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The Pseudorabies Virus Homolog of the Herpes Simplex Virus UL21 Gene Product Is a Capsid Protein Which Is Involved in Capsid Maturation

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We mutagenized, mapped, and sequenced the pseudorabies virus (PRV) homolog of gene UL21 of herpes simplex virus type 1. A polyclonal mouse antiserum against the protein encoded by the UL21 homolog was generated and used to monitor the expression and subcellular localization of the UL21-encoded protein. We found that the protein is identical to a previously detected PRV capsid protein. We analyzed viable PRV strains encoding mutant UL21 homologs, truncated by insertion of an oligonucleotide that contains stop codons in all reading frames. In two PRV mutants carrying the oligonucleotide at two sites within the gene, processing of newly replicated viral DNA was impaired. In addition, we show that one of the UL21 mutants has strongly reduced virulence for mice.

Herpesviruses are large enveloped viruses containing double-stranded linear DNA genomes with sizes of between 120 and 200 kbp (30). Replication of the viral genome via a rolling-strand mechanism, cleavage of viral DNA to unit-length molecules, capsid assembly, and encapsidation of the processed genome into immature capsids are intertwined in a strictly regulated process (12, 20–22, 34, 35, 39). With the use of temperature-sensitive (*ts*) mutants of herpes simplex virus type 1 (HSV-1), several HSV-1 genes have been identified that are involved in this process. At the nonpermissive temperature, these mutants are unable to process and package the viral genome into immature (pre-B type [35]) capsids, resulting in accumulation of both concatemeric viral genomes and immature capsids in the nucleus of the infected cell. HSV-1 genes involved in DNA processing and encapsidation include UL6 (40), UL25 (1), UL26 (encoding the scaffolding protein [8, 28, 29, 35]), UL28 (2), and UL33 (3).

We recently described oligonucleotide insertion mutagenesis of a 41-kbp subgenomic fragment of the porcine alphaherpesvirus pseudorabies virus (PRV) (4, 6) with an oligonucleotide that contains stop codons in all reading frames (14, 15). Here we describe the characterization of two viable PRV strains, each carrying the oligonucleotide at a distinct site in the PRV homolog of HSV-1 gene UL21 (17, 24, 25) and varicella-zoster virus (VZV) gene 38 (10). This gene was mapped to the viral *Bam*HI-4 restriction fragment, where PRV *ts* mutations that caused failure to synthesize any capsids were also mapped. These mutants belong to four complementation groups (18, 20, 21). A subsequent study (23) showed that this PRV restriction fragment encoded at least three genes that encode proteins of 142, 62, and 32 kDa which are found in PRV capsids. We sequenced the PRV UL21 homolog and demonstrated in an in vitro expression system that it encodes the 62-kDa capsid protein. We generated antibodies against the protein, expressed in *Escherichia coli*; these antibodies were used to demonstrate the presence of the protein in the cytoplasmic and nuclear fractions of infected cells and in PRV virions. Analysis of processing of newly replicated viral DNA revealed a defect in cleavage to unit-length molecules in PRV UL21 mutants. This demonstrates that the PRV UL21 gene product is involved in the process of capsid maturation, placing the PRV UL21 gene product in the same category as the HSV-1 UL6, UL25, UL26, UL28, and UL33 gene products.

MATERIALS AND METHODS

Cells and viruses. PRV mutants were derived from PRV strain NIA-3 (4). Porcine kidney cell lines PK15 and SK6 were used for transfections and for virus growth and RNA isolation, respectively. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum.

Construction of PRV mutants. The method of oligonucleotide insertion into and characterization of mutant derivatives of PRV cosmid c-448, which carries a 41-kbp segment of the PRV genome (Fig. 1B), has been described recently by us (14, 15). The inserted 20-bp oligonucleotide contains translational stop codons in all six reading frames as well as an *Eco*RI site which is not present in wild-type (wt) PRV. Insertion of the oligonucleotide in an open reading frame (ORF) leads to termination of translation. The positions of the oligonucleotide insertions on c-448 that are relevant to this study are indicated in Fig. 1C. Viable virus mutants carrying the inserted oligonucleotide were generated by cotransfection of the mutated c-448 derivative with overlapping cloned viral fragments (Fig. 1B) that together include the entire viral genome, as described before (38).

DNA sequencing. Overlapping fragments of both DNA strands of the region of interest were subcloned into M13mp18 and M13mp19. The precise sites of insertion of the oligonucleotide were determined after subcloning of the sequence flanking the oligonucleotide as *Eco*RI-*Sau*3A fragments. Dideoxy sequencing (32) was performed with the T7 sequencing kit from Pharmacia, Uppsala, Sweden, with 7-deaza-2′-dGTP substituting for dGTP. Sequencing prod-

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ucts were analyzed on 4.5 or 6% polyacrylamide gels containing 8 M urea at 55°C with a Macrophor electrophoresis unit (LKB Instruments).

Computer analyses. Sequence data were analyzed with the PC/gene software package (Intelligenetics Inc. and Genofit SA). Searches of the GenBank (version 70.0) and SWISS-PROT (version 19.0) data bases were performed with the program FastA from the University of Wisconsin Genetics Computer Group software (13).

Isolation of mRNA, gel electrophoresis, and blotting. Total RNA was isolated from SK6 cells that had been infected with wt PRV 2 or 6 h before at a multiplicity of infection of 10. RNA was subjected to electrophoresis on 1% agarose–formaldehyde gels and blotted to nitrocellulose membranes (Schleicher & Schuell). Probes were labeled with [α-^{32}P] dATP by random priming. For these procedures and for hybridization of Northern (RNA) blots, we used protocols essentially as described before (31).

