JOURNAL OF VIROLOGY, Aug. 1993, p. 4549–4556
0022-538X/93/084549-08$02.00/0

Vol. 67, No. 8

African Swine Fever Virus Encodes a Serine Protein Kinase Which Is Packaged into Virions

SALLY A. BAYLIS,[1]† ALISON H. BANHAM,[1]‡ SOOPAYAH VYDELINGUM,[2] LINDA K. DIXON,[2] AND GEOFFREY L. SMITH[1]*

Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE,[1] and Pirbright Laboratory, Agriculture and Food Research Council Institute for Animal Health, Pirbright, Woking, Surrey GU24 ONF,[2] United Kingdom

Received 26 February 1993/Accepted 11 May 1993

Nucleotide sequencing of the *SalI* j region of the virulent Malawi (LIL20/1) strain of African swine fever virus (ASFV) identified an open reading frame (ORF), designated *j9L*, with extensive similarity to the family of protein kinases. This ORF encodes a 35.1-kDa protein of 299 amino acids which shares 24.6% amino acid identity with the human *pim*-1 proto-oncogene and 21.0% identity with the vaccinia virus *B1R*-encoded protein kinase. The ASFV ORF contains the motifs characteristic of serine-threonine protein kinases, with the exception of the presumed ATP-binding site, which is poorly conserved. The ORF was expressed to high levels in *Escherichia coli*, and the recombinant enzyme phosphorylated a calf thymus histone protein on serine residues in vitro. An antibody raised to an amino-terminal peptide of the ASFV protein kinase was reactive with the recombinant protein in Western immunoblot analyses and was used to demonstrate the presence of the protein kinase in ASF virions.

African swine fever virus (ASFV) causes an economically important disease of swine which is highly contagious and may devastate domestic pig populations. ASFV replicates not only in swine but also in several species of soft tick belonging to the genus *Ornithodoros* which act as vectors for transmission of the virus (reviewed in references 44 and 71). Replication of ASFV occurs in the cytoplasm of infected cells, although some evidence suggests that the host cell nucleus plays a role in the early stages of virus DNA replication and appears to be necessary for productive infection (20, 45). The ASFV genome is a linear, double-stranded DNA molecule with terminal cross-links and inverted terminal repeats which, depending on the strain of virus, varies in length between 170 and 190 kbp (reviewed in references 17 and 70). The genome is packaged within an icosahedral virus particle. Virus gene expression is temporally regulated, and genes are divided into three classes. The first contains genes which are expressed only early during infection, the second includes genes expressed both early and late, and the third consists of genes expressed late following DNA replication (18, 59, 68). Owing to its cytoplasmic mode of replication, ASFV is likely to encode a number of enzymes necessary for transcription and replication of the virus genome. Several ASFV genes that encode enzymes have been identified. These include enzymes involved in nucleotide metabolism, such as thymidine kinase (8, 25) and both large and small subunits of ribonucleotide reductase (9). Other enzymes which may play roles in virus DNA replication include a DNA ligase (22) and a type II DNA topoisomerase (7, 19). The virus also encodes a ubiquitin-conjugating enzyme (26). Other ASFV genes that have been identified are members of two multigene families (3, 21) and those encoding structural polypeptides p37, p72, p22, and p12 (1, 10, 39, 40).

Transcription of the ASFV genome is independent of host cell RNA polymerase II, and ASFV packages within virions a DNA-dependent RNA polymerase (34); mRNA capping, methylation, and polyadenylation enzymes (54); and a DNA topoisomerase (55). Several other enzyme activities are found in ASF virions; these include a single-strand-specific DNase (6), nucleoside triphosphohydrolases (35), an acid phosphatase (69), and a protein kinase (47).

The protein kinases are a family of phosphotransferases that transfer the phosphate groups onto protein substrates. The processes of protein phosphorylation and dephosphorylation serve to regulate many diverse cellular activities, including protein synthesis, cell division, and modulation of metabolic enzymes (reviewed in reference 28). The fundamental nature of protein phosphorylation and the evidence that ASFV packages a protein kinase within virions and that several ASFV polypeptides are phosphorylated (47, 56) collectively suggest that protein phosphorylation in ASFV is likely to represent an important regulatory mechanism in the replication of the virus. Like ASFV, members of the family *Poxviridae* replicate within the cytoplasm of infected cells and the prototypical member of this family, vaccinia virus, has been shown to contain protein kinase activity within virions (30–32). This activity is of at least two distinct types, one of which is encoded by the vaccinia virus *B1R* gene (4, 38).

Here we report the identification and initial characterization of an ASFV gene (*j9L*) which shows homology to members of the family of protein kinases. The ASFV protein kinase is enzymatically active, phosphorylating serine residues on a histone protein in vitro, and the protein is packaged into ASF virions.

MATERIALS AND METHODS

Recombinant DNA methodology. Restriction endonuclease digestions, DNA ligations, and plasmid DNA preparations

* Corresponding author.
† Present address: Department of Cell Biology, Wellcome Research Laboratories, Beckenham Kent BR3 3BS, United Kingdom.
‡ Present address: Plant Sciences Department, University of Oxford, Oxford OX1 3RB, United Kingdom.

were performed by standard procedures (57). DNA-modifying enzymes were obtained from Boehringer Mannheim and GIBCO-BRL. Oligonucleotide primers were synthesized on an Applied Biosystems 380-B DNA synthesizer. DNA was amplified by the polymerase chain reaction with AmpliTaq DNA polymerase (Perkin-Elmer Cetus) in accordance with the manufacturer's instructions.

Cloning and sequencing of the ASFV protein kinase gene. ASFV DNA isolated from plasmid clone pLMw18 (16, 16a), which contains sequences from *Sal*I restriction endonuclease fragment j of the Malawi LIL20/1 strain of ASFV, was sonicated, end repaired, and cloned into M13mp18 as previously described (5). Single-stranded DNA was sequenced by the dideoxynucleotide chain termination method (58) with [α-^{35}S]dATP and Sequenase (U.S. Biochemical, Cleveland, Ohio). A contiguous sequence was assembled with the SAP computer program (64, 65). The consensus sequence was translated into six frames with the ORFFILE program, and files for individual protein sequences were created with the DELIB program (both kindly provided by Mike Boursnell, Cantab Pharmaceuticals, Cambridge, United Kingdom). Protein sequences were compared against the SWISSPROT data base (release 22.0) with the FASTA program (46). Further protein and DNA sequence analysis was done with the University of Wisconsin Genetics Computer Group package of programs (15). The multiple protein alignments were created with the Pileup program and adjusted for best fit, where necessary, with the Lineup program.

