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A Conserved African Swine Fever Virus IκB Homolog, 5EL, Is Nonessential

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An African swine fever virus (ASFV) gene with similarity to the cellular inhibitor of NFκB (IκB) was described in the pathogenic African isolate Malawi Lil-20/1 (ORF 5EL) and a cell-culture-adapted European virus, BA71V (ORF A238L). Recently, this gene was shown to be a functional IκB homolog capable of downregulating NFκB-regulated gene expression. This observation suggests the gene may be of significance to aspects of ASFV pathogenesis and virulence in domestic swine by interfering with a normal antiviral host response. Here we show, using nucleotide sequence analysis, that *5EL* is highly conserved among various African and European pathogenic field isolates and that in all cases its similarity to IκB genes is limited to the presence of four low complexity ankyrin repeats in the ASFV gene. The *5EL* gene of Malawi Lil-20/1 encodes a 28-kDa protein which was expressed early in virus-infected macrophage cell cultures with maximum levels observed at 3 to 5 hr postinfection. To study gene function, a Malawi Lil-20/1 *5EL* gene deletion mutant (Δ5EL) was constructed. Growth characteristics of Δ5EL in porcine macrophage cell cultures were indistinguishable from those of the parental virus. And, Δ*5EL* exhibited an unaltered parental Malawi Lil-20/1 disease and virulence phenotype in domestic swine. Thus, although highly conserved among ASFV isolates, *5EL* is nonessential for growth in porcine macrophages *in vitro* and for viral virulence in domestic swine. A possible role for this gene in transmission of ASFV in nature, a setting which involves the cycling of ASFV between two highly adapted hosts, *Ornithodoros* ticks and warthogs or bush pigs, in sub-Saharan Africa is discussed.

INTRODUCTION

African swine fever virus (ASFV), the causative agent of ASF, is a large icosahedral virus with a linear double-stranded DNA genome of 170 to 190 kilobase pairs (kbp). Like poxviruses, the ASFV genome contains terminal crosslinks, inverted terminal repeats, a central conserved region, and variable regions at each end of the genome. Also, virus replication occurs in the cell cytoplasm and mature virus particles are thought to contain all the necessary enzymatic machinery for synthesis and processing of early mRNA (Costa, 1990; Gonzalez *et al.,* 1986; Kuznar *et al.,* 1980; Sogo *et al.,* 1984).

ASFV is a unique and complex DNA virus; it is the sole member of an unnamed family of animal viruses, and it is the only known DNA arbovirus (Brown, 1986; Costa, 1990; Dixon *et al.,* 1995). In nature, 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 (Plowright *et al.,* 1969; Thomson *et al.,* 1981; Wilkinson, 1989). An important aspect of this natural virus–host interaction is persistent infection, where virus persists in both ticks and wild pigs following infection (Carrillo *et al.,* 1994; DeKock *et al.,* 1994; DeTray, 1957; Sanchez-Botija, 1963; Thomson *et al.,* 1981).

The severity of ASF in pigs ranges from highly lethal to subclinical infections depending on contributing viral and host factors. The virus infects cells of the mononuclear-phagocytic system, including fixed tissue macrophages, and specific lineages of reticular cells in spleen, lymph node, lung, kidney, and liver (Colgrove *et al.,* 1969; Konno *et al.,* 1971; Mebus, 1988; Moulton and Coggins, 1968). The ability of ASFV to replicate and induce marked cytopathology in these cell types *in vivo* appears to be a critical factor in ASFV virulence. The nature of viral and host factors responsible for the differing outcomes of infection with highly virulent and strains of lesser virulence is unknown.

Other large DNA viruses such as pox-, herpes-, and adenoviruses encode nonessential, virulence-associated genes which play significant roles in downregulation of host cytokine-modulated immunological and inflammatory responses (Spriggs, 1994). Generally these viral genes are expressed early in infection and encode atypi-

Sequence data from this article was deposited with GenBank under the Accession Numbers AF014472 (Chiredzi/83/1), AF014473 (Crocodile/96/1), AF014474 (Crocodile/96/3), AF014475 (E70), AF014476 (E75), AF014477 (Haiti 811), AF014778 (Wildebeeslaagte/96/1), AF014779 (Malawi Lil-20/1), AF014780 (MS16), AF014781 (Pretoriuskop/96/5), AF014782 (Tengani), AF014783 (Uganda '61), AF014784 (Fairfield/96/1), and AF014485 (Lisbon '60).

