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Vpr of Simian Immunodeficiency Virus of African Green Monkeys Is Required for Replication in Macaque Macrophages and Lymphocytes

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The genomes of simian immunodeficiency viruses isolated from African green monkeys (SIVagm) contain a single accessory gene homolog of human immunodeficiency virus type 1 (HIV-1) *vpr*. This genomic organization differs from that of SIVsm–SIVmac–HIV-2 group viruses, which contain two gene homologs, designated *vpr* and *vpx*, which in combination appear to share the functions of HIV-1 *vpr*. The in vitro role of the SIVagm homolog was evaluated with molecularly cloned, pathogenic SIVagm9063-2. These studies revealed that this gene shares properties of HIV-1 *vpr*, such as nuclear and virion localization. In addition, SIVagm mutants with inactivating mutations of *vpr* are unable to replicate in nondividing cells, such as macaque monocyte-derived macrophages, but replicate to almost wild-type levels in a susceptible human T-cell line. The transport of virus preintegration complexes into the nucleus in primary macrophages, as measured by the production of unintegrated circular viral DNA, is less efficient for the mutant viruses than it is for the wild-type virus. SIVagm mutants also replicate inefficiently in primary macaque peripheral blood mononuclear cells, with a propensity for substitutions that remove the inserted inactivating stop codon. These data, in conjunction with recent findings that the Vpr protein is capable of inducing G_2 arrest, are consistent with designation of this SIVagm accessory gene as *vpr* to reflect its shared functions and properties with HIV-1 *vpr*.

Human and simian immunodeficiency virus (HIV and SIV) genomes contain several accessory genes, *tat*, *rev*, *vif*, *vpr*, *vpx*, and *vpu*, in addition to the *gag*, *pol*, and *env* genes common to all retroviruses. While genes such as *tat* and *rev* are essential for viral replication, the functions of the other accessory genes are less well defined, particularly since deletions of many of these genes are not lethal to in vitro viral replication. The prototype accessory gene that falls into this category is the *nef* gene. Nef is essential for virulence of SIVmac in vivo (26) and does not appear to be required for growth of either SIVmac or HIV type 1 (HIV-1) in vitro, even in primary lymphocytes and macrophages (15, 44). *vpr*, an additional accessory gene which is not absolutely required for viral replication, is highly conserved in all primate lentivirus subtypes, which is consistent with this gene possessing a conserved and essential function.

Most of the information about *vpr* has been derived from studies with HIV-1 which revealed that although Vpr is not required for virus growth in rapidly dividing T-cell lines (1, 9, 17), it is required for replication in primary macrophages (1, 9, 17, 46), although the reported degree of impairment in this cell type varies (12, 17). The HIV-1 Vpr is packaged through association with the $p6^{gag}$ protein (33, 37) within the virion (8, 48) and appears to localize to the nucleus in infected cells in culture (32, 34, 48). The requirement for Vpr for efficient replication in primary macrophages is associated with its function in facilitating nuclear localization of the reverse transcriptase (RT) complex (18). This function is apparently duplicated by the HIV-1 matrix (MA) protein, which also appears to mediate nuclear transport through a nuclear localization signal (NLS) (4, 5, 13). HIV-1 lacking both Vpr as well as the NLS in MA is more severely impaired for replication in nondividing cells, such as macrophages. Another seemingly unrelated function of HIV-1 Vpr which has been reported is the ability of Vpr to induce cell cycle arrest leading to the accumulation of cells in the G_2 phase of the cell cycle (2, 16, 21, 39). This latter effect may explain earlier observations that HIV-1 Vpr influences cellular differentiation of human rhabdosarcoma cells (29). A number of other poorly defined properties of HIV-1 Vpr have been reported (30), including association with a cellular uracil DNA glycosylase (3), a weak transactivating effect (45), and interaction with the glucocorticoid receptor complex (40). In summary the HIV-1 *vpr* gene appears to be multifunctional, and the relative significance and contribution of these various functions or activities to viral pathogenesis are unknown.

SIV infection of macaques is a relevant animal model in which to address the function of various genes shared by SIV and HIV-1. Although SIVmac and SIVsm contain a gene analog of HIV-1 *vpr* (15, 49), also designated *vpr*, this gene appears to exhibit only a portion of the properties of the HIV-1 gene. Although Vpr of SIVmac or SIVsm induces a cell cycle arrest in the G_2 stage of the cell cycle (38), as does HIV-1 Vpr, the SIVmac gene is not required for replication in nondividing cells, such as monocyte-derived macrophages (MDM) (11). Instead, it appears that another gene, *vpx*, is essential for growth of SIVmac and SIVsm in macaque MDM (11, 15, 25, 49) and has no affect on the progression of cells through the cell cycle (38). Vpx of HIV-2 is packaged within virions (27, 46, 47), and as for SIVmac239, Vpx is required for efficient replication in primary peripheral blood mononuclear cell (PBMC) cultures (15, 35, 36, 49). However, defects in replication in PBMCs were not observed for SIVsmPBj Vpx mutants (11) or HIV-1 Vpr mutants (9). Because of the structural and phylogenetic similarities of the *vpr* and *vpx* genes, it has been suggested that these genes arose by gene duplication (43) or nonhomologous recombination (41). Regardless of their origin,

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these two genes appear to complement one another to provide the functions of the single HIV-1 *vpr*.

SIVmac with deletions of *vpr* and/or *vpx* genes can be used to dissect the relative contribution of the two major effects of these genes, nuclear transport and cell cycle arrest. Surprisingly, such studies have demonstrated that SIVmac *vpr* and *vpx* genes individually are apparently not essential for in vivo pathogenesis in infected macaques (14, 20, 28). For example, although inactivating mutations in SIVmac Vpr revert in vivo, those Vpr-deficient viruses which do not revert are still capable of inducing AIDS (28). Other more recent studies using SIVmac239 Vpr deletion mutants suggest that the Vpr protein has very little effect on SIV pathogenesis (14, 20). Vpx mutant viruses appear to be more attenuated than Vpr-deficient SIVmac (14); however, animals infected with such Vpx mutants of SIVmac still infrequently develop AIDS after a long clinical latency. However, SIVmac239 with deletions in both Vpr and Vpx, while still capable of establishing an infection in macaques, is severely attenuated (14).

The genomic organization of other SIV subtypes, such as SIVmnd (mandrill monkey), SIVsyk (Sykes monkey), and SIVagm (African green monkey), is more analogous to that of HIV-1 with respect to the *vpr* gene (19). Although the analogous SIVagm gene was initially identified as *vpx*, it is actually equidistant in identity to SIVmac *vpr* and *vpx* and thus also shares the common genomic organization (19). Therefore, these latter viruses may provide a more relevant model for the study of the role of *vpr* in viral pathogenesis. However, with the exception of SIVagm infection of pigtailed macaques (20), the majority of these viruses do not induce immunodeficiency. For these reasons, we chose to study the in vitro function of Vpr in an infectious pathogenic SIVagm molecular clone, SIVagm9063-2. Planelles et al. (38) recently reported that SIVagm Vpr (from the SIVagm/gri-1 clone) induces a G_2 arrest of CV-1, an African green monkey cell line, but has no effect on human cell lines such as HeLa. Therefore, it appears that SIVagm Vpr may possess a repertoire of functional capabilities similar to those of HIV-1 Vpr. However, the potential role of SIVagm Vpr in nuclear localization and in facilitating the growth of virus in macrophages has not been studied. In the present study, the biological properties of SIVagm Vpr and its requirement for replication in nondividing cells, such as macrophages, were studied in the context of a pathogenic SIVagm clone.

