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Terminal Repetitive Sequences in Herpesvirus Saimiri Virion DNA

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The H-DNA repeat unit of Herpesvirus saimiri strain 11 was cloned in plasmid vector pAGO, and the nucleotide sequence was determined by the dideoxy chain termination method. One unit of repetitive DNA has 1,444 base pairs with 70.8% G+C content. The structural features of repeat DNA sequences at the termini of intact virion M-DNA (160 kilobases) and orientation of reiterated DNA were analyzed by radioactive end labeling of M-DNA, followed by cleavage of the end fragments with restriction endonucleases. The termini appeared to be blunt ended with a 5′-phosphate group, probably generated during encapsidation by cleavage in the immediate vicinity of the single *Apa*I recognition site in the H-DNA repeat unit. The sequence did not reveal sizeable open reading frames, the longest hypothetical peptide from H-DNA being 85 amino acids. There was no evidence for an mRNA promoter or terminator element, and H-DNA-specific transcription could not be found in productively infected cells.

Herpesvirus saimiri, a ubiquitious agent of squirrel monkeys, is oncogenic in a number of New World primates. The complete genome (M-genome) of herpesvirus saimiri is linear, double-stranded DNA of approximately 160 kilobase pairs (10, 12, 13). Most of the genetic information is contained in the 112-kilobase (kb) L-DNA segment (20) of low G+C content (14) which is located between two stretches of repetitive DNA with high G+C content (H-DNA). Restriction enzyme analysis has shown that each repeat has approximately 1.44 kb (2). The number of repeats varies at both ends of the genome; the reiterations are arranged in tandem, and repetitive DNA of the two termini is oriented in the same direction (13). A similar genome structure was found in herpesvirus ateles (a tumor virus from spider monkey) (10), herpesvirus aotus type 2 from an owl monkey (15), and bovine herpesvirus type 4 (30). The herpesviruses are unique in that they possess repetitive DNA which can amount to one third of the entire genome. However, the functional role of repetitive H-DNA, perhaps in transcription, replication, or encapsidation, remains to be elucidated. In this study we describe the topology and nucleotide sequence of herpesvirus saimiri H-DNA, and discuss the possibility for transcriptional, translational, and packaging signals within H-DNA.

MATERIALS AND METHODS

Virus and cell culture. Herpesvirus saimiri strain 11 was isolated originally from peripheral leukocytes of a squirrel monkey (9). The virus was propagated on owl monkey kidney (OMK) cells strains 210 or 637 (6, 31) as described previously (17). Virus particles were purified from infected cell cultures by velocity sedimentation in sucrose gradients (11).

Purification of nucleic acids, molecular cloning procedures, and blot hybridizations. To obtain viral M-DNA, purified herpesvirus saimiri particles were dissociated by incubation in 20 mM Tris-hydrochloride (pH 8.5)–2% (wt/wt) sodium lauroyl sarcosinate for 60 min at 60°C, and virion DNA was banded by isopycnic centrifugation in CsCl density gradients (14). Restriction fragments of virion DNA were cloned in plasmid vectors pACYC 184 (3), pAGO (5), and pWD7 (20). Extraction of RNA from lytically infected cells with guanidinium isothiocyanate and centrifugation through a CsCl cushion has been described in detail (19). Also, procedures for size determination of DNA restriction fragments by Southern blot hybridizations and detection of RNA with Northern blots have been reported previously (19).

Nucleotide sequencing. The nucleotide sequences of virion DNA fragments were mostly determined by the dideoxynucleotide procedure (27). Restriction fragments generated by *Alu*I, *Hae*III, *Hpa*II, and *Taq*I were cloned in the bacteriophage vectors M13 mp 8/9 (24). Sequence data were analyzed in a VAX computer (Digital Equipment Co.) with the programs of Staden (28, 29). For some experiments, DNA sequencing was performed by a base-specific chemical degradation method (23).

