Characterization of the genes, including that encoding the viral proteinase, contained in BamHI restriction fragment 9 of the pseudorabies virus genome

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We describe the nucleotide sequence, transcription pattern and open reading frames (ORFs) located on BamHI restriction fragment 9 (0.406-0.435 map units) in the unique long segment of the pseudorabies virus (PRV) genome. The fragment contains three nested genes with a common 3' end. The 5' ends of the corresponding 0.9, 1.7 and 3.3 kb mRNAs have been mapped. Fragment BamHI-9 contains three complete ORFs, ORF1, ORF2 and ORF2.5. ORF1, which is within the 3.3 kb transcript, encodes a protein with an apparent molecular mass of 60 kDa which is homologous to the product of the herpes simplex virus type 1 UL25 gene. The 1.7 kb mRNA contains ORF2, whose product is homologous to the herpesvirus proteinases, while the 0.9 kb transcript contains ORF2.5, which probably encodes the assembly protein precursor. ORF2 was identified as the PRV proteinase gene following expression in E. coli, using the product of ORF2.5 as the substrate.

Introduction

Pseudorabies virus (PRV), a member of the subfamily Alphaherpesvirinae (genus Varicellovirus), is an important swine pathogen. Its genome is a linear, double-stranded DNA molecule of 140 kb with a 74 mol% G + C content (Ben-Porat & Kaplan, 1985). The complete nucleotide sequence of the PRV genome has not yet been determined, although approximately 40 PRV genes have been mapped and sequenced.

The virion contains four distinct components: the core, an icosahedral capsid, the tegument and a lipid envelope. Its morphogenesis is similar to that of herpes simplex virus type 1 (HSV-1) (McCrae & Clarke, 1971). In the assembly of herpesvirus capsids, there is a proteolytic cleavage which appears to be an essential event in the formation of infectious virions (Gao et al., 1994; Preston et al., 1983). Two cleavage sites have been described: the procapsid assembly protein precursor and the proteinase itself (Welch et al., 1991b; Liu & Roizman, 1992). This proteinase is encoded by a viral gene, which also contains the gene for the assembly protein precursor (Welch et al., 1991a; Liu & Roizman, 1991).

We report here the nucleotide sequence and arrangement of the genes contained in fragment 9 of the BamHI PRV restriction map (Ben-Porat & Kaplan, 1985) and the predicted amino acid sequence of the gene products. Northern blot analyses, primer extension and SI protection experiments were performed to identify and map the transcripts encoded by this DNA fragment. The results revealed an overlapping pattern of three RNAs with a common 3' end. We designate the open reading frames (ORFs) contained in the transcripts ORF1, ORF2 and ORF2.5 to reflect the organizational relationship among them and their homologous counterparts in other herpesviruses. The predicted ORF1 product exhibits amino acid sequence homology with a structural protein whose gene is located upstream of the virus proteinase gene in all the alphaherpesvirus genomes sequenced so far. The ORF2 product shows amino acid sequence homology with the herpesvirus proteinases. The protein encoded by ORF2.5 is the in-frame C-terminal half of the polypeptide encoded by ORF2. Finally, we have studied the ability of the PRV proteinase to autoprocess and cleave the protein encoded by ORF2.5.

Methods

- **Synthetic oligonucleotides.** The oligonucleotides designed for this study were:
  1. 5' CCCCTGCTGGTGTTCGT; 2. 5' AGAGCTGGCGTCGATGGTG; 3. 5' TGCGTACCTGCTGAGCAA; a3, 5' TTGCTCAGCAGGTAC-
Nucleotide sequence and computer analysis. Plasmid pRA8, containing restriction fragment BamHI-9 of PRV DNA (strain NIA3), was obtained from the library of Prieto et al. (1991). pRA8 was digested with the restriction enzymes BamHI, SmaI, NcoI, PstI, Sall, XhoI and NarI (Fig. 1), and the resulting DNA segments were cloned into pUC18. The nucleotide sequence of both strands of each DNA segment was determined by the dideoxy nucleotide chain-termination method (Sanger et al., 1977), using the universal direct and reverse primers or primers 1, 2, 3, 4, 5, 6 and Eco (described above and in Fig. 1), and Sequenase (USB). The sequence data were analysed with the GelStart program. Searches of the GenBank and Swiss-Prot databases were performed with the TFASTA program from the University of Wisconsin Genetics Computer group (Devereux et al., 1984). Multiple protein sequence alignments were done using the PILEUP program.