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cal protein homologs of cellular cytokines, their receptors, or activator proteins. For example, Epstein–Barr virus encodes a homolog of the cellular cytokine IL-10, which downregulates INF-γ while stimulating the proliferation and antibody secretion from B cells (Ho and Moore, 1994; Hsu *et al.,* 1990). In some instances, viruses express soluble analogs of various cytokine receptors, such as the IL-1 β binding protein homolog encoded by vaccinia and cowpoxvirus (Smith and Chan, 1991; Spriggs *et al.,* 1992), the Shope fibroma virus-secreted type II TNF receptor (Hu *et al.,* 1994; Spriggs *et al.,* 1992), and the INF γ binding protein in myxoma virus (McFadden *et al.,* 1995). Viruses also encode cytokine receptors that are expressed on the membrane of infected cells as in the case of the capripox virus ORF Q2/3L, swinepox virus ORF K2R, and human cytomegalovirus ORF US28 (Cao *et al.,* 1995; Gao and Murphy, 1994; Massung *et al.,* 1993). And, viral-encoded inhibitors of cytokine activator proteins such as the cowpox virus CrmA protein, which inhibits interleukin-8 converting enzyme (ICE), have been described (Pickup, 1994).

Downregulation of proinflammatory cytokine gene expression (TNF-α and INF-α) has been observed in mitogen or phorbol myristic acid-activated porcine macrophages within 4 hr of ASFV infection (Powell *et al.,* 1996). Expression of these cytokines is in part regulated by the transcription factor, nuclear factor kappa beta (NFκB) protein complex.

An African swine fever virus gene with similarity to the inhibitor of NFκB (IκB), has been identified in the pathogenic African isolate Malawi Lil-20/1 and a cell-culture-adapted European virus BA71V (*A238L*) (Lu *et al.,* unpublished data; Yanez *et al.,* 1995). Expression of A238L in pig kidney cells (PK15) inhibited both the expression of an NFκB reporter gene and NFκB binding to DNA, indicating that *A238L* had functional IκB activity (Powell *et al.,* 1996). Since the NFκB protein complex functions as a pleiotrophic transcription factor which modulates a variety of genes in cells of the immune system (T cells, B cells, and monocytes/macrophages), including those encoding immunoreceptors, cell adhesion molecules, acute phase proteins, proinflammatory cytokines, hematopoetic growth factors, and nitric oxide synthase (Baeuerle and Henkel, 1994), a viral-encoded IκB could conceivably play a highly significant role in counteracting an antiviral response in the ASFV-infected swine host.

MATERIALS AND METHODS

Viruses and cell culture

African swine fever viruses used in this study were the following: pathogenic tick isolates — Chiredzi/83/1, Fairfield/96/1, Wildebeeslaagte/96/1, Pretoriuskop/96/5, Crocodile/96/1, Crocodile/96/3, Malawi Lil-20/1; pathogenic pig isolates — Tengani, Uganda'61, Lisbon'60, E75, E70, Haiti 811; and a nonpathogenic cell-culture-adapted virus, MS16.

Vero cell lines used were obtained from the American Type Culture Collection, propagated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and incubated at 37° with 5% CO_2. Primary porcine macrophage cell cultures were prepared from heparinized swine blood as previously described (Genovesi *et al.,* 1990; Neilan *et al.,* 1997).

DNA manipulation, PCR cloning, and DNA sequencing

Viral DNA was isolated from purified virions using Proteinase K and sodium dodecyl sulfate (SDS) lysis followed by phenol extraction and ethanol precipitation (Wesley and Tuthill, 1984). Southern blot, radiolabeling, and hybridization analyses were carried out using standard methods (Sambrook *et al.,* 1989). Plasmid DNA was prepared and manipulated essentially as described by Sambrook *et al.* (1989).

