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Arrangement of Repetitive Sequences in the Genome of Herpesvirus Sylvilagus

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Herpesvirus sylvilagus is a lymphotropic (type gamma) herpesvirus of cottontail rabbits (*Sylvilagus floridanus*). Analysis of virion DNA of herpesvirus sylvilagus has revealed that the genome consists of one stretch of about 120 kilobase pairs of internal, unique DNA flanked by a variable number of 553-base-pair tandem repeats. The G + C content of the repetitive DNA is extremely high (83%), as determined by sequencing. The organization of the herpesvirus sylvilagus genome is, therefore, similar to that of the primate lymphotropic viruses herpesvirus saimiri and herpesvirus ateles.

Important biological properties of lymphotropic (also called gamma) herpesviruses include their abilities to infect lymphoid cells, establish latency, and cause tumors or lymphoproliferative disorders. The host range of these herpesviruses is relatively narrow. Well-studied lymphotropic herpesviruses include Epstein-Barr virus of humans, herpesvirus saimiri and herpesvirus ateles of New World monkeys, and Marek's disease virus of chickens (12, 13, 17).

Herpesvirus sylvilagus has been isolated from apparently healthy cottontail rabbits (*Sylvilagus floridanus* [4]). This rabbit virus can cause an acute lymphoproliferative disease in adult cottontail rabbits (5). This lymphomalike disease usually resolves spontaneously; however, it can be fatal in some cases, predominantly in young animals (5). Therefore, herpesvirus sylvilagus is classified as a member of the gammaherpesvirus group (17).

Epstein-Barr virus and the primate herpesviruses (herpesvirus saimiri and herpesvirus ateles) can infect B and T cells, respectively; however, herpesvirus sylvilagus can latently infect both T and B cells (7). Lymphoid cells from herpesvirus sylvilagus-infected animals contain covalently closed circular (latent) genomes and a small number of linear viral genomes (representing lytic infection), suggesting that the lymphoid organs are predominantly latently infected (7, 10). Similarly, the genomes of Epstein-Barr virus, herpesvirus saimiri, and Marek's disease virus form predominantly latent circular episomes in immortalized cell lines (1, 6, 14, 19, 22).

The genomes of herpesviruses display a great variety of arrangements of unique and repetitive DNA sequences. On the basis of the relationship of repetitive and unique sequences, the genomes of herpesviruses can be classified into five groups (17). This study was designed to reveal the architecture of the herpesvirus sylvilagus genome and to determine whether unique or repetitive DNA is located at the two termini of the linear virion genome, since the terminal sequences are probably involved in the circularization process during latent infection. Since lymphocytes transformed by these herpesviruses carry the viral genome as circular episomes, it is possible that circularization of the DNA is a structural requirement for oncogenic conversion. The results of this study show that repetitive DNA is at both ends of the genome.

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Herpesvirus sylvilagus can be grown to high titers in cottontail rabbit kidney cells, and virions, as a source of viral DNA, can be readily purified (10). We have isolated herpesvirus sylvilagus virion DNA from purified virions by pronase digestion followed by phenol and chloroform-isoamyl alcohol extraction and ethanol precipitation as described previously (10). Digestion of virion DNA with restriction endonuclease *Pst*I or *Not*I resulted in the appearance of a 0.55-kilobase-pair (kb) band present in about 20-fold molar excess. *Xho*I and *Sma*I enzymes produced two supermolar bands of lower molecular weight (0.33 plus 0.22 kb and 0.52 plus 0.03 kb, respectively; data not shown). These patterns suggested that repetitive DNA is present in the genome of herpesvirus sylvilagus in repeat units of 0.55 kb.

