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Circular *Herpesvirus sylvilagus* DNA in Spleen Cells of Experimentally Infected Cottontail Rabbits

PETER MEDVECZKY,[1,3]* WILLIAM J. KRAMP,[2] AND JOHN L. SULLIVAN[2,3]

Departments of Pharmacology,[1] Pediatrics,[2] and Molecular Genetics and Microbiology,[3] University of Massachusetts Medical School, Worcester, Massachusetts 01605

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Cottontail rabbits (*Sylvilagus floridanus*) were infected with *Herpesvirus sylvilagus*, and spleen cells were analyzed for the presence of virus-specific, covalently closed circular, and linear DNA molecules by a simple electrophoretic technique, followed by transfer to nitrocellulose filters and hybridization with cloned viral DNA (Gardella et al., J. Virol. 50:248–254, 1984). Approximately 0.2 copies per cell of circular DNA and 0.2 copies per cell of linear DNA were detected by hybridization with a cloned viral DNA fragment. The size of the viral DNA was estimated at ca. 158 kilobase pairs. Restriction endonuclease patterns suggested structural similarities to cottontail herpesvirus DNA.

Herpesvirus sylvilagus (*H. sylvilagus*) has been isolated from primary kidney cells of apparently healthy cottontail rabbits (*Sylvilagus floridanus*) trapped in southern Wisconsin (11). The virus can be easily propagated in primary newborn cottontail rabbit kidney cells (11). Experimental infection of cottontail rabbits induces a malignant lymphoproliferative disease in 27% of young and 10% of adult animals, and a benign lymphoid hyperplasia develops in the majority of infected animals (12). This benign disease is thought to be similar to infectious mononucleosis caused by Epstein-Barr virus (EBV) in man (12). Like EBV, *H. sylvilagus* has a very narrow host range; experimental infection has been only successful in cottontail rabbits (12). Limited information has been published about the molecular biology of *H. sylvilagus*. The viral DNA has reportedly a low 33% guanine-plus-cytosine content (9), and the polypeptides of the virion have been studied in some detail (3).

The virion DNA of herpesviruses is always found in a linear duplex form. However, multiple copies of covalently closed circular viral DNA molecules have been shown in EBV-infected B-lymphoblastoid cell lines derived from African Burkitt's lymphoma or infectious mononucleosis patients. Some Burkitt cell lines produce infectious virus; in these cells, a variable amount of linear DNA is also detectable (for a review, see reference 1). Other lymphotropic herpesviruses such as *Herpesvirus saimiri*, *Herpesvirus ateles*, and Marek's disease virus are T-cell-tropic viruses, and viral DNA is found in an episomal circular form in tumor cells (13, 17, 19, 22).

The state of EBV DNA in lymphoid tissues of infectious mononucleosis patients is unknown. For detection of viral DNA in patient samples, a considerable amount of tissue would be needed if conventional techniques were used. This report presents evidence for both circular and linear *H. sylvilagus* DNA in spleen tissues of experimentally infected cottontail rabbits by a simple electrophoretic technique which requires a small number of virus-infected cells.

To obtain cloned viral DNA fragments for hybridization, we purified virions and cloned the viral DNA as follows. Virus was pelleted from cell-free supernatants of infected newborn cottontail rabbit kidney cells and purified by sucrose gradient centrifugation, and the viral DNA was extracted from virions (7). The DNA was suspended in 10 mM Tris-hydrochloride–1 mM EDTA (pH 7.6) and cleaved with *Bam*HI restriction endonuclease, and the resulting DNA fragments were cloned in the *Bam*HI site of pBR322 by standard cloning procedures (14). A clone (pSYB8.5) containing an 8.5-kilobase pair (kbp) *H. sylvilagus Bam*HI DNA fragment was selected. This fragment did not hybridize to uninfected cellular DNA but did hybridize to *H. sylvilagus Bam*HI fragment F (data not shown).