The *5EL* gene was amplified by PCR from purified virion DNA using a mixture of oligonucleotide primers based on the BA71V (Yanez *et al.,* 1995) and Malawi Lil-20/1 gene sequences (Lu *et al.,* unpublished data) (forward primers 5′-GAATTCCGGCGATGGATACGATAGGCCTGTTTCAG-3′ and 5′-GAATTCCGGCGATGGAACACATGTTTCCAG-3′; reverse primer, 5′-GAATTCGAGATTACTTTCCATACTTG-3′). PCR was performed for 40 cycles of thermal denaturation (90° for 30 sec), reannealing (45° for 30 sec), and extension (70° for 45 sec), and amplified products were cloned into the TA cloning vector pCR 2.1 (Invitrogen, San Diego, CA). Two independent PCR clones from each isolate were sequenced completely using M13 forward and reverse primers and internal primers based on the consensus sequences from the 12 isolates (forward sequence primer, 5′-GGAAACTCTGCTTTACATTAT-3′ and reverse sequence primer, 5′-GAGTCATCCCATTAAAATTCGG-3′). The nucleotide and predicted amino acid sequences were compared to each other using various alignment, similarity, and phylogenetic computer programs (Chao *et al.,* 1997; Devereux *et al.,* 1984; Galtier *et al.,* 1996; Takezaki *et al.,* 1995; Tamura and Nei, 1993).

5EL protein expression and immunoprecipitation

5EL was amplified from Malawi Lil-20/1 viral DNA with a gene-specific primer pair which introduced synthetic *Eco*RI sites at either end of the gene (forward primer 5′-GAATTCCGGCGATGGATACGATAGGCCTGTTTCAG-3′; reverse primer, 5′-GAATTCGAGATTACTTTCCATACTTG-3′), cloned into the pCR 2.1 cloning vector (Invitrogen) and the insert was completely sequenced for verification. A 672-base pair *Eco*RI fragment, encoding 224 amino acid residues from the amino terminus of the 5EL ORF, was cloned into the expression vector pGEX-2T (Pharmacia, Piscataway, NJ) yielding a glutathione *S*-trans-

ferase (GST)-5EL plasmid construct pGEX5EL. *Escherichia coli* BL3 pGEX5EL transformants were induced by the addition of isopropyl-β-D-thio-galactopyranoside (IPTG) (Stratagene, La Jolla, CA) to a 1 m*M* final concentration and the GST–5EL fusion protein was affinity purified on glutathione–Sepharose beads. New Zealand white rabbits were inoculated subcutaneously with 1 mg of the glutathione–Sepharose bound GST fusion protein in Freund's complete adjuvant and boosted five times, at 2-week intervals, with the fusion protein extract emulsified in incomplete Freund's adjuvant. 5EL immune serum specifically immunoprecipitated an L-[^{35}S]methionine-labeled *in vitro*-translated 5EL protein (data not shown).

Primary swine macrophages were infected with Malawi Lil-20/1 (m.o.i. = 10), pulse radiolabeled for 2-hr intervals at various times postinfection with L-[^{35}S]-methionine in methionine-deficient RPMI medium and immunoprecipitated with the 5EL monospecific antibody as previously described (Afonso *et al.,* 1992).

