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Simian Immunodeficiency Virus (SIV) from Sun-Tailed Monkeys (*Cercopithecus solatus*): Evidence for Host-Dependent Evolution of SIV within the *C. lhoesti* Superspecies

BRIGITTE E. BEER,[1] ELIZABETH BAILES,[2] ROBERT GOEKEN,[1] GEORGE DAPOLITO,[1] CHEIK COULIBALY,[3] STEPHEN G. NORLEY,[3] REINHARD KURTH,[3] JEAN-PIERRE GAUTIER,[4] ANNIE GAUTIER-HION,[4] DOMINIQUE VALLET,[4] PAUL M. SHARP,[2] AND VANESSA M. HIRSCH[1]*

Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, Maryland 20852[1]; Institute of Genetics, University of Nottingham, Queens Medical Centre, Nottingham NG7 2UH, United Kingdom[2]; Paul-Ehrlich-Institute, 63225 Langen, Germany[3]; and UMR 6552 (CNRS), Station Biologique, 35380 Paimpont, France[4]

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Recently we reported the characterization of simian immunodeficiency virus (SIVlhoest) from a central African l'hoest monkey (*Cercopithecus lhoesti lhoesti*) that revealed a distant relationship to SIV isolated from a mandrill (SIVmnd). The present report describes a novel SIV (SIVsun) isolated from a healthy, wild-caught sun-tailed monkey (*Cercopithecus lhoesti solatus*), another member of the l'hoest superspecies. SIVsun replicated in a variety of human T-cell lines and in peripheral blood mononuclear cells of macaques (*Macaca* spp.) and patas monkeys (*Erythrocebus patas*). A full-length infectious clone of SIVsun was derived, and genetic analysis revealed that SIVsun was most closely related to SIVlhoest, with an amino acid identity of 71% in Gag, 73% in Pol, and 67% in Env. This degree of similarity is reminiscent of that observed between SIVagm isolates from vervet, grivet, and tantalus species of African green monkeys. The close relationship between SIVsun and SIVlhoest, despite their geographically distinct habitats, is consistent with evolution from a common ancestor, providing further evidence for the ancient nature of the primate lentivirus family. In addition, this observation leads us to suggest that the SIVmnd lineage should be designated the SIVlhoest lineage.

Simian immunodeficiency viruses (SIV) and the closely related human immunodeficiency viruses (HIV-1 and HIV-2) belong to the lentivirus subfamily of retroviruses. At present these primate lentiviruses can be classified into five lineages based upon the sequence and functional similarity of their genes (31, 62). These five lineages are represented by (i) SIVcpz from chimpanzees (*Pan troglodytes*) together with HIV-1 (19, 34, 35, 54, 68), (ii) SIVsm from sooty mangabeys (*Cercocebus torquatus atys*) together with HIV-2 (10, 33, 46, 55, 58), (iii) SIVagm from four species of African green monkeys (members of the *Chlorocebus aethiops* superspecies) (4, 6, 12, 16, 32, 36, 38, 47), (iv) SIVsyk from Sykes' monkeys (*Cercopithecus mitis albogularis*) (13, 30), and (v) SIVmnd from a mandrill (*Mandrillus sphinx*) together with SIVlhoest from l'hoest monkeys (*Cercopithecus lhoesti lhoesti*) (28, 66, 67).

The phylogenetic relationships of these viruses exhibit little correlation with those of the host primate species, indicating that cross-species transmissions have occurred on numerous occasions (62). Most notably, the two groups of viruses giving rise to AIDS in humans both appear to have resulted from multiple independent transmissions from other species. It has been clear for some time that feral sooty mangabeys are the source of the HIV-2 epidemic in West Africa (10, 20, 21, 46), while very recent work has implicated chimpanzees of the subspecies *P. troglodytes troglodytes* in Central Africa as the natural reservoir of HIV-1 (19, 34, 35, 53, 54, 68). Other transmissions have occurred both in captivity and in the wild. For example, SIV infection of several species of macaques (*Macaca* spp.) has resulted from accidental introduction of SIVsm in North American primate centers (22, 27, 50), while a yellow baboon (*Papio hamadryas cynocephalus*) in Tanzania (37), a chacma baboon (*Papio ursinus*) in South Africa (69), and a patas monkey (*Erythrocebus patas*) in Senegal (8) have each become infected in the wild by viruses derived from the local sympatric species of African green monkeys. These examples all reflect comparatively recent cross-species transmission events, readily identifiable by virus-host phylogenetic discordance within the major primate lentivirus lineages. In addition, deeper branchings within the phylogeny are at odds with the relationships among the species inferred to be the natural host for each lineage. The five major viral lineages are approximately equidistant, and yet four comprise viruses naturally infecting *Cercopithecidae* (Old World monkeys) while the fifth is found in apes. Furthermore, it is remarkable that the recently characterized SIVlhoest falls within the same lineage as SIVmnd (28), since l'hoest monkeys are closely related to other members of the genus *Cercopithecus* while mandrills are more closely related to mangabeys (*Cercocebus* spp.) (25). Thus, cross-species transmissions greatly complicate any attempts to determine the evolutionary origins of this group of viruses or the timescale of primate infection. For this reason, it is of interest to identify groups of viruses that may have evolved in a host-dependent fashion.

There is evidence of host-dependent evolution of lentiviruses among African green monkeys (4, 32, 38, 47). However, the viruses identified to date may represent only a small fraction of the lentiviruses present in African primates. Recently, SIVs from red-capped mangabeys (*Cercocebus torquatus torquatus*) (SIVrcm) and drills (*Mandrillus leucophaeus*) (SIVdrl) have been partially characterized (11, 24). In addition, serological surveys have indicated that DeBrazza monkeys (*Cercopithecus neglectus*), moustached monkeys (*Cercopithecus ce-*

* Corresponding author. Mailing address: LMM, NIAID, NIH, 12441 Parklawn Dr., Rockville, MD 20852. Phone: (301) 496-2976. Fax: (301) 480-2618. E-mail: vhirsch@nih.gov.

TABLE 1. Serological survey of different wild-caught primate species

Species (common name)	Sex[a]	Origin	Age (yr)	Result of:				
				SIVagm ELISA (titer)	SIVagm Western blot	HIV-1/2 Western blot[b]	SIVsmH4 RIPA	HTLV Western blot[c]
Cercopithecus campbelli (Campbell's monkey)	M	Sierra Leone	12	−	−	−	−	−
	F	Sierra Leone	26	+ (3,768)	+ (p24)	+ (p24)	+ (p24, gp120/160)	−
	F	Sierra Leone	11	+ (2,925)	−	−	+ (gp160)	−
	F	Sierra Leone	11	ND[d]	+ (p24)	ND	+ (p24, gp120/160)	−
	F	Sierra Leone	14	ND	−	ND	−	−
Cercopithecus nictitans (greater white-nosed monkey)	F	Gabon	21	−	ND	−	ND	−
	M	?	12	−	ND	−	ND	−
Cercopithecus erythrotis (red-eared monkey)	M	Cameroon	13	−	−	−	−	−
	F	Cameroon	16	−	−	−	−	−
Cercopithecus lhoesti solatus (sun-tailed monkey)	M	Gabon	13	−	ND	−	−	−
	M	Gabon	9	+ (4,463)	ND	+ (gp160)	+ (gp160)	−
Lophocebus albigena (grey-cheeked mangabey)	F	Gabon	23	−	ND	−	ND	−
Cercocebus torquatus torquatus (red-capped mangabey)	F	?	16	ND	ND	−	ND	−

[a] M, male; F, female.
[b] Genelabs Diagnostics, Singapore, Singapore.
[c] Murex Biotech Ltd., Dartford, England. RIPA, radioimmunoprecipitation.
[d] ND, not done.

phus), Diana monkeys (*Cercopithecus diana*), greater white-nosed monkeys (*Cercopithecus nictitans*), talapoins (*Miopithecus talapoin*), Allen's swamp monkeys (*Allenopithecus nigroviridis*), and colobus monkeys (*Colobus guereza*) may all harbor lentiviruses (45, 51, 65).

