

The Evolutionary Rate of Nonpathogenic Simian Immunodeficiency Virus (SIVagm) Is in Agreement with a Rapid and Continuous Replication *in Vivo*

MICHAELA C. MÜLLER-TRUTWIN,* SYLVIE CORBET,* MARISA DIAS TAVARES,*† VINCENT M. A. HERVÉ,‡
ERIC NERRIENET,*¹ MARIE-CLAUDE GEORGES-COURBOT,‡¹ WILLIAM SAURIN,§
PIERRE SONIGO,|| and FRANÇOISE BARRÉ-SINOUSI*²

*Unité de Biologie des Retrovirus, Institut Pasteur, Paris, France; †Institut Pasteur, Bangui, Central African Republic; §Unité de Programmation Moléculaire, CNRS UAD1444, Institut Pasteur, Paris, France; ||Génétique des Virus, ICGM-CNRS UPR0415, Institut Cochin de Génétique Moléculaire, Paris, France; and †Universitary Hospital CFF, UFRJ, Brazil

Received February 23, 1996; accepted June 28, 1996

African green monkeys (AGMs) are divided into four species (*Cercopithecus aethiops*, *C. pygerythrus*, *C. sabaesus*, *C. tantalus*), each harboring a species-specific simian immunodeficiency virus (SIVagm). Little is known about the host and/or viral factors that are responsible for the apathogenicity of SIVagm infections in their natural hosts. In order to analyze the specific selective pressures exerted by the host on the virus *in vivo*, we compared the genetic evolution of SIVagm.tan in its natural host (*C. tantalus*) and in a foreign host species (*Erythrocebus patas*), in which we could obtain a reproducible and persistent infection by SIVagm.tan. As in AGMs, patas monkeys do not develop any disease following SIVagm infection. Our longitudinal study in *env* (V3-C3-V4-C4-V5) of SIVagm.tan from three AGMs and three patas monkeys revealed a high ratio of synonymous to nonsynonymous mutation frequencies (1.5–6.2). These data indicate that the selective pressures for stability exerted by AGMs and patas monkeys on SIVagm override positive selection for change reported in pathogenic HIV-1 infections. The rapid accumulation of mutations observed in AGMs and patas monkeys ($0.4\text{--}7.2 \times 10^{-2}$ nucleotide substitutions per site per year) suggests a continuous replication of SIVagm viruses *in vivo*. We thus propose that nonpathogenic SIVagm infections are the result of a long-term selection of SIVagm variants whose dissemination can be controlled in the host, rather than being explained by a low ability of the virus to replicate *in vivo*. © 1996 Academic Press, Inc.

INTRODUCTION

African monkeys can be naturally infected by simian immunodeficiency viruses (SIV). These viruses were named according to the species from which they were isolated. The lentiviruses SIVsm, SIVmnd, SIVcpz, SIVsyk, SIVtal, and SIVagm have thus been respectively obtained from sooty mangabeys (*Cercocebus atys*), mandrills (*Papio sphinx*), chimpanzees (*Pan troglodytes*), sykes monkeys (*Cercopithecus mitis*), talapoin monkeys (*Miopithecus talapoin*), and African green monkeys (*Cercopithecus aethiops*) (Bosch *et al.*, 1994; Emau *et al.*, 1991; Fukasawa *et al.*, 1988; Fultz *et al.*, 1986; Lowenstine *et al.*, 1986; Peeters *et al.*, 1989; Tsujimoto *et al.*, 1988). The African green monkey (AGM) superspecies is further subdivided into four species, the vervet (*Cercopithecus pygerythrus*), grivet (*Cercopithecus aethiops*), sabaesus (*Cercopithecus sabaesus*), and tantalus monkey (*Cercopithecus tantalus*) (Lernould, 1988). Each of those harbors a species-specific SIVagm subtype, respectively named

SIVagm.ver, SIVagm.gri, SIVagm.sab, and SIVagm.tan (Allan *et al.*, 1990, 1991; Fomsgaard *et al.*, 1991; Hirsch *et al.*, 1993; Jin *et al.*, 1994a; Müller *et al.*, 1993). These viruses seem to have coevolved with their natural host species and may represent the oldest primate lentiviruses (Allan *et al.*, 1991; Johnson *et al.*, 1990; Muller *et al.*, 1993). In addition, SIVs genetically related to SIVagm viruses were isolated from three other African monkey species, the white-crowned mangabey (*Cercocebus torquatus lunulatus*), the yellow baboon (*Papio hamadryas cynocephalus*) (Jin *et al.*, 1994b; Tomonaga *et al.*, 1993), and the patas monkey (*Erythrocebus patas*) (Bibollet-Ruche *et al.*, 1996). The SIV infections in the yellow baboon and the patas monkey probably result from simian-to-simian cross-species transmissions *in natura* of SIVagm.ver and SIVagm.sab, respectively. In contrast, New World and Asian monkeys are not infected by SIV in the wild (Hendry *et al.*, 1986; Lowenstine *et al.*, 1986; Ohta *et al.*, 1988). Phylogenetic studies indicated that SIVmac resulted from SIVsm cross-species transmission between sooty mangabeys and macaques in captivity approximately 30 years ago (Hirsch *et al.*, 1989).

Persistent SIV infections in naturally infected African nonhuman primates are not associated with clinical signs of immunodeficiency, in contrast to HIV or SIV infections in humans or macaques, respectively. The rea-

¹ Present address: Centre International de Recherches Médicales, Franceville, Gabon.

² To whom reprint requests should be addressed at Unité de Biologie des Retrovirus, Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris, Cedex 15, France. Fax: 33 1 45 68 89 57.

sons for the lack of pathogenicity of SIVs in their natural hosts in Africa are far from being fully understood. It was shown that experimental passages of SIVsm or SIVagm.ver into an Asian nonhuman primate species, the pig-tailed macaque (*Macacca nemestrina*), can result in an immunodeficiency syndrome similar to human AIDS (Fultz *et al.*, 1989; Hirsch *et al.*, 1995). These viruses are therefore not nonpathogenic per se, but can induce disease in a specific foreign species. Recent studies strongly indicate that pathogenicity correlates with the extent of viral load *in vivo*, both in humans (Cao *et al.*, 1995; Piatak *et al.*, 1993) and in monkeys (Hirsch *et al.*, 1995; Marthas *et al.*, 1989). Viral burden depends both on the ability of the virus to multiply in the host and on the capacity of the host to control the viral infection. According to the error-prone replication mechanism of retroviruses, the accumulation of mutations in the viral genome is related to the number of replication cycles (Temin, 1993). A low level of viral replication in the natural host should therefore be characterized by a slow accumulation of mutations.

Only few comparative data are available on the genetic evolution of nonpathogenic primate lentiviruses in their natural hosts and after experimental cross-species transmissions into a foreign monkey species (Cournaud *et al.*, 1992; Fomsgaard *et al.*, 1993). We report here such comparative data for the SIVagm.tan in its natural host, i.e., the AGM from the *tantalus* species, and in a distinct simian species from Africa, the patas monkey. The patas monkey apparently does not carry any patas-specific SIV in the wild (Bibollet-Ruche *et al.*, 1996; Hervé *et al.*, 1992; Lowenstine *et al.*, 1986), but we show that it can be reproducibly and persistently infected by SIVagm. Since the patas monkey is not the natural environment of SIVagm, we speculate that the selective pressures exerted on the virus in this foreign host might be different from that in AGMs, to which it is adapted. In order to test this hypothesis, we focused our comparative analysis on one *env* region, which seems to be submitted to a high positive selection for change during pathogenic primate lentivirus infections (Balfe *et al.*, 1990; Burns and Desrosiers, 1991; Myers *et al.*, 1994).

MATERIALS AND METHODS

Animals

AGMs (*C. tantalus*) and patas monkeys (*E. patas*) were wild caught in the Central African Republic (C.A.R.) and held in animal facilities at the Pasteur Institute in Bangui. One of the AGMs (AGMB14) was naturally infected by SIVagm.tanB14 as previously described (Müller *et al.*, 1993). All the other animals were SIV-seronegative as assessed by Western blot analysis (New Lav Blot I, New Lav Blot II, Diagnostic Pasteur) with 0.2 ml of serum. The naturally infected AGM was approximately 3 years old when he was captured and obviously no information

about the date of his exposure to the virus in the wild is available.

Experimental inoculation of AGMs and patas monkeys with SIVagm.tan

Two AGMs and four patas monkeys were inoculated intravenously with SIVagm.tanB14 infectious material obtained from the naturally infected AGMB14, from one experimentally infected AGM, or from one experimentally infected patas monkey (Table 1). The experimental infections were done as follows. Two milliliters of plasma and 10^6 PBMC from the naturally infected AGMB14, collected 5 months after its capture, were inoculated into a patas monkey (PAT5). Nine months later, 5 ml of blood and spleen cells from PAT5 were injected into a second patas monkey (PAT40). A third patas monkey (PAT63) was inoculated with 10^6 PBMC which were previously infected *in vitro* with a SIVagm.tanB14 supernatant. This supernatant resulted from a short-term *in vitro* culture of AGMB14 PBMCs obtained 1 year after its capture. A fourth patas monkey (PAT87) and one AGM (AGMB86) each received 2 ml of plasma collected from the AGMB14 three years after its capture. Two months later, 2 ml of plasma and 10^6 PBMC from AGMB86 were inoculated into a second AGM (AGMB87). The serological status of the animals was regularly followed by commercialized HIV-2 Western blots (New Lav Blot II, Diagnostic Pasteur), according to the cross-reactivities between SIVagm.tan and HIV-2 antigens (Müller *et al.*, 1993).