Construction of ASFV recombinant virus Δ5EL

Flanking DNA fragments mapping to the left (0.95 kbp) and right (1.25 kbp) of *5EL* were amplified by PCR using Malawi Lil-20/1 genomic DNA as a template. Primer sets, each of which introduced a *Bam*HI restriction site adjacent to *5EL* and *Bgl*II and *Kpn*I sites at the other end for the left and right flank, respectively, were left flank forward primer 5′-GCTTAAGTCTTGGCGCTAGATCTCC-3′; reverse primer 5′-GGATCCGGAAAGTAATCTCTTGATTATC-3′; right flank forward primer 5′-GGATCCTCGTGCTCTCACTC-3′ and reverse primer 5′-GGTACCGAGGGGGTAGAGGTACCAGAAC-3′. The fragments were digested with *Bam*HI/*Bgl*II or *Bam*HI/*Kpn*I and systematically cloned into pCR 2.1 (Invitrogen) to give pT5EL. A reporter gene cassette containing the *β-Glucuronidase* gene with the ASFV p72 late gene promoter, p72GUS (Neilan *et al.,* 1997), was inserted into *Bam*HI-digested pT5EL to yield the p72GUSΔ5EL transfer vector which removed the complete 5EL open reading frame. Macrophage cell cultures were infected with Malawi Lil-20/1 and transfected with p72GUSΔ5EL as described previously (Neilan *et al.,* 1997; Zsak *et al.,* 1996). Four recombinant viruses representing independent primary plaques were purified to homogeneity by plaque assay and verified as products of a double crossover recombination event.

Animal infections

Yorkshire pigs (30 to 35 kg) were inoculated intramuscularly with 10^2 $TCID_{50}$ of the parental Malawi Lil-20/1 virus or the *5EL* null mutant, Δ5EL. Clinical signs of ASF infection, i.e., fever (a rectal temperature greater than 40°), anorexia, lethargy, shivering, cyanosis, and recumbency, were monitored daily. Blood samples were collected every other day for the course of the experiment. Virus isolation and titration of ASFV in blood samples were performed as previously described (Onisk *et al.,* 1994). Virus titers were calculated using the method of Spearman–Karber and expressed as $TCID_{50}$ (Finney, 1984).

RESULTS AND DISCUSSION

5EL is highly conserved among ASFV isolates

To assess the degree of *5EL* gene conservation, the *5EL* gene was amplified from virion DNA purified from one nonpathogenic cell-culture-adapted virus (MS16), three European pathogenic isolates (E70, E75, Lisbon'60), one Caribbean pathogenic isolate (Haiti 811), two additional African pathogenic isolates from pigs (Uganda'61, Tengani), and six pathogenic South African field isolates from *Ornithodoros* ticks (Chiredzi/83/1, Fairfield/96/1, Wildebeeslaagte/96/1, Pretoriuskop/96/5, Crocodile/96/1, Crocodile/96/3) and then cloned and sequenced completely.

In contrast to Malawi Lil-20/1 ORF 5EL, which encoded a protein of 239 amino acids (predicted molecular mass of 28.2 kDa, PI 10.1), all other isolates encoded proteins of 238 amino acids (Fig. 1). Overall the Malawi Lil-20/1 isolate was 86% identical at the amino acid level when compared with the remaining isolates which were 97 to 100% identical when compared to each other with an 0.023 average change per residue. Most amino acid differences in Malawi Lil-20/1 occured at the amino terminus of the protein before residue 23, and approximately one-half were conservative substitutions (PAM250 cutoff = 0.5). Based on amino acid identity, four groupings of highly related proteins were apparent: (1) Fairfield/96/1 and Wildebeeslaagte/96/1; (2) Crocodile/96/1, Crocodile/96/3, and Pretoriuskop/96/5; (3) BA71V, E75, Haiti 811, Lisbon'60, MS16, E70, and Tengani; (4) Chiredzi/83/1 and Uganda'61.

All isolates contained four highly conserved low complexity ankyrin-like repeat motifs similar to the ankyin consensus motif [Nx3(D/N)x2Gx(T/S)(P/A)L(H/M)(L/W)A(A/V)x2G(H/N)x2(L/V)Vx2LLx(2,3)GAN] (Blank *et al.,* 1992) near the amino terminus (residues 41 to 77, 81 to 114, 118 to 150, and 153 to 183 in 5EL of Malawi Lil-20/1). And while the first ankyrin region of the ASFV protein was weakly similar to the ankyrin consensus pattern, the amino terminal region of the protein from residue 25 to 183 most closely resembled the amino terminus and first four low complexity ankyrin repeats in ankyrin containing proteins (Blank *et al.,* 1992; Wooton and Federman, 1993) such as those found in transcription factors (Peters and Lux, 1993) and their inhibitors which have four to seven ankyrin repeats (Bennett, 1992; Liou and Baltimore, 1993) and are less than 600 amino acid residues long, e.g., NFκB (Fasta score 132, 27% identity over 117 residues), swine IκB (Fasta score 120, 30% identity over 130 residues), GA binding transcription factor (Fasta score 123, 34% identity over 100 residues). The ASFV ankyrin repeat

