Compartmentalization and Clonal Amplification of HIV-1 Variants in the Cerebrospinal Fluid during Primary Infection[▽]

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Human immunodeficiency virus type 1 (HIV-1)-associated dementia (HAD) is a severe neurological disease that affects a subset of HIV-1-infected individuals. Increased compartmentalization has been reported between blood and cerebrospinal fluid (CSF) HIV-1 populations in subjects with HAD, but it is still not known when compartmentalization arises during the course of infection. To assess HIV-1 genetic compartmentalization early during infection, we compared HIV-1 populations in the peripheral blood and CSF in 11 primary infection subjects, with analysis of longitudinal samples over the first 18 months for a subset of subjects. We used heteroduplex tracking assays targeting the variable regions of *env* and single-genome amplification and sequence analysis of the full-length *env* gene to identify CSF-compartmentalized variants and to examine viral genotypes within the compartmentalized populations. For most subjects, HIV-1 populations were equilibrated between the blood and CSF compartments. However, compartmentalized HIV-1 populations were detected in the CSF of three primary infection subjects, and longitudinal analysis of one subject revealed that compartmentalization during primary HIV-1 infection was resolved. Clonal amplification of specific HIV-1 variants was identified in the CSF population of one primary infection subject. Our data show that compartmentalization can occur in the central nervous system (CNS) of subjects in primary HIV-1 infection in part through persistence of the putative transmitted parental variant or via viral genetic adaptation to the CNS environment. The presence of distinct HIV-1 populations in the CSF indicates that independent HIV-1 replication can occur in the CNS, even early after HIV-1 transmission.

Human immunodeficiency virus type 1 (HIV-1) infection of the central nervous system (CNS) can lead to neurological disease in a subset of HIV-infected individuals and may include the development of HIV-1-associated dementia (HAD) (2, 18). HAD is characterized by severe neurological dysfunction, and affected individuals generally have impaired cognitive and motor functions. HIV-1 enters the CNS during primary infection, most likely via the migration of infected monocytes and lymphocytes across the blood-brain barrier (33, 37, 42). The main cell types in the CNS that HIV-1 can productively infect are the perivascular macrophages and microglial cells, which express low receptor densities of CD4, CCR5, and CXCR4 (7, 18, 60, 63). Previous studies have also reported that neurotropic HIV-1 variants are generally macrophage tropic (19, 20, 32, 45, 52, 61). Although cells in the CNS may be infected with HIV-1 during the course of disease, it is still unclear whether productive HIV-1 replication occurs in the CNS early during infection.

Genetically compartmentalized HIV-1 variants have been detected in the brains of HAD subjects at autopsy (13, 14, 43, 48, 52) and in the cerebrospinal fluid (CSF) of HAD subjects sampled over the course of infection (26, 46, 51, 59). Extensive compartmentalization between the periphery and the CNS has been reported in subjects with HAD; however, it is not yet

known when compartmentalization occurs during the course of HIV-1 infection. Primary HIV-1 infection refers to the acute and early phases of infection, during which peak plasma viremia often occurs and a viral "set point" may be reached (8, 34), within the first year after HIV exposure (64). Studies examining compartmentalization between the blood plasma and CSF during primary infection have been limited, and extensive compartmentalization has not been detected in primary infection subjects (26, 50).

In this study, we examined HIV-1 genetic compartmentalization between the peripheral blood and CSF during primary HIV-1 infection. Cross-sectional and longitudinal blood plasma and CSF samples were analyzed for viral compartmentalization using the heteroduplex tracking assay (HTA) and single genome amplification (SGA). We used the HTA to differentiate between HIV-1 variants in the CSF that were either compartmentalized to the CSF or equilibrated with the peripheral blood. Previous studies have used the HTA to separate HIV-1 genetic variants in different anatomical compartments (10, 24, 27, 51) and to follow HIV-1 evolutionary variants over the course of infection (9, 25, 31, 41, 49, 50). We also conducted SGA on a subset of subjects to further examine viral genetic compartmentalization during primary infection. Here we report the detection of compartmentalized and clonally amplified HIV-1 variants in the CSF of subjects in the primary stage of HIV-1 infection. Our results suggest that minor to extensive HIV-1 genetic compartmentalization can occur between the periphery and the CNS during primary HIV-1 infection and that viral compartmentalization, as measured in the CSF, is transient in some subjects.

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TABLE 1. Subject population characteristics and inflammatory markers

Subject ID[a]	Sample date (mo/day/yr)	Days p.i.[b]	CD4[c]	CSF WBC[d]	Plasma HIV RNA[e]	CSF HIV RNA[e]	Albumin ratio[f]	CSF neopterin[g]	% Difference[h]
9003	3/1/06	78	1044	2	26,400	49	3.38	7.3	
9010	10/10/06	235	497	11	1,030	206	5.55	9.5	
9012	2/8/07	103	601	8	74,131	398	11.09	14.3	
9014	3/20/07	169	751	12	35,481	537	4.13	8.5	
9016	4/24/07	143	353	1	776,247	49	5.44	6.6	
9017	6/20/07	49	361	14	17,378	151	4.51	8.6	
9001	12/14/05	206	488	3	153,000	246	4.55	14.3	NR
	3/22/06	305	573	4	194,000	1,180	3.50	19.0	19.0
	6/21/06	393	575	5	74,800	4,120	4.33	20.5	10.4
9002	2/15/06	86	770	6	7,670	19	6.47	8.9	0
	4/6/06	137	686	2	16,500	208	6.69	11.3	0
	8/22/06	273	539	8	44,800	4,560	7.35	21.0	0
	9/26/06	306	542	14	32,400	4,630	9.29	21.3	0
	10/26/06	336	501	14	37,200	12,000	9.61	17.0	0
	12/14/06	384	640	12	198,000	8,130	9.67	15.1	0
	5/8/07	528	546	11	13,800	1,280	9.79	9.0	0
	7/17/07	597	459	2	40,000	515	8.73	10.1	6.6
9006	9/14/06	150	435	8	27,700	1,300	4.64	12.9	0
	11/1/06	197	512	6	29,000	1,310	4.31	NA	0
	3/20/07	336	453	6	65,600	1,620	3.75	NA	15.9
9007	9/18/06	149	256	4	233,000	426	10.85	33.1	NR
	11/2/06	193	336	9	183,000	3,250	7.88	23.5	14.4
	6/5/07	406	292	4	29,500	3,900	6.87	22.9	7.4
9019	8/13/07	58	750	13	49,300	1,390	5.58	13.9	2.2
9025	4/21/08	50	752	7	101,000	15,200	3.91	NA	NA
9037	5/14/09	46	884	26	507,652	2,682	6.35	NA	NA
9039	6/1/09	36	539	53	367,728	19,911	9.31	NA	NA
9018	8/2/07	198	350	11	404,000	37,500	6.45	11.6	29.2
9040	6/15/09	165	705	4	16,710	6,176	7.67	NA	NA
7146	9/23/03	156	552	86	429,000	168,000	14.35	56.6	23.9
	10/14/03	177	476	97	217,000	24,900	14.35	41.2	19.1
	11/10/03	203	310	53	103,000	26,000	7.12	23.5	41.1
	4/29/04	372	380	1	13,399	19	4.39	14.5	NA
	6/24/04	427	337	2	225,000	36	7.31	NA	NA
	10/7/04	530	305	2	308,000	2,630	5.40	20.0	15.9

