Recombination

Evidence of recombination between HBV/A and HBV/E was observed in one of the Cameroonian strains (CMR151, marked by an asterisk in Fig. 2). The result of the bootscan analysis for the complete genome sequence of the strain is presented in Fig. 3(a). The phylogenetic tree constructed using the corresponding sequence segment confirmed the grouping of the CMR151 strain together with the HBV/E strains, with strong bootstrap support (Fig. 3b). The recombinant segment corresponded to a part of the non-overlapping HBV DNA polymerase in the reverse transcriptase (RT) domain and a part of the enhancer I–X promoter. (Fig. 3c). The breakpoints at nucleotide positions 882 and 1060 were estimated by mapping the informative sites and using χ^2 confirmation (Robertson *et al.*, 1995).

Enhancer/promoter elements and amino acid characteristics of the HBV/Ac (A3) strains

A comparison of the nucleotide substitutions within the *cis*-acting elements among the four HBV/Ac (A3) strains and the consensus sequences of the HBV/Aa (A1) and HBV/Ae (A2) subtypes (subgenotypes) as well as the other HBV genotypes (including HBV/Ba, Bj, B2, B1) are summarized in Table 2. Nine specific nucleotide substitutions were found in HBV/Ac (A3) strains: $G^{1173}A$ (enhancer I–X promoter), $C^{1473}G$, $G^{1512}A$ and $C^{1703}T$ (enhancer II–core promoter), $A^{2742}G$ (S1–promoter), $C^{3021}T$, $C^{3042}T$,

Table 2. Subtype (subgenotype) specific sites (bold) within enhancers and promoter regions of HBV/Aa (A1), HBV/Ac (A3) and HBV/Ae (A2)

Nucleotide positions correspond to the HBV genome reference sequence, GenBank accession no. NC_003977. Consensus sequences were composed according to 60% or higher incidence at the corresponding nucleotide position.

Region	Position (nt)	HBV/Aa (A1)	HBV/Ac (A3)				HBV/Ae (A2)
			CMR711	CMR82	CMR983	CMR151	
Enhancer I–X promoter (950–1350)	963	**C**	T	T	T	T	T
	1041	T	A	A	A	G	**A**
	1173	G	–	**A**	**A**	**A**	–
	1320	A	A	A	A	A	**G**
	1350	**T**	–	–	–	–	C
Enhancer II–core promoter (1400–1850)	1404	**T**	–	–	–	–	C
	1464	**G**	T	T	T	T	T
	1473	C	**G**	**G**	–	**T**	–
	1484	A	–	–	–	–	**C**
	1511	G	–	–	–	–	**A**
	1512	**T**	**A**	**A**	–	**A**	**G**
	1703	C	T	T	T	A	–
	1727	A	–	–	–	–	**G**
	1740	T	–	–	–	–	**C**
	1809–1812	**TCAT**	-TC-	--C-	----	G--C	G--C
Encapsidation signal (1846–1908)	1888	**A**	G	G	G	G	G
S1–Promoter (2716–2806)	2720	**A**	T	T	T	T	**G**
	2742	**A**	**G**	**G**	**G**	**G**	–
	2744	C	**A**	**A**	–	–	–
	2777	G	C	T	T	T	T
S2–Promoter (2999–3219)	3013–3014	**CA**	--	--	--	--	**GC**
	3021	C	T	T	T	T	–
	3042	C	T	T	T	–	–
	3052	T	–	–	–	–	C
	3057/60	T/C	C/T	C/T	C/T	C/T	C/T
	3069	A	–	–	–	–	**C**
	3072–3073	TG	A-	C-	--	--	-A
	3076	T	**C**	**C**	**C**	**C**	–
	3111	T	T	T	T	T	C
	3118	C	–	–	–	–	T
	3121	G	–	–	–	–	A
	3124	G	–	–	–	–	A

$T^{3076}C$ and $C^{3111}T$ (S2–promoter). Interestingly, three of four strains had substitutions in the Kozak sequence (1809–1812) (Ahn *et al.*, 2003; Tanaka *et al.*, 2004), and two had a basal core promoter double mutation (1762/1764).

