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Identification and Characterization of the Bovine Herpesvirus 1 UL7 Gene and Gene Product Which Are Not Essential for Virus Replication in Cell Culture

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The UL7 gene of bovine herpesvirus 1 (BHV-1) strain Schönböken was found at a position and in a context predicted from the gene order in the prototype alphaherpesvirus herpes simplex virus type 1. The gene and flanking regions were sequenced, the UL7 RNA and protein were characterized, and 98.3% of the UL7 open reading frame was deleted from the viral genome without destroying productive virus replication. Concomitant deletion of nine 3′ codons from the BHV-1 UL6 ORF and 77 amino acids from the carboxy terminus of the predicted BHV-1 UL8 protein demonstrated that these domains are also not essential for function of the respective proteins. The UL7 open reading frame encodes a protein of 300 amino acids with a calculated molecular mass of 32 kDa. Comparison with UL7 homologs of other alphaherpesviruses revealed a high degree of homology, the most prominent being to the predicted UL7 polypeptide of varicella-zoster virus, with 43.3% identical amino acids. A monospecific anti-UL7 serum identified the 33-kDa (apparent-molecular-mass) UL7 polypeptide which is translated from an early-expressed 1.7-kb RNA. The UL7 protein was localized in the cytoplasm of infected cells and could not be detected in purified virions. In summary, we describe the first identification of an alphaherpesviral UL7-encoded polypeptide and demonstrate that the UL7 protein is not essential for replication of BHV-1 in cell culture.

Bovine herpesvirus 1 (BHV-1) is a natural pathogen of cattle and causes respiratory and genital diseases and abortions (9, 15, 34). It is a member of the subfamily *Alphaherpesvirinae* with a genome of approximately 138 kbp. Other members of this subfamily are pseudorabiesvirus, Marek disease virus, varicella-zoster virus (VZV), equine herpesvirus 1 (EHV-1), and the prototype herpes simplex virus type 1 (HSV-1). The entire genomes of HSV-1 (19), VZV (3), and EHV-1 (31) have been sequenced, and the complete BHV-1 DNA sequence will be available in the near future (32). The expression of alphaherpesvirus genes is temporally controlled and coordinated in a cascade fashion (10). At least three classes can be differentiated: immediate early, or α; early, or β, and late, or γ. Genomes of alphaherpesviruses encompass approximately 70 open reading frames (ORFs) which, with few exceptions, are to various degrees homologous among each of the viruses mentioned above. Therefore, the genes identified in BHV-1 are named according to their counterparts in HSV-1, although the prototype orientations of both the U_S and U_L segments are inverted relative to the original prototypic genome arrangement (14, 17, 27, 32). It should be noted, however, that the common nomenclature for the alphaherpesvirus genes does not implicate identical functions or activities of sequence elements or gene products.

Functions have been assigned to the products of most of the HSV-1 ORFs (18, 33). The UL7 gene, located near the left end of the U_L segment of HSV-1, codes for one of the so-far-uncharacterized alphaherpesvirus proteins (24, 27, 33). For HSV-1, UL7 is thought to be essential (33). Recently, Patel and MacLean reported that UL7 of HSV-1 is expressed as a delayed-early gene from which a 1.3-kb RNA is transcribed (24). They identified the 5′ cap site and showed that UL6 and UL7 transcripts are coterminal at their 3′ ends.

In the present study, we report the nucleotide sequence of the UL7 gene of BHV-1 strain Schönböken, analyze the UL7 transcript, and show that the UL6 RNA overlaps the UL7 ORF. We demonstrate that, in contrast to HSV-1, the BHV-1 UL7 gene is regulated as an early gene. We also identify the UL7 protein, which accumulates in the cytoplasm of BHV-1-infected cells but could not be found in purified virions. By constructing a viable UL7 deletion mutant, we show that UL7 is not essential for productive replication of BHV-1 in cell culture as are the nine carboxy-terminal amino acids of the putative UL6 protein and the 77 3′ codons of the UL8 ORF.

MATERIALS AND METHODS

Cell culture and viruses. Madin-Darby bovine kidney cell clone Bu100 (MDBK-Bu100; kindly provided by W. Lawrence and L. Bello, Philadelphia, Pa.) and primary bovine embryonic lung, embryonic kidney, and embryonic heart cells (obtained from R. Riebe, Insel Riems, Germany) were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 5% fetal calf serum, 350 μg of glutamine per ml, 100 U of penicillin per ml, and 100 μg of streptomycin per ml.

BHV-1 wild-type strains Aus12 and Schönböken (obtained from O. C. Straub, Tübingen, Germany) were propagated on MDBK-Bu100 cells.

Sequencing and S1 analysis. The nucleic acid sequences of both strands were determined by the method of Maxam and Gilbert (16), with modifications as previously described (12).

Sequences were analyzed by using the Genetics Computer Group software package, version 8.0-UNIX (4). Nucleotide and amino acid sequences were compared by using the program BestFit. S1 analysis was performed as described elsewhere (12). Briefly, 5 μg of cytoplasmic RNA was hybridized to 5′-end-labeled DNA fragments and digested with nuclease S1. After precipitation with ethanol, samples were resuspended and separated on 6% urea sequencing gels. Protected fragments were visualized by autoradiography.

RNA isolation and Northern blot hybridization. Cytoplasmic RNA was isolated essentially as described previously (26). Glyoxal-treated RNA (5 μg) was separated in 1% formaldehyde gels and transferred to nitrocellulose filters (22). To detect transcripts encompassing the UL7 ORF, the 506-bp *Sma*I-*Mlu*I fragment was eluted from the agarose gel after cleavage of pSub3 (Fig. 1). The same

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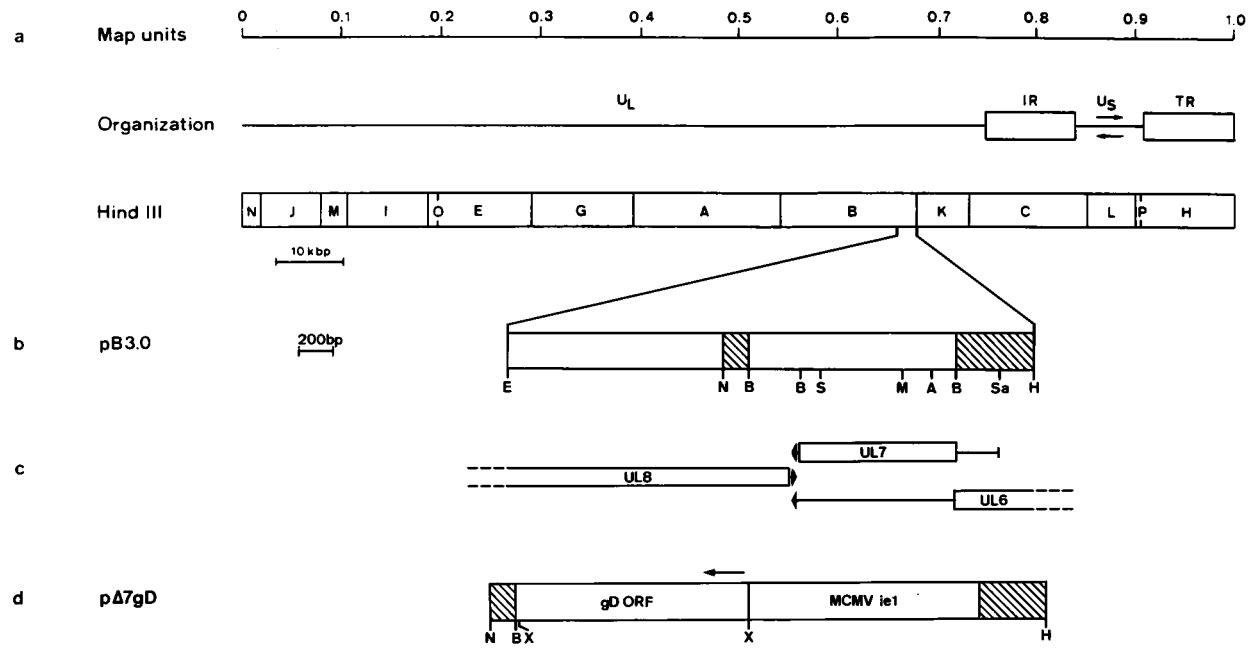

