African swine fever virus NL gene is not required for virus virulence

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Previously, we described a highly conserved non-essential African swine fever virus (ASFV) right variable region gene, NL. Deletion of NL from the European pathogenic isolate E70 resulted in almost complete attenuation of the virus in domestic swine. To study gene function further, NL gene deletion mutants were constructed from two pathogenic African ASFV isolates, Malawi Lil-20/1 (Mal) and Pretoriuskop/96/4 (Pr4). Unexpectedly, both Mal (Mal-ΔNL) and Pr4 (Pr4ΔNL) null mutants remained highly virulent when inoculated in swine. Mal-ΔNL exhibited a disease and virulence phenotype indistinguishable from its revertant, Mal-NLR, which caused 100% mortality. Mortality among Pr4ΔNL-infected animals was also high; however, a significant delay in onset of fever and viraemia and in time to death was observed. These data indicate that NL gene function is not required for ASFV virulence and that other yet-to-be identified viral determinants perform significant virulence functions in these African field isolates.

African swine fever (ASF) is a highly lethal and significant disease of domestic pigs, for which there is no vaccine or disease control strategy other than animal slaughter. The causative agent, a large enveloped double-stranded DNA virus (ASFV), is the sole member of the newly named Asfarviridae (Dixon et al., 1995; L. Dixon, personal communication). ASFV genomic organization and its cytoplasmic replication strategy share similarities with the Poxviridae (Costa, 1990; González et al., 1986; Ortín et al., 1979; Sogo et al., 1984).

ASFV is the only known DNA arbovirus (Brown, 1986; Costa, 1990; Dixon et al., 1995). In nature, the perpetuation and transmission of this virus involves the cycling of virus between two highly adapted hosts, Ornithodoros ticks and wild pig populations (warthogs and bushpigs) in sub-Saharan Africa (Plowright et al., 1969a, b; Thomson et al., 1983; Wilkinson, 1989). In the warthog host, infection is subclinical, characterized by low-titre viraemias (Plowright et al., 1994; Thomson et al., 1980). Persistent infection of both ticks and pigs occurs following infection (Carrillo et al., 1994; de Kock et al., 1940; DeTray, 1957; Heuschele & Coggins, 1969; Kleiboeker et al., 1998; Plowright et al., 1969a; Sanchez-Botija, 1963; Thomson et al., 1983).

In domestic pigs, ASF occurs in several disease forms, ranging from highly lethal to subclinical infections, depending on contributing viral and host factors (Colgrove et al., 1969; Mebus et al., 1981). ASFV infects cells of the mononuclear-phagocytic system, including highly differentiated fixed-tissue macrophages and reticular cells; affected tissues show extensive damage after infection with highly virulent virus strains (Colgrove et al., 1969; Konno et al., 1971a, b; Lord et al., 1990; Mebus, 1988; Moulton & Coggins, 1968). ASFV strains of lesser virulence also appear to infect these cell types, but tissue involvement and resulting tissue damage are much less severe (Hess, 1981; Mebus et al., 1981; Mebus, 1988). The abilities of ASFV to replicate and to induce marked cytopathology in these cell types in vivo appear to be critical factors in ASFV virulence. The nature of viral factors responsible for the differing outcomes of infection with strains of high virulence and of lesser virulence are largely unknown.

