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Genetic Characterization of New West African Simian Immunodeficiency Virus SIVsm: Geographic Clustering of Household-Derived SIV Strains with Human Immunodeficiency Virus Type 2 Subtypes and Genetically Diverse Viruses from a Single Feral Sooty Mangabey Troop

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It has been proposed that human immunodeficiency virus type 2 (HIV-2) originated from simian immunodeficiency viruses (SIVs) that are natural infections of sooty mangabeys (*Cercocebus torquatus atys*). To test this hypothesis, SIVs from eight sooty mangabeys, including six new viruses from West Africa, were genetically characterized. *gag* and *env* sequences showed that while the viruses of all eight sooty mangabeys belonged to the SIVsm/HIV-2 family, each was widely divergent from SIVs found earlier in captive monkeys at American primate centers. In two SIVs from sooty mangabeys discovered about 100 miles (ca. 161 Km) from each other in rural West Africa, the amino acids of a conserved *gag* p17-p26 region differed by 19.3%, a divergence greater than that in four of five clades of HIV-2 and in SIVs found in other African monkey species. Analysis of *gag* region sequences showed that feral mangabeys in one small troop harbored four distinct SIVs. Three of the newly found viruses were genetically divergent, showing as much genetic distance from each other as from the entire SIVsm/HIV-2 family. Sequencing and heteroduplex analysis of one feral animal-derived SIV showed a mosaic genome containing an *env* gene that was homologous with other feral SIVsm *env* genes in the troop but having a *gag* gene from another, distinct SIV. Surprisingly, a *gag* phylogenetic tree based on nucleotide sequences showed that the African relatives closest to all three household-derived SIVs were HIV-2 subtypes D and E from humans in the same West African areas. In one case, the SIV/HIV-2 cluster was from the same village. The findings support the hypothesis that each HIV-2 subtype in West Africans originated from widely divergent SIVsm strains, transmitted by independent cross-species events in the same geographic locations.

AIDS is caused by two distinct retroviruses, human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) (2, 7, 10). On the basis of phylogenetic and biological properties, both types of HIV are in the lentivirus family of retroviruses. However, because both HIV-1 and HIV-2 are more closely related to the simian immunodeficiency virus (SIV) group found in African and Asian nonhuman primates (4, 11, 24, 26, 31, 44) than to lentiviruses from other species, such as horses, cats, goats, and bovines (5, 20, 21, 48), a simian origin for HIV has been hypothesized.

SIV is widely distributed in African primates, having been found in four subspecies of African green monkeys (SIVagm) (1, 33, 43, 47) and in the mandrill (SIVmnd) (59), Syke's monkey (SIVsyk) (11), sooty mangabey (SIVsm) (6, 16, 24, 36, 41, 44, 50), and chimpanzee (SIVcpz) (49), but is not naturally found in Asian macaques (36, 47, 62). These SIV-positive simian species, which inhabit all parts of sub-Saharan Africa (61), provide evidence that African simians and not Asian macaques are the natural hosts of SIV. HIV-1 and HIV-2 are most closely related to SIVcpz and SIVsm, respectively (24, 26). Therefore, HIV-1 and HIV-2 may have originated from two or more separate cross-species transmissions of SIV from chimpanzees and mangabeys.

The range of the sooty mangabey, *Cercocebus torquatus atys*, includes Liberia, Sierra Leone, and the western third of the Ivory Coast (61). The sooty mangabey is a subspecies of the *C. torquatus* group, which includes two other subspecies, the white-collared mangabey, *C. torquatus lunulatus*, and the red-capped mangabey, *C. torquatus torquatus* (61). Thus far, no unique SIVs have been found in the other two mangabey subspecies or in any other species of African mangabey (45, 58). SIV found in *C. torquatus lunulatus* was probably acquired in captivity from SIV-infected African green monkeys housed in the same facility (58). Therefore, the sooty mangabey remains the only known natural simian host of HIV-2-like viruses.

Sooty mangabeys were brought to America during the 19th century for zoos and private collections (37). Establishment of American primate centers in the 1960s (3), however, led to the introduction of SIV into macaques. SIVmac was identified in rhesus and cynomolgus macaques after an outbreak of lymphoma at the New England Regional Primate Research Center from 1972 to 1980 (8, 29). Although there were no sooty mangabeys at that primate center, SIVmac was later traced to the California Primate Center, which did house sooty mangabeys at that time (38). An AIDS-like disease in a macaque colony at Tulane Regional Primate Research Center was also traced to sooty mangabeys (44). Furthermore, surveys of macaques in Asia have not yielded evidence of SIV infections in

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TABLE 1. Species distribution of feral monkey troops and household pets in Sierra Leone and Liberia

Subjects	Monkey species	Total no. observed[a]	No. positive/ no. tested
Feral troop 1	*Cercocebus atys*	22	4/10
	Colobus verus	1	NT[b]
Feral troop 2	*Cercocebus atys*	9	0/4
	Cercopithecus campbelli	11	0/11
	Colobus verus	1	0/1
Feral troop 3	*Cercocebus atys*	17	0/4
Household pets	*Cercocebus atys*	96	4/91
	Papio papio	6	0/3
	Cercopithecus aethiops sabeus	18	0/14
	Erythrocebus patas	2	NT
	Pan troglodytes	8	NT
	Cercopithecus diana	1	0/1
	Cercopithecus petaurista	1	0/1
	Cercopithecus campbelli	4	0/4

[a] Samples could not be obtained from some primates.
[b] NT, not tested.

the genus *Macaca* (36, 47, 62), indicating that macaques are not a natural host. Additional evidence of the sooty mangabey origin of SIVmac is provided by the fact that an HIV-2-related SIV was found in a West African household pet sooty mangabey (41) that had no known laboratory contact. Finally, more recently, we documented the first SIV isolate (SIVsmSL92a) from a free-ranging feral sooty mangabey in West Africa (6). Because SIVsmSL92a and SIVsmC12, an isolate from two captive sooty mangabeys in an Ivory Coast zoo (50), are both genetically divergent from HIV-2 and SIVsm found in the United States, sooty mangabeys appear to have been infected with SIV in their natural habitat before being brought to North America.

Significant evolutionary relationships have been discerned between SIVsm/SIVmac and one of five HIV-2 subtypes identified thus far in humans. That one, subtype D (HIV-2F0784), found in a Liberian, was closer to SIVsm than to other HIV-2 isolates (18). Since all five HIV-2 subtypes were almost equidistant from each other, with about 17 to 24% nucleotide intersubtype diversity, it was hypothesized that HIV-2 subtypes emerged from multiple introductions of genetically diverse sooty mangabey lentiviruses (17, 54). Thus far, however, no such diversity has been found in SIVsm from sooty mangabeys in their natural West African habitat.

To study the phylogenetic relationship between SIVsm and HIV-2 and to determine if SIV divergence is indicative of multiple cross-species transmission to humans in West Africa, we collected specimens from feral and household pet sooty mangabeys in Sierra Leone and Liberia. We report on eight distinct SIVsm strains and present evidence for new clusters within the SIVsm/HIV-2 family. Interestingly, one new SIVsm was a recombinant virus that occurred naturally in a wild sooty mangabey. We observed highly divergent SIVs in both free-ranging feral and household pet sooty mangabeys. Finally, we found that viruses from household pets in Liberia and Sierra Leone clustered with those in persons from nearby villages within the same country, the first evidence of a geographic association between naturally occurring SIVsm and HIV-2 in West Africa.

MATERIALS AND METHODS

Specimens. Samples were collected from most of the animals listed in Table 1. In Liberia, household pet samples were collected from 1988 to 1991 and in Sierra Leone from 1992 to 1993. Peripheral blood mononuclear cells and plasma were separated in the field by Ficoll centrifugation using lymphocyte separation medium (Organon Teknika, Inc., Durham, N.C.) as previously described (41). Animal cells were preserved in liquid nitrogen and shipped on dry ice for viral isolation and DNA analysis. All animals were tattooed with a unique number so that no animal was inadvertently sampled more than once. Specimens from the Brookfield Zoo colony of sooty mangabeys were collected in 1985 (36).