Plasmid construction. Plasmid pLMw18 (16) was digested with *Eco*RI, and a 1.574-kbp fragment containing the ASFV protein kinase gene was purified, end filled with Klenow polymerase, and ligated into the *Sma*I site of pUC118 to create plasmid pPK1. To introduce *Eco*RI and *Nde*I restriction endonuclease sites at the 5′ terminus of the ASFV gene, the original 5′ coding sequence was replaced with a fragment amplified from pLMw18 by PCR with the oligonucleotide primers 5′-CCCGAATTCATATGTCCAGGCCGGAACAAC (5′ primer) and 5′-GTAGGATAAACTGCATATTG (3′ primer). Plasmid pPK1 was digested with *Eco*RI and *Bsu*36I to remove the 5′ 57 nucleotides of the ASFV protein kinase gene, and the large fragment was purified and ligated with the 81-bp polymerase chain reaction fragment, which had been digested with the same restriction endonucleases, to create plasmid pPK2. Plasmid pGMT7, derived from bacteriophage T7 expression vector pET-3c (52), contains a multiple cloning site immediately downstream of the T7 gene *10* translational start site and was used to express the ASFV protein kinase gene in *Escherichia coli*. pPK2 was digested with *Nde*I and *Bam*HI, and a 0.95-kbp fragment containing the ASFV protein kinase gene was purified and ligated with vector pGMT7 digested with the same enzymes. The resulting plasmid, pPK3, contained the ASFV protein kinase gene positioned downstream from the T7 gene *10* promoter, allowing expression from the first methionine codon in the ASFV open reading frame (ORF).

Bacterial strains. *E. coli* TG1 [*supE hsd*Δ*5 thi*Δ(*lac-proAB*) F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)] was used for DNA cloning. *E. coli* BL21(DE3)pLysE (66), transformed with plasmid pPK3, was used for expression of the ASFV protein kinase gene. Bacteria were grown at 37°C in either 2× TY medium or M9 medium (57) supplemented with 50 μg of ampicillin per ml for pUC-based plasmids or 200 μg of ampicillin per ml and 25 μg of chloramphenicol per ml for selection of plasmids derived from pGMT7 in *E. coli* BL21(DE3)pLysE.

Assay of ASFV protein kinase activity in *E. coli* cell extracts. *E. coli* cells transformed with plasmid pPK3 were grown to an optical density at 550 nm of 0.4, and expression of the ASFV protein kinase was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested at 2 h postinduction, and extracts were prepared as described previously (4). Bacterial extracts (5 μg of total protein) were assayed for protein kinase activity in a 10-μl volume containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM dithiothreitol, 5 μM ATP, 1 μCi of [γ-^{32}P]ATP, and 10 μg of protein substrate. The reaction was started by addition of the bacterial extract and stopped after 10 min at 37°C by addition of 10 μl of 2× protein sample buffer (125 mM Tris-HCl [pH 6.8], 4% sodium dodecyl sulfate [SDS], 40% glycerol, 1 M β-mercaptoethanol, 0.002% bromophenol blue). Samples were analyzed by SDS-polyacrylamide gel electrophoresis (36) and autoradiography of the dried gel.

Phosphoamino acid analysis. Calf thymus histones phosphorylated by the ASFV protein kinase, by using the in vitro protein kinase assay described above, were resolved on a 15% polyacrylamide gel and electrophoretically transferred to Immobilon-P. The ^{32}P-labeled histone band was excised and briefly rinsed with distilled water before hydrolysis in 5.7 M HCl for 1 h at 110°C. The supernatants were lyophilized and redissolved in pH 1.9 buffer (50:156:1,794 ratio of 88% formic acid-glacial acetic acid-H_2O), and phosphoamino acids were resolved by one-dimensional electrophoresis on a thin-layer cellulose plate at pH 3.5 as described previously (14, 29) and detected by autoradiography. Unlabeled phosphoamino acid standards were located by ninhydrin staining.

Production and purification of antibody to the N terminus of the ASFV protein kinase. An N-terminal peptide comprising the first 20 amino acids (CMSRPEQQFKKVLKNPQAQ YA) from the ASFV protein kinase was synthesized by the solid-phase technique. A New Zealand White rabbit was inoculated once intramuscularly and once subcutaneously with 1 mg of peptide in complete Freund's adjuvant at each site and then boosted via the same routes with 125 μg of peptide in incomplete Freund's adjuvant after 21, 42, and 56 days. Immune sera were collected after 21, 45, 56, and 63 days and tested for antipeptide activity by enzyme-linked immunosorbent assay with the peptide as the target antigen. Briefly, the peptide was adsorbed to 96-well plates and incubated with doubling dilutions of antiserum. After washing, bound antibody was detected by incubation with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Sigma Chemical Co.) and then with the substrate *para*-nitrophenylphosphate, and the A_{410} was determined. Serum showing a reciprocal of the endpoint dilution in excess of 64,000 was used for purification of antibody reactive with the immunizing peptide. The peptide (10 mg) was coupled by amino linkage to Proton 1 columns (Multiple Peptide Systems) in accordance with the manufacturer's instructions. Antipeptide serum (5 ml) was initially fractionated by ammonium sulfate precipitation and then applied to the Proton 1 column to which the peptide had been coupled. The column was washed, and purified antipeptide antibody was eluted by using procedures recommended by the manufacturer.

Western immunoblot analyses. (i) Bacterial extracts. Cultures of *E. coli* transformed with pPK3 were grown to an optical density at 550 nm of 0.4 and induced to express the ASFV protein kinase by addition of 1 mM IPTG for 1 h. The cells were then harvested by centrifugation and resuspended in 250 μl of 2× protein sample buffer. Ten-microliter volumes of induced or noninduced samples were electrophore-

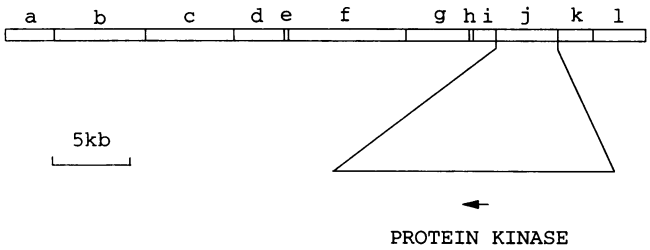

FIG. 1. *Sal*I restriction endonuclease map of the 180-kb ASFV Malawi LIL20/1 genome. The *Sal*I j fragment is expanded to show the position and orientation of the protein kinase gene. The scale refers to the expanded *Sal*I j restriction fragment.

sed on SDS–10% polyacrylamide gels and electrophoretically transferred to Immobilon-P (Millipore). Antipeptide antibody was incubated with blots overnight at 4°C at a dilution of 1 in 200 in Tris-buffered saline containing 5% Marvel (BLOTTO). Blots were then washed and reacted with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G at a dilution of 1 in 1,000 for 3 h at room temperature. After washing in Tris-buffered saline containing 0.05% Tween 20, protein reactivity was detected with a naphthol AS-MX Fast Blue substrate (Sigma Chemical Co.)

