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# Circular viral DNA detection and junction sequence analysis from PBMC of SHIV-infected cynomolgus monkeys with undetectable virus plasma RNA

Andrea Cara,[a] Maria Teresa Maggiorella,[a] Roberta Bona,[a] Leonardo Sernicola,[a] Silvia Baroncelli,[a] Donatella R.M. Negri,[a] Pasqualina Leone,[a] Zahra Fagrouch,[b] Jonathan Heeney,[b] Fausto Titti,[a] Aurelio Cafaro,[a] and Barbara Ensoli[a,*]

[a] *Laboratory of Virology, Istituto Superiore di Sanità, 00161, Rome, Italy*

[b] *Department of Virology, Biomedical Primate Research Center, 2280 GH Rijswijk, The Netherlands*

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**Abstract**

Extrachromosomal forms of human immunodeficiency virus (HIV)-1 can be detected in peripheral blood mononuclear cell (PBMC) from HIV-infected patients in the absence of detectable viral replication and are thought to be a sign of active but cryptic virus replication. No information, however, are available on whether these forms are also present in animal models for acquired immunodeficiency syndrome (AIDS) and on their relation with other methods of detection of virus replication. To this aim, a polymerase chain reaction (PCR) approach was used to detect and analyze unintegrated circular 2-LTR-containing forms in PBMC of simian human immunodeficiency virus (SHIV)89.6P infected cynomolgus monkeys with RNA levels ranging between $1.8 \times 10^6$ and less than 50 copies/ml of plasma. 2-LTR forms were detected in 96.5% of monkeys' samples above 50 copies/ml of plasma, whereas they were present in 75.8% of monkeys' samples below 50 copies/ml of plasma. Persistence of unintegrated viral DNA in monkeys with undetectable plasma RNA could indicate either stability in non-dividing cells or ongoing low levels of viral replication in dividing cells.


*Keywords:* 2-LTR; E-DNA; SHIV; Unintegrated DNA; Circular forms; Episomal DNA

## Introduction

During human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) infections, unintegrated forms of extrachromosomal DNA (E-DNA) readily accumulate in the infected cells or tissues throughout the course of the disease (reviewed in Cara and Reitz, 1997; De Milito et al., 2003; Sharkey and Stevenson, 2001). The E-DNA is constituted of both fully reverse-transcribed linear DNA and closed circular forms of DNA. Circular E-DNA is considered a marker of nuclear entry because it is detected only in the nucleus of the infected cell and, after circularization of the double-stranded linear DNA, contains either a single copy or a tandem double copy of the long terminal repeat (1-LTR and 2-LTR forms, respectively) (Bukrinsky et al., 1992). The 1-LTR and 2-LTR circular forms of DNA are considered terminal products of the reverse transcription process with a short half-life (Bowerman et al., 1987; Brown et al., 1989; Bukrinsky et al., 1992; Lobel et al., 1989; Pauza et al., 1994; Sharkey et al., 2000; Varmus and Swanstrom, 1984). Because of this instability, E-DNA has been regarded as a convenient marker for ongoing replication in vitro and in vivo. In particular, recent work from several groups has shown the persistence of 2-LTR circular forms in peripheral blood mononuclear cell (PBMC) of HIV-infected individuals in the absence of detectable plasma viremia levels. In some instances, this has been correlated with residual cryptic viral replication (Brussel et al., 2003; Cara et al., 2002; Morlese et al., 2003; Nunnari et al., 2002; Sharkey et al., 2000). Nevertheless, the stability of E-DNA

* Corresponding author. Laboratory of Virology, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161, Rome, Italy. Fax: +39-06-49903002.
*E-mail address:* ensoli@iss.it (B. Ensoli).

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in nonreplicating cells has not yet been characterized and, as such, could represent a potential reservoir of persistent E-DNA during lentiviral infections. In this respect, work from several groups has shown that E-DNA is longer lived in non-dividing than in dividing cells (Bushman, 2003; Butler et al., 2002; Pierson et al., 2002).

The infection models of *Macaca fascicularis* (cynomolgus monkey) and *Macaca mulatta* (rhesus monkey) with the SIV or SHIV provide experimental HIV-infection animal models of acquired immunodeficiency syndrome (AIDS) and are widely used for the development of HIV vaccine strategies (Stott and Almond, 1995; Warren, 2002). Therefore, it is important to evaluate virological parameters during infection and to compare them with those obtained from humans during infection with HIV-1.

In SHIV89.6P model of infection, after the initial acute phase of viral replication, virus may phase out below detectable levels as determined by a highly sensitive method capable of detecting as low as 50 copies/ml of blood (Ten Haaft et al., 1998). However, infected monkeys harbor reservoirs of replication-competent virus because they sporadically experience rebound of replicating virus (Igarashi et al., 2001). So far, it is unknown whether E-DNA is present and its molecular status in cynomolgus monkeys after infection with SHIV89.6P and in the absence of detectable viral replication. As it had been found in humans, the presence of E-DNA during covert infection might be indicative of low-level residual viral replication.

