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Species-Specific Diversity among Simian Immunodeficiency Viruses from African Green Monkeys

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The prevalence, natural history, and genetic characteristics of simian immunodeficiency virus (SIV) infections in most feral African monkey species are presently unknown, yet this information is essential to elucidate their origin and relationship to other simian and human immunodeficiency viruses. In this study, a combination of classical and molecular approaches were used to identify and characterize SIV isolates from West African green monkeys (*Cercopithecus sabaeus*) (SIVagm isolates). Four SIVagm viruses from wild-caught West African green monkeys were isolated and analyzed biologically and molecularly. Amplification, cloning, and sequencing of a 279-bp polymerase fragment directly from uncultured peripheral blood mononuclear cells was facilitated by the use of nested polymerase chain reaction. The results indicated that West African green monkeys are naturally infected with SIVs which are closely related to East African SIVagm isolates. However, structural, antigenic, and genetic differences were observed which strongly suggest that the West African green monkey viruses comprise a phylogenetically distinct subgroup of SIVagm. These findings support our previous hypothesis that SIVagm viruses may have evolved and diverged coincident with the evolution and divergence of their African green monkey host. In addition, this study describes a polymerase chain reaction-based approach that allows the identification and molecular analysis of divergent SIV strains directly from primary monkey tissue. This approach, which does not depend on virus isolation methods, should facilitate future studies aimed at elucidating the origins and natural history of SIVs in feral African green monkey populations.

Simian immunodeficiency viruses (SIVs) have been isolated from a number of African nonhuman primates, including African green monkeys (SIVagm), sooty mangabeys (SIVsm), mandrills (SIVmnd), macaques (SIVmac), and chimpanzees (SIVcpz) (3, 9, 16, 35, 40, 43, 46, 47, 59). These viruses are similar to the human immunodeficiency viruses (HIVs) in tropism and cytopathology for CD4-bearing T lymphocytes, ability to establish persistent infections, genomic structure and organization, and phylogenetic ancestry. SIVs from African nonhuman primates, however, generally fail to cause disease in their natural hosts (3, 16, 40, 43, 46). Macaques infected with SIVmac or SIVsm, on the other hand, develop immunodeficiency and a fatal disease similar to that of humans infected with HIV type 1 (HIV-1) or HIV-2 (7, 8, 38, 43). Since feral macaques are not infected with SIV in their natural habitat (40, 46) and since SIVmac is very similar to SIVsm in its nucleotide sequence (6, 24), it has been proposed that macaques acquired their SIV infection in captivity by cross-species transmission from naturally infected sooty mangabeys (24, 43). A similar event may also explain the pathogenic nature of HIV in humans, since close genetic relatives of HIV-1 and HIV-2 have been identified in chimpanzees and sooty mangabeys, respectively (21, 24, 26, 50). Cross-species transmission of nonpathogenic SIVs to an "unnatural" primate host may thus represent an important factor in the generation of pathogenic lentiviruses (10, 12). The probability and frequency of such events, however, are difficult to evaluate since little information is presently available concerning the prevalence, transmissibility, and genetic properties of naturally occurring SIVs in wild-living African primates.

Among all African nonhuman primates known to be infected with SIVs in the wild, African green monkeys (*Cercopithecus aethiops*) comprise the largest reservoir, with seroprevalence rates approaching 50% in some areas of Africa (2, 22, 29, 40, 46). African green monkeys have been divided into four species, which are commonly termed vervets (*Cercopithecus pygerythrus*), grivets (*Cercopithecus aethiops*), tantalus monkeys (*Cercopithecus tantalus*), and green monkeys (*Cercopithecus sabaeus*) (31, 37, 45). These four species of African green monkeys are both phenotypically and geographically distinct and are believed to have diverged following the migration and segregation of an ancestral monkey into distinct regions of Africa. Natural infection of each species with SIV therefore provides a unique opportunity to study the evolution of SIVagm viruses in relation to their respective monkey groups.

Several isolates of SIVagm, all derived from East African green monkeys, have recently been described and shown to comprise a phylogenetically distinct group of viruses that is equidistant from other SIVs and HIVs (3, 9, 15, 35, 46). These SIVagm isolates display significantly greater genotypic variability than HIV-1, HIV-2, and SIVmac and SIVsm viruses, suggesting that SIVagm strains evolved and diverged within their host species over a much longer period of time (5, 15, 27, 28, 39). West African SIVagm viruses have not been isolated or genetically characterized, yet they may be of particular interest because of the geographic proximity

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of their host species to SIVsm-infected sooty mangabeys and HIV-2-infected humans.

In this article, we report the isolation and characterization of SIV from four wild-caught West African green monkeys (*C. sabaeus*) and, independently, the direct amplification and molecular analysis of SIV sequences from uncultured peripheral blood mononuclear cells by nested polymerase chain reaction (PCR) and generic oligonucleotide primer. Our results demonstrate the existence of a genetically distinct subtype of SIVagm in West Africa and describe a PCR-based approach for rapidly identifying novel strains of human and simian retroviruses.

MATERIALS AND METHODS

Animals. Ten wild-caught West African green monkeys were imported to the United States from Senegal, West Africa, in July 1989. The animals were captured in Senegal and housed individually prior to their export to the United States. All 10 animals were randomly selected by a professional animal handler, and their serological status with respect to SIV infection was unknown at the time of purchase. While on study, all animals (six females and four males) were housed in individual cages and fed a diet of commercial monkey ration supplemented with fresh vegetables and water ad libitum according to the National Institutes of Health (NIH) guidelines for the humane care of laboratory animals. All 10 animals were screened for antibodies to SIVagm, HIV-1, and HIV-2, and four animals were identified as seropositive for SIVagm(tyo-1) (animals SAB381, SAB384, SAB385, and SAB386, all females). Regardless of their serological status, all animals have remained clinically healthy since importation.

Cells and viruses. All cell lines, including the immortalized T-cell lines Molt4(clone8) (30), CEMx174 (53), SupT1 (57), HUT78 (18), and AA2 (AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases), were maintained in RPMI 1640 supplemented with 15% fetal bovine serum, 2 mM L-glutamine, 50 U of penicillin per ml, and 50 μg of streptomycin per ml (complete medium). The viral isolates SIVagm(tyo-1), SIVagm(gri-1), HIV-1/IIIB, and HIV-2/ST have been described previously (3, 33, 46, 48). SIVmac251(7908) is an isolate from an experimentally infected rhesus macaque (animal 7908) who received a low-passage stock of SIV mac251 (7). During the viremic stage of infection, peripheral blood mononuclear cells from animal 7908 were cocultivated with Molt4(clone8) cells, resulting in a productively infected cell culture. For the preparation of virus stocks, SIVagm-(tyo-1), SIVagm(gri-1), and SIVmac251(7908) were propagated in Molt4(clone8) cells, while HIV-1/IIIB and HIV-2/ST were grown in Molt3 and CEMx174 cells, respectively. Virus replication in infected cultures was monitored by reverse transcriptase and HIV-1 p24 antigen capture assays (Coulter Immunologics).

Virus isolation. Blood samples were obtained from four SIVagm-seropositive West African green monkeys (SAB381, SAB384, SAB385, and SAB386) and two seronegative animals (SAB378 and SAB379). For venipuncture, monkeys were anesthetized to minimize discomfort in accordance with NIH guidelines for handling of nonhuman primates. Peripheral blood mononuclear cells (PBMC) were isolated from 5 ml of heparinized whole blood by Ficoll-Hypaque gradient centrifugation, washed, resuspended at a concentration of 10^6 per ml, and stimulated with phytohemagglutinin (PHA; 5 μg/ml) for 48 h. Cultures were subsequently washed free of lectin, maintained in complete medium containing 10% interleukin-2 (IL-2) for 3 additional days, and then cocultivated (1:1) with Molt4(clone8) cells and with CEMx174 cells. Supernatants were harvested every 3 to 4 days and monitored for virus production by an HIV-1 p24 antigen capture assay (Coulter Immunologics). In addition, cultures were observed daily for the appearance of virus-induced syncytia. Antigen- and syncytium-positive cultures were confirmed by Western immunoblot analysis of culture supernatants.

