Preliminary Characterization of the Alcelaphine Herpesvirus 1 Genome

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SUMMARY

Alcelaphine herpesvirus type 1 (AHV-1) is a causative agent of the fatal lymphoproliferative disease malignant catarrhal fever in deer and cattle. The genomes of the attenuated WC11 isolate and the virulent C500 isolate have been studied. The genome of WC11 comprises a region of unique DNA of approximately 130 kbp, which has a G+C content of 50%, and approximately 30 kbp of additional tandem direct repeat sequences with a G+C content of 72%. WC11 possesses a major repeat sequence of 950 bp interspersed with a small number of related sequences of different length; these sequences are probably terminal in location. DNA from the C500 isolate has a similar restriction profile to that of WC11 in the unique region, but only one repeat sequence of 1050 bp is present. We propose, on the basis of biological and structural properties, that AHV-1 be included within the γ2 group of herpesviruses of which herpesvirus ateles is the prototype.

INTRODUCTION

Alcelaphine herpesvirus type 1 (AHV-1) is a herpesvirus of the blue wildebeest, Connochaetes taurinus, which is an inapparent carrier of the virus. In free-living animals the virus spreads through ocular and nasal secretions (Mushi et al., 1980) such that all wildebeest calves over the age of 7 months are infected (Plowright, 1967). Infection of ruminants other than wildebeest leads to a fatal lymphoproliferative and degenerative disease known as malignant catarrhal fever (MCF) (Plowright et al., 1960; Plowright, 1968, 1982). Economic losses in Africa and in zoological parks can be severe. The disease is a world-wide phenomenon, which indicates that another agent must exist. Epidemiological evidence implicates sheep as a second carrier of infection. However, attempts to isolate a virus capable of inducing MCF from sheep or sheep agent-affected animals have failed repeatedly (Storz, 1976). Little characterization of AHV-1 has been reported to date, and we provide the first account of the genomic organization of this virus.

METHODS

Viral isolates. Experiments were generally performed using the attenuated isolate WC11 (Plowright et al., 1963) which produces cell-free virus. Two varieties of the virulent C500 isolate described by Plowright et al. (1975) were employed for comparative purposes: cell-associated C500, C500 ca, and a cell-free derivative, C500 cf.

Preparation of virion DNA. Virus particles were prepared as described by Herring et al. (1989). DNA was extracted by incubation in SDS-protease K followed by phenol and chloroform extractions. Viral DNA was concentrated by ethanol precipitation using mussel glycogen (Boehringer) as carrier.

Preparation of viral DNA from infected cells. In the single experiment in which viral DNA was prepared from the C500 ca virus isolate, susceptible cells (bovine kidney) were cocultivated with cells from the lymph nodes of a C500 ca-infected rabbit. Following the onset of c.p.e. cells were harvested, washed in phosphate-buffered saline and lysed by resuspension in 0.5% (v/v) NP40 in RSB (RSB is 10 mM-NaCl, 1 mM-MgCl2, 10 mM-Tris-HCl pH 7.5) using gentle homogenization. Chromatin and cellular debris were removed by centrifugation for 10 min at 800 g

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and 4 °C. The supernatant fluid was layered over 25% (w/v) sucrose in RSB and the nucleocapsids were pelleted by centrifugation at 114000 g for 30 min at 4 °C. Viral DNA was then prepared from the pellet using the selective extraction procedure of Hirt (1967). The supernatant fraction from this extraction was digested with proteinase K and phenol-extracted as described.

Source of other viral DNAs. We thank R. W. Honess for herpesvirus saimiri (HVS) DNA and clones, P. Medveczky for herpesvirus sylvilagus clones, R. Jarrett for DNA from cells infected with human herpesvirus 6 (HHV-6), J. Arrand for cloned Epstein–Barr virus (EBV) BamHI/W DNA and E. Thiry for bovine herpesvirus 4 (BHV-4) DNA.

Agarose gel electrophoresis. Electrophoresis was performed using gel strengths of 0.4 to 2% (w/v) agarose and TBE buffer (1 × TBE is 134 mM-Tris base, 44 mM-boric acid, 2.6 mM-EDTA). Fragments were transferred to nitrocellulose (Schleicher & Schuell) or nylon (Amersham Hybond) membranes by the bidirectional blotting method of Smith & Summers (1980). DNA fragments of greater than 20 kb were separated by orthogonal field-alteration gel electrophoresis (OFAGE; Carle & Olson, 1984).