To this aim, we have analyzed the presence of 2-LTR forms of DNA in PBMC of SHIV89.6P-infected cynomolgus monkeys with detectable or undetectable levels of plasma viral RNA using a single-round polymerase chain reaction (PCR)-based assay. In addition, to evaluate sequence variation and viral quasi-species at the 2-LTR junctions, we analyzed the sequence heterogeneity at the LTR-LTR junction in this population of nonhuman primate isolates.

## Results

### *Presence of 2-LTR junctions in SHIV89.6P-infected cynomolgus monkeys in the absence of plasma viremia*

Twenty-one SHIV89.6P-infected monkeys with plasma viral RNA levels ranging between $1.8 \times 10^6$ and less than

Table 1A
2-LTR circle junction analysis in PBMC from SHIV89.6P-infected monkeys with plasma viremia levels above 50 copies/ml

| Monkey code | Week of E-DNA analysis from SHIV89.6 challenge | Viral load (RNA Eq/ml plasma) | E-DNA presence | Class of sequence found at the 2-LTR junction | CD4+ lymphocyte % (cells/μl) |
|---|---|---|---|---|---|
| 37 | 69 | 73,000 | + | | 3.67 (269) |
| 54963 | 4 | 198,900 | + | | 3.1 (190) |
| 55123 | 4 | 1,268,200 | + | | 7.43 (380) |
| 55129 | 4 | 79,800 | + | | 3.26 (210) |
| 55396 | 4 | 341,900 | + | | 1.07 (90) |
| | 65 | 1,100 | + | | 12.24 (1280) |
| | 71 | 1,600 | + | | 17.75 (1360) |
| | 92 | 11,000 | + | | 14.4 (942) |
| 61636 | 38 | 6,600 | + | | 22.8 (876) |
| 61756 | 34 | 84,000 | + | | 1.5 (86) |
| 61760 | 33 | 1,800,000 | + | | 25 (1767) |
| | 42 | 260,000 | + | | 29.4 (1711) |
| 61763 | 33 | 53,000 | + | | 0.2 (9) |
| | 38 | 86,000 | + | | 0.4 (25) |
| 61766 | 80 | 1,300 | + | | 19.9 (609) |
| 61771 | 42 | 4,300 | + | | 23 (886) |
| 61774 | 33 | 17,000 | + | | 0.3 (10) |
| | 42 | 29,000 | + | | 0.5 (18) |
| 61775 | 33 | 2,600 | + | | 28.2 (1090) |
| | 38 | 7,300 | + | III-V | 29.7 (2331) |
| | 42 | 3,200 | + | | 31 (1428) |
| | 61 | 4,200 | + | | 32 (1784) |
| | 80 | 980 | + | I-II-IV | 29.9 (1476) |
| 61779 | 33 | 39,000 | + | | 5.7 (182) |
| 61782 | 33 | 50,000 | + | | 4.2 (262) |
| | 38 | 95,000 | + | | 7.0 (390) |
| | 42 | 49,000 | + | | 5.7 (188) |
| | 33 | 9,200 | + | | 16.5 (1042) |
| 61783 | 38 | 1,100 | - | | 19.3 (1081) |

Monkeys were infected intravenously with either 10 or 20 $MID_{50}$ of SHIV89.6P and viral E-DNA was analyzed by single-round PCR at the week indicated. Presence of E-DNA was confirmed by sequencing (grey boxes) or hybridization.

50 copies/ml were evaluated in this study (Tables 1A and 1B). In particular, plasma viremia levels and the presence of 2-LTR E-DNA was analyzed multiple times on 17 monkeys, and only once on 4 monkeys. For analysis purposes, in Table 1A are shown data on samples with viral load above 50 copies/ml of plasma, whereas in Table 1B are shown data on samples with viral load below 50 copies/ml of plasma. The median CD4+ T cell counts for the samples with more than 50 copies/ml of plasma was 609 cells/mm$^3$ (average 9–1767 cells/mm$^3$), whereas the median CD4+ T cell counts for the samples with less than 50 copies/ml of plasma was 926 cells/mm$^3$ (average 260–1933 cells/mm$^3$), and the mean duration for viral loads at levels below 50 copies/ml was 31 weeks.

Analysis of the presence of 2-LTR was performed on DNA extracted from selected PBMC samples starting from week 4 and up to week 92 from SHIV89.6P infection for the samples with viral load above 50 copies/ml of plasma (Table 1A) and from week 33 and up to week 92 postinfection for the samples with viral load below 50 copies/ml of plasma (Table 1B). In all cases, the LMW-enriched DNA was extracted from a starting cell count of $2 \times 10^6$ to $5 \times 10^6$ PBMC and single-round PCR analysis was performed with a primer pair spanning the junction between the two LTR, as described in Materials and methods (Fig. 1). All PCR products were validated by either hybridization (data not shown) or nucleic acid sequence analysis (Fig. 2). As shown in Tables 1A and 1B, a total of 62 determinations were performed and 2-LTR E-DNA was detected in 53 out of 62 samples (85.5%) regardless of the plasma viremia status. In particular, 2-LTR E-DNA was detected in 28 out of 29 monkeys' PBMC with plasma viremia above 50 copies/ml (96.5%) (Table 1A) and in 25 out of 33 monkeys' PBMC with plasma viremia below 50 copies/ml (75.8%) (Table 1B). Concerning the analysis performed at week 38 on monkey 61783 in which the 2-LTR E-DNA signal was absent despite the presence of 1100 RNA Eq/ml of plasma, one explanation might be that the levels of 2-LTR circles

