JOURNAL OF VIROLOGY, Sept. 1992, p. 5200–5209
0022-538X/92/095200-10$02.00/0

Vol. 66, No. 9

# Herpesviruses Encode an Unusual Protein-Serine/Threonine Kinase Which Is Nonessential for Growth in Cultured Cells

NIELS DE WIND, JOS DOMEN, AND ANTON BERNS*

*Division of Molecular Genetics of The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands*

Received 16 March 1992/Accepted 22 May 1992

**We have performed large-scale random oligonucleotide insertion mutagenesis on a 41-kbp genomic segment derived from the unique long (UL) region of the alphaherpesvirus pseudorabies virus (PRV). This procedure has resulted in the generation of a series of PRV strains, each carrying a single gene whose termination of translation is induced by the inserted oligonucleotide. To relate the genes that were involved in the mutagenization to genes previously identified in herpes simplex virus type 1, the prototype alphaherpesvirus, we have performed cross-hybridization studies. In this way, we have mapped the location of the homolog of a gene which was described to have sequence characteristics of a eukaryotic phosphotransferase. We characterized the phenotype of a mutant PRV strain lacking this putative phosphotransferase and also the phenotype of a PRV strain lacking, in addition to the UL-encoded putative phosphotransferase, the protein kinase encoded within the unique short region of the virus. To assess the enzymatic activity of the UL region-encoded phosphotransferase, we expressed the gene transiently in a eukaryotic expression system. Immunoprecipitation of the protein followed by kinase assays and phosphoamino acid analyses revealed protein-serine/threonine kinase activity. Implications of sequence divergence of this protein from classical protein-serine/threonine kinases for kinase structure and function are discussed in view of the recent resolution of the structure of the catalytic domain of cyclic AMP-dependent protein kinase.**

Herpesviruses are large enveloped viruses containing a linear double-stranded DNA genome. They can be divided into three subgroups; examples of human herpesviruses from these groups include the alphaherpesviruses herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) and varicella-zoster virus (VZV), the betaherpesviruses human cytomegalovirus (HCMV) and human herpesvirus 6 (HHV6), and the gammaherpesvirus Epstein-Barr virus (EBV). The genomes of HSV-1, VZV, HCMV, and EBV have been sequenced completely (1, 8, 15, 40, 42, 43, 46). The alphaherpesviruses and EBV encode about 70 genes, whereas HCMV encodes approximately 200 genes. These analyses also revealed the presence of large genomic regions that are partially syntenic, especially within subgroups (7, 8, 16, 35, 40; see references 38 and 39 for reviews). This finding made it possible to use data obtained on gene organization and function in a single herpesvirus as the basis for the study of gene structure and function in other herpesviruses. Functional analyses of genes, using virus strains with the gene of interest disrupted, have been performed predominantly with HSV-1 as a model system. Mutagenesis is generally performed by cotransfecting naked viral DNA together with a DNA fragment containing a copy of the gene with an inserted marker gene. This procedure is followed by screening for virus mutants that have incorporated the marker gene in the cognate locus by homologous recombination. Commonly used marker genes for this purpose are *lacZ* (23) and the thymidine kinase gene (reviewed in reference 48).

We recently described a novel approach for the mutagenesis of large genomic regions of a herpesvirus yielding, in a single experiment, a large series of virus mutants (19). Each of these mutants bears an oligonucleotide, containing translational stop codons in all reading frames, at a different site. Here, we report on the large-scale mutagenesis of a 41-kbp-long segment of the porcine alphaherpesvirus pseudorabies virus (PRV). We cloned short sequences flanking the inserted oligonucleotide from all mutants and used these sequences as probes to investigate colinearity between PRV and HSV-1. In this way, we traced a gene which has been found in herpesviruses of all subfamilies (9) and which is reported to have sequence characteristics of a phosphotransferase (9, 53).

We studied the phenotype of PRV strains carrying the mutagenic oligonucleotide within the gene. In addition, we sequenced the gene and expressed it transiently in mammalian cells. Immunoprecipitated protein was shown to have protein-serine/threonine (S/T) kinase activity on a number of protein substrates. On the basis of clustering alignments with other, classical S/T kinases, we show that the herpesvirus proteins form a distinct subgroup. On the basis of these alignments and of the recently resolved structure of the catalytic domain of the alpha form of cyclic AMP-dependent kinase (cAPK-α [32, 33]), a prototype S/T kinase (see reference 56 for a review), we discuss possible implications of the divergencies of the PRV-encoded S/T kinase for the structure and function of the enzyme.

## MATERIALS AND METHODS

**Cells and viruses.** PRV mutants were derived from PRV strain NIA-3 (2). Porcine kidney cell lines PK15 and SK6 were used for transfections and for virus growth and RNA isolation, respectively. Simian virus 40 large-T-antigen-transformed cell line COS-7 was used for transient expression studies. All cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

**Construction of PRV mutants.** Oligonucleotide insertion mutants were generated as described previously (19), using

* Corresponding author. Electronic mail address: TBERNS@NKI.NL.

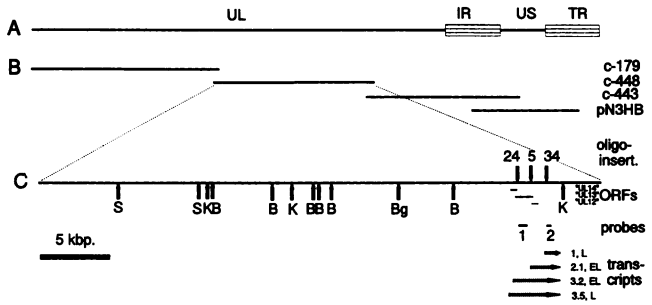


FIG. 1. (A) Schematic genomic map of PRV. The length of the total genome is estimated to be 140 kbp (3). UL, unique long region; US, unique short region; IR and TR, internal and terminal repeats. (B) Cloned fragments used to regenerate PRV by cotransfection (adapted from reference 57). C, c-448, a derivative of c-27, subjected to oligonucleotide insertion mutagenesis. Locations of the PRV homologs of HSV-1 UL12 and UL13 and the 5′ end of UL14 are indicated. Arrows indicate oligonucleotide insertion sites relevant to this study. B, *Bam*HI; Bg, *Bgl*II; K, *Kpn*I, S, *Sca*I. The locations of probes 1 and 2, used for Northern blotting, are indicated. The putative locations of the transcripts found in this region are shown below. EL, early-late transcript; L, late transcript.

as a target for mutagenesis cosmid c-448 (Fig. 1B), which is a derivative of cosmid clone c-27 (57) with *Hin*dIII linkers instead of *Eco*RI linkers attached to the insert. Briefly, c-448 was linearized at quasi-random sites, using restriction enzymes with 4-bp specificity, in the presence of ethidium bromide. Subsequently, a palindromic oligonucleotide containing stop codons in all three reading frames as well as an *Eco*RI restriction site, which is not present in the wild-type (wt) PRV genome, was ligated to the fragment. After *Eco*RI digestion and recircularization, *Escherichia coli* DH5 was transformed. The site of insertion of the mutagenic oligonucleotide was mapped by double digestions with *Eco*RI and either *Bam*HI, *Kpn*I, *Sca*I, or *Bgl*II. Mutant PRV strains were generated by cotransfection of PK15 cells with the mutagenized fragment, together with overlapping insert fragments (derived from c-179, c-443, and pN3HB; Fig. 1B), together comprising the complete PRV genome, as described previously (57). Viable PRV mutants were plaque purified three times on SK6 cells. DNA was isolated from all mutants; the site of insertion of the oligonucleotide and integrity of the viral genome were investigated by restriction enzyme analysis.

A double mutant bearing the oligonucleotide insertion in both PRV protein kinase genes was constructed by cotransfection of the inserts of mutant clones pN3HB-549 (mutant for the protein kinase encoded within the unique short [US] region of the virus [19]) and c-448-B24 (mutant for the PRV UL13 homolog [this work]) with c-179 and c-447 (a derivative of c-443 [19]) inserts.