(ii) ASF virions. The ASFV Uganda isolate was grown in IBRS2 cells, and virus was purified from the supernatant fraction by Percoll gradient centrifugation (11). Purified virus was disrupted in protein sample buffer, and polypeptides were separated by electrophoresis on SDS–12% polyacrylamide gels. Proteins were blotted onto Hybond C super membrane (Amersham). The purified antipeptide antibody was preabsorbed with uninfected IBRS2 cell extracts and reacted, at a dilution of 1 in 50 for 1 h at 37°C, with protein blots which had been blocked in phosphate-buffered saline containing 1% Tween 20 and 3% bovine serum albumin. Blots were then washed and reacted with swine anti-rabbit serum coupled to horseradish peroxidase at a dilution of 1 in 2,000 for 1 h at 37°C. After being washed in PBS-Tween 20, blots were reacted with ECL reagents (Amersham) and bound antibody was detected by autoradiography.

Nucleotide sequence accession number. The nucleotide sequence of the gene for the ASFV protein kinase appears under accession number X72954 in the EMBL data base.

RESULTS

Homology of the 35.1-kDa ASFV ORF with protein kinases. Translation of the ASFV DNA sequence from clone LMw18 revealed the presence of 16 complete ORFs of greater than or equal to 65 amino acids. One of these ORFs, predicted to encode a 35.1-kDa polypeptide of 299 amino acids, is the subject of this report and is called *j9L*. The *Sal*I restriction endonuclease map of the Malawi LIL20/1 strain of ASFV is shown in Fig. 1, and the *Sal*I j fragment is expanded to show the position and predicted direction of transcription of the 35.1-kDa ORF. The nucleotide sequence of the gene and its encoded amino acid sequence are shown in Fig. 2. The sequence that surrounds the first ATG of the ORF is AACATGT, which is likely to be an efficient site for translational initiation since it conforms reasonably well to the eukaryotic translation consensus sequence ACCATGG (33).

Recent transcriptional analysis of ASFV multigene family 110 has revealed that these genes are expressed early before

```
1    TTTTTTACCAAAAAAACAAAGAGGTAGGATTAGGCTATACCTTTTATGAATACAATCCCATCATTAAGGCATATATTGAAATTAAACCTTCTAATCCTTTATATATAAAGTTAATAAAAC  120

     M  S  R  P  E  Q  Q  F  K  K  V  L  K  N  P  Q  A  Q  Y  A  V  Y  P  T  I  K  V  E  R  I  S  T  T  E  H  M  Y  F  I  A
121  ATGTCCAGGCCGGAACAACAGTTTAAAAAAGTGTTAAAAAACCCTCAGGCACAATATGCAGTTTATCCTACAATAAAGGTGGAAAGGATCTCCACAACGGAGCACATGTACTTCATTGCA  240

     T  K  P  M  F  E  G  G  R  R  N  N  V  F  L  G  H  Q  V  G  Q  P  V  V  F  K  Y  V  S  K  K  E  I  P  G  N  E  V  V  V
241  ACGAAACCTATGTTTGAGGGTGGTAGAAGAAATAACGTATTTTTGGGTCATCAAGTAGGGCAACCAGTTGTTTTTAAGTATGTATCTAAAAAAGAAATTCCAGGAAATGAGGTCGTCGTG  360

     M  K  A  L  Q  D  T  P  G  V  I  K  L  I  E  Y  T  E  N  A  M  Y  H  I  L  I  I  E  Y  I  P  N  S  I  D  L  L  H  Y  H
361  ATGAAAGCCCTGCAAGATACTCCCGGAGTTATTAAGCTTATTGAATATACTGAAAATGCCATGTATCATATACTGATCATAGAATATATTCCAAATAGTATTGATCTACTTCATTATCAT  480

     Y  F  K  K  L  E  E  N  E  A  K  K  I  I  F  Q  M  I  L  I  I  Q  N  I  Y  E  K  G  F  I  H  G  D  I  K  D  E  N  L  I
481  TACTTTAAAAAACTTGAGGAAAACGAAGCCAAAAAAATAATATTTCAGATGATTCTTATTATACAAAACATTTATGAGAAGGGCTTTATCCACGGGGATATTAAGGATGAGAACCTTATT  600

     I  D  I  D  Q  K  I  I  K  V  I  D  F  G  S  A  V  R  L  N  E  T  H  P  Q  Y  N  M  F  G  T  W  E  Y  V  C  P  E  F  Y
601  ATAGATATAGATCAAAAGATCATTAAGGTCATTGACTTTGGAAGCGCTGTTCGATTAAATGAAACCCATCCTCAATATAATATGTTTGGAACATGGGAATACGTGTGCCCAGAATTTTAT  720

     Y  Y  G  Y  Y  Y  Q  L  P  L  T  V  W  T  I  G  M  V  A  V  N  L  F  R  F  R  A  E  N  F  Y  L  N  D  I  L  K  G  E  N
721  TATTATGGTTATTACTACCAGCTTCCTCTAACGGTGTGGACGATAGGTATGGTGGCGGTTAATCTCTTTAGATTTCGTGCAGAAAATTTTTATTTAAATGATATCCTGAAAGGAGAAAAC  840

     Y  I  P  D  N  I  S  E  T  G  K  Q  F  I  T  D  C  L  T  I  N  E  N  K  R  L  S  F  K  G  L  V  S  H  P  W  F  K  G  L
841  TATATTCCCGATAACATTTCAGAGACGGGAAAACAGTTTATCACGGATTGTTTAACAATTAATGAAAATAAGCGGCTTTCTTTTAAGGGTCTTGTATCACATCCTTGGTTTAAAGGGCTT  960

     K  K  E  I  Q  P  I  S  E  L  G  V  D  Y  K  N  V  I  T  *
961  AAAAAAGAAATCCAGCCTATTTCAGAACTAGGAGTAGATTATAAAAATGTGATTACTTAGACGTTTTTAAAGATTTAAAGTAAAGTGGAATTCTGATTCGATTTTTTGGTATATATAGAT  1080

1081 TTATTTTTTCTGATAAAAGCGGTGGTATACATTTGATAAAAATCGTGTTATTTTTAT 1137
```

FIG. 2. Nucleotide and deduced amino acid sequences of the ASFV protein kinase gene from the Malawi LIL20/1 strain. Nucleotides are numbered starting 120 bp upstream of the first methionine codon of the ORF.