Table 1B
2-LTR circle junction analysis in PBMC from SHIV89.6P-infected monkeys with plasma viremia levels below 50 copies/ml

| Monkey code | Week of E-DNA analysis from SHIV89.6 challenge | Duration in weeks of viral load (<50 copies/ml) | E-DNA presence | Class of sequence found at the 2-LTR junction | CD4+ lymphocyte % (cells/μl) |
|---|---|---|---|---|---|
| 12 | 65 | 24 | + | | 12.21 (340) |
| | 71 | 30 | – | | 12.67 (570) |
| | 92 | 51 | + | I | 14.4 (316) |
| 55123 | 65 | 32 | + | | 23.46 (1190) |
| | 92 | 24 | + | II - III | 23.4 (1913) |
| 54963 | 65 | 37 | + | | 7.76 (320) |
| | 71 | 43 | + | | 13.43 (260) |
| | 92 | 64 | + | | 13.3 (436) |
| 55129 | 65 | 19 | + | | 12.36 (890) |
| | 71 | 25 | – | | 14.92 (510) |
| | 92 | 46 | – | | 10.8 (388) |
| 61636 | 61 | 19 | + | | 26.5 (937) |
| | 80 | 38 | + | I - III | 27.3 (926) |
| 61640 | 61 | 45 | + | I - IV | 31.9 (1668) |
| 61754 | 34 | 9 | + | I | 23.8 (646) |
| | 44 | 19 | + | III - V | 27.7 (989) |
| | 71 | 46 | – | | 21.8 (955) |
| | 82 | 57 | – | | 27.4 (1578) |
| 61766 | 33 | 17 | + | | 18.3 (729) |
| | 38 | 22 | + | II - V | 19.9 (1024) |
| | 61 | 45 | + | I - II | 19.6 (795) |
| 61776 | 33 | 13 | + | | 23.7 (813) |
| | 38 | 18 | + | | 25.2 (1234) |
| | 61 | 41 | + | II - IV - V | 28.3 (1141) |
| | 80 | 10 | + | I - II - III | 25.4 (827) |
| 61783 | 42 | 4 | + | | 6.8 (324) |
| | 52 | 14 | + | I - III | 16.6 (1271) |
| | 61 | 23 | – | | 18.5 (1447) |
| | 80 | 42 | – | | 19.1 (1100) |
| 61785 | 34 | 13 | + | I - II | 27.2 (1142) |
| | 44 | 23 | + | | 29.7 (824) |
| | 71 | 50 | + | I - III - IV | 21.5 (1221) |
| | 82 | 61 | – | | 32.3 (1933) |

Monkeys were infected intravenously with either 10 or 20 $MID_{50}$ of SHIV89.6P and viral E-DNA was analyzed by single-round PCR at the week indicated. Presence of E-DNA was confirmed by sequencing (grey boxes) or hybridization.