In our efforts to elucidate the origins and evolution of the primate lentiviruses, we obtained blood samples from 13 wild-caught monkeys, representing six different species, from West Africa. Plasma samples of three Campbell's monkeys (*Cercopithecus campbelli*) and one sun-tailed monkey (*Cercopithecus lhoesti solatus*) contained antibodies that were cross-reactive with SIVagm antigens. However, a virus with a Mg^{2+}-dependent reverse transcriptase (RT) activity could be isolated only from the SIV-positive sun-tailed monkey. The new viral isolate was designated SIVsun according to its species of origin. The sun-tailed monkey is a rare species, inhabiting the Forêt des Abeilles (Forest of the Bees), a restricted area of about 10,300 km^2 in Gabon (9), and was first described in 1984 (26). SIVsun is of particular interest because sun-tailed and l'hoest monkeys are classified within the same superspecies, indicating that they are considered to be closely related. This paper describes the biological and genetic characterization of SIVsun.

MATERIALS AND METHODS

Animals, serologic testing, and virus isolation. Blood samples from five Campbell's monkeys (*Cercopithecus campbelli*), two greater white-nosed monkeys (*Cercopithecus nictitans*), two red-eared monkeys (*Cercopithecus erythrotis*), two sun-tailed monkeys (*Cercopithecus lhoesti solatus*), one grey-cheeked mangabey (*Lophocebus albigena*), and one red-capped mangabey (*Cercocebus torquatus torquatus*) were obtained. All the animals had been caught in the wild in West and Central Africa (Table 1). Plasma samples from the animals were tested by enzyme-linked immunosorbent assay (ELISA) with whole SIVagm lysate, by Western blot analysis against SIVagm or HIV-1 and HIV-2 (Genelabs Diagnostics, Singapore, Singapore), and by radioimmunoprecipitation (RIPA) with SIVsmH4 virus and cell lysate. Subsequently, peripheral blood mononuclear cells (PBMC) from the animals that appeared to be seropositive by at least one of these assays were cocultivated with the human T-cell lines C8166 or MOLT4clone8 (M4C8) to isolate SIVs. Antibodies to human T-lymphotropic virus (HTLV) types 1 and 2 were tested with commercial Western blot strips (Murex Biotech Ltd., Dartford, United Kingdom).

Cells, production of virus stocks, and in vitro infectivity studies. The human $CD4^+$ cell lines, MT2, MT4, H9, U937, SupT1, C8166, CEMss, CEM174, PM1, Hut78, and M4C8 were maintained in RPMI complete medium (RPMI 1640, supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10 mM HEPES). PBMC were separated by Ficoll-Hypaque density gradient centrifugation of whole blood, and 10^7 cells were stimulated with 2 μg of phytohemagglutinin (PHA; Sigma, St. Louis, Mo.) per ml for 3 days and then maintained in RPMI complete medium, supplemented with 5 half-maximal units of human interleukin-2 (Advanced Biotechnologies, Columbia, Md.) per ml. Virus stocks were produced on CEMss cells by harvesting the supernatant at the peak of RT activity, filtering it through a 0.45-μm-pore-size filter, and cryopreserving the stocks in the vapor phase of liquid nitrogen. Titers of virus stocks were determined on CEMss and C8166 cells by using threefold dilutions and used to infect human $CD4^+$ cell lines or monkey PBMC at a multiplicity of infection (MOI) of 0.01. The cells were infected for 4 h, washed three times with Hanks' buffered salt solution (HBSS), and then resuspended in 3 ml (cell lines) or 10 ml (PBMC) of RPMI complete medium. Cell-free supernatants were taken at regular intervals for the measurement of RT activity (52) combined with medium exchange.

Lymphocyte immunophenotyping. Lymphocyte subsets (CD3, CD4, CD8, and CD20) were measured by fluorescence-activated cell sorter analysis with a Coulter EPICS XL-MCL instrument (assays were performed by FAST Systems, Inc., Gaithersburg, Md.). Monkey peripheral blood leukocytes were stained with monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), or PerCP. EDTA whole-blood samples were incubated for 30 min in the dark at 4°C in the presence of sodium azide with the appropriate monoclonal antibody conjugate. Following staining, the erythrocytes were lysed and the leukocytes were fixed in 1% paraformaldehyde and analyzed with the Coulter EPICS XL-MCL instrument. Several human monoclonal antibodies to cell surface markers were tested, and the ones with the highest cross-reactivity were used for immunophenotyping: rhesus anti-CD3–PE (from M. Rosenzweig, Harvard University) to identify total T lymphocytes, OKT4A-FITC (Ortho Diagnostic Systems, Raritan, N.J.) to identify $CD4^+$ lymphocytes, Leu2A-PerCP (Becton Dickinson, Franklin Lakes, N.J.) to identify $CD8^+$ lymphocytes, and Leu16-

FITC (Becton Dickinson) to identify CD20-expressed on B lymphocytes. Leukocyte counts were measured with a Technicon H1*E hematology instrument. Absolute lymphocyte counts were determined by differentiating the lymphocytes, monocytes, and granulocytes by light scattering on the Coulter EPICS XL-MCL flow cytometer and multiplying the lymphocyte percentage by the leukocyte count.

Neutralizing-antibody assay. Heat-inactivated plasma (56°C for 20 min) from the sun-tailed monkeys were serially diluted threefold in tissue culture microtiter plates (20 μl per well; four replicates and six dilution steps) beginning with a 1:2 dilution. A total of 100 50% tissue culture infective doses ($TCID_{50}$) of SIVsun, grown in CEMss cells, was added in an equivalent volume (20 μl) per well, mixed with the virus by pipetting, and incubated at 37°C for 1 h. A 160-μl volume of PHA-stimulated pigtailed macaque PBMC (10^5 cells) was added to each well, and the plates were incubated for 7 days before they were scored for RT activity. The following controls were included in the assay: (i) virus and cells (no-plasma control) (ii) cells alone (no-virus control), and (iii) virus alone (no-cell control).

RIPA. CEMss or CEM174 cells were infected with SIV and, at the peak of RT activity, labeled overnight with L-[^{35}S]methionine and L-[^{35}S]cysteine (Amersham, Arlington Heights, Ill.). The next day, the labeled cells were lysed with 1 ml of RIPA buffer (20 mM Tris-HCl [pH 7.5], 5 mM EDTA [pH 8.0], 312.5 mM NaCl, 1.0 g of sodium deoxycholate, 1% Nonidet P-40) and centrifuged, and the cell lysate supernatant was preabsorbed with 50 μl of protein A-agarose beads (Gibco BRL, Gaithersburg, Md.) for 1 h. A 10-μl volume of plasma was combined with 50 μl of the protein A-agarose beads and incubated with shaking for 1 h at 4°C. The protein A-agarose bead-antibody complex was washed once with phosphate-buffered saline, combined with 100 μl of the cell lysate, incubated with shaking for 1 h at 4°C, and then washed five times with 1 ml of RIPA buffer. The pellet was resuspended in 50 μl of RIPA buffer and 50 μl of 2× sodium dodecyl sulfate (SDS) gel-loading buffer (100 mM Tris-HCl [pH 6.8], 200 mM 2-mercaptoethanol, 4% SDS, 0.2% bromophenol blue, 20% glycerol), boiled for 4 min, and then loaded onto an SDS–10% polyacrylamide gel. The dried gel was exposed to Bio-Max MR film (Kodak, Rochester, N.Y.) for 3 days. The polyclonal plasma used for precipitation originated from the naturally SIV-infected sun-tailed monkey (α-SIVsun, two different blood draws); from macaques experimentally infected with SIVlhoest-P (α-SIVlhoest), SIVagmVer(3) (α-SIVagm), SIVsmF236 (α-SIVsm), or SIVmac251/32H (α-SIVmac); and from HIV-infected humans (α-HIV-1 and α-HIV-2). The α-HIV-1 plasma consisted of a pool collected from several individuals.

