AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 16, Number 2, 2000, pp. 139–151
Mary Ann Liebert, Inc.

Near-Full-Length Genome Sequencing of Divergent African HIV Type 1 Subtype F Viruses Leads to the Identification of a New HIV Type 1 Subtype Designated K

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ABSTRACT

We recently reported a high divergence among African subtype F strains. Three well-separated groups (F1, F2, and F3) have been shown based on the phylogenetic analysis of the p24 gag and envelope sequences with genetic distances similar to those observed for known subtypes. In this study, we characterized the near-full-length genomes of two strains from epidemiological unlinked individual belonging to each of the subgroups: F1 (96FR-MP411), F2 (95CM-MP255 and 95CM-MP257), and F3 (96CM-MP535 and 97ZR-EQTB11). Phylogenetic analysis of the near-full-length sequences and for each of the genes separately showed the same three groups, supported by high bootstrap values. Diversity plotting, BLAST subtyping, and bootstrap plotting confirmed that the divergent F strains correspond to nonrecombinant viruses. The divergence between F1 and F2 is consistently lower than that seen in any other intersubtype comparison, with the exception of subtypes B and D. Based on all the different analyses, we propose to divide subtype F into two subclades, with F1 gathering the known subtype F strains from Brazil and Finland, and our African strain (96FR-MP411), and F2 containing the 95CM-MP255 and 95CM-MP257 strains from Cameroon. The F3 strains, 97ZR-EQTB11 from the Democratic Republic of Congo and 96CM-MP535 from Cameroon, meet the criteria of a new subtype designated as K. The equidistance of subtype K to the other subtypes of HIV-1 suggests that this subtype existed as long as the others, the lower distance between B and D, and between F1 and F2 suggest a more recent subdivision for these latter strains.

INTRODUCTION

ONE OF THE MAJOR CHARACTERISTICS of human immunodeficiency viruses type 1 (HIV-1) is their remarkable genetic variation. Phylogenetic analysis of many isolates from different geographic regions allowed HIV-1 to be subdivided into three groups, M, N, and O. The group O viruses represent a minority of the HIV-1 strains and seem to be endemic in West Central Africa[1–4] and group N has been recently identified in two Cameroonian patients.[5] In contrast, group M comprises the majority of the HIV-1 strains responsible for the AIDS epidemic worldwide. The group M viruses can be subdivided into at least 10 envelope subtypes A to J approximately equidistantly related.[6,7] The geographic distribution of the different subtypes is very heterogeneous and in Africa all the subtypes have been documented. However, as the HIV/AIDS pandemic grows, viral strains are becoming more geographically dispersed and the simultaneous presence of multiple subtypes in many other regions of the world is now common. Therefore, subtype designations have been powerful molecular epidemiological markers to follow the course of the HIV global pandemic.

The genetic diversity mainly results from the error-prone na-

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ture of the viral reverse transcriptase and from the *in vivo* selection of variants.[8,9] In addition, recombination events among sequences of different genetic subtypes of HIV-1 group M have been frequently identified. Since the first report on recombinant HIV-1 viruses, increasing numbers of recombinant HIV-1 genomes have been recognized and recombinant forms of epidemiologic importance have been identified. All representatives of subtypes E and G, sequenced to date, represent mosaic genomes, with parts of the genome clustering with subtype A viruses and other parts forming clearly distinguishable clades designated as E and G.[10–12] In certain populations and regions, where multiple HIV-1 subtypes cocirculate, many combinations of intersubtype recombinant viruses have been documented (A/C, A/D, B/F, A/G/H, A/G/I, A/G/J).[11,13–15] At the present time, subtypes A, B, C, D, F, H, and J are well defined: for each of them at least one full-length nonrecombinant isolate has been sequenced.[13,16]

Recombination can be missed if large portions or different regions of the viral genome are not examined. Therefore, full-genome sequencing is necessary to determine if new HIV-1 variants correspond to new subtypes or to new circulating recombinant forms.

We recently reported a high variability among African subtype F strains, and characterized three phylogenetic subgroups preliminarily called F1, F2, and F3.[17] These three subgroups were identified in the p24 region of the *gag* gene, and in the complete envelope gene they were supported by high bootstrap values. In addition, the intragroup and intergroup distances were comparable to those obtained for the other known subtypes in the corresponding regions, although F2 was more closely related to F1, analogous to B and D. All these data suggested the possibility that subgroups F2 and F3 might be new subtypes. Whereas the subgroup F1 gathered together all the known subtype F viruses from Brazil, Romania, and also some African strains, subgroup F2 contained exclusively Cameroonian strains from our study and previous studies,[15,18] and in subgroup F3, strains from several Central African countries clustered together with a previously described divergent F strain, VI354, from Gabon.[17,19] In this study we describe the first full-length genome sequences from representatives of the divergent Cameroonian F2 and Central African F3 viruses, and we demonstrate that the F3 viruses correspond to a new HIV-1 subtype, designated as subtype K.

MATERIALS AND METHODS

Virus isolates

Five HIV-1 viruses, from African origin and collected between 1995 and 1997 from epidemiologically unlinked individuals, were studied. Table 1 summarizes the geographic origin of the samples, the year of sample collection, and whether sequences were done on primary peripheral mononuclear cells (PBMCs) or on short-term cultured PBMCs. Based on previously described p24 gag and gp160 sequences, four of them were reported as being divergent subtype F viruses clustering into one of the two well-defined F subgroups called F2 and F3.[17] Strain 96FR-MP411 was isolated from a French patient infected with HIV-1 in Chad or Yugoslavia after overseas deployment and clustered into subgroup F1 with previously known Brazilian and Romanian F strains. The 95CM-MP255, 95CM-MP257, and 96CM-MP535 strains were isolated between 1995 and 1996 from Cameroonian patients attending the military hospital in Yaounde. For the 97ZR-EQTB11 virus, only primary PBMCs were available and the sample was collected in 1997 from a tuberculosis patient in the Equateur province from the Democratic Republic of Congo (DRC, former Zaire) during an HIV seroprevalence survey.[20] The 95CM-MP255 and 95CM-MP257 viruses were preliminarily classified in subgroup F2 and 96CM-MP535 and 97ZR-EQTB11 in subgroup F3.

Polymerase chain reaction conditions and sequencing

DNA was extracted from short-term cultured (96FR-MP411, 95CM-MP255, 95CM-MP257, and 96CM-MP535) or primary PBMCs (97ZR-EQTB11) with the Qiagen DNA extraction kit. Overlapping polymerase chain reactions (PCRs) were done to obtain the near-full-length genome sequence of each strain.