In vitro transcription and translation. Transcription in vitro by SP6 RNA polymerase in the presence of m^{7}G(5')ppp(5')G was performed as suggested by the manufacturer (Promega) with minor modifications. The vector was pSP73 (Promega). We used covalently closed templates and a GTP concentration of 200 μM instead of 50 μM to improve transcription of the GC-rich PRV DNA. In vitro translation in rabbit reticulocyte lysates was performed in the presence of [^{35}S]methionine according to the manufacturer's (Promega) recommendation. Synthesized proteins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and detected by autoradiography.

Generation of antibodies. A *Bgl*II site was fused to the 5' end of the PRV UL21 gene by treatment of *Nco*I-digested PRV UL21 with mung bean nuclease and ligation to the *Eco*RV site in the polylinker of pSP73. The PRV UL21 homolog was excised as a *Bam*HI-*Bgl*II fragment and fused in frame to the glutathione *S*-transferase gene in vector pGEX-2T (36) (Pharmacia). Fusion protein was induced in *Escherichia coli* JM109 by vigorous shaking in 2×LB medium for 8 h at 37°C in the presence of 0.5 mM isopropylthiogalactoside. Insoluble aggregates containing the fusion protein (inclusion bodies) were purified as described before (31). Protein (100 μg per mouse) was resuspended in complete Freund's adjuvant and injected subcutaneously at four sites in two BALB/c and two FVB mice. Mice were boosted with 100 μg of antigen resuspended in incomplete Freund's adjuvant 1 and 2 months after the primary immunization. One month after the final booster, the mice were bled. Antiserum was pooled and purified by overnight adsorption with inclusion bodies containing (unfused) glutathione *S*-transferase protein, and inclusion bodies were removed with bound immunoglobulin by centrifugation.

Labeling and fractionation of infected cells, and purification of labeled virions. From 3 to 24 h after infection at multiplicity of infection of 10, SK6 cells were labeled with 50 μCi of [^{35}S]methionine per ml in methionine-free DMEM–10% dialyzed fetal calf serum. Infected cells were fractionated into cytoplasmic and nuclear fractions as described before (27). Virus was purified from the infected-culture supernatant by low-speed clarification and centrifugation through a 30% sucrose cushion as described before (5). For direct analysis on SDS-PAGE gels, virions were precipitated with acetone, as described before (27).

Immunoprecipitations. PRV proteins were immunoprecipitated from purified labeled virions and cell fractions in RIPA buffer (27) with 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, 1 μg of pepstatin per ml, and 10 μg of leupeptin per ml. Lysates were precleared twice on ice with normal mouse serum and protein A-Sepharose beads (Pharmacia). Immunoprecipitation was done by incubation on ice for 1 h with anti-PRV UL21 antibodies (this study) or with the anti-glycoprotein gII monoclonal antibody 75N10 (16); protein A-Sepharose beads were added, and the mixture was shaken gently for 30 min at 4°C. The beads were washed three times in NET buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5% Nonidet P-40), pelleted, and resuspended in SDS sample buffer. Proteins were analyzed on 10 or 12.5% polyacrylamide–SDS gels.

Isolation of DNA from infected cells and determination of processing of viral DNA. SK6 cells were infected for 18 h with PRV mutants or with wt PRV. DNA was isolated by lysis of infected cells, proteinase K treatment, extraction with phenol-chloroform, and ethanol precipitation. DNA was digested with *Bam*HI, electrophoresed, and blotted. The blot was hybridized with the radiolabeled PRV *Bam*HI-14' fragment, derived from the left terminus of the unique long (UL) region (Fig. 1A).

Virulence of PRV mutants for mice. Virus suspension (100 μl) containing 10^6 PFU was injected subcutaneously in the neck of six 6-week-old BALB/c mice. The mice were monitored for clinical signs and death every 8 h.

Nucleotide sequence accession number. The GenBank accession number for the DNA sequence described here is M95285.

RESULTS

Mapping and sequence analysis of the PRV UL20 and UL21 genes. We mapped the PRV homolog of HSV-1 gene UL21 to the *Bam*HI-4 fragment of PRV by sequence analysis of PRV DNA flanking the oligonucleotide insertions in PRV mutants B59 and B9 (Fig. 1). Subsequently, we sequenced a 2.5-kbp region containing the complete ORF for PRV UL21 (Fig. 2). The DNA sequence also includes an ORF encoding the PRV homolog of HSV-1 UL20 (11, 24) and VZV gene 39 (10). Although the recently published DNA sequence of the major capsid protein of PRV (41) also included the DNA sequence of the PRV UL20 gene, the authors have not identified the PRV UL20 ORF. A few differences, likely due to strain divergencies, are present between their and our sequences (not shown).

Sequence features of the PRV UL21 gene and its encoded protein. The ORF that encodes the PRV homolog of HSV-1 UL21 (17, 24, 25) and VZV gene 38 (10) is 1,569 bp long (Fig. 2) and has the capacity to encode a protein of 523 amino acids with a predicted mass of 55 kDa. This ORF is preceded by a TATA box-like sequence. A putative poly(A) addition signal is present at the 3' end of the ORF (Fig. 2). In agreement with the distance of 1.6 kbp between these elements, we detected a late transcript of 1.8 kb [including the putative poly(A) stretch; Fig. 3]. The location of the PRV UL21 probe used for this hybridization is shown in Fig. 1C. In PRV mutants B59 and B9 (Fig. 1C), the mutagenic oligonucleotide is inserted within this ORF, 3' of position 859 in mutant B59 and 3' of position 1723 in mutant B9. The presence of the inserted oligonucleotide, represented by a unique *Eco*RI site within both PRV UL21 mutants, is visualized by *Bam*HI plus *Eco*RI digests of total DNA isolated from SK6 cells infected with strains B59 and B9. An agarose gel demonstrating this is shown in Fig. 4. The predicted lengths of the truncated proteins that are encoded by these mutants are only 4 amino acids in mutant B59 and 291 amino acids in mutant B9 (Fig. 5).