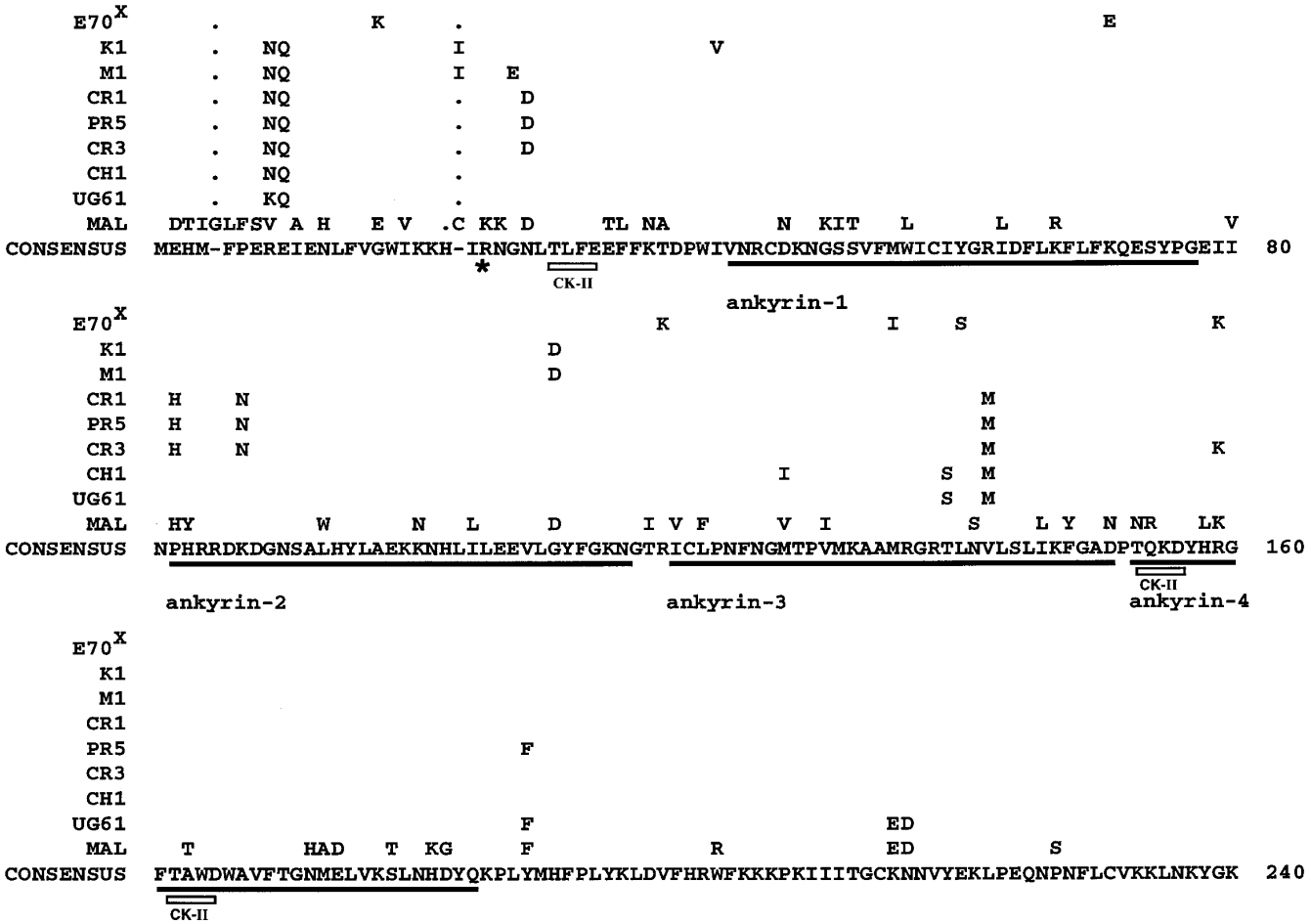

FIG. 1. Alignment of the predicted amino acid sequences encoded by *5EL* homologs in pathogenic ASFV isolates: Malawi Lil-20/1 (MAL), Uganda'61 (UG61), Chiredzi/83/1 (CH1), Crocodile/96/3 (CR3), Pretoriuskop/96/5 (PR5), Crocodile/96/1 (CR1), Wildebeeslaagte/96/1 (M1), Fairfield/96/1 (K1). The amino acid sequences of E70, E75, Haiti 811, MS16, Lisbon'60, Tengani, and BA71V (GenBank Accession No. U18466) were identical to each other and are represented here by $E70^{X}$. The consensus sequence was constructed with the Wisconsin GCG computer program Pileup (Devereux *et al.,* 1984) using the Dayhoff PAM-250 symbol comparison table with a 0.5 cutoff value. Differences in residues are shown above the consensus sequence. A period (.) denotes missing amino acids. Ankyrin repeat consensus sequences are underlined. An asterisk (*) denotes the amino terminal residue from which these genes show sequence similarity to the first ankyrin repeat region in other ankyrin containing proteins. Open boxes (□) highlight potential casein kinase II phosphorylation sites.

units also were similar to the membranous bound erythrocytic ankyrins which have up to 24 repeat units (Lux *et al.,* 1990). In contrast, the ASFV ankyrin-like repeat region was least similar to the ankyrin repeats of vaccinia virus (ORF B18R, B4R, C9L, M1L, host range 27-kDa protein), shope fibroma virus (ORF T5), fowlpox virus (ORF B7), and cowpox host range protein which also have a low number (one to five) of ankyrin-like repeats (Peters and Lux, 1993). There were no PEST regions (Rogers *et al.,* 1996) in the ASFV gene as are found in some other ankyrin containing proteins, e.g., porcine IκB or *Drosophila* cactus (DeMartin *et al.,* 1993; Geisler *et al.,* 1992). There were three