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Extensively Deleted Simian Immunodeficiency Virus (SIV) DNA in Macaques Inoculated with Supercoiled Plasmid DNA Encoding Full-Length SIVmac239

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Using long-distance DNA PCR, we prospectively followed rhesus monkeys that had been inoculated intramuscularly with supercoiled plasmid DNA encoding intact simian immunodeficiency virus (SIV). From 4 to 10 weeks postinoculation onward, we detected extensively deleted proviral genomes along with full-length viral genomes in peripheral blood mononuclear cells (PBMC) in adult macaques. During their chronic asymptomatic phase of infection, the frequency of deleted proviral genomes was similar in PBMC and lymph nodes. The latter, however, harbored significantly more full-length proviral DNA than PBMC, consistent with the lack of effective antiviral cytotoxic T-cell activity in lymph nodes described by others during human immunodeficiency virus infection. After the macaques progressed to AIDS, full-length proviral DNA became equally abundant in lymph nodes and in PBMC. We have demonstrated that although a single molecular species of proviral DNA was inoculated, genomic diversity was detected within a short time, thus confirming the genetic instability of the SIV genome *in vivo.*

Key Words: truncated SIV DNA; intramuscular inoculation of proviral DNA; SIV replication *in vivo;* lymph nodes.

INTRODUCTION

Extensively deleted forms of human immunodeficiency virus type 1 (HIV-1) DNA accumulate in peripheral blood mononuclear cells (PBMC) of HIV-1-infected individuals and may represent over 90% of intracellular HIV-1 DNA (Sanchez *et al.,* 1997). In contrast, only rare subgenomic HIV-1 DNA fragments are present in cells infected *in vitro* (Sanchez *et al.,* 1997). The biological significance of defective viruses in the pathogenesis of HIV-1 infection is not clear. Interference of defective HIV-1 provirus or defective interfering (DI) particles with HIV-1 helper virus was suggested by experiments *in vitro* (Trono *et al.,* 1989; Little *et al.,* 1994; Bernier and Tremblay, 1995; Dropulic *et al.,* 1996) but was not observed *in vivo.* Defective viruses are generally thought to interfere with the replication of coinfecting viruses, and they may limit the establishment and maintenance of a persistent infection *in vivo* and the viral disease process (Holland and Villareal, 1975; Huang and Baltimore, 1977; Holland *et al.,* 1980). However, in the retrovirus family, defective murine leukemia virus was identified as the pathogen that induced severe immunodeficiency in mice (Aziz *et al.,* 1989). In cats, the appearance of a deleted feline leukemia virus correlated with the onset of disease (Overbaugh *et al.,* 1988). In addition to the interference phenomenon, integrated defective genomes of avian leukosis-sarcoma viruses and mammalian B-, C-, and D-type viruses provide a classical example of the phenotypic change in host cells caused by chromosome rearrangements at the integration site, *cis*- and *trans*-activation, superinfection immunity, and aberrant expression of cellular oncogenes.

In this report, we describe the formation of full-length and extensively deleted simian immunodeficiency virus (SIV) DNA in a longitudinal follow-up study of two adult rhesus macaques and one infant rhesus macaque after intramuscular inoculation of supercoiled plasmid DNA encoding full-length SIVmac239 (Liska *et al.,* 1999). Proviral DNA was amplified by long-distance (LD) polymerase chain reaction (PCR) from genomic PBMC, lymph node, and different organ DNA. In comparison to natural or experimental infection with live virus particles, DNA inoculation avoids the original bias caused by coinfection of the mixture of replication-competent and defective virus. In cell culture systems, the accumulation of cell-associated defective viruses is the consequence of fre-

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quent sequential passages of virus at high multiplicities of infection and of the substantially longer lifetime of nonproductively compared to productively infected cells (Coffin, 1996; von Magnus, 1954). According to this general mechanism, cells harboring deleted SIVmac239 proviruses might accumulate during persistent infection *in vivo* as the cells retain their typical life span. We studied the accumulation of deleted proviral DNA in peripheral blood and in lymph nodes, where the majority of the virus population replicates. The ratio of deleted to full-length proviruses in lymph nodes and peripheral blood can change during the course of infection due to a dynamic process of SIV-target cells trafficking in diverse lymphoid compartments (Veazey *et al.,* 1998, 2000) and different actions of virus-specific cytotoxic lymphocytes (CTL) during the early asymptomatic phase and during progressive disease (Soudeyns and Pantaleo, 1999; Kuroda *et al.,* 2000). The lack of expression of CCR7 and CD62L lymph node homing receptor molecules on effector $CD8^+$ CTL, specific for the chronic HIV infection, could be a major reason for inadequate immune protection against HIV replication in secondary lymphatic organs (Chen *et al.,* 2001).

We detected deleted SIVmac239 DNA isolated from PBMC of adult macaques as early as 4 weeks postinoculation (p.i.) and found that their titers generally followed the curve of plasma viremia. During the chronic asymptomatic phase of infection in adult monkeys, the titer of full-length SIVmac239 DNA was higher in lymph nodes than in PBMC, whereas the titer of deleted proviruses in both compartments was similar. However, after both animals had developed AIDS, full-length provirus DNA no longer predominated in lymph nodes. Our results demonstrate rapid development of SIV genomic diversity *in vivo* after inoculation of supercoiled proviral DNA.