**DNA sequencing.** Overlapping fragments of both DNA strands of the region of interest were subcloned into M13mp18 and M13mp19. Sequencing according to the method of Sanger et al. (51) was performed by using the T7 sequencing kit from Pharmacia (Uppsala, Sweden), with 7-deaza-2′-dGTP substituting for dGTP. Sequencing products were analyzed on 4.5 or 6% polyacrylamide gels containing 8 M urea at 55°C, using a Macrophor electrophoresis unit (LKB Instruments).

**Computer analyses.** Sequence data were analyzed by using the PC/Gene software package (Intelligenetics Inc. and Genofit SA) and the GCG software package of the University of Wisconsin Genetics Computer Group (18). Searches of the GenBank (version 70.0) and SWISS-PROT (version 19.0) data bases were performed by using the GCG program FastA. The alignment shown in Fig. 5 was performed by using the GCG programs Gap and PileUp and was modified by eyeball scanning to optimize grouping of residues in kinase subdomains as defined previously (25, 26). Multiple sequence alignments, yielding phylogenetic relationships, were performed by using the GCG program PileUp.

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

**Isolation of mRNA, gel electrophoresis, and blotting.** RNA was isolated from SK6 cells that had been infected with wt PRV 2 or 6 h previously with a multiplicity of infection of 10. Cells were extracted with acid phenol and then subjected to ethanol precipitation and poly(A)$^+$ selection on oligo(dT)-cellulose columns. RNA was subjected to electrophoresis on 1% agarose–formaldehyde gels and blotted to Nytran membranes (Schleicher & Schuell). Probes were labelled with [α-$^{32}$P]dATP by random priming. For these procedures and for hybridization of the Northern (RNA) blots, we used protocols essentially as described elsewhere (49).

**PCR and site-directed mutagenesis.** The high (80%) percentage of G · C base pairs in the 5′ part of the PRV gene interfered with amplification by the polymerase chain reaction (PCR) technique. Therefore, standard PCR conditions were adjusted to 60 cycles (1 min at 95°C, 1 min at 60°C, and 2 min at 72°C) in the presence of 0 to 5% dimethyl sulfoxide (depending on the fragment to be amplified). Ten nanograms of linearized plasmid DNA was used per amplification reaction. PCR primers used for fusion of a *Bam*HI site to the 5′ end of the PRV UL13 homolog were CCGCGTGGATCC ATGGCTGCTGGAGGAGGCGGAG and CGCGTACTGC AGGTCGTCCGGC. Site-directed mutagenesis of the PRV UL13 Lys-103 residue by using PCR was performed essentially as described previously (27) after subcloning of a 315-bp *Pst*I-*Sac*I DNA fragment into the polylinker of pSP72 (Promega). Oligonucleotides carrying the desired mutation were CGGTGGCCGTGATGACGCTCCG and CGGAGCG TCATCACGGCCACCG. External oligonucleotides, hybridizing to the SP6 and T7 promoters of the vector, were ATA CACATACGATTTAGGTGACAC and AAATTAATACGA CTCACTATAGGG, respectively. All PCR products were sequenced prior to use.

**Transfections and immunoprecipitations.** Ten-centimeter-diameter dishes with $5 \times 10^6$ cells of the COS-7 cell line were transfected by DNA-calcium phosphate coprecipitation as described previously (24), using covalently closed plasmid DNA that was purified by two successive cesium chloride-ethidium bromide density gradient centrifugations. The vector used for transient expression studies was pMT2 (31). Thirty-six hours after transfection, cells were labelled for 12 h with 50 μCi of [$^{35}$S]methionine per ml in methionine-free Dulbecco's modified Eagle's medium containing 10% dialyzed fetal calf serum and then subjected to lysis on ice in Nonidet P-40 (NP-40) lysis buffer (50 mM Tris-HCl [pH 7.5],

150 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.5% NP-40, 7 mM β-mercaptoethanol) containing 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 spun for 5 min in a microcentrifuge to remove nuclei, precleared twice with normal mouse serum and protein A-Sepharose beads (Pharmacia), and immunoprecipitated with 100 μl of culture supernatant of hybridoma P5D4 (34) and 4 mg of protein A-Sepharose beads. Beads were washed twice in NP-40 lysis buffer containing protease inhibitors and three times in NET buffer (50 mM Tris/HCl [pH 7.5], 150 mM NaCl, 0.5% NP-40, 7 mM β-mercaptoethanol), transferred to new tubes onto a 20% sucrose cushion in NET buffer, and pelleted. After two additional washings in NET buffer, immunoprecipitates were used either for direct analysis on SDS-polyacrylamide gels or for kinase assays.

**Kinase assays.** Protein A-Sepharose beads containing immunoprecipitated protein kinase were given a final wash in 20 mM Tris-HCl (pH 7.5)–150 mM NaCl–7 mM β-mercaptoethanol, resuspended in 10 μl of the same buffer containing 10 mM $MgCl_2$ or $MnCl_2$, and preincubated for 5 min on ice, after which 5 μCi of [γ-$^{32}$P]ATP was added. The final reaction volume was 15 μl; the phosphorylation reaction was for 30 min at 30°C. Phosphorylated proteins were analyzed by PAGE and autoradiography. Exogenous kinase substrates sometimes included were bovine histone mix and purified bovine histone H2B (Boehringer), salmon protamine (free base; Sigma), casein (Sigma), and the oligopeptide PLSRTLSVAAKK, from glycogen synthase (28; a gift from T. Eichholz), as S/T kinase substrates and angiotensin I peptide (Sigma), Src peptide (Sigma), and enolase (Sigma) as protein-tyrosine kinase substrates. Of the exogenous proteins, 1 μg was included per reaction; the final concentration of included peptides was 0.5 mM.

**Phosphoamino acid analysis.** Phosphorylated amino acids were identified by hydrolysis of phosphorylated proteins after elution from polyacrylamide gels, followed by two-dimensional electrophoresis on thin-layer cellulose plates and autoradiography, as described previously (6).

**Nucleotide sequence accession number.** The sequence data reported in this paper have been submitted to GenBank and assigned accession number M94870.

## RESULTS

**Mutagenesis of PRV.** Cosmid c-448, containing 41 kbp of PRV sequences (Fig. 1B), was mutagenized at quasi-random sites by insertion of a palindromic oligonucleotide containing stop codons in all reading frames and an *Eco*RI site which is not present in the PRV genome (3). Mutant PRV strains were generated by cotransfecting the c-448-derived viral DNA fragments with the inserts from PRV clones c-179, c-443, and pN3HB (Fig. 1B) (57). All resulting mutant virus strains were verified for the integrity of the viral genome and the presence of the inserted oligonucleotide by restriction enzyme analysis (not shown). Small *Eco*RI-*Sau*3A fragments, flanking the insertion sites of all mutagenized c-448 derivatives, were cloned and used as probes to detect cross-hybridization with cloned HSV-1 fragments under low-stringency conditions. The HSV-1 clones used were selected on the basis of earlier cross-hybridization studies (4, 17). In this way, we were able to map the PRV gene, homologous to HSV-1 gene UL13 (and to VZV gene 47, EBV gene BGLF4, HCMV gene UL97 or HSRF3, and HHV6 gene 15R [9]). The mapping data were confirmed by sequencing the clones flanking oligonucleotide insertion sites 24 and 5 (Fig. 1C); in these mutants, the oligonucleotides were found to be inserted in the 5′ and 3′ ends of the gene, respectively (see also below).

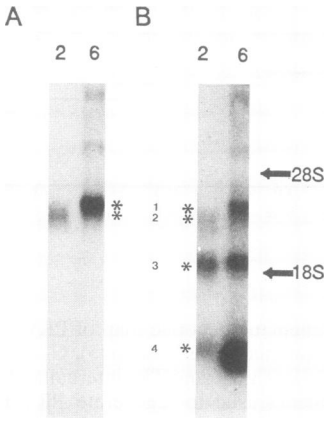


FIG. 2. Northern blot analysis of transcripts in the PRV UL14-UL13-UL12 region. Poly(A)$^+$ RNA was derived from wt NIA-3-infected SK6 cells isolated 2 and 6 h after infection. (A) Blot hybridized to probe 1 (Fig. 1C); (B) blot hybridized to probe 2 (Fig. 1C). The bands are numbered as follows: 1, approximately 3.5 kb late; 2, approximately 3.2 kb early-late; 3, 2.1 kb early-late; 4, approximately 1 kb late. Arrows indicate the positions of 28S and 18S rRNAs (4.7 and 1.9 kb, respectively).

