Analysis of bovine herpesvirus 4 genomic regions located outside the conserved gammaherpesvirus gene blocks

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Bovine herpesvirus 4 (BHV-4) DNA sequences located outside the gene blocks conserved among the gammaherpesviruses BHV-4, herpesvirus saimiri (HVS) and Epstein–Barr virus (EBV) were analysed. Twelve potential open reading frames (ORFs) were found. Protein database comparisons showed that no ORF translation products were similar to proteins encoded by alpha- or betaherpesviruses. Nevertheless, six of the ORFs were homologous in amino acid sequences to proteins encoded by HVS but apparently not to those encoded by EBV. Furthermore, the location and orientation of these six ORFs in the BHV-4 genome were similar to the corresponding ORFs in the HVS genome. No genes homologous to known cellular genes were found in the BHV-4 genome; this feature is the major difference between the BHV-4 and HVS genomes with regards to the overall gene content.

Bovine herpesvirus 4 (BHV-4) has a worldwide distribution and has been isolated from cattle with a variety of clinical diseases as well as from healthy cattle (for review see Thiry et al., 1990, 1992). BHV-4 strains are designated as Movar 33/63-like or DN 599-like in reference to the two prototype strains (Thiry et al., 1989). A very close antigenic relationship (Dubuisson et al., 1989) and a great similarity in the restriction DNA patterns (Bublot et al., 1990) have been shown for these two strains. The BHV-4 virion contains a dsDNA of 144 ± 6 kb (Ehlers et al., 1985). Molecular data have shown a clear relationship between BHV-4 and the members of the subfamily gammaherpesvirinae (Honess, 1984; Bublot et al., 1990, 1991, 1992; van Santen, 1991; Thiry et al., 1992). Therefore, the Herpesvirus Study Group of the International Committee on Taxonomy of Viruses recently assigned BHV-4 to this subfamily (Roizman et al., 1992).

Data from the genomes of completely (Baer et al., 1984; Davison & Scott, 1986; McGeoch et al., 1985, 1986, 1988; Perry & McGeoch, 1988; Chee et al., 1990; Albrecht et al., 1992a; Telford et al., 1992) or partially sequenced (Gompels et al., 1988; Bublot et al., 1992; Nicholas, 1994) mammalian herpesviruses have revealed genes specific to either a particular virus or a virus subfamily as well as conserved genes. Interestingly, the conserved genes are clustered in blocks which have an identical overall arrangement for members of a subfamily but are different between subfamilies (Davison & Taylor, 1987; Kouzarides et al., 1987; Gompels et al., 1988; McGeoch, 1989; Chee et al., 1990; Albrecht et al., 1992a; Bublot et al., 1992).

The analysis of the complete sequence of Epstein–Barr virus (EBV) and herpesvirus saimiri (HVS), and part of the BHV-4 sequence identified genes conserved among the three viruses. The conserved genes are clustered into five gene blocks (Bublot et al., 1992) with a similar location and orientation. The genomic space between the blocks is similar in the BHV-4 and HVS genomes but differs in the EBV genome.

Regions not conserved between HVS and EBV genomes contain genes coding for proteins responsible for such biological properties as latency, immortalization, lytic-cycle transactivation and other virus-host interactions. EBV genes coding for latently expressed proteins such as EBNA and latent membrane proteins (LMPs), the transactivator ZEBRA (coded by the BZLF gene) and the major glycoprotein complex (gp350/220; coded by the BLLF1 gene) have no counterpart in HVS (Baer et al., 1984; Kieff & Liebowitz, 1990; Nicholas et al., 1992b; Albrecht et al., 1992a). Furthermore, genes involved in lymphocyte immortalization, i.e. those coding for EBNA-2, LMP and BARF1 in
Fig. 1. Location and orientation of BHV-4 and HVS ORFs in the regions located outside the conserved gammaherpesvirus gene blocks. The five gene blocks which are conserved within gammaherpesviruses are shaded. Horizontal arrows indicate the position of ORFs present in BHV-4 and HVS genomes outside the blocks. Vertical arrows show the position of HVS genes coding for proteins homologous to cellular proteins. These genes are marked by their abbreviations and are numbered according to the nomenclature used in Albrecht et al. (1992a): DHFR, dihydrofolate reductase; CCPH, complement control protein homologue; CD59, cluster designation 59 homologue; TS, thymidylate synthase; Cyc, cyclin family member homologue; GCR, G-coupled receptor homologue. Dotted lines link homologous BHV-4 and HVS ORFs (see Table 1). The position of the major immediate-early gene of BHV-4 (IE1; van Santen, 1991) and HVS (IE-G; Nicholas et al., 1990) is also represented but no amino acid sequence homology is apparent between these two genes. Terminal repeats (TR) are represented by open rectangles; small internal repeats are indicated by vertical lines or black rectangles. The star indicates the incompletely sequenced zone in the interblock region E.