Although HBV/Ac (A3) amino acid motifs in general were more similar to HBV/Aa (A1) than to HBV/Ae (A2) motifs, HBV/Ac (A3) strains had some specific sites: Thr^{84} in preS1, Ala^{146} in Pol/terminal protein, Ser^{239}, Trp^{246}, Ser^{257} in Pol/spacer, Asp^{356}, Arg^{501}, Ser^{607} in Pol/RT, and Thr^{47} in X proteins when compared with consensus sequences composed according to 60 % or higher incidence at the corresponding amino acid position (Table 3). Pre-core/core amino acid patterns had no specific substitutions among HBV/A subtypes (subgenotypes).

Table 3. Subtype (subgenotype) specific sites (bold) in amino acid sequences of HBV/Aa (A1), HBV/Ac (A3) and HBV/Ae (A2)

Consensus sequences were composed according to 60 % or higher incidence at the corresponding amino acid position.

ORF	Position (aa)	HBV/Aa (A1)	HBV/Ac (A3)				HBV/Ae (A2)
			CMR711	CMR82	CMR983	CMR151	
PreS1	54	Q	–	–	–	–	**A**
	67	F	–	–	–	–	**L**
	74	V	–	–	–	–	I
	84	I	**T**	**T**	**T**	–	–
	86	A	T	T	T	T	T
	89	P	–	–	–	–	**S**
	90	A	–	–	–	–	**T**
	91	V	–	–	–	–	**I**
PreS2	32	L	V	V	V	V	V
	47	S	–	–	–	–	**A**
S	209	L	–	–	–	–	**V**
Pol/terminal protein	17	E	G	G	G	G	**G**
	33	E	–	–	A	A	**A**
	74	P	**Q**	**Q**	–	–	–
	102	T	**N**	**N**	–	–	–
	120	N	–	–	–	–	**T**
	146	T	**A**	**A**	**A**	**A**	–
Pol/spacer	236	T	–	–	–	–	S
	239	P	**S**	**S**	**S**	**S**	–
	246	R	**W**	**W**	**W**	–	–
	257	F	**S**	**S**	**S**	**S**	–
	269	Y	–	–	–	–	H
	271	A	–	–	–	–	**V**
	273	S	–	–	–	–	**N**
	308	S	K	K	–	–	**C**
	334	Q	–	–	K	K	K
	338	K	E	E	E	E	E
	348	L	–	–	–	–	**R**
Pol/RT	356	E	**D**	–	**D**	**D**	–
	501	W	**R**	**R**	**R**	**R**	–
	607	T	**S**	**S**	**A**	**S**	–
	617	I	**L**	**L**	–	–	–
	619	H	–	–	**D**	**D**	–
	666	K	**R**	**R**	–	–	–
X region	11	S	–	–	–	–	P
	31	A	S	S	S	S	S
	34	L	**V**	**V**	–	**F**	–
	47	S	**T**	**T**	–	**T**	A
	146	S	F	–	–	A	A
	147	S	–	–	–	P	P

DISCUSSION

A previous study carried out in Cameroon among the Bantus and the Pygmies (Kowo *et al.*, 1995) demonstrated a high (18·6 %) overall seroprevalence of HCV, which was significantly higher in Bantus (31·7 %) than in Pygmies (11·1 %). The results of the present study also indicate the very high HCV seroprevalence (14·5 %), and support the difference between the two populations. However, in our study, HCV seroprevalence among the Pygmies was lower (2·3 %), which might be attributed to the younger age of examined subjects compared with the cohort previously studied (Kowo *et al.*, 1995). The difference in HCV seroprevalence between the two populations might be explained by exposure of the Bantus to transmission routes such as medical procedures and blood transfusion, to which the Pygmies are not exposed. However, HBV seroprevalence (HBsAg and anti-HBc) was equally high among the two populations and different regions of the country, which is concordant with previous data (Ndumbe *et al.*, 1993). Further epidemiological investigation is required to evaluate factors contributing to the difference in HBV and HCV transmission in the Pygmies, in contrast with neighbouring Bantus.