PCR amplification and plasmid cloning. Total cellular DNA was extracted from infected C8166 cells by conventional methods. For amplification of the *gag* sequence, the following primer pairs, which were chosen from regions that were conserved between SIVlhoest and SIVmndGB1, were used. The primers contained *Xho*I and *Csp*45I restriction sites (underlined) to facilitate cloning: lhoest *gag* F (5′-CTAGCTCGAGGCGCCCGAACAGGGACTTCAAG-3′) and lhoest *gag* R (5′-ATTCATTCGAACTATTGGTCGTCTGGAAAGAG-3′). The conditions for the amplification were 30 cycles of 94°C for 1 min, 55°C for 1.5 min, and 72°C for 2 min. The resulting 1,856-bp (*gag*) fragment was cloned into the pGEM-7Zf plasmid vector (Promega, Madison, Wis.) and subsequently used as a probe for Southern hybridization and screening of a bacteriophage lambda library.

Bacteriophage lambda cloning. A variety of restriction enzymes were evaluated by Southern blot hybridization of total DNA from infected C8166 cells by using the 1,856-bp *gag* fragment as a probe. All enzymes useful for lambda cloning had restriction sites within the SIVsun genome, and thus *Eco*RI, which cleaved once within the genome, was chosen for lambda cloning to yield a 8.6-kb viral fragment. Total cellular DNA was digested to completion with *Eco*RI, fractionated over a 20 to 60% sucrose gradient to obtain 9- to 20-kb fragments, and ligated into *Eco*RI-cleaved arms of the λDASHII vector (Stratagene, La Jolla, Calif.). Ligation products were packaged in vitro (Gigapack Gold III; Stratagene) and subjected to titer determination on *Escherichia coli* K802. A total of 8.3×10^5 recombinant plaques were screened, using a [^{32}P]dCTP-labeled *gag* fragment (Multiprime DNA labeling system; Amersham), and the hybridized blotting filters were exposed to Kodak X-Omat AR film. Six positive clones were detected and plaque purified. The restriction enzyme *Sma*I was used to reduce the cellular sequences flanking the 5′ long terminal repeat (LTR) to 4 kb, and the complete clone was ligated as a *Sma*I-*Eco*RI fragment (L14) into the pGEM7Zf plasmid vector to facilitate further cloning strategies. The missing 1.4-kb 3′ part of the full-length virus was generated by PCR with primers 3322 (5′-AAGTAG TACCAGCTCCACTGC-3′) and 3323 (5′-ATGCCTCGAGTGCTAGCCGTC AGCCCCGGAT-3′). Primer 3323 contained a *Xho*I restriction site (underlined). The PCR fragment (S2) was digested with *Xho*I and partially with *Eco*RI (since the fragment contained two *Eco*RI sites) and cloned into pGEM7Zf plasmid vector. Both virus fragments (8.6 and 1.4 kb) were sequenced by automated fluorescence sequencing (*Taq* amplification/termination; Perkin- Elmer Applied Biosystems, Warrington, United Kingdom). Finally, the 1.4-kb fragment was ligated into the linearized plasmid containing the 12.6-kb fragment to achieve the full-length replication-competent molecular clone SIVsunλ20(L14/S2).

Sequence comparisons. The predicted protein sequences encoded by SIVsun were compared to the following representatives of the major lentivirus lineages: HIV-1 subtype A (isolate U455; GenBank accession no. M62320), subtype B (BRU; K02013), and group O (MVP5180; L20571); SIVcpz strains Gab (X52154) and Ant (U42720); SIVsm (PBj; M31325); HIV-2 subtype A (ROD; M15390) and subtype B (EHOA; U27200); SIVagm from vervets (ver155; M29975), grivets (gri-1; M58410), and tantalus (tan-1; U58991); SIVsyk (173; L06042); SIVmnd (GB1; M27470); and SIVlhoest (AF075269). Protein sequences were aligned by using ClustalX (64), with minor manual adjustment with SEAVIEW (18). Sites that could not be aligned unambiguously, as well as sites containing a gap in any of the sequences, were excluded from the analyses. The extent of sequence difference along the genome between SIVsun and other primate lentiviruses was examined in diversity plots of concatenated Gag, Pol, Vif, Env, and Nef protein sequences; in the regions of overlap between the Gag and Pol, Pol and Vif, and Env and Nef coding sequences, the carboxy termini of the Gag, Pol, and Env proteins were excluded. The fractional amino acid sequence difference was calculated for a window size of 200 residues, moved in steps of 10 residues.

Phylogenetic relationships among the sequences were estimated by the neighbor-joining and maximum-likelihood approaches. The neighbor-joining method (59) was applied to protein distances with Kimura's correction (40) and 1,000 bootstrap replicates and was implemented by using ClustalX (64). The maximum-likelihood method was implemented with PROTML (1) by using the JTT model (39) with data frequencies. The order of sequence input was shuffled five times, with the same best tree being found each time.

Nucleotide sequence accession number. The sequence of SIVsunλ20L14/S2 has been submitted to GenBank under accession no. AF131870.

RESULTS

Plasma samples from 13 wild-caught African monkeys, including 2 sun-tailed monkeys, were tested for SIV-specific antibodies. As shown in Table 1, plasma from three Campbell's monkeys (*Cercopithecus campbelli*) and one sun-tailed monkey (*Cercopithecus lhoesti solatus*) had cross-reactive antibodies to SIVagm, SIVsm, or HIV-1 and HIV-2, with ELISA titers against SIVagm ranging from 1:3,000 to 1:5,000. Whereas plasma from the seropositive sun-tailed monkey cross-reacted solely with gp160 of SIVsm and HIV-1/HIV-2, plasma from two of the three seropositive Campbell's monkeys additionally cross-reacted with the major core protein p27 of SIVagm and SIVsm. Interestingly, all the monkeys were negative for antibodies to HTLV-1 and HTLV-2 (Table 1). The seropositive sun-tailed monkey was of particular interest since this species is a member of the same superspecies as the l'hoest monkey, the source of the recently described SIVlhoest isolate (28). Isolation of SIV from PBMC of this sun-tailed monkey was therefore attempted by cocultivation with the human $CD4^+$ cell lines, C8166 or M4C8. Virus was successfully isolated, as evidenced by the presence of RT activity in culture supernatants by 2 weeks after cocultivation with either of these two human T-cell lines, and the new virus isolate was designated SIVsun. Several attempts to isolate SIV from the three Campbell's monkeys by cocultivation of whole PBMC with different human T-cell lines (C8166, M4C8, and CEMss) and of CD8-depleted PBMC with a herpesvirus saimiri-transformed $CD4^+$ cell line from a seronegative Campbell's monkey were unsuccessful.

Host range of SIVsun in human $CD4^+$ cell lines and monkey PBMC. The tropism of SIVsun for human T-cell lines was compared with that of SIVlhoest and SIVagm. The human $CD4^+$ cell lines, MT2, MT4, H9, U937, SupT1, C8166, CEMss, CEM174, PM1, Hut78, and M4C8 were infected with an MOI of 0.01 of uncloned, cell-free SIVagmVer90, SIVlhoest, and SIVsun produced in CEMss cells, and culture supernatants were evaluated for RT activity for 4 weeks. After infection with SIV and extensive washing with HBSS, all RT activities were below 300 cpm per μl. Subsequent RT activity of less than 1,000 cpm per μl was considered negative. The RT activities shown in Table 2 represented peak levels 1 or 2 weeks after infection. All three viruses replicated in CEMss, M4C8, MT4, SupT1, and C8166 cell lines with variable efficiency. None of the viruses replicated in H9, U937, Hut78, or PM1 (a Hut78 derivative) cell lines. Some of the cell lines, such as MT2

TABLE 2. Host range of uncloned SIVagm90, SIVlhoest, and SIVsun in different human T-cell lines

Cell line	RT activity[a] of:		
	SIVagm90	SIVlhoest	SIVsun
MT2	+	−	−
C8166	++	+	+
H9	−	−	−
MT4	+	+++	+++
U937	−	−	−
SupT1	+++	+	++
PM1	−	−	−
Hut78	−	−	−
Molt4cl8	+	+	++
CEMss	+++	++	+++
CEM174	−	+	−

[a] RT activities represent peak values 1 or 2 weeks after infection with a MOI of 0.01. −, 0 to 1,000 cpm/μl; +, 1,001 to 5,000 cpm/μl; ++, 5,001 to 20,000 cpm/μl; +++, >20,000 cpm/μl.

