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Molecular characterization of a novel simian immunodeficiency virus lineage (SIVtal) from northern talapoins (*Miopithecus ogouensis*)

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Abstract

Simian immunodeficiency viruses (SIVs) are found in an extensive number of African primates, and humans continue to be exposed to these viruses by hunting and handling of primate bushmeat and following occupational exposures to captive nonhuman primates. Here, we report the molecular characterization of a new SIV lineage, SIVtal, from wild-caught and captive talapoin monkeys (*Miopithecus ogouensis)* from Cameroon and U.S. zoos, respectively. Phylogenetic tree analyses of a small fragment in the *pol* gene indicated that all SIVtal strains clustered together forming a single species-specific lineage. Full-length sequence analysis for two strains, SIVtal-00CM266 and SIVtal-01CM8023, from wild-caught animals in Cameroon confirmed that SIVtal was distinct from all primate lentiviruses isolated so far and represents a new SIV lineage. Phylogenetic analyses in different viral genes showed a significant clustering of the SIVtal lineage with the *Cercopithecus*-specific SIVs. In addition, SIVtal and *Cercopithecus*-specific SIVs share functional motifs in Gag and Env that distinguish them from other primate lentiviruses. Like SIVsyk and SIVdeb, a *vpu* gene homologue was also absent in SIVtal. Although northern talapoins belong to the *Miopithecus* genus, their SIVs belong to the *Cercopithecus* SIV lineage, suggesting evolution from a common ancestor or cross-species transmission between both primate genera.

Keywords: SIV; Non-human primate; Talapoin; HIV; Evolution; Cameroon

Introduction

SIVs are a large group of viruses that are found naturally in an extensive number of African primate species and serological and/or molecular evidence for SIV infection have been reported in at least 36 African nonhuman primates (NHPs) (Bailes, 2002; Peeters and Courgnaud, 2002; Reed et al., 2004; Takemura et al., 2005; Verschoor et al., 2004). Both HIV-1 and HIV-2, the etiologic agents for AIDS in humans, are the result of cross-species transmissions from SIVs from primates in Africa. Their closest relatives are respectively, SIVcpz in chimpanzees (*Pan troglodytes troglodytes*) from West central Africa and SIVsm in sooty mangabeys (*Cercocebus atys*) from West Africa (Corbet et al., 2000; Gao et al., 1992, 1999).

Although SIVs are called immunodeficiency viruses, these viruses do not typically induce an AIDS-like disease in their natural hosts, suggesting that they have been associated and evolved with their hosts over an extended period of time (Kestens et al., 1995; Muller-Trutwin et al., 1996; Pandrea et al., 2003; Silvestri et al., 2003). A high genetic diversity is observed among the known SIVs but, generally each primate species is infected with a species-specific virus which form monophyletic lineages in phylogenetic trees. In addition, several "major SIV lineages" have been identified which represent groups of SIVs from different primate species that are more closely related to one another than they are to other

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SIVs (Bibollet-Ruche et al., 2004). For some of these groups, virus and host phylogenies seem to match, suggesting virus/host co-speciation, but there are also numerous examples of cross-species transmission and recombination (Allan et al., 1991; Bailes et al., 2003; Beer et al., 1999, 2001; Clewley et al., 1998; Courgnaud et al., 2001, 2003; Jin et al., 1994; Souquiere et al., 2001). Among the African primates the *Cercopithecus* genus harbors the largest number of species known to be infected with related SIVs and recently a *Cercopithecus*-specific SIV lineage has been described (Bibollet-Ruche et al., 2004). Despite examples of co-evolution between viruses and hosts, and cross-species transmission within this genus, the SIVsyk, SIVdeb, SIVgsn, SIVmus, SIVmon, and SIVden viruses which are all derived from different *Cercopithecus* species, consistently form one highly supported group in phylogenetic trees and share functional motifs in *gag* and *env* that distinguish them from other primate lentiviruses (Bibollet-Ruche et al., 2004; Dazza et al., 2005). These results suggest that these SIVs evolved from a common ancestor that most likely infected a *Cercopithecus* host in the distant past. Interestingly, it has also been shown that one primate species can be infected with two different SIVs. For example, mandrills from central and southern Gabon are infected with SIVmnd-1, whereas those living in northern Gabon and the south of Cameroon are infected with SIVmnd-2 (Souquiere et al., 2001). The genetic diversity among NHP lentiviruses is thus extremely complex. The current knowledge of SIV/HIV phylogeny indicates that both co-evolution and cross-species transmissions with concomitant genetic recombination have driven lentiviral evolution.

Given that viruses from chimpanzees and sooty mangabeys have both crossed the species barrier on multiple occasions resulting in HIV-1 and HIV-2, it is thus important to identify and characterize SIVs that circulate in African primates species to estimate which lentiviruses could potentially infect the human population. In a recent survey of bushmeat markets in Cameroon, we found evidence suggesting that a substantial proportion of wild-living monkeys are SIV infected and PCR analysis on a subset of samples led to the discovery of new SIV strains not previously known to infect primates (Peeters et al., 2002). This study thus documented for the first time that humans who hunt and butcher primates are routinely exposed to a plethora of genetically divergent SIV strains. In addition, persons who work with NHPs in captivity are also at risk for SIV infection (Khabbaz et al., 1994; Sotir et al., 1997). Therefore, to gain further insights into the diversity of SIVs to which humans are exposed and to SIV evolution overall, full-length SIVtal sequences were derived from two wild-caught northern talapoin monkeys (*Miopithecus ogouensis*) which were identified as SIV-infected during a survey of primate bushmeat in Cameroon. In addition, to characterize SIVs recently identified in captive talapoins (Ndongmo et al., 2004), partial DNA sequences were also amplified from four captive seropositive talapoins housed in different U.S. zoos. The talapoin monkey, the smallest species of Old World monkeys, belongs to the *Miopithecus* genus in which two species have been identified (Groves, 2001). The northern talapoin (*M. ogouensis*) are found in southern Cameroon south of the Sanaga River, and in Equatorial Guinea and Gabon, while the southern talapoin (*M. talapoin*) occurs south of the Congo River in Angola and northwest of the Democratic Republic of Congo (DRC, formerly Zaire) (Gautier-Hion et al., 1999; Groves, 2001). Our results indicate that the SIVtal strains obtained from wild and captive talapoins, all belonging to the *M. ogouensis* species, form a separate species-specific lineage which consistently falls in the recently described *Cercopithecus* SIV cluster.