[a] ID, identifier. Antibody (Ab) data were as follows: subject 9003, 1 band (p24) on 12/20/05; subject 9010, negative Ab on 8/2005; subject 9012, not known; subject 9014, negative Ab on 8/4/06, positive Ab on 12/6/07; subject 9016, positive HIV RNA, negative Ab on 12/27/06; subject 9017, not known; subject 9001, negative Ab on 5/25/05, 10 bands on 11/1/05; subject 9002, 10 bands on 1/3/06; subject 9006, 2 bands (p24 and gp160) on 5/5/06; subject 9007, not known; subject 9019, positive HIV RNA, negative Ab on 6/2007; subject 9025, negative HIV RNA on 1/14/08, HIV RNA 10,000 cps/ml and negative Oraquick on 3/13/08, and positive Ab on 3/20/08; subject 9037, negative EIA with positive ELISA on 4/19/09; subject 9039, not known; subject 9018, last negative Ab on 11/4/06; subject 9040, negative Ab on 11/4/08, positive Ab on 2/24/09; subject 7146, negative Ab on 3/25/03.
[b] Estimated.
[c] Cells/μl.
[d] Cells/μl.
[e] Copies/ml.
[f] CSF/plasma albumin ratio.
[g] Nanomoles per liter; NA, not applicable.
[h] Percent differences between plasma and CSF viral populations as measured by HTA. The reported values are the averages calculated from two independent HTA replicates (see references 31 and 49 for the methods). Percent differences were not measured for subjects 9003, 9010, 9012, 9014, 9016, and 9017 due to low CSF HIV-1 RNA levels. NR, not reported due to poor sampling.

MATERIALS AND METHODS

Subject population. The study subjects were individuals enrolled in a neurological study of acute and early HIV-1 infection at the University of California at San Francisco. Diagnosis of primary HIV-1 infection at enrollment followed the previously described Serologic Testing Algorithm for Recent HIV Seroconversion (STARHS) (40). In brief, subjects had positive HIV-1 nucleic acid tests and either had negative antibody tests in the past 12 months or had a less sensitive enzyme immunoassay (EIA) result supportive of infection within the previous 6 months. Further estimation of the number of days since HIV-1 exposure (days postinfection [p.i.]) was based upon the presence of symptoms of an acute seroconversion reaction or taken as the halfway point between the last negative and first positive antibody tests (35, 36). Serial blood plasma and cerebrospinal fluid samples were collected for study purposes as early as possible after initial diagnosis and at various intervals thereafter. Plasma and CSF HIV-1 RNA concentrations were determined using the Amplicor HIV Monitor kit (Roche). The study was approved by the Committee for Human Research at the University of California at San Francisco, and all subjects provided written informed consent for the collection of samples.

Markers of inflammation. Entry blood and CSF determinations, including cell counts, protein, and albumin, were performed by the San Francisco General Hospital Clinical Laboratories. CSF neopterin was assayed using a commercially available enzyme-linked immunosorbent assay (ELISA) according to the vendor's methods (Henning Berlin GMBH, Berlin, Germany).

Viral RNA isolation, RT-PCR, HTA, and PhosphorImager analysis. Procedures for viral RNA isolation, RT-PCR, and HTA have been previously described (11, 12, 24, 31, 41, 49). HIV-1 RNA was isolated from blood plasma and CSF samples (140 μl), using a QIAmp viral RNA kit (Qiagen). All CSF samples were centrifuged at $1,000 \times g$ for 5 min prior to RNA isolation to remove contaminating cellular debris. To increase template number, samples with viral RNA levels less than 10,000 copies/ml were pelleted (0.5 to 1.0 ml) by centrifugation at $25,000 \times g$ for 1.5 h prior to RNA isolation. Reverse transcription and PCR amplification of the V1/V2 and V4/V5 regions of *env* were conducted with
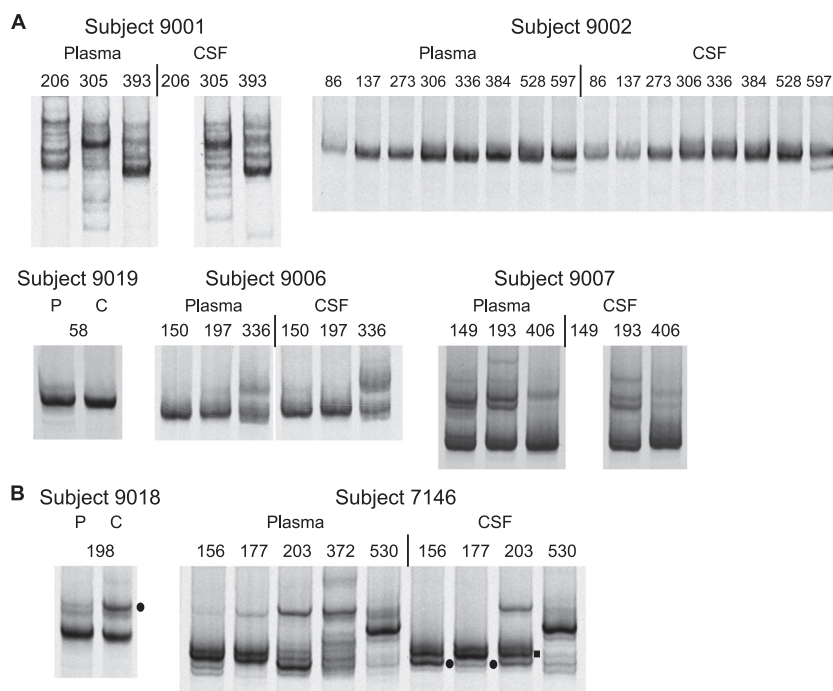
FIG. 1. Cross-sectional and longitudinal HTA analysis of HIV-1 in the blood plasma and CSF of primary infection subjects. (A) Longitudinal V1/V2 or V4/V5 HTA analysis of HIV-1 using paired blood plasma and CSF samples from 5 subjects without compartmentalized CSF variants. The HTA shown for subject 9007 targeted the V1/V2 region of *env*, and the V4/V5 HTA is shown for all other subjects. Sample time points are listed above each HTA gel as days p.i. The HTA gel images for the CSF viral population of subject 9001 at 206 days p.i. and subject 9007 at 149 days p.i. are not included because there was poor reproducibility between the HTA replicates due to low CSF viral loads. (B) Longitudinal V4/V5 HTA analysis of HIV-1 using paired blood and CSF samples from 2 subjects with compartmentalized CSF variants. CSF-compartmentalized variants are indicated by filled black circles next to the gel images. A variant enriched in the CSF is indicated with a filled black square next to the gel image.

5 µl of purified RNA (from a 60-µl-column elution volume), using a Qiagen one-step RT-PCR kit per the manufacturer's instructions and using previously described primers for V1/V2 (31, 49) and V4/V5 (49).

The heteroduplex annealing reactions have been previously described (31, 41). Heteroduplexes were separated by 6% native polyacrylamide gel electrophoresis for V1/V2 and V4/V5 HTA (24, 31). The HTA probes used in these studies included the V1/V2 Ba-L probe (31, 49), the V1/V2 JRFL probe (31, 49), the V4/V5 NL4-3 probe (24), and the V4/V5 YU2 probe (49). The HTA gels were dried under vacuum, and bands were visualized by autoradiography. The dried HTA gels were exposed to a PhosphorImager screen, and the relative abundance of each detected viral variant (heteroduplex) was calculated using ImageQuant software (Molecular Dynamics). The variant RNA concentration was calculated by multiplying the relative abundance of each individual variant by the total HIV-1 RNA concentration for that sample. Template sampling was validated by analyzing duplicate RT-PCR products by HTA for each sample. For any time point where the HTA patterns differed significantly between the two replicates (>20%), the samples were not used in data analysis. Percent difference values between plasma and CSF viral populations were calculated as previously described (31, 49).