RNA isolation. Total RNA was extracted from PK15 cells that were uninfected or infected with PRV (strain NIA3) at an m.o.i. of 5 p.f.u. per cell, as described by Martín Hernández & Tabarés (1991). Cytoplasmic RNA was extracted from cells infected 14 h earlier with PRV at an m.o.i. of 10 p.f.u. per cell. Cells were washed with TSM (0·15 M-NaCl, 5 mm-MgCl₂, 10 mm-Tris–HCl, pH 7·6) and lysed with TSM containing 0·2% NP40. Nuclei were pelleted by centrifugation, and the RNA from the cytoplasmatic fraction was extracted by adding 3% SDS, 31 mM-EDTA, 0·5 M-NaCl, 78 mm-Tris–HCl, pH 8, and 8 mM-urea, followed by phenol extraction and alcohol precipitation.

Northern hybridization. For Northern blot assays, 20 μg of total RNA from infected or uninfected cells was subjected to electrophoresis on 1% agarose–formaldehyde gels and blotted onto a Hybond-N nylon membrane (Amersham). The probes were derived from pUC18 clones containing PRV fragments (Fig. 3). Probe A5 contained the 134 and 128 bp SmaI-generated fragments from clone pUC18A5 (nucleotides 1–1211), probe C10 was a 496 bp fragment generated by Sall–PstI digestion of clone pUC18C10 (nucleotides 1212–2033) and probe C4 was the PstI 782 bp fragment from pUC18C4 (nucleotides 2130–2912). The DNA fragments were separated on agarose gels and purified using the Magic PCR Preps purification system (Promega). Each double-stranded probe (10 ng) was labelled in a volume of 25 μl using random primers. Klenow fragment (Boehringer Mannheim), 1 mM-deoxynucleotides (other than dCTP) and 25 μCi [α-32P]dCTP. More than 5 × 10⁶ c.p.m. of probe were used in each blot. When oligo 2 was used as the probe, 50 ng oligonucleoside was labelled with 50 μCi [α-32P]ATP and T4 kinase (Biolabs). Northern blots were performed as described by Cistue & Tabarés (1992). Hybridization was carried out overnight at 37 °C in 6 x SSC (1 x SSC is 0·15 M-NaCl, 0·015 M-sodium citrate) containing 50% formamide. Filters were washed with 1 x SSC–0·5% SDS at 20 °C, then with 0·1 x SSC–0·1% SDS at 50 °C, followed by several washes at 65 °C with 0·1 x SSC–0·1% SDS.

Primer extension. Primer extension experiments were performed...
by the method of Kingston (1987). The synthetic oligonucleotides p1, p2, p1.1 and p2.2, indicated in Fig. 2, were 5' end-labelled with [α-32P]dCTP and polynucleotide kinase. Cytoplasmic RNA (50 μg) and 10^4 c.p.m. of labelled primer were mixed and ethanol precipitated. Pellets were dissolved in 20 μl of hybridization buffer (10 mM-Tris–HCl, pH 7.5, 0.35 M-NaCl and 1 mM-EDTA), heated for 5 min at 90 °C and allowed to anneal at 42 °C overnight. After ethanol precipitation, extension reactions were carried out at 42 °C for 90 min with 30 U avian myeloblastosis virus reverse transcriptase (Promega). The extension products were denatured and electrophoresed on 6% polyacrylamide–8 M-urea sequencing gels.

**S1 mapping.** S1 protection assays were performed as described by Sambrook et al. (1989). Plasmid pUC18A5 was digested with NcoI and EcoRI (vector restriction site) and the resulting 1005 bp fragment was purified and labelled at the 3' end with [α-32P]dATP and Klenow fragment. Using this protocol, only the mRNA complementary strand was labelled. DNA (4 × 10^4 c.p.m., 0.09 pmol) and 50 μg of cytoplasmic RNA or tRNA were mixed and ethanol precipitated. Pellets were resuspended in 15 μl hybridization buffer (40 mM-PIPES, pH 6.4, 0.4 M-NaCl, 1 mM-EDTA and 70% 5 formamide), heat denatured, and incubated overnight at 52 °C. S1 nuclease (Boehringer; 400 U) was added and incubation was carried out at 37 °C for 45 min in a final volume of 200 μl. After alcohol precipitation, the RNA was hydrolysed with 0.2 M-NaOH, the solution was neutralized with 0.2 M-ammonium acetate, pH 4.5, and the DNA was precipitated with ethanol. Pellets were resuspended in loading buffer and electrophoresed on a 4% polyacrylamide–8 M-urea gel.

