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Importance of *vpr* for Infection of Rhesus Monkeys with Simian Immunodeficiency Virus

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The importance of the *vpr* gene for simian immunodeficiency virus (SIV) replication, persistence, and disease progression was examined by using the infectious pathogenic molecular clone called SIVmac239. The ATG start codon of the *vpr* gene was converted to TTG by site-specific mutagenesis. The constructed Vpr$^-$ mutant virus is identical with the parental SIVmac239/*nef*-stop virus with the exception of this one nucleotide. These viruses replicated with similar kinetics and to similar extents in rhesus monkey lymphocyte cultures and in the human CEMX174 cell line. Five rhesus monkeys were inoculated with the Vpr$^-$ variant of SIVmac239/*nef*-stop, and two monkeys received SIVmac239/*nef*-stop as controls. Both controls showed reversion of the TAA stop signal in *nef* by 2 weeks postinfection, as has been observed previously. Reversion of the TAA stop codon in *nef* also occurred in the five monkeys that received the Vpr$^-$ variant, but reversion was delayed on average to about 4 weeks. Thus, the mutation in *vpr* appeared to delay the rapidity with which reversion occurred in the *nef* gene. Reversion of the TTG sequence in *vpr* to ATG was observed in three of the five test animals. Reversion in *vpr* was first observed in these three animals 4 to 8 weeks postinfection. No *vpr* revertants were found over the entire 66 weeks of observation in the other two test animals that received the *vpr* mutant. Antibodies to *vpr* developed in those three animals in which reversion of *vpr* was documented, but antibodies to *vpr* were not observed in the two animals in which reversion of *vpr* was not detected. Antibody responses to *gag* and to whole virus antigens were of similar strength in all seven animals. Both control animals and two of the test animals in which *vpr* reverted maintained high virus loads and developed progressive disease. Low virus burden and no disease have been observed in the two animals in which *vpr* did not revert and in the one animal in which *vpr* reversion was first detected only at 8 weeks. The reversion of *vpr* in three of the five test animals indicates that there is significant selective pressure for functional forms of *vpr* in vivo. Furthermore, the results suggest that both *vpr* and *nef* are important for maximal SIV replication and persistence in vivo and for disease progression.

Human and simian immunodeficiency viruses (HIV and SIV), members of the lentivirus subfamily, have, in addition to the genes common to all retroviruses, a number of accessory genes (4, 15). Two of the respective gene products, Tat and Rev, are necessary for synthesis and cytoplasmatic targeting of viral mRNA (1, 31, 39). The Vif protein appears to be required for efficient infectivity of cell-free virus and viral spread in cell culture (12, 38, 43). Four genes, *vpr*, *vpx*, *vpu*, and *nef*, are termed nonessential, since they can be deleted without abolishing the ability of virus to replicate in standard cell culture systems (6, 16–18, 24, 26, 30, 34, 44, 47).

SIV parallels HIV in genomic organization and numerous biological properties (7), thus providing an appropriate model with which to study genetic determinants for the pathogenesis of AIDS (2, 8, 29). Molecularly cloned SIV strain 239 (SIVmac239), isolated from a rhesus monkey, was shown to cause AIDS in rhesus monkeys within a time suitable for studies of pathogenesis (22). By using this model, the importance of the *nef* gene for development of AIDS was convincingly demonstrated (24). SIVmac239 has a *nef* gene truncated by a single in-frame stop signal at the codon 93; selective pressure for an open, functional *nef* gene in vivo results in selection of reverted forms at this position. Animal studies with SIVmac239 carrying a deletion in *nef* showed that *nef* is required for efficient virus replication in vivo, for maintaining high virus loads in vivo, and for full pathogenic potential of the virus.

The *vpr* gene is present in HIV as well as in SIV isolates from sooty mangabeys (9, 19), mandrills (45), a chimpanzee (21), and various macaque species (23). Vpr proteins were shown to be virion associated (3, 47, 48). They are among the best-conserved proteins of immunodeficiency viruses (46) and are immunogenic in AIDS patients (36) as well as in SIVmac-infected rhesus monkeys (47). Although virus mutated in *vpr* replicates quite well in cell culture, the high conservation of *vpr* suggests that *vpr* gene products are likely to be important for some aspects of the virus life cycle. To address the question of the extent to which *vpr* is necessary in the natural life cycle of immunodeficiency viruses, we constructed a Vpr$^-$ mutant of SIVmac by converting the ATG start codon to TTG and infected five rhesus monkeys. Here we show that spontaneous reversion to a Vpr$^+$ phenotype does occur in at least some monkeys infected with *vpr* mutant virus. The results suggest that *vpr*

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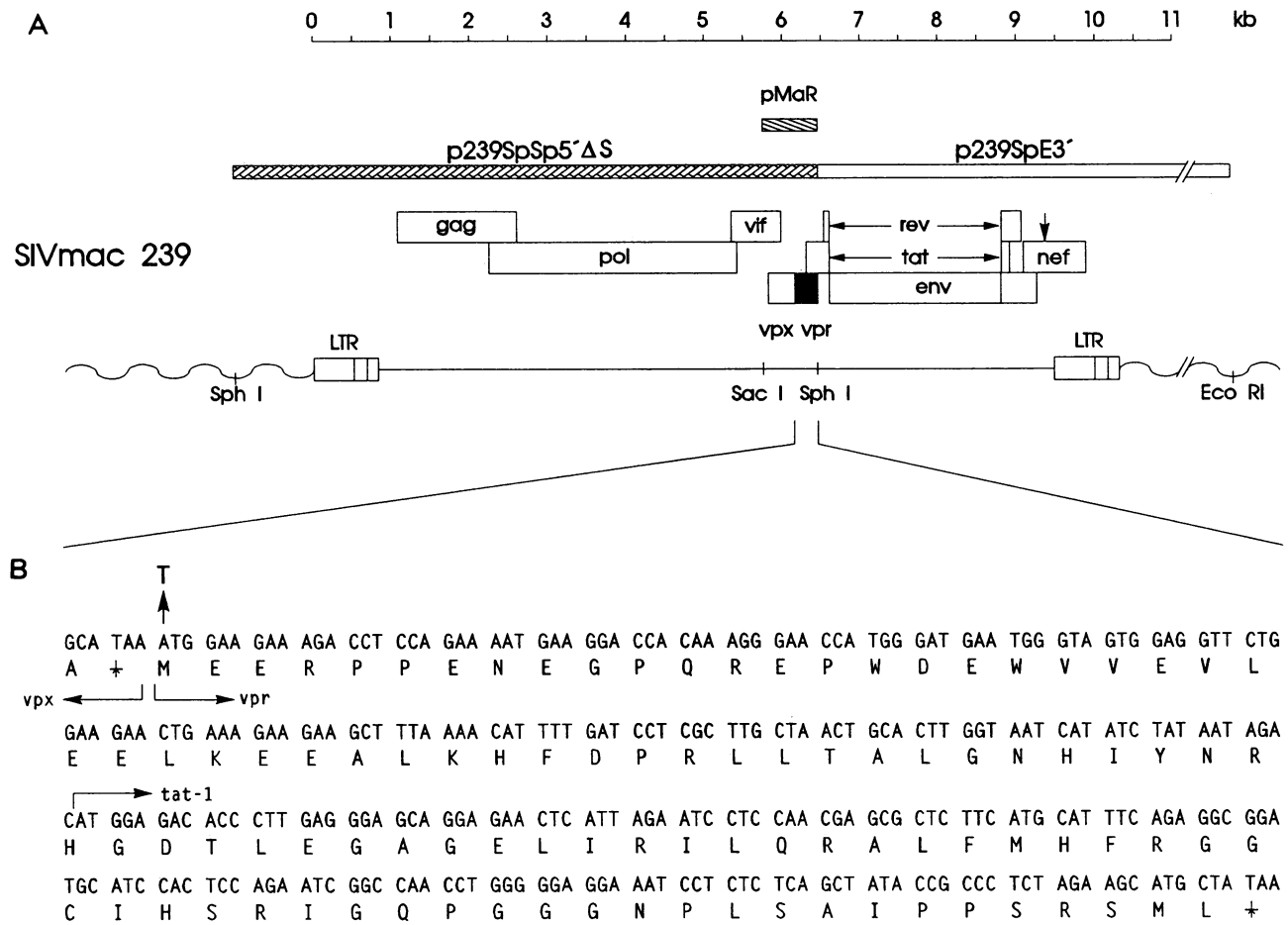

