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Genome Organization of Herpesvirus Aotus Type 2

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Herpesvirus aotus type 2, a virus commonly found in owl monkeys without overt disease, has a similar genome structure to the oncogenic herpesviruses of nonhuman primates (herpesvirus saimiri, herpesvirus ateles). Virion DNA of herpesvirus aotus type 2 (M-DNA) has an unique 110-kilobase-pair region of low G+C content (40.2%, L-DNA), inserted between stretches of repetitive H-DNA (68.7% G+C, about 41 kilobase pairs per molecule) that are variable in length. A minority of virions contain defective genomes that consist of repetitive H-DNA only. The H-DNA is composed of various types of repeat units that are related in sequence with each other. The two dominant types of repeats (2.3 and 2.7 kilobase pairs) were cloned and compared by restriction enzyme cleavages and partial nucleotide sequencing. They are homologous in at least 1.3 kilobase pairs. The two forms of repeat units are randomly arranged and oriented in tandem. Reassociation kinetics did not allow detection of sequence homologies between H- and L-DNA of herpesvirus aotus type 2 and the respective sequences of oncogenic primate herpesviruses.

Herpesviruses of New World primates appear to be relevant for several aspects of viral oncology. Herpesvirus saimiri, a common virus of squirrel monkeys, induces malignant lymphoproliferative diseases in a number of South American monkeys and in rabbits. Similarly herpesvirus ateles, a natural inhabitant of spider monkeys (*Ateles* sp.), causes acute lymphocytic leukemias and lymphyomas in a similar spectrum of animals upon experimental infection (8, 12, 14). The two viruses share some unusual characteristics in their molecular genome structure. Unlike all other herpesviruses described so far, herpesvirus saimiri and herpesvirus ateles DNA molecules (M-DNA) have repetitive G+C-rich DNA (H-DNA) sequences flanking a unique 110-kilobase-pair (kbp) section of L-DNA (low G+C). The repeats are oriented in tandem, and they amount to approximately one-third of the viral genome (12, 15). There is no indication that H-DNA codes for proteins during virus growth (29; W. Bodemer, personal communication). Nevertheless, repetitive H-DNA sequences are required for replication, conceivably because they contain a signal sequence required for packaging. Also, H-DNA sequences are consistently found in tumor biopsies and lymphoid tumor cell lines, in spite of the observation that the majority of viral genes can be missing in transformed lymphoid cells (18, 26). A certain segment of L-DNA is not required for viral replication, however, correlated with the oncogenic phenotype of herpesvirus saimiri (10, 20; R. C. Desrosiers, A. Bakker, J. Kamine, R. D. Hunt, and N. W. King, submitted for publication).

To understand the evolution and epidemiology of herpesvirus saimiri and herpesvirus ateles, we extended the studies of genome structure to some additional New World primate herpesviruses. Three independent isolates from owl monkey (*Aotus trivirgatus*) kidney cell cultures were differentiated by cross-neutralization tests and were accordingly designated herpesvirus aotus types 1, 2, and 3 (2, 5, 6). Preliminary seroepidemiology suggested that all of these viruses are very common in owl monkeys, the natural hosts; however, no pathogenic properties have been attributed to these agents in monkey species so far (M. D. Daniel, personal communication). Herpesvirus aotus types 1 and 3 appear closely related, as judged from nucleic acid hybridizations and comparison of structural viral polypeptides; the genome structure of these viruses resembles that of human cytomegalovirus DNA (11). Herpesvirus aotus type 2, however, appears clearly distinct from types 1 and 3 with regard to several biological criteria and molecular characteristics. We found an extreme intramolecular G+C heterogeneity and highly repetitive DNA, akin to the sequence organization in herpesvirus saimiri and herpesvirus ateles DNA. In this paper, we describe the overall genome structure and repeat arrangements in herpesvirus aotus type 2 DNA and give a comparison with oncogenic herpesviruses of nonhuman primates.

