African swine fever virus gene j13L encodes a 25–27 kDa virion protein with variable numbers of amino acid repeats

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The African swine fever virus (ASFV) j13L gene encodes a 177 amino acid protein (19.0 kDa) with a putative transmembrane domain between residues 32 and 52. There is a potential signal peptide cleavage site at residue 54 and several possible motifs for phosphorylation and myristylation. Rabbit antisera raised against a synthetic peptide from the C terminus of the j13L ORF identified proteins of 25–27 kDa in cells infected with a recombinant vaccinia virus expressing the j13L ORF, in ASFV-infected cells and in purified extracellular ASF virions. In ASFV-infected cells the j13L protein was expressed late during infection and exhibited size variation (25–27 kDa) between the different ASFV strains. Nucleotide sequence analysis of the gene in these strains showed that these size differences were due to variation in the number and sequence of tandemly repeated amino acid repeats. Although ASFV-infected animals made antibodies to the j13L protein, no protection was observed when pigs were vaccinated with a recombinant vaccinia virus expressing the j13L ORF.

Introduction

African swine fever (ASF) was first reported in 1921 in Kenya as a highly contagious swine disease which caused high mortality (Montgomery, 1921). The disease was epidemic in many European and African countries in the 1950s and 1960s and caused heavy losses in the swine industry. Presently, ASF is endemic in Spain, Italy and many African countries and remains one of the most serious viral diseases threatening the swine industry due to lack of effective prophylactic measures (for reviews see Costa, 1990; Viñuela, 1987; Wilkinson, 1989).

The causative agent of ASF is a large, cytoplasmically located, icosahedral DNA virus which was originally classified as a member of the Iridoviridae because of their morphological similarities and cytoplasmic location (Gorrha & Granoff, 1979; Matthews, 1982). However, recent evidence has shown that both the genome structure and replication strategy of ASFV are more similar to that of the Poxviridae and thus ASFV has been removed from the Iridoviridae (Brown, 1986). The virus has a large, double-stranded, DNA genome of 170–190 kb which encodes more than 100 open reading frames (ORFs). More than 100 proteins are induced in ASFV-infected cells and extracellular virions contain more than 50 proteins, although none of these are major structural glycoproteins (Carvalho & Rodrigues-Pousada, 1986; Esteves et al., 1986; Schloer, 1985; Del Val et al., 1986; Del Val & Viñuela, 1987). Only a few structural proteins have been mapped to specific genes on the virus genome. These include proteins of molecular masses 72 kDa, 12 kDa, 10 kDa, 11.5 kDa and polyprotein pp220 which gives rise to four major structural proteins, p150, p37, p34 and p14, after proteolytic processing (López-Otin et al., 1990; Cistué & Tabarés, 1992; Alcamí et al., 1993; Angulo et al., 1993; Muñoz et al., 1993; Simón-Mateo et al., 1993).

The most striking aspect of the immune response against ASFV is the lack of production of traditional virus-neutralizing antibodies, both by pigs recovered from natural infection and by laboratory animals inoculated with virus preparations (Costa, 1990; Forman et al., 1982). However, some immune protection does occur since pigs that have recovered from infection are resistant to homologous virus challenge. Although the exact mechanism(s) of the immune protection is unclear, antibodies may play some role as transfer of serum from recovered animals can delay the onset of the disease (Schlafer et al., 1984; Onisk et al., 1994).

ASF virions have a complex structure consisting of a nucleoprotein core surrounded by a lipid membrane and the capsid which is enveloped by an external lipid membrane (Carrascosa et al., 1984). Virus proteins inserted into either the external virion membrane or the plasma membrane of infected cells may play an im-
portant role through interacting with and stimulating the host’s immune response. These proteins are predicted to contain at least one transmembrane domain. Here we report the identification of one ASF virus protein which is encoded by ORF j3L on the Malawi LIL 20/1 isolate genome and contains a putative transmembrane region.