**In vitro transcription and translation.** The vector used in these experiments was pGEM-3Z (Promega). Plasmid pGEM-4K contains the 4093 bp BamHI fragment. Plasmids pGEM-3K, pGEM-2K and pGEM-1.5K contain the DNA fragments between restriction sites BamHI–XhoI (nucleotides 1–2860), BamHI–SalI (nucleotides 1–1708) and BamHI–PstI (nucleotides 1–1211), respectively. Plasmid pGEM-2.35K was constructed from pGEM-2K by addition of the PstI–Pst fragment corresponding to clone pUC18C10 (nucleotides 1212–2033) (Fig. 1).

In vitro transcription of plasmid inserts was performed with Sp6 RNA polymerase in the presence of m7G(5')ppp(5')G, as described by Martín Hernández & Tabarés (1991). In vitro translation of transcripts in rabbit reticulocyte lysates was performed in the presence of [35S]methionine. Synthesized polypeptides were separated by SDS–PAGE (17% gel).

**Expression in E. coli.** Plasmids pRSET2K1 and pRSET2K1 (derived from vector pRSETA (Invitrogen), were described by Kroll et al. (1993). pRSETA is a pUC-derived expression vector containing the T7 promoter. In addition, DNA inserts are positioned in-frame with a sequence that encodes an N-terminal fusion peptide. This sequence includes an ATG start codon at the 3' end of ORF1; however, an AATAAA sequence is located between the T7 promoter and the initiation codon.

Identification of three 3' co-terminal transcripts

Northern blot analyses were performed to characterize the size and class of transcripts encoded in the DNA region under study (Fig. 3). RNAs from cells infected with PRV for 3 and 8 h, or from mock-infected cells, were hybridized with double-stranded DNA probes A5, C10 and C4 and with oligo 2 (Fig. 3a). As shown in Fig. 3(b), probe A5 hybridized with four transcripts of 0.9, 1.7, 3.3 and 5.6 kb specific to infected cells, and a fifth of 4.5 kb, also present in mock-infected cells. RNAs of 1.7 and 3.3 kb also hybridized with probe C10. In contrast, probe C4 only hybridized to the 3.3 kb transcript. When the RNA complementary oligo 2 probe was used (Fig. 2), the hybridization pattern was similar to that of probe A5, except that the 5.6 kb RNA was not detected. This result indicates that the 5.6 kb RNA is encoded on the opposite strand of the BamHI-9 DNA fragment which encodes the other three RNAs.

The hybridization patterns observed are consistent with the existence of three overlapping mRNAs, each with its own 5' end and a common 3' end (Fig. 3). Identification of the RNA 5' ends was carried out by primer extension of labelled probes (Fig. 4a). The synthetic oligonucleotides used as probes (p1, p2, p2.1 and p2.2) are shown underlined in Fig. 2. Oligonucleotides p2.1 and p2.2, with sequences complementary to areas near the 5' end of the 0.9-kb RNA, yielded primer extension products consistent with an mRNA 5' end around 10 μCi/ml [35S]methionine. Cells were collected by centrifugation and extracts were prepared by freeze–thawing and sonication.

**Protease assay.** E. coli with or without pRSET2K1 (8 ml) was treated with IPTG for 1 h and collected by centrifugation. Cells were lysed by freeze–thawing and sonication in 500 μl protease buffer (10 mM-Tris–HCl, pH 7.5, 1 mM-DTT, 50 mM-NaCl). Labelled extracts of cells harbouiring plasmid pRSET1K1 were mixed with the unlabelled extracts from bacterial controls or E. coli(pRSET2K1) and incubated at 37 °C for 2 h.

