

Open Reading Frame *vpr* of Simian Immunodeficiency Virus Encodes a Virion-Associated Protein

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The genomes of simian immunodeficiency viruses isolated from rhesus macaques (SIV_{mac}) contain an open reading frame (ORF), *vpr*, which has a coding potential of 97 to 101 amino acid residues. In this study, a *vpr* ORF-encoded protein of approximately 11 kDa was identified, and anti-*vpr* antibodies were detected in rhesus macaques infected by SIV_{mac}. These results provide clear evidence that the *vpr* ORF is a coding gene of SIV_{mac}. The *vpr* protein, like the *vpx* protein which is encoded by another accessory gene of SIV_{mac}, was also found to be associated with viral particles. This observation demonstrates that more than one accessory gene product can be present in the virions of this family of retroviruses and raises the possibility that the *vpr* protein may have a role in early part of the virus life cycle.

In addition to three structural genes, *gag*, *pol*, and *env*, which are commonly found in replication-competent animal retroviruses, human immunodeficiency virus type 1 (HIV-1) has at least seven regulatory genes in its genome. These include *vif* (22, 26, 37), *vpr* (8, 32, 43), *vpu* (9, 30, 39), *tat* (3, 38), *rev* (12, 36), *tev/tnv* (4, 34), and *nef* (1). Many studies have been carried out to investigate the function of these regulatory genes, and some genes, such as *tat* and *rev*, have been reported to have more impact on virus replication than others, such as *vpr* and *nef*. Despite relatively extensive knowledge of how various HIV-1 regulatory genes function in vitro, the question of whether these regulatory genes have an indispensable role in disease pathogenesis remains unanswered.

To date, the only animal host found to be susceptible to infection by HIV-1 is the chimpanzee, and HIV-1-infected chimpanzees are not known to develop acquired immunodeficiency syndrome (AIDS) (2, 15, 17). Thus, this animal model has little value for studying the role of regulatory genes in disease pathogenesis. In contrast, infection of rhesus macaques by simian immunodeficiency virus (SIV_{mac}) can be a useful model to study the role of regulatory genes in disease pathogenesis. This is because SIV_{mac} can reproducibly induce AIDS-like diseases in rhesus macaques, and the genomic organization of SIV_{mac} appears to be similar to that of HIV-1.

Besides the three structural genes, the genome of SIV_{mac} also contains open reading frames (ORFs) analogous to the *vif*, *vpr*, *tat*, *rev*, and *nef* genes of HIV-1, and a *vpx* ORF which is unique to SIV_{mac}, HIV-2, and other related viruses (5, 19). Except for *tat* and *rev*, which have similar regulatory functions to those of HIV-1 (10, 29, 42) and the *vpx* ORF, which was shown to encode for a 12-kDa virion-associated protein (14, 20, 24, 44), no direct evidence is available to prove the hypothesis that all those HIV-1 analogous ORFs in SIV_{mac} are, in fact, coding genes. If some of the HIV-1 regulatory genes turn out not to have a counterpart in SIV_{mac}, then it will not be feasible to use the SIV_{mac} model to study the role of those regulatory genes in disease pathogenesis.

This study was designed to address the question of

whether the *vpr* ORF is a coding gene of SIV_{mac}. The predicted *vpr* product of SIV_{mac} shares 57% protein sequence identity with that of HIV-1 (5). This degree of sequence conservation supports the hypothesis that the *vpr* ORF of SIV_{mac}, like that of HIV-1, does encode a protein product. However, the possibility that the *vpr* ORF may not be a coding gene of SIV_{mac} was also raised by two other observations. First, SIV isolated from African green monkeys (SIV_{agm}) does not contain the *vpr* ORF in its genome (16). Second, mutations introduced into the *vpr* ORF of a SIV_{mac}-related virus, HIV-2, have no effect on virus replication (11, 35). In the present study, the *vpr* ORF of SIV_{mac} was found to encode a virion-associated protein, supporting the hypothesis that this ORF is a coding gene of SIV_{mac}.