MATERIALS AND METHODS

Virus and cell culture. Herpesvirus aotus type 2, isolate 68-69/E-166 E, was provided by M. D. Daniel. The virus was grown on owl monkey kidney (OMK) cells, strain 637 or 210 (7); the monolayer cultures were propagated in minimum essential medium with 10% heat-inactivated fetal calf serum. Pronounced cytopathic effects were usually seen 10 to 20 days after infection of cultures. Plaque purifications of virus stocks were done under 2% (wt/vol) methyl cellulose; infectious virus could be picked from single plaques after 10 to 12 days. Virus and cells were checked routinely for mycoplasma by isolation procedures as described elsewhere (16) and were found to be free of contamination.

Purification of viral DNA. Herpesvirus particles (herpesvirus aotus types 1, 2, and 3; herpesvirus saimiri; herpesvirus ateles) were concentrated from cell culture fluids by high-speed centrifugation and extracted from the cytoplasm fraction of infected cells as described previously (11). The particles were partially purified by centrifugation through 15 to 30% (wt/wt) sucrose gradients and lysed with 2% (wt/vol) sodium lauroyl sarcosinate; viral DNA was isolated by a single equilibrium centrifugation in CsCl (11). To obtain isolated fractions of H- and L-DNA, the viral DNA was mechanically sheared by passing 20 times through a 25-gauge syringe needle before analytical or preparative CsCl density centrifugation. The purity of virion DNA (<2% contamination with cellular DNA) was verified by UV scanning in an analytical ultracentrifuge.

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Reassociation kinetics. Viral DNA was radioactively labeled by adding about 15μCi of [^{3}H]thymidine (15 to 25 Ci/mmol; Amersham-Buchler, Braunschweig) per ml to infected OMK cell cultures. Details of liquid phase hybridization procedures (denaturation of DNA, reannealing conditions, hydroxyapatite chromatography, counting radioactivity) have been described previously (13, 16).

Analytical centrifugation. The buoyant densities of intact or fragmented viral DNA were measured in a Beckman model E analytical ultracentrifuge by following a protocol described earlier (11). Purified DNA from *Micrococcus lysodeikticus* (1.731 g/ml) (30) and *Mycoplasma orale* type 1 (1.685 g/ml) (19) was added to purified virion DNA to ascertain the slope of the internal density gradients.

Cloning of repetitive DNA units. DNA fragments of herpesvirus aotus type 2 generated by *Bam*HI or *Eco*RI digestion were eluted in the electrical field from agarose gels into dialysis bags and cloned into plasmid vector pBR322 (3) or pBR328 (27), respectively. After transformation of *Escherichia coli* HB101, clones containing viral DNA were detected by colony hybridization (17) with the ^{32}P-labeled virion DNA. Quick lysates were prepared from positive clones and digested with *Bam*HI or *Eco*RI; viral inserts were identified by coelectrophoresis with authentic viral fragments and by Southern blot hybridization. Physical maps were obtained by double cleavages and by partial digests of DNA that was end labeled with T4 polymerase.

DNA labeling and Southern blot hybridizations. DNA was labeled by the nick repair procedure (23) or by the replacement synthesis method with T4 polymerase (22). Blotting of DNA followed a published protocol (28). Nitrocellulose filters were preincubated with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% Denhardt solution, and 0.1 mg of yeast RNA per ml for 4 h at 65°C. Hybridization was performed with 0.3×10^5 cpm of nick-labeled probe per cm^2 of the filter for 2 days at 42°C (50% formamide, 5× SSC, 0.02% Denhardt solution, 0.02 M phosphate, 0.1 mg of yeast RNA per ml). The filters were successively washed with 2×, 1×, and 0.1× SSC at 65°C.

DNA sequencing. Cloned DNA was digested with the respective restriction enzymes; the resulting fragments were 5′ labeled with polynucleotide kinase, and the labeled ends were separated by a second cleavage with single-cut enzymes. Fragments of interest were isolated by agarose gel electrophoresis and electroelution. DNA sequencing was performed by base-specific chemical degradation (21).

