Arch Virol (2002) 147: 217–223

Archives of Virology

Clone B7 cells have a single copy of SIVsmB7 integrated in chromosome 20

Brief Report

I. Martínez[1], **L. Giavedoni**[2], and **E. Kraiselburd**[1,3]

[1]Department of Microbiology and Medical Zoology, School of Medicine, University of Puerto Rico, San Juan, Puerto Rico, U.S.A.
[2]Department of Virology and Immunology, Southwest Foundation for Biomedical Research and Southwest Regional Primate Research Center, San Antonio, Texas, U.S.A.
[3]Caribbean Primate Research Center, Division of Comparative Medicine, University of Puerto Rico, San Juan, Puerto Rico, U.S.A.

Accepted July 3, 2001

Summary. B7 is the designation of a cell clone derived from the human cell line CEMx174, which was infected with SIVsmH3 clone. B7 cells chronically produce high quantities of non-infectious virus-like particles (VLP) denominated SIVsmB7. Here we report the molecular characterization of the B7 cell line. We found that B7 cells have a single copy of the SIVsmB7 provirus integrated in a noncoding region of chromosome 20 (nt 24,957 of clone RP5-963K23 on human chromosome 20q 13.11–13.2). Similarly to HIV and SIVmac, we show that integration of SIVsm results in a characteristic five base pair sequence repeat of host DNA that flanks the proviral DNA genome. Since the SIVsmB7 genome has a deletion in the IN coding sequence, the generation of this defective proviral genome most likely occurred during a faulty process of reverse transcription. Thus, these studies reveal the molecular clonality of the SIVsmB7 VLP produced by B7 cells. These genetically homogeneous VLP are useful reagents for vaccine development. In addition, these particles have been used by others (Montelaro et al.) to study the maturation of immune system responses to SIV infection.

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The clone B7 is a CEMx174 human cell clone (NIH AIDS Research and Reference Program, Cat no 272, Rockville, Maryland) that chronically produces high quantities of non-infectious SIVsmB7 virus particles [1]. These viral particles have been shown to be immunogenic in monkeys, and animals immunized

with SIVsmB7 had lower viral loads than naive controls after challenge with pathogenic SIVsmE660 [2]. In addition, this virus has been used to study the development of protective immune responses induced by candidate SIV vaccines [3]. Previous DNA sequencing studies of SIVsmB7 have provided evidence that this virus has a ∼1.6 Kb deletion in the pol region (from nt 4654 to nt 6232) which includes integrase (IN) and Vpr/Vpx [2], and defects in Vpr/Vpx and Gag expression were reported in an earlier publication [1]. No major nucleotide differences have been observed between SIVsmH3 and SIVsmB7 in Gag, Env and in the LTR genome regions [2]. Here we report that clone B7 cells have a single copy of SIVsmB7 integrated in the chromosome 20.

Determination of a single proviral integration event in clone B7 cells: The first goal of our study was to determine whether the SIVsmB7 provirus was present in clone B7 cells as an episomal DNA or was integrated in the cellular chromosomes, and, in the latter case, the number of copies of such proviral DNA. In order to accomplish this task, we hybridized the clone B7 cellular DNA, previously digested with restriction enzymes, with a probe based in the LTR sequence of SIVsm. Sixty micrograms of clone B7 DNA were digested with either Eco RI or Hind III, were separated in a 1% agarose gel, transferred to nitrocellulose membranes and hybridized with a DIG-labeled LTR probe. The SIVsmH4 DNA clone (a kind gift of Dr. Vanessa Hirsch, NCl, NIH) was added to a cocktail of 50 μl containing dNTPs + DIG-labeled dUTP (Boerhinger Mannheim, Indianapolis, IN), 1 × *Taq* buffer, 0.5 units *Taq* enzyme (Promega, Madison, WI), and 20 pM each of LTR forward (521–538) 5′-CTCTGCCGAGAGGCTGGC-3′ and LTR reverse (776–759) 5′-GGGTCCTAACAGACCAGG-3′; the position of the nucleotides in the SIVsmH4 sequence is given in parenthesis. The DNA was amplified using a Thermo Cycler (Model 480, Perkin Elmer) as described in an earlier publication [1]. The DIG-labeled LTR probe was purified by Gene Clean (Bio 101, Vista, CA) and the hybridization reactions were carried out for 36 h at 62 °C. A chemiluminescent detection kit (Boehringer Mannheim, Indianapolis, IN) was used to identify the products of the hybridization reaction and membranes were exposed to an X-ray film overnight.

