Acta Medica Okayama

Volume 51, *Issue* 4 1997 *Article* 4

AUGUST 1997

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Abstract

A simian cell line, Si-IIA, harboring Epstein-Barr-virus (EBV) -related herpesvirus (Si-IIA-EBV), produces malignant lymphoma in rabbits when administered by intravenous inoculation. In this study, we analyzed the Si-IIA-EBV genome and compared it with human EBV and herpesvirus macaca fascicularis 1 (HVMF 1), which is associated with B-cell lymphoma developing in SIV-infected immunosuppressed monkeys. DNA from Si-IIA-EBV was amplified by the polymerase chain reaction using three different primer pairs complementary to human EBV (B95-8) DNA; two of the primer pairs covered part of the long internal repeat 1 region (IR 1) and the third covered part of the BRRF 1 region. Direct sequencing of the three PCR products revealed that Si-IIA-EBV DNA had about 82% nucleotide homology to the human EBV DNA in all three regions and 92.4% homology to HVMF1 in the IR1 region. The blotting pattern by Southern blot analysis was different between Si-IIA-EBV and human EBV.

KEYWORDS: Epstein-Barr virus, HVMF 1, lymphoma, ?monkey cell line, PCR

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*PMID: 9284968 [PubMed - indexed for MEDLINE]

Acta Med Okayama 1997; 51(4): 207-212

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A simian cell line, Si-IIA, harboring Epstein-Barr-virus (EBV) -related herpesvirus (Si-IIA-EBV), produces malignant lymphoma in rabbits when administered by intravenous inoculation. In this study, we analyzed the Si-IIA-EBV genome and compared it with human EBV and herpesvirus macaca fascicularis 1 (HVMF 1), which is associated with B-cell lymphoma developing in SIV-infected immunosuppressed monkeys. DNA from Si-IIA-EBV was amplified by the polymerase chain reaction using three different primer pairs complementary to human EBV (B95-8) DNA; two of the primer pairs covered part of the long internal repeat 1 region (IR 1) and the third covered part of the BRRF 1 region. Direct sequencing of the three PCR products revealed that Si-IIA-EBV DNA had about 82% nucleotide homology to the human EBV DNA in all three regions and 92.4% homology to HVMF1 in the IR1 region. The blotting pattern by Southern blot analysis was different between Si-IIA-EBV and human EBV.

Key words: Epstein-Barr virus, HVMF I, lymphoma, monkey cell line, PCR

Si-IIA is a simian cell line established by cocultivating Cynomolgus leukocytes with a human T-lymphotropic virus type-II (HTLV-II)-producing human cell line (1, 2). This cell line has been shown to produce HTLV-II virus and immortalize human T cells in cocultivation (3, 4). In addition to HTLV-II, Si-IIA cells harbor herpesvirus particles and express Epstein-Barr virus (EBV)-encoded RNA-1 (EBER-1) (5). This herpesvirus was tentatively named Si-IIA-EBV (5). Si-IIA-EBV DNA was amplified by polymerase chain reaction (PCR) using only some primers complementary to human EBV sequence, but not others (5). Therefore, Si-IIA-EBV is closely related to human EBV, but nonetheless different from it. We found that Si-IIA cells or their cell-free pellets induced malignant lymphoma in Japanese white rabbits when inoculated intravenously (5, 6). These lymphoma cells did not show integration of the HTLV-II provirus genome, but contained EBV-related DNA. Si-IIA cells did not contain proviral DNA of simian T-cell leukemia virus and did not react with antibody-positive sera against Herpesvirus saimiri or Herpesvirus ateles (6). This evidence suggests that Si-IIA-induced malignant lymphomas (ML) are not causally associated with HTLV-II or well-known simian oncogenic viruses (7), but with a human EBV-related simian herpesvirus, Si-IIA-EBV (5). Recentlty, it has been reported that B-cell lymphomas developing in SIV-infected immunosuppressed monkeys are closely associated with an EBV-related simian herpesvirus, herpesvirus Macaca fascicularis (HVMF 1) (8). In the present study, we analyzed nucleotide sequences of PCR products amplified from the genome of Si-IIA-EBV using three primer pairs and compared them with those of human EBV or HVMF 1 DNA.

Material and Methods

Cells. Si-IIA cells were used as a source of EBV-related simian herpesvirus, Si-IIA-EBV. Tumor cell lines established from Si-IIA-EBV-induced rabbit malignant lymphoma (6) were also used. B95-8 is an EBV-producing marmoset cell line and was used as an EBV-positive control. A human T-cell line, TALL-1 and Cynomolgus peripheral blood leukocytes were used as an EBV-negative control.