MATERIALS AND METHODS

Generation of SIVagm-specific antisera. Antisera to SIVagm9063-2 Vpr were produced by injection of rabbits with either a Vpr C-terminal keyhole limpet hemocyanin-conjugated peptide, CRRRQPFEPYEERR, or an N-terminal keyhole limpet hemocyanin-conjugated peptide, CMASGRGPRENRPGE, in incomplete Freund's adjuvant. Antisera to these peptides were used in indirect immunofluorescence or Western blotting of virion proteins. Other sera used for indirect immunofluorescence were plasma from an SIVagm-infected macaque (PT63), which reacted with all the major SIVagm antigens, including Vpr, and polyclonal rabbit antiserum produced by immunization of rabbits with purified, bacterial-expressed SIVagm9063-2 capsid (CA) protein (His-tag system; Novagen, Madison, Wis.).

Western blot and immunofluorescence detection of Vpr. To evaluate the cellular localization of Vpr in SIVagm-infected cells, the susceptible adherent sMAJI cell line (7) was plated on eight-well glass labteck slides (Nalgene Nunc, Naperville, Ill.) in RPMI complete medium (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml) and infected with 10 to 50 tissue culture infective doses (TCID) of SIVagm9063-2. Some wells were mock infected to provide a negative control. At 24, 48, and 72 h postinfection, the cells were washed with phosphate-buffered saline (PBS), fixed with a 1:1 ice-cold acetone-methanol mixture for 2 min, and subjected to indirect immunofluorescence. The slide was incubated for 1 h at 37°C with 1% goat serum in 1% nonfat milk-PBS (milky PBS), followed by a 1:100 dilution of either rabbit anti-SIVagm Vpr peptide in milky PBS, rabbit anti-CA, or whole macaque antiserum to SIVagm, with intervening washing steps with PBS. Finally, slides were incubated with a 1:100 dilution of fluorescein-conjugated goat anti-rabbit immunoglobulin G (IgG) (or mouse anti-human IgG) for detection of macaque primary antibody (heavy plus light chains) (Pierce, Rockford, Ill.), mounted with fluoromount-G (Southern Biotechnology Association, Inc., Birmingham, Ala.), and examined by fluorescence microscopy on a Zeiss Axiophot.

Virion-associated proteins were detected in whole pelleted SIVagm (both wild type and mutants) by a chemiluminescence Western blot assay with rabbit anti-Vpr peptide antibody or plasma from an SIVagm-infected macaque (PT63). Briefly, virus was pelleted over a 20% sucrose cushion and resuspended in a 0.05 M Tris-hydrochloride buffer containing 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, and 1% 7-deoxycholic acid. The amounts of virus were adjusted with a standard virus antigen test for SIV p27 (Coulter, Hialeah, Fla.), and aliquots containing approximately 10 ng of p27 were electrophoresed through an SDS–8 to 16% gradient polyacrylamide gel (Novex, San Diego, Fla.). Viral proteins were transferred to nitrocellulose by electroblotting, incubated for 10 min with Superblock (Pierce) in PBS, and reacted in a chemiluminescence assay. Briefly, the nitrocellulose was incubated for 1 h in a 1:100 dilution of primary antibody (in 5% bovine serum albumin–PBS–0.1% Tween 20 buffer), washed with 0.5 M NaCl–PBS–0.1% Tween 20 three times for 5 min each, and then incubated in a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Pierce) (in 0.25 M NaCl–PBS–0.1% Tween 20), and antibody complexes were detected by an enhanced chemiluminescence technique as described by the manufacturer (Amersham, Arlington Heights, Ill.).

Construction of Vpr mutant viruses. A derivative of the wild type, SIVagm9063-2 (19), was constructed by filling a second *Csp*45I site in the flanking genomic DNA with T4 DNA polymerase, and this clone was subcloned into 5′ and 3′ subgenomic clones. An *Sst*I-*Csp*45I fragment containing the *vpr* gene (bases 5884 to 6032) was subcloned into pGEM-7Zf+ and used for site-directed mutagenesis of *vpr*. To perform mutagenesis of nucleotides in the 5′ portion of *vpr*, an *Sph*I-*Sst*I fragment (bases 4130 to 5023) of the 5′ SIVagm9063-2 clone was subcloned into pGEM-7Zf+. Modified fragments were then substituted for wild-type fragments into the 3′ or 5′ half of the SIVagm9063-2 genome and confirmed by sequence analysis, and a full-length mutant clone was generated. SIVagm Vpr mutant clones were named according to the codon modified; e.g., R-S53 has a stop codon introduced at codon 53 of *vpr*. The oligonucleotide primers and types of mutagenesis used are shown in Table 1.

Cells and viruses. To evaluate infectivity and to generate virus stocks, full-length SIVagm9063-2 and Vpr mutant clones were transfected by a modified DEAE dextran method into the susceptible human T-cell line CEMss. CEMss cells were then propagated in RPMI complete medium, and the culture supernatants were monitored for RT activity. Cell-free supernatants containing the respective viruses were filtered (0.45-μm-pore-size filters) and stored in aliquots in liquid nitrogen, and the 50% TCID ($TCID_{50}$) of each was determined by limiting dilution infectivity for sMAJI cells (7). The infectivity and replication kinetics of equivalent TCIDs of these viruses were evaluated by infection of CEMss cells, primary macaque phytohemagglutinin (PHA)-stimulated PBMCs, or MDM. PBMCs were isolated by centrifugation on lymphocyte separation medium (Organon Teknika, Durham, N.C.) and cultured in RPMI complete medium supplemented with 5 μg of PHA (Sigma, St. Louis, Mo.) per ml and 1% recombinant interleukin-2 (IL-2) for 3 days prior to infection. Macrophages were derived from PBMCs by allowing them to adhere to the plastic of 24- or 48-well tissue culture plates (Corning Costar, Cambridge, Mass.) in RPMI complete medium supplemented with 10% normal rhesus serum for 5 to 10 days and then extensively washing the plates to remove nonadherent macrophages prior to infection, as previously described (18). The replication kinetics of the viruses were measured by quantitation of RT activity at different time points with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

Detection of revertant viruses. Total cellular DNA was isolated from PBMCs or CEMss cells at 15 to 20 days postinfection with an Isoquick kit (ORCA Research Inc., Bothell, Wash.) and used in a PCR–single-stranded conformation polymorphism (SSCP) assay for the detection of single or multiple base changes in the 5′, 3′, or entire *vpr* region. PCR-SSCP was performed essentially as described previously (6) with primers corresponding to the 5′ *vpr* (bases 5713 to 5934 of the SIVagm9063-2 genome; primer 2695, 5′ TCACTGCAGTACTTAG CCCTTCTG, and primer 2707, 5′ CCGTCTCTCTCCTTCCTCCTGACA), 3′ *vpr* (bases 5908 to 6115; primer 2708, 5′ TATTGTCAGGAAGGAGAGAGAC GG, and primer 2696, 5′ TTATATTGTCTAATCAAGTCCTGG), or the entire *vpr* region (primers 2695 and 2696).

