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An African Swine Fever Virus Virulence-Associated Gene *NL-S* with Similarity to the Herpes Simplex Virus *ICP34.5* Gene

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We described previously an African swine fever virus (ASFV) open reading frame, 23-NL, in the African isolate Malawi Lil 20/1 whose product shared significant similarity in a carboxyl-terminal domain with those of a mouse myeloid differentiation primary response gene, *MyD116*, and the herpes simplex virus neurovirulence-associated gene, *ICP34.5* (M. D. Sussman, Z. Lu, G. Kutish, C. L. Afonso, P. Roberts, and D. L. Rock, J. Virol. 66:5586–5589, 1992). The similarity of *23-NL* to these genes suggested that this gene may function in some aspect of ASFV virulence and/or host range. Sequence analysis of additional pathogenic viral isolates demonstrates that this gene is highly conserved among diverse ASFV isolates and that the gene product exists in either a long (184 amino acids as in 23-NL) or a short form (70 to 72 amino acids in other examined ASFV isolates). The short form of the gene, *NL-S*, encodes the complete highly conserved, hydrophilic, carboxyl-terminal domain of 56 amino acids common to 23-NL, MyD116, and ICP34.5. Recombinant *NL-S* gene deletion mutants and their revertants were constructed from the pathogenic ASFV isolate E70 and an E70 monkey cell culture-adapted virus, MS44, to study gene function. Although deletion of *NL-S* did not affect viral growth in primary swine macrophages or Vero cell cultures in vitro, the null mutant, E70/43, exhibited a marked reduction in pig virulence. In contrast to revertant or parental E70 where mortality was 100%, all E70/43-infected animals survived infection. With the exception of a transient fever response, E70/43-infected animals remained clinically normal and exhibited a 1,000-fold reduction in both mean and maximum viremia titers. All convalescent E70/43-infected animals survived infection when challenged with parental E70 at 30 days postinfection. These data indicate that the highly conserved *NL-S* gene of ASFV, while nonessential for growth in swine macrophages in vitro, is a significant viral virulence factor and may function as a host range gene.

African swine fever virus (ASFV), the causative agent of ASF, is a unique and complex DNA virus; it is the sole member of an unnamed family of animal viruses and is the only known DNA arbovirus (3, 10, 11). ASFV, a large icosahedral virus with a linear double-stranded DNA genome of 170 to 190 kbp, replicates in the cell cytoplasm (10, 49). In nature, the perpetuation and transmission of this virus involve the cycling of virus between *Ornithodoros* ticks and wild pig populations (warthogs and bushpigs) in sub-Saharan Africa (35, 36, 45, 53).

ASF occurs in several disease forms in domestic pigs, ranging from highly lethal to subclinical infections, depending on contributing viral and host factors (9, 29). Hemostatic and hemodynamic changes (hemorrhage, edema, ascites, and shock) resulting from intravascular activation of coagulation are observed in dying pigs infected with highly virulent strains of this virus (46–48). ASFV infects cells of the mononuclear-phagocytic system, including highly differentiated fixed-tissue macrophages and specific lineages of reticular cells; affected tissues show extensive damage after infection with highly virulent viral strains (9, 22, 23, 29, 31). Moderately virulent ASFV strains also appear to infect these cell types, but the degree of tissue involvement and the resulting tissue damage are much less severe (18, 29, 30). The abilities of ASFV to replicate and induce marked cytopathology in these cell types in vivo appear to be critical factors in ASFV virulence. The nature of viral and host factors responsible for the differing outcomes of infection with highly virulent strains and strains of lesser virulence is unknown.

Previously we described an ASFV open reading frame (ORF), 23-NL, which encodes an intermediate to late 23-kDa protein that shares significant similarity with a myeloid differentiation primary response gene, *MyD116* (25), a growth arrest DNA damage gene *GADD34* (13), a partial ORF from *Amsacta moorei* entomopoxvirus (15), and a neurovirulence-associated gene of herpes simplex virus (HSV), *ICP34.5* (1, 28, 40). All of the proteins encoded by these genes contain a centrally located acidic region with a highly conserved 56-amino-acid domain located at the carboxyl terminus. The functions of the cellular proteins MyD116 and GADD34 have not been established. *MyD116* is an immediate-response gene expressed early in myeloid cell differentiation (24), and *GADD34* is induced by growth arrest and DNA damage (13). The HSV ICP34.5 protein, while nonessential for growth in a variety of cell cultures, is a host range factor required for efficient replication and viral virulence in fully differentiated neuronal tissue (2, 5, 6, 28, 42). Additionally, this protein also appears to perform a viral host range function in the mouse cornea (2, 52). The similarity of the ASFV *23-NL* gene to these other genes suggests the *NL* gene may function in some aspect of ASFV virulence and/or host range.

Here we demonstrate the ASFV *NL* gene is highly conserved among viral isolates and that it exists in either of two forms, i.e., the one we described earlier (*23-NL*) and a shorter form, *NL-S*, which is found in the European and African pathogenic isolates examined here.

Experimental data from recombinant ASFV *NL-S* gene deletion mutants indicate that the ASFV *NL-S* gene, while nonessential for growth in macrophages and Vero cells in vitro, is

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a highly significant viral virulence factor and may function as a host range gene.

