Primary Structure of the Alcelaphine Herpesvirus 1 Genome

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Alcelaphine herpesvirus 1 (AHV-1) causes wildebeest-associated malignant catarrhal fever, a lymphoproliferative syndrome in ungulate species other than the natural host. Based on biological properties and limited structural data, it has been classified as a member of the genus Rhadinovirus of the subfamily Gammaherpesvirinae. Here, we report on cloning and structural analysis of the complete genome of AHV-1 C500. The low GC content DNA (L-DNA) region of the genome consists of 130,608 bp with low (46.17%) GC content and marked suppression of CpG dinucleotide frequency. Like in herpesvirus saimiri, the prototype of the rhadinoviruses, the L-DNA is flanked by approximately 20 to 25 GC-rich (71.83%) high GC content DNA (H-DNA) repeats of 1,113 to 1,118 nucleotides. The analysis of the L-DNA sequence revealed 70 open reading frames (ORFs), 61 of which showed homology to other herpesviruses. The conserved ORFs are arranged in four blocks collinear to other Rhadinovirus genomes. These gene blocks are flanked by nonconserved regions containing ORFs without similarities to known herpesvirus genes. Notably, a spliced reading frame with a coding capacity for a 199-amino-acid protein is located in a position homologous to the transforming genes of herpesvirus saimiri at the left end of the L-DNA. A gene with homology to the semaphorin family is located adjacent to this. Despite common biological and epidemiological properties, AHV-1 differs significantly from herpesvirus saimiri with regard to cell homologous genes, probably using a different set of effector proteins to achieve a similar T-lymphocyte-transforming phenotype.

Malignant catarrhal fever (MCF) is a usually fatal disease in various ruminants. It occurs in two distinct epizootological forms: (i) wildebeest-associated MCF (WA-MCF), which is widespread in southern and eastern Africa; and (ii) sheep-associated MCF (SA-MCF), which has an almost worldwide distribution. The causative agent of WA-MCF has been isolated from asymptotically infected wild wildebeest (Connochaetes taurinus taurinus) (61). It was classified as alcelaphine herpesvirus 1 (AHV-1). Preliminary characterization has shown that AHV-1 should be included in the genus Rhadinovirus of the subfamily Gammaherpesvirinae (15). Closely related herpesviruses were isolated from several other species of genuine antelopes (68). Attempts to isolate the agent of SA-MCF were unsuccessful, but partial genomic sequences of a virus with a close relationship to AHV-1 have been characterized from lymphoblastoid cells of diseased cattle, deer, and rabbits (16). This agent has been designated ovine herpesvirus 2. The agent is readily demonstrable by serology (42) and PCR (9, 78) in samples from SA-MCF. The symptoms of both forms include fever, ocular and nasal discharge, corneal opacities, and diarrhea. The pathology of MCF is characterized by a combination of lymphoproliferation and degenerative symptoms in the affected animals. A certain variation of the predominant morphological changes has been noted between different affected species, from lymphosarcoma-like changes in deer (12) to vasculitis and necrosis predominating in other species (64). The lymphoid and lymphoblastoid cells present in MCF have been shown to be T lymphocytes of a CD8 or CD4 phenotype (17, 22, 48, 49, 77). MCF and the diseases caused by MCF have been shown to be T lymphocytes of a CD8 or CD4 different affected species, from lymphosarcoma-like changes in more than 80% of the cells. AHV-1 strains C500 (62) and WC11 (63) were serially propagated by infection of fresh MDBK cells with aliquots of infected MDBK cells showing cytopathic changes in more than 80% of the cells. AHV-1 C500 was obtained from H. W. Reid (Moredun Research Institute, Edinburgh, United Kingdom), and the attenuated WC11 strain was obtained from D. W. Verwoerd (Veterinary Research Institute, Onderstepoort, South Africa). For the preparation of virions, tissue culture supernatant was precipitated by centrifugation at 2,000 × g. Strain C500 virions (passage 5) were pelleted by centrifugation at 50,000 × g with an SW28 rotor. The viral DNA was extracted from the pellet with phenol-chloroform and precipitated with ethanol. WC11 DNA was further purified by density gradient centrifugation as described previously (37).