Virus isolation. SIVsm isolation from one West African household pet mangabey has been reported in detail elsewhere (41). Viruses were recovered from mangabey samples either by cocultivating monkey cells with CEMx174 cells or by directly inoculating African monkey cells intravenously and without laboratory manipulations into SIV-seronegative rhesus macaques (*Macaca mulatta*) (41). The latter method minimized the possibility of contamination with known SIVs.

ELISA and Western blot (immunoblot) analysis. Techniques for indirect enzyme-linked immunosorbent assay (ELISA) and Western blot analyses have been described in detail elsewhere (40, 41). The antigen used for screening was gradient-purified SIVsmLib-1, a virus derived from a West African household pet (41). The negative controls were plasma from a pet sooty mangabey that was SIV negative (41), as confirmed by PCR (data not shown), and an SIV-negative rhesus macaque. Known positive plasma was from an SIVsmLib-1-positive pet mangabey or from SIVmac251-inoculated rhesus macaques. Positive ELISA results were confirmed by using HIV-1 and HIV-2 commercial Western blot kits (Cambridge Biotech, Boston, Mass.).

Genomic DNA extraction and PCR amplification. High-molecular-weight genomic DNA from uncultured lymphocytes was extracted by using a DNA-RNA extraction kit (United States Biochemical Corp., Cleveland, Ohio). Nested primers were used to amplify an 898-bp *gag* fragment covering part of the *gag* p17 and p26 regions and a 1,001-bp *env* fragment covering the transmembrane protein (gp43) region. The primers, whose lengths were included in both fragments, were designed both to be highly conserved among the HIV-2, SIVmac, and SIVsm sequences published in the Los Alamos database (45) and to amplify DNA fragments that allowed comparisons with other HIV-2/SIVmac/SIVsm family members (17). Primer sequences, corresponding to the SIVsmH4 genome, are shown in Table 2. PCR was done in a Perkin-Elmer 9600 thermocycler (Perkin-Elmer, Foster City, Calif.) in 100-μl volumes containing 0.5 to 1 μg of DNA, 10 mM Tris-HCl (pH 8.5), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 μM each dATP, dGTP, dCTP, and dTTP, 20 pmol of each primer,

TABLE 2. Nested PCR primer pairs for SIVsm proviral DNA amplification and quantification

Primers	Nested primer	Genome location[a]	Nucleotide sequence
env (gp43)			
EF1	Outer	7753–7781	5′-ACA TTC ATG TGG ACA AAT TGC AGA GGA GA-3′
EF2	Inner	7789–7818	5′-TAC TGC AAA ATG AAT TGG TTT CTT AAT TGG-3′
ER1	Outer/inner	8764–8790	5′-GGG AGG GGA AGA GAA CAC TGG CCT ATA-3′
gag (p17-p26)			
GF1	Outer	1043–1069	5′-TGG GAG ATG GGC GCG AGA AAC TCC GTC-3′
GR1	Outer	2279–2305	5′-TCC ACA TTT CCA GCA GCC CTG TCT TCT-3′
GF2A	Inner	1075–1099	5′-AGG GAA GAA AGC AGA TGA ATT AGA A-3′
GR3	Inner	2000–2031	5′-GCA TTT TGA ATC AGC AGT GTT TGA GTC ATC CA-3′
gag (p17)			
GRM1	Outer	1313–1338	5′-GTA TGT TTC ACT TTC TCT TCT GCG TG-3′
GRM2	Inner	1262–1284	5′-TAA AGG CTT TTT AAA TTT TCT GA-3′

[a] Positions in the SIVsmH4 complete genome sequence (45).

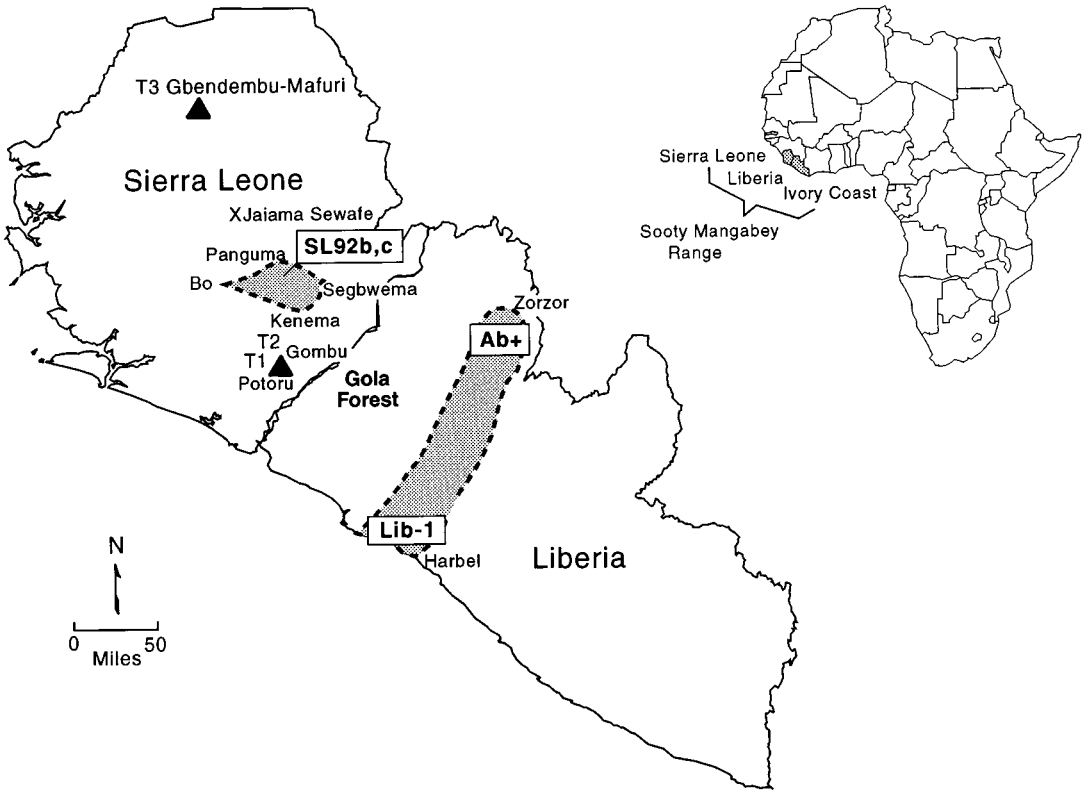

FIG. 1. Map indicating distribution of feral monkey troops and household pets tested for SIV in Sierra Leone and Liberia. The map shows the locations of the three wild sooty mangabey troops (T1, T2, and T3; ▲). T1 and T2 ranged freely within 5 miles of each other northeast of Potoru along the Moa River at the village of Gombu (11°22′40″W, 7°35′40″N). T3 was about 175 miles to the north of T1 and T2 in a rural forest along the Maboli River near the village of Gbendembu-Mafuri (12°19′20″W, 9°4′40″N). Four mangabeys from T1 were positive for HIV-2-related viruses (SIVsmSL92a, -SL92d, -SL92e, and -SL92f). All T2 and T3 members tested were SIV negative. The areas enclosed by dotted lines were surveyed for household monkey pets. Locations of virus strains from pets (Lib-1, SL92b, and SL92c) are shown. One pet was antibody positive (Ab+), but no virus was detected. HIV-2 subtype D was found in a person in Harbel. Jaiama Sewafe is the home village of the patient in whom HIV-PA subtype E was detected.

and 2.5 U of *Taq* polymerase. The first round of PCR consisted of an initial cycle at 95°C for 2 min followed by 30 cycles of 95°C for 20 s, 45°C for 1.5 min, and 72°C for 2 min, using *gag* or *env* primer pair GF1-GR1 or EF1-ER1, respectively. The second PCR round, using inner primer pair *gag* GF2A-GR3 or *env* EF2-ER1, consisted of another 30 cycles of 94°C for 20 s, 55°C for 1.5 min, and 72°C for 2 min. For both reaction rounds, the last cycle's extension was at 72°C for 8 min.