Figure 1 shows the strategy used to express a recombinant *vpr* protein. The plasmid pBK-S1 is a subclone of an infectious SIV_{mac} molecular clone, pBK-28 (25). A 1.1-kb *Nco*I- and *Pst*I-restricted DNA fragment derived from pBK-S1 was cloned into a previously described prokaryotic expression vector, p806 (40, 44) to generate a *vpr* recombinant protein expression vector, pR-2. The *vpr* recombinant protein expressed by this vector is a fusion peptide of 202 amino acid residues. The C terminus of this fusion peptide consists of 83 amino acid residues from the C terminus of the SIV_{mac} *vpr* gene, which is preceded by 111 amino acid residues from the N terminus of the *v-ras*^H gene and 8 amino acid residues from the polylinker sequence. The expression of this fusion protein is under the control of the *tac* promoter and can be induced by isopropyl-β-D-thiogalactopyranoside (IPTG). Upon induction by IPTG, two proteins of approximately 25 and 23 kDa were detected in lysates of the pR-2-bearing X-90 cultures (Fig. 2). These proteins were not detected in the lysates of the uninduced control cultures. The smaller species could have been derived from proteolytic cleavage of the larger species or from an alternative initiation using downstream initiator codons that are in the same translational frame as the *ras* gene, because the smaller species was also specifically recognized by the goat anti-*ras* serum. The latter possibility appears unlikely, because the use of either of the two initiator codons that are 66 and 71 amino acid residues downstream from the initiator codon for the *ras* gene is expected to produce a protein much smaller than 23 kDa.

The *vpr* recombinant protein was partially purified by

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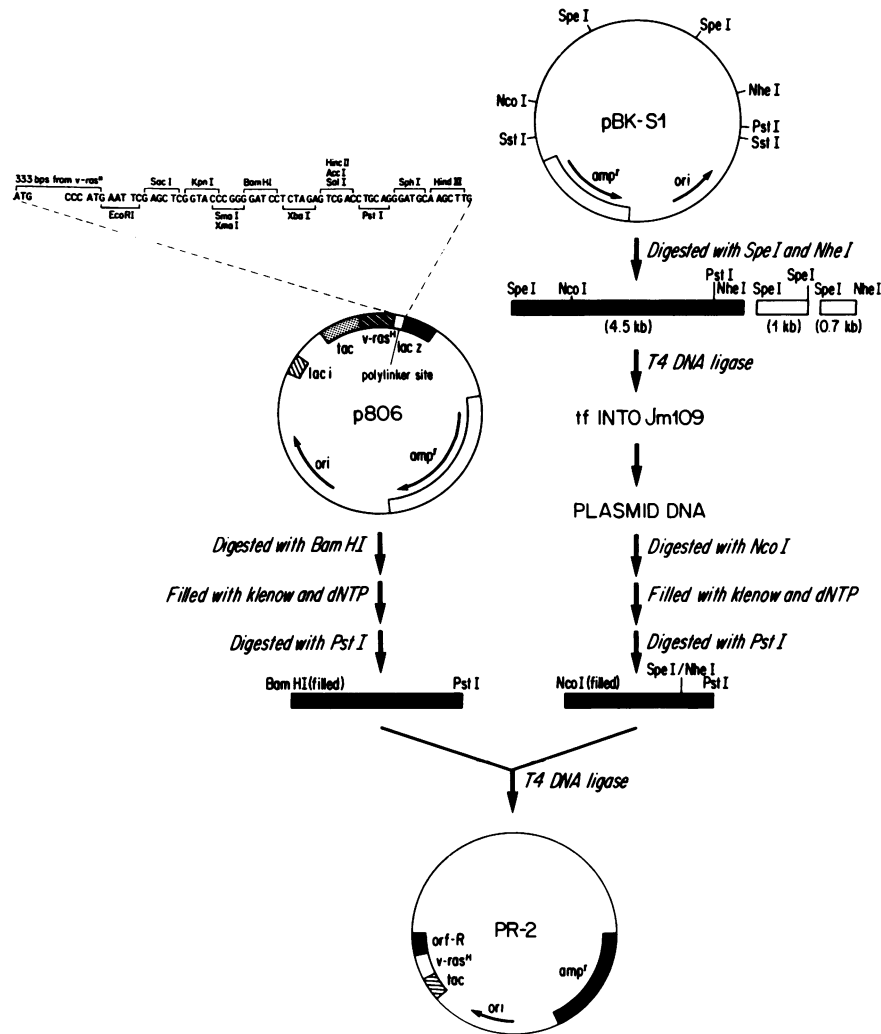