A long gag–pol PCR was performed using the XL PCR kit with G00 (5′GACTAGCGGAGGCTAGAG3′) and Vpu1 (5′GGTTGGGGTCTGTGGGTACACAGG3′) as outer primers. A seminested PCR with G00 and G25 (5′TCAAGGCAAGCTTTATTGAGGCTTAAGCAG3′) as inner primers allowed the gag gene to be amplified. The PCR conditions were as follows: a first denaturation step for 5 min at 94°C followed by 38 cycles for 20 sec at 94°C, 30 sec at 55°C, and 2 min at 72°C with a final extension for 7 min at 72°C; 10 pmol/liter of each primer, 2.5 U of Taq polymerase (Promega Corporation, Madison, WI), and 0.2 mmol/liter of dNTPs were used. An-

TABLE 1. ORIGIN OF THE STUDIED VIRUSES

Samples	*Origin*	*Year of collection*	*DNA source*	*Genetic subgroup*[b]	
				gag[a]	*gp160*
96FR-MP411	Yugoslavia or Chad	1996	C	F1	F1
95CM-MP255	Cameroon	1995	C	F2	F2
95CM-MP257	Cameroon	1995	C	F2	F2
96CM-MP535	Cameroon	1996	C	F3	F3
97ZR-EQTB11	DRC[c]	1997	U	F3	F3

[a]DNA source: U, uncultured PBMCs; C, short-term cultured PBMCs.
[b]Triques et al.[17]
[c]DRC, Democratic Republic of Congo (former Zaire).

other second round using G60 and Vpu2 (5′GCWTCTTTCCACACAGGTACCC3′) as inner primers allowed the complete *pol* gene to be amplified using the XL PCR kit.

The *nef* gene (600 bp) was amplified by a nested PCR with 5.1e (5′GTGCCTCTTCAGCTACCACCG3′) and 3.3e (5′ AGCATCTGAGGGTTAGCCACT3′) as outer primers and 5.1e (5′CTGCAGGACAGATAGGGTTATAGAA3′) and 3.7e (5′CATGGATCCACCTCCCCTGGAAAGTCCCC3′) as inner primers. The first round PCR conditions were as follows: a first denaturation step for 5 min at 94°C followed by 27 cycles for 45 sec at 94°C, 30 sec at 55°C, and 2 min at 72°C, with a final extension for 5 min at 72°C, in a final volume of 50 μl; 20 pmol/liter of each primer, 20 mmol/liter of dNTPs, and 2.5 U of Taq polymerase were used. Of this round 5 μl was used for the second round, which used 32 cycles with 50°C as the annealing temperature. This protocol was used for all the viruses except for the 97ZR-EQTB11 strain, for which the XL PCR kit (Perkin Elmer, Branchburg, NJ) with 41.1 (5′GGGTTCTTGGGAGCAGCAGGAAGCACTATGGGCG3′) and LsiGi (5′TCAAGGCAAGCTTTATTGAGGCTTAAGCAG3′) as inner primers was used.

The accessory genes (*Vif*, *Vpu*, *Tat*, *Rev*, and *Vpr*) (1300 pb) were amplified using Vif1 (5′GGGTTTATTACAGGGACAGCAGAG3′) and Vpu1 as inner primers and Vif2 (5′GCAAAACTACTCTGGAAAGGTG3′) and Vpu2 as outer primers previously described[21] as follows: a first denaturation step for 3 min at 94°C followed by 35 cycles for the two rounds for 20 sec at 94°C, 30 sec at 54°C, and 2 min at 94°C, with a final extension for 10 min at 72°C; 10 pmol/liter of primers, 20 mmol/liter of dNTPs, and 2.5 U of Taq polymerase were used.

The amplification of the envelope gene has been previously described[17]; briefly a nested PCR was performed with the outer primers A and N and the inner primers B and M and others encompassing the envelope gene, using the Expand™ High Fidelity PCR System (Boehringer Mannheim, Indianapolis, IN).

All the PCR fragments were detected by electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining. The amplified products were purified using a QiaQuik gel extraction (Qiagen, Qiagen SA, France).

Nucleotide sequences were obtained by direct sequencing of the amplified DNA, using the inner primers of the *nef* gene, the inner primers of the accessory genes, and several primers encompassing the *gag* and the *pol* genes. Cycle sequencing was performed using fluorescent dye terminator technology (dye terminator cycle sequencing with AmpliTaq DNA polymerase FS, Perkin Elmer, Roissy, France) according to the instructions of the manufacturer. Electrophoresis and data collection were done on an Applied Biosystems 373A (Stretch model) automatic DNA sequencer.

The sequenced fragments were assembled into contiguous sequences and a consensus of the two strands was formed by using the Seqed program (Applied Biosystems, Branchburg, NJ).

Phylogenetic tree analysis

Phylogenetic relationships of the newly derived viruses were estimated from sequence comparisons with previously reported representatives of group M. Nucleotide amino acid sequences of the near-full-length genome and for each of the genes were aligned using CLUSTAL W[22] with minor manual adjustments, bearing in mind the protein sequences. Sites where there was a gap in any of the sequences, as well as areas of uncertain alignment, were excluded from all sequence comparisons. Phylogenetic trees of the entire genome and the different genes were constructed with the neighbor joining method and reliability of the branching orders using the bootstrap approach was implemented by CLUSTAL W. TreeView was used to draw trees for illustrations. Genetic distances were calculated with Kimura's two parameter method.[23] The viral polyprotein sequences were joined end to end omitting the 5′ end of the overlapping genes, and were aligned using CLUSTAL W and phylogenetic analysis and genetic distances on the amino acid sequences were performed as described above.

Analysis for intersubtype mosaicism

Diversity plots. To determine whether the viruses were recombinant or not, several additional analysis were done. Diversity plots, using the DIVERT program available online (the ANRS website, http://193.50.234.246/~beaudoin/anrs/Diversity.html), determined the percentage diversity between selected pairs of sequences by moving a window of 400 bp along the genome alignment in 10-bp increments. The divergence values for each pairwise comparison were plotted at the midpoint of the 400-bp segment.

Blast subtyping. A web-based HIV-1 subtyping system that uses the BLAST algorithm was done on our sequences to identify their subtype (http://www.ncbi.nlm.nih.gov/retroviruses/HIV-1/). The subtyping method employs a BLAST comparison between the HIV-1 sequence to be subtyped, input sequences, and a panel of complete genome references for the subtypes A, B, C, D, E, F, G, and H of group M, as well as for group O and N available in the GenBank. This program detects the best local similarities between a query sequence and a set of HIV-1 subtype reference sequences without performing a global alignment. A sliding window along the query sequence allows the detection of possible intersubtype recombinants with interspersed regions from two or more subtypes.