Results and discussion

Species identification of the SIV-positive talapoins

Talapoins are among the smallest NHPs in Africa and inhabit only a restricted area in west central Africa. Currently, two different species of talapoin (*Miopithecus* species) monkeys are recognized, both of which inhabit different geographic locations. The northern talapoin (*M. ogouensis*) is found in Cameroon, south of the Sanaga River, in Equatorial Guinea and Gabon, while the southern talapoin (*M. talapoin*) occurs south of the Congo River in Angola and northwest of the Democratic Republic of Congo (DRC, former Zaire) (Gautier-Hion et al., 1999; Groves, 2001) (Fig. 1A). The wild-caught talapoins from Cameroon are thus northern talapoins, but the geographic origin and hence the species of the 4 captive talapoins was unknown. Thus, to determine the talapoin species, we PCR amplified and sequenced fragments of the mitochondrial 12S rRNA gene from the talapoins from Cameroon (CM-266, CM-271, and CM-8023) and the US zoos (US-742, US-416, US-1867) since it was recently shown that this particular gene fragment was suitable to distinguish primate species (Van der Kuyl et al., 2000). The six sequences were compared with the GenBank database and were found between 98.5% and 100% identity with 12S rRNA reference sequences from *M. ogouensis* and 95% to 96% with 12S rRNA sequences from *M. talapoin*, suggesting that all our samples are from the same species, *M. ogouensis*. Furthermore, phylogenetic tree analysis of 12S rRNA sequences showed that all our sequences cluster significantly with those from northern talapoins supporting further the origin of these animals (Fig. 1B).

Partial polymerase (pol) sequences from wild-caught and captive SIVtal-positive animals

Initial genetic characterization of the talapoin SIVs was performed with partial *pol* sequences from four captive monkeys (animals 416, 1511, 742, and 1867) and one wild-caught animal (01CM-8023) from Cameroon. Phylogenetic analysis showed that these five new SIVtal *pol* sequences clustered with the previously described SIVtal *pol* sequences from two wild-caught northern talapoins from Cameroon

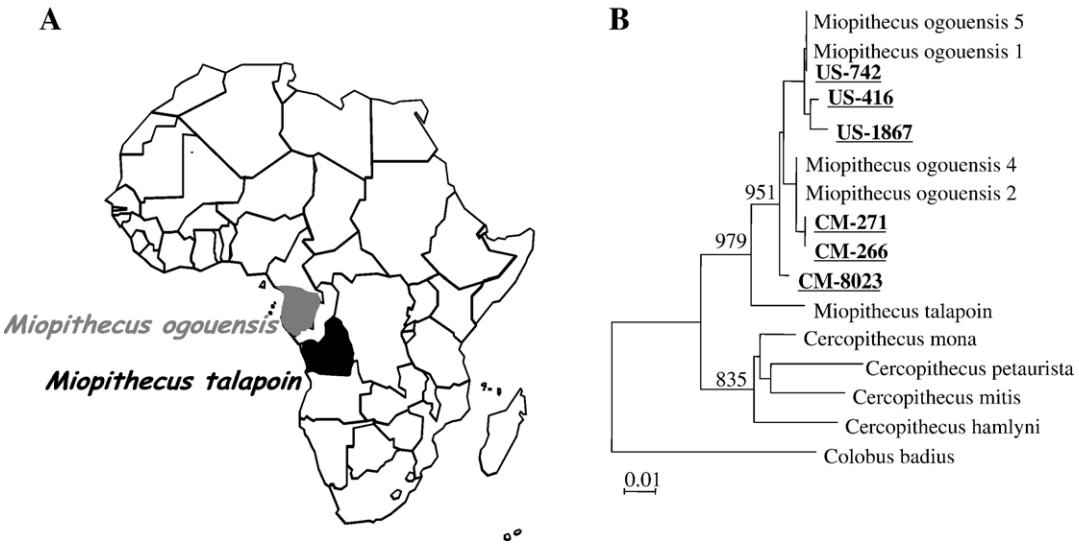

Fig. 1. (A) Geographical distribution of the two *Miopithecus* species (*M. ogouensis* and *M. talapoin*) in sub-Saharan Africa. (B) Neighbor-joining tree of 12S rRNA sequences from talapoins from Cameroon (CM-266, 00CM-271, and CM-8023) and US zoo's (US-742, US-416, and US-1867) plus selected sequences from Old World primate 12S rRNA from the Genbank database. *M. ogouensis* 1,2, 4, and 5 are reference 12S rRNA sequences from the genbank, the tree was rooted using a 12S sequence from *Colobus badius*. The significance of the branching order was estimated by the bootstrap method (1000 resampling).

identified in primate bushmeat, 00CM-266 and 00CM-271 (Peeters et al., 2002). However, the four SIVtal sequences from the captive animals were significantly more closely related to each other (95% to 97% nucleotide sequence identity) than to the SIVtal strains from Cameroon (82–84% identity) as would be expected from zoo animals in close contact (Fig. 2). Moreover, the genetic diversity among the Cameroonian strains was higher (84% identity) and comparable to the within-lineage diversity observed in other SIV lineages. These data suggest a more recent epidemiological link between the different captive animals studied, and that SIVtal could have been transmitted in captivity among different animals from U.S. zoos. This hypothesis is supported by the fact that 1867 is the offspring of 1511 and an unknown female that died in captivity; the genetic identity among the father–son talapoin pair was 97%.