Single-genome amplification. HIV-1 RNA was isolated as described above. Viral RNA was reverse transcribed using Superscript III reverse transcriptase (Invitrogen) and oligo(dT) according to the manufacturer's instructions. SGA of the full-length HIV-1 *env* gene through the 3′ U3 region was conducted as previously described (29, 44, 53). Briefly, cDNA was endpoint diluted, and nested PCR (15, 56) was conducted using Platinum *Taq* High Fidelity polymerase (Invitrogen) as described by Salazar-Gonzalez et al. (53). The primers B5853 UP0 (5′-TAGAGCCCTGGAAGCATCCAGGAAG-3′) and LTR DN1 (5′-GACTCTCGAGAAGCACTCAAGGCAAGCTTTATTGAG-3′) were used for the first round of PCR. The primers B5957 UP1 (5′-GATCAAGCTTTAGGCATCTCCTATGGCAGGAAGAAG-3′) and LTR DN1 were used for the second round of PCR. The SGA amplicons were then sequenced from the start of V1 through the ectodomain of gp41 (HIV HXB2 numbering of positions 6600 to 8000).

Phylogenetic and compartmentalization analyses. The nucleotide sequences of the *env* genes were aligned using Clustal W (4, 62) or MAFFT software (28). Maximum likelihood phylogenetic trees were generated using PhyML (22) with the following parameters: HKY85 nucleotide substitution model, four substitution rate categories, estimation of the transition/transversion rate ratio, estimation of the proportion of invariant sites, and estimation of the gamma distribution parameter (21). All sequences were subjected to quality control analysis to ensure that sequences from different subjects were not mislabeled.

Variants in the CSF were considered compartmentalized by the HTA if they were unique to the CSF or if they had a substantially higher copy number in the CSF than in the plasma. Compartmentalization of CSF viral populations by sequence was determined using the Slatkin-Maddison test for compartmentalization (57) implemented in the HyPhy software (47) and using 10,000 permutations.

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study have been deposited in GenBank under accession numbers GQ49667 to GQ497076 and GU206564 to GU206786.

RESULTS

Study population characteristics and inflammatory markers. Our study included 17 subjects enrolled in a neurological primary HIV-1 infection cohort for which we attempted to examine CNS compartmentalization during primary infection (Table 1 lists the clinical and laboratory characteristics). Eleven subjects had sufficient viral-RNA loads in both the plasma and CSF compartments for HTA and SGA analyses of the viral populations. All subjects in this study had higher HIV-1 RNA concentrations in the plasma than in the CSF, and a substantial proportion (35%; 6 of 17) of the subjects had
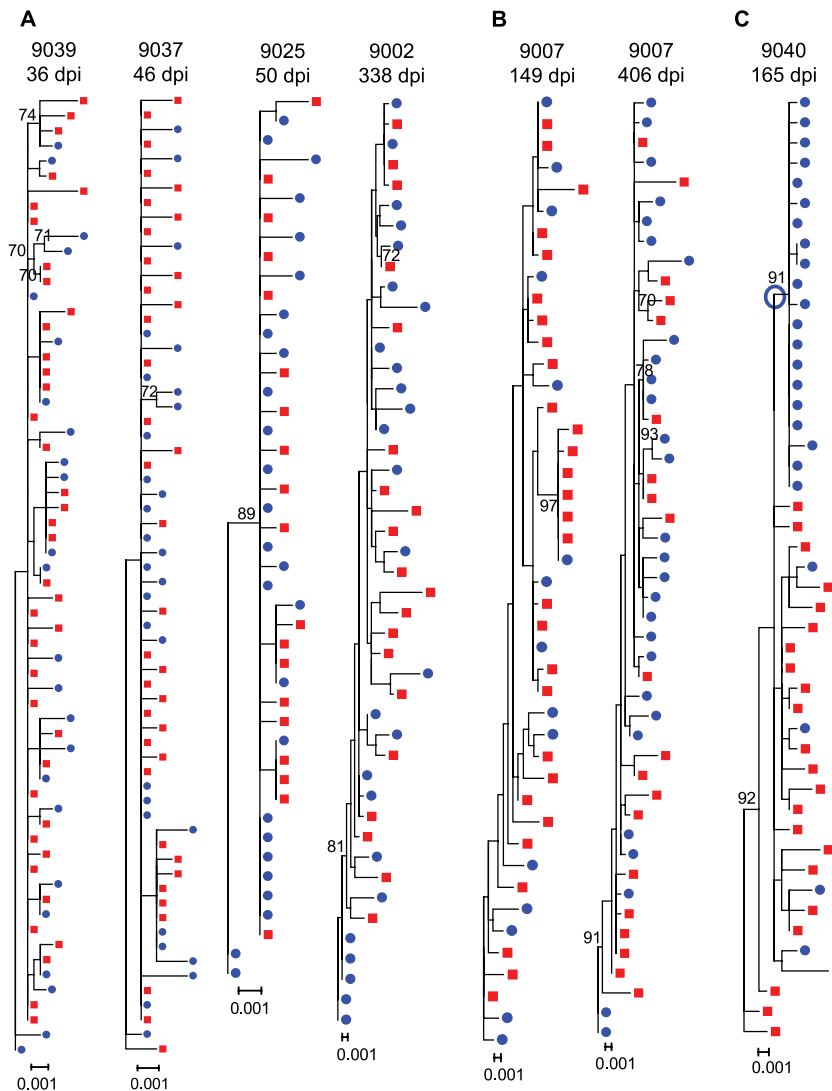
FIG. 2. Phylogenetic analysis of plasma and CSF HIV-1 populations. (A) Phylogenetic trees of HIV-1 *env* sequences for subjects 9002, 9025, 9037, and 9039, which display equilibration between blood plasma and CSF HIV-1 populations. (B) Phylogenetic trees of HIV-1 *env* sequences for subject 9007 at 149 days p.i. (dpi) and 406 days p.i. HIV-1 populations were equilibrated at 149 days p.i. but became slightly discordant at 406 days p.i. (C) Phylogenetic tree of HIV-1 *env* sequences for subject 9040, which displays significant compartmentalization in the CSF. The open blue circle indicates the node of divergence for the compartmentalized CSF sequences. SGA amplicons were first aligned, and a maximum likelihood phylogenetic tree was constructed using PhyML. Bootstrap numbers ≥70 are indicated at the appropriate nodes. Sequences obtained from the CSF are labeled with solid blue circles, and plasma sequences are labeled with solid red rectangles on the tree. The genetic distances between sequences are indicated by the distance scale bars at the bottoms of the trees.

extremely low CSF HIV-1 RNA concentrations at study entry (mean = 232 ± 197 copies/ml), in contrast to the other 11 subjects (mean = 22,986 ± 49,492 copies/ml). Viral population characteristics were not analyzed for six subjects with low CSF viral loads at entry (subjects 9003, 9010, 9012, 9014, 9016, and 9017).

