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In Vivo Sequence Variability of Human Immunodeficiency Virus Type 1 Envelope gp120: Association of V2 Extension with Slow Disease Progression

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According to the rate of depletion of CD4 cell counts, we grouped 12 cases of human immunodeficiency virus type 1 (HIV-1) infection as 6 rapid (21.0 to 33.8 cells per μl per month) and 6 slow (0.9 to 7.9 cells per μl per month) progressors and determined the individual viral quasispecies patterns by sequencing the genome region encoding the V1, V2, and V3 loops of envelope protein. Although the quasispecies structures varied widely from one individual to another, a strong correlation was observed between a low rate of disease progression and a high degree of genetic diversity of HIV-1. Furthermore, the V2 loop extension was observed specifically in individuals with slow or no disease progression, whereas basic amino acid substitutions in V3 characteristic of a viral phenotype shift from non-syncytium inducing to syncytium inducing were observed in patients with advanced stages of disease regardless of their rate of disease progression. Studies with recombinant viruses suggested that elongation of V2 potentially restricts the capacity of HIV-1 to replicate in macrophages. Thus, our results suggest the association of distinct sequence features of both V3 and V2 with particular patterns of disease progression. Elongation of the V2 loop may be a good predictor of slow disease progression, while basic substitutions of V3 without elongation of V2 are characteristic of rapid progression.

Human immunodeficiency virus type 1 (HIV-1) infection is generally characterized by a long-term, chronic disease course gradually progressing to AIDS (7, 24, 29). However, there are a few strikingly different scenarios. A small fraction of persons with HIV-1 infections remain normal both clinically and immunologically over 10 years or more after seroconversion (18, 22). Conversely, another marked fraction features an extremely rapid disease progression taking place even within 1 year (33). Determining the factors of these different disease courses would be extremely helpful to better understand and control AIDS, and thus they have been under extensive investigation. So far, involvement of the humoral and cellular immune responses (2, 57), specific virus-suppressing chemokines (37), genetic defect of the coreceptor CCR-5 for HIV-1 entry (8, 23, 43), and pathogenicity of the virus itself (1, 3, 5, 48, 49) has been suggested, but no pattern has been firmly established.

Rapid and continuous virus reproduction is commonly seen even in the clinically asymptomatic phase (10, 16, 36, 53). HIV-1 reverse transcriptase is highly error prone due to its lack of error correction mechanisms (13, 39, 41). Therefore, HIV-1 circulates in vivo as a mixture of heterologous populations termed quasispecies (28, 47), which include a variety of conditionally lethal or conditionally surviving variants and whose structure is thus subject to continuous changes responding to intrahost conditions. These arising in vivo variants sometimes display distinct phenotypes in cell cultures, and their significance in in vivo pathogenicity has been discussed elsewhere (1, 3, 5, 43, 48, 49). For instance, basic amino acid substitutions at the 11th and/or 25th position of V3 (the third hypervariable region) in gp120 of subtype B HIV-1 appeared to be important for changing the phenotype from non-syncytium inducing (NSI) to syncytium inducing (SI) (9, 12). The same amino acid substitutions may further determine whether the virus can infect macrophages or T-cell lines in vitro (4, 17, 46, 54) and are sometimes seen shortly before the decline of $CD4^+$ cell numbers (5, 21, 40). The sequence changes in the first and second hypervariable loops (V1 and V2) also affect the viral SI property and cellular host range (14, 19, 20, 51). However, more-systematic studies are required to establish the above correlation and to learn the significance of the sequence changes in disease progression.

In this study, we analyzed the quasispecies structures of V1, V2, and V3 as well as one constant region (C2) for 12 infected individuals in relation to their disease stages and disease progression rates. We show a marked correlation between particular sequence changes and disease progression, including close association of V2 elongation due to a sequence duplication with a low rate of disease progression, as well as an association of basic amino acid substitutions of V3 but no V2 elongation with rapid disease progression.

MATERIALS AND METHODS

Patients. We studied 12 HIV-positive individuals who were grouped according to their disease progression rates. Figure 1A shows data for six subjects who manifested rapid disease progression. For subjects 1, 2, 28, and 43, $CD4^+$ cell counts rapidly decreased below 200/μl within 2 years after infection through sexual transmission. Disease progression was extremely rapid in case 1, resulting in death within 8 months after infection, and details were described previously (33, 47). Subjects 8 and 9 were infected through HIV-contaminated blood products. Since contaminated blood products had been distributed widely in Japan between 1983 and 1985, their infections were assumed to have been contracted in this period. Until 1989 or 1990, their $CD4^+$ cell counts were satisfactorily high, but then they suddenly decreased to less than 200/μl in 1990. The plasma viral RNA load was generally high in all six cases. Figure 1B shows data for six cases which manifested a low progression rate. Subject 20 was infected through sexual transmission, while the other five individuals were infected through blood prod-

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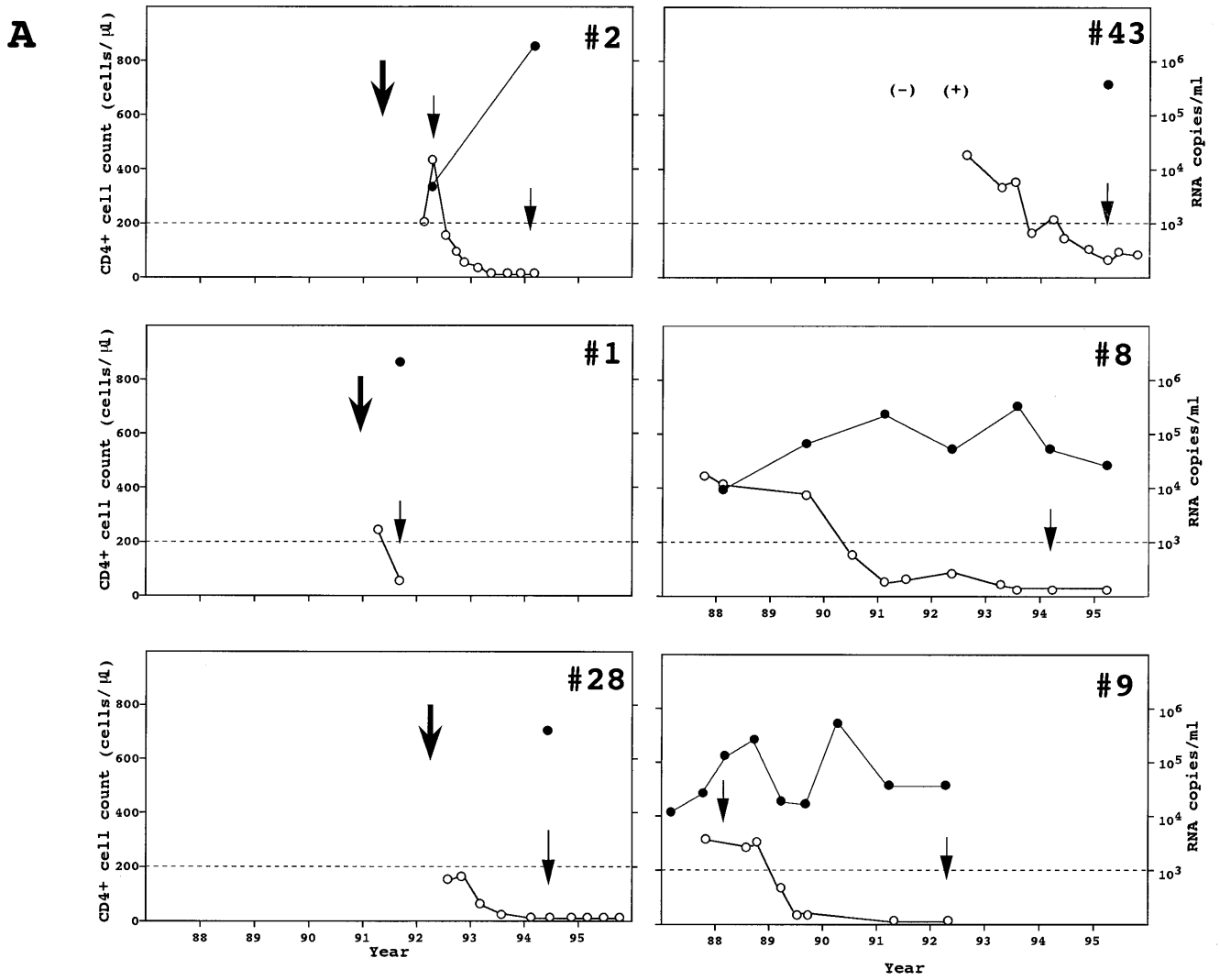