RESULTS

Structure of virion M-DNA. Isopycnic centrifugation of intact DNA from herpesvirus aotus type 2 virions in analytical CsCl gradients resulted in a sharp symmetrical band at ρ = 1.708 g/ml (Fig. 1A and B). Assuming that virion DNA does not contain modified bases, this corresponds to an average G+C content of 48.8% (25). A second small peak of absorption, representing 9.4 ± 1.1% of total virion DNA, became visible at ρ = 1.727 g/ml, equivalent to 68.7% G+C. However, a drastic change in absorption profiles was seen when virion DNA was degraded by mechanical shearing to average fragments ≤15 kbp (Fig. 1C and D). A fraction of high-density DNA, designated H-DNA, of herpesvirus aotus type 2, banding at ρ = 1.727 (68.7% G+C), represented 34 ± 4% of total virion DNA. The majority of fragmented viral DNA, 66 ± 4%, designated as L-DNA, was found at ρ = 1.699 g/ml, equivalent to 40.2% G+C. This experiment allowed the conclusion that herpesvirus aotus type 2 particles contain two types of high-molecular-weight DNA, a majority of M-genomes (M-DNA) and a small proportion of H-genomes (H-DNA). M-DNA has a pronounced intramolecular G+C content heterogeneity, consisting of 73% L-DNA and 27% H-DNA. This is strikingly similar to the high-

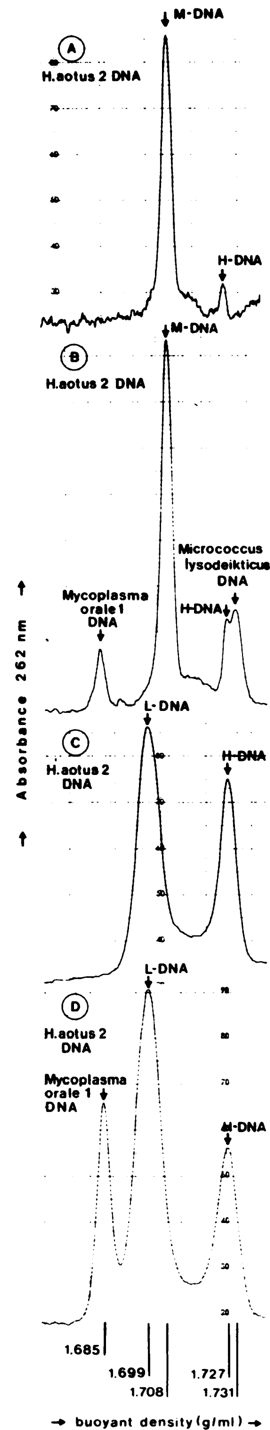

FIG. 1. Analytical scanning of herpesvirus aotus type 2 DNA banding in isopycnic CsCl gradients. (A) Intact viron DNA, (B) intact virion DNA with density markers (DNA from *M. lysodeikticus* and *M. orale* type 1), (C) mechanically fragmented herpesvirus aotus type 2 virion DNA, (D) mechanically fragmented virion DNA with the same density markers.

TABLE 1. Buoyant densities and base composition of virion DNA fractions

DNA	herpesvirus aotus type 2		herpesvirus saimiri		herpesvirus ateles	
	g/ml	% G+C	g/ml	% G+C	g/ml	% G+C
H-DNA	1.727	68.7	1.729	70.6	1.733	74.6
L-DNA	1.699	40.2	1.695	35.8	1.697	37.5
M-DNA	1.708	48.8	1.705	45.4	1.706	47.1

and low-G+C sequence arrangement in herpesvirus saimiri and herpesvirus ateles genomes (Table 1).