At day 23, PBMC DNA was isolated by Isoquick extraction for direct PCR sequencing of *vpr*. The *vpr* region was amplified by PCR (with a Perkin-Elmer Cetus 9600) from 250 ng of each DNA sample with primers 2695 and 2696 (see above). Thirty-five cycles of PCR amplification were conducted as follows: 95°C for 20 s, 55°C for 30 s, and 72°C for 30 s. The resulting PCR products were evaluated following electrophoresis on a 2% agarose gel, and approximately 20 ng of each product was sequenced in both directions, after purification through a Qiaquick PCR purification column according to the manufacturer's instructions (Qiagen, Chatsworth, Calif.), with internal primers, 5′ ATGGCATCAGG GAGAGGTCCC and 5′ TGGAGCACGGTTCGCTCTTCC, on the ABI 373 automated sequencer (Applied BioSystems, Inc., Foster City, Calif.).

Analysis of viral DNA synthesis in newly infected macrophages. Viral RT products were amplified by PCR from DNA extracted (Isoquick kit) from cells

TABLE 1. Primers and types of mutagenesis used to construct Vpr mutant clones

Clone[a]	Primers[b]	Type of mutagenesis
R-S53	5′CAGAGAGCTCCTCTTCCAAGTTT**A**GAATT3′ 5′GTAAGGTTCGAAGGGCTGTCTTCTACGACA3′	Direct PCR
R-S77	5′CACTCTTTGTTCATTTCCGTTGCT3′ 5′CTTTCTGAACCTACC***T***ATAGTATC3′	Inverse PCR
R-S92	5′GTTGTCGT***T***GAAGACAGCCCTTCG3′ 5′CACAACGGAAATGAACAAAGAGTG3′	Inverse PCR
R-MS3	5′CCATCAGAA***C***GGCAT***A***AGGGAGAG3′ 5′GCCAGAAGGGCTAAGTACTGCAGT3′	Inverse PCR
R+1	5′**CTAACATCA**TCTATAGGCCCTCTC3′ 5′**GCTTACTG**TACTATAGGTTGGTT3′	Inverse insertional PCR
R+2	5′CGAA**TGATGTTAGGCTTAGG**TT3′ 5′CGAA**CCTAAGCCTAACATCA**TT3′	*Csp*45I linker insertion

[a] The 5′ R-MS3 and 3′ R-S53 clones were combined to generate R-MS3,53. All mutations were confirmed by sequence analysis of the entire amplified region(s) and restriction junctions.

[b] Underlined bases in the primers correspond to the *Sst*I and *Csp*45I restriction sites, italic boldface type indicates nucleotide substitutions relative to the wild-type virus, and roman boldface type indicates insertions.

infected with wild-type or Vpr mutant viruses at 0, 8, 24, or 48 h postinfection. Early products were detected by amplification of strong-stop DNA with primers 2558, 5′ (bp 8737) TCTTACTAGGAGACCAGCTTGAGC, and 2559, 5′ (bp 8931) TGGGTCAGAGAGAGAACCCAGTAA. Production of full-length genomes was assessed by amplification of a region in *pol* with the primers 2556, 5′ (bp 3124) TACTGGCAAGTGAGCTGGATTCCT, and 2557, 5′ (bp 3627) ATG TTCTTCTTGAGCTTCTTCTAT. Finally, circular unintegrated viral DNA, found only in the nucleus, was detected by amplification by inverse PCR with primers in *nef* and *gag*: primer 2537, 5′ GAATGGCATGAATGGTCAGATG AT, and primer 2538, 5′ TACTTGCCCAGGGTAGTTATGGCT, respectively. The cell copy number was normalized by amplification of the β-globin gene from an equivalent amount of DNA and from serial dilutions of each sample with human β-globin gene primers (2500, 5′ GAAGAGCCAAGGACAGGTACGG CTGTCATC, and 2501, 5′ CACCACCAACTTCATCCACGTTCACCTTGC).

RESULTS

Nuclear localization of SIVagm Vpr. In order to study the cellular localization of SIVagm Vpr, the susceptible cell line sMAJI was infected with the wild-type virus, SIVagm9063-2, and subjected to indirect immunofluorescence. Three different antibody preparations were used: plasma from an SIVagm-infected macaque (PT63), rabbit antiserum to CA, and rabbit Vpr peptide antiserum. Utilizing the Vpr peptide antiserum, Vpr was detected within the nucleus of infected cells as early as 24 h postinfection, with increasing intensity by 48 h post-infection (Fig. 1A). Punctate fluorescence was also observed in

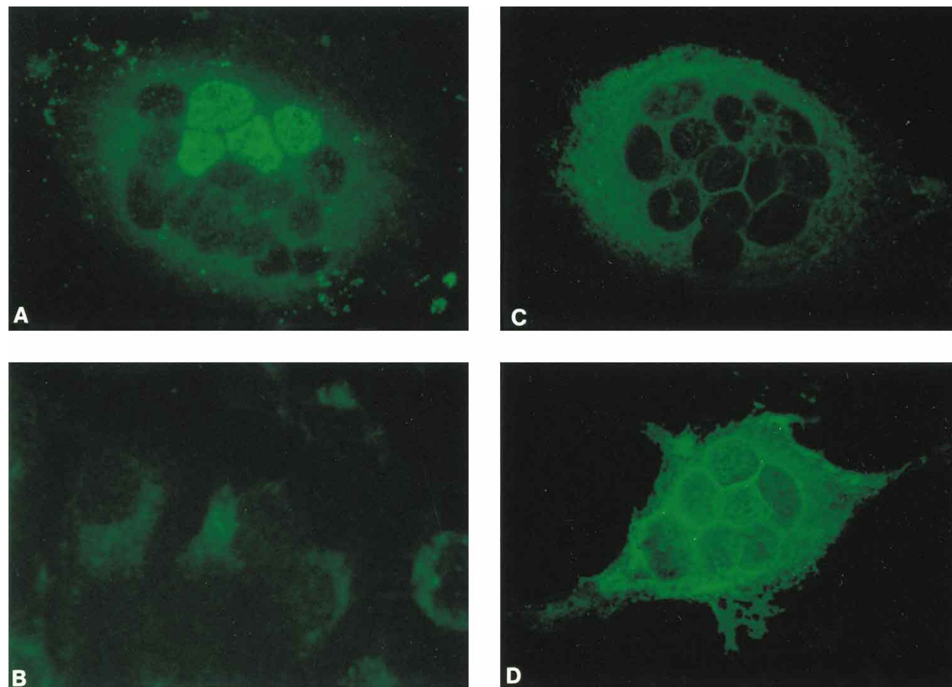


FIG. 1. Nuclear localization of SIVagm Vpr in infected sMAJI cells. Indirect immunofluorescence staining of sMAJI cells that were fixed 48 h after infection with 10 to 50 TCID of SIVagm9063-2 (A, C, and D) or mock infection (B) is shown. The primary antibody used was a 1:100 dilution of either antiserum to a carboxy-terminal peptide of Vpr (A and B), antiserum to a bacterial-expressed SIVagm CA protein (C), or whole macaque antiserum to SIVagm (D).