Cloning procedures. C500 DNA was restricted with BamHI, BamHI-SmaI, EcoRI, HindIII, PstI, NolI, SreI, and XbaI. The fragments were ligated into the respectively cut plasmids pBluescribe M13+ and pBluescript KSII+ (Stratagene, Cambridge, MA). These plasmids were used to transform E. coli strain XL1-Blue. Recombinant plasmids were isolated and used to infect H. W. Reid's MDBK cells. Plasmid DNA was extracted from infected cells and purified with QIAquick Plasmid isolation kit (Qiagen, Hilden, Germany). The purified DNA was sequenced with the dideoxy method (39) using the Sequenase 2.0 kit (United States Biochemical, Cleveland, OH).

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La Jolla, Calif.) and transformed into *Escherichia coli* K-12 DH5α (Life Technologies, Gaithersburg, Md.) by electroporation. Specific clones were identified by color selection with isopropyl-β-D-thiogalactopyranoside plus 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and a subsequent colony screening (6) with [α-32P]dATP-labelled (24), gradient-purified DNA of AHV-1 WC-11. The cloned fragments were mapped to the viral genome by Southern blotting (70) and sequencing of their ends. Clones containing terminal low GC content DNA (L-DNA) fragments were identified by hybridizing *Bam*HI-*Sma*I and *Hin*dIII clones with a radiolabelled WC11 repeat probe prepared from supermolar small fragments of *Sac*II-digested viral DNA. A fragment of 4 kb missing in the initial cloning procedure was amplified by PCR with specific primers and cloned in pBluescript KSII+.

Random subclones from suitable genomic clones were generated by sonication of inserts purified by preparative agarose gel electrophoresis (66). DNA from these shotgun clones was prepared either by modified alkaline lysis followed by precipitation with polyethylene glycol (Applied Biosystems, Foster City, Calif.) or by anion-exchange chromatography (Qiawell kit; Qiagen, Hilden, Germany).

**PCR applications.** PCR amplification of AHV-1 was done with 250 ng of DNA isolated from lytically infected MDBK cells. Fragments smaller than 2 kb were amplified with AmpliTaq (Perkin-Elmer, Weilderstadt, Germany); the missing 4-kb fragment (Fig. 1) was amplified with Vent-polymerase (New England Biolabs). Fragments larger than 2 kb and up to 8 kb were amplified with the Expand Long Template PCR System (Boehringer Mannheim). For the rapid amplification of cDNA ends (RACE), the Marathon kit (Clontech, Palo Alto, Calif.) was used. As starting material for RACE, poly(A)+ RNA from AHV-1-infected MDBK cells was isolated with ferromagnetic poly(dT) beads (Dynal, Hamburg, Germany).

**Nucleotide sequence determination.** DNA sequencing was performed with an ABI 373A automated sequencer and *Taq*-dye deoxy terminator chemistry (Applied Biosystems) in a combination of shotgun and primer-walking approaches (66). Random clones were sequenced with standard primers flanking the plasmid cloning sites. Regions of low redundancy and residual single-stranded regions were sequenced with virus-specific primers. All oligonucleotides were purchased from Eurogentec (Seraing, Belgium). The assembly of sequence readings was done with the program XBASE (21) on a Sparstation 10 (SUN Microsystems, Mountain View, Calif.). Oligonucleotides were selected with the program OSP (33) implemented in XBAP.