DNA isolation from culture supernatants.

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Culture supernatants obtained from 800–1000 ml of Si-IIA cell culture (5×10^5 cells/ml) were first centrifuged at 2,000 *g* for 30 min to remove cell debris and then at 100,000 *g* for 60 min to obtain the pellets. DNA was prepared from the pellets according to the standard procedures (10).

Polymerase chain reaction. Extracted sample DNAs were amplified by PCR using the following three primer pairs: Ws, 5'-CCACCTTCATCACCGTCGCTGACTCC-3' (B95-8 coordinates 14518 to 14543) and Was-3, 5'-ATGCAACTTGAGGCAGCCTAATCC-3' (14926 to 14949), covering part of the EBV long internal repeat 1 (IR1) sequence (BamHIW region) (11); No. 11 sense, 5'-ATGAGGAAGGTAATCGCGGA-3' (105662 to 105681) and No. 11 antisense, 5'-GGCTCGGTTATTTTGGTTCC-3' (106320 to 106339), covering part of the EBV BRRF1 region (5). No. 2 sense, 5'-TTGTCCAGATGTCCAGGGGT-3' (13806 to 13826) and No. 2 antisense, 5'-GGACCACTTTATACCAGGGG-3' (14345 to 14364), also covering part of the EBV IR 1 region. These primer pairs were employed according to the complete sequences of human EBV (B95-8) (5). PCR was performed with 500 ng of genomic DNA, 20 pmol of each primer, 10 mM Tris HCl (pH 8.0), 50 mM KCl, 25 mM MgCl 2, 10 mM dNTP and 2.5 units of Taq polymerase. Amplified PCR products were visualized by electrophoresis in 0.8 % agarose gels stained with 0.5 mg/ml ethidium bromide under ultraviolet transillumination.

Southern blot analysis. DNA (1 μg) of each sample was digested with EcoRI or BamHI, electrophoresed in 0.8 % agarose gels and blotted onto Nylon membranes. The membranes were hybridized overnight at 60 °C with the PCR-amplified Si-IIA-EBV probes labeled using the Fluorescein Gene Image Labeling System (Amersham kit; RPN 3540/3541, Little Calfont, Backinghamshire, UK). Hybridization, washing and detection were performed according to the manufacturer's instructions.

DNA sequencing and sequence analysis. PCR products were purified on Bio-spin chromatography columns (BIO RAD, Hercules, CA, USA) and directly sequenced using the Taq dye deoxy terminator cycle sequencing kit (Perkin-Elmer, Branchburg, NJ), according to the manufacturer's protocol. The templates were subjected to 25 cycles of amplification, each cycle consisting of 94 °C for 20 sec, 50 °C for 20 sec, and 60 °C for 30 min. Subsequent electrophoresis and analysis of the samples was performed on an ABI 373 A DNA sequencer with associated software (Applied Biosystem, Inc., Foster City, CA, USA). Sequence data were analyzed using the GENETYX-Mac version 8.0 program, and the GenBank 91.0 and EMBL 44.0 databases were searched for nucleotide homology.

Results

PCR of the Si-IIA-EBV DNA, using the primer pairs Ws and Was-3, No. 11 sense and No. 11 antisense, and No. 2 sense and No. 2 antisense, amplified the fragments of 432-bp, 678-bp, 559-bp, respectively (Fig. 1). Southern blot analysis with the labeled PCR product-probes of IR1 sequence revealed that Si-IIA pellet DNA showed the same pattern as DNA from Si-IIA-EBV-induced rabbit lymphoma cells (Ra-SLN) but a different pattern from human EBV of B95-8 cells (FIg. 2). Sequence analysis of the products amplified with the primer pair Ws and Was-3, showed that Si-IIA-EBV DNA had 81.6 % nucleotide homology to EBV DNA from B95-8 cells and 92.4 % nucleotide homology to HVMF1 DNA in the IR1 region (Fig. 3). The PCR products amplified from the Si-IIA-EBV DNA with the other primer pairs (No. 11 sense and No. 11 antisense, No. 2 sense and No. 2 antisense) showed 82.8 % nucleotide homology to B95-8 DNA in the corresponding region (Figs. 4A, B). The amino acid sequences predicted from Si-IIA-EBV DNA sequences in the BamHIW region and the BRRF1 region showed 74 % and 79 % homology to the respective reported amino acid sequences of human EBV.

