Structure and function of the prDNA and the genomic termini of the γ2-herpesvirus bovine herpesvirus type 4

Hermann Broll,† Hans-Jörg Buhk,1 Wolfgang Zimmermann2 and Michael Goltz1

1 Robert Koch-Institut, Nordufer 20, 13353 Berlin, Germany
2 Institut für Molekulare Biotechnologie, Abteilung für Genomanalyse, 07745 Jena, Germany

The linear virion DNA of bovine herpesvirus type 4 (BHV-4) is flanked by tandem repeats designated polyrepetitive DNA (prDNA). To investigate the structure and functional role of the prDNA for cleavage/packaging of progeny viral DNA, the complete nucleotide sequence (2267 bp) of a cloned prDNA unit of BHV-4 was determined. Moreover, the terminal fragments of the genome and the junctions between prDNA and the central unique DNA were analysed. In order to characterize the function of the prDNA of BHV-4, a transient packaging assay was developed. The prDNA has a G+C content of 71.1%. Its structure is composed of numerous internal repeats and every unit contains the conserved sequence of the cleavage/packaging signal. A fragment of 443 bp comprising the cleavage/packaging signal was found to be sufficient for cleavage and encapsidation of replicated concatemeric viral DNA. These results suggest that prDNA is a functionally important region of the genome of BHV-4.

Introduction

Bovine herpesvirus type 4 (BHV-4) has been classified as a member of the subfamily Gammaherpesvirinae due to its similarity in genome organization and other molecular features such as collinear gene arrangement with herpesvirus saimiri (HVS), Kaposis’s sarcoma-associated herpesvirus (HHV-8) and Epstein–Barr virus (Bublot et al., 1992).

BHV-4 has a B-type genome organization (Roizman, 1996). Its linear, double-stranded DNA of 144 ± 6 kbp consists of a long central unique part (~110 kbp) which is flanked by two stretches of tandem repeats with high G+C content (Ehlers et al., 1985) (Fig. 1). These stretches have been designated polyrepetitive DNA (prDNA). Using a prDNA unit as a molecular probe, we have (i) identified the genomic termini of BHV-4 as well as restriction fragments representing the junctions between prDNA and unique DNA and (ii) demonstrated that the frequencies of prDNA units at both genomic termini vary between individual molecules of a strain (Ehlers et al., 1985). For the investigated BHV-4 strain 66-p-347 (Storz, 1968), the overall number of prDNA units was estimated to be 15 on average. It was further shown that size and restriction maps of prDNA units of different BHV-4 strains vary according to the number of internal repeats within these units. The size of prDNA units is in general strain-specific; however, small fractions may vary within one strain (Bublot et al., 1990).

In vitro studies showed that the lytic cycle of herpesviruses is initiated by fusion of the termini of the linear viral genome. The resulting monomeric DNA circles probably serve as templates for a rolling circle type of DNA replication (Skaliter et al., 1996). The resulting concatemeric genomes are then monomerized through a specific cleavage step and packaged into preformed capsids (the cleavage/packaging signal) (Roizman & Sears, 1996).

In herpes simplex virus type 1 (HSV-1), cleavage occurs at the a sequence, which contains well-conserved cis-acting signals. These signals, designated pac-1 and pac-2, are responsible for cleavage and packaging of HSV-1 virion DNA and were detected at the genomic termini of all herpesviruses (Deiss et al., 1986; Roizman & Sears, 1996; Spaete & Frenkel, 1985). The high degree of conservation of the pac signals indicates a similar mechanism for the cleavage/packaging process for nearly all herpesviruses.

In this study, we have determined the nucleotide sequences of a prDNA unit and the prDNA borders of BHV-4. Furthermore, a functional assay has been established to assess the role of prDNA in the cleavage/packaging process.
Methods

- **Cells and virus growth.** BHV-4 strain 66-p-347 was propagated in Georgia bovine kidney (GBK) cells and BHV-4 particles were prepared from the supernatant of infected cells. Virus DNA isolation has been described previously (Ehlers et al., 1985).

- **Cloning of complete prDNA units.** Virion DNA was digested with EcoRI and Eagl, respectively, and separated on agarose gels. EcoRI and Eagl cut only once within a prDNA unit. Fragments representing complete prDNA units were isolated and ligated into the vectors pUC19 or pBluescript SK(+) (+). The resulting clones representing the sequence of one complete prDNA unit were used to generate subclones with a variety of restriction enzymes or with the Double-Stranded Nested Deletion kit (Amersham Pharmacia Biotech).