FIG. 1. Schematic representation of the *vpr* mutant of SIVmac239 used in this study. Positions of the premature in-frame stop codon in the *nef* gene (↓) and the *vpr* gene (■) are shown. (A) Extent of SIVmac239 sequences in plasmid subclones p239SpSp5′ΔS and p239SpE3′, determined by using *Sph*I and *Eco*RI restriction sites for cloning (22). *vpr* coding sequences were subcloned into plasmids of the pMa/c series (27), resulting in pMaR. Site-directed mutagenesis of the *vpr* gene was performed on plasmid pMaR, which contained a *Sac*I-*Sph*I fragment (positions 6011 to 6702 in SIVmac239). (B) Nucleotide sequence of the *vpr* open reading frame (positions 6401 to 6712 in SIVmac239) and predicted amino acid sequence. The *vpx* and *vpr* open reading frames and the first coding exon of *tat* are indicated. An arrow indicates the A→T transversion at the *vpr* start codon. Thus, the *vpr* mutant obtained is completely isogenic to the original SIVmac239 clone with the exception of the single nucleotide exchange.

contributes in an important way to SIV's ability to replicate and to establish a persistent high-level infection in rhesus monkeys.

MATERIALS AND METHODS

Plasmid construction and in vitro mutagenesis. The molecular clones of SIVmac239, p239SpSp5′ and p239SpE3′, represent nonoverlapping segments of the proviral DNA with a premature stop codon in *nef* (22) (Fig. 1). To remove duplicate recognition sites for *Sph*I and *Sac*I in the multiple cloning site, p239SpSp5′ was partially digested with *Sph*I and *Sac*I, and sticky ends were removed by T4 DNA polymerase treatment. Upon religation, plasmid p239SpSp5′ΔS was obtained. A 691-bp *Sac*I-*Sph*I fragment containing the *vpx* and *vpr* genes was cloned into plasmids of the pMa/c series, which are designed for site-directed mutagenesis (27). The ATG start codon of the *vpr* open reading frame was converted to TTG by using the oligonucleotide 5′-CCAGGACTAGCATAATTGGAAGAAAGACC-3′. The oligonucleotide is identical to the sense sequence except that the A residue at position 6407 was changed to a T (underlined nucleotide). The mutation was introduced into p239SpSp5′ΔS by substituting the viral 691-bp *Sac*I-*Sph*I fragment with the mutagenized fragment. Viral regulatory gene products were expressed as β-galactosidase fusion proteins, using the vector pROS in *Escherichia coli* for use on Western immunoblots (10); 375 N′-terminal residues of β-galactosidase were fused to Vpr (amino acids 11 to 101), Vpx (amino acids 3 to 110), Vif (amino acids 16 to 198), and Rev (amino acids 2 to 107).

Viruses, cell culture, and DNA transfection. Human T-cell lines HUT-78 and CEMX174 were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum and glutamine (300 μg/ml). Peripheral blood mononuclear cells (PBMC) were purified from citrated blood by banding over a sodium diatrizoate Ficoll gradient (Pharmacia, Freiburg, Germany). Isolated PBMC were stimulated with phytohemagglutinin (10 μg/ml) for 24 to 36 h, washed free of lectin, and maintained in RPMI 1640–25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-buffered medium supplemented with 10% heat-inactivated

