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Pseudorabies Virus and Equine Herpesvirus 1 Share a Nonessential Gene Which Is Absent in Other Herpesviruses and Located Adjacent to a Highly Conserved Gene Cluster

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We have determined the nucleotide sequence and transcriptional pattern of a group of open reading frames in the pseudorabies virus (PrV) genome located near the left end of the unique long region within *Bam*HI 5′ fragment at map positions 0.01 to 0.06. The 7,412-bp *Bam*HI 5′ fragment was found to contain five complete open reading frames and part of a sixth whose deduced amino acid sequences showed homology to the UL50 (partial), UL51, UL52, UL53, and UL54 gene products of herpes simplex virus type 1 (HSV-1) and corresponding genes identified in other alphaherpesviruses. Homologs to the UL55 and UL56 genes of HSV-1 were not detected. However, we identified a gene with homology only to the first open reading frame (ORF-1) of the equine herpesvirus 1 strain Ab4 (E. A. Telford, M. S. Watson, K. McBride, and A. J. Davison, Virology 189:304–316, 1992). Northern blot analyses revealed unique mRNAs for the UL51, UL54, and ORF-1 genes and a set of 3′-coterminal mRNAs for the UL52 to UL54 genes. A PrV mutant lacking ORF-1 was isolated after deletion of ORF-1 coding sequences and insertion of a *lacZ* expression cassette. The ORF-1$^-$ PrV mutant was able to productively replicate in noncomplementing cells to levels similar to those of wild-type PrV, proving that ORF-1 is not essential for replication of PrV in cell culture. The conservation of this gene between PrV and equine herpesvirus 1 documents the close evolutionary relationship between these animal herpesviruses and points to a possible function of the respective proteins in infection of the natural host.

Pseudorabies virus (PrV), the causative agent of Aujeszky's disease, which causes severe financial losses in the pig industry, belongs to the alphaherpesvirus subfamily of the *Herpesviridae* (reviewed in reference 45). Within the *Alphaherpesvirinae*, it is grouped in the genus *Varicellovirus* together with the human pathogen varicella-zoster virus (VZV) and other animal pathogens, such as equine herpesviruses 1 and 4 (EHV-1 and EHV-4) and bovine herpesvirus 1. Comparison of nucleic acid or protein sequences showed that this genus is separable from the *Simplexvirus* genus, which comprises, among others, herpes simplex viruses type 1 and 2 (HSV-1 and HSV-2) (41, 54).

Although the complete sequence of the PrV genome has yet to be elucidated, it is apparent that gene arrangement is mainly collinear to that in other sequenced alphaherpesvirus genomes, i.e., VZV (15), EHV-1 (62), and HSV-1 (42). A peculiarity of the PrV genome is the presence of a large inversion in the middle of the unique long (U_L) region compared with the other alphaherpesvirus genomes (4). Although most of the genes appear to be conserved between VZV, EHV-1, and HSV-1, differences are observed. A region of divergence is located at one end of the U_L region where the UL56 gene appears to be unique to HSV-1, whereas homologs to EHV-1 genes 2 and 3 are found in VZV but not in HSV-1. The first open reading frame (ORF-1) detected in EHV-1 strain Ab4 was described to be unique for EHV-1 (62). Given the high conservation of gene structure, the differences in biological behavior of the different viruses are surprising. PrV, for example, is able to lethally infect all mammals except higher primates including humans, whereas HSV-1 is a natural pathogen only for humans. PrV and HSV-1 both grow lytically in culture in a wide variety of cells. However, pig cells are highly susceptible to PrV but only poorly infected by HSV-1, whereas human cells are largely refractive to infection by PrV while efficiently supporting HSV-1 replication (24, 60, 65).

Since heterogeneity within the complement of genes appears to reside at one end of the U_L region and to continue towards the completion of the PrV genomic sequence, we analyzed the coding content of the *Bam*HI 5′ fragment located near the left end of the PrV U_L region. Our data show the presence of a conserved gene cluster containing ORFs homologous to the UL50 through UL54 genes of HSV-1 and corresponding genes in other alphaherpesviruses. The PrV genome, however, at this position lacks homologs of HSV-1 UL55 and UL56 but specifies an additional gene which is separated from the conserved gene block by a cluster of repeated elements. The deduced translation product shows amino acid homology and comparable secondary structure to the ORF-1 gene product of EHV-1. Conservation of this gene between these two animal pathogens points to a close evolutionary relationship. Neither gene is essential for replication of the respective virus in cell culture, which indicates a possible functional role of the gene products for replication in the natural host (61).

MATERIALS AND METHODS

Cells and virus. PrV strain Kaplan (Ka) (33) was grown on pig kidney (PSEK), bovine kidney (MDBK), or Vero cells cultivated in minimum essential medium with 5% fetal calf serum. Cotransfections were performed by using the calcium phosphate coprecipitation technique (25).