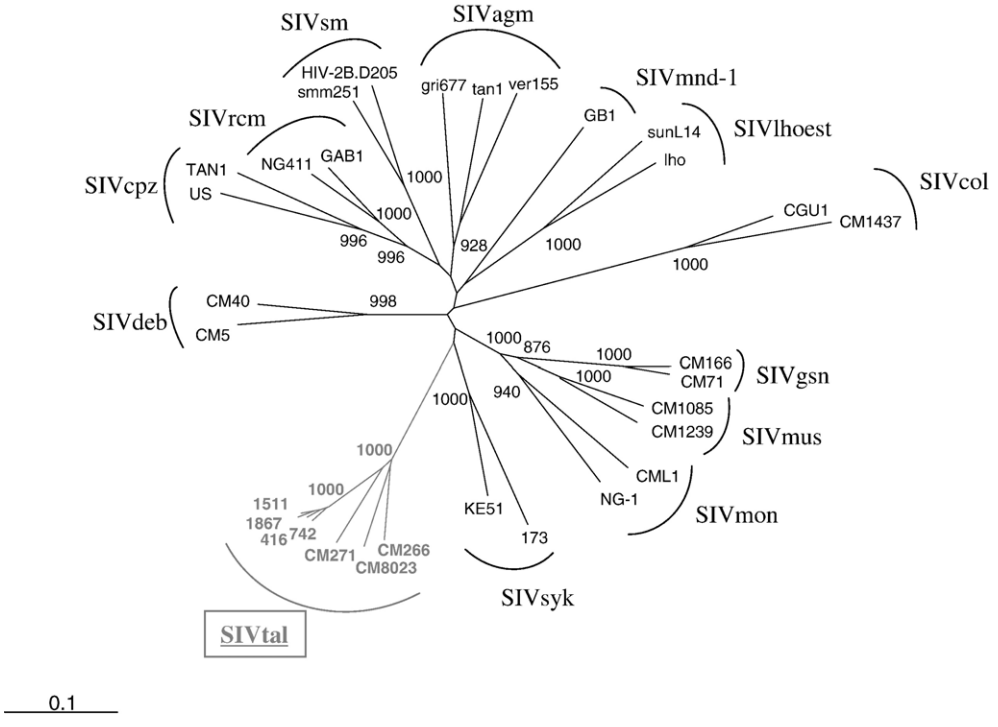

Fig. 2. Phylogenetic tree showing the relationships of the SIVtal *pol* sequences (650 bp) to equivalent sequences from representatives of other SIV lineages. Phylogenetic relationships were computed by the neighbor-joining method. The significance of the branching order was estimated by the bootstrap method (1000 resampling). Only values >80% are shown (0.1 = 10% difference).

Full-length genome sequence and genomic organization of SIVtal

To further characterize this virus group in more detail, we amplified complete genomes of two SIVtal strains (SIVtal-00CM266 and SIVtal-01CM8023) from uncultured whole blood of the two corresponding wild-caught animals from Cameroon. The animals were from two different geographic locations in southern Cameroon, situated 50 and 150 km south of Yaounde, the capital city. To amplify the full-length genome, circular unintegrated viral DNA was targeted for SIVtal-CM266, and overlapping linear PCR fragments were amplified for SIVtal-CM8023.

Previously described degenerate primers were used to amplify a 650-bp fragment of the *pol* gene from DNA extracted from a whole-blood sample from animal 00CM266. Based on the sequences of the *pol* fragments of SIVtal-00CM266, specific primers were designed to amplify unintegrated circular DNA in a primary PCR. The complete genome sequence of SIVtal-00CM266 was then obtained by successive nested PCRs as illustrated in Fig. 3A and using primers provided in Table 1. Amplified fragments were then cloned and sequenced. The SIVtal-01CM8023 genome was obtained by amplification of overlapping fragments using degenerate consensus and sequence-specific primers as shown in Fig. 3B and Table 1. The concatenated SIVtal-00CM266 and SIVtal-01CM8023 sequences (R-U5-gag-pol-env-U3) are 9158 and 8845 bp in length, respectively. For SIVtal-01CM8023, only the end of U5 and beginning of U3 regions in the LTR were sequenced giving the appearance of a shorter genome.

The SIVtal genome was compared to the other primate lentiviruses and displayed the expected reading frames for *gag*, *pol*, *vif*, *vpr*, *tat*, *rev*, *env*, and *nef*. As for most of the SIV lineages, SIVtal did not encode for a *vpu* or *vpx* analogue. The long terminal repeat (LTR) of SIVtal contained all the characteristic features of other primate lentiviruses. The U3 region contained two NF-kB sites and one potential SP-1 binding sites for SIVtal-00CM266, whereas one NF-kB sites and two potential SP-1 binding sites were observed for SIVtal-01CM8023. Similarly, as for all SIVs characterized to date except SIVdeb, the *vpr* gene and the first exon of *tat* in the SIVtal genome overlap.

SIVtal is related to Cercopithecus-specific SIVs: phylogenetic analysis of full-length SIVtal genomes and functional motifs

In order to compare the two SIVtal sequences to previously characterized SIV strains, we performed similarity plot analyses on full-length nucleotide sequences. Fig. 4 depicts the similarities between SIVtal-00CM266 and SIVtal-01CM8023 as well as between the individual SIVtal sequence to representatives of the other SIV lineages. The two SIVtal strains were quite similar to one another, sharing 80% to 90% nucleotide acid similarity according to the genes analyzed. All other SIV strains analyzed were considerably more distant. These analyses also revealed no evidence of recombination between SIVtal and any of the other SIV lineages. Overall, SIVtal was much more similar to SIVs from the *Cercopithecus* monkey virus lineage than to other SIVs. Table 2 shows the amino acid homologies between SIVtal and the other SIVs for