(SIVagm) and CEMx174 (SIVlhoest), were variably infected with one or more of the viruses.

The replication capacity of SIVsun was also assessed in primary PHA-stimulated PBMC from rhesus macaques (*Macaca mulatta*), pigtailed macaques (*M. nemestrina*), and patas monkeys (*Erythrocebus patas*). Culture supernatant was collected every 2 days with a complete medium change, and thus the measured RT activities in Fig. 1 represent newly produced virus. SIVsun replicated efficiently in PBMC from rhesus, pigtailed, and patas monkeys, with peak levels achieved as early as 4 days after infection (Fig. 1). PBMC from both rhesus macaques showed similar susceptibility to SIVsun, whereas PBMC from one each of the two pigtailed macaques and patas monkeys were considerably less susceptible to infection. Studies to determine the replication kinetics in human PBMC are in progress.

Characteristics of natural infection of sun-tailed monkeys with SIVsun. The sun-tailed monkey that was the source of the SIVsun isolate was born in 1989 in the Forêt des Abeilles in Gabon, caught at the age of 15 days, and shipped to the Primate Facility in France. During captivity, the monkey had contact with only two other sun-tailed monkeys, one of which is SIV seronegative and one of which had an unknown SIV status, thereby excluding cross-species transmission. As observed with other African monkeys naturally infected with SIV, this monkey was apparently healthy, and signs of immunodeficiency, such as weight loss, diarrhea, or opportunistic infections were not observed.

To gather information on natural SIV infection, the characteristics of the immune response and the distribution of lymphocyte subsets were evaluated. Fluorescence-activated cell sorter analysis was used to determine the lymphocyte subsets in both the seronegative and seropositive sun-tailed monkeys. The absolute numbers of $CD4^+$ cells were slightly smaller in the SIV-infected than in the noninfected monkey (391 and 516/μl of blood, respectively [Table 3]), but this difference was unlikely to be significant. $CD8^+$ lymphocyte numbers exceeded $CD4^+$ lymphocyte numbers, leading to an inverted CD4/CD8 ratio (0.9 and 0.7). Unlike African green monkeys, in which $CD4^+$ lymphocytes routinely coexpress the CD8a antigen (2, 7, 14, 48, 57), $CD4^+$ $CD8^+$ double-positive PBMC did not exceed 1% for either of these two sun-tailed monkeys.

In terms of serologic response, Western blot analysis of the plasma sample from the seropositive sun-tailed monkeys demonstrated only antibodies cross-reactive with envelope proteins

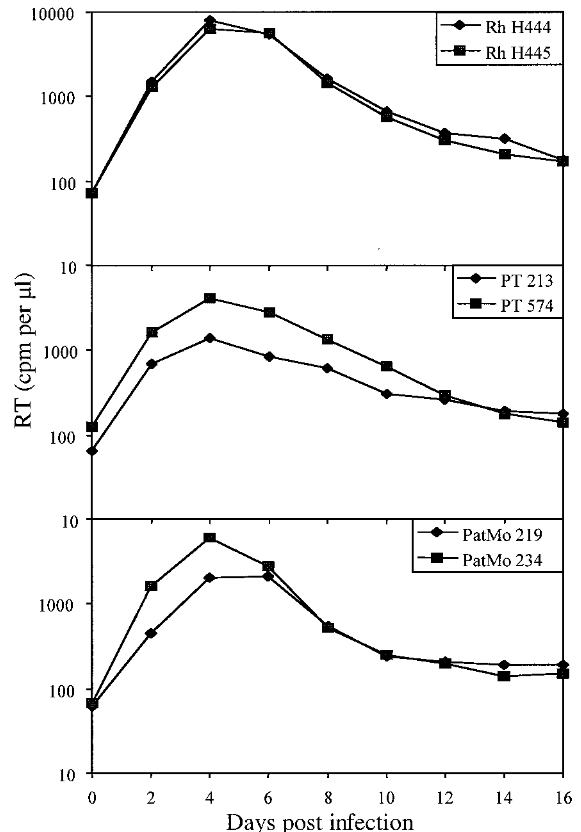

FIG. 1. Infection of PHA-stimulated PBMC from rhesus (Rh), pigtailed (PT), and patas (PatM.) monkeys with uncloned SIVsun (MOI, 0.01). The virus stock was produced on CEMss cells. PBMC were infected for 4 h, washed three times with HBSS, and then resuspended in 3 ml of RPMI complete medium. Cell-free supernatants were taken at regular intervals for the measurement of RT activity combined with a complete exchange of medium.

of SIVagm or HIV-2 (Table 1). Weak Gag-specific antibody responses are also characteristic of naturally infected African green monkeys. Since the sera of the naturally infected sun-tailed monkey showed strong binding to the SIVsun envelope proteins, the ability of these sera to neutralize 100 $TCID_{50}$ of SIVsun was evaluated by using PHA-stimulated PBMC from pigtailed macaques as target cells. Neutralizing activity in three different plasma samples was not demonstrable even with a 1:2 dilution of these samples (data not shown).

Antigenic cross-reactivity between SIVsun and other primate lentiviruses. Immunoprecipitation of SIV antigens from radiolabeled cell lysates of cells infected with either SIVsun (Fig. 2A) or SIVsm (Fig. 2B) was used to evaluate the antigenic relationship of SIVsun to other SIV and HIV isolates. A range of antisera from the SIV-infected monkeys (SIVsun, SIVlhoest, SIVsm, SIVmac, and SIVagm) and HIV-infected humans (HIV-2 and HIV-1) were used in this assay. Plasma of the SIVsun-infected monkey immunoprecipitated the envelope proteins of SIVsun but did not react with SIVsun Gag proteins. The Gag proteins (a 55-kDa precursor and a 27-kDa protein) of SIVsun could be seen by immunoprecipitation with plasma samples from a SIVlhoest-infected macaque. This sample also reacted with the envelope proteins of SIVsun. In contrast, antisera specific for SIVagm, SIVsm, SIVmac, HIV-1, and HIV-2 reacted primarily with the Gag proteins and only weakly with envelope proteins. The weakest antigenic cross-

TABLE 3. Lymphocyte subsets in two wild-caught sun-tailed monkeys

Subset	Value in:	
	SIV-negative monkey	SIV-positive monkey
Lymphocytes (%)	34.7	23.7
$CD3^+$ lymphocytes[a] (%)	54.6	56.1
$CD4^+$ lymphocytes[b] (%)	36.3	31.8
$CD8^+$ lymphocytes[c] (%)	40.8	46.2
$CD4^+$ $CD8^+$ lymphocytes (%)	1.0	1.2
$CD20^+$ lymphocytes[d] (%)	15.6	11.9
Leukocytes (cells/μl)	4,100	5,190
Lymphocytes (cells/μl)	1,423	1,230
$CD3^+$ lymphocytes (cells/μl)	777	690
$CD4^+$ lymphocytes (cells/μl)	516	391
$CD8^+$ lymphocytes (cells/μl)	580	568
$CD20^+$ lymphocytes (cells/μl)	222	146

[a] Rhesus anti-CD3–PE (M. Rosenzweig, Harvard University).
[b] OKT4A-FITC (Ortho Diagnostic Systems, Raritan, N.J.).
[c] Leu2A-PerCP (Becton Dickinson, Franklin Lakes, N.J.).
[d] Leu16-FITC (Becton Dickinson).

reactivity was observed for antisera specific to HIV-1 and HIV-2. These data suggest a closer antigenic relationship between SIVsun and SIVlhoest.