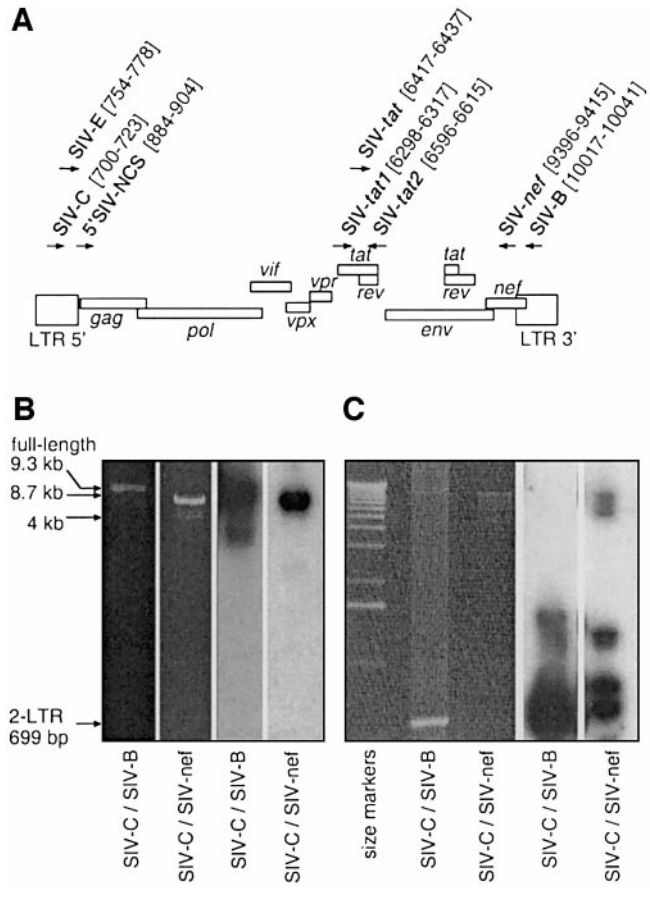

FIG. 1. Amplification of SIVmac239 DNA by LD-DNA PCR. (A) The organization of the SIV genome. Arrows indicate the primers and their orientation. Numbers in parentheses correspond to nucleotide positions in the SIVmac239 sequence. (B) LD-DNA PCR of 1000 copies of pBSIVmac239 linearized by *Aat*II using different primers and visualized by ethidium bromide staining of agarose gel (left) and by Southern blot using the SIV-E probe (right). (C) LD-DNA PCR of 600 and 200 ng of PBMC DNA of macaque 270W extracted 28 weeks after inoculation with plasmid DNA encoding full-length SIVmac239 and amplified by primers SIV-C/SIV-B and SIV-C/SIV-*nef,* respectively. Full-length products amplified by primers SIV-C/SIV-B and SIV-C/SIV-*nef* are composed of 9289 and 8716 nt, respectively. Two-LTR circles amplified by primer pair SIV-C/SIV-B are 699 nt long.

RESULTS

Clinical course of SIV infection in rhesus macaques after intramuscular inoculation of DNA plasmids encoding full-length SIVmac239

Two adult monkeys and one infant monkey were inoculated with SIVmac239 DNA as described (Liska *et al.,* 1999). All three animals became highly viremic and did not display typical acute peak viremia. Instead, there was only a minor drop in viral RNA levels consistent with high viral setpoints. Adult macaque 270W had a severe drop of its $CD4^+$ T cells to 59 cells/μl at week 20 p.i. The $CD4^+$ T cells subsequently recovered transiently and remained above 500 cells/μl until week 34 p.i., from which time on they remained persistently below 500 cells/μl. From week 46 p.i. onward, the absolute $CD4^+$ T-cell counts were consistently below 200 cells/μl, consistent with the diagnosis of AIDS. This animal also developed severe thrombocytopenia at week 25 p.i., which persisted with some fluctuations in the platelet counts. At necropsy at week 54, this animal had moderate to severe lymphoplasmocytic and eosinophilic gastroenterocolitis, mild eosinophilic bronchopneumonia, minimal glomerular sclerosis of the kidneys with minimal interstitial nephritis, and generalized lymphoid hyperplasia.

In adult 458U, the absolute $CD4^+$ T cells fell below 500 cells/μl at 30 weeks p.i., and from 46 weeks p.i. onward, the $CD4^+$ T-cell count remained below 200 cells/μl, which is consistent with the diagnosis of AIDS. This animal was also anemic. At necropsy at week 54, mesenteric lymphadenopathy, splenomegaly, and moderate multifocal interstitial pulmonary infiltrates were seen. The stomach, which showed mild gastritis, was infected

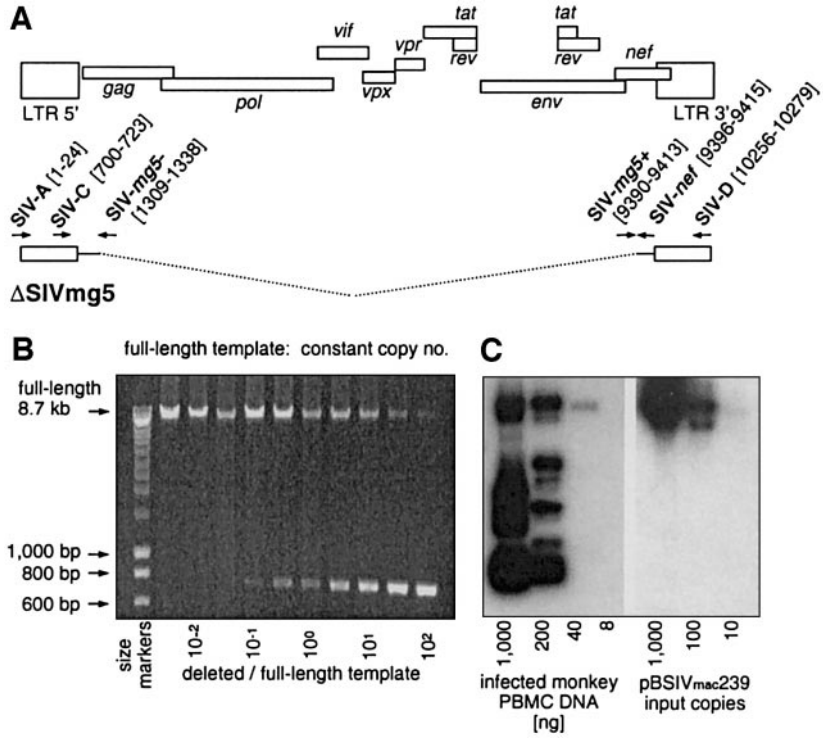

FIG. 2. Titration of full-length and truncated SIV DNA. (A) Schematic representation of a truncated ΔSIVmg5 standard deleted between SIVmac239 nt 1338 and 9390. Arrows indicate the primers used for its construction and their orientation. Numbers in parentheses correspond to nucleotide positions in the SIVmac239 sequence. (B) Ethidium bromide-stained gel for LD-DNA PCR analysis of the constant amount of 5×10^4 input molecules of full-length SIVmac239 DNA (8716-nt PCR product) amplified in the presence of the indicated molar ratios of ΔSIVmg5 DNA (709-nt PCR product) to full-length SIVmac239 DNA using the primer pair SIV-C/SIV-nef. Lane 1 contains SmartLadder size markers (Eurogentec). The relative efficiency of LD-DNA PCR for both amplification products was calculated as the ratio of complete to deleted input molecules resulting after amplification in an equimolar output (Piatak *et al.,* 1993; Sanchez *et al.,* 1997). The equimolar point ($\Delta SIVmg5_{con}$/SIVmac239 = 1) was reached when 5×10^4 SIVmac239 molecules were amplified with 3.8×10^3 ΔSIVmg5 molecules. This indicates that the relative efficiency of amplification of the truncated DNA was approximately 10-fold higher than that of full-length DNA. (C) Titration of SIVmac239 DNA extracted from PBMC of macaque 270W at 14 weeks postinoculation. Five- and 10-fold serial dilutions of PBMC genomic DNA of macaque 270W and of pBSIVmac239 external standard were amplified by LD-DNA PCR using primers SIV-C/SIV-nef in the presence of 1 μg of genomic PBMC DNA of an SIV-negative monkey.

with *Helicobacter* bacteria. The small bowel showed mild-to-moderate lymphocytic, plasmacytic, and eosinophilic enteritis. Most lymph nodes revealed reactive lymphadenitis, whereas follicular hyperplasia with prominent blastogenesis and lymphoid necrosis were found in the spleen.