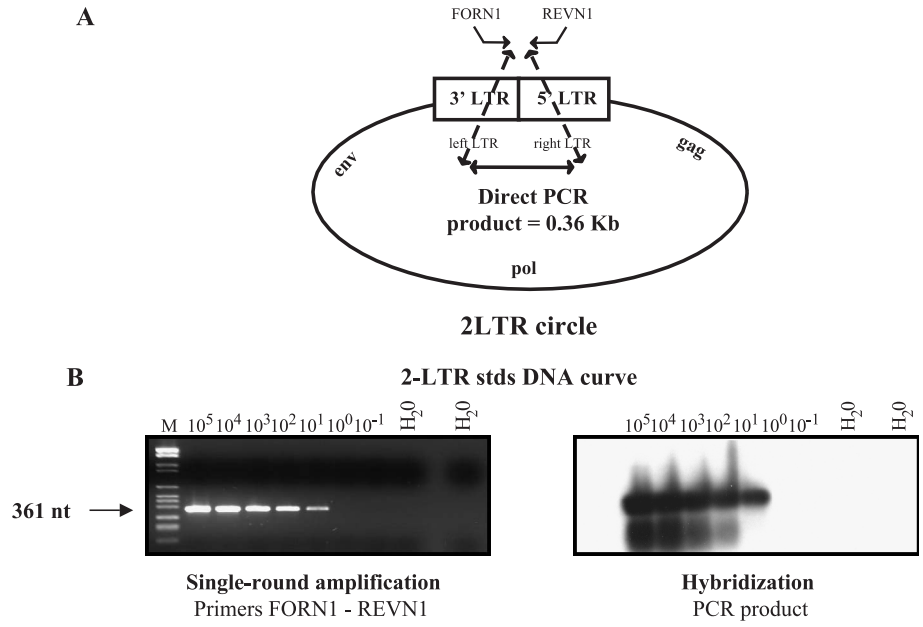


Fig. 1. DNA-PCR assay for SHIV89.6P 2-LTR circles. (A) Schematic diagram of single-round PCR strategy for amplification of the 2-LTR junction of SHIV89.6P DNA. Primer pair is FORN1/REVN1; the expected PCR product is 361 bp. (B) Sensitivity of the PCR assay and hybridization of the PCR product. Ten-fold dilution (starting at $10^5$ copies) of a plasmid containing known amounts of the SHIV89.6P 2-LTR junction target sequences were amplified with primer pair FORN1/REVN1 as described under Materials and methods (left). M, 100-bp ladder. Gel was blotted and hybridized with the 5′-end labeled purified PCR fragment (right) using standard methodologies (Sambrook et al., 1989).

were below the limit of detection of the single-round DNA-PCR. A second explanation might be that the cells in the peripheral blood are not representative of infected cells present elsewhere in the body that are actually producing virus. On the other hand, it cannot be excluded that the 2-LTR forms were not detected because of sequence variation at the 2-LTR junctions where either of the PCR primers would not be able to anneal to the target sequence and carry out the subsequent amplification.

*Sequence analysis of 2-LTR junctions*

For sequence analysis, PCR reaction products were gel-purified and, after insertion into the pCR2.1 vector, multiple clones from different PCR were examined. A total of 15 time points were sequenced at the 2-LTR junctions and analysis was performed on 10 different monkeys. In particular, sequencing was carried out once on five monkeys' PBMC and twice at different time points on five different monkeys' samples (Tables 1A and 1B). With regard to the PCR samples from monkeys with plasma viremia levels above 50 copies/ml, sequencing was carried out at two different time points (weeks 38 and 80) on monkey 61775 (Table 1A, grey boxes). Concerning the PCR samples from monkeys with plasma viremia levels below 50 copies/ml, sequencing was carried out at two different time points on four different monkeys (monkeys 61754, 61766, 61776, and 61785), whereas at a single time point on five monkeys' PBMC (monkeys 12, 55123, 61636, 61640, and 61783) (Table 1B, grey boxes). As shown in Fig. 2, next to the sequence corresponding to the joining of the unprocessed two LTR termini derived from the SHIV89.6P predicted circle junction (top of Fig. 2), we found a high number of miscellaneous sequences at the junction between the two LTR. For uniformity, we have decided to employ the classification used by Randolph and Champoux (1993), in which the sequences between the two LTR were divided into various classes (I–V) based on the most likely origin, features, and preponderance.

Class I junction sequences correspond to the expected sequence, representing the joining of the unprocessed two LTR termini. This might indicate the presence of an ineffective integrase (IN) protein in the infecting provirus. This class of sequences was found with a frequency of 18 out of 56 PCR clones (32%) in 10 out of 15 monkeys' samples that were analyzed (Fig. 2 and Table 2). Furthermore, class I junction sequences were found at both time points analyzed, weeks 34 and 71, in monkey 61785 (Fig. 2 and Table 1B).

Class II junction sequences is the very likely consequence of the incomplete removal of the tRNA primer

Fig. 2. Nucleotide sequences at the 2-LTR circle junction derived from PBMC DNA of SHIV89.6P-infected monkeys. The sequences were aligned to the corresponding U5-U3 2-LTR junction from the SHIV89.6P genomic sequence (Karlsson et al., 1997) using the VNTI suite 6.0 AlignX program from InforMax (Bethesda, MD, USA). Dashes represent gaps introduced in the figure for alignment purposes. Shown in bold are the invariant CA and TG nucleotides. Nucleotides in lower case are those derived from the tRNA primer binding site or from the PPT next to the U3.