To confirm the antigenic relationships between SIVsun and SIVsm/SIVmac, SIVsmH4-infected cell lysates were used as the source of antigen, as shown in Fig. 2B. The SIVsun and SIVlhoest-specific samples cross-reacted weakly with the Env proteins of SIVsm. Due to the lack of Gag-specific antibodies in the plasma sample from the SIVsun-infected monkey, Gag antigens were not observed for these immunoprecipitations. In contrast, sera specific for viruses of the SIVsm lineage (SIVsm, SIVmac, and HIV-2) precipitated the Gag, Pol, and Env proteins of SIVsm, demonstrating the close antigenic relationship between these viruses. Interestingly, the SIVagm-specific plasma also cross-reacted with SIVsm Env glycoproteins despite the distant antigenic and genetic relationship between SIVsm and SIVagm. As also observed for the SIVsun cell lysates in Fig. 3A, HIV-1-specific plasma cross-reacted weakly with the SIVsmH4 Env proteins. A faint reaction to p27 in the plasma of the SIV-positive sun-tailed monkey could be detected by SIVsun-specific Western blotting but the band was much weaker than that corresponding to the reaction of plasma of SIVlhoest- or SIVagm3-infected pigtailed macaques (data not shown).

SIVsun is a novel member of the SIVmnd/SIVlhoest lineage. Assuming a closer genetic relationship between SIVsun, SIVlhoest, and SIVmnd, primers spanning the primer binding site and the *gag* gene were chosen from sequences conserved between SIVlhoest and SIVmnd. A 1,856-bp fragment was amplified by PCR from cellular DNA extracted from infected C8166 cells and cloned. This *gag* fragment was used for subsequent Southern blot hybridization to identify restriction enzymes useful for cloning and as a probe to identify proviruses within a bacteriophage lambda library generated by *Eco*RI digestion of cellular DNA extracted from infected C8166 cells. Six clones hybridizing with the *gag*-specific viral probe were obtained, and one, which contained a 8.6-kb viral fragment, was used to generate a full-length replication-competent clone of SIVsun. The 3′ part of the virus (1.4 kb), which was absent from the lambda clone, was generated by PCR and ligated into the linearized recombinant plasmid containing the 8.6-kb viral fragment to generate the full-length molecular clone SIVsunλ20(L14/S2).

SIVsunλ20(L14/S2) was infectious after transfection of CEMss cells (data not shown). The clone was sequenced in its entirety (10,006 nucleotides [nt]) and compared to other primate lentiviruses. The genomic organization of SIVsun was similar to that of SIVlhoest, SIVmnd, SIVagm, and SIVsyk.

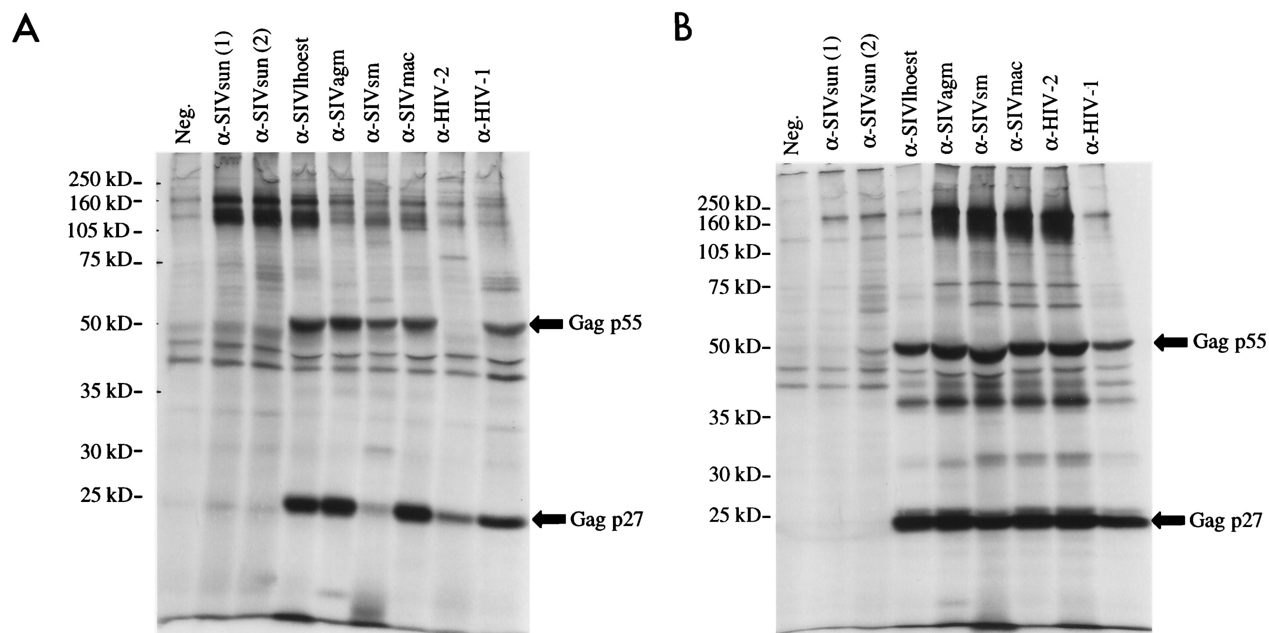

FIG. 2. RIPA of viral proteins. CEMss cells infected with SIVsun (A) or CEM174 cells infected with SIVsmH4 (B) were labeled overnight with L-[^{35}S]methionine and L-[35^{35}S]cysteine (Amersham), lysed, and precipitated with plasma from different monkeys. α-SIVsun plasma was derived from two separate bleedings of the naturally infected sun-tailed monkey [α-SIVsun(1) and α-SIVsun(2)]; α-SIVlhoest, α-SIVagm, α-SIVsm, and α-SIVmac plasma were collected from experimentally SIV-infected macaques; and α-HIV-1 and α-HIV-2 plasma originated from infected humans.