The clinical course and necropsy findings of infant BR-2 have been described (Liska *et al.,* 1999). It had rapidly progressive disease with severe anemia and was euthanized at week 10 p.i. Necropsy showed severe acute and subacute proliferative glomerulonephritis, severe ulcerative hemorrhagic gastritis, and moderate lymphoid depletion. The renal pathology resembled that seen in HIV nephropathy (Liska *et al.,* 1999).

Long terminal repeat (LTR)(U5) and *nef* gene primers amplify truncated SIVmac239 DNA

Oligonucleotide primers located in the LTR(U5) region (upstream primer) and in the LTR(R) region (downstream primer) were shown to amplify extensively deleted forms of HIV-1 DNA from PBMC of infected individuals by LD-DNA PCR (Sanchez *et al.,* 1997). Similarly positioned oligonucleotide primers in SIV DNA, SIV-B and SIV-C (Fig. 1A), were tested for amplification of the whole internal sequence of the provirus, flanked by parts of the LTRs. The expected 9.3-kb band was the major LD-DNA PCR product of the plasmid pSIVmac239 linearized by *Aat*II in the pUC sequence (Fig. 1B). In contrast, a 699-nt band corresponding to the LTR–LTR junction of the two-LTR circle (Fig. 1C) represented the major LD-DNA PCR amplification product in all PBMC samples tested that had been collected from macaques inoculated with plasmid DNA encoding full-length SIVmac239. No full-length SIV DNA was detected using these primers and Southern blot hybridization in any PBMC sample (Fig. 1C). A higher efficiency of LD-DNA PCR for shorter templates than for longer ones results in a preferential amplification of 646-nt over longer products by the primer pair SIV-C/SIV-B. Previously, we showed in the HIV-1 system that the relative intensity of the amplified bands ranging from 0.5

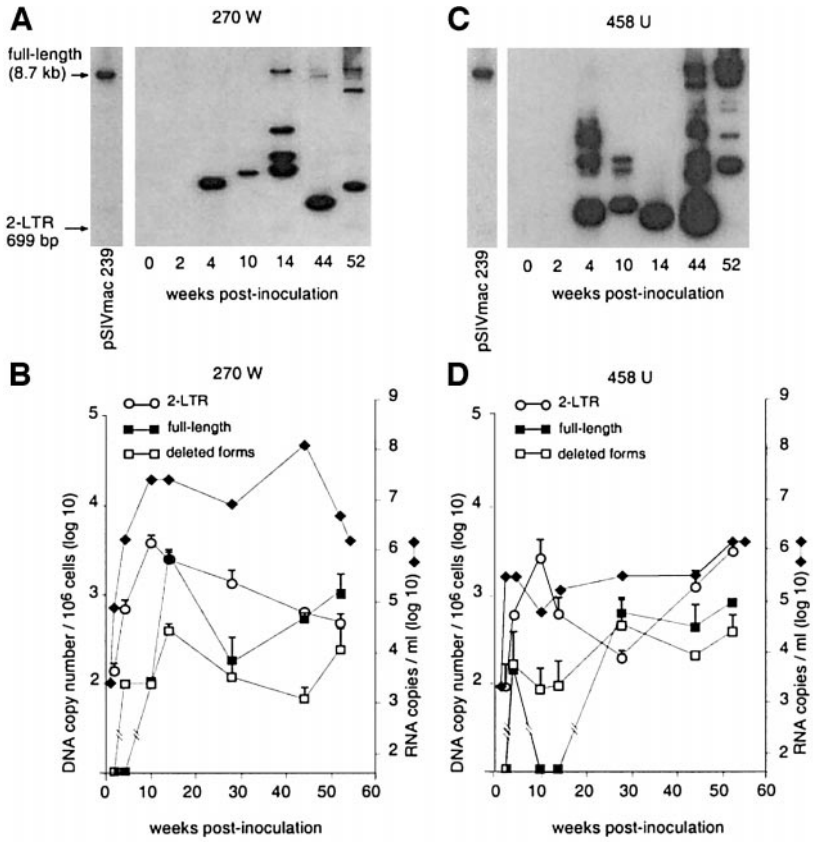

FIG. 3. Levels of full-length and deleted SIVmac239 DNA in PBMC of infected adult macaques 270W (A, B) and 458U (C, D). PBMC DNA (200 and 400 ng) of macaque 270W (A) and 458U (C), respectively, was amplified by LD-DNA PCR using primers SIV-C/SIV-*nef* and visualized by Southern blot with a ^{32}P-labeled SIV-E probe. Plasmid pBSIVmac239 linearized by *Aat*II and amplified under the same conditions is shown on left. (B, D) Plasmid viral RNA load (◆) was determined by quantitative competitive, reverse transcriptase-PCR, scale on the right (Liska *et al.,* 1999); two-LTR circular SIVmac239 DNA was amplified by standard PCR using primers SIV-B/SIV-C. Titers of full-length (■), deleted (□), and two-LTR circular (○) SIVmac239 DNA were calculated from end-point dilutions, starting with 1 μg of PBMC DNA, scale on the left; background values were arbitrarily set one dilution lower. Broken lines indicate points below the level of sensitivity. Shown are the means + SD of at least two independent experiments.

to 9 kb is inversely proportional to their size (Sanchez *et al.,* 1997, 1998).

To amplify full-length SIV DNA in PBMC of infected macaques in the presence of a large quantity of two-LTR circles, the primer pair SIV-C/SIV-*nef* was tested. Just the upstream oligonucleotide primer in this pair was localized in the LTR (Fig. 1A), thus precluding amplification of two-LTR circles. This pair of primers yielded a full-length amplification product using the linearized plasmid pSIVmac239 template (Fig. 1B). It resulted in highly specific amplification of full-length and deleted viral DNA from PBMC of an infected monkey (Fig. 1C) and was used in the majority of our experiments.