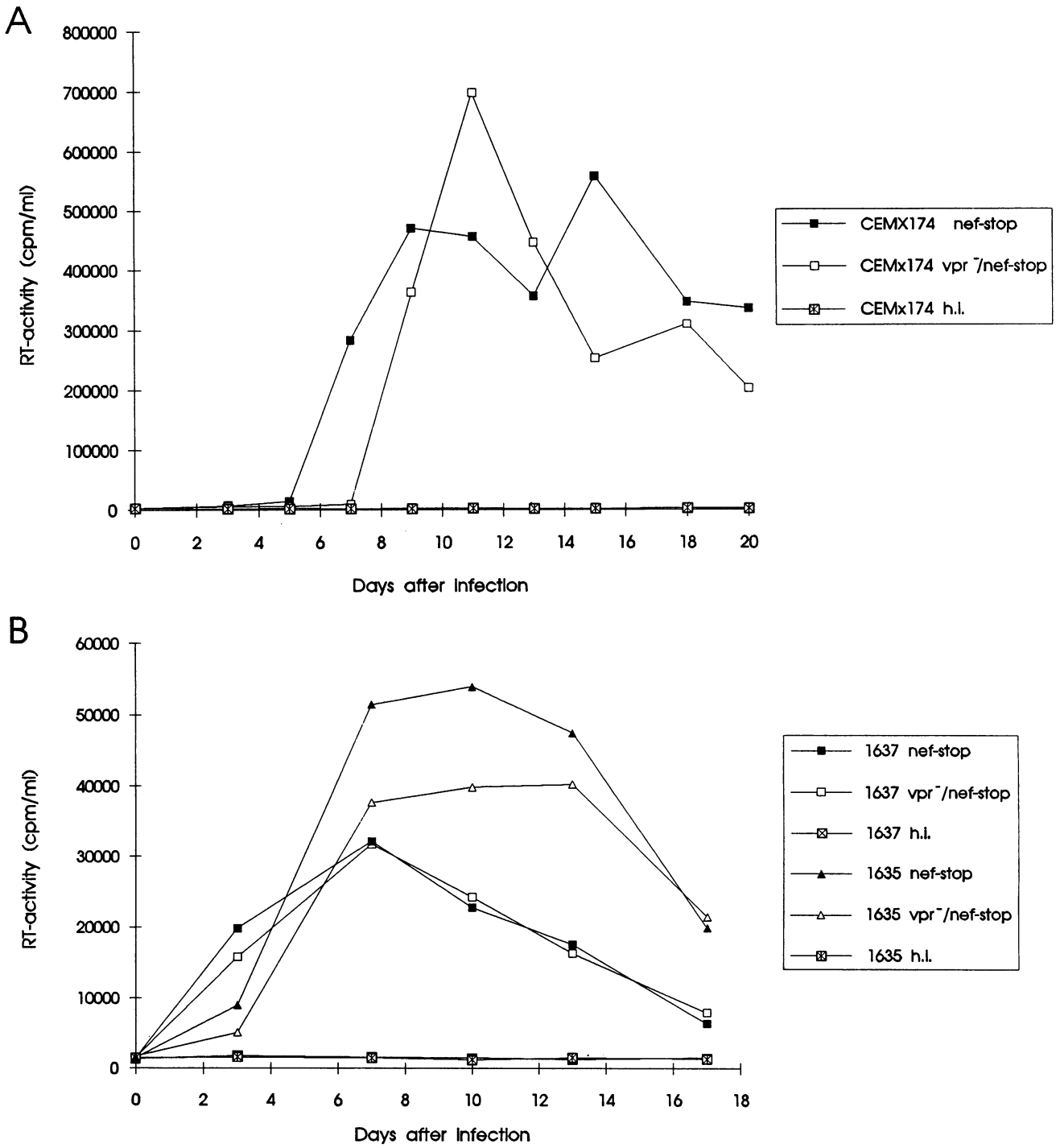

FIG. 2. Replication kinetics of SIVmac239/Vpr$^-$/*nef*-stop and SIVmac239/*nef*-stop in different cell culture systems. RT activity in culture supernatants (1 ml) was monitored (5). (A) Human cell line CEMX174 (MOI = 0.04); (B) macaque PBMC from two donors (MOI = 0.04 for donor 1637; MOI = 0.09 for donor 1635). As a negative control, heat-inactivated (h.i.) SIVmac239/*nef*-stop (30 min at 56°C) was used.

fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), glutamine (300 μg/ml), and interleukin-2 (100 U/ml; Eurocetus, Frankfurt, Germany). To generate biologically active SIV, plasmid clones of the 3′ half (p239SpE3′) and 5′ half (p239SpSp5′ΔS and the *vpr* mutant p239SpSp5′ΔSM32) of SIVmac239 were cleaved with *Sph*I and purified by phenol extraction. DNA of both 5′ clones was mixed with equimolar amounts of DNA derived from the 3′ clone and ligated. Ligated DNA (6 μg) was transfected into HUT-78 cells by using DEAE-dextran as described previously (32, 40). For virus stock preparation, uninfected HUT-78 cells were added to the transfected HUT-78 cultures at four times.

TABLE 1. Reversion of the mutagenized *vpr* start codon and of the *nef* stop codon in SIVmac239/Vpr$^-$/*nef*-stop- and SIVmac239/*nef*-stop-infected rhesus monkeys[a]

Wk post-infection	No. with sequence/no. tested													
	SIVmac239/Vpr$^-$/*nef*-stop infected										SIVmac239/*nef*-stop infected			
	Mm HR69		Mm HR70		Mm HR71		Mm HR72		Mm 44-86		Mm HR75		Mm HR76	
	vpr ATG/Σ	*nef* op/Σ	*vpr* ATG/Σ	*nef* op/Σ	*vpr* ATG/Σ	*nef* op/Σ	*vpr* ATG/Σ	*nef* op/Σ	*vpr* ATG/Σ	*nef* op/Σ	*vpr* ATG/Σ	*nef* op/Σ	*vpr* ATG/Σ	*nef* op/Σ
2	ND	0/6	ND	0/6	0/6	4/6	0/6	0/6	0/6	0/6	6/6	5/6	6/6	5/6
4	ND	6/6	ND	5/5	0/6	ND	2/6	6/6	0/6	6/6	ND	ND	ND	ND
6	0/6	ND	0/6	ND	1/6	ND	2/6	ND	ND	ND	ND	ND	ND	ND
8	ND	ND	ND	ND	4/6	ND	4/6	ND	1/6	ND	ND	ND	ND	ND
10	ND	ND	ND	ND	5/6	ND	5/6	ND	ND	ND	ND	ND	ND	ND
12	0/7	ND	0/6	ND	ND	ND	ND	ND	6/6	ND	ND	ND	ND	ND
14	ND	ND	ND	ND	6/6	ND	6/6	ND	ND	ND	5/5	ND	6/6	ND
16	0/3	6/6	0/6	6/6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
20	0/6	ND	0/5	ND	ND	ND	ND	ND	6/6[b]	ND	ND	ND	ND	ND
30	0/6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	—	
58	0/6	ND	0/6	ND	—		—		ND	ND	—			

[a] *vpr* and *nef* sequence data from reisolated viruses are summarized for animals Mm HR69, Mm HR70, Mm HR71, Mm HR72, Mm 44-86, Mm HR75, and Mm HR76 for the indicated weeks after infection. ATG/Σ, number of independent clones with *vpr* wild-type ATG start codon from total number of sequenced clones; op/Σ, number of independent clones with *nef* open reading frame from total number of sequenced clones; ND, not done; —, no longer available because animal had been euthanized.

[b] Sample obtained at week 22.

Culture supernatant was collected at day 70 after transfection and filtered (pore size, 0.2 μm), and aliquots were frozen. Virus stock titers were determined by limiting dilution as 10^4 50% tissue culture infective doses per ml, using CEMX174 cells. For in vitro infection studies, cells were incubated with virus for 2 h, washed two times, and subcultured as described above.

For virus recovery from infected macaques, PBMC were purified, stimulated as described above, and subsequently cocultured with CEMX174 cells or CD8$^+$-depleted human PBMC in a 1:1 ratio. For limiting-dilution coculture, the number of PBMC was determined by using a hemocytometer, and 10^6, 3×10^5, 10^5, 5×10^4, 2.5×10^4, and 10^4 cells were stimulated for 36 h and cocultured as described above.

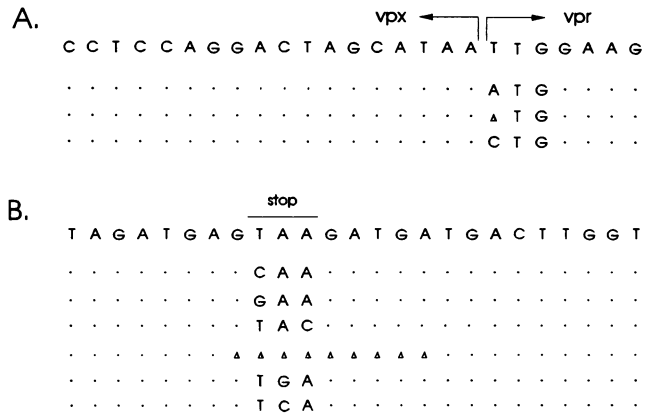

FIG. 3. Different mutations in Vpr$^+$ and *nef*-open revertant viruses isolated from SIVmac239/Vpr$^-$/nef-*stop*- and SIVmac239/*nef*-stop-infected rhesus monkeys. (A) SIVmac239/Vpr$^-$/*nef*-stop sequences (positions 6389–6413) together with sequences of reisolated viruses. The stop codon of *vpx* and the mutagenized start codon of *vpr* are indicated by arrows. (B) *nef* sequences (positions 9601 to 9625 of SIVmac239) with the premature stop codon. Sequences of *nef*-open virus variants obtained are shown below. △, deleted nucleotide.