End labeling of virion DNA. To label the 5′ ends of M-genomes, 1 μg of purified virion DNA was incubated with 40 U of calf intestine phosphatase (Boehringer Mannheim, Mannheim, West Germany) in 250 μl of 0.1 M glycine (pH 10.5)–1 mM $MgCl_2$–0.1 mM $ZnCl_2$ for 30 min at 37°C, followed by a second 30-min incubation at 56°C with an additional 40 U of calf intestine phosphatase. The reaction was stopped by adding 30 μl of 100 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid at 65°C. Viral DNA was extracted with phenol and phenol chloroform (1:1) under gentle agitation, precipitated with 2.5 volumes of ethanol, and dissolved in 30 μl of kinase buffer (50 mM Tris-hydrochloride [pH 7.6], 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidine, 0.1 M EDTA). The labeling reaction was carried out at 37°C for 60 min with 12 U of polynucleotide kinase (Boehringer Mannheim) in the presence of 20 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol, Amersham-Buchler, Braunschweig, West Germany) and concentrated by ethanol precipitation. The 3′ termini of virion DNA were radioactively labeled by the replacement synthesis method (26) with bacteriophage T4 DNA polymerase. Intact M-DNA (4 μg) was incubated at 37°C with 5 IU of T4 DNA polymerase for 5 min in 50 μl of TA buffer (33 mM Tris-acetate [pH 7.9] 66 mM potassium acetate, 10 mM magnesium acetate,

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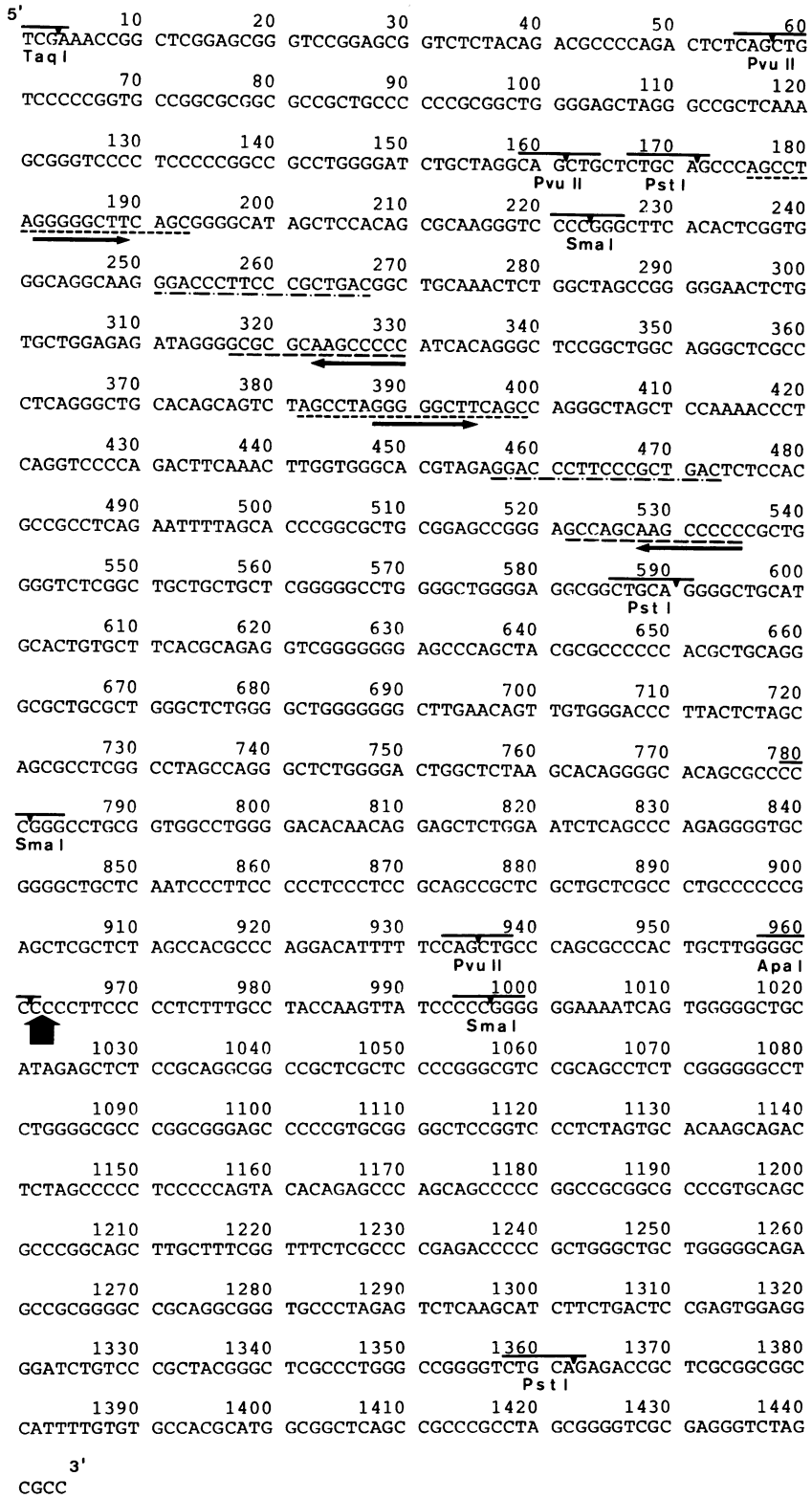