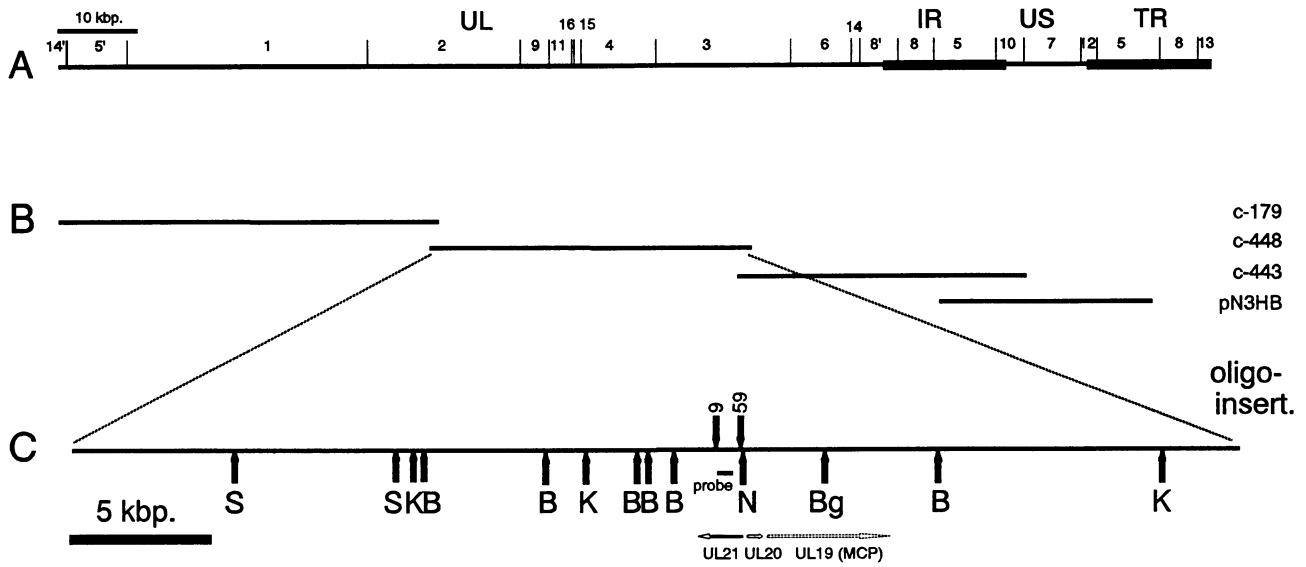

FIG. 1. (A) Schematic genomic map of PRV. The length of the total genome is estimated to be 140 kbp (6). UL, unique long region; US, unique short region; IR and TR, internal and terminal repeat, respectively. *Bam*HI sites and *Bam*HI fragments, labeled by the nomenclature of Ben-Porat and Kaplan (6), are indicated. (B) Cloned fragments used to regenerate PRV by cotransfection (38). (C) c-448, a derivative of c-27, used for oligonucleotide insertion mutagenesis (14). The locations of the PRV homologs of HSV-1 genes UL20 and UL21 are shown. MCP, major capsid protein (41), encoded by the PRV homolog of HSV-1 gene UL19 (11, 24). Arrows indicate oligonucleotide insertion sites relevant to this study. B, *Bam*HI; Bg, *Bgl*II; K, *Kpn*I; N, *Nco*I (not all *Nco*I sites are mapped); S, *Sca*I. The location of the probe used for Northern blotting is indicated.

Mutant B9, having plaques of wt size, showed a 10-fold reduction in titer compared with wt PRV after a single-step infection of SK6 cells. Mutant B59 grows very poorly and yields tiny plaques; this phenotype is rescued by growth on a cell line stably transfected with PRV UL21 under the control of the human cytomegalovirus immediate-early promoter (not shown).

Analysis of the deduced protein sequence of the PRV UL21 gene (Fig. 5) reveals a putative leucine zipper motif, involved in protein-protein interactions (amino acids 106 to