FIG. 1. Localization of UL7 gene. (a) The *Hin*dIII restriction fragment map of the genome of BHV-1 strain Schönböken is shown below a schematic representation of the prototype orientation (7, 17). (b) The 3-kbp *Hin*dIII-*Eco*RI fragment contained within plasmid pB3.0 is enlarged, and relevant restriction enzyme cleavage sites are indicated. (c) ORFs with homology to HSV-1 UL6, UL7, and UL8 are depicted by open bars; arrowheads indicate the directions of transcription and locations of consensus sequences for polyadenylation. The initiation site for UL7 transcription is marked by a vertical line. (d) Schematic representation of the replacement fragment contained in plasmid pΔ7gD used for the deletion of the UL7 ORF from the BHV-1 genome. In this construct, the expression of the BHV-1 gD ORF is directed by the MCMV-ie1 enhancer-promoter. Relevant restriction enzyme cleavage sites are indicated. Hatched bars in panels b and d mark sequences in the viral genome provided in pΔ7gD for homologous recombination. Abbreviations: A, *Ava*II; B, *Bsi*WI; E, *Eco*RI; H, *Hin*dIII; M, *Mlu*I; N, *Not*I; S, *Sma*I; Sa, *Sac*I; X, *Xba*I.

plasmid was cleaved with *Hin*dIII and *Sac*I to isolate the 206-bp fragment for the identification of the UL6 RNA. DNA probes were labeled with [α-^{32}P]dCTP by using the Ready To Go DNA labeling kit (Pharmacia, Freiburg, Germany). For estimation of RNA sizes, *Escherichia coli* 16S and 23S rRNAs and murine 18S and 28S rRNAs were used as size markers. Hybridization procedures were performed as described previously (11).

Construction of replacement plasmid pΔ7gD. Plasmid pSub3 was constructed by cleavage of the pSP73-based plasmid pB3.0 (Fig. 1) with *Eco*RI and *Not*I. The remaining viral sequences, linked to the cloning vector, were blunt ended, and the DNA was religated. The 1.8-kbp insert comprises the UL7 gene flanked by UL6 and UL8 sequences. The UL7 ORF, the last 9 codons of the UL6 ORF, and the 77 3′ codons of the UL8 ORF were removed from pSub3 by cleavage with *Bsi*WI (Fig. 1). After generation of blunt ends, a 2.7-kbp blunt-ended *Bam*HI-*Hpa*I DNA fragment of pie1gD was inserted. This fragment contains the ORF coding for BHV-1 glycoprotein D (gD) downstream from the enhancer–immediate-early 1 (ie1) promoter element of the murine cytomegalovirus (MCMV) (6). In the resulting plasmid pΔ7gD, the gD ORF is in the same orientation as the UL7 ORF in pSub3 (Fig. 1).

Deletion of UL7 ORF from BHV-1 genome. MDBK-Bu100 cells were cotransfected with 1 μg of purified DNA of gD$^-$ BHV-1 mutant 80-221 (8) and 5 μg of pΔ7gD by using the mammalian transfection kit of Stratagene (La Jolla, Calif.) according to the manufacturer's protocol. At 4 h after cotransfection, the cells were treated with glycerol as recommended to increase the transfection efficiency and incubated until a cytopathic effect appeared. Virus progeny from the culture supernatants was titrated on MDBK-Bu100 cells. Single plaques were picked and analyzed by dot blot hybridization with UL7- and gD-specific probes. UL7$^-$ gD$^+$ isolates were titrated on MDBK-Bu100 cells and again plaque purified. One of the substitution mutants, designated BHV-1/ΔUL7, was selected for further characterization.

DNA isolation and Southern blot hybridization. To isolate whole-cell DNA, cells were harvested at 20 h postinfection (p.i.) by trypsinization and low-speed centrifugation. The pellet was resuspended in 10 mM Tris-HCl (pH 8.0)–10 mM EDTA–150 mM NaCl–0.2% sodium dodecyl sulfate (SDS)–200 μg of RNase A per ml, incubated for 1 h at 37°C, and digested with proteinase K (final concentration, 200 μg/ml). After 1 h at 56°C, a 0.4 volume of a saturated NaCl solution was added. Debris was removed by centrifugation, and DNA in the supernatant was precipitated with 2.5 volumes of ethanol, washed in 70% ethanol, dried, and resuspended in 10 mM Tris-HCl–1 mM EDTA (pH 7.5).

For Southern blot analysis, 2 μg of whole-cell DNA was digested with *Hin*dIII, size fractionated in a 0.6% agarose gel, transferred to nitrocellulose filters, and hybridized following standard procedures (26). DNA probes used for hybridization were labeled with [α-^{32}P]dCTP by using the Ready To Go DNA labeling kit (Pharmacia).

In vitro transcription and translation. UL7 mRNA was in vitro transcribed from pSub3 after linearization with *Cla*I by T7 RNA polymerase in the presence of the cap analog m^{7}GpppG according to the manufacturer's protocol (Boehringer, Mannheim, Germany) and translated in vitro in the presence of 60 μCi of [^{35}S]methionine per reaction mixture as recommended by the supplier (Promega, Heidelberg, Germany).

UL7 sense RNA or RNA complementary to the UL7 mRNA was transcribed from pSub3 after linearization with *Cla*I or *Hin*dIII by T7 or SP6 RNA polymerase in the presence of 50 μCi of [α-^{32}P]UTP and used as hybridization probe.

Production of anti-UL7 serum. The expression vector pATH11, which contains a part of the *E. coli* TrpE ORF downstream of the inducible TrpE promoter (5), was digested with *Eco*RI and blunt ended with Klenow enzyme, and a blunt-ended 882-bp *Bsi*WI fragment (Fig. 1) encompassing codons 5 to 299 of the UL7 ORF was inserted in frame. The resulting plasmid, pFUSORF7, encodes a TrpE-UL7 fusion protein which consists of 334 amino acids of the TrpE protein followed by 295 amino acids of the putative UL7 protein.

Expression of the TrpE-UL7 fusion protein was induced in *E. coli* C600 with indolacrylic acid (10 μg/ml) in the absence of tryptophan as described previously (21, 28). Bacteria were harvested 4 h later, resuspended in protein sample buffer, and subjected to preparative denaturing gel electrophoresis. The TrpE-UL7 fusion protein was electroeluted by using a Biotrap device (Schleicher & Schuell, Dassel, Germany) and dialyzed against 20 mM ammonium carbamate. Rabbits were inoculated subcutaneously with 40 to 80 μg of fusion protein emulsified with complete Freund's adjuvant. The rabbits were boosted seven times at 3- to 4-week intervals with fusion protein in incomplete Freund's adjuvant. The anti-UL7 serum used in this study was obtained 4 weeks after the last immunization.