the EBV genome (Wei & Ooka, 1989; Kieff & Liebowitz, 1990) and gene 1 coding for the transformation-associated protein in the HVS genome (Murthy et al., 1989) are not conserved. It seems therefore that specific genes located outside the conserved gene blocks could be responsible for the different biological characteristics specific to a given gammaherpesvirus. In order to identify genes which could be responsible for BHV-4-specific properties, these particular regions were sequenced. Potential open reading frames (ORFs) were identified and their amino acid sequences analysed to determine whether or not they were homologous to cellular or other herpesvirus proteins.

Nucleotide sequences located outside the conserved gene blocks (Bublot et al., 1992) were obtained by sequencing fragments from the BHV-4 genome (Movar-like V.Test strain and DN 599-like strain). DNA sequencing was performed by the dideoxynucleotide chain termination method (Sanger et al., 1977) either by direct sequencing of dsDNA or phagemid ssDNA using oligonucleotide primers, or by sequencing single-stranded M13 clones as described by Bankier & Barrell (1983). The aim of this work was to determine BHV-4 genes and compare them to those of the two other gammaherpesviruses HVS and EBV, in relation to their location, orientation and translation products. Of a total of 23 kb of DNA sequenced, about 2 kb of potential coding sequences were derived from a single DNA strand. Sequencing was repeated to detect and minimize any errors so that the sequences obtained could be relied on to be accurate.

Sequence data were compiled and analysed using the PHYLIP software package version 7.0 (Unix) of the University of Wisconsin Genetics Computer Group (Devereux et al., 1984). Translated potential ORFs were compared to protein sequences present in the SWISS-PROT database (release 27.0 and 27.+) using the FASTA program (Ktuple of 2; Pearson & Lipman, 1988). The GAP program was used specifically for detailed comparisons based on amino acid similarities and identities. The PEPPLOT program was used to predict signal sequences, anchor sequences and potential N-linked glycosylation sites (NXT/S).

The total length of the BHV-4 sequenced regions was about 23 kbp (about 21% of the L-DNA) with a G+C content of 43%. Each region was sequenced from the end of a conserved gene block to the beginning of the next one. Regions situated at both ends of the L-DNA were sequenced so that partial putative polyrepetitive DNA (prDNA) sequences at both junctions between H-
Table 1. BHV-4 ORFs within the five sequenced regions and homology between BORF translation products and those of the gammaherpesviruses HVS and EBV*

