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Structure of Nonintegrated, Circular *Herpesvirus saimiri* and *Herpesvirus ateles* Genomes in Tumor Cell Lines and In Vitro-Transformed Cells

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Nonintegrated, circular DNA molecules of *Herpesvirus saimiri* and *Herpesvirus ateles* were found in five lymphoid cell lines originating from tumor tissues or established by in vitro immortalization of T lymphocytes. The arrangement of unique (L) and repetitive (H) DNA sequences in circular viral genomes was analyzed by partial denaturation mapping followed by visualization with an electron microscope. Three types of circular viral DNA structures were found. (i) The virus-producing cell line RLC, which is derived from an *H. ateles*-induced rabbit lymphoma, contains circular viral genomes which consist of a single L-DNA and a single H-DNA region, both the same length as in virion DNA. (ii) The circular viral genomes of the nonproducer cell lines H1591 and A1601, in vitro transformed by *H. saimiri* and *H. ateles*, respectively, have deletions in the unique L-DNA region and larger H-DNA regions. Cell line A1601 lacks about 8% of virion L-DNA, and H1591 cells lack about 40% of viral L-DNA information. (iii) The nonproducing *H. saimiri* tumor cell lines 1670 and 70N2 harbor viral genomes with two L-DNA and two H-DNA regions, respectively. Both types of circular molecules have a long and a short L segment. The sequence arrangements of circular DNA molecules from *H. saimiri*-transformed cell lines were compared with those of linear virion DNA by computer alignment of partial denaturation histograms. The L-DNA deletion in cell line H1591 was found to map in the right half of the virion DNA. Comparison of the denaturation patterns of both L regions of cell lines 1670 and 70N2 identified the short L regions as subsets of the long L regions. Thus, circular viral DNA molecules of all four nonproducer cell lines represent defective genomes.

Herpesvirus saimiri and *Herpesvirus ateles* are highly oncogenic agents, causing acute leukemias and rapidly progressing malignant lymphomas in various species of New World primates upon experimental infection (7, 14, 21, 32, 33). Despite a number of discrete differences in genome structure and tumor biology, *H. saimiri* and *H. ateles* are similar in some respects to the human Epstein-Barr virus (EBV) (reviewed in reference 17). EBV genomes persist, at least in part, as nonintegrated circular DNA molecules in transformed cells (1–3, 37). Circular viral DNA has been found in tumor tissue biopsies (26), tumor cell lines (31), and lymphoblastoid B cell lines established from healthy donors by in vitro transformation (28). The extent to which viral DNA may be covalently linked to cellular DNA sequences remained controversial.

H. saimiri and *H. ateles* virion DNAs have a structure different from that of all other herpesviruses described previously. A long segment of unique (L) DNA with a guanine plus cytosine (G+C) content of 36% is flanked at both ends by stretches of highly repetitive (H) sequences with 71% G+C, as can be visualized with an electron microscope after partial denaturation mapping (4, 17). An earlier study from this laboratory showed that a high proportion of resident viral genomes in the lymphoid tumor cell line 1670 persists as covalently closed circular molecules (40). The circular molecules found in this cell line have a molecular weight of 131.5×10^6, which is considerably higher than that of the linear DNA present in the virion. The circles from 1670 cells contain a long and a short region of L-DNA (57.8×10^6 and 33.7×10^6 molecular weight, respectively) separated by two segments of H-DNA. The short L-DNA region is a subset of the long L-DNA region; the long L-DNA region differs from virion DNA by the absence of a segment of a molecular weight of 13.0×10^6 (8, 41). Viral DNA in cell line 1670 is heavily

methylated in the cytosine residues of cytidylic-guanylate dinucleotides in both H- and L-DNA sequences (10, 27).

We wanted to investigate whether the persistence of nonintegrated circular viral DNA is a more general phenomenon in herpesvirus-transformed lymphoid cells of nonhuman primates. Electron microscopy and partial denaturation mapping were applied to investigate the structure of circular viral genomes in two additional tumor cell lines (70N2 and RLC) and in two in vitro-immortalized lymphoid cell lines (H1591 and A1601).

The observation of large deletions in the L regions of the four nonproducer cell lines indicates certain regions of the large herpesvirus genomes not required for persistence of circular viral DNA. The long-range goal of these studies is to help identify the regions of herpesvirus genomes which are required for maintenance of the growth-transformed state in lymphoid cells.

MATERIALS AND METHODS

Origin of lymphoblastoid cell lines. Cell lines 1670 and 70N2 were obtained from L. A. Falk (Primate Center of Harvard Medical School) (16) and grown in this laboratory continuously over several years. Cell line H1591 was obtained by in vitro immortalization of peripheral lymphocytes of a cotton top marmoset with *H. saimiri* strain OMI (B. Fleckenstein, unpublished data). Cell line A1601 was obtained in our laboratory by in vitro immortalization with *H. ateles* strain 73 according to published procedures (15). RLC cells were established by M. D. Daniel (Primate Center of Harvard Medical School) from *H. ateles*-induced rabbit tumors (M. D. Daniel, V. R. Zurawski, Jr., A. D. Strosberg, P. Marche, B. Vray, C. Kaschka-Dierich, B. Fleckenstein, R. Tamulevich, N. W. King, Jr., and L. A. Falk, Jr., submitted for publication). Cell line RLC produced *H. ateles* in an infectious center assay on owl monkey kidney (OMK) cell monolayers under 2% methylcellulose; all other cell lines used in this study did not produce infectious virus.

Virus and cell culture. *H. saimiri* strain 11 and *H. ateles* strain 810 were propagated on OMK monolayer cells as described previously (23). *H. saimiri*- and *H. ateles*-transformed cells were grown as suspension cultures in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum and 350 μg of glutamine, 100 U of penicillin, and 100 μg of streptomycin per ml. The cells were kept in closed Erlenmeyer flasks and subcultured at 3- to 4-day intervals. They were checked at regular intervals for mycoplasma by an anaerobic isolation procedure and were found to be free of contamination (22).

Isolation of circular viral DNA. Procedures for enrichment of *H. saimiri* DNA from transformed cells followed essentially the methods described for isolation of circular EBV genomes (31). To obtain a sufficient concentration of circular viral DNA molecules for electron microscopy, 1×10^8 to 3×10^8 actively growing cells were harvested from about 300 ml of suspension cultures. The cells were collected by low-speed centrifugation ($700 \times g$) for 10 min at 20°C and washed twice with isotonic phosphate-buffered saline (pH 7.0). The cell pellet was suspended in phosphate-buffered saline to a final concentration of 10^7 cells per ml. The release of highly viscous DNA was achieved by the addition of 0.5 volume of 3% Sarkosyl–0.075 M Tris-hydrochloride–0.025 M EDTA (pH 9.0). After incubation for 0.5 h at room temperature with gentle rolling, 1/10 volume of a 0.1% preincubated (1 h; 37°C) proteinase K solution (Merck, Darmstadt, West Germany) was added. The lysate was further incubated for 2 h at 37°C. After dilution of the lysate with 3 volumes of 50 mM Tris-hydrochloride buffer (pH 8.5), solid CsCl was added at 20°C to $n_D = 1.4007$. DNA gradients were formed by equilibrium centrifugation in a Spinco vertical-type (VTi 50) rotor for 20 h at 40,000 rpm and 20°C. Fractions (1.0 ml each) were collected from a hole punctured through the side of the tube near the bottom with the aid of a closed-system collection device (type no. E 274; Metrohm, Herisau, Switzerland). Total DNA was localized by reading the optical density at 260 nm of each fraction. Virus-specific DNA sequences were monitored by hybridization of each fraction with ^{3}H-labeled cRNA. Fractions containing the bulk of the viral sequences were pooled and centrifuged to equilibrium in a CsCl gradient with 350 μg of ethidium bromide per ml (VTi 50 rotor; 40,000 rpm; 20°C). The position of covalently closed circles was monitored by the inclusion of ^{3}H-labeled simian virus 40 (SV40) form I DNA. Usually, fractions located in the density range of SV40 form I DNA were pooled and run in a subsequent cesium chloride-ethidium bromide gradient. The fractions containing *H. saimiri* DNA were combined, and ethidium bromide was removed by repeated extraction with 90% isoamyl alcohol. The pooled fractions were dialyzed against 10 mM Tris-hydrochloride (pH 8.5)–1 mM EDTA. The DNA was precipitated with 2.5 volumes of ethanol at −20°C and could be stored in alcohol for several months without noticeable effect. For electron microscopy, precipitated DNA was collected in a Spinco SW60 rotor by centrifugation at 35,000 rpm for 60 min at 0°C and dissolved in 20 μl of 10 mM Tris-hydrochloride (pH 8.5)–1 mM EDTA.

