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Variability of the *env* Gene in Cynomolgus Macaques Persistently Infected with Human Immunodeficiency Virus Type 2 Strain ben

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The sequence variability of distinct regions of the proviral *env* gene of human immunodeficiency virus type 2 strain ben (HIV-2ben) isolated sequentially over 3 to 4 years from six experimentally infected macaques was studied. The regions investigated were homologous to the V1, V2, V3, V4, V5, and V7 hypervariable regions identified in the *env* genes of HIV-1 and simian immunodeficiency virus SIVmac, respectively. In contrast to findings with HIV-1 and SIVmac, the V1- and V2-homologous regions were found to be highly conserved during the course of the HIV-2ben infection in macaques. The V3-homologous region showed a degree of variation comparable to that of HIV-1 but not of SIV. In the V4-, V5-, and V7-homologous regions, mutation hot spots were detected in most reisolates of the infected monkeys. Most of these mutations occurred during the first 10 weeks after infection. After 50 weeks, new mutations were rarely detected. At most mutation sites, a dynamic equilibrium between the mutated viral isotype and the infecting predominant wild type was present. This equilibrium might prevent an accumulation of mutations in isolates later in the course of infection.

Human immunodeficiency virus type 2 (HIV-2) belongs to the lentivirus subgroup of retroviruses. HIV-2 isolates are more closely related to simian immunodeficiency virus (SIV) than to HIV-1 (8, 13, 17, 20, 26). HIV-2 causes AIDS in humans but is less pathogenic than HIV-1 (30). In contrast to HIV-1, HIV-2 can infect baboons and macaques (9, 37, 43). Therefore, infection of these monkeys with HIV-2 can be used to test the protective effect of a vaccine against viral infection. Several recent studies demonstrate that the *env* gene product is an essential part of a successful vaccine. We have found that vaccination of cynomolgus monkeys (*Macaca fascicularis*) with native gp130 of HIV-2 strain ben (HIV-2ben) partially protects against a homologous challenge (29). However, the antigenic variability of the *env* gene of immunodeficiency viruses is thought to be a major obstacle for developing protective vaccines. As numerous studies with HIV-1 and SIV have shown, the *env* gene is the most variable viral gene (1, 15, 18, 22, 33, 35, 44, 45). So far, however, only limited information is available on the variability of the *env* gene of HIV-2 during the course of infection. The consensus sequence of different HIV-2 isolates revealed variable and constant regions similar to those described for HIV-1 and SIV (26). Franchini et al. (14) reported on a low genetic drift in the *env* gene of macaques infected with cloned HIV-2sbl/isy. In contrast, a high variability was found recently in the V3 region of different HIV-2 field isolates (5), but there is no information on the variability of the remaining *env* gene regions. We have studied the major genotype variation of six putative variable *env* gene regions from HIV-2ben in six macaques. Five were infected with the primary isolate originating from a patient with predominantly neurological symptoms (41). One macaque was infected with virus originating from the molecular cloned HIV-2ben/MK6 (25).

HIV-2ben sequences are present in a low copy number in PBMC DNA of infected macaques. In a first set of experiments, we tried to sequence the regions of interest directly from PCR products obtained with peripheral blood monuclear cell (PBMC) DNA as the template. A minimum of 1 μg of genomic PBMC DNA was necessary to obtain detectable amounts of amplification products. However, in most cases, insufficient amounts of amplification products were obtained for direct sequencing or cloning. Cocultivation of PBMC from HIV-2ben-infected monkeys with Molt-4 clone 8 cells led to rapid virus replication. Results of PCR analysis with 0.5 ng of genomic DNA as the template after 2 to 4 weeks of cocultivation revealed products sufficient for direct sequencing. Thus, cocultivation increased the proviral concentration approximately 1,000-fold. As shown by titration experiments with DNA of HIV-2ben-infected Molt-4 clone 8 cells, one proviral copy could be detected in about 1×10^5 to 5×10^5 cells, comparable to 500 ng of DNA (data not shown). Thus, the virus load in infected cynomolgus monkeys was less than one HIV-2ben proviral copy in 100,000 cells, indicating a low replication rate of this virus in these animals. This low replication rate of HIV-2ben and the absence of pathogenicity in these monkeys (43) is reminiscent to the behavior of HIV-1 during the early asymptomatic phase of infection (12). A similarly low virus activity and pathogenicity is seen in long-term survivors (42) or in macaques infected with *nef*- or *vpr*-deleted SIV (24, 28).