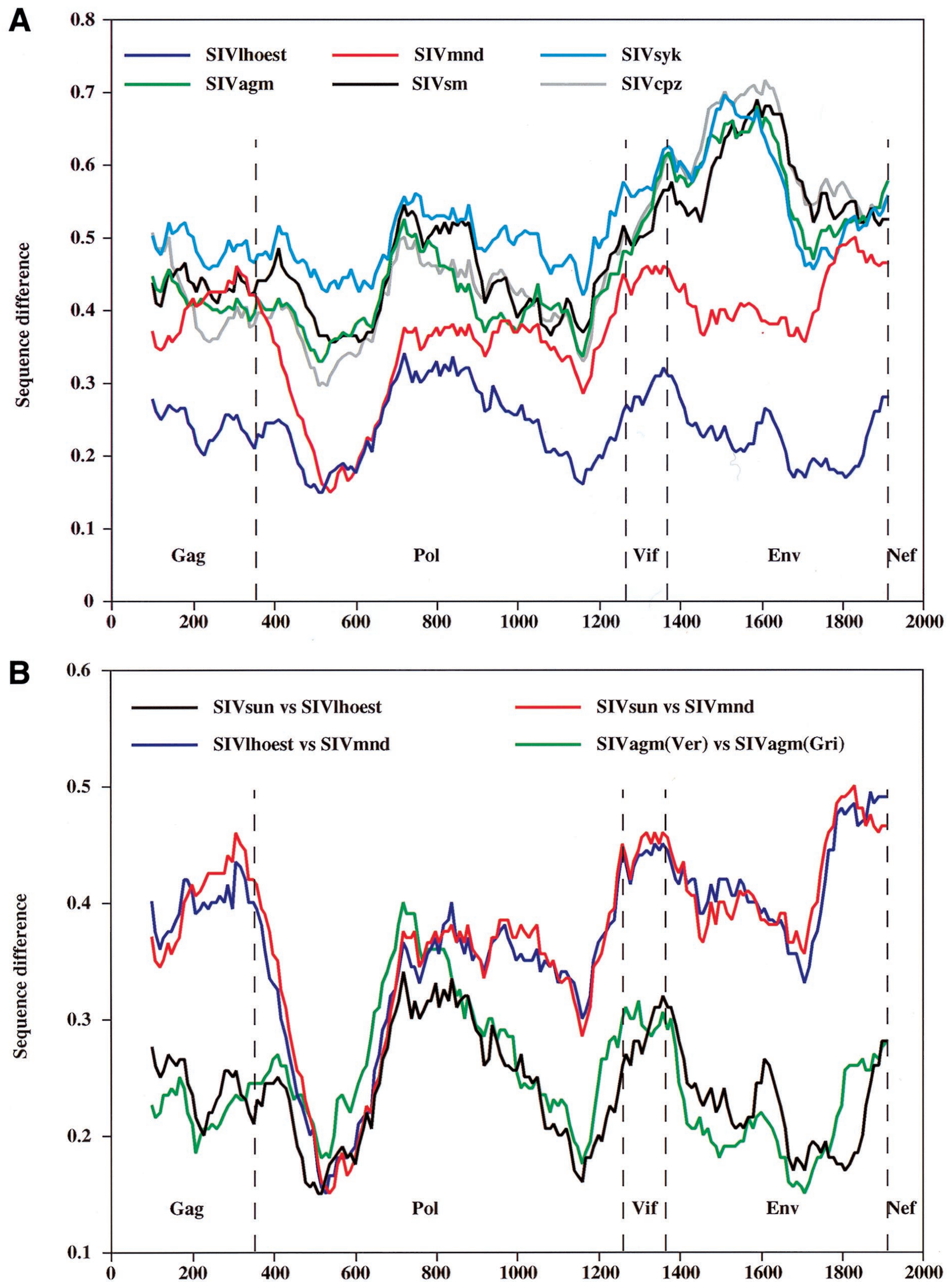

FIG. 3. Diversity plots. (A) Plots comparing SIVsun with representatives of the five major lineages of primate lentiviruses, i.e., SIVlhoest, SIVmnd, SIVsyk, SIVagm(Ver), SIVsm(PBj), and SIVcpz(Gab). (B) Plots comparing SIVsun with SIVlhoest, SIVsun with SIVmnd, SIVlhoest with SIVmnd, and SIVagm(Gri) with SIVagm(Ver). The protein sequence difference is plotted for windows of 200 amino acids moved in steps of 10.

TABLE 4. Percent protein sequence identities between SIVsun and other SIVs

SIV	% Identity to SIVsun in:							
	Gag	Pol	Vif	Vpr	Tat	Rev	Env	Nef
SIVlhoest	71	73	52	63	46	46	67	56
SIVmnd	56	66	34	50	32	39	48	48
SIVsyk	46	49	30	25	29	13	35	34
SIVagm	53	56	29	40	40	31	33	39
SIVsm	52	54	33	40	33	26	34	43
SIVcpz	50	57	31	38	27	17	30	43

Each of these viruses encodes *gag*, *pol*, and *env*, as well as the accessory genes *vif*, *vpr*, *tat*, *rev*, and *nef*, but lacks the additional genes *vpu* (found only among the members of the HIV-1/SIVcpz lineage) and *vpx* (specific to the members of the HIV-2/SIVsm lineage). The LTR of SIVsun (769 nt) contained all the characteristic features of other primate lentivirus LTRs. Similar to the LTRs of the other members of the SIVmnd lineage (SIVmnd and SIVlhoest), the LTR of SIVsun contained one NF-κB site and two potential SP-1 binding sites (data not shown).

Comparisons of the predicted protein sequences encoded by the eight common genes revealed that SIVsun was much more similar to SIVlhoest than to any other SIV (Table 4). For the larger proteins at least, the next most similar virus was SIVmnd; this is as expected, since SIVlhoest and SIVmnd were previously found to be members of the same major lineage (28). The nature of the divergence between SIVsun and other SIVs was also examined in diversity plots, depicting the extent of sequence difference for windows of 200 amino acids along the concatenated predicted gene products (or, for simplicity, the proteome). Such diversity plots are useful in highlighting viral sequences that may have undergone recombination in the past (19): mosaic sequences generated by ancestral recombination exhibit different relative extents of divergence from other sequences. The plots confirmed that SIVsun is consistently much less divergent from SIVlhoest than from other SIVs throughout most of the proteome. However, the plots also revealed one region, of about 100 residues near the beginning of the reverse transcriptase region of Pol, where SIVsun, SIVlhoest, and SIVmnd are approximately equidistant (Fig. 3A; see also Fig. 3B).

To investigate whether the SIVsun, SIVlhoest, and SIVmnd lineages had recombined at some point, phylogenetic analyses were conducted for the same moving windows of 200 amino acids. Fifteen HIV and SIV sequences were included (as in Fig. 4) and 100 bootstrap replicates were performed for each of the 182 windows examined, and so the neighbor-joining method was used. For all 182 windows, SIVsun, SIVlhoest, and SIVmnd formed a clade which in most cases (142 windows) was supported by high bootstrap values (>80%). In all but eight consecutive windows, SIVsun and SIVlhoest were more closely related to each other than to SIVmnd, with high bootstrap support in the vast majority of cases (160 of 174). Within the exceptional region of 270 residues highlighted in the diversity plot (Fig. 3A), alternative branching orders were found, with SIVmnd clustering with either SIVsun or SIVlhoest. The bootstrap values for these alternative topologies ranged between 48 and 71%. This exceptional region was investigated further by maximum-likelihood phylogenetic analysis of moving windows of 300 amino acids from just four sequences, namely, SIVsun, SIVlhoest, SIVmnd, and an outgroup (SIVagmVer). Moving in steps of 10 amino acids, there were only three

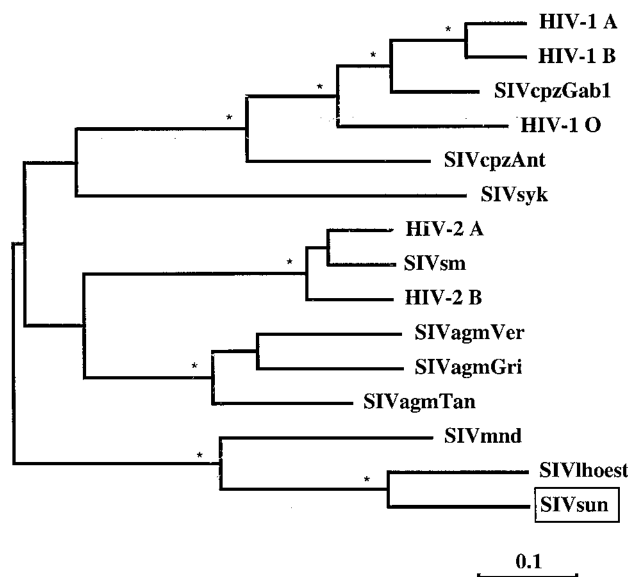

FIG. 4. Phylogenetic relationship of SIVsun (boxed) to other primate lentiviruses. The tree was derived by the maximum-likelihood analysis of a concatenated Gag-Pol-Vif-Env-Nef protein alignment (see the text for details). A tree derived by neighbor-joining analysis differed in no significant way. Stars indicate that the clade to the right was found in 100% of bootstrap replicates of the neighbor-joining analysis. Horizontal branch lengths are drawn to scale, with the bar indicating 0.1 amino acid replacement per site.

windows in which the tree clustering SIVsun and SIVlhoest was not optimal, and in each case the (suboptimal) tree in which SIVsun and SIVlhoest clustered was less than one standard error worse than the optimal tree. This Kishino and Hasegawa maximum-likelihood test (41) indicated that this change in branching order was not significant. Thus, there was no significant support for recombination, and we conclude that the diversity plots are highlighting a region that has been unusually highly conserved in the SIVmnd lineage. Therefore, to summarize the phylogenetic position of SIVsun, we performed a maximum-likelihood analysis on a concatenated Gag-Pol-Vif-Env-Nef protein alignment. As shown in Fig. 4, the results confirmed that SIVsun is a member of the same major lineage as SIVmnd and SIVlhoest and that within that lineage SIVsun and SIVlhoest are clearly the more closely related pair.