Western blot analysis. Viral antigen was prepared from culture supernatants of productively infected cell lines. Supernatants were precleared to remove residual cells by low-speed centrifugation (1,800 × *g*, 10 min), followed by filtration through a 0.45-μm filter. Virus was concentrated by centrifugation through a 20% sucrose cushion (71,500 × *g*, 2 h), resuspended in 400 μl of electrophoresis buffer (80 mM Tris-HCl [pH 6.8], 100 mM dithiothreitol, 2% sodium dodecyl sulfate [SDS], 10% glycerol, bromophenol blue), and boiled for 3 min. Proteins were separated on a 12% SDS–polyacrylamide discontinuous gel system and passively transferred to nitrocellulose sheets. Sheets were blocked with 3% bovine serum albumin and incubated with a 1:50 or 1:80 dilution of serum in phosphate-buffered saline (PBS) and 0.2% Tween 20. Detection of viral proteins was accomplished by the streptavidin-biotin system (Amersham Inc.) with diaminobenzedine as the substrate for color development.

Infectivity studies. Supernatants from cell lines productively infected with HIV-1/IIIB, HIV-2/ST, SIVagm(tyo-1), SIVmac251(7908), SIVagm(sab-1), and SIVagm(sab-2) were clarified by centrifugation (1,800 × *g*, 10 min), filtered (0.45-μm filter), and frozen at −80°C prior to use. All viral supernatants were initially titrated for tissue culture infectious doses (TCIDs) on susceptible cell lines. For viral infectivity studies, 250 μl of each virus stock (10^3 to 10^4 TCIDs) was incubated with 2×10^6 pelleted target cells [AA2, Hut78, Molt4(clone8), CEMx174, and SupT1]. After 2 h at 37°C, the volume was adjusted to a cell concentration of 0.5×10^6/ml in complete medium. The next day, cell cultures were washed four times in RPMI 1640, resuspended in complete medium, and grown for 28 days. The appearance of multinucleated giant cells was monitored on a daily basis, the supernatants were harvested every 3 to 4 days, frozen at −80°C, and tested for reverse transcriptase activity and SIV p27 antigen (Coulter Immunologics) upon termination of the cultures.

For reverse transcriptase assays, 50 μl of concentrated virus preparation was added to 96-well plates containing 50 μl of virus dilution buffer (50 mM Tris-HCl [pH 7.8], 100 mM NaCl, 150 mM dithiothreitol, 0.2% Triton X-100). Twenty-five microliters of this preparation was then incubated with 50 μl of reaction mixture [50 mM Tris-HCl, 150 mM KCl, 7.5 mM $MgCl_2$, 0.1% Triton X-100, 5 μg of poly(rA) per ml, 6.25 μg of oligo(dT) per ml, and 1 μCi of [^{3}H]TTP] for 60 min at 37°C. Following incubation, 50 μl of the mixture was applied to nitrocellulose filter paper and washed extensively with 5% trichloroacetic acid (TCA)–5% $NaPP_i$, followed by 5% TCA and then 50% ethanol. Filters were then dried and processed for scintillation counting.

PCR. Heparinized blood (5 ml) was collected in vacutainers from four seropositive (SAB381, SAB384, SAB385, and SAB386) and two seronegative (SAB378 and SAB379) West African green monkeys and sent under code to investigators at the University of Alabama at Birmingham for PCR analysis. Blood was diluted with 2 volumes of Hanks's balanced

TABLE 1. Nested PCR primers and their genomic locations

Primer pair	Sequence (5′→3′)	Specificity	Amplified fragment (bp)	Function	Gene	Location[a]
pol A pol B	AGGGGAGGCTATACATGGGCAAGTAAATGC CTGCCTTCTCTGAAATAGACCCGAAAA	HIV-2, SIVmac, SIVagm/sab	555	Outer set	*pol*	4642–5196 (SIVmac/BK28)
pol C pol D	CAGTACATGTTGCAAGTGGATTTATAGA CTTCTTTTAAAATTCATGCAATGAACTGCC	HIV-2, SIVmac, SIVagm/sab	337	Inner set	*pol*	4731–5067 (SIVmac/BK28)
pol C′ pol D′	CAGTACATGTTGCAAGTGAATTCATAGA CTTCTTTTAGAATTCATGCAATGAACTGCC	HIV-2, SIVmac, SIVagm/sab	337	Cloning primers	*pol*	4731–5067 (SIVmac/BK28)
LTR A LTR B	CTGAGACTGCAGGGACTTTCCAGAAGGG AAGCAGAAAGGGTCCTAACAGACCAGGGT	HIV-2, SIVmac	374	Outer set	LTR	403–776 (SIVmac/BK 28)
LTR C LTR D	AGGCTGGCAGATTGAGCCCTGGGAGGTTC CCAGGCGGCGACTAGGAGAGATGGGAGCAC	HIV-2, SIVmac	200	Inner set	LTR	522–721 (SIVmac/BK28)
LTR I LTR II	CAAGGATCCTTCCCTGATTGGCAGAACTAC TAACCAGAGAGACCCAGTACAGGCAAAAAG	HIV-1	406	Outer set	LTR	63–468 (HIV-1/HXB2)
LTR III LTR IV	GACCTTTGGATGGTGCTACAAGCTA CCTGGAAAGTCCCCAGCGGAAAGTC	HIV-1	255	Inner set	LTR	122–376 (HIV-1/HXB2)

[a] Sequences are numbered according to the published nucleotide sequence of SIVmac/BK28 (11a, 34) and HIV-1/HXB2 (50).

salt solution (HBSS) without calcium or magnesium and layered over lymphocyte separation medium. PBMC were isolated by gradient centrifugation at 800 × *g* for 20 min and washed twice with HBSS without calcium and magnesium. High-molecular-weight DNA was extracted from PBMC as follows. Approximately 20×10^6 PBMC were lysed in 500 μl of DNA extraction buffer (10 mM Tris-HCl [pH 7.6], 5 mM EDTA, 0.5% SDS, 100 μg of proteinase K per ml), incubated at 37°C overnight, extracted twice with phenol-chloroform (1:1) and once with chloroform, precipitated with 2 volumes of ethanol, and dissolved in TE buffer (10 mM Tris-HCl [pH 7.6], 1 mM EDTA).

Nested PCR amplifications were performed with uncultured PBMC DNA for template. Genomic DNA (1 to 3 μg/10 μl) was first amplified with an outer set of PCR primers under reduced stringency conditions (denaturing, 94°C, 1 min; annealing, 45°C, 1 min; extension, 68°C, 1 min; 30 cycles) to ensure amplification of divergent viral sequences. PCR products were subsequently analyzed by acrylamide gel electrophoresis (8% nondenaturing gels) for the presence of specific DNA fragments. In the absence of a recognizable amplification product, an aliquot (3 μl) of the initial amplification mixture was then reamplified under the same conditions (denaturing, 94°C, 1 min; annealing, 45°C, 1 min; extension, 68°C, 1 min; 30 cycles) with a second set of PCR primers designed to amplify a DNA fragment internal to the first primer pair (nested primer set). Following this second round of amplifications, products were reexamined for appropriately sized DNA fragments by polyacrylamide gel electrophoresis (PAGE). All positive specimens were then amplified a third time under increased stringency conditions (denaturing, 94°C, 1 min; annealing, 60°C, 1 min; extension, 72°C, 1 min; 30 cycles) with modified second-round primer pairs that contained restriction enzyme sites to facilitate subsequent M13 cloning and sequence analysis. Amplification reactions were performed in a total volume of 100 μl, containing 10 mM Tris hydrochloride (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM deoxynucleotide triphosphates, 20 pmol of each primer, and 2.5 U of *Taq* polymerase (Perkin Elmer Cetus). Samples were routinely overlaid with 100 μl of mineral oil to avoid evaporation. All DNA specimens were analyzed on three separate occasions along with the appropriate number of negative and positive control DNAs.