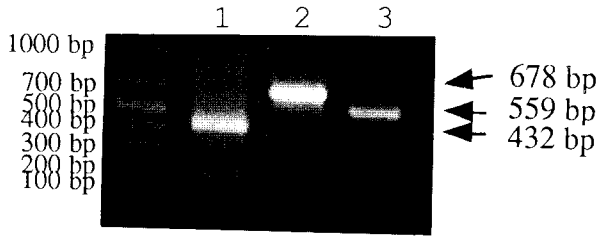

Fig. 1 Electrophoretic analysis of the PCR products of Si-IIA cell-free pellet DNA using three primer pairs derived from a sequence of human EBV DNA. Lane 1, Ws and Was-3; lane 2, No. 11 sense and No. 11 antisense; lane 3, No. 2 sense and No. 2 antisense.

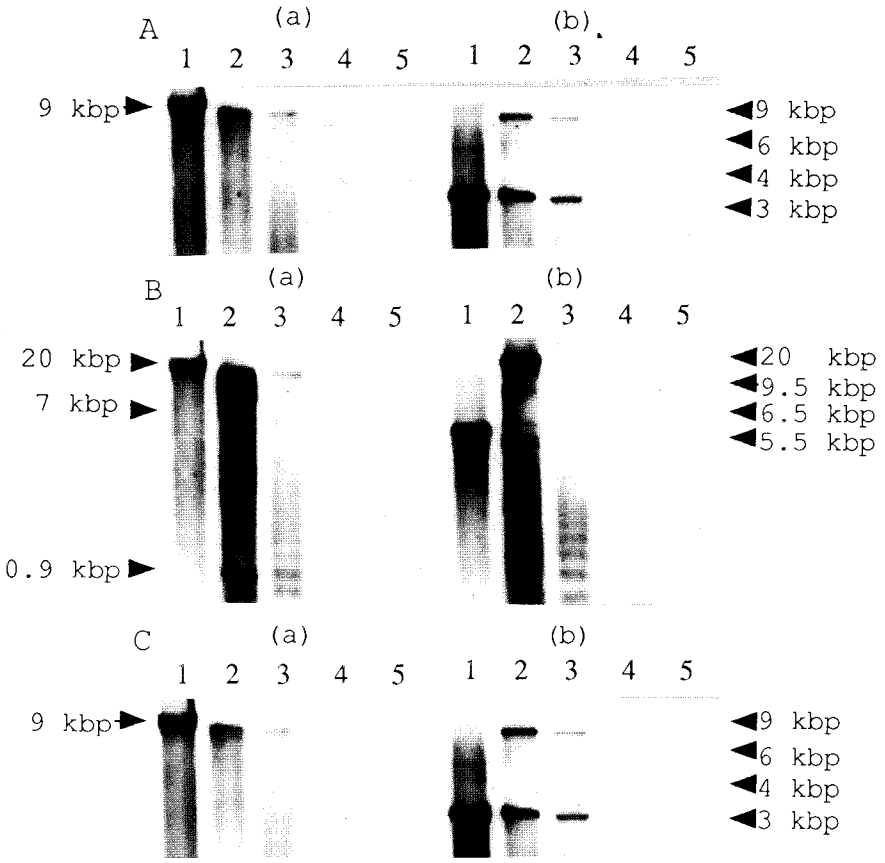

Fig. 2 Southern blot analysis of EBV-related DNA digested with EcoRI (**a**) or BamHI (**b**). Probes are the labeled PCR products amplified from Si-IIA-EBV DNA using the primer pairs: **A**, Ws and Was-3; **B**, No. 11 sense and No. 11 antisense; **C**, No. 2 sense and No. 2 antisense. Lane 1, B 95-8; lane 2, Si-IIA; lane 3, Ra-SLN; lane 4, TALL-1; lane 5, Cynomolgus PBL.