Hybridization techniques. Filter hybridizations and synthesis of cRNA were performed as described by Lindahl et al. (31). Briefly, purified viral DNA was transcribed in vitro by *Escherichia coli* RNA polymerase into labeled cRNA (specific activity of about 3×10^7 cpm/μg of cRNA was reached) with [^{3}H]CTP (Amersham-Buchler, Braunschweig, West Germany). Density gradient fractions were supplemented with salmon sperm DNA to obtain a final total amount of 10 μg of DNA. The denaturation of the DNA was achieved by the addition of an equal volume of 0.5 M NaOH and heating for 10 min at 80°C. The solutions were neutralized with 0.02 volume of concentrated HCl and 2 volumes of a solution containing 4× SSC (1× SSC, 0.15 M NaCl plus 0.015 M sodium citrate) and 0.2 M Tris-hydrochloride (pH 7.1). Denatured DNA was passed through nitrocellulose membrane filters (Schleicher and Schuell type BA 85; 13-mm diameter). After drying (20 h at room temperature and 4 h at 80°C under reduced pressure), the filters were soaked in 1 ml of 6× SSC–50% formamide for 1 h at 37°C. Thereafter, 300 μl of hybridization mixture containing about 3 ng of virus-specific cRNA (100,000 cpm) in 6× SSC–50% formamide was added to each

filter. Hybridization was performed at 45°C for 96 h. After extensive washing, the individual filters were incubated with 1 ml of a 30-μg/ml solution of pancreatic RNAse (Merck) in 2× SSC (heated at 80°C for 10 min before use) for 1 h at room temperature. Subsequently, the filters were washed three times with 2× SSC, dried, and counted in toluene supplemented with Permablend III (Packard Instrument Co., Inc., Rockville, Md.).

Estimation of viral genome equivalents by reassociation kinetics was done as described previously (25).

Partial denaturation and electron microscopy of DNA. The denaturation procedure followed the methods described previously by Bornkamm et al. (4), and DNA spreading was performed by the method of Davis et al. (6). DNA was incubated for 30 min at 30°C in 7 M sodium perchlorate, 0.6 or 0.8 M formaldehyde, 10 mM sodium phosphate (pH 7.5), and 1 mM EDTA. The DNA was passed through a Sephadex G-75 column (5 by 0.5 cm) and eluted with 1 mM EDTA. The hyperphase, consisting of 0.1 mg of cytochrome *c* per ml, 40% (vol/vol) formamide, 0.1 mM Tris-hydrochloride (pH 8.5), and 10 mM EDTA, was spread along a glass rod onto a hypophase containing 10% (vol/vol) formamide solution with 10 mM Tris-hydrochloride (pH 8.5) and 1 mM EDTA. Bacteriophage PM2 DNA (6.4×10^6 molecular weight) (34) was added as a length marker. DNA was picked up onto Parlodion-coated grids, stained with uranyl acetate, and rotary shadowed with platinum-palladium. Electron micrographs were taken in an EM 10 electron microscope equipped with an OKM internal measuring device (Zeiss, Oberkochen, West Germany).

Length measurement and computer analysis of partially denatured molecules. The external length determinations of double-stranded (ds) and single-stranded (ss) segments in partially denatured molecules were performed with a mechanoelectronic X-Y measuring stage (LM-1; Brühl, Nürnberg, West Germany), with a standard deviation of less than 0.4%. Partial denaturation data were evaluated with the computer program PART, written in Fortran IV on a Cyber 173 computer (Control Data Corp., Minneapolis, Minn.) equipped with a Benson plotter (type 122; Benson, Paris, France).

The PART program correlated ss and ds regions of individual molecules. Data obtained after length measurement of ss and ds regions of each molecule were stored on punch cards. Computing started with a correction of different magnifications by data transformation procedures. The different regions of each single molecule were multiplied by an individual factor *F* ($F = SL/SLB$, where SL = the mean length of all internal length standards measured and SLB = the mean length of all internal standards on a photograph with a partially denatured molecule). The same factor was used for ss and ds regions. The molecules were sorted according to their lengths. Multiplication by the factor *SF* (*SF* = standard length/length of the molecule) led to a length standardization of all molecules within a certain size range. The standard length was the mean length of all circular molecules or the maximum length of all linear molecules. Nonstandardized molecules could also be used in the PART computer program.

The transformation of ss and ds segments of every molecule to integer format facilitated the following steps. PART had a maximal capacity of 200 molecules with up to 300 transitions between ss and ds regions. Linear molecules were shifted against each other up to 10% of standard length; circular molecules were shifted along each other over the whole length. Optimal correlation of partial denaturation patterns was achieved by aligning each individual molecule with the sum histogram of previously aligned molecules. Linear virion DNA molecules (*N*) were shifted to both sides by defined steps of one segment length until a maximum was reached. Circular molecules were shifted first in steps with the length of 64 segments. At the position of optimal correlation, the length of the steps was gradually decreased until one step became equal to one segment. Linear and circular molecules were analyzed in both orientations by PART.

In aligning single molecules to a given number of optimally fitting molecules, the coefficient *S*, which reflects optimal correlation of denaturation patterns, was calculated. The number of molecules possessing the same signal (ds or ss) in the position of a given segment was designated c_i; the number of molecules possessing the opposite code to a given segment was designated n_i. *S* was defined as the sum of all differences $(c_i - n_i)$ for all segments *L* of *N*

$$\left[S = \sum_{i=1}^{L} (c_i - n_i) \right].$$

The *S* value was calculated for each possible position of *N*. Optimal position was characterized by the highest *S* value. PART is able to calculate 400 *S* values, each consisting of 1,000 differences $(c_i - n_i)$ according to the equation above. The procedure can be repeated many times.

RESULTS

Density of viral DNA in transformed lymphoid cell lines. The M genomes (L + H DNA) from *H. saimiri* strain 11 and *H. ateles* strain 810 were previously found to band in CsCl at buoyant densities of 1.7045 and 1.7061 g/ml, respectively (18, 19). *H. ateles* DNA sequences from the rabbit tumor cell line RLC banded at the position at which virion DNA would be expected (Fig. 1a). However, cell lines 1670, 70N2, H1591, and A1601 apparently contain viral DNA molecules with a lower buoyant density than virion DNA (Fig. 1b–d; data for cell line A1601 not shown). The densities of viral DNA were estimated to be 1.706 g/ml in RLC cells, 1.696 g/ml in 1670, H1591, and A1601 cells, and 1.702 g/ml in 70N2 cells (Fig. 1 and Table 1). The lower than expected buoyant densities of circular viral DNA in CsCl are probably due to extensive cytosine methylation in the repetitive DNA (see Discussion).