Oligonucleotide primers were synthesized on a Du Pont (model 300) DNA synthesizer and purified according to the manufacturer's recommendations. A list of the primer pairs tested for suitability to amplify West African viruses, along with their specificity, genomic location, and the predicted sizes of their amplification products, is given in Table 1. Both first- and second-round primer pairs were designed according to the most current compilation of HIV and SIV consensus sequences (44). All three sets of primer pairs were tested individually under various amplification conditions with a reference panel of uncultured HIV-1 and HIV-2 DNAs. The PCR conditions described above represent the lowest stringencies under which specific amplifications could still be detected by gel electrophoresis. Primer synthesis, primer purification, DNA extraction and storage, nested PCR, and subsequent molecular analyses were performed in physically separated laboratories to avoid PCR carryover and sample contamination.

Molecular cloning and DNA sequence analysis. The authenticity of the PCR products was confirmed by molecular cloning into M13mp18 and subsequent nucleotide sequence analysis. Third-round amplification products were cleaved with *Eco*RI and separated on a preparative 2% agarose gel. DNA fragments of appropriate size were identified by ethidium bromide staining and then excised and purified from agarose gel slices by using glass beads (MerMaid; Bio-Rad). Fragments were than subcloned into M13mp18 in both orientations and sequenced by the dideoxynucleotide chain termination method with the M13 universal primer (54). A total of three or four individual M13 clones were sequenced for each amplified DNA specimen. Nucleotide and amino acid sequence alignments were performed with the programs

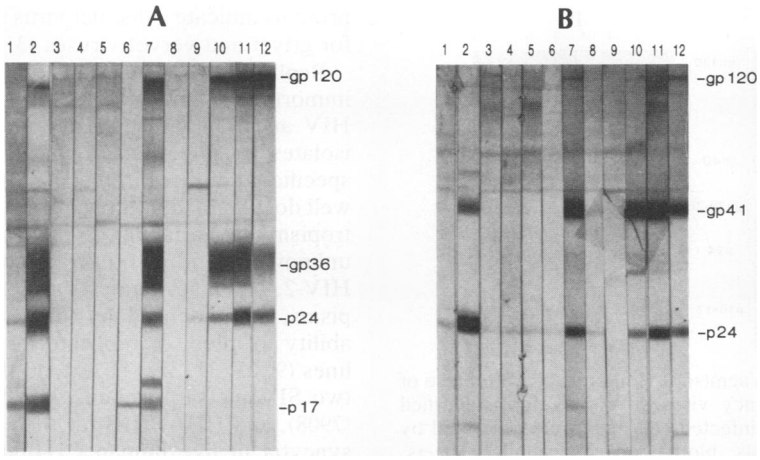

FIG. 1. Western blot analysis of serum samples from 10 randomly selected, wild-caught West African green monkeys. Immunoblot strips were prepared from purified preparations of SIVagm(tyo-1) (A) and HIV-2/ST (B). Individual strips were reacted with serum from a normal human control subject (lanes 1), a rhesus macaque experimentally infected with SIVagm(gri-1) (A, lane 2), a rhesus macaque experimentally infected with SIVmac251 (B, lane 2), as well as sera from 10 West African green monkeys (SAB377, lanes 3; SAB378, lanes 4: SAB379, lanes 5; SAB380, lanes 6; SAB381, lanes 7; SAB382, lanes 8; SAB383, lanes 9; SAB384, lanes 10; SAB385, lanes 11; and SAB386, lanes 12).

Microgenie (Beckman) and PCGene (Intelligenetics). Phylogenetic tree analyses were performed in collaboration with Gerald Myers and Kersti MacInnes, Theoretical Division, Los Alamos National Laboratory, with the MULPARS algorithm of PAUP (version 2.4.2) and the DNABOOT algorithm of PHYLIP (version 3.21) (13, 58).

RESULTS

Serological analysis of 10 wild-caught West African green monkeys. Upon importation, all 10 West African green monkeys were screened by Western blot analysis for antibodies reactive with SIVagm(tyo-1), HIV-1, and HIV-2 proteins. Four of the 10 animals had antibodies which strongly recognized the *env* gene products of SIVagm(tyo-1) and, to a lesser degree, its major core antigen, p24. However, no reactivity was observed to *pol*, and only one sample had slight reactivity to other *gag*-related SIVagm(tyo-1) proteins (Fig. 1A). To determine whether any of the animals harbored viruses more closely related to the HIVs, all sera were also screened for antibodies reactive with HIV-1/IIIB (not shown) and HIV-2/ST proteins (Fig. 1B). The four green monkeys with seroreactivity to SIVagm(tyo-1) also recognized the envelope, and to some extent, the major core antigens of HIV-2, but did not react with any of the HIV-1 envelope glycoproteins. This cross-reactivity with HIV-2 envelope proteins has been observed previously with sera from SIVagm-infected monkeys and presumably results from shared envelope determinants reflecting a closer antigenic relationship of the HIV-2 *env* gene with that of SIVagm (3). None of the SIVagm-seronegative monkeys recognized either HIV-1 or HIV-2 antigens.

Isolation and antigenicity of SIVagm from West African green monkeys. PBMC from the four seropositive monkeys (SAB381, SAB384, SAB385, and SAB386) and two seronegative monkeys (SAB378 and SAB379) were stimulated with PHA and IL-2, cocultivated with Molt4(clone8) cells and CEMx174 cells, and subsequently monitored for evidence of virus infection. Molt4(clone8) cells were selected because this cell line supports infection of most immunodeficiency viruses, including HIV-1, HIV-2, SIVmac, and East African SIVagm isolates (30, 46). CEMx174 cells were included because of their sensitivity for infection by SIVsm, SIVmac, HIV-2, and HIV-1 (25, 33). By day 28, virus was recovered from all Molt4(clone8) cultures derived from seropositive animals, as evidenced by reverse transcriptase activity, p24 antigen in culture supernatants, and characteristic giant-cell formation. No virus or cytopathic effects were detected in CEMx174 cell cultures, nor was virus isolated from the two seronegative animals in either cell line. The four virus isolates, designated SIVagm(sab-1, -2, -3, and -4) to reflect their species origin, represented animals SAB384, SAB385, SAB386, and SAB381, respectively.

The SIVagm(sab) viruses were examined by Western blot analysis to compare the apparent molecular weights and antigenicity of their structural gene products with those of HIV-1, SIVagm(tyo-1), SIVagm(gri-1), and SIVmac251 (7908). Serum from a rhesus macaque experimentally infected with SIVagm(gri-1) was selected for initial identification of SIVagm proteins because it recognized all structural viral proteins of SIVagm(gri-1) (Fig. 2A). Both envelope glycoproteins and *gag*-encoded p24 proteins were recognized from all four SIVagm(sab) isolates (lanes 5 to 8). In addition, SIVagm(gri-1) antibodies detected the $p24^{gag}$ gene product of SIVmac251, SIVagm(tyo-1), and HIV-1/IIIB, reflecting the conserved nature of the *gag* gene among all immunodeficiency viruses. The p24 protein of SIVagm(sab) isolates migrated slightly more slowly than that from the East African vervet (lane 3) and grivet (lane 2) viruses, while HIV-1 could be distinguished by its faster mobility (lane 4). This finding is consistent with our previous results demonstrating size differences among the major *gag*-encoded antigens correlating with the African green monkey species (3). Weaker *env* recognition of the West African viruses was observed with SIVagm(gri-1) antibodies, which suggests significant differences in antigenic determinants between West African and East African SIVagm viruses. While both SIVagm(tyo-1) and SIVmac251 are characterized by a truncated transmembrane protein (gp36 and gp32, respectively), all SIVagm(sab) isolates and the SIVagm(gri) isolate encoded a larger, nontruncated transmembrane glycoprotein (gp40), similar to the nontruncated gp41 expressed by HIV-1.