Full-length SIVmac239 provirus can be detected in the presence of a 100-fold molar excess of truncated SIV DNA

The higher efficiency of PCR for amplification of shorter fragments can reduce the ability to detect full-length SIVmac239 DNA amplified in the presence of deleted virus. To determine the efficiency of amplification of full-length SIVmac239 provirus in a background of deleted viral genomes, we performed a reconstitution experiment, in which a constant amount of full-length SIVmac239 templates was amplified in the presence of variable quantities of ΔSIVmg5, an SIVmac239 fragment missing 8 kb from the central region of the SIV genome (Figs. 2A and 2B). Using the primer pair SIV-C/SIV-*nef,* the full-length SIVmac239 and the truncated ΔSIVmg5 templates yielded amplification products of 8716 and 709 nt, respectively. No heteroduplexes were detected in repeated LD-DNA PCR runs. Despite the higher efficiency of LD-DNA PCR for amplification of shorter fragments, the full-length SIVmac239 DNA was detected in the presence of a 100-fold molar excess of truncated input DNA (Fig. 2B).

An example of the titration analysis is shown in Fig. 2C. Comparison of the limiting dilutions of PBMC genomic DNA from an infected monkey and plasmid pBSIVmac239 revealed that the sensitivity level for full-length proviral DNA was approximately 10 copies per microgram of DNA and that approximately 1700 and 170

copies of full-length and truncated SIVmac239 DNA, respectively, were present per one million PBMC of macaque 270W at 14 weeks postinoculation. The higher efficiency of LD-DNA PCR for amplification of shorter fragments can hide the presence of the full-length SIV DNA at a high concentration of deleted sequences (Sanchez *et al.,* 1997, 1998). As illustrated in Fig. 2C, this bias can be overcome at end-point dilutions; although multiple truncated forms were detected in the amplification product of 200 ng of PBMC DNA, only the full-length SIVmac239 DNA was amplified from 40 ng of PBMC DNA template.

Early appearance of deleted SIVmac239 DNA in PBMC

Formation of full-length, extensively deleted, and two-LTR circle SIV DNA was followed throughout the course of infection of the two adult macaques inoculated with supercoiled plasmid DNA encoding full-length SIVmac239 (Fig. 3). To titrate SIVmac239 DNA, fivefold serial dilutions of genomic DNA were amplified by long-distance DNA or standard DNA PCR and hybridized with the SIV-E probe. Distinct populations of extensively deleted SIVmac239 genomes of variable size were detected in PBMC of both macaques 270W and 458U as early as 4 weeks postinoculation (Figs. 3A–3D). LD-DNA PCR assays in all lanes of Figs. 3A and 3C were conducted with one constant amount of genomic PBMC DNA; consequently, average values of at least two serial end-point dilutions shown in Figs. 3B and 3D were not reached for most lanes. The titers of extensively deleted and full-length SIVmac239 proviruses as well as those of two-LTR circles resembled the levels of plasma viremia. This is in agreement with data showing that the titers of two-LTR circles reflect plasma viremia (Polacino *et al.,* 1995), similar to the HIV system (Pauza *et al.,* 1994). The titers of full-length SIVmac239 DNA at most time points were higher than those of truncated forms. Since end-point dilutions were conducted, full-length SIVmac239 DNA could be analyzed at these time points without interference of shorter fragments, which are amplified more efficiently as shown earlier.

Antibody responses were detected in both adult macaques from 4 weeks postinoculation onward by Western blot analysis (Fig. 4). While the anti-Env antibody responses became more intense with time, we noted a selective loss of anti-Gag antibody responses from weeks 14 and 28 postinoculation onward, respectively, from both adults. The selective loss of these antibody responses is a poor prognostic sign in HIV-infected individuals as well as in SIV-infected monkeys and heralds the onset of immunodeficiency (Binley *et al.,* 1997). Indeed, AIDS developed in both animals at week 46.

At early time points, plasma viral RNA levels were high, whereas relatively few full-length SIV proviral genomes were found in PBMC DNA. The peaks of full-length and defective SIV DNA in macaques 270W and 458U appeared 4 and 2 weeks after the onset of plasma viremia, respectively. In agreement with the titration of the *gag* gene performed by DNA PCR in PBMC of the same macaques (Liska *et al.,* 1999), the level of all three forms of SIVmac239 DNA was at least 1000 times lower than the plasma viral RNA load. The minimal titers of full-length SIV DNA during the chronic asymptomatic phase were approximately 10-fold lower than the titers at the peak of primary viremia or at end-stage disease (Figs. 3B and 3D). This correlates with the disappearance of the full-length proviral DNA signals in Southern blots 10 and 14 weeks p.i. in macaque 458U (Fig. 3C).

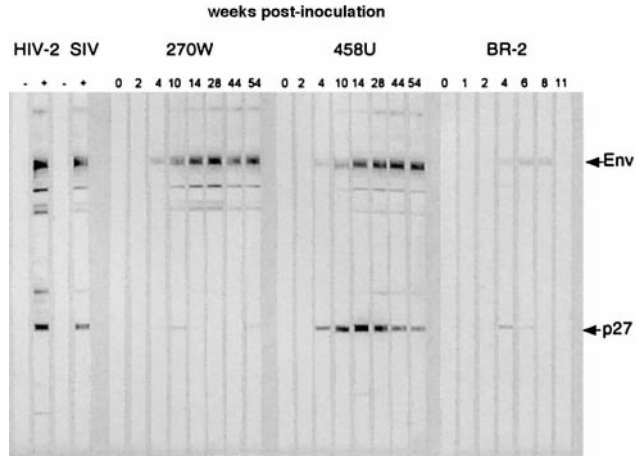

FIG. 4. Western blot analysis of antibody responses in two adult (458U and 270W) macaques and one infant (BR-2) macaque. The numbers above the lanes represent weeks postinoculation.

Prevalence of full-length SIV proviral DNA in lymph nodes during chronic, asymptomatic infection

LD-DNA PCR of paired DNA samples extracted 28 weeks p.i. from PBMC and lymph nodes of the two adult macaques resulted in amplification of both full-length and truncated SIV DNA (Fig. 5). The distribution pattern of proviral DNA forms in lymph nodes resembled that observed when peripheral blood lymphocytes were infected with SIV *in vitro,* with full-length SIVmac239 DNA being more prevalent than in matched PBMC DNA samples. To determine the absolute copy number for full-length and truncated forms, three to five independent LD-DNA PCRs were run for each sample. The efficiency of amplification was determined in each separate LD-DNA PCR run by amplification of serial dilutions of external standards as comparison (Figs. 5F and 5G). The amplification efficiency of full-length SIVmac239 DNA was 10 to 100 times lower than that of the 709-bp amplification product of plasmid ΔSIVmg5.