To map the putative repetitive DNA, we have cloned the 0.55-kb *Pst*I fragment into the *Pst*I site of the M13-SK+ vector (Stratagene, La Jolla, Calif.). Viral DNA was digested with various restriction enzymes, separated on agarose gels, blotted onto nitrocellulose filters by the vacuum technique (9), and hybridized with cloned repetitive DNA or virion DNA labeled with [^{32}P]dCTP by nick translation (15). The pattern obtained by hybridization with virion DNA was consistent with complete digestion of the samples (Fig. 1A). Two bands hybridized with repetitive probe in *Pst*I-digested DNA (3.1 and 2.0 kb, fragments L and V) with about equal intensities (Fig. 1B). The 0.55-kb *Pst*I supermolar band hybridized much more strongly than *Pst*I fragments L and V. These two unique fragments, containing some repetitive sequences, probably represent junction sequences between repetitive and unique DNA. In addition, two bands of approximately 0.2 and 0.15 kb were apparent after longer exposures. These small fragments could be either two more junction fragments or the two physical ends of the genome (the results presented below support the second hypothesis).

When the viral DNA was digested with *Bgl*II or *Bam*HI, the repetitive DNA probe hybridized to several bands which were sequentially about 0.55 kb smaller (Fig. 1B). *Bgl*II yielded one and *Bam*HI yielded two such ladderlike patterns. *Eco*RI yielded a high-molecular-weight smear. The features of the ladderlike structure suggested that the number of repetitive units within the viral genome is variable. In addition, the presence of a double *Bam*HI ladder is consistent with a model of the genome with two separate blocks of repetitive DNA.

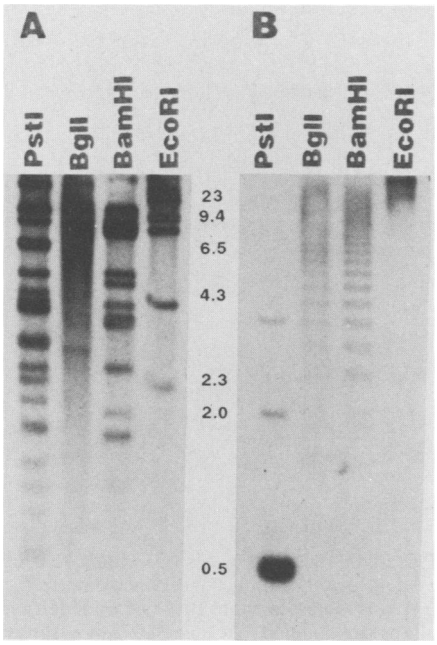

FIG. 1. Analysis of herpesvirus sylvilagus DNA by restriction enzymes and hybridization with repetitive DNA. About 0.1 μg of virion DNA was cleaved with restriction endonucleases *Pst*I, *Bgl*II, *Bam*HI, and *Eco*RI, as indicated, under conditions suggested by the manufacturer (Promega Biotec, Madison, Wis.). Samples were separated on a 1% agarose gel by electrophoresis followed by blotting by vacuum onto nitrocellulose (9). The filter was hybridized with ^{32}P-labeled (15) virion DNA probe (A). The virion probe was removed by 70% formamide at 80°C and then rehybridized with the repetitive probe (B).

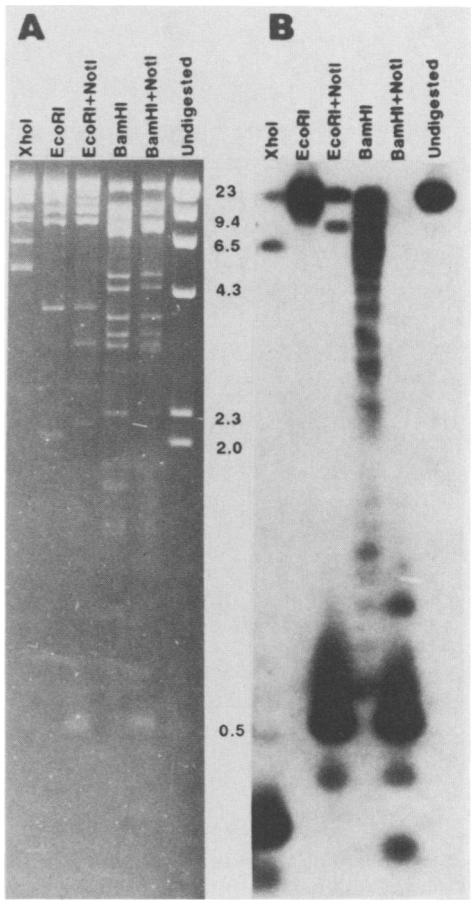

FIG. 2. Double digestion of viral DNA with *Not*I plus enzymes with no cleavage site within repetitive DNA. Viral DNA was digested with the enzymes indicated, and the samples were analyzed by blot hybridization with the repetitive probe as described in the legend to Fig. 1.