MATERIALS AND METHODS

Cell cultures and viruses. Vero cells were obtained from the American Type Culture Collection and propagated in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum (FBS). Macrophage cultures were prepared from defibrinated swine blood as previously described (14). Cells were cultured in plastic Primaria (Falcon; Becton Dickinson Labware, Franklin Lakes, N.J.) tissue culture flasks containing RPMI 1640 medium with 30% L929 supernatant and 20% FBS for 48 h (37°C in 5% CO_2). Adherent cells were detached from the plastic by using 10 mM EDTA in phosphate-buffered saline and then reseeded into Primaria T25 6-well or 96-well dishes at a density of 5×10^6 cells per ml for assays 24 h later.

The pathogenic European ASFV isolate E70 and the E70 cell culture-adapted variant MS44 (E70 passaged 44 times in MS monkey cells [41]) were provided by J. M. Escribano (INIA, Madrid, Spain). Pathogenic ASFV isolates Brazil (BR), Cameroon (CA), Kerita (KE), Victoria Falls (VI), Tengani (TE), Spencer (SP), Uganda (UG), and Zimbabwe (ZI) were obtained from the Plum Island Animal Disease Center ASFV reference collection (16, 21, 50).

Animal infections. Yorkshire pigs (30 to 35 kg) were inoculated intramuscularly with either 10^2 50% tissue culture infective doses ($TCID_{50}$) (experiment one) or 10^3 $TCID_{50}$ (experiment two) of parental E70 or recombinant viruses. A dose of 10^2 $TCID_{50}$ of E70 represents a challenge of between 10 and 100 100% lethal doses (LD_{100}). Clinical signs of ASF (fever [a rectal temperature greater than or equal to 40°C], anorexia, lethargy, shivering, cyanosis, and recumbency) were monitored daily. Blood samples were collected every other day for 30 days postinfection (DPI). Virus isolation and titration of ASFV in blood samples were performed as previously described (33). Virus titers were calculated by the method of Spearman-Karber and expressed as $TCID_{50}$ (12).

Construction of ASFV E70 genomic cosmid library and DNA sequencing. E70 viral DNA was purified from viremic pig blood by the method of Wesley and Tuthill (51). Genomic DNA was partially digested with *Sau*3A restriction enzyme and subjected to gel electrophoresis in 0.3% agarose gel, and DNA fragments of about 40 kb were isolated by phenol extraction and ethanol precipitation.

DNA fragments were ligated into the *Bam*HI site of the linearized cosmid vector, pHC79 (Boehringer Mannheim, Indianapolis, Ind.), packaged into bacteriophage lambda particles, and then used to infect DH5αF′ *Escherichia coli* cells as previously described (20). Cosmid clones were amplified and screened by dot blot hybridization by using an ASFV gene probe, ORF 23-BL (4), from the right end of the Malawi Lil 20/1 genome. A cosmid clone, H7, representing the right terminus of the E70 genome, was identified and purified. H7 was further subcloned, and a 5-kb *Eco*RI-*Sal*I fragment within it, H7E, was sequenced by using the dideoxynucleotide chain termination method (38). DNA sequences were assembled by using Staden's Sequence Assembly Program (39) and analyzed by using FASTA method (34) along with other phylogenetic programs (43, 44).

PCR and reverse transcription (RT)-PCR analysis. PCR amplification of the NL gene region from various ASF viruses was performed by using low-molecular-weight DNA extracts from virus-infected cells as targets (19). The NL gene region (approximately 1.2 kb), bracketed by ORF MR on the left and a 360 multigene family member, ORF OR, on the right (see Fig. 1A), was amplified with the primer pairs 5′-CTTTCACCCCACGACTTCTTA-3′ and 5′-CACTTG TAGAGTGGATGGCAT-3′ (nucleotide 729 and 1983 in the H7E clone, respectively). PCR was performed for 40 cycles of thermal denaturation (96°C for 15 s), reannealing (50°C for 30 s), and extension (60°C for 30 s). Amplified products were cloned into the TA cloning vector, pCR II (Invitrogen, San Diego, Calif.) and sequenced by using the T7 and Sp6 priming sites (37).

RT-PCR was performed by using protocols for reverse transcription of RNA and PCR amplification of cDNA provided with the *GeneAmp* Thermostable r*Tth* Reverse Transcriptase RNA PCR kit (Perkin-Elmer). Briefly, RNAs were extracted from ASFV-infected cells at 16 h postinoculation (multiplicity of infection [MOI] = 10) by using the Micro-Scale Total RNA Separator kit (Clontech, Palo Alto, Calif.) and treated with 100 U of DNase I (Boehringer Mannheim) per μg of RNA at 37°C for 90 min. One microgram of total infected-cell RNA was reverse transcribed for 15 min at 70°C by using 5 U of r*Tth* DNA polymerase and a gene-specific downstream primer. Resulting cDNAs were then amplified by PCR with a gene-specific primer pair: *NL-S* gene, forward primer, 5′-GTAT GGGAAGCCGACGACATC-3′; reverse primer, 5′-TTACTGCTGCTCCAG TAGCTT-3′; and *UK* gene, forward primer, 5′-TCATCTGTCGTGGATAATG CA-3′; reverse primer 5′-GTCTGCACTGGTCATTTTTCT-3′. Non-reverse-transcribed RNA samples were used in the PCR as a control to ensure absence of viral DNA contamination. PCR products were analyzed by gel electrophoresis and Southern blot hybridization (37) by using PCR-generated DNA probes whose sequences were contained within the primary amplification products.