Digestion of *H. sylvilagus* virion DNA with restriction endonuclease *Sma*I resulted in 26 DNA fragments ranging from 29 to 0.55 kbp (Fig. 1). Fragments smaller than 0.55 kbp were not scored. All DNA fragments appeared to be present in equimolar amounts except the smallest, *Sma*I fragment Y, which was present in 10- to 15-fold excess. The size of *H. sylvilagus* DNA was estimated at ca. 158 kbp, based on the sum of the sizes of all *Sma*I fragments; the Y fragment counted as a 10-fold repeat. In *Bam*HI-cleaved *H. sylvilagus* DNA (Fig. 1.), several fragments were observed that were present at apparently lower molarity; we observed four fragments (O, P, R, and T) that appeared at concentrations of ca. 0.5 M each and three fragments (D, E, and F) at concentrations of 0.25 M each.

Two adult cottontail rabbits (trapped in central Massachusetts) were infected intraperitoneally with 5×10^6 PFU of *H. sylvilagus* prototype strain obtained from H. C. Hinze (11). Leukocytosis (10,000 to 12,000 leukocytes per mm^3) was observed with relative lymphocytosis 16 days after infection. The animals were sacrificed 16 and 23 days after infection, and spleen cells were frozen by controlled-rate freezing to −100°C. Considerable splenomegaly was observed in infected animals as compared with uninfected controls. About 10^9 cells were obtained from infected rabbit spleens after thawing, whereas control spleens yielded only about 10^8 cells. About 100 PFU of infectious virus was detected in 10^6 infected spleen cells by infectious center assay on newborn cottontail rabbit kidney cells.

Cell suspensions were analyzed by the gel technique of Gardella et al. (8) for the presence of superhelical and linear viral DNA as briefly outlined below. One million cells from

* Corresponding author.

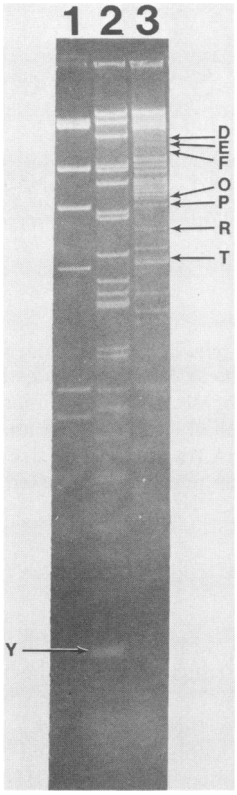

FIG. 1. Restriction endonuclease cleavage pattern of *H. sylvilagus* DNA. Viral DNA (100 ng) was digested with *Sma*I and with *Bam*HI (Promega-Biotec), and the resulting DNA fragments were separated on a 1% agarose gel. DNA bands were visualized by staining in 1 μg of ethidium bromide per ml and photography over UV light. *Hin*dIII fragments of bacteriophage lambda DNA were used as molecular weight standards (lane 1). *H. sylvilagus* DNA was digested with *Sma*I (lane 2) and *Bam*HI (lane 3).

each of the two infected spleens and the same amount from uninfected controls were washed and suspended in electrophoresis buffer containing 15% Ficoll. The cell suspensions were loaded into the wells of a vertical agarose gel and overlaid with buffered sodium dodecyl sulfate-pronase solution. The electrophoresis was started at low voltage when the sodium dodecyl sulfate-pronase mixture slowly migrated in the cell layer, resulting in gentle cell lysis. This was followed by high-voltage separation of putative circular and linear DNA molecules.

After electrophoresis, the gel was stained with ethidium bromide and photographed over UV light (Fig. 2A). The loading wells containing very high-molecular-weight cellular DNA and an area where partially degraded linear DNA migrates were brightly stained, but no visible bands were observed in the area of the gel where large superhelical DNA molecules were expected to be, except a faint band in lane 1, which contained Raji cells. These Burkitt lymphoma cells carry ca. 50 copies of episomal circular EBV DNA molecules (2). The appearance of circular DNA in lanes containing Raji cell lysate indicated that the lysis procedure and the electrophoretic separation were successful. Therefore, DNA from the gel was transferred to a nitrocellulose filter (21) hybridized with pSYB8.5 probe labeled with ^{32}P by nick translation (18). Two faint bands were hybridized with the cloned fragment (Fig. 2B, lanes 3 and 4). These bands comigrated with circular EBV DNA of Raji cells, indicating that in *H. sylvilagus*-infected rabbit spleen cells, the viral DNA is in a superhelical circular form, because only large covalently closed circular DNA molecules can be detected in this area of the gel, as shown previously (5, 8). The sizes of the circular EBV and circular *H. sylvilagus* DNA molecules were estimated to be very similar.