FIG. 1. Construction of SIV_{mac} vpr expression plasmid PR-2. A 1.7-kb SpeI-NheI fragment was deleted from pBK-S1 so that the NcoI site in the vpr coding region became unique. The 1.1-kb NcoI-PstI fragment that contains most of the vpr coding sequences was inserted into the polycloning site of p806.

using a modified protocol of Pallas et al. (33), and the recombinant protein dissolved in 8 M urea was used as antigens for Western blot (immunoblot) analysis as described previously (30). The vpr recombinant proteins were reactive with a postimmune goat anti-ras serum but not with a control preimmune goat serum (data not shown). This finding in conjunction with the result of DNA sequence analysis supported the conclusion that the IPTG-induced proteins were vpr and v-ras^H fusion proteins. The vpr recombinant proteins were also reactive with sera from SIV_{mac}-infected monkeys, HIV-2 seropositive donors, and HIV-1 seropositive donors. The reactivity appears to be directed against the vpr region of the fusion protein, because these sera have no reactivity to the v-ras^H protein (data not shown). No reactivity was seen with sera from either monkeys not infected with SIV_{mac} or HIV-1 and HIV-2 seronegative donors (data not shown).

To detect the native product of vpr gene, a radioimmuno-precipitation assay was used. The molecular clone of SIV_{mac} pBK-28 was used to create the vpr mutant pBK-r by site-directed mutagenesis. A 3.5-kb SstI fragment from pBK28 was first cloned into M13mp18. The recombinant DNA was

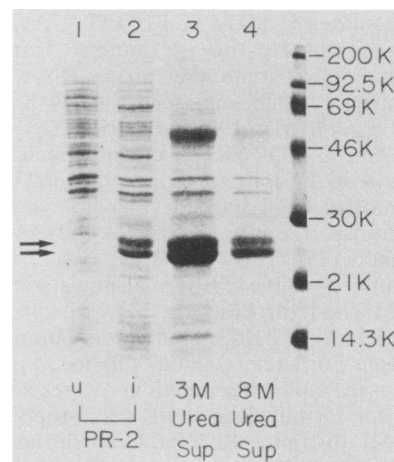


FIG. 2. Expression and partial purification of vpr recombinant protein. Coomassie blue staining of whole-cell lysates from PR-2 bearing X-90 cultures without (lane 1) or with IPTG induction (lane 2). Arrows indicate the induced vpr protein. Lane 3, recombinant vpr protein solubilized in 3 M urea; lane 4, recombinant vpr protein solubilized in 8 M urea.