**Methods**

**Cells and viruses.** IBRS2, BSC40 and Hu TK-143 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) (growth medium) or 2% (v/v) (maintenance medium) fetal calf serum (FCS). Pig bone marrow (PBM) cells were prepared from normal outbred pigs and grown in Eagle's medium supplemented with 12% (v/v) normal pig serum (Malmquist & Hay, 1960). Vaccinia virus (VV) Western Reserve (WR) strain was used to construct a recombinant vaccinia virus (rVV). The virulent ASFV Malawi (LIL 20/1) isolate has been described previously (Haresnape et al., 1988). Other virus isolates used were as follows: tissue culture-adapted Uganda (Ug) has been described previously (Hess et al., 1965); Lisbon 57 (Li 57), Lisbon 60 (Li 60) and Tomar 86 (Tom) were gifts of Dr J. D. Vigarrio (LNIV, Lisbon, Portugal); Tanzania 87 (Tanz) was a gift of Dr E. C. Anderson (Southern Highlands, Tanzania); Burundi 84/2 (Bur) was a field sample from Burundi; Malawi Bougara 83 (Bon) has been described previously (Sumption et al., 1990); Republic of South Africa 85/1 (RSA) was a gift of Dr G. Thomson (Veterinary Research Institute, Onderstepoort, RSA) and Lillie 148 (Lillie) was described previously (Pini & Wagenhaar, 1974). Extraacellular virus particles of Uganda strain were purified by Percoll gradient centrifugation (Carrascosa et al., 1985).

**Plasmid and bacterial strains.** Plasmid pGS53 (Chakrabarti et al., 1985) enables genes to be cloned downstream of the VV 7.5K promoter and to be recombinated into the TK locus of VV. DNA manipulations were performed using standard methods (Sambrook et al., 1989) and *E. coli* DH5α was used as the host for cloning.

**Polymerase chain reaction (PCR).** PCRs were performed in 50 or 100 μl volumes containing 10 pm of each primer, 0.4 mM-dNTPs, 5 or 10 μl of 10x reaction buffer (replenished by the enzyme supplier), 10–50 ng of template DNA and 1 unit of Taq DNA polymerase (Boehringer Mannheim) on a programmable thermal controller (MJ Research Inc.) using the following programme: cycle 1, 94 °C for 5 min, 50 or 55 °C for 2 or 3 min; cycles 2–29, 94 °C for 1 min, 50 or 55 °C for 2 min, 72 °C for 3 min; cycle 30, 94 °C for 1 min, 50 or 55 °C for 2 min, 72 °C for 10 min. For cloning and construction of recombinant VV, a BamHI restriction endonuclease site was incorporated into the forward primer (5′ AACGGTTCTGTGGATTCTGGAATTTTTCACGGC) and a Smal site into the reverse primer (5′ GGTCCAGCCGTTACAGGAGGTTCTGGAATTTTTCACGGC) and a SmaI site into the reverse primer (5′ GTGCGACCAGGCTTACAGGAGGTTCTGGAATTTTTCACGGC) and a SmaI site into the reverse primer (5′ GTGCGACCAGGCTTACAGGAGGTTCTGGAATTTTTCACGGC). Either bacteriophage λ clone LMW 18 (Dixon, 1988) which contains the Sall fragment of Malawi LIL 20/1 isolate genome, phenol-extracted DNAs from different ASFV strains or rVV-infected cell lysates were used as the DNA template.

**Construction of recombinant vaccinia virus.** Plasmid pGS53 was linearized by BamHI-Smal double digestion and then ligated with the PCR product of ASFV gene j3L which had also been digested at BamHI and Smal sites contained within the primers used for PCR. The recombinant plasmid pGSJ3L was transfected into VV WR-infected Hu TK–143 cells using lipofectin (GIBCO BRL, Life Technologies Inc.) according to the manufacturer’s instructions. Hu TK–143 cells were used to select TK– recombinant in the presence of 5-bromo-2′-deoxyuridine (BrDU). A recombinant was purified by three cycles of plaque assay on the Hu TK–143 cells. The presence of the inserted ASFV gene j3L was confirmed by PCR and j3L protein expression was initially confirmed by dot-blotting using anti-j3L peptide immunoglobulins (Igs).