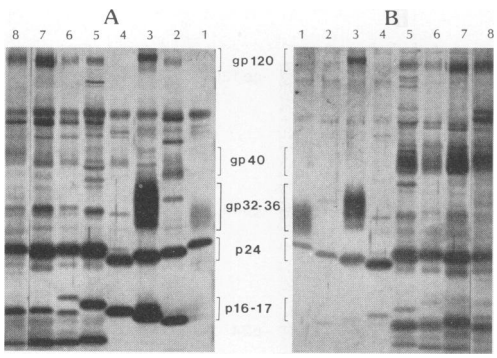

FIG. 2. Comparison of SIVagm(sab) viral proteins with those of other primate immunodeficiency viruses. Viral antigens purified from culture supernatants of infected cell lines were separated by SDS-PAGE gel electrophoresis, blotted on nitrocellulose sheets, and subsequently incubated with serum from a rhesus macaque experimentally infected with SIVagm(gri-1) (A) and a naturally infected West African green monkey (SAB384) (B). Individual lanes represent viral antigens from SIVmac251 (lanes 1), SIVagm(gri-1) (lanes 2), SIVagm(tyo-1) (lanes 3), HIV-1/IIIB (lanes 4), SIVagm(sab-1) (lanes 5), SIVagm(sab-3) (lanes 6), SIVagm(sab-4) (lanes 7), and SIVagm(sab-2) (lanes 8). Because of retarded migration rates in the two outer lanes during gel electrophoresis, proteins in lanes 1 and 2 appear at a relatively higher-molecular-weight position.

Viral proteins were also compared for reactivity with antibodies from an infected West African green monkey (Fig. 2B). Antibodies from SAB384 reacted strongly with the exterior envelope glycoprotein gp120 and the transmembrane envelope protein (gp40-1) of all four SIVagm(sab) isolates. The transmembrane proteins of SIVagm(tyo-1) and SIVmac251 were less reactive, and only slight reactivity to SIVagm(gri-1) *env*-encoded proteins was observed. There was no evidence of cross-reactivity to HIV-1 envelope glycoproteins. Similarly, antibodies from SAB384 also recognized the p24 proteins of all viruses tested, reacted weakly to the p17-like proteins of SIVagm(sab) isolates, and did not recognize those of SIVagm(gri-1), SIVagm(tyo-1), SIV mac251, and HIV-1. Significant differences in either the antigenicity or immunogenicity of the amino-terminal *gag* gene proteins from SIVagm(sab) viruses are likely. Together, these results place the SIVagm(sab) viruses within the SIVagm family of viruses; however, consistent differences in the structural and antigenic nature of the viral proteins indicate a distinct virus species, as noted previously for grivet and vervet viruses (3, 28).

Replication of SIVagm in human T-cell lines. CD4-bearing, immortalized human T-cell lines are routinely used to isolate HIV and SIV viruses. Differences in the ability of HIV-1 isolates to infect macrophages and T cells and isolate-specific differences in their cytopathic properties have been well documented, although the nature of this differential cell tropism and variability in induction of syncytia is presently unknown (14, 17, 19). Furthermore, studies have shown that HIV-2, SIVmac, and SIVagm have restrictive T-cell tropisms, as evidenced by virus-specific differences in their ability to infect or replicate in immortalized human T-cell lines (9, 25, 32, 35). To extend these findings, we compared two SIVagm(sab) isolates with SIVagm(tyo-1), SIVmac251 (7908), and HIV-1/IIIB for their ability to infect and induce syncytia in five human CD4-positive T-cell lines [SupT1, Molt4(clone8), CEMx174, HUT78, and AA2 cells]. As shown in Table 2, SIVagm(sab-1 and sab-2) exhibited in vitro growth characteristics and cell tropism similar to those of SIVagm(tyo-1). West African SIVagm viruses replicated efficiently in Molt4(clone8) cells, as did all other viruses evaluated, and formed characteristic giant cells, but failed to replicate or induce syncytia in CEMx174 cells. In addition, SIVagm(sab-1 and sab-2) as well as SIVagm(tyo-1) formed syncytia with HUT78 cells, but little reverse transcriptase activity was detected in culture supernatants. SIV mac251(7908), on the other hand, infected all cell lines, including CEMx174 cells, but did not fuse with or kill SupT1 cells despite high reverse transcriptase levels which appeared late in infection (day 28). Others have also shown that SIVmac does not readily infect SupT1 cells (25, 32). In comparison, HIV-1/IIIB replicated and induced syncytia in all CD4-bearing T-cell lines examined.

For those SIVagm cultures with low reverse transcriptase activity (below 1,000 dpm), we also analyzed supernatants for virus expression with an SIV p27 antigen capture assay (data not shown), which is known to be more sensitive than the reverse transcriptase assay for detecting SIVs, including both grivet and vervet viruses. No p24 was detected in the CEMx174 cells incubated with the two SIVagm(sab) isolates, and only a weak p24 response was detected with SIVagm(tyo). Furthermore, we failed to detect virus in HUT78 cells incubated with the West African green monkey viruses despite the occurrence of syncytia, while a weak p24 signal was detected with SIVagm(tyo). HUT78 cells therefore appear to be susceptible to SIVagm infection but express only very low levels of virus (46). CEMx174 cells,

TABLE 2. Differential tropism of SIV and HIV isolates for human T-cell lines[a]

Virus	SupT1		Molt4(clone8)		HUT78		CEMx174		AA2	
	RT (dpm)	Syncytia	RT (dpm)	Syncytia	RT (dpm)	Syncytia	RT (dpm)	Syncytia	RT (dpm)	Syncytia
HIV-1/IIIB	14,709	+	13,514	+	5,467	+	6,997	+	16,533	+
SIVmac251	19,874	−	44,171	+	12,188	+	16,816	+	16,279	+
SIVagm(tyo-1)	12,422	+	1,522	+	463	+	213	−	877	+
SIVagm(sab-1)	54,724	+	24,737	+	123	+	69	−	10,015	+
SIVagm(sab-2)	59,589	+	64,751	+	70	+	115	−	15,120	+

[a] Reverse transcriptase (RT) activity was determined from culture supernatants at 1, 4, 7, 14, 21, and 28 days after infection. Values represent the means of duplicate assays (minus background activity from uninfected cultures) and indicate the peak activities during the 28-day period. Values below 1,000 dpm were confirmed as either negative or positive by antigen capture assay for SIV p27. Supernatants from HUT78 and CEMx174 cells were weakly positive for SIVagm(tyo-1) core antigen, while both cell lines were negative for core antigens of sabaeus isolates. Syncytium formation was recorded on a daily basis. In general, cultures were considered positive when each microscopic field contained approximately 10 or more syncytia (magnification, 100×).