**Northern blot analysis.** To detect early and late PRV-encoded transcripts, Northern blots were made with poly(A)$^+$ mRNA that was isolated from SK6 cells after infection with wt PRV for 2 or 6 h. Probes flanking the oligonucleotide insertion sites 24 (probe 1) and 34 (probe 2; Fig. 1C) were used for hybridization. As shown in Fig. 2, a number of overlapping transcripts were found. A late transcript of approximately 3.5 kb and a slightly shorter early-late transcript, detected with both probes 1 and 2, most probably represent messengers for PRV homologs of HSV-1 genes UL14 and UL13 (40, 44) (designated PRV UL14 and PRV UL13, respectively). The 2.1-kb early-late transcript, which is detected with probe 2 only, is probably the mRNA for the PRV homolog of the HSV-1 UL12 (alkaline nuclease [AN] [20, 40, 44]) protein. The small (about 1-kb) late transcript, detected with probe 2, probably represents the mRNA for a PRV homolog of HSV-1 open reading frame (ORF) UL11 (20, 40). These data indicate that all transcripts terminate 3′ of the latter gene, downstream of the tandem of PRV homologs of HSV-1 UL14, UL13, UL12, and UL11 (Fig. 1C). This conclusion is in concordance with the absence of poly(A) addition signals downstream of PRV UL14 and PRV UL13 (Fig. 3). This transcriptional organization closely resembles that of the corresponding HSV-1 genomic region (12, 13, 20).

**Sequence analysis of PRV UL13 and expression of the gene in vitro.** We sequenced 2,202 bp of PRV DNA, containing the PRV homologs of HSV-1 UL14, UL13, and the 5′ end of UL12 (Fig. 3). The PRV UL14, PRV UL13, and PRV UL12 homologs partially overlap. In mutant 24, the oligonucleotide is inserted between positions 716 and 852, in the 5′ part of PRV UL13; in mutant 5, it is inserted between positions 1602 and 1617, in the 3′ part of PRV UL13. These are the only oligonucleotide insertion mutants that were found to be accompanied by a small deletion. The deduced molecular size of the PRV UL13 protein is 41.5 kDa; the calculated isoelectric point of the protein is 9.2. For the PRV UL14

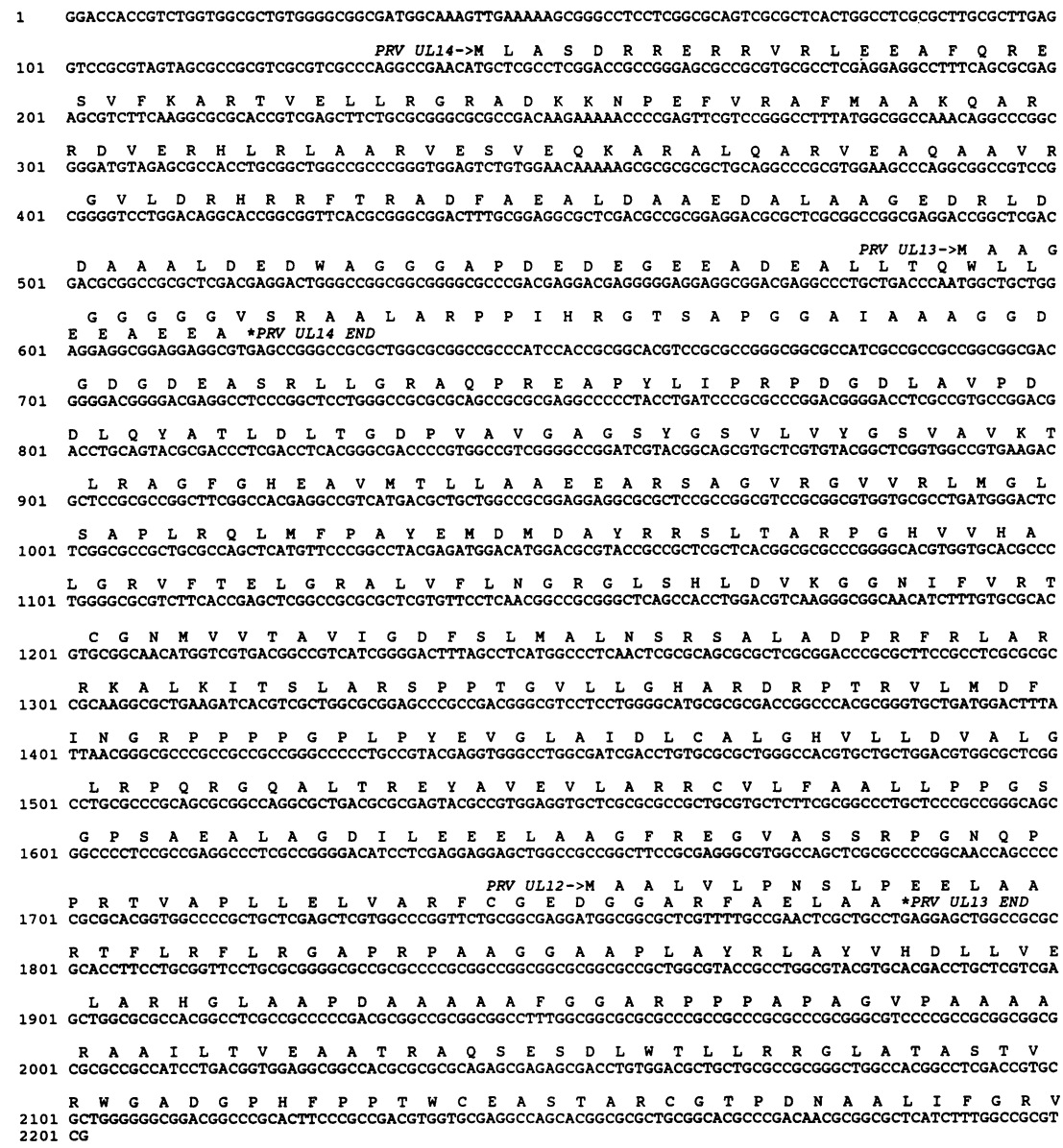


FIG. 3. Nucleotide and predicted amino acid sequences of the PRV UL14 and PRV UL13 (phosphotransferase) genes and part of PRV UL12 (AN) gene. The N and C termini of the ORFs are indicated. Amino acids are displayed in the one-letter code.

homolog, the calculated molecular size is 18 kDa. To verify that the PRV UL13 ORF was assigned correctly, a number of fragments were used as templates in an in vitro transcription-translation system. An autoradiogram demonstrating the correctness of the assignment is shown in Fig. 4. Lane 1 contains protein encoded by a clone containing a PRV DNA fragment starting 224 bp 5′ of the putative start codon of the PRV UL13 ORF (Fig. 3). Lane 2 demonstrates that the same protein is synthesized by a cloned PRV DNA fragment that starts at the putative start codon of PRV UL13 at position 590. These lanes both show the expected product of 41.5 kDa. As expected, no protein, except a version that is probably internally initiated, is synthesized from a clone starting at position 803 (lane 3). The product in lane 4, synthesized from a clone harboring oligonucleotide insertion 24, is most likely represented by a faint band that comigrates with the bromophenol blue dye. This result demonstrates that translation of the mutant gene product is terminated prematurely as expected.