The arrangement of high- and low-G+C sequences in H- and M-genomes was analyzed by cleavages with a number of restriction endonucleases. Some enzymes such as *AluI*, *BamHI*, *BglI*, *DdeI*, *EcoRI*, *HaeII*, *HpaII*, *PstI*, *SacII*, and *SmaI* were found to cleave within the H-DNA, whereas others did not (*BglII*, *HindIII*, *KpnI*, *SalI*, *TaqI*, *XbaI*, *XhoI*). Fig 2A (lanes a and b) shows the cleavage patterns of DNA from the H-genome fraction after digestion with *BamHI* and *EcoRI*. In both cases, two hypermolar bands of 2.1 and 2.3 kbp were seen, indicating that H-DNA is highly repetitive. Instead of this pattern, some virus stocks of higher passage level had two hypermolar bands of 2.3 and 2.75 kbp. When M-DNA was digested with enzymes cleaving in H-DNA, in addition to hypermolar repeat fragments a series of unique L-DNA fragments became apparent in the size range between 22 and 6.5 kbp. No hypermolar fragments of H-DNA became apparent when restriction endonucleases were applied that do not cleave within the H-DNA repeats. Thus, there was no indication for repeat structures in the L-DNA fragments. Compiling the sizes of L-DNA fragments generated by five restriction endonucleases, the entire length of L-DNA was estimated to correspond to a region of 109.4

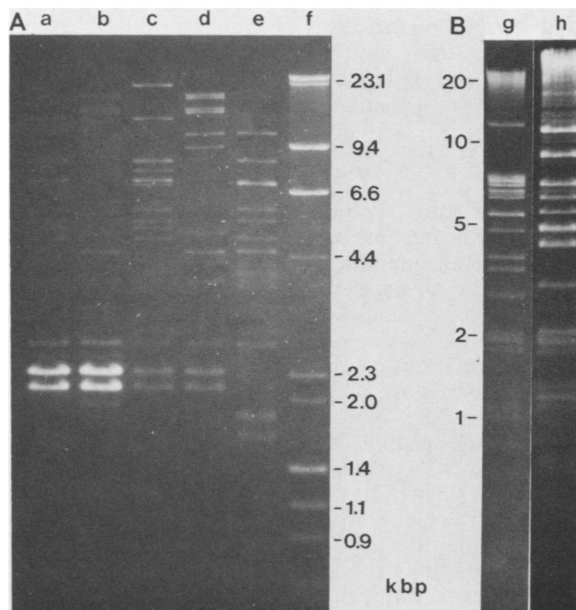

FIG. 2. Cleavage of herpesvirus aotus type 2 virion DNA with restriction endonucleases. DNA from H-genome fraction digested with *BamHI* (a) and *EcoRI* (b); M-DNA cleaved with *BamHI* (c), *EcoRI* (d), *BamHI* plus *EcoRI* (e), *XbaI* (g), and *HindIII* (h). Lane f contains DNA size markers.

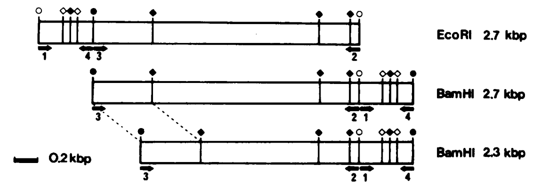

FIG. 3. Physical maps of *BamHI* (●), *NarI* (◇), *EcoRI* (○), and *DdeI* (◆) cleavage sites within three cloned repeat units of herpesvirus aotus type 2. The maps were aligned according to corresponding cleavage sites. Arrows represent sequenced DNA regions. Identical sequences are labeled by the same number.

± 1.4 kbp. Cleavage with *HindIII* or *XbaI* resulted in a heterogeneous population of high-molecular-weight molecules (Fig. 2B), suggesting that size heterogeneity of these DNA fragments is due to variable numbers of repeat elements in each H-DNA stretch. These analyses suggested that the M-genome of herpesvirus aotus type 2 consists of a single L-DNA region and one or two variable stretches of H-DNA repeats; this was confirmed by electron microscopy. Partial denaturation mapping indicated a unique low-G+C DNA region of about 110 kbp inserted between two terminal segments of G+C-rich DNA. Thus, the overall structure of herpesvirus aotus M-DNA appeared essentially identical with sequence arrangement in M-genomes of herpesvirus saimiri and herpesvirus ateles.