- **Cloning of junction fragments and genome termini.** Virion DNA was digested with BamHI or NaeI. According to previous data (Ehlers et al., 1985), BamHI fragments representing the junctions between prDNA and unique DNA and NaeI fragments representing the genome termini were isolated from preparative agarose gels. The BamHI junction fragments were ligated to BamHI-digested pUC19. One aliquot of the purified NaeI-terminal fragments was cloned directly into EcoRV-digested pBluescript SK(+). Another aliquot was treated with T4 DNA polymerase prior to ligation (Sambrook et al., 1989). In brief, DNA was incubated with 3 U T4 DNA polymerase in reaction buffer supplied by the manufacturer (NEB) and 0.5 mM dNTP for 15 min at 12 °C.

- **Nucleic acid sequence determination.** The nucleotide sequence of the junction elements between unique and prDNA and the entire DNA sequence was determined by a modified dideoxynucleotide termination method (Sanger et al., 1977) using the Prism Ready Reaction Dye Terminator Cycle Sequencing kit (PE Applied Biosystems).

<table>
<thead>
<tr>
<th>Plasmid construct</th>
<th>BHV-4 prDNA*</th>
<th>BHV-4 DNA origin of replication</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHB880</td>
<td>1 prDNA unit (EcoRI–EcoRI)</td>
<td>+</td>
</tr>
<tr>
<td>pHB881</td>
<td>2 prDNA units (EcoRI–EcoRI)</td>
<td>+</td>
</tr>
<tr>
<td>pHB923</td>
<td>443 bp (PstI–PstI), including the pac-1 and pac-2 sites</td>
<td>+</td>
</tr>
<tr>
<td>pHB922</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>pHB858</td>
<td>1 prDNA unit (EcoRI–EcoRI)</td>
<td>—</td>
</tr>
<tr>
<td>pHB988</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>pHB561</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* For cloning, the single EcoRI site at position 226 in a prDNA unit was chosen. Therefore, the putative cleavage/packaging signal is included in every prDNA unit. Position 1 coincides with the first prDNA nucleotide in the right junction between unique and prDNA.
The genomic termini of BHV-4

The genomic termini of BHV-4

Fig. 2. Reference sequence of a prDNA unit of BHV-4. Position 1 corresponds to the first prDNA bp in the right junction between unique and prDNA. The 25 bp repeats spanning the point of cleavage are underlined. The 196/197 bp repeats essentially responsible for size variation of prDNA units are underlined with arrows. ∆ indicates a truncated repeat.

Sequencing reactions were analysed with automated DNA sequencers (PE Applied Biosystems). This technique was also used for sequencing of the genomic termini. Sequence data were assembled with the Auto-Assembler software (PE Applied Biosystems) and further analysed with MacVector (Oxford Molecular Group) and the HUSAR GCG software package (Devereux et al., 1984; Pearson & Lipman, 1988).

All sequence data were verified by the results of shotgun sequencing of the complete BHV-4 genome, again using dye terminator chemistry and automated sequencers (W. Zimmermann and others, unpublished data).

Construction of amplicon plasmids. Amplicon plasmids pH880 and pH881 were constructed in three steps. BHV-4 DNA was digested with HindIII, which does not cleave within prDNA. Restriction fragments representing prDNA were purified from agarose gels. Subsequently, isolated terminal BHV-4 fragments (500 ng) were partially digested with 0-1 U EcoRI for 10 min and separated by agarose gel electrophoresis. Since EcoRI cleaves only once in a single prDNA unit, fragments with different numbers of prDNA units were generated. DNA fragments representing 1 or 2 prDNA units were ligated into an EcoRI-cut pUC18 vector plasmid to generate plasmids pH858 and pH859, respectively. Analysis of BamHI restriction digests of these plasmids revealed that pH858 contains the prDNA in the opposite orientation compared to pH859. Plasmid pH859, bearing the ori of BHV-4 (M. Goltz, unpublished data), was constructed by ligating a 5-0 kbp BamHI-BamHI fragment of BHV-4 into a BamHI-cut pBluescript SK(+) vector plasmid. Amplicon plasmids pH880 and pH881 were generated by HindIII and SacI digestion of the ori-carrying plasmid pH859, followed by ligation of the resulting 5-0 kbp fragment into HindIII-SacI cut pH858 and pH859, respectively.