We measured inflammation and cell migration into the CNS for each subject through several indirect analyses, including CSF/plasma albumin ratios, CSF white blood cell (WBC) counts, and CSF neopterin levels. The CSF/plasma albumin ratios were calculated as a measure of blood-brain barrier (BBB) disruption, while CSF WBC levels were measured as a simple marker of inflammation and inflammatory-cell migration into the CNS. Additionally, the level of CSF neopterin, a

pteridine produced by activated macrophages and associated with intrathecal immunoactivation, is elevated in HIV-infected subjects and is characteristically particularly high (>22 nmol/liter) in those with HIV-1-associated dementia (3, 17). All 17 subjects were enrolled within the first year of infection, with a median at baseline of 143 days p.i. (interquartile range, 58 to 165 days). The subjects were naïve to antiretroviral treatment at enrollment, and only one subject started treatment during the study intervals reported here (subject 7146, after 10 November 2003).

Based on the published normal values of CSF WBC (0 to 5 cells/µl) (16) and the CSF/albumin ratio (<6.8) (1), most subjects had mild to moderate CNS inflammation and blood-brain barrier disruption during this early stage of infection. Subject 7146 was

symptomatic at the time of enrollment, with clinical meningitis that was presumed to be secondary to HIV-1 seroconversion (extensive laboratory evaluations for standard bacteria, tuberculosis, spirochetes, and fungal organisms were negative), and had the highest CSF WBC level, CSF/plasma albumin ratio, and CSF neopterin level in the group (Table 1). All other subjects for whom HTA or SGA was performed were neurologically asymptomatic at study enrollment.

Compartmentalization of HIV-1 variants in the CSF during primary infection. The HTA is a useful tool for resolving and quantifying complex viral populations based on genotype and is able to detect minor variants within the viral population. We utilized HTAs targeting the hypervariable regions V1/V2 and V4/V5 of the *env* gene to detect and distinguish between compartmentalized and noncompartmentalized HIV-1 variants in the CSF and plasma of primary infection subjects. Cross-sectional HTA analyses were conducted on 7 primary infection subjects, and we were able to classify the relationship between the blood and CSF viral populations into two groups. Five subjects (9001, 9002, 9006, 9007, and 9019) (Fig. 1A) showed concordance (the difference between blood and CSF was <20%) between the blood plasma and CSF viral populations at the earliest time point sampled.

The second group (subjects 9018 and 7146) had evidence of compartmentalization when the blood and CSF viral populations at study entry were compared (Fig. 1B). In this small study, the two subjects with compartmentalized variants in the CSF had higher CSF viral loads than subjects with concordant populations in the plasma and CSF (Table 1). HIV-1 populations in the plasma and CSF were also discordant in subjects with CSF-compartmentalized variants as measured by percent difference (9018, 29.2% different at 198 days p.i.; 7146, 23.9% different at 156 days p.i.). In this group, subject 9018 had only mild CNS inflammation, while subject 7146 had evidence of significant inflammation in the CNS (Table 1). These results show that HIV-1 compartmentalization in the CSF can occur during primary infection and as early as 5 months after HIV-1 transmission.

Compartmentalization during primary HIV-1 infection can be transient. Longitudinal HTA analyses were conducted to examine compartmentalization in the CSF over the course of primary HIV-1 infection (subjects 9001, 9002, 9006, 9007, and 7146). HIV-1 populations in the plasma and CSF were concordant over time for subjects 9001, 9002, 9006, and 9007, with some minor variations (Table 1 lists percent differences). In subject 9001, viral populations in the plasma and CSF were concordant at each time point; however, the viral population underwent substantial divergence between time points, demonstrating equilibration between these compartments even as the virus was evolving in the periphery. Conversely, HTA analysis of subject 9002 revealed concordance between the plasma and CSF populations over time, with only a small amount of viral diversification detected at 597 days p.i. The results for subjects 9006 and 9007 are summarized in Fig. 1 and Table 1. We conclude that for these subjects most of the virus detected in the CSF was recently imported from the periphery and underwent little independent replication in the CNS.

Longitudinal HTA analysis of subject 7146 revealed one viral variant that was compartmentalized in the CSF at 156 and 177 days p.i., although by 177 days p.i. this variant comprised a

TABLE 2. Single genome amplification results

Subject ID	Sample date (mo/day/yr)	Days p.i.[a]	No. of plasma amplicons	No. of CSF amplicons	CSF compartment[b]
9002	10/26/06	338	20	26	Eq
9007	9/18/06	149	29	15	Eq
	6/5/07	406	20	28	Uneq
9018	8/2/07	198	29	22	Comp
9025	4/21/08	50	19	27	Eq
9037	5/14/09	46	37	29	Eq
9039	6/1/09	36	40	24	Eq
9040	6/15/09	165	23	24	Comp
7146	9/23/03	156	22	27	Comp, Amp
	10/14/03	177	36	29	Eq
	11/10/03	203	26	26	Comp, Amp
	10/7/04	530	33	22	Eq

[a] Estimated.
[b] HIV-1 population characteristics in the CSF compartment. Eq, equilibration with the blood plasma; Unequ, discordance between the CSF and blood plasma without substantial compartmentalization; Comp, compartmentalization in the CSF; Amp, clonal amplification of variants detected in the CSF.

small minority of the total CSF viral population. We also noticed that a new viral variant was significantly enriched in the CSF viral population at day 203 postinfection (Fig. 1B). However, the plasma and CSF viral populations had equilibrated again by 530 days p.i. At that time point, the CSF variant that was enriched at 203 days p.i. still seemed to be slightly enriched in the CSF compared to the plasma population, although the difference was minor. These results indicate that when compartmentalization occurs during primary HIV-1 infection, it can be transient, and the CSF viral population can change substantially over the course of primary HIV-1 infection.

Single genome amplification confirms compartmentalization and identifies clonal amplification of HIV-1 variants in the CSF of primary infection subjects. We examined the genetically compartmentalized viral variants in more detail by conducting SGA on plasma and CSF samples from subjects with detectable compartmentalization in the CSF by HTA (subjects 9018 and 7146). We also conducted SGA for two subjects (9002 and 9007) with HIV-1 populations equilibrated between the plasma and CSF and on 4 additional subjects who were not analyzed by the HTA (9025, 9037, 9039, and 9040) (Fig. 2 and Table 2). The structure of the phylogenetic trees and limited *env* sequence diversity suggest that subjects 9002, 9007, 9025, 9037, and 9039 were each infected with a single HIV-1 variant during transmission. We found that the plasma and CSF *env* sequences for subjects 9002, 9025, 9037, and 9039 mixed together throughout their respective phylogenetic trees, indicating that the two compartments were well equilibrated for each subject (Fig. 2A). In addition, the CSF *env* sequences for these subjects were not considered compartmentalized using the Slatkin-Maddison test (9002, $P = 0.56$; 9025, $P = 0.84$; 9037, $P = 0.057$; 9039, $P = 0.97$). Subject 9007 had plasma and CSF HIV-1 populations that were well equilibrated at 149 days p.i. (Slatkin-Maddison test; $P = 0.6928$), although the viral populations were somewhat discordant 406 days p.i. (Fig. 2B) (Slatkin-Maddison test; $P = 0.0397$). These data confirm the HTA analysis showing that the plasma and CSF can be fully equilibrated.