To estimate the copy number of viral DNA molecules in spleen cells, we mixed different dilutions of purified *H. sylvilagus* virion DNA with uninfected spleen cells (Fig. 2, lanes 6, 7, and 8). As expected, viral DNA was detected by hybridization in the area of linear DNA. Lane 9 contained 10^4 PFU of extracellular virus, and strong hybridization was found in the area of linear DNA. As with the positive controls, faint hybridization was observed at the level of linear DNA in lanes containing samples from spleen cells of infected rabbits (Fig. 2, lanes 3 and 4). We estimated that ca. 0.2 copies of circular and about the same amount of linear viral DNA were present per cell in spleen cells of infected rabbits as estimated by comparing intensities of control bands with those of bands of infected spleen cells. No hybridization was observed in lanes containing spleen cells from uninfected control animals (Fig. 2, lanes 2 and 5).

Little is known about the structure of *H. sylvilagus* DNA. The genome structure of a herpesvirus isolated from a cottontail rabbit (CTHV) has been shown to have some features found in *H. saimiri* as well as some properties of herpes simplex virus DNA (J. Cebrian, N. Berthelot, M. Laither, and P. Sheldrick, personal communication). CTHV

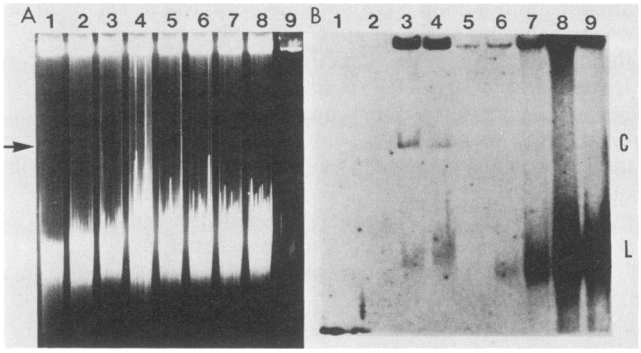

FIG. 2. Detection of circular (C) and linear (L) *H. sylvilagus* DNA by the method of Gardella et al. (8). (A) For fluorography, suspensions (0.1 ml) of 10^6 cells in 15% Ficoll buffered in 89 mM Tris–89 mM boric acid–2.5 mM EDTA (pH 8.2) were layered in wells of a vertical 0.75% agarose gel (12 by 12 by 0.25 cm). The cell suspensions were carefully overlaid with 0.1 ml of buffered 5% Ficoll containing 1% sodium dodecyl sulfate and 1 mg of pronase per ml. Electrophoresis was for 3 h at 0.8 V/cm and then for 15 h at 7.5 V/cm at 4°C. The gel was stained with 1 μg of ethidium bromide per ml and photographed over UV light with Polaroid type 665 film and a Kodak 23A filter. The DNA was transferred to nitrocellulose (21) and hybridized for 18 h with 2×10^6 cpm of pSYB8.5 DNA labeled to a specific activity of 5×10^8 cpm per μg of DNA (18). The hybridization was in 10% dextran sulfate containing 50% formamide, 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 20 mM sodium phosphate buffer (pH 6.5), 100 μg of salmon sperm DNA per ml, and Denhardt solution at 42°C for 18 h as published before (8). The filter was washed in five changes of 0.1× SSC–0.1% sodium dodecyl sulfate at 60°C for 5 min each. (B) For autoradiography, the filter was exposed to Kodak XAR-2 film for 5 days at −70°C. The following cells were used: Raji cells (lane 1); spleen cells from an uninfected rabbit (lanes 2 and 5); spleen cells 16 days (lane 3) and 23 days (lane 4) after infection; uninfected cells mixed with 8 pg (lane 6), 80 pg (lane 7), and 800 pg (lane 8) of virion DNA of *H. sylvilagus*; and 10^4 PFU of cell-free virus (lane 9).