The low concentration of HIV-2ben in the cynomolgus monkeys allowed direct sequencing of the PCR-amplified proviral DNA from PBMC only in a few cases. Therefore, it was necessary to expand the proviral DNA by cocultivation of the PBMC with Molt-4 clone 8 cells prior to our variability analysis.

No *env* gene variability detected in the first 12 weeks of coculture. To examine the influence of the coculture on the HIV-2 sequence, Molt-4 clone 8 cells were infected with the primary isolate of HIV-2ben and studied over 25 weeks. The major *env* genotype found in cocultures with monkey PBMC was very similar to that obtained from the primary isolate of HIV-2ben. Figure 1 summarizes the results of the in vivo studies for the V4-, V5-, and V7-homologous regions. In the

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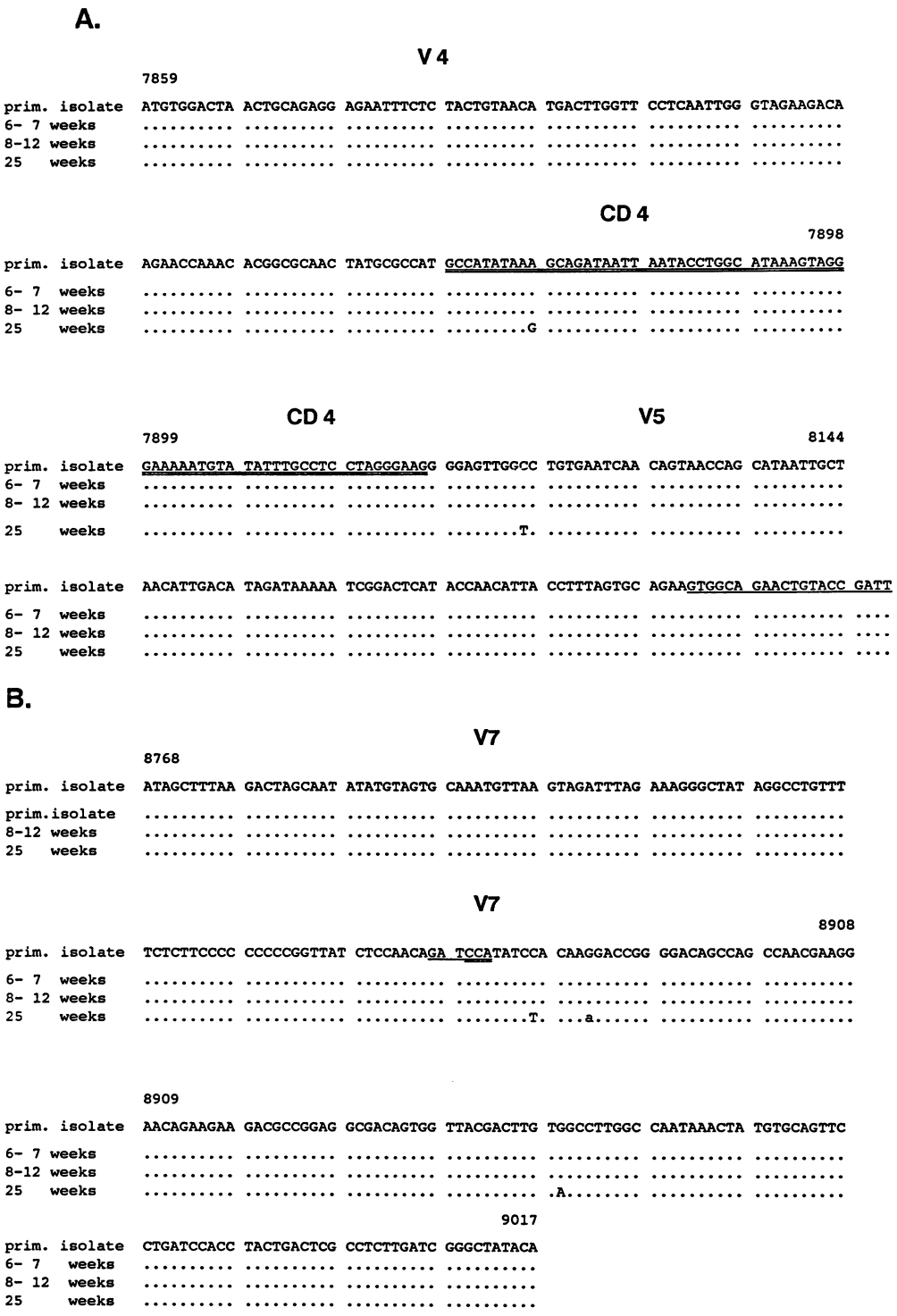