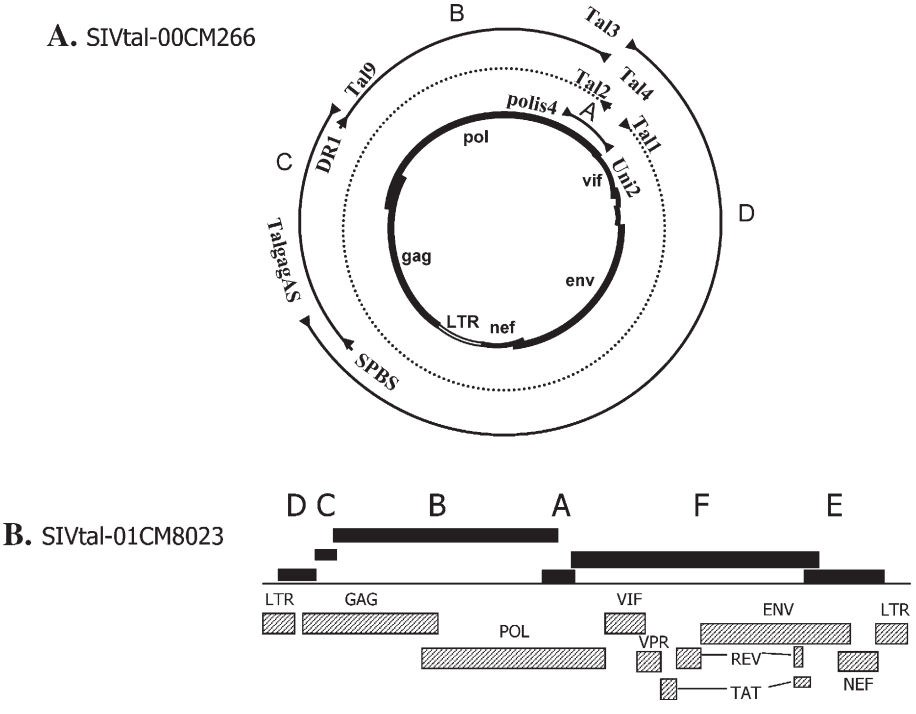

Fig. 3. Schematic representation of the PCR amplification of full-length SIVtal sequences from uncultured whole-blood DNA. The positions of the various amplification products are shown in relation to an unintegrated circular intermediate of SIVtal-00CM266 (A) (depicted in the center) with major genomic regions indicated or linear overlapping PCR fragments for SIVtal-01CM8023 (B). Primer sets and fragment designations are identical to those in Table 1.

Table 1
Primers used to amplify full-length genomes of SIVtal-00CM266 and SIVtal-01CM8023

	Fragment[a]	Primer	
SIVtal-OOCM266			
First round	A	NDR1-PolOR	
Second round		Polis4-Uni2	
First round		Tal1 (5′-GTGGCCAGCAGATGGCCTATAAG-3′)	s
		Tal2 (5′-CTTGGCAGAATCTTAGTCTCTATG-3′)	as
Second round	B	DR1	s
		Tal4 (5′-ATGATTTGTCCCTCCAAATGGGTG-3′)	as
	C	SPBS (5′-GGCGCCCGAACAGGGACTTG-3′)	s
		Tal9 (5′-TATACCCTGCCAATCCCCCTACTG-3′)	as
	D	Tal3 (5′-GAAATGCAGGCCATGGTATGGTGG-3′)	s
		TalgagAS1 (5′-TTCTACCGGGTGCTTGTCTGGAGG-3′)	as
SIVtal-01CM8023			
First round	A	NDR1-PolOR	
Second round		Polis4-Uni2	
First round	B	TDS-713s (5′-GAGAAAGTDAGAYTGAGRCCRAAAGG-3′)	s
		8023Pol1as (5′-GCATGCCCCAAATGTCAGGTCCAAG-3′)	as
Second round	B1	TDS1251s (5′-GGAGATCAWCAGGGGGCHATGCA-3′)	s
		TDS2225as (5′-ATTGGTCKTCTCCAAAGAGA-3′)	as
	B2	TDS2225s (5′-TCTCTTTGGAGAMGACCAAT-3′)	s
		8023pol2as (5′-GCAGTAGGGACATGGCAGATGGATT-3′)	as
	B3	8023RACF (5′-AACCCACCGCACCGCCGATAGAGCC-3′)	s
		8023RACR (5′-TATCCTGCCAATCCCCCTACTGTTT-3′)	as
First round	C	TDS-713s (5′-GAGAAAGTDAGAYTGAGRCCRAAAGG-3′)	s
		TDS1554as (5′-TAGAAYCTRTCTACATAGTCTTTGAA-3′)	as
Second round	C	TDS1251s (5′-GGAGATCAWCAGGGGGCHATGCA-3′)	s
		TDS1377as (5′-GCTKKTTGTYCCTGCTATGTC-3′)	as
First round	D	LTR1s (5′-GCTRGGAAGAGTGGCAGAGAGGC-3′)	s
		S8023gag1as (5′-ACTTGTTCTGCTGGGGTGCTGGTTG-3′)	as
Second round	D	LTR2s (5′-TGAGACTRAGGCCSAAAGGGAARAA-3′)	s
		S8023gag2as (5′-TTCCTGCTATGTCACTGCCTGCTG-3′)	as
First round	E	TDS7682s (5′-CTTTGYTGRSTGGGATAGTGCAGCA-3′)	s
		TDS9304as (5′-GGATAGCTACAGAGCATCDGCTTATATG-3′)	as
Second round	E	TDS7912s (5′-CCWCANTGGVANAATATGACMTGGCA-3′)	s
		TDS9304as (5′-GGATAGCTACAGAGCATCDGCTTATATG-3′)	as
First round	F	8023Pol1s (5′-GCATGCCCCAAATGTCAGGTCCAAG-3′)	s
		8023Env1as (5′-CTGCTATTCTAAGCCATCTAAACCAAGC-3′)	as
Second round	F	8023Pol2s (5′-GCAGTAGGGACATGGCAGATGGATT-3′)	s
		8023Env2as (5′-TGTGATAGTGTCTGAGGCATTGTCTACC-3′)	as

Letters correspond to the fragments labeled as such in Fig. 1.
[b]Y = C or T; W = A or T; R = A or G; H = A or C or T; B = C or G ou T; M = A or C S = G or C; K = G or T; V = G or A or C; D = G or A or T; N = A or G or C or T.
[a] TDS = Tal, Deb, Syk are consensus primers designed to amplify the SIVsyk, SIVdeb and SIVtal lineages.

the three major proteins, confirming the observations of the similarity plots based on the nucleotide alignment.