FIG. 1. Nucleotide sequence of one repeat unit from H-DNA of herpesvirus saimiri strain 11. Three tandemly oriented sequence duplications are underlined, a 18-bp repeat (– – – –), a 17-bp repeat (– . — . — . –), and a 14-bp repeat (— — —). The repeats motifs of 18 and 14 bp form two pairs of inverted repetitions with perfect matching over eight nucleotides (indicated by arrows). The packaging cleavage site is indicated by a bold arrow between bp 962 and 963.

100 μg bovine serum albumin per ml, 0.5 mM dithiothreitol) to excise up to 100 nucleotide per 3′ end. The polymerase reaction was started by adding 2.5 μl of a mix of dATP, dTTP, and dCTP (2 mM each) and 10^{-11} mol of [α-^{32}P]dGTP (800 Ci/mmol). The reaction was allowed to proceed for 10 min; then 1 μl of 2 mM dGTP was added, and the mixture was incubated for another 10 min to guarantee the formation of blunt ends. The reaction was stopped by heating to 70°C for 5 min.

RESULTS

Structure and nucleotide sequence of the H-DNA repeat unit. The initial cloning of the H-DNA repeat unit was started with high-molecular-weight H-genomes that were extracted from purified herpesvirus saimiri strain 11 virions. The viral DNA was cleaved to the size of single repeat units with the restriction endonuclease *Taq*I, which cleaves only once in each reiteration (13). The *Taq*I restriction fragments were cloned in the *Cla*I cleavage site of vector pAGO (5). To establish a library of H-DNA in bacteriophage M13 mp 8/9, the H-DNA repeat was excised with *Taq*I, isolated from agarose gels, and cleaved to small fragments with *Alu*I, *Hae*III, or *Hpa*II. Blunt-ended digestion products and fragments with cohesive ends were inserted into the *Sma*I and *Acc*I sites of vector M13 mp 8/9, respectively, and the entire nucleotide sequence of H-DNA was determined (Fig. 1). We found that one H-DNA repeat unit consists of 1,444 base pairs (bp) (13.5% T, 15.7% A, 34.1% C, and 36.7% G). The G+C content of 70.8% is in good agreement with earlier determinations by density centrifugation and high-pressure liquid chromatography (11, 12). Computer analysis of dinucleotide frequencies indicated that the number of possible eucaryotic methylation sites (CpG) in H-DNA is 13.96%; this corresponds to statistical expectations and deviates from the usual CpG depletion in eucaryotic DNA. The H-DNA unit contains three sets of tandemly oriented sequence duplications (18, 17, and 14 bp), each pair separated by a distance of 191, 189, and 188 bp, respectively. The 14- and 18-bp tandem reiteration contained a short inverted repeat motif (Fig. 1). Other inverted repeats of more than five base pairs could not be found. Reading frame analysis detected multiple stop codons in six possible translational phases. The longest open reading frame between the initiation codon ATG and a termination sequence would allow the synthesis of a polypeptide of 85 amino acids (Fig. 2). No canonical TATAA sequence was found that could be part of a transcriptional promoter. Thus, the primary structure of

