The most abundant protein in bovine herpes 1 virions is a homologue of herpes simplex virus type 1 UL47

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The bovine herpesvirus type 1 (BHV-1) protein VP8 is present, in large amounts, in the tegument of virions. As a preliminary step towards determining the function of VP8 and the biological relevance for its abundant presence, we describe the mapping of the location of its gene and determination of its nucleotide sequence. The gene for VP8 was located between 0.088 and 0.108 map units on the BHV-1 genome and contained a 2226 bp reading frame encoding a 742 amino acid protein. The protein, produced in vitro by transcribing and translating the reading frame, was precipitated by monoclonal antibodies and polyclonal serum directed against VP8. The primary structure of VP8 showed considerable homology with the product of the UL47 reading frame of herpes simplex virus type 1.

Introduction

Virions of bovine herpesvirus type 1 (BHV-1) comprise about 25 to 33 polypeptides (Pastoret et al., 1980; Misra et al., 1981). Cells infected with the virus contain an additional 15 polypeptides that are not represented in purified virions (Misra et al., 1981). The nucleotide sequences of the genes for some of these polypeptides have been determined. These include BHV glycoprotein B or gI (Misra et al., 1988; Whitbeck et al., 1988), BHV-gC or gII (Fitzpatrick et al., 1989), BHV-gD or gIV (Tikoo et al., 1990) and thymidine kinase (TK) (Mittal & Field, 1989; Smith et al., 1990). The location on the genome of other viral genes, including those for the DNA polymerase (Owen & Field, 1988) and the major DNA-binding protein (Bandyopadhyay et al., 1990), have also been mapped.

As with other herpesviruses the expression of BHV-1 genes is temporally regulated such that viral proteins can be categorized as immediate early (α), early (β) or late (γ) depending upon the order of their synthesis in the infected cell (Misra et al., 1981; Ludwig & Letchworth, 1987; Nelson et al., 1989). The γ gene products can be divided further into γ1 and γ2 subclasses. The γ1 genes are expressed earlier in infection than γ2 genes and are affected minimally if viral DNA replication is interrupted. The γ2 genes are expressed later in infection and are not expressed if viral DNA synthesis is blocked.

Virions of BHV-1 contain an abundance of a protein that has been designated VP8 (Misra et al., 1981), VP7 (Pastoret et al., 1980) or 107K (Marshall et al., 1986). The protein, which is also the most abundant viral protein in infected cells, has been shown to be located in the tegument of virions (Marshall et al., 1986) and preliminary results (G. Weinmaster, unpublished results) suggest that it is a γ phosphoprotein.

In this communication, as the first step towards determining the function of VP8 and the biological relevance for its abundant presence, we describe the mapping of the location of its gene and the determination of its nucleotide sequence. The amino acid sequence of VP8 bears considerable resemblance to that of the herpes simplex virus type 1 (HSV-1) gene UL47.

Methods

Cells and virus. BHV-1 strain P8-2 was isolated at the University of Wisconsin by J. R. Saunders. Details of virus propagation in Madin-Darby bovine kidney (MDBK) cells have been published (Misra et al., 1981). To obtain radiolabelled infected cell proteins, cells infected with BHV-1 at a multiplicity of infection of 10 p.f.u. per cell were maintained in either methionine-free medium to which [35S]methionine (100 μCi/ml) had been added or in phosphate-free medium supplemented with 500 μCi of H32PO4 per ml. Cells were harvested 20 h after infection.

Construction of a ßgt11 expression library. BHV-1 genomic DNA (2.5 μg) was partially digested with the restriction enzymes HaeIII (2.5 units) and Rsal (7 units) for 8 min. The digest was electrophoresed through a 0.8% agarose gel and DNA fragments, 500 to 2000 bp in length, were eluted from the gel. EcoRI linkers (Promega) were ligated to the fragments, which were then digested with EcoRI. The fragments were cloned into Protocline ßgt11 arms (Promega) and packaged using the Packagene in vitro packaging system (Promega). The library was grown on Escherichia coli host strain Y1090(r+) and expression of the β-galactosidase–VP8 fusion protein was induced with isopropyl thio-β-D-
galactoside (Bethesda Research Laboratories). Plaques were transferred to nylon membranes by the method of Maniatis et al. (1982).