HMA. A heteroduplex mobility assay (HMA), described by Delwart et al. (9), was used to identify viral variants both from total genomic DNA containing multiple proviral copies and from molecular clones (one proviral copy per clone). Nested PCR for HMA was done in the same way as for phylogenetic analysis. PCR products amplified from a single molecular clone were used as a standard reference, and the homologous PCR products amplified from other molecular clones or from total genomic DNA were hybridized and compared with the same standard. For heteroduplex formation, 5-μl aliquots (1/20) of the second-round PCR products of each molecular clone or genomic DNA were mixed separately with 5 μl of standard products, 8 μl of H_2O, and 2 μl of 10× HMA buffer (1 M NaCl, 100 mM Tris [pH 7.8], 20 mM EDTA). Each reaction mixture was boiled for 3 min, cooled on ice for 10 min, then applied to a 4% polyacrylamide gel in 1× Tris-buffered saline (53), and electrophoresed in an SE 400 apparatus (Hoefer, Inc., San Francisco, Calif.) for 4 h at a constant voltage of 200 V. The DNA duplexes were viewed and photographed after ethidium bromide staining. The heteroduplex bands were observed only in lanes containing genetic variants.

Measurement of proviral load in lymphocytes. Proviral DNA was measured by a previously described limiting-dilution method (55). PCR primers corresponding to the *gag* gene of both HIV-2 and SIVsm were selected. Proviral DNA was diluted to an endpoint at which less than 25% of replicated samples yielded PCR products. The number of proviral copies was estimated by $-\ln[F]$, where F is the fraction of negative reactions, assuming that the incidence of positive PCR products follows a Poisson distribution. Positive and negative controls were included in all of the quantitative assays. The reaction mixture was prepared as described above except that the total volume was reduced to 25 μl. The first PCR round consisted of an initial cycle at 95°C for 2 min followed by 30 cycles of 95°C for 15 s, 45°C for 30 s, and 72°C for 30 s, using *gag* primer pair GF1-GRM1 (Table 2). The second PCR round, using inner primer pair *gag* GF2A-GRM2, consisted of another 30 cycles of 94°C for 15 s, 50°C for 30 s, and 72°C for 30 s. For both reaction rounds, the last cycle's extension was at 72°C for 9 min.

DNA sequencing. PCR products were subjected to direct TA cloning using pCR vectors (version 2.0; Invitrogen), and the ligates were transformed into host cells supplied with the kit. Double-stranded recombinant plasmid DNA was purified by using a Wizard Miniprep DNA purification system (Promega, Madison, Wis.), and sequencing was performed with the *Taq* Track DNA sequencing system (Promega) or with a Sequenase kit (United States Biochemical Corp.).

Phylogenetic analysis of DNA sequences. Proviral DNA sequences were aligned by using the CLUSTAL V program, kindly provided by D. G. Higgins (23), and the final alignment was adjusted by eye. Pairwise genetic distances were calculated by using the DNADIST program implemented in PHYLIP (13, 14) or in the GCG (Genetics Computer Group) package (University of Wisconsin, Madison) under Kimura's two-parameter model of molecular evolution (32). No primer sequences were included for the phylogenetic analysis. The evolutionary relationship of primate lentiviruses was analyzed by various phylogenetic approaches, including the neighbor-joining (GCG package) (52) and parsimony methods in the PAUP (phylogenetic analysis using parsimony) package (56). The reproducibility of the branching orders was estimated by bootstrapping the same data set with 1,000 replicates, using a program in the Mega (molecular evolutionary genetics analysis) package (34).

Nucleotide sequence accession numbers. The nucleotide sequences obtained in this research were submitted to GenBank (accession numbers U48810 to U48824).

RESULTS

Feral mangabey troops and household pet mangabeys. The locations of the three feral troops containing sooty mangabeys and other species as well as the locations of household pets in Sierra Leone and Liberia are shown in Fig. 1. Troops 1 and 2 were polyspecific, containing more than one monkey species.

Troop 1 had 22 sooty mangabeys and 1 olive colobus monkey (*Colobus verus*). Troop 2 had 9 sooty mangabeys, 11 Campbell monkeys (*Cercopithecus campbelli*), and 1 olive colobus. Troop 3 was monospecific for sooty mangabeys and contained 17 animals. Table 1 shows the monkey species that were tested from West African feral troops and households. Only a portion of each troop was successfully sampled: 10 sooty mangabeys from troop 1; 4 sooty mangabeys, 11 Campbell monkeys, and 1 olive colobus from troop 2; and 4 sooty mangabeys from troop 3. Table 1 also shows that a total of 136 nonhuman primate household pets, including chimpanzees and seven different monkey species, were observed. With the permission of the owners, specimens were collected from 114 monkey pets. Chimpanzees were not sampled.

As shown in Fig. 1, the area in Liberia surveyed for pets was along the roads from the coastal town of Harbel inland to the village of Zorzor within 5 miles (1 mile = 1.609 km) of the Guinea border. The pet survey area in Sierra Leone was bordered by the towns of Bo, Kenema, Segbwema, and Panguma. According to all owners, all pets had been wild caught as infants. This information was probably accurate, because this is a common practice and we observed that the pets were tame. Had the monkeys been captured as adults, they would have been much less tame. In addition, they had close human contact, and most of them were juveniles.

Identification of seropositive feral and pet sooty mangabeys. All monkeys were tattooed at the time of sampling to ensure that each was sampled only once. Table 3 shows the positive results of SIV antibody testing in the monkeys. Screening was done by ELISA using sucrose gradient-purified SIVsmLib-1, HIV-1, and HIV-2 as the test antigens. Four of 30 feral and 4 of 114 pet monkeys were antibody positive for an SIV/HIV-2-like virus. Only sooty mangabeys were seropositive for HIV-2-like antibodies; all other species were antibody negative.

Two of the pets identified and confirmed as seropositive were from Panguma, Sierra Leone. These two monkeys, SM042 and SM051, were the first household SIV-positive pets identified from that country. The other two pets, SM1 and SM023 from Liberia, had previously been found to be seropositive (41). Since only 4 of 91 pet sooty mangabeys were SIV positive, the seroprevalence of SIVsm infection among mangabey pets was only 4.4%, compared with 22.2% found in sooty mangabeys of the feral troops. A possible explanation for the low seroprevalence in pets is that most or all of the animals were removed from the wild troops as infants, before becoming infected. Supporting this contention, of 10 feral animals tested from troop 1, only 4 were adults, and yet 3 of the 4 adults were SIV positive.

Previously, we described an SIV isolate from a zoo colony of sooty mangabeys in the United States (36). The zoo isolate was designated sooty mangabey lentivirus but here is renamed SIVsmBro85 (Table 3), in keeping with currently accepted nomenclature. Isolation of SIVsmLib-1 has also been reported previously (41). Both isolates are included in this analysis because neither has been phylogenetically analyzed and both may contain new genetic information on the SIVsm family.

To test for serological cross-reactivities with other primate lentiviruses, plasma samples from all positive sooty mangabeys were tested by Western blot analysis with SIVsmLib-1 viral lysate. As shown in Fig. 2A, animals found positive by ELISA had antibodies strongly reactive with core protein p26 and transmembrane protein gp43 of SIVsmLib-1 (data not shown for SM023 and SM085). Plasma samples from SM1, the autologous plasma in this assay, and from SM087 were the only samples with antibodies against envelope protein gp120. To determine if commercial Western blot kits would be useful for detecting SIVsm antibodies in feral animals, plasma samples from the four positive feral mangabeys were tested against HIV-1 and HIV-2 antigens (Fig. 2B and C). All four feral animals had HIV-2 antibodies reactive against core protein p26 and transmembrane protein gp34 and the gp34 trimer. In contrast, only two feral animals had antibodies that reacted with HIV-1 core protein p24. Therefore, only commercial HIV-2 immunoblots are useful for identifying antibodies in West African mangabeys.