In order to examine the phylogenetic relationships of SIVtal to other SIV lineages, phylogenetic trees were constructed for the three major genes *gag*, *pol*, and *env*. Fig. 5 shows clearly that SIVtal forms a separate cluster within the *Cercopithecus* SIV lineage in each of the three major genes. This genetic relationship was supported with high bootstrap values. Although SIVtal was not obtained from a *Cercopithecus* species, the SIVtal strains form a species-specific SIV lineage falling between the SIVgsn/mus/mon lineage and the SIVdeb, SIVden, and SIVsyk lineages of the *Cercopithecus*-specific SIVs. From these analyses, it appears that SIVtal, SIVgsn/SIVmon/SIVmus, SIVsyk, SIVden, and SIVdeb most likely evolved over long periods from a common SIV ancestor.

The secondary structure of the Tat transactivation response (TAR) element has previously been described to exhibit SIV lineage-specific differences (Bibollet-Ruche et al., 2004; Jin et al., 1994). Secondary structure predictions showed that all *Cercopithecus* SIVs have a duplicated stem-loop structure consisting of a single nucleotide bulge (C or U), a 3-bp stem, and a 6-bp terminal loop with the sequence 5′-CUGGGA-3′. Interestingly, the predicted TAR secondary structures for SIVtal also exhibited duplicated TAR elements containing a 3-bp stem between the bulge and the terminal loop (data not shown). It has also been previously shown that all *Cercopithecus* SIVs contain 18 conserved cysteine residues in the gp120 envelope glycoprotein surface subunit (Bibollet-Ruche et al., 2004; Dazza et al., 2005). Similarly, as for all *Cercopithecus* monkey SIVs, the same 18 cysteine residues are conserved in SIVtal and among other primate lentiviruses, but with other cysteine residues present at different positions in these other SIVs (data not shown). Finally, two different sites known to be critical for primate lentivirus budding have been identified in SIV Gag p6

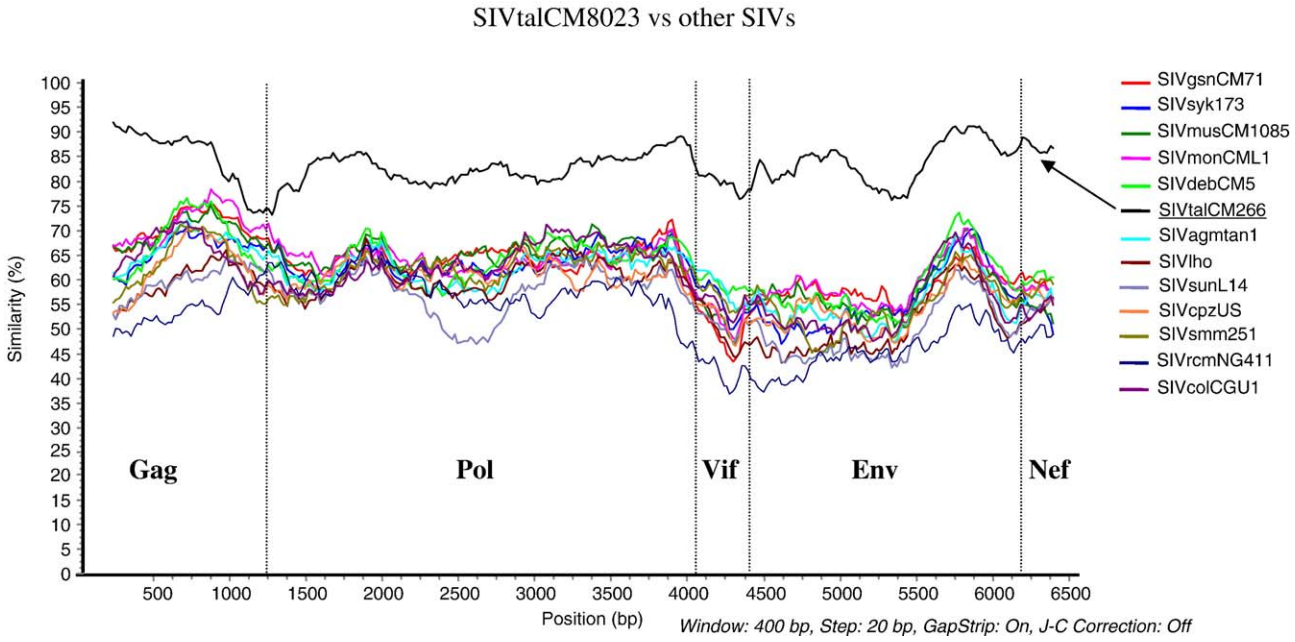

Fig. 4. Diversity plots of nucleotide sequences illustrating the extent of genetic diversity between SIVtal and other SIV lineages. The proportion of nucleotide acid sequence differences between SIVtal and other SIV strains is shown in different colors. Values are plotted at the midpoint of the sequence window on the *x*-axis, with the N-termini of *gag*, *pol*, *vif*, *env*, and *nef* denoted. The *y*-axis denotes the distance between the viral proteins.

protein sequences: PT/SAP and YPXL (Freed, 2002; Strack et al., 2003; Von Schwedler et al., 2003). Except SIVcol, SIVden, and SIVdeb, all other primate lentiviruses have a PT/SAP motif. In the absence of this motif, budding occurs via binding of the YPXL motif with high affinity to AIP1, a second host protein involved in endosomal sorting and retroviral budding (Martin-Serrano et al., 2001; Puffer et al., 1997). All *Cercopithecus* SIVs have both motifs, except SIVdeb and SIVden (Bibollet-Ruche et al., 2004; Dazza et al., 2005) which, like SIVcol, only have a YPXL motif. Interestingly, SIVtal also has both motifs which is thus an additional characteristic shared by SIVs infecting *Cercopithecus* and *Miopithecus* monkey species.