Construction of ASFV recombinant viruses. ASFV recombinant viruses were generated by homologous recombination between parental ASFV genomes and engineered recombination transfer vectors in primary swine macrophages or Vero cells.

The recombination transfer vector for introducing the *NL-S* gene deletion in E70 and MS44 was constructed by deleting a 162-bp *Sac*I-*Hin*dIII fragment from the cosmid subclone H7E (see Fig. 3A) and replacing it with the β-glucuronidase (GUS) reporter gene under the control of an ASFV late structural gene promoter, p72 (32). The *Sac*I site is a unique restriction site in the H7E subclone, while the *Hin*dIII was created by using PCR site-directed mutagenesis at the NL-S start site (position 962). This deletion removes all but 45 nucleotides of the NL-S ORF.

Primary swine macrophages were infected with E70 (MOI = 5), incubated at 37°C for 1 h, and then washed three times with Dulbecco's minimal essential medium containing 10% FBS. A transfection mixture containing 5 μg of the recombinant transfer vector DNA and 20 μl of LipofectAMINE in 1 ml of Opti-MEM medium (GibcoBRL, Gaithersburg, Md.) (incubated 30 min at room temperature prior to use) was then added to the infected cultures. After a 5-h incubation at 37°C, the transfection mixture was replaced with RPMI medium containing 20% FBS. Cell cultures were harvested when complete cytopathic effect was evident. Transfection/infection cultures were sonicated, and serial 10-fold dilutions of the lysate were plated on swine macrophages. GUS-expressing recombinant viruses were detected in a plaque assay by overlaying cultures with 0.5% agarose containing 100 μg per ml of X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) (GibcoBRL). Recombinant viruses were subjected to 6 to 8 additional plaque purifications until PCR and Southern blot analysis showed no evidence of contamination with parental virus. An MS44 *NL-S* gene deletion mutant was constructed by using an identical approach in Vero cells.

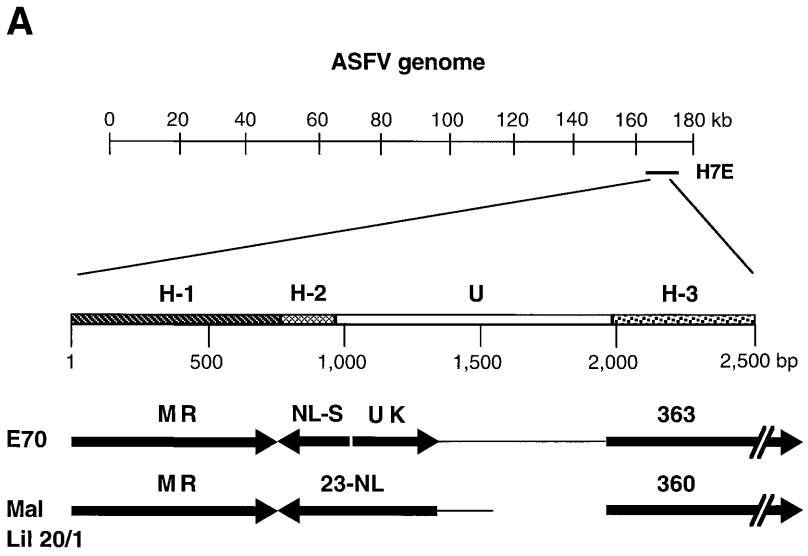
Revertant viruses were constructed from both the E70 *NL-S* gene deletion mutant E70/43 and the MS44 *NL-S* gene deletion mutant MS44/43. Cell cultures (swine macrophages or Vero cells) were infected with the deletion mutant and then transfected with a recombinant plasmid containing the intact H7E genomic region as described above. The revertant virus population present in the E70/43 transfection/infection mixture was enriched in vivo by intramuscularly inoculating pigs with 10^3, 10^4, and 10^5 $TCID_{50}$ of the transfection/infection mixture. At 3, 6, 8, and 11 DPI, blood samples were collected, and serial 10-fold dilutions of the samples were plated onto swine macrophages. At the time of complete cytopathic effect, X-Gluc was added and wells containing blue-staining cells were visualized. GUS-negative viruses (putative revertants) were further purified three times in macrophage cultures by limited dilution and analyzed in Southern blot, PCR, and RT-PCR assays to demonstrate the restoration of *NL-S* gene sequences to the revertant viral genome. The MS44/43 transfection/infection mixture was screened with a plaque assay in Vero cells overlaid with agarose containing X-Gluc. GUS-negative plaques were subjected to three additional plaque purifications, and revertant viruses were characterized as described above.

Nucleotide sequence accession numbers. The sequences in this study were assigned GenBank accession numbers U73713 (E70), U73715 (MS44), U73711 (BR), U73712 (CA), U73714 (KE), U73719 (VI), U73717 (TE), U73716 (SP), U73718 (UG), and U73720 (ZI).