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(a)  1' CCACCTTCATCACCGTCGCTGACTCCGCCATCCAAGCCTAGGGGAGACCCAAACGAAGCC
(b)     **************************************************G**GT****G*
(c)           ***********************************__*****__*C*******__
    61' CCCAGACCTTCCAGGCCCGGGCCCCCAGGTCTCGGGCCAGAGGTAAGTGGACTTTCAGTT
        **TG****AA**C************C***A************************A*T**
        -***********_____*CCGTT***********TT*********************
   121' ATTCTGCTCAGCCCACCACTCCACCACACCCAGGCACACACTACTCACAGCCACCCGTCT
        T*******A******A*****************************A****C**********
        ******************************************A*****************
   181' CAGGGCCCCTTCGGAGACCTCCTGAGAAGGCGCAGGCACAACAGCCCAGCCGGCGGAGAC
        *****T***C*****C*G*****A*******A*C**TCGCC***T**TA**A*A**G*G*
        *****_T**********************T*****************************
   241' CCACAGGGACCCACACGCGTTCGCAGGAGGGTCTTCCTTGAAGAAAAAGAACGGCCGGTA
        *A*G*___*****G***A**C**T**A******C**G*CC*GC**G****GGA*GT****
        ******************************************G*G**G*********
   301' AGCGGTCCACCAGTAAGGGTAGGTAACTTCACC--TCCAGGGCCCACATCATGGGAGGCC
        ******T****TTC*G*****A*****C*G***TC********T*****A*A*******T
        ***************************__**************************
   359' TAGTGCACACCCTTCTTGCTTTTCACAGGAACCTGGGGTCTAATCCGGGTGGGATTAGGC
        ****AT***TG*****************************G***G**T**************
        ****A*****G*********************************G**
   419' TGCCTCAAGTTGCAT
        ***************
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Fig. 3 Comparison of the nucleotide sequences corresponding to a part of the IR1 region of the B95-8 EBV (#14518-#14949; ref. 5) among Si-IIA-EBV (**a**), B95-8 human EBV (**b**), and HVMF1 (**c**). The sequences from the 5' end of the Ws primer region to the 3' end of the Was-3 primer region are shown. Tentative numbering starts from the 5' end of the sense primer. Available sequence data of HVMF1 (**c**) in the literature (8) is only from #7 to #403. Identical bases among the three sequences are shown by asterisks (*), and gaps generated by the computer to line up these sequences are shown by bars (−).