The simplot 2.5 software. The simplot 2.5 software, kindly provided by Stuart Ray,[24] was used to calculate similarity/diversity plots and bootstrap plots and to analyze informative sites. For similarity/diversity plots, Simplot calculates and plots the percentage identity/diversity of the query sequence to a panel of reference sequences, or to a consensus sequence from representatives of the different subtypes (with a threshold of 50%), in a sliding window, which is moved across the alignment in steps. The window and the size are adjustable. For the bootstrap plots we performed bootscanning on neighbor joining trees by using SEQBOOT, DNADIST (with Kimura's two parameter method and a transition/transversion ratio of 2.0), NEIGBOR, and CONSENSUS from the Phylip package for a 500-bp window moving along the alignment in increments of 50 bp (this approach is similar to the bootscanning method of Salminen et al.[25] We evaluated 100 replicates for each phylogeny. The bootstrap values for the studied sequences were plotted at the midpoint of each window. The Simplot program was used to identify informative sites as described by Robertson et al.[26] To estimate the location and the significance of

crossovers, each putative hybrid sequence was compared with a representative of each of the two subtypes inferred to have been involved in the recombination event and an appropriate outgroup. Phylogenetically informative sites in this context are those at which four taxa are divided equally into two groups, each of which has identity at that site. Each informative site supports one of the three possible phylogenetic relationships among the four taxa, and a cluster analysis maximizing the value of Chi square is then used to select breakpoints among the clusters. These breakpoints were used to divide the alignment into segments for phylogenetic tree construction as described above.

RESULTS

Sequence analysis of near-full-length HIV-1 genome

All the five HIV-1 genomes were sequenced in their entirety, with the exception of the LTR extremities. The genome structure was similar to all other HIV-1 subtypes, with the *gag*, *pol*, and *env* structural genes and *vif*, *vpr*, *vpu*, and *nef* single exon regulatory/accessory genes. The tat and rev exons 1 and 2 were also discernible at their usual positions. All reading frames were open and of complete length. None of the genomes had major deletions or rearrangements.

Phylogenetic analysis of the near-full-length genome

To determine the phylogenetic relationships of the newly characterized viruses, we constructed evolutionary trees from the near-full-length genome. The results confirmed the initial diversity observed among subtype F, and the three subgroups, F1 to F3, previously described in *gag* and envelope genes, were also observed by phylogenetic tree analysis of the entire genome (Fig. 1). They were supported by high bootstrap values (100%). The 96FR-MP411 strain clustered with the previously described subtype F sequences from Brazil (93BR020), Finland (FI.FIN9363), and Belgium (wife infected in the Democratic Republic of Congo) (VI850). The 95CM-MP255 and 95CM-MP257 strains of the previously identified F2 subgroup clustered together separately and have a branching pattern with the F1 subgroup similar to that observed between subtypes B and D. The 96CM-MP535 and 97ZR-EQTB11 strains, previously identified as F3, clustered together separately and equidistantly from other subtypes. The same results were obtained when phylogenetic analysis was done on amino acid sequences (2741 amino acids) (data not shown).

Phylogenetic analyses on *gag*, *pol*, *nef*, and accessory genes sequences are consistent in all these different genomic regions (Fig. 2). In the *gag*, *pol*, and accessory genes regions, the three F subgroups were supported by high bootstrap values, 100% for each subgroup. Only in the *nef* gene were bootstrap values lower, 85% for F1, 65% only for F2, and 100% for F3. An im-

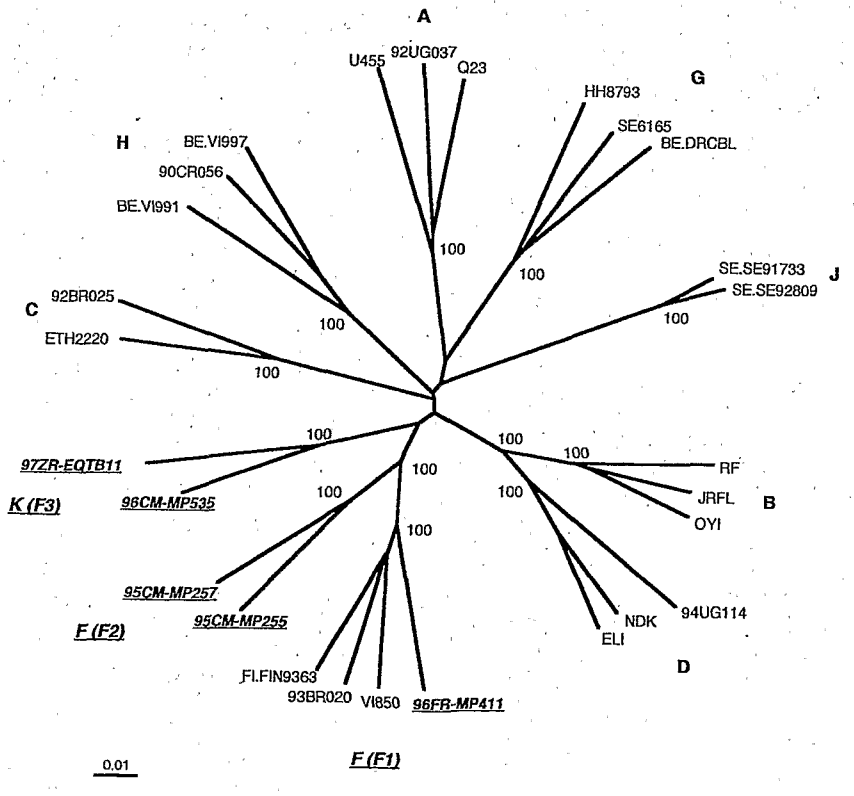

FIG. 1. Unrooted phylogenetic tree of the near-full-length nucleotide sequences constructed using the neighbor joining method (A) with the newly characterized F1 (F), F2 (F), and F3 (K) sequences, and reference strains representing the different genetic subtypes.