During the chronic phase of infection at 28 weeks p.i. of macaque 270W, full-length SIVmac239 DNA was mark-

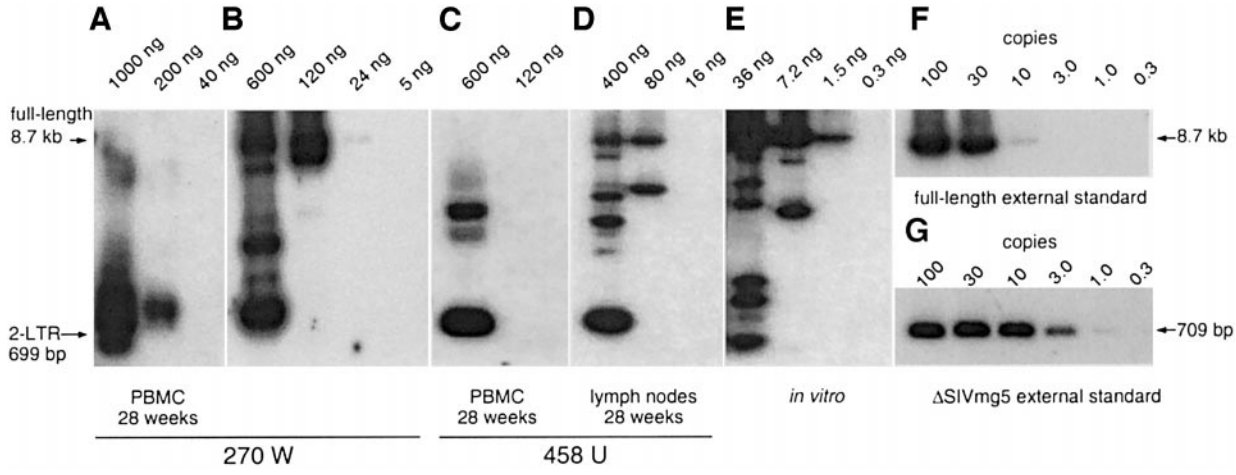

FIG. 5. Titration of SIVmac239 DNA in PBMC (A, C) and lymph nodes (B, D) of macaque 270W (A, B) and 458U (C, D) and *in vitro* infected PBL (E). Fivefold serial dilutions of genomic DNA amplified by LD-DNA PCR using primers SIV-C/SIV-*nef.* PBMC and lymph node DNA was extracted 28 weeks p.i.; DNA from *in vitro* infected PBL was extracted 10 days p.i. Serial dilutions of external standards, (F) full-length pBSIVmac239 and (G) ΔSIVmg5 DNA, both linearized by *Aat*II. The sensitivity levels for truncated SIVmac239 DNA were equivalent to 1 plasmid copy. The sensitivity levels for full-length proviral DNA determined by comparison with a dilution series of pBSIVmac239 external standard were 10 (A, B, D, E) and 100 (C) copies.

edly more prevalent in the lymph nodes than in the PBMC ($P = 0.001$; Fig. 6). Full-length SIVmac239 DNA in lymph node was also significantly more prevalent than deleted forms in the same tissue ($P = 0.0007$). These differences were statistically significant after Bonferroni correction for multiple comparisons. Borderline predominance of full-length SIVmac239 DNA in lymph nodes compared to PBMC was also observed in macaque 458U. In contrast, the relative frequency of deleted SIVmac239 proviruses was similar in both compartments of these animals. The marked predominance of full-length proviruses found in the lymph nodes at this time point resembled the predominance of full-length proviruses observed when PBL were infected *in vitro;* full-length DNA was the major product of SIV DNA amplified from these lymphocytes (Fig. 6). After progression to AIDS, the differences between the full-length SIVmac239 DNA in lymph nodes and in PBMC were no longer significant ($P \leq 0.14$). Full-length SIVmac239 DNA in lymph nodes during the chronic phase of infection of macaque 270W was significantly more prevalent than the full-length SIVmac239 DNA in lymph nodes after progression to AIDS ($P = 0.01$). The full-length SIVmac239 DNA of macaque 458U showed in the same comparison a similar trend ($P = 0.02$).

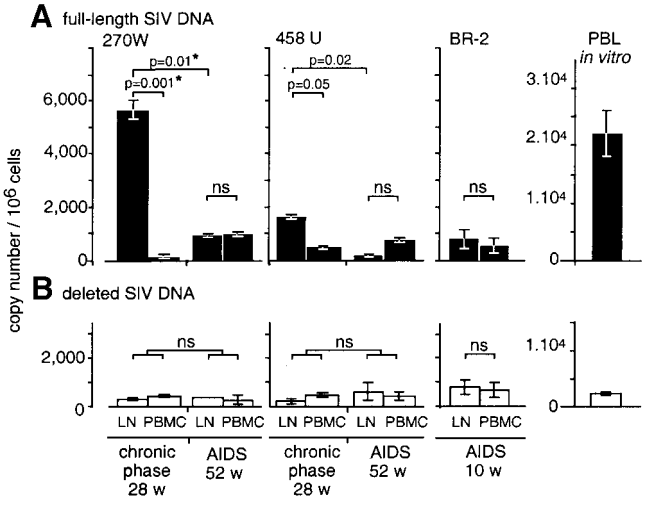

FIG. 6. Titers of full-length (A, black columns) and truncated (B, open columns) forms of SIVmac239 DNA in lymph nodes and PBMC of adult macaques 270W and 458U, infant BR-2, and PBL infected by SIVmac239 *in vitro.* To determine the absolute copy number for full-length and deleted forms, three to five independent LD-DNA PCRs were run for each genomic DNA sample. Titers were calculated as geometric mean titers of independent experiments in which DNA end-point dilutions were analyzed. Shown are the means ± SD of three to five independent experiments. Statistically significant differences between the titers of full-length SIVmac239 DNA in lymph node and the respective DNA sample are indicated (ANOVA followed by Student's *t* test; The numbers above the brackets represent *P* values. ns, not significant; *statistically significant after Bonferroni correction for multiple comparison).