FIG. 1. Alignment of the V4 (A)-, V5 (A)-, and V7 (B)-homologous regions of the *env* gene from HIV-2ben present in Molt-4 clone 8 cells infected with the primary (prim.) isolate. For sequence analysis, DNA was prepared from the Molt-4 clone 8 cells as described previously (3). The extracted DNA was used as the template in PCR to specifically amplify distinct regions from the proviral *env* gene. Primer pairs used in this study were as follows: V1- and V2-homologous regions, nucleotides 6986 to 7006 (5′GAGACATCAATAAAACCATG3′), 7377 to 7394 (5′CCGGT GCTCAGTATCTA3′), 6969 to 6984 (5′GAAGATGTCTGGCATC3′), and 7483 to 7497 (5′GCGTTTCCATCATCCT3′); V3-homologous region, nucleotides 7525 to 7543 (5′TAGAGCAGAAAATAGAAC3′), 7769 to 7789 (5′ATCTGGGATGTTGTACAAGG3′), 7569 to 7590 (5′AGATAATAGGACTATCATTAGC3′), and 7744 to 7763 (5′TTCACCTCCTGCATGGCTTC3′); V4- and V5-homologous regions, nucleotides 7858 to 7878 (5′ATGTGGACTAACTGCAGAGG3′) and 8117 to 8136 (5′AATCGGTACAGTTCTGCCAC3′); and the V7-homologous transmembrane region (2, 22), nucleotides 8718 to 8738 (5′CCTCCTGGGTCAAGTATATTC3′) and 9004 to 9024 (5′CAGATGTTGTATAGC CCGATC3′). About 0.2 μg of DNA was added to each PCR mixture containing 1.5 mM Mg^{2+}, 0.1% Triton X-100, 200 mM each deoxynucleoside

V4-homologous region, no mutation was found over a period of 25 weeks. In the other regions, changes appeared only 12 weeks after cocultivation. In the putative CD4 binding domain, one amino acid change, from lysine (K) to arginine (R), was observed. One substitution was also found in the V5-homologous region, changing the predicted amino acid from threonine (T) to valine (V). In the transmembrane region, a slightly higher mutation rate was found. Three base substitutions, leading to three changes in the amino acid sequence, were detected. After 25 weeks in culture, a stop codon truncating the transmembrane protein in viral particles released from Molt-4 clone 8 cells appeared in position 8946 (data not shown).

The influence of coculture was studied, since several groups reported an in vitro selection of distinct HIV-1 and SIV phenotypes (7, 19, 27, 32). With HIV-2ben during the first 12 weeks in coculture, changes of the predominant genotype were not detected and no shift of the major genotype used for the primary infection of the culture cells occurred. Adaptation to the cell culture conditions, such as the creation of the stop codon in the transmembrane region, occurred only later. This difference may be due to the methods used or reflect a distinct biological behavior of HIV-2 in Molt-4 clone 8 cells. We have studied the major genotype of HIV-2ben by direct sequencing of the PCR-amplified DNA. Analyses were performed in triplicate, and only mutations which occurred at least in two analyses were considered as true mutation events. Thereby, copying error rates by *Taq* polymerase or recombination artifacts that falsify the sequence of the major genotype were avoided. Such errors may have had an influence on studies in which the PCR products were cloned first and then sequenced (10, 11, 32, 33).