Immunoprecipitation and Western blotting. Immunoprecipitation, SDS–10% polyacrylamide gel electrophoresis (PAGE), and fluorography were carried out as described previously (13).

For Western blotting (immunoblotting), cells or purified virions (8) were lysed in sample buffer, and proteins were separated by SDS–10% PAGE under reducing or nonreducing conditions. Proteins were electrotransferred to nitrocellulose membranes, blocked in 3% skimmed milk powder, and reacted sequentially with the anti-UL7 serum and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Amersham, Buckinghamshire, United Kingdom, or Dianova, Hamburg, Germany). Immunoreactive proteins were visualized by enhanced chemiluminescence detection (Amersham).

Infection of cells for indirect immunofluorescence assays. Monolayers of MDBK-Bu100 cells were infected with BHV-1 at a multiplicity of infection of 1.

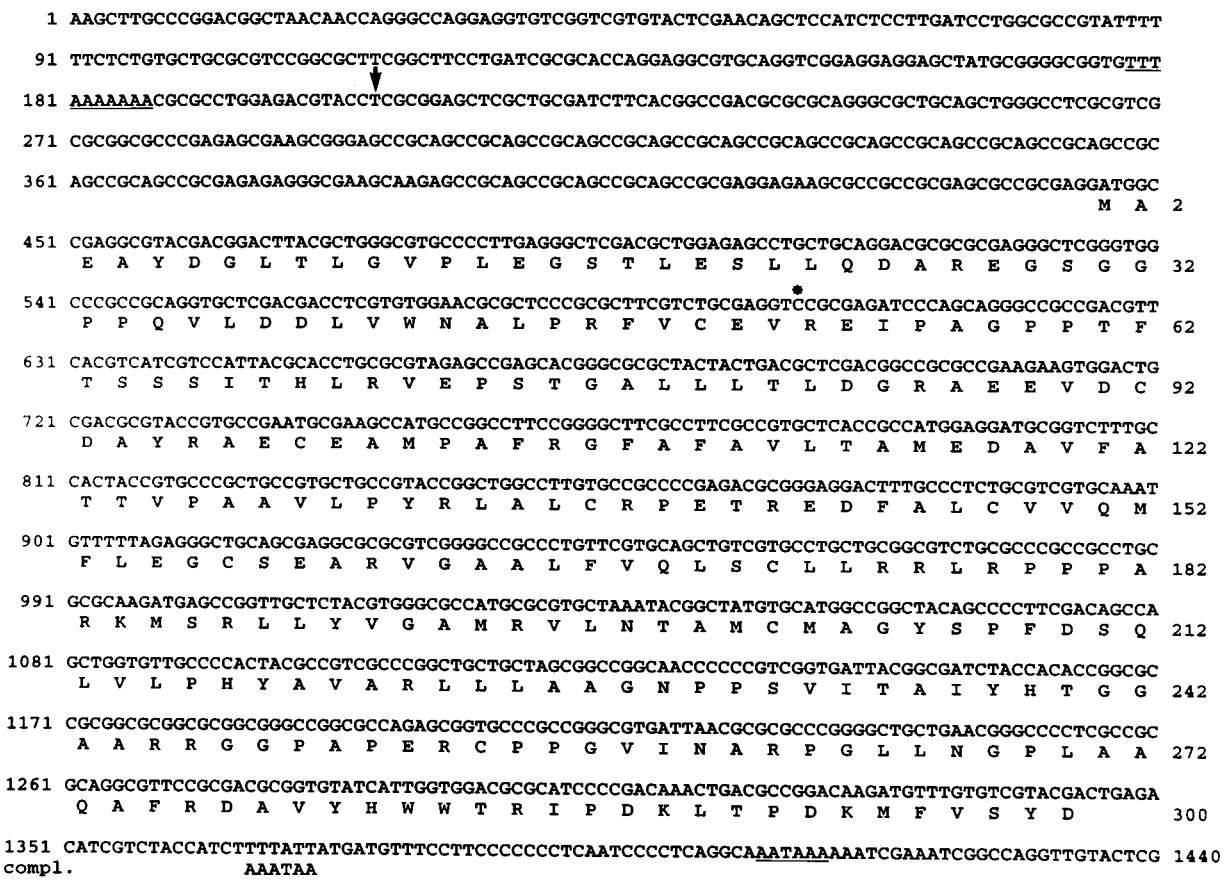

FIG. 2. Nucleotide sequence of BHV-1 strain Schönböken UL7 gene and deduced UL7 amino acid sequence. The nucleotide sequence is shown from the *Hin*dIII site at map unit 0.678 to the predicted polyadenylation site for UL6 and UL7 transcripts (underlined) at map unit 0.667. The deduced amino acid sequence is shown below the UL7 ORF in single-letter code. The 5′ cap site of the UL7 mRNA is marked by an arrowhead, and the *Ava*II site used for 5′ end labeling is indicated by an asterisk. The putative TATA box and the consensus sequence for polyadenylation of the UL6 and UL7 transcripts are underlined. The putative poly(A) signal for the UL8 transcript, located on the complementary (compl.) strand, is indicated.

At 8 h p.i., the cells were fixed with 3% paraformaldehyde, subjected to membrane permeabilization with 0.2% Triton X-100, and sequentially incubated with the anti-UL7 serum or preimmune serum and DTAF-conjugated goat anti-rabbit immunoglobulin G (Dianova).

Single step growth curves. MDBK-Bu100 cells were infected with 10 PFU per cell and incubated for 2 min with citrate buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl, [pH 3.0]) at 2 h p.i. to inactivate virus that had not penetrated into the cells. At the times indicated, supernatants were removed and stored at −70°C. Cells were washed with phosphate-buffered saline (PBS), incubated with citrate buffer for 2 min to inactivate cell-associated extracellular virions, washed again with PBS, and harvested by low-speed centrifugation after trypsinization. Cell pellets were resuspended in 1 ml of cell culture medium and stored at −70°C. Cells and supernatants were thawed and sonicated at 100 W in a Branson ultrasonic water bath. Serial dilutions were titrated on MDBK-Bu100 cells.

Nucleotide sequence accession number. The sequence reported in this article has been submitted to GenBank and assigned accession no. X91751.

RESULTS

Nucleotide sequence of BHV-1 UL7 gene. Assuming colinearity of the BHV-1 genome and the genome of EHV-1, VZV, or HSV-1, the BHV-1 UL7 gene was expected to be located near the right end of the *Hin*dIII-B fragment of BHV-1 strain Schönböken (Fig. 1a). The entire nucleotide sequence between map units 0.655 (*Eco*RI) and 0.678 (*Hin*dIII), contained within plasmid pB3.0 (Fig. 1b), was determined by the method of Maxam and Gilbert (16). Sequence analysis revealed three ORFs with high degrees of homology to the UL6, UL7, and UL8 ORFs of HSV-1 and corresponding homologs of EHV-1 and VZV. Their arrangement was identical to that reported recently for the BHV-1 Cooper strain (32). The *Hin*dIII-*Eco*RI fragment encompasses the complete UL7 gene, and the UL7 ORF extends from an ATG at positions 446 to 448 to a translational stop codon TGA at positions 1346 to 1348 (Fig. 2). The deduced amino acid sequence showed 43.3 (60.1), 41 (61.4), and 29.6% (48.8%) identity (similarity) to the predicted UL7 proteins of VZV, EHV-1 and HSV-1, respectively. A polyadenylation consensus sequence AATAAA (20) is located downstream from the ORF between nucleotide (nt) 1407 and 1412. The sequence motif GGTTGTA, 21 nt downstream from the poly(A) signal, shows similarity to the consensus sequence YGTGTTYY which is often found at mRNA 3′ ends (1, 20). Between nt 295 and 414, 17 copies of the hexanucleotide AGC CGC, interrupted after 13 repetitions by a unique segment of 17 bp (nt 374 to 390), are found. The sequence TTTAAAA AAA, located at positions 178 to 187, is preceded by an SP1 binding site (GGGGCGGTGT [2]) and could represent the TATA box of the UL7 promoter. The sequence shown in Fig. 2 also contains the codons for the 162 carboxy-terminal amino acids of the UL6 ORF which terminates in codon 15 of the UL7 ORF at positions 487 to 489. The probable polyadenylation signal for the BHV-1 UL8 gene is located upstream from the UL7 poly(A) signal between nt 1371 and 1366 on the complementary strand (indicated in Fig. 2).