Restriction endonuclease *Not*I cleaves repetitive DNA once and has no more than three sites within unique sequences. The *Not*I sites within the unique DNA are more than 10 kb from repetitive DNA (data not shown). Figure 2 shows double cleavages of the viral DNA with *Not*I plus *Bam*HI and with *Not*I plus *Eco*RI. When the viral genome was cleaved with *Bam*HI alone, the repetitive DNA probe hybridized with a ladderlike series of bands. However, when the viral DNA was digested with *Bam*HI plus *Not*I, the ladder was not seen but a 0.55-kb supermolar band, plus three much fainter bands of low molecular weights, was hybridized with the repetitive probe. This is consistent with the fact that *Bam*HI sites are near the blocks of repetitive DNA, since the two repetitive *Bam*HI DNA ladders start at the 2-kb range. The *Bam*HI ladders span between 2 and about 20 kb (Fig. 1 and 2), which could be explained by extreme variability in the number of repetitive units present in a given molecule within the blocks of repetitive DNA. The size of the repeat unit, 0.55 kb, and that of the *Bam*HI ladder, 2 to 20 kb, suggest that the number of repeat units can vary from 1 to at least 35 at each end. The equal hybridization to each band in the ladder indicates that molecules with each number of repeat units are about equally represented. *Eco*RI-cleaved DNA hybridized with the repetitive probe as a high-molecular-weight smear with a 9- to 23-kb span range. The *Not*I-plus-*Eco*RI-cleaved DNA yielded two high-molecular-weight junction fragments (Fig. 2B), suggesting that the *Eco*RI sites are at least 8 kb away from the repetitive DNA.

The data presented above are consistent with two models concerning the arrangement of the repetitive DNA within the herpesvirus sylvilagus genome. There are either two blocks of repetitive DNA (two *Bam*HI ladders) flanked by unique sequences or one stretch of the unique DNA flanked by two blocks of repetitive elements. Each model is compatible with the *Pst*I, *Not*I-plus-*Eco*RI, and *Not*I-plus-*Bam*HI analyses (one supermolar band plus repetitive sequences within four 1 M fragments).

To determine whether repetitive DNA is located at the physical ends of the genome, virion DNA was partially digested with BAL 31 exonuclease (Bethesda Research Laboratories, Gaithersburg, Md.) for different periods of time. BAL 31 exonuclease is capable of digesting 3′ and 5′ ends of double-stranded DNA in a progressive, stepwise manner. The reactions were stopped by sodium dodecyl sulfate and ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA); DNA was extracted with phenol and chloroform, precipitated with ethanol, and cleaved with *Bam*HI; and the samples were analyzed by blot hybridization with ^{32}P-labeled virion DNA (Fig. 3A). The sizes of unique *Bam*HI fragments were unaffected by previous BAL 31 exonuclease digestion (Fig. 3A). To determine whether fragments of the *Bam*HI repetitive DNA ladder were smaller, the same blot was treated with 70% formamide at 80°C to remove the virion probe (11) and the blot was reprobed with cloned repetitive DNA labeled with ^{32}P. The