Organization of repetitive DNA. To obtain a more detailed picture of the organization of the repetitive DNA, virus of high passage level was plaque purifed four times, and H-DNA was isolated; two *BamHI* repeats and the large *EcoRI* repeat were molecularly cloned in *E. coli*. The resulting plasmids were named pPF10 (2.7-kbp *BamHI* repeat), pPF11 (2.3-kbp *BamHI* repeat), and pPF12 (2.7-kbp *EcoRI* repeat). Physical maps of the cleavage sites for *BamHI*, *EcoRI*, *DdeI*, and *NarI* within the three repeats are shown in Fig. 3. The data suggest that large and small repeats have identical flanks, but differ within a variable region of at most 1.4 kbp. The homology between both types of repeats was confirmed by cross-hybridization of the cloned DNAs in Southern blot experiments (Fig. 4). The exact identity within 300 base pairs was established by DNA sequencing, which revealed identical nucleotide sequences around all *BamHI* and *EcoRI* cleavage sites (arrows in Fig. 3).

Cloned repeat DNA was labeled by nick repair and used as a probe for Southern blot hybridization with cleaved M-DNA (Fig. 4B). The two repeat bands that could be demonstrated by ethidium bromide staining (Fig. 2) led to prominent signals on the autoradiogram. In addition, a highly regular ladder-like pattern of weaker bands was observed. Two ladders of different intensity were seen. The first included the main repeats, the sizes of the DNA fragments increased by steps of about 400 base pairs. The second one showed identical intervals, having its bands shifted by about 200 base pairs after *BamHI* cleavage. Three other bands appeared apart from the ladder-like pattern. All of these bands must be derived from repetitive DNA; this could be shown by hybridization of radioactively labeled cloned H-DNA with M-DNA after digestion with enzymes that do not cut repetitive DNA. Uncleaved repetitive DNA of high molecular weight hybridized in this case, and none of the unique DNA bands was observed (data not shown). These data indicate that the H-DNA is composed of related subunits with different sizes which are clustered apart from