FIG. 1. Levels of $CD4^+$ cell counts (open circles) and HIV-1 RNA copy numbers (closed circles) during the disease course of HIV-1-infected individuals. (A) Results for six rapid progressors. (B) Results for four slow progressors (subjects 20, 47, 15, and 65) and two nonprogressors (subjects 19 and 63). The times of primary HIV-1 infection estimated from the onset of infectious mononucleosis-like symptoms (thick arrows), positive and negative seroreactivity [(+) and (−), respectively] to HIV-1 proteins as determined by immunoblot assay (Bio-Rad Laboratories), and sampling times for sequence determination (thin arrows), and $CD4^+$ cell counts of 200/μl (dashed lines) are indicated.

ucts. Subjects 19 and 63 showed virtually no CD4 depletion for more than 10 years and appear to fall into the category of nonprogressor. Although detailed clinical data for subject 15 before 1991 and for subject 65 before 1993 were not available, sera of both patients collected in 1987 showed positive seroreactivity to HIV-1 proteins by immunoblot assay (Bio-Rad Laboratories, Richmond, Calif.), confirming that the infections occurred before 1987. None of these slow progressors received antiviral drugs before 1994, and none of the nonprogressors have received antiviral drugs to date.

Viral RNA load in plasma. Copy numbers of HIV-1 RNA in plasma were quantitated with an Amplicor HIV Monitor test kit (Roche Diagnostic Systems, Somerville, N.J.) (30).

PCR, cloning, and sequencing. Total RNA was extracted from 50 μl of plasma or cell-free virus isolates and subjected to reverse transcription by using primer MK601 (5′-TTCTCCAATTGTCCCTCATATCTCCTCCTCCA-3′) according to a protocol described previously (33, 47). DNA fragments of 700 to 800 bp corresponding to the V1 to V3 loops of gp120 were amplified by nested PCR. The outer primers used were MK601 and E1 (5′-GGTAGAACAGATGCATG AGGAT-3′), and the inner primers were YT001 (5′-ACAATTTCTGGGTCCC CTCCTGAGGA-3′) and E2 (5′-ATCAGTTTATGGGATCAAAGCC-3′). To avoid sequencing multiple clones derived from a single viral RNA, 10 to 15 independent PCRs were simultaneously performed. The resultant PCR products were cloned into PCRII vector, and nucleotide sequences of single clones derived from each PCR product were determined with an automated DNA sequencer, ALFII (Pharmacia, Uppsala, Sweden). We used fluorescein isothiocyanate-labeled YT001 and E2 primers to determine the sequences of both strands.

Phylogenetic tree analyses. Nucleotide and amino acid sequences were edited and translated by SDC-GENETYX computer programs (Software Development, Tokyo, Japan). Nucleotide sequences were aligned by using CLUSTAL W, version 1.4 (50), and corrected to ensure that gaps did not alter the reading frame. Insertions and deletions were not counted. The nucleotide substitutions among each clone were estimated by Kimura's two-parameter method, and phylogenetic trees were constructed from the distance matrix by the neighbor-joining method (42). These procedures were computed by using PHYLIP, version 3.572 (11), and the phylogenetic trees were drawn with TREEVIEW, version 1.3 (35). Strain SF162 was used as an out-group. The reliability and topology of each tree branch were tested by bootstrap analysis (15) with 100 replicates.

Calculation of rate of nucleotide substitutions and MD. The numbers of synonymous and nonsynonymous substitutions relative to the consensus sequence at the first time point were calculated for each clone by a method described previously (31). The mean diversity (MD) of HIV-1 quasispecies in infected individuals was expressed as a percentage of mismatched bases and was calculated by pairwise calculation of all matched and mismatched nucleotides. Insertions and deletions were not counted.

Generation of recombinant viruses. A 136-bp *Hph*I-*Stu*I fragment of the infectious proviral DNA of $HIV\text{-}1_{SF162}$ was replaced with the corresponding fragment derived from plasma samples collected in 1988 (1988 plasma) or a 1994