Two new biological isolates were obtained. The first, SIVsmSL92a, was isolated from SM089, the largest male mangabey in troop 1, whose behavior was consistent with being the dominant male. Preliminary data on SIVsmSL92a isolation has previously been presented (6). The second new isolate, SIVsmSL92b, was obtained from a household pet, SM051, in Sierra Leone. All West African isolates, SL92a, SL92b, and Lib-1, plus the isolate from the American zoo, readily infected CEMx174 cells (Table 3). Repeated attempts to isolate viruses

TABLE 3. Geographic distribution and virological characteristics of SIV-infected sooty mangabeys in West Africa[a]

Seropositive animal	Sex[b]	Location[c]	Year of sampling	PCR	No. of proviral copies/10^6 cells	Virus isolation[d]	Virus[e]
Feral troop 1							
SM079	F	Gombu, SL	1992	+	39	−	SIVsmSL92d
SM085	F	Gombu, SL	1992	+	2,799	−	SIVsmSL92e
SM087	F	Gombu, SL	1992	+	633	−	SIVsmSL92f
SM089	M	Gombu, SL	1992	+	37	+	SIVsmSL92a
Pets							
SM1	F	Harbel, Lib	1988	+	ND[f]	+	SIVsmLib-1
SM023	Unk	Zorzor, Lib	1989	ND	ND	ND	
SM042	M	Panguma, SL	1992	+	ND	−	SIVsmSL92c
SM051	M	Panguma, SL	1992	+	ND	+	SIVsmSL92b
Zoo animal (Cinders)	F	Brookfield Zoo, United States	1985	+	ND	+	SIVsmBro85[g]

[a] All samples were positive in three of three assays, as described in Materials and Methods.
[b] F, female; M, male; Unk, unknown.
[c] SL, Sierra Leone; Lib, Liberia.
[d] Mangabey cells were cocultured with 2×10^6 CEMx174 cells as described elsewhere (41).
[e] Viruses were named after the place of origin and the year the specimen was collected. The name of SIVsmLib-1 was not changed to include the year (1988) because the original name is in use in the Los Alamos database (45).
[f] ND, not done.
[g] SIV isolate previously designated sooty mangabey lentivirus (36).

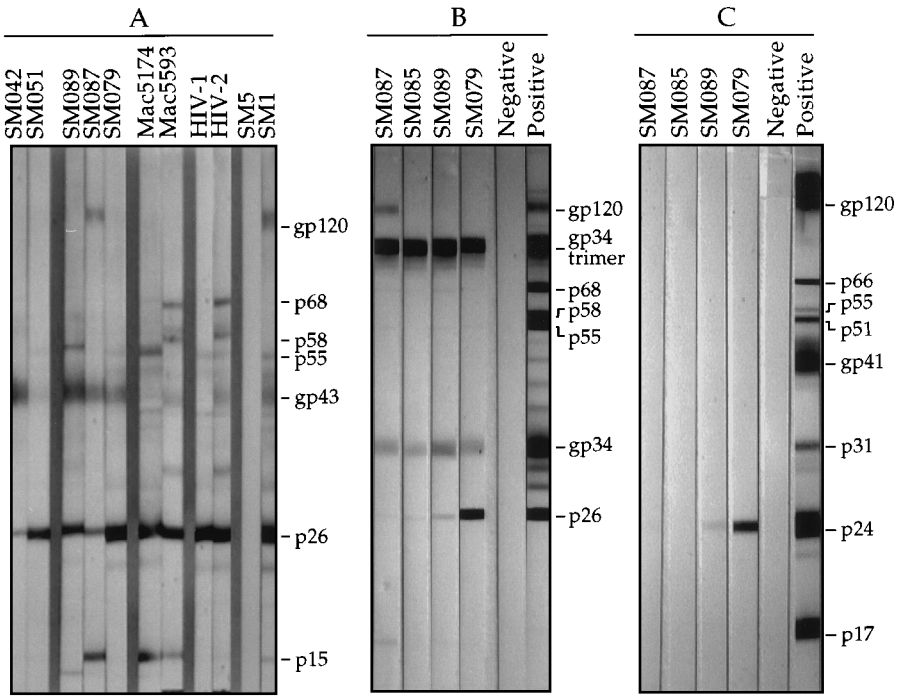

FIG. 2. Western blot analyses of antibodies of feral and household pet sooty mangabeys against SIVsmLib-1 and HIV-1 and HIV-2 antigens. (A) Western blot for plasma SIVsmLib-1-reactive antibodies from the following: SM042 and SM051, the two seropositive pet mangabeys found in Sierra Leone; SM089, SM087, and SM079, three seropositive feral mangabeys in Sierra Leone troop 1; Mac5174, a rhesus monkey infected with SIVsmSL92a, a virus of the feral mangabey SM089; Mac5593, a rhesus monkey infected with SIVmac251; HIV-1- and HIV-2-infected patients; SM5, a seronegative pet mangabey in Liberia; SM1, a pet mangabey from which SIVsmLib-1 was isolated (41). (B and C) HIV-1 and HIV-2 Western blots from commercial kits to test for HIV-1- and HIV-2-cross-reactive antibodies in the plasma of four seropositive feral sooty mangabeys from troop 1, this time including SM085. Negative and positive sera were supplied with the kit. Positions of standard viral proteins are indicated.

from the remaining three seropositive feral and two pet mangabeys were negative. However, SIV *gag* and *env* DNA fragments were amplified from seven of the eight seropositive mangabeys and were suitable for phylogenetic studies. As no cells were available from the ninth animal, SM023, no further studies were done.

Quantification of proviral DNA and amplification of SIVsm *env* and *gag* fragments from primary mangabey genomic DNA. To test the number of proviral copies in lymphocytes of sooty mangabeys infected in the wild, a nested PCR was designed to quantify a 215-bp *gag* fragment from the SIVsm genome. The sensitivity of the PCR was one proviral copy amplified from a plasmid containing only one SIV genome from SIVsmSL92a (data not shown). The 215-bp *gag* fragment was amplified from each of the four seropositive feral sooty mangabeys. The proviral DNA copy number, estimated by the limiting-dilution method (55), ranged from 2,799 to 37 copies per 10^6 cells (Table 3). The three feral adults, SM079, SM087, and SM089, had less proviral DNA copies than the juvenile (SM085). Since endogenous retroviruses have one proviral copy per somatic cell, SIVsm in feral sooty mangabeys does not appear to be germ line derived.

An 898-bp *gag* fragment and a 1,001-bp *env* fragment were also PCR amplified (Table 2). These two fragments were chosen because they contain regions previously characterized from all five known HIV-2 subtypes (17), allowing for phylogenetic comparisons. Both gene fragments were successfully amplified from each of four feral and three pet seropositive sooty mangabeys (Table 3). *gag* fragments were also amplified from the peripheral blood mononuclear cells of a rhesus monkey infected with SIVsmSL92a and from the original viral culture from which SIVsmBro85 was derived.

A 750-bp *gag* fragment and a 393-bp *env* gp43 fragment were sequenced from each animal, and Fig. 3 shows the amino acid sequence alignments. The proteinase cleavage site and one of two HLA-restricted epitopes in the *gag* genes (42) were conserved among viruses in the SIVsm/HIV-2 family. However, an identical amino acid change in the second HLA epitope was found in *gag* genes of only three viruses: HIV-2PA subtype E and SL92b and SL92c from the two household pets in Sierra Leone. In addition, the number of synonymous (silent [no amino acid change]) nucleotide substitutions is much more than that of nonsynonymous changes (amino acid change) (Fig. 3). Synonymous changes are probably distributed randomly and saturated in the *gag* gene (Fig. 3). However, to address the selective pressure on SIVsm structural genes, further statistic analysis should be carried out for both synonymous and nonsynonymous nucleotide substitutions.