Data bank computer searches yielded clear homology only between the deduced protein sequence encoded by the PRV UL13 gene and its HSV-1 and VZV homologs. Alignment between the protein encoded by the PRV UL13 gene and the HSV-1 UL13-encoded protein (Fig. 5) reveals that the HSV-1 protein has additional stretches of amino acid residues at a number of sites. These sites are particularly at the N terminus, which shows no homology, and at sites that coincide predominantly with the putative borders of protein kinase subdomains as defined by Hanks et al. (25, 26). For comparison, the sequence of the catalytic domain of cAPK-α is also included in Fig. 5. From this alignment and also from alignments between the herpesvirus homologs of the PRV UL13-encoded protein and a series of protein kinase sequences (9, 53; our results), it is apparent that a number of

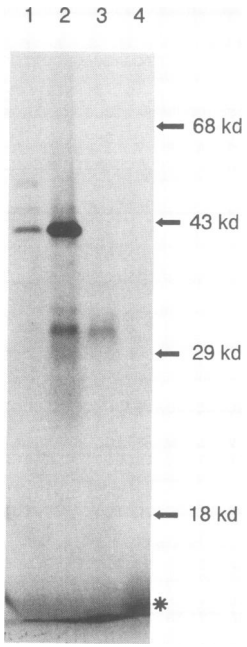


FIG. 4. In vitro transcription-translation of PRV UL13-containing DNA fragments, analyzed on an SDS–12.5% polyacrylamide gel. Lanes: 1, *Pst*I (position 366 in Fig. 3)-*Eco*RI (derived from an oligonucleotide insertion, about 2 kbp downstream of the *Pst*I site); 2, *Bam*HI (fused by PCR to the putative start codon at position 590; Fig. 3)-*Eco*RI; 3, *Pst*I (position 803; Fig. 3)-*Eco*RI; 4, *Pst*I (position 366; Fig. 3)-*Hin*dII (at about hypothetical position 2250 in Fig. 3) containing oligonucleotide insertion 24 within PRV UL13. The band comigrating with the bromophenol dye in this lane is indicated with an asterisk. Molecular sizes of the marker proteins are indicated on the right.

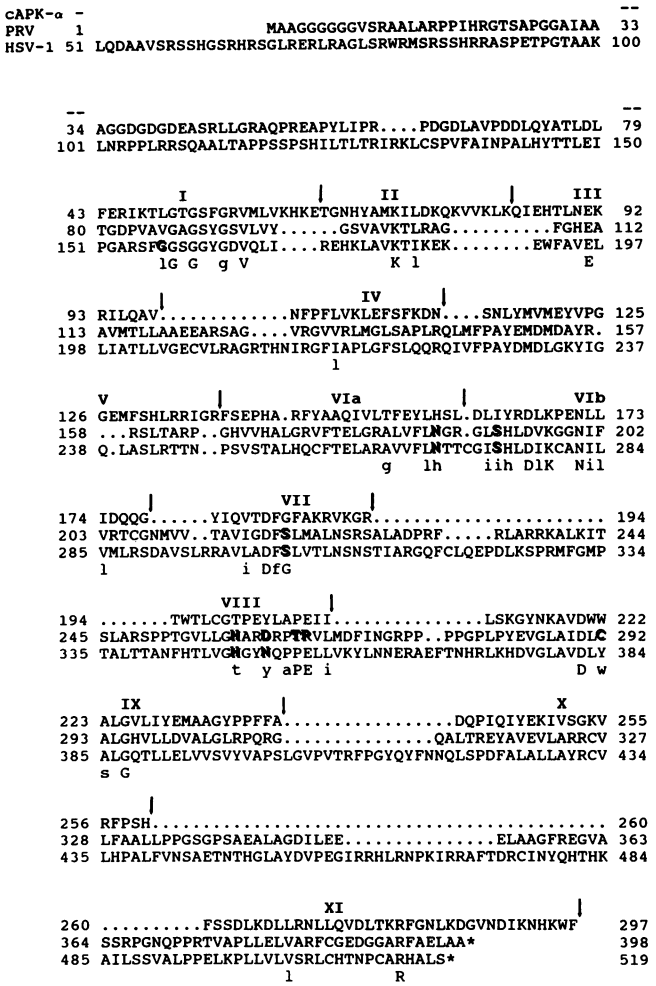


FIG. 5. Alignment between the catalytic domain of cAPK-α, of the PRV Ul13 gene product, and of the HSV-1 UL13 gene product. Kinase subdomains I to XI, as identified by the alignment of catalytic domains of 75 S/T kinases (25), are indicated. Arrows indicate the borders between the domains. Below the alignment, residues that are conserved in more than 72 of 75 S/T kinases are shown in capitals; residues that may be replaced by amino acids of similar nature are shown in lowercase. Groupings used for this purpose are nonpolar chain R groups (M, L, I, V, and C), aromatic or ring-containing R groups (F, Y, W, and H), small R groups with near-neutral polarity (A, G, S, T, and P), acidic and uncharged polar R groups (D, E, N, and Q), and basic polar R groups (K, R, and H). Amino acids deviating from the consensus are shaded.

the residues highly conserved in many kinases are absent in this protein and in the homologs from other herpesviruses (discussed below). In addition, many moderately conserved residues are absent from these proteins.

The phylogenetic relationship between the putative kinase catalytic subunit of the herpesvirus genes and that of classical kinase domains is illustrated in Fig. 6. A dendrogram that displays phylogenetic relationships among the putative catalytic domains of the herpesvirus proteins, a variety of protein kinase catalytic domains, and the catalytic domain of sea urchin guanylate cyclase, a protein related to protein kinases, was constructed by clustering computer alignment. This dendrogram demonstrates that PRV UL13 and its herpesvirus homologs form a subgroup distinct from classical protein kinases and even from the guanylate cyclase protein.

**Phenotype of PRV UL13 mutants.** Plaquing assays do not reveal significant differences in end titer and plaque size between the PRV UL13 mutant strain B24 and wt PRV (Table 1). To assess a role of the PRV UL13 gene in the viral infection cycle in cultured cells, we constructed a PRV UL13$^-$ double mutant that carries an additional oligonucleotide insertion in the protein kinase encoded by the US region of the virus (USPK [58, 60; see reference 36 for a review]). This double mutant yielded a strongly reduced end titer compared with that of wt PRV; in addition, the plaque size of the double mutant was very small (Table 1). The phenotype of the double mutant could be rescued by cotransfecting naked DNA of the double mutant with a 3-kbp intact DNA fragment encompassing the insertion site of B24, resulting in a PRV strain with a restored PRV UL13 gene. This result clearly indicates a role of the PRV UL13 gene product in the viral infection cycle.

**Immunoprecipitation of the PRV UL13 gene product, kinase assays, and phosphoamino acid analysis.** To determine whether the gene product of PRV UL13 is a protein kinase, we expressed the gene transiently in cultured cells. To be able to purify the protein from cells, we added an 11-amino-acid-long epitope (tag; derived from the vesicular stomatitis virus glycoprotein C-terminal end [34]) to the 3′ end of the PRV UL13-encoded protein. This was performed by removal of the TGA stop codon by *Sau*I digestion and mung bean nuclease treatment, followed by ligation to a DNA fragment containing the coding sequence for the epitope