RESULTS

Two forms of the *23-NL* gene are found in pathogenic ASFV isolates. Conservation of the *23-NL* gene in pathogenic ASFV isolates was examined by sequence analysis. A 5-kb fragment (clone H7E) (Fig. 1A) from the right variable region of the pathogenic European isolate E70 was sequenced in its entirety, and the NL gene region was analyzed and compared with the sequence from the same region of the African pathogenic isolate Malawi Lil 20/1. In contrast to the 23-NL ORF encoding 184 amino acids (predicted molecular mass of 21.3 kDa) present in Malawi, E70 contained a shortened NL ORF (NL-S) encoding only 71 amino acids (predicted molecular mass of 8.5 kDa), which represented the carboxyl-terminal portion of 23-NL (Fig. 1A). Like Malawi 23-NL, E70 NL-S was transcribed to the left, and it contained the complete highly conserved, hydrophilic, 56-amino-acid carboxyl-terminal domain with the 38-amino-acid motif [Vx(7,8)Rx3Wx5DRxRFxRRx11L] common to 23-NL, MyD116, GADD34, AmEPV, and ICP34.5 (Fig. 1B). E70 *NL-S* had 72% identity with *23-NL* over 170 nucleotides and the encoded products had 73% identity (100% similarity) over 71 amino acids. Both gene products contained a highly basic amino terminus composed of arginine and lysine residues; however, there was no sequence similarity in this region.

In both viruses, the NL gene region (Fig. 1, H-2 and U) was bracketed by conserved genomic regions (Fig. 1A, H-1 and H-3). Both genomes contained a highly conserved ORF, MR, 3′ to the NL ORF on the opposite strand (Fig. 1A, H-1). In



B

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HSV ICP34.5   186 ........tpatparVRFsphvrvrhLv.VWasAarvaRaG  217
MyD116        542 ........IplkarKVhFAekvtvhfLa.VWagpaqaaRrG  573
AmEPV           1...............KVRFsseppklhimyVWlyAakqtRKl   27
Malawi 23-NL  118 ........Irqrdv KVyFAtddi...LikVr.EADDIdRKG  146
E70 NL-S        1 mggrrrkkrtndtkhVRFAa......aveVW.EADDIeRKG   34

HSV ICP34.5   218 sWareraDRaRFrRRvAeaEavigpcLgpeararalar      255
MyD116        574 PWEQfArDRsRFaRRIAgaEekLgpyLtpdsrarawar     611
AmEPV          28 yWdkfAiDRhRFkRRInDidisiSwVLtphhrhkinet      65
Malawi 23-NL  147 PWEQaAVDRlRFQRRIADtEkiLSaVLLRkKlnpmEhe     184
E70 NL-S       35 PWEQvAVDRfRFQRRIAsvEelLStVLLRqK.kllEqq      71
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FIG. 1. (A) Structural diversity of the ASFV NL gene region in pathogenic virus isolates E70 and Malawi Lil 20/1. H-1, ORF MR region; H-2, ORF NL corresponding to the carboxyl-terminal region; U, unique region; H-3, multigene family 363/360 ORF OR region. (B) Alignment of the predicted amino acid sequences at the conserved carboxyl-terminal domains of HSV ICP34.5 (GenBank accession no. M33699), MyD116 (GenBank accession no. X51829), AmEPV (GenBank accession no. M77182), Malawi 23-NL (GenBank accession no. M95672), and E70 NL-S. Residues identical to 23-NL are shown as uppercase boldface letters.

Malawi Lil 20/1, the 23-MR ORF predicted a protein of 240 amino acids with a molecular mass of 27.5 kDa. The homologous ORF in E70 was 255 amino acid residues in length, sharing 80% identity and 95% similarity over 238 residues with the Malawi ORF. To the right of the NL gene region was a conserved member of the 363/360 multigene family, ORF OR (53.8% identity over the encoded 305 amino acids) (Fig. 1A, H-3). Apart from the similarity of 23-NL to NL-S described above, the remainder of the two NL gene regions, 1,071 bp in E70 and 663 bp in Malawi, were dissimilar (Fig. 1A, U). Within this region, the E70 genome contains a novel ORF, UK, which is immediately upstream from NL-S and transcriptionally oriented toward the right end of the genome. ORF UK predicts a protein of 96 amino acids with a molecular mass of 10.7 kDa.

The NL gene region was examined in eight additional pathogenic viruses representing African, European, and Caribbean isolates. The genomic region located between ORF MR on the left and the 363/360 multigene family member, ORF OR, on the right, was amplified by PCR, cloned, and sequenced completely. A DNA fragment of approximately 1.2 kb was amplified from each of the eight isolates (data not shown). Sequence analysis revealed an E70-type genome arrangement within this region for all eight viruses. All contained both a short NL ORF encoding either 70, 71, or 72 amino acids and ORF UK. Predicted amino acid sequences of these NL-S ORFs are shown in Fig. 2. Pathogenic isolates E70, BR, CA, KE, and VI contained identical NL-S ORFs encoding 71 amino acids. Identical NL-S ORFs encoding 70 amino acids were found in African isolates SP and UG. A 72-amino-acid sequence encoded by NL-S ORF was present in the African isolate ZI. It is interesting to note that most nucleotide changes in the *NL-S* gene sequences were silent mutations. There were 13 polymorphic nucleotide sites with 26 changes for a high 5.2 corrected average transition/transversion ratio and a low 0.01 average change per nucleotide position. While the TE isolate was most different from the others at the *NL-S* locus, there was no significant difference between isolates in the overall relationship at this locus (Kimura 2 parameter distance estimate by using a neighbor-joining branch length test and a cluster test with 1,000 bootstrap samples) or in mutation rate (chi-square = 2.4, 10 df). The *NL-S* genes of highly monkey cell culture-adapted viruses MS44 and BA71V (54) were identical at the nucleotide level with *NL-S* of E70. These data indicate that the *NL* gene is highly conserved among diverse pathogenic ASFV isolates