Previously, we described an ASFV gene, NL, with similarity to the neurovirulence gene ICP34.5 of herpes simplex virus (Chou et al., 1990; McGeoch & Barnett, 1991), a myeloid differentiation primary response gene, MyD116 (Lord et al., 1990), a growth arrest DNA damage gene, GADD34 (Fornace et al., 1989) and a partial ORF from Amsacta moorei entomopoxvirus (Hall & Moyer, 1991), and found it to be highly conserved among pathogenic ASFV isolates existing in either a long (23-NL, 184 amino acids) or short (NL-S, 70–72 amino acids) gene form (Sussman et al., 1992; Zsak et al., 1996). Deletion of NL-S from the highly virulent European isolate E70 resulted in marked attenuation of the virus in the swine host. Pig infections with the null mutant were characterized by a lack of clinical disease apart from a transient fever response, and by reduced viraemia titres (1000-fold) and no mortality (Zsak et al., 1996). Notably, pigs previously infected with the E70 NL-S gene deletion mutant E70/43 were resistant to infection when subsequently challenged with fully virulent parental E70.
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Fig. 1. (A-C) Characterization of a Mal NL gene deletion mutant, Mal-ΔNL, and its revertant, Mal-NLR. (A) Diagram of the NL gene regions in parental Mal, Mal-ΔNL and Mal-NLR. H, HindIII site. (B) Southern blot analysis of Mal-ΔNL (lane 1) and Mal (lane 2). Purified DNA was digested with HindIII, electrophoresed, blotted and hybridized to the parental 1-8 kbp HindIII fragment containing the 23-NL gene and adjacent flanking regions. (C) PCR of NL and GUS sequences from Mal (lane 1), Mal-ΔNL (lane 2) and Mal-NLR (lane 3) viral DNA. (D–F) Characterization of an ASFV Pr4 NL-S gene deletion mutant, Pr4-ΔNL-S. (D) Diagram of the NL gene regions in parental Pr4 and Pr4-ΔNL-S. B, BamHI site. (E) and (F) Southern blot analysis of Pr4 (lane 1) and Pr4-ΔNL-S (lane 2). Purified DNA was digested with BamHI, electrophoresed, blotted and hybridized to Pr4 DNA probes located at the left (E) and the right (F) of the engineered NL gene deletion. Positions of molecular size markers are indicated in kbp. LVR, left variable region; CVR, central variable region; RVR, right variable region.

virus (Zsak et al., 1996). These observations suggested that ASFV NL gene deletion mutants might be suitable for use as engineered live attenuated ASFV vaccines.

In this report, we demonstrate that NL gene deletion mutants of two pathogenic African ASFV isolates, Malawi Lil-20/1 (Mal) and Pretoriuskop/96/4 (Pr4), remained highly virulent when inoculated in swine. Thus, NL gene function is not an absolute requirement for virulence in all ASFV isolates, and other yet-to-be identified viral determinants must perform significant virulence functions.

To examine further the significance of the ASFV NL gene for pig virulence, recombinant NL gene deletion mutants were constructed of two additional pathogenic African ASFVs isolated from ticks (Dixon, 1988; Haresnape et al., 1988; Kleiboeker et al., 1998), Mal, which contained the long NL gene form (184 amino acids) and Pr4, which contained the short form of the gene (71 amino acids). ASFV recombinant viruses were generated by homologous recombination between parental ASFV genomes and engineered recombination transfer vectors following transfection-infection in primary swine macrophage cell cultures as previously described (Neilan et al., 1997; Zsak et al., 1996).

The recombination transfer vector for introducing the NL gene deletion into Mal was prepared by inserting a reporter gene cassette containing the β-glucuronidase (GUS) gene with the ASFV late gene promoter (p72GUS) (Neilan et al., 1997) into unique XbaI and Apol sites located within a subcloned 2-85 kbp SphI-SalI fragment (pNL1) from the right variable region of Mal (Dixon, 1988). This deletion removed all but 108 5′-terminal nucleotides of the NL ORF (Fig. 1A). Adjacent upstream sequences of the p72GUS cassette and the remaining NL gene sequences do not contain a start codon, so expression of a truncated NL protein was not possible. Transfection-infection assays were done as previously described (Zsak et al., 1996). A Mal NL gene deletion mutant, Mal-ANL (GUS-positive), was isolated, plaque-purified on macrophage cell
cultures and analysed by PCR and Southern blotting (Zsak et al., 1996). A revertant of Mal-ANL was also constructed. A reporter cassette containing the β-galactosidase (GAL) gene with the p72 promoter (p72GAL), was inserted at the unique XhoI site of pNL1 (Fig. 1A). This construct was then used in transfection-infection experiments to restore the NL ORF in the Mal-ANL genome. A revertant virus Mal-NLR, a GUS-negative and GAL-positive virus, was purified by plaque assay and characterized as described above. Characterization of ASFV recombinants Mal-ANL and Mal-NLR is shown in Fig. 1 (B–C). Viral DNA was purified from macrophage cell cultures infected with Mal, Mal-ANL and Mal-NLR as previously described (Wesley & Tuthill, 1984), digested with HindIII, separated by gel electrophoresis, Southern-blotted and hybridized to 32P-labelled DNA probes. A novel HindIII fragment with a predicted size of 3.4 kbp was observed for Mal-ANL when probed with sequences flanking the NL gene region. The net 1.6 kbp size increase resulted from the introduced deletion of 652 bp together with the insertion of the 2.2 kbp p72GUS cassette (Fig. 1B, lane 1). Deleted NL gene sequences were not detected in Mal-ANL by PCR (Fig. 1C, lane 2). The predicted 147 bp NL PCR fragment was, however, observed in both parental (lane 1) and revertant (lane 3) viruses.