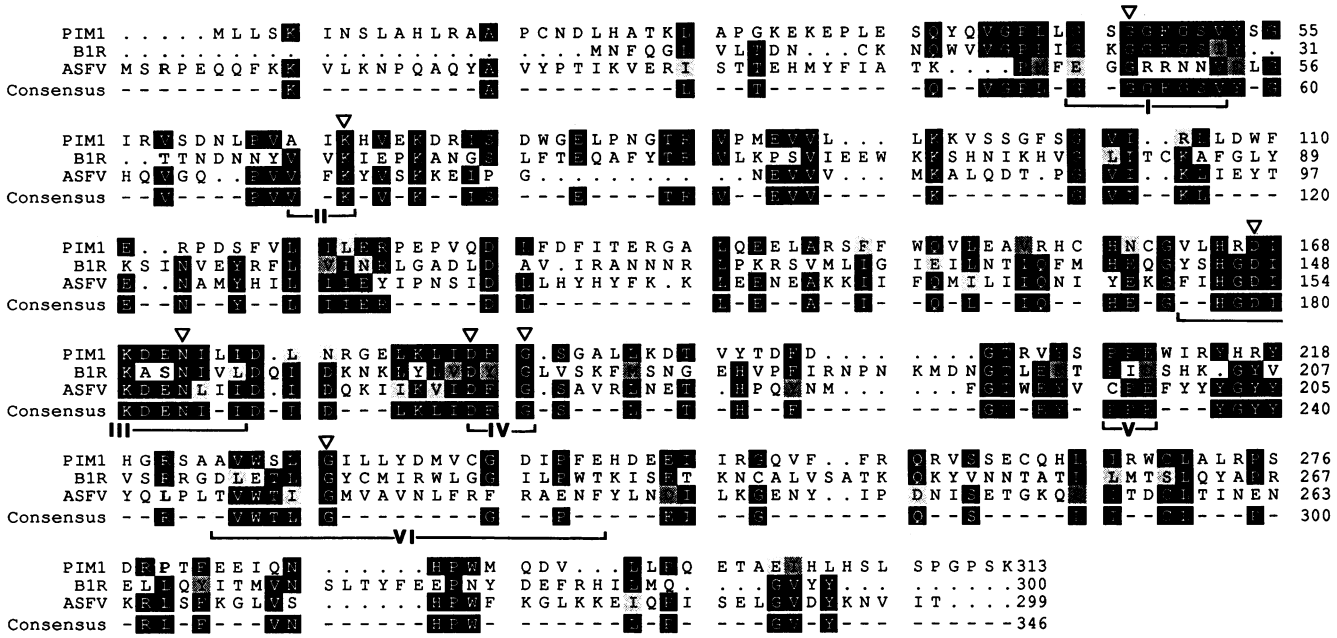

FIG. 3. Amino acid alignment of the ASFV protein kinase, the human *pim*-1 proto-oncogene (48), and the vaccinia virus *B1R*-encoded protein kinase (27, 67). The conserved catalytic domains are numbered I to VI, and residues conserved in all of the protein kinases are indicated by arrowheads (37). The alignment was created and edited by using the Pileup and Lineup programs and displayed by using Prettybox. Conserved, similar, and somewhat similar residues (defined previously [61]) are highlighted with black, dark, and light shading, respectively. Nonmatching amino acid residues are not highlighted. Where two or more sequences are identical, these and the consensus sequence are boxed.

the onset of virus DNA replication (2). Although ASFV promoter sequences have not been defined, Almazán et al. (2) found that the regions immediately upstream of the transcriptional initiation sites of the multigene family are significantly AT rich. The 3′ ends of these transcripts terminate a short distance from the translational termination codon and map within seven or more consecutive thymidylate residues (7T). However, in gene *j9L*, no 7T motif is present within 1,150 nucleotides downstream of the ORF.

A search of the SWISSPROT data base (release 22.0) revealed that the 35.1-kDa ORF showed homology to members of a family of protein kinases. The best matches were found against the mouse and human *pim*-1 proto-oncogenes, with optimal FASTA scores (with a KTUP of 1) of 296 and 287, respectively (48, 62). The ASFV 35.1-kDa ORF shows 30.1% amino acid identity with the murine enzyme over a 209-amino-acid region and 27.6% amino acid identity over a 210-amino-acid region with the human enzyme. Significant scores were found with several other protein kinases, and these include, for example, rat glycogen synthase kinase-3α (72), with 24.5% amino acid identity over a 241-amino-acid overlap, and the rat *mak*-encoded protein kinase (43), with 30.1% amino acid identity over a 153-amino-acid overlap. The ASFV ORF also shares 21.0% amino acid identity with the vaccinia virus *B1R*-encoded protein kinase (27, 67). In all cases, the homologies reflect the overall conservation of the catalytic domains of protein kinases.

An alignment of the 35.1-kDa ASFV ORF with the human *pim*-1 proto-oncogene and the vaccinia virus *B1R*-encoded protein kinase is shown in Fig. 3. The alignment was created with the program Pileup (15) and adjusted by hand. A number of conserved residues are present which indicate that the 35.1-kDa ORF is likely to function as a protein kinase with serine-threonine specificity. Here reference is made to the six major conserved regions found in catalytic domains of protein kinases (63), which are labeled I to VI in Fig. 3. Together, regions I and II form the ATP-binding site. The consensus for region I is G-X-G-X-X-G-X-V (23) where X may be any residue, and this is indicated on the multiple alignment (residues 50 to 57). This region is believed to fold around the bound nucleotide (24). Notably, the ASFV sequence deviates at two positions from the consensus. The first glycine is replaced by glutamate, a semiconservative change, and the third glycine is replaced by asparagine, a nonconservative change. In general, region I is highly conserved in protein kinases; however, there are exceptions, including casein kinase (60) and *nim*-1 (53), in both of which the third glycine residue in region I is replaced by a serine. In *mik1*, all three glycine residue are replaced, the first by histidine and the second and third by serine residues. Only the valine residue is conserved. Although the protein has not been shown to be a protein kinase, it is a functional homolog of *wee1*$^+$ in *Schizosaccharomyces pombe* (41), which suggests that the gene product is active. Within region II, A/I/V-X-K (residues 70 to 72 on the multiple alignment), there is an invariant lysine which is essential for protein kinase activity (13). This residue is generally 14 to 23 residues downstream of the third glycine residue in region I. For the vaccinia virus *B1R*-encoded protein kinase, site-directed mutagenesis has confirmed that lysine 41 is essential for protein kinase activity (38). This lysine residue aligns with lysine 66 in the ASFV ORF, and these residues lie 13 or 15 residues downstream of the third conserved glycine in the region I consensus (position 55 on the multiple alignment).

Regions III and IV are believed to play a role in nucleotide binding and phosphate transfer and are the most highly conserved regions in the three aligned sequences (positions 174 to 187 and 199 to 201, respectively). These regions have