**Peptide synthesis and antibody production.** On the basis of protein secondary structure predictions using the programs PeptideX and Pelestrucre a good antigenic domain was mapped at the C-terminus of ORF j3L (Fig. 1). An 18 amino acid (SNELDHKHTYNDRLNE) peptide was synthesized using the solid phase method (Merrifield et al., 1982). Each of two New Zealand White rabbits was immunized with 500 μg of the synthetic peptide in Freund’s complete adjuvant and boosted with a half dose of the peptide in Freund’s incomplete adjuvant on days 21 and 42. On day 64, the blood was collected for serum separation. The specific anti-peptide Igs were purified by affinity chromatography using ProtOn columns (Multiple Peptide System) according to the manufacturer’s instructions. Pig anti-ASFV antisera were collected from pigs recovered from experimental inoculation with different ASFV strains.

**Sequential extraction of virion proteins.** Percoll gradient-purified extracellular ASFV (Uganda strain) was treated first with 0.1% (w/v) n-octyl-β-D-glucopyranoside (NPG) at 4 °C for 1 h in a Centricon 100 concentrator (Amicon). After centrifugation at 3500 r.p.m. (Denley BR 401 refrigerated centrifuge) for 10 mins, solubilized proteins were collected and the virus was treated with 0.25%, 0.5%, 1% and 5% (w/v) NPG using the same conditions. The viral protein preparations were mixed with equal volumes of 2 × SDS-PAGE sample buffer (Laemmli, 1970) and boiled for 10 min prior to SDS–PAGE. Proteins were detected by silver staining and enhanced chemiluminescent (ECL) immunoblotting (Amersham) following transfer to Immobilon-P transfer membranes (Millipore).

**Immunoblotting.** PBM cells or BSC40 cells were infected with different ASFV strains or rVV in the absence or presence of cytosine arabinoside (50 μg/ml, Sigma). The infected cells were harvested at different times post-infection and lysed on ice with 50 mm Tris–HCl pH 8.0, 5 mm-EDTA, 1% (v/v) NP-40, 1% PMSF, 50 μg/ml N-tosyl-l-phenylalanine chloromethyl ketone (TPCK) and 50 μg/ml Nα-p-tosyl-l-lysine chloromethyl ketone (TLCK). Percoll gradient-purified extracellular viruses or infected cell lysates were separated by SDS–PAGE and the proteins were transferred to Immobilon-P transfer membranes according to the manufacturer’s instructions. The membranes were blocked at 4 °C overnight or at 37 °C for 1 h with PBS containing 10% (v/v) FCS or 5% (w/v) dry milk powder and 0.1% (v/v) Tween 20 and incubated at room temperature for 1 h with 1:100 purified rabbit anti-j3L peptide Igs (0.67 mg/ml) in the blocking buffer. After washing four times for 5 min in PBS containing 0.1% (v/v) Tween 20, the membranes were incubated at room temperature with 1:3000 goat anti-rabbit IgG–horseradish peroxidase (HRP) conjugate (DAKO) in the blocking buffer. Bound goat IgG–HRP conjugate was detected using ECL detection reagents (Amersham) according to the manufacturer’s instructions.