Amplicon pH923 was generated in a similar way to pH880 and pH881. Plasmid pH921 was constructed by cloning a 443 bp PstI-PstI fragment spanning the conserved jac-1 and jac-2 sites of a single prDNA unit in PstI-cut pUC18. From pH921, a 2-5 kbp EcoRV fragment was deleted to generate pH922. The resulting 2-5 kbp EcoRV-BamHI
fragment is sufficient for replication of BHV-4 (M. Goltz, personal communication). Amplicon plasmid pH923 was obtained by ligation of a 0.45 kbp KpnI–HindIII fragment from pH921 into the KpnI/HindIII-digested ori-carrying plasmid pH922.

Plasmid pH988 was constructed by subcloning the 3.0 kbp EcoRI K fragment of BHV-4 into pBluescript SK(+) in addition, pHB561 consists of the BamHI fragments G, N and F of BHV-4 subcloned in pUC18 (Fig. 1). The features of all plasmid constructs are summarized in Table 1.

**Transient packaging assay.** GBK cells (2 × 10^5) were transfected with 10 µg plasmid DNA by electroporation (Bio-Rad). Two transfections were combined in one 145 mm diameter tissue-culture dish and resuspended in 25 ml DMEM containing 10% foetal calf serum. At 24 h post-transfection, cells were superinfected with BHV-4 at an m.o.i. of 1 p.f.u. per cell. Four to five days after infection, the progeny viruses were prepared as described previously (Storz et al., 1984). To remove cellular nucleic acids, the virion pellet was resuspended in 0.5 ml DNase I buffer (40 mM Tris–HCl, 6 mM MgCl₂) and 35 U RNase A (5 µg/ml) were added. The mixture was incubated for 1 h at 37°C. To terminate the DNase I digestion, proteinase K (0.1 mg/ml) and SDS (1%) were added, and the mixture was incubated for an additional 2 h at 60°C. After treatment with phenol, the DNA was precipitated and resuspended in TE buffer (10 mM Tris–HCl, pH 8.3, 0.1 mM EDTA). Viral DNA was cleaved with DpnI and BamHI. DpnI was added to remove any non-replicated bacterial input DNA. BamHI was chosen to resolve the concatemeric structure of test plasmids isolated from viral capsids. The restriction fragments were subjected to agarose gel electrophoresis and blotted onto a nylon membrane (Boehringer Mannheim). Hybridization was performed with a DIG-labelled DNA probe that detected the bacterial plasmid portion of the constructs. Labelling was carried out with the PCR DIG labelling kit (Boehringer Mannheim) with primers comprising part of the β-lactamase gene of pUC18, resulting in a 432 bp amplified fragment in PCR.

Visualization was performed by chemiluminescence according to the manufacturer’s instruction (Boehringer Mannheim).

**Results and Discussion**

**Sequence of a BHV-4 prDNA unit**

The nucleotide sequence of a prDNA unit of the major fraction in strain 66-p-347 and the position of the large internal repeats are shown in Fig. 2 (GenBank accession no. AF092919). Its size is 2267 bp with a G+C content of 71.1%. For comparison, a H-DNA unit of the γ# reference virus, HVS strain 11, consists of 1444 bp with a G+C content of 70.8% (Bankier et al., 1985). A prDNA unit contains three complete and one incomplete direct repeat of 196–197 bp as well as two 25 bp direct repeats separated by an AGA or GGC motif. One of the 18 clones analysed contained three of these 25 bp repeats, separated by one AGA and one GGC motif. Open reading frame analysis with subsequent database analysis of the predicted proteins did not show any significant homologies to known proteins. This result is in agreement with results of Chang & Van Santen (1992), who did not detect transcripts from the prDNA of BHV-4.

**Characterization of genomic termini and junction fragments**

To characterize the borders of prDNA, we isolated the junction and terminal fragments after digestion of viral DNA with BamHI and NaeI, respectively, as described in Methods.
The genomic termini of BHV-4

Fig. 4. Alignment of the putative pac-1 and pac-2 sequences. (a) pac-1 signals and (b) pac-2 signals of BHV-4 are shown in comparison to 12 representatives of the α-, β- and γ-herpesviruses (EBV, Epstein–Barr virus; HHV-8, Kaposi’s sarcoma-associated herpesvirus; HHV-6 and -7, human herpesvirus type 6 and 7, respectively; HVS, herpesvirus saimiri; HCMV, human cytomegalovirus; MCMV, murine cytomegalovirus; HSV-1, herpes simplex virus type 1; VZV, varicella-zoster virus; PRV, pseudorabies virus; BHV-1, bovine herpesvirus type 1; EH-1, equine herpesvirus type 1). The sequences are presented 5’ to 3’. Conserved motifs in pac-1 and pac-2 are shadowed in boxes. The positions of pac-1 and pac-2 signals are specified for every herpesvirus. An asterisk indicates unknown genomic end structures. Interestingly, in the pac-2 sequence of HCMV strain AD169, no T-motif could be identified.