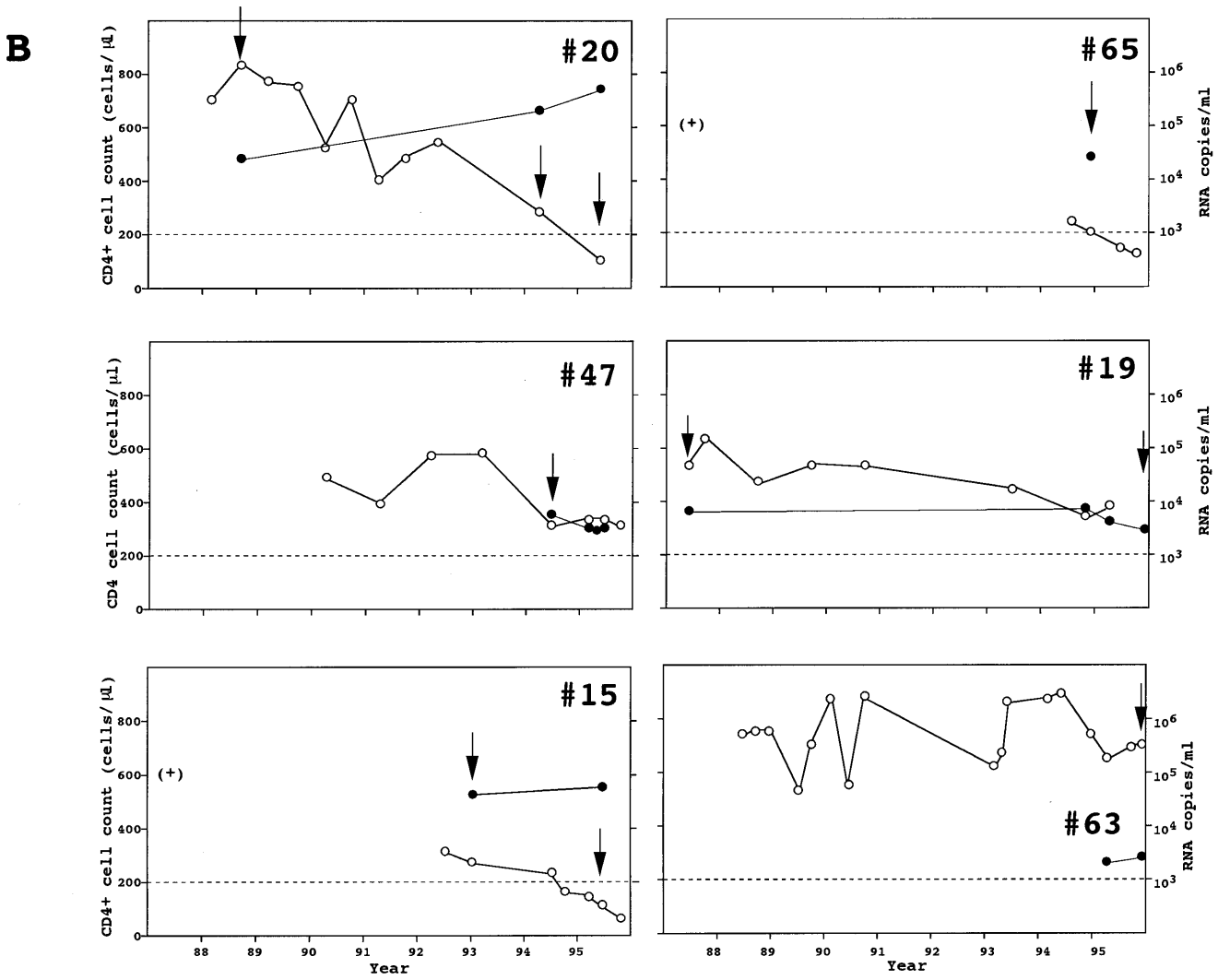

FIG. 1—*Continued.*

virus isolate from subject 20 (see Fig. 7A). Recombinant viruses were recovered by transfection of the resultant recombinant proviral DNAs into RD4 cells followed by cocultivation with phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMCs) from seronegative individuals. Culture supernatants were used as virus stocks for further investigations. Viruses carrying the V2 loop from 1988 plasma and the 1994 virus isolate were designated SV2/SF162 and LV2/SF162 (for short and long), respectively.

Cell culture. Hut78 and MT4 T-cell lines were grown in RPMI 1640 medium supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS). Human rhabdomyosarcoma (RD4) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS. PBMCs were obtained from healthy seronegative donors. The cells were stimulated with PHA (3 μg/ml) for 3 days before use and were then maintained in RPMI 1640 medium supplemented with 10% FBS and 50 U of interleukin 2 (Genzyme Corp., Cambridge, Mass.) per ml. Primary peripheral blood monocytes were obtained from PBMCs by the plastic adherence technique (3) and maintained in RPMI 1640 medium supplemented with 10% FBS and 5% heat-inactivated human serum to allow their differentiation into macrophages.

HIV-1 infection. PHA-stimulated PBMCs, peripheral blood macrophages, and T-cell lines were treated with Polybrene (2 μg/ml) for 30 min at 37°C and exposed to 1,000 50% tissue culture infective doses of virus for 2 h at 37°C. The cells were washed twice with RPMI 1640 medium and maintained in the culture medium for each cell type as described above. Culture supernatants of the infected cells were assayed for reverse transcriptase activity at intervals of 3 to 4 days (55). Data points are the means of duplicate cultures.

Primary HIV-1 isolation. Approximately 3×10^5 PBMCs obtained from HIV-1-infected individuals were cocultivated with 10^6 PHA-stimulated PBMCs from a healthy seronegative donor. Culture supernatants of the cells were assayed for levels of p24 core antigen by enzyme-linked immunosorbent assay (Abbott, Wiesbaden-Delkenheim, Germany) at intervals of 3 to 4 days.

Nucleotide sequence accession numbers. The viral sequences have been submitted to DDBJ under accession no. AB002829 through AB003019.

RESULTS

Sequence changes of HIV-1 in a slow progressor, subject 20. Figure 2A shows the predicted amino acid sequences of the V1, V2, and V3 loops obtained from plasma of a slow progressor, subject 20. In August 1988, when CD4 counts were over 600/μl and the HIV-1 RNA load in plasma was 2.2×10^4 copies/ml, the V3 sequences were highly homogeneous, with an MD of only 0.52%. They all possessed serine and glutamic acid residues at the 11th and 25th positions, respectively, thus presenting a structural feature of the NSI phenotype. However, 6 years later (1994), when CD4 counts had declined to 280/μl and the viral load in plasma had increased to 1.7×10^5 copies/ml, the V3 sequences in plasma displayed a high degree of heterogeneity (MD = 10.9%). Of 12 sequences, 3 possessed a basic arginine residue at the 11th position, as is often found in SI-type viruses. Indeed, simultaneously isolated virus was found to have the same basic amino acid substitution (Fig. 2A)