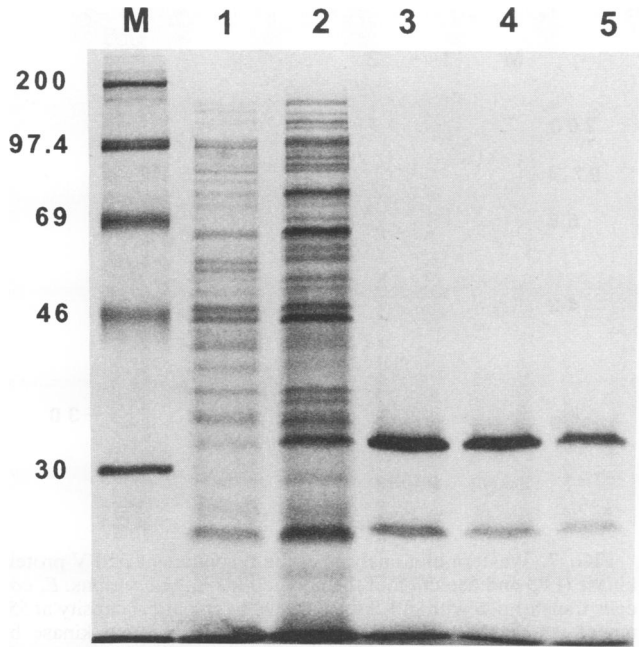

FIG. 4. Expression of the ASFV protein kinase in *E. coli*. Strain BL21(DE3)pLysE cells transformed with plasmid pPK3 were grown to an optical density at 550 nm of 0.4 in 2× TY medium and then transferred to M9 medium. Samples of the culture were removed and pulse-labeled for 5 min with 10 μCi of [^{35}S]methionine prior to induction with 1 mM IPTG (lane 1) or 30 min after induction (lane 2). Rifampin (200 μg/ml) was added to the remainder of the culture, and samples were pulse-labeled after 30, 60, and 90 min (lanes 3, 4, and 5, respectively). The labeled proteins were electrophoresed on an SDS–10% polyacrylamide gel and visualized by autoradiography. The molecular masses of the ^{14}C-labeled molecular size standards in lane M are indicated in kilodaltons.

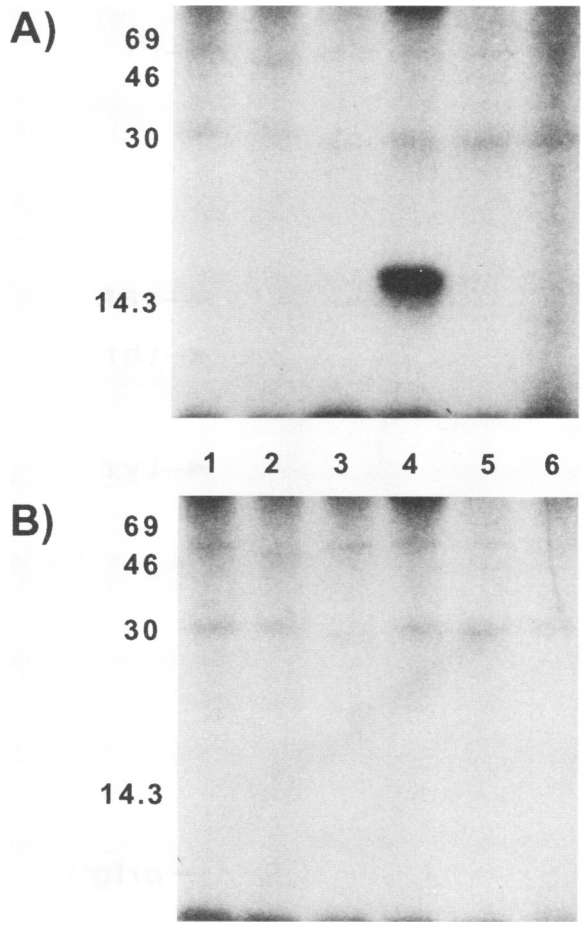

FIG. 5. In vitro substrate specificity of the ASFV protein kinase. Protein substrates (10 μg) were incubated with extracts from *E. coli* cells expressing the ASFV protein kinase from plasmid pPK3 (A) or cells containing parental plasmid pGMT7 (B). ^{32}P-labeled proteins were resolved by electrophoresis on SDS–15% polyacrylamide gels and visualized by autoradiography. No substrate, bovine serum albumin, casein, calf thymus histones, phosvitin, and protamine substrates are shown in lanes 1 to 6, respectively. The molecular masses of ^{14}C-labeled molecular size standards are indicated in kilodaltons.

previously been defined for the vaccinia virus *B1R*-encoded protein kinase (27). The aspartate and asparagine residues at positions 179 and 184 of region III and the aspartate and glycine residues at positions 198 and 200 are conserved in all protein kinases. Here the aspartate residues interact with the phosphate groups on ATP by formation of Mg^{2+} salt bridges (24). Region V, with the consensus A/G/P/S-P/A/I/L-E (12, 63), is present at positions 230 to 232 on the multiple alignment. The ASFV ORF is conserved at the second and third positions; however, a cysteine residue is present at the first position. Region VI (residues 246 to 265 on the multiple alignment) has a glycine residue conserved in all protein kinases (position 251) (24). Finally, the region downstream of motif VI contains a conserved hydrophobic residue 11 amino acids before an arginine (position 302 on the multiple alignment). The presence of lysine, glycine, and threonine residues at positions 181, 225, and 226, respectively, indicates that the ASFV protein kinase is likely to have serine-threonine specificity.

Expression of the protein kinase in *E. coli*. The ASFV protein kinase gene was expressed in *E. coli* as described in Materials and Methods. To analyze the gene product, bacterial cultures were pulse-labeled for 5 min with [^{35}S]methionine, in the presence or absence of rifampin, before and at various times after induction. Figure 4 shows that a 33-kDa protein was synthesized when cells were induced with IPTG and that this protein was the major translational product in the presence of rifampin, which inhibits the *E. coli*, but not the T7, RNA polymerase (66). The size of this protein is approximately that expected of the ASFV ORF (35.1 kDa). A lower-molecular-weight protein was also present and represents a degradation product of the larger species since it was recognized by antibody raised to the amino terminus of the ASFV ORF (data not shown). Two to four hours after induction, the ASFV protein kinase was present at high levels and represented the major protein in *E. coli* extracts seen by Coomassie brilliant blue staining of SDS-polyacrylamide gels. Moreover, the protein was readily soluble (data not shown).

Protein kinase activity. Crude extracts from bacteria transformed with plasmid pPK3 or parental plasmid pGMT7 were used in an in vitro enzyme assay for protein kinase activity (Fig. 5). The extract from cells expressing the ASFV protein (Fig. 5A) phosphorylated a calf thymus histone (lane 4) but not bovine serum albumin, casein, phosvitin, or protamine (lanes 2, 3, 5, and 6, respectively). The control extract from cells transformed with plasmid pGMT7 was unable to phos-