Polyacrylamide gel electrophoresis. DNA fragments of up to 1000 bp were separated by continuous PAGE in Loening's E buffer; fragments of 200 to 4000 bp were separated by discontinuous PAGE in Laemmli's buffer. Gels were prepared according to McClanaghan et al. (1984).

Silver staining of polyacrylamide gels. Silver staining provided a sensitive means for the detection of DNA fragments. The method used was a slight modification of that described by Herring et al. (1982). Staining with silver nitrate was performed for 20 min only, and sodium borohydride was omitted from the development step. The intensity of stained fragments in polyacrylamide gels was assessed using a Joyce-Loebl Chromoscan 3 densitometer.

Radiolabelling of DNA. DNA was labelled by nick translation (Rigby et al., 1977) or by random hexanucleotide primer extension (Feinberg & Vogelstein, 1983, 1984) using [α-32P]dCTP at 300 Ci/mmol and 10 mCi/ml.

Hybridization of DNA. Filters were pre-hybridized in 4 × SSC, 5 × Denhardt's solution, 0.1% (w/v) SDS, 200 µg/ml sonicated salmon sperm DNA and 50% (v/v) formamide for at least 30 min at 37 °C, and hybridized in a similar solution with 1 × Denhardt's solution for 12 to 18 h at 37 °C. Filters were rinsed in 4 × SSC, and washed in 50% formamide, 4 × SSC and 1 × Denhardt's solution for 2 × 2 h at 37 °C, followed by 30 min washes in 1 × SSC and 0.5 × SSC at 37 °C. Hybridization to heterologous DNA was performed as above but at 42 °C, and a series of hybridizations in 20%, 30%, 40% and 50% (v/v) formamide were performed. Filters were rinsed in 4 × SSC, and washed twice for 2 h in 20% formamide, 4 × SSC and 1 × Denhardt's solution before autoradiography.

CsCl density gradient centrifugation. Five ml gradients with an initial density of 1.72 g/ml (57% w/v CsCl) were formed in TE buffer pH 7.5 (TE is 10 mM-Tris–HCl, 1 mM-EDTA). The A260 profile was measured by upward displacement of the gradient using an ISCO model 184 density gradient fractionator with a u.v. monitor. Fractions of 0.2 ml were collected and the density was determined by refractometry.

Molecular cloning of AHV-1. Two AHV-1 clones were used in the experiments described in this paper, R65 consists of an 18 kbp WC11 fragment cloned into λEMBL4. λEMBL4 DNA was restricted with EcoRI and then with BamHI to digest the central fragment of the vector. The small EcoRI–BamHI fragments were removed by isopropanol precipitation, and the vector arms were ligated to EcoRI-restricted AHV-1 DNA. Phage were recovered by packaging, and recombinant molecules were identified by hybridization to whole viral DNA.

M30 comprises the WC11 950 bp major repeat sequence cloned into M13mpl8. WC11 HindII fragments were separated by agarose gel electrophoresis. The 950 bp sequence was isolated and purified by the freeze–squeeze method of Thuring et al. (1973), then ligated to SmaI-restricted M13mpl8 replicative form DNA.

Preparation of λ phage. Packaging of λ DNA was performed according to the method of Grosveld et al. (1981). Packaged recombinant EMBL phage were chosen by their ability to plate on P2 lysogens (Kaiser & Murray, 1985). Phage liquid lysates were prepared as described by Murray et al. (1977), and phage were purified on a CsCl step gradient. Phage DNA was isolated by phenol extraction.

Preparation of M13 phage. M13 phage were propagated in host strain NM522. Growth and selection of phage were as described by Messing (1983). Replicative form DNA was prepared as described by Maniatis et al. (1982).

RESULTS

Base composition of the genome

Early studies of AHV-1 included preparative CsCl density gradient centrifugation of viral DNA performed as the last stage of genome purification. The absorbance profiles of the gradients revealed a major peak at a density of 1.709 and a minor but reproducible satellite component at a density of 1.730 g/ml (Fig. 1). These densities correspond to G + C contents of 50% and 72%, respectively (Schildkraut et al., 1962). Such base sequence heterogeneity is typical of gammaherpesviruses. Subsequent restriction analysis has confirmed that the dense satellite peak comprises repeat sequences (discussed below).
Characterization of the AHV-1 genome

Digestion of the AHV-1 genome with restriction endonucleases has been used to compare isolates of the virus, and to analyse the genome structure.

