Virology **266,** 286–298 (2000)
doi:10.1006/viro.1999.9991, available online at http://www.idealibrary.com on IDEAL®

Implication of the C-Terminal Domain of Nef Protein in the Reversion to Pathogenicity of Attenuated SIVmacBK28-41 in Macaques

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Received April 2, 1999; returned to author for revision May 17, 1999; accepted September 1, 1999

We have analyzed the *nef* gene sequences amplified from 12 macaques presenting various patterns of infection with SIVmacBK28-41, a clone derived from attenuated SIVmacBK28. We have observed seven mutation hot spots at positions 56, 75, 432, 588, 680, 699, and 779. The major alteration was a thymidine insertion at position 699, leading to a frameshift in the SIVmacBK28-41 *nef* gene and changing the last 15 amino acids of Nef into a 31-amino-acid-long C-terminal domain nearly identical to that encoded by pathogenic SIVmac239 and SIVmac251. The insertion was found at early time points in proviruses obtained from rapid progressor macaques, after 2 years postinfection in progressors, and rarely or only after 4 years postinfection in nonprogressors. Fixation of the other mutations occurred only after insertion of thymidine 699. Phylogenetic analysis demonstrated that the *nef* genes isolated from progressors evolved from the allele present in SIVmacBK28-41 to alleles present in SIVmac239 or SIVmac251, whereas *nef* sequences from nonprogressors stayed clustered with that of the inoculated molecular clone. These data stress the importance of the C-terminal extremity of the Nef protein of SIVmac239 or SIVmac251 in viral pathogenesis.

INTRODUCTION

Monkeys infected with the simian immunodeficiency virus (SIV) provide one of the best system for modeling *in vivo* human immunodeficiency virus (HIV) infection and studying the functions of the viral proteins and their interactions with the host during the development of the disease. Pathogenic SIV strains, such as SIVmac251, induce an illness in macaques which closely resembles that of HIV-infected patients, characterized by a primary infection with a high viral load followed by an asymptomatic phase leading progressively to the development of an AIDS-like disease (Daniel *et al.,* 1987; Kestler *et al.,* 1988), whereas a more rapid disease can be observed with variants like SIVsmmPBj14 (Fultz *et al.,* 1989). Several infectious molecular clones have been obtained, some of them pathogenic, like the SIVmac239, but also many of them displaying an attenuated phenotype (Chakrabarti *et al.,* 1987; Dewhurst *et al.,* 1990; Franchini *et al.,* 1987; Hirsch *et al.,* 1987; Kestler *et al.,* 1988, 1990; Kornfeld *et al.,* 1987; Luciw *et al.,* 1992; Naidu *et al.,* 1988). For example, the SIVmacBK28 molecular clone, derived from the pathogenic SIVmac251 isolate after several passages on a human T-cell line, can easily infect macaques but the asymptomatic phase is far longer than that with a pathogenic virus (Edmonson *et al.,* 1998). One major difference between attenuated SIVmacBK28 and pathogenic SIVmac239 resides in the *nef* gene.

The Nef protein is now recognized as a key element of viral pathogenesis not only in the simian model but also during human infection (Harris, 1996; Kestler *et al.,* 1990; Spina *et al.,* 1994). The *nef* gene, unique to lentiviruses of primates, is localized in the 3′ part of the viral genome, overlapping the polypurine tract and a part of the U3 region in the LTR. This gene is massively transcribed early in the viral cycle and produces a 25- to 35-kDa protein which is subsequently myristylated and phosphorylated (Guy *et al.,* 1987). Nef protein is involved in downregulation of CD4 and MHC-I molecule surface expression (Aiken *et al.,* 1994; Garcia and Miller, 1991; Guy *et al.,* 1987; Hua and Cullen, 1997; Schwartz *et al.,* 1996), preventing superinfection of already infected cells (Benson *et al.,* 1993) and allowing them to resist killing by cytotoxic T lymphocytes (CTL) (Collins *et al.,* 1998). In addition, Nef increases the viral particles' infectivity as well as the efficiency of reverse transcription (Aiken and Trono, 1995; Luo *et al.,* 1997, 1998; Miller *et al.,* 1995; Schwartz *et al.,* 1995).

The *in vivo* importance of Nef was first observed during the infection of rhesus macaques inoculated with simian immunodeficiency virus (SIVmac) variants de-

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession Nos. AF128345–AF128391.

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0042-6822/00 $35.00

leted in the *nef* gene (Kestler *et al.,* 1991). Contrary to pathogenic SIVmac239 *nef* open, SIVmac239Δ*nef* demonstrated an attenuated phenotype in infected animals, characterized by a viral load 100 times lower during the primary infection, no plasmatic antigenemia, no $CD4^+$ T lymphocyte depletion, and no clinical symptoms. A similar discrepancy was also observed between macaques infected with either C8 attenuated or J5 pathogenic variants of SIVmac32H, two molecular clones differing only by point mutations and one in-frame deletion in the *nef* gene (Rud *et al.,* 1994). Likewise, *nef* genes obtained from some HIV-1-infected nonprogressor humans are altered by point mutations, small deletions, or the presence of a premature stop codon (Mariani *et al.,* 1996). Moreover, major alterations in the *nef* gene have been observed in some human long-term nonprogressors (LTNP) (Deacon *et al.,* 1995; Kirchhoff *et al.,* 1995; Salvi *et al.,* 1998). For example, eight individuals from the Sydney Blood Bank cohort, infected by blood transfusions from a single donor, presented a nonprogressive HIV infection for almost 10 years (Deacon *et al.,* 1995). The *nef* genes of viruses amplified from these patients and from the donor were shown to be partially deleted. By serological analysis with Nef peptides, Greenway *et al.* (1998) demonstrated that the single initial *nef* deletion present in the virus from the donor had evolved in the recipients with a loss of up to 80% of the *nef* sequence. It was proposed that the lack of a functional *nef* gene was responsible for the viral attenuation and the absence of, or slow, clinical evolution observed in these patients. Nevertheless, after a longer period of observation declining CD4 T-cell counts were reported for patients with detectable plasma viral load (Learmont *et al.,* 1999).

In this study, we describe the *in vivo* evolution of the *nef* gene amplified from rhesus macaques presenting different patterns of clinical evolution after infection with attenuated SIVmacBK28-41. The precise insertion of a single nucleotide into the SIVmacBK28-41 *nef* sequence allowed the expression of a different carboxyl terminal extremity. This alteration, followed by specific point mutations, is associated with an increase in cellular viremia and viral pathogenicity in macaques, demonstrating the importance of the C terminal extremity of SIVmac Nef protein in viral virulence.