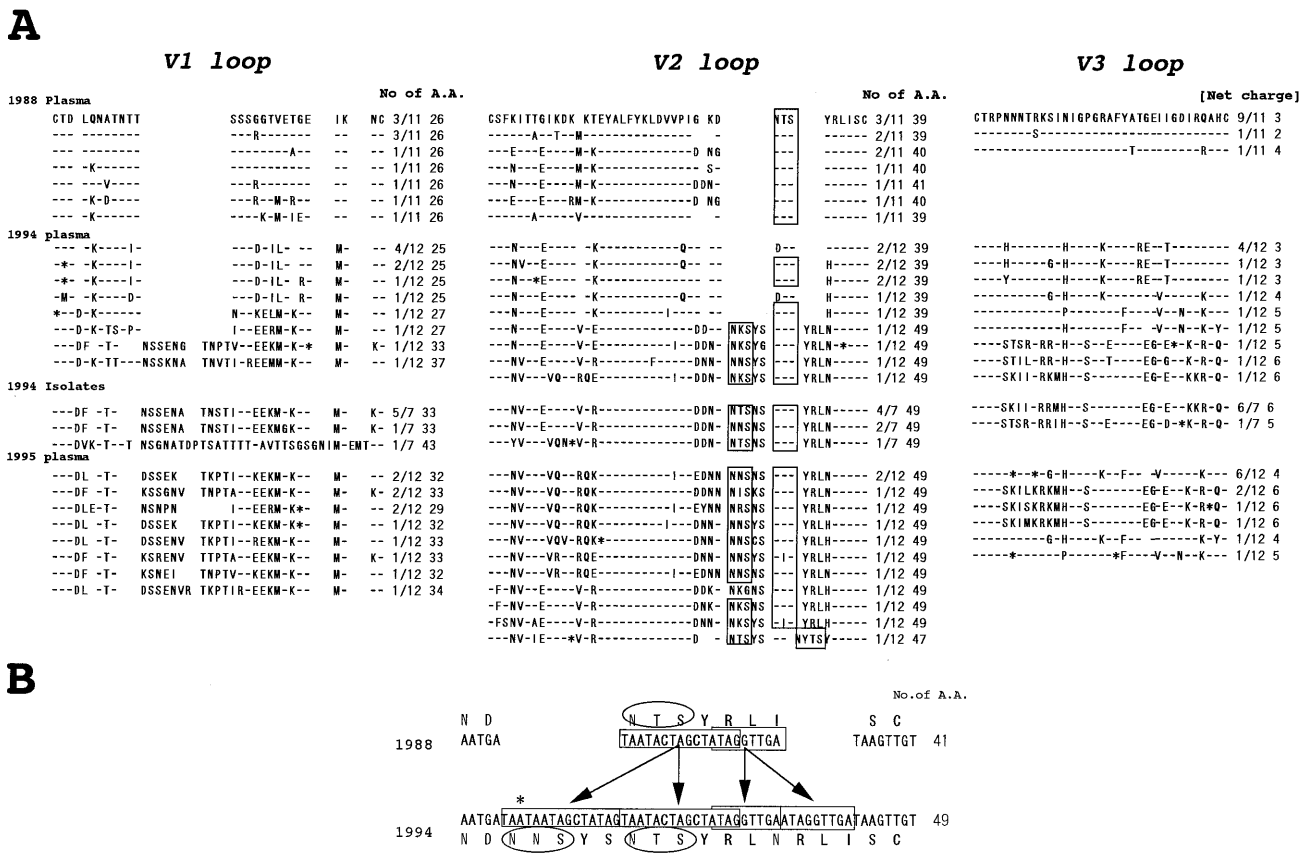

FIG. 2. (A) Amino acid (A.A.) sequences of V1, V2, and V3 loops of HIV-1 gp120 obtained from plasma and virus isolates of slow progressor subject 20. Amino acid sequences of V3 loops found in 1988 and 1994 plasma samples were published previously (44). Identity with the sequences shown at the top (dashes) and synonymous substitutions (asterisks) are indicated. Actual numbers of clones sequenced, numbers of amino acid residues of V1 and V2 loops, and net positive charges in V3 loops are shown at the end of each sequence. Potential asparagine-linked glycosylation sites in the C-terminal portion of V2 loops are also shown (boxed). (B) Duplication of 9- and 15-nucleotide sequences in V2 loops observed for subject 20. Nucleotide and amino acid sequences corresponding to the C-terminal portion of the representative V2 loops from 1988 and 1994 plasma are shown. Duplicated sequences (boxes), potential asparagine-linked glycosylation sites (ellipses), and nucleotide differences among duplicated units (asterisks) are indicated.

and was typically SI in PBMC cultures (data not shown). The remaining nine plasma clones possessed a neutral serine or glycine residue at the 11th position, like those found in 1988 plasma. However, none of the sequences were identical to any from 1988. The high degree of heterogeneity of V3 loops was maintained 1 year later (May 1995, MD = 12.4%), when CD4 counts dropped further to 118/μl and the viral RNA load in plasma increased further to 5.2×10^5 copies/ml. Although putative NSI and SI genotypes were still present, all but one NSI clone were different in sequence from those found in 1994 plasma. The nearly gross population shift of V3 sequences described above is clearly illustrated by the clustering of the putative NSI genotypes at each time point (see Fig. 4). Sequences with the putative SI genotype showed longer branch distances, suggesting that the V3 region of virus with SI genotypes evolves more rapidly than that of virus with NSI genotypes.

The V1 and V2 loops in 1988 plasma displayed an MD (3.9%) slightly higher than that of V3 loops. A variation in length of 1 or 2 residues was observed in the V2 loop, whereas the V1 lengths were identical. In 1994, when the MD increased to 6.3%, a marked extension of the V2 loop by 10 residues was observed in 4 of 12 clones. These extensions of the V2 loop were obviously due to duplication of 9 or 15 nucleotides and created an additional asparagine-linked glycosylation site (Fig. 2B). Two of the clones with V2 elongation further displayed an extension of the V1 loop to 33 or 37 residues. This extension appeared to be caused by an insertion of a sequence of 7 or 10 residues whose origin is unidentifiable. In stark contrast, the V1 and V2 populations became totally occupied by elongated sequences during the subsequent year. The degree of heterogeneity (MD) of V1 and V2 simultaneously increased up to 10%.

The mutations within the V1 to V3 region in slow progressor subject 20 were highly biased to nonsynonymous ones, since the calculated rate of accumulation of nonsynonymous substitutions from 1988 to 1994 (0.943% per year) was more than 12 times higher than that of synonymous substitutions (0.076% per year).

In summary, in vivo sequence changes of the virus of this slow progressor featured rapid accumulation of nonsynonymous point mutations through V1 to V3, including the presumably important basic amino acid substitutions in V3, elongation of V1 and V2, and a generally high degree of heterogeneity at each time point. Thus, the virus in this patient could be under strong selection pressures.

Poor sequence changes of HIV-1 in a rapid progressor, subject 2. Subject 2, a rapid progressor, was recognized by infectious mononucleosis-like symptoms in May 1991 before seroconversion. The primary infection was suspected to have

occurred early in 1991. A year later, $CD4^+$ cell counts were already below 200/μl, and the patient died of *Pneumocystis carinii* pneumonia in 1994. The in vivo sequence features were considerably different from those of subject 20 in that sequence heterogeneity was far less prominent (Table 1), the MDs of V3 were 1.3% in 1992 and 3% shortly before death in 1994, and the MDs of V1 and V2 were 2% in 1992 and 5.1% in 1994. However, the virus load in plasma was markedly high. Moreover, the most prevalent V3 sequence in 1992 plasma (4 of 11 clones) was also the most prevalent sequence in 1994 plasma (3 of 12 clones) (Fig. 3), indicating no population replacement as seen in subject 20. This was confirmed by a phylogenetic comparison (Fig. 4) of V3 sequences of subjects 2 and 20. Weaker selection pressure in subject 2 than in subject 20 was also suggested by a significantly higher ratio (0.168) of synonymous to nonsynonymous substitutions for subject 2 than for subject 20 (0.081). Furthermore, in contrast to the findings for subject 20, virus from subject 2 displayed no elongation of V1 or V2. Its V2 was constantly 40 residues long and possessed no or only one N-linked glycosylation site in the C-terminal portion. The V1 length differed at most by 2 residues. However, one sequence feature shared by subjects 2 and 20 was a typical basic amino acid substitution in V3, which was presumably associated with a phenotypic change from NSI to SI. This was found in 2 of 12 clones in 1994 and was absent in 1992.

Generalization of HIV-1 evolutionary patterns associated with rapid progressors and slow progressors. To determine whether the above-described differences in sequence changes between subjects 2 and 20 were generalized, we studied five more rapid progressors and five more slow progressors or nonprogressors (Fig. 5). A total of 100,000 nucleotides spanning V1, V2, C2, and V3 were determined and compared. Rapid progressor subjects 1, 28, and 43, who were infected through sexual transmission and developed AIDS within 2 years, displayed exactly the same features of sequence variation found for subject 2. These included a high degree of sequence homogeneity with a small MD ranging from 0.27% (subject 1) to 2.12% (subject 43), a lack of V2 elongation, and a basic amino acid substitution in V3 (Fig. 5 and Table 1). The HIV-1 isolates from subjects 28 and 43 were subtypes B_B and E, respectively, which are epidemic in Thailand (34), and putative SI genotypes were predicted from the basic amino acid substitutions in V3. In addition, the length of V1 varied only by 1 or 2 residues, if detected at all, within each of the three patients, and by 12 residues, at most, between the cases. There appeared to be no further extension as seen in subject 20.