Comparison of the profiles produced by restriction endonuclease digestion of the DNA from the three available isolates was hindered by the difficulty of preparing sufficient DNA for agarose gel electrophoresis. This has necessitated the use of polyacrylamide gels and silver staining. Comparisons have been made using HindIII (data not shown), HindII and SacI (Fig. 2a); the results showed that C500 ca and cf are essentially identical (not shown) and that the genome of WC11 differs only slightly from these, particularly in the repeated sequences (discussed below).

Genomic DNA from the WC11 isolate has been analysed using a number of restriction endonucleases. Several enzymes produced supermolar fragments such as those shown in Fig. 3(a) and (b) for Smal. Similar supermolar fragments were seen using AluI, ApalI, AvaII, HaeIII, HhaI, HindII, HpalI, MspI and SacI. No such fragments were seen following digestion of WC11 DNA with BamHI, BglII, EcoRI, HindIII, KpnI, PstI, PvuII, SalI or XhoI. Supermolar fragments are a feature of genomes containing repeated sequences.

The supermolar fragments of C500 are much simpler than those of WC11. MboI, HindII and SacI all produced a single supermolar fragment with C500 cf and ca DNA (Fig. 2 and unpublished results). In contrast, HindII and SacI produced three clearly supermolar fragments with WC11 DNA, and these occurred at different levels of abundance, that is at non-molar ratios (Fig. 2a). The fragments shown in Fig. 2(a) were separated by PAGE using a discontinuous buffer system. This system maximizes fragment resolution, but has been shown to give aberrant size estimates. More reliable estimates were provided by agarose gel electrophoresis and continuous PAGE, which indicated a size of 1050 bp for the single supermolar fragment of C500 cf, and 1800, 1050 and 950 bp for the three WC11 supermolar fragments. This anomaly in size estimation probably results from an unusually high G + C content of the supermolar fragments.

The difference in abundance of the different WC11 repeat sequences can also be seen in Fig. 3(b), which shows three major repeat Smal fragments with mobilities corresponding to 165, 275 and 525 bp, and minor fragments with mobilities of 285, 360 and 430 bp. The 950 bp HindII repeat sequence from WC11 has been cloned into M13mp18 (clone M30); hybridization
experiments using this clone showed that all the supermolar fragments of C500 and WC11 produced by HindII, SacI or SmaI digestion hybridize with the cloned sequence. Restriction analysis of M30 using SmaI showed the insert to consist of the 165, 275 and 525 bp fragments as expected (with the HindII site within the 525 bp fragment). Direct SmaI analysis of C500 cf DNA showed the 1050 bp repeat to consist of fragments of 165, 275, 285 and 360 bp (data not shown). The two additional fragments appear as minor fragments in the WC11 repeats (Fig. 3b) suggesting that the C500 and WC11 HindII and SacI 1050 bp fragments are equivalent. The 1800 bp WC11 HindII fragment has been shown to contain a 430 bp SmaI fragment (data not shown), thus accounting for all the minor WC11 SmaI fragments.

Evidence that these repeat sequences reside in the genome as tandemly arranged blocks of various lengths was provided by the following observations. Digestion of WC11 and C500 ca DNA with enzymes such as HindIII or XhoI which do not cut in the repeat sequences, followed by hybridization using the repeat sequence probe M30 showed homology to large fragments of indistinct resolution. Hybridization to EcoRI digests showed a ladder of fragments with homology to M30 ranging in size from 8 to 20 kbp with a periodicity of 950 bp. A complex ladder was revealed when WC11 DNA was digested with MboI and probed with M30 (Fig. 4a). This figure resolves at least 15 sets of fragments with a periodicity of 950 bp. Above 3 kbp the
Characterization of the AHV-1 genome

fragments became increasingly less resolved as their size increases; between 3 kbp and the 1050 bp repeat fragment the ladder was well resolved.

A complex ladder of well defined fragments was observed when WC11 DNA was digested with HindIII or SacI and probed with M30 (Fig. 2b and 4b). The MboI ladder was also obtained on restriction of the $\rho = 1.730$ fraction from the CsCl density gradient and from digestion of the indistinct large HindIII fragments of WC11 isolated from an agarose gel (data not shown).

Digestion patterns using the restriction endonucleases MspI and HpaII were identical, indicating that there is no significant methylation of cytosine at CpG dinucleotides.