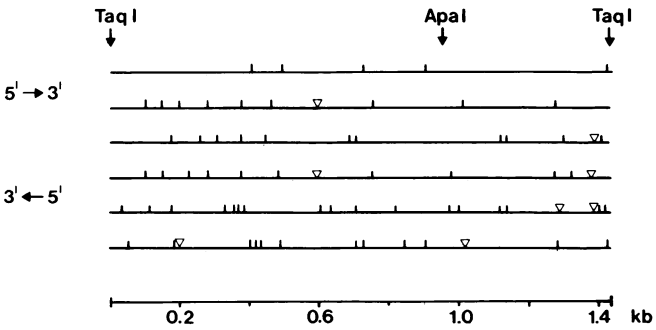

FIG. 2. The six possible translational reading frames of herpesvirus saimiri H-DNA. Vertical bars indicate stop signals; triangles correspond to ATG codons.

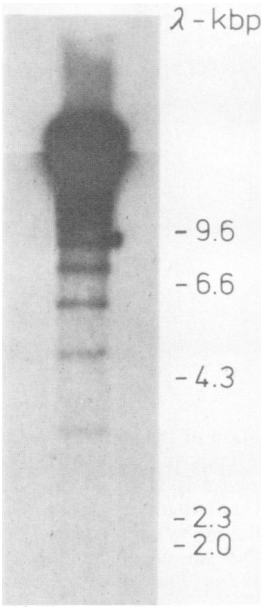

FIG. 3. Detection of the discrete-sized terminal H-DNA segments in herpesvirus saimiri M-DNA. Virion DNA was cleaved with *Eco*RI; the fragments were separated on 0.8% agarose gels, transferred to nitrocellulose filters, and hybridized with ^{32}P-labeled cloned H-DNA. Each of the sizeable restriction fragments (≧10 kb) corresponds to a unique L-sequence (right-terminal *Eco*R-J fragment of L-DNA) plus an integer number of H-DNA repeat units. kbp, Kilobase pairs.

H-DNA from herpesvirus saimiri strain 11 does not give obvious evidence that it possesses capacity to code for a functional RNA polymerase II transcript. Also there was no evidence for RNA polymerase III transcription in vitro (W. Bodemer and J. Arrand, unpublished data). No significant sequence similarities could be found in the comparison with the known repeat sequences of herpes simplex virus, Epstein-Barr virus, and human cytomegalovirus of the EMBO data base.

Packaging cleavage site in H-DNA. Earlier studies on M-genome structure, mostly partial denaturation mapping in the electron microscope, had indicated that the lengths of repetitive DNA stretches at both ends of virion DNA are highly variable, i.e., between less than 2 kb and approximately 50 kb. In general, molecules with few H-DNA repeats at one terminus had a high number of repeats at the opposite end, resulting in a relatively constant overall length of M-genomes (2). This suggested that herpesvirus saimiri genomes arise from concatemeric forms during virus replication, using a packaging system that cleaves monomeric genomes from newly replicated chains by a head-full mechanism. Taking advantage of the known nucleotide sequence of herpesvirus saimiri H-DNA, we searched for possible signals in H-DNA that may be recognized as cleavage sites during encapsidation. Virion M-DNA was digested with restriction endonuclease *Eco*RI which is known to find at least 14 recognition sites in the L-DNA of herpesvirus saimiri strain 11 without cleaving in the repetitive DNA. The right-most *Eco*RI fragment of L-DNA has 1.8 kb, whereas the large left-terminal L-DNA fragment contains about 22 kb, both defined by the distance to the closest *Sma*I site. The fragments were separated by agarose gel electrophoresis, transferred to nitrocellulose filters, and hybridized with ^{32}P-labeled cloned H-DNA. Figure 3 shows an autoradio-