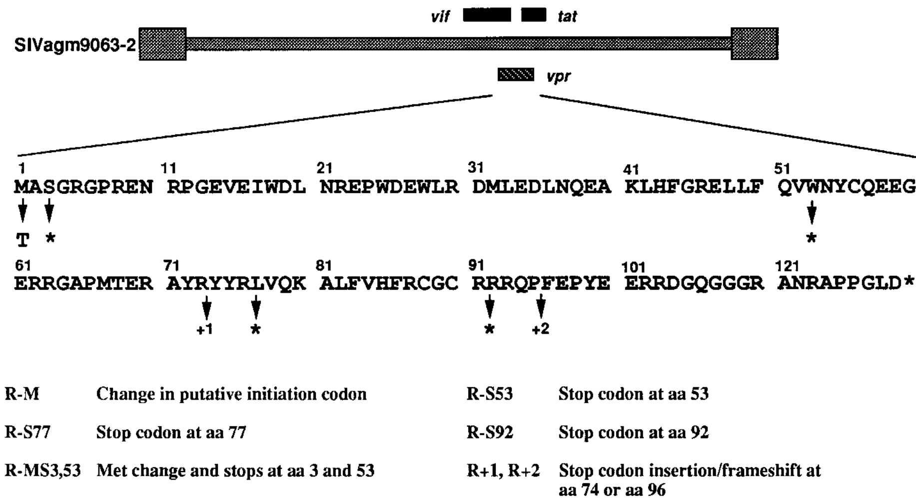


FIG. 2. Diagram of Vpr mutant clones derived from SIVagm9063-2. The entire amino acid sequence of the putative Vpr protein is shown in single-letter amino acid code. Substitutions introduced into various mutants are indicated by arrows and are summarized in the lower half of the figure. Derivation of the clones is described in detail in Materials and Methods. *, stop codon insertions in the coding sequence.

apparent association with the plasma membrane at the later time points, but specific cytoplasmic fluorescence was not observed. Interestingly, there was a distinct pattern of intense focal nuclear fluorescence in all positive cells, although frequently less than half of the nuclei within infected multinucleated cells were positive. This contrasted with the pattern observed with antisera to either CA or the whole virus. As expected from the predicted localization of CA, immunofluorescence detection with antiserum to CA resulted in a diffuse cytoplasmic staining pattern (Fig. 1C) with no apparent nuclear staining. The whole SIVagm antisera produced diffuse cytoplasmic as well as nuclear staining (Fig. 1D) consistent with the broad reactivity of this plasma with multiple viral antigens. The specificity of each of these antibody reactions was demonstrated by lack of fluorescence in uninfected sMAJI cells (Fig. 1B).

Generation of Vpr-deficient SIVagm clones. A panel of clones with substitutions that introduced one or more premature stop codons (clones R-S53, R-S77, and R-S92), a threonine substitution (ACG) for the initiating methionine of *vpr* (clone R-M), or combinations of these mutations (clones R-MS3 and R-MS3,53) were also generated (Fig. 2). None of the substitutions altered the predicted amino acid sequence of the overlapping *vif* or *tat* genes. To provide mutants that might be more stable for in vivo experiments, two additional mutants were generated by the insertion of nucleotides either immediately 3′ to *vif* (clone R+1) or near the region of the *vpr* gene corresponding to the C terminus (amino acid [aa] 96) of Vpr (clone R+2). These insertions resulted in multiple premature stop codons as well as shifting of the reading frame to produce viruses which had more stable inactivating mutations. The phenotypes of these various mutants for growth in CEMss cells and in primary macaque PBMC and macrophage cultures are summarized in Table 2. Following transfection or subsequent infection of CEMss cells, these various mutant clones were infectious as indicated by production of RT activity in culture supernatants (Fig. 3A). However, the kinetics of virus production in CEMss cells was consistently delayed compared to the kinetics observed with the wild-type virus, with an occasional exception, such as the kinetics observed with the R+1 mutant (Fig. 3A). In addition, the peak levels of virus production as indicated by supernatant RT activity or the $TCID_{50}$ (data not shown) were consistently lower than that observed for the wild-type virus.

To evaluate the composition of progeny viruses of these various clones and to confirm the presence or absence of Vpr protein in mutant viruses, virions were evaluated by Western blot assay with plasma from an SIVagm-infected macaque (PT63) or antiserum to the C-terminal region of Vpr. As shown in Fig. 4, the profiles of viral antigens in virions of either wild-type or mutant viruses, as detected with polyclonal infected-macaque plasma, were almost indistinguishable, with the exception that there was no 14-kDa antigen in any of the preparations of the Vpr mutant viruses. This antigen has the predicted molecular mass of Vpr, and its identity was confirmed by reactivity with rabbit anti-Vpr serum in the wild-type virus preparations but in none of the mutant virus preparations (Fig. 4). The lack of Vpr protein in mutant viruses was confirmed by immunoprecipitation with rabbit antiserum generated against the N-terminal Vpr peptide, which precipitated Vpr from the supernatant of cells transfected with the wild-type virus but not from the supernatants of cells transfected with mutant virus clones (data not shown). Although present in cell lysates from wild-type-virus-transfected cultures, Vpr was also not detectable within cell lysates from cultures transfected

TABLE 2. Mutations and phenotypes of SIVagm Vpr mutants

Mutant	Mutation	Replication in[b]:			Reversion[c] in PBMCs
		CEMss	PBMC	MDM	
RS53	W 53 stop[a]	+	−	−	+
RS77	L 77 stop	+	−	−	+
RS92	R 92 stop	+	±	±	−
RMS3,53	M 1 T; S 3 stop; W 53 stop	+	−	−	+
R+1	Insertion at aa 77	+	−	−	+
R+2	Insertion at aa 96	+	±	±	−

[a] The original amino acid, followed by the amino acid position in the protein and either the substituted amino acid or the substituted codon.

[b] +, replication in the cell type as determined by detectable RT activity in culture supernatant; −, lack of detectable replication in the absence of reversion; ±, low level of viral replication.

[c] Reversion was assessed by direct sequence analysis of the *vpr* region in RT-positive cultures.