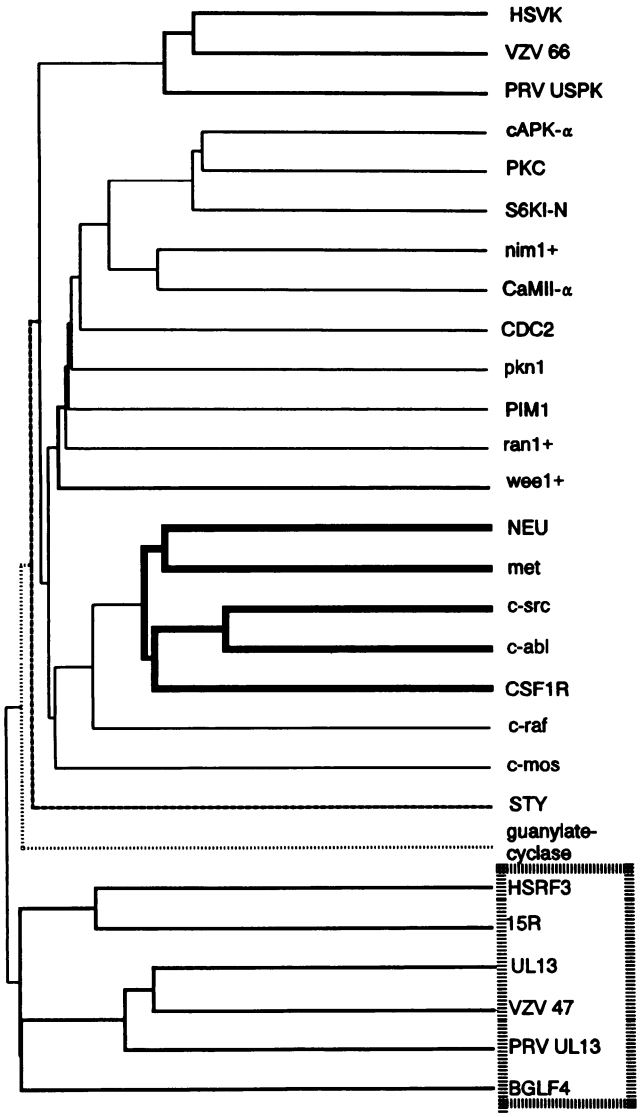


FIG. 6. Dendrogram displaying clustering relationships between catalytic domains of a variety of S/T kinases (solid lines), protein-tyrosine kinases (bold lines), a kinase with dual specificity (interrupted line), and sea urchin guanylate cyclase. Herpesvirus homologs of PRV UL13 are boxed. See reference 25 for references on the kinases (and guanylate cyclase) with the exception of PRV USPK and VZV 66, the PRV (58, 60) and VZV (15, 41) homologs of the alphaherpesvirus S/T kinase encoded in the US region (HSVK in HSV-1 [22, 25, 41, 47]), Pkn1 (45), STY (29), and the herpesvirus homologs of UL13 (this report).

preceded by codons for glycine, arginine, and proline (as a helix breaker).

To exclude the possibility that phosphorylation is performed by a contaminating kinase which could be physically associated with the PRV UL13 gene product or fortuitously recognized by the antibody, we constructed a mutant that has no intrinsic protein kinase activity but has probably retained its overall structure. To this end, we changed Lys-103 to the structurally similar amino acid methionine by site-directed mutagenesis. By analogy with known protein kinases, Lys-103 is predicted to be involved in ATP binding (5, 30, 32, 33, 54, 61); the mutation to Met was previously

TABLE 1. Virus end titers in a plaquing assay of PRV strains

| Strain | Genotype | Titer ($10^6$ PFU/ml) | Plaque size ± SD (mm) |
|---|---|---|---|
| B24 | PRV UL13$^-$ | 250 | 1.42 ± 0.09 |
| FH549 | USPK$^-$ | 60 | 1.44 ± 0.08 |
| B24/FH549 | PRV UL13$^-$ USPK$^-$ | 15 | 0.40 ± 0.07 |
| NIA-3 | wt | 300 | 1.66 ± 0.23 |

shown to abolish enzymatic activity of the epidermal growth factor receptor protein-tyrosine kinase (10) and the Pim-1 S/T kinase (52). Both the tagged PRV UL13 product and its mutant tagged derivative were cloned in expression vector pMT2. Immunoprecipitations of lysates from transfected COS-7 cells labelled with [$^{35}$S]methionine revealed the expected product for both wt and mutant proteins (Fig. 7, lanes 1 and 2). When these products are incubated with [$\gamma$-$^{32}$P] ATP, label is incorporated only in the presence of the wt protein (Fig. 7, lanes 5 and 6). In addition to the PRV UL13 gene product, several proteins of higher molecular weight that are present as [$^{35}$S]methionine-labelled minor contaminants in the immunoprecipitated material are phosphorylated as well. The phosphorylating activity is dependent on the presence of $Mn^{2+}$ ions; in the presence of only $Mg^{2+}$, no phosphorylation is found (Fig. 7, lanes 3 and 4). The exogenous substrates Src peptide, angiotensin I peptide, enolase (protein-tyrosine kinase substrates), and protamine (S/T kinase substrate) were not detectably phosphorylated by the kinase (not shown). In contrast, several histones, including H2B, are readily phosphorylated, as are casein and an oligopeptide substrate for protein kinase, derived from glycogen synthase. The latter peptides are all substrates for S/T kinases.

To determine the amino acid specificity of the kinase, we performed phosphoamino acid analysis on the autophosphorylated kinase and on histone H2B phosphorylated by the protein. Figure 8 shows that phosphorylated amino acids are serine and threonine in the autophosphorylated protein and serine in histone H2B. This result demonstrates that the protein is an S/T kinase.

## DISCUSSION

We performed random oligonucleotide insertion mutagenesis of a 41-kbp cloned PRV genomic fragment. Mutant virus is reconstituted by cotransfecting the mutagenized fragment with overlapping cloned fragments, together comprising the entire viral genome. Short DNA fragments flanking oligonucleotide insertion sites were cloned and used as probes to map viral transcripts and in cross-hybridization experiments with HSV-1 clones. This procedure enabled us to delineate colinear regions between the mutagenized part of the viral genome and the prototype alphaherpesvirus HSV-1. In this way, we traced the PRV gene homologous to the HSV-1 UL13 gene. The latter gene and homologs of this gene in other herpesviruses were recently reported to have characteristics of eukaryotic phosphotransferases (9, 53).

In two of the oligonucleotide insertion mutants, B24 and B5, expression of the PRV UL13 gene product is affected by premature termination of translation as a result of the presence of stop codons in the inserted oligonucleotide. Analysis of the end titer and plaque size of mutant B24 revealed almost no reduction in comparison with wt PRV. To reveal a role of the protein in the viral infection cycle in

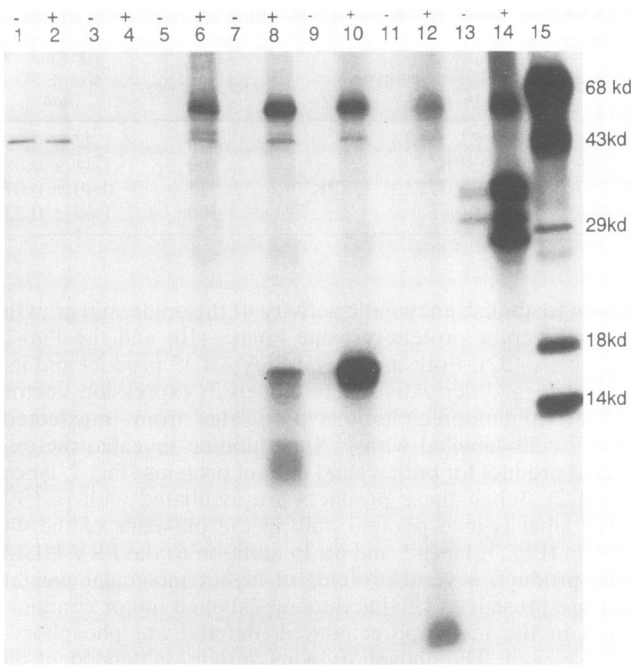


FIG. 7. SDS–20% polyacrylamide gel of the immunoprecipitated Lys-103→Met mutant of tagged PRV UL13 protein (−) and of wt tagged PRV UL13 protein (+). Lanes: 1 and 2, [$^{35}$S]methionine-labelled protein; 3 and 4, autophosphorylation in the presence of $Mg^{2+}$; 5 and 6, autophosphorylation in the presence of $Mn^{2+}$; 7 to 14, phosphorylation in the presence of $Mn^{2+}$ and added peptide or protein; 7 and 8, containing added histone mixture; 9 and 10, containing added histone H2B; 11 and 12, containing added glycogen synthase peptide; 13 and 14, containing added casein; 15, molecular weight markers (sizes indicated on the right). In lanes 3 to 14, approximately 3% of the amount of PRV UL13-encoded protein of lanes 1 and 2 is present.