Phylogenetic analysis and geographic clustering of SIVsm and HIV-2. To define the evolutionary relationships of the newly identified SIVsm strains to previously reported lentiviruses, phylogenetic trees were constructed by using nucleotide and amino acid sequences from PCR-derived *gag* p17 and p26 regions. When compared with all five lineages of primate lentiviruses (46, 54), the new SIVsm strains clustered only within the SIVsm/HIV-2 lineage (data not shown). To define the relationships within the SIVsm/HIV-2 lineage, *gag* and *env* trees based on nucleotide sequences were constructed by using known SIVsm and HIV-2 isolates and the newly found and previously uncharacterized viruses (boxed in Fig. 4). In both *gag* (Fig. 4A) and *env* (Fig. 4B) trees, all HIV-2 isolates segregated into distinct subtypes, as reported by Gao et al. (17). In addition, previously characterized SIV strains derived from

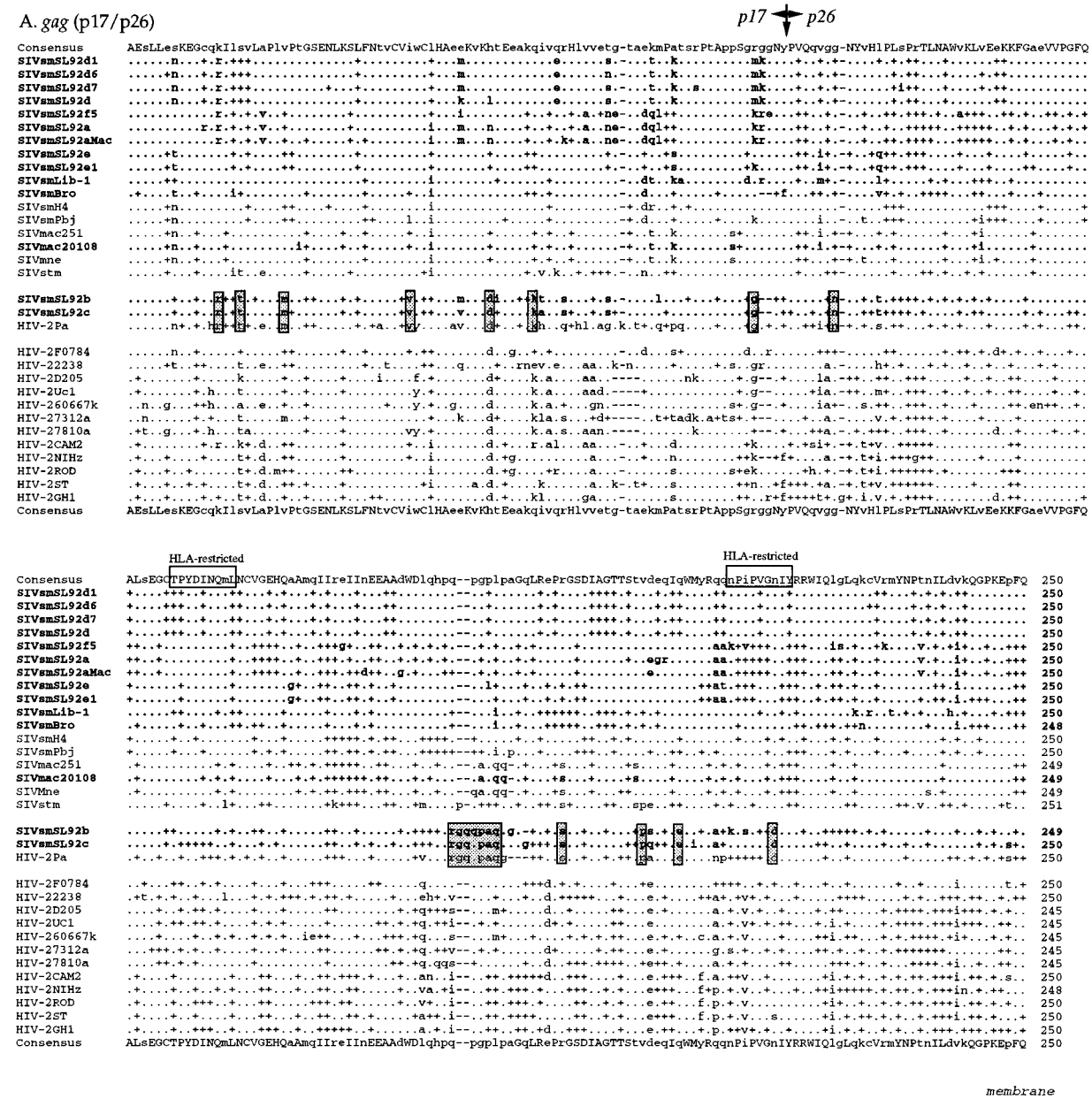

FIG. 3. Multiple sequence alignments of deduced amino acids based on partial *gag* (A) and *env* gp43 (B) regions. Alignments and consensus sequences were compared between newly characterized SIVsm strains (boldface) and the existing lentiviruses of the SIVsm/HIV-2 lineage in the database (45). Dots denote amino acids identical with the consensus sequence, and dashes denote amino acid deletions. Capital letters in the consensus indicate amino acid identity among all viruses compared. Lowercase letters indicate variable amino acid sites, and plus symbols indicate synonymous mutations. The *gag* p17-p26 precursor cleavage site and the beginning of the transmembrane domain in *env* gp43 are indicated (17). Two HLA-restricted CTL epitopes in the *gag* region are boxed (42). A signature sequence (RGQPPAQ) and multiple informative sites for identical nonsynonymous changes of HIV-2PA, SIVsmSL92b, and SIVsmSL92c are boxed and shaded.