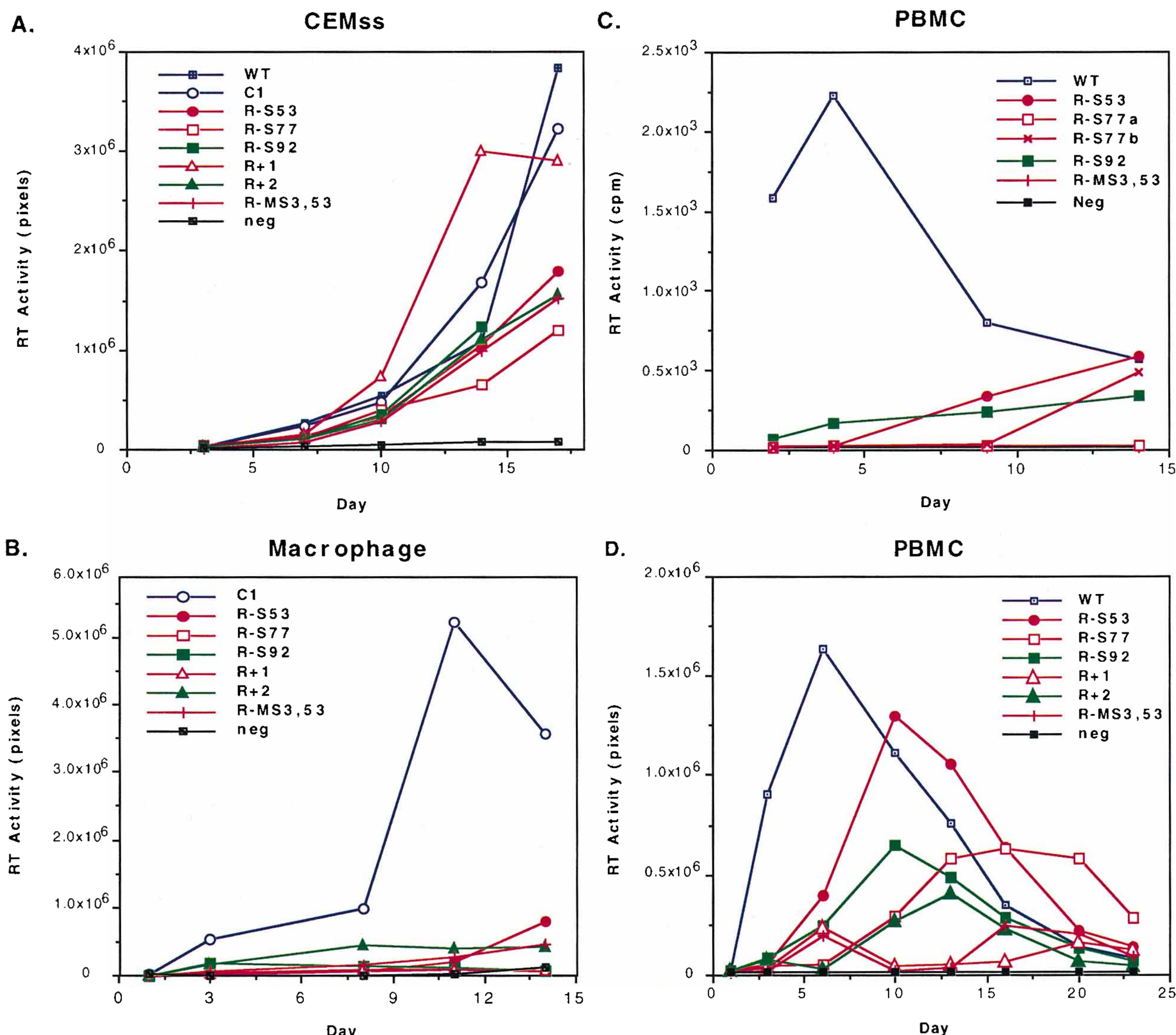


FIG. 3. Replication of SIVagm9063-2 Vpr mutant viruses in CEMss cells (A), primary macaque MDM (B), or primary PBMCs from three different donor macaques (C and D). The indicated cell type was infected with equivalent amounts (as measured by RT activity) of wild-type or mutant viruses. Samples from each set of infections were taken for measuring RT activity at the indicated times after infection and measured at the same time. RT levels were assessed with a PhosphorImager (Molecular Dynamics) (A, B, and D) and with Betagen (C). Wild-type virus is shown in blue, Vpr mutants which are stable in PBMCs are shown in green, and those mutants which are unstable in PBMCs are shown in pink. Each of the RT-positive cultures shown in panel D were assessed by SSCP or sequence analysis for reversion.

with mutant virus (data not shown). These data are consistent with instability of the truncated Vpr proteins within infected cells, indirectly leading to lack of virion incorporation.

Vpr is required for efficient virus replication and nuclear import in macrophages. Replication of the mutant viruses was then evaluated in primary MDM. With the exception of viruses with mutations near the 3′ end of *vpr* (R-S92 and R+2), the Vpr mutants produced little or no RT activity in MDM from three different donor macaques; a representative experiment is shown in Fig. 3B. Data from other donors showed similar results (data not shown). The inability of SIVagm *vpr* mutants to replicate in primary macrophages suggested that, as in HIV-1, the *vpr* gene might be required for transport of the preintegration complex into the nucleus of nondividing cells.

By using a modification of a protocol used to evaluate the HIV-1 Vpr protein (17), the stages of reverse transcription were evaluated in MDM at early time points postinfection (Fig. 5A). Levels of strong-stop DNA (R-U5) were used as a measure of viral input and first strand synthesis, a *pol* product was used to evaluate completion of reverse transcription, and circular viral DNA was used as a measure of nuclear import. The results of the experiment were within the linear range of the assay, as indicated by the PCR assays of a series of dilutions of SIV (Fig. 5D). As shown in Fig. 5A, similar levels of strong-stop DNA were observed in DNA extracted from MDM infected with either the wild-type virus or one of two Vpr mutants (R-MS3,53 and R+1), indicative of equivalent input and entry of virus into MDM. In general, a minor but consistent reduction in the level of *pol* product was observed in cells infected with the Vpr mutants compared to levels in wild-type-infected cultures. This relatively minor reduction of *pol* product contrasted with a more marked reduction in circular DNA forms in Vpr mutant-infected MDM when compared to the levels observed in cells infected with the wild-type virus. Dis-