Cocultures were maintained for 4 weeks; only cultures obtained from animals HR69 and HR70 16 weeks after infection were maintained for 6 weeks. Virus replication became apparent by syncytium formation and was confirmed by measuring the reverse transcriptase (RT) activity in cell-free culture supernatant (5) or the detection of p27 SIV core protein by an antigen capture assay (Coulter, Luton, England). Cocultures were regarded as virus negative if no RT activity or cytopathic changes were detected by week 4 or 6 (animals HR69 and HR70). At the time when virus replication was first detected, proviral DNA was extracted according to the protocol of Hirt (20).

Experimental infection of rhesus monkeys. Six rhesus monkeys (*Macaca mulatta*) of Indian origin, 2.5 to 3 years old, and one 6-year-old animal (Mm 44-86) were selected for this study. They were negative for SIV, as shown by antibody determination and polymerase chain reaction (PCR). Five rhesus monkeys were inoculated intravenously with SIVmac239/Vpr$^-$/*nef*-stop, and two monkeys inoculated with SIVmac239/*nef*-stop served as positive controls. Six animals received 1 ml of cell culture supernatant derived from transfected HUT-78 cells and containing 10^4 50% tissue culture infective doses of the respective virus; test animal Mm 44-86 was inoculated with supernatant from a short-term culture of transfected CEMX174 cells. Animal husbandry, handling of the monkeys, and collection of specimens have been described elsewhere (41). All inoculated animals were monitored at regular intervals by virus isolation from PBMC, semiquantitative PCR determination of SIV DNA in PBMC, quantitation of the *gag* protein p27 in plasma, and determination of SIVmac-specific antibodies.

Serology and neopterin determination. The humoral immune response of infected monkeys to either whole SIVmac antigen or viral polypeptides was determined by enzyme-linked immunosorbent assay (ELISA) and Western blotting as described previously (42), using pelleted whole SIVmac239 as antigen. To measure antibodies reactive to SIVmac regulatory proteins, Western blotting was performed, using purified recombinant β-galactosidase fusion proteins

TABLE 2. SIV recovery from rhesus monkeys inoculated with SIVmac239/Vpr$^-$/*nef*-stop or SIVmac239/*nef*-stop during the time course of infection by limiting-dilution coculture or qualitative coculture only

Wk post-infection	SIV recovery[a]					
	SIVmac239/Vpr$^-$/*nef*-stop infected				SIVmac239/*nef*-stop infected	
	Mm HR69	Mm HR70	Mm HR71	Mm HR72	Mm HR75	Mm HR76
2–14	+ (2.8×10^6–6.5×10^6)	+ (2.8×10^6–4.6×10^6)	+ (2.0×10^6–5.1×10^6)	+ (5.3×10^6–6.9×10^6)	+ (2.5×10^6–6.8×10^6)	+ (1.4×10^6–8.4×10^6)
16	+ (5×10^6)	+ (6×10^6)	+ (10^4)	+ (10^4)	+ (10^4)	+ (10^4)
20	+ (10^6)	+ (10^6)	+ (10^4)	+ (10^4)	+ (10^4)	+ (10^4)
24	+ (8×10^6)	− (3.5×10^6)	+ (10^4)	+ (10^4)	+ (10^4)	+ (10^4)
28	− (5.5×10^6)	− (3.5×10^6)	+ (10^4)	+ (10^4)	+ (10^4)	—[b]
32	+ (4.2×10^6)	− (2.6×10^6)	+ (10^4)	+ (10^4)	+ (10^4)	
40	− (7.1×10^6)	− (3.3×10^6)	+ (10^4)	—	+ (10^4)	
46	+ (20.3×10^6)	− (19.6×10^6)	+ (10^4)		+ (10^4)	
50	− (12.6×10^6)	− (4.4×10^6)	—		—	
58	+ (25.2×10^6)	+ (17.1×10^6)				

[a] Virus was recovered (+) or was not recovered (−); numbers in parentheses are numbers of PBMC used.
[b] —, death of the animal (Mm HR76, week 27 after infection; Mm HR72, week 40 after infection; Mm HR71, week 47 after infection; Mm HR75, week 49 after infection).

as antigen. Recombinant proteins were purified as described by Kiyoshi and Thorgersen (25).

Neopterin, known to be a nonspecific marker for immune activation (13, 14), was investigated in early-morning urine samples of the monkeys (11). Differences in the specific weight of the urine were corrected by means of creatinine concentrations. Neopterin concentrations obtained after inoculation were related to preinfection values and expressed as a percentage of the individual baseline levels.

PCR amplification and DNA sequencing. PCR was used to amplify *nef*- and *vpr*-spanning fragments from 1 μg of Hirt DNA (37). The sequences of the SIVmac-specific oligonucleotides used were derived from isolate 239. For amplification of *vpr* sequences, oligonucleotides SL14 (5′-ACCAGG TACCAAGCCTACAGTAC-3′, nucleotides 6023 to 6045, sense) and SL15 (5′-CCTTTTTCGAGCTCTTCTTCTC-3′, nucleotides 6822 to 6800, antisense) gave rise to an 800-bp fragment. Oligonucleotides SL16 (5′-GGATTAGACAAGG GCTTGAGCTCAC-3′, nucleotides 9465 to 9489, sense) and SL17 (5′-GTCCCTGCAGTTTCAGCGAGTTTCC-3′, nucleotides 10136 to 10112, antisense) were used to amplify a 672-bp *nef* fragment. Underlined nucleotides correspond to single base changes that were introduced into the oligonucleotides to create two restriction sites for cloning: a *Sac*I site in SL15 and a *Pst*I site in SL17. Oligonucleotides SL14 and SL16 contained naturally occurring restriction sites for *Asp* 718 and *Sac*I, respectively. PCRs were run separately for *vpr* and *nef* amplification for 30 cycles in a thermal cycler (first step, 94°C for 1 min; second step, 60°C for 2 min; third step, 72°C for 1 min; 2 s was added to the third step after each cycle, and 10 min at 72°C was added after the last cycle). Amplified DNA was precipitated, digested with restriction endonucleases, and cloned into pBS(−). Double-stranded DNA was sequenced by the dideoxy-chain termination method, using T7 DNA polymerase (Pharmacia) for polymerization. To determine *vpr* sequences, a SIVmac239-specific primer (5′-GCATTGCAAGAAAGGCTGTAG-3′, nucleotides 6310 to 6330, sense) was used.

For semiquantitative PCR, PBMC were purified as described above and total nucleic acid was extracted (49). SIV long terminal repeat (LTR) sequences were amplified together with β-globin sequences as an internal control. An SIV-specific fragment of 352 bp was obtained upon PCR using oligonucleotides SL3 (5′-TCGGGAACGCCCACTT TCTTGATGT-3′, nucleotides 720 to 744, U3 region, sense) and SL4 (5′-CTAGGGATTTTCCTGCTTCGGTTTC-3′, nucleotides 1072 to 1048, U5 region, antisense). Amplification with oligonucleotides SL10 (5′-ACACAACTGTGTTCAC TAGC-3′, sense) and SL11 (5′-CAACTTCATCCACGTT CACC-3′, antisense) gave rise to a 110-bp β-globin fragment (28, 49). Total nucleic acid (corresponding to 5×10^5 cell equivalents) was assayed by 30 cycles of semiquantitative PCR as follows. One hundred nanograms of 5′-end-^{32}P-labeled SL3 (usually 5×10^6 cpm) and 30 ng of 5′-end-^{32}P-labeled SL10 (about 2×10^6 cpm) were used together with 200 ng of unlabeled SL4 and 60 ng of unlabeled SL11 for each amplification reaction. The conditions for amplification were as follows: first step, 94°C for 1 min; second step, 60°C for 2 min; third step, 72°C for 30 s; 1 s added to the third step after each cycle, and 2 min at 72°C added after the last cycle. SIV DNA standards were run in parallel, using cloned SIVmac142 proviral DNA equivalent to 10, 100, 1,000, and 10,000 copies of SIV genomes. Various amounts of total nucleic acid isolated from human cord blood lymphocytes were used to standardize β-globin signals. Labeled PCR products were analyzed by electrophoresis on 5% polyacrylamide gels (1.5 mm, 300 V, 2 h) and exposed for autoradiography.