Two other rapid progressors, subjects 8 and 9, were infected through contaminated blood products. About 5 or 6 years after infection, their CD4 counts suddenly and rapidly decreased (>200/μl per year). Their viral sequence patterns were similar

TABLE 1. V1, V2, and V3 variations

Patient no.	Infection yr	CD4 depletion		Sequencing				V1 length(s)[d]	V2 length(s)[d]	V3 charge	MD (%)			
		Yr	Rate[a]	Yr	CD4[b]	Viral load[c]	Source				V1/V2	C2	V3	Total (SD)
1	1991	1991	33.7	1991	28	1.3×10^6	Plasma	19	40	3, 5[e]	0	0.25	0.75	0.27 (0.16)
							Isolate	18, 19	40	5[e]	1.10	0.37	0.32	0.58 (0.26)
2	1991	1992	33.8	1992	434	4.2×10^3	Plasma	17	40	3	2.10	0.95	1.34	1.36 (0.49)
				1994	2	1.6×10^6	Plasma	15, 16, 17	40	3, 6[e]	5.18	1.91	3.03	3.07 (1.48)
							Isolate	16, 17	40	3, 6[e]	3.36	1.04	3.16	2.10 (1.51)
28	1992	1992	27.3	1994	8	4.4×10^5	Plasma	29	42	3	1.00	0.78	2.18	1.09 (0.71)
							Isolate	29	42	5[e]	2.13	0.70	0	1.08 (0.37)
43	1991	1993	21.0	1995	75	1.1×10^5	Plasma	29, 30, 31	42	3, 4, 5[e]	2.33	0.95	5.05	2.12 (1.15)
8	1983–85[g]	1990	25.6	1994	20	2.0×10^5	Plasma	18, 20	39, 41	3	6.50	2.38	1.03	3.33 (1.30)
							Isolate	22	42	5[e]	0.60	0	0	0.17 (0.15)
9	1983–85[g]	1989	29.2	1988	262	1.3×10^5	Plasma	25, 30	40	3, 4	2.63	2.64	1.71	2.47 (0.91)
				1992	17	5.8×10^4	Plasma	25, 27, 30	40	3, 6[e]	4.28	3.23	4.35	3.76 (2.73)
							Isolate	30	40	6[e]	0.32	0	0	0.11 (0.08)
20	1986	1994	7.9	1988	832	2.2×10^4	Plasma	26	39, 40, 41	2, 3, 4	3.88	1.52	0.52	2.08 (0.73)
				1994	285	1.7×10^5	Plasma	25, 27, 33, 37	39, 49[f]	3, 4, 5,[e] 6[e]	6.37	3.10	10.94	5.50 (3.40)
							Isolate	33, 43	49[f]	5,[e] 6[e]	5.35	1.82	2.45	3.10 (4.13)
				1995	118	5.2×10^5	Plasma	29, 32, 33, 34	47,[f] 49[f]	4, 5, 6[e]	10.00	2.65	12.41	6.59 (3.08)
47	1983–85[g]	1994	3.2	1994	314	8.2×10^3	Plasma	37, 40, 43, 44, 45	40, 41, 42, 44[f]	6[e]	9.73	3.35	3.60	5.68 (1.97)
65	1983–85[g]	1994	6.4	1994	198	2.9×10^4	Isolate	42, 45, 47	39, 40, 42[f]	4[e]	8.88	1.32	1.52	4.15 (4.29)
15	1983–85[g]	1994	7.9	1993	236	8.0×10^3	Plasma	21, 23	38, 41	4, 6[e]	3.70	1.38	2.48	2.27 (0.84)
				1995	108	6.8×10^4	Isolate	34	54[f]	8[e]	0	0	0.40	0.06 (0.14)
19	1983–85[g]		1.3	1987	515	8.8×10^3	Plasma	26	42	3	2.57	1.62	1.92	1.74 (0.92)
				1995	393	2.8×10^3	Plasma	25, 26	42, 44,[f] 47[f]	3	5.64	3.39	0.82	3.58 (1.47)
							Isolate	26	47[f]	3	1.16	0.22	0.48	0.59 (0.20)
63	1983–85[g]		0.9	1995	703	2.4×10^3	Plasma	27	40, 41, 43, 45,[f] 49[f]	3	6.38	2.10	2.64	3.58 (4.07)
							Isolate	27	49[f]	3	0.34	0.44	0	0.33 (0.11)

[a] Number of cells per microliter per month.
[b] Number of cells per microliter.
[c] Number of copies per milliliter.
[d] Number of amino acid residues.
[e] Putative SI genotype.
[f] Duplication of N-glycan.
[g] Infections of these individuals are assumed to have been contracted between 1983 and 1985.

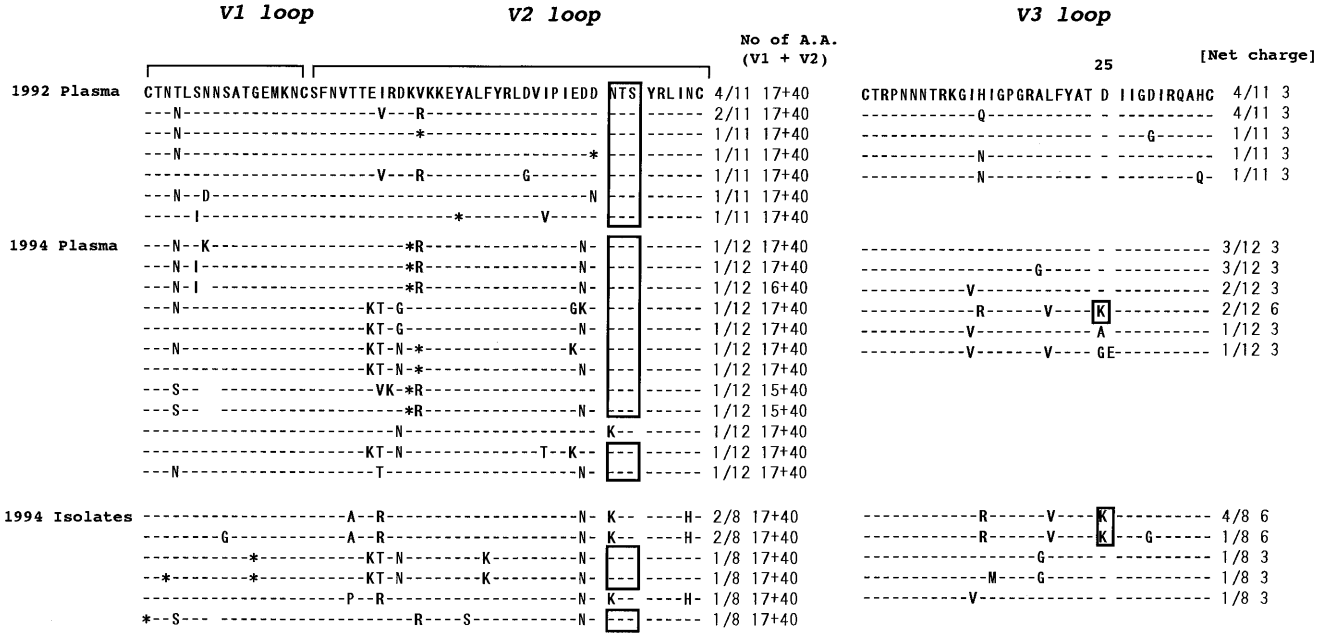

FIG. 3. Amino acid (A.A.) sequences of V1, V2, and V3 loops of HIV-1 gp120 obtained from plasma and virus isolates of rapid progressor subject 2. Actual numbers of clones sequenced, numbers of amino acid residues of V1 and V2 loops, and net positive charges in V3 loops are shown at the end of each sequence. Identity with the sequences shown at the top (dashes), synonymous substitutions (asterisks), and potential asparagine-linked glycosylation sites in the C-terminal portion of V2 loops and basic lysine residues at the 25th position of V3 loops (boxes) are indicated.