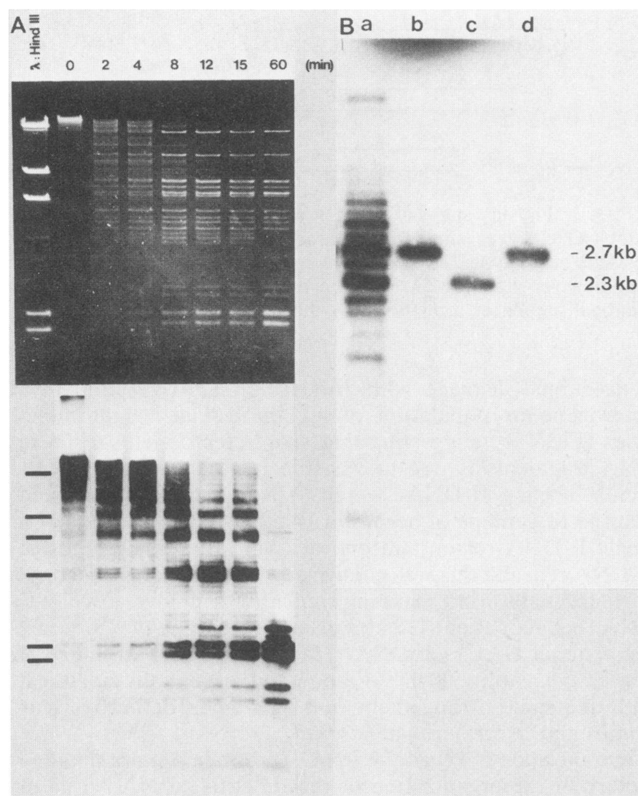

FIG. 4. Herpesvirus aotus M- and H-DNA sequences as revealed by hybridization to the ^{32}P-labeled, large *Bam*HI repeat DNA. (A). Partial *Bam*HI digestion of herpesvirus aotus M-DNA. DNA (14 μg) was incubated with 7 U of enzyme, and samples were removed at the indicated time points. The reaction was stopped by the addition of electrophoresis sample buffer and heating at 70°C for 10 min. The top shows the gel stained with ethidium bromide; the bottom shows the Southern blot of this gel hybridized to the ^{32}P-labeled large *Bam*HI repeat DNA. (B) Hybridization of ^{32}P-labeled *Bam*HI repeat DNA with (a) herpesvirus aotus M-DNA cleaved with *Bam*HI, (b and c) cloned *Bam*HI repeat units, and (d) a cloned *Eco*RI repeat unit.

the unique DNA. Double cleavages of M-DNA in conjunction with the physical map of cloned repeats (Fig. 2 and 3) suggested a head-to-tail arrangement of the subunits, although a few inversions could not be excluded.

To analyze the arrangement of the individual subunits relative to each other, partial *Bam*HI digests of M-DNA were examined by blot hybridization (Fig. 4A). In the range of subunit dimers three major bands of roughly equal intensity were seen, which correspond to the following combinations of the most prominent repeat units: large-large, large-small, and small-small. Diffuse bands were observed at the positions of trimers, tetramers, pentamers, etc., corresponding to the multiples of subunits with an average size of 2.5 kbp. These data indicated that the different subunit classes are oriented in the same direction and are randomly interspersed with each other throughout the stretches of repetitive DNA. Even after prolonged exposure, the largest oligomers observed were nonamers, corresponding to a total length of about 23 kbp.

In view of the results of the partial digest it was not surprising to find that four rounds of plaque purification of herpesvirus aotus did not affect the complex pattern of the repeats, although the relative amount of individual repeat classes varied between virus stocks of different passage levels. This confirmed that units of different size are mixed within single genomes.

DNA sequence comparisons with related viruses. In the absence of sizeable lengths of known nucleotide sequences, we preferred to obtain preliminary information on the taxonomic relationship between herpesvirus aotus type 2 and oncogenic primate herpesviruses by a series of liquid phase hybridization experiments. Virion L-DNA of herpesvirus aotus type 2 was labeled in cell culture with [^{3}H]thymidine to a final specific activity of 550,000 cpm/μg. Reassociation of 100 ng of labeled herpesvirus aotus type 2 DNA per ml, conducted at 26°C below melting temperature, was significantly accelerated by the presence of 5 μg of unlabeled purified homologous viral DNA per ml; however, there was no elevation above background reassociation with 5μg of unlabeled M-DNA from herpesvirus saimiri, herpesvirus ateles, and herpesvirus aotus types 1 and 3 per ml (Fig. 5A and B). Inversely, hybridization reactions of labeled L-DNA from herpesvirus saimiri (100 ng/ml; 158,000 cpm/μg) and herpesvirus ateles (100 ng/ml; 160,000 cpm/μg) could not be driven by 5 μg of unlabeled M-DNA from herpesvirus aotus type 2 per ml (Fig. 5C and E). Also no significantly increased reassociation velocity was seen when ^{3}H-labeled H-DNA from herpesvirus saimiri (300,000 cpm/μg, 50 ng/ml) or herpesvirus anteles (490,000 cpm/μg, 50 ng/ml) was hybridized with an excess (5 μg/ml) of purified M-DNA from herpesvirus aotus type 2 (Fig. 5D and F). The relatively high initial C_0/C_t values of herpesvirus ateles H-DNA are probably due to short inverted repeats causing hairpin loops or due to tertiary structures in the extremely high (75%)-G+C DNA sqeuences. In conclusion, cross-hybridization experiments indicated consistently that herpesvirus aotus type 2 does not share sequence homologies with herpesvirus saimiri or herpesvirus ateles over sizable genome lengths. Short arrays of sequence homology, representing less than about 5% of the respective DNA fractions, were not expected to be found by this method. Homologous L-DNA sequences with less than about 19% base pair divergence would have been detected under the conditions of stringency used in these experiments, and H-DNA homologies would have been found up to 27% mismatch.