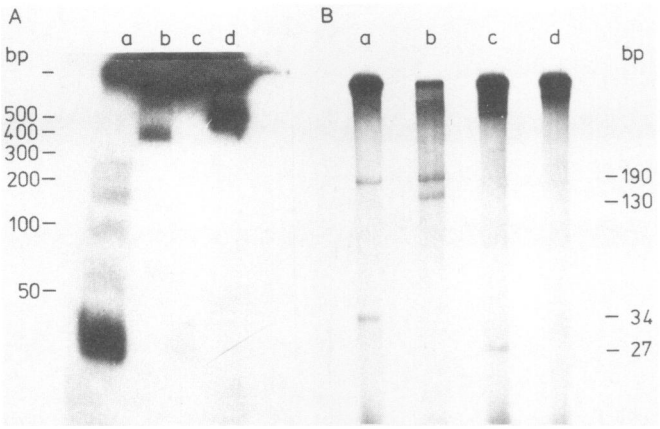

FIG. 4. Determination of the terminal sequences of H-DNA in M-genomes. (A) The ends of intact M-DNA were radioactively labeled with bacteriophage T4 DNA polymerase I. Virion DNA was cleaved with *Xho*I, and fragments larger than 44 kb (from left terminus) were isolated by agarose gel electrophoresis and electroelution. The DNA was cleaved with *Hpa*II (lane a), *Pst*I (lane b), *Sma*I (lane c), and *Taq*I (lane d). Sizes of radioactive fragments were determined by 7.5% (wt/vol) polyacrylamide electrophoresis gel. (B) The ends of intact M-DNA were treated with calf intestine phosphatase and ^{32}P-labeled by polynucleotide kinase. DNA was cleaved with *Sma*I (lane a), *Hin*fI (lane b), *Pvu*II (lane c), and *Apa*I (lane d). Sizes of fragments were determined by electrophoresis in 10% (wt/vol) polyacrylamide gels.

gram which demonstrates the sizes of *Eco*RI-generated restriction fragments which contain H-DNA sequences. A series of discrete fragments became visible that appeared in about equimolar representation. The smallest *Eco*RI-generated M-DNA terminus corresponded to a 3.4-kb fragment, and all other bands were found at positions equivalent to regular increments of 1.4 kb, thus representing the right-hand termini of M-DNA. These results corroborated the earlier observation that H-DNA termini of M-DNA have discrete lengths (13) and indicated that each H-DNA repeat unit possesses a single packaging cleavage signal. Remarkably, the smallest fragment had a larger size (3.4 kb) than the sum of the *Eco*RI-J fragment (1.8 kb) and one H-DNA (1.444 kb) repeat. Apparently adjacent to the *Eco*RI-J fragment (defined at the right end by an *Sma*I site; see reference 20) there is a short sequence of at least 200 bp which is not genunine H-DNA and does not have a packaging cleavage signal.

Initial experiments to define the outermost terminal M-DNA sequences were performed by 3'-end labeling with T4-DNA polymerase. Double-stranded, labeled terminal fragments from both molecular ends, generated by *Hpa*II, *Pst*I, *Sma*I, and *Taq*I, were determined in size by 7.5% (wt/vol) polyacrylamide gel electrophoresis. All of these experiments placed consistently the M-DNA terminus around bp 960 of H-DNA (Fig. 1). The 3'-end-labeled M-DNA also allowed determination of the orientation of H-DNA in M-genomes relative to the published cleavage maps of L-DNA. After being labeled with T4-DNA polymerase, M-DNA was cleaved with *Xho*I, and the large (44-kb) left-terminal *Xho*I-A fragment of L-DNA with flanking H sequences was isolated by agarose gel electrophoresis. The single radioactive fragments obtained with *Hpa*II and *Sma*I had ~25 bp; *Pst*I and *Taq*I fragments had ~400 and ~500 bp, respectively (Fig. 4A). On the other hand, the 3'-labeled right-end fragment of M-DNA generated by *Pvu*II had ~30 bp (data not shown). This indicated that for the maps of H-DNA repeat units to correspond to the published L-DNA cleavage map they should be oriented as shown in Fig. 1 and 5.