**VP8 polyclonal antiserum.** Antisera to VP8 were raised in rabbits. The polypeptides of virions purified from infected cells in eight 100 mm diameter culture plates were separated by electrophoresis through a preparative 7.5% (w/v) SDS-PAGE gel (Misra et al., 1981). The outer lanes of the gel were stained with Coomassie blue to visualize the proteins. The segment of the unstained gel, which corresponded to the VP8 band, was excised and dialysed in water to remove SDS. The gel slices were homogenized to a slurry with a Polytron homogenizer (Brinkman Instruments). Freund's complete adjuvant (1 ml) and the slurry (1 ml) were mixed and 0.4 ml inoculant was injected intramuscularly and subcutaneously at several sites. At 2 week intervals, 1 ml slurry and 1 ml Freund's incomplete adjuvant were mixed and injected into the rabbits. Eight weeks after the initial immunization, the rabbits were bled and serum was collected. The rabbit serum was absorbed with ProtoBlot extract (Promega) to remove cross-reacting antibodies. The resulting serum, diluted 1:500, was used to detect VP8-expressing plaques from the λgt11 library. Bound antibody was visualized with horseradish peroxidase-labelled anti-rabbit antibody (Bio-Rad).

**Mapping of the VP8 gene on the BHV-1 viral genome.** Viral genomic DNA (2 μg) was digested with the restriction enzymes EcoRI, HpaI, HindIII and BglII (5 units, 1 h at 37°C) then electrophoresed through a 0.8% agarose gel. The DNA fragments were transferred to a nylon membrane (Amersham) and hybridized to a radiolabelled DNA fragment isolated from the λgt11 clone that reacted with the anti-VP8 serum.

S1 nuclease mapping of the start of transcription. A variation of the procedure of Berk & Sharp (1977) was used to map the start of transcription of the VP8 gene. The VP8 gene was mapped, by S1 nuclease digestion, in two segments. The 3.0 kb EcoRI G fragment (Fig. 2) was cloned into the EcoRI site of the multiple cloning site of pEMBL18. The orientation of the insert was determined from the location of an internal HindIII site. A HindIII digest would excise a 2.0 kb fragment if the insert was cloned in one direction or a 1.0 kb fragment if it was cloned in the other direction. A clone that released a 2.0 kb fragment upon HindIII digestion was mapped with ApaI and HindIII. A 1300 bp ApaI-EcoRI fragment of BHV-1 DNA, with the entire multiple cloning site adjacent to the EcoRI end, was eluted from the gel and was labelled at the 5' end with [γ-32P]ATP using alkaline phosphatase (Promega). The fragment was then digested with EcoRI to remove the labelled multiple cloning site. The result was a 1300 bp EcoRI-ApaI fragment labelled on one strand at the ApaI site. A similar strategy was used to label the 1700 bp EcoRI-HindIII portion of the 3.7 kb HindIII M fragment. The entire 3.7 kb HindIII M fragment was cloned into the HindIII site of the multiple cloning site. An internal EcoRI restriction site was used to determine the orientation. The 1700 bp EcoRI-HindIII portion with the entire multiple cloning site at the HindIII end was released with an EcoRI digest. This was eluted from a gel, labelled and the multiple cloning site was removed with a HindIII digest. This resulted in a 1700 bp EcoRI-HindIII fragment labelled S' on one strand at the EcoRI site. Labelled DNA was hybridized to total infected cell RNA at a ratio of 1:5. Total infected cell RNA was isolated from MDBK cells, by the method of Cuthala et al. (1983), 20 h after BHV-1 infection. Products of S1 digestion were electrophoresed on a urea-containing 6% (w/v) acrylamide gel. A 32P-labelled BstEII digest of λ DNA was denatured and electrophoresed to determine the size of the fragments protected from digestion with S1 nuclease.

In vitro transcription and translation. The coding sequence for the VP8 gene was cloned between the Accl and HindIII sites of pGEM-3Z (Promega) in two step cloning reaction. First the HindIII M fragment was cloned into the HindIII site of pGEM-3Z. An EcoRI digest was used to determine the fragment orientation. A clone that would release a 1.7 kb fragment upon EcoRI digestion was isolated. This clone was digested with Accl which released a 600 bp fragment and a 1100 bp fragment from the vector which still contains 2000 bp of the VP8 gene. The 1100 bp fragment and the pGEM partial VP8 fragment were eluted from a gel and ligated. Regeneration of the VP8 gene coding sequence in the pGEM vector was screened by EcoRI digestion. RNA was transcribed from the T7 promoter using the Riboprobe system (Promega). During transcription the transcripts were capped with the cap analogue pGpp (Pharmacia). The capped RNA was translated in a rabbit reticulocyte lysate containing [35S]methionine (Pelham & Jackson, 1976). The reticulocyte lysate was a gift from A. J. Pawson (University of Toronto).