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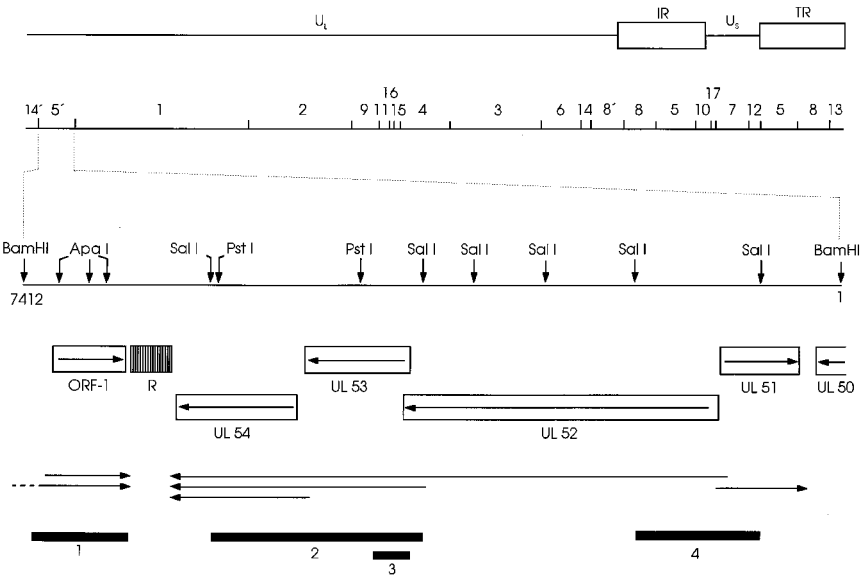

FIG. 1. Gene organization in PrV *Bam*HI 5′ fragment. A *Bam*HI restriction fragment map is shown beneath a schematic diagram of the PrV genome. Open boxes represent inverted repeat regions (IR and TR) that bracket the unique short (U_S) region and separate it from the U_L part. Enlarged is the *Bam*HI 5′ region of the PrV genome. Also shown are relevant restriction enzyme cleavage sites. The locations and orientations of the identified ORFs are indicated, as is the position of the repeat cluster (R). The arrows indicate the locations and transcriptional direction of the identified mRNAs. Closed boxes show the positions of hybridization probes.

Plasmids and sequence determination. Plasmid pBR325-5′ containing *Bam*HI 5′ fragment of PrV Ka was kindly provided by T. Ben-Porat, Nashville, Tenn. The 5′ fragment was recloned into the *Bam*HI site of plasmid TN77, a pBR322 derivative containing the polylinker site of phage M13mp18 (45), giving rise to plasmid TN5′. For further subcloning, TN5′ was digested with *Bam*HI and *Pst*I, resulting in fragments of 4.3 kb (*Bam*HI-*Pst*I), 1.8 kb (*Bam*HI-*Pst*I), and 1.3 kb (*Pst*I-*Pst*I). Fragments were separated on a 0.8% agarose gel, and *Bam*HI-*Pst*I fragments were subcloned into *Bam*HI-cleaved TN77 after fill-in of noncompatible overhangs. The 1.3-kb *Pst*I fragment was inserted into *Pst*I-cut pUC19, excised with *Eco*RI-*Hin*dIII, and ligated to *Eco*RI-*Hin*dIII-cleaved TN77. Plasmids carrying fragments in both orientations were isolated.

For sequencing, a nested set of overlapping subclones was prepared (28). Sequences were determined by the dideoxy chain termination method (57) with T7 polymerase, using pBR322-specific primers (New England Biolabs, Schwalbach, Germany). Both DNA strands were sequenced at least twice. Sequence ambiguities which resulted from the high G+C content of PrV DNA of 73% (5) were resolved by substituting 7-deaza-2′-deoxy-GTP for dGTP in the sequencing reaction and separating the sequencing products on denaturing polyacrylamide gels containing 40% formamide (48). To verify the orientations of the subfragments and the *Pst*I restriction sites, TN5′ was cleaved with *Sal*I and subcloned in TN77. Again, a nested set of subclones was prepared, and clones containing the junction sites were sequenced.

Sequences were analyzed using the University of Wisconsin Genetics Computer Group software package (18) in VAX/VMS version 7.1. ORFs were detected by using the programs Frames and Codonpreference, and comparison of amino acid sequences was performed with the program Gap. The values given represent the percentages of identical amino acids. The protein secondary structure was predicted according to the method of Chou and Fasman (12).

RNA and DNA isolation and blot hybridizations. For RNA analysis, PSEK cells were infected at a multiplicity of infection of 5 and harvested 1, 2, 3, 4, and 5 h postinfection (p.i.). Cells were lysed, and RNA was isolated as described previously (35). Control RNA was isolated from mock-infected cells. For DNA analysis, DNA was isolated from PrV-infected cells as reported previously (37). Hybridization probes were labeled with [^{32}P]dCTP, and Northern (RNA) and Southern blot hybridizations were performed as described previously (35, 37).