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E70          .
BA71V        .
MS44         .
BR           .
CA           .
KE           .
VI           .
TE           .                                          A
SP           ..          A
UG           ..          A
ZI           G           V
CONSENSUS    M-GGRRRKKRTNDTKHVRFAAAVEVWEADDIERKGPWEQV   40

E70
BA71V
MS44
BR
CA
KE
VI
TE                              A
SP                              AM
UG                              AM
ZI                              A
CONSENSUS    AVDRFRFQRRIASVEELLSTVLLRQKKLLEQQ           72
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FIG. 2. Alignment of the predicted amino acid sequences encoded by NL-S ORFs from the pathogenic ASFV isolates E70 (Spain, 1970), BR (Brazil, 1979), CA (Cameroon, 1982), KE (Kerita, 1967), VI (Victoria Falls, 1967), TE (Tengani, 1961), SP (Spencer, 1951), UG (Uganda, 1961), and ZI (Zimbabwe, 1967) and from attenuated viruses MS44 and BA71V (GenBank accession no. U18466). Differences in residues are shown above the consensus sequence. •, missing amino acids.

with gene products existing in either a long (184 amino acids) or short form (70 to 72 amino acids).

Construction and analysis of recombinant ASFV *NL-S* gene deletion mutants and their revertants. ASFV *NL-S* gene deletion mutants were constructed from the pathogenic European isolate E70 and its cell culture-adapted variant MS44 by homologous recombination between parental genomes and engineered recombination transfer vectors in swine macrophages and Vero cells as described in Materials and Methods. The introduced deletion removed a 162-bp *Sac*I-*Hin*dIII fragment (Fig. 3A) from the ASFV genome and inserted in its place a 2.4-kb p72GUS reporter gene cassette. This deletion removed all but the 45 nucleotides corresponding to the carboxyl terminus of NL-S. Revertant viruses were constructed for both the E70 *NL-S* null mutant, E70/43, and the MS44 *NL-S* null mutant, MS44/43, as described in Materials and Methods. These viruses were referred to as E70/43R and MS44/43R (Fig. 3). Genomic DNAs from parental, deletion mutant, and revertant viruses were analyzed by Southern blot and PCR. Viral DNAs were purified from infected cells, digested with *Eco*RI, gel electrophoresed, Southern blotted, and hybridized with the 5-kb *Eco*RI-*Sal*I fragment contained in clone H7E (Fig. 3A). The right terminal *Eco*RI fragment of the ASFV genome, which contained the NL gene region, was 7-kb long in E70 and 9-kb long in MS44 (Fig. 3B, lanes 2 and 6). A predicted *Eco*RI fragment of increased size, 9.2 kb for E70/43 and 11.2 kb for MS44/43, was observed for *NL-S* deletion mutants (Fig. 3B, lanes 3 and 7). The net 2.2-kb size increase resulted from deletion of 162 bp from *NL-S* together with insertion of the 2.4-kb p72GUS reporter gene cassette. As expected, terminal *Eco*RI fragments of revertant viruses E70/43R and MS44/43R were of parental size (Fig. 3B, lanes 4 and 8). Deleted *NL-S* gene sequences were not detected in E70/43 and MS44/43 virus stocks by using PCR (Fig. 4A, lane 3 and 6). The predicted 146-bp PCR fragment was, however, observed in both parental (lanes 2 and 5) and revertant virus stocks (lanes 4 and 7). *NL-S* gene transcripts were detected by RT-PCR in macrophages