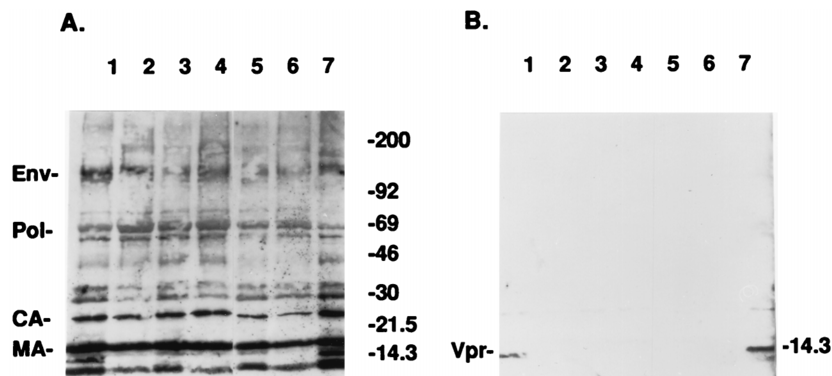


FIG. 4. Western blot detection of virion proteins reveals that Vpr is detected in significant levels in wild-type but not Vpr mutant virions. Virus was pelleted from RT-positive supernatants of CEMss cells and run through an SDS–8 to 16% polyacrylamide gel, transferred to nitrocellulose, and reacted in a chemiluminescence Western blot assay with a 1:100 dilution of whole macaque antiserum to SIV (A) or antiserum to a carboxy-terminal peptide of Vpr (B) as the primary antibody. Lanes 1 and 7 correspond to approximately 10 ng of SIVagm9063-2 virus (as indicated by SIV p27 antigen capture; see Materials and Methods); lanes 2 through 6 correspond to equivalent amounts of Vpr mutant viruses (R-S53, R-S77, R-S92, R+2, and R-MS3,53, respectively). Molecular weights (in thousands) are shown on the right.

proportionate reduction in circular DNA is consistent with impaired nuclear import of preintegration complexes of Vpr-deficient SIVagm.

Vpr is required for replication of SIVagm in PBMCs. Replication of the *vpr* mutants in PBMCs from three different donor macaques appeared to be more variable than the tightly restricted replication observed in macrophages. However, duplicate infection experiments with the same mutant virus frequently yielded conflicting results. Thus, as shown in Fig. 3C, significant replication of mutant virus RS77 was observed in only one of two identically inoculated cultures. In addition, when RT production from cultures infected with *vpr* mutant viruses was observed, the kinetics of appearance was consistently delayed over that observed for the wild-type virus (Fig. 3C). This inconsistent pattern and delayed kinetics of viral replication in PBMCs was suggestive of reversion or repair of the inactivating mutation in RT-positive cultures. We therefore used PCR-SSCP to examine proviral DNA in these cultures for stability of the original mutation (Fig. 6A). SSCP analysis of the Vpr fragment amplified by PCR from this culture and the RS53 RT-positive culture (Fig. 6A) demonstrated a clear alteration in the SSCP pattern, consistent with reversion.

Reversion of Vpr mutations occurs at high frequency in PBMC cultures. To identify the specific mutations generated in revertant cultures, DNA was isolated at 20 days postinfection of PBMCs with various *vpr* mutant viruses (Fig. 4D) and the *vpr* region was amplified by PCR and directly sequenced with the ABI automated sequencer. With the exception of the PBMCs infected with R+2 or RS92, there was evidence of

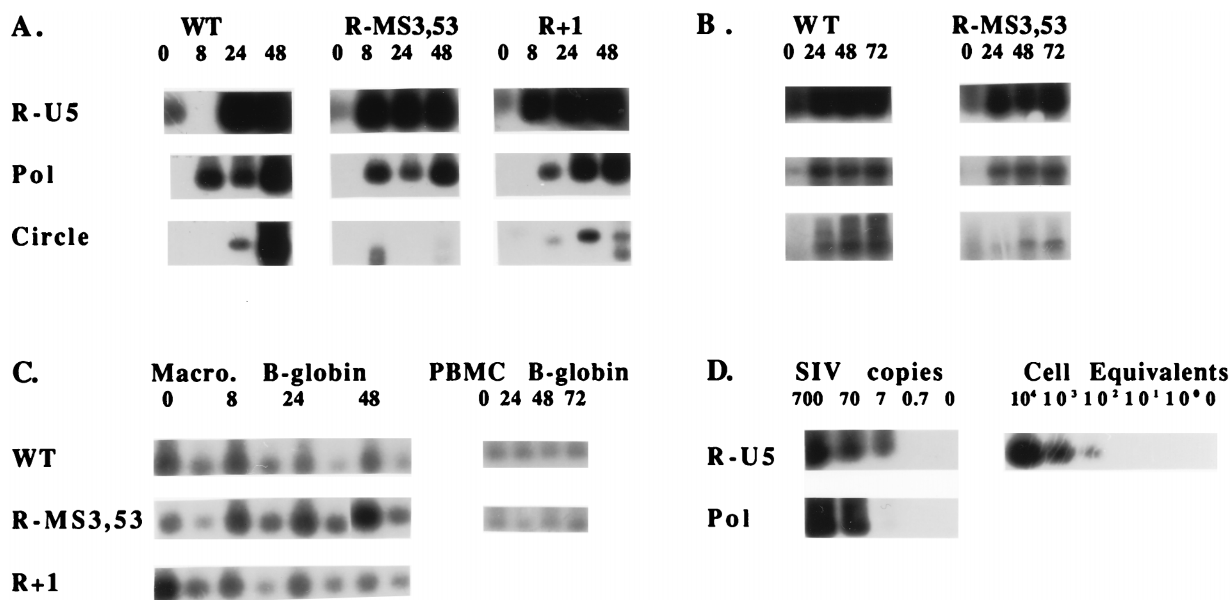
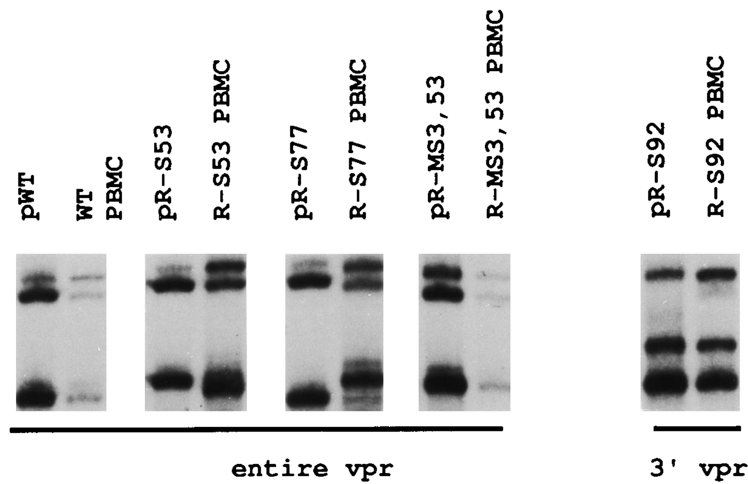