**Immunoprecipitation.** Immunoprecipitations were carried out using the method of Misra et al. (1982) using monoclonal antibodies (MAbs) or polyclonal antisera developed in our laboratory.

**Sequencing.** Sequences and 7-deaza-dGTP reagents (United States Biochemicals) were used to determine the nucleotide sequence of the VP8 gene. Nested deletions of the DNA or subclones, constructed using convenient restriction sites, were used to sequence both strands of the entire VP8 coding region. Regions downstream from the VP8 coding region were sequenced on one strand only.

**Protein sequence alignment.** VP8 and UL47 protein sequences were aligned using the CLUSTAL computer program of Higgins & Sharp (1988).

**Results**

**VP8 is a γ2 phosphoprotein**

The protein we have designated VP8 (Misra et al., 1981) is made in the later stages of infection and is the most abundant of the virion proteins of BHV-1 (Misra et al., 1981; Pastoret et al., 1980; Marshall et al., 1986). VP8 has also been shown to reside in the viral tegument (Marshall et al., 1986) and G. Weinmaster (unpublished results) demonstrated that BHV-1-infected cells contain a phosphoprotein with an electrophoretic mobility similar to that of VP8.

To confirm that VP8 is a γ class phosphoprotein, BHV-1 infected cells were labelled with either [35S]methionine or H3[32]PO4, in the presence or absence of 250 μg phosphonoformic acid (PFA) per ml medium. VP8 and BHV-gB, a β class protein (Nelson et al., 1989), were then immunoprecipitated from the cell lysates. Monoclonal antibodies were used for immunoprecipitation.

Fig. 1 shows that although [35S]-labelled intact BHV-gB (130K) and its cleavage products (70K and 50K) were immunoprecipitated from PFA-treated and untreated cells, VP8 and 60K proteolytic degradation product were immunoprecipitated only from cells in which viral DNA synthesis had not been interrupted by PFA. VP8, immunoprecipitated from untreated infected cells, was labelled with 32P. These results confirm that VP8 is a γ2 class phosphoprotein.
**Screening the λgt11 library**

A BHV-1 expression library was constructed to isolate a DNA probe for the VP8 gene. To determine which phage in the expression library contained a portion of the VP8 gene, polyclonal antiseras to the protein were raised in rabbits. Serum from rabbits, immunized three times with VP8 protein, bound to the VP8 protein in Western blot analysis. The VP8 antisera detected a plaque from the λgt11 BHV-1 genomic library. This clone contained a 600 bp fragment of BHV-1 DNA. The 5' end of this fragment was sequenced using a primer homologous to the coding sequences of β-galactosidase in λgt11 to determine in which reading frame this fragment was being expressed.

**Mapping the location of VP8 on the BHV-1 genome**

The 600 bp DNA fragment from the expression library was used to probe fragments in EcoRI, HpaI, BglII and HindIII digests of the BHV-1 genome. The location of the sites for these enzymes on the P8-2 strain of the BHV-1 genome have been mapped (M. A. N. Beckie, unpublished results). The probe hybridized to the 3 kb EcoRI G fragment (0-096 to 0-119 map units), the 15 kb HpaI D fragment (0-032 to 0-143 map units), the 7-5 kb HindIII M fragment (0-083 to 0-111 map units) and the 12-5 kb BglII E fragment (0-026 to 0-119 map units). These fragments all map at the left-hand end of the BHV-1 genome (Fig. 2).