The only data available on HBV genotypes in Cameroon demonstrated the predominant prevalence of HBV/A in human immunodeficiency virus-positive cohort (Mulders *et al.*, 2004). The present study revealed that both HBV/A and HBV/E are distributed equally in both native populations in Cameroon. The phylogenetic analysis revealed a close relationship in the large S coding region among the Cameroonian strains sequenced in this study and those from the same country available from previous reports (Mulders *et al.*, 2004; Norder *et al.*, 1992). Based on phylogenetic analysis of the complete genome, including four sequences in this study, the presence of a third phylogenetic cluster was confirmed within HBV/A in this study. The cluster was distinct from known HBV/Aa (A1) and HBV/Ae (A2) subtypes (subgenotypes), and designated HBV/Ac (A3) (where 'c' stands for Cameroon and Central Africa). The inter-subtype (subgenotype) nucleotide divergence over the complete genome sequences falls within the 4–8 % range that justifies the classification of HBV/Ac (A3) into a distinct subtype (subgenotype) according to the recent proposals on HBV nomenclature (Kato *et al.*, 2005; Kramvis *et al.*, 2005). The high intra-subtype (subgenotype) nucleotide divergence of four HBV/Ac (A3) complete genomes suggests a long natural history of this subtype (subgenotype) within the native population of Cameroon, as has been reported for subtype (subgenotype) HBV/Aa (A1) in southern African Blacks (Kimbi *et al.*, 2004). On the other hand, HBV/E strains obtained from the Pygmies did not group together separately from the strains isolated in different geographical regions, even though the Pygmies represent an isolated population in Africa. The presence of low divergent HBV/E genotype among the Pygmies might not support the hypotheses proposed previously that HBV/E has a very short history in humans (Mulders *et al.*, 2004).

The newly described subtype (subgenotype) HBV/Ac (A3) possesses a combination of the sites specific for either HBV/Aa (A1) or HBV/Ae (A2) within the corresponding enhancer/promoter elements and amino acid motifs (Kimbi *et al.*, 2004; Sugauchi *et al.*, 2004; Tanaka *et al.*, 2004). Moreover, the subtype (subgenotype) also has HBV/Ac (A3) unique substitutions. The recombination affecting a short, non-overlapping segment of the polymerase RT domain found in one of the Cameroonian strains is the first event documented to have occurred between HBV/A and HBV/E. The sequencing data generated in the present study could be used to design assays that can discriminate between HBV/Ac (A3) and the other subtypes (subgenotypes) of HBV/A in order to characterize its clinical–virological features. Cohort studies are required to investigate a possible association of HBV/Ac (A3) infection with early HBeAg/anti-HBe seroconversion and low HBV DNA levels in carriers indicated by the tendencies observed on the small number investigated in present study.

At the present time, investigation of HBV molecular heterogeneity, global distribution of HBV genetic forms, including recombination and mutations as well as efficient implications of the data, is one of the major directions in the field of virus research (Kramvis *et al.*, 2005). In this respect, further standardization of the HBV nomenclature and, an efficient and logical classification should be based on a consensus of the accumulated data including recent studies.

In conclusion, the complete genome of the third subtype (subgenotype) of HBV/A, identified in Cameroon, has been analysed and unique nucleotide/amino acid substitutions have been identified within this subtype (subgenotype). The high intra-group divergence suggests that this subtype (subgenotype) represents an indigenous HBV strain with a long natural history. Recombination between this subtype (subgenotype) and genotype E is described.

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Short Communication

A novel simian immunodeficiency virus from black mangabey (*Lophocebus aterrimus*) in the Democratic Republic of Congo

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In order to understand primate lentivirus evolution, characterization of additional simian immunodeficiency virus (SIV) strains is essential. Here, an SIV from a black mangabey (*Lophocebus aterrimus*) originating from the Democratic Republic of Congo was analysed phylogenetically. The monkey had cross-reactive antibodies against human immunodeficiency virus type 1 (HIV-1) and HIV-2. The viral *pol* region sequence was amplified by nested PCR and sequence analysis confirmed that it was related to known SIV sequences. This is the first report to characterize genetically an SIV from the monkey genus *Lophocebus*. Phylogenetic analysis of the *pol* region revealed that this novel SIV, designated SIVbkm, fell into the SIVsyk and SIVgsn virus group, containing viruses isolated from the genus *Cercopithecus*, and suggests that cross-species transmission has occurred between species of the genera *Lophocebus* and *Cercopithecus*.