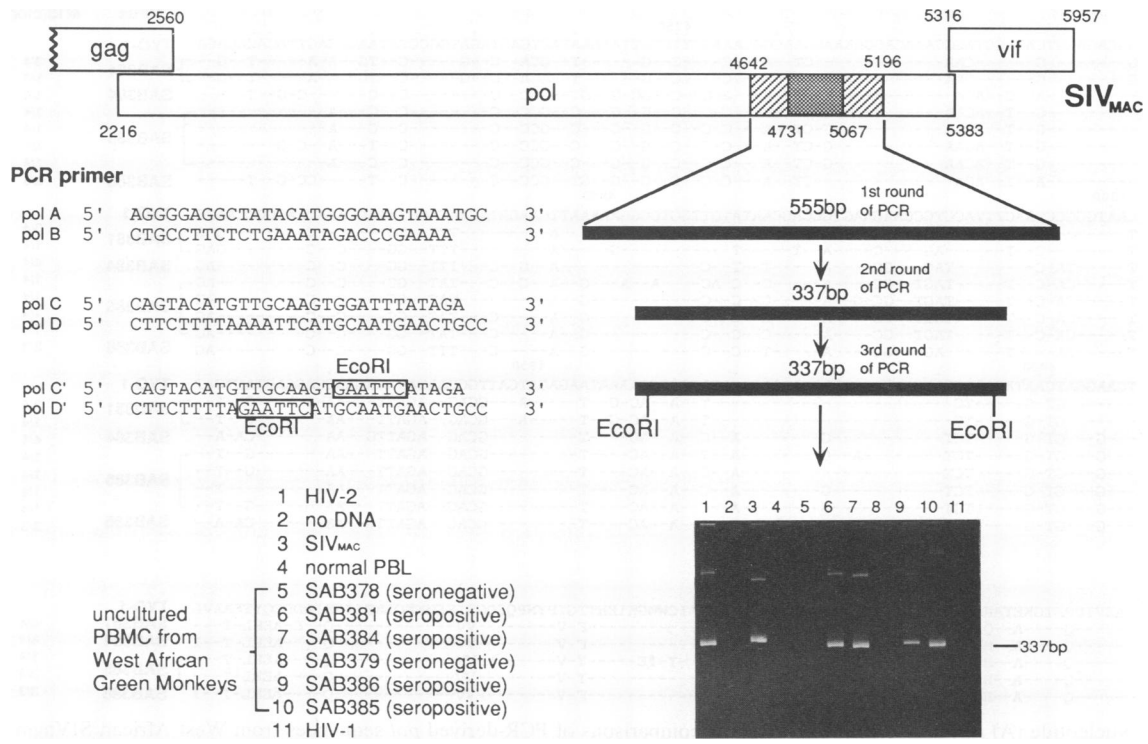

FIG. 3. PCR amplification of SIV sequences from uncultured PBMC DNA from four seropositive West African green monkeys. Three sets of nested PCR primers are depicted, along with their relative locations within the SIVmac *pol* open reading frame and the predicted sizes of their respective amplification products (light and dark shaded boxes indicate first- and second-round amplification products, respectively). *polC'* and *polD'* are cloning primers with *Eco*RI sites for M13 cloning and sequence analysis. An ethidium bromide-stained polyacrylamide gel identifies second-round amplification products from uncultured PBMC DNA from four seropositive West African green monkeys (SAB381, lane 6; SAB384, lane 7; SAB386, lane 9; and SAB385, lane 10). Amplification of positive and negative control DNAs is also depicted (HIV-2/ST, lane 1; no DNA, lane 2; SIVmac/BK28, lane 3; normal human PBMC, lane 4; HIV-1/IIIB, lane 11).

however, appear to be resistant to West African SIVagm viruses.

Direct amplification of SIVagm(sab) sequences from uncultured PBMC by nested PCR. Derivation of persistently infected T-cell lines is thought to artificially select only a subpopulation of the virus strains present in vivo (41) and, on occasion, fails to propagate primate immunodeficiency viruses to high titers. For these reasons, we investigated the use of nested PCR analysis to identify and characterize SIV infections directly in primary tissue specimens (1, 42, 56). As a template for the PCR reaction, we chose DNA extracted from uncultured PBMC from the West African green monkeys under study. Three different sets of nested primer pairs were synthesized according to HIV and SIV consensus sequences. Two sets of primers were designed to target highly conserved *pol* and long terminal repeat (LTR) sequences in divergent HIV-2/SIVmac/SIVsm viruses, while a third set was designed to amplify highly conserved LTR sequences in divergent HIV-1 viruses (Table 1). We selected these particular primers pairs for initial screening purposes because SIVagm consensus sequences from divergent isolates were not available. In addition, we speculated that the West African SIVagm viruses might be relatively more closely related to other West African HIV and SIV viruses because of their geographic proximity.

DNA specimens from six study animals were analyzed under code along with negative and positive control DNAs so as not to bias PCR results based on the known serological status of the animals (Fig. 3). Using between 1 and 3 μg of total genomic DNA, we found that only the HIV-2/SIVmac *pol* primer pairs successfully amplified West African SIVagm sequences. Neither of the other two primer sets yielded specific amplifications, including the HIV-2/SIVmac LTR primers (data not shown). After two rounds of PCR, a 337-bp fragment was reproducibly detected in uncultured SAB381, SAB384, SAB385, and SAB386 DNAs but not in SAB378 and SAB379 PBMC DNAs (Fig. 3). First-round amplifications repeatedly failed to produce DNA fragments visibly detectable by ethidium bromide staining (data not shown). Amplification of SIVagm sequences also required substantially lower primer annealing and extension temperatures than HIV-2- and SIVmac-containing specimens. This, along with the failure to achieve amplification with LTR primer pairs specific for HIV-1, HIV-2, and SIVmac, suggested that the West African SIVagm sequences were likely to be genetically quite divergent. Importantly, our ability to amplify DNA from uncultured green monkey PBMC DNAs correlated 100% with the presence or absence of SIVagm antibodies in the same animals.

Sequence analysis of PCR-amplified *pol* fragments. To confirm that the amplified PCR fragments indeed represented independent viruses and not endogenous primate sequences or inadvertent PCR contamination products, we introduced *Eco*RI restriction enzyme sites in the inner set of the *pol* primer pairs to facilitate subsequent M13 cloning and sequence analysis (Fig. 3). Second-round amplifications with these modified primers, however, failed to amplify viral DNA from known-positive PBMC DNAs. We concluded

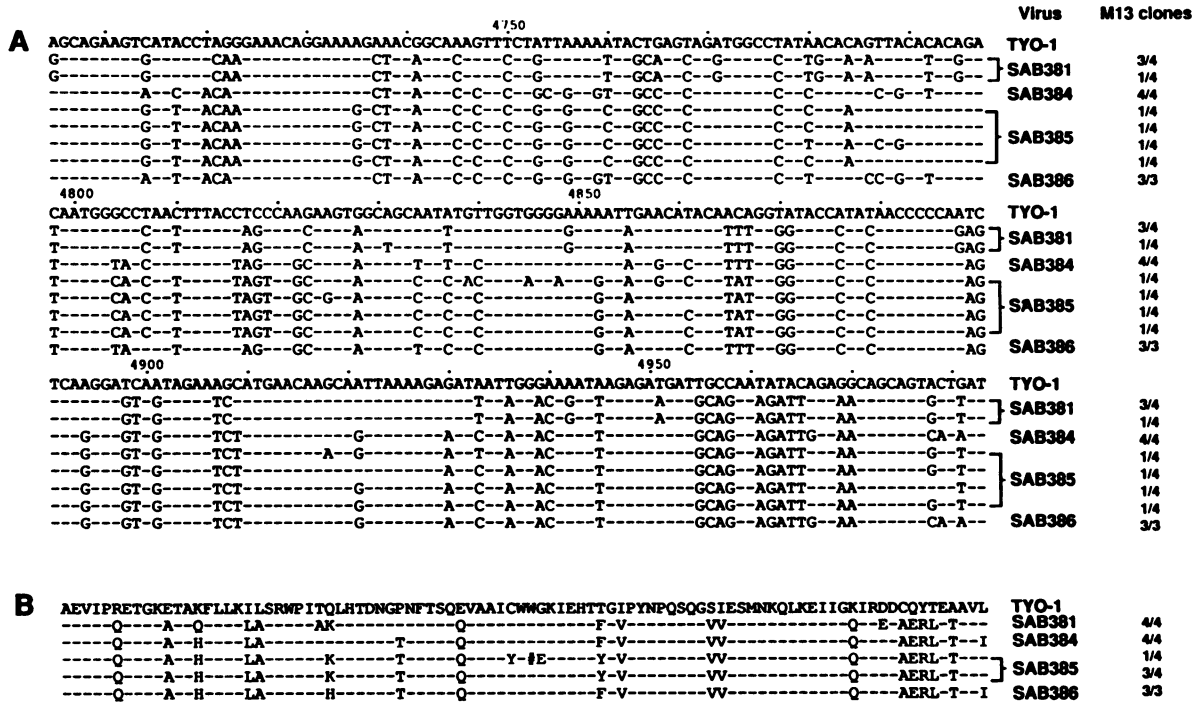

FIG. 4. Nucleotide (A) and amino acid (B) sequence comparisons of PCR-derived *pol* sequences from West African SIVagm viruses. A 279-bp *pol* sequence from SAB381, SAB384, SAB385, and SAB386 is compared with the corresponding *pol* region in SIVagm(tyo-1) (primer sequences *pol*C′ and D′ are not included). Sequence changes shown are indicated in reference to the published SIVagm(tyo-1) sequence (15). Dashes represent sequence identity. The frequencies of M13 clones with identical sequences, along with the total number of clones analyzed, are shown. A pound sign marks the position of an in-frame stop codon in one of the four SAB385 sequences.

that the nucleotide sequence changes introduced by the generation of *Eco*RI cloning sites had significantly altered the annealing potential of the primers to native West African SIVagm sequence. We therefore subjected second-round amplification products to a third round of PCR with the *Eco*RI-containing primer pairs under increased stringency. Because of the unknown effect of repeat amplifications on the overall frequency of PCR-induced sequence changes, we also subjected DNA from an SIVmac control cell line, originally transfected with a single provirus of known nucleotide sequence (34), to the same number of PCR amplification cycles (Fig. 3, lane 3).