to those of subjects 1, 2, 28, and 43 in that there was no extensive elongation of V2, V1 length variations were within a relatively narrow range, and there were basic amino acid substitutions in V3 (Fig. 5A and Table 1). However, their MDs were a little higher than those of the latter four cases, probably reflecting the longer disease courses of subjects 8 and 9. In case 9, the ratio of synonymous to nonsynonymous substitutions between the sequences of 1988 and 1992 was 0.224, which was higher than that for another rapid progressor, subject 2 (0.168). This suggested that the high MD of subject 9 was at least partly due to synonymous substitutions without strong selection pressures.

With the plasma samples obtained from the five slow progressors or nonprogressors in 1994 or 1995, PCR amplification was successful for a single slow progressor (subject 47) and two nonprogressors (subjects 19 and 63) but was unsuccessful for the remaining two cases (15 and 65). However, we were able to isolate viruses from the latter two subjects and used these primary isolates instead of plasma for sequencing. All these slow progressors or nonprogressors were hemophiliac patients without any other risk factors and were infected with HIV-1 between 1983 and 1985. In contrast to the six rapid progressors, who had relatively similar features, these slow progressors and nonprogressors differed markedly from one another. However, as for subject 20, high MDs ranging from 3.58 to 5.68% were commonly observed for the plasma of subjects 47, 19, and 63 (Table 1). Although virus isolates obtained after tissue culture selection generally showed more homogeneous sequences than plasma sequences (Table 1), isolates from subject 65 still exhibited a considerably high MD. Furthermore, virus from all these slow progressors or nonprogressors displayed extremely long V2 loops, which were at least 44 residues long and even 54 residues long in one case (Fig. 5B and Table 1). The potential N-linked glycosylation site was doubled in at least three sequences for each patient (Fig. 5B and Table 1). This highly contrasts with the finding that virus from all six rapid progressors possessed short V2 loops with constant or only slightly different numbers of residues and with only one glycosylation site. V1 also appeared to be longer for the slow progressors or nonprogressors, except for cases 19 and 63, than for rapid progressors. The mean length of the longest V1 loops for six slow progressors or nonprogressors was 36.2 residues, and that for six rapid progressors was 24.7. The mean length of the longest V2 loops for six slow progressors or nonprogressors was 47.5, while that for six rapid progressors was 41.

To determine whether the extended version of V2 evolved from a short V2 in these infected individuals, we tried to further analyze the previously obtained plasma specimens of subjects 15, 19, and 63. PCR amplification was successful for 1993 plasma from subject 15 and 1987 plasma from subject 19. Sequence analyses of the resultant PCR products revealed that all the V2 sequences obtained from these plasma samples were short versions with only one N-linked glycosylation site in the C-terminal portion. Although we could not exclude the possibility that those plasma samples may contain undetectable levels of HIV-1 carrying an extended V2, these results support the idea that the extended V2 sequences observed in 1995 samples from subjects 15 and 19 evolved from a short version of V2.

On the other hand, basic amino acid substitutions in the V3 loop were observed in the slow progressor subjects 15, 47, and 65 but not in the nonprogressor subjects 19 and 63, consistent with the notion that the basic substitutions of V3 are associated with an advanced stage of the disease.

Phylogenetic analyses for all cases showed that the sequences obtained from each case clustered together most tightly and were divergent from those of other cases (Fig. 6). Sequences from cases 1 and 2 are relatively similar to each other, and the diversity between cases 1 and 2 was even less than that in case 20. We suspect that cases 1 and 2 were

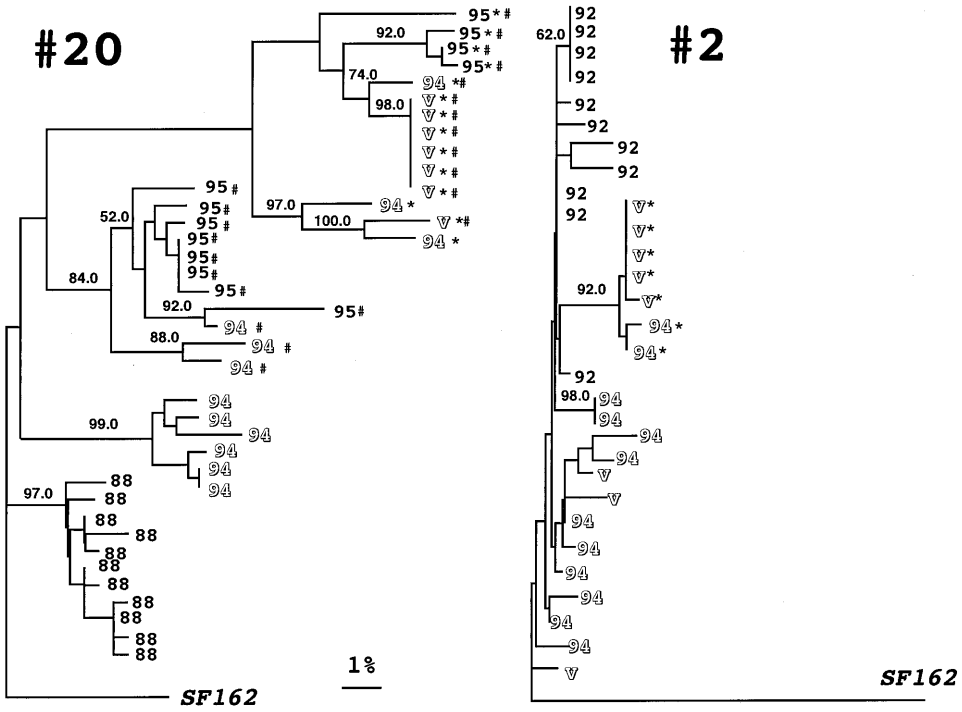

FIG. 4. Phylogenetic analyses of V3 sequences from rapid progressor subject 2 and slow progressor subject 20. Sequences of 279 nucleotides, corresponding to positions 7049 to 7327 of HIV-1_{SF2} (46), were used to construct the phylogenetic trees. Each sequence is indicated by boldface or outlined characters according to the sampling time. The sequence of HIV-1_{SF162} was used as an out-group. V3 sequence with a putative SI genotype (asterisks) and V3 sequences connected with extended V2 loops (#) are indicated. Each bootstrap probability is shown on the corresponding tree branch.

epidemiologically linked. Nevertheless, the case 1 sequences derived from 14 independent PCRs formed a distinct cluster with a bootstrap value of 92%. These results excluded the possibility of contamination during the PCR procedure and supported the idea that the sequence variations observed were indeed derived from a single or minimum number of clones in the respective individuals and not due to infections with multiple different clones.