The junction fragments were ligated into BsmHI-digested pUC19, cloned and sequenced. In order to determine if the termini consist of blunt or sticky ends, two cloning assays were performed. For both assays, the vector was cleaved with EcoRV, resulting in blunt ends. As mentioned above, terminal fragments were generated by digestion of viral DNA with the restriction enzyme NaeI, which also generates blunt ends.

Subsequently, one aliquot of the prepared terminal fragments was treated with T4 DNA polymerase prior to ligation with the vector. Another aliquot of the purified genomic termini was cloned directly into EcoRV-digested pBluescript SK(+) 

If the genomic termini of BHV-4 have a blunt-end structure, both cloning assays should be successful. If, on the other hand, the genomic termini consist of sticky ends, efficient cloning should be expected only after T4 DNA polymerase treatment of the genomic termini. No significant differences in the cloning efficiency between these two assays could be detected, suggesting a blunt-end structure for the genomic termini.

To investigate the right genomic terminus, 31 clones were analysed by sequencing. In 16 clones with untreated inserts and 14 clones with T4 DNA polymerase-treated inserts, the sequences ended with the 25 bp repeat, separating AGA or GGC motifs followed by the initial C of the next 25 bp repeat (Fig. 3). One insert, derived from the T4 DNA polymerase treatment, ended with GGC only and might be considered a cloning artefact.

For characterization of the left genomic terminus, 45 clones were sequenced. The sequences ended with 5–6 C nucleotides of the C stretch of the 25 bp repeats. The distribution was as
follows: C<sub>i</sub> in 16 untreated and 26 T4 DNA polymerase-treated inserts; and C<sub>e</sub> in one untreated and two T4 DNA polymerase-treated inserts.

The results of the analysis of the genomic termini and the junctions between prDNA and unique DNA of BHV-4 are summarized in Fig. 3. The sequences of the termini were arranged to simulate joined termini of a circular BHV-4 genome and aligned to a corresponding internal prDNA– prDNA junction. The sequence of the termini in a joined condition shows a 1 bp deletion (42 out of 45 clones) or no deletion (3 out of 45 clones) when compared to the internal junction.

The missing base pair could be explained as 3′ overhangs of one base at each of the two termini, removed by the T4 DNA polymerase treatment. Such overhangs are described for some herpesviruses (Chowdhury et al., 1990; Davison, 1984; Hammerschmidt et al., 1988; Mocarski & Roizman, 1982; Tamashiro & Spector, 1986). But this is contradictory to the finding that ligating untreated termini of BHV-4 was as efficient as ligating T4 DNA polymerase-treated termini indicating blunt-ended termini as mentioned above.

Combining these results, we were able to exactly map the position of the genomic termini resulting from the monomerization/linearization process following virus replication. Although the results indicate a single base 3′ overhang, the detailed structure of the genomic termini, however, remains unclear.

One prDNA unit is sufficient for cleavage and packaging

As a result of sequencing of one prDNA unit, two well-conserved cis-acting signals for the cleavage/packaging process of herpesvirus DNA were identified. These signals, designated pac-1 and pac-2 sequences of BHV-4, were compared with those of some other herpesviruses (Fig. 4).

Although the overall homology between different herpesvirus pac-1 sites is low, the presence of stretches of both C and G nucleotides at defined distances from the viral genome termini is well-conserved among herpesviruses (Fig. 4a). In the pac-2 site, a stretch of T nucleotides 27–33 nt away from the termini and a G-C-rich region are common motifs among all herpesviruses (Fig. 4b). It has been suggested that a putative herpesvirus-encoded terminase cleaves the concatemeric genomes of herpesviruses at a defined distance from these cleavage/packaging signals, as observed in the cleavage/packaging mechanism described for bacteriophages (Baines et al., 1994; Black, 1989; Davison, 1992).

As with HHV-8 and HVS, the putative pac-1 site within the prDNA of BHV-4 is located near the left end of the genome, whereas the putative pac-2 site is located near the right end.