Presence and size of covalently closed circular viral DNA in transformed cells. The viral DNA from transformed cell lines, banded in CsCl density gradients, was subjected to subsequent gradient centrifugation in cesium chloride-ethidium bromide. The majority of viral DNA se-

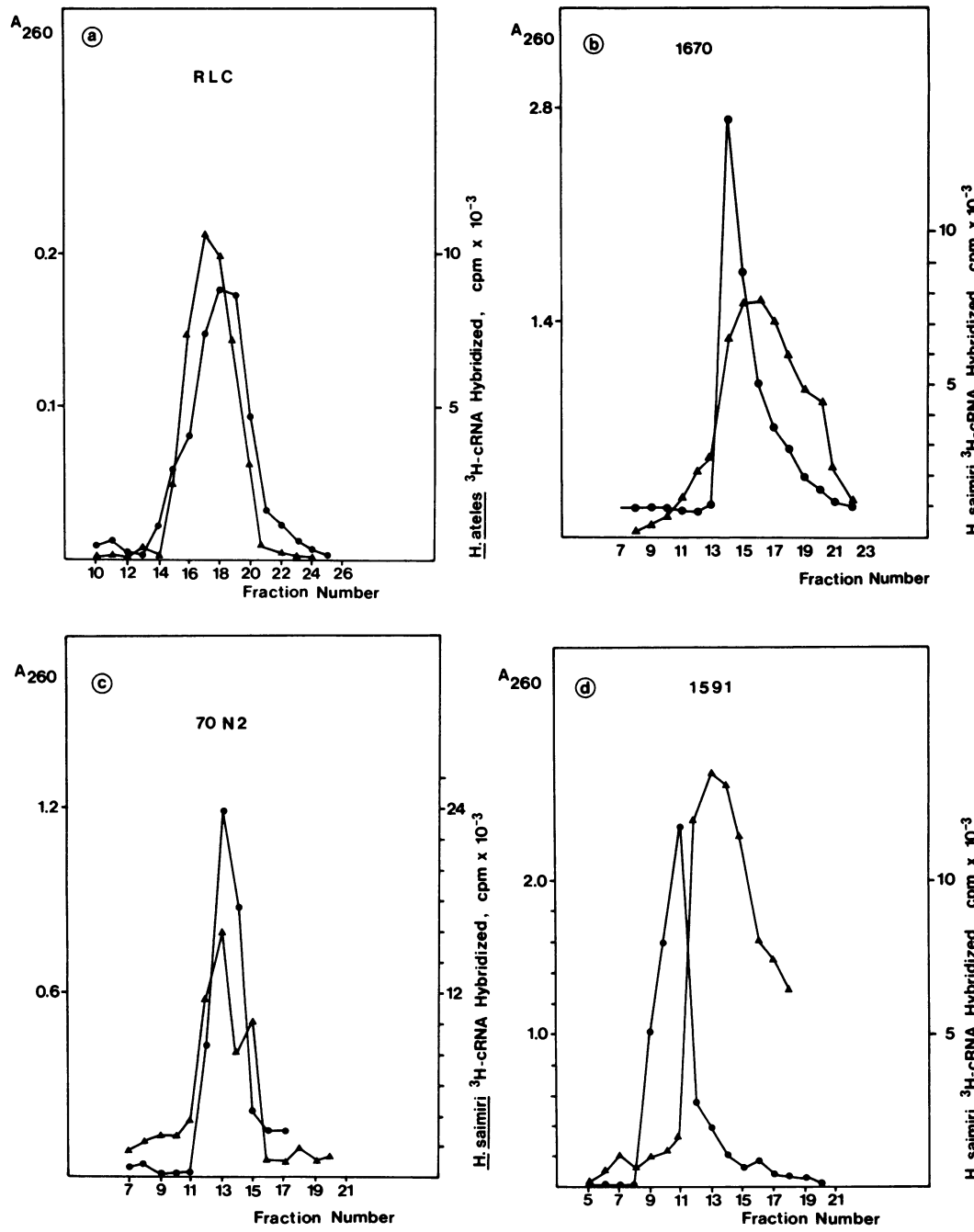

FIG. 1. Density profiles of *H. saimiri* and *H. ateles* DNA sequences from five lymphoid cell lines in CsCl equilibrium gradients. Virus-specific sequences in each fraction were detected by filter hybridization with [^{3}H]cRNA (▲). The position of cellular DNA was measured by reading the optical density at 260 nm (A_{260}) (●). Cell lines: (a) RLC, (b) 1670, (c) 70N2, (d) H1591.

quences from all cell lines was found at 1.57 g/ml, the approximate density expected of covalently closed superhelical DNA. One such gradient from the DNA of cell line RLC is shown in Fig. 2. No significant amount of viral DNA was found at the position of RLC cell DNA. However, the dye-salt gradients with DNA from the four other cell lines showed variable amounts of viral DNA in the density range of cellular DNA. These viral DNA sequences banding with cellu-

TABLE 1. Viral DNA in five cell lines of different origin

Cell line	Origin			Virus strain	Viral DNA in cells			
	Species	Cell type	Producer status[a]		Viral genome equivalents per cell (22, 25)	Molecular size of circular viral DNA (Md)[b]	Density in CsCl (g/cm^3)	Methylation of viral H-DNA
1670	White lip marmoset	Tumorous spleen	NP	*H. saimiri* 11	210	129.5	1.696	2+
70N2	Cotton top marmoset	Tumorous thymus	NP	*H. saimiri* 11	270	115.4	1.702	+
H1591	Cotton top marmoset	In vitro-transformed lymphocytes	NP	*H. saimiri* OMI	40	94.4	1.696	2+
A1601	Cotton top marmoset	In vitro-transformed lymphocytes	NP	*H. ateles* 73	240	99.4	1.696	2+
RLC	Rabbit strain ACCRB	Tumorous spleen	P	*H. ateles* 810	330	90.7	1.706	–

[a] NP, Nonproducer; P, producer.
[b] Determined from nondenatured molecules.

lar DNA could be mostly open circular structures, but it is not known to what extent they might represent entangled superhelical molecules or linear viral DNA associated with the host cell genomes. The peak of viral DNA in cesium chloride-ethidium bromide gradients coincided in each case with the peak of SV40 form I DNA as determined from parallel gradients with [^{3}H]thymidine-labeled SV40 DNA.

Electron microscopy of viral DNA banding at high density in cesium chloride-ethidium bromide gradients confirmed the circular nature of these molecules. Three large circles from 1670 cells were found to have contour lengths corresponding to a molecular weight of 129.5 ± 5.3 × 10^6. This is fairly consistent with the earlier size estimation of circular DNA from 1670 cells, which gave a value of 131.5 ± 3.6 × 10^6 molecular weight (40, 41). The molecular weight of each of five circular DNA molecules of 70N2 cells was estimated to be 115.4 ± 5.4 × 10^6. The length of 21 circular DNA molecules of cell line H1591 was measured and determined to correspond to a molecular weight 95.4 ± 2.9 × 10^6. A

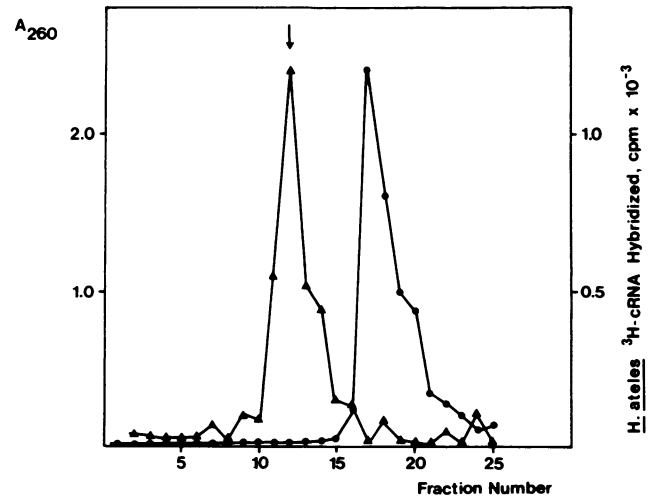

FIG. 2. Density of *H. ateles*-specific sequences from cell line RLC in a cesium chloride-ethidium bromide gradient. Viral DNA (▲); cellular DNA (●) (see Fig. 1). The arrow indicates the position of SV40 form I DNA.