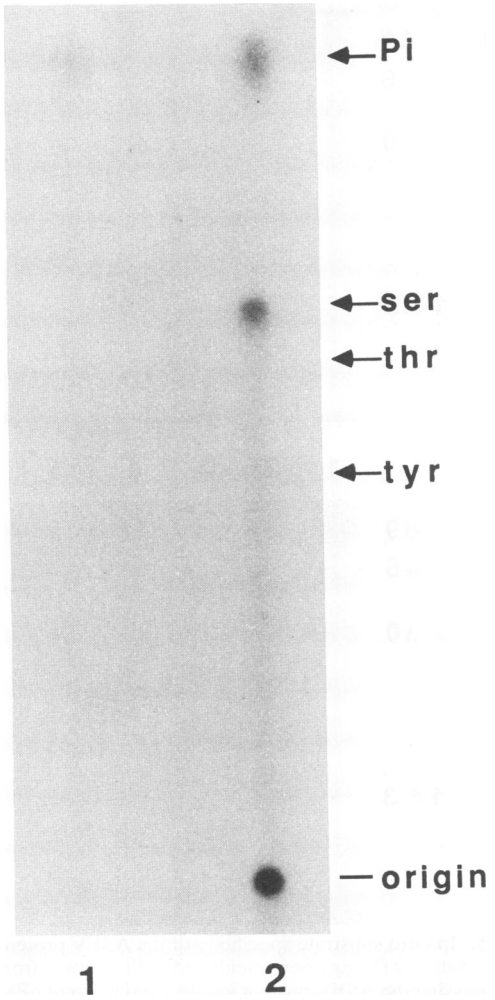

FIG. 6. Amino acid specificity of the ASFV protein kinase. Crude *E. coli* extracts from cells transformed with plasmid pGMT7 (lane 1) or pPK3 (lane 2) were used to phosphorylate calf thymus histones in vitro. Phosphoamino acids were prepared and separated by electrophoresis on thin-layer cellulose plates as described in Materials and Methods. The positions of the phosphoamino acid standards phosphoserine (ser), phosphothreonine (thr), and phosphotyrosine (tyr) are shown. Pi, free phosphate.

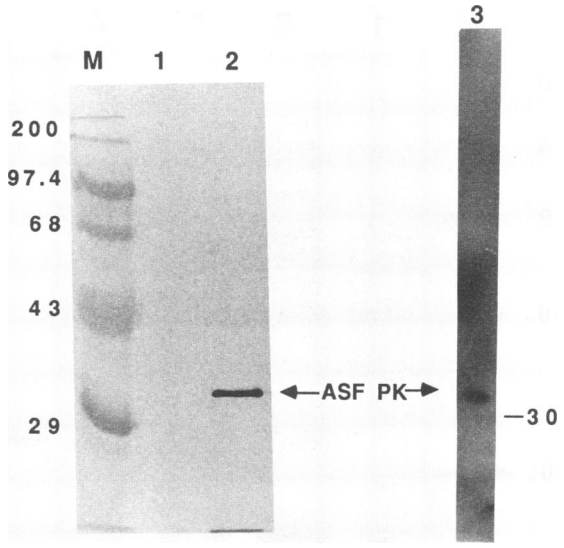

FIG. 7. Western blot analysis of the recombinant ASFV protein kinase (PK) and detection of the polypeptide in ASF virions. *E. coli* cells transformed with pPK3 were grown to an optical density at 550 nm of 0.4 and induced to express the ASFV protein kinase by addition of 1 mM IPTG. Extracts from these cells or purified ASF virions were resolved by SDS-polyacrylamide gel electrophoresis prior to Western blot analysis and detection of bound immunoglobulin complexes as described in Materials and Methods. Lanes: 1 and 2, extracts from noninduced or induced *E. coli* cells, respectively; 3, purified ASF virions. The molecular masses of the molecular size standards in lane M are indicated in kilodaltons.

phorylate any of these substrates (Fig. 5B). Phosphorylation of a 30-kDa *E. coli* protein by a bacterial protein kinase, seen previously when assaying the vaccinia virus *B1R*-encoded protein kinase (4), was observed weakly in all reactions.

Amino acid specificity of the ASFV protein kinase. The amino acid specificity of the ASFV protein kinase was determined by using calf thymus histones phosphorylated in vitro and transferred to Immobilon-P membrane. The ^{32}P-labeled histone band was excised, and polypeptides were acid hydrolyzed under conditions which favored the recovery of phosphotyrosine rather than the more acid-stable phosphoserine and phosphothreonine (14). Phosphoamino acids were separated by electrophoresis on cellulose thin-layer plates, and the positions of labeled spots were compared to those of unlabeled phosphoamino acid standards located by ninhydrin staining. The results shown in Fig. 6 demonstrate that the ASFV protein kinase phosphorylates a histone protein on serine but not on threonine or tyrosine residues.

The ASFV protein kinase is packaged into virions. Antibody reactive against the N terminus of the ASFV protein kinase (Materials and Methods) was used in Western blot analyses to confirm the authenticity of the ASFV protein expressed in *E. coli* and to examine ASF virions for the presence of the enzyme. Figure 7 shows the presence of a 33-kDa protein in extracts from *E. coli* transformed with pPK3 and induced with IPTG (lane 2). This band was not detected without IPTG induction (lane 1) nor when preimmune serum was used to probe identical blots (data not shown). The size of the protein correlated with the size of the protein radiolabeled in the presence of rifampin (Fig. 4). The purified antibody identified a similar-size protein in ASF virions purified from the supernatant of tissue culture cells by two Percoll gradients (Fig. 7, lane 3). This virus preparation lacked polypeptides of greater than 150 kDa that are indicative of vesicle contamination, but to confirm that the protein was intrinsic to the virion and not a surface contaminant, the virus preparation was treated with octylglucoside in 0.5 M NaCl (a process that removes external virion proteins), the particles were sedimented through sucrose, and blotting was repeated. The antibody detected the same protein (data not shown), confirming that the ASFV protein kinase is packaged into virions in vivo.

DISCUSSION

This report describes the identification, sequencing, expression, and amino acid specificity of a protein kinase encoded by ASFV. The 35.1-kDa ORF (*j9L*) contains all of the major conserved catalytic domains of the protein ki-

nases, with the exception of region I, where the sequence deviates from the consensus at the first and third glycine residues, and also the first position of region V. The enzyme is predicted to phosphorylate protein substrates on serine-threonine residues. Despite the variations in regions I and V, the ASFV ORF expressed in *E. coli* was an active protein kinase in vitro and specifically phosphorylated serine residues on a calf histone protein. The demonstration that ASFV encodes a serine protein kinase is consistent with previous findings showing that ASFV packages a protein kinase activity within virions (47, 56) which phosphorylates virion proteins p9 and p10 on serine residues (56). The origin of the ASF virion protein kinase was uncertain, however, since it could have been encoded by ASFV or the host cell. Analysis of ASF virions by Western blotting with the antibody specific for the N terminus of the protein kinase demonstrated the presence of the same polypeptide in ASF virions. This finding suggests that the 35.1-kDa ORF may encode the serine protein kinase packaged into ASF virions. Confirmation of this awaits purification of the virion protein kinase, further biochemical analysis, and determination of reactivity with the specific antibody. The system which we describe here provides large amounts of soluble ASFV protein kinase that may be used for more extensive biochemical and structural studies to elucidate the function of this enzyme in ASFV replication.

Although ASFV and poxviruses are structurally distinct, they replicate in the cytoplasm of infected cells and share several other properties. For example, their genomes are large, linear, double-stranded DNA molecules with inverted terminal repeats and terminal cross-links which may be expected to replicate by similar mechanisms. Both ASFV and the poxviruses package or express several transcriptional and DNA-replicative enzymes. The prototypical poxvirus, vaccinia virus, packages more than one protein kinase activity into virions (30–32, 38), and one of these is encoded by the vaccinia virus *B1R* gene (4, 38). Although the function of the *B1R*-encoded protein kinase has not been determined, conditional lethal mutants show a DNA-negative phenotype under nonpermissive conditions, suggesting a direct or indirect role in virus DNA replication (49, 50, 67). The protein is expressed early during infection and is found in cytoplasmic factories, the sites of virus DNA replication and virion morphogenesis (4, 49). The temporal regulation of the ASFV protein kinase is unknown, but it may function analogously to the vaccinia virus enzyme. This may be studied by using molecular genetics to exchange the protein kinase genes between these viruses (42, 51).