Effect of V2 extension on viral cellular host range. The findings described above indicate close association of V2 elongation with slow disease progression. In vitro studies with cell cultures showed that elongation of V2 is one of the requirements for the SI phenotype (14), although this might not always be reproducible (6, 52). On the other hand, V2 loops were constantly short in macrophage-tropic HIV-1 strains and possessed only one N-linked glycosylation site in the C-terminal portion (19, 51). Therefore, we attempted to determine whether the V2 extension in vivo observed here disrupts the viral macrophage tropism. Two infectious DNA clones which differ from each other only in the C-terminal portion of the V2 loop were generated by replacing the 136-bp *Hph*I-*Stu*I fragment in the macrophage-tropic HIV-1 strain SF162 with the corresponding fragments derived from 1988 plasma samples and 1994 isolates from subject 20 (Fig. 7A). The nucleotide sequences of the entire gp120 gene of the constructs were determined to confirm that there were no sequence changes other than the C-terminal portion of V2. Viruses were recovered from these two DNAs by transfection into RD4 cells followed by cultivation with PHA-stimulated PBMCs and were named SV2/SF162 and LV2/SF162, respectively. In PBMC cultures, both recombinant viruses showed slightly slower replication kinetics than the parental SF162 (Fig. 7B). None of the three viruses replicated in T-cell line MT2, MT4, M8166, or Hut78 (data not shown). In primary macrophages, however, LV2/SF162 replicated much less efficiently than SV2/SF162 or the parental virus, whereas the difference in titers between the latter two was only about twofold (Fig. 7B). These findings clearly demonstrate that the elongated version of V2 that arose in the virus in subject 20 has the capacity to restrict the ability of SF162 to replicate in macrophages.

DISCUSSION

In this study, we examined HIV-1 *env* gene quasispecies from 12 infected individuals in various disease stages with different disease progression rates. Although the quasispecies structures varied widely from each other, a strong correlation between a low rate of disease progression and a high degree of genetic diversity of HIV-1 was observed. Furthermore, V2 loop extension was observed specifically in individuals with slow or no disease progression, whereas basic amino acid substitutions in V3 characteristic of a viral phenotype shift from NSI to SI were observed in patients with advanced disease stages, regardless of their rate of disease progression. The latter observations confirmed and extended the previous notion that SI variants with basic amino acid substitutions at particular positions in V3 evolve and become a distinct population during the disease course (5, 21, 40).

HIV-1 infection is recognized as a continuous battle between vigorous virus replication and the host defense against the virus. During the course of infection, HIV-1 has been shown to escape the cellular and humoral immune responses in vivo (26, 27, 38). On the basis of these findings, Nowak et al.

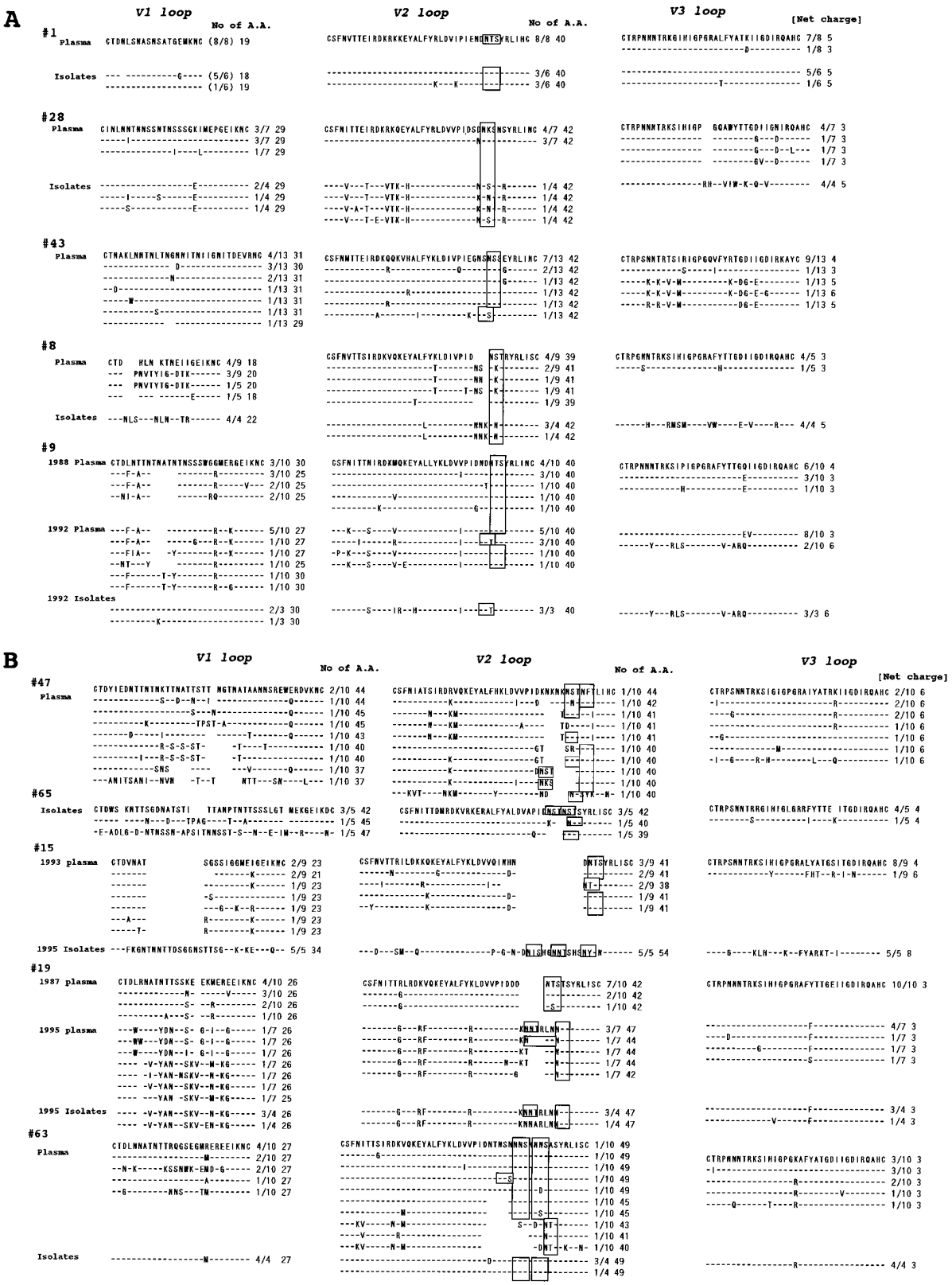

FIG. 5. Amino acid (A.A.) sequences of V1, V2, and V3 loops of HIV-1 gp120 in plasma or virus isolates obtained from 10 infected individuals. Parts of the amino acid sequences of V3 loops from subject 1 were published previously (31, 44). Identity with the sequences shown at the top (dashes) and potential asparagine-linked glycosylation sites in the C-terminal portion of V2 loops (boxes) are indicated. Actual numbers of clones sequenced, numbers of amino acid residues of V1 and V2 loops, and net positive charges in V3 loops are shown at the end of each sequence. (A) Rapid progressor subjects 1, 28, 43, 8, and 9. (B) Slow progressor subjects 47, 65, and 15 and nonprogressor subjects 19 and 63.