RESULTS

Infection of rhesus macaques with attenuated SIVmacBK28-41

Eight rhesus macaques of a vaccination trial were inoculated intravenously with 10 MID_{50} of molecular clone SIVmacBK28-41 grown on macaque PBMC. This attenuated SIV is derived from molecular clone SIVmacBK28, in which the premature stop codon present in the *env* gene was mutated in order to restore the complete ORF. The animals were monitored for cell-associated viral load and for $CD4^+$ and $CD8^+$ T lymphocyte counts, and all eight were found to be infected. During primary infection they had a detectable viral load ranging from 10 to 31,250 infected cells per million PBMC (Table 1). No reduction in the initial viral load was observed for the immunized animals compared to the control monkeys. Moreover, during follow up, some monkeys suffered from a progressive immunodeficiency and three of them died from infection. Independently of the scheme of immunization, the rhesus macaques could be separated into three groups according to their clinical evolution and frequency of virus isolation (Table 1).

The first group comprises macaques who died within 2.5 years postinfection and for which virus isolation was always positive. In group II, animals survived for more than 3 years and had a frequently positive virus isolation and a reduced CD4/CD8 lymphocyte ratio, whereas virus was not recovered for years, and no significant lymphocyte depletion or symptoms were observed in group III.

Macaque PH440 was classified in group I. Its cell-associated viral load varied between 10 and 250 infected cells per million PBMC. The animal suffered a progressive $CD4^+$ T lymphocyte depletion (from 1156 to 130 cells/μl, CD4/CD8 ratio from 1.07 to 0.03) and a severe loss of weight (more than 50%) and ultimately died at 100 weeks postinfection with a characterized immunodeficiency.

Group II was composed of macaques 33593, 35830, 49114, and P2. The virus was isolated from these animals during the primary infection and was punctually recovered over the following 2 years. The cell-associated viral load increased thereafter. Macaques 33593 and 49114 died with a partial depletion in $CD4^+$ T lymphocytes (273 and 566 $CD4^+$ cells/μl, respectively) and a drop in the CD4/CD8 ratio to 0.4 and 0.8, respectively. Another monkey was sacrificed because of TB positivity (35830). The last macaque (P2) was still alive 281 weeks postinfection in spite of an immunosuppression (from 1010 to 160 $CD4^+$ T lymphocytes/μl) and a CD4/CD8 ratio of 0.15.

Group III contained one macaque from each immunization regimen (33598, 49113, P6). During the primary infection, the cell-associated viral load was similar to that observed for the other macaques (10–6250 infected cells per million PBMC). However, it decreased thereafter and the virus could never, or only on rare occasions at very late time, be isolated by coculture over the following 4 years. These animals had no significant changes in the $CD4^+$ T lymphocyte population and had no clinical symptoms.

We have also included in this study four naive macaques inoculated with SIVmacBK28-41 to establish whether a similar difference of evolution could be observed in the absence of previous immunization. The follow up was less frequent during the asymptomatic phase but on the basis of their immunological and virological parameters, one of these macaques (51190), who

TABLE 1

PBMC-Associated Viral Load in Macaques Inoculated with SIVmacBK28-41

(A) Vaccinated macaques

	Group I	Group II				Group III		
Macaques:	PH440	33593	35830	49114	P2	33598	49113	P6
Time pi								
1.5	50	10	10	250	31250	10	250	6250
4	250	10	10	10	1250	50	2	10
9	50	1	1	1	2	1	—	—
13	250	1	—	—	—	2	1	—
21	10	10	—	—	1	—	—	—
44.5	50	—	2	—	—	—	—	—
72	10	50	10	50	1	—	—	—
100	ND	10	2	50	2	—	—	—
150		—	—	10	50	—	—	—
195					1	—	—	—
216					50	1	—	—
231					50	—	—	0.25
Clinical status:	Death	Death	Sacrifice	Death	Alive	Alive	Alive	Alive
Time:	W100	W152	W173	W179	W281	W281	W281	W28

(B) Naive macaques

	Group I	Group II		
Macaques:	51190	51181	51184	51192
Time p.i.				
2	400	—	40	4000
4	4000	40	40	40
8	400	400	4	4
18	2	2	0.4	—
83	250	—	ND	ND
97	ND	ND	10	10
Clinical status:	Death	Alive	Alive	Alive
Time:	W110	W141	W155	W155

Note. The animals were classified according to their cell-associated viral load and clinical evolution in three groups. The cell-associated viral load is expressed as the number of infected cells per million. Samples for which the virus could not be isolated from 4 million PBMC are indicated by a dash. Times postinfection are expressed in weeks. Tissue samples were collected at necropsy on macaques PH440 (week 100) and 49114 (week 179) for *nef* gene sequencing without concomitant determination of the cell-associated viral load. Clinical status was given at time of death or at the last time point for the surviving macaques. ND, not determined.

died 110 weeks postinfection, was included in group I. Three others (51181, 51184, 51192), showing a reduced CD4/CD8 ratio (0.7, 0.1, 0.9, respectively, at 3 years postinfection), were included in group II.

nef gene sequence

As SIVmacBK28 was reported to be an attenuated virus, we wanted to determine the reasons for the differences in the evolution of the infection in individual animals and, particularly, the bases for the reversion to pathogenicity. We focused our analysis on the *nef* gene. DNA was purified from PBMC or lymphoid organ cells from the infected monkeys and was submitted to PCR amplification with SIV *nef*-specific primers. Sequencing reactions were performed on PCR products and provide data on the predominant strain, although variant strains present in the sample in sufficient amount could also be detected (Lang *et al.,* 1997; Larder *et al.,* 1993). The PCR amplification and *nef* sequencing were performed on samples obtained at 4, 100, 195, 216, and 231 weeks postinfection.

In the 42 samples analyzed, corresponding to 33,243 sequenced nucleotides, we found 241 mutations in the *nef* gene (792 bp from the start codon), among which 21 were an insertion at a unique position (Table 2). Among the other 220 mutations, we observed a majority of transitions (transition/transversion ratio of 2.85), with the A to G and G to A transitions being the most represented. No

TABLE 2

Distribution of the Mutations between the Groups or in Function of the Insertion in Position 699

	Number of				Sequence variability (%)[b]	Insertion in position 699[c]	Insertion frequency (%)
	Macaques	Sequences	Nucleotides	Mutations[a]			
Group I	2	5	3959	48	1.21	4/5	80
Group II	7	20	15,833	169	1.07	13/20	65
Group III	3	17	13,833	24	0.18	4/17	23
With insertion	NA	21	16,632	239	1.44	21/21	100
Without insertion	NA	21	16,611	2	0.01	0/21	0

	P value for insertion only	*P* value for seven hot spots
Group I versus group II	0.47	0.25
Group I versus group III	$P < 0.05$	$P < 0.005$
Group II versus group III	$P < 0.05$	$P < 0.001$

Note. The proviral sequence was determined on a tissue sample (PBMC, lymph node, or spleen cell) taken at a time point from one animal. When a mixed population was observed at a precise location, like the insertion in 699, only the majority was considered and the subpopulations were excluded, as we did not evaluate the proportion precisely. NA, not applicable.

[a] The insertion in position 699 of the *nef* gene was considered as one mutation event.

[b] The variability (%) was calculated on the nucleotide sequence taking into account the number of insertions.