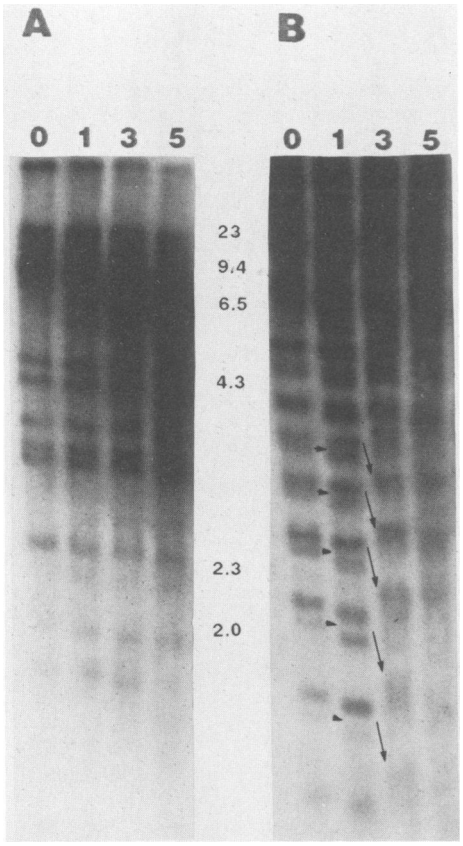

FIG. 3. Exonuclease digestion of viral DNA with BAL 31 followed by cleavage with *Bam*HI. Approximately 0.3 μg of purified virion DNA was digested with 0.1 U of BAL 31 (Bethesda Research Laboratories) in 100 μl of reaction buffer containing 12 mM $CaCl_2$, 12 mM $MgCl_2$, 0.2 M NaCl, 20 mM Tris hydrochloride (pH 8.0), and 1 mM EDTA at 30°C for 0, 1, 3, and 5 min as indicated for each lane. After incubation the reactions were stopped by EGTA (final concentration, 30 mM) and 0.5% sodium dodecyl sulfate and extracted with phenol and chloroform, and the DNA was precipitated by ethanol. After centrifugation (16,000 × *g*, 30 min), pellets were air dried, dissolved in 10 mM Tris hydrochloride–1 mM EDTA, and digested with *Bam*HI. Samples were separated on a 1% agarose gel by electrophoresis followed by blotting by vacuum onto nitrocellulose (9). The filter was hybridized with ^{32}P-labeled (15) virion DNA probe (A). The virion probe was removed by 70% formamide at 80°C (11) and then rehybridized with the repetitive probe (B). Arrows indicate changes in size of repetitive *Bam*HI ladders.

size of each band in the *Bam*HI ladder was decreased by about 50 base pairs (bp) after 1 min of BAL 31 pre-digestion and by about 0.4 kb after 3 or 5 min of predigestion (Fig. 3B). Further predigestion with BAL 31 has resulted in extensive smearing of the repetitive sequences (data not shown).

Under conditions employed in this work, the rate at which exonuclease BAL 31 can remove nucleotides from the ends of double-stranded DNA was calculated to be no more than 50 bp/min (8). Our experimental data showed that the sizes of both *Bam*HI repetitive-DNA ladders were decreased at about the calculated rate after BAL 31 pretreatment. The results of the BAL 31 experiment strongly suggest that the model which places repetitive sequences at both ends of the viral DNA is the correct one and that the number of the repeats attached to the unique DNA is variable.

To test whether the repeats at one end of the viral genome are inverted or tandem relative to those at the other end,