<table>
<thead>
<tr>
<th>ORF</th>
<th>Location in the genome†</th>
<th>Orientation in the genome‡</th>
<th>Nucleotide length</th>
<th>Amino acid length</th>
<th>Calculated molecular mass (kDa)</th>
<th>Homologous ORFs§</th>
<th>Similarity/identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BORFA1</td>
<td>Region A</td>
<td>r</td>
<td>3864</td>
<td>1288</td>
<td>142</td>
<td>ORF 3</td>
<td>55.6/35.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ORF 75</td>
<td>47.7/25.3</td>
</tr>
<tr>
<td>BORFA2</td>
<td>Region A</td>
<td>r</td>
<td>270</td>
<td>90</td>
<td>10</td>
<td>BORF1</td>
<td>46.5/24.8</td>
</tr>
<tr>
<td>BORFB1</td>
<td>Region B</td>
<td>r</td>
<td>1164</td>
<td>388</td>
<td>43</td>
<td>ORF 10</td>
<td>53.8/28.1</td>
</tr>
<tr>
<td>BORFB2</td>
<td>Region B</td>
<td>r</td>
<td>654</td>
<td>218</td>
<td>24</td>
<td>ORF 16</td>
<td>46.5/25.8</td>
</tr>
<tr>
<td>BORFD1†</td>
<td>Region D</td>
<td>r</td>
<td>573</td>
<td>191</td>
<td>19</td>
<td>ORF 51</td>
<td>38.2/25.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(529) + (290)</td>
<td>(273)</td>
<td>(28)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BORFE1</td>
<td>Region E</td>
<td>l</td>
<td>159</td>
<td>53</td>
<td>**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BORFE2</td>
<td>Region E</td>
<td>l</td>
<td>546</td>
<td>182</td>
<td>22</td>
<td>ORF 71</td>
<td>45.2/23.8</td>
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<tr>
<td>BORFE3</td>
<td>Region E</td>
<td>l</td>
<td>759</td>
<td>253</td>
<td>28</td>
<td>ORF 73</td>
<td>44.5/19.4</td>
</tr>
<tr>
<td>BORFF1</td>
<td>Region F</td>
<td>l</td>
<td>495</td>
<td>165</td>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BORFF2</td>
<td>Region F</td>
<td>r</td>
<td>213</td>
<td>71</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BORFF3</td>
<td>Region F</td>
<td>r</td>
<td>669</td>
<td>223</td>
<td>26</td>
<td></td>
<td></td>
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<tr>
<td>BORFF4</td>
<td>Region F</td>
<td>r</td>
<td>684</td>
<td>228</td>
<td>26</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The values given for protein comparisons are based on GAP alignments with gap and length weights set at 3.0 and 0.1, respectively.
† Region A, left end to block 1; region B, block 1 to block 2; region D, block 3 to block 4; region E, block 4 to block 5; region F, block 5 to right end, according to the nomenclature used in Bublot et al. (1992).
‡ r indicates that the ORF is rightward-directed; l indicates that the ORF is leftward-directed.
§ Nomenclature of HVS genes is that used in Albrecht et al. (1992 a); alternative names of ORFs 51, 71 and 73 (EDRF2, ECLF3 and ECLF1, respectively) have been used in Nicholas et al. (1992 b). EBV ORF nomenclature follows the conventions described by Baer et al. (1984).
|           |                  |                           |                   |                  |                                 |                  |                         |
|           |                  |                           |                   |                  |                                 |                  |                         |
|           |                  |                           |                   |                  |                                 |                  |                         |
|           |                  |                           |                   |                  |                                 |                  |                         |
| Numbers given in brackets correspond to the data obtained by analysis of a putative double exon gene.
| Location in the EBV genome different from the putative homologous BHV-4 and HVS genes in their respective genomes.
** This ORF corresponds to the first exon of a putative double exon spliced gene.

DNA and L-DNA were obtained. A region of about 800 nucleotides (nt) located just after the end of the fourth conserved gene block, within the non-conserved region E, could not be completely sequenced. Analysis of the sequenced regions showed the presence of 12 potential ORFs (designated BORFs to indicate BHV-4 ORFs) excluding those coding for the major immediate early IE1 protein and a late protein, already described by van Santen (1991). The similarities of potential ORF amino acid sequences to already described proteins in HVS and EBV were investigated.