Despite the fact that SIVtal was isolated from a primate species belonging to another primate genus, our study shows, based on full-length sequence analysis of two SIVtal strains obtained from wild-caught northern talapoins, that SIVtal always falls within the cluster of the *Cercopithecus*-specific SIVs and shares also functional motifs characteristic of *Cercopithecus* SIVs. Among the *Cercopithecus* SIVs, some harbor a *vpu* gene (SIVgsn, SIVmus, SIVmon and SIVden), whereas in others (SIVdeb and SIVsyk), such a gene is absent. The latter was also the case for SIVtal. Overall, our results suggest that the new SIVtal lineage evolved from a common ancestor with SIVsyk, SIVdeb, SIVden and SIVgsn/mon/mus. However, the high genetic divergence between these species-specific SIV lineages (SIVsyk, SIVdeb, SIVden, SIVgsn/mon/mus, and SIVtal) indicates that their common origin did not occur recently. The relationship of SIVtal with the *Cercopithecus*-specific SIVs can therefore be explained by ancient cross-species transmissions between these species because the geographic ranges of talapoin monkeys overlap those of greater spot-nosed, mustached, and De Brazza's monkeys (Gautier-Hion et al., 1999; Groves, 2001). Alternatively, it could also be possible that the common ancestor of these SIVs was already present before the last common ancestor of *Miopithecus* and *Cercopithecus* monkeys diverged. However, the hypothesis that SIVs co-speciated with their primate hosts is also controversial because attempts to estimate the time of lentivirus phylogeny

Table 2
Percentage amino acid identity between SIVtal-01CM8023 and SIV strains representative to other SIV lineages in the 3 major genes, Gag, Pol and Env

	GAG	POL	ENV
SIVtal-00-CM266	83	87	81
SIVdeb.CM5	59	58	40
SIVdeb.CM40	58	58	42
SIVmon.CML1	65	58	46
SIVgsn99CM166	64	59	45
SIVgsn99CM71	64	59	44
SIVmus1239s	65	58	46
SIVmus.1085	66	58	45
SIVsykKE51	57	57	46
SIVsyk.173	58	58	48
SIVagm.TAN1	56	55	37
SIVagm.GRI677	55	53	40
SIVagm.VER155	53	54	40
SIVsmm.251	54	53	38
HIV-2-D205	54	53	38
SIVrcm.Ngm	56	55	37
SIVrcmGAB1	55	55	37
SIVcpzUS	50	52	38
SIVcpz.TAN1	50	53	40
SIVsun	46	49	31
SIVlhoest	47	51	30
SIVmnd.GB1	51	53	31
SIVcol-CGU1	41	48	26

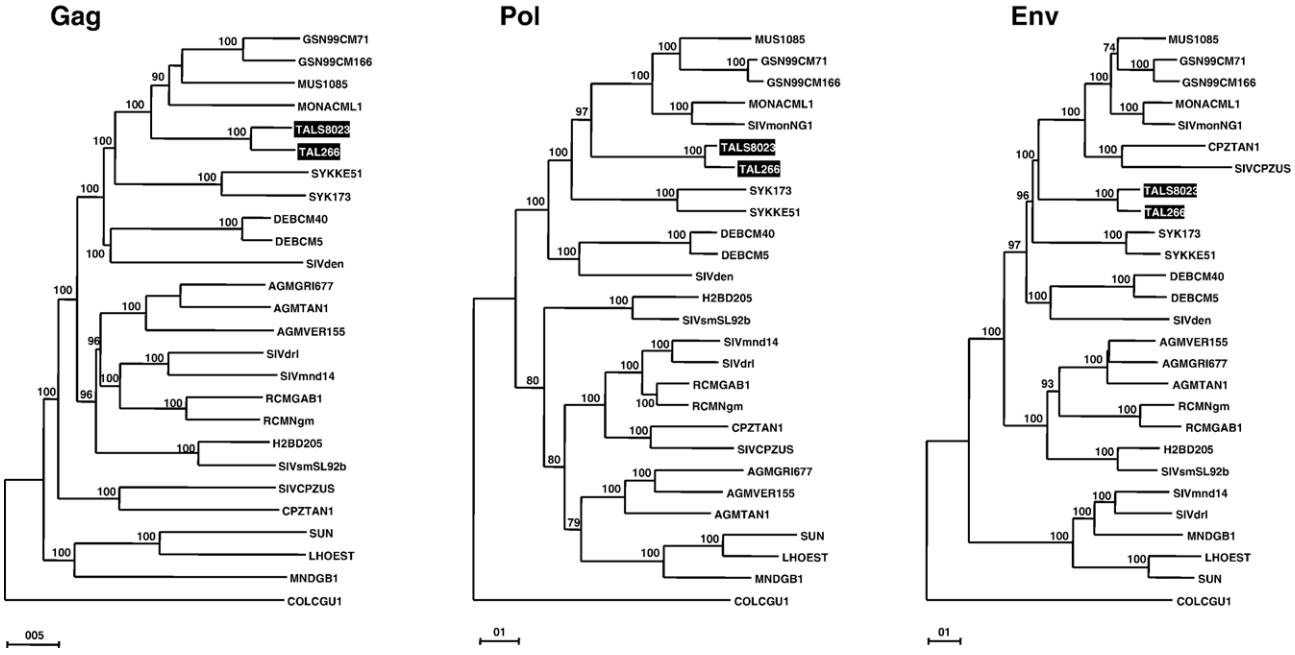

Fig. 5. Phylogenetic relationships of the newly derived SIVtal sequences to other SIV lineages. Maximum likelihood trees were inferred from protein sequences of the major SIV genes, Gag, Pol, and Env, using Bayesian estimation, and then midpoint rooted. The numbers on nodes are estimated posterior probabilities (only values of 95% or greater are shown). Horizontal branch lengths are drawn to scale, with the bar indicating 0.1 amino acid replacements per site.

using molecular clocks do not correspond to the estimated time for species phylogeny (Sharp et al., 2000). Importantly, it has also to be noted that speciation and subspeciation in talapoin monkeys has been puzzling to taxonomists for a long time. Earlier primate classification listed *Miopithecus* as a subgenus of *Cercopithecus* but phylogenetic studies using mtDNA and endogenous retroviruses as a marker, indicated that *Miopithecus* is a true primate genus, well separated from the *Cercopithecus* genus (Gautier-Hion et al., 1999; Groves, 2001; Van der Kuyl et al., 2000).