The V1- and V2-homologous regions were highly conserved in cynomolgus PBMC during the time of infection. The genetic stability of the V1- and V2-homologous regions was studied after infection of three cynomolgus monkeys, Mf723, Mf778, and Mf5172, over 2 to 4 years. During this investigation period, this sequence did not change in the three monkeys investigated (Fig. 2A). This result was confirmed when the two regions were sequenced directly with the DNA of PBMC from these animals as the template without coculture. The genomic stability of HIV-2ben in the V1- and V2-homologous regions in vivo is remarkable since this sequence is quite divergent among different HIV-2 isolates (Fig. 2A). It is also in contrast to the high variability of HIV-1 and SIVmac in these regions (2, 18, 21, 39, 45). This finding suggests an evolutionary pressure on these regions in the case of the development of an asymptomatic long-term persistence of HIV-2ben in macaques. The functions of the V1 and V2 regions are unknown. However, we have found a correlation between the low replication competence, low virus load, and lack of HIV-2ben pathogenicity in these macaques on the one hand with an extremely low variability of the V1- and V2-homologous regions on the other. Investigations of the variability of the V1 and V2 regions in HIV-1-infected long-term survivors may elucidate the role of this region for pathogenicity.

The V4-, V5-, and V7-homologous regions vary during the course of infection. Compared with the conservation of the V1- and V2-homologous regions of HIV-2ben during infection, the V4-, V5-, and V7-homologous regions are variable (Fig. 2B and C). In the V4-homologous region, we observed a clustering of mutation sites near the 3′ end. In summary, in the six monkeys investigated, we detected five mutation sites over a short stretch of seven amino acids, leading to an altered polypeptide sequence. The number of mutation sites is variable between the reisolates. Consequently, in each monkey, a distinct predominant genotype had developed. This site of variability at the 3′ end of the V4 region is also present among different original HIV-2 isolates (Fig. 2B). In the V5-homologous region, a cluster of mutation sites similar to that in V4 was observed (Fig. 2B). In this case, five mutation sites were detected over a short stretch of seven amino acids. Again, this region is highly variable among different original HIV-2 isolates. Two additional mutation sites were observed in the 5′ direction. These positions are also variable among HIV-2 isolates (Fig. 2B). In the V7-homologous region, no mutation cluster was observed. Moreover, the overall variability with a total of three mutation sites over a stretch of 50 amino acids is very low. In contrast, among different original HIV-2 isolates, 13 mutation sites are present in this region.

In summary, we found 25 mutation sites in the V4-, V5-, and V7-homologous regions. The vast majority of the mutation sites are detectable early after infection (Table 1). Only a small number of new mutation sites occurred in the second year after infection or later. However, there appeared to be a dynamic equilibrium between the wild type and the variants at most of the mutation sites. Only in a limited number of cases were the mutations found in all reisolates of a single monkey. The detected mutation sites were not randomly dispersed throughout the regions investigated. This is demonstrated by a comparison of the proviral sequences of the infected monkeys as well as of different original HIV-2 isolates. Moreover, the amino acid exchanges observed at most mutation sites were similar in all macaques investigated (Fig. 2B and C). Perhaps a high genetic pressure on the proviral sequences allows mutations to only a limited degree. This assumption is supported by findings of a comparison of the number of mutation sites among infected individuals (Table 2). No significant differences were observed in the number of mutation sites found in reisolates of the six monkeys investigated except in the V4-homologous region. In this region, the proviral genomes of Mf760 and Mf778 were more variable than those of the other monkeys investigated.

The V3-homologous region is highly variable in the course of infection. Much attention has been focused on the variability of the V3 region of HIV-1 containing the principal virus neutralizing domain (16, 31, 36, 40). Recently it has been reported that the V3-homologous region of HIV-2 field isolates varies to a similar extent as that of HIV-1 (5). We have studied the V3-homologous region of four monkeys (Mf723, Mf778, Mf5172, and Mf5173) 2 to 4 years after infection. In the monkeys investigated, the V3-homologous region was highly variable. In this region comprising 188 bp, 30 mutation sites were detected (Table 2), and most of them initiate a change in

triphosphate, 50 pmol of each oligonucleotide primer, and 1 U of *Taq* polymerase. DNA was amplified for 35 cycles with the following cycle profile: denaturation at 92°C for 30 s, annealing at 55°C for 30 s, and extension for 30 s at 72°C. PCR with HIV-negative DNA was carried out simultaneously to detect contamination. Each PCR was carried out in triplicate, and products were independently sequenced directly (3). The in vitro-obtained sequences were compared with the corresponding sequence of the primary isolate. The identification of variable and putative CD4 binding regions is based on information for HIV-2 gp130 (25, 31). Capital letters indicate mutations found in all reisolates examined; lowercase letters indicate mutations present in only a few reisolates. The putative CD4 binding region is underlined.