DISCUSSION

Herpesvirus aotus type 2 is an indigenous agent of owl monkeys that, after preliminary studies, appears to be apathogenic in the natural hosts and a number of related primates. In the absence of obvious oncogenic properties, we were unable to immortalize leukocytes of tamarin marmosets (*Saguinus* sp.) in vitro by herpesvirus aotus type 2. However, the genome organization of this virus is strikingly similar to the structure of DNA from the highly oncogenic herpesviruses of New World primates. The dominant type of virion DNA, designated M-genome, consists of a unique 110-kilobase L-DNA region that is constant in length. It is flanked at both sides by variable stretches of H-DNA repeats that are, mostly or entirely, in head-to-tail arrangement. In addition, a minority of virus particles contain defective genomes that probably consist of H-DNA repeats solely (Fig. 6). However, given all these structural similarities with herpesvirus saimiri and herpesvirus ateles, there remain some clear differences. The L-DNA in herpesvirus aotus type 2 is higher in average GC content than corresponding sequences of herpesvirus saimiri and herpesvirus ateles; inversely, the G+C content of repetitive herpesvirus aotus DNA is significantly lower (Table 1). Herpesvirus aotus type

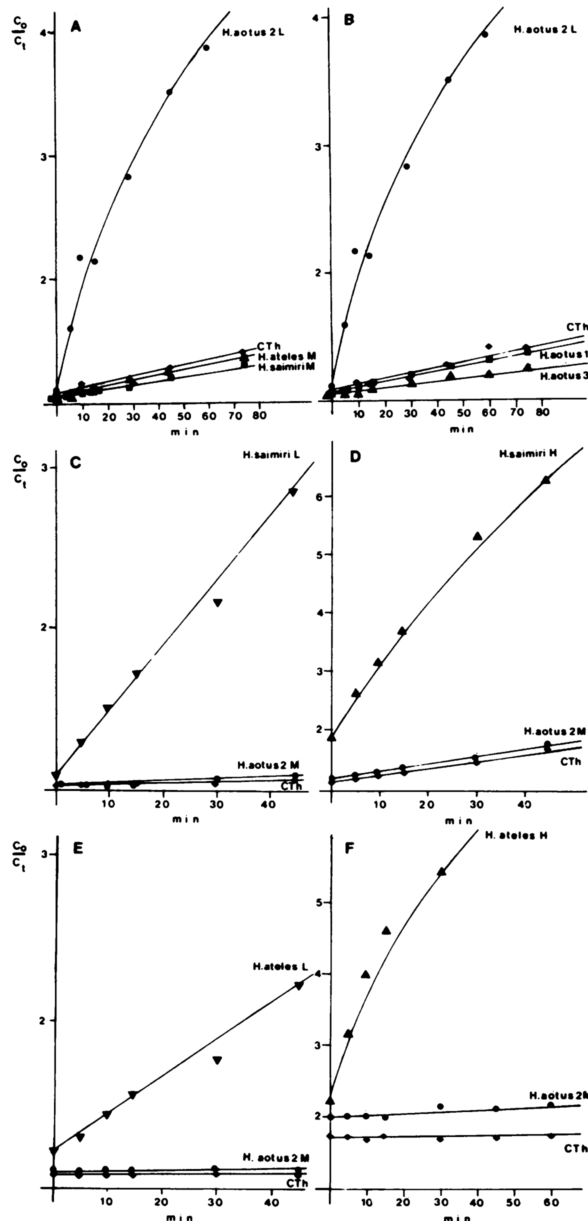

FIG. 5. Reassociation kinetics of ^{3}H-labeled virion DNA fraction with an excess of purified homologous or heterologous labeled DNA. (A) Labeled herpesvirus aotus type 2 L-DNA hybridizing with unlabeled herpesvirus aotus type 2 L-DNA (●), herpesvirus saimiri M-DNA (■), herpesvirus ateles M-DNA (▲) and an equal amount of calf thymus DNA (◆). (B) Labeled herpesvirus aotus type 2 L-DNA hybridizing with excess homologous DNA (●), herpesvirus aotus type 1 DNA (■), herpesvirus aotus type 3 DNA (▲), and an equivalent amount of calf thymus DNA (◆). (C) Labeled herpesvirus saimiri L-DNA hybridizing with excess of homologous unlabeled DNA (▼), herpesvirus aotus type 2 M-DNA (●), and an equal amount of calf thymus DNA (◆). (D) Labeled herpesvirus saimiri H-DNA hybridizing with an excess of unlabeled homologous DNA (▲), herpesvirus aotus type 2 M-DNA (●), and an equal amount of calf thymus DNA (▲). (E) Labeled herpesvirus ateles L-DNA hybridizing with an excess of homologous unlabeled DNA (▼), herpesvirus aotus type 2 M-DNA (●), and an equal amount of calf thymus DNA (◆). (F) Labeled herpesvirus ateles H-DNA hybridizing with an excess of unlabeled homologous DNA (▲), herpesvirus aotus type 2 M-DNA (●), and an equal amount of calf thymus DNA. C_0/C_t is counts per minute in total DNA divided by the counts per minute in single-stranded DNA. The concentrations of DNA and specific activities are given in the text.

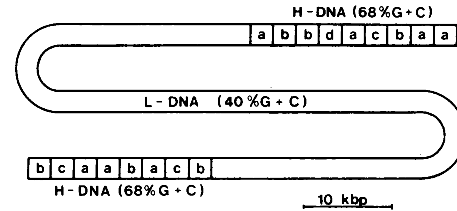