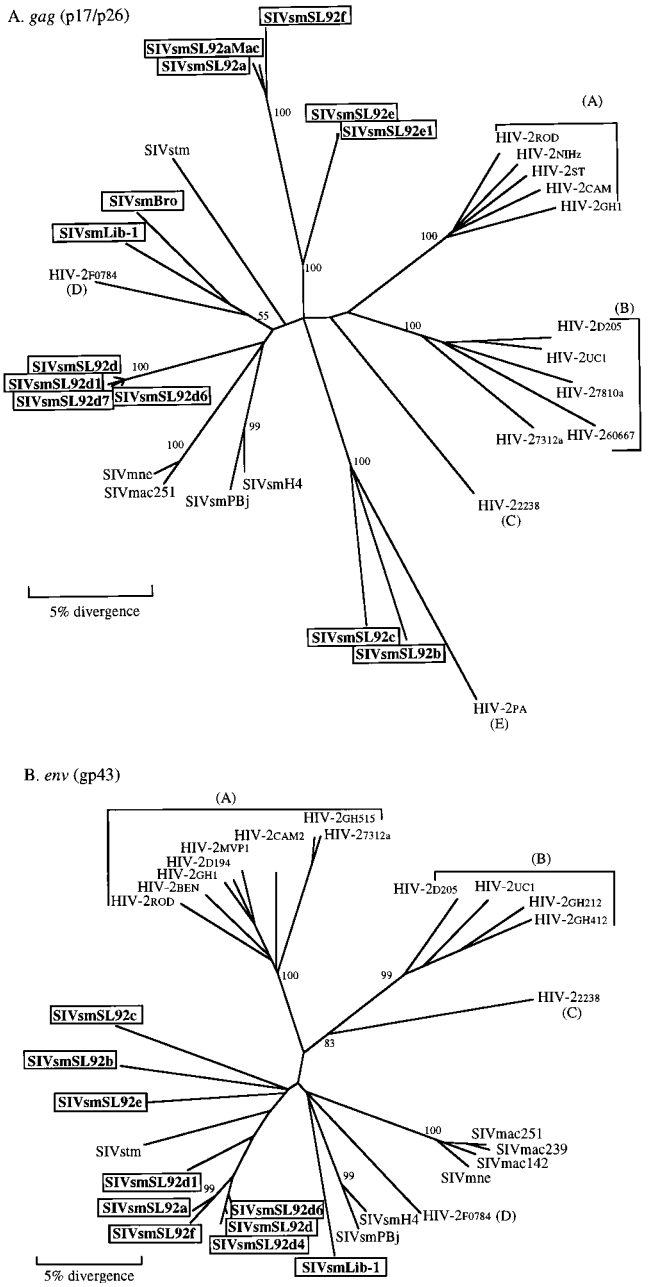

FIG. 4. Phylogenetic relationship between newly characterized SIVsm (boxed) and existing primate lentiviruses (unboxed) within the SIVsm/HIV-2 lineage. (A) A tree based on *gag* sequences; (B) a tree based on *env* gp43 sequences. Both trees were unrooted and constructed by the neighbor-joining method, using nucleotide distance data sets calculated with Kimura's two-parameter approach (32). No sequence gaps were included for analysis. In the *gag* tree, all five HIV-2 subtypes are indicated as A, B, C, D, and E; in the *env* gp43 tree, HIV-2 subtypes A to D are indicated, but subtype E was not available (17). The branch length is additive and proportional to the genetic distances between virus pairs. Values on the branches represent the percentage of 1,000 bootstrap replicates. Bootstrap values over 50% are marked in the trees.

captive monkeys in the United States segregated into three distinct groups: SIVmac/mne, SIVsmH4, and SIVstm (Fig. 4).

Among the newly identified SIVsm strains, none grouped with HIV-2 subtypes A and B, which contain the largest number of known viruses. Using neighbor-joining, parsimony, and bootstrap analyses, we found *gag* and *env* topologies to be consistent in all of the new SIVsm strains except SL92d, which segregated differently in each tree (Fig. 4). SL92d was obtained from an adult female in feral mangabey troop 1, the same troop in which SIV strains SL92a, SL92e, and SL92f were found. As shown in Fig. 4B, SL92d clustered closely with SL92a and SL92f in the *env* tree but formed a separate and unique single branch in the *gag* tree (Fig. 4A), indicating that SL92d may contain a recombinant genome. The cluster between SL92a/f and SL92d in *env* genes was also supported by phylogenetic analysis of the V4-C4 regions (data not shown). Further analysis of SL92d by HMA is presented below. Of the four viruses from feral troop 1, only SL92a and SL92f clustered tightly in both trees. The fourth virus from troop 1, SL92e, did not cluster with any other virus. Instead, SL92e was genetically

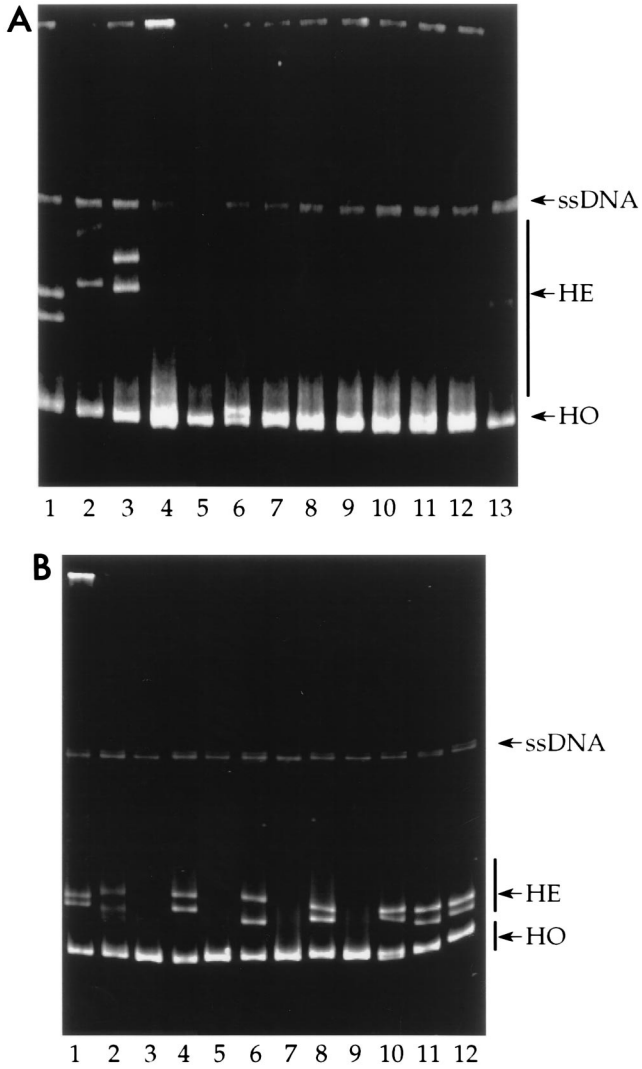

FIG. 5. HMA analysis of SIVsmSL92d viral quasispecies, using *gag* (A) and *env* gp43 (B) fragments. Single-strand DNA (ssDNA) and heteroduplex (HE) and homoduplex (HO) formations are marked in a 4% native acrylamide gel. (A) Heteroduplexes between SL92d molecular clone 1 (lane 5) and molecular clones of SL92e (lane 1), SL92f (lane 2), and SL92a (lane 3), which served as quasispecies controls. The SL92d clone was also used to probe for viral variants in the total genomic DNA derived from the host of SL92d (lane 4) and in the other SL92d clones (lanes 6 to 13). (B) A clone of SL92a (lane 1), probed by using SL92d clone 1 (lane 3), served as the control. SL92d clone 1 was also used to probe for viral variants in the total genomic DNA (lane 2) and in the other SL92d clones (lanes 4 to 12).

equidistant from SL92a, SL92f, and SL92d, forming a unique branch in both *gag* and *env* trees. These data show that mangabeys from a single, small feral animal troop harbored three highly divergent viruses, suggesting a long natural history for SIV in African sooty mangabeys and the possibility that coinfection can account for the mosaic genome of SL92d.

Next we assessed the effect of cross-species transmission on virus diversity. Cells from SM089, the natural host of SIVsmSL92a, were directly inoculated into a rhesus macaque (Mac5174). The virus obtained, SL92aMac, remained phylogenetically closest to SL92a (Fig. 4A), showing that cross-species transmission of a feral SIV into a rhesus macaque does not result in selection or significant viral divergence in the portion of *gag* gene tested.

The three viruses from household pet mangabeys, Lib-1, SL92b, and SL92c, were also divergent from each other and from all known SIVsm and HIV-2 strains, forming distinct branches in their *gag* and *env* trees (Fig. 4). Importantly, the branch lengths between each of these diverse mangabey viruses were comparable to those found between HIV-2 subtypes. Moreover, a comparison of amino acids between *gag* genes showed that feral and pet mangabey virus diversity is high, up to 19.3% for SL92b and Lib-1 (Table 4), which is higher than that found between HIV-2 subtypes A, B, C, and D (13.9 to 15.2%). Only HIV-2 subtype E has comparable divergence. This broad genetic diversity in individual sooty mangabeys provides evidence that multiple HIV-2 subtypes could have originated from sooty mangabeys through multiple cross-species transmissions in West Africa.

Besides marked viral divergence, we surprisingly found geographic correlations between pet-derived viruses and HIV-2. SIVsmSL92b and SIVsmSL92c, from pets in Sierra Leone, clustered with HIV-2PA (Fig. 4A), a virus known to have infected a Sierra Leonian (25). Moreover, sequence alignments of deduced Gag amino acids support this grouping. HIV-2PA, SL92b, and SL92c share multiple informative sites (boxed in Fig. 3A), especially a unique signature sequence, RGQPPAQ, in the p26 protein and in the HLA-restricted peptide (Fig. 3A) (42). Lib-1 sequences also showed evidence of geographic clustering. Lib-1, a virus from a Liberian pet mangabey, was closely related to HIV-2F0784 (Fig. 4), a virus from a Liberian rubber plantation worker (18). These findings further support the hypothesis that multiple introductions of SIVsm in West Africa resulted in the emergence of distinct HIV-2 subtypes.