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1     CAGCGAGAGCGTGCTGCAGTAGGTGCCGAGCAGCGCGTCGAACTCGGCACCGTAGAGGTTGTTGTCGTCCGAGCGGAAGCGCTTGAAGATGTCGAACAGC
                                                                                <-- MCP N-term.
101   GCGCGGTGCGAGTGCACCTCGATGTTGGACAGGATCTGCCCCGACGGCAGGATGGCCGGGCGCTCCATCGTGTGGCGACGACCGCGTGCGGGCGCGTGTG
                                                  PRV UL20 C-term. *
201   CGCGGTCGCTGGGGAGCAGGGGGGAGAGTGGCGCGGGCGCGCCGCGGCCGCCTTTTAATGCATGATGGAGCGCGCGTACGCGCGCGGCAGCAGGAAGGCG
301   AGCGCGTCGCAGACGCAGTCGGCGCCGTACAGCGCCCCGGCGAGGGCGGGCGCGTAGTGGCGGCCCGCGTCCAGGGCGACGTACAGGAAGGCCAGCCGCG
401   CCGCCGCCAGGAGCCCCAGCGCCGCCTGCTGCGCCGGCGAGAGCGGCATCCGGTCCCGGGCCACGTTCCGCGCCACGAGGACGGCGCAGACGGCCGCGCG
501   CGCGTAGAAGGCCAGCGCGGTGAGGAGCGCCGCGAGGGTGGCCCGCGAGCCGTGGAGCGCCAGGACGAGGCAGCACGCGGGCCGGAGCGCGAGCGCCAGG
601   GCCAGGAGGCTCACCACGTGCGAGCTGAAGCGCGGGCGCGGCGGGCGCGCGCCCACAAAGGCCGAGCTCAGCAGCGCGTCGTTCTCCCCCGTCAGCTTCG
                                                  <-- PRV UL20 N-term.                          "TATA"
701   CGTCCGCCGCCGCGTCCACGTCCGCCGCCGCGTCCTCCATCCCCGCGCCGCCGCGTGTGTGCGGCGGGAGGCGCCCCCCGGCGGGTTTAAAGGAGTGGTC
                                        PRV UL21 N-term. -->
801   GCGCCGCCTCGCTCACCCATTTGCCGCGCCGCGTGTGCCCGCCGCCATGGAGTTTGAGTACCAGAGCACGATCGTGCACCAGGGGGTGCTGTTCTACGTC
901   GCCGACGGCGGGGACCGCGCGTACTTTGTGCACGGGGGCTGCATCGTGTCCGTGCACCGGCGCTCGCGGGAGATCGGCAAGTTCGGGCTCACGCTGCGCG
1001  GGAACGCGCCCGGGAACCGCGTCGTCGCCAACTACGTGCGCACGGAGCTGGCGCGCCTCGGCCGCGCGTGGGCCGCCCCGCAGGGGAGCGACGACGTCTT
1101  CGTGGACGCCCTGGGGCTGCTGCTGCCGCTGACGGAGCTGGACCTCTGCGGCCGCGCGGAGCTGGACGTGTACGACCCCTACCTCGTCGAGTGCATGGTC
1201  TCGCTGCCGGCGTCGGCGCTCTCGCTGACGCTCGTGCACGACCGCCAGCAGGACCGCGTCCTGGAGCTCCTGGCCGAGCCCGCCATCGTGCACCCCTCCT
1301  CGGGCTTCGTGTACGCCGTGAACGAGGCCTGCTTCGCGCTGGTGCAGGCGTACCTCTCCGAGCTGCCCAGCTCGCTGCAGGTGCTGACGGAGGGGCTCTT
1401  TGACGGCATCCCCGGCGTGCGCCCCCCGCTCAGCGGCGAGACGCGGCCCACGGCCGTGGTGGTGAAGGGCGGGCGCGCGGCCCCTACGCTGAGCGTGCGC
1501  CCGCGCCGGTACGCCGAGCGCGCGCTGCGCGCGACGGTGGTCAGCGACTTTGTGCAGGTGCGCTACATCCCGGCGACGCGGCGCATCTGGGCGACGCGCG
1601  GCGGGAGCCTGTCCCTGCAGATGCTCTGCGACCTCGTGGCCGGGGCGGACGCCATCCTGCGGCGGGCCGCGGGCGCCTCGGACGACGCCTCGGCCGCCGT
1701  GGTCGAGGCCGTGTCCGCCGTCGCGGCGGACCCCTTCTTCGGGACGGGCTCCACGTCGCTCACGGGCGCGCAGCGGTTCGCGCTGTACCAGTTCATCCTG
1801  GCCCGCTGGCACCTGCCGAGCTGCTACGCCGCGCTGGAGGGCATGCTCGACAGGCTGGACGAGCGCCCCGGGGCGGGCGCGGGCGACGACGACGACGGAG
1901  GAGAAGGGGGAGGAGGGGGCGGCCACGGCGGCTCTCGGGCCGCGAGCGCCGTGGCCCACGCCGTCAACCGCGTGCTGCGCGAGGCGACCGTCTTCGGCGA
2001  GGTGATGCGGATGCTCGTGAACGCCGCCGTGGTGCACGCCCCCGCGATCGCGGACCCGGCGGGGGCCTCGCCGCCGACGACGAAGCACGCCCGGGAGGAC
2101  GCGGCGACGGGCCTGGAGCTGGCGGTCATGATGAGCGACGCGGAGACGAACGCGCTGGACGCCGACGCGTGCGAGCTGGTGGAGGCGGCCGGGGCGCGGG
2201  TGCTGGACGGGCTCTACGCCGGCCGGGGCCTCGTCGCCGCGACGGCGCCCGTGGGGCGCGCGCTGCGGCCCACGTCCGCCGTCTGCGCCGAGGCGGCGCT
2301  CCTGACCGCCTTCGGCGACTCGCCCGCGGCCCTCCGCGGGGCCCAGTACCTGTTTCAGCTGTTTCGGGCGCGCCTGACCCGCGCCAACATCTCCATCGTC
      Poly(A)        * PRV UL21 C-term.
2401  CTCAATAAAAACCGTTAACCAAACACCACGTCCGCGCCCCCGTCCGTTCTCTGTCCGCGACACGCACACACGCGCGCGCCCGCCCGCCTCGGTCTCGGTG
2501  CCCCCCCGGA
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FIG. 2. DNA sequence containing the 5′ end of the gene for the major capsid protein (MCP) (41) and of the PRV UL20 and PRV UL21 homologs. The putative TATA box and poly(A) addition site of PRV UL21 are indicated. Note that the sequence is inverted with respect to the prototype genomic arrangement (Fig. 1A). term., terminus.