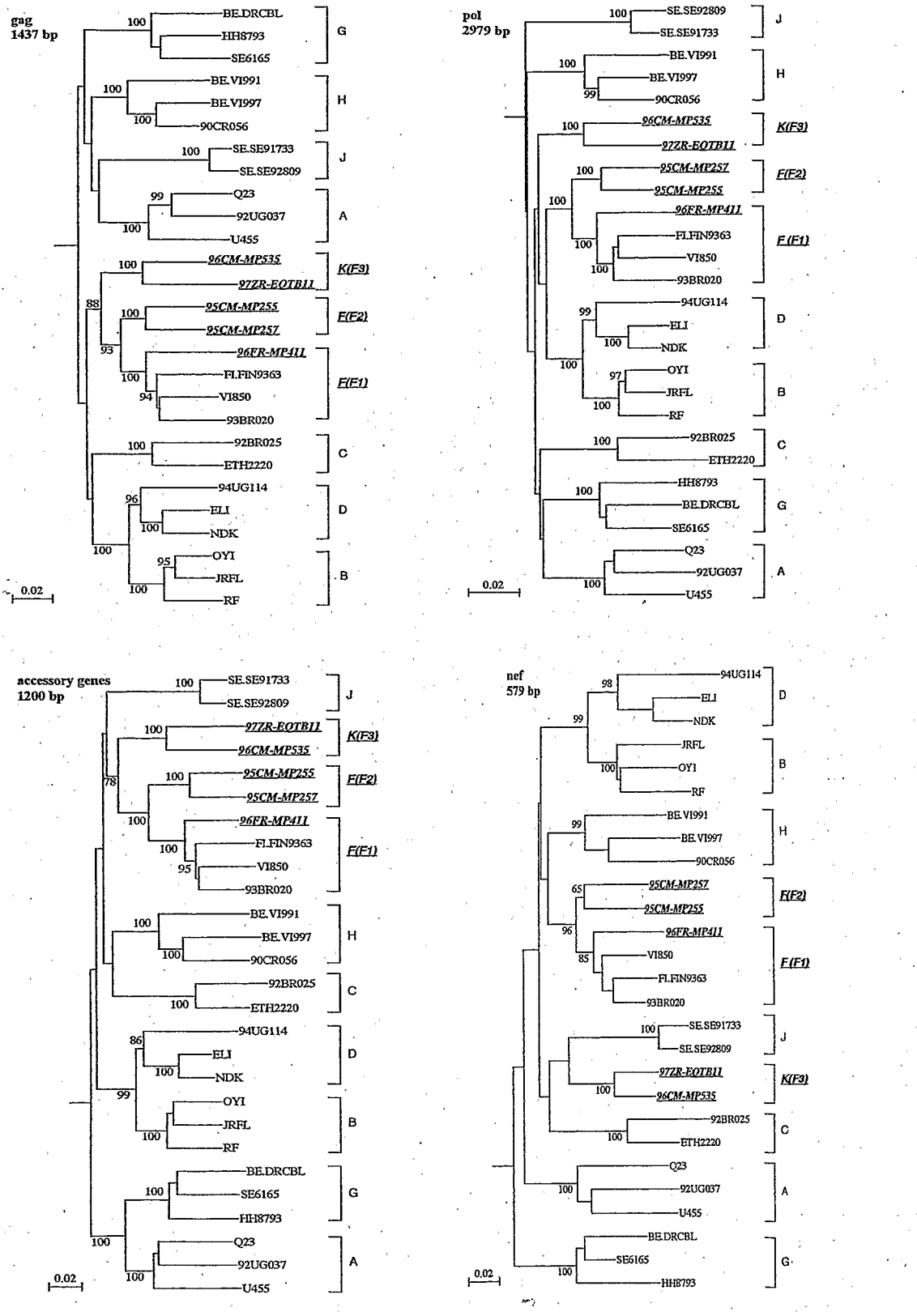

FIG. 2. Phylogenetic trees based on aligned nucleotides from the *gag* (1437 bp), *pol* (2979 bp), *nef* (579 bp), and accessory genes (1200 bp) from F1 (F), F2 (F), and F3 (K) sequences and sequences of reference strains representing the different genetic subtypes. The trees were rooted with the corresponding region of the $SIV_{CPZ\text{-}gab}$ isolate, which was used as an outgroup. The analysis was performed as described in Materials and Methods.

portant feature of the F1 and F2 viruses is that they form two separated well-defined clusters but always starting from a common node, as is observed for subtype B and D in the different genomic regions. On the other hand, the F3 viruses cluster independently from the F1 and F2 subgroups in the *pol* and *nef* genes. This was also previously seen in the envelope gene.

The genetic distances were calculated on *gag*, *pol*, *nef*, and accessory genes with Kimura's two parameter method, and with the same strains as those used for the phylogenetic tree analysis in Fig. 2. The results are presented in Table 2. The mean intersubtype distance based on nucleotide sequences from the near-full-length genomes (A, B, C, D, G, H, and J) was 15.1% and ranged from 10.2% for B versus D to 16.4% for G versus C (Table 2). The distances observed between subgroups F1 and F3 and between subgroups F2 and F3 were 12.9%, whereas the distance observed between F1 and F2 was lower (11%) and closer to the intersubtype distance seen between subtypes B and D (10.2%).

For all the studied genes, the distances within each F subgroup were comparable to intrasubtype distances observed for the known subtypes A, B, C, D, G, H, and J. Also distances between the different F subgroups were comparable to distances observed between the subtypes in the different genes. However, it is noteworthy that the intersubtype genetic distances observed between subgroups F1 and F2 are overall lower and more closely related to those seen between subtypes B and D, especially in *gag*, *pol*, and the accessory genes (9.4 and 8.4%, 7.8 and 6.7%, and 11.6 and 10.3% for F1 versus F2 and B versus D in *gag*, *pol*, and accessory genes, respectively). The exception is the *nef* gene, where the distance between F1 and F2 is lower than the intersubtype distance and corresponds to intrasubtype distances observed in this gene, confirming the phylogenetic position of these two subtypes in the *nef* gene. Moreover, the genetic distances in all the studied genes between F3 and F1 and F3 and F2 were close to the mean intersubtype distances obtained for the reference subtypes, subtypes B and D excluded.

Diversity plots

To determine more precisely whether the F1, F2, and F3 viruses correspond to different nonrecombinant subtypes, we performed pairwise sequence comparisons between the near-full-length genome sequence of the five new isolates and those available from the database, using the same alignment of nucleotide sequences used for the phylogenetic tree construction of the near-full-length sequences. Importantly, distance values were calculated only after removing all sites with a gap in any of the sequences, ensuring that all comparisons were made across the same sites.

The F2-95CM-MP255, F2-95CM-MP257, F3-96CM-MP535, and F3-97ZR-EQTB11 viruses were roughly equidistantly related to the reference strains for subtype A to J, including the F-93BR020, F-FI.FIN9363, and F-VI850 strains, over the entire length of their genome. These data suggest that they could represent members of new nonrecombinant subtypes (data not shown). When the F2-95CM-MP255 virus was plotted against the A-U455, F-93BR020, F3-96CM-MP535, and F2-95CM-MP257 virus, a lower level of sequence divergence was seen with the F2-95CM-MP257 virus (ranging from 1.3% in *pol* to 12% in *env*). The divergence between F2-95CM-MP255 and F2-95CM-MP257 was slightly higher at the 5′ end of the envelope, whereas in the *nef* gene a similar degree of low divergence was seen between each of the F2 viruses (95CM-MP255 and 95CM-MP257) and the F-93BR020 strain. A similar diversity profile was seen when F2-95CM-MP257 was plotted against the A-U455, F-93BR020, F3-96CM-MP535, and the F2-95CM-MP255 virus (Fig. 3A).