potential casein kinase II phosphorylation sites at residues 30, 153, and 162 in the consensus sequence (Fig. 1); but there were no protein kinase C nor tyrosine phosphorylation sites as are present in other IκB proteins (DeMartin *et al.,* 1993). The hydrophilic carboxyl terminal region of the ASFV gene was highly conserved among ASFV isolates (4 conservative substitutions over 56 residues); however, no similarity of this region to any of the ankyrin containing proteins nor to any other protein in the current databases was observed.

At the nucleotide level the 5EL region of the Malawi Lil-20/1 isolate was 82% identical when compared with the remaining isolates which were 97 to 100% identical when compared to each other (Lisbon'60, MS16, BA71V and Haiti 811 were identical to each other). The *5EL* gene sequences contained 147 polymorphic nucleotide sites with 173 changes for a high 4.4 corrected average transition/transversion ratio and a low 0.016 average change per nucleotide position. While the Malawi Lil-20/1 isolate was most different from the other isolates at the *5EL* locus, there was no significant difference between isolates in the overall relationship at this locus (Kimura 2

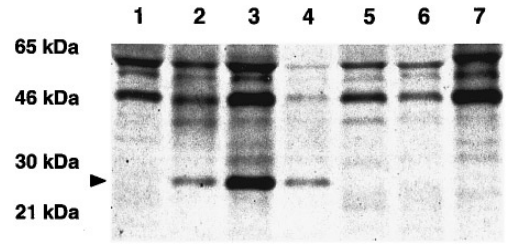

FIG. 2. Expression of p28 in ASFV-infected cell cultures. Cell extracts of mock-infected (lane 1) and Malawi Lil-20/1-infected swine macrophage cell cultures (lanes 2 to 7), pulse labeled with L-[^{35}S]methionine at 1 to 3 hr (lane 2), 3 to 5 hr (lane 1, 3 and 7), 5 to 7 hr (lane 4), 7 to 9 hr (lane 5), and 9 to 24 hr (lane 6) postinfection were immunoprecipitated with anti-5EL monospecific serum (lanes 1 to 6) or a preimmune serum (lane 7). Positions of the molecular mass markers are given on the left and the position of p28 is highlighted by the arrow.