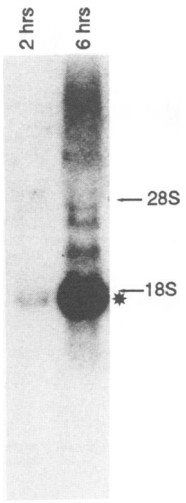

FIG. 3. Northern blot of RNA isolated from SK6 cells 2 and 6 h after infection with wt PRV. The blot was probed with a DNA fragment from the PRV UL21 gene flanking the inserted oligonucleotide in mutant B9 (Fig. 1C). The transcript for the PRV UL21 gene is indicated with an asterisk. The positions of the 18S (1.9 kb) and 28S (4.7 kb) rRNAs are indicated.

127). Another salient feature is a stretch very rich in aspartic acid and glycine residues (amino acids 342 to 362), which is predicted to form a flexible hinge in the protein. The significance of these stretches is unclear, although the Asp- and Gly-rich sequence may be a site of proteolytic processing (see below and Discussion). Alignment of the PRV UL21 protein with the protein sequences of HSV-1 UL21 and VZV gene 38 shows homology, which is predominant in the N-terminal half of the proteins and also near the C termini (Fig. 5). The overall homology between the PRV and VZV proteins is higher than that between the PRV and HSV-1 proteins.

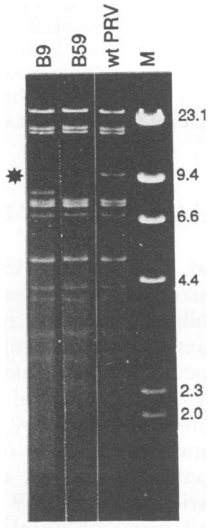

FIG. 4. Agarose gel of *Bam*HI plus *Eco*RI digests of total DNA isolated from cells infected with the PRV mutants described in this study and with wt PRV. The *Bam*HI-4 fragment (9.4 kbp) is indicated (asterisk). Lane M, marker fragments (sizes in kilobase pairs).

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                    ↓
PRV UL21    MEFEYQSTIVHQGVLFYVADGGDRAYFVHGGCIVSVHR----RSREIGKF  46
HSV-1 UL21  MELSYATTMHYRDVVFYVTTDRNRAYFVCGGCVYSVGRPCASQPGEIAKF  50
VZV gene 38 MEFPYHSTVSYNGVTFYFNERATRAYFICGGCLISIPR---KHGGEIAKF  47
            **. * .*. ...* ** ..  .****. ***. *. *    .  **.**

            GLTLRGNAPGNRVVANYVRTELARLGRAWAAPQGSDDVFVDALGLLLPL-  95
            GLVVRGTGPDDRVVANYVRSELRQRGLQDVRPIGEDEVFLDSVCLLNPNV 100
            GHVVRGVGPGDRSVASYVRSELNRTGKTWAVSSNNNCVFLDRVALLAAGS  97
            * ..** .*..* **.***.** . *   . . ... **.* . ** .

            -T-ELDLCGRAELDVYDPYLVECMVSLPASALSLTL-VHDRQQDRVLELL 142
            SS-ELDVINTNDVEVLDECLAEYCTSLRTSPGVLISGLRVRAQDRIIELF 149
            GAVDRDLCGTFDVEVEDPTLADYLVSLPVTHLTLVAGVDVTRENK-LKLF 146
             . . *. .  ...* *  *... .**...   *.  .    ... ..*.

            AEPAIVHPSSGFVYAVNEACFALVQAYLSELPSSLQVLTEGLFDGIPGVR 192
            EHPTIVNVSSHFVYTPSPYVFALAQAHLPRLPSSLEALVSGLFDGIPAPR 199
            PTPTAINTTNGFMYVPNEASFSLVYMRMLELPESLQELVSGLFDGTPEIR 196
              *. .. .. *.*. .   *.*.    .  **.**. *..*****.*. *

            PPLSGET----------RPTAVVVKGGRAAPTLSVRPRRYAERALRA--- 229
            QPLDAHN----------PRTDVVITGRRAPRPIAGSGAGSGGAGAKR--- 236
            DALNGSNDDEKTSIIVSRRAADVVTEDVKADDVPISGEPYSEKQPRRRKK 246
             .*.. .           .... *...  .  .. .    ..   .

            ---TVVSDFVQVRYIPATRRIWATRGGSLSLQMLCDLVAGAD------AI 270
            ---ATVSEFVQVKHIDRVGPAGVSPAPPPNNTDSSSLVPGAQDSAPPGPT 283
            SDHITLSNFVQIRTIPRVMDIWDPRHKATTH----------------C 278
                ..*.***.. *  .     ..  . .
                                    ↓
            LR----------RAAGASDDASAAVVEAVSAV---------------AAD 295
            LRELWWVFYAADRALEEPRADSGLTREEVRAVRGFREQAWKLFGSAGAPR 333
            IRALSCAVFFADEVIFKARKWPGLEDELNEARETIYTAVVAVYGERGEL- 327
            .*          .  .   ..  *  *              .

            PFFGTG-STSLTGAQRFALYQFILAR-WHLPSCYAALEGMLDRLDERPGA 343
            AFIGAA--LGLSPLQKLAVYYYIIHRERRLSP-FPALVRLVGRYTQRHGL 380
            PFFGHAYGRDLTSCQRFVIVQYILSR-WEAFNCYAVIEDLTRSYVN---- 372
            .*.* .   .*.  *.... .*. * .     ....  .  .  .

            GAGDDDDGGEGGGGGGHGGSRAASAVAHAVNRVLREATVFGEVMRMLVNA 393
            YVPRPDD----------------PVLADAINGLFRDALAAGTTAEQLLMF 414
            ALPSDDDTDQVAQD-------LIRTIVDTANSLLREVGFIGTLAETLL-- 413
                 **               ..... * ..*..   *.    *.