**DNA sequencing and sequence analysis.** DNA sequence co-ordinates were from a 55 kbp region at the right end of the Malawi (LIL 20/1) isolate genome (Dixon et al., 1994). PCR fragments were generated from the genomes of ASFV isolates described above using primer p10 (5′ CATTGGCGGCAACAG) which corresponds to a sequence 92 bp upstream from the first ATG codon of ORF j2L from LIL 20/1 isolate (co-ordinate 30774) and primer p11 (5′ GCTGGCGGCAACAG) which is complementary to a sequence 47 bp downstream from the stop codon (co-ordinate 30106). PCR fragments containing the j3L ORF were sequenced on both strands by cycle sequencing using the fmol sequencing kit (Promega). DNA and protein sequences were analysed using the University of Wisconsin Genetics Computer Group programs (Devereux et al., 1984). Protein secondary structures were predicted using PeptideX and plotted with Plotstructure. Consensus amino acid sequences were identified using Motifs and sequence analysis programs (Devereux et al., 1984). Protein secondary structures were predicted using PeptideX and plotted with Plotstructure.
Results

Selection and structure of ORF j13L

Computer analysis of the DNA sequence of 55 kb from the right end of the ASFV (Malawi LIL 20/1 isolate) genome revealed 67 major ORFs (Dixon et al., 1994). Several of these were predicted to encode proteins with putative transmembrane regions and one such ORF, j13L, located between positions 30199 and 30726 on the 55 kb sequence read towards the left DNA terminus, encodes a 177 amino acid peptide with a predicted relative molecular mass of 19.0 kDa (Dixon et al., 1994). Database searches against the SwissProt protein library (version 28) did not find significant homology between the j13L sequence and other published protein sequences. However, a search of the Prosite database using the program Motifs identified one casein kinase II phosphorylation motif at residue 168, six protein kinase C phosphorylation motifs at residues 53, 54, 109, 114, 119 and 168, and three myristylation motifs at residues 93, 123 and 127 (Fig. 1). In addition, the predicted j13L protein contains a 21 amino acid hydrophobic region near the N terminus between residues 32 and 52 (Fig. 1). A possible signal peptide cleavage site was predicted using the program Sigcleave at position 54 with a score of 3.8. This program uses the method of Von Heijne (1986) to locate signal sequences, and identify the cleavage site. This method is 95% accurate in resolving signal sequences from non-signal sequences with a cutoff score of 3.5, and 75–80% accurate in identifying the signal cleavage site. This suggests that ORF j13L may encode a cleavable signal peptide and could be secreted into the medium from infected cells. If the cleavage site is not recognized, the protein could be inserted into membranes as a type II or type III transmembrane protein. Three antigenic domains (from residues 72 to 84, 107 to 119 and 145 to 176) were also predicted.

Expression of j13L in ASFV- and recombinant VV-infected cells

To obtain antisera specific for the j13L product, an 18 amino acid peptide was synthesized containing the amino acid sequence of the third antigenic domain (residues 160 to 176) and was used to immunize rabbits. Without coupling to any carrier protein, the peptide elicited a good antibody response (positive at a dilution of 1:3000 by ELISA). The specific Igs were purified by affinity chromatography for characterization of the j13L gene product.

To identify the j13L gene product, a recombinant vaccinia virus, VVj13L, was constructed which expressed the whole j13L ORF from the Malawi LIL 20/1 isolate under control of the early/late VV 7.5K promoter. BSC40 cells were infected with either wild-type VV WR strain, recombinant VVj13L or the tissue culture-adapted Uganda ASFV. In addition, PBM cells were infected with either the virulent LIL 20/1 ASFV isolate, VV WR strain or VVj13L. Immunoblotting using the anti-j13L Igs identified a protein of 25 kDa in BSC40 cells infected with the VVj13L and in PBM cells infected with Malawi LIL 20/1 ASFV isolate but not in mock-infected cell lysates or in wild-type VV-infected cell lysates (Fig. 2). The same 25 kDa protein was detected in BSC40 cells infected with the recombinant VVj13L, but infection of these cells with ASFV Uganda strain produced a 27 kDa protein. These 25 kDa and 27 kDa proteins were not detected using the preimmune serum (data not shown). These results demonstrated that the anti-peptide sera were specific for the j13L product, and identified this as a polypeptide of 25 to 27 kDa depending on the ASFV strain.