**Results**

**Sequence analysis and identification of ORFs**

Fragment BamHI-9 comprises 4093 bp, with a 75 mol% G+C content. As shown in Figs 1 and 2, sequence analysis revealed two complete ORFs (ORF1 and ORF2) and a third incomplete ORF whose ATG is located 28 nucleotides from the BamHI site in the adjacent fragment 11 homologous to the HSV-1 UL24 gene. The PRV gene encoding glycoprotein C is located between BamHI restriction fragments 2 and 9, with its 3' end in fragment 9 (Fig. 1 and Robbins et al., 1986). ORF1 has the capacity to encode a 534–540 amino acid polypeptide with a predicted molecular mass of 58 kDa; ORF2 encodes a polypeptide of 526 amino acids with a predicted molecular mass of 56 kDa. No apparent polyadenylation signal exists at the 3' end of ORF1; however, an AATAAA sequence is present 39 nucleotides downstream of the ORF2 termination codon.

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nucleotide 1150. When p2, the oligonucleotide complementary to the 5' end region of the 1.7 kb RNA, was used, a major transcription initiation site was detected approximately 127 nucleotides upstream of a possible translation initiation codon. The presence of the 0.9 kb RNA indicates a third gene product. To define the 3' ends of the three mRNAs, 51 protection experiments were carried out with a probe which encompasses the polyadenylation signal. Cytoplasmic RNA annealed with the probe protected a fragment of approximately 760 nucleotides (Fig. 4b), which is fairly close to the 753 nucleotide size expected to be protected if the poly(A) sequence was the 3' transcript end.

**Characterization of gene products**

Translational analysis of the sequence of the BamHI-9 4093 bp fragment revealed two complete ORFs, ORF1 and ORF2, which are in-frame with ORF2. If ORF2.5 has its initiation codon at positions 3516 and 3534, could initiate translation of ORF2.5 within ORF2. If ORF2.5 has its initiation codon at position 1008, the products of ORF2 and ORF2.5 would be 107 amino acids shorter than ORF2.5 and start at position 1008. The presence of the 0.9 kb RNA indicates a third gene product. Locations of 5' end RNAs are indicated by dots. Initial methionines for each ORF are in bold; potential TATA boxes are in italics.

Fig. 2. Nucleotide sequence of fragment BamHI-9. The predicted amino acid sequences corresponding to the ORF1 and ORF2 gene products are indicated. Synthetic oligonucleotides p1, p2, p2.1, oligo 2 and the polyadenylation signal (AATAAA) are underlined. Locations of 5' end RNAs are indicated by dots. Initial methionines for each ORF are in bold; potential TATA boxes are in italics.
To characterize the polypeptides encoded by these ORFs, in vitro transcription and translation experiments were performed. When the entire BamHI fragment was used, a polypeptide with a molecular mass of 60 kDa was produced (Fig. 5a, lane 2). This polypeptide corresponds well to the 58 kDa predicted product of ORF1. With pGEM-2.35K, containing ORF2, two polypeptides were detected: one of 70 kDa, larger than that predicted from the amino acid sequence, and another of about 65 kDa (lane 1). With plasmid pGEM-1.5K as template (containing ORF2.5), a 42 kDa polypeptide was obtained (lane 3). No polypeptides were detected when RNA from plasmid pGEM-3K was translated (data not shown).

The products of ORF2 and ORF2.5 were also expressed in E. coli. In this case, the DNA insert was positioned downstream and in-frame with a sequence that encoded an N-terminal fusion peptide which includes the ATG. Two plasmids were constructed: pRSETA1K1, containing ORF2.5, and pRSETA2K1, containing ORF2. In cells harbouring pRSETA1K1, a single protein band with a molecular mass of 42 kDa was labelled after rifampicin addition, whereas several bands appeared in bacteria harbouring pRSETA2K1 (Fig. 5b, lanes 2 and 5). These polypeptides have molecular masses of approximately 72, 67, 45, 41 and 27 kDa and two minor components of 63 and 37 kDa. If the 72 kDa polypeptide has the N-terminal amino acid addition due to vector sequences, it could correspond to the 70 kDa band present in the eukaryotic system. Similarly, the 67 kDa polypeptide could correspond to the 65 kDa polypeptide observed in vitro. Since there are no methionines in ORF2 to account for the 65 (67) kDa polypeptide, these could be derived from the 70 kDa polypeptide by proteolysis. When proteins translated from pGEM-2.35K were incubated in protease buffer and analysed by electrophoresis, there was a decrease in the 70 kDa band and an increase in two minor bands of 65 and 61 kDa (data not shown).