Sequence analysis was performed on 15 individual M13 clones containing *pol* fragments from four West African SIVagm viruses (Fig. 4A) as well as on three M13 clones containing SIVmac *pol* fragments (data not shown). Each of the clones comprised 279 bp of viral *pol* sequence, excluding the primer pairs. Comparison of the SIVmac clones revealed only minor sequence variability (two base-pair changes in two clones, and no change in the third clone) with respect to the sequence of the parental SIVmac/BK28 provirus. M13 clones representing the four West African SIVagm viruses, on the other hand, differed by as many as 40 nucleotides from each other and by as many as 80 bp from SIVagm-(tyo-1) (Fig. 4A). These data confirmed that the Senegalese green monkeys were infected with independent SIVagm strains which, as a group, differed considerably from East African SIVagm viruses. The contribution of repeated PCR amplifications to the number of sequence differences observed among independent SIVagm viruses was not significant.

PCR analysis of uncultured PBMC DNA also allowed us to evaluate the extent of SIV sequence variability within the same animal. Interestingly, individual M13 clones derived from SAB384 and SAB386 exhibited no sequence changes, while individual clones derived from SAB385 differed from each other by as much as 3.6% in nucleotide sequence. Most sequence differences within the same isolate did not result in corresponding amino acid sequence changes (Fig. 4B), and there was only one M13 clone derived from SAB385 which contained an in-frame stop codon. The results thus suggest that West African green monkeys, like humans (41), can be infected in vivo with a mixture of genotypic variants which include defective proviral genomes.

Identification of a genetically unique subtype of SIVagm in West African green monkeys. Pairwise alignments of the deduced *pol* product amino acid sequences determined the degree of genetic variability among different SIVagm strains and revealed their relatedness to HIV-1 and HIV-2 (Fig. 5). West African sabaeus viruses were highly related to each other, with amino acid sequence differences ranging between 1.1 and 6.5%. Other strains of SIVagm differed significantly more, although the degree of diversity was not uniformly distributed among them. While one virus strain, SIVagm(gri-1), exhibited a particularly high degree of sequence variation with respect to all other African green monkey viruses (greater than 20% amino acid sequence differences), considerably less variation was noticed among the other four SIVagm virus sequences (3.3 to 7.6% amino acid sequence changes). Interestingly, SIVagm(gri-1) was the only isolate derived from a grivet monkey (3, 27; unpublished data), while the other four SIVagm viruses either represented known vervet viruses (ver-2 [3]) or likely vervet viruses (tyo-1, agm155, agm3) based on their geographic origin (5,

		SAB384	SAB385	SAB386	SAB381	TYO-1	AGM155	VER-2	AGM3	GRI-1	HIV-1
sabaeus	SAB384	—									
	SAB385	96.7	—								
	SAB386	98.9	96.7	—							
	SAB381	93.5	94.6	93.5	—						
vervet	TYO-1	80.4	80.4	79.3	79.3	—					
	AGM155	78.3	79.3	79.3	78.3	92.4	—				
	VER-2	81.5	81.5	80.4	80.4	93.5	94.6	—			
	AGM3	81.5	81.5	80.4	80.4	96.7	95.7	96.7	—		
grivet	GRI-1	80.4	80.4	81.5	79.3	80.4	79.3	81.5	80.4	—	
	HIV-1	70.7	69.6	70.7	69.6	62.0	62.0	64.1	62.0	69.6	—
	HIV-2	68.5	68.5	69.6	69.6	60.9	62.0	63.0	63.0	62.0	62.0

FIG. 5. Amino acid sequence homologies among primate immunodeficiency viruses. The predicted amino acid sequence of a 279-bp PCR-derived *pol* fragment from West African SIVagm viruses (SAB384, SAB385, SAB386, and SAB381) is compared with the corresponding region of East African vervet (tyo-1, agm155, ver-2, and agm3) and grivet (gri-1) viruses as well as with HIV-1/HXB2 and HIV-2/ST. The numbers indicate the percentage homology among pairwise aligned sequences. Boxes differentiate the degree of sequence variability among SIVagm strains derived from different African green monkey species (open, less than 10% sequence variation; shaded, between 18 and 22% sequence variation). SIVagm(tyo-1), SIVagm155, SIVagm3, HIV-1/HXB2, and HIV-2/ST sequences have been reported (5, 15, 28, 36, 50). SIVagm(ver-2) and SIVagm(gri-1) and their nucleotide sequences have been described elsewhere (3, 14a, 21a).

27, 28, 35, 46). These data thus suggested that the vervet, grivet, and sabaeus species each harbored their own individual SIVagm type. The sequence comparison also placed the various SIVagm viruses in relationship with the human viruses. Despite their similar geographic origin, West African SIVagm viruses differed from HIV-2 to the same degree as they differed from HIV-1 (30% amino acid sequence differences).

To better define the evolutionary distances of West African SIVagm viruses to other primate lentiviruses, we undertook a phylogenetic tree analysis of their PCR-derived *pol* sequences (Fig. 6). The analysis confirmed an overall branching order consistent with the division of viruses into four major subgroups (group 1, HIV-1 and SIVcpz; group 2, HIV-2, SIVsm, and SIVmac; group 3, SIVmnd; and group 4, SIVagm). The resulting phylogenetic tree, however, also revealed a distinct separation of West and East African SIVagm sequences within the SIVagm superfamily and in addition indicated that vervet and grivet viruses were evolutionarily more closely related to each other than to the sabaeus viruses. Because of the limited sequence data available, the computer predicted six equivalent tree diagrams (data not shown). Differences were most pronounced among the relative positions of the mandrill sequence, which clustered both with East and West African SIVagms and with the HIV-2/SIVmac/SIVsm group of viruses. The position of the grivet sequence was also variable in two of the six tree diagrams, where it clustered within the group of vervet viruses rather than as a separate group. The separate grouping of vervet and sabaeus sequences, however, remained unchanged in all six alternative diagrams. Since bootstrap analysis, which represents an alternative method to determine branching orders (13), confirmed the branching order depicted in Fig. 6, we selected this particular tree to represent our results.

DISCUSSION

Phylogeny of SIVagm viruses: evidence for evolution coincident with the host species. In the present report, we examined a previously unrecognized strain of SIVagm from West African green monkeys and compared the biological, antigenic, structural, and molecular properties of four of these viruses with those of isolates from East African green monkeys. Our results show that seroprevalence rates in wild-caught West African animals appear to be comparable to those identified in other African green monkey populations (3, 22, 29, 40, 46). Antibodies from infected African green monkeys detected the viral proteins of every SIVagm strain tested regardless of geographic location, whereas significantly less cross-reactivity to HIV-2 envelope proteins and only *gag* gene reactivity to HIV-1 were observed, suggesting a close antigenic relationship among SIVagm strains. Furthermore, the SIVagm isolates, whether from East or West African green monkeys, displayed similar restrictions in human T-cell tropism which were distinguishable from HIV-1 and SIVmac cell tropisms (Table 2), indicating a similarly close biologic relationship between SIVagm viruses (3, 9, 46). Our results, however, also demonstrate that West African SIVagm viruses are characterized by structural and molecular properties which distinguish them from previously studied East African SIVagm strains. These include characteristic differences in the electrophoretic profiles of their major structural core proteins and significant nucleotide sequence variation. Phylogenetic analysis of available sequence data strongly suggests the existence of a distinct species of SIVagm unique to West African green monkeys.