We selected Eco RI to digest the cellular DNA because there are only two Eco RI restriction sites in the 10,276 nucleotides long SIVsmH4 viral genome (positions 3,205 and 7,781), whereas Hind III sites are not present in this viral sequence [5]. Thus, if a single integrated copy of SIVsmB7 DNA was present in clone B7 cells, a labeled SIV LTR probe would identify only two DNA bands in an EcoRI-digested DNA, one band of more than 3.2 Kb and another one of more than 2.4 Kb (10,276 − 7,781 ∼2.4 Kb). Since the SIVsmB7 genome contains a ∼1.6 Kb deletion in its Pol region, a single band of more than 8.7 Kb (the size of the SIVsm B7 genome) would be expected after hybridization of the SIV LTR probe with Hind III-restricted clone B7 DNA. On the other hand, if the viral DNA was present as a circular episomal DNA, hybridization with the SIV LTR probe of EcoRI-digested cellular DNA would reveal a single band of ∼5.6 Kb (3.2 Kb + 2.4 Kb) whereas the Hind III-digested DNA would show a single band smaller than 8.6 Kb, due to the faster migration of circular DNA with respect

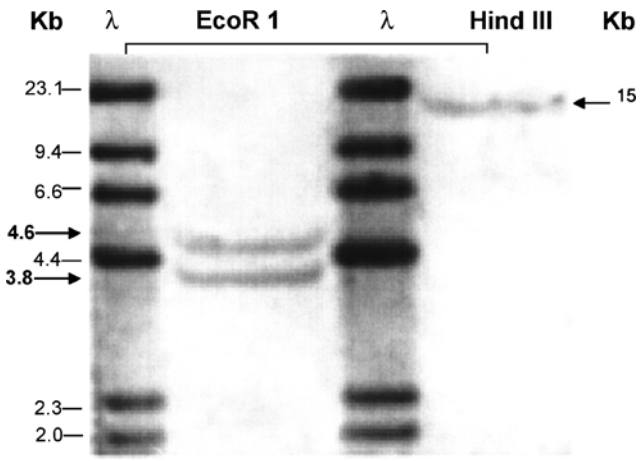

Fig. 1. Hybridization of EcoR I- and Hind III-restricted clone B7 DNA with an SIV LTR probe. Enzyme-restricted DNA was separated by gel electrophoresis, transferred to a nitrocellulose filter and allowed to hybridize with a DIG-labeled SIVsmH4 LTR probe. Molecular weight markers (λ DNA/Hind III fragments) are shown

to linear DNA. As shown in Fig. 1, the SIV LTR probe hybridized with two bands of ∼4.6 Kb and of ∼3.8 Kb, respectively for the EcoRI-digested clone B7 DNA, and a single band of ∼15 Kb was revealed by the SIV LTR probe when Hind III-restricted cell DNA was used for hybridization. These results provided strong evidence for the presence of a single SIVsmB7 proviral DNA copy in clone B7 cells.

Identification of the 5′ integration site of SIVsmB7: The identification of the 5′ integration site was carried out with the use of both degenerate and SIVsm-specific primers, according to a two-PCR procedure described by Sørensen et al. [4], with minor modifications. The following primers were used: FP1 (5′-CAGTTCAAGCTTGTCCAGGAATTCNNNNNNNGGCCT-3′), FP2: (5′-CAGTTCAAGCTTGTCCAGGAATTCNNNNNNNGCGCT-3′), FP3 (5′-bio-CAGTTCAAGCTTGTCCAGGAATTC-3′), SIVsmGag1 (5′-bio-GGGAACTCAGTCCTATGACTTTTC-3′, position 994–1017), SIVsmLTR1 (5′-GGGTCCTAACAGACCAGGGT-3′, position 757–776), and SIVsmLTR2 (5′-GCCAGCCTCTCCGCAGAGCG-3′, position 519–538). The first PCR reaction was done in a 50-μl reaction volume containing 1 × TaqGold Reaction buffer (P-E Corp.), 0.3 μM (each) deoxynucleoside triphosphates, 2.5 μM Mg^{++}, 400 nM of the SIVsmGag1 primer, 200 nM each of either primers FP1 or FP2, and 2.5 u of TaqGold DNA polymerase (P-E). DNA from clone B7 or CEM-x-174 cells (1 μg) was used as a template. The PCR conditions were 94 °C and 90 sec for denaturation, 62 °C and 90 sec for annealing, and 72 °C and 90 sec for extension, and this cycle was repeated 35 times. One volume of the first PCR reaction was combined with one volume of M-2809 streptavidin microbeads (Dynal Corp.), and the suspension was incubated at room temperature for 15 min. The microbeads were washed three times with 1 × B&W buffer (5 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 1 M NaCl),