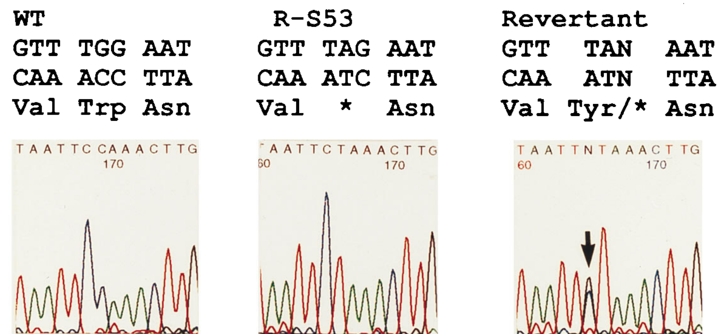
FIG. 5. Synthesis of viral DNA and nuclear import of SIVagm9063-2 and SIVagm Vpr mutant viruses in primary macaque cells. Cells were harvested at the indicated times (days) after virus infection, and equal amounts of isolated DNA were subjected to one round of PCR with primers specific for R-U5, *pol*, and *nef-gag* (circle forms). Synthesis of viral DNA in MDM (A) or actively dividing PBMCs (B) is shown. Control of levels of DNA was measured with PCR primers specific for β-globin (C). For the MDM (Macro.) β-globin controls, both undiluted samples (left gels) and 1:10 dilutions (right gels) of the DNA are shown, while for the PBMCs, only undiluted samples are shown. The sensitivities of R-U5 and *pol* primers were measured with dilutions of plasmid viral DNA (D, left), while relative levels of cell equivalents were measured with dilutions of cellular DNA and β-globin primers (D, right). WT, wild type.

A.



B.

R-S53 (aa 52-54)



R-S77 (aa 76-78)

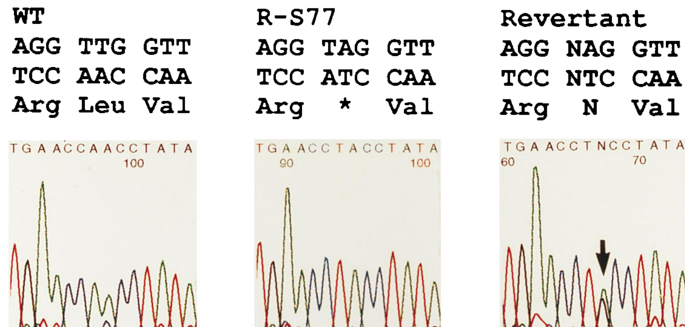


FIG. 6. Reversion of single-point Vpr mutants after infection of PBMCs. (A) SSCP of the entire or the 3′ *vpr* coding region, as indicated. DNA amplified by PCR with primers corresponding to the indicated region in the presence of radiolabel was subjected to polyacrylamide electrophoresis under native conditions (see Materials and Methods). The first lane of each pair contains bands corresponding to the single-stranded bands of the indicated plasmid control (wild type [WT], R-S53, R-S77, R-MS3,53, and R-S92), and the second lane corresponds to the single-stranded bands of DNA isolated from PBMCs at 15 to 20 days postinfection. Changes in the migration patterns of the bands from the plasmid lanes are indicative of nucleotide sequence differences (R-S53 and R-S77). (B) Direct sequence analysis of PCR-amplified DNA from two different Vpr mutant viruses (R-S53 and R-S77). Viral RNA isolated from CEMss viral stocks of wild-type virus or R-S53 (or R-S77) and from PBMCs 15 to 20 days after infection (Revertant) was amplified by an RT-PCR assay, and the entire *vpr* coding region was sequenced. The reverse complement sequence of 13 bases surrounding the nucleotide change (indicated by the arrow) is shown. Above the sequence is the coding sequence of the three putative amino acids surrounding the changed nucleotide (aa 52 to 54 of R-S53 or aa 76 to 78 of R-S77). The asterisk (*) indicates a stop codon, while the single N is a mixture of two amino acids.

reversion in each of the mutant virus infections analyzed in this way. Examples of reversion of the RS53 and RS77 mutations are shown in Fig. 6B. Even in the triple mutant RMS3,53, which might be expected to be more stable, reversion of all three inactivating mutations was observed. Sequence alterations were confined to the original mutated codon. As shown in Fig. 6B, a mixture of genotypes was observed as indicated by the unidentifiable nucleotide (N) at position 65 in the PCR product amplified from cells infected with R-S53. While the original introduced mutation was still present (C to T), the adjacent nucleotide was different from that found in both the wild-type and mutant viruses. Based upon peak colors, a mixture of viruses encoding G (wild-type sequence) or A at this position were observed. The G-to-A substitution resulted in replacement of the stop codon with the codon for tyrosine at aa 53 in a portion of the viruses in the culture. A similar substitution occurred with the R-S77 mutant, in which the mutated nucleotide remained stable but the adjacent nucleotide consisted of a mixture of the wild-type A and C, resulting in a virus encoding Gln at position 77. Interestingly, these substitutions always altered a nucleotide in the codon different than the one altered by the originally introduced substitution. As summarized in Table 2, with the exception of the C-terminal mutants R-S92 and R+2, measurable replication of *vpr* mutant viruses was associated with reversion of the inactivating mutation. The instability of these mutations even in vitro is consistent with an essential function of Vpr in viral replication in vitro and may be predictive of a propensity for reversion in vivo.

Since the Vpr mutants were also impaired for replication in PBMC cultures, the level of the block to replication was evaluated by PCR for reverse transcription products and unintegrated circular DNA, as performed for macrophage cultures. Similar levels of strong-stop DNA were observed in PBMC cultures infected with either the wild-type virus or a particularly stable *vpr* mutant (R-MS3,53), suggesting that entry of the Vpr mutants was not impaired. A minor but consistent reduction in the level of *pol* product was observed, suggestive of some degree of impairment in the process of reverse transcription. Similar to the levels in macrophage cultures infected with the Vpr mutant viruses, the levels of circular unintegrated viral DNA (Fig. 5B) were also decreased in PBMCs infected with the mutant virus compared to cultures infected with wild-type virus.

TABLE 3. In vitro characteristics of SIV and HIV-1 Vpr and Vpx

Characteristic	HIV-1 Vpr[a]	SIVmac and SIVsm Vpx[b]	SIVmac and SIVsm Vpr[c]	SIVagm Vpr[d]
Packaged in virion	Yes	Yes	Yes	Yes
Nuclear localization	Yes	Yes	Yes	Yes
Required for replication in:				
Macrophages	Yes	Yes	No	Yes
PBMCs	No	Yes	No	Yes
T-cell lines	No	No	No	No
Induction of G_2 cell cycle arrest in:				
HeLa	Yes	No	Yes	No
CV-1	Yes	No	Yes	Yes

[a] References for HIV-1 Vpr: 1, 2, 8, 9, 12, 16, 17, 18, 21, 23, 32, 33, 34, 37, 39, 46, and 48.
[b] References for SIVmac Vpr: 11, 15, 36, and 38.
[c] References for SIVmac Vpx: 11, 15, 24, 27, 31, 35, 36, 38, 46, and 49.
[d] References for SIVagm Vpr: 38 and 42.