FIG. 6. Genome organization of herpesvirus aotus M-DNA. The letters a, b, c, and d represent different size classes of repeats that are randomly mixed throughout H-DNA.

2 has longer H-DNA repeats than all strains of herpesvirus saimiri or herpesvirus ateles that have been investigated (9, 13). The reiterations of herpesvirus aotus are much more variable; two types of units were found, a larger repeat of 2.75 kbp and a small element of 2.3 kilobases, that appear to share at least 1.3 kilobases of identical DNA sequence. To form chains of H-DNA, both units are oriented in the same direction and appear intermixed at random within a single molecule. Additional repeat units are found, and changing molar representation of repeats is seen at different passage levels. Hybridizations, even at low stringency, did not allow to detect base sequence homologies between herpesvirus aotus type 2 DNA and its structural counterparts. Studies on DNA structure and sequence homologies place herpesvirus aotus type 2 at larger evolutionary distance from herpesvirus saimiri and herpesvirus ateles than those two viruses from each other.

The phylogenetic relationship of the host species within the family of *Cebidae* is not clearly defined so far. However, based on morphology (1, 24) and serological cross-reactivity of serum proteins (16), squirrel monkeys are assumed to have diverged from the common ancestor of owl and spider monkeys. This contrasts with the relatedness of the viruses and may indicate that the herpesviruses of New World primates crossed species barriers at some time of their evolution. So far, there was no indication that herpesviruses of the respective genome organization occur in other animals than *Platyrrhinae*, though a similar DNA structure may exist in a herpesvirus of cattle (bovine herpesvirus type 4) (H. Ludwig, personal communication).

Herpesvirus aotus type 2 gives another example that overall genome structure and pathogenic properties of herpesviruses do not necessarily correlate. Herpesviruses that are known to induce lymphoproliferative disease (herpesvirus saimiri, herpesvirus ateles, Epstein-Barr virus) usually possess tandemly repeated sequences in their genomes. A remarkable exception, however, is the virus of Marek's lymphomatosis, which possesses two pairs of inverted repeats (4). For herpesvirus aotus type 2, there is no indication of oncogenicity; yet it shares genome structure with the oncogenic herpesviruses of nonhuman primates. It will be of interest to search for genes of herpesvirus aotus type 2 that are functionally equivalent to the genomic region of herpesvirus saimiri that is required for oncogenicity (20; Desrosiers et al., submitted).

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