The degree of protein sequence similarity between SIVsun and SIVlhoest was also compared to that seen in the other cases where different lineages of SIV have been found within a species or superspecies, namely, in African green monkeys and in chimpanzees (Table 5). Overall, the extent of sequence identity between SIVsun and SIVlhoest was very similar to that found among SIVagm isolates from vervet, grivet, and tantalus monkeys. The extent of divergence between SIVcpz from dif-

TABLE 5. Percent protein sequence identities within primate lentivirus lineages

Comparison	% Identity in:							
	Gag	Pol	Vif	Vpr	Tat	Rev	Env	Nef
SIVsun vs SIVlhoest	71	73	52	63	46	46	67	56
SIVagmTan vs SIVagmVer	73	69	60	82	54	53	66	63
SIVagmGri vs SIVagmVer	71	71	53	70	52	50	68	70
SIVagmTan vs SIVagmGri	78	67	54	69	62	44	63	69
SIVcpzGab vs SIVcpzAnt	67	73	54	63	59	43	49	52

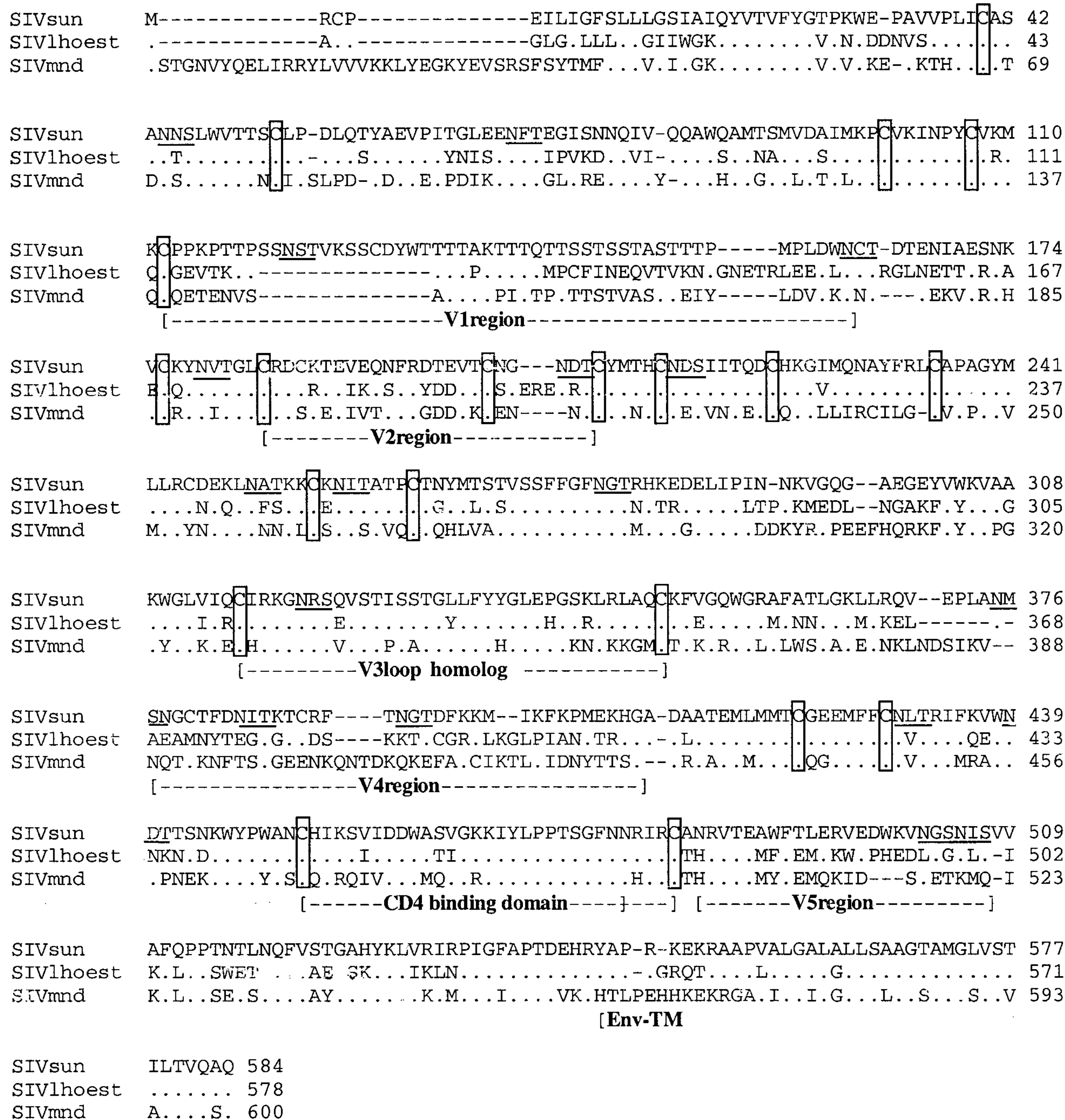

FIG. 5. Comparison of the predicted protein sequence of the surface subunit of the envelope of SIVsun, SIVlhoest, and SIVmnd reveals remarkable conservation of cysteine residues and regions such as the V3 loop homolog and the CD4 binding. Conserved cysteines are boxed. Potential N-linked glycosylation sites are underlined. The predicted sequence of gp120 of SIVsunλ20L14/S2 molecular clone is shown at the top. Substitutions relative to this sequence in the predicted sequence of gp120 of SIVlhoest and SIVmnd are aligned below. Dots indicate amino acid identity at a residue, and dashes indicate gaps introduced to optimize alignment. Variable regions analogous to those observed in HIV-1 and other SIVs are indicated, and the cleavage site for the transmembrane glycoprotein (TM) is shown.

ferent subspecies of chimpanzee was also similar, except in the envelope protein. In diversity plots (Fig. 3B), the extent of sequence difference between SIVsun and SIVlhoest was remarkably similar to that between two SIVagm lineages (the plot shown is for vervet versus grivet SIV). Parallel fluctuations in the two plots along the proteome are consistent with divergence of the two pairs of sequences over approximately the same timescale and suggest that similar selective constraints have operated on the genome within the two different SIV lineages.

The envelope proteins of SIVsun and SIVlhoest are particularly well conserved, being little more divergent overall than the Pol protein (Table 4 and Fig. 3A). The similarity among SIVsun, SIVlhoest, and SIVmnd is illustrated in an alignment of the surface unit (gp120) portion of the Env protein (Fig. 5). Although scattered amino acid replacements are evident

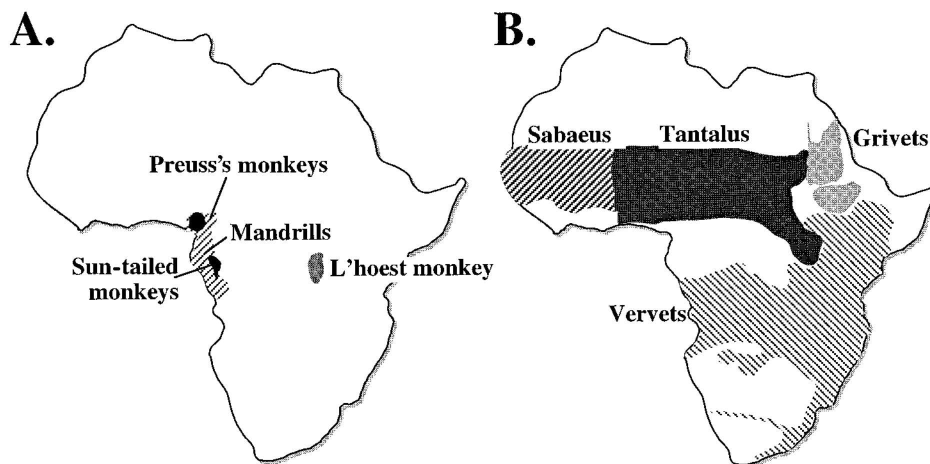

FIG. 6. Schematic views of Africa showing the ranges occupied by l'hoest monkeys (*C. lhoest lhoesti*) and their close relatives, Preuss's monkeys (*C. lhoest preussi*), and sun-tailed monkeys (*C. lhoesti solatus*) (the distribution of mandrills [*Mandrillus sphinx*] is indicated by cross-hatching) (A) and the geographic distribution of the four African green monkey species, i.e., the grivet (*C. aethiops*), tantalus (*C. tantalus*), vervet (*C. pygerythrus*), and sabaeus monkeys (*C. sabaeus*) (B) (44).