When the F3 viruses, 96CM-MP535 and 97ZR-EQTB11, were plotted against each other together with the A-U455, F-93BR020, and F2-95CM-MP255 virus, a low level of sequence divergence comparable to viruses from the same subtype was seen, and this time there were no ambiguities in certain parts of the genome, divergence ranged from 3% in *pol* to 18% in

TABLE 2. AVERAGE OF INTRACLUSTER AND INTERCLUSTER GENETIC DISTANCES[a]

	Genetic distances (%)					
	gag	*pol*	*Accessory genes*	*env*[b]	*nef*	*Full-length genome*
			Intracluster			
F1	7.3	5.8	6.9	9.6	7.0	7.4
F2	5.9	4.4	6.5	12.9	8.3	7.7
F3	8.2	5.3	9.1	13.4	5.6	7.7
Intrasubtypes	6.1 (2.4–7.6)	4.9 (2.5–6.4)	7.5 (3.1–10.3)	10.9 (5.4–15.5)	8.9 (3.2–12.3)	7.1 (2.9–8.8)
			Intercluster			
F1 vs F2	9.4	7.8	11.6	15.8	9.5	11.0
F1 vs F3	12.6	9.8	14.8	16.3	15.0	12.9
F2 vs F3	10.9	9.0	15.3	17.2	15.4	12.9
B vs D	8.4	6.7	10.3	13.7	14.4	10.2
Intersubtypes (excluding B vs D)	13.7 (12.7–15.2)	11.3 (10.7–12.5)	16.9 (13.6–19.7)	18.0 (16.2–19.0)	20.1 (17.6–22.7)	15.1 (13.2–16.4)

[a]Average of intracluster and intercluster genetic distances (%) in *gag*, *pol*, *env*, *nef*, and accessory genes. Distances were calculated according to Kimura's two parameter method as described in Materials and Methods based on nucleotide sequence alignment used to construct the phylogenetic trees of Figs. 1 and 2.

[b]Triques et al.[17]

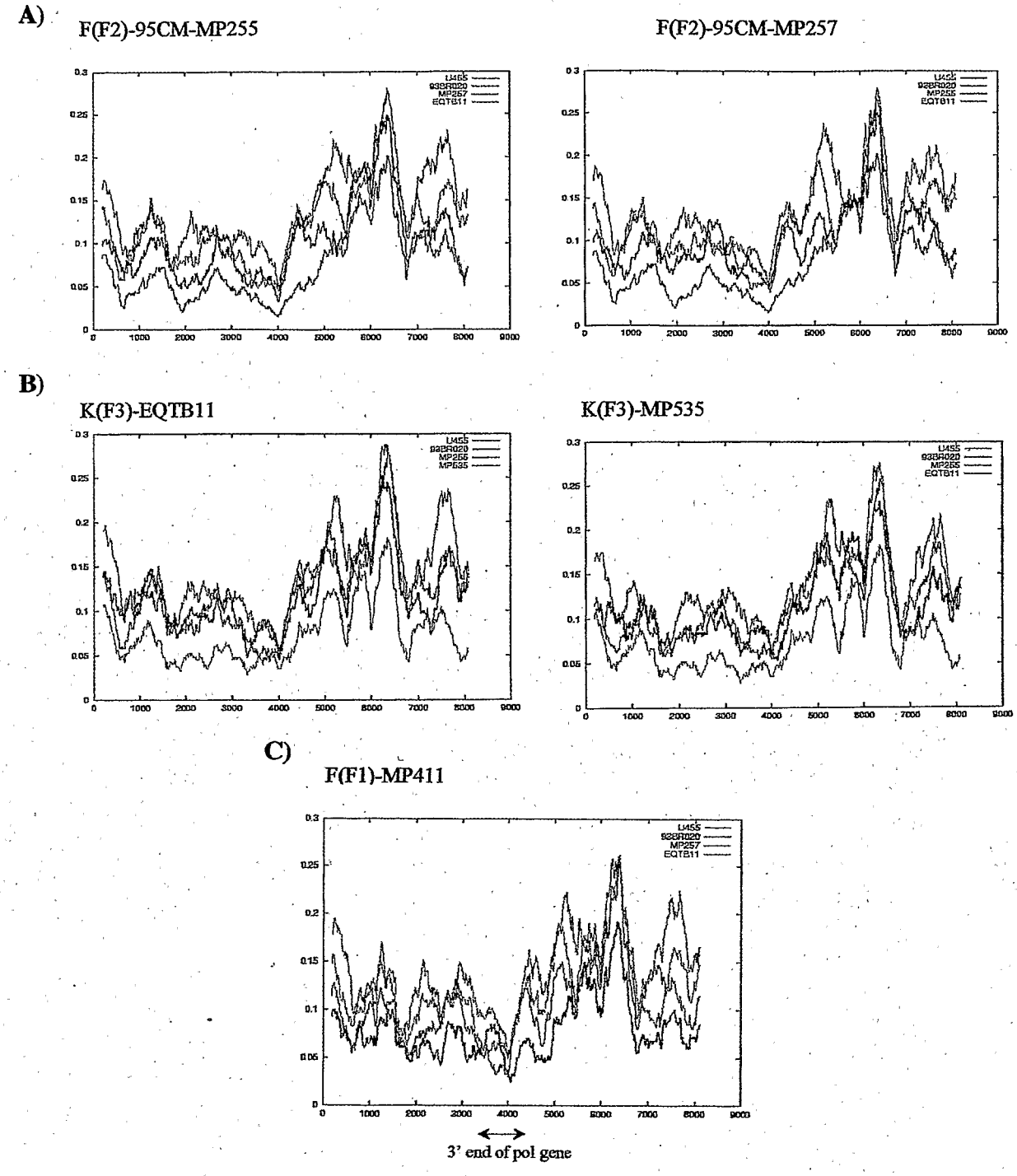

FIG. 3. Diversity plots comparing the sequence relationships of the newly characterized (A) F3 (K), (B) F2 (F), and (C) F1 (F) strains to each other and to reference sequences from the database. The sequences named above the plots are compared to the sequences listed on the right (sequences are colored code). U455 and 93BR020 are published sequences for subtypes A and F. Distance values were calculated for a window of 400 bp moving in increments of 10 nucleotides. The *x* axis indicates the nucleotide position along the alignment (gaps were removed from the alignment). The *y* axis shows the distance between the viruses compared (0.05 = 5% divergence).

the envelope gene (Fig. 3B). Based on the diversity plots, there was no evidence for mosaicism in the genome of these four viruses (F2-95CM-MP255, F2-95CM-MP257, F3-96CM-MP535, and F3-97ZR-EQTB11) at the level of both intra- and intersubtype comparisons. However, in the *nef* gene the F2 viruses were very close to the F1 viruses.

When the F-96FR-MP411 virus was plotted against the A-U455, F-93BR020, F2-95CM-MP255, and F3-96CM-MP535

viruses, the lowest divergence was seen with the F-93BR020 strain, except in a small region of the *pol* gene where the lowest divergence was seen with the F2-95CM-MP255 sequence (Fig. 3C). The same was observed when F2-95CM-MP255 was replaced by F2-95CM-MP257. These data suggest that the F-96FR-MP411 genome could represent a mosaic of F1 and F2 sequences. Diversity plots of five new near-full-length genome sequences, using consensus sequences from the different known subtypes and the two new subtypes instead of one particular strain, lead to the same conclusions as the diversity plot results with individual strains (data not shown).