TABLE 1. First appearance of mutations in the variable *env* regions in proviral DNA from PBMCs of infected *M. fascicularis*

Region	Mutation site	Animal(s) with mutation at indicated earliest time of detection[a]		
		<10 wpi	11–50 wpi	>50 wpi
V4	7897	Mf760, Mf778		
	7921	Mf5172	Mf760, Mf5173	
	7926		Mf760	
	7927	Mf5172		
	7930	Mf760, Mf778		
	7938	Mf5171		
	7941	Mf778	Mf760, Mf5173	
	7944	Mf778	Mf760	Mf723
V5	8036	Mf723, Mf778, Mf5171 Mf5172	Mf5173	
	8042			Mf760
	8079			Mf723
	8084	Mf723, Mf760, Mf778 Mf5171, Mf5172	Mf5173	
	8088	Mf760, Mf778		
	8094		Mf5173	
	8104	Mf723, Mf760	Mf778	
V7	8780	Mf778, Mf5171	Mf760	
	8795	Mf778		
	8797	Mf760	Mf778	
	8801	Mf778, Mf5171	Mf760	
	8848		Mf760	
	8874		Mf778	
	8881	Mf760		
	8922	Mf723, Mf5171, Mf5172, Mf5173		

[a] Animals Mf5171 and Mf5172 were not investigated. Underlined animal numbers indicate stable mutations. wpi, weeks postinfection.

the predicted amino acid sequence (Table 3). This finding indicates a biological function of the V3 loop of HIV-2 similar to that discussed for HIV-1. This suggestion is supported by results of Björling et al. (4), who described V3 peptides of HIV-2 inducing neutralizing antibodies. However, another study described the failure of linear HIV-2 V3 region peptides to induce such antibodies (38). Further investigations are required to resolve these contradictory results.

Lymph nodes and PBMC harbor similar HIV-2ben proviral genotypes. PCR analysis with lymph node DNA as the template revealed provirus concentrations about 5 to 10 times higher than in PBMC (data not shown). This made direct sequencing of amplified DNA possible. The genotype found in the lymph nodes was similar to that in PBMC. Again, the V1- and V2-homologous regions appeared to be very stable. In general, the same mutation sites as with PBMC were detected in the V4-, V5-, and V7-homologous regions. The results of direct sequencing of PCR amplification products from lymph node DNA emphasize that the data obtained from the PBMC cocultures reflect the in vivo situation.

TABLE 2. Numbers of mutations described in the variable regions of the proviral *env* gene from PBMC of HIV-2ben-infected *M. fascicularis*

Animal	No. of mutation sites detected				
	V1/V2 (350 bp)	V3 (118 bp)	V4 (80 bp)	V5 (80 bp)	V7 (190 bp)
Mf723		10	1	4	1
Mf760	ND[a]	ND	6	4	5
Mf778		13	4	4	5
Mf5171	ND	ND	1	2	3
Mf5172		8	2	2	1
Mf5173	ND	5	2	1	1

[a] ND, not determined.