and were resuspended in 15 μl of 0.1 M NaOH for 10 min. The microbeads were separated with a magnet, and the supernatant containing the non-biotinylated DNA was transferred to a new tube, where the solution was neutralized with 7.5 μl of 0.2 M HCl and 2 μl of 1 M Tris-HCl pH 8.0. Finally, the solution volume was adjusted to 60 μl with water. For the second PCR reaction, 5 μl of the purified DNA from the first PCR were combined with 1 × TaqGold Reaction buffer, 0.3 μM (each) deoxynucleoside triphosphates, 2.5 μM Mg^{++}, 400 nM of either primer SIVsmLTR1 or SIVsmLTR2, 400 nM primer FP3, and 2.5 u of TaqGold DNA polymerase (P-E). The conditions of the reaction were the same as for the first PCR. Finally, the PCR products were visualized in a 1.2% agarose gel, DNA fragments 1.0 Kb or larger were purified from the gel and their sequence was determined (Lark Technologies Inc., Houston, TX).

Two DNA products were found with this methodology. One band of ∼1.2 Kb was obtained using primers FP3 and SIVsmLTR1, and another of ∼1 Kb was obtained using primers FP3 and SIVsmLTR2 (data not shown). Sequence analysis of these DNA fragments showed that the ∼1.2 Kb DNA band contained sequences known to be present in human chromosome 20 (Gene Bank accession # AL031685) as well as SIVsm 5′LTR sequences. Specifically, the ∼1.2 Kb DNA contained a 0.32 Kb fragment (nt 24,637 to nt 24,957, present in clone RP5-963K23 on human chromosome 20q13.11–13.2) contiguous to SIVsm 5′LTR sequences (nt 1–738, Gene Bank accession # X14307). The ∼1.0 Kb DNA fragment contained human DNA sequences from the same chromosome region (nt 24,590–24,957) linked to SIVsm 5′LTR (nt 1–538). To corroborate these findings, clone B7 DNA was PCR-amplified using a forward primer specific for the RP5-963K23 clone and a reverse primer specific for SIVsm, and the single ∼1.4 Kb DNA obtained by this PCR reaction was sequenced. Results obtained confirmed the findings described above, and there was 100% homology with the reported human sequences obtained using primers FP3 with either LTR1 or LTR2. When comparing SIVsmH4 and SIVsmB7, a single substitution of G to A was found at position 130, and this substitution was observed in all DNA products obtained by the three independent PCR reactions described above.

Identification of the 3′ integration site of SIVsm B7: Since we showed that the 5′ LTR of SIVsmB7 was integrated at position 24,957 of the sequences present on human chromosome 20, it was implied that sequences from this region should be contiguous to the 3′ end of the SIVsmB7 genome. Data generated by our laboratory [2] determined more than 99% homology between SIVsmB7 DNA and SIVsmH4 genomic sequences from nucleotide position 7781 to nucleotide position 10,277. Thus, to determine the cellular sequences flanking the SIVsmB7 3′ LTR, clone B7 DNA was PCR-amplified using a forward primer derived from the SIV env region (primer ENV9189, 5′-GATACTCGCAATCCCTAGG-3′, position 9,189 in the SIV genome), and a reverse primer derived from the human DNA sequences present on clone RP5-96K23 (primer GEN 553, 5′-GGATGGCTCAAGTATCTGC-3′, position 25,245 in clone RPS-963K23 on chromosome 20q13.11–13.2). The expected DNA band of ∼1.4 Kb obtained was sequenced as described above. Data obtained showed that this DNA band

was consisted of a fragment (nt 9,190–10,277) that contained the same mutation observed in the 5′ LTR (G → A, nt 9592) followed by a region that was 100% homologous to clone RF5 963K23 (from nt 24,953 to nt 25,245). Interestingly, a repeat of 5 nucleotides of human DNA sequences (GTAAC) was present at both ends of the proviral genome.