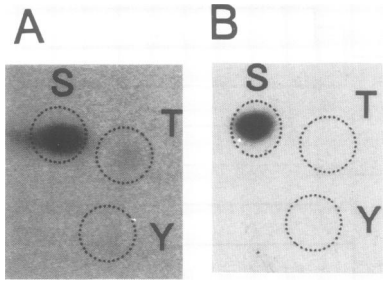


FIG. 8. Phosphoamino acid analysis of autophosphorylated tagged PRV UL13 protein (A) and of histone H2B phosphorylated by tagged PRV UL13 (B). Ninhydrin staining of marker phosphoamino acids is indicated by dotted circles; amino acid names are indicated in the one-letter code.

cultured cells, we constructed a double mutant in which the expression of both the PRV UL13 gene and the S/T kinase encoded in the US region of PRV (58, 60) and of other alphaherpesviruses (22, 41, 47; see reference 36 for a review) was abolished. This double mutant showed a strongly reduced end titer and plaque size. This result suggests synergism between both kinases, although it is also possible that the two kinases have the same substrate(s) and are able to take over each other's function so that only one of the genes is required for the virus. On the basis of the differences in substrate specificity in vitro (36), the latter possibility seems not very likely. At present, we have no clear indications as to the exact function of the PRV UL13 protein in the viral infection cycle. Determining the natural substrate(s) of the protein will be instrumental in this respect.

The derived amino acid sequence of the PRV gene reveals a basic protein (pI 9.2) of 398 amino acids with a molecular size of 41.5 kDa, considerably smaller than its herpesvirus homologs (9). Alignments show that most differences are found in the N-terminal parts of the proteins, outside the catalytic domain. In addition, we find a PRV homolog of HSV-1 UL14 at the 5′ of, and overlapping with, PRV UL13 (Fig. 1). The PRV homolog for the AN (UL12) gene is also found; the ORF starts at position 1752 within the 3′ end of the PRV UL13 ORF. This gene organization closely resembles that of herpesviruses of all other subgroups (although EBV possibly has no UL14 homolog [35, 44]). Northern blot analysis indicates that in PRV, the transcripts for the UL14, UL13, UL12, and UL11 homologs probably all terminate downstream of the UL11 homolog. This transcriptional organization is similar to that of the corresponding segment of the HSV-1 genome except for the absence of one of the two transcripts for the AN gene in PRV (probably the longer, late AN transcript [12, 13, 20]). The assignment of the size and location of the PRV UL13 gene and the absence of the protein as a result of the inserted oligonucleotide in mutant B24 were confirmed by expression of the PRV UL13-encoded protein in vitro.

Comparison of a series of 75 catalytic domains of S/T kinases has demonstrated the presence of many residues that are conserved in almost all kinases. However, a significant number of these highly conserved residues and many less well conserved residues are absent in the herpesvirus UL13 homologs (see also below). Moreover, a phylogenetic tree based on a multiple alignment of the catalytic domains of the herpesvirus proteins and those of classical S/T and protein-tyrosine kinases (and sea urchin guanylate cyclase) shows the herpesvirus proteins to belong to a very distinct branch (Fig. 6).

To directly assess the activity of the PRV UL13-encoded protein, we fused a tag to the C terminus and transiently expressed the protein in cultured cells. Immunoprecipitations followed by kinase assays demonstrated that the protein has intrinsic $Mn^{2+}$-dependent protein kinase activity, both in an autophosphorylation reaction and in phosphorylation of added substrates. Autophosphorylation is weak compared with the phosphorylation of contaminants in the immunoprecipitate. In this context, it is worthwhile to note that immunoblotting has shown that the major phosphorylated contaminant is recognized by antibody P5D4, suggesting that the contaminant may be a modified form of the PRV UL13-encoded protein. Surprisingly, both acidic (casein) and basic (histones and the glycogen synthase peptide but not protamine) polypeptides were substrates for the kinase. Phosphoamino acid analysis of autophosphorylated protein and of phosphorylated histone H2B revealed phosphoserine and phosphothreonine. This finding is in agreement with the presence of some S/T kinase-specific amino acid residues in the protein (see also below). Recently, Cunningham et al. (14) demonstrated the protein encoded by HSV-1 UL13 to be a virion protein (Vmw57) that is phosphorylated by a protein kinase which is induced in cells after virus infection. In view of the results described here, it is likely that this phosphorylation represents autophosphorylation of Vmw57; moreover, the HSV-1-induced kinase activity shows a specificity

for exogenous substrates similar to that of the PRV UL13 gene product (casein is a substrate, but not protamine).

Besides the S/T kinase encoded in the US region in alphaherpesviruses and the kinase domain present in the large subunit of the ribonucleotide reductase protein in HSV-2 (11), this is the third protein kinase found in herpesviruses. The product of the UL13 homolog is, however, the only kinase found in all (α, β, and γ) subgroups of herpesviruses (9). This finding suggests that the gene was acquired at an early stage of herpesvirus evolution, and this may also be the reason for the extensive sequence divergence from classical eukaryotic S/T kinases. In light of the recent resolution of the structure of the catalytic subunit of cAPK-α as a prototype S/T kinase (32, 33), the role of many of the amino acid residues in the kinase catalytic domain is resolved. Alignments between the deduced protein sequences of the herpesvirus UL13 homologs and classical S/T kinases (Fig. 5) (9, 53) provide additional information on the relevance of protein subdomains and conserved residues for kinase function. (i) In the PRV protein, the C-terminal part of subdomain I and the loop between subdomains I and II are absent. As in cAPK-α, these subdomains likely reside within the small lobe of the catalytic subunit and constitute the nucleotide-binding domain. Residues 57 to 63 and 67 to 75 in cAPK-α form antiparallel β strands. The sequence absent in the PRV protein (and some of its herpesvirus homologs), between subdomains I and II, is distal to the actual nucleotide-binding site. For this reason, the deletion is not expected to affect nucleotide binding directly. (ii) cAPK-α residues 78 to 87, which are absent in the PRV kinase, are close to the phosphate anchor in cAPK-α, although they extend somewhat from the protein core. Apparently, these residues are not essential for the conformation of the nucleotide-binding site, at least in the PRV UL13 gene product. (iii) Inserts as found between subdomains III and IV and between several other subdomains are present in more protein kinase catalytic domains (25) and generally form loops between regions of secondary structure in cAPK-α; inserts at these sites do not interfere with the activity of the enzyme (see also reference 32). Most divergences between the PRV kinase and classical S/T kinases reside in the large lobe of the catalytic subunit which extends from subdomains V (residue 126 in cAPK-α) to XI. This lobe is responsible for substrate recognition and binding. (iv) Subdomain VIb, containing a loop (residues 165 to 171 in cAPK-α) involved in orienting the substrate and in catalysis and an indicator of specificity towards S/T phosphorylation (26), essentially corresponds to the consensus in the herpesvirus proteins. (v) In subdomain VII, the highly conserved triplet DFG (positions 184 to 186 in cAPK-α) is DFS in PRV and HSV-1 (DYS in HHV6, HCMV, and VZV and DYG in EBV). Asp-184 is conserved in all 75 protein kinases aligned by Hanks and Quinn (25) and thought to be involved in interaction with MgATP (or MnATP) during catalysis. Its carboxylate interacts with the backbone amide of Gly-186, leading to stabilization of the local secondary structure. Tyr and Ser (in some of the herpesvirus proteins) are structurally very similar to Phe and Gly, respectively, so the function of the triplet is probably unchanged in the herpesvirus kinases. (vi) In subdomain VIII, dramatic differences are found between the herpesvirus sequences and the consensus. The conserved triplet Ala-Pro-Glu (positions 206 to 208) in this subdomain is considered to be an important protein kinase catalytic domain indicator. In the cAPK-α tertiary structure, this region is in close contact with the catalytic center and is involved in substrate binding. In addition, Thr-201 is an indicator of specificity toward S/T phosphorylation (26). Glu-208, which is invariant among all 75 S/T kinases aligned by Hanks and Quinn (25), strongly interacts with Arg-280 to stabilize this region of the protein. In our alignments with the other herpesvirus homologs, residues homologous with amino acids 201 to 208 (in cAPK-α) have also diverged (HGT NQPPE in VZV, KDTYKPLC in EBV, HPAFRPMP in HCMV, and NPGFRPLV in HHV6). The influence of these divergences on kinase function is unclear, although they demonstrate that the triplet APE that is found in the vast majority of the classical kinases is not pivotal for the activity of the enzyme. (vii) Subdomain IX, involved in stabilization of the catalytic center, largely conforms in our alignments to the consensus in all herpesvirus homologs (DLYALG in VZV, DLQSLG in EBV, ELSALG in HCMV, and DLQSL G in HHV6). (viii) We were unable to unambiguously align subdomain XI with the consensus. Hallmarks of this subdomain are an invariant arginine residue (Arg-280, interacting with Glu-208 in the substrate-binding domain), preceded at 11 amino acids by a hydrophobic residue and often followed, at 12 to 18 amino acids, by a 4-amino-acid-long stretch consisting of His, Pro, aromatic, and hydrophobic residues (25). We were not able to trace this pattern in the herpesvirus proteins, although we found conserved arginine residues preceded by a hydrophobic residue at −11 in all virus proteins except the HHV6 homolog. These aberrancies are not surprising since Glu-208 is also absent in most of the proteins. It is likely that the tertiary structure in the herpesvirus kinases is stabilized by another mechanism, although we cannot exclude, in light of the divergences in the substrate-binding domain, that these proteins bind to their substrates in a way fundamentally different from that of classical S/T kinases.