SGA was also conducted on the cross-sectional plasma and CSF samples for subject 9018, which had one compartmental-
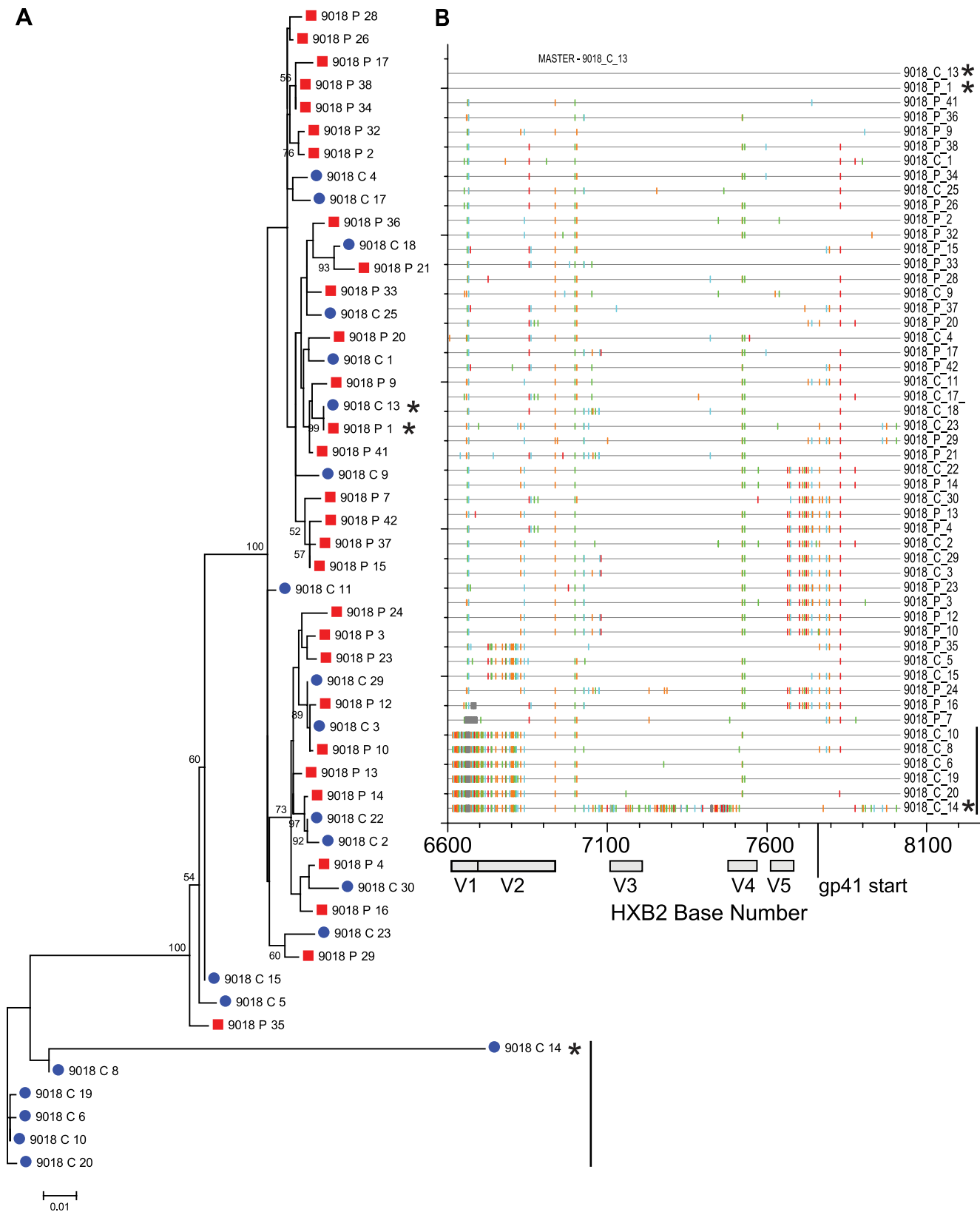
FIG. 3. Phylogenetic and sequence analyses of plasma and CSF HIV-1 populations at 198 days p.i. for subject 9018. (A) Maximum likelihood phylogenetic tree. Sequences from the CSF (C) are labeled with solid blue circles, and plasma sequences (P) are labeled with solid red rectangles. Genetic distances between sequences are indicated by the scale bars located at the bottom of the tree. Bootstrap values of >50 are labeled at the appropriate nodes. Putative transmitted viruses are labeled with asterisks, and CSF populations that were considered compartmentalized based on the phylogenetic analysis are indicated with a solid black line. (B) Highlighter plot of aligned *env* plasma and CSF sequences. The HXB2 base number is indicated on the *x* axis, and the sequence identifier is indicated on the *y* axis. Base changes are indicated by the following ticks on the highlighter plot: A, green; T, red; G, orange; C, light blue; and gaps, gray.
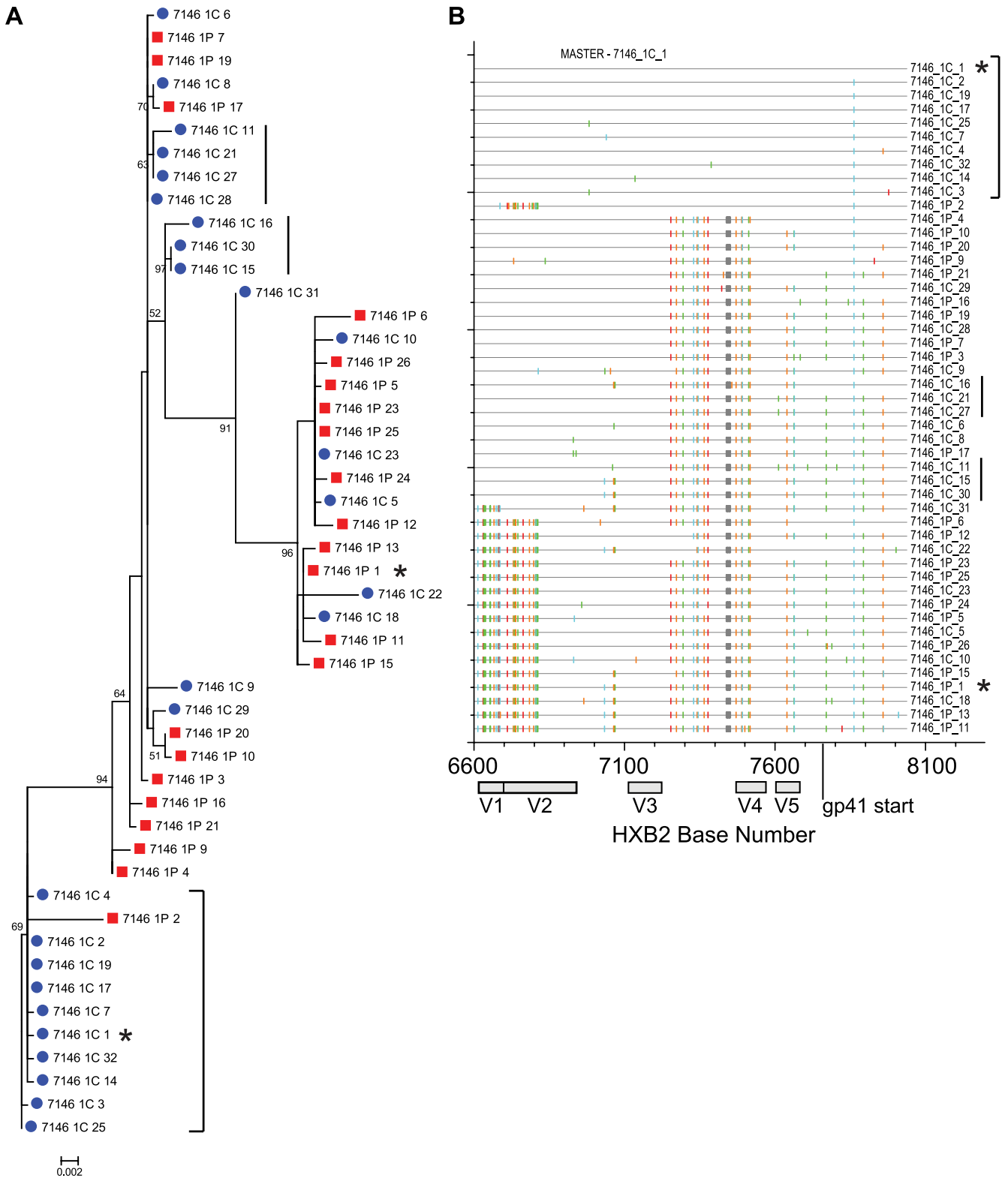
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FIG. 4. Phylogenetic and sequence analyses of subject 7146 HIV-1 populations in the plasma and CSF at 156 days p.i. (A) Maximum likelihood phylogenetic tree. Sequences from the CSF (C) are labeled with solid blue circles, and plasma sequences (P) are labeled with solid red rectangles. Bootstrap values of >50 are labeled at the appropriate nodes. Genetic distances between sequences are indicated by the scale bar located at the bottom of the tree. The clonally amplified HIV-1 variants in the CSF population are indicated by the black bracket. Putative transmitted viruses are labeled with asterisks, and CSF populations that were compartmentalized are indicated with solid black lines. (B) Highlighter plot of aligned plasma and CSF *env* sequences. The HXB2 base number is indicated on the *x* axis, and the sequence identifier is indicated on the *y* axis. Base changes are indicated by the following ticks on the highlighter plot: A, green; T, red; G, orange; C, light blue; and gaps, gray.