Virus isolations

Blood samples were regularly collected from each animal. The PBMCs were isolated on Ficoll-Hypaque gradients and stimulated for 3 days with staphylococcal enterotoxin A (SEA) at a concentration of 0.5 μ g/ml. The cells were then maintained in the presence of Interleukin-2 and the supernatants monitored twice weekly for reverse transcriptase activity (Barré-Sinoussi *et al.*, 1983). The isolation of SIVagm.tanB14 from the naturally infected animal was previously reported (Müller *et al.*, 1993).

DNA extraction and PCR amplification

Since the number of PBMCs carrying proviral DNA seemed to be low (unpublished observations), the cells were cultured *in vitro* for 6 days to increase the number of SIV-infected cells before DNA extraction. For the longitudinal study of SIVagm in the naturally infected AGMB14, we analyzed the proviral DNA obtained 1, 3, and 4 years after the capture of the animal. For all experimentally infected animals, at least one time point between seroconversion and 6 months p.i. was analyzed. The later time points studied were 1½ years p.i. for AGMB86, AGMB87, and PAT63 and 3–3½ years p.i. for AGMB87

TABLE 1

Follow-up of SIVagm-Infected Tantalus and Patas Monkeys

Animal	Source of SIV inoculum ^a	Follow-up (years p.i.)	Seroconversion	Persistent infection ^b	Proviral DNA ^c
AGMB14	Natural infection	4	+	+	+
AGMB86	AGMB14(B)	1,5	+	+	+
PAT5	AGMB14	1,5	+	+	nd
PAT63	AGMB14(A)	1,5	+	+	+
PAT87	AGMB14(B)	1,5	+	+	+
AGMB87	AGMB86(A)	3,5	+	+	+
PAT40	PAT5	3,5	+	+	+

^a The inocula specified by an (A) or (B) designate biological material from which corresponding viral isolates had been obtained for the genetic study.

^b The presence of a persistent infection was assessed regularly by virus isolation from monkey PBMCs.

^c Proviral DNA was repeatedly detected in monkey PBMCs by nested PCR during the follow-up. nd, not determined.

and PAT40. The first, second, and third samples of each animal were called respectively, A, B, and C. The patas PAT5 died with symptoms unrelated to AIDS 9 months after SIVagm inoculation and no PBMCs from this animal were available for the longitudinal study. The PBMCs were lysed and genomic DNA was extracted in a separate room using standard techniques.

One microgram of DNA was used for nested PCRs with first NS3s (TG TAGGAG ACCAGGAAACAAGACA)/NS3as (AAGCCTAAGAACCCTAGCACAAA) as outer primer pair and S1 (CAACAGTCCGACCCAGTAACCATC-ATGGC)/S4 (TCTGTTGGAGTCCGACCCTATTGGT) as inner primer pair. The outer primers correspond to HIV2/SIVmac/SIVagm consensus sequences and the inner primers to SIVagm.tan consensus sequences. The inner primers span the nucleotides from positions 796 to 1436 within the *env* gene of SIVagm.tanB14. The underlined nucleotides indicate terminal *SalI* restriction sites which were used for subsequent cloning of PCR amplified fragments. Cycling parameters of the PCR were as follows: 35 cycles at 94° for 1 min (3 min in the first cycle), 55° for 1 min, and 72° for 1 min (5 min for the last cycle). PCRs were performed under standard buffer conditions (Müller *et al.*, 1993). One to 5 µl from the first-round reaction was used for the second-round PCRs. Products from repeated PCR reactions with a same DNA sample were pooled before cloning. In addition, products from three repeated PCRs on PBMCs of three animals (AGMB14, AGMB87, and PAT63) were not pooled, but were independently cloned and sequenced in order to evaluate the eventual variations related to PCRs.

Cloning of PCR products

PCR products were extracted with chloroform, ethanol precipitated, and then digested with *SalI*. *SalI* restriction sites were chosen because they are absent in all known SIVagm.tan sequences of the selected *env* region. The DNA fragments were then separated on low-melting-

point agarose gels and purified with centrifuge filters (Spin-X, Costar). Fragments of the expected size, but also fragments of a smaller size, were purified because of possible *SalI* restriction sites in not yet identified viral variants. The fragments were then ligated to *SalI*-digested, dephosphorylated pUC18 vectors. After transformation of competent JM109 *Escherichia coli* cells, plasmids were purified on anion-exchange columns (Qiagen Plasmid Purification, Qiagen).

Heteroduplex mobility assays (HMA)

HMA (Delwart *et al.*, 1993) was performed on two inocula that were available and on two sequential viral isolates from each of the respective recipient animals (AGM87 and PAT63). Five independent PCR reactions were first performed for each viral sample to be analyzed according to the PCR conditions described above. Then, 250 ng of each nested PCR product was denatured in an annealing buffer (0.1 M NaCl, 10 mM Tris-HCl, pH 7.8, 2 mM EDTA) at 94° for 2 min. In addition, 125 ng of nested PCR products corresponding to two different viral samples was mixed before denaturation. Heteroduplexes were obtained by reannealing PCR-amplified sequences through cooling on wet ice. Heteroduplex mobility was analyzed on a neutral, 6% polyacrylamide gel (250 V for 3 hr). Signals were visualized by staining with ethidium bromide.

Sequence determination and analysis

Double-stranded plasmid DNA was denatured with alkaline and sequenced by the dideoxynucleotide chain termination method (Sequenase 2.0 Sequencing kit, U.S.B.). Both sense and antisense sequences were determined using forward and reverse pUC-sequencing primers as well as the inner primers AE5s (AGACAATGGGAGACCCAG) and MMas (TTCAGATAATTTAGAAAC-CAATC).

TABLE 2
SIVagm Intraisolate Variability

Viral isolate ^a	time p.i. (years)	Mean divergence (%) ^b	
		Nucleotides	Amino acids
AGMB14(A)	>1	1.1	4.8
AGMB14(B)	>3	2.5	4.0
AGMB14(C)	>4	2.5	4.6
AGMB86(A)	<0.5 (8 weeks)	2.3	1.7
AGMB86(B)	1.5	0.2	0.4
AGMB87(A)	<0.5	2.0	3.9
AGMB87(B)	1.5	1.6	2.8
AGMB87(C)	3	1.9	2.3
PAT40(A)	<0.5 (6 weeks)	1.1	1.9
PAT40(B)	3.5	1.2	1.9
PAT63(A)	<0.5	0.5	0.9
PAT63(B)	1.5	1.2	1.1
PAT87(A)	<0.5 (4 weeks)	1.1	1.3
PAT87(B)	<0.5 (15 weeks)	0.5	0.7

^a (A), (B), and (C) refer respectively to the first, second, or third isolate.

^b Mean intraisolate divergences were determined between the variants identified in sequential isolates from each animal. Only the pairs of sequences which are related in the minimal trees that were previously established for each individual isolate are considered.

Sequences were aligned with CLUSTAL (Higgins *et al.*, 1992) and corrected manually. Phylogenetic trees were performed by using the PHYLIP package (Felsenstein, 1990).

The minimal trees were constructed with a program using Prim's algorithm (Prim, 1957). For this, spanning trees are first constructed in which pairs of sequences are directly connected to each other and not through a theoretical ancestor as in classical phylogenetic trees. These trees are then evaluated by the sum of nucleotide substitutions between pairs of connected sequences. The minimal tree corresponds to the spanning tree showing the lowest sum of substitutions. Only the distances between the connected sequences in the minimal tree are used for the determination of the genetic variability. This method avoids a pairwise comparison of all sequences present in the isolates and which are not necessarily phylogenetically related. This method also excludes the possibility of counting the same mutation more than once. It therefore avoids an overestimation of the genetic variability. It may even in some cases lead to an underestimation of the genetic distances since identical mutations which emerged independently are not taken into consideration.

The nonparametric *U* test of Mann–Whitney was used to evaluate the significance of the differences in the variability between SIVagm isolates from AGM and patas monkeys. The values were obtained by considering all the distances that give rise to the mean intraisolate divergences shown in Table 2.