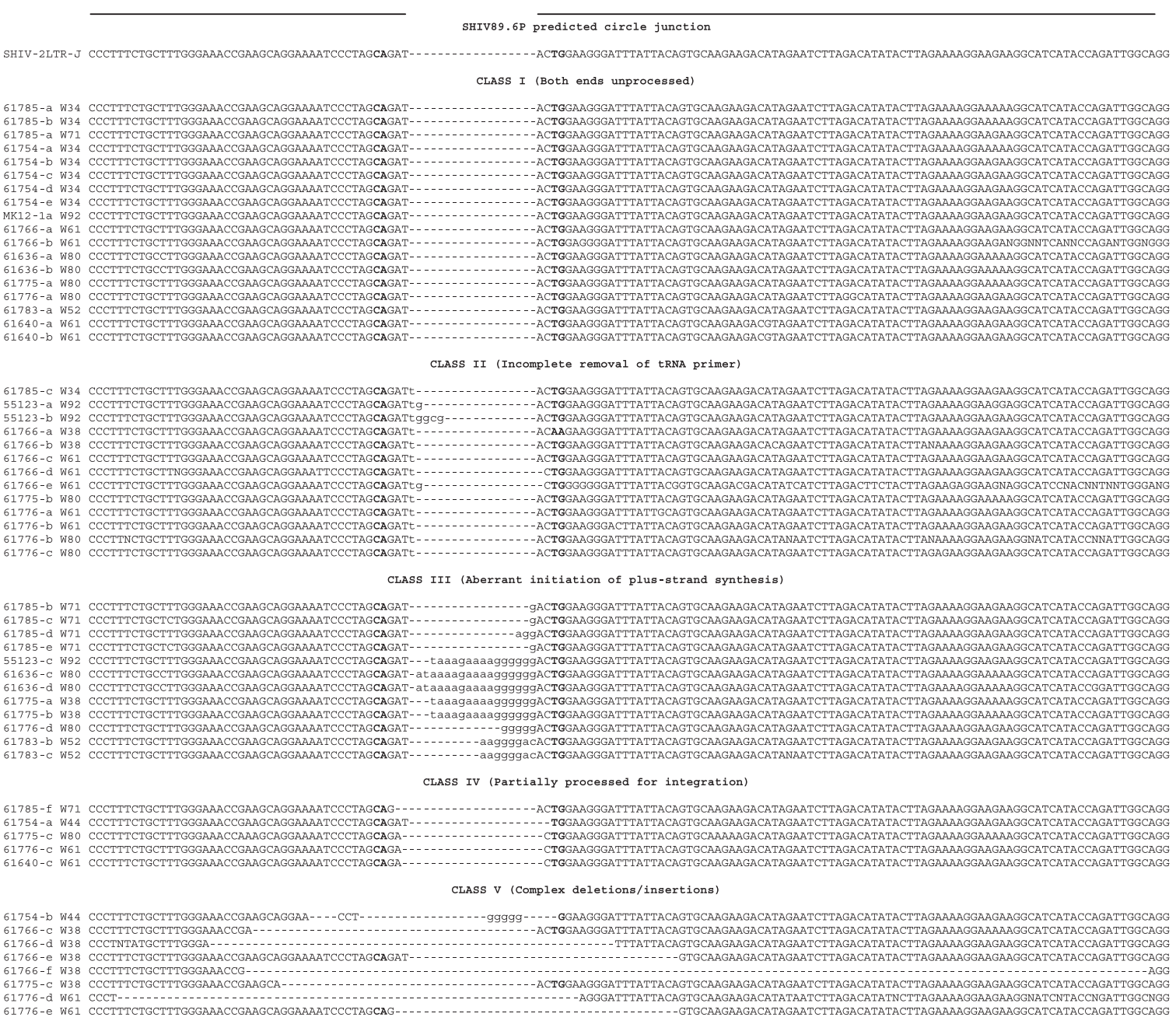
3´LTR - U5
U3 - 5´LTR
SHIV89.6P predicted circle junction
SHIV-2LTR-J CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGAT----------ACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
CLASS I (Both ends unprocessed)
61785-a W34 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGAT----------ACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAAAAGGCATCATACCAGATTGGCAGG
61785-b W34 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGAT----------ACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAAAAGGCATCATACCAGATTGGCAGG
61785-a W71 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGAT----------ACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
61754-a W34 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGAT----------ACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAAAAGGCATCATACCAGATTGGCAGG
61754-b W34 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGAT----------ACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
61754-c W34 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGAT----------ACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
61754-d W34 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGAT----------ACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
61754-e W34 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGAT----------ACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
MK12-1a W92 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGAT----------ACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
61766-a W61 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGAT----------ACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
61766-b W61 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGAT----------ACTGGAGGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGANGGNNTCANNCCAGANTGGNGGG
61636-a W80 CCCTTTCTGCCTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGAT----------ACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAAAAGGCATCATACCAGATTGGCAGG
61636-b W80 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGAT----------ACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
61775-a W80 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGAT----------ACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAAAAGGCATCATACCAGATTGGCAGG
61776-a W80 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGAT----------ACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGGCATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
61783-a W52 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGAT----------ACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
61640-a W61 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGAT----------ACTGGAAGGGATTTATTACAGTGCAAGAAGACGTAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
61640-b W61 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGAT----------ACTGGAAGGGATTTATTACAGTGCAAGAAGACGTAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
CLASS II (Incomplete removal of tRNA primer)
61785-c W34 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGATt---------ACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
55123-a W92 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGATtg---------ACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGGAGGCATCATACCAGATTGGCAGG
55123-b W92 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGATtggcg---------ACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
61766-a W38 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGATt---------ACAAGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
61766-b W38 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGATt---------ACTGGAAGGGATTTATTACAGTGCAAGAAGACACAGAATCTTAGACATATACTTANAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
61766-c W61 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGATt---------ACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
61766-d W61 CCCTTTCTGCTTNGGGAAACCGAAGCAGGAAATTCCCTAGCAGATt---------CTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
61766-e W61 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGATtg---------CTGGGGGGGATTTATTACGGTGCAAGACGACATATCATCTTAGACTTCTACTTAGAAGAGGAAGNAGGCATCCNACNNTNNTGGGANG
61775-b W80 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGATt---------ACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAAAAGGCATCATACCAGATTGGCAGG
61776-a W61 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGATt---------ACTGGAAGGGATTTATTGCAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
61776-b W61 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGATt---------ACTGGAAGGGACTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
61776-b W80 CCCTTNCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGATt---------ACTGGAAGGGATTTATTACAGTGCAAGAAGACATANAATCTTAGACATATACTTANAAAAGGAAGAAGGNATCATACCNNATTGGCAGG
61776-c W80 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGATt---------ACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAGAAGGAAGAAGGCATCATACCAGATTGGCAGG
CLASS III (Aberrant initiation of plus-strand synthesis)
61785-b W71 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGAT----------gACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
61785-c W71 CCCTTTCTGCTCTGGGAAACCGAAGCAGGAAAATCCCTAGCAGAT----------gACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
61785-d W71 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGAT----------aggACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
61785-e W71 CCCTTTCTGCTCTGGGAAACCGAAGCAGGAAAATCCCTAGCAGAT----------gACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
55123-c W92 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGAT---taaagaaaaggggggACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
61636-c W80 CCCTTTCTGCCTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGAT-ataaaagaaaaggggggACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAAAAGGCATCATACCAGATTGGCAGG
61636-d W80 CCCTTTCTGCCTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGAT-ataaaagaaaaggggggACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAAAAGGCATCATACCGGATTGGCAGG
61775-a W38 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGAT---taaagaaaaggggggACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAAAAGGCATCATACCAGATTGGCAGG
61775-b W38 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGAT---taaagaaaaggggggACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAAAAGGCATCATACCAGATTGGCAGG
61776-d W80 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGAT------------gggggACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
61783-b W52 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGAT---------aaggggacACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
61783-c W52 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGAT---------aaggggacACTGGAAGGGATTTATTACAGTGCAAGAAGACATANAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
CLASS IV (Partially processed for integration)
61785-f W71 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAG----------ACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
61754-a W44 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGAT----------TGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
61775-c W80 CCCTTTCTGCTTTGGGAAACCAAAGCAGGAAAATCCCTAGCAGA----------CTGGAAGGGATTTATTACAGTGCAAAAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
61776-c W61 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGA----------CTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
61640-c W61 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGA----------CTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
CLASS V (Complex deletions/insertions)
61754-b W44 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAA----CCT----------ggggg-----GGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
61766-c W38 CCCTTTCTGCTTTGGGAAACCGA----------ACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAAAAGGCATCATACCAGATTGGCAGG
61766-d W38 CCCTNTATGCTTTGGGA----------TTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
61766-e W38 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGAT----------GTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
61766-f W38 CCCTTTCTGCTTTGGGAAACCG----------AGG
61775-c W38 CCCTTTCTGCTTTGGGAAACCGAAGCA----------ACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG
61776-d W61 CCCT----------AGGGATTTATTACAGTGCAAGAAGACATATAATCTTAGACATATNCTTAGAAAAGGAAGAAGGNATCNTACCNGATTGGCNGG
61776-e W61 CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAG----------GTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGG