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Simian immunodeficiency viruses (SIVs) naturally infect a wide range of wild African non-human primates (Hahn *et al.*, 2000; Hayami *et al.*, 1994; Peeters *et al.*, 2002). On the basis of their genomic sequences, SIVs are currently classified into six major phylogenetic lineages: (i) SIVcpz in chimpanzees (*Pan troglodytes*), which clusters with human immunodeficiency virus type 1 (HIV-1) (Corbet *et al.*, 2000; Gao *et al.*, 1999; Huet *et al.*, 1990); (ii) SIVsm in sooty mangabeys (*Cercocebus atys*), which clusters with HIV-2 (Chen *et al.*, 1996; Hirsch *et al.*, 1989); (iii) SIVagm in four species of African green monkeys (*Chlorocebus aethiops*) (Allan *et al.*, 1991; Fukasawa *et al.*, 1988); (iv) SIVmnd-1 in mandrills (*Mandrillus sphinx*), which forms a cluster with SIVlhoest in l'Hoest monkeys (*Cercopithecus lhoesti*) and SIVsun in sun-tailed monkeys (*Cercopithecus solatus*) (Beer *et al.*, 1999; Hirsch *et al.*, 1999; Tsujimoto *et al.*, 1988, 1989); (v) SIVsyk in Sykes' monkeys (*Cercopithecus albogularis*) (Hirsch *et al.*, 1993); and (vi) SIVcol in guereza colobus monkeys (*Colobus guereza*) (Courgnaud *et al.*, 2002). SIVmus from moustached monkeys (*Cercopithecus cephus*) and SIVmon from mona monkeys (*Cercopithecus mona*) are relatives of SIVgsn from greater spot-nosed monkeys (*Cercopithecus nictitans*), which carry the *vpu* gene in their genome (Barlow *et al.*, 2003; Courgnaud *et al.*, 2002, 2003a). They have been designated as a new SIV lineage, although Biollet-Ruche *et al.* recently suggested that the clusters of SIVgsn and SIVsyk including SIVdeb should be considered as one ancestral SIV lineage that infected monkeys of the genus *Cercopithecus* (Bibollet-Ruche *et al.*, 2004; Courgnaud *et al.*, 2003a; Verschoor *et al.*, 2004). In addition, some novel SIV strains such as SIVtal from talapoins (*Miopithecus*

The GenBank/EMBL/DDBJ accession number for the nucleotide sequence determined in this work is AY518534.

talapoin), SIVwrc from western red colobus (*Piliocolobus badius*) and SIVolc from olive colobus monkeys (*Procolobus verus*) have been reported, although their sequences have only been partially characterized (Courgnaud *et al.*, 2003b; Peeters *et al.*, 2002).

It is now widely accepted that HIV-1 originated from SIVcpz (Gao *et al.*, 1999; Hahn *et al.*, 2000). Bailes *et al.* (2003) suggested that SIVcpz could be a recombinant virus between SIVrcm from red-capped mangabeys (*Cercocebus torquatus*) and SIVgsn. Furthermore, viruses of the above-mentioned six major lineages may also have complex mosaic genomes (Salemi *et al.*, 2003). To understand better the evolutionary relationships among primate lentiviruses, a search for additional SIVs from other non-human primate species is essential. Thus, our research efforts have focused on non-human primates whose habitats are adjacent to or overlap the habitat of common chimpanzees in the forests of central Africa. In the present study, we genetically characterized a new SIV strain from a black mangabey (*Lophocebus aterrimus*) originally isolated in the Democratic Republic of Congo (DRC).

The animal was caught in the wild in 2001 in the Bas-Congo region, in the south-eastern part of the DRC, and kept in a separate cage for 1 year at the Kinshasa Zoo. Peripheral blood was collected in 2002 using acid citrate glucose anticoagulant solution. A plasma sample was initially tested for antibodies using a commercial particle agglutination kit (Genedia HIV-1/2, Fujirebio) and showed positive reactivity, with an antibody titre of 1:64. We then conducted a Western blot assay using commercial Western blot kits (HIV-1 and -2 LAV blot; Bio-Rad) and demonstrated strong reactivity against p18 and p25 (HIV-1 core antigens), p26 (HIV-2 core antigens) and gp105 (HIV-2 glycoprotein) (data not shown). These data suggested that this monkey was infected with an SIV strain related to HIV-1 or -2.