[c] Insertion: number of sequences having the insertion of one thymidine in position 699 of the *nef* gene, restoring the *nef* ORF to a SIVmac239-like *nef* length. Insertion frequency was calculated with the sequences collected at all time points. Probabilities were determined with Pearson's χ^2 test for independence using StatXact-3 software.

mutation created a premature stop codon and they were unequally distributed on the *nef* gene (Fig. 1). Some regions were preserved, such as the 5′ extremity, the polypurine track, or the sequences corresponding to putative structural domains (Lee *et al.,* 1996). In contrast, other areas concentrated a lot of mutations, such as the regions between nucleotides 103–159, 301–357, and 562–636, representing ~42% of the mutations. Moreover, 7 mutation hot spots were found in positions 56, 75, 432, 588, 680, 699, and 779, representing nearly 40% of all mutations. The mutation in position 75 was silent, whereas mutations in positions 56, 432, 588, 680, and 779 were nonsynonymous. The mutation in position 699 was an insertion of one thymidine leading to a frameshift

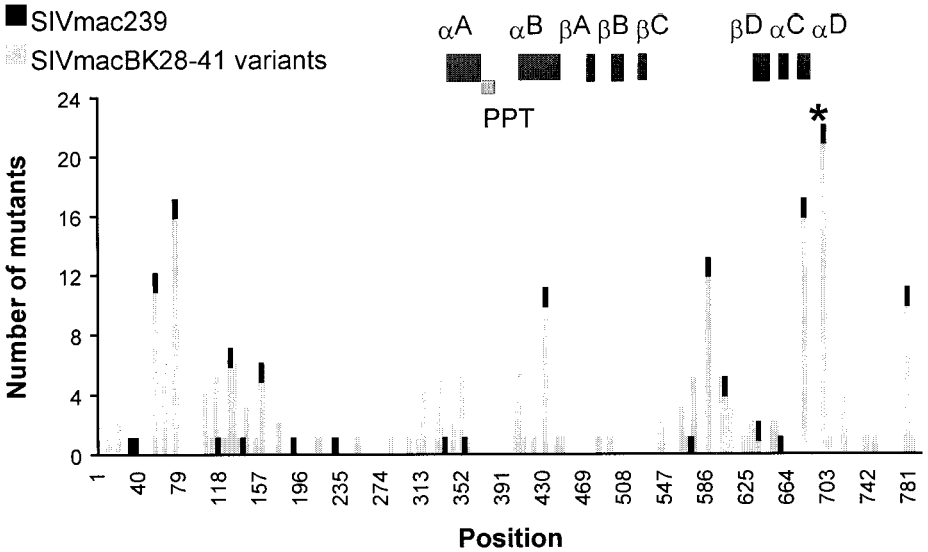

FIG. 1. Distribution of the mutations on the SIVmacBK28-41 *nef* gene. The mutations are distributed on a 792-bp SIVmacBK28-41 sequence starting from the *nef* start codon. Gray bars represent localization of the mutations found in the *nef* gene of the infected macaques. Black bars localize sequence differences between SIVmacBK28-41 and SIVmac239 *nef* open. The star marks the position of the insertion in 699 changing the open reading frame. Sequences encoding the structural domains described by Lee *et al.* and the polypurine track (PPT) are depicted, respectively, by eight black and one gray horizontal bars (Lee *et al.,* 1996).