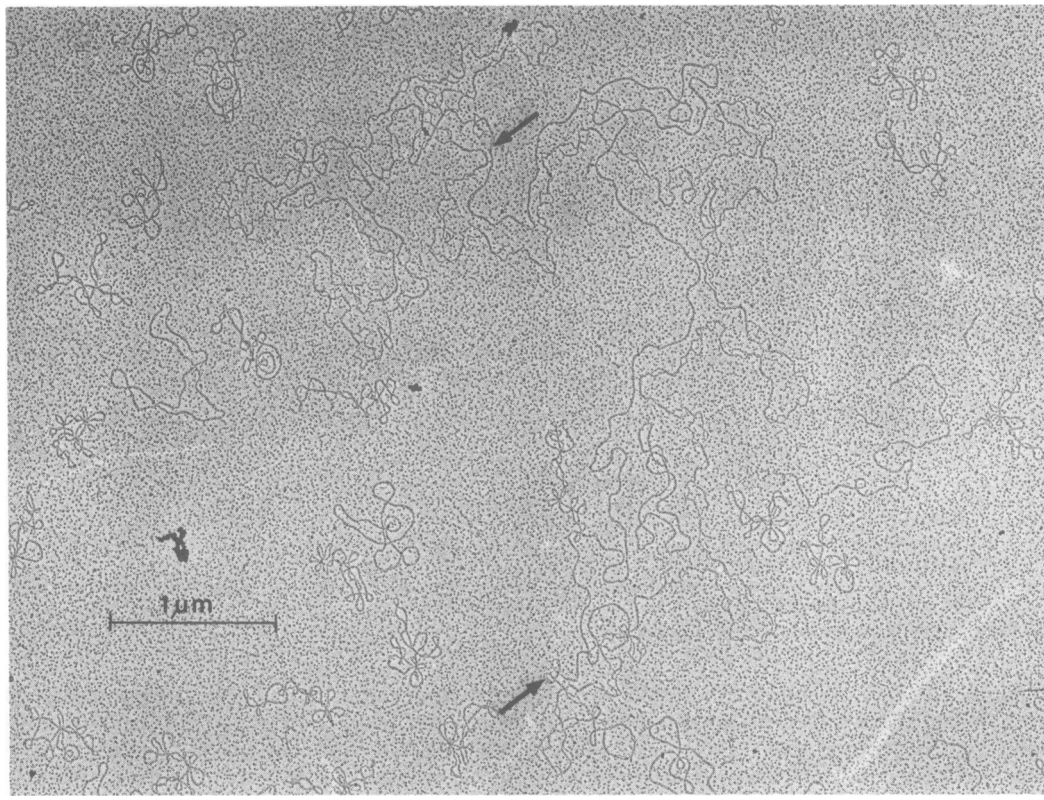

FIG. 3. Electron micrograph of a circular molecule from cell line RLC partially denatured by sodium perchlorate and formaldehyde. The arrows indicate the transitions between H- and L-DNA regions. The small circular DNA molecules are phage PM2 DNA added as a size marker.

total of 22 double-stranded *H. ateles* DNA molecules isolated from cell line RLC had an average molecular weight of $90.7 \pm 2.9 \times 10^6$. Double-stranded DNA circles of cell line A1601 had a size corresponding to a molecular weight of $99.4 \pm 6.8 \times 10^6$, as determined from six molecules.

Arrangements of L- and H-DNA sequences in circular *H. ateles* genomes of cell lines RLC and A1601. Cell line RLC, derived from an *H. ateles*-induced rabbit tumor, contains about 330 genome copies per cell (25). Cell line A1601, which was established by in vitro immortalization of peripheral leukocytes of cotton top marmosets, harbors about 240 genome equivalents per cell (Table 1). The partial denaturation of circular viral DNA from both cell lines revealed molecules consisting of one H- and one L-DNA region. One such molecule from each cell line is shown in Fig. 3 and 4, respectively. The length of total circular molecules and the sizes of H- and L-DNA regions are shown in two histograms summarizing the data from 19 partially denatured RLC circles and 13 partially denatured molecules from cell line A1601 (Fig. 5a and b). Circular molecules from cell line RLC had an average total molecular size of 94.7 ± 4.4 megadaltons (Md); the L region was found to be 71.2 ± 3.8 Md, and the size of the H region was 23.5 ± 3.0 Md. Cell line A1601 harbored 98.9 ± 3.8-Md circles consisting of a 63.7 ± 2.8-Md L-DNA segment and a 35.1 ± 3.8-Md H-DNA segment. One denatured circle from cell line A1601 was considerably shorter (77.4 Md) than all other molecules found in this cell line; it possessed a 52.9-Md L-DNA segment and a 24.5-Md H-DNA stretch. This molecule was not included in the histogram in Fig. 5b. The proportion of H- and L-DNA sequences in circular viral DNA molecules from cell line RLC was the same as that in virion DNA (19). In contrast, L-DNA in cell line A1601 was shorter by 8 Md than the L-DNA region of virion H-DNA. This deletion was compensated by H-DNA sequences, resulting in a total size of circular molecules which rather exceeded the size of virion DNA (19). We have not determined from which region the missing 11.2% was derived.

Arrangement of L- and H-DNA sequences in *H. saimiri* circles of cell line H1591. Cell line H1591, the first line obtained by immortalization of

lymphoid cells by *H. saimiri*, appeared to contain approximately 40 genome equivalents per cell, as determined by reassociation kinetics with tritium-labeled *H. saimiri* DNA (Fleckenstein, unpublished data). As demonstrated in Fig. 6, circular viral DNA molecules isolated from this cell line consist of one L-DNA and one H-DNA region. The L-DNA region of H1591 circles (42.5 ± 3.4 Md) was strikingly shorter than L-DNA of *H. saimiri* virion DNA (71.6 Md) (4). However, the segment of H-DNA (51.2 ± 1.4 Md) far exceeded the total amount of H-DNA present in virion genomes (approximately 30.0 Md; Fig. 5c). This results in a total molecular size of 93.7 ± 4.2 Md, which is close to the values found for DNA from virus particles. Figures 7 and 8 show computer alignments and sum histograms of 17 partially denatured circular molecules. It is evident that the majority of missing L-DNA sequences in cell line H1591 correspond primarily to the right half of the L region in virion DNA.

Comparison of H- and L-DNA arrangements in *H. saimiri* circles of cell lines 70N2 and 1670. Cell lines 1670 and 70N2 are derived from monkey tumors inoculated with *H. saimiri* strain 11. The 1670 and 70N2 cell lines contained approximately 210 and 270 genome copy equivalents per cell, respectively, as determined by reassociation kinetics with complete labeled L-virion DNA (22).

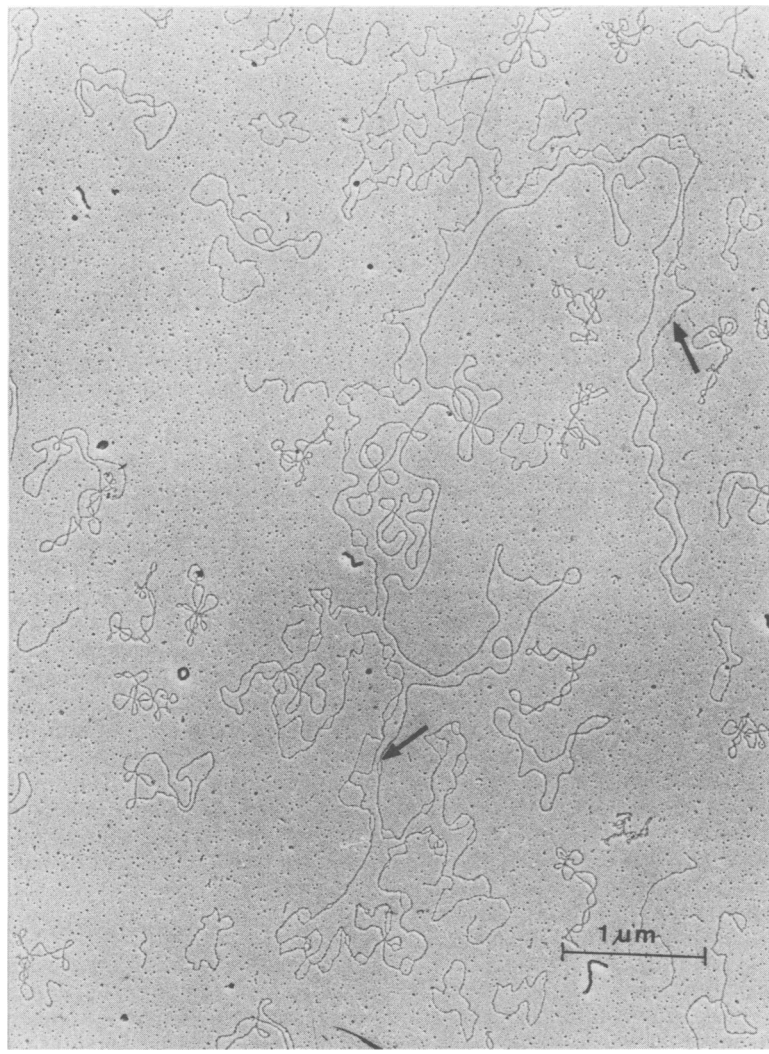

FIG. 4. Electron micrograph of a partially denatured circular molecule from cell line A1601 (see legend to Fig. 3). The small double-stranded circular DNA molecules represent phage PM2 DNA, and the single-stranded circles are phage φX 174 DNA (36).