Blast-subtyping

The Blast-subtyping method employs a BLAST comparison between the HIV-1 sequence to be subtyped and a panel of complete genomic references for the subtypes A, B, C, D, E, F, G, H, and J of group M, as well as for groups O and N. This program detects the best local similarities between a query sequence and a set of HIV-1 subtype reference sequences from the GenBank and our new input sequences without performing a global alignment. A sliding window of 300 bp with an increment of 100 bp along the query sequences (F2-95CM-MP255, F2-95CM-MP257, F3-96CM-MP535, F3-97ZR-EQTB11, and F-96FR-MP411) was done to identify the subtype or to detect possible recombinants with interspersed regions from two or more known subtypes. If there are identical scores in a specific window with more than one subtype, the window bars are divided diagonally and the different subtypes are represented. The comparison between F2-95CM-MP255 as the query sequence and F2-95CM-MP257 included with the reference sequences revealed a higher similarity with F2-95CM-MP257. When F2-95CM-MP255 was replaced by F2-95CM-MP257 as query sequence, the same profile was obtained, with nevertheless slight ambiguities in the 5′ envelope gene, the same region observed with divert plots (data not shown). The comparison between F3-96CM-MP535 as query sequence and F3-97ZR-EQTB11 and reference sequences showed identical scores with F3-97ZR-EQTB11 along all the genome. When F3-97ZR-EQTB11 was compared to F3-96CM-MP535 and to other known sequences, a similar profile was observed. The F-96FR-MP411 was compared with F2 and F3 strains and with the known subtypes A, B, C, D, E, F, G, H, and J of group M, as well as for groups O and N. As expected, the Blast graph showed a higher similarity with the F1 strains along the genome, except at the 3′ end of the *pol* gene, where there are identical scores with the F2 strains.

Bootstrap plots

To evaluate systematically the bootstrap values supporting independent monophyletic phylogeny in different parts of the genome, the alignment was bootscanned in regard to the clustering of the F2 and F3 sequences, with a sliding window of 500 bp advancing with 50-bp increments. The same multiple genome alignment generated for the phylogenetic analysis of the near-full length genomes shown in Fig. 1 was used to calculate the consensus reference sequences (50% threshold) for the eight groups of nonrecombinant subtypes corresponding to subtypes A, B, C, D, F, G, H, and J. The magnitudes of the bootstrap values supporting the clustering of our strains with consensus sequences from members of each subtype, and one strain from each of the F2 and F3 subgroups, were determined along the genome. The results of the bootstrap analysis are shown in Fig. 4.

Bootstrap plots of the F2-95CM-MP255 and F2-95CM-MP257 strains showed that almost over the entire genome, these strains form a monophyletic group, supported with more than 70% of the bootstrap values, except for about 400 bp in the *gag* gene and a 600-bp fragment in the 5′ end of the envelope. In the *gag* gene, the diversity plots revealed no ambiguities in this region. This fragment was extracted from the alignment and phylogenetic tree analysis on it showed clearly that both F2-95CM-MP255 and F2-95CM-MP257 formed a separate cluster supported with 86% as bootstrap values. In the 5′ end of the envelope, the problem was more complex. Phylogenetic tree analysis from this same region revealed, however, the presence of the three subgroups, F1, F2, and F3; however, the two F2 strains clustered together only with very low bootstrap values (27%). Also on the diversity plots, a higher diversity was seen between the F2-95CM-MP255 and F2-95CM-MP257 strains than in the other parts of the genome. Additional exploratory tree analysis and phylogenetically informative sites in this region of the envelope did not give more exact information on an eventual recombination event with another subtype (data not shown).

The bootscan analysis of the F3-96CM-MP535 and F3-97ZR-EQTB11 viruses showed without ambiguities that the two strains formed a monophyletic cluster with a high bootstrap value, more than 80% over almost the entire genome. Nevertheless, in small regions the bootstrap values dropped to 70%.

Bootscan analysis of the F-96FR-MP411 strain showed that this virus formed a monophyletic group with the previously described subtype F viruses F-93BR020, F-FI.FIN9363, and F-VI850 (data not shown) and more particularly with the F1 strains. Similarly, as observed on the diversity plots, a small region of approximately 500 bp at the 3′ end of the *pol* gene was associated with F2 in the bootscan.

Reevaluation of the phylogenetic position of the F2 and F3 strains

Diversity plotting, Blast subtyping, and bootstrap plotting confirmed that the divergent F strains, preliminarily called F2 and F3, correspond to nonrecombinant viruses. Phylogenetic tree analysis and distances calculated in the different genomic regions showed that the divergence between F1 and F2 is consistently lower than that seen in any other intersubtype comparison, with the exception of subtypes B and D. Based on all the different analyses, we propose to divide subtype F into two subclades, with F1 gathering the known subtype F strains from Brazil and Finland, and our African strain (96FR-MP411), and F2 containing the 95CM-MP255 and 95CM-MP257 strains from Cameroon. The phylogenetic relationship and genetic distances observed between the F3 strains, 97ZR-EQTB11 from the Democratic Republic of Congo and 96CM-MP535 from Cameroon, show that these strains are equidistantly related to the other known subtypes. The F3 viruses should be elevated with the status of a new nonrecombinant subtype, designated K.