Region A, located at the left-most end of the L-DNA, is about 5500 nt long and contains two potential ORFs, BORFA1 and BORFA2 (Fig. 1). The start codon of the BORFA1 gene is located 441 nt after the putative junction between the prDNA and the L-DNA, represented by a PstI site (Bublot et al., 1990). The protein database comparisons revealed a close similarity between BORFA1 and the HVS ORF 3 (gene 3) translation product (1246 aa), the HVS ORF 75 (gene 75) translation product (1299 aa) and the EBV BNRF1 protein (1318 aa) (Table 1). The homologous genes EBV BNRF1 and HVS gene 75 (Cameron et al., 1987), located near the right end of the L-DNA, and a similar BHV-4 gene at a similar location (Bublot et al., 1992) constitute the fifth conserved gene block in each of the three virus genomes. Moreover, HVS gene 3 located near the left end of the L-DNA and in the reverse orientation to HVS gene 75 is also homologous to EBV BNRF1. By direct analogy, BHV-4 BORFA1 is therefore the counterpart of HVS gene 3. The similarity in location and orientation of these two genes supports this observation. Thus, while two similar proteins in BHV-4 and HVS are encoded by two different genes, only one of these two proteins is present in EBV. The putative BORFA2 translation product did not show homology to any protein in the database.

Region B is about 6600 nt long and is located between the first and second conserved gene blocks. Two rightward-directed ORFs, BORFB1 and BORFB2, lay at the beginning and the end of this region, respectively (Fig. 1). Four exons which code for the major immediate early IE1 protein and a probable late protein lie in the complementary strand as already described by van Santen (1991). The BORFB1 amino acid sequence and the translation product of HVS ORF 10 (407 aa) were found to be homologous (Table 1), with the corresponding genes identically located and oriented in their respective genomes (Fig. 1). Homology was also found between the BORFB2 protein and the HVS ORF 16 translation product (160aa) (Table 1). This observation
is consistent with the two genes being located in a similar position in their respective genomes (Fig. 1).

Region C, which is less than 100 nt long, is located between the second and the third conserved gene blocks. It has been previously found to contain no ORFs (Bublot et al., 1992).

Region D, located between the third and fourth conserved gene blocks is about 1400 nt long. It contains one rightward-directed ORF, BORFD1 (Fig. 1). The BORFD1 translation product, of which 58% of its amino acid residues were serine and threonine, showed similarities to several serine/threonine-rich proteins, although many of these comparisons were not plausible. Nevertheless, BORFD1 showed a similarity with the HVS ORF 51 translation product (269 aa) (Table 1), with both ORFs having the same location and orientation in their respective genomes (Fig. 1). The BORFD1 hydrophobicity profile showed the presence of a potential signal sequence without any anchor sequence (data not shown). Eight potential N-linked glycosylation sites were detected in the amino acid sequence. BORFD1 also showed a similarity with the EBV BDLF3 gene product (Table 1) which is located in the second conserved gene block in the EBV genome. The EBV BDLF3 protein has been shown to be a heavily O-linked glycoprotein with an apparent molecular mass in gel electrophoresis of 150 kDa, instead of the predicted 24 kDa (L. Hutt-Fletcher, personal communication).

A 324 nt sequence, without a stop codon, lay immediately after the BORFD1 stop codon. Analysis of the corresponding translated sequence showed the presence of a large hydrophobic region (data not shown). Interestingly, the nucleotide sequences located immediately upstream and downstream of the putative BORFD1 stop codon showed the presence of potential exon donor (AGGTAAC) and acceptor (PyCCAG) sequences (Fig. 2) which conformed to the GT-AG rule and to the consensus sequence for splice donor and acceptor sites (Mount, 1982). The putative spliced mRNA which could result from the cleavage of this intron would encode a 273 aa protein with potential signal and anchor sequences. The hydrophobic profile of this putative protein would then be similar to that of the HVS ORF 51 and EBV BDLF3 translation products.