Construction of PrV ORF-1$^-$ mutant. For construction of an ORF-1 mutant, 433 bp of the ORF-1 coding sequence were deleted by *Apa*I digestion of plasmid pSal1 containing the left *Bam*HI-*Sal*I subfragment of the *Bam*HI 5′ fragment. After insertion of *Bam*HI linkers (Gibco-BRL, Eggenstein, Germany), a *Sal*I-*Bam*HI glycoprotein G–β-galactosidase expression cassette was introduced in parallel transcriptional orientation (47) after fill-in of the noncompatible end with Klenow polymerase, resulting in plasmid pORFGAL. Cotransfection of pORFGAL with wild-type PrV DNA resulted in the appearance in the transfection progeny of plaques which stained blue under a Bluo-Gal (Gibco-BRL) agarose overlay. They were picked by aspiration and purified to homogeneity.

One-step growth curve. One-step growth kinetics were established as described previously (37). MDBK cells in 24-well dishes were infected at a multiplicity of infection of 5 with either wild-type PrV or PrV ORF-1$^-$ for 1 h at 4°C. After this attachment period, the inoculum was removed, and the cells were overlaid with prewarmed medium to initiate penetration. Cells and supernatant were harvested immediately and 4, 8, 12, 24, and 36 h thereafter and titrated. The values for intra- and extracellular virus titers were added and plotted.

Nucleotide sequence accession number. The *Bam*HI 5′ fragment sequence identified in this study has been assigned GenBank accession no. X87246.

RESULTS

Nucleotide sequence of *Bam*HI 5′ fragment. After cleavage with *Pst*I, the genomic *Bam*HI 5′ fragment of PrV strain Kaplan was cloned in three subfragments with sizes of 4.3, 1.8, and 1.3 kb and sequenced. For verification of the orientations and locations of the subfragments, appropriate *Sal*I subclones of the *Bam*HI 5′ fragment were sequenced. The *Bam*HI 5′ fragment comprises 7,412 bp, with an overall G+C content of 75% (GenBank accession no. X87246). Numbering starts at the first nucleotide of the *Bam*HI site separating *Bam*HI fragments 1 and 5′ and ends at the last nucleotide of the *Bam*HI site separating fragments 5′ and 14′ (Fig. 1). Computer analyses revealed several ORFs whose deduced amino acid sequences showed significant homology to those of polypeptides predicted to be encoded by the UL50 through UL54 genes of HSV-1 (42) and corresponding homologs in VZV (15) and EHV-1 (62). The deduced translation product of one of the predicted ORFs did not reveal homology to any HSV-1 or VZV protein but only to the ORF-1 gene product of EHV-1 strain Ab4 (62). Properties of the deduced proteins are compiled in Table 1. Genes were named according to their counterparts in HSV-1 (UL50 to UL54) or EHV-1 (ORF-1).

At the extreme right end of the *Bam*HI 5′ fragment, 224 bp of the 3′-terminal part of an ORF were found whose deduced translation product showed homology to the carboxy terminus of the UL50 product of HSV-1 (Table 1). The 5′ end of this ORF is located in the adjacent *Bam*HI fragment 1 (32). HSV-1 UL50 encodes a dUTPase (21, 51), and the deduced PrV UL50 protein also contains dUTPase motifs (32). An ORF in opposite orientation to UL50 is predicted to encode a protein with a size of 25 kDa comprising 236 amino acids (aa) which shows 39% identity to the deduced HSV-1 UL51 protein. A putative

TABLE 1. Properties of ORFs in *Bam*HI 5′ fragment

Designation	Position of[a]:			Bp	No. of amino acids	Molecular mass of deduced protein (kDa)	Homology to (value)[b]:				
	TATA	ATG	Stop codon	Poly(A)			HSV-1	VZV	EHV-1	HCMV	EBV
UL50 (partial)[c]			224	303–308			UL50	Gene 8	Gene 9	UL72	BLLF2
UL51	1257–1253	1088	378	318–313	711	236	25	UL51 (39%)	Gene 7 (42%)	Gene 8 (47%)	UL71 (21%)	BSRF1 (19%)
UL52	1030–1035	1087[d]	3963		2,877	958	103	UL52 (47%)	Gene 6 (45%)	Gene 7 (48%)	UL70 (28%)	BSLF1 (28%)
UL53	3861–3866	3918[d]	4856		939	312	34	UL53 (33%)	Gene 5 (32%)	Gene 6 (42%)	—	—
UL54	4845–4850	4936[d]	6021	6021–6026	1,086	361	40	UL54 (41%)	Gene 4 (33%)	Gene 5 (44%)	UL69 (23%)	BMLF1 (22%)
ORF-1	7167–7162	7115	6492	6489–6484	624	207	22	—	—	ORF-1 (35%)	—	—

[a] Locations of putative TATA boxes and poly(A) addition signals as well as initiation and stop codons relative to the *Bam*HI 5′ fragment are indicated.
[b] HCMV, human cytomegalovirus; EBV, Epstein-Barr virus; —, no homologous gene present.
[c] Since only the 3′ part of the ORF for UL50 (PrV) has been sequenced, the positions of the TATA box and initiation codon as well as the molecular size for the deduced protein and overall homology values cannot be given.
[d] Location of the most probable start codon is given.