            AVVHAPAIADPAGASPPTTKHAREDAATGLELAV-MMSDAETNALDADAC 442
            DLLPPKDVPVGSDVQADSTALLRFIESQRLAVPGGVISPEHVAYLGA--- 461
            -FLPLPQLP----CYKETSHLAKKEGVRILRLAKTGVGLSDTVPVDVSVT 458
              ..  ...        ..  .  .  * ..   .. ...  ...

            ELVEAAGARVLDGLYAGRGLVAATAPVGRALRPTSAV--CAEAALLTAFG 490
            ---------FLSVLYAGRGRMSAATHTARLTGVTSLVLAVGDVDRLSAFD 502
            ERHEYEISRYLDTLYSGDPCYNGAVRLCRLLGSSIPIALYYNTISGNAFE 508
                     *. **.*    .....  *   .  .   ..  .**.

            DSPAAL----RGAQYLFQLFRARLTRANISIVLNKNR              523
            RGAAGAASRTRAAGYLDVLLTVRLARSQHG----QSV              535
            PYFAG----RRYIAYLGALFFGRVHQTPFGDGKKTQR              541
              *.    *  **  *.  *. ..  .
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FIG. 5. Alignment between the PRV UL21 protein sequence and those of its HSV-1 and VZV homologs. The putative leucine zipper and the Gly- and Asp-rich stretch in the PRV UL21 gene product are underlined. The sites of insertion of the mutagenic oligonucleotide in mutants B59 (C-terminally of amino acid 4) and B9 (C-terminally of amino acid 291) are indicated. Amino acids that are identical among the three proteins are indicated with an asterisk; similar amino acids are indicated with a dot. Dashes indicate gaps introduced to maintain alignment.

Expression of the PRV UL21 gene in vitro. To verify whether the PRV homolog of HSV-1 UL21 is able to encode a protein of the predicted size, several PRV DNA fragments were used as templates in an in vitro transcription-translation assay. In this assay, a single protein species with an apparent mass of 60 kDa is detected after expression from templates starting at position 846 or 5′ thereof (Fig. 6, lanes 1 to 3). This apparent mass is slightly larger than the deduced mass (55 kDa) of the PRV UL21 gene product. A construct in which the *Nco*I site, including the putative ATG start codon at position 847 (Fig. 2), was removed by mung bean nuclease digestion does not yield protein in the in vitro transcription-translation assay (Fig. 6, lane 4). From these data, we infer that translation of the protein starts at the ATG codon at position 847.

These data indicate that the PRV UL21 protein is identical to a 62-kDa (or 63-kDa [37]) capsid protein that was previously found to be encoded by the PRV *Bam*HI-4 fragment by hybrid selection followed by in vitro translation (23).

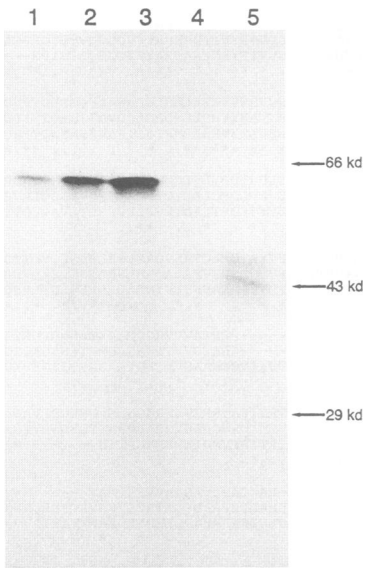

FIG. 6. In vitro transcription-translation of PRV UL21-containing DNA fragments cloned in pSP73. Radiolabeled proteins were analyzed on a 12.5% PAGE–SDS gel. Lane 1, *Sac*I (position 664 in Fig. 2)-*Bam*HI (downstream of PRV UL21, Fig. 1C) fragment; lane 2, *Dra*I (position 786 in Fig. 2)-*Bam*HI fragment; lane 3, *Nco*I (position 845 in Fig. 2; filled in with Klenow enzyme)-*Bam*HI fragment; lane 4, *Nco*I (position 845 in Fig. 2; removed with mung bean nuclease)-*Bam*HI fragment; lane 5, *Sac*I (position 1264 in Fig. 2)-*Bam*HI fragment. Positions and sizes of marker proteins are indicated on the right.