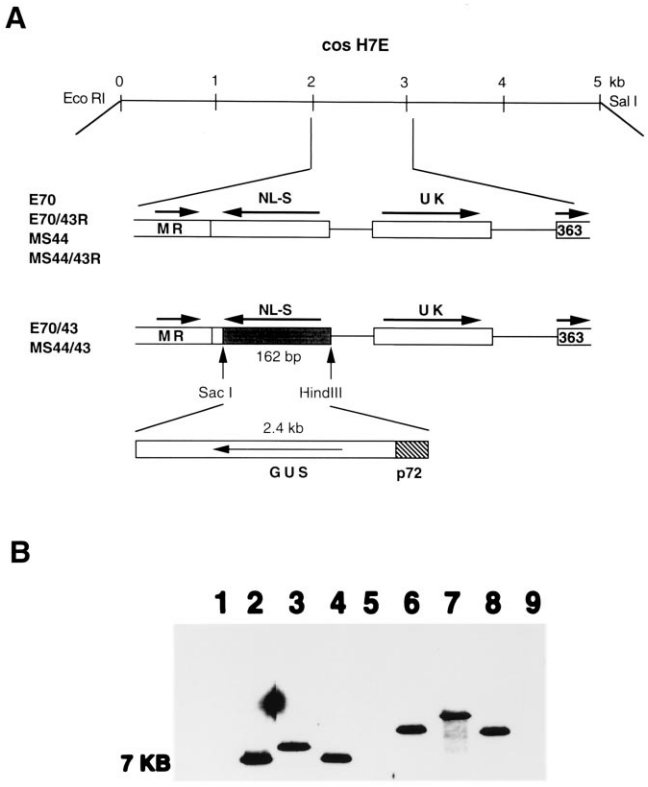

FIG. 3. Construction of recombinant *NL-S* gene deletion mutants and revertants from E70 and MS44 ASF viruses. (A) Transfer vectors and recombinants were constructed as described in Materials and Methods. (B) Southern blot analysis of NL-S gene region of E70 (lane 2), MS44 (lane 6), deletion mutants E70/43 (lane 3) and MS44/43 (lane 7), and revertant viruses E70/43R (lane 4) and MS44/43R (lane 8).

infected with E70 and E70/43R (Fig. 4B, lanes 3 and 5) but were absent in cells infected with the E70 *NL-S* deletion mutant, E70/43 (Fig. 4B, lane 4). RT-PCR analysis of the adjacent *UK* gene indicated that the *NL-S* deletion introduced into E70/43 did not affect *UK* gene transcription in infected mac-

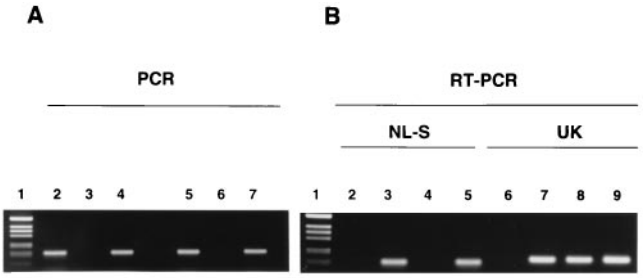

FIG. 4. (A) PCR analysis of *NL-S* gene sequences in E70 (lane 2), deletion mutant E70/43 (lane 3), revertant E70/43R (lane 4), MS44 virus (lane 5), deletion mutant MS44/43 (lane 6), and revertant MS44/43R (lane 7). PCR amplification was performed by using *NL-S*-specific oligonucleotides (see Materials and Methods); amplified DNAs were subjected to gel electrophoresis and ethidium bromide staining. A 1-kb DNA ladder as marker is shown in lane 1. (B) RT-PCR amplification of RNAs from E70 (lanes 3 and 7), deletion mutant E70/43 (lanes 4 and 8), and revertant E70/43R (lanes 5 and 9)-infected macrophages. One hundred nanograms of total RNA was used in the assay with either *NL-S*-specific (lanes 2, 3, 4, and 5) or with *UK* gene-specific oligonucleotides (lanes 6, 7, 8, and 9). A 1-kb DNA ladder (lane 1) and control, non-reverse-transcribed, DNase-treated RNA samples from E70 and E70/43R infected macrophages (lanes 2 and 6, respectively) are also shown.