We carried out nested PCR to amplify the viral *pol* region using chromosomal DNA extracted from peripheral blood mononuclear cells. DNA was extracted using the QIAamp Blood DNA mini kit (Qiagen) according to the manufacturer's instructions. We first amplified a fragment of 150 bp in the *pol* region using oligonucleotide primers DR1 (5′-TRCAYACAGGRGCWGAYGA-3′) and DR2 (5′-AIADRT-CATCCATRTAYTG-3′) for the first round and primers DR4 (5′-GGIATWCCICAYCCDGCAGG-3′) and DR5 (5′-GGIGAYCCYTTCCAYCCYTGHGG-3′) for the second round (Clewley *et al.*, 1998; Courgnaud *et al.*, 2002). Amplification was carried out with an initial denaturation at

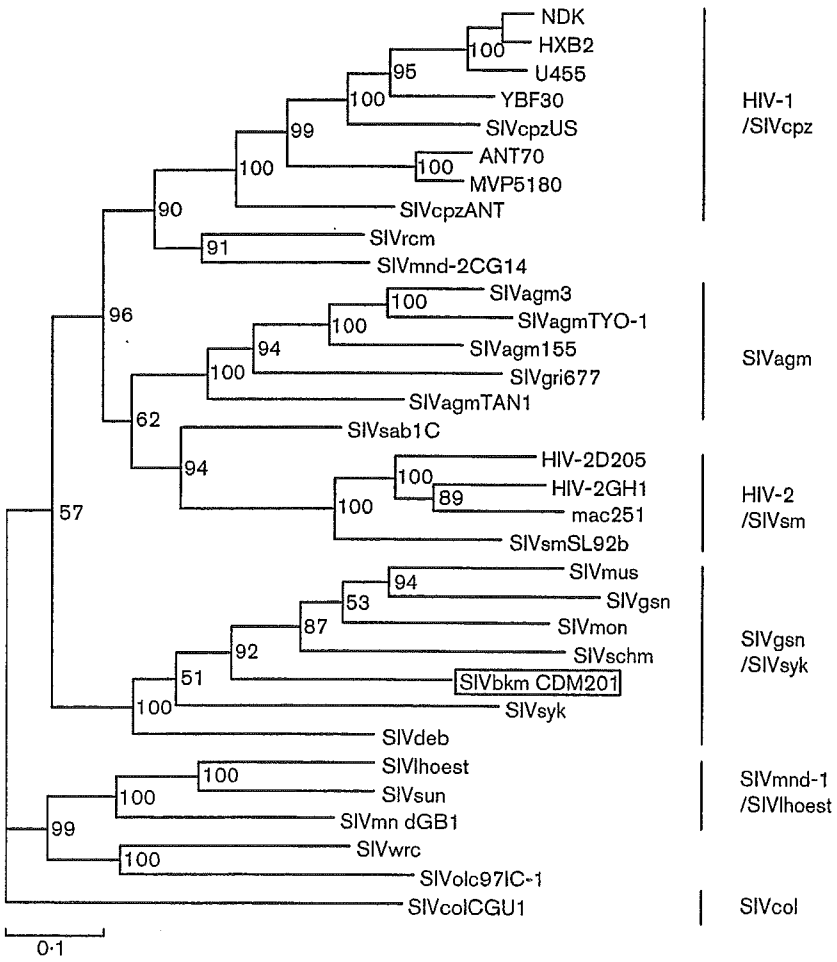

Fig. 1. Phylogenetic tree of HIV and SIV based on the RT–IN region, which is part of the *pol* gene. The new isolate, SIVbkm CDM201 (boxed), was composed of other strains representing each group. The numbers at the nodes indicate estimated posterior probabilities. Bar, number of nucleotide substitutions per site. A neighbour-joining tree (Saitou & Nei, 1987) gave almost identical results (not shown).