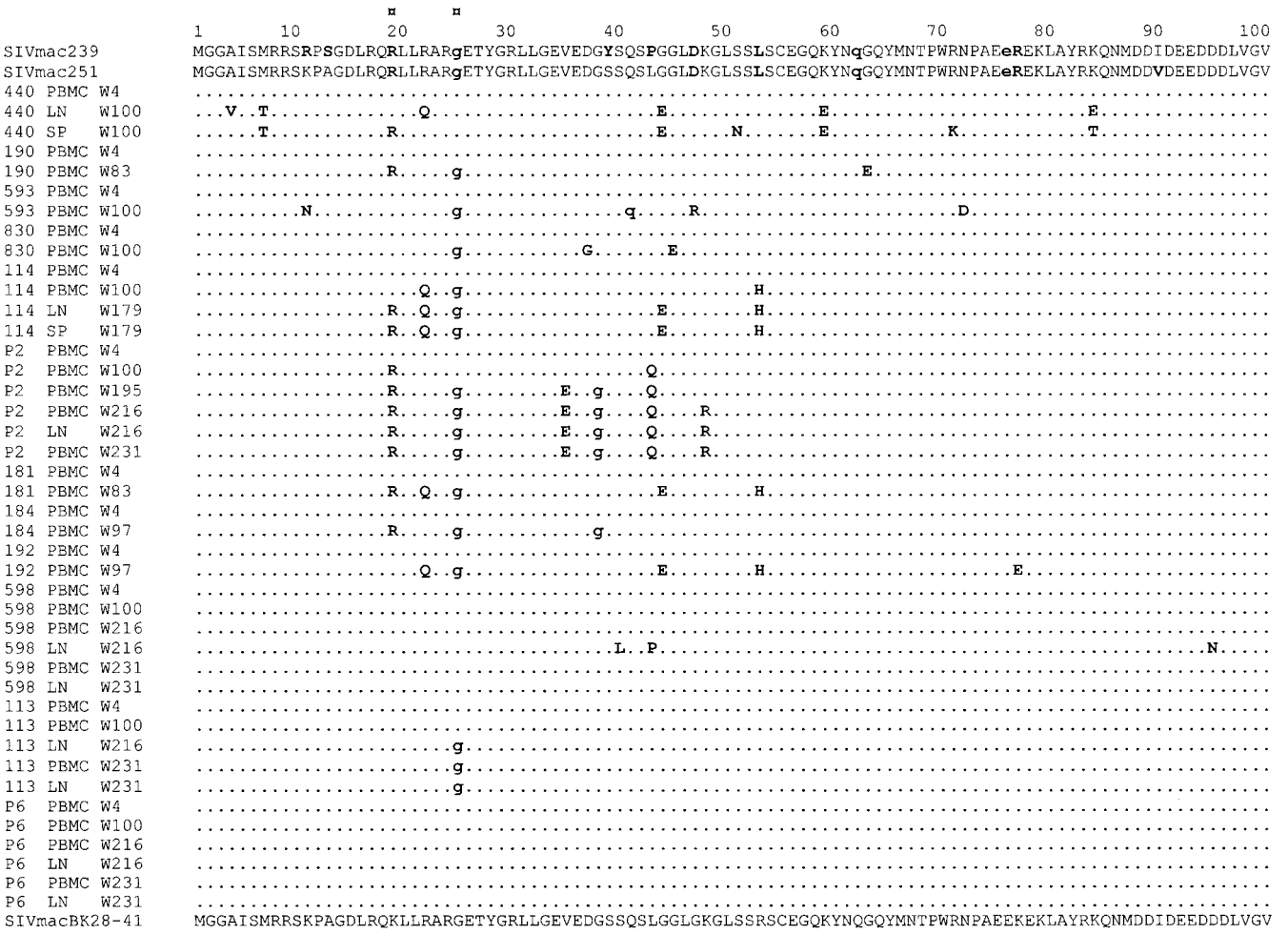

FIG. 2. Alignment of the predicted Nef protein sequences obtained from the macaques infected with SIVmacBK28-41. The sequences are classified chronologically per animal. Each sequence denomination is composed of the three last digits of the animal's tattoo, the tissue sample (PBMC, peripheral blood mononuclear cell; LN, lymph node; or SP, spleen cells) and the time postinfection expressed in weeks. For example, 440-PBMC-W4 identified the *nef* sequence obtained from macaque PH440 with PBMC taken at 4 weeks postinfection. When on a precise location, such as the insertion in 699, a mixed population was observed, only the majority was considered, and the subpopulations were excluded as the proportion was not evaluated precisely. We used the sequence of SIVmacBK28-41 for reference. The mutations are indicated in bold capitals for the nonsynonymous mutations and in bold lowercase letters for the synonymous mutations. The dots and stars indicate amino acids identical to SIVmacBK28-41 and the stop codon, respectively. ◘ localizes the seven mutation hot spots. For the sequences having the insertion in 699, the protein sequence after the insertion is written in normal capitals, with the mutation written in bold characters. The structural domains, four α helix and four beta sheets, similar to those described by Lee *et al.* for HIV-1 Nef protein, are localized over the numeration. PPII indicates the PXXP motif which interacts with the cellular protein kinases.

in the *nef* ORF and changing the last 15 amino acids of SIVmacBK28-41 Nef protein into a 31-amino-acid-long C-terminal extremity nearly identical to that encoded by SIVmac239 (Fig. 2). Interestingly, the majority of mutations was found in provirus sequences already harboring the insertion in position 699 (Table 2).

We thereafter sequenced the *nef* gene of SIVmac251 virions produced on macaque PBMC. We observed 23 mutations compared with the sequence of SIVmacBK28, 16 being identical to mutations found in the SIVmac239 *nef* gene. The insertion in position 699 was present, as well as 5 of the 6 other hot spots (the nucleotide in position 432 was the same as that in SIVmacBK28). Moreover, we carried out *nef* sequencing on RT–PCR fragments obtained from virus isolated from macaques PH440 (week 97 postinfection), 49114 (week 100), 35830 (week 100), and P2 (week 231). In all cases, the sequences were similar to that of the provirus analyzed at the same time, which demonstrates that our proviral *nef* data reflected the sequences of infectious viruses.

Impact of thymidine insertion in position 699 on clinical evolution

We then studied whether the distribution of the thymidine insertion was similar in the three groups. Four weeks postinfection, the proviruses bore a *nef* gene identical to the SIVmacBK28 gene for all animals with