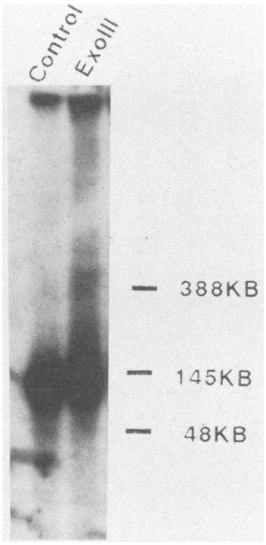

FIG. 4. Pulse-field electrophoresis of virion DNA digested with exonuclease III to detect circular and concatemeric forms. Virion DNA (0.2 μg) was digested with 10 U of exonuclease III (New England BioLabs) for 5 min at 37°C. The DNA was self-annealed for 5 h in 0.1 ml of 50% formamide–5× SSC–20 mM sodium phosphate buffer (pH 6.5) at 42°C. The size of DNA was analyzed by pulse-field agarose gel electrophoresis (21) by using a programmable power converter (PPI-200; MJ Research Inc., Cambridge, Mass.). Electrophoresis was in a 0.8% agarose gel for 14 h with constant recirculation of the buffer at 6 V/cm; the buffer was 45 mM Tris–45 mM boric acid–0.5 mM EDTA. The power converter was set at program 5; reverse time was 0.1 to 10 s, forward time was 0.3 to 30 s, one cycle was 10:25.2 s (ratio of total reverse time to forward time in one cycle), and slowdown was 2. Viral DNA was detected by Southern blotting with cloned repetitive DNA pSY0.55 as a probe. Ligated concatemers of lambda phage DNA were run in adjacent lanes as size markers; relevant sizes are indicated in kilobase pairs. Lane ExoIII, Viral DNA predigested with exonuclease III. Lane Control, Viral DNA treated under conditions identical to those for lane ExoIII except that no exonuclease III was added.

viral DNA was digested with exonuclease III, which catalyzes release of 5′ nucleotides from the 3′-hydroxy end of double-stranded DNA (16). If the repeats of the two ends are in tandem orientation, the single strands generated by exonuclease III digestion should be complementary. Virion DNA (0.2 μg) was digested with 1 U of exonuclease III for 5 min at 37°C; we calculated that at least 0.5 kb of the single-stranded region was generated at both ends under these reaction conditions. The DNA was self-annealed for 5 h in 0.1 ml of 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–20 mM sodium phosphate buffer (pH 6.5) at 42°C and analyzed by pulse-field agarose gel electrophoresis (21) followed by Southern blotting. Although the majority of DNA migrated as the undigested control DNA, two bands of about 300 and 450 kb and a smear continuing to the loading well were present for the exonuclease-treated sample. These large molecules were absent in control undigested DNA (Fig. 4). Therefore, a portion of DNA molecules have joined as concatemers or circles, which is possible only if the orientation of the repeats at one end is tandem relative to the orientation of the repeats at the other end.

DNA sequence of the *Pst*I repeat cloned in Bluescript M13-SK+ vector (Stratagene) pSYP0.55 was determined. DNA was sequenced from denatured plasmid preparations

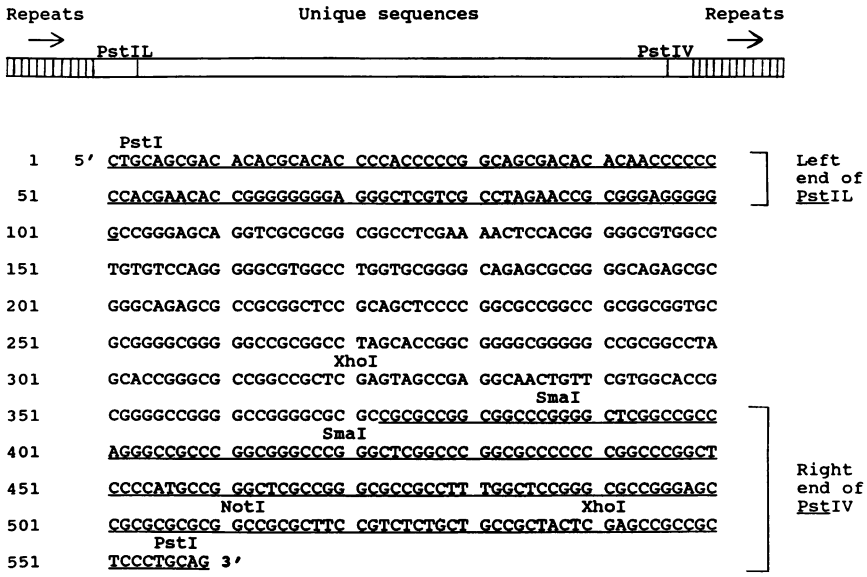

FIG. 5. Proposed structure of the herpesvirus sylvilagus genome and nucleotide sequence of one repeat unit. DNA sequences of the *Pst*I repeat cloned in Bluescript M13-SK+ vector (Stratagene) pSYP0.55 and of a series of subclones of pSYP0.55 with nested deletions were determined by the dideoxy method with avian myeloblastosis virus reverse transcriptase (20, 23). DNA was sequenced from denatured plasmid preparations with M13 universal primer. Brackets indicate sequences (underlined) which are common in the 553-bp repeat unit and in the *Pst*I junction fragments L and V.

of a series of subclones of pSYP0.55 with nested deletions (3). M13 universal primer was annealed with denatured DNA; sequencing was by the dideoxy method with avian myeloblastosis virus reverse transcriptase (20, 23). The suggested structure of the genome with nucleotide sequences and restriction endonuclease sites of the 553-bp repetitive DNA are shown in Fig. 5. The G+C content of the repetitive DNA is extremely high (83%).