SIV proviral DNA patterns in a macaque infant with fulminant disease

The infection of infant macaque BR-2 progressed rapidly; its plasma viral load increased steadily without a noticeable peak (Liska *et al.,* 1999). When the animal became moribund and was euthanized 10 weeks postinoculation, its plasma viral RNA loads were approximately 10^9 RNA copies/ml (Liska *et al.,* 1999). This infant macaque had only a weak, transient seroconversion 4 weeks postinoculation, which is indicative of fulminant disease (Fig. 4). Even though the lymph node specimens of the infant were taken at 10 weeks postinoculation, the differences between the full-length SIVmac239 DNA in

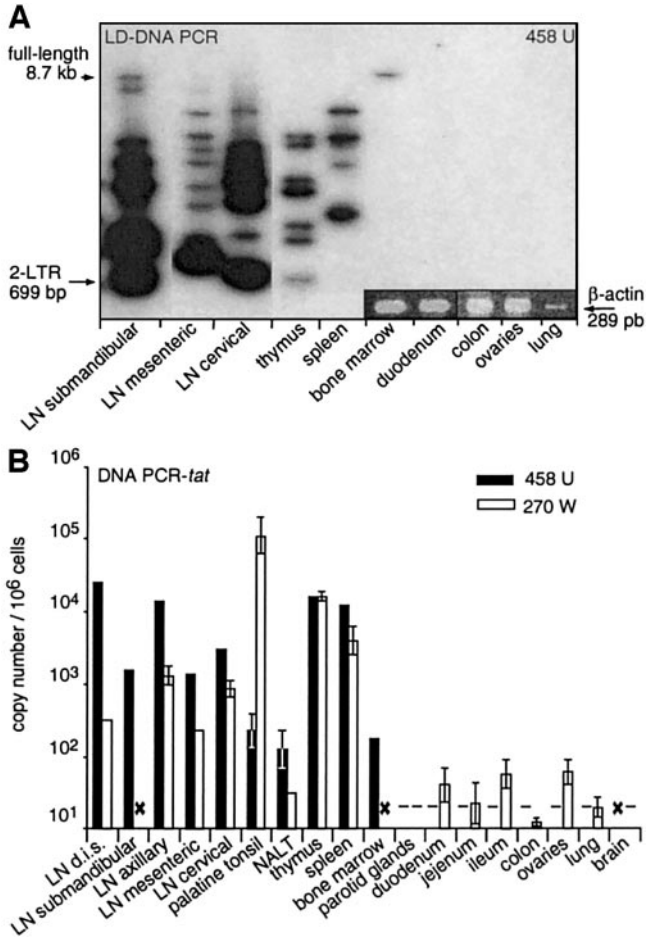

FIG. 7. Amplification of SIVmac239 DNA extracted from different necropsy tissues of macaque 458U by LD-DNA PCR (A) and of macaque 270W (black columns) and macaque 458U (open columns) by standard DNA PCR of *tat* gene sequences (B). Titers of *tat* were calculated from end-point dilution of DNA amplified by primers SIV-*tat1*/SIV-*tat2* and visualized by Southern blot with ^{32}P-labeled SIV-*tat* probe. Shown are the means ± SD of two independent experiments. −, no detectable signal; ×, no available sample.

lymph nodes and that in PBMC were not significant, and the full-length viral DNA forms were not more prevalent than truncated forms (Fig. 6). In contrast, the full-length proviral DNA in lymph node biopsies taken from adult animals 270W and 458U at 28 weeks postinoculation, during the clinically asymptomatic phase, was significantly more prevalent than its deleted form. Thus, ratios of full-length to deleted proviral DNA in lymph nodes of infant macaque BR-2 resembled the results obtained during end-stage disease in the adult macaques.

Proviral DNA forms in tissues obtained at necropsy

Extensively deleted SIVmac239 DNA segments were amplified by LD-DNA PCR in different tissues obtained from the necropsy samples. Full-length SIVmac239 DNA was detectable in tissue DNA of submandibular lymph nodes and bone marrow of macaque 458U (Fig. 7A). As expected, the highest titers of extensively deleted SIVmac239 DNA were found at the sites of virus replication, in lymphoreticular tissue, lymph nodes, palatine tonsils, thymus, and spleen (Fig. 7A). No LD-DNA PCR signal was obtained from genomic DNA isolated from duodenum, colon, ovaries, and lung of macaque 458U, and only a sporadic amplification was detected from genomic DNA of these tissues of macaque 270W (not shown). Amplification of the β-actin gene indicates that DNA from these tissues was not degraded. The distribution of proviral DNA titers determined in different tissues by LD-DNA PCR was similar to that obtained by more sensitive standard DNA PCR of the *tat* gene (Fig. 7B). An approximately 100 times higher titer of the *tat* gene was found in lymphoreticular tissue than in gastrointestinal tract, lung, and ovaries of macaque 270W. The latter tissues of macaque 458U remained free of detectable *tat* gene signals. No signals were detected in parotid glands and brain.

Analysis of SIV proviral DNA by standard PCR for *tat* sequences and two-LTR circles

Loss of full-length SIVmac239 DNA from lymph nodes during disease progression could artificially be caused by degradation of genomic DNA from necropsy samples. This does not seem to be the case because the necropsy samples were collected immediately after euthanasia and frozen at −70°C, and the extracted DNA comigrated in agarose gels together with high-molecular-weight DNAs extracted from PBMC and other biopsies. In addition, the same inverse distribution of SIVmac239 DNA between lymph nodes and PBMC in the asymptomatic period of infection and during disease progression was observed when provirus titration was performed by standard PCR of *tat* and two-LTR circles (Table 1), i.e., techniques that are less sensitive to DNA degradation than LD-DNA PCR.

DISCUSSION

In the present study, we have addressed the dynamics of SIV proviral DNA distribution in lymph nodes and in PBMC during the course of infection. During the chronic, asymptomatic phase, significantly more full-length proviral DNA was present in lymph nodes than in PBMC in adult monkeys. This confirms the finding that lymph nodes represent the site of preferential SIV replication at this stage of infection (Smit-McBride *et al.,* 1998; Stahl-Henning *et al.,* 1999; Veazey *et al.,* 1998). The titers of deleted SIVmac239 variants have been adjusted in this study by incorporating information about the number of bands that are discernible at the end-point dilution. This might underestimate the number of templates in case of overlapping bands, and the titers were reported would represent a minimum estimate. On the other hand, if heteroduplexes were formed, the number of templates

TABLE 1

Standard PCR Analysis for SIV *tat* and Two-LTR Sequences

Macaque	Titer	Chronic phase of infection[a]		Progressive disease[b]	
		Lymph node	PBMC	Lymph node	PBMC
270W	*tat*[c]	7810	2120	870	4050
	Two-LTR[d]	2100	695	865	625
	CD4 (%)[e]	ND	7	ND	4
458U	*tat*[c]	7810	290	6250	3125
	Two-LTR[d]	884	200	874	3125
	CD4 (%)[e]	ND	12	ND	4
BR-2[f]	*tat*[c]			15625	3125
	Two-LTR[d]			1670	1670
	CD4 (%)[e]			ND	6

Note. ND, not determined.