The frequencies of synonymous and nonsynonymous

substitutions were calculated in a classical pairwise comparison (Nei and Gojobori, 1986) between related sequences defined by the minimal trees. We calculated first the number of sites where synonymous and nonsynonymous substitutions can occur (Nei and Gojobori, 1986). The ratio of synonymous (and nonsynonymous) mutations per site was then determined by dividing the observed number of synonymous (and nonsynonymous) mutations (*M*_s and *M*_a) by the calculated number of synonymous (and nonsynonymous) sites (*S*_s and *S*_a). The *K*_s/*K*_a ratio for sequences from a same isolate was calculated according to the formula $K_s/K_a = (\sum M_s / \sum S_s) / (\sum M_a / \sum S_a)$. The methods used in the literature to calculate *K*_s/*K*_a ratios are slightly heterogenous. In order to better compare our data to *K*_s/*K*_a values of the literature, we analyzed published HIV-1 *env* sequences from a hemophilic patient sequentially obtained over a 5-year period (Simmonds *et al.*, 1991) and from 9 symptomatic patients of Central Africa infected by HIV-1 subtype A (Murphey *et al.*, 1993). HIV-1 subtype A *env* sequences from 14 early seroconvertors were also analyzed (manuscript in preparation). For these three groups of HIV-1 sequences, the *K*_s/*K*_a ratios we obtained ranged from 0.8 to 2.1 (data not shown) and were therefore comparable to those established by many other groups for HIV-1 (Balfe *et al.*, 1990; Lukashov *et al.*, 1995; Wolinsky *et al.*, 1992).

Nucleotide sequence Accession numbers

The nucleotide sequences described in this paper have been submitted to the GenBank database under Accession numbers U28506–U28640.

RESULTS

Cross-species transmission of SIVagm.tan can occur into patas monkeys

Four monkeys, belonging either to the natural host species of SIVagm.tan or to a foreign species, the patas monkey, were experimentally inoculated with blood derivatives from a naturally SIVagm-infected monkey (Table 1). For one AGM and one patas monkey, the virus was then passaged to a second animal of the same species. We used infectious material containing whole viral populations rather than molecular clones for the inoculations because they might be more representative of natural infections *in vivo*. After 2 months, both AGMs and patas monkeys developed an antibody response as indicated by reactivities with HIV-2 antigens on commercial Western blots (data not shown). These antibodies persisted in the animals throughout their follow-up. All the animals were presenting antibodies against the surface glycoprotein (SU), but reactivities with the transmembrane glycoprotein were weak. As already reported for SIVagm-infected AGMs (Daniel *et al.*, 1988; Müller *et al.*, 1993; Ohta *et al.*, 1988), the four infected patas monkeys also

showed only a low immune response to the p26 major core protein.

SIVs were repeatedly isolated from the PBMCs of all seroconverted AGM and patas monkeys (Table 1). Proviral DNA was persistently detected by PCR in the sequentially obtained PBMCs from all tested AGM and patas monkeys (AGMB14, AGMB86, AGMB87, PAT40, PAT63, and PAT87) after seroconversion (Table 1). However, a nested PCR was always required to obtain detectable amplification signals of proviral DNA by ethidium bromide staining (data not shown).

The animals were followed until 1½ to 4 years p.i. (Table 1). During this period, we did not observe any significant decline of CD4 cells in AGMs nor in patas monkeys (data not shown). None of the SIVagm-infected AGM and patas monkeys showed clinical signs of immunodeficiency.

Sequential SIVagm isolates from both AGM and patas monkeys contain a high proportion of distinct variants

To compare the genetic evolution of SIVagm in AGMs and patas monkeys, viral variants present in simian PBMCs at different time points after infection were analyzed. In the case of the naturally infected animal (AGMB14), the analysis was performed on three sequential samples obtained 1, 3, and 4 years after its capture. For each experimentally inoculated AGM or patas monkey, we analyzed at least two different time points lying between 1.5–6 months p.i. and/or 18–39 months p.i. The *env* region spanning the conserved and variable V3, C3, V4, C4, and V5 domains was chosen for the comparative study. The localization of these domains corresponded to that of HIV-1, except that V3 is localized 3' of the HIV-1 V3 loop (Müller *et al.*, 1993). In total, 136 clones containing the selected 540-bp *env* fragments were sequenced. Only one clone, corresponding to one AGMB87 variant, was shorter because of an internal *SalI* restriction site. Its sequence was very similar to the other AGMB87 variants and was not included in the genetic analysis.

Among the sequential SIVagm isolates from the donor monkey AGMB14, no dominant variant was identified. The alignment of the amino acid sequences (Fig. 1) was therefore performed with regard to a consensus sequence of all AGMB14 clones. Eight isolates obtained from two AGMs (AGMB14, AGMB87) and two patas (PAT40, PAT87) essentially contained unique *env* nucleotide sequences. The remaining five additional isolates obtained from two AGMs (AGMB14, AGMB86) and one patas monkey (PAT63) were containing identical clones, but a high proportion of unique *env* sequences was also identified. The variants differed from each other by 1 to 58 nucleotide substitutions. Since the majority of viruses showed a distinct *env* sequence, it is unlikely that we analyzed several clones resulting from PCR amplification of an identical proviral copy, but we rather amplified dis-

tinct variants originally present in PBMC-extracted DNA. In addition, the amplified sequences which were obtained from independent PCR reactions on the same PBMC DNA sample always showed an identical HMA pattern and also did not phylogenetically cluster together according to the PCR reaction (data not shown). Thus, although the number of clones analyzed per sample is too limited to identify every variant present in PBMCs, the clones should be representative of the overall variability.

The viral populations therefore appear heterogeneous in naturally and experimentally infected AGMs as well as in SIVagm-infected patas. However, the cysteines and the potential N-glycosylation sites are conserved among the variants of each viral population from both AGM and patas monkeys (Fig. 1). There is no evidence of specific amino acid mutations emerging in patas monkeys compared to AGMs. The sequence motifs in the V3 to V5 region, which were reported as specific for SIVagm from AGMs of the *tantalus* species (Jin *et al.*, 1994a; Müller *et al.*, 1993), are conserved even when the virus is transmitted into patas monkeys.

Twelve of the 79 analyzed AGM clones (15%) were presenting a high level of G to A substitutions. This phenomenon was already described for other primate lentiviruses (Daniel *et al.*, 1988; Johnson and Hirsch, 1992; Ohta *et al.*, 1988). According to the genetic code, the G to A substitutions induce the appearance of charged amino acids like asparagine (N), glutamic acid (E), and lysine (K) and also modify tryptophan coding codons (TGG) into stop codons (TAA) (Fig. 1). Among the 16 *env* clones from AGMs with stop codons, 75% of them indeed presented a high level of G to A substitution. Stop codons were also found in 4 of 56 patas clones (0.7%), but G to A hypermutated sequences were not identified in the patas isolates. However, we may not have analyzed enough clones to be able to exclude the existence of this phenomenon in patas monkeys.

SIVagm variants transmitted to two AGMs cluster irrespective of the recipient animal, in opposition to those transmitted to three patas monkeys

The nucleotide sequence alignment was used to construct a phylogenetic tree shown in Fig. 2. SIVagm *env* variants identified over time in the same AGM monkey were found in different clusters. The variants from the naturally infected animal and the two experimentally infected AGMs are very close to each other and do not cluster together according to the animal. The position of the variants within a same cluster, but not the position of the clusters themselves, could change according to the method used to construct the phylogenetic tree (data not shown). In contrast to the AGM viruses, the viral populations identified in the three patas monkeys are clearly separated into three single groups, each corresponding to a distinct recipient animal. We never identi-