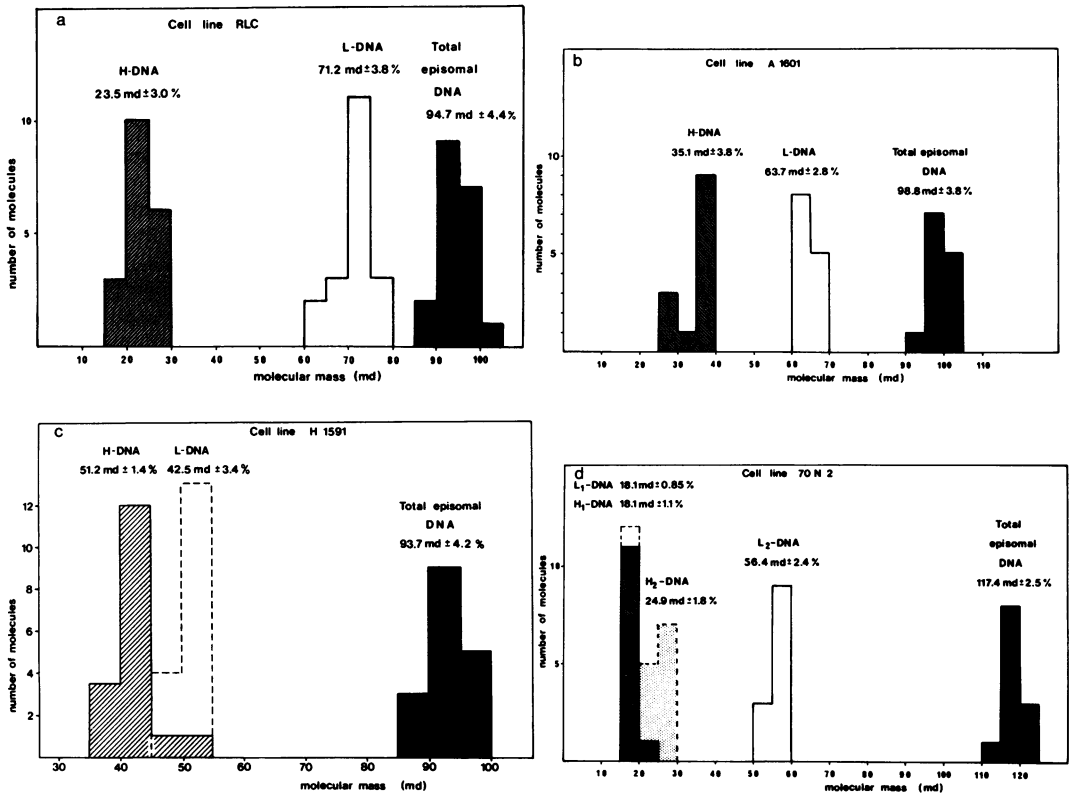

FIG. 5. Histograms showing the lengths of H-DNA and L-DNA regions in (a) 19 molecules of cell line RLC, (b) 13 molecules of cell line A1601, (c) 17 molecules of cell line H1591, and (d) 12 molecules of cell line 70N2.

The structure of circular viral DNA in cell line 1670 has been described by partial denaturation mapping (40) and blot hybridization with ^{32}P-labeled virion DNA (8). Like 1670 cells, the circular viral DNA molecules from 70N2 cells were found to have a molecular weight exceeding that of linear M genomes. Partial denaturation showed that 70N2 circles possessed a long (L_1) and a short (L_2) L-DNA region (L_1 = 56.4 ± 2.4 Md and L_2 = 18.1 ± 0.85 Md) and a long (H_1) and a short (H_2) H-DNA region (H_1 = 24.9 ± 1.8 Md and H_2 = 18.1 ± 1.1 Md) (Fig. 9 and 10). Thus, the overall structure of 1670 and 70N2 circular molecules is very similar. Both cell lines have a higher relative proportion of H-DNA to L-DNA than virion M genomes. The L_1, H_1, and H_2 regions of 1670 and 70N2 cells are virtually identical in length. However, the L_2 region of 70N2 cells is significantly shorter than the L_2 segment of 1670 cells (Fig. 5d, 11, and 12).

The partial denaturation histogram shown in Fig. 12, which was drawn after independent computer alignment of the denaturation maps of the L region of virion no. 11 DNA and the L_1 and L_2 regions of 70N2 circles, indicates that the short L segment is a subset of the long L-DNA segment. Since the 13-Md deletion of 70N2 cells is identical with the deletion in the corresponding region of 1670 (8), it suggested that L_2 of 70N2 cells represents approximately the L-DNA between map units 0.0 and 0.35. Thus, it appeared that the L-DNA between map units of about 0.0 and 0.35 occurs twice in the circles, whereas DNA sequences from map units 0.35 to 0.52 and 0.70 to 1.0 are represented once. Virion DNA between map units 0.52 and 0.70, about 18% of the genetic information, was found to be missing in the circles. Thus, the genetic complexity of cell lines 70N2 and 1670 is the same. The computer alignment indicated that the two L regions of 70N2 cells are in tandem polarity, as shown previously for 1670 cells (40).

DISCUSSION

H. saimiri- and *H. ateles*-transformed lymphoid cell lines contain high amounts of persisting viral DNA with genome copy numbers up to 340 per cell (Table 1). The present study shows that viral DNA is able to persist as large, covalently closed circular DNA molecules in all cell lines examined. Circularized viral DNA was

found in tumor-derived cells as well as in cell lines obtained by immortalization in vitro. Such structures were found to occur in monkey and rabbit cells and in virus-producing as well as in nonproducing cell lines. Thus, including EBV and Marek's disease virus, four herpesviruses are known so far which are able to form circular molecules in lymphoid cells from a broad spectrum of mammalian and avian animal species (26, 31, 38, 40).

In view of the high amount of nonintegrated viral DNA present in transformed cells, it remains a difficult problem to determine definitively whether any herpesvirus DNA is integrated into the cellular DNA of immortalized or transformed lymphoid cells. Entanglement of circular molecules or density changes by modified bases could simulate an association with the cellular genome upon preparative separation steps. This study shows that at least 95%, if not all, of *H. ateles* DNA can be separated from genomic DNA of rabbit tumor cell line RLC by density centrifugation in cesium chloride-ethidium bromide (Fig. 2); however, when DNA from other *H. saimiri*- and *H. ateles*-transformed cells was analyzed, a minor proportion of viral sequences was usually found at a lower density position than superhelical DNA (data not shown). Since relaxation of the large superhelical herpesvirus circles can be due to trace amounts of intracellular nucleases or shear forces during preparation, isopycnic centrifugation will probably be inappropriate to determine whether integrated DNA of oncogenic herpesviruses exists. Experiments are in progress to attempt to prove or exclude *H. saimiri* integration by sensitive blot hybridizations of restriction enzyme-cleaved cellular DNA with viral DNA probes that are cloned in plasmids and lambda phages.

Viral DNA from four of the five cell lines

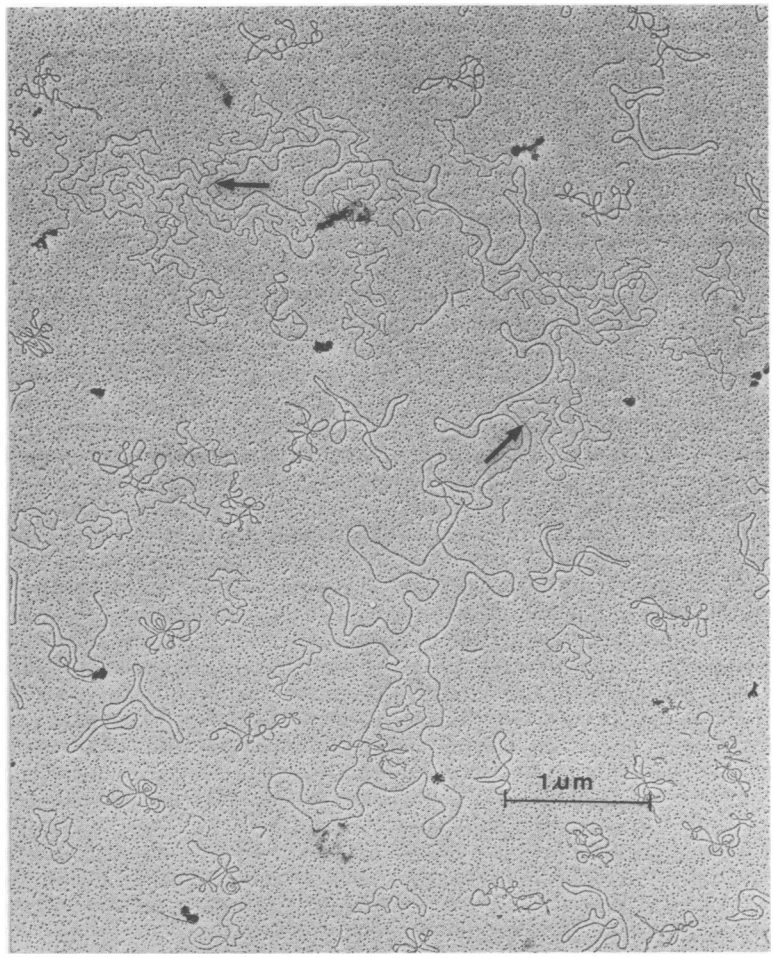

FIG. 6. Electron micrograph of a partially denatured circular molecule from cell line H1591 (see legends to Fig. 3 and 4).