S/T kinases with highly aberrant sequences have been found to be encoded by some RNA viruses (hepatitis B virus transcriptional activator [59] and L proteins of vesicular stomatitis virus [50] and Sendai virus [21]); a mammalian S/T kinase (BCR) lacking a classical kinase domain has been described recently (37). The sequences of these S/T kinases have no clear homology with those of classical kinase catalytic domains. The S/T kinase family described here, however, is likely to be transduced by an ancient herpesvirus from a classical cellular kinase, as has been found to be the case with a number of other herpesvirus genes (see reference 55 for a review). The divergent evolution between the cellular proteins and their herpesvirus descendants allows further refinements of structure-function relationships of S/T kinases. Our analysis illustrates that a number of conserved kinase residues are not required for the catalytic activity of an S/T kinase.

## ACKNOWLEDGMENTS

We thank D. McGeoch for HSV-1 clones; Alice Zijderveld, Hanneke van der Gulden, and Ellen Wientjens for oligonucleotide synthesis and for helping with some of the experiments; and Jaqueline Alblas, Thomas Eichholz, Martijn Gebbink, Dick Schaap, and Loes van der Voorn for the VZV tag, P5D4 antibodies, vector pMT2, cell line COS-7, and protein kinase substrates. In addition, we thank Chris Saris for helpful discussions and Maarten van Lohuizen for critically reading the manuscript.

## REFERENCES

1. **Baer, R., A. T. Bankier, M. D. Biggin, P. L. Deininger, P. J. Farrell, T. J. Gibson, G. Hatfull, G. S. Hudson, S. C. Satchwell, C. Séguin, P. S. Tuffnell, and B. G. Barrell.** 1984. DNA sequence and expression of the B59-8 Epstein-Barr virus genome. Nature (London) **310**:207–211.

2. **Baskerville, A., J. B. McFerran, and C. Dow.** 1973. Aujeszky's disease in pigs. Vet. Bull. **43**:465–480.
3. **Ben-Porat, T., and A. S. Kaplan.** 1985. Molecular biology of pseudorabies virus, p. 105–173. *In* B. Roizman (ed.), The herpesviruses, vol. 3. Plenum Press, New York.
4. **Ben-Porat, T., R. A. Veach, and S. Ihara.** 1983. Localization of regions of homology between the genomes of herpes simplex virus, type 1, and pseudorabies virus. Virology **127**:194–201.
5. **Bhatnager, D., F. T. Hartl, R. J. Roskoski, R. A. Lessor, and N. J. Leonard.** 1984. Adenosine cyclic 3',5'-monophosphate dependent protein kinase: nucleotide binding to the chemically modified catalytic subunit. Biochemistry **23**:4350–4357.
6. **Boyle, J. W., P. van der Geer, and T. Hunter.** 1991. Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. Methods Enzymol. **201**:110–149.
7. **Chee, M., and B. Barrell.** 1990. Herpesviruses: a study of parts. Trends Genet. **6**:86–91.
8. **Chee, M. S., A. T. Bankier, S. Beck, R. Bohni, C. M. Brown, R. Cerny, T. Horsnell, C. A. Hutchinson III, T. Kouzarides, J. A. Martignetty, E. Preddie, S. C. Satchwell, P. Tomlinson, K. M. Weston, and B. G. Barrell.** 1990. Analysis of the protein-coding content of the sequence of huma cytomegalovirus strain AD169. Curr. Top. Microbiol. Immunol. **154**:125–169.
9. **Chee, M. S., G. L. Lawrence, and B. G. Barrell.** 1989. Alpha-, beta-, and gamma-herpesviruses encode a putative phosphotransferase. J. Gen. Virol. **70**:1151–1160.
10. **Chen, W. S., C. S. Lazar, M. Poenie, R. Y. Tsien, G. N. Gill, and M. G. Rosenfeld.** 1987. Requirement for intrinsic protein-tyrosine kinase in the immediate and late actions of the EGF receptor. Nature (London) **328**:820–823.
11. **Chung, T. D., J. P. Wymer, C. S. Smith, M. Kulka, and L. Aurelian.** 1989. Protein kinase activity associated with the large subunit of herpes simplex virus type 2 ribonucleotide reductase (ICP10). J. Virol. **63**:3389–3398.
12. **Costa, R. H., K. G. Draper, L. Banks, K. L. Powell, G. Cohen, R. Eisenberg, and E. K. Wagner.** 1983. High-resolution characterization of herpes simplex virus type 1 transcripts encoding alkaline exonuclease and a 50,000-dalton protein tentatively identified as a capsid protein. J. Virol. **48**:591–603.
13. **Costa, R. H., K. G. Draper, L. Banks, K. L. Powell, G. Cohen, R. Eisenberg, and E. K. Wagner.** 1985. An unusual spliced herpes simplex virus type 1 transcript with sequence homology to Epstein-Barr virus DNA. J. Virol. **54**:317–328.
14. **Cunningham, C., A. J. Davison, A. Dolan, M. C. Frame, D. J. McGeoch, D. M. Meredith, H. W. Moss, and A. C. Orr.** 1992. The UL13 virion protein of herpes simplex is phosphorylated by a novel virus-induced protein kinase. J. Gen. Virol. **73**:303–311.
15. **Davison, A. J., and J. E. Scott.** 1986. The complete DNA sequence of varicella-zoster virus. J. Gen. Virol. **67**:1759–1816.
16. **Davison, A. J., and P. Taylor.** 1987. Genetic relations between varicella-zoster virus and Epstein-Barr virus. J. Gen. Virol. **68**:1067–1079.
17. **Davison, A. J., and N. M. Wilkie.** 1983. Location and orientation of homologous sequences in the genomes of five herpesviruses. J. Gen. Virol. **64**:1927–1942.
18. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387–395.
19. **de Wind, N., A. Zijderveld, K. Glazenburg, and A. Berns.** 1990. Linker insertion mutagenesis of herpesviruses: inactivation of single genes within the US region of pseudorabies virus. J. Virol. **64**:4691–4696.
20. **Draper, K. G., G. Devi-Rao, R. H. Costa, E. D. Blair, R. L. Thompson, and E. K. Wagner.** 1986. Characterization of the genes encoding herpes simplex virus type 1 and type 2 alkaline exonuclease and overlapping proteins. J. Virol. **57**:1023–1036.
21. **Einberger, H., R. Mertz, P. H. Hofschneider, and W. J. Neubert.** 1990. Purification, renaturation, and reconstituted protein kinase activity of the Sendai virus large (L) protein: L protein phosphorylates NP and P proteins in vitro. J. Virol. **64**:4274–4280.
22. **Frame, M. C., F. C. Purves, D. J. McGeoch, H. S. Marsden, and D. P. Leader.** 1987. Identification of the herpes simplex virus protein kinase as the product of viral gene US3. J. Gen. Virol. **68**:2699–2704.
23. **Goldstein, D. J., and S. K. Weller.** 1988. Herpes simplex type 1-induced ribonucleotide reductase activity is dispensable for virus growth and DNA synthesis: isolation and characterization of an ICP6 *lacZ* insertion mutant. J. Virol. **62**:196–205.
24. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
25. **Hanks, S. K., and A. M. Quinn.** 1991. Protein kinase katalytic domain sequence database: identification of conserved features of primary structure and classification of family members. Methods Enzymol. **200**:38–62.
26. **Hanks, S. K., A. M. Quinn, and T. Hunter.** 1988. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science **241**:42–52.
27. **Higuchi, R., B. Krummel, and R. K. Saiki.** 1988. A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucleic Acids Res. **16**:7351–7367.
28. **House, C., R. E. H. Wettenhall, and B. E. Kemp.** 1987. The influence of basic residues on the substrate specificity of protein kinase C. J. Biol. Chem. **262**:772–777.
29. **Howell, B. W., D. E. H. Afar, J. Lew, E. M. J. Douville, P. L. E. Icely, D. A. Gray, and J. C. Bell.** 1991. STY, a tyrosine-phosporylating enzyme with sequence homology to serine/threonine kinases. Mol. Cell. Biol. **11**:568–572.
30. **Kamps, M. P., S. S. Taylor, and B. M. Sefton.** 1984. Direct evidence that oncogenic tyrosine kinases and cyclic AMP-dependent protein kinase have homologous ATP-binding sites. Nature (London) **310**:589–592.
31. **Kaufman, R. J., M. V. Davies, V. K. Pathak, and J. W. B. Hershey.** 1989. The phosphorylation state of eukaryotic initiation factor 2 alters translational efficiency of specific mRNAs. Mol. Cell. Biol. **9**:946–958.
32. **Knighton, D. R., J. Zheng, L. F. Ten Eyck, V. A. Ashford, N. Xuong, S. S. Taylor, and J. M. Sowadsky.** 1991. Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. Science **253**:407–414.
33. **Knighton, D. R., J. Zheng, L. F., Ten Eyck, N. Xuong, S. S. Taylor, and J. M. Sowadski.** 1991. Structure of a peptide inhibitor bound to the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. Science **253**:414–420.
34. **Kreis, T. E.** 1986. Microinjected antibodies against the cytoplasmic domain of vesicular stomatitis virus block its transport to the cell surface. EMBO J. **5**:931–941.
35. **Lawrence, G. L., M. Chee, M. A. Craxton, U. A. Gompels, R. W. Honess, and B. G. Barrell.** 1990. Human herpesvirus 6 is closely related to human cytomegalovirus. J. Virol. **64**:278–299.
36. **Leader, D. P., and F. Purves.** 1988. The herpesvirus protein kinase: a new departure in protein phosphorylation? Trends Biochem. Sci. **13**:244–246.
37. **Maru, Y., and O. N. Witte.** 1991. The BCR gene encodes a novel serine/threonine kinase activity within a single exon. Cell **67**: 459–469.
38. **McGeoch, D. J.** 1989. The genomes of the human herpesviruses—contents, relationships, and evolution. Annu. Rev. Microbiol. **43**:235–265.
39. **McGeoch, D. J.** 1990. Evolutionary relationships of virion glycoprotein genes in the S regions of alphaherpesvirus genomes. J. Gen. Virol. **71**:2361–2367.
40. **McGeoch, D. J., M. A. Dalrymple, A. J. Davison, A. Dolan, M. Frame, D. McNab, L. J. Perry, J. E. Scott, and P. Taylor.** 1988. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. **69**:1531–1574.
41. **McGeoch, D. J., and A. J. Davison.** 1986. Alphaherpesviruses possess a gene homologous to the protein kinase family of eukaryotes and retroviruses. Nucleic Acids Res. **14**:1765–1777.
42. **McGeoch, D. J., A. Dolan, S. Donald, and D. H. K. Brauer.**