Analysis of these host DNA sequences revealed EcoRI restriction sites at several positions, including those closest to the integration site, at nt 23,536 and nt 26,316. Based on those sequences, Eco RI digestion of clone B7 DNA should have produced two LTR-containing DNA bands. One of MW of ∼4.6 Kb [(24,957–23,536) + 3206], containing the 5′ LTR, and the other of ∼3.8 Kb containing the 3′LTR sequences. This was indeed observed (Fig. 1). In addition, the host genome sequences flanking the provirus contain 23 Hind III sites. Those closest to the provirus are present at nt 20,285 and at nt 26,019. Since there are no Hind III sites in the 8.7 Kb SIVsmB7 DNA, restriction of clone B7 DNA with this enzyme should have produced a single DNA band of ∼14 Kb, which was also observed (Fig. 1).

Clone B7 cells harbor an integrated SIVsm that lacks integrase: Replication of retroviruses (including SIV) requires the insertion of a DNA copy of the virus genome into the host cell chromosome. The integration of the proviral template, which is used to transcribe the different virus products, is mediated by the viral integrase (IN) protein, encoded by the virus *pol* gene. Upon infection of cells, IN associates with the preintegration complex, which contains the newly synthesized virus DNA, Gag and Pol proteins, the accessory virus products Vpr and Vpx, as well as host factors [6–8]. The preintegration complex migrates to the nucleus where integration into the host cell genome takes place. The two ends of the virus DNA join the target host DNA in a staggered fashion, leading to a duplication of the host sequences that immediately flank the inserted provirus. The length of this duplicated host DNA sequences is virus-specific, and in the case of HIV-1 and SIVmac239 is a 5 base pair repeat [9–11].

Even though SIVsmB7 contains deletions in gene functions critical for integration, it is reasonable to assume that SIVsmB7 DNA integrated in the host genome using a complex that contained all the necessary preintegration virus and cell products. SIVsmB7 deletions could have arisen by a faulty process of retrotranscription of a replication-competent virus. Deletions are known to occur when template exchanges are made during the process of cDNA synthesis [12]. Strand transfer of retrotranscriptase enzyme to non-contiguous virus genome regions during cDNA synthesis could produce a (Pol/Vpr/Vpx) deleted virus genome that is able to integrate into the host chromosome by means of a fully functional preintegration complex. Alternatively, since it has been shown that integration can be complemented in *trans* [7], the necessary virus products could have been provided in *trans*, during coinfection of the same cell with SIVsmB7 and a wild type virus. However, the presence of a single, defective proviral genome in the B7 chromosomes strengthens the hypothesis of the faulty reverse transcription. Of interest, chronically infected cells producing Pol-deleted/mutated HIV have been reported by others [13, 14]. Nevertheless, the mechanisms by which a

Pol/Vpr-defective genome (such as SIVsmB7 DNA) can integrate into the host cell genome have yet to be elucidated.

In conclusion, a single integrated copy of SIVsmB7 DNA was found to be present in a noncoding region of chromosome 20 of clone B7 cells. The characteristic five base pair sequence repeat in host DNA flanked the replication-defective SIVsmB7 provirus DNA. Thus, clone B7 cells constitutively produce high levels of homogeneous, replication-defective VLP (SIVsmB7). Others laboratories have established cell lines harboring defective proviruses [13, 14]. B7 cells are also clonal in nature, and produce non-infectious particles that have been shown to be immunogenic. The defect of its resident proviral DNA has been characterized [2]. These characteristics provide investigators with a useful reagent to study specific aspects of the immune response against SIV as well as aspects of its biology. SIVsmB7 preparations are currently being used in molecular and biological studies as well as in vaccine efficacy protocols in our laboratory.

Acknowledgements

We acknowledge the skillful assistance of Mr. Angel Salaman Bayrón and Mr. Alejandro Rodríguez in the Southern blot procedures. These studies were supported by grants RO1 Al 44592 and RO1 Al 34209, and the RCMI Program of UPR-MSC. We thank Mrs. Ingrid Acevedo for typing this manuscript.

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Author's address: Prof. E. N. Kraiselburd, Unit of Comparative Medicine, UPR Medical School, GPO Box 365067, San Juan, PR 00936-5067, U.S.A.; e-mail: ekraiselburd@rcm.upr.edu

Received April 20, 2001