Further experiments were aimed at determining more closely the termini of M-DNA relative to the H-DNA sequence of herpesvirus saimiri strain 11. Entire M-DNA molecules could be ^{32}P-labeled by polynucleotide kinase after treatment with calf intestine phosphatase. The labeled DNA was cleaved with a number of restriction endonu-

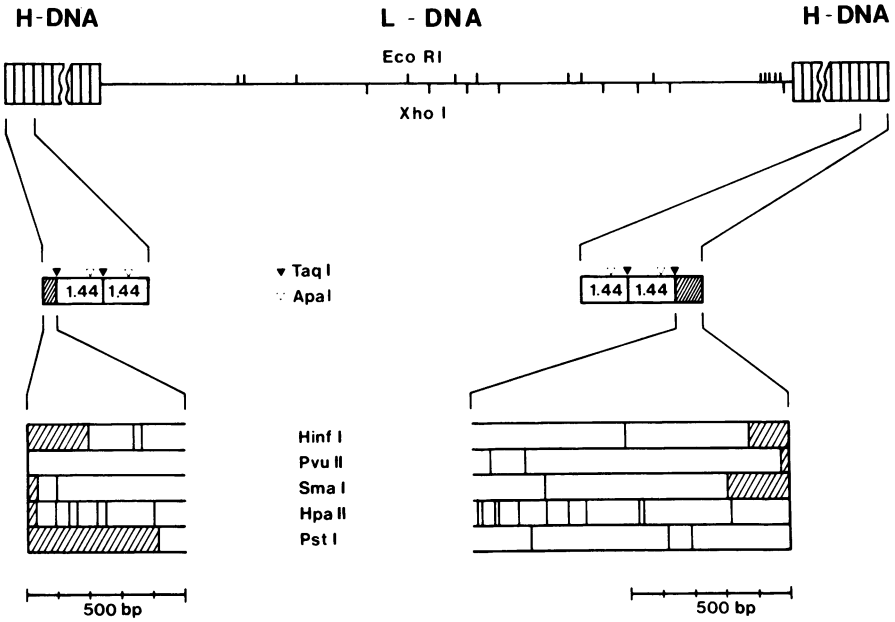

FIG. 5. Cleavage maps of the outermost H-DNA repeats units between packaging site and first *Taq*I-cleavage site. Hatched boxes represent terminal restriction fragments that could be identified at either of the two ends of M-DNA (see Fig. 4).

cleases (e.g., *Apa*I, *Hin*fI, *Pvu*II, *Sma*I), and the size of terminal double-stranded fragments was determined in 10% (wt/vol) nondenaturing polyacrylamide gels. The terminal *Hin*fI-fragments appeared to have ~130 and 190 bp, the *Sma*I-generated termini had about 30 and 190 bp, and a single sizeable fragment of approximately 25 bp became visible after cleavage with *Pvu*II (Fig. 4B). No discrete bands were seen after cleavage with *Apa*I. These results suggested that the M-DNA terminus is around bp 960 of H-DNA, close to or coinciding with the *Apa*I restriction site (Fig. 1). It was not possible to label M-DNA with polynucleotide kinase when the molecule was not treated with calf intestine phosphatase before the phosphorylation reaction. This probably indicates that the termini of virion DNA have free, accessible single 5'-phosphate groups and are not protected by covalently bound protein.

The accurate localization of the M-DNA terminus was obtained by electrophoresis of 5'-labeled end fragments in denaturing sequencing gels, using restriction endonucleases *Pvu*II and *Sma*I. The *Pvu*II-generated 5'-labeled single strand has 27 bases, whereas the position of the corresponding *Sma*I fragment was found to have 34 bases (Fig. 6). The distance between the respective restriction endonuclease cleavage sites which are in close proximity of the packaging cleavage sequence, is equal to 61 bp (Fig. 1). This apparently indicates that, monomeric M-DNA is cut from concatemers before or during encapsidation in such a way as to produce blunt ends, using a recognition signal in H-DNA.