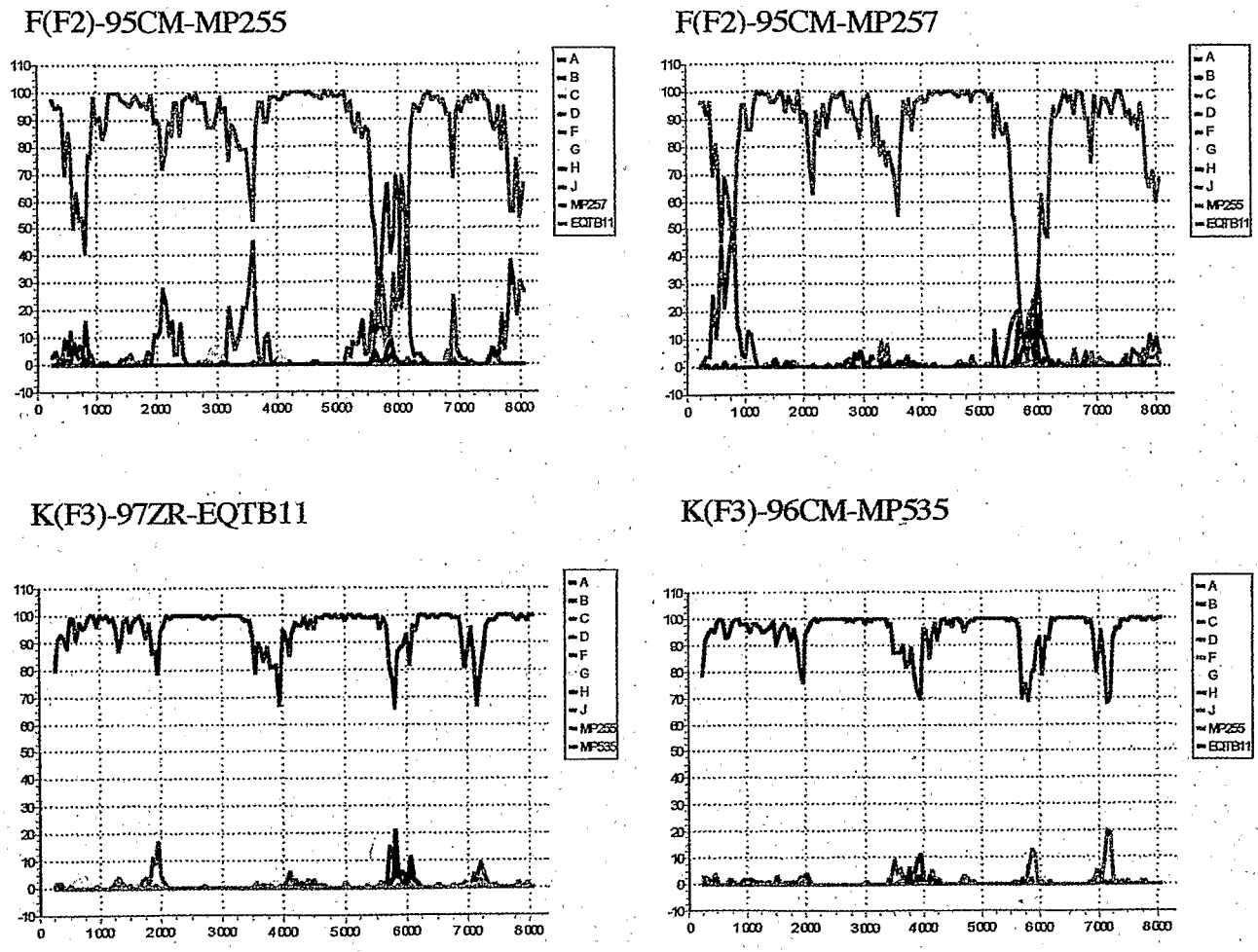

FIG. 4. Bootscan plots of strains from subclades F2 (F) and F3 (K). The new sequences were bootscanned against the consensus sequences and a representative from both subclades F2 and F3, and the genome was scanned in segments of 500 nt moving in increments of 50 bp along the alignment. The *x* axis indicates the nucleotide position along the alignment (gaps were removed from the alignment). The *y* axis shows the bootstrap values (%).

Recombination analysis in 96FR-MP411

Diversity plots, Blast subtyping, and bootstrap plots all indicate that in a small region of the *pol* gene the 96FR-MP411 strain is closer to F2 than to F1. However, more detailed analysis provided no real significant results to conclude a recombination event between F1 and F2. The *p* values, obtained by maximizing the chi square values in the permutation tests on the informative sites, are between 0.032 and 0.046. A 546-bp fragment covering this region was extracted and compared to all subtypes by neighbor-joining phylogenetic tree analysis, and the F-96FR-MP411 strain was shown to form a cluster with the F2 sequences in this region, but supported only by 52% of the bootstrap values.

Relationship to previously reported divergent subtype F strains from Africa

The degree to which these sequences represent variants circulating in Africa or other parts of the world was studied by a series of phylogenetic trees that included subtype F sequences previously obtained by other groups. These strains were reported from Brazil, Romania, Cameroon, Democratic Republic of Congo, Central African Republic, and Gabon.[15,17–19,27–31] The phylogenetic analysis was performed as described above on a 272-bp fragment encompassing the V3 region of the gp120 envelope gene. The results showed clearly the three well-separated subgroups within subtype F, F1 to F3 (data not shown). As expected, the Brazilian sequences all clustered in the F1 subgroup with the 93BR020, FI.FIN9363, and VI850 viruses together with some strains from Romania and with our previously described strain from Central African Republic (MP84) and 96FR-MP411 from Chad or Yugoslavia. The majority of Cameroonian strains clustered separately with the F2-95CM-MP255 and F2-95CM-MP257 sequences. The VI354 strain from Gabon clustered with the K(F3)-96CM-MP535 virus from Cameroon and the K(F3)-97ZR-EQTB11 virus from the Democratic Republic of Congo, together with strains previously described from the Democratic Republic of Congo (ZR36). The divergent VI325 subtype F was reported to cluster in the F3 subtype in the p24 region of the *gag* gene with K-96CM-MP535 and K-97ZR-EQTB11. The VI354 and VI325 viruses should be renamed subtype K in *env* and *gag*, respectively. Table 3

TABLE 3. NEW SUBTYPE DESIGNATION OF THE PREVIOUSLY DIVERGENT SUBTYPE F STRAINS

Samples	*Reference*	*Origin*	*Regions sequenced*		*New subtype designation*
MP446	17	Cameroon		Partial env	F2
CA4	18	Cameroon		Partial env	F2
CA16	18	Cameroon		Partial env	F2
CA20	18	Cameroon		Partial env	F2
CMR304	15	Cameroon		Partial env	F2
CMR158	15	Cameroon		Partial env	F2
CMR275-F	15	Cameroon		Partial env	F2
VI354	19	Gabon		Partial env	K (F3)
VI325	27	Belgium	Gag		K (F3)
MP584	17	Cameroon	Partial gag	Partial env	Recombinant gagA–envF2
EQS16	17	DRC[a]	Partial gag	Partial env	Recombinant gagF2/F1–envF1
ZR36	17	DRC	Partial gag	Partial env	Recombinant gagA–envK (F3)

[a]DRC, Democratic Republic of Congo.

summarizes the new subtype designation of the previously described divergent subtype F strains.

DISCUSSION

In this paper, the near-full-length genome sequences of five African HIV-1 viruses previously identified as subtype F or divergent F strains are described. Based on partial genome sequencing in gag and the envelope, we preliminarily subdivided subtype F into three subgroups called F1, F2, and F3.[17] Sequencing of the near-full-length genome of representative strains obtained from epidemiologically unlinked individuals from each of these subgroups allowed us to determine that the divergent subtype F strains preliminary called F3 correspond to a new nonrecombinant subtype, designated K. Phylogenetic tree analysis, diversity plotting, and bootscanning of strains K-96CM-MP535 and K-97ZR-EQTB11 showed that subtype K is evolutionary equidistant to previously described subtypes A to J.

Overall the genetic distances observed between F1 and F2 are lower and close to those observed between subtype B and D. As with subtypes B and D, F1 and F2 viruses cluster consistently together in phylogenetic trees of different genomic regions. The genetic divergence between B and D, as well as for F1 and F2, is hardly any greater than the diversity seen within some other subtypes, especially subtype A.[11] Therefore we prefer to consider the F2 strains as a subclade of subtype F instead as an equidistantly related new subtype.