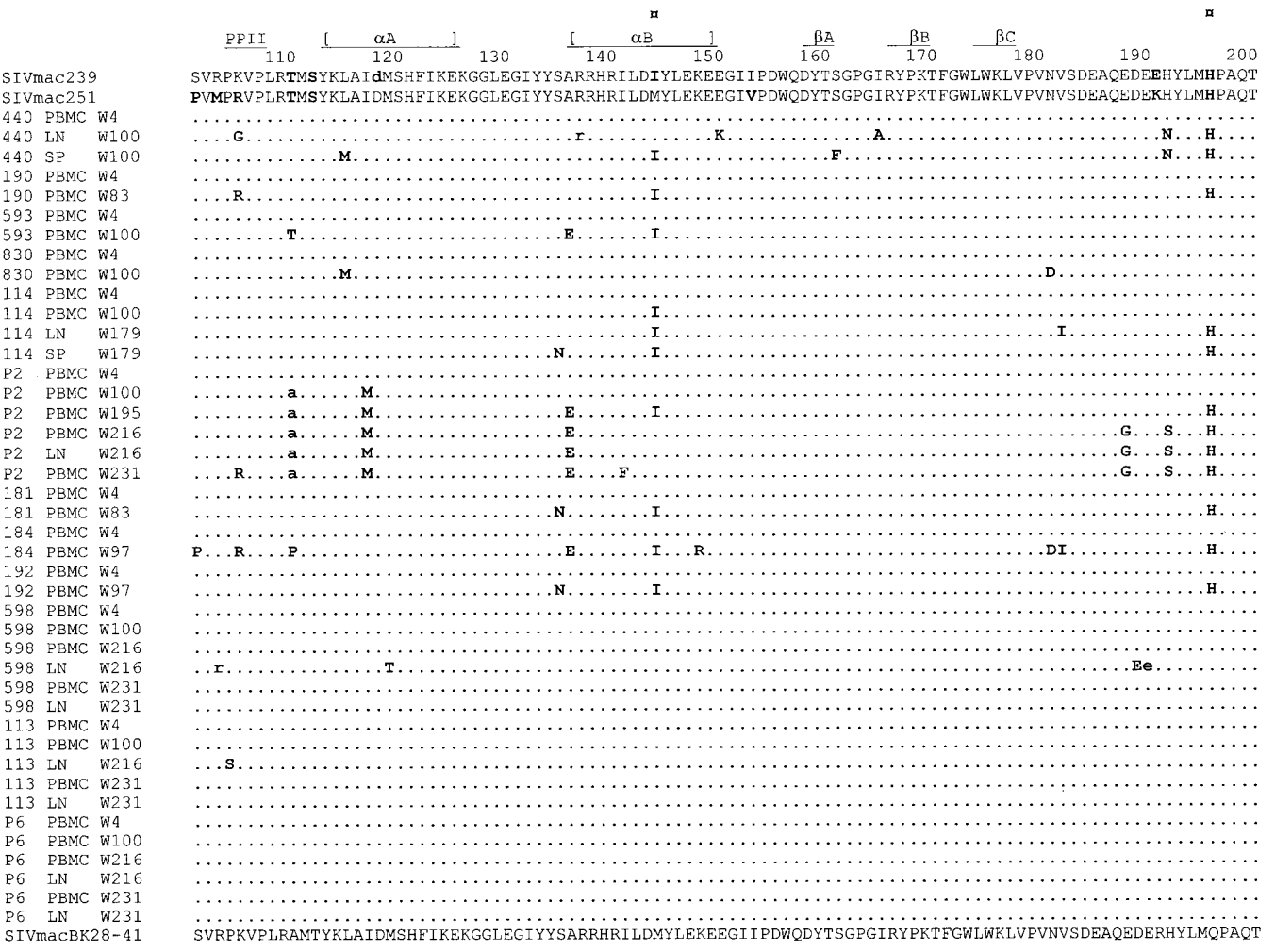

FIG. 2—*Continued*

two exceptions (Table 1, Fig. 2). One monkey of group II (P2) had a mixed population of *nef* genes, the major population being identical to that of the *nef* gene of the other animals, whereas the minor population already had the insertion of one thymidine in position 699 which restored the ORF to a length similar to that of SIVmac239 *nef* open. The *nef* genes obtained from macaque PH440 (group I) were also a mixed population, with the majority carrying the thymidine insertion. Sequences obtained from spleen and lymph node samples collected at necropsy on this macaque (100 weeks postinfection) showed that only the provirus with the T insertion could be recovered from these two organs. At the same time, the *nef* sequences obtained from macaque PBMC of group II had reverted, whereas no change was found on the *nef* gene in group III. Later during infection two animals from group II died and the evolution of *nef* sequences obtained from lymph node and spleen cells of macaque 49114 was similar to that of macaque PH440. The original *nef* allele was undetectable and only provirus harboring the T insertion was found. In addition, some nonconservative mutations accumulated. Similar observations were made with the *nef* sequences of P2, with a more pronounced effect of mutation fixation (Fig. 2). The situation is more complex in group III. In one macaque (P6), where the viral load remained below the threshold of quantitation after 4 weeks postinfection, except on one occasion, the *nef* gene conserved a SIVmacBK28-type sequence in the PBMC as well as in the lymph nodes. In a second animal (49113), the insertion was found first in the lymph node at week 216 (the *nef* sequence could not be obtained at that time from the PBMC, presumably because of very low viral load) and 15 weeks later in PBMC and lymph nodes. In macaque 33598, the virus was detected at two time points. The *nef* sequences were of the SIVmacBK28 type in PBMCs and of the SIVmac239 type in a lymph node at week 216, whereas at week 231 the *nef* population was mixed in PBMCs and without T699 insertion in another lymph node. It is possible that, as viral replication resumed after 4 years, the distribution of virus carrying the SIVmac239 type *nef* gene was heterogeneous in the animal's body. The sequence data showed that the insertion occurred early in group I, later in group II, with the exception of P2

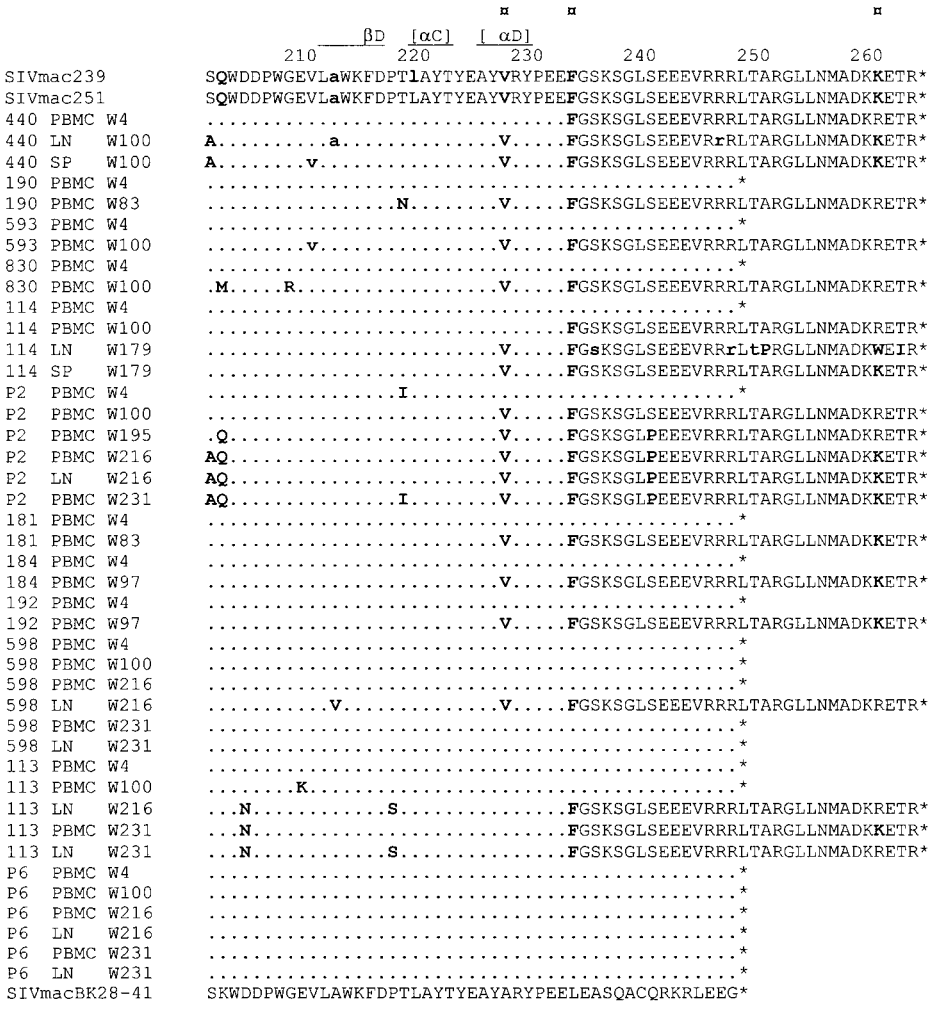

FIG. 2—*Continued*

(4 weeks), and more than 4 years postinfection or it has not occurred after this time in group III. Moreover, the overall *nef* sequence variability decreased from 1.21% in group I to 0.18% in group III (Table 2).

A phylogenetic analysis was performed with the 46 *nef* sequences using SIVmacBK28 and SIVmac239 *nef* sequences as references (Fig. 3). The sequences obtained at an early stage clustered around SIVmacBK28, reflecting the homogeneity of the molecular clone inoculated. Later sequences of group III were also found in the same cluster, whereas sequences of group I (PH440, 51190) and group II (33598, 49114, 35830, P2, 51184, 51181, 51192) tended to evolve toward the SIVmac239 *nef* sequence. A progressive evolution could be observed for macaques 49114, PH440, and P2. The analysis of the protein sequences encoded by the *nef* gene demonstrated that the fixation of mutations occurred with a fixed order (Table 3). The first event was the insertion in 699 of a thymidine leading to a frameshift and expression of a SIVmac239-type Nef C-terminal domain. The second mutation was a C to T transition at position 680, converting the Ala 227 into Val. The third event was an A to G transition at position 779, changing the Arg 260 into Lys. All these mutations were found in the macaques of groups I and II. In group III the thymidine insertion was found on rare occasions and at most one of the two other mutations (680, 779) was observed on the *nef* sequences.

DISCUSSION

In this study, we have demonstrated the importance of the C-terminal domain of the SIVmac Nef protein by analyzing the *in vivo* evolution of the *nef* gene. We have shown that recovery of virulence in the macaque is associated with the insertion of one nucleotide converting the C-terminal extremity of the SIVmacBK28-41 Nef protein into the domain of pathogenic SIVmac239.

Indeed, we have monitored two independent groups of rhesus macaques infected by the SIVmacBK28-41 molecular clone, derived from the attenuated molecular clone SIVmacBK28 by restoration of a full-length gp41.