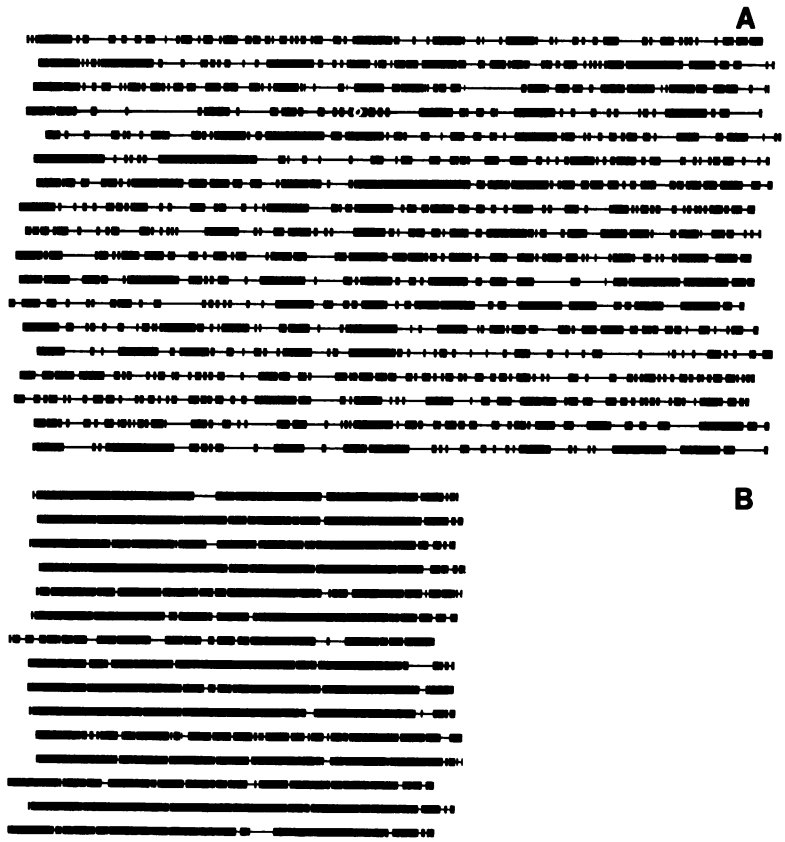

FIG. 7. Computer alignment of (A) 18 partially denatured L regions of *H. saimiri* virion DNA strain 11 with (B) 15 partially denatured L regions of cell line H1591. Duplex regions are represented by thin lines, and denatured regions are represented by bold lines.

investigated in our experiments had a significantly lower density in salt gradients (Fig. 1b–d) than expected from the average G+C contents calculated from the relative amounts of L- and H-DNA in partial denaturation histograms (Fig. 5 and Table 2). Circular DNA molecules from all cell lines, except the virus-producing rabbit tumor cell line RLC, have a higher proportion of G+C-rich H-DNA sequences than virion DNA (Table 2); however, they possess a lower buoyant density in CsCl gradients than M genomes from virus particles (Fig. 1 and Table 1). It is known that the buoyant density of DNA is decreased by 5-methylation of cytosine (12, 29, 30). The DNA of *H. saimiri* in cell line 1670 was found to be heavily methylated in the cytosine of the 5'CpG3' dinucleotide (9, 10). Viral DNA in 70N2 cells is methylated in H-DNA to a considerably lower extent. This parallels the observation that viral DNA in 70N2 cells underwent a density shift which is less pronounced than that in cell line 1670 (27). Thus, the low-density values observed in these studies in two additional nonproducing cell lines (H1591 and A1601) may be indicative of a high proportion of 5-methylcytosine in the G+C-rich H-DNA in nonintegrated *H. saimiri* and *H. ateles* genomes. The low density of viral DNA in CsCl proved to be advantageous for the isolation of circular virus genomes, since the relative concentration of viral DNA was enriched as much as 10 times by one preparative density centrifugation. Remarkably, circular viral DNA from the single virus-producing cell line RLC did not give evidence for a density shift in CsCl, indicating the absence of significant proportions of 5-methylcytosine.

Three patterns of sequence arrangements were found in circular viral DNA from lymphoid cell lines (Fig. 13): (i) molecules consisting of one stretch of repetitive H-DNA and a single L-DNA region with the same length as the unique L region of virion M genomes (cell line RLC); (ii) circular viral genomes consisting of one L-

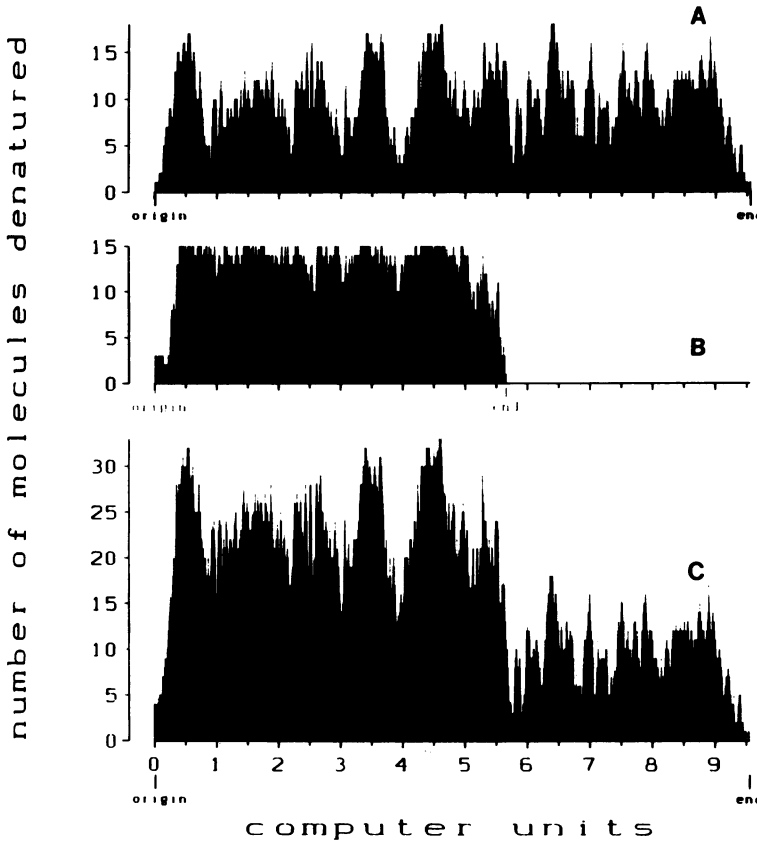

FIG. 8. Sum histograms of partially denatured molecules (A and B; corresponding to Fig. 7) after optimal fitting. Compilation of (A) and (B) results in (C).