Comparison of SIVtal sequences from M. ogouensis and M. talapoin

As mentioned above, in the *Miopithecus* genus two species are described which are geographically separated (see Fig. 1). A 550-bp SIVtal *pol* sequence has been previously reported from a captive talapoin described as *Miopithecus* talapoin housed at the Zoo of Amsterdam, The Netherlands (Osterhaus et al., 1999). The reported 550-bp fragment in *pol* does not correspond to the 650-bp partial *pol* fragment that was sequenced in our study. In order to compare this previously reported sequence with the SIVtal sequences from captive and wild-captured *M. ogouensis* from our study, a 2450-bp fragment corresponding to part of the reverse transcriptase and integrase of the *pol* gene was sequenced for two additional samples from captive *M. ogouensis* in U.S. zoos, SIVtal1867 and SIVtal742. Phylogenetic tree analysis on the common 550-bp fragment showed that all SIVtal *pol* sequences, from both *Miopithecus* species cluster together forming a single species-specific lineage (Fig. 6). Moreover, the SIVtal sequence from *M. talapoin* appears to be slightly closer to the SIVtal sequences from the other captive *M. ogouensis* than to those from the wild-caught northern talapoins from Cameroon.

This close relationship between SIVtal from two different *Miopithecus* species may suggest that a related virus was already present in the ancestor of both species. Alternatively, each *Miopithecus* species may have become infected unknowingly

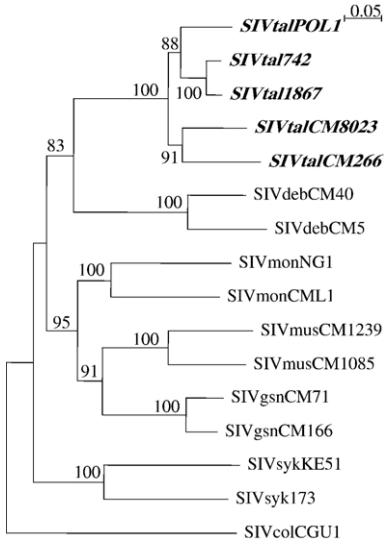

Fig. 6. Phylogenetic relationships of SIVtal from *M. ogouensis* and *M. talapoin*. The tree was derived from a 550-bp *pol* sequence by using the neighbor-joining method and rooting with SIVcolCGU1. The significance of the branching order was estimated by the bootstrap method (1000 resampling). Only values >80% are shown.

with the same virus by close contact in a zoo exhibit shared by both species. The close genetic relationship of these viruses supports the latter hypothesis. Nevertheless, identification of SIV-infected, wild-captured southern talapoins and full molecular characterization of their corresponding viral genomes are needed to fully elucidate the diversity and evolution of SIVs in this genus.

In this paper, we showed that northern talapoins are naturally infected with a new species-specific primate lentivirus, termed SIVtal. Phylogenetic tree analysis shows a significant clustering of the SIVtal viruses with the *Cercopithecus*-specific SIVs. Overall, our data add further evidence of the complex evolutionary history of primate lentiviruses. Full-length sequences of SIVs from additional *Cercopithecus* species and from other nonhuman primate genera are needed for a better understanding of the overall evolution of primate lentiviruses. More sequences from the same species but sampled at distant geographic locations are also necessary to understand whether the identified viruses are the real species-specific SIVs or whether they represent a strain naturally infecting sympatric primate species that were introduced by cross-species transmission. Further studies of the lentiviruses in NHP species are not only required to understand the origin and evolution of primate lentiviruses but may also yield important insights into the risks for human exposure to SIV-infected primates and the potential for the introduction of new retroviral zoonoses into the general population. African NHP species represent a large reservoir for simian lentiviruses, and we recently showed that humans are exposed to a large diversity of SIV through hunting and handling of primate bushmeat (Peeters et al., 2002). Although talapoins are less frequently hunted due to their small size, humans may also be exposed to SIVs from these monkeys.

Materials and methods

Animals and serologic testing

Whole-blood samples from two wild-caught and four captive talapoin monkeys, previously identified as SIV-positive by the presence of cross-reacting antibodies with HIV antigens using INNOLIA-LIA HIV confirmation test (Innogenetics, Ghent, Belgium) (Peeters et al., 2002) or peptide EIA and Western Blots (Ndongmo et al., 2004), were available for study. The two talapoins from Cameroon (00CM-266 and 02CM-8023) were obtained from wild-caught animals sampled as bushmeat with government approval from the Cameroonian Ministry of Environment and Forestry as previously described (Peeters et al., 2002). Blood specimens from four talapoins (416 (female), 1867 (male), 1511 (male), and 742 (female) housed in North American zoos were obtained on an opportunistic basis in accordance with the animal care and use committees at each institution. Of note, 1867 is the offspring of 1511 and a female (1009) that died in captivity and 416 and 742 both had wild-born sires. Peripheral blood mononuclear cells (PBMC) and plasma were obtained by Ficoll-Hypaque centrifugation.

PCR amplification and sequencing of pol

Total DNA was isolated from whole blood or PBMCs using the QIAamp blood kit (Qiagen) according to the manufacturer's instructions. A small fragment of *pol* (650 bp) was amplified for all samples with degenerate consensus primers (DR1-PolOR for the first round and Polis4-UNIPOL2 for the second round) as previously described (Clewley et al., 1998; Courgnaud et al., 2001; Peeters et al., 2002). For a subset of samples, we also amplified a 2450-bp *pol* fragment, using DR1-PolOR primers for the first round and DR4-UNIPOL2 for the second round and by using PCR conditions as previously reported (Courgnaud et al., 2003). After purification, the PCR products were then directly sequenced on both strands.