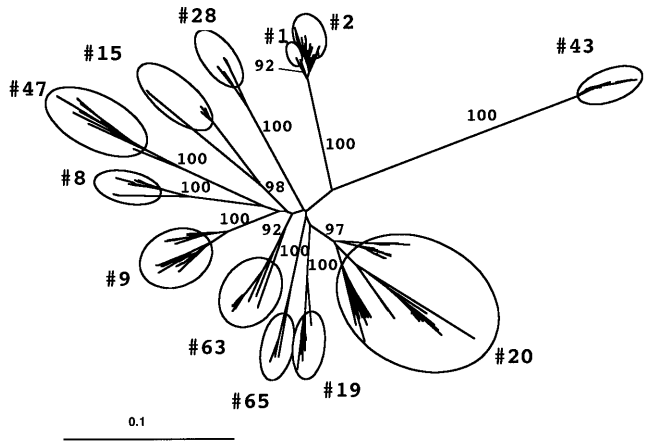

FIG. 6. Phylogenetic analyses of all viral sequences. The sequences obtained from each case most tightly clustered together and were divergent from those of other cases. Each bootstrap probability is shown on the corresponding tree branch.

proposed the antigenic diversity threshold theory to explain how HIV-1 finally defeats the host immune system (32). In this hypothesis, the growing antigenic diversity driven by the host immune responses gradually exceeds the potential repertoire of the immune responses; hence, the immune system can no longer suppress HIV-1 replication in vivo, leading to the decline of $CD4^+$ cell counts and disease progression. This view seems to be supported in part by our findings showing extensive accumulation of changes in the entire region spanning V1 to V3 in such cases as 20, 47, and 65, slowly but inevitably progressing to AIDS. In addition, compared with these cases, the in vivo MDs of HIV-1 in two genuine nonprogressors (subjects 19 and 63) were considerably lower. However, the virus in all rapid progressors showed less efficient sequence changes and less diversity than that in slow progressors or nonprogressors, contradicting the threshold theory. The theory seems valid only if it is assumed that the threshold differs among individuals and is low in rapid progressors and high in slow progressors. Thus, a more plausible explanation for the evolutionary pattern characteristic of rapid progressors is that because the immune responses are too weak to control virus replication, the original viral population proliferates without encountering pressures strong enough to generate mutants to escape immune responses. Similar findings for intrahost evolution of V3 (25) or regions spanning V3 to V5 (56) of HIV-1 also seemed inconsistent with the antigenic diversity threshold theory.

At present, it is difficult to define any causal relationship between V2 extensions and a low rate of disease progression. V2 extensions could be the consequence of prolonged disease courses. It is also possible that V2 extension with duplication of N-linked glycan causes alteration in the antigenic structure of V2. As described above, HIV-1 gp120 in slow progressors has been exposed to a stronger selection pressure than in rapid progressors. Therefore, V2 may extend as a result of escape from the specific humoral and/or cellular immune responses against the V2 loop. If so, the V2 extensions observed in virus isolates from slow progressors or nonprogressors are most likely a reflection of their long immunocompetent period. Alternatively, the V2 extensions may have caused the reduced rate of disease progression. As shown above, V2 extensions potentially reduced the viral ability to grow in macrophages. Since heterozygous defects of the CCR-5 gene, a coreceptor for the macrophage-tropic HIV-1 strains, are known to reduce the rate of disease progression (8), it is also possible that loss of macrophage tropism of HIV-1 infected individuals leads to reduced viral load and, thus, to a prolonged disease course. In any case, V2 extensions may be a good predictor of slow disease progression.

As for viral phenotype, V2 extensions with duplication or relocation of the N-linked glycosylation site were previously reported to be associated with the SI phenotype of HIV-1 (14). However, other researchers who examined more than 30 viral isolates for their ability to cause syncytia in MT2 T-cell line cultures failed to find any correlation between a particular V2 structure and the SI and NSI phenotypes of HIV-1 (6, 52). The reason for this discrepancy is unclear, but recombinant-virus studies using two molecular clones of HIV-1 strains showing a distinct cellular host range suggested that the HIV-1 macrophage tropism required shorter V2 loops with only one N-linked glycosylation site in the C-terminal portion (19, 51). Our present findings obtained with recombinant viruses clearly confirmed this concept.

V2 extensions were also noted to precede the basic amino acid substitutions of V3 in the course of infection on the basis of the sequence analysis of virus isolates obtained from four individuals (14). This might be the case in nonprogressor subjects 19 and 63, since these individuals displayed long V2 loops connected with V3 without any basic amino acid substitutions at the 11th or 25th position. However, all six rapid progressors

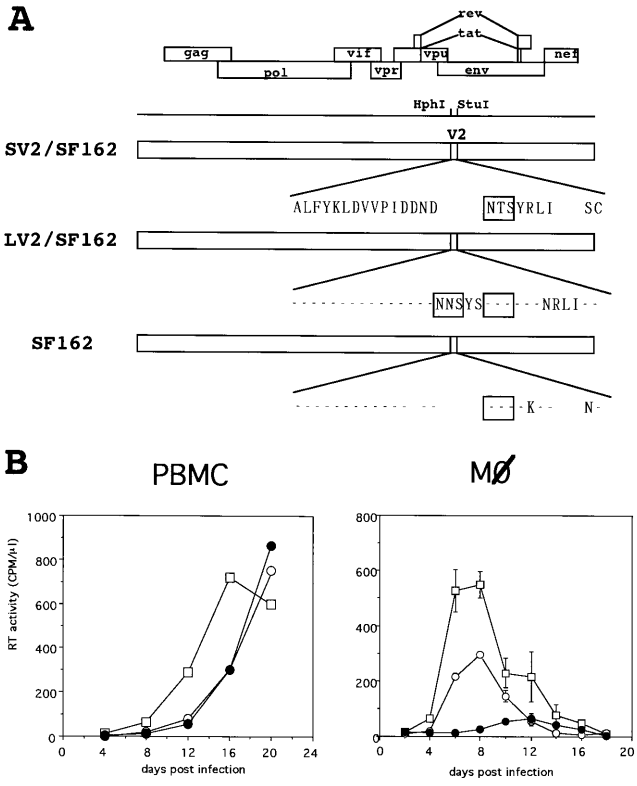

FIG. 7. (A) Construction of recombinant viruses carrying the V2 loops recovered from the 1988 plasma samples and 1994 isolates of subject 20. The amino acid sequence of the C-terminal portion of the V2 loop of each recombinant construct is shown. Sequence identity with SV2/SF162 is indicated (dashes). (B) Replication of recombinant viruses SV2/SF162 (○) and LV2/SF162 (●) and the parental macrophage-tropic HIV-1_{SF162} (□) in PBMCs and primary macrophages. Supernatants of infected cultures were assayed for reverse transcriptase (RT) activity at 2- to 4-day intervals.

examined so far, subjects 1, 2, 8, 9, 23, and 43, displayed short V2 and V3 loops with basic amino acid substitutions. Although we could not exclude the possibility that the V2 loop transiently extended but returned to the original length shortly after the basic amino acid substitutions of V3 (45), we reasonably assumed that the V2 loop did not extend at all in these rapid progressors. Detailed analysis of samples from rapid progressors, especially those at earlier time points, is needed to clarify this point.

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