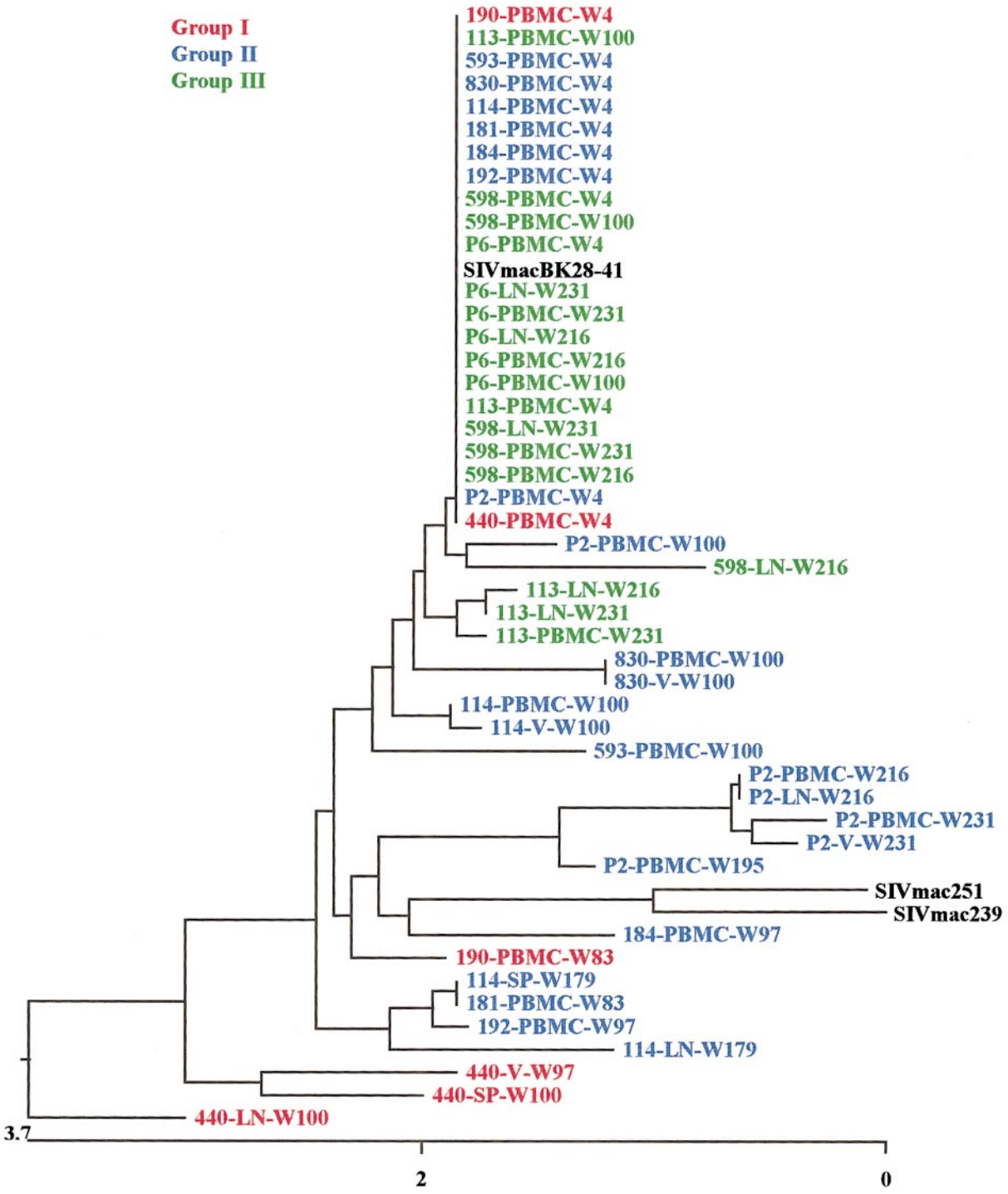

FIG. 3. Phylogenetic analysis of the *nef* sequences. The phylogenetic analysis was performed on the 792-bp sequences of the *nef* genes (791 bp for sequences without the insertion in position 699) starting at the Nef start codon. The sequences were aligned with the Clustal method from the Megalign program (Lasergene package, DNASTAR Inc., Wisconsin). Denomination of the sequences was identical to that of Fig. 2. Reference sequences of SIVmac239 and of the inoculated SIVmacBK28-41 are in black. Sequences of groups I, II, and III are, respectively, in red, blue, and green.

This modification has been shown to increase slightly the pathogenicity of the virus by shortening the median survival time from 7 years to less than 3 years (2/3 animals) (Edmonson *et al.,* 1998).

The clinical, hematological, and virological data allowed us to classify the infected monkeys into three separate groups. The first group was composed of macaques developing an AIDS-like disease in less than 2 years (rapid progressors). The outcome of the illness was delayed for the second group (progressors), whereas no clinical evo-

TABLE 3

Order of Mutation Fixation in the SIVmacBK28 *nef* Gene

Macaque	Time p.i[a]	Mutation position and amino acid change		
		680	699	779
SIVmacBK28		GCT Ala	TTG Leu	AGG Arg[b]
PH440	4	GCT Ala	TTT>TTG Phe>Leu	AGG Arg
	100	GTT Val	TTT Phe	AAG Lys
P2	4	GCT Ala	TTG>TTT Leu>Phe	AGG Arg
	100	GTT>GCT Val>Ala	TTT Phe	AGG Arg
	195	GTT Val	TTT Phe	AGG Arg
	216	GTT Val	TTT Phe	AAG Lys
	231	GTT Val	TTT Phe	AAG Lys
49114	4	GCT Ala	TTG Leu	AGG Arg[b]
	100	GCT Ala	TTT Phe	AGG Arg
	179 LN	GTT Val	TTT Phe	AAG Lys
	179 SP	GTT Val	TTT Phe	TGG Trp
SIVmac239		GTT Val	TTT Phe	AAG Lys
SIVmac251		GTT Val	TTT Phe	AAG Lys

Note. Examples of three hot spots (680, 699, and 779) are given for three macaques (PH440, group I; P2 and 49114, group II). For macaque 49114, the letters LN (lymph node) and SP (spleen) specify the tissue from which the sequences were obtained. The insertion in position 699 converts the TTG codon into TTT G, changing the open reading frame. When a mixed population was observed, the major and the minor populations are specified by the > sign.

[a] The time postinfection of the sequences is given in weeks.

[b] This codon encoding an arginine is located outside the Nef ORF in SIVmacBK28-type sequences.

lution was observed in the third group (nonprogressors), at least up to 281 weeks postinfection.