TATA box (13) is located at nucleotides (nt) 1257 to 1253, and a GC box (7, 8) at nt 1268 to 1263. A poly(A) addition signal (6, 22) is found 60 bp downstream of the stop codon followed by a GT cluster (43) which could act to direct correct mRNA 3′ processing (nt 278 to 271). For PrV UL52, which is transcribed in opposite orientation to PrV UL51, two possible start codons were found (nt 1075 and 1087). Since the second ATG is in a favorable context for translation initiation (39), it seems the more probable UL52 start site. Assuming that translation starts at this ATG, the predicted gene encodes a 103-kDa protein of 958 aa. No consensus sequence for polyadenylation is present immediately downstream of PrV UL52. Downstream of UL52 in the same transcriptional orientation lies an ORF whose deduced translation product exhibits homology to the HSV-1 UL53 product, glycoprotein K (gK) (29). For PrV UL53, two possible initiation sites are present (nt 3918 and 3963). Computer analysis of the predicted translation product favors the first ATG. The deduced UL53 protein comprises 312 aa, with features typical for multiply membrane-spanning glycoproteins, including a putative signal sequence (64), four possible transmembrane domains, and two consensus sequences for addition of N-linked glycans (38, 40). Preliminary results indicate that the PrV UL53 protein is indeed glycosylated (3). Two in-frame start codons were also detected for PrV UL54 (nt 4828 and 4936). The stop codon is immediately followed by a poly(A) addition signal, which represents the first such signal present downstream of the UL52, UL53, and UL54 genes. Since the second ATG represents the more probable start codon (39), PrV UL54, as predicted, encodes a 40-kDa protein of 361 aa with 41% homology to the corresponding HSV-1 polypeptide, the essential immediate-early protein ICP27.

Downstream from the UL54 gene, collinearity in gene arrangement between PrV and the human pathogens VZV and HSV-1 ceased. ORFs with homology to HSV-1 UL55 and UL56 were not detected. Instead, a region of reiterated sequences in which the sequence 5′-ATCTCCTCTCCCCATCTCCCC-3′ is repeated 17 times is present. These repeats are followed by an ORF which is transcribed in opposite orientation to the UL52 to UL54 genes. Upstream from this ORF, putative TATA (nt 7167 to 7162) and GC elements (nt 7244 to 7239) are present. Immediately downstream of the ORF, a perfect poly(A) addition signal is followed by a GT cluster. This ORF is predicted to encode a 22-kDa protein of 207 aa which shows significant homology of 35% only to the EHV-1 strain Ab4 gene 1 product

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PrV    1 MPPQ...RARGAPPRRRGSDPPDPGSLAGRL..............SPGG 32
EHV    1 MRPEGVSRGRASSVSISMCPPPPNGARRASLGCAPPLNSRPVCCAPSSVS 50
PrV   33 RSGGGSRRTL.....SRSSSLTSVASAPVETPVAAEALGLGAPGSRPPSY 77
EHV   51 LSSSSSRRSMPSLGSSRSSSLPSTGSLRSITR.DPERL.....PSRPPSY 94
PrV   78 GDV......VRAGPRPHRSPDTPLFARGPPPSYSETLLFDPPAYAVTIPD 121
EHV   95 TAINPECLLERGAERPRAWTASVMTA...PPSYSEALCQAPPAYEL.VPE 140
PrV  122 PPAYEAHRHRTAAAEARDWISSPSVVQPSLLGPFSQCLPQLTCFDCRYPE 171
EHV  141 LSYHPTQ.......DPRGVYSSRSD.............PHQTSR....RR 166
PrV  172 DRPMVLVGFLWGGLLLLVGLVFLILLPVLRESVVFP 207
EHV  167 QNPICIFIIVVATMLLILGLLLTITLSSLTNGKKEK 202
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FIG. 2. Comparison of predicted amino acid sequences of PrV (this paper) and EHV-1 ORF-1 (62) gene products. The amino acid sequences were compared by using the program Gap. Vertical bars show identical amino acids. Related amino acids are indicated by dots. The 207-aa PrV ORF-1 and the 202-aa EHV-1 ORF-1 gene products show 35% sequence identity.

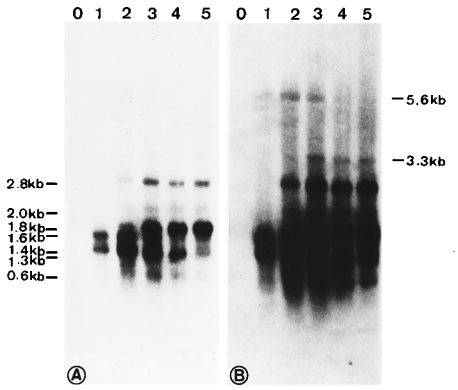

FIG. 3. Mapping of transcripts in *Bam*HI 5′ by Northern blot analysis. Northern blots of total RNA from PrV-infected PSEK cells harvested at 1, 2, 3, 4, and 5 h p.i. were hybridized to *Bam*HI 5′ fragment. Lane 0 contains RNA from mock-infected cells. Panels A and B show different exposures of the same blot. The sizes of the transcripts are indicated.