Amplification of complete SIVtal genomes

For two animals, 00CM-266 and 01CM-8023, the full-length SIVtal sequence was obtained either by amplification of unintegrated circular DNA or by overlapping PCR fragments, respectively. The amplification strategies and primers used to obtain the different fragments are summarized in Fig. 3 and Table 1. For SIVtal-00CM266, a 650-bp *pol* fragment was obtained as described above. To obtain the full-length genomic sequence of SIVtal-00CM266, two specific primers were generated based on the known *pol* sequence to amplify in the first round unintegrated circular viral forms: Tal1 and Tal2. Several nested PCRs were then performed to generate overlapping fragments spanning the entire genome, using combinations of SIVtal-266-specific and/or SIV consensus primers: DR1 and Tal4, SPBS positions 248–267 and Tal9; positions 2358–2381, and Tal3 positions 4568–4591, and TalgagAS1 positions 576–600. PCRs were performed using a Long Expand or Expand High Fidelity PCR kit (Roche Molecular Biochemicals), including a hot start (92 °C for 3 min) with the following cycle conditions: 10 cycles of denaturation at 92 °C for 10 s, annealing at 56 °C for 30-s extension at 68 °C for 7 min, followed by 20 cycles with extension at 68 °C for 7 min with an increment of 20 s per cycle. Amplification was completed by a final extension at 68 °C for 10 min. Cycling conditions for inner PCRs were 10 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C (with primers SPBS and Tal9 or at 58 °C with primers Tal3 and TalgagAS1) for 30-s extension at 72 °C for 2.5 min or 3.5 min, followed by 20 cycles with an increment of 5 s per cycle of the extension time at 72 °C, followed by 1 cycle of 7-min extension at 72 °C. PCR products were purified and cloned into pGEM-TEasy vector (Promega). DNA was sequenced using cycle sequencing and dye terminator methodologies (ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq FS DNA polymerase (PE Biosystems, Warrington, England) on an automated sequencer (ABI 373, model Stretch, Applied Biosystems) using the Genome Priming System GPS™-1 (New England BioLabs, Beverly, MA, USA). To reconstitute the full-length genome sequence, overlapping sequences were assembled into contiguous sequences by using the Sequencher software (Gene Codes Corp., USA).

For SIVtal-CM8023, unintegrated circular DNA could not be amplified, and the full-length sequence was obtained by amplification of overlapping PCR fragments with consensus primers designed on the newly obtained SIVtal-00CM266 sequence and other SIVs available from the database and GenBank. The primers used are shown in Table 1, and the corresponding amplification strategy is depicted in Fig. 3B. All amplifications were performed using the Expand High Fidelity PCR kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. Each amplification reaction included a manual hot-start and 35 cycles. Annealing temperatures were 50 °C except for the amplification of fragments B and D for which 53 °C was used. Extension times varied depending on the size of the expected fragment and were typically set at 1 min/kb. Amplified fragments were agarose gel purified and directly sequenced using Big-Dye Chemistry (Applied Biosystems, France) according to the manufacturer's instructions. Electrophoresis and data collection were done on an Applied Biosystems 3100 Genetic Analyzer. To reconstitute the full-length genome sequence, overlapping sequences were assembled into contiguous sequences by using SEQMAN DNASTAR software (Lasergene, DNASTAR, Inc., Madison, WI).

Talapoin species identification

To determine the species of talapoin monkeys, we amplified in a single round PCR, a 390-bp segment of the mitochondrial 12S rRNA gene using primers 12S-L01091/12S-H01478 as described previously (Van der Kuyl et al., 2000). PCR products were then directly sequenced and compared to the GenBank database by using the BLAST program. Phylogenetic relationships of the primate mitochondrial 12S rRNA sequences were estimated using the neighbor-joining method with the Kimura's 2 parameter model implemented in the Clustal X program (Thompson et al., 1997). The reliability of branching orders was tested using the bootstrap approach (1000 replicates).

Genetic analyses

Nucleotide and protein sequences were aligned using ClustalX, with minor manual adjustments. Sites that could not be unambiguously aligned and sites with a gap in any sequence were excluded. Proteome sequences were generated by joining deduced Gag, Pol, Vif, Env, and Nef amino acid sequences; the carboxy terminal Gag, Pol, and Env amino acid sequences that overlapped with Pol, Vif, and Nef amino acid sequences, respectively, were excluded. The nucleotidic and predicted protein sequences encoded by SIVtal were compared to representatives of known SIV/HIV lineages. In order to study whether the newly characterized SIVtal sequences were recombinant with any of the other SIV lineages, similarity plot analysis was performed on the nucleotide alignment with the new SIVtal sequences and known SIV strains with the SIMPLOT package version 2.5 (Ray, 1999; http://www.med.jhu.edu/deptmed/sray) using a sliding window of 400 nucleotides (nt) moved in steps of 20 nt.

Phylogenetic tree analysis was done on amino acid sequences. Maximum likelihood trees were inferred by Bayesian estimation of phylogeny, based upon the posterior probability distribution of trees. The method was implemented in MrBayesv3.1 (Huelsenbeck et al., 2001) using the Jones–Taylor and Thornton model of protein evolution (Jones et al., 1992) with gamma distributed rates at sites (Ziheng, 1993). The program was run for 500,000 generations, including a "burn in" of 500 generations. The trees shown are majority rule consensus trees.

Secondary structure predictions

The TAR RNA secondary structure was predicted and drawn using the GENQUEST DNASTAR package (Lasergene, DNASTAR, Inc., Madison, WI).

Nucleotide sequence accession number

The complete sequences (SIVtal-00CM266 and SIVtal-01CM8023) and partial *pol* sequences (SIVtal742, SIVtal1867, SIVtal416, and SIVtal1511) are available GenBank (accession numbers AY655740-AY655744 and AM182197).

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