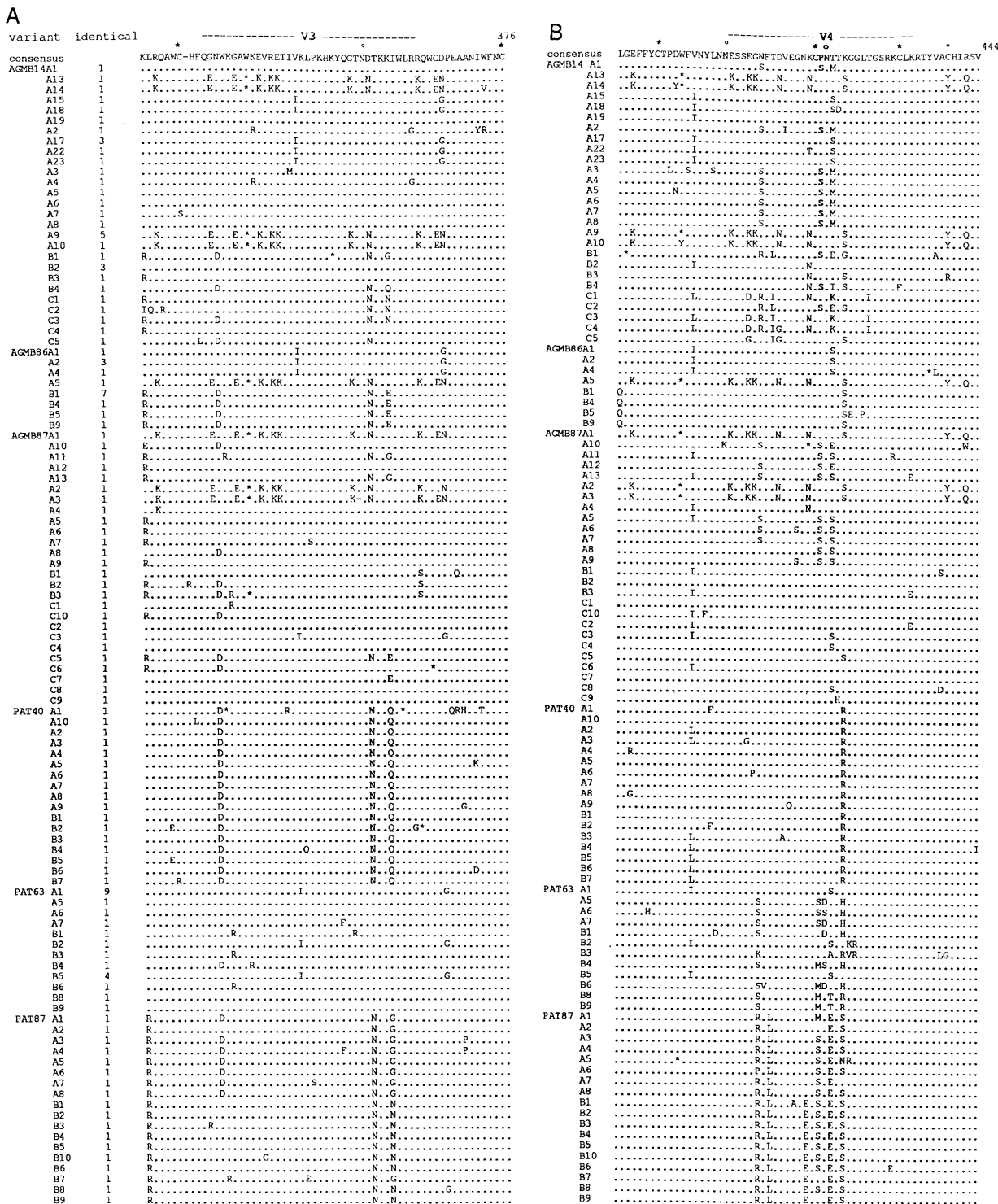


FIG. 1. Alignment of deduced amino acid sequences (positions 328–508 in SIV_{agm}-TYO-1 (Fukasawa *et al.*, 1988) from SIV_{agm} variants present in sequentially obtained PBMC samples designated A, B, and C. The number of nucleotide sequences that were found identical in the same isolate is indicated. Each of such identical sequences is presented once on the alignment. V3, V4, and V5 refer to the hypervariable regions previously determined for SIV_{agm} (Muller *et al.*, 1993). The localization of V3 corresponds thus to that of V3 of SIV_{mac} (Burns and Desrosiers, 1991), but not to V3 of HIV-1 (Modrow *et al.*, 1987). Asterisks mark conserved cysteines and circles indicate conserved potential N-glycosylation sites. Dots represent amino acid identity and dashes designate gaps. Stars indicate stop codons.

fied patas variants from two distinct monkeys in the same cluster whatever method we used to construct the phylogenetic tree. Patas variants were sometimes close to

SIV_{agm} from AGMs. For instance, all the PAT63 variants except one (PAT63B2) are close to those from isolate A of AGM14. This is not surprising since this isolate

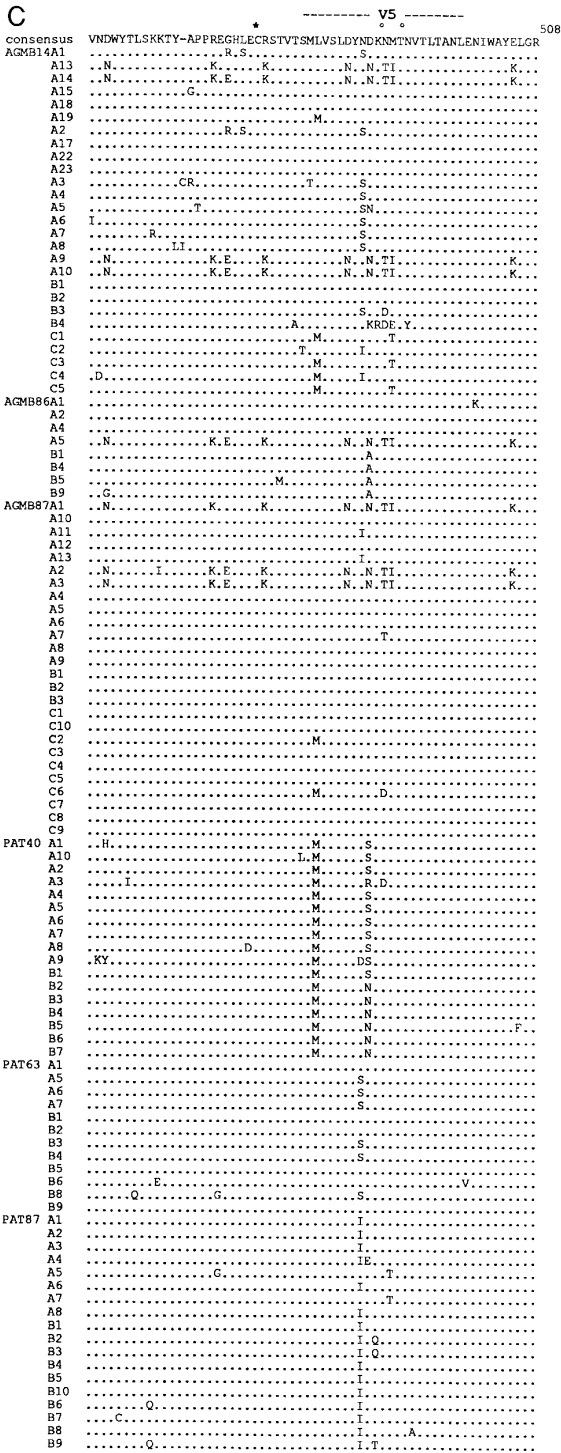


FIG. 1—Continued

viral populations in the inocula may thus have been different. They also may have contained fewer variants than those used to inoculate the AGMs. However, one AGM (AGMB86) and one patas monkey (PAT87) received exactly the same infectious plasma sample, but only the patas variants were found to cluster according to the host animal in this experiment.

SIV_{agm} inraisolat variability is higher in AGMs than in patas monkeys

The mean nucleotide divergences between variants of a same isolate ranged from 0.2 to 2.5% in AGMs and from 0.5 to 1.2% in patas monkeys (Table 2). No correlation exists between the number of clones analyzed per sample and the extent of variability. The maximal distances were always found among clones of AGM isolates. For example, two variants of the first AGMB86 isolate diverged by 58 nucleotide substitutions (10.9%) due to G to A hypermutation in one of those variants. Since this distance to the hypermutated sequence is considered only once in the minimal trees used for the calculation of the variability, the hypermutated sequences do not alter significantly the mean divergences. The mean inraisolat amino acid variability varied from 0.4 to 4.8% for AGMs and from 0.7 to 1.9% for patas monkeys. The culture of monkey PBMCs during 6 days *in vitro* before DNA extraction should be considered as a possible source of mutations that may not be representative of the initial material. However, according to a study on the SIV_{agm}.ver-derived reverse transcriptase, the rate of errors introduced during the replication *in vitro* is about 5.4×10^{-5} base substitutions per nucleotide and per replication cycle (Manns *et al.*, 1991). A replication cycle *in vitro* occurs approximately every 24 hr (Kim *et al.*, 1989). According to this information, no more than 0.18 substitutions may have been introduced into our 540-bp *env* fragment during the short-term culture *in vitro*. Indeed, such an estimate corresponds to the experimentally determined molecular data previously reported (Burns and Desrosiers, 1991; Tolle *et al.*, 1994). The variation rate we observed is also significantly higher than the frequency of errors due to the *Taq* polymerase that was reported to correspond to 0.4 substitutions/540 bp (0.07%) for 60 amplification cycles of PCR (Ennis *et al.*, 1990; Johnson *et al.*, 1991). We can thus consider our values, which indicate much more divergences than those due to experimental conditions, as representative of the viral populations circulating in the infected animals.

Based on these values, the inraisolat variability was significantly lower in patas monkeys than in AGMs. The differences were significant even when the G to A hypermutated sequences were not considered ($P = 0.0216$). It is interesting to notice that in four of five animals, the heterogeneity early after infection (<0.5 year p.i.) is not

corresponds to the PAT63 inoculum. Similarly, one SIV_{agm} variant from isolate B of AGMB14, which corresponds to the PAT87 inoculum, is mixed within the group of the PAT87 variants. Unfortunately, the PAT40 variants could not be compared to the inoculum, which was not available for sequencing. The three patas monkeys were not inoculated with the same infectious material and the

TABLE 3

Fixation Rate of Nucleotide Substitutions per Site and per Year in SIVagm *env* Sequences *in Vivo*^a

AGMB14	AGMB86	AGMB87	PAT40	PAT63	PAT87
2.6×10^{-2}	3.2×10^{-2}	1.8×10^{-2}	0.4×10^{-2}	1.0×10^{-2}	7.2×10^{-2}

^a Those variants in isolates A (or B) were selected that show the minimal distance to a variant in isolates B (or C) from the same monkey. The nucleotide fixation rate was determined by calculating the mean value of nucleotide substitutions between these pairs of variants respective to the time interval between the sequential isolates. The G to A hypermutated sequences were not considered.

lower than later after infection (Table 2), as if the degree of genetic polymorphism does not increase over time during the infection, neither in AGM nor in patas monkeys.