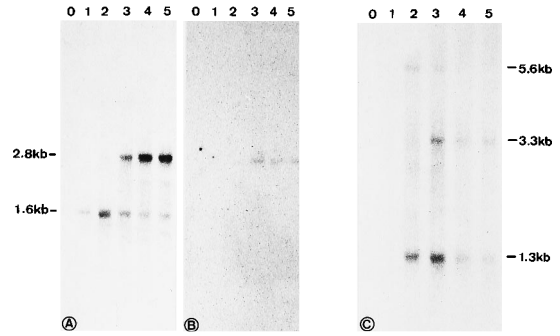

FIG. 5. Mapping of UL51 to UL54 transcripts by Northern blot analyses. Northern blots of RNA from PrV-infected PSEK cells harvested at 1, 2, 3, 4, and 5 h p.i. were performed with probes 2 (A), 3 (B), and 4 (C). Lane 0 contains RNA from mock-infected cells. The locations of the probes are shown in Fig. 1. The sizes of the transcripts are indicated.

(Fig. 2). Conservation of primary structure is particularly pronounced between aa 33 and 121 of the PrV protein corresponding to aa 51 to 140 of the EHV-1 polypeptide (Fig. 2). Chou-Fasman prediction (12) of the secondary structure indicates that both proteins contain a long hydrophobic carboxy-terminal domain and a hydrophilic amino-terminal part.

Transcriptional analysis of *Bam*HI 5′ fragment. To determine the transcriptional pattern of the *Bam*HI 5′ fragment region, Northern blot analyses of RNA isolated from PrV-infected PSEK cells at various times p.i. were performed. As shown in Fig. 3, the *Bam*HI 5′ fragment hybridized to nine RNAs of 5.6, 3.3, 2.8, 2.0, 1.8, 1.6, 1.4, 1.3, and 0.6 kb. Panels A and B show different exposures of the same blot. The 1.6- and 1.4-kb transcripts are detectable as early as 1 h p.i. The less abundant 5.6-kb RNA appears at 2 h p.i. but vanishes after 3 h p.i. The 2.8-kb RNA is also first detectable at 2 h p.i. but remains until 5 h p.i., as does the 2.0-kb RNA. The less abundant 3.3-kb transcript is detectable from 3 to 5 h p.i.

To identify specific transcripts for the predicted genes, different hybridization probes were used. The locations of these fragments are shown in Fig. 1. Probe 1 (Fig. 4) hybridizes with the 1.8-, 1.4-, and 0.6-kb mRNA. The 1.4-kb RNA is detectable from 1 until 5 h p.i. This RNA, which is not detected by the *Bam*HI 14′ fragment (data not shown), probably represents the ORF-1 message and fits in size with the prediction. The 1.8-kb RNA is recognized by probe 1 (Fig. 4) as well as the *Bam*HI 14′ fragment (data not shown), which indicates that this RNA starts further upstream from the ORF-1 gene within the *Bam*HI 14′ fragment. This transcript appears later in the infectious cycle and can be detected from 3 to 5 h p.i. So far, we have no clues as to the provenance of the 0.6-kb RNA.

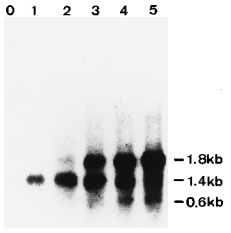

FIG. 4. Northern blot analysis of the left part of *Bam*HI 5′ fragment. Probe 1 (Fig. 1) was hybridized to RNA from PrV-infected PSEK cells harvested at 1, 2, 3, 4, and 5 h p.i. Lane 0 contains RNA from mock-infected cells. The sizes of the transcripts are indicated on the right.

Probe 2 (Fig. 5A) reacts with the 2.8- and 1.6-kb RNAs. The 1.6-kb mRNA is first detectable at 1 h p.i., increases in amount until 2 h p.i., and then decreases. The 2.8-kb RNA is detectable from 3 to 5 h p.i. Probe 3 (Fig. 5B) hybridized with the 2.8-kb RNA. These data combined with the sequencing results indicate that the 2.8-kb RNA encompasses the UL53 and UL54 genes, whereas the 1.6-kb RNA most likely represents the monocistronic UL54 transcript. Probe 4 (Fig. 5C) recognized the low abundant 5.6-kb RNA, which probably encompasses the UL52, UL53, and UL54 genes. This is corroborated by the fact that no consensus poly(A) addition signal has been found downstream from either the UL52 or UL53 genes but only behind the UL54 gene. So far, the provenance of the 3.3-kb RNA is unclear, whereas the 1.3-kb RNA, which is present from 2 to 3 h p.i. and largely disappears thereafter, might represent the UL51 transcript. None of the smaller hybridization probes contained UL50 sequences.