As described above, in two different systems (E. coli and rabbit reticulocytes), the expression of ORF2.5 gave rise to a unique polypeptide, while ORF2 produced various polypeptides with similar molecular masses in both systems. These
Fig. 4. Mapping of gene transcripts. (a) Primer extension assays were performed to map the transcript 5' ends. The sizes of the extended probes were estimated from an M13mp18 sequence (M13 lanes). XE1 is an unrelated sequence electrophoresed in parallel to the M13mp18 sequence. Lines in the sequence are ACGT. The calculated locations of the three transcriptional start sites are shown in Fig. 2. (b) S1 mapping. End-labelled probe covering the poly(A) signal was annealed to cytoplasmic RNA from infected cells (+) or tRNA (-) and digested with S1 nuclease. The size of the probe protected from digestion was estimated using inserts of the sequenced clones labelled with Klenow fragment as molecular size markers in nucleotides.

Fig. 5. Polypeptides produced by in vitro transcription and translation in reticulocyte lysates and in vivo in E. coli. (a) In vitro transcription and translation assays. Polypeptides translated in reticulocyte lysates from mRNAs transcribed from plasmids pGEM-4K (lane 2), pGEM-2.35K (lane 1) and pGEM-1.5K (lane 3). (b) Expression in E. coli. Bacterial cells containing plasmids pRSETA1K1 (lanes 1 and 2), pRSETA2K1 (lanes 4 and 5) or pRSET2K6 (lane 3) were grown and induced as described in Methods. Polypeptides were 35S-labelled before (lanes 1 and 4) or 30 min after (lanes 2, 3 and 5) rifampicin addition. Induced polypeptide molecular masses in kDa are indicated. (c) Trans-cleavage assay. Labelled lysates of bacteria harbouring pRSET1K1 were mixed with unlabelled lysates of pRSET2K1 (lanes 2-4), or with lysates without plasmid (lane 1), at substrate:enzyme ratios of 1:6, 1:60 and 1:120 (lanes 2-4, respectively). Mixtures were incubated in protease buffer. Molecular mass markers (kDa) were proteins translated from brome mosaic virus mRNA and unlabelled stained protein markers (BSA, ovalbumin, aldolase and lysozyme).

results suggested that ORF2 could encode a proteinase that undergoes autoproteolytic cleavage. It is known that the viral proteinase of the herpesviruses can process the assembly protein, encoded by a gene present in-phase in the 3' half of the protease gene (Liu & Roizman, 1993; Welch et al., 1991a). To confirm that the product of ORF2 is the PRV proteinase, un-
labelled proteins from bacteria harbouring pRSETA2K1 were mixed with the labelled proteins induced in the pRSETA1K1 lysate. As shown in Fig. 5(c), the product of ORF2 is able to cleave the 42 kDa ORF2.5 product to a 34 kDa polypeptide. An enzyme:substrate ratio as high as 6:1 was necessary to detect trans-proteolysis under the conditions used in these experiments.

Discussion

Transcripts encoded by the PRV BamHI-9 DNA fragment

We have found that PRV restriction fragment BamHI-9 contains three complete ORFs, ORF1, ORF2 and ORF2.5, with transcript sizes of 3-3, 1-7 and 0-9 kb, respectively. The gene homologous to HSV-1 UL24 and the gene encoding gpC are also partially included. Transcript maps of this DNA fragment have been reported previously. Deatly et al. (1984) described three major species of approximately 1-75, 2-2 and 3-8 kb, a minor RNA of 4-1 kb and a 6 kb RNA which was not chased to the minor species. In addition, De Wind et al. (1994) described RNAs with sizes of 1-3, 1-8, 3-5 and 4-0 kb. They assigned the 4-0 kb RNA to the gene homologous to HSV-1 UL24. These authors suggested a map of four overlapping genes. Since they used rRNA as molecular mass markers, the three smaller species probably correspond to the mRNAs that we assigned for the genes. We only detected a 4 kb transcript with two of the probes used, rather than with all four, and this 4 kb RNA is also found, although in lesser amounts, in mock-infected cells. Therefore, our 4 kb mRNA cannot be considered to be overlapping with the three RNAs described here.