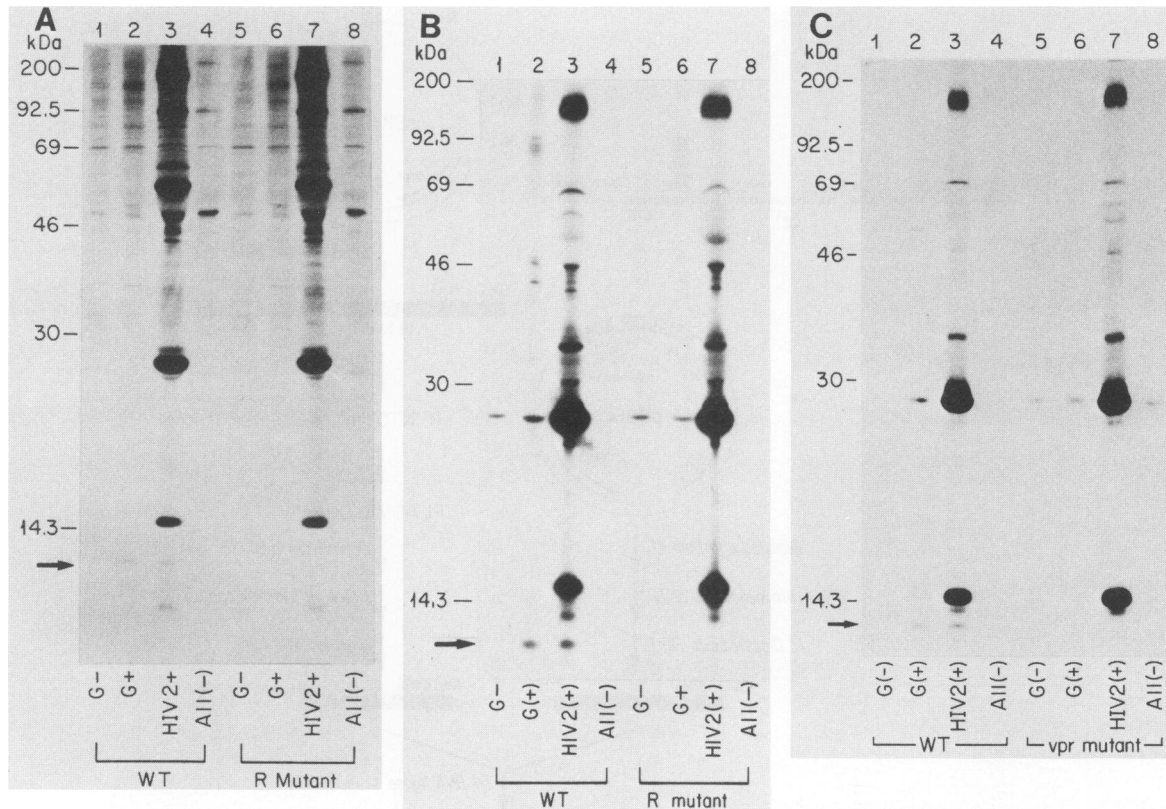


FIG. 3. Detection of *SIV_{mac}* *vpr* protein in virus-infected cells and cell-free virions. WT, Wild type; R mutant, *vpr* mutant. (A) RIPA analysis of cell lysates from wild-type- and *vpr* mutant-infected CEMX174 cells. Lanes 1 to 4, cell lysates from wild-type virus-infected CEMX174 cells; lanes 5 to 8, cell lysates from *vpr* mutant-infected CEMX174 cells; lanes 1 and 5, preimmune goat serum (G⁻); lanes 2 and 6, postimmune goat anti-*vpr* serum (G⁺); lanes 3 and 7, HIV-2-positive serum; lanes 4 and 8, serum from an HIV-seronegative donor (All⁻). (B) RIPA analysis of viral lysates. Lanes 1 to 4, wild-type virus; lanes 5 to 8, *vpr* mutant virus; lanes 1 and 5, preimmune goat serum; lanes 2 and 6, postimmune goat anti-*vpr* serum; lanes 3 and 7, HIV-2-positive serum; lanes 4 and 8, serum from an HIV seronegative donor. (C) RIPA analysis of sucrose gradient-purified virions. Lanes 1 to 4, wild-type virus; lanes 5 to 8, *vpr* mutant virus; lanes 1 and 5, preimmune goat serum; lanes 2 and 6, postimmune goat anti-*vpr* serum; lanes 3 and 7, HIV-2-positive serum; lanes 4 and 8, serum from an HIV seronegative donor.

transformed into *Escherichia coli* CJ236, and the single-stranded uracil-containing bacteriophage DNA was purified. An oligonucleotide (5'-TCTTCTCCGTTTATGCTAG-3') was used to synthesize the *vpr* mutant strand using the single-stranded uracil-containing phage DNA as the template. The phages which contained the *vpr* mutation were selected on *E. coli* MV1190 and verified by direct DNA sequencing. The 3.5-kb *Sst*I fragment which contained a point mutation at the initiator codon of *vpr* ORF (ATG to ACG) was cloned back into the proviral vector. Plasmid DNA were transfected into CEMX174 cells by the DEAE-dextran method (25). Ten million cells were washed with phosphate-buffered saline (PBS) once and suspended in 2 ml of TD (25 mM Tris hydrochloride [pH 7.4], 140 mM NaCl, 5 mM KCl, 0.7 mM K_2HPO_4) buffer containing 400 μ g of DEAE-dextran. For each recipient cell line, 5 μ g of plasmid DNA was used, and the reactions were kept at room temperature for 15 min. Reactions were stopped by adding 10 ml of RPMI 1640 plus 10% fetal calf serum and washed twice with RPMI 1640. Cells were suspended in 10 ml of complete medium and incubated at 37°C. Ten days after transfection, cell-free supernatant was obtained by spinning down cells at 800 \times *g* for 10 min and filtering through 0.45- μ m-pore-size filter units. These supernatants were used as virus stocks. The wild-type and *vpr* mutant viruses