Isolation of a PrV ORF-1$^-$ mutant. Of particular interest was the detection of a gene conserved between EHV-1 and PrV which is not present in HSV-1 and VZV. This ORF-1 has been shown to be nonessential for EHV-1 replication in vitro, and no phenotype could be associated with its inactivation in strain Ab4 (61). Furthermore, it has been found to be absent in the apathogenic Kentucky strain of EHV-1 (66). To assay for the importance of the PrV ORF-1, a deletion mutant was constructed by insertional mutagenesis. To this end, plasmid pORFGAL, which contained a 433-bp deletion in the ORF-1 gene and concomitant insertion of a gG–β-galactosidase expression cassette (47), was cotransfected with wild-type PrV DNA into Vero cells. Transfection progeny was harvested and screened under a Bluo-Gal agarose overlay for the appearance of blue-staining plaques. These were picked and purified to homogeneity. Genotypic characterization of the ORF-1$^-$ PrV mutant is shown in Fig. 6. DNA of wild-type PrV (Fig. 6, lanes 1) or the ORF-1$^-$ mutant (Fig. 6, lanes 2) was cleaved with *Bam*HI, and the fragments were separated in an 0.8% agarose gel. Figure 6A shows the ethidium bromide-stained gel. Hybridization with the *Bam*HI 5′ fragment (Fig. 6B) demonstrated the presence of a slightly smaller 5′ fragment, which comigrates with fragment 7 in mutant virus DNA compared with DNA of wild-type PrV. Insertion of the gG–β-galactosidase cassette into the *Bam*HI site introduced into the partially deleted ORF-1 resulted in the restoration of the *Bam*HI site proximal to the 3′ end of the ORF-1 and inactivation of the

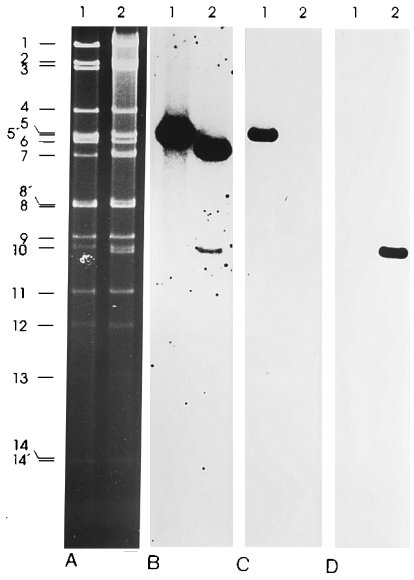

FIG. 6. Genomic characterization of PrV ORF-1$^-$. DNA was purified from cells infected with either wild-type PrV Ka (lanes 1) or PrV ORF-1$^-$ (lanes 2). After cleavage with *Bam*HI, fragments were separated in an 0.8% agarose gel. (A) Ethidium bromide-stained gel. (B to D) Hybridization with radiolabeled *Bam*HI 5′ fragment (B), *Apa*I fragments from ORF-1 (C), or β-galactosidase-specific probe (D). The numbers in panel A denote *Bam*HI fragments of wild-type PrV DNA.

distal *Bam*HI site. Therefore, after cleavage with *Bam*HI, two fragments of 6.6 and 4.0 kb should arise, comprising most of *Bam*HI 5′ (6.6 kb), and the 3.6-kb gG–β-galactosidase cassette plus 0.4 kb of the remaining *Bam*HI 5′ sequence, respectively. As shown in Fig. 6B, the *Bam*HI 5′ probe hybridized strongly to the 6.6-kb fragment and rather weakly to the 4.0-kb fragment. In contrast, the β-galactosidase-specific probe detected only the 4.0-kb fragment (Fig. 6D). To confirm the absence of ORF-1 sequences, hybridization with radiolabeled *Apa*I fragments originating from the ORF-1 (Fig. 1) was also performed (Fig. 6C). Whereas the wild-type *Bam*HI 5′ fragment was recognized, these probes did not hybridize to DNA from the ORF-1$^-$ mutant. In summary, these data show correct insertion of the gG–β-galactosidase cassette into the partially deleted ORF-1 in PrV ORF-1$^-$.

In vitro replication of PrV ORF-1$^-$. The fact that the ORF-1$^-$ mutant could be isolated on noncomplementing cells indicated that ORF-1 is not essential for growth of PrV in cell culture. To analyze replication of the mutant virus in more detail, one-step growth curves were established. The results shown in Fig. 7 demonstrate that deletion of ORF-1 in PrV did not lead to a recognizable defect in replication in MDBK cells. In addition, mutant virus plaques had the same size as wild-type PrV plaques (data not shown). We conclude that PrV ORF-1 is nonessential for viral replication in cell culture and that deletion of this gene did not lead to impairment of PrV replication.