The recombination transfer vector for introducing the NL gene deletion into the Pr4 ASFV isolate was prepared as follows. Flanking DNA fragments mapping to the left (800 bp) and right (1024 bp) of NL-S were amplified by PCR using Pr4 genomic DNA as a template. Primer sets, each of which introduced a BamHI site (underlined below) adjacent to NL-S were:

- left flank forward primer
  5’ TGCAAAATAGACTCTAAATT 3’;
- left flank reverse primer
  5’ AGGGGATCCATCGCGTGTC 3’;
- right flank forward primer
  5’ CATGGATCCCTGTGGATTGT 3’;
- right flank reverse primer
  5’ GCACCTTGAGGGATGGC 3’.

The fragments were cloned into pCRII to give pTNL-S and were sequenced to verify ASFV sequences. The nucleotide sequences of the cloned flanking regions were identical over their entire length to the Pr4 genomic DNA sequence. The p72GUS reporter gene cassette was inserted into BamHI-digested pTNL-S to yield p72GUSANL-S. This deletion removed all but the 5’-terminal 45 nucleotides of NL-S (Fig. 1D). A Pr4 NL-S gene deletion mutant, Pr4ANL-S (GUS-positive), was prepared and analysed as described above. Pr4 and Pr4ANL-S viral DNA was purified from infected macrophage cell cultures, digested with BamHI, Southern-blotted and hybridized to 32P-labelled probes containing sequences which flanked the deleted region. The right-terminal BamHI fragment of the Pr4 genome, which contained the NL gene region, was 12.7 kbp (Fig. 1E, F, lanes 1). Novel BamHI fragments of predicted size 8.0 and 4.5 kbp were observed for Pr4ANL-S when probed with the two parental flanking fragments from this region (Fig. 1E, F, lanes 2).

Growth characteristics of Mal-ANL and Pr4ANL-S were compared with those of parental and/or revertant viruses by infecting primary macrophage cell cultures (m.o.i. 0.01) and determining titres at various times post-infection as previously described (Zsak et al., 1996). Growth kinetics and virus yields of Mal-ANL were indistinguishable from those of Mal and Mal-NLR (Fig. 2). Similarly, as previously shown for the E70 NL-S gene deletion mutant E70/43 (Zsak et al., 1996), Pr4ANL-S exhibited parental growth characteristics on macrophages (data not shown). Thus, like the short gene NL-S, the longer NL gene, which encodes an additional 113 amino acids at the amino terminus of the protein, is nonessential for virus growth in swine macrophage cell culture.

To examine the virulence phenotype of Mal-ANL and Pr4ANL-S in domestic swine, Yorkshire pigs (30 to 35 kg) were inoculated intramuscularly with 10^5 TCID50 null mutants or parental and/or revertant viruses. A dose of 10^5 TCID50 Mal or Pr4 represents between 10 and 100 lethal doses (Neilan et al., 1997). Clinical signs of ASF (fever (a rectal temperature of greater than or equal to 40 °C), anorexia, lethargy, shivering, cyanosis and recumbency] were monitored daily. Blood samples were collected every other day for the duration of the experiment. Virus isolation and titration of ASFV in blood samples were performed as previously described (Onisk et al., 1994). Data from these experiments are shown in Table 1.

Unexpectedly, and unlike our prior observations for the E70 NL gene deletion mutant E70/43 (Zsak et al., 1996), Mal-ANL remained highly virulent in domestic pigs. Apart from a modest reduction in mean viraemia titres of Mal-ANL infected animals, disease onset, disease course and mortality were similar for Mal-ANL and its revertant Mal-NLR (Table 1, Expt 1). Pr4ANL-S also remained virulent with an 86% mortality
rate; however, aspects of virus infection were altered (Table 1, Expt 2 and 3). Significant delays in the onset of fever and viraemia (1 to 3 days) and in time to death (approximately 6 days) compared with parental Pr4-infected animals were observed. Mean and maximum viraemia titres were unaffected by the gene deletion.