ACKNOWLEDGMENTS

We thank Tim Doel and Bob Staple for peptide synthesis and Stephen Twigg for help with multiple protein alignment.

This work was supported by the Agriculture and Food Research Council and The Wellcome Trust.

REFERENCES

1. **Alcamí, A., A. Angulo, C. López-Otín, M. Muñoz, J. M. P. Freije, A. L. Carrascosa, and E. Viñuela.** 1992. Amino acid sequence and structural properties of protein p12, an African swine fever virus attachment protein. J. Virol. **66**:3860–3868.
2. **Almazán, F., J. M. Rodríguez, G. Andrés, R. Pérez, E. Viñuela, and J. F. Rodriguez.** 1992. Transcriptional analysis of multigene family 110 of African swine fever virus. J. Virol. **66**:6655–6667.
3. **Almendral, J. M., F. Almazán, R. Blasco, and E. Viñuela.** 1990. Multigene families in African swine fever virus: family 110. J. Virol. **64**:2064–2072.
4. **Banham, A. H., and G. L. Smith.** 1992. Vaccinia virus gene B1R encodes a 34-kDa serine/threonine protein kinase that localizes in cytoplasmic factories and is packaged into virions. Virology **191**:803–812.
5. **Bankier, A. T., K. M. Weston, and B. G. Barrell.** 1987. Random cloning and sequencing by the M13/dideoxynucleotide chain termination method. Methods Enzymol. **155**:51–93.
6. **Barros, M. F., C. V. Cunha, and J. V. Costa.** 1986. Single-stranded deoxyribonucleic acid nuclease induced by African swine fever virus and associated to the virion. Virology **155**: 183–191.
7. **Baylis, S. A., L. K. Dixon, S. Vydelingum, and G. L. Smith.** 1992. African swine fever virus encodes a gene with extensive homology to type II topoisomerases. J. Mol. Biol. **228**:1003–1010.
8. **Blasco, R., C. López-Otín, M. Muñoz, E.-O. Bockamp, C. Simón-Mateo, and E. Viñuela.** 1990. Sequence and evolutionary relationships of African swine fever virus thymidine kinase. Virology **178**:301–304.
9. **Boursnell, M., K. Shaw, R. J. Yáñez, E. Viñuela, and L. Dixon.** 1991. The sequences of the ribonucleotide reductase genes from African swine fever virus show considerable homology with those of the orthopoxvirus, vaccinia virus. Virology **184**:411–416.
10. **Camacho, A., and E. Viñuela.** 1991. Protein p22 of African swine fever virus: an early structural protein that is incorporated into the membrane of infected cells. Virology **181**:251–257.
11. **Carrascosa, A. L., M. del Val, J. F. Santarén, and E. Viñuela.** 1985. Purification and properties of African swine fever virus. J. Virol. **54**:337–344.
12. **Chee, M. S., G. L. Lawrence, and B. G. Barrell.** 1989. Alpha-, beta- and gammaherpesviruses encode a putative phosphotransferase. J. Gen. Virol. **70**:1151–1160.
13. **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.
14. **Cooper, J. A., B. M. Sefton, and T. Hunter.** 1983. Detection and quantification of phosphotyrosine in proteins. Methods Enzymol. **99**:387–402.
15. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387–395.
16. **Dixon, L. K.** 1988. Molecular cloning and restriction enzyme mapping of an African swine fever virus isolate from Malawi. J. Gen. Virol. **69**:1683–1694.

16a. **Dixon, L. K.** Unpublished data.

17. **Dixon, L. K., P. J. Wilkinson, K. J. Sumption, and F. Ekue.** 1990. Diversity of the African swine fever virus genome, p. 271–295. *In* G. Darai (ed.), Molecular biology of iridoviruses. Kluwer Academic Publishers, Boston.
18. **Esteves, A., M. I. Marques, and J. V. Costa.** 1986. Two-dimensional analysis of African swine fever virus proteins and proteins induced in infected cells. Virology **152**:192–206.
19. **García-Beato, R., J. M. P. Freije, C. López-Otín, R. Blasco, E. Viñuela, and M. L. Salas.** 1992. A gene homologous to topoisomerase II in African swine fever virus. Virology **188**:938–947.
20. **García-Beato, R., M. L. Salas, E. Viñuela, and J. Salas.** 1992. Role of the host cell nucleus in the replication of African swine fever virus DNA. Virology **188**:637–649.
21. **González, A., V. Calvo, F. Almazán, J. M. Almendral, J. C. Ramírez, I. de la Vega, R. Blasco, and E. Viñuela.** 1990. Multigene families in African swine fever virus: family 360. J. Virol. **64**:2073–2081.
22. **Hammond, J. M., S. M. Kerr, G. L. Smith, and L. K. Dixon.** 1992. An African swine fever virus gene with homology to DNA ligases. Nucleic Acids Res. **20**:2667–2671.
23. **Hanks, S. K., and A. M. Quinn.** 1991. Protein kinase catalytic domain sequence database: identification of conserved features of primary structure and classification of family members. Methods Enzymol. **200**:38–62.
24. **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.
25. **Hernandez, A. M. M., and E. Tabares.** 1991. Expression and