Three reasons prompted us to examine the *nef* gene from the provirus carried by these animals. First, a complete Nef protein is an important factor for the maintenance of a high viral load (Kestler *et al.,* 1991), a now well-recognized prognostic criterion indicative of a rapid development of AIDS and death (Ho, 1996; Mellors *et al.,* 1996; Ten Haaft *et al.,* 1998). Second, the follow up of some LTNP has indicated that HIV-1 with *nef* alteration could be responsible for the clinical nonprogression of certain infected individuals. Third, the *nef* sequence of attenuated SIVmacBK28-41 differs from the sequence of the pathogenic SIVmac239 *nef* open molecular clone by 23 point mutations and the deletion of one nucleotide generating a Nef protein with all structural domains intact but carrying a different C-terminal extremity. As the crystal and solution structures of HIV-1 Nef protein have been determined recently (Grzesiek *et al.,* 1996; Lee *et al.,* 1996), it is possible to identify the putative structural domains in the SIV Nef protein on the basis of the conserved amino acids. The majority of the mutations described in our study cluster in two unstructured domains localized in the N-terminal domain and between the C and D beta sheets (see Figs. 1 and 2). This is not surprising since similar data were obtained for the HIV-1 *nef* gene in humans and for the SIVmac239 *nef* gene in macaques (Shugars *et al.,* 1993; Zhu *et al.,* 1996). No mutation altered the domains indispensable for viral replication, such as the polypurine track, or regions involved in protein maturation, such as the myristylation site, the phosphorylation sites, or the PXXP kinase-binding domain. Nevertheless, the sequence adjacent to the PXXP domain was found to be mutated in one macaque (51184 at 97 weeks postinfection), creating a $(PXXP)_2$ domain similar to the kinase interaction site of HIV-1 Nef. The mutation converting Ala 136 into Glu may be due to the selection of a CTL escape mutant in the infected macaque, as it altered a CTL epitope describe in SIVmac251 Nef protein (Mortara *et al.,* 1998). Interestingly, Mortara *et al.* suggested that the CTL response in Nef-immunized macaques challenged with SIVmac251 is responsible for the selection of a minor population with Ala 136 changed into Thr. Furthermore, seven hot spots of mutation were identified, two of them are located in sequences encoding the unstructured N-terminal extremity, two in the B and D α helix, one in the unstructured loop between the C and D beta sheets, and one in the C-terminal domain. All these mutations can also be found in the sequence of the SIVmac239 *nef* gene. In their study on the evolution of SIVmacBK28 pathogenicity, Edmonson *et al.* (1998) have observed, in a variant having acquired an increased virulence, mutations residing in six of the seven hot spots of mutation described here.

The major alteration in the *nef* sequences analyzed was the insertion of one thymidine in position 699, creating a frameshift and allowing synthesis of a Nef protein with a SIVmac239 type C-terminal extremity. This phenomenon was observed for all animals in groups I and II and for some of the animals in group III at a very late time

after infection. Following this, a series of mutations occurred in the D α helix and in the C-terminal extremity. The frequencies of insertion and *nef* variability were higher in macaques from group I and decreased from group II to group III. As lentiviral variability is related to viral replication, the presence of the insertion in 699 and the expression of the C-terminal domain may be associated with an increase in viral replication followed by an acceleration in viral pathogenesis.

In vivo restoration of Nef protein is not an uncommon phenomenon. The initial SIVmac239 molecular clone possesses a premature stop codon at position 93 (Regier and Desrosiers, 1990), but *in vivo* the TAA mutates into GAA (Glu), restoring the *nef* ORF and the viral capacity to induce an AIDS-like disease in macaques (Kestler *et al.,* 1991; Zhu *et al.,* 1996). Two molecular clones, the J5 pathogenic and the C8-attenuated viruses, were obtained from the SIVmac32H isolate. They differ only by a 12-bp-long in-frame deletion in the sequence encoding the B α helix of C8 Nef protein. However, after a certain time of infection, some animals inoculated with the attenuated C8 virus evolve clinically to AIDS, the restoration of viral pathogenesis being linked to the restoration of the 12 bp by a sequence duplication process (Whatmore *et al.,* 1995). In contrast to these two examples, where structural domains were completely (SIVmac239 *nef* stop) or partially (C8 virus) lost, SIVmacBK28-41 had all the structural elements, and after evolution the last 15 amino acids were replaced by 31 amino acids, due to the frameshift. Despite being altered and encoding a shorter protein, no deletion was detected in the *nef* gene of SIVmacBK28-41. This suggests that the Nef protein of this virus is at least partially functional. Indeed, progressive extension of *nef* deletion was observed for viruses with nonfunctional *nef* genes (Deacon *et al.,* 1995; Kirchhoff *et al.,* 1994). The mechanism involved in the restoration of the *nef* gene in SIVmacBK28-41 is probably the stuttering of the reverse transcriptase allowing the insertion, next to two thymidines, of a third one in position 699.

The unique site of insertion stressed the functional difference between SIVmacBK28 and the SIVmac239-type Nef C-terminal extremity. Moreover, the order of mutation fixation suggests that expression of the C-terminal domain requires some structural adjustments, leading to selection of the *nef* gene with the other mutations identified. The synthesis of a Nef protein with a SIVmac239-type C-terminal extremity confers a structural and/or functional advantage to the virus. It has been shown previously that replacing the last 38 amino acids of SIVmac239 Nef with the last 11 residues of HIV-1 SF2 Nef dramatically decreases the stability of the transiently expressed protein (Garcia and Foster, 1995).

Stabilisation of the Nef protein in infected cells could thus increase its *in vivo* activity and, consequently, viral pathogenicity. However, we cannot exclude the fact that other variations in the lentiviral genome, in particular the *env* gene, contribute to increased virulence. Nevertheless, we can rule out the involvement of a fourth SP1 binding site in the LTR of the SIVmac251 isolate and SIVmac239, as it was never observed in our sequence analysis of SIVmacBK28-41 variants.

MATERIAL AND METHODS

Virus

The molecular clone SIVmacBK28-41 is derived from SIVmacBK28 (kindly provided by Dr. J. Mullins), in which the premature stop codon present in the *env* gene was mutated in order to restore a complete *env* ORF (Dunn *et al.,* 1997). This premature stop codon, probably due to virus cultivation in human cells, was shown to revert spontaneously *in vitro* in simian cells and *in vivo* in infected macaques (Hirsch *et al.,* 1989; Kodama *et al.,* 1989). This restoration, however, only marginally increases the virulence of SIVmacBK28 (Edmonson *et al.,* 1998). The virus stock was produced on macaque PBMC and titrated in rhesus macaques (Dunn *et al.,* 1997).

The SIVmac251 isolate, kindly provided by Dr. R. Desrosiers, was propagated on macaque PBMC.

Animals

Rhesus macaques were maintained according to the conditions stipulated in the European guidelines. All the animals were handled only after sedation with ketamine HCl (10 mg/kg; Imalgene, Mérieux). The animals were inoculated with cell-free virus stock in the saphenous vein. Blood samples were collected by femoral venipuncture.

A total of 12 rhesus macaques, forming two independent groups, were studied: 8 were included in a vaccination protocol and 4 were used for *in vivo* titration of the SIVmacBK28-41 stock. For the vaccination trial, 6 macaques were immunized with recombinant vaccinia viruses expressing either *gag* (vvTG4137—macaques PH440, 33598, 35830, P2) or *gag-pol-env* of SIVmac251 (vvTG6131—macaques 49113, 49114). Two control monkeys were immunized with wild-type vaccinia virus (macaques P6, 33593) and all the animals were challenged intravenously with 10 MID_{50} of SIVmacBK28-41 grown on macaque PBMC. The second monkey group, used for titration of the SIV stock, was formed by macaques 51192, 51184, 51190, and 51181. They were inoculated, respectively, with 32, 3.2, 1.6, and 0.8 MID_{50} of SIVmacBK28-41 (Dunn *et al.,* 1997).