94 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 45 °C for 30 s and 72 °C for 1 min, with a final extension at 72 °C for 5 min. We succeeded in amplifying a 150 bp fragment from this seropositive sample. The PCR product purified from an agarose gel was subcloned into the pUC119 (Takara Bio) vector and sequenced using a cycle sequencing kit and automated sequencers (BigDye Terminator Cycle Sequencing Ready Reaction, ABI 373 and 3100; Applied Biosystems). We then performed a semi-nested PCR to amplify a 1800 bp fragment using the primers DR1 and Unipol2 (5′-CCCCTATTCCTCCCCTTCTTTTAAAA-3′) for the first-round PCR and bkmpol1 (5′-GGATAT-GAAAGATGCTTACTATTCAG-3′) and Unipol2 for the second-round PCR (Miura *et al.*, 1990). The primer bkmpol1 was specifically designed on the basis of the sequence of the 150 bp fragment. PCR was carried out as described above but with cycle conditions of 94 °C for 1 min, 45 °C for 1 min and 72 °C for 3 min. The sequence of the obtained fragment was determined as described above.

The SIV derived from the black mangabey was designated SIVbkm strain CDM201. We constructed a phylogenetic tree to compare the sequence of SIVbkm CDM201 with sequences of representative isolates of SIVs and HIVs. Sequences were aligned using CLUSTAL W with minor manual modifications and a phylogenetic tree was constructed using the maximum-likelihood method with the MOLPHY program (Higgins & Sharp, 1989; Adachi & Hasegawa, 1996; Yamaguchi-Kabata & Gojobori, 2000; Yamaguchi-Kabata *et al.*, 2004). A phylogenetic tree based on 1930 bp fragments of the *pol* region showed that SIVbkm was divergent from other SIV strains, but relatively close to SIVgsn and SIVsyk group isolates (Fig. 1). Subsequently, we carried out a distance plot analysis to investigate the extent of sequence differences (Takehisa *et al.*, 1999). The reference strains used were HIV-1 groups M (strain HXB2), O (ANT70), SIVcpz (US), SIVsm (SL92L), SIVagm (TYO-1), SIVmnd-1 (GB1), SIVsyk (syk173), SIVcol (CGU-1), SIVgsn (99CM71), SIVrcm (Ngm), SIVmon (NG1) and SIVmus (01CM1085). The genetic distance (estimated using Kimura's two-parameter method) between each selected pair of sequences was determined by moving a window of 300 bp along the genome alignment in 10 bp increments and the distance was plotted at the midpoint. SIVmon exhibited a relatively close distance to SIVbkm throughout the *pol* reverse transcriptase (RT) to integrase (IN) region and, interestingly, HIV-1 group O strain ANT70 showed a close relationship to SIVbkm, with the shortest distance in the RNase H region (Fig. 2). At the amino acid level, SIVbkm CDM201 showed moderate similarities with three other primate lentiviruses, SIVgsn (62·5 %), SIVmon (62·0 %) and SIVsyk (59·3 %) (Table 1). However, in the RNase H region, SIVbkm CDM201 showed relatively high similarities to HIV-1 group O (ANT70) (68·6 %), SIVrcm