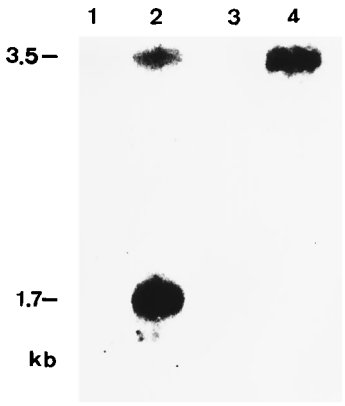

FIG. 3. Identification of transcripts encompassing UL7 ORF. Cytoplasmic RNA was isolated from noninfected cells (lanes 1 and 3) or at 4 h (lanes 2 and 4) after infection of MDBK-Bu100 cells with BHV-1 and transferred to nitrocellulose filters after agarose gel electrophoresis. The filters were hybridized to ^{32}P-labeled probes from the UL7 (lanes 1 and 2) or UL6 (lanes 3 and 4) ORF as described in Materials and Methods. The bound radioactive fragments were visualized by autoradiography. Sizes of the transcripts are indicated on the left.

Northern (RNA) blot analysis. From the sequence analysis it was expected that the UL6 and UL7 transcripts be 3′ coterminal and thus that at least two mRNAs encompass the UL7 ORF. To identify these transcripts, cytoplasmic RNA was isolated from BHV-1-infected cells at 4 h p.i., size separated by agarose gel electrophoresis, and transferred to nitrocellulose. A ^{32}P-labeled DNA probe representing the UL7 ORF identified two RNAs of 1.7 and 3.5 kb (Fig. 3, lane 2). A single-stranded ^{32}P-labeled cRNA probe complementary to the coding sequences of UL6 and UL7 hybridized to both the 1.7- and 3.5-kb transcripts. No specific signals were obtained with ^{32}P-labeled cRNA transcribed in the sense direction (data not shown). Hybridization with a ^{32}P-labeled probe from the UL6 ORF hybridized to the 3.5-kb RNA but not to the 1.7-kb transcript (Fig. 3, lane 4). These results are in good accordance with the predictions from the sequence analysis and led to the conclusion that the 1.7-kb RNA represents the UL7 mRNA whereas the 3.5-kb RNA constitutes a UL6- and UL7-encompassing structural bicistronic transcript.

To determine the kinetics of the steady-state levels of the UL7 and UL6 mRNAs, cytoplasmic RNA was isolated from BHV-1-infected cells at different times after infection and analyzed by Northern blotting. Hybridizations with ^{32}P-labeled DNA from the UL7 ORF revealed that the 1.7- and 3.5-kb RNAs were detected at 3 h p.i. and increased in abundance until 5 to 6 h p.i. (Fig. 4). Thereafter, signals generated by both RNAs weakened. In contrast to a 3.5-kb RNA which was reexpressed at 14 h p.i., the 1.7-kb transcripts were no longer detectable after 10 h p.i. Hybridizations with ^{32}P-labeled UL7 sense and antisense cRNAs showed that also the late-expressed 3.5-kb RNA originates from the UL6 and UL7 genes (data not shown). Thus, the presence of UL7 mRNA in the cytoplasm of infected cells appears to be restricted to the early phase of infection, whereas the putative UL6-UL7 structural bicistronic transcript is also present during the late phase. Beginning at 8 h p.i. the hybridization probe detected additional transcripts of 2.1, 4.2, 5.2, and 6.3 kb, the origin of which was not further analyzed.

Determination of 5′ end of UL7 transcripts. To locate the 5′ end of the UL7 mRNA, nuclease S1 analysis was performed. A 608-nt DNA fragment, which spans the putative UL7 promoter region was 5′ end labeled at an *Ava*II cleavage site at position 601. This fragment includes 601 nt of BHV-1 DNA, extending from the *Hin*dIII site to the *Ava*II site (Fig. 1), and 7 nt from the cloning vector in front of the *Hin*dIII site. Labeled DNA was hybridized to cytoplasmic RNA isolated from infected cells at 6 and 14 h p.i., and nuclease S1-resistant fragments were visualized by autoradiography after denaturing acrylamide gel electrophoresis. As shown in Fig. 5, lane 2, two fragments were protected by RNA isolated early in infection. The 396-nt fragment indicates a transcription start site at position 206, 19 bp downstream from the putative TATA box. The 601-nt fragment protected by early RNA corresponds to the full length of the labeled viral sequences and most probably results from protection by the overlapping UL6 transcript. The same fragment was protected by RNA from cells harvested at 14 h p.i., whereas no signal corresponding to the 396-nt fragment was detected. Although analysis to determine which of the late transcripts detected by the UL7 probes in Northern blots contribute to the protection of the 601-nt fragment has not been done, this result confirms our finding that the UL7 gene is not substantially transcribed into stable mRNA during the late phase of the infection and supports the conclusions drawn from the Northern blot analyses.

A comparison of the predicted promoter region of BHV-1 UL7 with corresponding sequences of HSV-1, EHV-1, and VZV is depicted in Fig. 6. The alignment shows a high conservation of the nucleotide sequence. An even higher degree of homology is found among the deduced amino acid sequences, with considerable divergence only within the transcription factor SP1 binding site consensus sequence preceding the putative

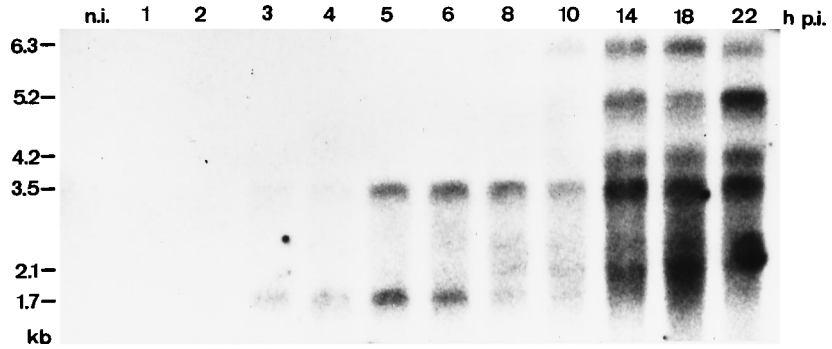

FIG. 4. Expression kinetics of UL7 transcripts. MDBK-Bu100 cells were infected with BHV-1, and cytoplasmic RNA was isolated at the indicated times after infection. After agarose gel electrophoresis and transfer to nitrocellulose filters, RNA was hybridized to a ^{32}P-labeled probe from the UL7 ORF. The bound radioactive fragments were visualized by autoradiography. Transcript sizes are indicated on the left. n.i., no infection.