Region E, which is about 5400 nt long (V. Test strain), is located between the fourth and fifth conserved gene blocks. It contains three leftward-directed potential ORFs, BORFE1, BORFE2 and BORFE3 (Fig. 1). Repeats were present in the left part of the sequence starting about 1500 nt after the end of the fourth conserved gene block. BORFE1, which lay just after the repeat nucleotide sequence region (Fig. 1), had a potential TATA box upstream of its ATG codon without any poly(A) signal sequence downstream. Preliminary analysis of BHV-4 RNA indicates that BORFE1 could be the first ORF of a two exon spliced gene. The repeat nucleotide sequence located just before the leftward-directed ORF may be part of the two exon spliced gene intron (R. Bermudez & V. van Santen, personal communication). BORFE2, whose protein was homologous to the HVS ORF 71 translation product (167 aa) (Table 1) is located and oriented in a manner similar to the HVS ORF 71 gene (Fig. 1). The BORFE3 protein showed similarities to the C-terminal sequence of the HVS ORF 73 translation product (407 aa) (Table 1). These similarities were located after a stretch of glutamic acid and alanine residues present in tandem repeats in the polypeptide encoded by the HVS genome. No repeat was detected in the BORFE3 protein sequence. BORFE3 was found to belong to a proline-rich protein family with 43 prolines, representing about 17% of its total amino acid residues. The location and orientation of BORFE3 in the BHV-4 genome is the same as ORF 73 in the HVS genome (Fig. 1).

The nucleotide sequence of region F, which is about 4000 nt long and located at the right-most end of the genome, was totally derived from a DN 599-like BHV-1 strain. This region contained four potential ORFs (Fig. 1). BORFF1, which ended 85 nt before the beginning of the ORF constituting the fifth conserved gene block (the gene homologous to HVS ORF 75 and EBV BNRF1) was leftward-directed. BORFF2, BORFF3 and BORFF4 were all rightward-directed, with the latter two having 43 overlapping nucleotides. No amino acid sequence similarity was found for the four ORF translation products with proteins catalogued in the database. The BORFF1 protein, like that of BORFE3, was rich in proline, representing 21.7% of its amino acid.
residues. BORFF1 and BORFE3 showed a high similarity with several proline-rich proteins including EBV EBNA-2, which has a proline content of 28%. Region F is probably a specific protein encoding region unique to BHV-4 as no ORF has been found in similar regions of HVS and EBV, which are 249 nt and 1.5 kb long, respectively.

Twelve potential ORFs were found by analysis of the five sequenced regions. Six of the ORFs showed a close similarity to genes present in the HVS genome both in amino acid sequence and in position and orientation. The six BHV-4 ORFs, BORFA1, BORFB1, BORFB2, BORFD1, BORFE2 and BORFE3 were homologous to HVS ORF 3, ORF 10, ORF 16, ORF 51, ORF 71 and ORF 73, respectively. Only BORFD1 was thought to be also homologous to an EBV gene (BDLF3) located in a different position in the EBV genome. However this homology remains to be clarified. The six other BHV-4 ORF translation products, BORFA2, BORFE1, BORFF1, BORFF2, BORFF3 and BORFF4, did not show any plausible amino acid sequence similarity to proteins catalogued in the database and they might therefore be unique to BHV-4.

BORFD1, which contains eight potential N-linked glycosylation sites, has a hydrophobicity profile with a potential signal sequence suggesting that its translation product could be a glycoprotein. The BORFD1 gene was found to be homologous to HVS gene 51. In addition, EBV BDLF3 was thought to be the homologue of BHV-4 BORFD1 and HVS gene 51 despite its different location in the EBV genome. Indeed, although EBV BDLF3 and HVS gene 28 have a similar position and orientation (in the second conserved gene block), the two genes are not related at the amino acid level (Albrecht et al., 1992a). The corresponding region in the BHV-4 genome has not yet been sequenced. Nevertheless, equine herpesvirus 2, which is a fully sequenced gammaherpesvirus closely related to HVS (Telford et al., 1993), does not possess a gene homologous to the EBV BDLF3. However, it possesses a gene homologous to HVS ORF 51 and both genes are located in the same position in their respective genomes (in the corresponding BHV-4 non-conserved region D; E. Telford, personal communication). The BORFD1 protein is rich in serine and threonine residues which may represent O-linked glycosylation sites. This putative O-linked glycosylation could result in the synthesis of a glycoprotein with a molecular mass much higher than that predicted (19 kDa). This phenomenon has already been described for other O-linked glycoproteins (Sun et al., 1994). Moreover, the translation product of EBV BDLF3 gene is a serine/threonine-rich glycoprotein with a predicted molecular mass of about 24 kDa but an apparent mass in gel electrophoresis of about 150 kDa (L. Hutt-Fletcher, personal communication). Analysis of the nucleotide sequence located upstream and downstream of the BHV-4 BORFD1 stop codon showed the presence of putative exon donor and acceptor sequences (Fig. 2). The putative in-frame splicing which may occur in the pre-mRNA would produce a mRNA encoding a 273 aa protein containing both a signal sequence and an anchor sequence.