Nucleotide sequence accession number. The nucleotide sequence of the *vpr* open reading frame has been taken from the SIVmac239 proviral sequence from the GenBank data base (accession number M33262).

RESULTS

Construction and characterization of a *vpr* mutant of SIVmac239. The molecular clone of SIVmac239 was found to carry a stop signal (TAA) at codon 93 in the *nef* reading frame (35). This truncation of the *nef* gene has no relevance for in vitro growth properties of the virus in a number of standard cell culture systems, but the stop codon was shown to quickly revert into a coding triplet in infected animals (24). We used this clone to generate a *vpr* mutant as described in Materials and Methods. The translational start codon of *vpr* was changed by site-directed mutagenesis, converting the ATG to TTG (Fig. 1). In contrast to the 101 amino acids

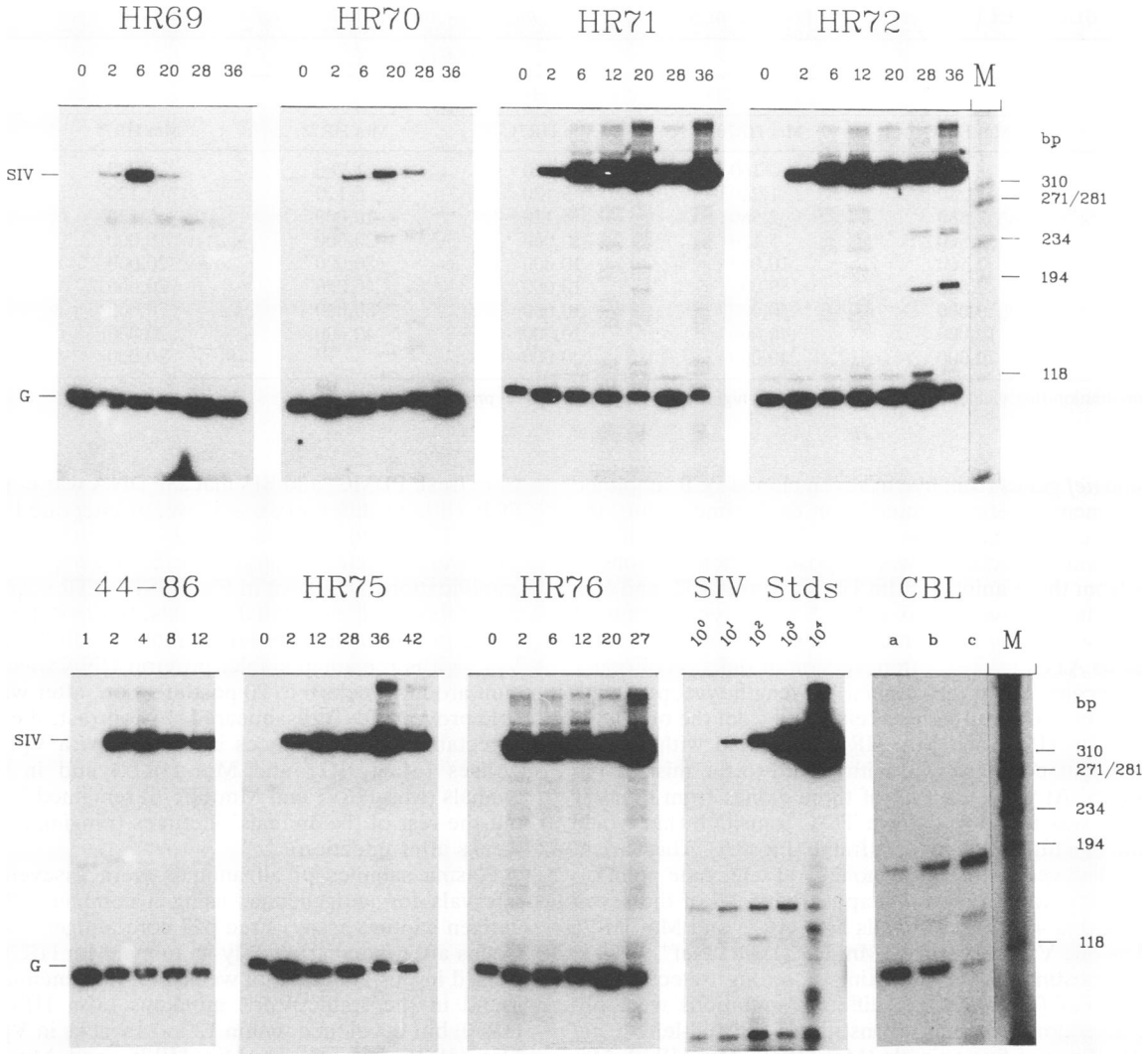

FIG. 4. PCR analysis of SIV-specific DNA in PBMC of SIVmac239/Vpr$^-$/*nef*-stop- and SIVmac239/*nef*-stop-infected rhesus monkeys. Animal designations and numbers indicating the week after infection when blood was obtained are shown above the lanes. SIV Stds, SIV DNA standards (equivalent to 0 to 10,000 copies) included to indicate the sensitivity of the PCR amplification; CBL, β-globin standards derived from 2 μg (a), 1 μg (b), and 0.02 μg (c) of total nucleic acid from human cord blood lymphocytes to allow comparison between different amplification reactions; M, DNA size marker; SIV, SIV LTR-specific amplification product (352 bp); G, β-globin-specific amplification product (110 bp).

encoded by the full-length *vpr* open reading frame, only 31 C-terminal amino acids could be expressed by initiation of translation at a downstream methionine at position 71 (Fig. 1). The 3′ clone of SIVmac239, p239SpE3′, was ligated either with the 5′ clone, p239SpSp5′ΔS, or its derivative, *vpr* mutant p239SpSp5′ΔSM32, to generate SIVmac239/*nef*-stop or SIVmac239/Vpr$^-$/*nef*-stop, respectively. Stability of the introduced mutation in cell culture was verified by PCR amplification of a *vpr*-spanning fragment from proviral DNA of SIVmac239/Vpr$^-$/*nef*-stop-producing cells, subsequent cloning, and sequencing of 50 independent plasmid clones. For infection studies in cell culture, equal multiplicities of infection (MOIs) were used for both viruses. Growth kinetics were analyzed in different T-cell lines, human cord blood lymphocytes, and rhesus monkey PBMC by measuring RT activity (Fig. 2). Both viruses displayed a pronounced cytopathic effect, with ballooning and multinucleated giant cells in human T-cell lines. Replication kinetics of the Vpr$^-$/*nef*-stop double mutant were similar to those of *nef*-stop virus in the human CEMX174 cell line and in rhesus monkey lymphocyte cultures (Fig. 2). The same replication kinetics were observed in CEMX174 cells when 10- or 100-fold-lower MOIs were used (data not shown). This result is consistent with an earlier report on the effect of SIVmac *vpr* (47).