![Fig. 1. Computer-based sequence analysis of the ASFV (Malawi LIL 20/1 isolate) protein encoded by ORF j13L. Hydrophilicity analysis was performed according to the Kyte and Doolittle method using the program Peptidestructure; consensus amino acid sequences (P, phosphorylation sites; M, myristylation sites) were identified using the program Motifs and the signal peptide cleavage site (C) using the program Sigcleave (Devereux et al., 1984). The darkened area represents the putative transmembrane domain, the hatched area the potential antigenic domain from which sequence j13L peptide was synthesized; arrows represent tandem repeat arrays.](image-url)
To determine the temporal control of j13L expression, IBRS2 cells were infected with ASFV strain Uganda in the absence or presence of cytosine arabinoside, which inhibits viral DNA replication and late gene expression. The infected cells were harvested at different times post-infection and cell extracts separated by SDS-PAGE followed by immunoblotting using anti-j13L Igs. As Fig. 3(a) shows, a 27 kDa protein was detected at 6 h post-infection in the ASFV-infected IBRS2 cell lysate. The expression of the 27 kDa protein increased significantly from 10 h post-infection, indicating that it was synthesized mainly late during ASFV infection. This was confirmed since expression of the protein was inhibited by cytosine arabinoside (Fig. 3b).

The j13L product is present in extracellular ASFV virions

To analyse whether the j13L gene product was present in ASFV virions, ASFV Uganda strain was grown in IBRS2 cells and the virus was purified from the extracellular medium by two rounds of Percoll gradient centrifugation. Virion proteins were separated by SDS-PAGE and detected by Coomassie blue, silver staining and immunoblotting. Coomassie blue and silver staining showed that proteins had a typical profile for ASFV virion proteins (Fig. 4). Immunoblotting using the anti-j13L Igs but not the preimmune serum identified a 27 kDa protein in the purified extracellular ASFV virions (Fig. 5a). The molecular mass of this protein was identical to that of the protein detected in the Uganda isolate-infected IBRS2 cell extracts (Figs 2 and 3).

To exclude the possible non-specific association of the j13L protein with ASFV virions, purified extracellular ASFV (Uganda strain) was treated with low concentrations of the mild detergent NOG and the liberated proteins were separated by SDS-PAGE followed by immunoblotting and silver staining. The 27 kDa protein was liberated from virions by treatment with 1% NOG (Fig. 5b), but was not among those proteins first extracted by lower concentrations of NOG and detected by silver staining (Fig. 5c). This indicates that the j13L protein is an integral virion protein.

Variation of j13L product between different ASFV strains

To investigate the variation of the j13L peptide sequence among different ASFV strains, synthetic peptide j13L was coated onto ELISA plates and reacted with antisera...
from pigs recovered from experimental inoculation with different ASFV strains. As Fig. 6(a) shows, synthetic peptide j13L was recognized by the pig antiserum against different ASFV strains tested compared to the normal pig serum although there is variation in antibody titre between individual animals. These results indicate that the predicted j13L antigenic epitope is immunogenic and conserved between different ASFV isolates.

Next, the j13L protein expressed by different ASFV strains was investigated by immunoblotting using rabbit Igs against the j13L synthetic peptide. Fig. 6(b) shows that all eight strains of ASFV tested expressed the j13L protein but that this varied in size between 25 and 27 kDa as had been observed previously for Uganda and LIL 20/1 isolates (Fig. 2). A faint band visible in tracks containing extracts infected with Lis 57 and Bon isolates is probably a cross-reactive cellular protein which was detectable sometimes in uninfected PBM cells.

**Molecular basis for the variation in the j13L protein**

To investigate the reason for the variation in the j13L protein, PCR analyses were performed using oligonucleotides flanking the j13L ORF (Fig. 7a). It was evident that there was size variation in the PCR products from different ASFV strains and that this correlated with the size of j13L proteins expressed. For instance, proteins from strains with the largest PCR products (Tanz and RSA) migrated more slowly than those from strains with the smallest PCR products (LIL 20/1 and Bon).