The SIVsm strains discussed above were identified from naturally infected West African sooty mangabeys. Another SIV, SIVsmBro85, from an American zoo mangabey (36), was phylogenetically characterized, and its genetic distances from other SIV strains in the United States and Africa were compared. As shown in Fig. 4A, SIVsmBro85 forms a distinct branch that is closer to the West African SIVsm strain Lib-1 than to any other domestic SIV isolate. These results demonstrate further divergence in the SIVsm family and also prove that genetically distinct SIVsm strains were introduced to the United States through mangabey importation.

HMA analysis of SIVsmSL92d quasispecies. Viral variants of SL92d were further analyzed by HMA assay. Phylogenetic analysis data (Fig. 4) suggested that SL92d may consist of a mosaic viral genome or, alternatively, its quasispecies may be a result of multiple infection. Products derived from six independent PCRs for both the *gag* (Fig. 5A) and *env* (Fig. 5B) regions of SL92d were subjected to HMA analysis before (Fig. 5A, lane 4, for *gag*; Fig. 5B, lane 2, for *env*) and after (Fig. 5A, lanes 5 to 13, for *gag*; Fig. 5B, lanes 3 to 12, for *env*) TA DNA cloning. These products were amplified from a total of 700 proviral copies, based on proviral genome quantification (Table 3). HMA analysis detected only minor variants in *gag* DNA derived from total genomic DNA containing 700 proviral genomes (Fig. 5A, lane 4). To confirm this result, nine randomly selected molecular clones were also tested (Fig. 5A, lanes 5 to 13). Compared with heteroduplex controls (Fig. 5A, lanes 1 to 3), which consisted of mixtures of SL92 viruses in the *gag* gene, SL92d was a homogeneous group of viral quasispecies. In the *env* transmembrane region, SL92d contained viral quasispecies that were more variable (Fig. 5B, lane 2) than those in the *gag* region. Heterogeneous viral quasispecies were identified from PCR products derived both from total genomic DNA (Fig. 5B, lane 2) and from six molecular clones (Fig. 5B, lanes 4, 6, 8, 10, 11, and 12). After sequencing of multiple clones, all SL92d viral quasispecies clustered closely with SL92a and SL92f (Fig. 4). Therefore, on the basis of HMA quasispecies analysis of 700 proviral genomes, SL92d was determined to be a recombinant with an *env* transmembrane protein gene that was ho-

TABLE 4. Amino acid diversity (%) of *gag* genes of lentiviruses within the SIVsm/HIV-2 lineage[a]

Virus	HIV-2 subtypes					SIV									
						Household pet mangabeys			Free-living feral mangabeys			American captive monkeys			
	A	B	C	D	E	SL92c	SL92b	Lib-1	SL92a/f	SL92e	SL92d	smBro	smH4	Mac	stm
A	7.7														
B	13.9	10.9													
C	14.4	15	NA[b]												
D	12.4	13.5	15.2	NA											
E	24.1	21.7	24.3	21.5	NA										
SL92c	18.1	17.4	18.8	16.2	10.7	NA									
SL92b	19.4	18.4	19.9	18.3	12.7	4.3	NA								
Lib-1	16.3	17.8	18.3	9.3	24.9	17.8	19.3	NA							
SL92a/f	15.4	17.1	17.3	12.2	23.3	16.8	16.4	13	4.5						
SL92e	12.3	13.8	14.3	7.4	20.4	14.1	15.7	9.8	10.3	NA					
SL92d	13.3	15.3	13.5	9.1	20.2	13	14.2	8.7	10	7.5	NA				
smBro	12.9	15.2	14.7	7.4	23.2	15.2	16.7	7	10.6	6.5	8.2	NA			
smH4	13.1	14.8	14.4	7	14.2	14.4	15.9	6.1	9.9	6.7	6.4	4.3	NA		
Mac	15.7	17.7	16.5	10.7	21.9	16.2	17.4	8.5	14.4	9.6	7.6	8.3	6.1	2.3	
stm	14.7	16.9	14.1	10.7	21.5	14.6	16.7	12.2	12.3	11.7	11.1	9.3	9.3	11.5	NA

[a] *gag* gene regions are shown in Fig. 3A.
[b] NA, not available, only one isolate known.

mologous with two viruses in the feral troop and a *gag* gene that was both distinct and divergent.

DISCUSSION

Survey of HIV-2-related SIV in naturally infected monkeys in West Africa. To study the evolution of SIV in West Africa, 114 pet and 30 feral monkeys encompassing seven nonhuman primate species from Sierra Leone and Liberia were tested by ELISA and Western blot analysis for SIVsm- and HIV-like viruses. All six newly found positive monkeys were sooty mangabeys, and 35 monkeys representing six other species were seronegative for HIV-2, HIV-1, and SIVsm antibodies. Because all six positive sooty mangabeys had antibodies against SIVsmLib-1 core and transmembrane proteins and one also contained antibodies against the surface protein (Fig. 2A), the viruses that they harbored share some antigenic epitopes with SIVsmLib-1. Furthermore, Western blot analysis revealed that all four positive feral mangabeys had antibodies that reacted to HIV-2 in both its core and transmembrane proteins, but only two of them had antibodies that reacted against HIV-1 core protein, and none of them had antibodies that reacted against HIV-1 transmembrane protein (Fig. 2B and C). Therefore, as with SIVsm found thus far (16, 24, 44), feral mangabey SIVsm strains were antigenically closer to HIV-2 than to HIV-1.

New SIVsm strains were discovered in two household pets in Sierra Leone. Although we found that monkeys were often kept as pets in West Africa, SIV-infected pets were much less common. Pet seroprevalence was 4.4%, much lower than that in the feral mangabey troops (22.2%). Among 30 feral sooty mangabeys tested, only 4 were adults, and 3 of these 4 were infected, showing a correlation between infection and sexual maturity as has been reported for a captive breeding group of sooty mangabeys (15). The data suggest that, since most or all of the pets had been removed from wild troops as infants, SIVsm is primarily transmitted between older animals and may be sexually spread.

Previously, we reported the isolation of SIVsmLib-1 from a pet mangabey in Liberia (41) and of SIVsmSL92a from a feral mangabey in Sierra Leone (6). Here we describe a third infectious isolate, SIVsmSL92b, from a pet sooty mangabey in Sierra Leone (Table 3). Our results, plus those of others (50), indicate that infectious SIVsm strains can be found in the entire sooty mangabey range, from Sierra Leone to the Ivory Coast (Fig. 1). As sooty mangabeys are often kept as pets and used for food in West Africa (41) and SIV is known to be transmissible to humans (30), the wide distribution of naturally infected sooty mangabeys poses a risk to persons exposed to SIV-infected animals throughout their natural habitat.

Molecular epidemiology of SIVsm in a feral monkey troop. Surprisingly DNA sequence and HMA analyses revealed three genetically distinct SIV strains in a single, small feral animal troop composed of 22 animals. The four newly identified viruses from troop 1, SL92a, SL92d, SL92e and SL92f, had complex evolutionary relationships. Phylogenetic analysis of all four indicated that only SL92a and SL92f clustered in *gag* and *env* trees (Fig. 4) as the most homologous strains in troop 1. As SL92a was found in the dominant male mangabey and SL92f was found in an adult female, SIV sexual transmission may have occurred between these two mangabeys. The third feral animal-derived SIV, SL92e, was from a juvenile female and had a separate lineage within the SIVsm/HIV-2 group (Fig. 4). Since juvenile males and females are known to wander between feral mangabey troops (60), this animal may have been infected with a distinct SIV before joining the feral troop. These results are consistent with our observation and those of local hunters that juveniles usually move between feral troops or gather to form a separate bachelor troop (57). The presence of these divergent viruses in a single, small group of breeding mangabeys provides the opportunity for coinfection that enables SIV recombination in the wild.