Reversion of *vpr* and *nef*-stop mutation in rhesus monkeys. Five rhesus monkeys, Mm HR69, Mm HR70, Mm HR71, Mm HR72, and Mm 44-86, were inoculated intravenously with SIVmac239/Vpr$^-$/*nef*-stop; two monkeys, Mm HR75 and Mm HR76, received SIVmac239/*nef*-stop. Blood samples were collected at regular intervals following inoculation for virus recovery. All monkeys that received either type of virus, Vpr$^+$ or Vpr$^-$, were successfully infected; virus could be reisolated from all seven animals on multiple occasions up to at least 20 weeks postinfection. The nucleotide sequences

TABLE 3. ELISA titers in sera taken from SIVmac239/Vpr$^-$/*nef*-stop- and SIVmac239/*nef*-stop-infected rhesus monkeys

Wk post-infection	ELISA titer[a]					
	SIVmac239/Vpr$^-$/*nef*-stop infected				SIVmac239/*nef*-stop infected	
	Mm HR69	Mm HR70	Mm HR71	Mm HR72	Mm HR75	Mm HR76
4	160	160	320	1,280	1,280	160
8	1,280	1,280	5,120	5,120	5,120	5,120
12	2,560	2,560	5,120	10,000	5,120	5,120
16	2,560	5,120	10,000	20,000	10,000	20,000
20	2,560	10,000	10,000	20,000	20,000	10,000
27/28	2,560	40,000	10,000	40,000	20,000	10,000
32	10,000	40,000	10,000	80,000	20,000	
40	10,000	40,000	20,000	40,000	20,000	
47/48	20,000	40,000	20,000		20,000	

[a] Serum dilution that yielded an absorbance twofold-higher than that of sera taken prior to infection.

of *vpr* and *nef* genes from five to seven cloned PCR-amplified gene fragments were examined for each time point; the findings are summarized in Table 1. First signs of *vpr* reversion were detected by week 4 postinfection. Reisolated viruses from three animals, Mm HR71, Mm HR72, and Mm 44-86, gradually reverted to a Vpr$^+$ phenotype within 14 weeks. Consistently, the mutagenized codon TTG was changed to ATG, by T→A transversion or deletion of one T residue, reconstituting the original full-length *vpr* open reading frame (Fig. 3A). All viruses reisolated from the other two animals (Mm HR69 and Mm HR70), infected with the *vpr* mutant, remained unchanged with regard to the missing *vpr* start codon. At week 16, one of three clones from monkey Mm HR69 was found to carry a T→C transition that would not result in a functional reading frame (Fig. 3A). This variant was not observed in the same monkey at later time points.

nef reading frame revertants appeared in all of the seven animals within 4 weeks. Animals Mm HR75 and Mm HR76 as well as one Vpr$^+$ revertant (Mm HR71) had Nef$^+$ virus at 2 weeks postinfection, indicating a strong selection for full-length *nef* (Table 1). Six different mutations were observed at codon 93; four reconstituted the full-length *nef* (Fig. 3B). Four of five animals (Mm HR69, Mm HR70, Mm HR72, and Mm 44-86) inoculated with SIVmac239/Vpr$^-$/*nef*-stop showed *nef* revertants only after 4 weeks, suggesting that the *vpr* mutation may delay reversion in *nef* in the animals (Table 1).

Virus load in rhesus monkeys. Virus was readily recovered during the first 10 to 12 weeks postinfection from all seven rhesus monkeys, but often virus could not be reisolated from animals Mm HR69 and Mm HR70 (nonrevertants for *vpr*) after more than 20 weeks (Table 2). Therefore, we performed limiting-dilution cocultures to measure the level of SIV in PBMC of infected animals at weeks 16 to 32 after infection. In four rhesus monkeys, the two animals carrying Vpr$^+$ revertants and the two SIVmac239/*nef*-stop-inoculated animals, virus was usually isolated from 10^4 PBMC (Table 2). In contrast, animals Mm HR69 and Mm HR70, which had not reverted to Vpr$^+$ but carried a full-length *nef*, had far lower (at least by a factor of 100) virus loads. Vpr$^-$ virus could not be recovered consistently after 22 weeks, even if 10^6 to 10^7 PBMC were used for coculture. However, virus isolation from Mm HR70 was positive at week 58 postinfection after a long period of unsuccessful isolation attempts. This result indicates that even if no virus could be reisolated over a long period, the Vpr$^-$ virus can persist in rhesus monkeys.

To further quantitate viral DNA in PBMC of infected animals during persistence, total nucleic acid was prepared from fresh PBMC, and SIVmac239 DNA was determined by PCR without differentiating between integrated and unintegrated DNA (49). LTR sequences between positions 720 and 1072 were selected as targets for SIVmac-specific sequence amplification. As shown in Fig. 4, SIV LTR sequences were detectable in all inoculated animals, but to different extents. In the two animals (Mm HR69 and Mm HR70) in which the Vpr$^-$ virus remained stable, proviral DNA was most abundant around weeks 6 to 20 postinfection, after which detectable proviral DNA disappeared. In contrast, the amounts of detectable LTR sequences in animals with Vpr$^+$ revertant viruses (Mm HR71 and Mm HR72) and in the control animals (Mm HR75 and Mm HR76) remained high throughout the rest of the animals' lifetimes (ranging from 27 to 49 weeks after infection).

Plasma samples of all animals were assayed at regular intervals for antigenemia, using a commercially available antigen capture assay. Free p27 core antigen was seen at 2 weeks after inoculation only in animal Mm HR76 but disappeared in this animal until week 14. Antigenemia was never found in the stable Vpr$^-$ monkeys (Mm HR69 and Mm HR70) but developed within 12 to 24 weeks in Vpr$^+$ animals Mm HR71, Mm HR72, Mm HR75, and Mm HR76 and remained detectable until they were sacrificed.

Clinical alterations and pathology. The two monkeys infected with the stable *vpr* mutant virus developed a mild and transient lymphadenopathy early after infection but remained healthy throughout the entire observation period of more than 1 year (66 weeks). Three animals, the two controls infected with Vpr$^+$ virus (Mm HR75 and Mm HR76) and Mm HR71 (Vpr$^+$ revertant), developed a more pronounced and progressing lymphadenopathy and needed to be euthanized in extremis at different times after infection. Upon autopsy, animal Mm HR76 showed atrophy of the lymph nodes, diarrhea caused by *Giardia lamblia*, and cachexia. Pathological findings of Mm HR75 included enterocolitis due to microfilariasis and lymphadenopathy. Mm HR71 suffered from colitis due to microfilariasis and chronic ascites and died of *Pneumocystis carinii* pneumonia. One monkey with Vpr$^+$ reversion (Mm HR72), which showed persistent lymphadenopathy, had to be sacrificed because of an injury unrelated to AIDS.