BORFE3 and BORFF1 encode two highly hydrophilic proteins, rich in proline residues (17% and 22%, respectively). Protein database comparisons showed that the two BHV-4 proteins had good identity scores with many proline-rich proteins, although their similarity was limited to the proline residues (data not shown). Among them was the EBV EBNA-2 protein (28% proline residues) which is expressed during the latent state of the virus life-cycle and has been shown to play an important role in the immortalization of B lymphocytes (Cohen et al., 1989). A class of transcriptional activators has been described in which the transcriptional activity resides in a domain of high proline content (Mermod et al., 1989). These findings suggest that BORFE3 and BORFF1 proteins could belong to such protein families.

Many potential non-coding regions were found outside the conserved gene blocks. Direct nucleotide sequence repeats were found in the B and E regions. These regions, without ORFs, have been shown to vary in size between BHV-4 isolates (Bublot et al., 1989, 1990; Thiry et al., 1992). A variable number of repeats is probably responsible for this size variation as is the case with many other herpesviruses (McGeoch et al., 1988; Kinoshita et al., 1988; Simon et al., 1989). The sequence of an 800 nt region situated just after the end of the fourth conserved gene block, within the non-conserved region E, could not be completely sequenced due to difficulties in obtaining clones containing the whole sequence corresponding to this region. Recent results have shown that this region would contain A+T-rich sequences. This region of the HVS genome corresponds to a non-coding sequence found in the promoter and 5' untranslated region of the thymidylate synthase gene (ORF 70). It contains dyad symmetries similar to those found in the origins of DNA replication of other herpesviruses (Albrecht et al., 1992a). In the Movar 33/63-like BHV-4 strains this region is different from that of the DN 599-like BHV-4 strains. Indeed, it contains an additional HindIII site and is around 500 nt smaller than that of the DN 599-like strains (Bublot et al., 1990). Further analyses are in progress to define a putative role for this particular region in the BHV-4 genome.

The EBV and HVS genomic regions located outside the conserved genes contain genes responsible for the specific biology of these viruses. It is therefore likely that one or several genes described in this work could play an
important role in the specific biology of BHV-4. In addition, the HVS genome also contains in these particular regions several ORFs coding for polypeptides homologous to cellular proteins: ORF 2 (dihydrofolate reductase homologue; Trimble et al., 1988), ORF 4a/b (complement control protein homologue; Albrecht & Fleckenstein, 1992), ORF 15 (human CD59 homologue; Albrecht et al., 1992b), ORF 70 (ECLF4; thymidylate synthase homologue; Honess et al., 1986), ORF 72 (ECLF2; cyclin family member homologue; Nicholas et al., 1992a) and ORF 74 (ECRF3; protein G-coupled receptor; Nicholas et al., 1992a). Genes coding for polypeptides homologous to cellular proteins are also present in the EBV genome: BCRF 1 (IL-10 homologue; Moore et al., 1990) and BHRF1 (bcl-2 homologue; Cleary et al., 1986). BHV-4 genes which could encode proteins homologous to these or any other presently described cellular proteins were not detected either previously by analysis of BHV-4 small random DNA sequences (Bublot et al., 1992) or in the present study. However, sequencing of regions located outside the conserved gene blocks showed that six homologous genes are present in similar positions and orientations in the HVS and BHV-4 genomes, whereas at least five of them are not conserved in EBV. Thus, to date the main genomic difference between BHV-4 and HVS remains the apparent absence of cellular gene homologues in the BHV-4 genome. Although little information is available on the expression of these genes in HVS, it is plausible to assume that the presence of these proteins in the virion may partially explain the differences in the pathogeneses of the two viruses.

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