Transcriptional activity of H-DNA. Previous transcription studies with ^{3}H-labeled cytoplasmic RNA in filter hybridizations did not allow detection of H-DNA-encoded transcripts in lytically infected cells (33). It made it unlikely that H-DNA is instructing mRNA or a viral polypeptide. This is consistent with the absence of a detectable canonical TATA box or a polyadenylation site (Fig. 1). To search for other transcripts, a series of Northern blots were done with the cloned H-DNA. Total cellular RNA, in some studies fractionated into polyadenylated and nonpolyadenylated transcripts, was transferred to nitrocellulose filters and hybridized with radioactive H-DNA probes. None of these experiments gave any detectable hybridization signal, whereas appropriate L-DNA fragments regularly traced immediate-early, early, or late transcripts (Fig. 7). Therefore, we conclude that the repetitive DNA of herpesvirus saimiri is not appreciably transcribed during virus replication.

DISCUSSION

M-genomes of herpesvirus saimiri are double-stranded DNA molecules of about 160 kb which represent the entire viral genome; repetitive DNA (H-DNA) accounts for approximately one third of the molecule. The tandemly oriented repeats, variable in their number, are located at the termini of M-DNA flanking the unique L-DNA region of 112 kb (2, 20). This study has shown that the H-DNA repeat unit has 1,444 bp with 70.8% G+C. End-labeling experiments indicated the orientation of H-DNA repeats relative to the L-DNA region; it was consistent with the orientation obtained by restriction mapping of the left H/L-DNA transition described in an earlier study (21). The H-DNA repeat does not have apparent signals for initiation or termination of mRNA synthesis. The longest open reading frame could allow synthesis of a peptide of 85 amino acids only; however, there is no indication for transcription of H-DNA during replication. In this study the structural peculiarities of herpesvirus saimiri M-DNA facilitated the analysis of the molecular ends. The terminal base pairs of M-DNA appear to correspond to nucleotides 962 and 963 of H-DNA at the right or left end, respectively. The ability to label both the 5' and 3' ends of the molecules indicates that there is no protection by a covalently bound protein.

For several herpesviruses, concatemeric replicative intermediates were demonstrated (4, 7, 8, 16, 22, 34; A. J. Davison and F. J. Ricon, *in* Y. Becker, ed., *Cloning of the DNA of Alphaherpesvirinae*, *vol. 5*, in press). The structural