DNA consists of two segments of unique sequences flanked and joined by tandem repeats of different length. Variability in the number of terminal repeat units has been found in *H. saimiri* DNA (for a review, see reference 6). Like herpes simplex virus (10, 20), CTHV genome displays four isomeric forms. Although both *H. sylvilagus* and CTHV have been isolated from the same rabbit species (*Sylvilagus floridanus*), no direct evidence is available to suggest that the two viruses belong to the same species. Our results, however, suggest that these viruses are related in overall structure: (i) we have found a supermolar 0.55-kbp *Sma*I fragment in *H. sylvilagus* DNA, and Cebrian et al. (Cebrian et al., personal communication) have detected a supermolar *Sma*I fragment of the same size in CTHV DNA, indicating that similar repetitive DNA elements are present in both genomes; and (ii) both 0.5 and 0.25 M fragments have been found in *Bam*HI digests of *H. sylvilagus* DNA, which could be interpreted as isomerization of the genome as described for herpes simplex virus (10, 20) and is consistent with electron microscopical studies and the proposed structure of the CTHV DNA by Cebrian et al. (Cebrian et al., personal communication).

The main observation of this study is that *H. sylvilagus*-infected lymphoid tissues contain superhelical circular viral DNA molecules. We have evidence that both B and T lymphocytes from spleens of infected rabbits are simultaneously infected by the virus and that both cell types carry circular viral DNA molecules (W. J. Kramp, P. Medveczky, C. Mulder, H. C. Hinze, and F. L. Sullivan, submitted for publication). The small amount of virus detected in spleen cells of infected animals correlated with the small amount of linear viral DNA detected by the method of Gardella et al. (8) (Fig. 2). These biological properties of *H. sylvilagus* are very similar to those of EBV: (i) both viruses carry covalently closed circular genomes, and (ii) both viruses infect B lymphocytes. A further similarity between these viruses has already been pointed out by Hinze: both viruses can cause benign lymphoproliferative diseases in their natural host (12). Both viruses are associated with malignant lymphoid tumors, although their significance in tumor formation remains unclear. Other lymphotropic herpesviruses such as *Herpesvirus saimiri* and *Herpesvirus ateles* differ from *H. sylvilagus* and EBV. *H. saimiri* and *H. ateles* are T-cell-tropic viruses and do not infect B lymphocytes. They do not induce benign lymphoproliferative disease in their natural hosts but are highly tumorigenic in other New World monkey species (15, 16). On the other hand, *H. saimiri* and *H. ateles* tumor cells also carry multiple copies of circular viral DNA (13, 22). Another intriguing question is the biological significance of circular viral DNA in these virus-host cell systems. Exposure of EBV-transformed lymphocytes to either of the antiviral drugs acyclovir or phosphonoacetic acid reduces the number of copies of linear viral DNA but has no effect on circular genomes (4, 23). Since these drugs are selective inhibitors of viral DNA polymerase, it has been suggested that circular EBV DNA is synthesized by cellular enzymes which are refractory to acyclovir or phosphonoacetic acid.

We are attempting to exploit *H. sylvilagus* as an animal model for EBV. *H. sylvilagus* offers some unique advantages: (i) *H. sylvilagus*, unlike EBV, can be propagated to high titers in newborn cottontail rabbit kidney cells; (ii) as shown, circular DNA can be easily detected in vivo, and with simple immunological techniques the carrier cell(s) could be identified; (iii) *H. sylvilagus* could be a model for latency and malignant lymphoma research; and (iv) effectiveness of potential antiviral drugs against circular genomes can be tested in rabbits in vivo.

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