A

B
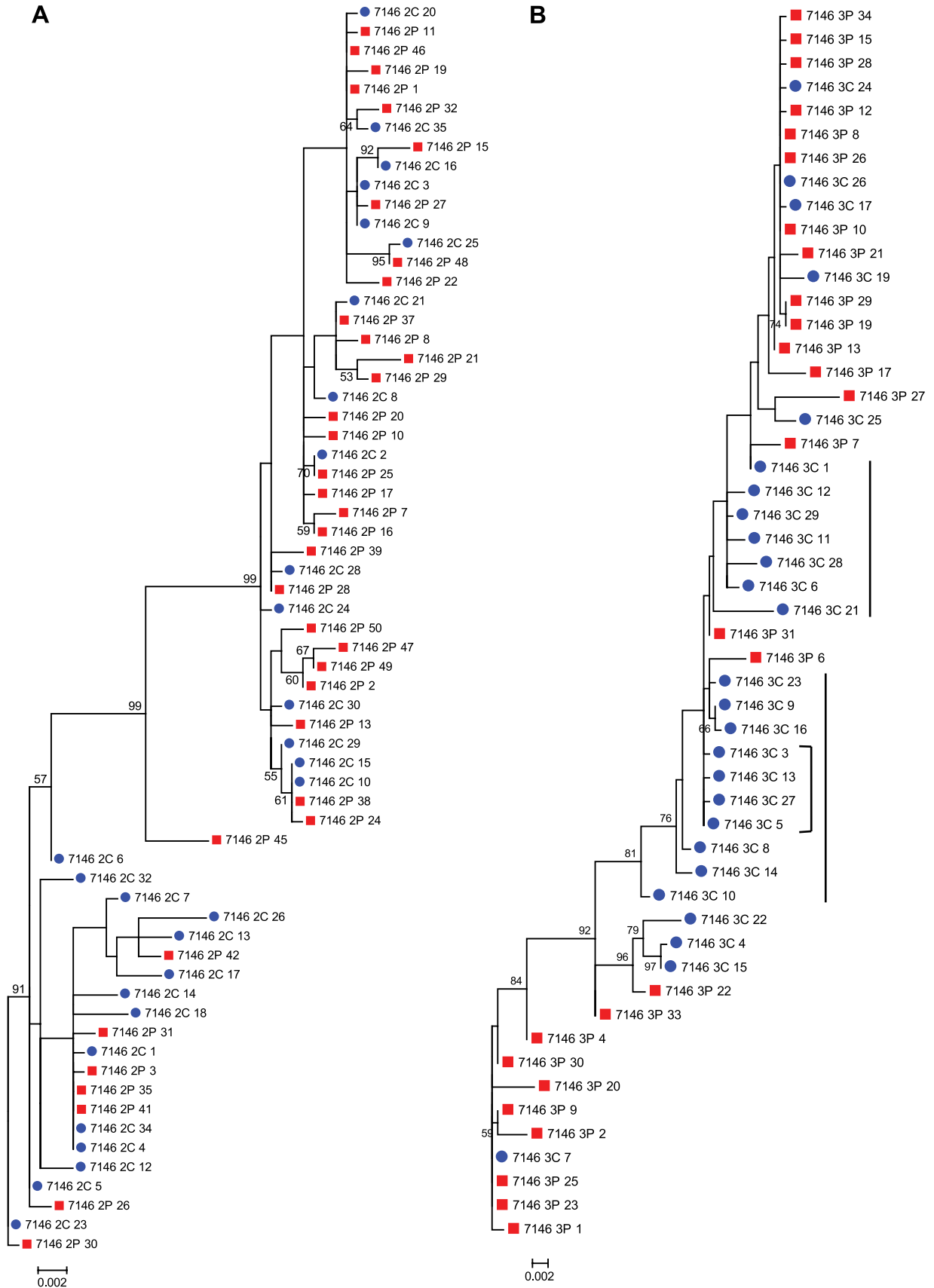
ized variant detected by HTA, and subject 9040. We detected significant compartmentalization between the blood plasma and CSF HIV-1 populations for subject 9040 (Fig. 2C) (Slatkin-Maddison test; $P < 0.0001$), and further sequence analysis demonstrated that all of the compartmentalized variants encoded a common V169R amino acid substitution in the V1/V2 stem region of the Env protein. Similarly, phylogenetic analysis of subject 9018 revealed a subset of *env* sequences derived from the CSF that branched separately from the rest of the sequences in the tree (Fig. 3A). In addition, the compartmentalized population in the CSF had a unique Q170R amino acid substitution in the V1/V2 stem of the envelope. However, the CSF viral population was not considered compartmentalized by the Slatkin-Maddison test ($P = 0.1078$). Even though the entire CSF viral population was not considered compartmentalized, a small HIV-1 population in the CSF appeared to be compartmentalized based on the phylogenetic tree structure and good bootstrap support for this node (Fig. 3A).

Based on the sequence and phylogenetic data, subject 9018 was likely infected with at least two viral variants during transmission. The two sequences that are likely closest to the parental viruses are 9018 C 13/9018 P 1 and 9018 C 14 (Fig. 3B), and recombination between the transmitted viruses appears to account for much of the *env* genetic diversity detected in both the plasma and CSF populations. This interpretation also leads to the conclusion that one of the putative transmitted sequences was underrepresented in the periphery and maintained in the CNS.

Longitudinal SGA analysis of subject 7146 revealed transient compartmentalization and clonal amplification in the CSF HIV-1 population. Based on the phylogenetic tree structure and *env* sequence diversity at the first time point sampled (156 days p.i.), we believe that subject 7146 was infected with two HIV-1 variants during the transmission event (Fig. 4A). The viral variants that appear to be most closely related to the parental viruses are 7146 1P 1 and 7146 1C 1 (Fig. 4B), and extensive recombination between the two transmitted viruses can account for most of the variants. Similar to what we detected in the phylogenetic analysis of subject 9018, one of the transmitted variants was maintained in the CSF but largely absent from the plasma viral population.