**Nucleotide and protein sequence analysis.** The GCG package (28) with the FASTA (60) and the BLAST programs (4, 29) was used for analysis of nucleotide and amino acid sequences and for comparison with GenBank and SwissProt databases (versions 97.0 and 34.0, respectively). Potential coding regions were identified with the program GENEMARK (13) using matrices for human and gammaherpesvirus DNA (kindly compiled by William Hayes, Georgia Institute of Technology, Atlanta). Putative signal sequences were analyzed with the SignalP server with a neuronal network trained on eukaryotic sequences (58).

**Nucleotide sequence accession number.** The complete L-DNA sequence is available from GenBank as AF005370. The different repeat sequences are available under accession no. AF005363 to AF005368. The right-terminal H-L junction sequence with rearranged high GC content DNA (H-DNA) is available as AF005508. The RACE-amplified cDNA sequences of open reading frames (ORFs) A2 and 57 are available as AF005369 and AF005362, respectively. The left-terminal region is also separately available in the database under accession no. U18243.

**RESULTS**

**Molecular cloning and sequencing of AHV-1 genome.** The positions of cloned fragments used for sequencing of the genome are shown in Fig. 1. Sequencing of numerous short stretches indicated that the AHV-1 genome is largely collinear to herpesvirus saimiri and that the previously published mapping of AHV-1 strain WC11 (14) used the opposite orientation of the viral genome with respect to herpesvirus saimiri. For
convenience, we have reversed the orientation of the genome and adapted the orientation of the L-DNA to the published genomes of herpesvirus saimiri (3), EHV-2 (73), and HHV-8 (51, 65). Altogether, 1,022 kb of raw data from 2,130 single readings were assembled into 130,608 bp of L-DNA sequence with an average redundancy of 7.83. The average content of GC nucleotides is 46.17%, with some significant local variations, particularly in the repetitive GC-rich regions. Like in herpesvirus saimiri, there is a marked suppression in the frequency of CpG dinucleotides. Junctions between adjacent, nonoverlapping restriction fragments were verified by direct sequencing of appropriate junction-spanning PCR fragments. The arrangement of regions represented by several smaller clones was also verified by PCR as indicated in Fig. 1.

Repetitive regions in the AHV-1 genome. A region of sequence heterogeneity was represented by clones c145H and c380P. A Southern blot showing multiple bands of genomic DNA suggested a repeat sequence of approximately 2 kb varying by ±500 bp. Random sequencing yielded 60 gel readings consisting exclusively of 29-bp repetitions, and there were only two types of junction sequences to the regular L-DNA. This indicated a uniform 29-bp GC-rich repeat region without other sequences interspersed. Because the exact number of repeats could not be determined, we have deliberately inserted a total of 59 units of this 29-bp element into the genome (positions 114100 to 115820 [R3] (Fig. 2). Each 29-bp unit contains an amber codon. Consequently, only a multiply spliced transcript from that region could possess coding potential. Several distinct repeats are localized within ORF73, as in the homologous genes of herpesvirus saimiri and HHV-8 (R4-6). All major repeats are shown in Fig. 2 (R1 to R7) and specified in the GenBank entry.

Eight independent H-DNA 1.1-kb SseI repeat units were cloned and sequenced. The C500 repeat sequence varies from 1,113 to 1,118 bp between the individual clones. Differences occurred at two positions in the H-DNA, either with a variable number of TG dinucleotides (position 125) or with eight or nine thymidines (position 1075). The GC content of H-DNA is 71.83%, and there is no obvious CpG suppression.

The coding capacity of the virion DNA. Potentially protein-coding ORFs in the L-DNA sequence were defined by criteria similar to those previously applied to other herpesvirus genomes (3, 53): (i) ORF size larger than 60 amino acids (aa), (ii) presence of potential transcriptional start sites and polyadenylation sites, (iii) high GENEMARK score (>0.5), and (iv) homology to previously described herpesvirus or other genes. The longest of several overlapping ORFs was considered significant. The identified potential ORFs are shown in Fig. 2 and Table 1. The nomenclature of ORFs was also adapted to other rhadinovirus sequences (3, 65, 73). Thus, ORFs with homologs in herpesvirus saimiri were assigned the number of the related herpesvirus saimiri gene. ORFs with no homolog in herpesvirus saimiri were consecutively numbered, beginning from the left end of the L-DNA (with the prefix A for alcelaphine).