Humoral immune response and urinary neopterin. Six animals, regardless of *vpr* reversion, developed about equal titers of antibodies as measured by ELISA using whole SIVmac as antigen after 28 weeks postinfection (Table 3). In animals carrying revertant Vpr$^+$ virus, antibody titers increased more rapidly in the first 16 weeks postinfection

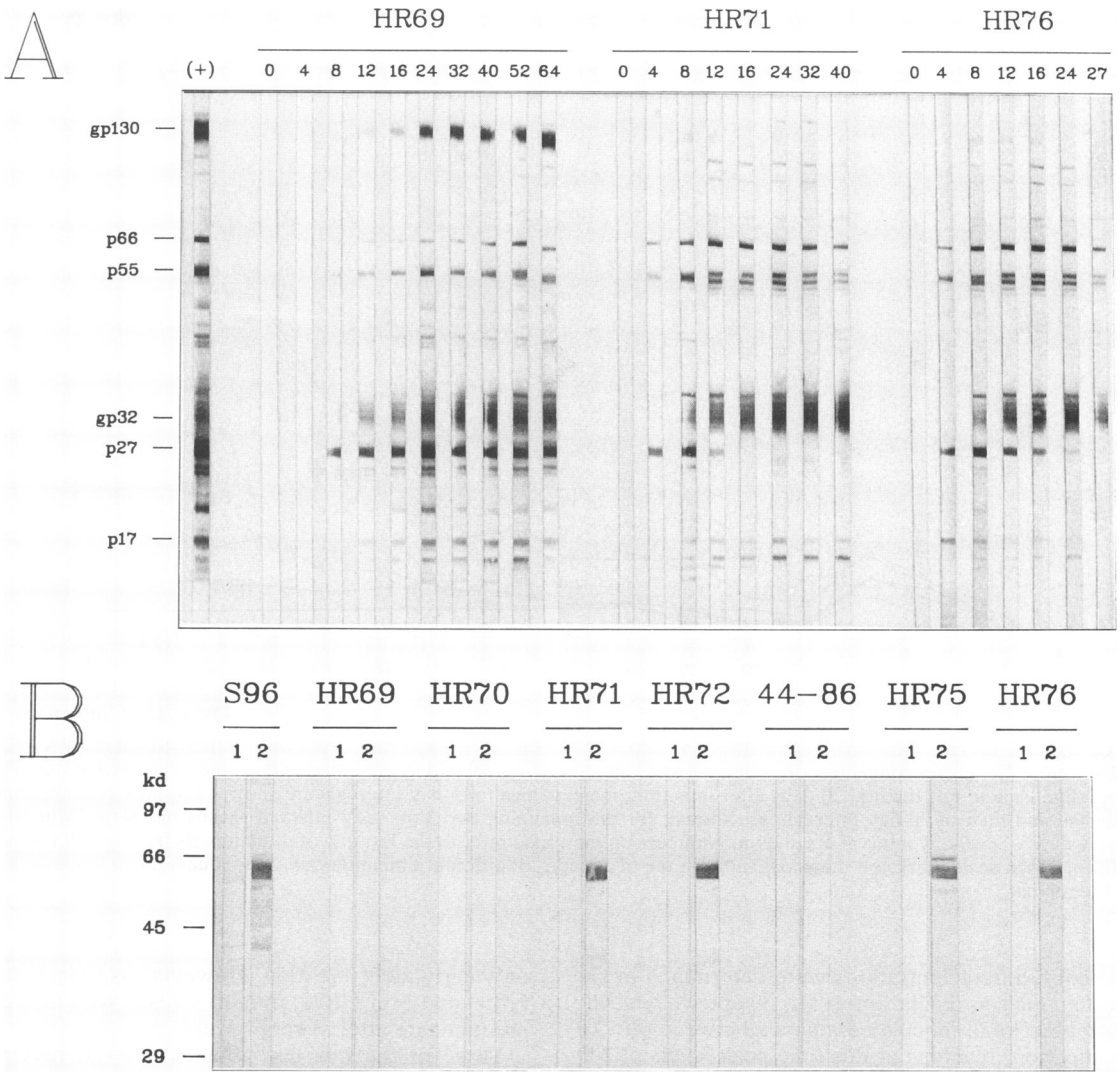

FIG. 5. Antibody response against SIVmac-specific polypeptides and recombinant Vpr protein in plasma from SIVmac239/Vpr$^-$/*nef*-stop- and SIVmac239/*nef*-stop-infected rhesus monkeys during the time course of infection by Western blotting. (A) Developed antibodies of one representative of each group (stably Vpr$^-$ [HR69], Vpr$^+$ revertant [HR71], and Vpr$^+$ control [HR76]) against structural proteins, using whole SIVmac239 as antigen. As a positive control (+), plasma from a rhesus monkey experimentally infected with SIVmac251 was included. To avoid overloading of the strips with antibodies, plasma samples obtained from week 0 to 24 were assayed in a 1:25 dilution; samples obtained thereafter were assayed in a 1:100 dilution. (B) Antibodies against the regulatory gene product Vpr. Lanes: 1, vector-encoded β-galactosidase (48 kDa); 2, Vpr–β-galactosidase fusion protein of SIVmac142 (58 kDa). Each lane contained 5 μg of purified protein. Plasma samples of infected rhesus monkeys were assayed in a 1:25 dilution. The earliest plasma samples that gave a positive result are shown for animals Mm HR71 (week 12), Mm HR72 (week 12), Mm 44-86 (week 12), Mm HR75 (week 24), and Mm HR76 (week 6). For animals Mm HR69 and Mm HR70, Western blots of plasma samples obtained at week 58 after infection are shown. As control, rabbit serum raised against recombinant Vpr protein was included (S96).

compared with stable *vpr* mutant-infected monkeys. Antibody responses against core proteins also seemed delayed in stable *vpr* mutant-infected rhesus monkeys when investigated by Western blotting. One representative of each group (Vpr$^-$, Vpr$^+$ revertant, and Vpr$^+$) is shown in Fig. 5A. However, later sera of these stable Vpr$^-$ virus-harboring monkeys showed a stronger response than did those of monkeys infected Vpr$^+$ revertants. Sera obtained from Mm HR69 and Mm HR70 exhibited a clear reaction of increasing intensity with gp130 and all major viral polypeptides during the time course of infection. In contrast, animals carrying Vpr$^+$ virus (Mm HR71, Mm HR72, Mm HR75, and Mm HR76) exhibited a progressive decline of antibodies against p27, with a complete loss of these antibodies by the time of sacrifice.

Monkeys in which Vpr$^+$ revertants appeared developed antibodies against Vpr protein, as measured by Western blotting with prokaryotically expressed Vpr antigen (Fig. 5B). No anti-Vpr antibodies were observed in these two animals (Mm HR69 and Mm HR70) in which no *vpr* reversion was observed (Fig. 5B). All rhesus monkeys developed antibodies against recombinant *vpx* and *rev*, and animals Mm HR71, Mm HR72, Mm HR75, and Mm HR76 seroconverted against *vif* (data not shown).