Phylogenetic analysis of the first time point for subject 7146 (156 days p.i.) revealed compartmentalization between the plasma and CSF viral populations (Fig. 4A), and this was associated with increased inflammation in the CNS (Table 1). In particular, one HIV-1 variant was clonally amplified in the CSF population (Fig. 4A, bracket). Compartmentalization was confirmed using the Slatkin-Maddison test ($P = 0.0047$), and the CSF population was still considered compartmentalized when the clonally amplified viral variants were removed from the analysis ($P = 0.0437$). The plasma and CSF populations in subject 7146 were equilibrated by 177 days p.i. (Fig. 5A) (Slat-

kin-Maddison test; $P = 0.2964$), coincident with elevated measures of BBB disruption (Table 1). The CSF viral population was considered compartmentalized again at 203 days p.i. based on phylogenetic analysis (Fig. 5B) (Slatkin-Maddison test; $P = 0.0048$) and in the setting of a reduced CSF/plasma albumin ratio. We also detected a small cluster of clonally amplified sequences in the CSF-compartmentalized population at 203 days p.i., although the viral populations were equilibrated again by 530 days p.i. (data not shown) (Slatkin-Maddison test; $P = 0.877$).

A phylogenetic tree containing representative data from all four time points sampled for subject 7146 revealed that the clonally amplified variants in the CSF at 156 days p.i. had died out of the CSF population by 177 days p.i. and did not give rise to any additional CSF viral variants (Fig. 6, bracket). In addition, HIV-1 variants in the CSF that were compartmentalized at 203 days p.i. (Fig. 6, arrows) arose from one main branch in the phylogenetic tree, and these variants were lost by 530 days p.i.. Clonally amplified variants detected in the CSF at 203 days p.i. were also lost from the viral population by 530 days p.i. We noticed that CSF sequences compartmentalized at 203 days p.i. branched with sequences detected in the CSF at 156 and 177 days p.i., even though no compartmentalized variants were detected in the CSF at 177 days p.i. This indicates that CSF variants detected at entry were maintained over the course of several months in the CNS of this subject. These data suggest that compartmentalization during primary infection can be resolved and recur later during infection. Additionally, transient clonal amplification can occur in the CSF-compartmentalized viral population.

DISCUSSION

Extensive HIV-1 genetic compartmentalization between the periphery and the CNS has been reported in subjects with HAD (13, 14, 26, 43, 46, 48, 51, 52, 59). Compartmentalized viral variants present in the CSF of HAD subjects are thought to originate from long-lived cells in the CNS (54) and are maintained in the population by independent viral replication in the brain. The time at which compartmentalization occurs during the course of HIV-1 infection has been a subject of debate, and the absence of CSF-compartmentalized variants during primary infection has supported the idea that compartmentalization occurs later during chronic infection (26, 51). The goal of our current work was to determine whether compartmentalized HIV-1 variants are present in the CSF of primary infection subjects and to examine the evolution of CSF variants over the course of early HIV-1 infection.

In this study, we used heteroduplex tracking assays (HTAs) and single-genome amplification (SGA) to identify CSF-compartmentalized variants and to examine the compartmentalized viral populations. Cross-sectional HTA analysis revealed

FIG. 5. Phylogenetic analysis of subject 7146 HIV-1 populations at days 177 and 203 postinfection. (A) Phylogenetic tree of plasma and CSF HIV-1 *env* sequences at 177 days p.i. (B) Phylogenetic tree of HIV-1 populations at 203 days p.i. Sequences from the CSF (C) are labeled with solid blue circles, and plasma sequences (P) are labeled with solid red rectangles. The CSF sequences that were considered compartmentalized are indicated by the solid black lines. Clonally amplified HIV-1 variants in the CSF population are indicated by the black bracket. Bootstrap values of >50 are labeled at the appropriate nodes. Genetic distances between sequences are indicated by the scale bars located at the bottoms of the trees.
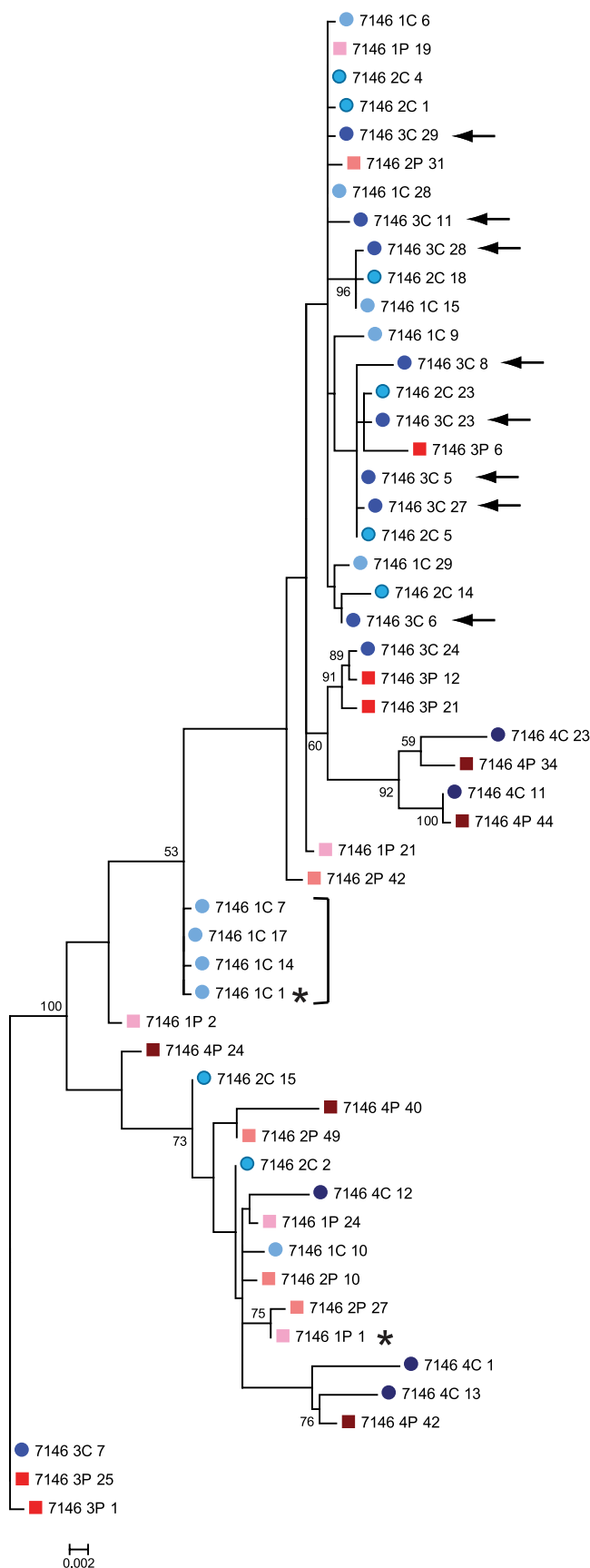
several examples of compartmentalized HIV-1 variants in the CSF early during HIV-1 infection. We also observed that compartmentalization during primary HIV-1 infection could be transient, and the compartmentalized variants in the CSF changed over time. Our SGA analyses confirmed the detection of compartmentalized variants in the CSF and identified clonal amplification of CSF variants in one subject with primary infection (subject 7146).