The molecular basis of the j13L gene variation was determined by directly sequencing PCR fragments amplified from the different ASFV strains. The 31 nucleotides upstream of the j13L start codon were conserved between all isolates. The 5' 300 nucleotides of the coding region were well conserved in both length and sequence between the different isolates, with not less than 91% identity, but diverged thereafter. The deduced amino acid sequences of the j13L proteins across the complete protein were compared. An alignment of these sequences is shown (Fig. 7b) and a dendrogram constructed (Fig. 7c) using program Pileup (Devereux et al., 1984). LIL 20/1 and Bon amino acid sequences were identical and most closely related to the Tanz isolate. Since two of these isolates were from Malawi and the other from a neighbouring country this result was as expected. The Lis 57 and Lis 60 isolates were also very closely related (98% amino acid similarity) and both Lillie and RSA were similar to these. The tissue culture-adapted Uganda isolate was most distantly related to the others. The position and sequence context surrounding the start and stop codons was conserved in all isolates as were the putative signal sequence and cleavage site apart from a Y to C substitution at position 32 and 1 to V substitution at position 36 in the Ug isolate and a V to L substitution at position 39 in the Lillie isolate. A number of the post-translational modification motifs were conserved between all of the isolates. These include the casein kinase II phosphorylation motif at residue 168, and protein kinase C phosphorylation motifs at positions 53, 54, 109, 119 and 168.

Although the j13L protein sequence was generally well conserved between different isolates, considerable variation in both sequence and length was observed between sequences in two arrays of tandem repeats (Fig. 7b). The first array was between residues 101 and 142 on the multiple sequence alignment and consisted of a five amino acid repeat unit which was present at between four (LIL 20/1 and Bon) and eight copies (Tanz and Ug). Repeat units varied in sequence between each other and the most common unit (RPATN) appeared at least once in all isolates except Tanz and Ug. Most of the other types of repeat unit varied by up to two residues from the two most common repeat units. The second variable part of the j13L protein was between residues 151 and 166 which contained a four amino acid repeat unit present at between one and three copies. Variation in sequence of this repeat unit was also observed. The predicted sizes of the primary translation products were
Fig. 5. Presence of j13L product in extracellular ASF virions. (a) Detection of j13L product in extracellular ASFV. Percoll gradient-purified extracellular ASFV strain Uganda was separated by SDS-PAGE and the j13L protein was detected by immunoblotting using preimmune serum (lane 1) or rabbit anti-j13L peptide Igs (lane 2). (b) Extraction of ASF virion proteins with the nonionic detergent NOG. Percoll gradient-purified extracellular ASFV strain Uganda was sequentially extracted at 4 °C for 1 h with increasing concentrations of NOG and the j13L protein was detected as in (a). (c) Detection of ASFV structural proteins. The NOG-extracted virion proteins were separated by SDS-PAGE and the gel was stained with silver solution.
Variable ASFV virion protein

Fig. 6. Variation of the j13L protein. (a) Immune recognition of synthetic peptide j13L by pig antisera to different ASFV strains. Synthetic peptide (5 µg/ml) was coated onto ELISA plates and reacted with normal pig serum (NPS), rabbit anti-j13L peptide serum (PRS) and pig antisera to two Malta strains (RR1 and RR4), a Belgium strain (PM81) and two Uganda strains (M192 and P375). (b) Immunoblot showing size variation of j13L gene products in different ASFV strains. Mock-infected PBM cells (M) or PBM cells infected with LIL 20/1 (1), Lis 57 (2), Lis 60 (3), Tom (4), Bon (5), Bur (6), RSA (7) or Tanz (8) isolate were harvested at 24 h post-infection and cell extracts treated as in Fig. 2.