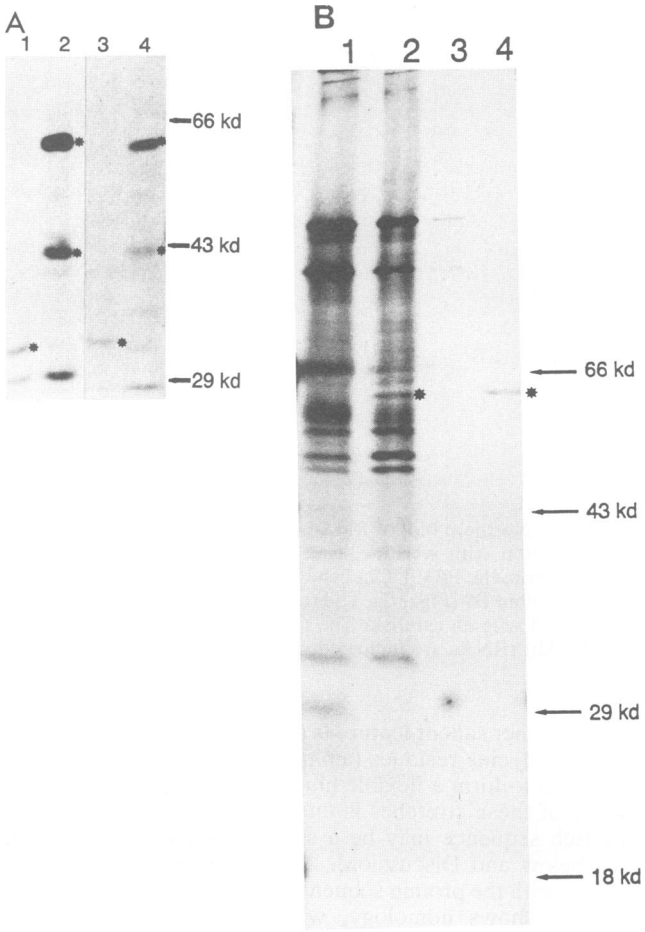

FIG. 7. Immunoprecipitations of the PRV UL21-encoded protein. (A) Fractionated infected cells. Lanes 1 and 2, cytoplasmic fractions; lanes 3 and 4, nuclear fractions. Lanes 1 and 3, truncated PRV UL21 protein precipitated from mutant B9; lanes 2 and 4, PRV UL21 protein precipitated from wt PRV. PRV UL21 protein-specific bands are indicated with asterisks. (B) Purified virions. Virions from mutant B9 (lane 1) and wt PRV (lane 2) precipitated with acetone; PRV UL21 protein precipitated from B9 virions (lane 3) and wt virions (lane 4). PRV UL21 protein is indicated with an asterisk. Note that 10 times more material was loaded on the gel in lanes 1 and 3 than in lanes 2 and 4, respectively. The sizes and positions of marker proteins are indicated on the right of each panel.

Detection of the PRV UL21 gene product in infected cells. We generated a polyclonal mouse antiserum against the PRV UL21-encoded protein produced in *E. coli*. This antiserum was used to immunoprecipitate the PRV UL21 protein from cytoplasmic and nuclear fractions of infected cells. The 60-kDa protein was detected in both the cytoplasm and the nucleus (Fig. 7A). Besides the 60-kDa band, a second major band of 41 kDa is precipitated from both cell fractions derived from wt-PRV-infected cells. The PRV UL21 gene product which is encoded by mutant B9 is truncated by premature termination of translation due to the inserted translational stop codon, has the expected mass of 32 kDa, and is also precipitated from both cytoplasmic and nuclear fractions. No 60-kDa or 41-kDa bands are visible in this lane, demonstrating the specificity of the antibody. This result supports the hypothesis that the 41-kDa product represents a processed form of the full-length PRV UL21 gene product. The presence of a secondary translational initiation site as the source of the 41-kDa product is unlikely, since we did not find the 41-kDa product in an in vitro translation reaction primed with a template in which the start codon at position 847 is deleted (Fig. 6, lane 4). That the nuclear fraction was not contaminated with cytoplasmic protein was evident from the inability to immunoprecipitate significant amounts of the (cytoplasmic) PRV glycoprotein gII from the nuclear fraction (not shown). A small fraction of glycoprotein gII is normally associated, during synthesis and processing, with the rough endoplasmatic reticulum, which is contiguous with the outer nuclear membrane (27). The PRV UL21 gene product is also detected in and immunoprecipitated from purified wt virions (Fig. 7B). Truncated PRV UL21 gene product is not found in virions of mutant B9 cells. Upon longer exposure, the 41-kDa cross-reacting protein is also visible in immunoprecipitates of PRV UL21 protein from wt virions (not shown).

Processing of viral DNA. Like HSV-1 genomes, PRV genomes are synthesized, at late stages of infection, as large concatemers in a rolling-circle-type replication (7). These genomes are subsequently cleaved to unit-length linear DNA molecules, in a process that is linked to encapsidation. Processing of newly replicated viral DNA to unit-length genomes can therefore be assessed by determining the ratio between free and concatemeric ends of the UL region. We hybridized a blot containing *Bam*HI digests of total DNA from cells infected with mutants B59 and B9 and wt PRV with the radiolabeled terminal *Bam*HI-14′ fragment (Fig. 1A). Processed viral DNA gives a band with a length of the *Bam*HI-14′ fragment (1.1 kbp), and concatemeric DNA gives a joint-spanning band consisting of the terminal *Bam*HI-14′ plus *Bam*HI-13 (1.6 kbp, Fig. 1A) fragments (a total of 2.7

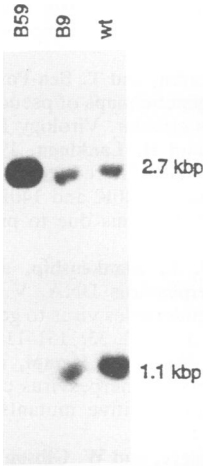

FIG. 8. Processing of viral DNA of PRV UL21 mutants and wt PRV. Lanes: 1, mutant B59; 2, mutant B9; 3, wt PRV. The lower (1.1-kbp) band represents processed DNA; the upper (2.7-kbp) band represents unprocessed (concatemeric) DNA. Note that approximately twice the amount of DNA was loaded of PRV strain B59 compared with that of strain B9 and wt PRV. After longer exposure, a minor processed (1.1-kbp) band is visible for mutant B59.

kbp). The resulting autoradiogram (Fig. 8) shows that DNA processing is very efficient in wt PRV. In mutant B59, lacking almost the entire PRV UL21 protein, DNA processing is nearly absent. In mutant B9, retaining 56% of the PRV UL21 protein, DNA processing is affected moderately. This result implies a role for the PRV UL21 gene product in capsid maturation.