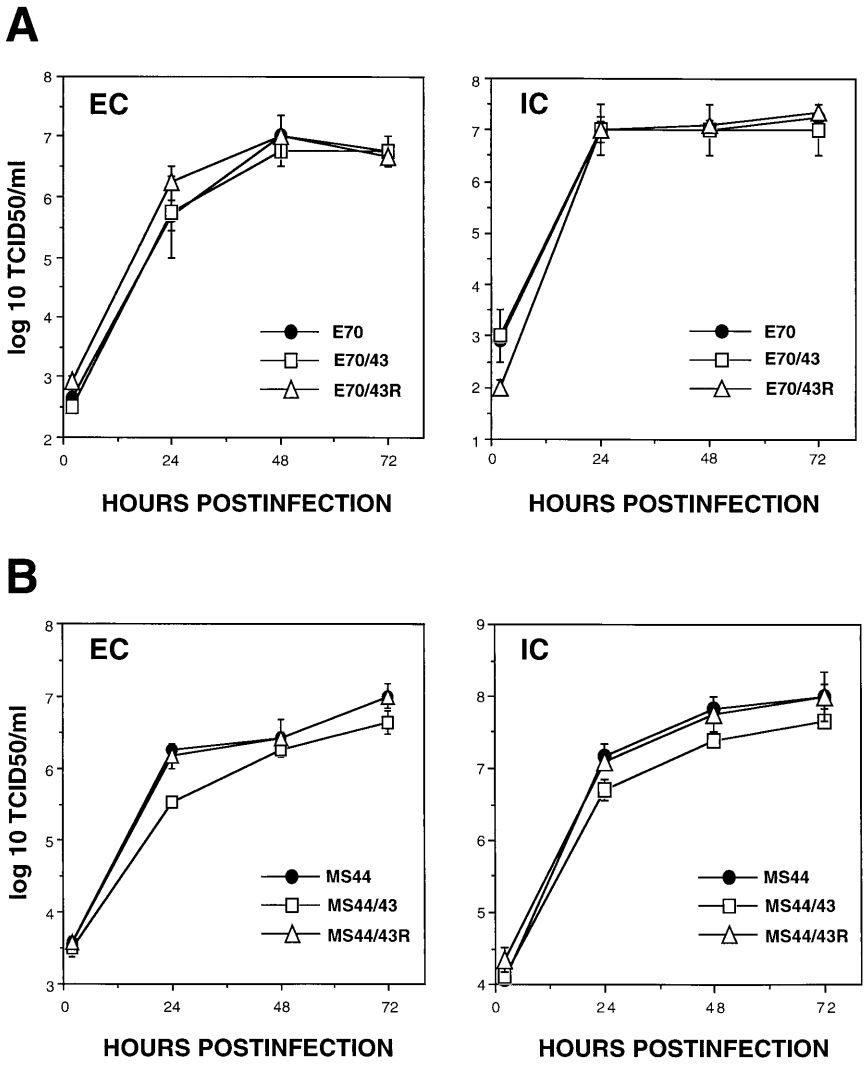

FIG. 5. (A) Growth characteristics of ASFV isolate E70, deletion mutant E70/43, and revertant E70/43R viruses in swine macrophage cultures. Primary swine macrophages were infected (MOI = 1) with E70, E70/43, and E70/43R viruses. At indicated times duplicate samples were collected and titrated for extracellular (EC) and intracellular (IC) virus yield. Data represent the means and standard errors of two independent experiments. (B) Growth characteristics of ASFV MS44, deletion mutant MS44/43, and revertant MS44/43R viruses in Vero cell cultures. Vero cell cultures were infected (MOI = 5) with MS44, MS44/43, and MS44/43R viruses. At indicated times duplicate samples were collected and titrated for EC and IC virus yields. Data represent the means and standard errors of two (MS44 and MS44/43R) or four (MS44/43 mutant) independent experiments.

rophages (Fig. 4B, lane 8). Inactivation of ASFV *23-MR* gene, located to the left of *NL-S*, did not affect the growth of virus in porcine macrophages or viral virulence in pigs (26, 27).

***NL-S* is nonessential for growth of ASFV in vitro.** Growth kinetics and viral yields of the *NL-S* null mutant, E70/43, in primary porcine macrophage cell cultures were statistically indistinguishable from those of the parental virus E70 and the revertant E70/43R (Fig. 5A). Likewise, MS44/43 exhibited unaltered growth characteristics in Vero cells compared with the parental virus MS44 and the revertant MS44/43R (Fig. 5B). In plaque assays on Vero cells, MS44/43 formed plaques that were indistinguishable in size and morphology from those of MS44 and MS44/43R (data not shown).

***NL-S* is a significant viral virulence factor.** To examine the role of *NL-S* in viral virulence, Yorkshire pigs were inoculated intramuscularly with 10^2 $TCID_{50}$ (experiment 1) or 10^3 $TCID_{50}$ (experiment 2) of the parental virus E70, the NL-S null mutant E70/43, and its revertant E70/43R. A 10^2 $TCID_{50}$ of E70 represents a challenge of between 10 and 100 LD_{100}. Results of these experiments are shown in Table 1. In contrast to E70 and E70/43R viruses, where mortality was 100%, all E70/43-infected animals survived infection. The disease pattern observed for E70- and E70/43R-infected animals contrasted markedly with that seen following infection with E70/43. With the exception of a transient fever response, E70/43-infected animals remained clinically normal following infection, whereas animals infected with E70 and E70/43R presented with clinical signs of ASF on 4 DPI, and these signs progressed until death in all cases. Both mean and maximum viremia titers in E70/43-infected animals were significantly reduced approximately 1,000-fold over control values ($P = 0.013$ to 0.0001) during this acute disease period. Duration of viremia in E70/43-infected pigs ranged from 0 to approximately 30 DPI. In experiment 2, all five animals had low levels of viremia (10^2 to 10^4 $TCID_{50}$ per ml) for 16 to 18 DPI. At 30 DPI, recovered E70/43-infected animals ($n = 9$) were challenged intramuscularly with 10^2

TABLE 1. Swine survival, viremia, and fever response following infection with E70, E70/43, and E70/43R[a]

Group	No. surviving	Days to death	Fever		Viremia		
			Days to onset	No. of days of fever	Days to onset	Mean titer (log_{10} $TCID_{50}$/ml)	Max titer (log_{10} $TCID_{50}$/ml)
Expt 1							
E70 ($n = 7$)	0/7	8.0 ± 0.4	4.7 ± 0.2	3.2 ± 0.6	3.8 ± 0.4	6.8 ± 0.5	7.8 ± 0.3
E70/43 ($n = 4$)	4/4		6.0 ± 1.0	1.2 ± 0.4*	9.0 ± 2.5	3.4 ± 1.0†	3.8 ± 0.8‡
Expt 2							
E70/43R ($n = 5$)	0/5	9.0 ± 1.1	3.6 ± 0.4	4.4 ± 1.2	3.8 ± 0.4	6.5 ± 0.2	7.5 ± 0.5
E70/43 ($n = 5$)	5/5		4.8 ± 0.5	1.2 ± 0.4§	4.2 ± 0.5	3.3 ± 0.05‖	4.3 ± 0.1#

[a] Values significantly different from those of the E70 or E70/43R group are indicated as follows: *, $P = 0.048$; †, $P = 0.013$; ‡, $P = 0.002$; §, $P = 0.03$; ‖, $P = 0.0001$; and #, $P = 0.0001$.