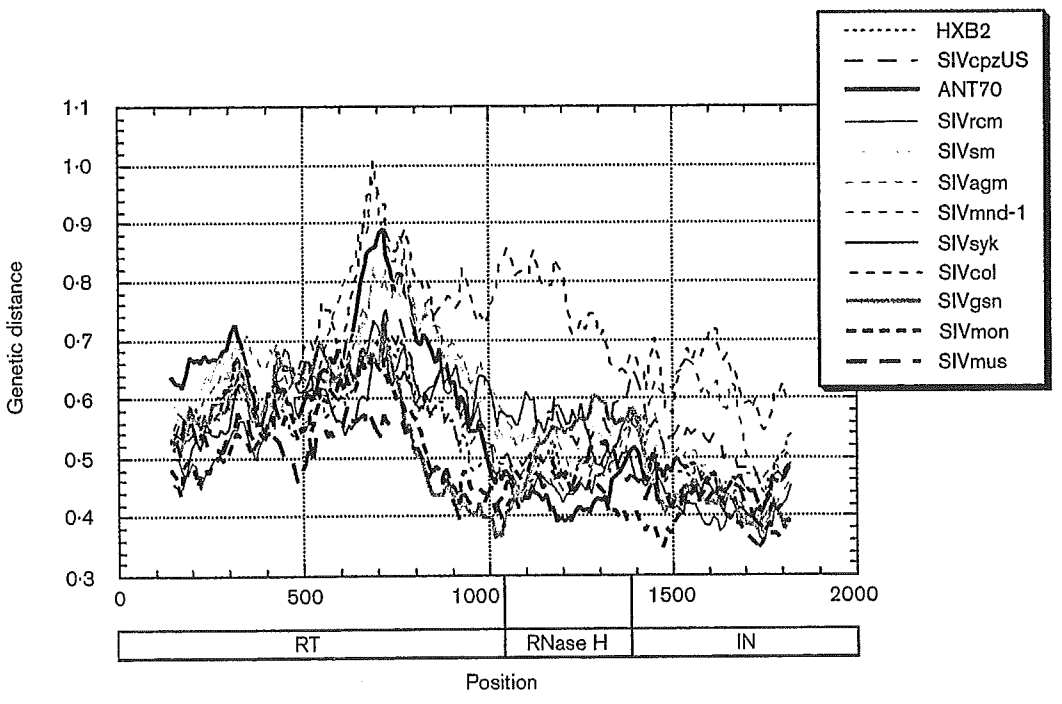

Fig. 2. Distance plots comparing SIVbkm CDM201 with representative primate lentiviruses. The sequences used were HIV-1 groups M (HXB2) and O (ANT70), SIVcpzUS, SIVrcm, SIVsm, SIVagm, SIVmnd-1, SIVsyk, SIVcol, SIVgsn, SIVmon and SIVmus. The genetic distance was estimated using Kimura's two-parameter method (Kimura, 1980). The alignment was sectioned into 300 bp fragments, which were moved along the genome in 10 bp increments. The distance value for each segment was plotted at the midpoint.

Table 1. Percentage amino acid identity in the *pol* region between SIVbkm CDM201 and representatives of other HIV/SIV lineages

Numbers in bold indicate the three highest identities in each respective region.

HIV/SIV strains	Amino acid identity (%)			
	RT–IN	RT	RNase H	IN
HXB2	55·7	52·2	65·3	55·7
YBF30	55·4	52·2	62·8	56·3
ANT70	56·0	49·6	**68·6**	59·6
SIVcpz	55·4	51·0	63·6	57·9
SIVrcm	57·4	51·3	**70·3**	60·1
SIVmnd-2	57·1	50·7	**68·6**	61·2
HIV-2 D205	53·6	46·8	62·8	60·1
SIVsm	55·0	48·4	66·9	59·6
SIVagm	55·0	47·8	63·6	62·8
SIVmnd-1	53·8	48·1	62·0	59·0
SIVsyk	**59·3**	**55·6**	62·0	**64·5**
SIVcol	46·5	47·2	39·7	49·7
SIVolc	47·1	44·0	53·7	48·6
SIVgsn	**62·5**	**58·2**	63·6	**70·0**
SIVmon	**62·0**	**58·4**	67·8	**65·0**

(70·3 %) and SIVmnd-2 (68·6 %). In the RT and IN regions, the similarities between SIVbkm CDM201 and these three viruses were relatively low.

In the present study, we have described a novel primate lentivirus, SIVbkm, from a black mangabey in the DRC. This is the first report to characterize genetically an SIV from monkeys of the genus *Lophocebus*. SIVbkm was found to be related to the SIVgsn and SIVsyk lineages, all of which were isolated from monkeys of the genus *Cercopithecus* (Barlow *et al.*, 2003; Courgnaud *et al.*, 2002, 2003a; Bibollet-Ruche *et al.*, 2004). These relationships were supported by high bootstrap values. Bibollet-Ruche *et al.* (2004) stated that the SIVsyk and SIVgsn lineages have the same ancestor and evolved with host-species specialization. The phylogenetic relationships between SIVbkm and the SIVsyk/SIVgsn cluster suggest that cross-species transmission occurred between species of the genus *Cerocopithecus* and a species of the genus *Lophocebus*. Clarifying the cross-species transmission between black mangabeys and *Cercopithecus* monkeys and the spread of the ancestral virus of this cluster may lead to an understanding of the origin of the *vpu* gene, since the SIVgsn lineage carries *vpu*, whereas the SIVsyk lineage does not. A better understanding of these events may also help to understand the origin and evolution of HIV-1.

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