DNA sequences of the termini of cloned *Pst*I junction fragments (fragments L and V, clones pSYP3.1 and pSYP2) were also determined from both ends of the viral inserts (about 150 bp was sequenced from both ends). The left end of the *Pst*I L fragment was homologous with the 5' end of the sequence of the repetitive DNA (Fig. 5). The right end of the *Pst*I V fragment was homologous with the 3' end of the repetitive DNA (Fig. 5). The right end of *Pst*I fragment L and left end of *Pst*I fragment V contained sequences with much lower G+C contents and had no homology at all with the repetitive DNA (results not shown). These results strongly suggest, again, that orientation of repeats at both ends is tandem, since the orientation of long stretches of repetitive sequences in the *Pst*I L and V junction fragments are tandem.

Recently, a study on the genomic structure of herpesvirus sylvilagus using the virus isolate which was involved in our work was reported (18); this report concluded that the repetitive elements are located internally. This conclusion appears to be an error and is not supported by the data obtained in the present study. After lambda exonuclease digestion, Rouhandeh and Cohrs (18) observed a decreased ethidium bromide staining of *Xho*I fragments A and E. However, their map shows B and E as terminal fragments and locates *Xho*I fragment A more internally than the repetitive sequences are placed. The authors do not explain why the terminal B fragment would not and the central A fragment would be affected by lambda exonuclease digestion.

We have also cleaved viral DNA with *Xho*I and analyzed the digest by blot hybridization (Fig. 2). Two bands, corresponding to *Xho*I fragments A and E, hybridized weakly with the cloned repetitive DNA, and a low-molecular-weight band hybridized strongly (Fig. 2B). *Xho*I fragments A and E are junction fragments, and we have demonstrated that the number of repetitive-DNA units attached to the ends is extremely variable. Therefore, *Xho*I fragments A and E are relatively close to the physical ends of the genome in DNA molecules with a small amount of repetitive DNA attached, which could explain why lambda exonuclease digestion has altered *Xho*I fragments A and E as previously reported (18).

The arrangement of repetitive elements within the genome of the oncogenic primate herpesviruses herpesvirus saimiri and herpesvirus ateles is very similar to that of herpesvirus sylvilagus; the size of one repeat unit (termed H DNA) is 1.4 kb, its G+C content is 71%, the number of terminal H DNA units is variable, and repeat units are in tandem orientation (2).

The size of unique sequences was estimated at about 120 kb by combining the sizes of unique restriction fragments of the herpesvirus sylvilagus genome (*Pst*I and *Sma*I fragments were used for the estimation). The total genome length is more difficult to estimate, since there is great variability in the number of repetitive units attached (1 to at least 40). Herpesvirus saimiri DNA molecules also feature great variability in length of repetitive DNA. However, the length of the genome is constant; molecules with short repetitive DNA at one end of the genome have a long stretch of repetitive DNA at the other end (2). We do not have evidence that the overall number of repetitive units is constant in the genome of herpesvirus sylvilagus.

The genome of Epstein-Barr virus also features terminal repeats similar in size to that of herpesvirus sylvilagus. The Epstein-Barr virus genome also contains several internal repeats, but we have no evidence for internal repeats of herpesvirus sylvilagus. Latency of the human, monkey, chicken, and rabbit lymphotropic herpesviruses is associated with covalently closed circular forms of the genome in

lymphocytes (1, 6, 14, 19, 22). The process of circularization probably involves joining the ends of the linear virion DNA by an unknown mechanism during latent infection. Further work is required to understand how the joining of terminal repeats of lymphotropic herpesviruses is mediated.

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