$TCID_{50}$ of parental E70 virus. Solid protective immunity to E70 challenge was observed in all animals; animals remained clinically normal following challenge, with no fever or detectable viremia. These data indicate that the ASFV *NL-S* gene is a significant viral virulence factor in E70; deletion of this gene leads to almost complete attenuation of the virus in the domestic swine host.

DISCUSSION

These data indicate the ASFV *NL-S* gene is nonessential for growth in swine macrophages in vitro, and is a significant viral virulence factor. To our knowledge, this is the first ASFV gene directly associated with pig virulence.

Sequence analysis of pathogenic ASFV isolates demonstrates that the gene is highly conserved among diverse viral isolates and the respective gene products exist in either a long (184 amino acids as in 23-NL) or short form (70 to 72 amino acids). With the exception of the Malawi Lil 20/1 isolate, all examined viruses contain the short form of the gene, i.e., *NL-S*. Whether this observation, which is based on limited sampling, reflects the actual frequency of the two gene forms in nature remains to be determined. The *NL-S* gene does not appear to be a truncated form of the longer *23-NL* gene that resulted from a simple deletion event. The NL gene regions of Malawi and E70 are quite distinct from each other, each containing unique sequences (Fig. 1A, H-2 and U). The E70 genotype could have resulted from a partial NL gene deletion together with a subsequent insertion event, or less plausibly, the two forms of the gene may have been independent acquisitions from cellular sources. Interestingly, the *NL-S* gene encodes little more than the highly conserved 56-amino-acid carboxyl-terminal domain which is also encoded by the *23-NL*, *MyD116*, *GADD34*, *AmEPV*, and *ICP34.5* genes. This C-terminal domain of ICP34.5 is necessary for preventing the total shutoff of protein synthesis in HSV-infected neuroblastoma cells and other human cell lines (7, 8), and the corresponding C-terminal domain of MyD116 can functionally substitute for the ICP34.5 domain in HSV-infected cells (17). The results presented here with the E70 *NL-S* gene deletion mutant indicate this C-terminal domain alone functions in ASFV virulence, and, further, they suggest that the functional domain of the longer 23-NL protein is also this conserved carboxyl-terminal region.

Although highly conserved, the ASFV *NL-S* gene is nonessential for growth in pig macrophages or Vero cells in vitro. *NL-S* gene deletion mutants exhibit normal replication kinetics and virus yields in these two cell types (Fig. 5). This suggests a host range function for the gene in either the pig or tick host. Similarly, HSV *ICP34.5* performs a host range function in corneal and neuronal tissues; viruses lacking the gene replicate poorly in these tissues, presumably because of their inability to prevent early shutoff of host cell protein synthesis which occurs prior to progeny production (2, 7, 52). Given that macrophages are the major target cells for the virus in vivo (9, 29) and that deletion of *NL-S* from the viral genome markedly attenuates the virus, it is interesting that *NL-S* does not affect virus growth in primary pig macrophages in vitro.

The E70 *NL-S* gene deletion mutant E70/43 was markedly attenuated compared with either the parent E70 or the revertant E70/43R where the LD_{100} was $\leq$10 $TCID_{50}$ (Table 1). We have infected animals with approximately 10^6 $TCID_{50}$ of E70/43 without observing any evidence of clinical disease apart from a transient fever response (55). Thus, the *NL-S* gene deletion increases the LD_{100} of E70 by at least 100,000-fold. The approximately 1,000-fold reduction in viremia observed for E70/43-infected animals suggests that the mutant replicates less efficiently in infected animals and/or that generalization of infection is impaired.

Our observations using pig inoculation to select the revertant virus E70/43R from a transfection/infection mix containing high titers of the deletion mutant E70/43 suggest that *NL-S* gene-containing virus had a distinct growth advantage in vivo. Pigs inoculated with either 10^4 or 10^5 $TCID_{50}$ of the transfection/infection mix had a mixture of mutant (GUS+, blue plaques) and revertant viruses (GUS−, white plaques) present in peripheral blood at 6 DPI, but at 8 and 11 DPI, only revertant virus could be isolated (55). ASFV preferentially infects cells of the mononuclear-phagocyte system, including highly differentiated fixed-tissue macrophages, reticular cells, and possibly endothelial cells (9, 29). The NL-S host range function may involve allowing efficient replication of virus in these differentiated cell populations in vivo. Attenuation of the E70/43 deletion mutant may result from reduced viral replication allowing time for an effective immune response to be mounted, or more simply, it may reflect the inability of the mutant virus to replicate in critical target cells. In common with these data, HSV ICP34.5 performs a host range function in differentiated cells of the cornea and nervous system (2, 52).

While *NL-S* may be necessary for E70 viral virulence, the gene alone is not sufficient. The *NL-S* gene is present and transcribed in MS44, an avirulent cell culture-adapted variant of E70, and in another highly passaged avirulent ASFV isolate, BA71V (54) (Fig. 2. and data not shown). Thus, other viral determinants besides the *NL* gene must play significant roles in ASFV virulence.

Pigs previously infected with the E70/43 deletion mutant were resistant to ASFV infection when challenged with the parental virus E70. Identification and further characterization of the *NL-S* gene and other ASFV virulence and swine host range genes may allow for rational design of engineered live ASFV vaccines.

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