The fourth virus, SL92d, segregated differently in *gag* and *env* trees and had properties of a recombinant viral genome, as described by others (17, 27, 51). The mosaic genome of SL92d contained an *env* gene that clustered closely with SL92a and SL92f (Fig. 4B), demonstrating that these three viral *env* genes must have originated from a common ancestor virus. The *gag* gene of SL92d, however, was unique among and distant from all lentiviruses in the SIVsm/HIV-2 lineage. Furthermore, HMA analysis of SL92d quasispecies revealed that in a total of 700 genome copies, both *env* and *gag* genes of SL92d consisted of only minor viral variants (Fig. 5). SL92d, therefore, resulted from a recombination between the *env* gene of a troop 1 virus and the *gag* gene of an unknown SIVsm strain. This finding of an SIVsm mosaic genome indicates that sooty mangabeys in the forest can be simultaneously infected with two divergent SIVsm strains. This result corresponds to similar findings for HIV-1 and HIV-2 (17, 27, 51, 63). The data also suggest that recombination may play a role in the generation of new SIV strains under natural conditions in wild mangabeys.

The natural history of SIVsm in West Africa and the United States. The finding of six new SIVsm strains raises the question of the origin of these diverse viruses. The hypotheses are as follows: (i) they were transmitted relatively recently from another animal species in Africa; (ii) they were transmitted from HIV-2-infected humans (12); and (iii) they descended from an ancient virus. The first hypothesis is unsupported thus far, because the sooty mangabey is the only animal species found to be infected with HIV-2-like lentiviruses (references 36 and 41 and Table 3). Moreover, SIVagmSab, from the only other known positive monkey species in this region, *Cercopithecus aethiops sabaeus*, the West African green monkey (27), did not cluster with any of the new SIVsm strains in the genes characterized (data not shown). The second hypothesis is unlikely because we found that forest-dwelling sooty mangabeys in a single, small feral troop without human contact were naturally infected with three divergent viruses diverse SIVsm strains, and transmission of all three from human beings into a group of wild sooty mangabeys is difficult to envision. Furthermore, SIV seroprevalence in feral troop 1 is high (40%), but HIV-2 seroprevalence in West Africans of the same geographic area is very low (0.02%, or 2 of 9,309) (39). The third hypothesis is most plausible because SIVsm appears to follow a gradual evolutionary process. As shown in the phylogenetic trees, especially in the *env* tree (Fig. 4B), the intertypic pattern for SIVsm is not as obvious as for HIV-2. Compared with HIV-2 subtypes A and B, most SIVsm internode lengths are short and not supported by high bootstrap values.

Widely divergent SIVsm strains in African sooty mangabeys suggest a prolonged natural history of SIVsm in West Africa. Our findings show amino acid diversity up to 19.3% in the *gag* gene of a single mangabey subspecies. This level is larger than that of SIVagm, found in an individual subspecies of the African green monkey (1, 28, 43, 46). Therefore, substantial SIV divergence can arise in mangabeys without either significant geographic isolation or the selective pressures that occur during monkey speciation. From the observed divergence of SIVagm and the large distances between the ranges of each African green monkey species in Africa, it has been inferred that SIVagm is old and has existed in these species since animal speciation (1, 28, 35, 43, 46). This same argument, however, may not apply to sooty mangabeys, in whom high

divergence was seen in a small group of monkeys in the same geographic location without evidence of subspeciation. If SIVagm and SIVsm have similar rates of amino acid replacement (35, 46), then SIVsm in sooty mangabeys is at least as old as SIVagm in African green monkeys. Our findings, therefore, support the hypothesis that five lineages of primate lentiviruses, including SIVsm, SIVagm, SIVmnd, SIVsyk, and SIVcpz, originated from a common ancient ancestor and further evolved in their own natural hosts (46, 54).

Multiple divergent African SIVsm strains were introduced to the United States during sooty mangabey importation, which led to SIV infection in Asian macaques. Two distinct SIV strains in Asian macaques, SIVmac/mne and SIVstm, are genetically equidistant from SIVsmH4 (4, 31), which was from mangabeys kept at Yerkes Primate Center since the 1960s (16, 24). Although SIVs in Asian macaques are known to have been transmitted from SIV originating in the sooty mangabeys (44), the limited diversity of the SIVsmH4 group still does not explain the genetic diversity of macaque viruses. In this study, we found that SIVsmH4, SIVmac/mne, and SIVstm are relatively close to SIVs found in West African sooty mangabeys (Fig. 4; Table 4), indicating the African origins of American SIV isolates. Moreover, SIVsmBro85, isolated from a zoo-housed sooty mangabey in a separate colony in the United States, like macaque SIVs, shows a similarly distant relationship to the SIVsmH4 virus. These results, plus our finding that no two SIVs cluster closely except for the probable mated pair in feral troop 1, support our hypothesis that multiple, diverse SIVsm strains were introduced to the American sooty mangabey colonies during monkey importation. Therefore, SIVstm and SIVmac/mne arose from SIVsm strains through at least two independent cross-species transmission events.

Divergence of SIVsm compared with that of HIV-2. If SIVsm is the origin of HIV-2, the SIVsm group should be at least as diverse as the HIV-2 family. Thus far, only limited genetic diversity has been found in SIVsm, making it difficult to firmly establish such a link (17, 18). Of special importance in this study is the striking similarity in genetic divergence between the newly characterized SIVsm strains and HIV-2 subtypes. Six new SIVs, SL92a to SL92f, plus two additional viruses, Lib-1 and smBro, which had not yet been characterized, add broad divergence to the SIVsm group. Two viruses, for example, SL92b and Lib-1 (Table 4), have more divergence than all previously known SIVsm strains and are more divergent than four of the five known HIV-2 subtypes. Moreover, the distinct new SIVsm strains were genetically about equidistant from all other SIVs and HIV-2 subtypes. Therefore, these data support the hypothesis that distinct HIV-2 subtypes originated from SIVsm strains through multiple cross-species transmission events (17, 54).

Geographic clustering of pet-derived SIV and HIV-2. In addition to their phylogenetic relationship, we found a geographic correlation between pet-derived SIVsm and HIV-2. Phylogenetically, the Lib-1 isolate, from a household pet in Harbel, Liberia, was relatively close to HIV-2F0784 (subtype D) and distant from the other six African SIVsm strains (Fig. 4; Table 4), as supported by 55% of the bootstrap trees in the *gag* gene. Remarkably, HIV-2F0784 was from a patient who worked at a rubber plantation in Harbel, Liberia (22). Moreover, this correlation was not the only such case we observed. SL92b and SL92c, pet-derived SIVs from Sierra Leone, clustered in the *gag* tree with HIV-2PA (subtype E), the most divergent subtype of all HIV-2 isolates, and this clustering was supported by 100% of the bootstrap trees (Fig. 4A). The amino acid diversities among HIV-2PA, SL92b, and SL92c (10.7 to 12.7%) were about twofold less than those found between HIV-2PA and all other HIV-2 subtypes (21.5 to 24.3%) (Table 4). Sequence alignments of deduced *gag* amino acids further supported a cluster of HIV-2PA, SL92b, and SL92c (Fig. 3A), because all three shared multiple informative sites, including a unique signature sequence, RGQPPAQ, in the p26 protein and an identical change in a peptide reported to be HLA restricted (Fig. 3A) (42). Further investigation revealed that the HIV-2PA patient's home village was only 50 miles (19) from the village of Panguma, where the two SIV-infected pets were found (Fig. 1). It is not known if the HIV-2PA-positive person was infected in that person's home village, but it appears certain that the infection occurred in Sierra Leone (25). Therefore, a geographic correlation was demonstrated in West Africa between HIV-2 and all three pet-derived SIV isolates.

The newly found divergence indicates that SIVsm group is the likely progenitor of all HIV-2 subtypes. Furthermore, the close relationship between SIVs in household pet mangabeys and humans suggest a scenario in which SIVs occurring regionally may have crossed the species barrier from household mangabeys to humans. However, exactly how this transmission may have occurred and resulted in the epidemic spread of new HIV-2 subtypes is still undetermined and requires further research.

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