To identify the cis-acting signals essential for cleavage/packaging of BHV-4 DNA, a transient cleavage/packaging assay was developed that offered the possibility of testing cleavage and packaging of recombinant plasmid DNA in the
The genomic termini of BHV-4

Fig. 6. Results of the transient cleavage/packaging assay with plasmid constructs pHB923, pHB881 and pHB880. Autoradiography of the blot is shown. After transfection with plasmids, GBK cells were infected with BHV-4 (left side of the blot). DNA isolated from the resulting virion particles was digested with BamHI and DpnI, separated by gel electrophoresis, blotted to nylon membranes and hybridized as described in the Methods. Additionally, pHB880 was analysed in the same way but without enzyme digestion (lane pHB880*). DNA isolated from virion particles after infection of cells transfected without DNA was also analysed (mock lane). As size markers, BamHI-digested plasmids are shown on the right side of the blot. In contrast to the negative-control plasmids pHB858, pHB561, pHB988 and pUC18, only plasmid constructs bearing both the ori and the cleavage/packaging site were efficiently packaged into viral capsids. Without enzyme digestion prior to gel electrophoresis, the concatemeric shape of the plasmid construct pHB880 is demonstrated due to the reduced electrophoretic mobility (lane pHB880*).

A prDNA region of 443 bp contains the cis-acting signals essential for cleavage and packaging

To map the cleavage/packaging signals within a prDNA unit, we constructed an amplicon with a 443 bp PstI–PstI fragment bearing the junction between two prDNA units with the putative pac-1 and pac-2 sites. This construct was tested for cleavage and packaging. As shown in Fig. 6, pHB923 was encapsidated and therefore contains all cis-acting signals vector DNA. Therefore, only internal fragments of 2·90 kbp and no terminal fragments were detected with a labelled vector probe used in this assay (Fig. 5). As expected, no fragments were detected with plasmids pHB858, pHB561, pHB988 and pUC18 due to the absence of replication and/or cleavage/packaging signals. For plasmid pHB922, a very weak signal was also observed. This may be the result of unspecific interaction of the cleavage/packaging complex with the ori region of BHV-4. The genomic DNA of BHV-4 formed the major fraction of packaged DNA but was not detected with the DNA probe used in this assay.

presence of BHV-4 (Spaete & Frenkel, 1985; Zimmermann & Hammerschmidt, 1995). Table 1 summarizes the features of all constructs used in this study.

GBK cells were transfected with the recombinant plasmids to be tested and superinfected with BHV-4. After lytic replication, the viral capsid DNA was prepared and analysed. To investigate whether one prDNA unit is sufficient for cleavage and packaging, plasmid construct pHB880, containing a single prDNA unit, and pHB881, containing two prDNA units, were used in this assay.

As shown in Fig. 5, cleavage of concatemeric head-to-tail arranged pHB880 by terminase activity and subsequent digestion with BamHI should result in hypermolar internal fragments of 4·5 kbp. The 1·8 kbp BamHI–EcoRI fragment of prDNA comprising the putative cleavage/packaging signal is adjacent to the 2·7 kbp EcoRI–BamHI vector fragment. According to the fact that the cleavage/packaging signal is located at 0·2 kbp from the EcoRI site in prDNA, an additional unimolar terminal fragment of 2·90 kbp is produced (Figs 5 and 6). Due to the opposite orientation of prDNA in pHB881, the 0·2 kbp BamHI–EcoRI prDNA fragment is adjacent to the
essential for cleavage and packaging of concatemeric plasmid DNA by BHV-4. As with plasmid pHB881, only internal fragments of 2-95 kbp were detected with the labelled vector probe. Cleavage of DNA isolated from virion preparation with S*I, a unique cutting enzyme in pHB923, resulted in predicted terminal fragments (data not shown) which indicate the concatemeric shape of the replicated plasmid DNA.

Again, to demonstrate the concatemeric nature of the replicated plasmid DNA, the unrestricted plasmid construct pHB880 was separated on the agarose gel along with the restricted DNA. The hybridization signal showed an electrophoretic mobility comparable to that of viral genomic DNA (Fig. 6).

In summary, encapsidated amplicon plasmid DNA was isolated from virion preparation derived from supernatants of transfected and subsequently infected cell cultures. DpnI analysis of isolated DNA demonstrated that amplicon plasmids were newly synthesized. The detection of the predicted terminal fragments after restriction analysis of the isolated amplicon DNA showed clear evidence that the encapsidated amplicon DNA was generated by processing of concatemeric DNA (probably by a hypothetical terminase activity).

In this study, the structure and function of prDNA of BHV-4 have been analysed. For the first time, we have demonstrated for a herpesvirus with a B type genome organization that a prDNA region of 443 bp is sufficient for cleavage and packaging of BHV-4. These findings confirm the hypothesis that one role of prDNA is a genome-size buffer, which ensures the packaging of a complete copy of the unique DNA flanked by prDNA (Ehlers et al., 1985).

The authors thank Cornelia Walter and Sonja Liebmann for technical assistance.

References


Received 30 September 1998; Accepted 15 December 1998