No single restriction endonuclease has been found to give a profile suitable for genomic size estimation. The most informative profiles are provided by EcoRI, XhoI and SmaI. Restriction
Fig. 4. *MboI* repeat ladders. (a) Autoradiogram showing WC11 DNA digested with *MboI*, separated on 1% agarose and probed with the repeat sequence clone M30. (b) Autoradiogram showing WC11 DNA digested with *SacI* (lane 1), *HindII* (lane 2) or *MboI* (lanes 3 and 4), separated on 1.2% agarose and probed with M30. The 1050 bp repeat sequence common to both isolates is arrowed.

digestion of WC11 DNA with *EcoRI* produced fragments of 1.5, 3.5, 4.8, 5.2, 23 and two of 50 kbp (with the 50 kbp fragments visualized by OFAGE) in addition to the 8 to 20 kbp variable fragment associated with the repeat DNA. Thus the *EcoRI* fragments summate to approximately 150 kbp. Accurate sizes for the *SmaI* and *XhoI* fragments have not been obtained because of the difficulty of preparing sufficient viral DNA for analysis by agarose gel electrophoresis. The *SmaI* fragments summated to approximately 155 to 165 kbp, depending on the exact number of repeat sequences, and the *XhoI* fragments to approximately 160 kbp.
Characterization of the AHV-1 genome

The number of repeat sequences can be estimated from densitometry or the analysis of the MboI ladders. Densitometric scanning of silver-stained polyacrylamide gels has yielded estimates of genome copy numbers of 20 to 25 for the single SacI/HindIII repeat of C500. The WC11 isolate produced supermolar fragments at two abundances; the 950 bp SacI/HindIII fragment was present at 15 to 20 copies per genome and the 1800 and 1050 bp SacI/HindIII fragments were each present at 4 or 5 copies per genome.

Taking all these data into account it is likely, therefore, that the genome size is of the order of 160 kbp.

Homology to other herpesviruses

Two AHV-1 DNA clones were used to assess the homology of AHV-1 to a range of herpesviruses. These were M30, the WC11 950 bp HindIII repeat clone, and the unique region clone R65. Southern blot analysis suggests that R65 represents a truncated form of the 23 kbp EcoRI fragment. The two AHV-1 clones were used to probe clones of herpesvirus sylvilagus derived from both the unique and repeated regions of the genome, genomic DNA from HVS and BHV-1 (infectious bovine rhinotracheitis virus), HHV-6-infected cellular DNA, cloned BamHI fragment W of EBV, and DNA from a Belgian isolate of BHV-4, V/Test strain.

M30 labelled by nick translation failed to hybridize to EBV BamHI W, but hybridized to a 2-6 kbp EcoRI fragment of BHV-4 under conditions of low stringency. R65 labelled by primer extension did not show homology with HHV-6-infected cellular DNA restricted with HindIII or cloned herpesvirus sylvilagus DNA at low hybridization stringency. There was clear homology at medium stringency of hybridization to the 11.25 kbp MspI C fragment of HVS (data not shown). There was far weaker hybridization to BHV-1 DNA under the same conditions. BHV-1 is an alphaherpesvirus with a G + C content of 71% (Goodheart & Plummer, 1975), which is considerably higher than the G + C content of the unique DNA of HVS.

DISCUSSION

A method of classification of the herpesviruses into alpha, beta and gamma subfamilies on the basis of biological properties has been described by Roizman (1982). Gammaherpesviruses are lymphotropic, and may be further subdivided into B-lymphotropic or γ1, for example EBV, and T-lymphotropic or γ2 herpesviruses, for example HVS (Honess, 1984). The γ2 herpesviruses HVS, herpesvirus ateleis, (HVA), herpesvirus aotus type 2 and herpesvirus sylvilagus possess a similar genomic organization, comprising one unique region of DNA bounded by terminal tandem direct repeats of high G + C DNA (Stamminger et al., 1987; Fleckenstein et al., 1978; Fuchs et al., 1985; P. Medveczky, personal communication). In HVS, the total number of repeat sequences is constant, but the number present at either terminus varies from 1 to 33 (Stamminger et al., 1987).

The γ2 herpesviruses also share numerous biological properties. By definition, all members of the group are T-lymphotropic. Herpesvirus sylvilagus, however, infects both T and B lymphocytes in vivo (Kramp et al., 1985). Lymphoblastoid cell lines with the characteristics of large granular lymphocytes have been cultured from animals affected with HVS-induced disease (Ortaldo et al., 1985). Lymphoblastoid cells of this type have also been cultured from rabbits and rats experimentally infected with AHV-1 (Reid et al., 1984; Jacoby et al., 1988), suggesting that these cells may also be important target cells for this virus. It has been suggested that dysfunction of this cell type following infection is responsible for the pathological changes observed in MCF (Reid & Buxton, 1984). Numerous authors have commented upon the similarity of the pathology of AHV-1 to that of the T-lymphotropic gammaherpesviruses (Hunt & Billups, 1979; Patel & Edington, 1980, 1981; Plowright, 1982). The major difference lies in the essentially neoplastic response of animals reacting with HVS or HVA, as compared with the response in MCF-affected animals which is both lymphoproliferative and degenerative. However, rats experimentally infected with AHV-1 develop lesions resembling neoplasia (Jacoby et al., 1988).