The fixation rate of nucleotide substitutions in SIVagm is high in AGM and patas monkeys

In order to compare mutation rates of SIVagm in AGMs and patas monkeys, we estimated the number of mutations fixed in the V3 to V5 *env* region after a specific time interval in each animal. In AGMs, the mean fixation rate of nucleotide substitutions ranged from 1.8×10^{-2} to 3.2×10^{-2} per site and per year (Table 3). Similar values (0.4×10^{-2} to 7.2×10^{-2} nucleotide substitutions per site and per year) were obtained for patas monkeys, although a higher level of interindividual variation was observed.

It must be remembered that our sequence analysis does not allow us to identify every genotype contained in one PBMC sample. Some variants identified in the animals may already have been present in the inoculum or in previous isolates without being detected. Differences between variants from two samples would therefore not reflect real changes in the viral populations. In order to further evaluate the bias of PCR and cloning in the selection of variants present in a viral population and to further demonstrate a turnover of individual genetic variants in the SIVagm populations of AGMs and patas monkeys studied, we realized HMA. HMA has already been described as a first approach to rapidly determining the pattern of viral variants in a swarm and to detecting the presence of minor variants in a background of distinct molecules (Delwart *et al.*, 1993). Two sequential SIVagm isolates from either AGM87 or PAT63 were compared to each other and to their respective inoculum (Fig. 3). Five independent PCRs were realized on each inoculum and each sequential isolate. The quasispecies pattern was identical for each PCR product from a same sample (data not shown), but different samples showed distinct heteroduplex patterns (Fig. 3). For example, the inoculum used to infect the AGMB87 showed two slow migrating heteroduplexes (Fig. 3A, lane 1), which were not observed in two sequential isolates of AGMB87 (Fig. 3A, lanes 2 and 3). Two unique, very slowly migrating heteroduplexes were identified within the AGMB87 C isolate (Fig. 3A,

lane 3), indicating the presence of some variants that are not detected in the inoculum and the previous sequential isolate. The mixing of the PCR products from the inoculum and the two AGMB87 isolates with each other always resulted in a unique profile (Fig. 3A, lanes 4, 5, and 6), confirming that the viral populations are not identical. Distinct HMA patterns were also observed for the two PAT63 sequential isolates, but to a lower extent. The PAT63 B isolate indeed shows a diffused DNA band that migrated more slowly than the homoduplexes (Fig. 3B, lane 3) and that could be detected neither in the inoculum (Fig. 3B, lane 1) nor in the PAT63 A isolate (Fig. 3B, lane 2). After mixing the PCR products of the inoculum and the PAT63 A isolate, no heteroduplexes could be detected indicating that the viral populations in these two samples are similar (Fig. 3B, lane 4). The mixing of the PCR products from the PAT63 B isolate with the PAT63 inoculum (Fig. 3B, lane 5) or the PAT63 A isolate (Fig. 3B, lane 6), however, revealed again the diffused migrating heteroduplexes, showing that the second PAT63 isolate is more

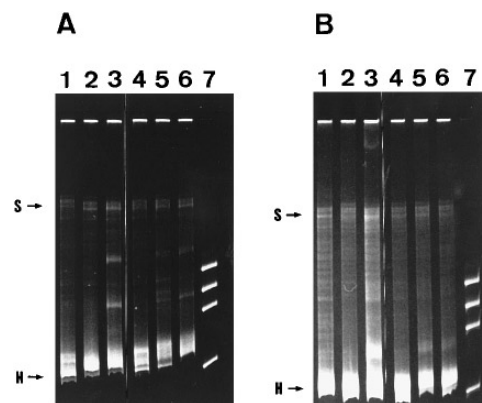


FIG. 3. Comparison of the viral populations present in the inoculum of AGM87 and PAT63 and in two respective sequential SIVagm isolates from these monkeys by heteroduplex mobility assays. Single-strand DNA (S) and homoduplexes (H) are indicated by arrows; the bands in between correspond to heteroduplexes. Lane 7, molecular weight marker. (A) AGMB87. Lanes 1, 2, and 3, PCR products of, respectively, inoculum, isolate B, and isolate C; lanes 4, 5, and 6, mixtures of PCR products, respectively, of inoculum and isolate B, inoculum and isolate C, isolate B and isolate C. (B) PAT63. Lanes 1, 2, and 3, PCR products of, respectively, inoculum, isolate A, and isolate B; lanes 4, 5, and 6, mixtures of PCR products, respectively, of inoculum and isolate A, inoculum and isolate B, isolate A and isolate B.

TABLE 4

Ratios of Synonymous to Nonsynonymous Substitutions (Ks/Ka) in the V3 to V5 *env* Region during Nonpathogenic SIVagm Infections^a

AGMB14			AGMB86		AGMB87			PAT40		PAT63		PAT87	
A	B	C	A	B	A	B	C	A	B	A	B	A	B
1.8	2.1	1.7	2.1	4.6	2.6	4.4	4.9	1.8	2.1	3.0	2.0	6.2	1.5

^a The ratios were determined between SIVagm *env* sequences that were found to be related to each other in the minimal trees established for each isolate. A, B, and C refer to the first, second, or third isolate.

heterogenous than the first one. These results confirm that changes in the viral populations take place over time in one SIVagm-infected AGM and in one SIVagm-infected patas monkey. They support our estimates of the fixation rate of nucleotide substitutions in SIVagm viral populations *in vivo*, which are similar to that previously established for clones of SIVagm.ver *in vivo* (Baier *et al.*, 1991; Fomsgaard *et al.*, 1993).

The fixation rate of nonsynonymous substitutions in the V3-V5 *env* region is low during nonpathogenic SIVagm infections

Changes in viral populations do not reflect accumulation of neutral mutations, but rather the response of a sufficiently large and divergent population to a variety of selective pressures (Coffin, 1995). In order to determine whether SIVagm is submitted to distinct selective pressures in its natural host and in patas monkeys, we evaluated the ratios of the synonymous to nonsynonymous substitution frequencies (Ks/Ka). The results are shown in Table 4. The ratios ranged from 1.7 to 4.9 for AGMs and from 1.5 to 6.2 for patas monkeys. The mean values were, respectively, 3.04 and 2.77 in the experimentally infected AGM and patas monkeys. The Ks/Ka ratios are therefore not significantly different for SIVagm *env* sequences in these two distinct monkey species.

If we assume that the synonymous substitutions are not under a selective pressure, these values mean that only a portion of the emerging nonsynonymous mutations in *env* (20–57% in AGMs and 16–67% in patas) are fixed in both species. These fixation rates of nonsynonymous mutations are lower for SIVagm in the natural host and in patas monkeys than those during pathogenic HIV-1 and SIVmac infections (Balfe *et al.*, 1990; Burns and Desrosiers, 1991; Chackerian *et al.*, 1994; Johnson *et al.*, 1991; Kodama *et al.*, 1993; Overbaugh *et al.*, 1991; Pelletier *et al.*, 1995). It is also interesting to notice that in the experimentally infected AGMs, the Ks/Ka ratios seem to increase over time, but decrease in two of the three experimentally infected patas monkeys (Table 4).

DISCUSSION

Up to now, the only foreign monkey species that were shown to be highly susceptible to experimental SIVagm

infection were Asian monkeys, in particular pigtailed macaques (Gravell *et al.*, 1989; Hartung *et al.*, 1990; Hirsch *et al.*, 1995). We succeeded in reproducibly and persistently infecting an African nonhuman primate species, the patas monkey, with SIVagm.tan (Table 1). One SIVagm.tan cross-species transmission through bites from an AGM to a patas monkey in captivity was also observed (unpublished observation). Cross-species transmissions of SIVagm probably also occur *in natura*, since SIVagm-related lentiviruses were recently identified in one wild-caught patas monkey (Bibollet-Ruche *et al.*, 1996) and in naturally infected baboons (Jin *et al.*, 1994b). It is, however, surprising that SIVagm infections were not identified in many other wild-caught patas (Hervé *et al.*, 1992; Lowenstine *et al.*, 1986), although patas monkeys share the same habitat as AGMs and interspecies contacts are known to occur occasionally (Galat-Luong, 1991). Although SIVagm can easily be experimentally transmitted to patas monkeys, it appears that cross-species transmission events *in natura* are rare.

Our experimental transmission study shows that SIVagm-infected patas monkeys do not develop any clinical signs of immunodeficiency, at least during a 3½-year follow-up. Patas monkeys are large animals with long limbs and the capacity for running fast (van der Kuyl *et al.*, 1995). Because of its characteristics, the patas monkey is generally excluded from the *Cercopithecus* group and classified into a separate genus (*Erythrocebus*). However, they are one of the species most closely related to AGMs (Cronin and Sarich, 1976). It has even been suggested to rename them *Cercopithecus patas*. This close relationship between patas and AGMs may explain why, like AGMs, the infected patas monkeys do not develop any disease.