Table 2
Summarized results of the 2-LTR junction fragments obtained after DNA-PCR and nucleotide sequence analysis

| Class | Frequency (%) | Number of PCR reactions in which present | Features |
|---|---|---|---|
| I | 18/56 (32%) | 10 | both ends unprocessed (ineffective integrase) |
| II | 13/56 (23%) | 7 | incomplete removal of tRNA primer |
| III | 12/56 (21.5%) | 6 | aberrant initiation of plus-strand synthesis |
| IV | 5/56 (9%) | 5 | partially processed for integration |
| V | 8/56 (14.5%) | 4 | complex deletions (nonspecific Dnase activity) |

during reverse transcription. Incomplete removal of the primer generates junction sequences of different length with the majority having an extra T at the U5 termini of the sequence in the right LTR. This class of sequences was found with a frequency of 13 out of 56 PCR clones (23%) in 7 out of 15 sequenced monkeys' samples (Fig. 2 and Table 2). Furthermore, class II junction sequences were found at both time points analyzed in monkeys 61766 (weeks 38 and 61) and 61776 (weeks 61 and 80) (Fig. 2 and Table 1B).

Class III junction sequences contain an anomalous sequence in the left U3-LTR termini, which very likely arose from an aberrant plus-strand DNA synthesis. In fact, all the clones maintained the conserved TG residues, and the plus-strand synthesis after the first jump most probably initiated within the polypurine tract (PPT) leaving from 1 to 17 nucleotides, in addition to the canonical AC nucleotides, for IN removal before integration (Fig. 2). Conversely, it might be possible that the sequences derive from a partial digestion of the PPT primer by the RNase H during reverse transcription. As such, the subsequent steps of reverse transcription would carry the additional sequence in the reverse-transcribed viral genome. This class of sequences was found with a frequency of 12 out of 56 PCR clones (21.5%) in 6 out of 15 sequenced monkeys' samples (Fig. 2 and Table 2).