throughout gp120, 20 cysteine residues (boxed) and 10 potential N-linked glycosylation sites (underlined) are conserved among all three sequences. In addition, the regions homologous to the V3 loop and the CD4 binding domain are remarkably highly conserved, with only four and three differences, respectively, between SIVsun and SIVlhoest. The most variable regions are V1 and V4, which exhibit characteristic insertion-deletion differences.

DISCUSSION

Recently we described SIVlhoest isolated from the l'hoest monkey, the nominal member of the l'hoest superspecies inhabiting mountain and lowland forests along the right bank of the Congo River (Uganda to Congo) (28). SIVlhoest unexpectedly showed the closest genetic relationship to SIVmnd-GB1, the only full-length SIV characterized so far from a naturally infected mandrill (*Mandrillus sphinx*) from Gabon (67). The relationship between SIVlhoest and SIVmnd presented an enigma. It could not be explained by host-dependent evolution, since mandrills and l'hoest monkeys are phylogenetically distant species within the Cercopithecinae; mandrills are closer to the mangabeys (*Cercocebus* species) than to the guenons (*Cercopithecus* and *Chlorocebus*) (25). Nor could it be explained by recent cross-species transmission, since mandrills and l'hoest monkeys presently inhabit geographically separate regions of Africa. However, SIVmnd could have been derived from cross-species transmission of SIV from one of the other members of the l'hoest superspecies, either *C. lhoesti preussi* or *C. lhoesti solatus*, whose ranges overlap that of the mandrill (Fig. 6A). Such a scenario is plausible since mandrills and these guenons share some ecological characteristics, including a mainly terrestrial way of life. In the present study, we have isolated an SIV from one of these other members of the l'hoest superspecies, the sun-tailed monkey, *C. lhoesti solatus*. This virus, SIVsun, was distinct from but most closely related to SIVlhoest. Therefore, SIVsun is more distantly related to SIVmnd and could not have been the proximal source of SIVmnd.

It is important to recognize that the fairly distant relationship between naturally occurring African SIV isolates within a lineage underscores the ancient nature of these viruses. The genetic relationship between SIVsun and SIVlhoest is similar to the diversity among the four different subtypes of SIVagm (vervet, grivet, tantalus, and sabaeus) (4, 6, 12, 15, 16, 32, 36, 38, 47). However, while the four species of African green monkeys inhabit different geographic regions of Africa, there are still areas of overlap between vervet, grivet, and tantalus species that could potentially allow transmission of viruses between species (Fig. 6B). The separation between the habitats of l'hoest monkeys, sun-tailed monkeys, and Preuss's monkeys is far more pronounced. To date, *C. lhoesti solatus* has an isolated distribution within Gabon and is separated by 600 km from *C. lhoesti preussi* in the northwest and by 1,600 km from *C. lhoesti lhoesti* in the east. These forested areas in Gabon, eastern Zaire, and the highlands of Cameroon are believed to have been connected in the past, and several theories have been proposed for how they were separated by the contraction and disintegration of tropical rainforest in Africa. These events are believed to have resulted in the isolation and speciation of the guenons over the last 1 million years (23, 26). The genetic relationship between SIVlhoest and SIVsun suggests that an ancestral SIV most probably infected an ancestral monkey before the vegetation changes and subsequent speciation events. The distant relationship of SIVmnd to SIVlhoest and SIVsun within the same lineage could be explained by the transmission of SIV to mandrills from (i) an ancestor of the l'hoest superspecies before the division into the three distinct species occurred, (ii) Preuss's monkey (mandrills and Preuss's monkeys share the same habitat), or (iii) a yet unidentified species. It may be possible to discriminate among these three hypotheses by characterizing SIVs from Preuss's monkeys and other monkeys that share the same habitat as mandrills and by characterizing more SIVs from mandrills.

The host-dependent divergence of SIVsun and SIVlhoest suggested above has implications for the timescale of HIV and SIV evolution. The date of emergence of the common ancestor of the various major lineages of primate lentiviruses has been estimated previously by molecular clock extrapolations, yielding estimates ranging from the 1940s (63) to 150 to 200 years ago (56, 61). The demonstration of HIV-1 infection in 1959, with the implication that the M group of HIV-1 arose even earlier, indicates that the former estimate is much too recent (70). The findings of related but divergent SIV lineages within the *C. lhoesti* superspecies, as well as within the African green monkey superspecies (4, 6, 12, 15, 16, 32, 36, 38, 47) and in different subspecies of the chimpanzee *Pan troglodytes* (19), are

all consistent with divergence events within major SIV lineages having occurred tens or even hundreds of thousands of years ago. Thus, even the estimates of 150 to 200 years ago may be out by several orders of magnitude. Variations in the rate of evolution among different HIVs and SIVs could contribute to the error in these molecular clock estimates (42, 60), but they seem inadequate to explain the magnitude of the discrepancy. Alternatively, the extent of genetic divergence among lineages of SIV might have been underestimated because standard methods of "correcting" for multiple hits ignore variation in the rates at which substitutions accumulate at different residues (43, 60).

The identification of SIV infection in another African monkey species provided the opportunity to examine some of the virologic and immunologic characteristics of asymptomatic natural infection. One characteristic of SIVsun infection, which is frequently observed in other naturally infected African monkeys, was the very weak anti-Gag antibody response in the SIV-infected sun-tailed monkey. It has been reported that naturally SIV-infected sooty mangabeys, Sykes monkeys, one mandrill, and especially African green monkeys frequently exhibit a low reactivity to Gag antigens by ELISA, RIPA, and/or Western blotting (3, 13, 17, 47, 49, 58, 66). The low anti-Gag response in SIV natural monkey hosts was particularly striking compared to the strong anti-Gag response that develops when heterologous hosts, such as macaques, are inoculated with the same virus (Fig. 2A) (17, 29, 49). The reason for the small amount of Gag-specific antibodies in SIV-infected African monkeys is still unclear. Since SIV-infected sooty mangabeys have been reported to show relatively high cellular and plasma virus loads (10^5 to 10^7 RNA copies per ml of plasma) (17, 58), this difference cannot simply be explained by differences in virus load. Another serologic similarity to naturally SIV-infected sooty mangabeys and African green monkeys (5, 17, 49) was the apparent lack of neutralizing antibodies against the homologous SIV isolate despite the presence of envelope-specific antibody and long-term infection with the virus without clinical disease development. In addition, despite lifelong SIV infection, the SIV-positive sun-tailed monkey did not exhibit signs of immunodeficiency or opportunistic infections.

In summary, we demonstrated that SIVsun is a novel member of the SIVmnd/SIVlhoest lineage, with closer relationship to SIVlhoest than to SIVmnd. The identification of highly related viruses within two members of a superspecies suggests that the lineage previously thought to originate in mandrills actually appears to have evolved within monkeys of the *C. lhoesti* superspecies. We therefore propose that the SIVmnd lineage should be redesignated the SIVlhoest lineage. This new SIV-host system offers an additional model for studying host-virus adaptation and may give insights into the age, origin, and evolution of SIVs in Africa.

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