Genes with homology to other herpesvirus genes and non-conserved genes. Conserved herpesvirus genes are arranged in four blocks collinear to herpesvirus saimiri (Fig. 2 and Table 1). Interspersed are ORFs without homology to known herpesvirus genes located in the nonconserved regions. The left-terminal region is comprised of four ORFs. The short ORF A1
The position of the respective ORF on the viral L-DNA is given from the first nucleotide of the first methionine codon to the last nucleotide of the stop codon.

Possible functions for the AHV-1 proteins are deduced from the homologous ORFs described in other herpesviruses (3, 7, 18, 44, 51, 53, 65, 73).

The homologous ORFs of selected nonmembers of the subfamily Gammaherpesvirinae are given from the first nucleotide of the first methionine codon to the last nucleotide of the stop codon.

### TABLE 1. AHV-1 ORFs and homologs to other herpesviruses

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### Additional Notes:
- Blocks of conserved herpesvirus genes are separated by spaces and indicated by Roman numerals.
- The first nucleotides of putative TATA boxes and polyadenylation signals (AATAAA or AATTTAA) are given, SD, splice donor; SA, splice acceptor.
- %id, percent identity of herpesvirus ORFs to the respective AHV-1 ORF calculated with the GAP program [GGC, version 9.0] according to the parameters gapweight = 4 and lengthweight = 12 [matrix BLOSUM62].
- ORFs in parentheses designate weak positional homologs or functional homologs at different positions on the viral genomes.
- The homologous ORFs of selected nonmembers of the subfamily Gammaherpesvirinae are provided for orientation. HCMV, human cytomegalovirus.
- Data for AHV-1 proteins are deduced from the homologous ORFs described in other herpesviruses (3, 7, 18, 44, 51, 53, 65, 73).
- After correction of a frameshift in the HVS sequence (G inserted after position 9917).
has a TATA box-like sequence upstream and a polyadenylation site downstream. ORF A2 may encode a 199-aa protein which has local similarity to the transcription factor ATF3 (19). ORF A3 is homologous to the semaphorin gene family (23). ORF A4 is located between the semaphorin and ORF 3 homologs, and it codes for a protein of 121 aa with a putative signal peptide cleavage site at position 19. The first conserved gene block consists of ORFs 6 to 11. ORF A5 is a homolog of...
Epstein-Barr virus (EBV) BILF1 and EHV-2 E6. It is followed by the weakly conserved ORFs 10 and 11. Analysis of the 7.6-kb region between ORFs 11 and 17 failed to detect any similarities to cellular genes or herpesvirus immediate-early genes described in other gammaherpesviruses (3, 52, 56, 65, 73, 75). The next conserved gene block includes ORFs 17 to 27. AHV-1 is the only gammaherpesvirus devoid of at least a positional equivalent to ORF 28. The third large block is comprised of ORFs 29b to 50. ORF 37 is a putative phosphotransferase containing a perfect tyrosine kinase motif, LxHxDxCxx-(L,I,V)3. ORF 48 lacks the repetitive region present in herpesvirus saimiri, and an ORF 49 homolog is absent. The putative R transactivator (RTA) homolog ORF 50 (BRLF1 in EBV) is only weakly conserved. ORF A6 codes for a protein with a size of 210 aa, and ORF A7 shows weak similarity to ORF 51 of herpesvirus saimiri and EHV-2, including a potential signal peptide cleavage site between the arginine and leucine residues at positions 19 and 20. ORF A8 is predicted to be a highly glycosylated protein of 683 aa. The following fourth gene block contains ORFs 52 to ORF 69. Between ORFs 67 and 68 is the short but significant ORF 67A, which was also described in EHV-2, human cytomegalovirus (UL51), HHV-6 and -7 (U35), and herpes simplex virus (HSV-1) 1 (UL33). The respective regions of EBV and HHV-8 also contain this ORF, but it was not described in the original publications (7, 65). In herpesvirus saimiri, resequencing of this region confirmed that this ORF was conserved among the herpesviruses (Table 1).