Although the patas monkeys did not show clinical symptoms in response to a SIVagm infection, we were interested to see whether the genetic evolution of SIVagm is nonetheless different in this foreign host species and in the natural host. The longitudinal evolution of SIVagm variants present in PBMCs of three AGMs and three patas monkeys was thus analyzed. Proviral DNA in PBMCs probably does not represent the predominant viruses expressed at the time point of analysis (Simmonds *et al.*, 1991), but it should nonetheless be globally

representative of the genetic evolution of the viral population in the host. It was reported indeed that the PBMC proviral DNA reflects the viral RNA population, but with a delay (Lineberger *et al.*, 1995; Simmonds *et al.*, 1991; Wei *et al.*, 1995) and, as a consequence, phylogenetic lineages can be established over longer time periods with PBMC DNA than with virion RNA (Delwart *et al.*, 1995). The phylogenetic pattern of the SIV_{agm} variants that we identified in donor and recipient AGMs (Fig. 2) suggests the transmission and persistence of multiple SIV_{agm} variants in the natural host. In contrast, only genetically very closely related variants were detected in patas monkeys. Our recent data on the comparative viral load in AGMs and patas monkeys indicate that the lower genetic heterogeneity of SIV_{agm} in patas does not correlate with a lower cellular viremia in these monkeys (manuscript in preparation). These observations may be consistent with a selection of a single or a few closely related variants present in the inoculum during or soon after transmission of SIV_{agm} into patas monkeys, similarly to what has been reported for HIV-1-infected adults and children (Wolinsky *et al.*, 1992; Zhang *et al.*, 1993; Zhu *et al.*, 1993). We cannot exclude that the 6-day PBMC *in vitro* culture before analysis reduced the SIV_{agm} genetic complexity in a more important manner for patas than for AGM PBMCs. But in either case, distinct selective pressures would act on SIV_{agm} according to the host species environment.

The differences in the degree of SIV_{agm} genetic heterogeneity according to the host species does not interfere with the rates at which the viruses accumulate mutations. They appear indeed similar for both species ($0.4\text{--}7.2 \times 10^{-2}$ substitutions per site and per year). These mutation fixation rates are also similar to the ones reported previously for pathogenic SIV_{mac} or SIV_{sm} clones in macaques and HIV-1 in humans ($0\text{--}11 \times 10^{-2}$ substitutions per site and per year) (Balfe *et al.*, 1990; Burns and Desrosiers, 1991; Johnson *et al.*, 1991; Pelletier *et al.*, 1995). If we assume that most mutations are introduced by the reverse transcriptase during the replication cycle, only a continuous replication of SIV_{agm}, as reported for HIV (Ho *et al.*, 1995; Wain-Hobson, 1993; Wei *et al.*, 1995), could explain the mutation rates we observed in infected AGMs and patas. According to recent data, SIV_{agm}-infected vervets (Hirsch *et al.*, 1995) and tantalus monkeys (manuscript in preparation) present a low-level viremia and only a very few virus-expressing cells in their lymph nodes. A low viral burden is not inconsistent with a continuous viral multiplication of SIV_{agm} *in vivo*, if *de novo* infection constantly occurs, but at a much lower level than that in humans (Ho *et al.*, 1995; Wei *et al.*, 1995). It was suggested that host factors like IL-16 contribute to the low viral load in AGMs by suppressing viral replication (Baier *et al.*, 1995). The mechanisms responsible for the low viral burden in AGMs remain, however, far from being fully understood up to now.

Synonymous to nonsynonymous mutation ratios (Ks/Ka) are indicative of the selective pressures exerted in the host on the virus. Our data on the V3 to V5 *env* sequences of SIV_{agm} indicate similar Ks/Ka ratios in AGMs (1.8–4.9) and patas monkeys (1.5–6.2) (Table 4). The low frequencies of nonsynonymous mutations we determined for SIV_{agm.tan} in AGM and patas monkeys contrast with those reported for another *env* region (V1/V2 region) in one AGM (vervet) infected with a SIV_{agm.ver} molecular clone (Baier *et al.*, 1991). The authors reported a higher percentage of nonsynonymous mutations. Such a discrepancy might be explained by distinct selective pressures acting on the N- and C-terminal regions of the SU. Our Ks/Ka ratios in the SU C-terminal half are significantly higher than that described for pathogenic SIV_{mac}, SIV_{sm}, and HIV-1 in equivalent *env* regions (Balfe *et al.*, 1990; Burns and Desrosiers, 1991; Chackerian *et al.*, 1994; Johnson *et al.*, 1991; Kodama *et al.*, 1993; Overbaugh *et al.*, 1991; Pelletier *et al.*, 1995). Studies based on interhost variability already suggested a lower fixation rate of nonsynonymous mutations during nonpathogenic infections (Johnson *et al.*, 1990; Myers *et al.*, 1994; Shpaer and Mullins, 1993). Intrahost studies in HIV-1-infected adults showed Ks/Ka values ranging from 0.35 to 1.9 for the V3 and V4–V5 regions (Balfe *et al.*, 1990; Lukashov *et al.*, 1995; Wolinsky *et al.*, 1992). Surprisingly, the lowest Ks/Ka ratios were reported for slow progressors (0.35) (Lukashov *et al.*, 1995). Although from a clinical point of view, the slow progressors resemble more the asymptotically infected African monkeys than the rapid progressors do, the differences in the Ks/Ka ratios indicate that the intrahost evolution in pathogenic and nonpathogenic infections is not driven by the same forces. It was suggested that in HIV-infected slow progressors, the higher fixation rate of nonsynonymous mutations is related to the persistent cytotoxic T-lymphocyte response that maintains strong positive selective pressures for change (Lukashov *et al.*, 1995). In AGMs, the pressures for change appear less strong. The intraspecies viral evolution in the long run may have led to the selection of SIV_{agm} viruses that are highly adapted to their environment (Shpaer and Mullins, 1993). Under these circumstances, the selective pressures are more conservative since virus and host are in an apparent optimum state of balance.

The results presented here gave an insight into the genetic evolution of nonpathogenic SIV_{agm} *in vivo*. They support that SIV_{agm} is highly adapted to its natural host. They also suggest that the differences between nonpathogenic SIV_{agm} and pathogenic HIV/SIV infections are not related to a lower ability of nonpathogenic viruses to replicate *in vivo*. Our study highlights the necessity of further investigations on nonpathogenic lentivirus models, in particular on the host factors that are involved in the absence of progression to AIDS.

ACKNOWLEDGMENTS

We thank Evelyne Leclerc for excellent technical work, Colombe Chappay for her precious assistance in the sequence analysis, and Alain Rein for helpful discussions. M.C.M.-T. and M.D.T. are respectively recipients of fellowships from the French Agency for AIDS Research (ANRS) and from the CAPES Ministry of Education in Brazil. This work was supported by grants from ANRS.