**S1 nuclease mapping the VP8 transcriptional start site**

The transcriptional start site of the VP8 gene was located on the 3-7 kb HindIII M fragment by S1 nuclease mapping. The HindIII M fragment was digested into a 1300 bp ApaI-EcoRI fragment and a 1700 bp EcoRI-
Fig. 3. Mapping the transcriptional start site of the VP8 gene. S1 nuclease protection analysis was performed on two different pieces of the HindIII M fragment. (a) Result of S1 mapping the 1300 bp Apal–EcoRI fragment. Radiolabelled 18BrEII size markers (lanes 5) were electrophoresed to determine the size of protected fragments and the sizes are indicated in the centre. Lanes 1, labelled DNA (5' end-labelled at the Apal site); lanes 2, DNA/RNA hybrid (labelled DNA hybridized to RNA); lanes 3, S1-treated (labelled DNA hybridized to RNA and treated with S1 nuclease); lanes 4, second S1-treated sample incubated for twice the time period. RNA-protected bands are indicated by << in lanes 3 and 4. (b) Result of S1 mapping the 1700 bp EcoRI–HindIII fragment. The lane numbers are the same as for (a) except that the 1700 bp EcoRI–HindIII fragment, 5' end-labelled at the EcoRI site, was used. RNA-protected bands are again indicated by <<. (c) Summary of results. The solid black bar represents the HindIII M fragment. Relevant restriction sites are indicated and the sizes of restriction fragments are given below. The small dark shaded box represents the portion of the HindIII fragment originally isolated from the λgt11 library and used as a probe for the VP8 gene. The large lightly shaded boxes represent the DNA fragments protected from S1 degradation when the 1300 bp Apal–EcoRI and 1700 bp EcoRI–HindIII portions of the HindIII M fragment were subjected to S1 nuclease protection analysis. The major transcriptional start site is indicated.

HindIII fragment (Fig. 3c). RNA from BHV-1-infected cells protected the entire 1300 bp Apal–EcoRI portion of the 3-7 kb HindIII fragment from S1 digestion (Fig. 3a) suggesting that the fragment was included, in its entirety, within a transcript. The size of the 1700 bp EcoRI–HindIII subfragment, labelled with 32P at the EcoRI site, was reduced in size by 600 bp by S1 nuclease, indicating that the beginning of the main transcriptional unit in this fragment was about 1100 bp from the EcoRI site (Fig. 3b). A minor transcription start site mapped about 650 bp from the EcoRI site.

Immunoprecipitation

To confirm that the transcription start site, identified by the S1 protection experiments, was that of VP8, a 3-1 kb AccI-HindIII piece of DNA from the HindIII M fragment was cloned between the AccI and HindIII sites of pGEM-3Z. The AccI site in the HindIII M fragment was located immediately upstream from the putative start site of the VP8 transcript (Fig. 3c). In vitro transcription, from the T7 promoter of pGEM-3Z, resulted in a single 3-1 kb RNA transcript. In vitro translation of this RNA produced a 90K protein which could be immunoprecipitated from the reticulocyte lysate by the anti-VP8 sera used initially to screen the λgt11 BHV-1 genomic library but not with monoclonal antibodies to BHV-gB (Fig. 4). Monoclonal antibodies to VP8 also immunoprecipitated the in vitro translated protein (data not shown). The VP8 antisera and monoclonal antibodies to VP8 and BHV-gB immunoprecipitated the appropriate proteins from lysates of BHV-1.
infected cells. The \textit{in vitro} translation of VP8 also yielded an approx 50K protein. This protein was translated from RNA endogenous to the lysate and appeared even if no external RNA was added to the lysate.

\textbf{Sequencing}

After confirming that the DNA from the \textit{HindIII} M fragment contained the gene for VP8, the nucleotide sequence of both strands of the fragment from the \textit{AccI} site to 2500 bp downstream was determined (Fig. 5). The fragment contained a 2226 bp open reading frame which would encode a protein 742 amino acids in length. This coding sequence was located between 0-088 and 0.108 map units on the BHV-1 genome.

\textbf{Protein sequence alignment}

A comparison of the VP8 amino acid sequence with the sequence of HSV-1 (McGeoch \textit{et al.}, 1988) revealed considerable homology with the protein encoded by the open reading frame UL47. A more detailed alignment, using the CLUSTAL program (Higgins & Sharp, 1988)

\textbf{Fig. 4.} Immunoprecipitation of the VP8 protein from \textit{in vitro} translation reaction and infected cell lysate. Lanes: 1, reticulocyte lysate with no exogenous RNA added; 2, \textit{in vitro} translation of \textit{in vitro} transcribed RNA containing the VP8 coding region; 3, immunoprecipitate of \textit{in vitro} translated VP8 using the polyclonal VP8 antisera originally used to screen the \textit{pgtl 1} library; 4, immunoprecipitate of BHV-1-infected cell lysate with VP8 MAb; 5, immunoprecipitate of BHV-1-infected cell lysate with polyclonal VP8 antisera; 7, immunoprecipitate of BHV-1-infected cell lysate with gB MAb. M, markers are indicated.