19.0 kDa (LIL 20/1 and Bon), 20.0 kDa (Lis 57 and Lis 60), 20.3 kDa (Lillie), 20.5 kDa (RSA), 20.8 kDa (Tanz) and 21.2 kDa (Uganda). Each of these is smaller than the apparent size of the j13L protein from SDS–PAGE and this discrepancy might be due to either post-translational modification or aberrant electrophoretic migration of the protein. The observed variation in the size of the j13L proteins was accounted for by variation in number of amino acid repeats and not other substantive charges in the coding region.

Immune potentials of j13L gene product

Different dilutions of the tissue culture-adapted ASFV strain Uganda were mixed with an equal volume (100 µl) of undiluted rabbit antiserum against j13L peptide and incubated at 37 °C for 1 h. The antiserum–virus mixture was then incubated with an equal volume (1–1.4 x 10⁶ cells) of IBRS2 cells for 2 days. No significant neutralization activity was demonstrated compared to a normal rabbit serum.

Each of two inbred miniature (dd haplotype) pigs was inoculated subcutaneously with 5 x 10⁹ p.f.u. of recombinant VVj13L on days 1 and 14 post-inoculation. On day 42, immune sera were collected and titrated against the j13L peptide by ELISA and against VV WR strain by dot-blotting. A low titre of antibody response to j13L gene product was demonstrated by ELISA (data not shown). The two pigs responded well to the VV WR strain with a dot-blot titre of 1:160. On day 49 post-inoculation, each of the two pigs was challenged intramuscularly with 2 ml (lg8 of mean haemadsorption
Fig. 7. (a) PCR analysis. PCR products were generated using virus DNAs from LIL 20/1 (1), Lis 57 (2), Lis 60 (3), Tom (4), Bon (5), Bur (6), RSA (7), Tanz (8), Ug (9) and a clone LMW18 (10) which contains the SalI fragment of the Malawi LIL 20/1 isolate genome and compared by 1.4% agarose gel electrophoresis. (b) Amino acid sequence comparisons of proteins encoded by j13L from different ASFV strains. PCR products containing the complete coding region of j13L were sequenced directly using the fmol sequencing kit (Promega). Sequences generated were translated and aligned using the program Pileup and plotted using Prettybox (Devereux et al., 1984). (c) Dendrogram showing relationship between j13L amino acid sequences. The amino acid sequences of j13L protein from different strains were compared using Pileup and a dendrogram was plotted to show the relationship between the sequences.

dose, HAD_{50}, of virulent ASFV strain Malawi LIL 20/1. The two pigs died on days 6 and 7 post-challenge respectively with typical ASF postmortal changes.

Discussion

The j13L ORF encodes a protein with a predicted transmembrane domain and possible signal peptide cleavage site. If cleaved at this site, the protein could be secreted from infected cells, but if not the protein may be inserted into membranes as a type II or type III transmembrane protein. The presence of j13L protein in purified extracellular ASF virions suggests that at least a proportion of the protein is not secreted from cells. This was supported by another experiment where no expected protein was detected in highly concentrated extracellular
media of ASFV-infected cells (data not shown). It was also demonstrated that about 60% of the j13L protein was present in the vesicle fraction of ASFV-infected cells, but the protein was very sensitive to protease K digestion and easily extracted from the vesicles by treatment with 1% Triton X-100 (data not shown), suggesting that the j13L protein was not translocated and thus was unlikely to be cleaved.

Although the exact location of the j13L protein within ASF virions has not been determined, extraction of virions with the mild detergent NOG showed that the j13L protein was not solubilized at low NOG concentrations (0.1–0.5%) with the first proteins detected by silver staining but required relatively high NOG concentrations to be solubilized. This suggests that most of the j13L protein may be located in the lipid membrane of the virion and not nonspecifically associated with virions. Investigation into the subcellular localization of j13L protein is currently being studied at the electron microscopy level. Preliminary results, however, unequivocally demonstrate the selective presence of the j13L protein in viral factories (our unpublished data).