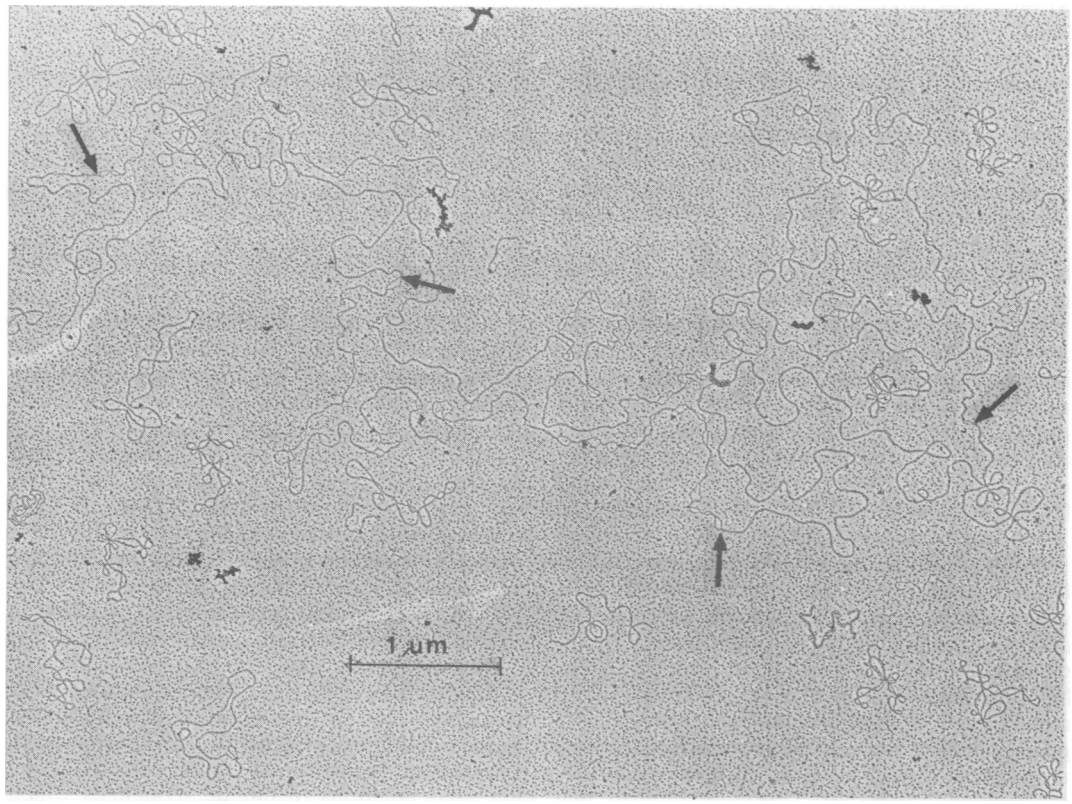

FIG. 9. Electron micrograph of a partially denatured circular viral DNA molecule isolated from cell line 70N2. Small circles are phage PM2 DNA.

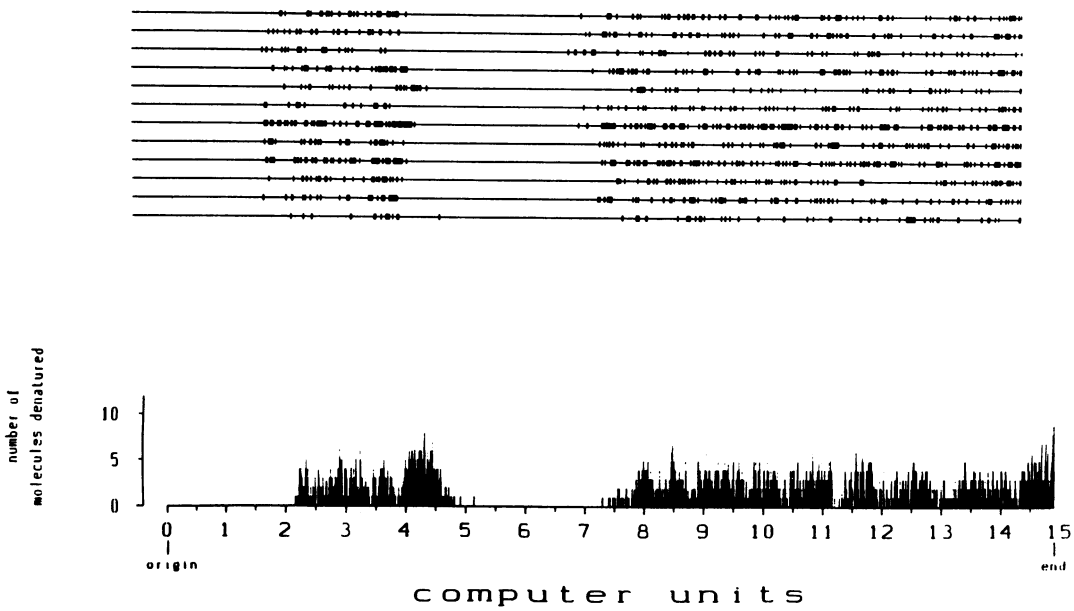

FIG. 10. Computer alignment of 12 partially denatured circular DNA molecules isolated from cell line 70N2.

DNA region which bears a deletion, compensated by an elongated region of H-DNA (cell lines H1591 and A1601); and (iii) oversize circles with two L- and two H-DNA regions which represent defective genomes, since an internal L-DNA fragment of 13 Md is deleted. The overall sizes of circular molecules and the lengths of individual H- and L-DNA segments were usually found to be homogeneous in each single cell line, as partial denaturation histograms did not show any significant length heterogeneity for a given line. There was only one exception: 1 of 19 circles from cell line A1601 appeared to be shorter by about 30% than all other molecules.

We found an identical sequence arrangement in two cultures of cell line 1670 which differed from each other by 4 years of continuous passage history. The apparent stability of *H. saimiri* and *H. ateles* in established lymphoid cell lines after multiple culture passages is remarkable, since the very high proportion of reiterated DNA consisting of repeats in tandem orientation could easily be available for recombination events that would result in altered sizes of H-DNA stretches. This may hint at mechanisms which can prevent recombination processes of persisting nonintegrated viral genomes in lymphoid cells. On the other hand, studies in our laboratory and in that of R. C. Desrosiers (Primate Center of Harvard Medical School) have given evidence for a pronounced structural heterogeneity of viral DNA at different early passages of cell line H1591. This could indicate that a high variability in DNA sequence arrangement is characteristic of the early cell lines; in contrast, long-term selection by continuous passage over several years may result in cell lines with a stable form of persisting viral genomes (8, 40) or, if rearrangements occur at low frequency, in continuous selection of the predominant structure.

Cell lines 1670 and 70N2 are similar in that their deleted internal L-DNA segments (13 Md) are virtually identical (8). Circles of cell line 1670 are larger in size (41) and possess a much higher proportion of methylated cytosine (10, 27), and, as shown in this study, the small L-DNA region (L_2) of 70N2 cells is much shorter than the L_2 segment of 1670 cells. There may be spots in L-DNA, possibly short tandem repeats, that predispose to recombination-excision during a certain period of circle formation.

All cell lines, except line RLC, have a higher relative proportion of repetitive H-DNA than the linear virion M genomes (Table 2). The function of repetitive sequences in the DNA of oncogenic primate herpesviruses in productive or transforming infection is unknown (20, 39). The increase in the proportion of H-DNA in circular molecules from transformed lymphoid cells parallels the deletions in L-DNA and the inability of the cells to produce infectious virus particles. This is reminiscent of EBV-transformed cell lines. In general, EBV persisting in established transformed lymphoblastoid cell lines has deletions of unique sequences which

