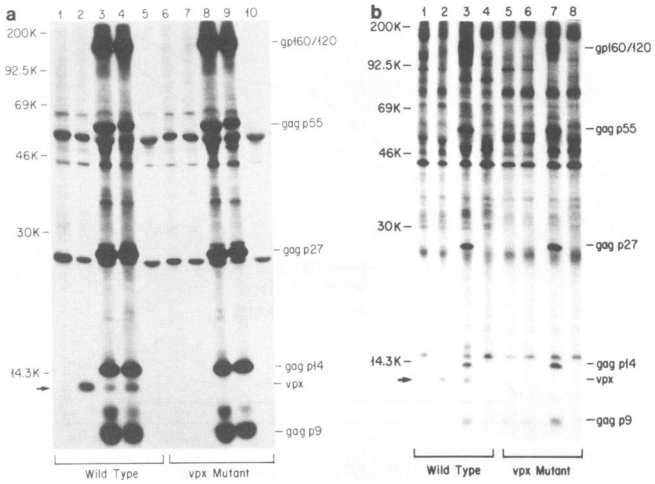

FIG. 4. Radioimmunoprecipitation assay analysis of cell lysates from wild-type- and *vpx* mutant-infected CEMX174 cells (a) and human PBLs (b). (a) Lanes 1 and 6, preimmune goat serum; lanes 2 and 7, postimmune goat anti-*vpx* serum; lanes 3 and 8, HIV-2-positive human serum; lanes 4 and 9, HIV-2-positive human serum; lanes 5 and 10, HIV-seronegative human serum. (b) Lanes 1 and 5, preimmune goat serum; lanes 2 and 6, postimmune goat anti-*vpx* serum; lanes 3 and 7, HIV-2-positive human serum; lanes 4 and 8, HIV-seronegative human serum. Molecular weight markers (in thousands) are shown at the left.

data suggest that the *vpx* gene of SIV_{mac} may be more critical for efficient viral replication in macrophages than in lymphocytes.

To demonstrate that reverse mutation in the *vpx* gene did not occur in the cell lines in which *vpx* mutant virus replicated or in PBLs, the expression of *vpx* protein was analyzed. Chronically infected CEMX174 cells and PBLs were metabolically labeled with [^{35}S]cysteine(100 μCi/ml) for 12 h. Cell lysates were subject to radioimmunoprecipitation assay analysis as previously described (24). The *vpx* protein could be detected in wild-type-infected CEMX174 cells (Fig. 4a) or PBLs (Fig. 4b) by a goat anti-*vpx* or HIV-2-positive serum containing anti-*vpx* antibody (23). However, this protein was not detected in the cell lysates of *vpx* mutant-infected cells (Fig. 4). No significant differences between wild-type and *vpx* mutant viruses with regard to major viral *gag* and *env* proteins could be detected. Analysis using DNA from *vpx* mutant-infected PBLs indicated that the *Xba*I site, which introduced the in-frame stop codon in the *vpx* gene, was still present 25 days after infection (data not shown). These data confirmed that reverse mutation of the *vpx* gene did not occur in the cell lines in which *vpx* mutant virus replicated or in PBLs.

The exact role of *vpx* in the viral life cycle is not clear at the moment. A structural function has been suggested by the data because the *vpx* protein is present in SIV_{sm} virions at a molar ratio equal to that of the *gag* proteins (11). The *vpx* protein contains a conserved cysteine-histidine motif similar to that of the NC protein of retroviruses and can bind to single-stranded nucleic acid (11).

Recently, it has been suggested that the *vpx* gene arose by duplication of the *vpr* gene (21). The *vpr* proteins of HIV-1 and HIV-2 were reported to act as promiscuous transcriptional activators and are required for productive infection in macrophages (2, 10). This raises the question of whether the virion-associated *vpx* protein can also act as a transcriptional

Zactivator which enhances viral replication before the synthesis of another potent transactivator, *tat*. Another possibility is that the *vpx* protein activates the expression of cellular factors that facilitate viral replication. This possibility is consistent with our finding that the defect of the *vpx* mutant is cell type dependent.

Our observations suggest that the *vpx* gene of SIV_{mac} may have an as yet unidentified function in vivo. The *vpx* gene is expressed in vivo because antibodies to the *vpx* protein have been detected in HIV-2-infected humans and SIV-infected monkeys (23). All of the isolates of HIV-2, SIV_{mac}, and SIV_{sm} sequenced to date contain a complete *vpx* gene (1, 4, 9, 12), and these *vpx* genes have a relatively high degree of sequence homology. This degree of conservation is consistent with our finding that the *vpx* gene facilitates viral replication in fresh lymphocytes and macrophages. Further study of the mechanism of *vpx* function may help us to understand why other groups of primate lentiviruses, such as HIV-1, do not require a *vpx* gene in their replication cycle.

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