The j13L protein varied in size by up to 2 kDa when extracts from cells infected with different ASFV strains were compared. The molecular basis for this size variation was investigated by sequencing PCR products containing the j13L coding region. This identified two regions within the coding region which contained tandemly repeated sequences. The ORFs from different isolates varied in number and sequence of repeat units within these repeat arrays. Such size variation due to variable numbers of repeated sequences may be a relatively common property of ASFV-encoded proteins since four more protein sequences which contain repeated sequences have been described (Dixon et al., 1994; Rodriguez et al., 1992, 1993; Martins et al., 1994; Borca et al., 1994).

Tandemly repeated regions within proteins are common in many eukaryotic parasites, including Plasmodium, Trypanosoma, Leishmania and Toxoplasma. In parasites, particularly in Plasmodium, repeats are widespread among proteins from different developmental stages with apparently divergent functions (Schofield, 1991). To date, several theories of the function of tandemly repeated sequences have been forwarded. In the first, each repeat unit is proposed to act as a ligand for host structures such as red blood cell receptors and hepatocytes (Nussenzweig & Nussenzweig, 1989). In this theory, the binding units are proposed to be repeated because this allows a multimeric high-avidity interaction between ligand and receptor. In the second theory, the repeats are proposed to enable the parasite to evade immunity by presenting to the host an extensive network of cross-reactive epitopes that abort affinity maturation of the response to 'protective' epitopes (Anders, 1986). A third theory (Schofield, 1991) proposes that repetitive domains have evolved as a mechanism of immune evasion by their ability to induce thymus-independent B cell activation. This theory suggests that repeated domains provide B cells with a thymus-independent activation signal by cross-linking surface immunoglobulins on B cells and this is the first step of a strategy to force them down an inappropriate (T cell-independent) differentiation pathway. A T cell-independent response is generally considered to be inferior to a T cell-dependent response. Thus the immune response to viruses or parasites containing proteins with repeated domains may be reduced in effectiveness. In addition, these repeated domains are often immunodominant and may suppress the formation of antibody to important adjacent areas on the same molecule (i.e. epitopic suppression) (Schofield, 1991). Repeated sequences in ASFV proteins may play a similar role to those in parasite proteins and might therefore be important in immune evasion or interaction of the virus with the receptor. At present, as we do not know whether the ASFV j13L-encoded tandem repeats are or are not exposed on the surface of the virus, we are unable to favour any of the above theories regarding the j13L protein.

Although the number of repeat units in arrays within j13L proteins was found to vary when different isolates were compared, PCR products from each individual isolate were homogeneous in length and sequence, suggesting that gain or loss of repeat units did not occur frequently during virus passage in pigs. This was supported by immunoblotting using anti-j13L IgGs since only one protein band was detected in infected PBM cells. Interestingly, there is some correlation between the number of repeats and virulence of the virus since the virulent LIL 20/1 isolate had the smallest number of tandem repeats and the tissue culture-adapted avirulent Uganda strain had the most repeated sequences. The significance of this is unclear.

Although traditional neutralizing antibodies against ASFV have not been demonstrated (Costa, 1990; Forman et al., 1982; Viñuela, 1987), it is evident that antibodies and cytotoxic T lymphocytes are involved in the immune protection of pigs from homologous ASFV challenge or reinfecction (Onisk et al., 1994; Martins et al., 1993). The j13L gene product was immunogenic as recovered pig sera contained antibodies to the predicted antigenic peptide epitope. An important question is whether such antibodies may play a role in protection from ASFV infection. A preliminary in vitro neutralization test, however, showed that rabbit antisera to synthetic peptide j13L had no significant neutralization activity against ASFV (Uganda strain) infection. Con-
sistent with this, vaccination of pigs with a vaccinia virus recombinant expressing the j3L ORF failed to protect against challenge with the virulent ASFV Malawi LIL 20/1 isolate. Thus the role of the j3L product in the virus–host interaction remains to be clarified.

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