In order to fully understand the significance of genotypic and phenotypic variability among SIVagm viruses, it is important to address the phylogenetic relationships among their respective primate hosts to clues to the origins of these viruses. The taxonomic classification scheme for African green monkeys (*C. aethiops*) and the *Cercopithecus* genus in general has changed frequently in recent years. Disagreement among primatologists regarding the exact species and subspecies classification of African green monkeys is common because current classification schemes rely primarily on phenotypic markers, including facial features and coloration, while more definitive genetic data are lacking (31, 37, 45). In this article, we have adopted the most recent classification scheme described in a review by Lernould (37). The term African green monkey has been used loosely by primatologists and retrovirologists and can taxonomically be consid-

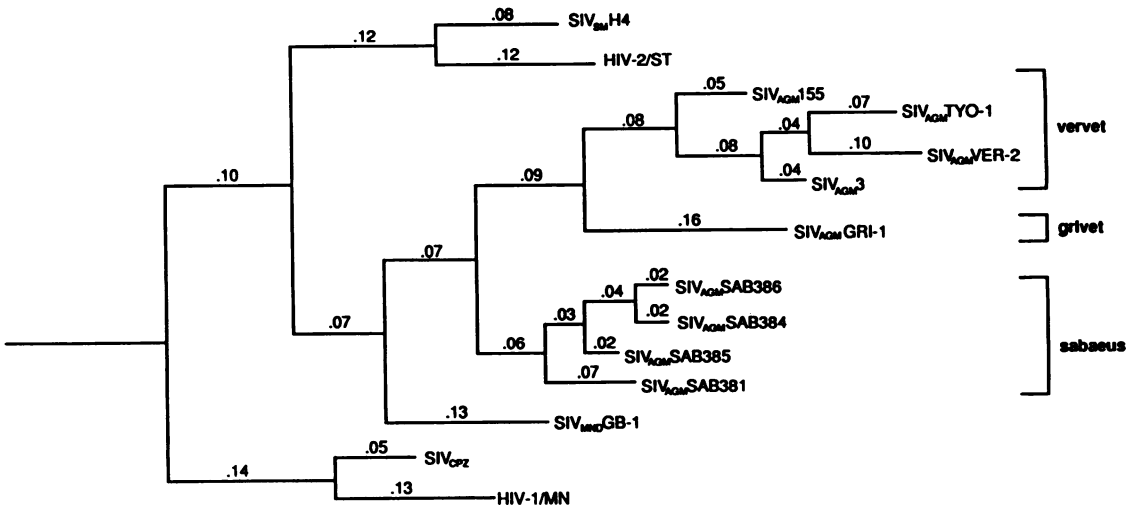

FIG. 6. Minimum-length evolutionary tree depicting the relationship of West African SIVagm viruses to other known HIV and SIV viruses. PCR-derived West African green monkey *pol* sequences were analyzed along with the corresponding *pol* sequences of other lentiviruses. The total number of sites examined was 279, of which 175 were variable. The consistency index was 0.542. The lengths of the horizontal lines are proportional to the minimum number of single-nucleotide sequence substitutions required to generate the observed variation (also expressed by the ratio between branch length and total number of sites above each line). The length of the vertical lines is for clarity only. The tree was constructed by using the multiple parsimony (MULPARS) algorithm of PAUP (version 2.4.2) as described previously (13, 58). The tree was rooted on a random sequence (not shown) generated by the MARKOVALL program. Five other equivalent minimal trees were also obtained, the principal differences stemming from the branching positions of mandrill and grivet sequences. The tree shown above agreed best with an alternative tree diagram generated by DNABOOT, a different algorithm belonging to the PHYLIP version 3.21 package (13). Sequences in addition to those referred to in Fig. 5 are SIVsmH4 (24), SIVmndGB-1 (60), SIVcpz (26), and HIV-1/MN (20).

ered a superspecies (*C. aethiops*) encompassing four separate species which are phenotypically and genetically distinct. Regardless of the precise taxonomic classification, their geographical distribution is restricted to specific areas of sub-Saharan Africa and is depicted in Fig. 7.

It has been hypothesized that these four species have evolved from a common ancestral African green monkey (superspecies) which migrated throughout sub-Saharan Africa in the distant past and has only recently (10,000 years ago) diverged into separate species (31). Considerable heterogeneity within each species of African green monkey has also been observed and necessitates further classification into a variety of subspecies (45). The largest species of African green monkeys is the vervet (*C. pygerythrus*), which comprises 14 separate subspecies ranging from Southern Ethiopia to South Africa and Angola. The grivet monkey (*C. aethiops*) has four subspecies which are limited to Ethiopia and the Sudan, while tantalus monkeys (*C. tantalus*) comprise three subspecies ranging from Uganda to the Volta River. In this study, we analyzed SIVagm viruses from a fourth species of African green monkey which resides exclusively in West Africa (*C. sabaeus*) and which comprises only a single subspecies.

Speculating that different African green monkey species might harbor different SIVagm types, we examined available biological and molecular data from West and East African green monkey viruses with respect to species-specific differences. Our laboratory had previously identified characteristic size differences between the p24 proteins of vervet and grivet viruses, which allowed the determination of their species origin in a blind study (3). Based on these findings, we hypothesized that the SIVagm group of viruses had evolved and diverged coincident with the evolution of their African green monkey host. The present study of West African SIVagm isolates also demonstrated similar core protein size differences (Fig. 2), and molecular analysis of West African SIVagm further confirmed the existence of species-specific genetic differences. Individual members of the same African green monkey species consistently differed by less than 8% in amino acid sequence within the PCR-derived *pol* fragment, while members of different species varied by at least 19% (Fig. 5). This correlation between sequence and species origin was substantiated by phylogenetic tree analysis, which reproducibly identified distinct sequence clusters for sabaeus and vervet sequences (Fig. 6). The most convincing data in support of parallel evolution of virus and host, however, resulted from a published phylogenetic study of the entire *Cercopithecus* genus. Comparing the electrophoretic mobility of 14 different protein markers, Rufolo constructed a tree diagram of 18 different *Cercopithecus* species which also depicted the phylogenetic position of vervet, grivet, and sabaeus monkeys (51). Importantly, the branching order of these three African green monkey species was identical to the branching order of their respective viruses. Like their viruses, vervet and grivet monkeys were evolutionarily more closely related to each other than they were to the sabaeus monkeys. Together, these results strongly support our previous hypothesis that African green monkey viruses have evolved coincident with their natural primate host.