Virulence of a PRV UL21 mutant for mice. Lomniczi et al. (23) mapped a mutation that reduced virulence to the *Bam*HI-4 fragment of attenuated PRV vaccine strain Bartha. We therefore assayed PRV UL21 mutant B9 for virulence for mice. The mean time to death of mice inoculated with PRV strain B9 was 145 ± 4 h, versus 54 ± 8 h for mice inoculated with a wt PRV strain, constructed by cotransfection of overlapping cloned wt viral fragments (38). This result clearly implies a role for the PRV UL21 gene product in determining the virulence of PRV.

DISCUSSION

Characterization of the PRV UL21 protein. The PRV UL21 protein appears to be identical to the 62- to 63-kDa protein found previously in PRV capsids (21, 23, 37). The transcript of the gene (1.8 kb) was also detected by Yamada et al. (41), although these authors estimated its size as 1.5 kb. The PRV UL21 transcript is most probably identical to the 2.1-kb transcript previously found to be encoded by the *Bam*HI-4 fragment (23). We have mapped the 1.5-kb transcript previously detected by Lomniczi et al. (23) to the right end of the *Bam*HI-4 fragment (not shown).

Analysis of the deduced PRV UL21 protein sequence does not provide insight on the function of the protein. Computer analysis predicts a putative leucine zipper (Leu-6aa-Leu-6aa-Leu-6aa-Leu, Fig. 5), found in many transcriptional regulators. Three of four leucine residues are conserved between PRV, HSV-1, and VZV (Leu-106 in PRV is Val in HSV-1 and VZV; Fig. 5). However, no stretches of basic residues are found in the vicinity of the Leu-6aa-Leu-6aa-Leu-6aa-Leu motif, as is the case with prototype leucine zippers (9). A second typical motif, the Gly- and Asp-rich stretch (amino acids 342 to 362 in Fig. 5) is predicted to form a hinge-like structure in the protein. The size of the protein that coprecipitates in immunoprecipitations of the PRV UL21-encoded protein (41 kDa) correlates well with the size expected if the protein were cleaved at the Gly- and Asp-rich stretch. This suggests that this stretch may be a signal for proteolytic processing. The absence of a shortened version of the 41-kDa protein encoded by mutant B9 (not shown), which lacks the Gly- and Asp-rich stretch, corroborates this notion. Remarkably, the N-terminal domain of the large subunit of the HSV-1 ribonucleotide reductase enzyme, which is sensitive to proteolytic cleavage (19), contains two similar stretches of small, nearly neutral amino acids (Ser in this case) combined with Asp residues (amino acids 189 to 206 and 223 to 235 [26]).

Virulence of mutant B9. PRV UL21 mutant B9 has a clearly reduced virulence for mice. Interestingly, Lomniczi et al. (23) found that vaccine strain Bartha contains a lesion affecting virulence, which maps in the *Bam*HI-4 fragment. Preliminary electron microscopic analysis of cells infected with the Bartha strain and PRV UL21 mutants show a similar maturation defect (not shown). Since PRV UL21 is thus far the only gene in the *Bam*HI-4 region of PRV which is implicated in capsid maturation (this work; see also below), strain Bartha may well have a defect in the UL21 gene. However, this hypothesis will have to be substantiated by marker rescue.

Role of PRV UL21 in capsid maturation. Both in HSV-1 (12, 34, 35, 39) and in PRV (20, 21), encapsidation of viral DNA is intrinsically linked to processing of newly replicated concatemeric DNA to unit-length viral genomes. The viability of PRV mutant B59, lacking almost the entire PRV UL21 protein, is severely compromised. This phenotype is rescued in a cell line stably expressing wt PRV UL21, demonstrating that the PRV UL21-encoded protein is important but not essential for viral viability in swine kidney cells. Processing of DNA to unit-length genomes is strongly impaired but not absent. This indicates a role for the protein in capsid maturation. Preliminary electron microscopic analysis of infected SK6 cells and nasal explant cultures supports a role for the protein in capsid maturation (not shown).

The finding that the PRV UL21 gene is not essential for virus viability indicates that the PRV UL21-encoded protein has a regulatory or accessory role in processing of viral DNA, in packaging, or in coupling of these processes (see also below). In mutant B9, which encodes a protein consisting of 56% of the wt protein, processing is affected only moderately. This suggests that the N-terminal part of the protein determines most of its function. It is tempting to speculate that the 41-kDa form of the protein, which may be derived by cleavage at the Gly- and Asp-rich stretch at amino acids 342 to 362, functions in capsid maturation. This is supported by the finding that predominantly the N-terminal halves of the PRV, HSV-1, and VZV homologs are conserved.

Previously, six PRV *ts* mutants that are unable to process and package viral DNA were found (20, 21). These mutants belong to different complementation groups, but none of the lesions map to the *Bam*HI-4 fragment of PRV (21). These mutants are also clearly different in phenotype from the mutants carrying mutations in the *Bam*HI-4 fragment of the viral genome, which do not synthesize any capsids (see the introduction). In the six PRV maturation mutants, the 62-kDa (UL21-encoded) protein is still found associated with the capsid at the nonpermissive temperature. The location of

the protein again supports a role in DNA processing, encapsidation, or coupling DNA processing to encapsidation.

A number of genes have now been identified in HSV-1 whose encoded proteins are involved in DNA processing and capsid maturation. Among these are the products of genes UL6 (39) (the mutation in the gene has not been identified yet), UL25 (1), UL26 (encoding the scaffolding protein [8, 28, 29, 35]), UL28 (2), and UL33 (3). An HSV-1-specific monoclonal antibody has been described which detects proteins with sizes similar to those of the PRV UL21 proteins (60 and 39 kDa) and that are associated with viral capsids as well (antibody Z2A9 [33]). This further supports the notion of functional homology between PRV- and HSV-1-encoded proteins that are involved in capsid maturation.

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