DISCUSSION

The present study demonstrates that the *vpr* gene of SIVagm shares many of the properties identified for the analogous HIV-1 gene. These properties include localization to the nucleus of infected cells, virion association, and a role in the transport of preintegration complexes into the nucleus of nondividing cells, such as macrophages. In addition to these functions, previous studies demonstrate that the SIVagm *vpr* gene arrests African green monkey (CV-1) but not human (HeLa) cells in the G_2 stage of the cell cycle (38). This latter function was confirmed to be preserved in the *vpr* allele of SIVagm9063-2 (42). As shown in Table 3, the *vpr* gene of SIVagm appears to perform functions similar to those of HIV-1 *vpr*. The significance of these in vitro functions for in vivo pathogenesis of either HIV-1 or SIVagm still requires investigation. However, studies with SIVmac239, in which the nuclear import and cell cycle arrest functions segregate between *vpx* and *vpr*, suggest that SIVmac can dispense with each of these functions alone and still retain some degree of virulence (14, 28). However, combined *vpr* and *vpx* mutants of SIVmac are severely attenuated in vivo (14). Lack of the cell cycle arrest function may have a less significant effect on pathogenesis since SIVmac239 derivatives with *vpr* deletions are still capable, albeit with slower kinetics, of inducing AIDS. In contrast, mutants that lack *vpx* are more attenuated.

While the majority of the functions of HIV-1 and SIVagm *vpr* appear to be well conserved, there are some minor differences between SIVagm and HIV-1 *vpr* mutants. First, while both HIV-1 and SIVagm *vpr* mutants were deficient for replication in macrophages, the defect in SIVagm mutants appeared to be more profound than that observed in HIV-1 *vpr* mutants. The inability of SIVagm *vpr* mutants to replicate in nondividing cells implies that this gene is sufficient to mediate nuclear transport of SIVagm. In contrast, a redundant nuclear localization function of the NLS of HIV-1 MA protein complements the activity of HIV-1 *vpr*. Thus, HIV-1 viruses with either a *vpr* or an NLS MA mutation replicate with impaired kinetics in macrophages but are still capable of inefficient replication (17). In contrast, mutants in which both the NLS of MA and the Vpr of HIV-1 are inactive are incapable of replication in macrophages (17), similar to single SIVagm mutants that lack Vpr. In preliminary attempts to identify the putative NLS (NGKKKY) within MA, SIVagm viruses with single or double amino acid substitutions of Thr for Lys were generated. The double mutant (NGKTTY) was biologically inactive in T-cell lines, whereas a virus with a single Lys-to-Thr substitution replicated with wild-type kinetics in PBMCs and macrophages, even in the context of a virus lacking *vpr* (data not shown). More extensive mutagenesis of the analogous signal in SIVsmPBj MA also suggests that this signal is not required for nuclear transport of SIVsm in nondividing cells (11). Clearly, more mutagenesis will be required to determine whether either SIVagm or SIVmac encodes a redundant NLS signal in MA, as observed in HIV-1.

A second difference between HIV-1 and SIVagm *vpr* mutants was the profound defect of SIVagm mutants for replication in macaque PBMCs. Due to a strong propensity for reversion in this cell type, the kinetics of viral replication of the mutants in PBMCs was difficult to document. However, based on analysis of RT products produced early after infection with *vpr* mutants (prior to reversion), the major block to replication in PBMCs appeared to be at the level of nuclear import, similar to our findings for macrophages. This finding suggests that the inability of the *vpr* mutants to replicate in PBMCs may be due, in part, to defects in nuclear import. While PBMCs are

generally considered to be dividing cells, their limited capacity for cell division and the asynchronous nature of PBMC cultures (few cells in mitosis at the time of infection) could impact the efficiency of nuclear transport and thus impair virus replication. In a representative experiment, we evaluated the cell cycle characteristics of these cultures; as expected of PHA–interleukin 2-stimulated PBMCs, approximately 30% of the cells were in the G_2-to-M phase of the cell cycle at the time of virus infection (data not shown). The high propensity for reversion of SIVagm Vpr mutants following replication in PBMCs is consistent with a low level of replication in PBMCs, and the lack of reversion in the macrophage, a cell incapable of cell division, is internally consistent with more a profound defect for replication in this cell type. In addition to a defect in nuclear import in PBMCs, a minor but significant reduction in *pol* product in these cells was consistent with an additional early block in the process of reverse transcription. Since the assay for reverse transcription products was only semiquantitative, it was not possible to definitively assess whether the decrease in production of circular DNA was proportional to the decreased levels of *pol* product (i.e., whether the decrease in circle production was a secondary effect of a decrease in reverse transcription). However, the consistently decreased levels of *pol* product in MDM infected with Vpr mutants suggest that there may be additional functions supplied by this gene that are required for efficient reverse transcription, possibly stabilization of the RT complex.

The inefficient replication of SIVagm Vpr mutants in PBMCs is reminiscent of reports of the replication of SIVmac and HIV-2 Vpx mutants in this cell type (16, 36, 49). In three separate studies, Vpx mutants of SIVmac (36, 49) or HIV-2 (16) showed profound defects in replication in PBMCs as well as in macrophages. In the study by Yu et al. (49), lack of Vpx expression in cell lysates of infected human PBMCs was consistent with stability of the introduced mutation; however, replication of these mutants in macaque PBMCs was not evaluated. In contrast, stop codon mutations in *vpx* were observed to revert even after replication in T-cell lines (36). These studies are consistent with a significant effect of Vpx upon viral replication in PBMCs as well as in macrophages, although the underlying mechanisms were not elucidated. However, in other studies, mutations in SIVmac Vpx (15) or HIV-2 Vpx (22) had minimal effects on replication in PBMCs. In a recent study, SIVsmPBj Vpx mutants were observed to have impaired replication in macrophage cultures but not in PBMCs (11). However, this particular virus may not be highly representative of SIV and HIV-2 Vpx function, since it induces cell activation and proliferation through the functions of a unique SH2 domain in the Nef protein (10). Therefore, the ability of this virus to replicate in PBMCs may be due to its ability to promote proliferation of lymphocytes. Overall, there appears to still be considerable controversy concerning the role of SIVmac, SIVsm, and HIV-2 Vpx in virus replication in PBMCs, which could be related in part to the use of different virus constructs and alleles of *vpx*.

In summary, SIVagm Vpr combines properties of both the Vpr and Vpx proteins of SIVmac and has all of the known functions of the HIV-1 Vpr protein. This is not surprising, given its genetic relatedness to both the SIVmac Vpr and Vpx proteins. The characteristics of the SIVagm Vpr protein seem to correlate more closely with the HIV-1 Vpr protein, and thus it would be more appropriate for the SIVagm Vpr-encoding gene to be classified as *vpr*. As also observed for SIVsmPBj, the SIVagm *vpr* gene is both necessary and sufficient for nuclear import of RT complexes in nondividing cells. The shared properties of the SIVagm and HIV-1 *vpr* genes and the pathogenic nature of SIVagm in pigtailed macaques provide an excellent model system to determine the role of these genes in viral pathogenesis and to dissect the relative contributions of the nuclear import and cell cycle arrest functions of Vpr.

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