**DISCUSSION**

Preliminary characterization and partial cloning of the viral genome of the tissue culture-adapted AHV-1 strain WC11 have been reported (14, 15), suggesting that AHV-1 should be included in the genus *Rhadinovirus* of the subfamily *Gammaherpesvirinae*. We have cloned the complete viral genome of low-passage strain C500. The DNA sequence was unambiguously determined in all but one highly repetitive region, which does not affect the coding potential of the DNA. The H-DNA repeat sequence of strain C500 is represented by a 1.1-kb *Sst*I fragment, in contrast to strain WC11, in which two distinct repeat subunits have been described (15). Minor variations of the C500 repeat occur at two positions: (i) in the right terminus of L-DNA, a region of 5.4 kb contains two ORFs, A9 and A10. ORF A9 is homologous to the Bcl2 family of apoptosis-regulating proteins. It contains only the well-conserved Bcl2 homology (BH) region BH1, whereas BH2 is hardly recognizable. The predicted protein also shares a hydrophobic carboxy terminus with the other Bcl2 homologs. ORF A10 has the potential to encode a 472-aa glycoprotein. The transcripts for viral terminase ORF 29 and regulatory protein ORF 50 (31, 54, 76) homologs are spliced in other herpesviruses, and the locations of potential splice donor and acceptor sites were deduced by comparative sequence analysis (Fig. 2, Table 1). ORF 57 is also known to be encoded by a spliced message in EBV (55). A 193-bp 5′RACE amplify was directly sequenced and shown to encode the 5′ end of the spliced ORF 57 message (Fig. 3b and Table 1).

**FIG. 3.** RACE-amplified cDNAs from AHV-1 C500. The amplified cDNA sequences are shown in boldface letters on the genomic sequence. The numbering corresponds to the nucleotide positions on the genomic sequence and the amino acid residue numbers of putative translation products, respectively. Polyadenylation sites, translational start codons, and stop codons are indicated by capital letters. Positions of synthetic oligonucleotides used for RACE are shaded. (a) ORF A2. (b) ORF 57.
number of TG repeats and (ii) in the length of a stretch of thymidines. Sequences surrounding this second variable motif are reminiscent of the processing site for the viral terminase in herpesvirus saimiri H-DNA (71). Thus, the minor differences observed may reflect processing errors at viral genomic termini.

Homologs to 60 conserved herpesvirus ORFs are arranged collinear to herpesvirus saimiri (Table 1). The highly conserved ORFs 6 to 9 are followed by ORF A5, which has homologs in EBV BRLF1 and EHV-2 E6 at the respective positions, but which is missing in herpesvirus saimiri and HHV-8; this hints at the presence of this ORF in an ancestral gammaherpesvirus and subsequent loss in the primate rhadinoviruses. It may code for a protein with characteristics of G protein-coupled receptors (GPCRs) with seven putative transmembrane domains. GPCR-like ORFs are generally present in the nonconserved regions of gamma-2 herpesviruses; they may serve as modulators of the host immune response (2) or even candidate onco-genes by delivering constitutive activation signals to transformed cells (5).