containing 20 \times 10⁶ cpm of reverse transcriptase (RT) activity were incubated with 5 \times 10⁶ CEMX174 cells at 37°C for 2 h. Cells were washed three times with PBS and suspended in 10 ml of complete medium. Six days postinfection, 20 \times 10⁶ CEMX174 cells infected with wild-type or *vpr* mutant viruses were washed once with PBS and then cultured in 20 ml of cysteine-free RPMI 1640 containing 10% fetal calf serum and [³⁵S]cysteine (100 μ Ci/ml) for 8 h. Cells were washed with PBS twice and lysed in 2 ml of RIPA lysis buffer (27). Insoluble cell lysates were removed by centrifugation at 40,000 rpm (Beckman 70 Ti rotor) for 1 h. Ten-microliter serum samples were incubated with 100 μ l of 10% protein A-Sepharose beads at 4°C for 4 h and washed with PBS twice. One hundred microliters of cell lysate was added to each reaction and incubated at 4°C overnight. The reactions were washed three times with RIPA washing buffer (27) and once with 0.05 M Tris (pH 7.2)–0.15 M NaCl. A 60- μ l sample buffer (27) was added to each reaction and boiled for 2 min. Fifteen-microliter samples were analyzed on 15% polyacrylamide–sodium dodecyl sulfate gels. A protein of approximately 11 kDa (arrow) was specifically recognized by a postimmune anti-*vpr* serum, but not by the preimmune serum (Fig. 3A). The goat anti-*vpr* serum was raised against *vpr* recombinant proteins purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously

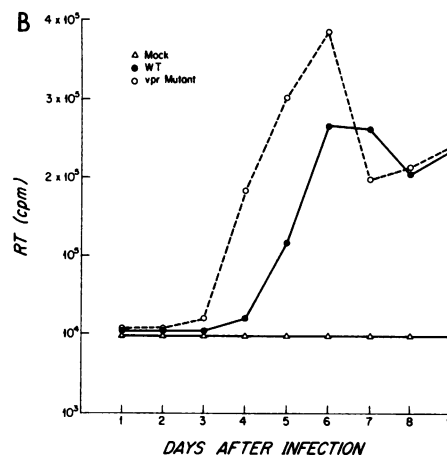
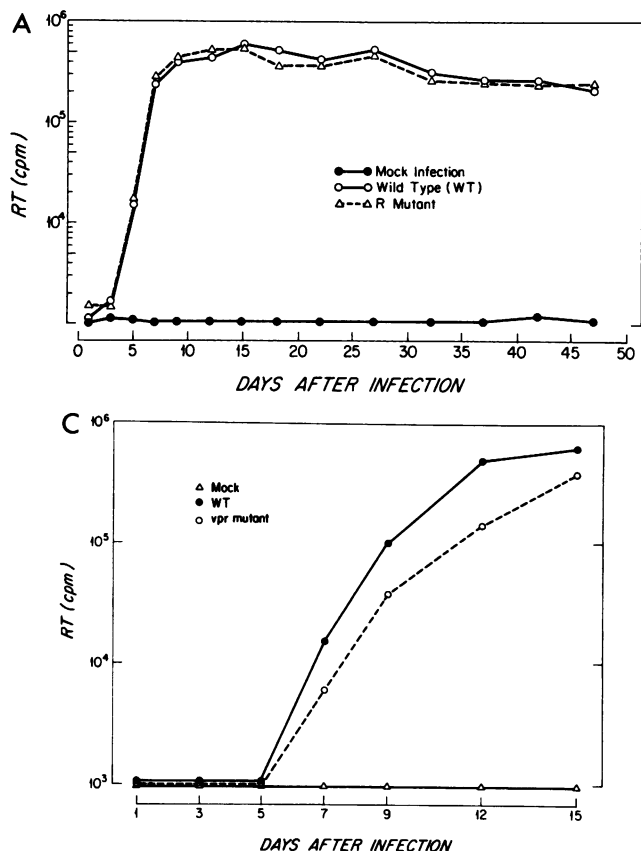