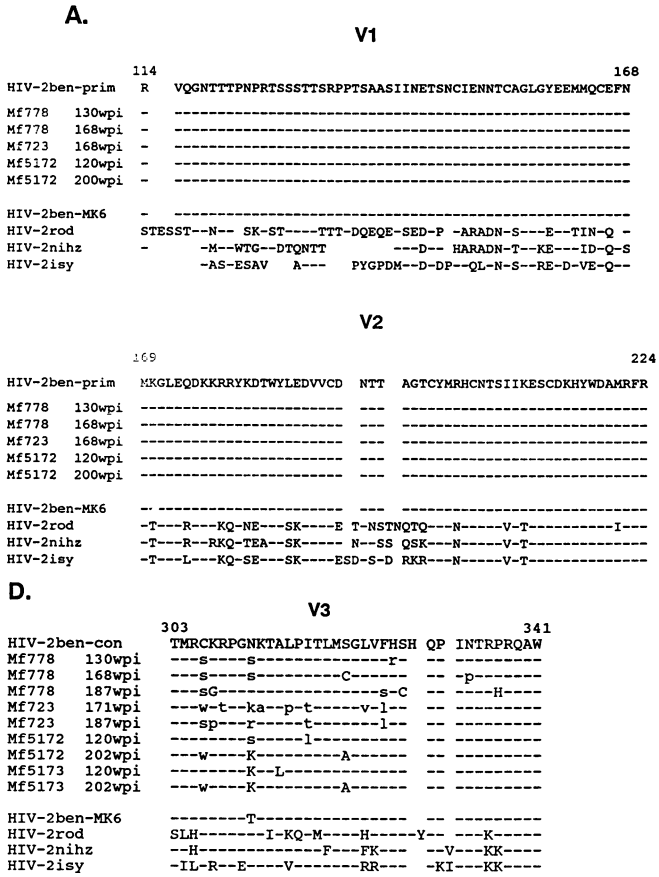

FIG. 2. Alignment of the V1 (A)-, V2 (A)-, V4 (B)-, V5 (B)-, V7 (C)-, and V3 (B)-homologous regions of the *env* gene from HIV-2ben present in vivo. Sequencing reactions were performed with PCR products from DNA which was prepared from PBMC directly or after cocultivation with Molt-4 clone 8 cells, as well as lymph node DNA prepared from homogenized tissue. Conditions for the PCR and sequencing are given in the legend to Fig. 1. Nested PCRs with DNA from PBMC as the template for amplification of the V1-, V2-, and V3-homologous regions were performed under the same conditions. The predicted amino acid sequence of the primary isolate (HIV-2ben-prim) is shown on the top line. Only amino acids which differ from those of the primary isolate are shown below; dashes indicates no changes in the amino acid sequence. The amino acids are shown in the one-letter code. Capital letters indicate mutations detected in all reisolates examined; lowercase letters indicate mutations present in only a few reisolates. The predicted amino acid sequence for each monkey is derived from nucleotide consensus sequences which resulted from several sequencing analyses of HIV-2ben reisolates from the infected monkeys. In panels B and C, the inferred amino acid sequences of the lymph nodes (ln) from two monkeys (Mf760 and Mf778) are aligned. The identification of variable and putative CD4 binding regions is based on information for HIV-2 gp130 (24, 32) over up to 3 years. Alignment of the inferred amino acid sequences from the HIV-2 isolates HIV-2_{ROD}, HIV-2_{NIHZ}, and HIV-2_{ISY} (24, 32) is also shown. wpi, weeks postinfection.

At most mutation sites, the predicted amino acid changed. At most mutation sites in the investigated *env* region, transi-