The putative transcriptional regulators encoded by ORF 50 (BRLF1 in EBV) and ORF 57 (55, 57) are only weakly conserved, presumably reflecting the changes necessary in the process of adaption of regulatory genes to different host environments. This may also apply to ORF 73, which shares structural similarity to herpesvirus saimiri and HHV-8 ORF 73. A partial cDNA encompassing the 3’ end of ORF 73 terminates in the 29-bp repetitive region, and a fusion protein derived from this cDNA is recognized by antiserum from AHV-1-infected animals (40). The potential membrane protein with homology to herpesvirus saimiri ORF 75 and EBV BHRF1 has highly significant similarities to FGARAT (EC 6.3.5.3) (8), an enzyme catalyzing the fourth step in the de novo synthesis of purine bases. The human native protein is 150 kDa (74), in the same range as the respective EBV p140 and herpesvirus saimiri p160 proteins, thus supporting an analogous function for this protein. It is tempting to speculate on the therapeutic potential of produgs specifically metabolized by the viral proteins.

The nonconserved regions of most rhadinoviruses contain several homologs to cellular genes (51). These are thought to be responsible for modulating the host immune response to viral infection and for the establishment of lymphoid transformation. The ORFs detected in the left-terminal and other positions of AHV-1 show no sequence similarity to these reading frames. The positionally equivalent ORFs A1, A2, and A4 especially show no similarity to the herpesvirus saimiri transformation-associated proteins (10, 27, 47) and their presumed functions (11, 34, 35, 38, 46). The ORF A2 gene product is encoded by a spliced transcript and has a motif similar to nuclear localization signals and the basic domain of the stress-induced transcription factor ATF3 (19). ORF A3 is a gene with homology to the semaphorin family and a related gene of poxviruses (23). Semaphorins are a growing gene family of chemoeffectors and/or repulsive factors with important roles in neuronal and lymphocyte development. The human T-cell semaphorin CD100 was recently shown to augment CD40/CD40-ligand interactions (30). The region including ORFs A6, A7, and A8 is similar in its arrangement of genes to EBV. Consequently, ORF A6 may be a regulatory protein like the BZLF-1 gene of EBV (59), whereas the putative glycoprotein A8 may be a candidate for interaction with a host cell receptor for AHV-1. Its positional equivalent EBV gp220 mediates attachment to the B-cell EBV receptor (CD21/CR2, C3d receptor) (25).

ORF A9 likely encodes a protein of 168 aa with homology to the Bcl2 family of regulators of programmed cell death or apoptosis (69). Bcl2 homologs have been described for iridoviruses (1) and the gammaherpesviruses EBV (BHLF1) (32, 41), herpesvirus saimiri (ORF 16) (50, 69), HHV-8 (ORF 16) (20), and bovine herpesvirus 4 (ORF B2) (43). Although located at a different genomic position, ORF A9 is similar in size and contains a well-conserved BH1 region as well as a conserved hydrophobic carboxy terminus.

We found no ORFs or indications for putative exons in a region of 7.6 kb between the ORF 11 and 17 homologs and in the 7.3-kb region between ORFs 69 and 73. These regions contain homologs to cellular genes and regulatory genes in herpesvirus saimiri, HHV-8, and bovine herpesvirus 4. Large nonrepetitive regions of the viral genome that have no coding potential are unusual among the herpesviruses. Such regions occur only in EHV-2, in which large regions between ORFs 9 and E5, ORFs 13 and 17, and the region between the interleukin-10 homolog (E7) and the right terminus of the L-DNA display a relative paucity of genes. It appears that all rhadinovirus genes to the left of the conserved gene block ORFs 6 to 11 and those to the right of ORF 69 are to some extent derived from cellular homologs. Even the ORF 73 coding sequence has similarity to pyrimidine-rich regions found in various cellular genes, making cellular origin a possibility. The terminal regions of gammaherpesviruses are a hot spot for the integration of cellular genes, probably facilitated by the process of viral replication that originates in these areas of the genome (39, 67).

Thus, despite sharing many biological and epidemiological properties, AHV-1 differs from herpesvirus saimiri (and all other gammaherpesviruses) in the content of virus cell homologs, suggesting that different viral effector molecules achieve a similar phenotype. Taken together, the biological properties, genome structure, and organization of rhadinovirus ORFs confirm the classification of AHV-1 as a rhadinovirus.

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