FIG. 4. In vitro growth property of wild-type and *vpr* mutant viruses in different cell lines. (A) Hut-78 cells; (B) MT-4 cells; (C) Human PBL cells. Two-milliliter cell-free supernatants were harvested and mixed with 1 ml of 30% polyethylene glycol to precipitate virion particles at 4°C. The pellets were dissolved in 100 μ l of RT buffer (0.5% Triton X-100, 15 mM Tris [pH 7.5], 3 mM dithiothreitol, 500 mM KCl, 30% glycerol), and 10 μ l was used to incubate with 90 ml of ³H-labeled TTP cocktail (40 mM Tris [pH 7.8], 8 mM dithiothreitol, 10 mM MgCl₂, 0.44 U of poly(rA)-oligo(dT) [optical density at 260 nm] per ml, 25- μ Ci/ml ³H-labeled dTTP) at 37°C for 2 h. The RT values shown in this figure represent 50 μ l of culture supernatant.

(30). In contrast, this 11-kDa protein was not detected in the cell lysates derived from cells infected by the *vpr* mutant virus. The 11-kDa protein was also reactive with sera from HIV-2 seropositive donors which had antibody reactivity to the *vpr* recombinant protein. Sera from donors not infected with HIV-1 or HIV-2 were not reactive with this protein. Reactivity to the native *vpr* protein was also observed with sera from HIV-1-infected people or SIV_{mac}-infected monkeys which had antibodies to the *vpr* recombinant proteins (data not shown). The identification of the protein product directly proves that the *vpr* ORF is a coding gene of SIV_{mac}. The observed size of 11 kDa for the *vpr* protein is in agreement with what is predicted from the nucleotide sequence of the *vpr* ORF (5). This result suggests that the *vpr* gene product is likely to be translated from only one coding exon and probably does not undergo extensive posttranslational modification. It is possible that a canonical splicing acceptor site which is located 11 nucleotides upstream from the *vpr* initiation codon is used for the *vpr* transcript.

To determine whether the *vpr* protein is associated with cell-free viral particles, cell-free supernatants from SIV_{mac} wild-type- and *vpr* mutant virus-infected CEMX174 cells, which were metabolically labeled with [³⁵S]cysteine, were collected and filtered through Nalgene 0.45- μ m-pore-size units. The supernatants were then centrifuged at 20,000 rpm (Beckman SW28 rotor) for 2 h through a 20% (wt/vol) sucrose cushion. The virus pellets were dissolved in the RIPA lysis buffer and subjected to radioimmunoprecipitation analysis. The 11-kDa *vpr* protein was specifically precipitated by postimmune goat anti-*vpr* serum and an HIV-2-positive serum, which had reactivity to the *vpr* recombinant protein, from lysates prepared from the wild-type virus (Fig.

3B). The 11-kDa protein in the lysates of the wild-type virus was not precipitated by a preimmune goat serum or negative control human serum. The specificity of the 11-kDa protein was further demonstrated by the finding that, while other viral proteins detected in the wild-type virus were readily detectable in the *vpr* mutant virus, the 11-kDa *vpr* protein was absent in lysates prepared from the *vpr* mutant virus (Fig. 3B). Similar analysis was also carried out with cell-free virions banded in sucrose gradients. Virus pellets purified through 20% sucrose cushions were dissolved in 3 ml of RPMI 1640 medium and overlaid on the discontinuous sucrose gradient. The discontinuous sucrose gradient was prepared by sequentially overlaying 11 layers of 3-ml sucrose with the following concentrations (wt/vol): 60, 55, 50, 45, 42.5, 40, 37.5, 35, 30, 25, and 20%. Twelve fractions were collected drop by drop from the bottom of the centrifuge tubes after centrifugation in a Beckman SW28 rotor at 20,000 rpm for 20 h. The fraction with the highest RT activity, which has a density of approximately 1.16 g/cm³, was used for RIPA analysis. Both the postimmune goat serum and the same HIV-2-positive serum described above precipitated the 11-kDa *vpr* protein from the lysates of the wild-type virus but not the *vpr* mutant (Fig. 3C). Neither preimmune goat serum nor a negative control human serum precipitated the *vpr* protein.