While present data point toward the possibility of species-specific SIVagm strains, definitive conclusions will require further study. Most importantly, the amount of sequence information currently available for SIVagm isolates from different species is limited, and a fourth species (tantalus monkey) has yet to be analyzed. In addition, there is evidence for strain-specific variation even within the same African green monkey species. Recent comparisons of LTR and *gag* sequences from East African SIVagm viruses identified a vervet isolate which differed substantially from grivet

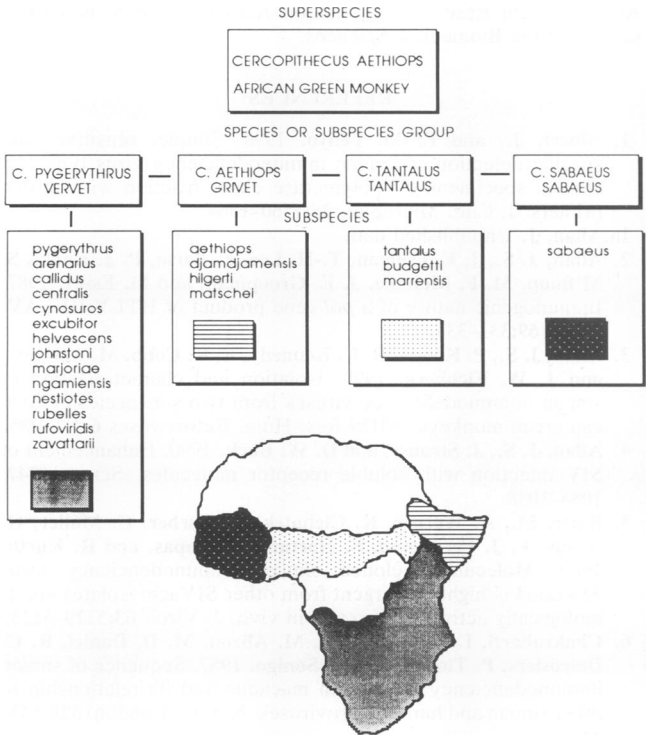

FIG. 7. Geographic distribution of major African green monkey species on the African continent. The approximate locations were compiled from Kingdon (31), Napier (45), and Lernould (37).

as well as other vervet isolates (27). Since most species of African green monkeys are represented by a number of subspecies, it is possible that significant variation may exist among viruses isolated from monkeys within a given species but representing different subspecies. In addition, genotypic recombination between SIVagm proviruses could further obscure comparisons of observed sequence variations. It should be pointed out that the suitability of different genomic regions for correct predictions of evolutionary distances and their minimum lengths are still unknown. Moreover, information on most SIVagm viruses reported thus far includes their geographic location but not their species or a subspecies origin (5, 9, 28, 35, 46). Complete nucleotide sequences of several well-defined vervet, grivet, sabaeus, and tantalus viruses will be necessary to ultimately determine their evolutionary relationship. SIV-like viruses have also been identified in many of the primate species that comprise the *Cercopithecus* genus, including DeBrazza's monkeys, Sykes monkeys, talapoins, patas monkeys, and others (1a, 40). Genetic comparisons of these monkey viruses may provide further evidence for virus-specific sequence variation coinciding with the phylogeny of the host species.

It should be pointed out that our estimate of the time of SIVagm virus divergence (approximately 10,000 years) differs from that in previously published reports on lentivirus evolution. Fukasawa et al. suggested that HIV and SIV viruses diverged in concert with the evolution of their primate host, implying a time period of millions of years (15). Querat et al. estimated that the radiation of all lentiviruses occurred some 400 years ago, with primate lentiviruses having diverged within the last 200 years (49). Similar time spans are proposed by Sharp and Li, who estimated that HIV-1, HIV-2, and SIVagm diverged 140 to 160 years ago (55). An inherent problem with these calculations lies in a number of assumptions for which there are presently no experimental data. All mathematical models assume that mutational rates are constant over time. Genetic variation among viruses in different animal species is also assumed to be identical. Mutational rates may also differ depending on the pathogenicity of the virus in its host. Natural infections with little or no pathogenicity and "unnatural" infections, in which the virus is transmitted to a nonadapted species, may influence mutational frequencies. In addition, there are certain practical considerations which are not addressed in the interpretation of most computer-driven models. In many instances, it is assumed that HIV-1 and HIV-2 have been present in the human population as long as SIVagm has been present in monkeys and diverged at the same time, although cross-species transmission from an animal reservoir is regarded as a more likely possibility, which affects the dating of phylogenetic trees (12, 55). Furthermore, given the geographical distances and the relative isolation of certain African nonhuman primate species, it is also difficult to imagine how an ancestral virus infected all species of green monkeys in only 200 years. Sabaeus monkeys, for example, live in an isolated area and do not interbreed with other African green monkey species. In fact, their speciation results from this isolation. In order to account for such a rapid spread of SIVagm in isolated regions of Africa, one would need to invoke other modes of transmission, including insect vectors, although present data indicate that these viruses are strictly transmitted by close physical contact. We believe that the time estimates currently available for lentivirus evolution are biased toward lentivirus radiation in an unnatural host (HIV-1, HIV-2, SIVmac). Mutational rates may be much lower in a natural host and dependent on differences in replication rates, viral latency, transmission frequencies, and host selection pressures, all of which contribute to the emergence of virus variants. As an example, we have observed significant immunological differences between HIV- and SIVagm-infected hosts. African green monkeys lack circulating neutralizing antibodies yet remain asymptomatic, whereas infected humans generate neutralizing antibodies yet develop AIDS (4). Similarly, the *pol* gene products of HIV-1 are highly immunogenic in the humans (2), while naturally or experimentally infected African green monkeys lack humoral responses to these proteins (1a, 3). Since genetic variation is influenced by host immunologic selection pressures, it is easy to see how host-related differences might translate into significant differences in the mutational rates of these viruses. There are many factors that might influence mutational rates in various host species, and until these questions have been addressed adequately, the evolutionary history of primate lentiviruses will remain an area for debate.

Nested PCR analysis with generic oligonucleotide primers provides a rapid means to identify novel HIV and SIV strains in uncultured tissue specimens. Recent advances in PCR technologies have opened new avenues for the molecular and biological characterization of human retroviruses (52). PCR amplification of small viral fragments for diagnosis (1), phylogenetic studies (27), and the elucidation of viral determinants of pathogenicity (11) represent only a few examples of a successful application of this method for the study of primate lentiviruses. In this study we used a nested PCR protocol (42), incorporating generic oligonucleotide primers, to detect and genetically characterize highly divergent SIV viruses directly from uncultured monkey PBMC DNA.

We selected the nested amplification procedure for our

purposes because this technique offers several advantages over the conventional (single-round) PCR technique (42). First, nested PCR amplifies viral sequences in quantities that can be visualized by ethidium staining of agarose or polyacrylamide gels, and does not require radioactive probes or end-labeled primers for the detection of amplification products (1, 11, 56). In addition, viral sequences can be amplified even from low-level-infected primary material in quantities sufficient for subsequent molecular cloning and sequence analysis (24, 41, 41a, 56). Nested PCR is also highly specific and ensures that only desired sequences are amplified (1, 56). Samples can be subjected to reduced-stringency PCR conditions without accumulation of unspecific amplification products. This is particularly important when amplification of highly divergent sequences is being attempted (27). Finally, this technique does not depend on the success or failure of virus isolation by tissue culture methods. Nested PCR amplification of DNA from uncultured material represents an alternative to virus propagation in cell culture for obtaining sufficient amounts of viral sequences for genetic and biological analysis (23, 41, 56). In addition, this technique facilitates the retrieval of viral DNA as it exists in vivo and excludes any bias which may have been introduced in the analysis of viral sequences from culture-selected variants (41).

For genetic analysis of the West African SIVagm viruses, we PCR amplified, cloned, and sequenced a 279-bp DNA fragment from a highly conserved region within the viral *pol* gene. Although this is a small fragment, nucleotide sequence analysis indicated that each isolate had unique sequences which could be used to estimate the genetic relationship of these viruses to previously characterized SIVagm isolates. Importantly, a PCR fragment of similar size and location was recently reported by other investigators to accurately reflect the degree of genotypic variation in East African SIVagm strains (39). The combination of nested PCR, generic oligonucleotide primer, and detailed serology should thus be of considerable value for future studies in Africa, where highly divergent strains of both HIV and SIV are known to exist. This approach is likely to prove particularly useful in field studies of feral animals where the infrastructure necessary for virus culture is often not available. PCR amplification and sequence analysis of small viral fragments directly from PBMC DNA will allow investigators to quickly estimate the genetic properties of newly identified viruses and will facilitate decisions as to whether additional experiments, including viral culture, proviral cloning, etc., should be attempted. M13 cloning can be avoided since direct sequencing of double-stranded PCR products has been described (56). In light of the simplicity of this PCR-based approach, it is not unreasonable to expect that molecular epidemiological data on feral African monkey populations, greatly needed for the understanding of the origin and natural history of all simian and human immunodeficiency viruses, will soon be forthcoming.

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