characterization of the thymidine kinase gene of African swine fever virus. J. Virol. **65**:1046–1052.
26. **Hingamp, P. M., J. E. Arnold, R. J. Mayer, and L. K. Dixon.** 1992. A ubiquitin conjugating enzyme encoded by African swine fever virus. EMBO J. **11**:361–366.
27. **Howard, S. T., and G. L. Smith.** 1989. Two early vaccinia virus genes encode polypeptides related to protein kinases. J. Gen. Virol. **70**:3187–3201.
28. **Hunter, T.** 1987. A thousand and one protein kinases. Cell **50**:823–829.
29. **Kamps, M. P., and B. M. Sefton.** 1989. Acid and base hydrolysis of phosphoproteins bound to Immobilon facilitates analysis of phosphoamino acids in gel-fractionated proteins. Anal. Biochem. **176**:22–27.
30. **Kleiman, J., and B. Moss.** 1973. Protein kinase activity from vaccinia virions: solubilization and separation into heat-labile and heat-stable components. J. Virol. **2**:684–689.
31. **Kleiman, J. H., and B. Moss.** 1975. Purification of a protein kinase and two phosphate acceptor proteins from vaccinia virions. J. Biol. Chem. **250**:2420–2429.
32. **Kleiman, J. H., and B. Moss.** 1975. Characterization of a protein kinase and two phosphate acceptor proteins from vaccinia virions. J. Biol. Chem. **250**:2430–2437.
33. **Kozak, M.** 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell **44**:283–292.
34. **Kuznar, J., M. L. Salas, and E. Viñuela.** 1980. DNA-dependent RNA polymerase in African swine fever virus. Virology **101**: 169–175.
35. **Kuznar, J., M. L. Salas, and E. Viñuela.** 1981. Nucleoside triphosphate phosphohydrolase activities in African swine fever virus. Arch. Virol. **69**:307–310.
36. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
37. **Leader, D. P.** 1988. Identification of protein kinases by computer. Nature (London) **333**:308.
38. **Lin, S., W. Chen, and S. S. Broyles.** 1992. The vaccinia virus B1R gene product is a serine/threonine protein kinase. J. Virol. **66**:2717–2723.
39. **López-Otín, C., J. M. P. Freije, F. Parra, E. Méndez, and E. Viñuela.** 1990. Mapping and sequence of the gene coding for protein p72, the major capsid protein of African swine fever virus. Virology **175**:477–484.
40. **López-Otín, C., C. Simon, E. Mendez, and E. Viñuela.** 1988. Mapping and sequence of the gene encoding protein p37, a major structural protein of African swine fever virus. Virus Genes **1**:291–303.
41. **Lungdren, K., N. Walworth, R. Booher, M. Dembski, M. Kirschner, and D. Beach.** 1991. mik1 and wee1 cooperate in the inhibitory tyrosine phosphorylation of cdc2. Cell **64**:1111–1122.
42. **Mackett, M., G. L. Smith, and B. Moss.** 1982. Vaccinia virus: a selectable eukaryotic cloning and expression vector. Proc. Natl. Acad. Sci. USA **79**:7415–7419.
43. **Matsushime, H., A. Jinno, N. Takagi, and M. Shibuya.** 1990. A novel mammalian protein kinase gene (*mak*) is highly expressed in testicular germ cells at and after meiosis. Mol. Cell. Biol. **10**:2261–2268.
44. **Mebus, C. A.** 1988. African swine fever. Adv. Virus Res. **35**:251–269.
45. **Ortin, J., and E. Viñuela.** 1977. Requirement of cell nucleus for African swine fever virus replication in Vero cells. J. Virol. **21**:902–905.
46. **Pearson, W. R., and D. J. Lipman.** 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85**:2444–2448.
47. **Polatnick, J., I. C. Pan, and M. Gravell.** 1974. Protein kinase activity in African swine fever virus. Arch. Gesamte Virusforsch. **44**:156–159.
48. **Reeves, R., G. A. Spies, M. Kiefer, P. J. Barr, and M. Power.** 1990. Primary structure of the putative human oncogene, *pim*-1. Gene **90**:303–307.
49. **Rempel, R. E., M. K. Anderson, E. Evans, and P. Traktman.** 1990. Temperature-sensitive vaccinia virus mutants identify a gene with an essential role in viral replication. J. Virol. **64**:574–583.
50. **Rempel, R. E., and P. Traktman.** 1992. Vaccinia virus B1 kinase: phenotypic analysis of temperature-sensitive mutants and enzymatic characterization of recombinant proteins. J. Virol. **66**:4413–4426.
51. **Rodríguez, J. M., F. Almazán, E. Viñuela, and J. F. Rodriguez.** 1992. Genetic manipulation of African swine fever virus: construction of recombinant viruses expressing the β-galactosidase gene. Virology **188**:67–76.
52. **Rosenberg, A. H., B. N. Lade, D.-S. Chui, S.-W. Lin, J. J. Dunn, and F. W. Studier.** 1987. Vectors for selective expression of cloned DNAs by T7 RNA polymerase. Gene **56**:125–135.
53. **Russell, P., and P. Nurse.** 1987. The mitotic inducer *nim-1*$^+$ functions in a regulatory network of protein kinase homologs controlling the initiation of mitosis. Cell **49**:569–576.
54. **Salas, M. L., J. Kuznar, and E. Viñuela.** 1981. Polyadenylation, methylation, and capping of the RNA synthesized *in vitro* by African swine fever virus. Virology **113**:484–491.
55. **Salas, M. L., J. Kuznar, and E. Viñuela.** 1983. Effect of rifamycin derivatives and coumermycin A1 on *in vitro* RNA synthesis by African swine fever virus. Arch. Virol. **77**:77–80.
56. **Salas, M. L., J. Salas, and E. Viñuela.** 1988. Phosphorylation of African swine fever virus proteins *in vitro* and *in vivo*. Biochimie **70**:627–635.
57. **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.
58. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
59. **Santarén, J. F., and E. Viñuela.** 1986. African swine fever virus-induced polypeptides in Vero cells. Virus Res. **5**:391–405.
60. **Saxena, A., R. Padmanabha, and C. V. C. Glover.** 1987. Isolation and sequencing of cDNA clones encoding alpha and beta subunits of *Drosophila melanogaster* casein kinase II. Mol. Cell. Biol. **7**:3409–3417.
61. **Schwartz, R. M., and M. O. Dayhoff.** 1979. *In* M. O. Dayhoff (ed.), Atlas of protein sequence and structure, p. 353–358. National Biomedical Research Foundation, Washington, D.C.
62. **Selten, G., H. T. Cuypers, W. Boelens, E. Robanus-Maandag, J. Verbeek, J. Domen, C. van Beveren, and A. Berns.** 1986. The primary structure of the putative oncogene *pim*-1 shows extensive homology with protein kinases. Cell **46**:603–611.
63. **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.
64. **Staden, R.** 1982. Automation of computer handling of gel reading data produced by the shotgun method of DNA sequencing. Nucleic Acids Res. **10**:4731–4751.
65. **Staden, R.** 1990. An improved sequence handling package that runs on the Apple Macintosh. Comput. Appl. Biosci. **6**:387–393.
66. **Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. **185**:60–89.
67. **Traktman, P., M. K. Anderson, and R. E. Rempel.** 1989. Vaccinia virus encodes an essential gene with strong homology to protein kinases. J. Biol. Chem. **264**:21458–21461.
68. **Urzainqui, A., E. Tabarés, and L. Carrasco.** 1987. Proteins synthesized in African swine fever virus-infected cells analyzed by two-dimensional gel electrophoresis. Virology **160**:286–291.
69. **Valdeira, M. L., M. C. Duque-Magalhães, and A. Geraldes.** 1990. Evidence for an acid phosphatase in African swine fever virus. Arch. Virol. **113**:125–131.
70. **Viñuela, E.** 1985. African swine fever virus. Curr. Top. Microbiol. Immunol. **116**:151–170.
71. **Wilkinson, P. J.** 1989. African swine fever virus, p. 17–35. *In* M. B. Pensaert (ed.), Virus infections of porcines. Elsevier Science Publishers, Amsterdam.
72. **Woodgett, J. R.** 1990. Molecular cloning and expression of glycogen synthase kinase-3/factor A. EMBO J. **9**:2431–2438.