Immunophenotyping

A standard whole blood staining method was used. Briefly, whole blood samples were incubated with anti-CD4-PE (OKT4-PE, Ortho, Roissy, France) and anti-CD8-FITC (Leu2a-FITC, Becton–Dickinson, Le Pont de Claix, France). Red blood cells were lysed with the "lyse and fix

reagents" according to the manufacturer's instructions (Immunotech, Luminy, France). Cell suspensions were washed, centrifuged, and then analyzed on a FACScan flow cytometer (Becton–Dickinson) in a lymphocyte gate defined by the FSC–SSC parameters, containing at least 5000 events.

Cell-associated viral load determination

Macaque PBMC were purified from heparinized blood by density gradient centrifugation on a Ficoll–Hypaque cushion (Eurobio, France). The quantitative determination of the cell-associated viral load was performed by cocultivating 2.5×10^6, 10^6, 5×10^5, and fivefold serial dilutions of the primary cells with 1.5×10^5 CEMx174 cells in 24-well plates. The cocultures were maintained for 4 weeks and treated twice weekly, once to change the culture medium (RPMI 1640 + 10% fetal calf serum heated at 56°C for 30 min + 2 mM Glutamax + 100 μg/ml streptomycin + 100 IU/ml penicillin) and once to divide the cells. The reverse transcriptase (RT) activity was determined at each time (Moog *et al.,* 1994) and its increase was taken as evidence of viral replication. The cell-associated viral load was expressed as the number of infected cells per 10^6 cells.

Lymph nodes were dilacerated with scissors and cells were dissociated by vigorous pipetting and then filtrated through a 70-μm gauze to obtain a single-cell suspension. The cells were pelleted and washed twice in PBS before being counted and cocultured as described above for the PBMC.

nef amplification and sequencing

DNA of PBMC, spleen, or lymph node cells was extracted by a phenol–trichloroethane technique following proteinase K digestion. Briefly, pellets of 10^6 cells were resuspended in 1 ml of lysis buffer (50 mM Tris–HCl, pH 7.4, 5 mM EDTA, 0.25% SDS, 150 mM NaCl, and 200 μg/ml Proteinase K) and incubated for 2 h at 56°C. The DNA was then extracted with phenol/trichloroethane and was ethanol-precipitated. After centrifugation, the DNA pellet was washed once with ethanol 70%, dried, and solubilized in 100 μl water.

DNA was submitted to PCR amplification with SIVmac *nef*-specific primers. A first PCR reaction was performed with NEF1 sense and NEF2 reverse primers. When a signal could not be detected, two heminested PCR reactions were performed in order to increase the amplification. NEF1 was used with reverse primer BO7096, whereas sense primer BO7095 was used with NEF2. This procedure was applied instead of a nested PCR reaction in order to avoid the loss of sequence data due to internal localization of the nested primers in the *nef* gene sequence. The PCR amplifications were carried out in a DNA thermal cycler (Perkin–Elmer) in a 100-μl reaction volume consisting of 75 mM Tris–HCl, pH 9.0, 20 mM $(NH_4)_2SO_4$, 0.01% (w/v) Tween 20, 2.5 mM $MgCl_2$, 200 μM dNTP, 35 pmol each sense and reverse primer, and 1.5 U of Gold Star DNA polymerase (Eurogentec, Serain, Belgium). The amplification program with NEF1 and NEF2 primers was as follows: 5 min denaturation at 95°C, 40 cycles of 1 min at 95°C, 1 min at 66°C, and 1 min at 72°C, and finally 10 min at 72°C. The heminested reactions were performed similarly: 25 cycles were used for these PCR amplifications and the hybridization temperatures were 66°C for NEF1/BO7096 and 64°C for the NEF2/BO7095. The primer sequences were the following: NEF1, 5′-AGGCTCTCTGCGACCCTACGA-3′, nucleotide positions 8995–9015 on SIVmacBK28 genome (GenBank Accession No. M19499); NEF2, 5′-AGAACCTCCCAGGGCTCAATCT-3′, positions 9994–9973; BO7095, 5′-CCTACCTACAATATGGGTGGAGC-3′, positions 9047–9069; and BO7096, 5′-CCTCTGACAGGCCTGACTTGCTTCC-3′, positions 9781–9756. The PCR products were separated on a 2% agarose gel and purified with the Wizard PCR preps DNA purification System (Promega Corp., Madison, WI) according to the manufacturer's specifications. The sequencing reactions were performed directly on the PCR products by Genome Express Company (Grenoble, France) using the fluorescent dideoxynucleotide terminator protocol and analyzed on an automated 377 ABI Prism sequencer.

Determination of major and minor populations was based on the fluorescence intensity of each nucleotide signal. As demonstrated by sequencing PCR products of mixtures of matrix with and without insertion in different proportions, our PCR system allows the detection of a minor population representing 25% of the mixture but not if present at 10%.

nef RT–PCR amplification from viral suspension

Genomic RNA of SIVmac251 or viral isolates was extracted by mixing 250 μl viral suspension with 750 μl Tri Reagent (Molecular Research Center Inc., Cincinatti, OH). After 5 min, 200 μl of trichloroethane was added and 5 min later the aqueous phase was recovered after centrifugation. The viral RNA was isopropanol-precipitated, pelleted by centrifugation, and washed with 70% ethanol. The RNA pellet was solubilized in 25 μl water.

Reverse transcription was carried out in 20 μl containing 35 pmol NEF2 reverse primer, 2 μl of 10× PCR Buffer II (Perkin–Elmer Applied Biosystem Division, Foster City, CA), 5 mM $MgCl_2$, 1 mM dNTP, 20 U RNAsin (Pharmacia Biotech, Uppsala, Sweden), and 50 U MuLV reverse transcriptase (Perkin–Elmer) and incubated for 30 min at 42°C. The *nef* sequence was amplified by PCR as described above for genomic DNA except that the NEF2 reverse primer, already present in the sample, was not added again in the PCR mix.

Sequence analysis

The *nef* sequences were reconstructed with the Editseq and Seqman softwares of the Lasergene package (Dnastar, Wisconsin). The *nef* sequences, covering 792 bp from the start codon (791 bp for SIVmacBK28), were aligned and a phylogenetic analysis was obtained using the Clustal method of the Megalign software. As references for our analysis, we have used the sequences of SIVmacBK28 and SIVmac239 (GenBank Accession No. M33262). For SIVmac239, the *nef* open sequence was chosen since the premature stop codon present at codon 93 reverts spontaneously in infected macaques (Kestler *et al.,* 1991; Regier and Desrosiers, 1990; Zhu *et al.,* 1996).

Statistical method

Pearson's χ^2 test for independence was used to compare the distribution of the 699 insertion using the StatXact-3 software (Cytel Software Corp., Massachusetts). For hot spot mutations the presence of zero to seven mutations was compared between the groups using Pearson's χ^2 test for independence with the Monte Carlo estimation.

ACKNOWLEDGMENTS

This work was supported by grants from INSERM, the French National Agency for AIDS Research (Agence Nationale de Recherche sur le SIDA), Institut Pasteur, and Synthelabo. Y.R. is an Elisabeth Glaser Scientist. We thank M. Methali for the plasmid recombinant pTG668 used to produce SIVmacBK28-41. We thank C. Spenlehauer and T. Kirn for critical reading of the manuscript.

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