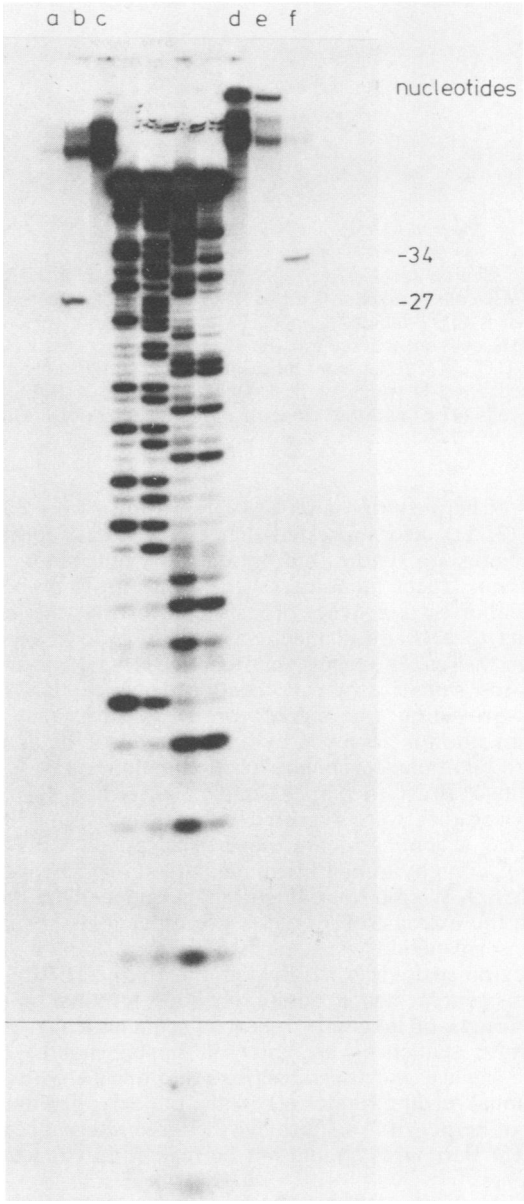

FIG. 6. Mapping of terminal bases of M-genomes with the H-DNA repeat units. The 5' ends of intact M-DNA were ^{32}P-labeled, and virion DNA was cleaved with *Hin*fI. A 190-bp fragment from the left end and a 130-bp fragment from the right end were isolated through 10% (wt/vol) polyacrylamide gels. The 130-bp fragment was cleaved with *Sma*I (lane a), *Pvu*II (lane b), and *Apa*I (lane c); the 190-bp fragment was cleaved with *Apa*I (lane d), *Pvu*II (lane e), and *Sma*I (lane f). Sizes of radioactive fragments were determined in a denaturing sequencing gel.

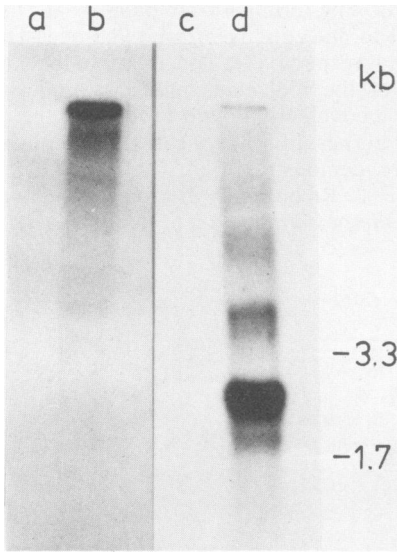

FIG. 7. Search for H-DNA specific transcripts in lytically infected OMK cells. Lanes: a, 5 μg of RNA from mock-infected cells hybridized with ^{32}P-labeled cloned H-DNA; b, 5 μg of total RNA from OMK cells after infection for 47 h, hybridized with cloned H-DNA; c, 5 μg of RNA from mock-infected cells hybridized with the cloned *Kpn*I-D fragment of L-DNA; d, 5 μg of RNA from infected cells (47 h) with the cloned *Kpn*I-D fragment of L-DNA.

features of herpesvirus saimiri M-DNA known from earlier studies (2, 11) also suggested that concatemeric forms of intermediates are synthesized during DNA replication. One would then expect monomers generated during or before encapsidation by a cleavage process that restricts the length according to a head-full mechanism. The experiments reported in this paper suggest that the H-DNA repeat unit of herpesvirus saimiri strain 11 contains a single DNA sequence providing the signal for DNA cleavage, and monomerization is achieved by an endonucleolytic activity similar to blunt-end-forming restriction endonucleases. It is in certain contrast to herpes simplex virus and varizella-zoster virus that were reported to possess 3'-single base extentions. A common feature of herpesviruses is the very high G+C content around the monomerization cleavage site (1, 7, 25, 32). We did not find striking sequence homologies between the extreme herpesvirus saimiri genome sequence and the corresponding sequences of herpes simplex virus, human cytomegalovirus and Epstein Barr virus. The Epstein Barr virus genome has, in addition to short terminal repeats, several arrays of tandemly repeated sequences (1). However, those sequences are internal, appear not to have cleavage signals, and are transcribed into mRNA with open translational reading frames (1). Thus, the extended tandem repeats of herpesvirus saimiri and the large internal repeats of Epstein Barr virus should not be regarded as functional equivalents.

Isolated intact L-DNA of herpesvirus saimiri generated by cleavage with *Sma*I, is not infectious in culture (18). This emphasizes that the repetitive DNA is necessary for virus replication. It is possible that the function of H-DNA is not more than to provide a signal sequence for monomerization-encapsidation. It is remarkable that very few functions for virus replication are provided by the H-DNA which amounts to one third of the entire (M) genome. This may reflect a certain trait of evolution where certain gene functions were lost, whereas the sizes of nucleocapsids were preserved and a certain minimum genome size remained to be required for encapsidation.

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