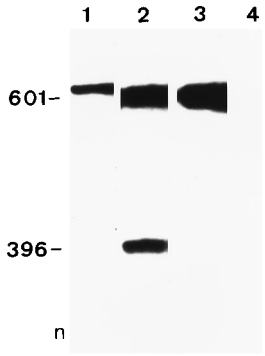

FIG. 5. Localization of UL7 mRNA start site. The 5′ end of the UL7 transcript was determined by nuclease S1 analysis. Lanes: 1, 608-nt fragment before nuclease treatment; 2 and 3, nuclease-resistant fragments after hybridization to RNA from infected cells isolated 6 and 14 h p.i., respectively; 4, reaction products after hybridization to RNA from noninfected cells. The 5′-end-labeled *Hpa*I restriction fragments of pBR322 (with sizes [in nucleotides] indicated on the left) and 123- and 1-kbp ladders (GIBCO-BRL, Eggenstein, Germany) were used as size markers. Fragment sizes (in nucleotides [n]) are on the left.

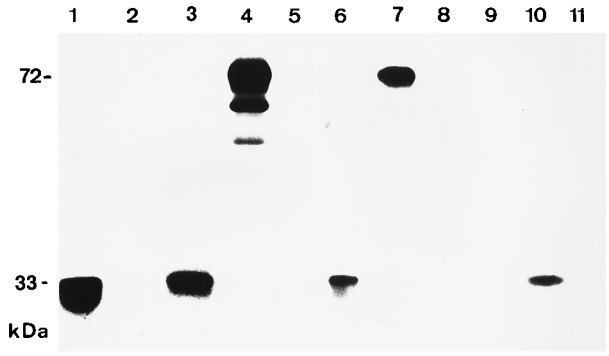

FIG. 7. Reactivity of polyclonal anti-UL7 serum with proteins from in vitro translation of in vitro-transcribed UL7 RNA, from infected cells, and from purified virions. ^{35}S-labeled proteins that were in vitro translated from in vitro-transcribed UL7 cRNA (lanes 1 to 3), from infected cells (lanes 4 to 6), or from purified virions (lanes 7 to 9) were incubated with a rabbit anti-UL7 serum against a bacterially expressed fusion protein (lanes 3, 6, and 9), the corresponding preimmune serum (lanes 2, 5, and 8), or with the gD-specific MAb 21/3/3 as a control (lanes 4 and 7). Precipitated proteins were analyzed by SDS-PAGE and fluorography. Lane 1 shows total in vitro translation products. Lanes 10 and 11 demonstrate reactivity of the anti-UL7 serum in immunoblots with proteins from infected cells (lane 10) and purified virions (lane 11) after SDS-PAGE and transfer to nitrocellulose. The apparent molecular masses of the proteins are indicated on the left.

TATA box. This might indicate an evolutionary pressure on protein sequence preservation, since the UL7 promoter region is contained within the UL6 ORF.

Identification of UL7-encoded protein. To identify the UL7 gene product, a rabbit antiserum (anti-UL7) was raised against an *E. coli*-expressed TrpE-UL7 fusion protein. The anti-UL7 serum and the corresponding preimmune serum were incubated with [^{35}S]methionine-labeled proteins in vitro translated from in vitro-transcribed cRNA (Fig. 7, lanes 1 to 3), from infected cells (Fig. 7, lanes 4 to 6), or from purified virions (Fig. 7, lanes 7 to 9). In vitro translation of the UL7 ORF results in a polypeptide with an apparent molecular mass of 33 kDa which is recognized by the anti-UL7 serum (Fig. 7, lane 3) but not by the preimmune serum (Fig. 7, lane 2). Inclusion of canine pancreatic membranes during in vitro translation had no effect on the mobility of the 33-kDa protein (data not shown). From infected cells, the immune serum specifically precipitated a protein with a size of 33 kDa (Fig. 7, lane 6) which was not found in purified virions (Fig. 7, lane 9). Similar reactivity was observed when the anti-UL7 serum was used to detect proteins from infected cells or from purified virions after SDS-PAGE and transfer to nitrocellulose (Fig. 7, lanes 10 and 11). Immunoprecipitations with the gD-specific monoclonal antibody (MAb) 21/3/3 demonstrated the presence of labeled gD in both infected cells and purified virions (Fig. 7, lanes 4 and 7). The migration of the 33-kDa protein in SDS-polyacrylamide gels was independent of the presence of reducing agents in the sample buffer, indicating that this protein does not form covalently linked homo- or heterooligomers (data not shown).

From these results, we conclude that the 33-kDa protein represents the UL7 gene product and that the UL7 protein is not a (major) constituent of virus particles. The apparent molecular mass is in close agreement with the M_r of 32 predicted from the deduced amino acid sequence.

Expression kinetics of UL7 protein. From the Northern blot analyses and nuclease S1 protection experiments, it was concluded that the UL7 promoter does not direct transcription of stable mRNAs during the late phase of infection. To elucidate the expression kinetics of the UL7 protein, infected cells were harvested at different times p.i., and proteins were transferred to nitrocellulose after SDS-PAGE. Filters were probed with the anti-UL7 serum. Figure 8 shows that the UL7 protein was first detectable in cell lysates at 4 h p.i., increased in intensity until 8 h p.i., and remained at a similar level until 16 h p.i., indicating stability of the 33-kDa protein.

To support this conclusion and to exclude substantial expression of the UL7 protein by mRNAs larger than the 1.7-kb transcript, infected cells were labeled with [^{35}S]methionine-cysteine from 2 to 10 h p.i. and either harvested or further incubated without ^{35}S-amino acids until 20 h p.i. In parallel, infected cell proteins were labeled from 10 to 20 h p.i. Immunoprecipitations were done with anti-UL7 serum or gD-specific MAb 21/3/3. In contrast to gD, which was abundantly expressed between 10 and 20 h p.i. (Fig. 9, lane 6), no labeled

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Sp1 binding site      KGGGCGGRRY
                                                    ↓
BHV-1   GAGGAGGAGCTATGCGGGGCGGTGTTTAAAAAAACGCGCCTGGAGACGTACCTC
                                                    ↓
HSV-1   GAGGAGGAGTTATGGGATGCGGTGTTTAAGAAAACCCGCCTGCAAACGTACCTG
EHV-1   GAGGAGGAGCTGTGTGTGGCGGTGTTCAAAAAAACCCGGCTCGAGGCTTATCTT
VZV     GAGGAAGAGTTATGCCAGGCTATTTTTAAAAAGGCGAGAACAGAGTCCTATTTA
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FIG. 6. Conservation of UL7 promoter regions. Sequences flanking the probable UL7 TATA boxes of BHV-1 and HSV-1 (19, 24) and the corresponding regions of VZV (3) and EHV-1 (31) are aligned, and codons for identical amino acids are shaded. The transcription initiation sites for BHV-1 and HSV-1 (24) are marked by arrows. The consensus sequence for SP1 binding (2) is aligned to a nearly identical sequence in front of the postulated BHV-1 UL7 TATA box. The code for alternate bases is K for T or G, R for A or G, and Y for T or C.