DISCUSSION

We present here the analysis of a 7,412-bp fragment of the PrV genome located near the left end of the U_L region. Since a complete genomic sequence for PrV is not available yet, these results represent a major step toward our goal to gain complete sequence information for the PrV genome. Within this region we identified ORFs which showed homology to the UL50 through UL54 genes of HSV-1 (42) and corresponding genes in VZV (15) and EHV-1 (62), the other members of the *Alphaherpesvirinae* whose genomes have been completely sequenced. PrV UL50 only partly resides in the *Bam*HI 5′ fragment and is predicted to encode a protein with homology to dUTPase encoded by HSV-1 UL50 (21, 51). Recently, direct evidence that PrV UL50 also encodes a dUTPase was obtained (32). No function has yet been assigned to any of the UL51 homologous proteins which are conserved in all three subfamilies of herpesviruses (Table 1), but HSV-1 UL51 was shown to be nonessential for replication in cell culture (2). Conservation is particularly pronounced in the N-terminal and middle part of the proteins, which includes four conserved cysteine residues (data not shown). The UL52 proteins are also highly conserved, exhibiting 48% identity between PrV and EHV-1, and are found in all herpesvirus subfamilies. HSV-1 UL52 encodes the primase of the helicase-primase complex (34), which also contains the UL5 and UL8 gene products (14). The PrV UL5 gene has been sequenced and predicted to encode a helicase (17). The PrV UL8 gene has also recently been identified (19).

Of particular interest are the UL53 and UL54 gene products. HSV-1 UL53 encodes gK, a very hydrophobic protein which is predicted to be multiply membrane spanning (29). This feature is conserved in the PrV UL53 gene product, which, besides a possible signal sequence, contains four regions with characteristics of membrane-spanning domains. The hydrophilic part immediately following the putative signal sequence contains two consensus sequences for N glycosylation (38). One of them is conserved in UL53 homologs of alphaherpesviruses. In vitro transcription-translation studies indicate that the PrV UL53 product is also glycosylated (3). Comparison of amino acid sequences of UL53 homologs shows a remarkable degree of conservation ranging from 42% between PrV and EHV-1 to 32% between PrV and VZV. This homology is higher than that of gH homologs, and within the glycoproteins is second only to the values for gB (36). HSV-1 gK has been found to play a role in virus-induced cell fusion (20, 29, 30). Whether the PrV UL53 product displays similar characteristics is under investigation.

HSV-1 UL54 encodes ICP27, which represents one of the five immediate-early proteins (27). ICP27 is a 63-kDa phosphoprotein which is involved in downregulation of viral immediate-early genes and acts in combination with ICP0 and ICP4 as an activator of late genes (56, 58). The transrepressor and transactivator functions are separable, but both appear to be located within the carboxy-terminal part of the protein (26, 44, 53). Genes encoding ICP27 homologs have been identified in VZV (50), EHV-1 (59, 67), and Marek's disease virus (52), which has recently been shown to resemble alphaherpesviruses in gene content (9, 10), as well as in human cytomegalovirus (11) and Epstein-Barr virus (1). In PrV, only one immediate-early protein, IE180, which is homologous to HSV-1 ICP4, has been described (31). The PrV UL54 transcript was first detectable at 1 h p.i., and high levels were present at 2 h p.i. but decreased thereafter, indicating early regulation, as was also found for the EHV-1 homolog gene 6 (67). The PrV UL54 protein as well as respective gene products in EHV-1, HSV-1, and VZV contain a putative zinc-finger motif at the carboxy terminus which in the case of HSV-1 ICP27 has been shown to be involved in binding DNA, RNA, and other proteins (63).

Apart from the genes conserved between HSV-1, VZV, EHV-1, and PrV, which are found in a collinear arrangement in all four viruses, we identified an ORF with homology only to the first gene identified in the U_L region of the pathogenic EHV-1 strain Ab4 (62). Surprisingly, this gene is absent in the