The *vpr* gene of SIV_{mac} appears to be dispensable for virus replication in CEMX174 cells because viral proteins (Fig. 3) and virion particles (data not shown) were detected in *vpr* mutant virus-infected cells. Aliquots of wild-type and mutant viruses from the same stocks, adjusted by RT activity, were also used to infect Hut-78 cells, MT-4 cells, human peripheral blood lymphocytes, and monocytes in a paralleled experiment. In Hut-78 cells, the wild-type and *vpr* mutant viruses produced comparable levels of virus with similar kinetics (Fig. 4A). Comparable levels of transient and limited syncytium formation and cytopathic effects were also ob-

served (data not shown). In MT-4 cells, the wild-type virus appeared to replicate slightly slower than the *vpr* mutant (Fig. 4B). In human PBL cells, the wild-type virus appeared to replicate slightly faster than the *vpr* mutant (Fig. 4C), while both the wild-type and *vpr* mutant did not show significant cytopathic effect (data not shown). Similar results were also observed in the enriched human monocytes (data not shown). Since it was reported that HIV-1 *vpr* could accelerate virus replication (8, 32), and this effect could only be observed with a low multiplicity of infection in some cells (32), we also studied the effect of *vpr* mutation in CEMX174 cells using 100-fold-diluted input viruses. No significant difference in virus growth kinetics was observed between the wild-type virus and the *vpr* mutant (data not shown).

Similar to the *vpr* mutants of HIV-1 and HIV-2 (8, 11, 35), the SIV_{mac} *vpr* mutant, pBK-r, appears to be dispensable for virus replication in several human lymphoid cell lines as well as fresh human PBL cells in vitro. Since the molecular clone of SIV_{mac} pBK-28 does not replicate well in macaque PBL cells, the effect of *vpr* mutation in these cells cannot be directly assessed. The *vpr* gene of the molecular clone pBK-28 also lacks four amino acid residues, including highly conserved arginine and methionine, which are found in the C terminus of some SIV_{mac}, SIV_{smm}, and HIV-2 isolates (31). It is possible that these four amino acids are essential for the function of the *vpr* protein. Thus, the lack of phenotypic differences between pBK-28 and pBK-r could be due to the attenuation of the *vpr* gene in pBK-28. However, it should be noted that a previous study using an HIV-2 clone, which has a *vpr* ORF containing the four amino acid residues not found in pBK-28, also did not observe significant phenotypic differences between the wild-type HIV-2 and its *vpr* mutant (35).

Evidence presented here strongly supports the conclusion that SIV_{mac} contains a *vpr* gene in its genome. Like the *vpr* gene of HIV-1 (7; X. Yuan, Z. Matsuda, M. Matsuda, M. Essex, and T.-H. Lee, AIDS Res. Hum. Retroviruses, in press), the *vpr* gene of SIV_{mac} also encodes a virion-associated protein. Until now, the only known virion-associated accessory protein of SIV_{mac} is Vpx (14, 20, 24, 44), which is unique to HIV-2 and most of SIV. It appears that the signal for packaging of SIV_{mac} *vpr* protein into the virion is independent of that for the *vpx* protein, since the *vpr* protein is detectable in the *vpx* mutant virus, and the *vpx* protein is detectable in the *vpr* mutant virus (data not shown).

The presence of the *vpr* gene product in the viral particles raises the possibility that this protein may have a role in the early stages of virus infection before the de novo synthesis of viral proteins. For instance, the virion-associated *vpr* protein may activate cellular factors that support subsequent viral replication. The observation that the product of the HIV-1 *vpr* gene can act as a promiscuous transcriptional activator is consistent with this hypothesis (8). However, the possibility that SIV_{mac} may also be able to replicate in other target cells which are known to support the replication of HIV-1, such as macrophages (18, 21), fibroblasts (41), glioma cells (6), neuronal cells (28), and bone marrow-derived precursor cells (13), and that the *vpr* gene may be required for efficient viral replication in these cells remain to be addressed.

Among the genes of HIV-1, HIV-2, and SIV_{mac}, it is known that the coding sequence of the *vpr* gene is one of the most conserved. Thus, the lack of such as a conserved gene in the genome of SIV_{agm} is quite unusual (16). Moreover, the lack of a *vpr* gene in SIV_{agm} and the absence of AIDS in SIV_{agm}-infected monkeys (23) sharply contrasts with the

presence of a *vpr* gene in HIV-1, HIV-2, and SIV_{mac} and the abilities of these viruses to cause AIDS in their respective hosts. Clearly, further studies using the SIV_{mac} model may help us to understand whether the *vpr* gene is indispensable for the induction of AIDS.

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