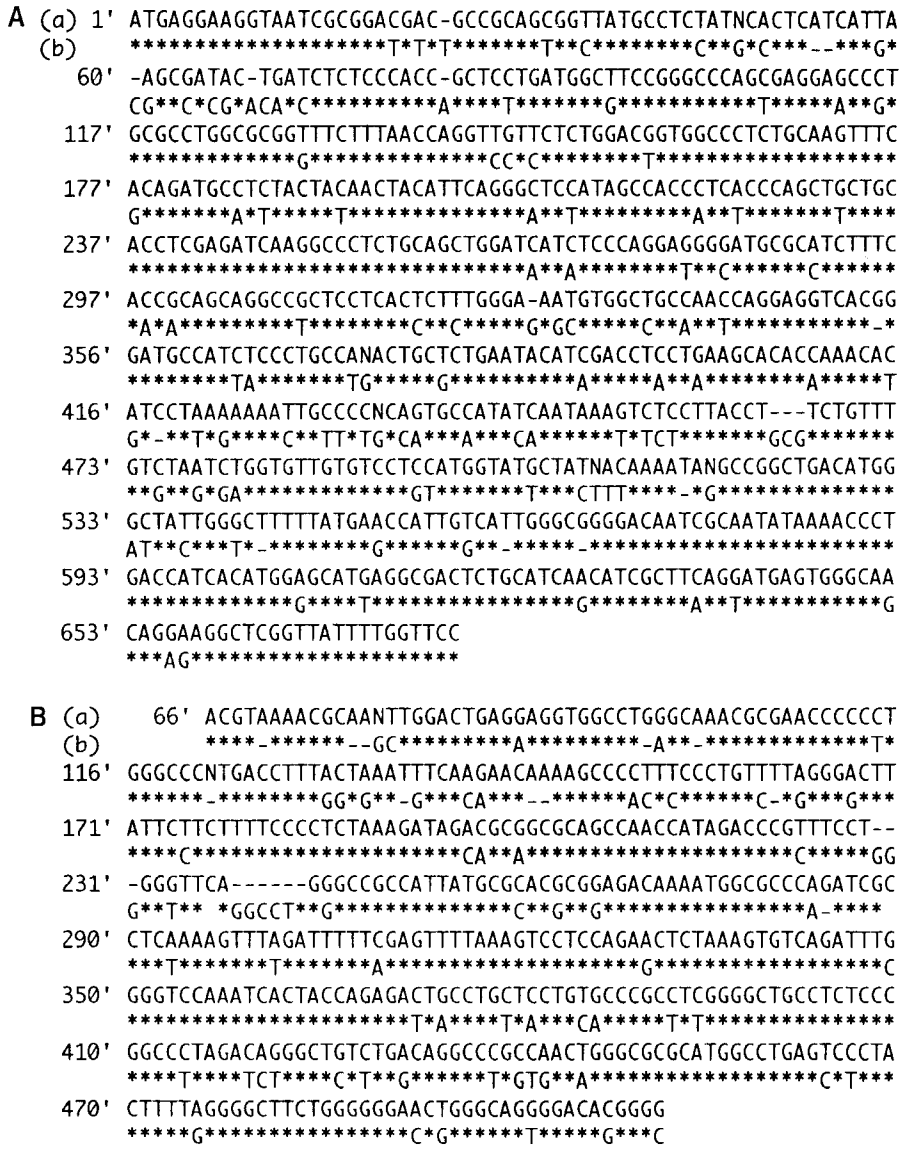

Fig. 4 Comparison of the nucleotide sequences corresponding to a part of the BRRF I region (**A**) and a part of the other IR I region (**B**) between Si-IIA-EBV (**a**) and B95-8 human EBV (**b**). In Fig. 4A, the sequences from 5' end of the No. 11 sense primer region (#105662) to the 3' end of the No. 11 antisense primer region (#106339) are shown. In Fig. 4B, only a middle part (475 bp #13872 to #14346, Ref. 5) of the sequence of the PCR product amplified with No. 2 sense and antisense primers is shown. Tentative numbering starts from the 5' end of the sense primer. Identical bases among the three sequences are shown by asterisks (*), and gaps generated by the computer to line up these sequences are shown by bars (−).

Discussion

Primary EBV infection usually results in establishment of asymptomatic latency, but occasionally develops infectious mononucleosis. EBV is also associated with various diseases, such as some non-Hodgkin's lymphomas related or unrelated to immune deficiency, about half of the cases of Hodgkin's disease and nasopharyngeal carcinoma (12–21). In a previous study, we found that intravenous inoculation with Si-IIA cells and S-IIA cell-free pellets induced ML in rabbits and that Si-IIA

cells harbored EBV-related herpesvirus (Si-IIA-EBV) (6). Absence of an HTLV-II genome in the Si-IIA-induced tumors excludes the possibility of ML induction by HTLV-II (5, 6). Therefore, we have concluded that MLs of rabbits are induced by a kind of EBV-related herpesviruses derived from Si-IIA cells but not by HTLV-II.

PCR analysis revealed that DNA of Si-IIA-EBV is amplified by some primers complementary to human EBV DNA but not by others (5). In this study, we analyzed the DNA of Si-IIA-EBV by PCR and Southern blotting and compared it with human EBV (B95-8) DNA. Direct DNA sequencing of the three homologous regions amplified by PCR showed only about 82% homology between Si-IIA-EBV DNA and human EBV (B95-8) DNA. The Southern blotting profiles of Si-IIA-EBV DNA were also different from those of human EBV DNA. On the other hand, it was shown that DNA of Si-IIA-EBV and HVMF1 had an extremely high sequence homology in the IR1 (BamHIW) region.

HVMF1 is an EBV-like herpesvirus associated with lymphomas in SIV-infected cynomolgus monkeys (8, 9, 11). The results of sequence analysis of the IR1 region raised the possibility that Si-IIA-EBV might be a variant of HVMF1. However, it is not presently possible to say that these two herpesviruses belong to the same virus species because the sequence data of HVMF1 DNA is now available only for the IR1 (BamHIW) region, meaning that the other two regions could not be compared. Besides HVMF1, EBV-related herpesviruses have been isolated in apes (22-25) and Old World monkeys including a Cynomolgus monkey (26-30). Information on genomic sequences of these viruses is not presently available. Therefore, molecular genetic comparison of simmian EBV-related herpesviruses including HVMF1 and Si-IIA-EBV is very difficult. One of these simian EBV-related herpesviruses from a Cynomolgus monkey isolated by Fujimoto *et al.* (31) also induced ML in rabbits. It showed close similarity to Si-IIA-EBV by comparison of the PCR products of viral DNA in some homologous regions (unpublished data), although the monkey serum reacting with this virus-bearing cells did not react with Si-IIA cells (5). Considering these results, EBV-related herpesviruses from a Cynomolgus monkey may be closely related herpesviruses which are lymphomagenic in rabbits under exprimental conditions.

Acknowledgments. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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Received January 10, 1997; accepteol March 21, 1997.