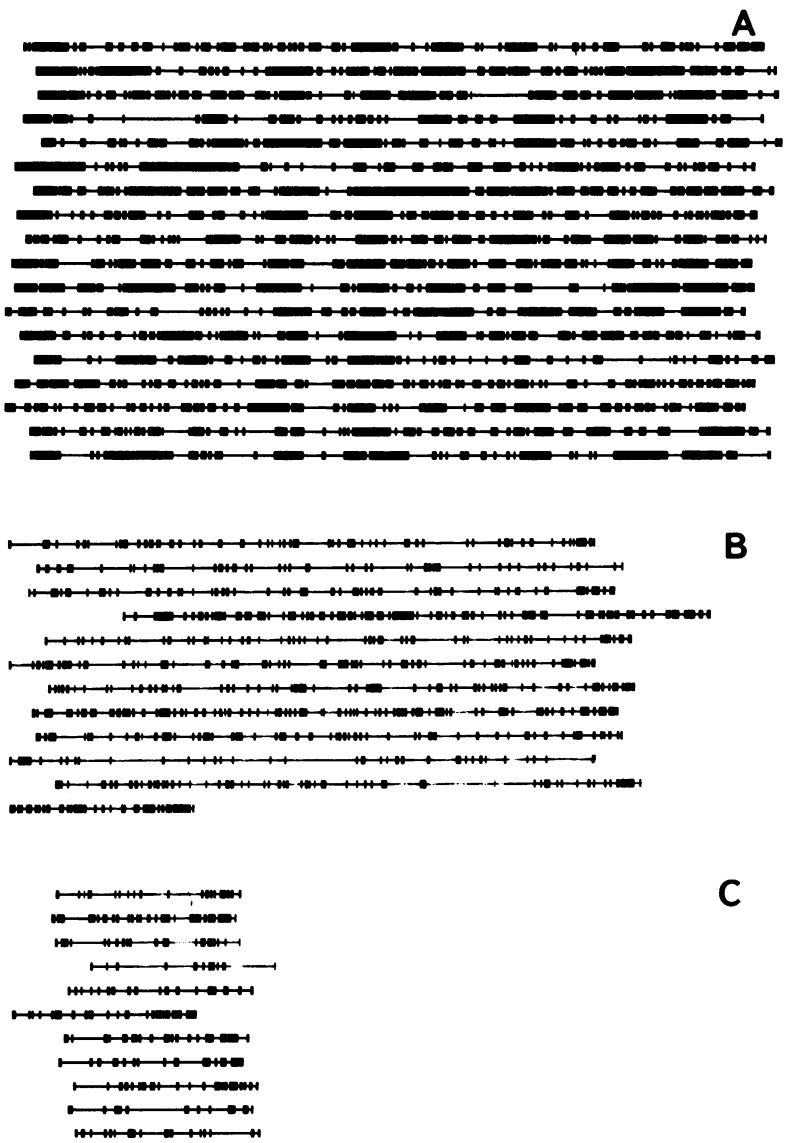

FIG. 11. Computer alignment of (A) partially denatured L regions of virion DNA, (B) partially denatured long L regions of 70N2 circles, and (C) partially denatured short L regions of 70N2 circles.

may be required for certain functions of replication or for initiation of transformation in vitro (5, 24). Our experiments suggest that about 40% of L-DNA from *H. saimiri*, mainly located in the right half of the L segment, can be dispensable in circular viral DNA molecules of transformed lymphoid cells. Perhaps some short deletions of the left half of the L region are also possible in transformed cells. This question will be better answered by a series of blot hybridizations with cloned viral DNA probes. The preferential presence of DNA sequences from the left half of the L region in three independent transformed cell lines is in accordance with recent observations of specifically hypomethylated CpG sites in the DNA of fresh tumor biopsies from *H. saimiri*-infected owl and marmoset monkeys (9). Experiments in several virus systems indicate that methylation of certain CpG sites in inversely correlated with gene expression (11, 13, 35). Hypomethylated CpG sites in tumor tissues were found only in those L-DNA segments that were consistently present in all lymphoid cell lines carrying *H. saimiri* DNA. Apparently, only

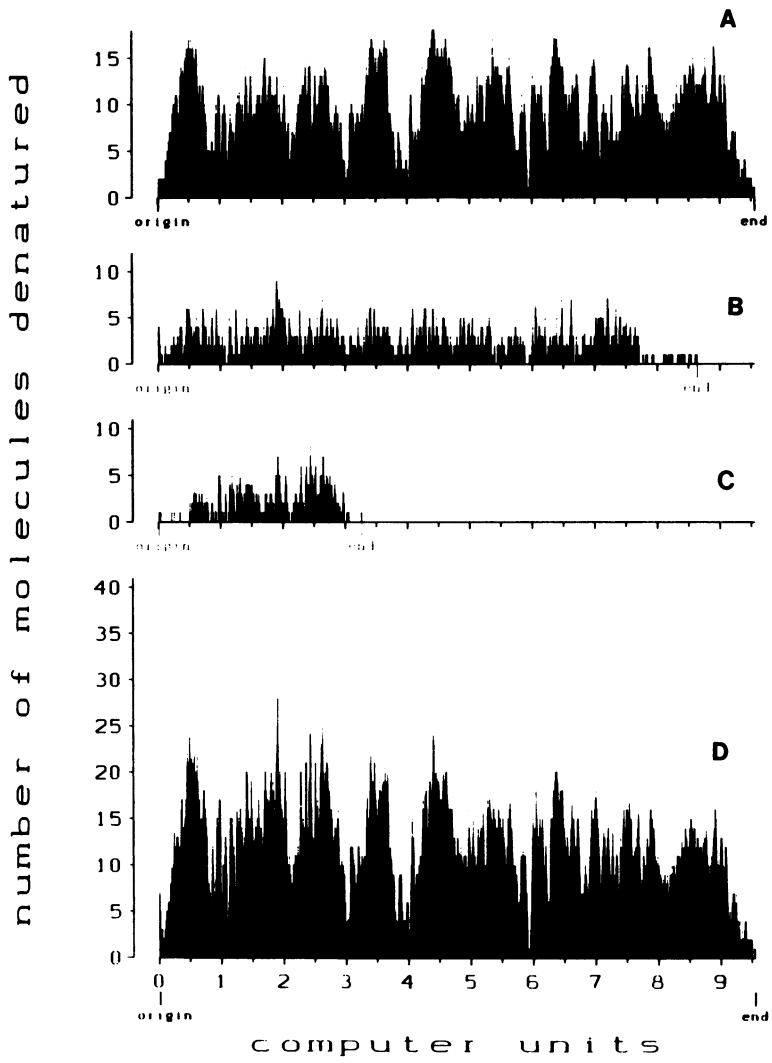

FIG. 12. Sum histograms (A), (B), and (C) corresponding to Fig. 13. Compilation of (A), (B), and (C) results in (D).

TABLE 2. Proportions of H- and L-DNA in virion M-DNA and in circular viral genomes of *H. saimiri*- and *H. ateles*-transformed lymphoid cell lines

DNA from:	% H-DNA	% L-DNA
H. saimiri 11 (19)	28	72
Cell line 1670	34.8	65.2
Cell line 70N2	36.6	63.4
Cell line H1591	54.6	45.4
H. ateles 810 (19)	25.8	74.2
Cell line RLC	24.8	75.2
H. ateles 73 (19)	26.4	73.6
Cell line A1601	35.5	64.5

parts of herpesvirus genomes must persist as circles and be available for gene expression in transformed cells.

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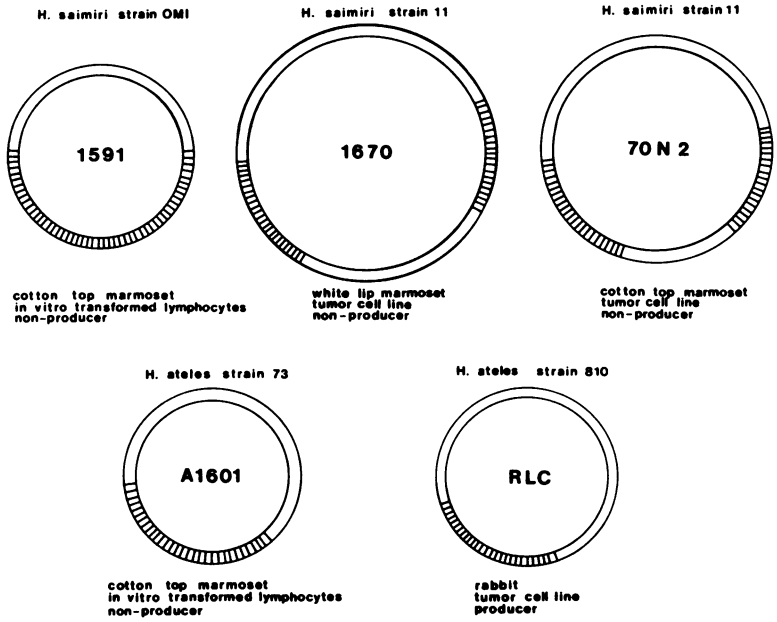

FIG. 13. Schematic representation of the structures of circular *H. saimiri* and *H. ateles* DNA isolated from five different cell lines. Area with bars represents H-DNA; area without bars represents L-DNA sequences.

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