These data indicate that NL gene function is not an absolute requirement for ASFV virulence and that the contribution of the NL gene to virus virulence is strain (isolate) dependent. For the three ASFV NL gene deletion mutants examined for pig virulence, there was a continuum of effect on virulence ranging from near complete attenuation (E70/43) (Zsak et al., 1996) through delayed disease onset and death (Pr4ANL-S) to little or no effect (Mal-ANL). In the case of the two African ASFV isolates described here, a yet-to-be identified viral determinant(s) must perform significant virulence functions. This might involve an additional viral gene(s) capable of directly complementing the NL defect or alternatively, a viral gene(s) encoding a dominant virulence factor absent or inactive in the E70 isolate. In either event, the relative importance of NL for virus virulence may depend on the complement of other virulence and swine host range genes encoded by a given ASFV isolate.

Importantly, these data indicate that NL gene deletion alone is not sufficient to engineer live attenuated host range-restricted ASFV vaccines. Identification and further characterization of the virulence determinant(s) present in African isolates will be necessary.

These data also suggest that although still highly pathogenic, the European E70 isolate and perhaps other European pathogenic ASFV isolates may be more attenuated and/or adapted in domestic pigs than either of the two African viruses examined here. In nature, perpetuation of ASFV involves the cycling of virus between highly adapted hosts, Ornithodoros ticks and warthogs and bushpigs in sub-Saharan Africa (Plowright et al., 1969a; Thomson et al., 1983; Wilkinson, 1989), and it is under these conditions with their attendant pressures that virus fitness is selected for. The ASF E70 virus was isolated in Spain in 1970, 10 years after the introduction of virus into the Iberian peninsula (Tabárès et al., 1987). During this period, transmission of virus largely occurred by spread within domestic pig populations. Therefore, it is possible, either due to the lack of selective pressure under these conditions or to virus-host adaptation, that the function of viral genes important in the natural cycle of infection may have been lost or altered. If this is the case, other findings on aspects of virus virulence and protective immunity against European ASFV isolates may not necessarily be applicable to African viruses found in nature.

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References


**Table 1. Swine survival, viraemia and fever response following infection with Mal-NLR, MalΔNL, Pr4 and Pr4ΔNL viruses**

<table>
<thead>
<tr>
<th>Expt</th>
<th>Group</th>
<th>Number surviving</th>
<th>Days to death</th>
<th>Days to onset</th>
<th>Days to onset</th>
<th>Mean titre</th>
<th>Maximum titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mal-NLR (5)</td>
<td>0/5</td>
<td>9±0.4</td>
<td>4±0.4</td>
<td>3±0.4</td>
<td>7±0.1</td>
<td>8±0.1</td>
</tr>
<tr>
<td></td>
<td>MalΔNL (5)</td>
<td>0/5</td>
<td>11±0.3</td>
<td>5±0.2</td>
<td>4±0.0</td>
<td>6±0.4</td>
<td>7±0.2</td>
</tr>
<tr>
<td>2</td>
<td>Pr4 (3)</td>
<td>0/3</td>
<td>8±0.7</td>
<td>3±0.3</td>
<td>2±0.0</td>
<td>7±0.6</td>
<td>9±0.3</td>
</tr>
<tr>
<td></td>
<td>Pr4ANL (3)</td>
<td>1/3</td>
<td>15±0.9</td>
<td>6±1.1</td>
<td>5±1.3</td>
<td>6±1.0</td>
<td>7±1.0</td>
</tr>
<tr>
<td>3</td>
<td>Pr4 (4)</td>
<td>0/4</td>
<td>9±0.4</td>
<td>4±0.2</td>
<td>2±0.0</td>
<td>6±0.2</td>
<td>8±0.1</td>
</tr>
<tr>
<td></td>
<td>Pr4ANL (4)</td>
<td>0/4</td>
<td>15±0.3</td>
<td>4±0.5</td>
<td>4±0.0</td>
<td>7±0.2</td>
<td>8±0.1</td>
</tr>
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</table>

Values significantly different from those of the Mal-NLR- or Pr4-infected group are indicated as follows: a, P < 0.05; b, P < 0.001; c, P < 0.0001. Data from experiments 2 and 3 were pooled for statistical analysis.

* log₁₀ haemadsorption units (HAD₉₀)/ml (mean ± SE).