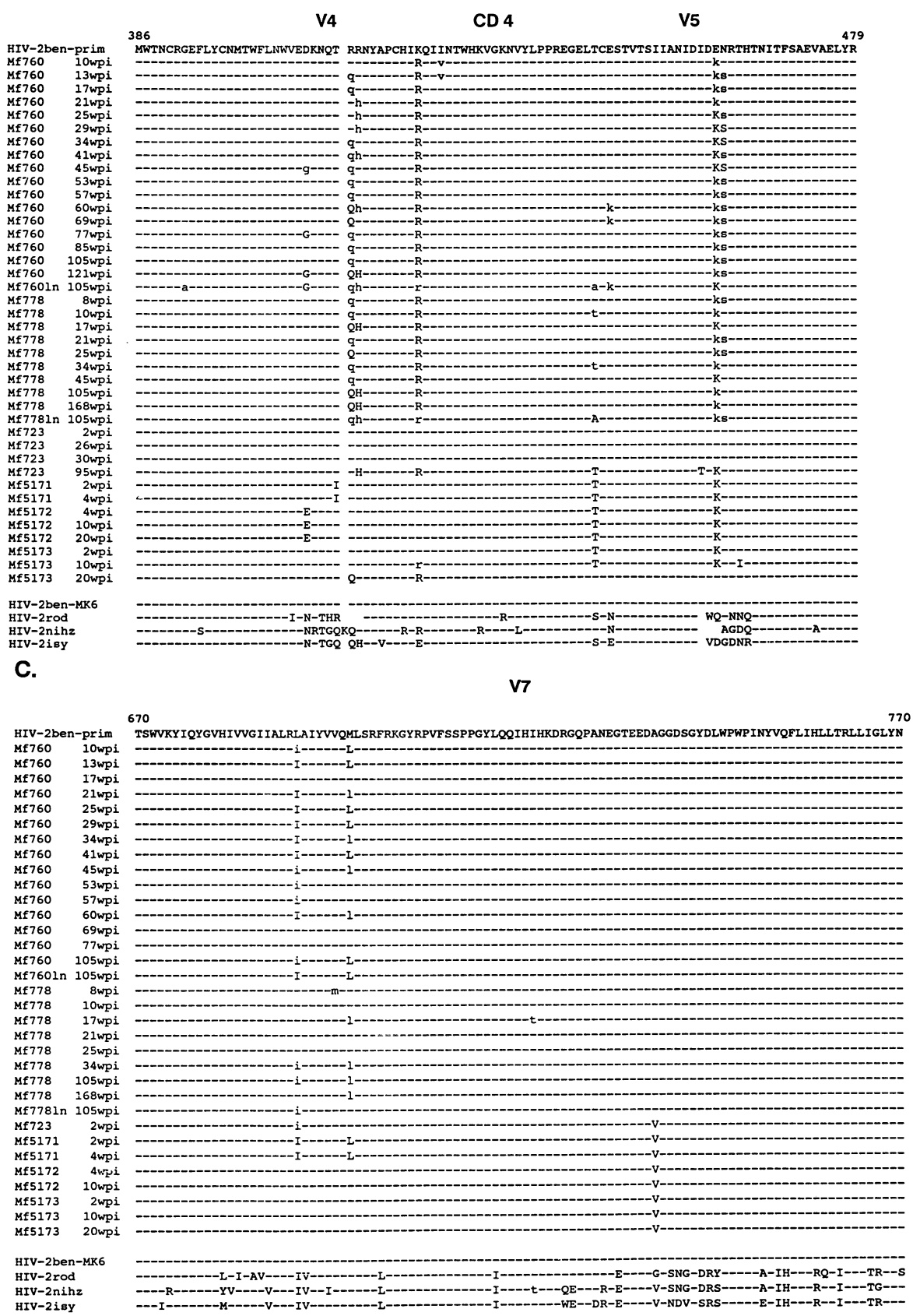

FIG. 2—*Continued.*

TABLE 3. Mutations with respect to changes in the predicted amino acid sequence

Predicted aminio acid sequence	No. of mutations in[a]:			
	V3 (118 bp)	V4 (80 bp)	V5 (80 bp)	V7 (190 bp)
None	8	3	1	1
Synonym	10	2	1	6
Nonsynonym	12	3	5	1

[a] V1/V2-homologous sequences were obtained only from Mf723, Mf778, and Mf5172; no mutations were found. V3-homologous sequences were obtained only from Mf723, Mf778, Mm5172, and Mm5173.

tions from G to A (40%) or from A to G (20%) occurred. Similarly, the level of mutations from C to T (20%) or from T to C (10%) was elevated. All other nucleoside exchanges were rare. Dependent on the region investigated, these mutations distinctly changed the predicted amino acid sequences (Table 3). In the V4-homologous region, roughly equivalent numbers of silent mutations as well as those leading to a homologous or heterologous amino acid exchange were detected. In the V5-homologous region, mutations leading to a heterologous amino acid exchange were very frequent, whereas most of the mutations in the V7-homologous region led to homologous amino acid exchanges.

As expected from the low replication rate of HIV-2ben in infected cynomolgus monkeys, we have detected a low genetic variability except in the V3-homologous region. Despite the low variability, specific differences in the degree of variability were found between the regions investigated. In contrast to the conservation of the V1- and V2-homologous sequences in our infected animals, the V4-, V5-, and V7-homologous sequences were moderately variable. The clustering of mutations in a limited number of sites in these regions and the appearance of certain amino acid exchanges indicate a strong selection for specific viral genotypes in the infected animals. HIV-2ben became highly adapted in the animals. After 1 year of infection, new mutation sites were rarely detected. This is in contrast to the behavior of SIVmac in rhesus monkeys, in which case increased amino acid substitutions were found over the time of infection and disease development (6, 7). Rather than being an epiphenomenon, the level of virus replication particularly early after infection followed by the accumulation of specific mutations appears to be an important factor for the pathogenicity of immunodeficiency viruses.

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