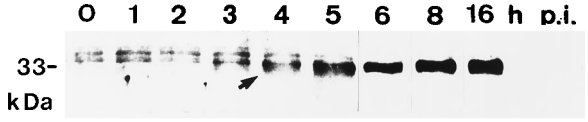

FIG. 8. Expression kinetics of UL7 protein. MDBK-Bu100 cells were infected with BHV-1, and cultures were lysed at the times indicated. Proteins were analyzed by immunoblotting with the anti-UL7 serum. The arrowhead points to a barely visible protein band at 4 h p.i. which is not detectable at earlier times. Proteins from cells lysed after 5 h were run on a separate gel. The enhanced chemiluminescence exposure time was identical for all samples.

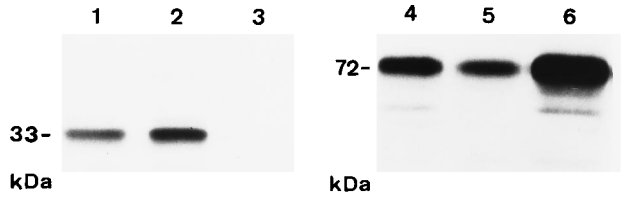

FIG. 9. Stability of UL7 protein. MDBK-Bu100 cells were infected, labeled with [^{35}S]methionine-cysteine from 2 to 10 h p.i. (lanes 1 and 4) and from 2 to 10 h p.i., and further incubated without radioactive amino acids until 20 h p.i. (lanes 2 and 5) or from 10 to 20 h p.i. (lanes 3 and 6). Cells were lysed, and proteins were immunoprecipitated by using the anti-UL7 serum (lanes 1 to 3) or gD specific MAb 21/3/3 (lanes 4 to 6). Labeled proteins were visualized by fluorography after SDS-PAGE. The molecular masses of the proteins are indicated on the left.

33-kDa protein could be detected (Fig. 9, lane 3). On the other hand, both the UL7 protein and gD, labeled from 2 to 10 h p.i. (Fig. 9, lanes 1 and 4), remained detectable without signs of degradation after the 10-h chase period (Fig. 9, lanes 2 and 5), supporting the conclusion that the UL7 protein is stable.

Subcellular distribution of UL7 protein. The subcellular distribution of the 33-kDa protein was examined by indirect immunofluorescence. The anti-UL7 serum reacted with antigens localized predominantly in the cytoplasm of infected cells, which appear to accumulate around the nucleus (Fig. 10, right panel). Probing of infected cells with the preimmune serum resulted in background fluorescence (Fig. 10, left panel).

UL7 is dispensable for BHV-1 replication in cell culture. To determine whether UL7 is required for productive replication, DNA from the gD$^-$ *lacZ*$^+$ BHV-1 mutant BHV-1/80-221 (8) was cotransfected with plasmid pΔ7gD into MDBK-Bu100 cells. Since BHV-1/80-221 cannot productively replicate in MDBK-Bu100 cells, generation of infectious virus requires recombination of the gD gene into the BHV-1/80-221 genome. As can be deduced from Fig. 1, homologous recombination of pΔ7gD should result in the replacement of codons 5 to 300 of the UL7 ORF, of the last 9 codons of the UL6 ORF, and of 77 3′ codons of the UL8 ORF by the ie1-gD cassette. Infectious progeny from the cotransfection was repeatedly plaque purified. One of the isolates that did not bind to a labeled UL7-specific probe in dot blot hybridizations, named BHV-1/ΔUL7, was used for further characterization. Figure 11 shows the results of genome analysis of wild-type BHV-1 (lanes 1 and 2), BHV-1/80-221 (lanes 3 and 4), and BHV-1/ΔUL7 (lanes 5 and 6) cleaved with *Hin*dIII (lanes 1, 3, and 5) and *Hin*dIII-*Xba*I (lanes 2, 4, and 6). Cleaved DNA from virus-infected cells was separated in 0.6% agarose gels and transferred to nitrocellulose filters. Panel a shows the ethidium bromide stained gel. Hybridization with the ^{32}P-labeled UL7 probe that bound to the 20-kbp *Hin*dIII-B fragment of wild-type BHV-1 and BHV-1/80-221 (Fig. 11b, lanes 1 to 4) confirmed the absence of the UL7 ORF in BHV-1/ΔUL7 (Fig. 11b, lanes 5 and 6). The gD probe detected the gD gene within the expected 8-kbp fragment of wild-type BHV-1 (Fig. 11c, lanes 1 and 2) did not hybridize to BHV-1/80-221 DNA (Fig. 11c, lanes 3 and 4) but bound to a 21.7-kbp *Hin*dIII fragment of BHV-1/ΔUL7 (Fig. 11c, lane 5). The size of this fragment is as expected, since replacement of the UL7 ORF by the ie1-gD expression unit within the 20-kbp *Hin*dIII-B fragment should result in an increase in size of about 1.7 kbp. After cleavage of BHV-1/ΔUL7 DNA with *Hin*dIII-*Xba*I, the gD probe hybridized exclusively to the 1.3-kbp fragment that contains the entire gD ORF (Fig. 11c, lane 6). Hybridization of BHV-1/ΔUL7 DNA with labeled DNA from the MCMV-ie1 promoter after cleavage with *Hin*dIII and *Hin*dIII-*Xba*I showed hybridization to the 21.7-kbp *Hin*dIII fragment (Fig. 11d, lane 5) and to a 2-kbp *Hin*dIII-*Xba*I fragment (Fig. 11d, lane 6) containing the MCMV-ie1 enhancer-promoter and adjacent sequences from the *Hin*dIII-B fragment provided in plasmid pΔ7gD for homologous recombination. We conclude that the UL7 ORF in BHV-1/ΔUL7 had been deleted and that sequences upstream and downstream from the envisaged mutation were not visibly affected.

Staining of BHV-1/ΔUL7-infected cells for the determination of β-galactosidase activity demonstrated that the *lacZ* gene was not impaired by the recombination. Thus, BHV-1/ΔUL7 is UL7$^-$ LacZ$^+$ ie1 gD$^+$.

BHV-1/ΔUL7 does not express the 33-kDa UL7 protein. Immunoprecipitations of ^{35}S-labeled proteins from wild-type

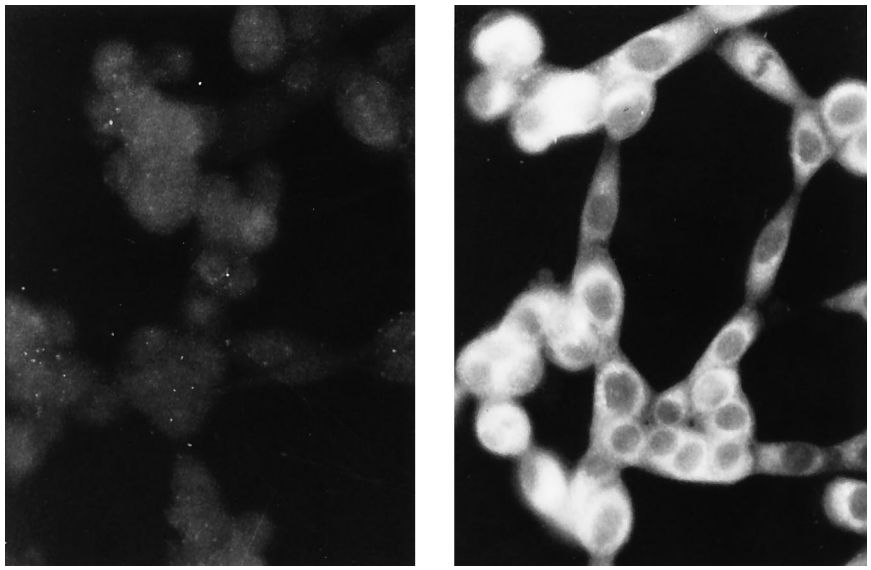

FIG. 10. Intracellular distribution of UL7 protein. Subconfluent MDBK-Bu100 cells were used at 8 h p.i. for indirect immunofluorescence with the anti-UL7 serum (right panel) or the matching preimmune serum (left panel) and stained with DTAF-conjugated goat anti-rabbit immunoglobulin G.