In each of the two F2 isolates there are genomic regions that seem less related to each other, however, these are inconsistently distributed and cannot be attributed to areas of recombination with other known subtypes. Based on the bootstrap plots, the associations between the F2-95CM-MP255 and F2-95CM-MP257 strains were lower in certain regions of the genome, especially in *gag* and in the 5′ end of the envelope gene. However diversity plots and phylogenetic tree analysis showed that also in these regions the closest relationship was observed between the F2 strains as compared to representatives of the other subtypes, and the F2-95CM-MP255 and F2-95CM-MP257 strains clustered together although supported with lower bootstrap values, especially in the 5′ end of the envelope gene. This can be due to lower phylogenetic information in this region. In the *nef* gene, F1 and F2 have a closer relationship but still form separate clusters in this region. A similar ambiguity is also observed for subtype A/G-IBNG, G, and A/E viruses in the proximal portion of the gp41 cytoplasmic domain, where the relationship between these viruses is close to intrasubtype divergence but these subtypes still form separate groups within this supercluster.[10,32]

The results presented on the K-95CM-MP535 and K-97ZR-EQTB11 strains show clearly that these strains represent members of a new subtype of HIV-1, subtype K. These viruses were isolated from patients living in two different countries and both patients denied traveling into another African country. These data indicate a generalized spread of subtype K in Central Africa.

Clearly defined subtype F samples have been documented in several South American countries, cases have been described in Europe, and subtype F strains have also been isolated in Africa or from Europeans infected in Africa or with an African partner.[33–38] In Romania, subtype F was responsible for an explosive HIV-1 outbreak among hospitalized children.[28,31] The close relationship of these European and South American samples with samples from Africa confirms an African origin of the subtype F epidemic in Romania and South America. Divergent subtype F samples have been mainly reported from Cameroon, and as has been shown in our study, these samples correspond to the F2 subclade within subtype F. All the samples previously described as subtype F from Cameroon by Nkengasong et al.[18] and Takehisa et al.[15] should be reconsidered as F2. The data from their studies and unpublished results from our group indicate also that F2 is relatively prevalent, representing 7 to 17% of the circulating strains in Cameroon. Up to now, this subclade has been described only in Cameroon. A large survey on the genetic distribution of HIV-1 subtypes revealed, however, that F2 viruses circulate also in other African countries, like Nigeria, Gabon, and the Democratic Republic of Congo.[39] Preliminary data on recombinant viruses in Cameroon indicated several examples of recombination events with F2 and other subtypes. Indeed, the MP584 strain belonging to F2 in the V3–V5 region of the envelope gene was identified as subtype A in *gag*.[17]

The VI354 strain described in Gabon,[19] and initially classi-

fied as the most divergent subtype F virus in the envelope, forms a highly supported cluster with the K-96CM-MP535 and K-97ZR-EQTB11 strains, therefore, its subtype designation should be reconsidered and this virus is a representative of the newly described HIV-1 subtype K viruses, at least in the envelope region. The same is true for the ZR36 strain from the Democratic Republic of Congo. This subtype circulates in at least three different Central African countries and preliminary data on samples from Cameroon and the Democratic Republic of Congo show that this subtype is also involved in recombination events. Indeed, the ZR36 strain has been found to cluster into subtype A and gag.[17]

The equidistance of subtype K to the other subtypes of HIV-1 and their presence in mosaic genomes suggest that this subtype existed as long as the other subtypes. The lower divergence between subtype B and D as compared to the values observed between other subtypes led to the hypothesis of a common ancestry and a more recent subdivision for B and D viruses.[40] As for these viruses, our observations also suggest a common ancestor for F1 and F2 viruses.

The global epidemic has been extensively characterized by genetic analysis. The various genetic subtypes differ in their geographic spread and so the subtype designations have been powerful molecular epidemiological markers for tracking the course of the global pandemic. Subtype classification would be of greater interest if members of the various subtypes were found to differ in biological differences. There are several indications that there may be biological differences among HIV-1 groups: the group O viruses do not have cyclophilin A to produce infectious virions[41] and *in vitro* data showed that HIV-1 group O viruses are naturally resistant to nonnucleoside reverse transcriptase (RT) inhibitors, as is HIV-2.[42,43] In addition, within group M, some subtype F samples are less susceptible to the nonnucleoside reverse transcriptase inhibitor tetrahydroimidazo[4,5,1-*jk*][1,4]-benzodiazepin-2-(1*H*)-one and -thione derivates (TIBO), and some subtype G strains are less susceptible to protease inhibitors.[44,45] The CXCR4-positive rapid/high phenotype is underrepresented among subtype C isolates and the syncytium-inducing phenotype is rare among subtype C-infected patients.[46,47] The genetic subtype can also have an impact on the efficiency of testing for HIV antibodies.[48] However, it will be difficult to show a consistent association between subtype and correlates of transmission and pathogenesis and a single characteristic such as subtype will most probably not account for significant differences in transmission and disease progression. Nevertheless, it would be premature to conclude that there are no subtype-specific differences in virus biology, transmission, or disease development.

Given the wide dispersal of HIV-1 subtypes internationally and the occurrence of international travel, the geographic distribution of subtypes is evolving, and intermixing of HIV-1 variants is inevitable. If differences exist among different subtypes, it is important that these subtypes are well defined and it remains significant to know the underlying molecular epidemiology of HIV-1. Since HIV-1 strains of different subtypes have been shown to recombine, the complete genome should be analyzed to verify that all regions of the virus cluster independently before considering a new subtype. In this study we sequenced the first near-full-length HIV-1 genomes from a new nonrecombinant HIV-1 subtype, designated K, as well as from two representatives of the F2 subclade within subtype F.

SEQUENCE DATA

The new sequences have been deposited in the GenBank Data Library under the following Accession Numbers: AJ249235–AJ249239.

ACKNOWLEDGMENTS

This work was supported by grants from the European Union (INCO-DC n°IC18CT970216), the Agence Nationale de Recherches sur le SIDA (ANRS, Projet Sidak), and Karine Triques has a Doctoral fellowship from SIDACTION.

We thank Bette Korber for helpful discussions and comments, and Paul Sharp for his assistance with the informative sites analysis and his instructive comments.

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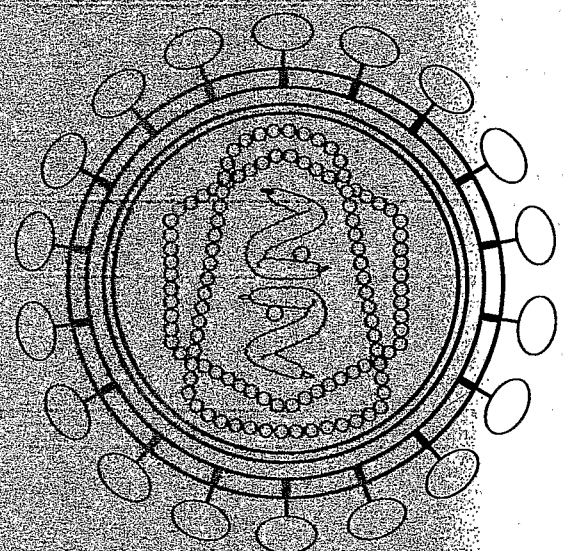
Now the Official Journal of the INTERNATIONAL RETROVIROLOGY ASSOCIATION

Volume 16 Number 2 January 20, 2000

ISSN: 0889-2229

AIDS RESEARCH AND HUMAN RETROVIRUSES

Editor: ERIC HUNTER, Ph.D.



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