1986. Complete sequence of the short repeat region of the genome of herpes simplex virus type 1. Nucleic Acids Res. **14**:1727–1747.

43. **McGeoch, D. J., A. Dolan, S. Donald, and F. J. Rixon.** 1985. Sequence determination and genetic content of the short unique region in the genome of herpes simplex virus type 1. J. Mol. Biol. **181**:1–13.
44. **McGeoch, D. J., A. Dolan, and M. Frame.** 1986. DNA sequence of the region in the genome of herpes simplex virus type 1 containing the exonuclease gene and neighbouring genes. Nucleic Acids Res. **14**:3435–3448.
45. **Muñoz-Dorado, J., S. Inouye, and M. Inouye.** 1991. A gene encoding a protein-serine/threonine kinase is required for normal development of *M. xanthus*, a gram-negative bacterium. Cell **67**:995–1006.
46. **Perry, L. J., and D. J. McGeoch.** 1988. The DNA sequences of the long repeat region and adjoining parts of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. **69**:2831–2846.
47. **Purves, F. C., R. M. Longnecker, D. P. Leader, and B. Roizman.** 1987. Herpes simplex virus 1 protein kinase is encoded by open reading frame US3 which is not essential for virus growth in cell culture. J. Virol. **61**:2896–2901.
48. **Roizman, B., and F. J. Jenkins.** 1985. Genetic engineering of novel genomes of large DNA viruses. Science **229**:1208–1214.
49. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
50. **Sanchez, A., B. P. De, and A. K. Banerjee.** 1985. In vitro phosphorylation of the NS protein by the L protein of vesicular stomatitis virus. J. Gen. Virol. **66**:1025–1036.
51. **Sanger, F. S., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
52. **Saris, C. J. M., J. Domen, and A. Berns.** 1991. The *pim-1* oncogene encodes two related protein-serine/threonine kinases by alternative initiation at AUG and CUG. EMBO J. **10**:655–664.
53. **Smith, R. F., and T. F. Smith.** 1989. Identification of new protein kinase-related genes in three herpesviruses, herpes simplex virus, varicella-zoster virus, and Epstein-Barr virus. J. Virol. **63**:450–455.
54. **Sternberg, M. J. E., and W. R. Taylor.** 1984. Modelling the ATP binding site of oncogenic products, the epidermal growth factor receptor and related proteins. FEBS Lett. **175**:387–392.
55. **Sugden, B.** 1991. Herpes viruses: human transducing viruses. Trends Biochem. Sci. **16**:45–46.
56. **Taylor, S. S., J. A. Buechler, and W. Yonemoto.** 1990. cAMP-dependent protein kinase: framework of a diverse family of regulatory enzymes. Annu. Rev. Biochem. **59**:971–1005.
57. **Van Zijl, M., W. Quint, J. Briaire, T. de Rover, A. Gielkens, and A. Berns.** 1988. Regeneration of herpesviruses from molecularly cloned subgenomic fragments. J. Virol. **62**:2191–2195.
58. **Van Zijl, M., H. van der Gulden, N. de Wind, A. Gielkens, and A. Berns.** 1990. Identification of two genes in the unique short region of pseudorabies virus; comparison with herpes simplex virus and varicella-zoster virus. J. Gen. Virol. **71**:1747–1755.
59. **Wu, J. Y., Z.-Y. Zhou, A. Judd, C. A. Cartwright, and W. S. Robinson.** 1990. The hepatitis B virus-encoded transcriptional *trans*-activator hbx appears to be a novel protein-serine/threonine kinase. Cell **63**:687–695.
60. **Zhang, G., R. Stevens, and D. P. Leader.** 1990. The protein kinase encoded in the short unique region of pseudorabies virus: description of the gene and identification of its product in virions and infected cells. J. Gen. Virol. **71**:1757–1765.
61. **Zoller, M. J., N. C. Nelson, and S. S. Taylor.** 1981. Affinity labelling of cAMP-dependent protein kinase with p-fluorosulfonylbenzoyl adenosine. Covalent modification of lysine 71. J. Biol. Chem. **256**:10837–10842.