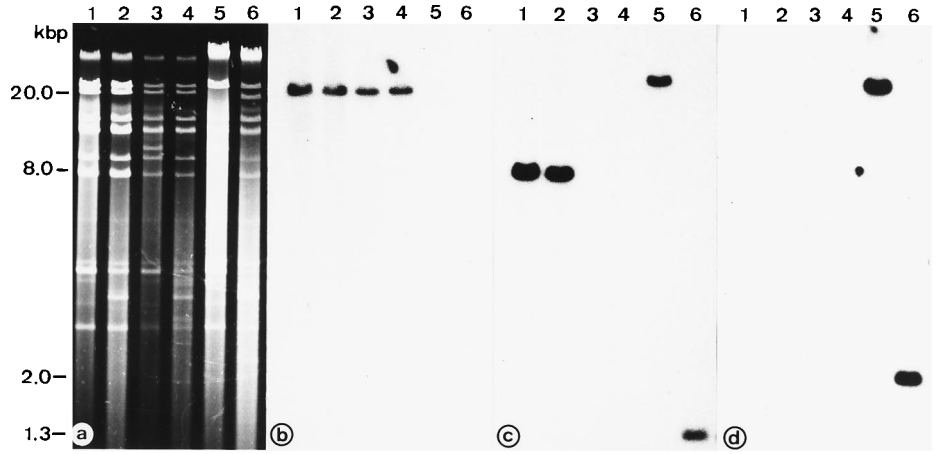

FIG. 11. The UL7 ORF is replaced by the ie1-gD cassette in BHV-1/ΔUL7. MDBK-Bu100 cells were infected with wild-type BHV-1 (lanes 1 and 2), gD$^-$ BHV-1/80-221 (lanes 3 and 4), or BHV-1/ΔUL7 (lanes 5 and 6). Whole-cell DNA was prepared 20 h p.i. and cleaved with *Hin*dIII (lanes 1, 3, and 5) or *Hin*dIII-*Xba*I (lanes 2, 4, and 6). After separation in a 0.6% agarose gel, fragments were stained with ethidium bromide (a), transferred to nitrocellulose filters and hybridized with ^{32}P-labeled DNA from the UL7 ORF (b), gD ORF (c), or MCMV-ie1 promoter (d). The bound radioactive fragments were visualized by autoradiography. Fragment sizes are indicated on the left.

(Fig. 12, lanes 1 and 3) and mutant BHV-1 (Fig. 12, lanes 2 and 4)-infected cells with the anti-UL7 serum (Fig. 12, lanes 3 and 4) or gD-specific MAb 21/3/3 (Fig. 12, lanes 1 and 2) demonstrated expression of gD and the absence of the 33-kDa protein after infection with BHV-1/ΔUL7 (Fig. 12, lanes 2 and 4) and further confirmed the conclusion that this protein is the UL7 gene product.

Single step growth curve of BHV-1/ΔUL7. The isolation and repeated plaque purification of BHV-1/ΔUL7 on noncomplementing MDBK-Bu100 cells (Fig. 13) as well as infectious replication of the mutant in primary bovine embryonic lung, embryonic kidney, and embryonic heart cells infected with a multiplicity of infection of 0.1 (data not shown) demonstrated that the UL7 ORF is not required for growth of BHV-1 in cultured cells. The replication behavior of BHV-1/ΔUL7 in MDBK-Bu100 cells compared with that of wild-type BHV-1 is illustrated in Fig. 13, in which titers of intracellular and released infectious virus are depicted. MDBK-Bu100 cells in 3.5-cm-diameter dishes were infected with 10 PFU per cell, and cell-associated extracellular virions were inactivated at 2 h p.i. by low-pH treatment to assure that only progeny virus contributed to extracellular infectivity. At the times indicated,

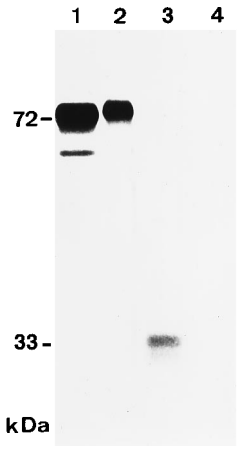

FIG. 12. Lack of UL7 expression in BHV-1/ΔUL7-infected cells. MDBK-Bu100 cells were infected with wild-type BHV-1 (lanes 1 and 3) and BHV-1/ΔUL7 (lanes 2 and 4). [^{35}S]methionine-cysteine was added at 2 h p.i., and labeled proteins were immunoprecipitated from cells lysed at 10 h p.i. by using the gD-specific MAb 21/3/3 (lanes 1 and 2) or the anti-UL7 serum (lanes 3 and 4). The apparent molecular masses of the proteins are indicated on the left.

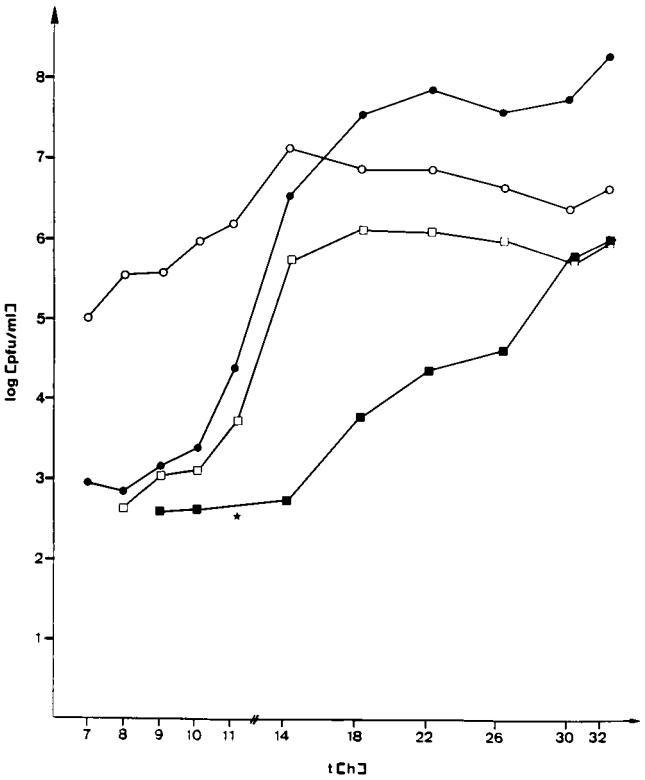

FIG. 13. Release of BHV-1/ΔUL7 from infected cells is impaired. MDBK-Bu100 cells were infected with wild-type BHV-1 (circles) and BHV-1/ΔUL7 (squares) at a multiplicity of infection of 10. The culture medium (closed symbols) and the cells (open symbols) were collected at the indicated times after infection, and infectivity was determined by double titration. The star denotes that no plaque was detected in the culture medium at 11 h p.i., indicating that the titer was below the detection limit of 10^2 PFU/ml. Data from a representative experiment are shown.

the amounts of intracellular infectious virus and released infectivity in the culture medium were determined independently. The overall pattern of intracellular infectivity for the mutant virus was similar to that found for wild-type BHV-1, although infectious virus was detected 1 h later and a lower infectious titer was obtained throughout the time course. Significant differences were observed regarding the appearance of progeny virus in the culture. In comparison with wild-type BHV-1, released BHV-1/ΔUL7 virions appeared only at later time points. In addition, already at 18 h p.i., infectivity of wild-type BHV-1 in the supernatants surpassed that found in infected cells, whereas a similar ratio was obtained by the mutant virus only after 30 h p.i. This could be indicative of a release defect of the BHV-1/ΔUL7 mutant virus, although the truncations of the 3′ codons of the UL6 and UL8 ORFs might also contribute to this phenotype. Nevertheless, these results show that BHV-1/ΔUL7 is able to productively replicate in MDBK-Bu100 cells, which confirms that UL7 is not essential for propagation of BHV-1 in cell culture.