REFERENCES

- Allan, J. S., Kanda, P., Kennedy, R. C., Cobb, E. K., Anthony, M., and Eichberg, J. W. (1990). Isolation and characterization of simian immunodeficiency viruses from two subspecies of African green monkeys. *AIDS Res. Hum. Retroviruses* **6**, 275–285.
- Allan, J. S., Short, M., Taylor, M. E., Su, S., Hirsch, V. M., Johnson, P. R., Shaw, G. M., and Hahn, B. H. (1991). Species-specific diversity among simian immunodeficiency viruses from African green monkeys. *J. Virol.* **65**, 2816–2828.
- Baier, M., Dittmar, M. T., Cichutek, K., and Kurth, R. (1991). Development *in vivo* of genetic variability of simian immunodeficiency virus. *Proc. Natl. Acad. Sci. USA* **88**, 8126–8130.
- Baier, M., Werner, A., Bannert, N., Metzner, K., and Kurth, R. (1995). HIV suppression by Interleukin-16. *Nature* **378**, 563–564.
- Balfe, P., Simmonds, P., Ludlam, C. A., Bishop, J. O., and Leigh-Brown, A. J. (1990). Concurrent evolution of human immunodeficiency virus type I in patients infected from the same source: Rate of sequence change and low frequency of inactivating mutations. *J. Virol.* **64**, 6221–6223.
- Barré-Sinoussi, F., Chermann, J.-C., Rey, F., Nugeyre, M.-T., Chamaret, S., Gruest, F., Dauguet, C., Axler-Blin, C., Brun-Vezinet, F., Rouzioux, C., Rozenbaum, W., and Montagnier, L. (1983). Isolation of a T-lymphotropic retrovirus from a patient at risk of acquired immunodeficiency syndrome. *Science* **220**, 868–871.
- Bibollet-Ruche, F., Galat-Luong, A., Cuny, G., Sarni-Manchado, P., Galat, G., Durand, J.-P., Pourrut, X., and Veas, F. (1996). Simian Immunodeficiency virus infection in a patas monkey (*Erythrocebus patas*): Evidence for cross-species transmission from African green monkeys (*Cercopithecus aethiops sabaeus*) in the wild. *J. Gen. Virol.* **77**, 773–781.
- Bosch, M. L., Pedersen, N., Amerongen, G. v., Reay, E., Marthas, M., Rose, T., Frankenhuis, M., and Osterhaus, A. D. M. E. (1994). Identification and partial characterization of a lentivirus from talapoin monkeys. *J. Med. Primatol.* **23**, 233.
- Burns, D. P. W., and Desrosiers, R. C. (1991). Selection of genetic variants of simian immunodeficiency virus in persistently infected rhesus monkeys. *J. Virol.* **65**, 1843–1854.
- Cao, Y., Qin, L., Zhang, L., Safrit, J., and Ho, D. D. (1995). Virologic and immunologic characterization of long-term survivors of human immunodeficiency virus type 1 infection. *N. Engl. J. Med.* **332**, 201–208.
- Chackerian, B., Morton, W. R., and Overbaugh, J. (1994). Persistence of simian immunodeficiency virus Mne variants upon transmission. *J. Virol.* **68**, 4080–4085.
- Coffin, J. M. (1995). HIV population dynamics *in vivo*: Implications for genetic variation, pathogenesis, and therapy. *Science* **267**, 483–489.
- Courgnaud, V., Lauré, F., Fultz, P. N., Montagnier, L., Bréchet, C., and Sonigo, P. (1992). Genetic differences accounting for evolution and pathogenicity of simian immunodeficiency virus from sooty mangabey monkey after cross-species transmission to a pig-tailed macaque. *J. Virol.* **66**, 414–419.
- Cronin, J. E., and Sarich, V. M. (1976). Molecular evidence for dual origin of mangabeys among Old World monkeys. *Nature* **260**, 700–702.
- Daniel, M. D., Letvin, N. L., Sehgal, P. K., Schmidt, D. K., Silva, D. P., Solomon, K. R., Hodi, F. S. J., Ringler, D. J., Hunt, R. D., King, N. W., and Desrosiers, R. C. (1988). Prevalence of antibodies to three retroviruses in a captive colony of macaque monkeys. *Int. J. Cancer* **41**, 601–608.
- Delwart, E. L., Busch, M. P., Kalish, M. L., Mosley, J. W., and Mullins, J. I. (1995). Rapid molecular epidemiology of human immunodeficiency virus transmission. *AIDS Res. Hum. Retroviruses* **11**, 1081–1093.
- Delwart, E. L., Shpaer, E. G., Louwagie, J., McCutchan, F. E., Grez, M., Rubsamen-Waigmann, H., and Mullins, J. (1993). Genetic relationships determined by a DNA heteroduplex mobility assay: Analysis of HIV-1 env genes. *Science* **262**, 1257–1261.
- Emau, P., McClure, H. M., Ishakia, M., Else, J. G., and Fultz, P. N. (1991). Isolation from African Sykes Monkeys (*Cercopithecus mitis*) of a lentivirus related to human and simian immunodeficiency viruses. *J. Virol.* **65**, 2135–2140.
- Ennis, P. D., Zemmour, J., Salter, R. D., and Parham, P. (1990). Rapid cloning of HLA-A, B cDNA by using the polymerase chain reaction: Frequency and nature of errors produced in amplification. *Proc. Natl. Acad. Sci. USA* **87**, 2833–2837.
- Felsenstein, J. (1990). PHYLIP (Phylogeny inference package). University of Washington, Seattle, WA.
- Fitch, W. M., and Margolish, E. (1967). Construction of phylogenetic trees. *Science* **155**, 279–284.
- Fomsgaard, A., Hirsch, V. M., Allan, J. S., and Johnson, P. R. (1991). A highly divergent proviral DNA clone of SIV from a distinct species of African Green Monkey. *Virology* **182**, 397–402.
- Fomsgaard, A., Johnson, P. R., London, W. T., and Hirsch, V. M. (1993). Genetic variation of the SIVagm transmembrane glycoprotein in naturally and experimentally infected primates. *AIDS* **7**, 1041–1047.
- Fukasawa, M., Miura, T., Hasegawa, A., Morikawa, S., Tsujimoto, H., Miki, K., Kitamura, T., and Hayami, M. (1988). Sequence of simian immunodeficiency virus from African Green Monkey, a new member of the HIV/SIV group. *Nature (London)* **333**, 457–461.
- Fultz, P. N., McClure, H. M., Anderson, D. C., and Switzer, W. M. (1989). Identification and biologic characterization of an acutely lethal variant of simian immunodeficiency virus from sooty mangabeys (SIV/SMM). *AIDS Res. Hum. Retroviruses* **5**, 397–409.
- Fultz, P. N., McClure, H. M., Anderson, D. C., Swenson, B., Anand, R., and Srinivasan, A. (1986). Isolation of a T-lymphotropic retrovirus from naturally infected Sooty-Mangabey monkeys (*Cercocebus atys*). *Proc. Natl. Acad. Sci. USA* **83**, 5286–5290.
- Galat-Luong, A. (1991). Observation de contacts interindividuels interspécifiques avec échanges de fluides corporels entre singes verts, *Cercopithecus aethiops*, et patas, *Erythrocebus patas*. 6th International Conference on AIDS in Africa. Dakar, Senegal T.A.124.
- Gravell, M., London, W. T., Hamilton, R. S., Stone, G., and Monzon, M. (1989). Infection of macaque monkeys with simian immunodeficiency virus from African green monkeys: Virulence and activation of latent infection. *J. Med. Primatol.* **18**, 247–254.
- Hartung, S., Norley, S., Kraus, G., Werner, A. J., Vogel, M., Bergmann, L., Baier, M., and Kurth, R. (1990). Infection of non-human primates with SIVagm and HIV-2. In "Animal Models in AIDS" (H. Schellekens and M. Horzinek, Eds.), pp. 73–79. Elsevier, Amsterdam.
- Hendry, R. M., Wells, M. A., Phelan, M. A., Schneider, A. L., Epstein, J. S., and Quinlan, G. V. (1986). Antibodies to simian immunodeficiency virus in African Green Monkeys in Africa in 1957-1962. *Lancet* **ii**, 455.
- Hervé, V., Manière, G., Le Gall-Campodonico, P., Mathiot, C., Georges, A. J., and Barré-Sinoussi, F. (1992). Natural infection by STLV and double infection by SIV and STLV in feral monkeys in Central African Republic. VIII International Conference on AIDS/III STD World Congress, Amsterdam, The Netherlands. PuA6096, 27.
- Higgins, D. G., Bleasby, A. J., and Fuchs, R. (1992). CLUSTAL V: Improved software for multiple sequence alignment. *Comp. Appl. Biosci.* **8**, 189–191.
- Hirsch, V., Dapolito, G., Johnson, P. R., Elkins, W. R., London, W. T., Montali, R. J., Goldstein, S., and Brown, C. (1995). Induction of AIDS by simian immunodeficiency virus from an African green monkey:

- Species-specific variation in pathogenicity correlates with the extent of in vivo replication. *J. Virol.* **69**, 955–967.
- Hirsch, V. M., Olmstedt, R. A., Murphey-Corb, M., Purcell, R. H., and Johnson, P. R. (1989). An African primate lentivirus (SIV_{sm}) closely related to HIV-2. *Nature* **339**, 389–392.
- Hirsch, V. M. H., McGann, C., Dapolito, G., Goldstein, S., Ogen-Odoi, A., Biryawho, B., Lakwo, T., and Johnson, P. R. (1993). Identification of a new subgroup of SIV_{agm} in tantalus monkeys. *Virology* **197**, 426–430.
- Ho, D. D., Neumann, A. U., Perelson, A. S., Chen, W., Leonard, J. M., and Markowitz, M. (1995). Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature* **373**, 123–126.
- Jin, M. J., Hui, H., Robertson, D. L., Muller, M. C., Barré-Sinoussi, F., Hirsch, V. M., Allan, J. S., Shaw, G. M., Sharp, P. M., and Hahn, B. H. (1994a). Mosaic genome structure of simian immunodeficiency virus from West African monkeys. *EMBO J.* **13**, 2935–2947.
- Jin, M. J., Rogers, J., Phillips-Conroy, J. E., Allan, J. S., Desrosiers, R., Shaw, G. M., Sharp, P. M., and Hahn, B. H. (1994b). Infection of a yellow baboon with simian immunodeficiency virus from African green monkeys: Evidence for cross-species transmission in the wild. *J. Virol.* **68**, 8454–8460.
- Johnson, P. R., Fomsgaard, A., Allan, J., Gravell, M., London, W. T., Olmstead, R. A., and Hirsch, V. M. (1990). Simian immunodeficiency viruses from African Green monkeys display unusual genetic diversity. *J. Virol.* **64**, 1068–1092.
- Johnson, P. R., Hamm, T. E., Goldstein, S., Kitov, S., and Hirsch, V. M. (1991). The genetic fate of molecularly cloned simian immunodeficiency virus in experimentally infected macaques. *Virology* **185**, 217–228.
- Johnson, P. R., and Hirsch, V. M. (1992). Genetic variation of simian immunodeficiency viruses in non-human primates. *AIDS Res. Hum. Retroviruses* **8**, 367–372.
- Kim, S., Byrn, R., Gropman, J., and Baltimore, D. (1989). Temporal aspects of DNA and RNA synthesis during human immunodeficiency virus infection: Evidence for differential gene expression. *J. Virol.* **63**, 3708–3713.
- Kodama, T., Mori, K., Kawahara, T., Ringler, D. J., and Desrosiers, R. (1993). Analysis of simian immunodeficiency virus sequence variation in tissues of rhesus macaques with simian AIDS. *J. Virol.* **67**, 6522–6534.
- Lernould, J. M. (1988). Classification and geographical distribution of guenons: A review. In "A Primate Radiation: Evolutionary Biology of the African Guenons," pp. 54–35. Cambridge Univ. Press, Cambridge.
- Lineberger, D. W., Kessler, J. A., Waterbury, J. A., Byrnes, V. W., Massari, F., Staszewski, S., and Emini, E. A. (1995). Turnover of circulating viron RNA and of cell-associated viral DNA reflects active viral replication in human immunodeficiency virus type 1-infected individuals. *J. Virol.* **69**, 2637–2639.
- Lowenstine, M. L., Pederson, N. C., and Higgins, J. (1986). Seroepidemiologic survey of captive old world primates for antibodies to human and simian retroviruses and isolation of a lentivirus from Sooty-Mangabeys (*Cercocebus atys*). *Int. J. Cancer* **38**, 563–575.
- Lukashov, V. V., Kuiken, C. L., and Goudsmit, J. (1995). Intra-host human immunodeficiency virus type 1 evolution is related to length of the immunocompetent period. *J. Virol.* **69**, 6911–6916.
- Manns, A., König, H., Baier, M., Kurth, R., and Grosse, F. (1991). Fidelity of reverse transcriptase of the simian immunodeficiency virus from African Green Monkey. *Nucleic Acids Res.* **19**, 533–537.
- Marthas, M. L., Banapour, B., Sutjipto, S., Siegel, M. E., Marx, P. A., Gardner, M. B., Pedersen, N. C., and Luciw, P. A. (1989). Rhesus macaques inoculated with molecularly cloned simian immunodeficiency virus. *J. Med. Primatol.* **18**, 311–319.
- Modrow, S., Hahn, B. H., Shaw, G. M., Gallo, R. C., Wong-Staal, F., and Wolf, H. (1987). Computer-assisted analysis of envelope protein sequences of seven human immunodeficiency virus isolates: Prediction of antigenic epitopes in conserved and variable regions. *J. Virol.* **61**, 570–578.
- Muller, M. C., Saksena, N. K., Nerrienet, E., Chappey, C., Herve, V. M. A., Durand, J.-P., Legal-Campodonico, P., Lang, M.-C., Digoutte, J.-P., Georges, A. J., Georges-Courbot, M.-C., Sonigo, P., and Barré-Sinoussi, F. (1993). Simian immunodeficiency viruses from Central and Western Africa: Evidence for a new species-specific lentivirus in tantalus monkeys. *J. Virol.* **67**, 1227–1235.
- Murphey, E., Korber, B., Georges-Courbot, M.-C., You, B., Pinter, A., Cook, D., Kieny, M.-P., Georges, A., Mathiot, C., Barré-Sinoussi, F., and Girard, M. (1993). Diversity of V3 region sequences of human immunodeficiency viruses type 1 from the Central African Republic. *AIDS Res. Hum. Retroviruses* **10**, 997–1006.
- Myers, G., Berzofsky, J. A., Korber, B., and Smith, R. F. (1994). "Human Retroviruses and AIDS." Los Alamos National Laboratory, Los Alamos, NM.
- Nei, M., and Gojobori, T. (1986). Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. *Mol. Biol. Evol.* **3**, 418–426.
- Ohta, Y., Masuda, T., Tsujimoto, H., Ishikawa, K., Kodama, T., Morikawa, S., Nakai, M., Honjo, S., and Hayami, M. (1988). Isolation of simian immunodeficiency virus from African green monkeys and seroepidemiological survey of the virus in various non-human primates. *Int. J. Cancer* **41**,
- Overbaugh, J., Rudensey, L. M., Papaenhausen, M. D., Benveniste, R. E., and Morton, W. R. (1991). Variation in simian immunodeficiency virus env is confined to V1 and V4 during progression to simian AIDS. *J. Virol.* **65**, 7025–7031.
- Peeters, M., Honore, C., Huet, T., Bedjabaga, L., Ossari, S., Bussi, P., Cooper, R. W., and Delaporte, E. (1989). Isolation and partial characterization of an HIV-related virus occurring naturally in chimpanzees in Gabon. *AIDS* **3**, 625–630.
- Pelletier, E., Saurin, W., Cheyrier, R., Letvin, N. L., and Wain-Hobson, S. (1995). The tempo and mode of SIV quasispecies development in vivo calls for massive viral replication and clearance. *Virology* **208**, 644–652.
- Piatak, J. M., Saag, M. S., Yang, L. C., Clark, S. J., Kappes, J. C., Luk, K.-C., Hahn, B. H., Shaw, G. M., and Lifson, J. D. (1993). High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR. *Science* **259**, 1749–1754.
- Prim, R. C. (1957). Shortest connection networks and some generalizations. *Bell. Syst. Tech. J.* **36**, 1389–1401.
- Saitou, N., and Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**, 406–425.
- Shpaer, E. G., and Mullins, J. I. (1993). Rates of amino acid change in the envelope protein correlate with pathogenicity of primate lentiviruses. *J. Mol. Evol.* **37**, 57–65.
- Simmonds, P., Qi Zhang, L., McOmish, F., Balfe, P., Ludlam, C., and Leigh Brown, A. (1991). Discontinuous sequence change of Human Immunodeficiency virus (HIV) type 1 env sequences in plasma viral and lymphocyte associated proviral populations in vivo: Implication for models of HIV pathogenesis. *J. Virol.* **65**, 6266–6276.
- Swofford, D. L. (1984). Phylogenetic analysis using parsimony (PAUP). Illinois Natural History survey, Champaign.
- Temin, H. M. (1993). Retrovirus variation and reverse transcription: Abnormal strand transfers result in retrovirus genetic variation. *Proc. Natl. Acad. Sci. USA* **90**, 6900–6903.
- Tolle, T., Petry, H., Bachmann, B., Hunsmann, G., and Luke, W. (1994). Variability of the env gene in cynomolgus macaques persistently infected with human immunodeficiency virus type 2 strain ben. *J. Virol.* **68**, 2765–2771.
- Tomonaga, K., Katahira, J., Fukasawa, M., Hassan, M. A., Kawamura, M., Akari, H., Miura, T., Goto, T., Nakai, M., Suleman, M., Isahakia, M., and Hayami, M. (1993). Isolation and characterization of simian immunodeficiency virus from African white-crowned mangabey monkeys (*Cercocebus torquatus lunulatus*). *Arch. Virol.* **129**, 79–92.
- Tsujimoto, H., Cooper, R. W., Kodama, T., Fukasawa, M., Miura, T., Ohta, Y., Ishikawa, K., Nakai, M., Frost, E., Reolants, G. E., Roffi, J., and

- Hayami, M. (1988). Isolation and characterization of simian immunodeficiency virus from mandrills in Africa and its relationship to other human and simian immunodeficiency viruses. *J. Virol.* **62**, 4044–4050.
- van der Kuyl, A. C., Kuiken, C. L., Dekker, J. T., and Goodsmid, J. (1995). Phylogeny of African monkeys based upon mitochondrial 12S rRNA sequences. *J. Mol. Evol.* **40**, 173–180.
- Wain-Hobson, S. (1993). Viral burden in AIDS. *Nature* **366**, 22.
- Wei, X., Gosh, S. K., Taylor, M. E., Johnson, V. A., Emini, E., Deutsch, P., Lifson, J. D., Bonhoeffer, S., Nowak, M., Hahn, B., Saag, M. S., and Shaw, G. M. (1995). Viral dynamics in human immunodeficiency virus type 1 infection. *Nature* **373**, 117–122.
- Wolinsky, S. M., Wike, C. M., Korber, B. T. M., Hutto, C., Parks, W. P., Rosenblum, L. L., Kunstman, K. J., Furtado, M. R., and Munoz, J. L. (1992). Selective transmission of human immunodeficiency virus type-1 variants from mothers to infants. *Science* **255**, 1134–1137.
- Zhang, L. Q., McKenzie, P., Cleland, A., Holmes, E. C., Leigh Brown, A. J., and Simmonds, P. (1993). Selection for specific sequences in the external envelope protein of human immunodeficiency virus type 2 upon primary infection. *J. Virol.* **67**, 3345–3356.
- Zhu, T., Mo, H., Wang, N., Nam, D. S., Cao, Y., Koup, R. A., and Ho, D. D. (1993). Genotypic and phenotypic characterization of HIV-1 in patients with primary infection. *Science* **261**, 1179–1181.