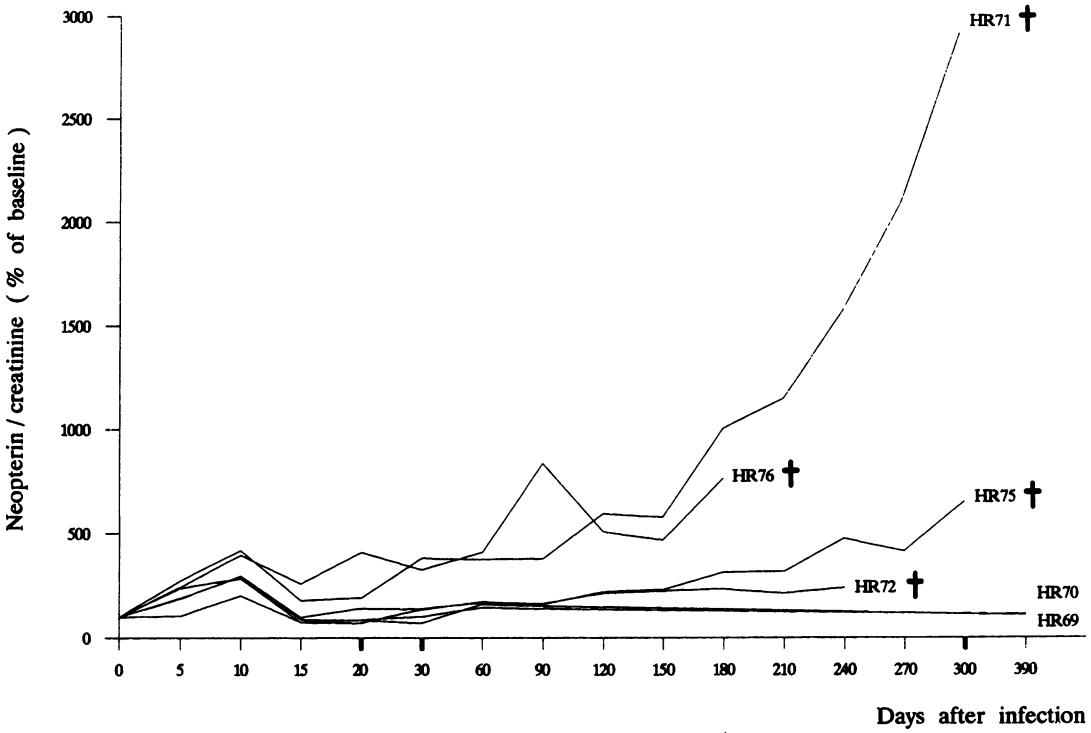

FIG. 6. Course of urinary neopterin concentrations after infection of rhesus monkeys with either SIVmac239/Vpr$^-$/*nef*-stop or SIVmac239/*nef*-stop. Neopterin concentrations obtained after infection were related to preinfection data and expressed as a percentage of the individual baseline values. For the first 20 days after infection, mean values for 5-day intervals are shown; for days 21 to 30, mean values of a 10-day interval and then of 30-day intervals are shown; for the remaining two Vpr$^-$ SIV-infected rhesus monkeys (Mm HR69 and Mm HR70), mean values of a 90-day interval are shown. Numbers on the *x* axis refer to the last day of the intervals from which mean values were calculated from. Thick scale marks on the *x* axis indicate the beginnings of different time intervals. Individual animal numbers are indicated. †, death of the animal.

Urinary neopterin concentrations were determined in six of seven test animals at regular intervals. Neopterin concentrations increased in all inoculated animals between 6 to 10 days after infection, reaching at this time peak levels of 200 to 400% above the baseline level (Fig. 6). After this moderate peak, the neopterin concentrations in the two monkeys stably infected with the *vpr* mutant virus rapidly declined to baseline values. In contrast, neopterin concentrations increased constantly in the two control animals and in animals Mm HR71 and Mm HR72, in which *vpr* revertants were observed.

DISCUSSION

Previous studies on HIV and SIV have shown that neither *vpr* (6, 18, 34, 47) nor *nef* is necessary for viral replication in a variety of T-cell lines and primary PBMC (17, 24). We have found that simultaneous mutation of *vpr* and *nef* also does not impair replication of SIV appreciably under standard cell culture conditions. No reversion of the mutagenized *vpr* initiator codon or *nef* stop codon was observed in long-term cultures of our Vpr$^-$/*nef*-stop virus (up to 70 days). In contrast, a strong selective pressure for a functional *nef* gene was observed in rhesus monkeys, resulting in *nef* reversion within 2 weeks in animals infected with SIVmac239/*nef*-stop (24). The pattern of *nef* reversion that we found in the current study is similar to the changes seen previously (24). The TAA of *nef* codon 93 was converted to GAA, CAA, TCA, or TAC; a deletion of codons 92 to 94 was observed only early after infection. However, as seen in animals Mm HR69 and Mm HR70, mutant viruses with GAA seemed to predominate after several weeks.

Three of the five test animals showed reversion in *vpr* beginning 4 to 8 weeks after infection. The *vpr* revertants gradually overgrew the *vpr* mutant and eventually predominated. The reversion to wild-type *vpr* in these three animals provides strong evidence for an important role for *vpr* in the replication or persistence of SIV in rhesus monkeys. Although reversion in *vpr* was delayed relative to reversion in *nef* and two of the monkeys showed no evidence for reversion in *vpr*, we cannot conclude at this time that the selective pressure for *vpr* reversion is weaker than that for *nef* reversion. There are at least seven ways in which the TAA stop codon in *nef* can revert by single point mutation. There is only one way that the *vpr* mutation can revert to create an appropriate *vpr* reading frame. Thus, slower and variable reversion of *vpr* in these experiments may be due at least in part to these statistical considerations.

The two control animals that received SIVmac239/*nef*-stop showed nearly complete reversion in *nef* by 2 weeks after infection. Similarly, Kestler et al. observed complete reversion in *nef* by 2 weeks after infection in the one animal examined at this early time (24). In contrast, four of the five rhesus monkeys infected with Vpr$^-$/*nef*-stop double mutant in this study showed no evidence for reversion of *nef* at 2 weeks, but reversion in *nef* was observed at 4 weeks. These results suggest that mutation in *vpr* may delay the appearance of *nef* revertants. Such a delay would be expected if

mutation of *vpr* slowed virus replication during these early weeks, since the appearance of reversional mutations is dependent on replication of the virus.

The persistence of a mutated *vpr* gene in the context of this experiment seems to have had a dramatic effect on the levels of persisting virus. The two monkeys in which *vpr* did not revert showed dramatic declines in the level of persisting virus, and these monkeys developed no significant signs of disease during 66 weeks of observation. As in previous studies (24, 33), the level of persisting virus correlated with disease progression. We cannot be certain at this time, however, that a nonfunctional *vpr* gene is directly responsible for the low virus loads and absence of disease progression in Mm HR69 and Mm HR70. The *vpr* gene also reverted in Mm 44-86, but this animal has maintained very low virus loads and has shown no signs of disease progression. The rapidity of *nef* reversion could conceivably play a key role in the eventual outcome. In view of the relative small number of animals used in this study, additional experiments with a different study design will be needed to clarify the role of *vpr* in maintaining high virus loads and in facilitating disease progression. In any event, our experiments provide evidence for fundamental effects of *vpr* on the biology of SIV in rhesus monkeys.

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