DISCUSSION

The UL7 gene of BHV-1 strain Schönböken was localized and characterized, and the gene product was identified. The results indicate that the gene organization of BHV-1 Schönböken in this region is identical to that of the prototype alphaherpesvirus HSV-1 and to the corresponding region of the BHV-1 Cooper strain (32). The BHV-1 Schönböken UL7 ORF encodes a polypeptide of 300 amino acids whereas Vlček et al. recently reported a 299-amino-acid UL7 protein for BHV-1 Cooper (32). Detailed comparisons between these two BHV-1 strains have to be postponed because the sequence of BHV-1 Cooper is not yet available to the public (32). Alignment of the deduced amino acid sequences of the UL7 proteins from BHV-1, HSV-1, EHV-1, and VZV showed considerable homology. The highest degree of identity was found between the UL7 proteins of BHV-1 and VZV (43.3%), followed by EHV-1 (41%) and HSV-1 (29.6%).

Northern blot analyses and nuclease S1 protection experiments showed that transcription of the 1.7-kb UL7 mRNA initiates 19 bp downstream from the sequence motif TTAAA AAAA which is preceded by the decanucleotide GGGGCG GGTGT, a putative binding site for the transcription factor SP1 (2). So far, HSV-1 is the only other alphaherpesvirus for which UL7 transcription has been characterized, and Patel and MacLean (24) determined the 1.3-kb HSV-1 UL7 mRNA transcription initiation site at exactly the same position as we report for the BHV-1 UL7 transcripts. Similar to the situation in HSV-1 (24), the UL7 gene of BHV-1 is also contained within the UL6-UL7 structural bicistronic transcript, which probably coterminates at the polyadenylation signal downstream from the UL7 ORF. Nuclease protection experiments to precisely locate the poly(A) addition site of the UL6 and UL7 mRNA invariably resulted in protected 3′-end-labeled fragments with sizes placing an RNA discontinuity immediately upstream from the putative UL6-UL7 poly(A) signal. On the basis of the size, it appears unlikely that the UL7 mRNA does not contain a poly(A) tract. Because this sequence does not encompass a splice donor motif (23), we concluded that insufficient stability of the heteroduplexes due to AT-rich sequences around the poly(A) signal (Fig. 2) resulted in overdigestion of the DNA-RNA hybrids (25). Thus, the exact 3′ end of the BHV-1 UL6 and UL7 mRNAs remains to be determined, as does the corresponding poly(A) addition site for HSV-1 (24). Analysis of the steady-state levels of UL6 and UL7 transcripts in the cytoplasm of infected cells revealed that both transcripts are expressed early after infection. In contrast to the UL6 RNAs, the UL7 transcripts are no longer detected after about 10 h p.i. Both RNAs are expressed in the presence of phosphonoacetic acid and were not detected when de novo protein synthesis following infection was inhibited by cycloheximide (data not shown). Thus, the UL6 gene can be classified as a delayed-early gene whereas the UL7 gene appears to be a true early gene. The latter conclusion is supported by protein expression experiments which gave no indication that the UL7 protein might be newly synthesized after 10 h p.i. Therefore, the regulation of the BHV-1 UL7 gene expression is different from that of the HSV-1 UL7 gene, which was classified as delayed early (24). It is tempting to speculate that this difference is correlated with the sequence divergence in the putative transcription factor SP1 binding site upstream from the UL7 TATA box of HSV-1.

In this report, the first demonstration of a UL7-encoded protein is presented. The polypeptide was identified by using a rabbit polyclonal antiserum against an *E. coli*-expressed TrpE-UL7 fusion protein. This serum detected a 33-kDa protein in BHV-1-infected cells but did not react specifically with proteins from purified virions. Within BHV-1-infected cells, the UL7 protein is localized in the cytoplasm and accumulates around the nucleus. The migration of the UL7 polypeptide in SDS-polyacrylamide gels was independent from the presence of reducing agents, demonstrating that it does not form covalently linked homo- or heterooligomers.

In vitro translation with or without inclusion of canine pancreatic microsomal membranes of in vitro-transcribed UL7 mRNA also resulted in a 33-kDa protein which was recognized by the anti-UL7 serum, suggesting that the first AUG of the predicted UL7 ORF is used for initiation of translation in infected cells and that the UL7 protein does not undergo major co- or posttranslational modifications. Immunoprecipitations of proteins from cells infected in presence of $^{32}P_i$ indicated that the 33-kDa protein might be phosphorylated. However, signals were weak, and a more detailed analysis is required to support this observation.

Isolation of an infectious UL7 deletion mutant BHV-1/ΔUL7 on noncomplementing MDBK-Bu100 cells and their infectious replication on primary bovine embryonic cells show that the UL7 protein is not required for productive replication of BHV-1 in cell culture. This appears to contrast with the situation in HSV-1 in which UL7 was proposed to be an essential gene (33). Determination of the biological properties of BHV-1/ΔUL7 in cell culture showed that the mutant grows to lower titers than wild-type BHV-1 and that release of infectious virions is impaired. However, because of the genotype of the mutant virus, it is unclear whether this phenotype is due to the absence of the UL7 gene or might be due to concomitant deletion of nine 3′ codons of the UL6 ORF and 77 amino acids from the carboxy-terminal end of the UL8 protein.

To date the functional properties of BHV-1 UL7 and its homologs are unknown (24, 32). Searching the data bank sequences by using the Genetics Computer Group TFasta program for the nucleotide sequence and the Genetics Computer Group Wordsearch program for the amino acid sequence did not yield any clues. Computer-aided analysis of the deduced amino acid of the BHV-1 UL7 protein showed that it contains 49 negatively charged and 31 positively charged amino acids, resulting in an predicted isoelectric point of 5.78 for the entire protein. Charged groups, however, are not evenly distributed within the protein, which has an isoelectric point of 4.04 for amino acids 1 to 160 and an isoelectric point of 11.26 for amino acids 161 to 300. A similar bipolarity has been described, e.g., for the ORF4 protein of potato leafroll luteovirus that binds to

single-stranded nucleic acids (29) and oligomerizes via an amphipathic alpha-helix in the acidic amino terminus (30). It has to be tested whether the BHV-1 UL7 protein exhibits similar properties.

In summary, our results show that the BHV-1 UL7 ORF is contained in an early mRNA of 1.7 kb and translated into a 33-kDa protein which is not essential for replication. BHV-1 UL7 deletion mutants should help in further analyses of UL7 function(s) and will be used to elucidate whether the UL7 homologs of alphaherpesviruses, thus far defined by genome location and sequence similarity, are also functional homologs.

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