Restriction data on the virus have been published in only two reports: Ludwig (1983) and Osorio et al. (1985). These papers include restriction profiles for several enzymes, but make no observations concerning genome organization. Osorio et al. found marked differences in
Table 1. *Mole percentage G + C*^*

<table>
<thead>
<tr>
<th>Herpesvirus</th>
<th>Unique DNA</th>
<th>Repeat DNA</th>
<th>Whole genome</th>
</tr>
</thead>
<tbody>
<tr>
<td>saimiri</td>
<td>35.8</td>
<td>70.6</td>
<td>45.4</td>
</tr>
<tr>
<td>ateles</td>
<td>37.5</td>
<td>74.6</td>
<td>47.1</td>
</tr>
<tr>
<td>aotus type 2</td>
<td>40.2</td>
<td>68.7</td>
<td>48.1</td>
</tr>
<tr>
<td>AHV-1</td>
<td>~</td>
<td>72</td>
<td>50</td>
</tr>
</tbody>
</table>

* Adapted from Fleckenstein & Mulder (1980).

Table 2. *Physical properties of γ2 herpesvirus genomes*

<table>
<thead>
<tr>
<th>Herpesvirus</th>
<th>Genome size (kbp)</th>
<th>Repeat size (bp)</th>
<th>Repeat number</th>
</tr>
</thead>
<tbody>
<tr>
<td>saimiri</td>
<td>153-161</td>
<td>1444</td>
<td>30-34</td>
</tr>
<tr>
<td>ateles</td>
<td>106</td>
<td>1600</td>
<td>15-20</td>
</tr>
<tr>
<td>aotus type 2</td>
<td>151</td>
<td>1400, 2700 related</td>
<td>4-5, 20-25</td>
</tr>
<tr>
<td>sylvilagus</td>
<td>108-129</td>
<td>500</td>
<td></td>
</tr>
<tr>
<td>AHV-1 (WC11 isolate)</td>
<td>155-165</td>
<td>1800, 1050 related</td>
<td>4-5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>950</td>
<td>15-20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1050</td>
<td>20-25</td>
</tr>
</tbody>
</table>

* References as in text and Cohrs & Rouhandeh (1987).

...restriction profiles between WC11 and a virus, isolated from an Indian gaur, which also causes MCF (Oklahoma strain). It seems likely therefore that WC11 and the Oklahoma virus represent distinct viruses.

The results presented here provide the first molecular evidence for inclusion of AHV-1 within the γ2 herpesvirus group. The physical properties of the AHV-1 genome are compared with those of the characterized members of this subfamily in Tables 1 and 2, and are shown to be very similar.

Several of the results require further clarification. It cannot be discounted that the virus preparations included defective particles, containing almost entirely high G + C repeat DNA. Such particles have been called H genomes (heavy genomes) and occur with HVS preparations (Fleckenstein et al., 1975). Fig. 1 shows a density profile of WC11 DNA which was not deliberately sheared. It was assumed that a low level of accidental shearing of the DNA gave rise to the small peak corresponding to the high G + C DNA, but the presence of H genomes provides another explanation. It seems very likely that the repeated sequences are terminal as with the other γ2 herpesviruses. However, attempts to establish the terminal location of the repeated sequences by digestion of WC11 DNA with the exonuclease Bal 31 have consistently failed; this could be explained by the presence of H genomes.

The organization of the repeated sequences in the WC11 isolate is clearly complex. The hybridization results produced with M30 following MboI, HindIII and SacI digestion are consistent with the existence of a number of divergent versions of the basic repeat sequence interspersed within blocks of this major 950 bp repeat. This is a similar situation to that observed in herpesvirus aotus type 2 (Fuchs et al., 1985).

Epidemiological evidence indicates that sheep carry an agent capable of inducing MCF in other ungulates. This is supported by immunological evidence (Rossiter, 1981; Herring et al., 1989; H. W. Reid, unpublished results). Preliminary experiments using cloned WC11 unique region DNA as hybridization probes for a related agent in lymphoblastoid cell lines derived from deer and cattle putatively infected with the sheep agent have yielded positive results. We are currently characterizing the homologous sequences.

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