[a] Lymph node biopsy and PBMC collected at 28 weeks p.i.

[b] For adult macaques 270W and 458U, lymph node tissue collected at necropsy 54 weeks p.i. and PBMC were collected at 52 weeks p.i. For infant BR-2, tissues were collected at necropsy at 10 weeks p.i.

[c] Copy number of *tat* sequence per 10^6 cells. Cut-off value, 10 copies/10^6 cells.

[d] Copy number of two-LTR circles per 10^6 cells. Cut-off value, 50 copies/10^6 cells.

[e] Percentage of CD4-positive cells in the total PBMC population. This value permits restandardization of the proviral titers, expressed as copy numbers per million PBMC, to natural target cells of SIV.

[f] BR-2 was a rapid progressor that never had a clinically asymptomatic phase; viral loads never fell after the initial infection. Consequently, no peak of viremia was noted.

would be overestimated. The proportionality of the titers of deleted subgenomic fragments and dilution factors and the absence of artificially formed heteroduplexes in the reconstitution experiment, in which the full-length SIVmac239 was coamplified with the truncated one, suggest that these factors have not confounded our titration analysis. Although some deleted SIVmac239 genomes could be formed by the impact of rearrangements upon uptake and integration of intramuscularly (im) inoculated DNA (Bartsch *et al.,* 1997; Merrihew *et al.,* 1996), we do not think that this mechanism had seriously biased the interpretation of our results. Random recombinants have a very low chance to replicate and to disseminate to peripheral blood and different organs.

During the acute infection of adult macaques, plasma viremia preceded the appearance of full-length SIVmac239 DNA in PBMC, and only low titers of their proviral DNA were detected in PBMC. During the chronic asymptomatic phase of infection, full-length proviral DNA predominated in lymph nodes. This is indirect evidence that viral RNA is generated predominantly in lymphoid tissue at these time points. Striking predominance of HIV-1 replication in lymph nodes as compared with PBMC of HIV-1-infected patients has been demonstrated by the excess of full-length, singly spliced, and doubly spliced HIV-1 RNA in the former compartment (Pantaleo *et al.,* 1993; Meylan *et al.,* 1996). After the adult macaques in our study progressed to AIDS, full-length provirus DNA no longer predominated in lymph nodes. These results correlate with the distribution and selective depletion of SIV-target cells in different lymphoid compartments and peripheral blood (Veazey *et al.,* 2000).

In fact, the titer ratio of full-length to deleted forms of SIVmac239 in lymph nodes during the asymptomatic phase of infection was similar to that seen in peripheral blood leukocytes (PBL) infected *in vitro.* Assuming that in both cases the rate of the formation of defective genomes during one replication cycle of SIV is similar, this suggests that lymphocytes harboring full-length SIVmac239 DNA in lymph nodes are inefficiently eliminated by the immune system. In contrast, lymphocytes containing full-length SIV DNA may be efficiently targeted in peripheral blood. Thus, specific antiviral CTL responses seem to be ineffective in lymph nodes in comparison to the peripheral blood (Soudeyns and Pantaleo, 1999). Exciting new findings have revealed a lack of CCR7 receptors, molecules required for efficient trafficking to lymphoid tissue, on CD8 T cells specific for chronic HIV infection (Chen *et al.,* 2001). Essentially, this lack of lymph node homing molecules on antiviral $CD8^+$ CTL would spare infected viral target cells in lymph nodes but not in peripheral blood. This could result in higher loads of replication-competent virus in lymphoreticular tissue than in PBMC and hence higher numbers of viral target cells harboring intact proviral DNA at the early stages of infection and during the chronic, asymptomatic phase. Indeed, in HIV-infected individuals, infectious cells were 50-fold more frequent in lymph nodes than in paired PBMC samples (Meylan *et al.,* 1996). However, once host immunity collapses and CTL activity is lost during end-

stage disease, full-length proviral genomes appear with equal frequency in PBMC, as we postulate for adult macaques in our study.

We have studied SIV replication in infected macaques by focusing on the formation of extensively deleted DNA derived from pathogenic SIVmac239. Accumulation of deleted lentiviral DNAs in PBMC of infected animals, in contrast to *in vitro* infection, is related to continuous delivery of fresh target cells by the hematopoietic system. Cells harboring defective provirus could accumulate during repeated virus/cell cycles if proviral DNA is transcriptionally silent. In this case, the cells could escape detection and killing by cytotoxic T cells. Furthermore, defective viral genomes may not be able to induce host cell killing. In contrast, virus cytotoxicity *in vitro* in the absence of renewal of noninfected cells limits the accumulation of defective HIV-1 to a few cell divisions (Coffin, 1996). This might theoretically suggest that the titer of defective viruses continuously rises over the period of infection *in vivo.* However, instead of continuous growth, the titers of deleted proviruses followed titers of full-length SIVmac239 DNA, including their decline after the peak of primary viremia. This indicates a limited life span and dynamic renewal of lymphocytes harboring truncated SIVmac239.

In sum, we have shown that deleted SIV genomes appear rapidly in animals inoculated with pure, cloned supercoiled plasmid DNA encoding the full-length SIV genome. Although the number of monkeys evaluated was small, this study demonstrates clearly that diversity of SIVmac239 DNA is generated rapidly and confirms the genetic instability of the SIV genome *in vivo.*

MATERIALS AND METHODS

Animals and animal care

The animal experiments were approved by the Animal Care and Use Committees at Bioqual, Inc. (Rockville, MD) and the Dana-Farber Cancer Institute (Boston, MA). Ketamine anesthesia was used before all procedures that required the removal of animals from their cages.

DNA inoculation of monkeys

Two adult rhesus monkeys (270W and 458U) were inoculated im with 500 μg of supercoiled pBSIVmac239 as described in Liska *et al.* (1999). The neonatal macaque, BR-2, received a DNA dose of 400 μg im (Liska *et al.,* 1999).