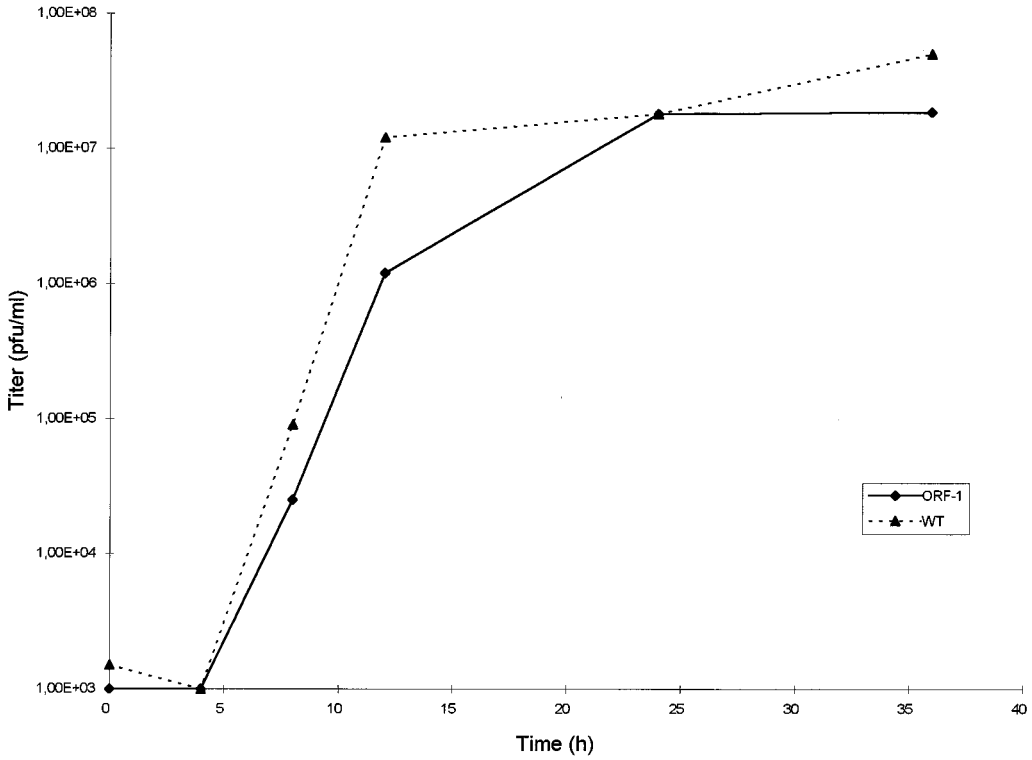

FIG. 7. One-step growth curves. MDBK cells were infected at a multiplicity of infection of 5 with wild-type (WT) PrV or PrV ORF-1$^-$ for 1 h at 4°C. The inoculum was then removed, and the cells were overlaid with prewarmed medium. Immediately after the temperature shift and at the indicated times thereafter, cells and supernatant were harvested. Infectivity was determined in plaque assays on MDBK cells. The values for the titers of intra- and extracellular virus were added and plotted. Data from a representative experiment are shown.

avirulent laboratory EHV-1 strain Kentucky (66). The conservation of this nonessential gene between EHV-1 Ab4 and PrV Ka, which itself represents a laboratory strain with intermediate virulence for PrV's natural host, swine, may indicate an important role for this gene or protein in viral virulence. Studies to analyze the behavior of the PrV ORF-1$^-$ mutant in vivo are under way. Despite the limited amino acid sequence homology of 35% between the EHV-1 and PrV ORF-1 proteins, both are predicted to contain a hydrophobic domain at the carboxy terminus which might mediate membrane association and a hydrophilic amino-terminal part. It is tempting to speculate that these proteins represent membrane constituents. In fact, the high prevalence of serine and threonine residues within the ORF-1 proteins would allow extensive O glycosylation to occur. However, respective proteins have so far not been identified and analyzed in either EHV-1 or PrV.

PrV ORF-1 is separated from the region with complete collinearity to HSV-1 and VZV genomes by a cluster of short reiterated sequences. This area of the genome is one of the few regions where differences in the genetic content of the different alphaherpesviruses are observed. Homologs of the HSV-1 UL56 gene, which has been associated with pathogenicity (55), are missing in PrV, VZV, and EHV-1, whereas PrV lacks a UL55 homolog which is present in EHV-1, VZV, and HSV-1. Homologs to gene 2 of EHV-1 and gene 1 of VZV are absent in PrV, at least at a collinear position. In contrast, ORF-1 is conserved between PrV and EHV-1 but absent in VZV and HSV-1. The presence of ORF-1, therefore, separates the two animal alphaherpesviruses EHV-1 and PrV within the *Varicellovirus* genus from the human pathogen VZV and also clearly distinguishes them from HSV. Different calculated evolutionary trees based on amino acid homologies have placed EHV-1 and PrV in close relationship (41), which is supported by the conservation of ORF-1. In fact, another PrV gene, UL3.5, previously described as PrV specific (16), also has a homolog in EHV-1 gene 59 (23), which further emphasizes the relationship between the two viruses.

Results from sequencing and Northern blot hybridizations indicated that UL51 is transcribed into a monocistronic 1.3-kb mRNA, whereas UL52 to UL54 give rise to a set of three presumably 3′-coterminal mRNAs that are tricistronic, bicistronic, and monocistronic in nature, respectively. In HSV-1, UL52 and UL53 are translated into two 3′-coterminal mRNAs which terminate at a poly(A) signal behind UL53 (49). In VZV and EHV-1, no poly(A) addition signal is present behind the UL52 and UL53 homologous genes (15, 62). In addition, EHV-1 UL53 and UL54 homologs have been shown to be transcribed into two 3′-coterminal mRNAs (67), paralleling the situation in PrV. The PrV ORF-1 is transcribed into an RNA of 1.4 kb, although another transcript of 1.8 kb which probably starts in the adjacent *Bam*HI 14′ fragment also contains ORF-1 sequences. We are currently determining the complete sequence of *Bam*HI 14′ to check whether there is another gene located upstream from ORF-1.

In summary, we analyzed a 7,412-bp fragment of PrV DNA encompassing genes homologous to the UL50 to UL54 genes of HSV-1 and homologs in other herpesviruses in a collinear arrangement. Separated from this conserved gene cluster by reiterations of a 21-bp motif is an ORF with homology to only the first gene identified in the U_L region of EHV-1 strain Ab4. The deduced protein products exhibit features conserved between PrV and EHV-1, and both are nonessential for viral

replication in cell culture (reference 61 and this paper). Experiments in PrV's natural host, swine, will hopefully elucidate a function for this protein in an in vivo situation.

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