\textbf{Fig. 5.} Nucleotide and amino acid sequences of the coding region for the VP8 gene. The sequence for the segment of the DNA containing a large open reading frame, from which \textit{in vitro} transcription/translation produced the VP8 protein, is listed above. The \textit{AccI} restriction site used to clone the fragment into pGEM-3Z is indicated. The \textit{Apal} restriction site located within the fragment isolated as a probe is also indicated between 2260 bp and 2270 bp. The putative promoter consensus sequences (i.e. TATA box and SPI sites) for a gene that shares a 3' terminus with the VP8 gene are indicated between 2340 and 2376 bp (underlined). The region >60 bp downstream from the VP8 coding sequence was sequenced on one strand only.
Fig. 6. Comparison of the amino acid sequence of BHV-1 VP8 and HSV-1 UL47 as determined by the CLUSTAL sequence alignment computer program. An asterisk below amino acids indicates identical residues, and a point below corresponding amino acids indicates conservative substitutions.

showed that regions of homology were located throughout the two molecules (Fig. 6). The CLUSTAL alignment program’s method for deciding conservative replacements is based on the log odds matrix of Dayhoff (1978). This relies on the frequency at which various amino acids are replaced by each other.

Discussion

Several lines of evidence suggest that the BHV-1 protein VP8 is a homologue of the product of the HSV-1 gene UL47. First, the genomic map location of VP8 is similar to UL47 with respect to other shared homologues, (e.g. gB, gC, DNA polymerase, TK and capsid protein) of the viruses (Fig. 7). Varicella-zoster virus (VZV) also has a gene arrangement collinear to those of BHV-1 and HSV-1, with the open reading frame 11 mapping in the same relative location as VP8 and UL47 (Wirth et al., 1989; McGeoch et al., 1988; Davison & Scott, 1986). Additional evidence is found in RNA transcript mapping. UL47 is transcribed as a 4-7 kb y2 transcript which shares a 3’ terminus with a 2-5 kb y1 transcript (Hall et al., 1982). This is very similar to the transcript map of the corresponding region of the BHV-1 genome. Wirth et al (1989) showed that the 3.7 kb HindIII M fragment hybridizes to a 4-5 kb y2 and a 2-7 kb y1 transcript. In Northern blot analysis, we have found the 1300 bp Apal–EcoRI fragment, containing the distal half of the VP8 gene, hybridized to a 4-5 kb transcript (data not shown) of which only the first 2700 bp would encode the VP8 protein. Immediately downstream from the VP8 coding sequence there is a promoter consensus sequence (Fig. 5) which may be the regulatory sequence for the transcript that shares a 3’ terminus with VP8.

Finally, we have sequenced the BHV-1 VP8 gene and shown that the derived amino acid sequence has considerable homology to the derived amino acid sequence of HSV-1 UL47 (McGeoch et al., 1988). Blocks of identical amino acids appear throughout the sequences; however, the carboxy half of the molecules appear to have maintained greater conservation. The proteins differ in size by 49 amino acids. This size difference generally seems to be due to changes in the amino terminal half of the proteins. VP8 also shares considerable homology with VZV open reading frame 11. Once again there seems to be more conservation of the carboxy half of the proteins with variability in the amino half of the molecule.

In HSV-1-infected cells, viral gene expression is initiated when the major structural tegument protein
Vmw65 (α-trans-inducing factor or α-TIF) forms a complex with cellular proteins which then bind to cis-acting elements in the α gene promoters (Roizman et al., 1988). A review of Vmw65 interaction with cellular proteins is presented by Stern et al. (1989) and Goding & O'Hare (1989).

The gene products of the HSV-1 open reading frames UL47 and UL46 were initially implicated as modulators of the α-TIF trans-activation in transient expression systems (McKnight et al., 1987). A more recent paper indicates that deletion of UL46, within the context of the virus, has no apparent effect on α-TIF trans-activation but deletion of UL47 has a significant effect (Zhang et al., 1991). It is not known by what mechanism the product of UL47 affects α-TIF trans-activation. The coding sequence for UL47 is reportedly not required for viral replication in cell culture (Barker & Roizman, 1990; BARKER et al., 1983). Upon viral infection, UL47, a modulator of α-TIF, is carried into the cell. UL47 may act as well as the α-TIF is carried into the cell. U'L47 may act in its tegument. Initial results suggest this kinase activity may be attributed to the VP8 protein and we are investigating this possibility further.

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References


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