Class IV junction sequences probably derived from partial processing by IN of either or both the LTR termini, with consequent failure of integration and 2-LTR circles formation. This class of sequences was found with a frequency of 5 out of 56 PCR clones (9%) in 5 out of 15 sequenced monkeys' samples (Fig. 2 and Table 2). Only one clone, corresponding to monkey 61754-1 W44, seems to have been correctly processed at the left U3-LTR termini, although leaving unprocessed the right U5-LTR termini. All the other clones possess extra nucleotides at both the left and right LTR termini, corresponding to unprocessed or partially processed termini.

Class V junction sequences, containing deletions or insertions at the junction between the two LTRs, are all of uncertain origin and, due to their characteristics, are classified as complex. This class of sequences was found with a frequency of 8 out of 56 PCR clones (14.5%) in 4 out of 15 sequenced monkeys' samples (Fig. 2 and Table 2). It is possible to speculate that nonspecific IN activity or cellular enzymes like DNases might be responsible for the appearance of these particular E-DNA forms.

## Discussion

Previous works by others and us on HIV patients have shown that 50–99% of the patients' samples with viral plasma RNA levels below the limits of detection had detectable 2-LTR circular E-DNA in PBMC (Brussel et al., 2003; Cara et al., 2002; Morlese et al., 2003; Nunnari et al., 2002; Sharkey et al., 2000). Patient populations, treatment regimen, PCR conditions, and HIV-1 sequence variations at the LTR, which would ultimately affect the PCR conditions, might be responsible for such reported differences. In the present work, we extended this analysis during SHIV-89.6P infection of a well-defined population of cynomolgus monkeys, a nonhuman primate model for AIDS, and found comparable results. Indeed, 75.8% of the samples with viral plasma RNA levels below 50 copies/ml of plasma were positive for 2-LTR circles. This form of DNA is found in the nucleus of the infected cells and it might be indicative of either ongoing virus replication with the generation of newly infected cells or with the presence of a reservoir in which this form remain stable. Consistent with the former hypothesis, several groups have shown that unintegrated circular DNA forms are labile in dividing cells and their half-life does not exceed 48 h. In fact, following in vitro infection of replicating T-cells, E-DNA rapidly disappears, thus suggesting that any persistence must be due to active viral replication (Pauza et al., 1994; Sharkey et al., 2000). However, in agreement with the latter hypothesis, recent work has pointed out that in non-dividing cells unintegrated DNA is stable over a period of several days (Bushman, 2003; Butler et al., 2002; Pierson et al., 2002). Because unintegrated DNA is unable to replicate due to the absence of an origin of replication, it is possible that both hypotheses are correct. As such, newly infected actively replicating T-cells would dilute and loose the circular form of the virus while replicating, whereas in non-dividing cells, like terminally differentiated macrophages, non-dividing memory T-cells or as a yet unidentified cell population, 2-LTR circles would be maintained for a longer period of time. Nevertheless, the presence of 2-LTR forms in monkeys' samples in the absence of detectable plasma viremia for 1 year or more would be indicative of low levels of ongoing viral replication. Concerning the presence of E-DNA in non-dividing cells such as macrophages, it should be noted that although the HIV-1 *env* present in the SHIV-89.6P derives from a virus that used both the CCR5 and CXCR4 coreceptors for entry into monkeys CD4+ T-cells, further passages in

macaques have selected a SHIV89.6P that uses only CXCR4 (Zhang et al., 2000). As such, at least at the early stages of infection, the virus would replicate exclusively in CXCR4-bearing cells such as CD4+ T-cells. However, we have analyzed the presence of E-DNA over an extended period of time (up to 92 weeks from challenge) and during this period it cannot be excluded that the virus has reverted to the dual-tropic background acquiring the ability to infect macrophages, as described in humans accidentally infected with HIV-1 (Cornelissen et al., 1995; Moore et al., 2004). In any case, as for HIV-infected patients, if there were a population of cells within PBMC where these forms are more stable, the utility of the assay for monitoring ongoing SHIV infection would be limited and should be further explored. In this setting, it would be interesting to determine whether E-DNA in aviremic animal is more abundant in certain cell subsets such as resting cells.

A minority of monkeys' samples did not have detectable 2-LTR forms and, with one exception (monkey 61783, week 38), these samples had no detectable plasma RNA as well. Moreover, E-DNA-negative samples were those farther away from the day of SHIV infection. This seems convincing for monkeys 55129, 61754, 61783, and 61785 although with one exception (monkey 12), in which the presence of 2-LTR junctions could represent a reactivation of viral replication after a period of absence of detectable viral replication. Although it is possible that the absence of 2-LTR forms may merely represent a sampling error due to the starting cell number, this would also reflect the viral status in the infected monkeys. In fact, the longer infected monkeys remained with plasma viremia below detection, the higher was the likelihood that 2-LTR circles were undetectable.