A proportion of the primary infection subjects enrolled in our study had low baseline CSF viral loads, even though the plasma viral loads were high. One explanation is that plasma virus is not efficiently entering the CNS in these subjects, while another possibility is that HIV-1 that enters the CSF/CNS is being rapidly cleared. In either case, our data suggest that substantial autonomous viral replication is not occurring in the CNS of this subset of primary infection subjects. This is consistent with our finding that the majority of primary infection subjects in our study had concordant viral populations between the plasma and CSF compartments. The detection of equilibrated viral populations in the CSF early during HIV-1 infection indicates that the CSF compartment is accessible to most of the peripheral HIV-1 variants and supports an absence of sustained viral replication in the CNS, although independent viral replication in the CNS may be occurring at a level below our limit of detection.

Three of the primary infection subjects that we analyzed had compartmentalized HIV-1 variants present in their CSF viral populations, indicating that local viral replication can occur in the CNS at this stage. Subjects 9018 and 7146 had a multiple-variant transmission event based on our sequence and phylogenetic data, where one HIV-1 lineage was detected in both the plasma and CSF while a second transmitted virus lineage was detected only in the CSF virus population (Fig. 3 and 4). These data suggest that some CSF variants have properties that permit enhanced replication in the CNS, even early after viral transmission.

Phylogenetic and sequence analysis of subject 9040 revealed a V169R single-amino-acid substitution in the V1/V2 stem region of Env for CSF variants that were compartmentalized, and this change was not detected in any of the plasma *env* sequences. The compartmentalized HIV-1 variants in the CSF of subject 9018 had a Q170R substitution similar to a basic amino acid in the same region of the Env protein, and HIV-1 variants in the plasma and CSF of 7146 had an R/K170 genotype. This suggests that perhaps basic amino acid substitutions in the V1/V2 stem of Env enhance viral replication in the CNS

FIG. 6. Longitudinal phylogenetic analysis of subject 7146 HIV-1 populations. Shown is a phylogenetic tree of a representative set of plasma and CSF HIV-1 *env* sequences from 156 days p.i. (1P, light-pink square; 1C, light-blue circle), 177 days p.i. (2P, dark-pink square; 2C, bright-blue circle with dark outline), 203 days p.i. (3P, bright-red square; 3C, royal blue circle), and 530 days p.i. (4P, dark-red-brown square; 4C, navy blue circle). CSF sequences that were clonally amplified at 156 days p.i. are indicated by the black bracket, and CSF sequences that were considered compartmentalized at 203 days p.i. are indicated with arrows. Bootstrap values of >50 are labeled at the appropriate nodes. Genetic distances between sequences are indicated by the scale bar located at the bottom of the tree.

environment. One possibility is that additional basic amino acids in the V1/V2 stem could enhance the binding and entry of these viral variants into cells with low receptor densities on the surface, such as perivascular macrophages and microglia in the CNS. However, basic amino acid substitutions in the V1/V2 stem do not completely explain compartmentalization during primary infection, since several subjects without compartmentalized variants in the CSF had additional basic amino acid substitutions within their plasma and CSF HIV-1 populations (data not shown).

Studies examining viral populations in the CSF of primary HIV-1 infection subjects have been limited; however, a small study by Harrington et al. (23) examined the population dynamics of simian immunodeficiency virus SIVsm strain E660 in the blood and CSF of macaques over the course of infection. A comparison of the blood and CSF viral populations revealed two distinct patterns of evolution: viral genetic concordance between the blood and CSF populations in two macaques and discordance in the blood/CSF populations in a third macaque (23). Notably, the blood/CSF population discordance that was detected in animal C002 arose early after transmission (day 27) and persisted over the course of infection (23). Similarly, we detected concordance between the blood plasma and CSF viral populations for eight of the primary infection subjects in our study. Three subjects in our study showed discordance and compartmentalization in the CSF viral population, and this disturbance was apparent early after transmission (5 months). Additionally, Harrington et al. (23) reported increased levels of CSF monocyte chemoattractant protein 1 (MCP-1) and statistically significant numbers of infiltrating CD68$^+$ macrophages in brain sections of macaque C002 compared to other animals in the study (23). Human studies have also shown that the levels of chemokines, such as gamma interferon-inducible protein 10 (IP-10) and MCP-1, are elevated in the CSF of HAD subjects compared to asymptomatic subjects (5, 6, 30, 55). Although our measures of CNS inflammation differ from those in the macaque study, we detected elevated levels of CSF neopterin and pleocytosis in one subject with moderate CSF compartmentalization.

One easily measured marker of inflammation in the CNS is pleocytosis, defined as CSF WBC counts > 5 cells/μl (38, 58). Although pleocytosis can occur at any stage of neurological disease, during chronic infection, increased CSF WBC counts are correlated with CSF HIV-1 RNA concentrations (39, 58). Subject 7146 had elevations in CSF WBC, as well as CSF neopterin and the albumin ratio, prior to initiation of antiretroviral therapy (Table 1). We believe this inflammatory environment in the CNS resulted from both independent HIV-1 replication and the correlated meningitis detected at entry in subject 7146. The detection of BBB disruption also suggests an increased ability of lymphocytes to traverse the BBB and move into the CNS. Subjects 9018 and 9040 also had evidence of compartmentalization of CSF variants; however, CSF WBC counts, CSF neopterin levels, and albumin ratios were not distinct from those in subjects without compartmentalization. Thus, marked inflammation at a given time point is not necessary for the production or maintenance of compartmentalized variants.

One specific viral lineage in subject 7146 was clonally amplified in the CSF viral population at 156 days p.i., and our longitudinal SGA analysis determined that this viral lineage died out in both the CSF and plasma populations by 177 days p.i. We believe that an influx of inflammatory cells may have resulted in the clonal amplification of specific CSF viral variants at entry. Monocytes and CD4$^+$ T cells migrating into the CNS would provide a larger target cell population for HIV-1 infection and might stimulate increased HIV-1 replication in the CNS. A small population of clonally amplified variants was also detected in the CSF at 203 days p.i., when CSF pleocytosis was still apparent. These data suggest that independent HIV-1 replication may occur in the CNS of primary infection subjects with detectable compartmentalized variants, and this viral replication may cause increased inflammation in the CNS that can result in the clonal expansion of CSF variants.

In conclusion, our study illustrates a complex pattern of HIV-1 evolution in the CNS during primary/early infection. The majority of subjects in the primary stage of HIV-1 infection have HIV-1 populations equilibrated between the blood plasma and CSF; however, we detected compartmentalized HIV-1 variants in the CSF populations of three primary infection subjects. Two subjects with compartmentalized variants in the CSF were asymptomatic and did not show elevated levels of inflammatory markers in the CSF, suggesting that compartmentalized virus in the CSF/CNS can occur during primary HIV-1 infection in the absence of overt neurological symptoms. Additionally, sequence data from *env* genes obtained from subject 9040 (165 days p.i.) suggest that HIV-1 can rapidly adapt to enhance viral entry and growth in the context of the CNS environment. Early detection of CSF-compartmentalized variants may identify subjects that will have HIV-associated neurological problems later during infection, since similar compartmentalization late in infection likely indicates a loss of control of viral replication in the periphery and is associated with HAD (reference 26 and unpublished observation). Our results demonstrate that compartmentalization in the CNS compartment can arise shortly after HIV-1 transmission and suggest that subjects with compartmentalization of viral variants in the CSF during early infection should be considered for early antiretroviral therapy to reduce viral replication in the CNS.

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