Cell culture, virus preparation, and infection of tissue cultures

Human PBMC of healthy donors were separated on Ficoll-Hypaque gradients. Monocyte-depleted PBL were activated with phytohemagglutinin (PHA, Difco, Franklin Lakes, NJ) for a period of 3 days and infected at a multiplicity of infection of 0.1 with SIVmac239 obtained by transfection of HeLa cells with pBSIVmac239. Virus production was monitored as described by Barré-Sinoussi *et al.* (1983).

DNA extraction

PBMC ($\geq 10^6$ cells), the lymph nodes biopsies (about 50 μg) and the necropsy samples (100–300 μg), collected immediately after euthanasia, were frozen and kept at −70°C until DNA extraction. High-molecular-weight genomic DNA was isolated using DNAzol (Molecular Research Center, Cincinnati, OH) as described (Liska and Ruprecht, 1999). PCR amplification of the β-actin gene was used to test the ability of the DNAs to be amplified and to ensure that approximately equal amounts of genomic DNA were included in the reactions.

PCR amplification of viral DNA

To amplify SIV sequences, DNA samples in a background of 100 ng of carrier human PBL DNA were subjected to LD PCR in a 50-μl reaction mixture containing 50 mM Tris–HCl, pH 9.2, 14 mM $(NH_4)_2SO_4$, 1.75 mM $MgCl_2$, 350 μM each dNTP, and 45 pmol each of SIV-C, 5′-AGTGTGTGTTCCCATCTCTCCTAG-3′ (nt 700–723, sense) or SIV-NCS, 5′-AGTAAGGGCGGCAGGAACCAA-3′ (nt 884–904, sense) and SIV-B, 5′-TACCTGCTAGTGCTGGAGAGAACCT-3′ (nt 558–582, antisense) or SIV-*nef,* 5′-TAACTCATTGTTCTTAGGGG-3′ (nt 9396–9415, antisense) primers (Fig. 1A), with 1.75 units of enzyme (Expand Long Template PCR System, Roche, Basel, Switzerland) as described (Sanchez *et al.,* 1997). Reaction mixtures were then subjected to 40 amplification cycles. The first 10 amplification cycles comprised a denaturing step of 10 s at 94°C, a primer-annealing step of 30 s at 62°C, and a primer extension step of 8 min at 68°C. The primer extension step of the following 15 amplification cycles was gradually extended by 20 s at each cycle and was kept constant at 13 min for the last 15 cycles. A final extension step of 7 min at 72°C was performed. In addition to the whole internal sequence of SIV provirus, SIV-C and SIV-B primers span the LTR–LTR junction of the two-LTR circle and may generate a shorter amplification product (699 nt in the case of SIVmac239). The quantity of DNA molecules bearing either central or two-LTR circle sequences of the SIVmac239 genome was estimated by semiquantitative PCR using *tat* or SIV-C and SIV-B primers, respectively. Fivefold dilutions of DNA were assayed in a standard PCR with *Taq* polymerase and SIV-*tat*I primer, 5′-TAGACATGGAGACACCCTTG-3′ (nt 6298–6317, sense) and SIV-*tat2* primer, 5′-CAAGTAAGTATGGGATGTCT-3′ (nt 6596–6615, antisense) for the presence of a 317-nt amplification product. Tenfold dilutions of a known concentration of plasmid pBSIVmac239 standard DNA were amplified in a control experiment.

PCR products were transferred to a Hybond–N^+ membrane (Amersham, Life Science, Uppsala, Sweden) and

hybridized with ^{32}P-labeled oligonucleotide probes SIV-E, 5′-TAAGAAGACCCTGGTATGTTAG-3′ (nt 754–778, sense), and SIV-*tat,* 5′-GCGGTATAGCTGAGAGAGGAT-3′ (nt 6417–6437, antisense).

Preparation of truncated standard SIVmac239 DNA

To standardize LD-DNA PCR, a truncated form of SIVmac239 provirus missing the central region of the SIV genome (nt 1339–9389) was constructed. For this purpose, terminal parts of the SIV genome were amplified by PCR using primers SIV-A, 5′-TGGAAGGGATTTATTACAGTGCAA-3′ (nt 1–24, sense), and SIV-*mg5*−, 5′-CTTTCTCTTCTGCGTGAATGCACC-3′ (nt 1309–1338, antisense), for the 5′-end, and primers SIV-*mg5*+, 5′-AAGTTCCCCTAAGAACAATGAGTT-3′ (nt 9390–9413, sense), and SIV-D, 5′-TGCTAGGGATTTTCCTGCTTCGGT-3′ (nt 10,256–10,279), for the 3′-end (Fig. 1B). Both PCR products were cloned into the pGEM-T Easy Vector (Promega Inc., Paris, France). The 3′-terminal fragment of SIVmac239 DNA was excised by *Eco*RI and blunt-ligated into the *Sal*I-linearized pGEM-T Easy Vector containing the 5′-terminal fragment of SIVmac239 DNA. The selected clone ΔSIVmg5 contained the truncated SIVmac239 sequence with LTRs at both termini.

Titration of full-length and truncated SIV DNA

To titrate full-length and deleted SIV DNA, at least two parallel fivefold serial dilutions of genomic DNA were amplified by LD-DNA PCR and analyzed by gel electrophoresis followed by Southern hybridization by using ^{32}P-end-labeled SIV-E probe. The number of full-length proviral SIV DNA copies at the end-point dilution was determined by comparison with a dilution series of the target pBSIVmac239 external standard linearized by *Aat*II. Linearization precludes amplification of the vectorial part of the plasmid by primers localized in LTR sequences and a possible bias in amplification of the mixture of circular and linear forms present in plasmid preparations. The sensitivity level for full-length proviral DNA, determined at each LD-DNA PCR run, varied between 10 and 100 molecules. The sensitivity level for the truncated standard ΔSIVmg5 DNA was between 1 and 10 molecules, i.e., 10 times higher than that for the full-length SIV DNA. The titers of full-length and deleted SIV DNA, adjusted to 10^6 PBMC, were calculated from the number of copies at the end-point dilution, taking into account that 1 μg of DNA is contained in 150,000 PBMC.

The titer of deleted proviral forms was calculated by multiplying the dilution factor by the number of bands at the end-point dilution. Taking the number of bands into account improves the accuracy of the limiting dilution technique. Proportionality between the titer of deleted subgenomic fragments and the dilution factor was limited by the detection of about 10 bands per LD-DNA PCR, apparently because of competition among deleted fragments. Fewer than five deleted bands were detected at the end-point dilutions in titration experiments. That indicates proportionality of the fragment dose and the dilution factor.

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