Analysis of the nucleotide sequence revealed a single polyadenylation signal, suggesting that the fragment could give rise to three co-terminal transcripts. This was confirmed by several lines of evidence. First, we have detected RNAs with sizes close to those expected for transcripts derived from ORF1 (3-3 kb), ORF2 (1-7 kb) and ORF2.5 (0-9 kb) with a unique polyadenylation site. Second, the overlapping relationship of the transcripts has been demonstrated with specific probes. Finally, the size of the nucleotide fragment protected by RNA from S1 nuclease hydrolysis was that expected if the mRNAs have a common 3' end.

Proteins encoded by the fragment

Genes similar to those described here have been characterized in other herpesviruses. These PRV genes are located in the same position and relative orientation to the thymidine kinase gene, as are their homologous genes in all alphaherpesvirus genomes sequenced to date.

The gene between PRV BamHI fragments II and 9 is homologous to gene 37 of equine herpesvirus type 1 (EHV), gene 35 of varicella-zoster virus (VZV) and gene BXRF1 of Epstein–Barr virus (EBV). The predicted amino acid sequence of the product is 38.5% identical to the product of HSV-1 gene UL24. Fig. 6(a) shows the sequence alignments. In the alphaherpesviruses, homology in this protein is found throughout the entire molecule, with two stretches of amino acids conserved in all the herpesviruses, one at amino acids 47–62 and the other at 126–134. We have not found functional homology for these stretches.

ORF1 presents identities ranging from 16.7% with an overlap of 466 amino acids with the herpesvirus saimiri (HSVSA) gene 19 product to 47.1% (with 191 amino acids overlap) with the EHV gene 36 product and 47.0% with the HSV-1 UL25 product. The latter has been characterized as a structural protein (Addison et al., 1984). A striking homology of conserved stretches in the three herpesvirus subfamilies has been detected (Fig. 6(b)) with different chains of the mitochondrial NADH-ubiquinone oxidoreductase. The functional significance of this identity, if any, is at present unknown. ORF1 has two possible initiation codons; it could therefore encode a protein with 534 or 540 amino acids. In vitro translation experiments have shown that ORF1 encodes a 60 kDa polypeptide.

The ORF2 product is homologous to the herpesvirus proteinases, with 65.7% amino acid identity (with 230 amino acids overlap) to the EHV gene 35 product and 22.1% identity (with 516 amino acids overlap) to the human cytomegalovirus (HCMV) UL80 gene product. The predicted amino acid sequence of the PRV proteinase encoded by ORF2 is 47.5% identical to HSV-1 proteinase. PRV proteinase is similar to the alphaherpesvirus proteinases in the N-terminal half of the sequence, characterized in HSV-1 as responsible for the protease activity (Burck et al., 1994). It has the conserved domains CD1–5, with the potential active site residues of His in CD1 and CD2 and Ser in domain CD3 (Welch et al., 1993; Liu & Roizman, 1992) and the cleavage-site domains R and M (Fig. 6(c)). The C-terminal half of the proteinase, which also corresponds to the ORF2.5 product, and has been characterized in HSV-1 as the assembly precursor protein (Newcomb & Brown, 1991), is not conserved. PRV proteinase is composed of 526 amino acids with a molecular mass in SDS–PAGE gels of 70 kDa. The disagreement between the predicted size of the ORF2 product and that obtained in E. coli or by in vitro translation could be due to abnormal mobility in SDS–PAGE gels. Expression of ORF2 in E. coli resulted in the synthesis of several bands with molecular masses of 72, 67, 63, 45, 41, 37 and 27 kDa, whereas expression in this system of ORF2.5 (encoding the 236 C-terminal amino acids) produced a unique band of 42 kDa. Since we had demonstrated that ORF2 encodes the PRV proteinase, the 72 kDa polypeptide should be the proteinase precursor and the other polypeptides could be produced by autoproteolysis. Further studies are needed to identify the origin of each polypeptide.

The product of ORF2.5 has homology with a few short amino acid sequences of the HSV-1 assembly protein. Besides
the M domain, the sequence Tyr/Phe-Pro-Gly-Glu (amino acids 322-325) conserved in the three families should be noted. The substitution of the herpesvirus conserved Ser (in HSV-1, amino acid 248) in the cleavage position of the R domain for Thr in the PRV sequence is of interest, as this Ala-Ser pair is conserved in all but one (infectious laryngotracheitis...
References


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