Sequence analysis at the junction between the two LTR indicated that miscellaneous sequences were present in the monkeys' samples. Very likely, all the junctions resulted from ligation of integration-defective viral DNA ends. Of the 15 samples analyzed, 13 contained more than one class of sequence at the time of analysis. This would suggest that multiple forms of circular 2-LTR co-existed in the PBMC populations. Moreover, although 18 out of 56 class I junction sequences contained the predicted circular junction GAT↓AC, the majority (38/56) showed heterogeneous insertions or deletions. These classes of junctions (II–V) do not result from the joining of normal unprocessed LTR termini but most likely from errors incorporated during the reverse transcription process. These various LTR termini sequences lack the conserved residues that act as substrates for integration and, therefore, end up as substrates for the formation of closed circular DNA. On the other hand, a poorly active IN might account for the formation of class IV junction sequences, in which the LTR termini have been partially processed for integration. Similar studies involving HIV-1 infection in vivo also showed some sequence heterogeneity at the 2-LTR circle junctions (Cara and Reitz, 1997; Jurriaans et al., 1992; Sharkey et al., 2000). However, in these studies on HIV-1, the analysis was performed on a limited number of patients and PCR-derived clones. Overall, the sequence data derived from our monkey population displayed sequence heterogeneity across the 2-LTR junction with the majority of clones lacking the normal unprocessed ends for ligation as seen in vivo with HIV infection and in vitro with SIV and HIV infections (Buckman et al., 2003; Cara et al., 2002; Randolph and Champoux, 1993; Sharkey et al., 2000). Unfortunately, this junction sequence heterogeneity precludes the use of a junctional probe to selectively detect the presence of 2-LTR circular DNA in infected monkeys.

Larger studies with long-term follow-up will be needed to determine if the absence of 2-LTR DNA in PBMC of SIV/SHIV-infected monkeys predicts a better outcome, for example, after challenge, during a vaccination protocol. Moreover, longitudinal in vivo and in vitro studies are required to address whether these circular forms are stable in different cell types, and to determine if the presence or absence of unintegrated DNA might be prognostic of the duration of inhibition of viral replication.

## Materials and methods

### *Animals and samples collection*

Twenty-one adult male cynomolgus monkeys (*M. fascicularis*) were housed in single cages within level-three biosafety facilities according to the European guidelines for nonhuman primate care (EEC, Directive No. 86-609, November 24, 1986). All animals were infected intravenously with either 10 or 20 $MID_{50}$ of the SHIV89.6P virus. Clinical observation, weight measurements, and bleeding for hematological and virological analysis were taken while under Ketamine Hydrocloride anesthesia (10 mg/kg, im). Bleedings were performed by collection of 10 ml of whole blood in an acid citrate-containing tube.

### *Plasma SHIV RNA levels*

For RNA analysis, plasma was separated by centrifugation and viral RNA levels were determined utilizing an ultrasensitive QC-RNA-PCR with a lower limit of detection of 50 copies/ml of plasma as already described (Ten Haaft et al., 1998).

### *2-LTR detection*

For E-DNA analysis, PBMC were isolated from blood samples using Ficoll–Hypaque gradient centrifugation. After separation, PBMC were pelleted by centrifugation into $2 \times 10^6$ to $5 \times 10^6$ aliquots and cell pellets were kept frozen at −80 °C until analysis. To enrich for unintegrated E-DNA, low-molecular-weight (LMW) DNA was extracted from the cell pellet using the QIAprep Spin miniprep kit from QIAGEN (Valencia, CA) as described (Cara et al.,

2002). A single-round DNA-PCR reaction was performed to detect the 2-LTR forms of E-DNA using the primer pair FORN1/REVN1, amplifying a fragment of DNA of expected 361 bp encompassing the 2-LTR junction between the 3′ and 5′ LTR, respectively (Fig. 1). The PCR parameters were as follows: one step of denaturation for 5 min at 94 °C followed by 94 °C, 30 s; 60 °C, 30 s; and 72 °C, 30 s, for 40 cycles with a final elongation step of 10 min at 72 °C, using 10 pmol of each primer: FORN1: 5′-GTG ACT CCA CGC TTG TTT GC-3′, corresponding to nt 10320–10339 in the SHIV89.6P DNA sequence (R region of the 3′ LTR), in the sense orientation; REVN1: 5′-CTC CTG TGC CTC ATC TGA TAC C-3′, corresponding to nt 157–178 in the SHIV89.6P nucleotide sequence (U3 region of the 5′LTR), in the antisense orientation. PCR products were then analyzed by electrophoresis on 2% agarose gels. Bands were confirmed by hybridization using the FORN1/REVN1 PCR product as probe (Fig. 1 and data not shown) or by nucleic acid sequence analysis (see below) following standard methodologies (Sambrook et al., 1989).

*Cloning and sequencing of the PCR products*

For cloning, 2-LTR containing PCR products were size-selected and gel purified using the QIAGEN gel purification kit (QIAGEN). After purification, 2-LTR containing PCR products were cloned into the pCR2.1 vector (Invitrogen, California). After cloning, plasmids were isolated with the QIAGEN miniprep purification kit (QIAGEN) and digested with *Eco*RI to confirm insertion of the PCR products. Plasmid DNAs were sequenced with T7AS primer using an ABI/Prism-377 DNA sequencer (Perkin Elmer).

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