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 = 10.36, 11 *df*). These data indicate that the *5EL* gene is highly conserved among a diverse group of pathogenic ASFV isolates, including both pig and tick field isolates. This suggests a significant function for it in some aspect of ASFV infection.

5EL encodes an early viral protein, p28

5EL protein expression in ASFV-infected macrophage cell cultures was examined by immunoprecipitation analysis using a monospecific antiserum prepared against a bacterial-5EL fusion protein. Primary macrophage cell cultures were pulse radiolabeled for 2-hr intervals at various times postinfection with L-[^{35}S]methionine in methionine-deficient RPMI medium and immunoprecipitated with the 5EL monospecific antibody. A specific protein with an apparent molecular mass of 28 kDa was first detected in ASFV-infected cell extracts labeled from 1 to 3 hr postinfection (hpi) (Fig. 2). The protein was expressed at maximum rate from 3 to 5 hpi, decreasing to undetectable levels by 7 hpi. Thus, Malawi Lil-20/1 *5EL* encodes an early protein, p28.

Construction and analysis of a recombinant ASFV Δ5EL gene deletion mutant

To examine gene function, a *5EL* gene deletion mutant of Malawi Lil-20/1 was constructed by homologous recombination between parental virus and an engineered recombination transfer vector. Four recombinant viruses representing independent primary plaques were purified to homogeneity by plaque assay and verified as products of a double crossover recombination event. Two of these viruses were selected for further analysis. Genomic DNA from parental virus and the Δ5EL viruses was digested with *Eco*RI, gel electrophoresed, Southern blotted, and hybridized with ^{32}P labeled DNA probes. As expected, a 5EL gene probe failed to hybridize with genomic DNA from Δ5EL viruses (Fig. 3, IIA, lanes 6 and 7). Novel *Eco*RI fragments of predicted size 5.0 and 3.6 kbp were observed for the Δ5EL isolates when hybridized with probes for the parental 4.34- and 2.55-kbp *Eco*RI fragments from this region (Fig. 3, IIB, lanes 1 to 3). PCR analysis failed to detect any contaminating parental virus in the DNA preparations from either of the two *5EL* deletion mutants (Fig. 3, III, lanes 1 and 2), and, as expected, p28 was not detected in Δ5EL-infected macrophages (Fig. 3, IV, lane 2). Together these data indicate that *5EL* deletion mutants were of the expected genomic structure and were free of contaminating parental virus.

5EL is nonessential for growth of ASFV in primary porcine macrophage cell cultures

Growth characteristics of Δ5EL were compared to those of Malawi Lil-20/1 by infecting primary macrophage cultures (m.o.i. = 1) in duplicate, and then titrating both intracellular and extracellular virus at various times postinfection. In two independent experiments, growth kinetics and viral yields of Δ5EL were statistically indistinguishable from those of parental virus Malawi Lil-20/1 (Fig. 4), indicating that 5EL did not affect viral replication in swine macrophage cell cultures.

5EL does not affect disease course nor viral virulence in domestic pigs

To examine the role of the *5EL* in viral virulence, Yorkshire pigs were inoculated intramuscularly with 10^2 $TCID_{50}$ of the parental virus Malawi Lil-20/1 and the *5EL* null mutant, Δ5EL. A 10^2 $TCID_{50}$ of Malawi Lil-20/1 represents a challenge dose of between 10 and 100 LD_{100} (Neilan *et al.,* 1997). Both groups of animals presented with clinical signs of ASF 4 days postinfection, and in all cases these signs progressed until death (Table 1). No differences in the onset or course of clinical disease, viremia, nor time to death were noted for the two virus groups. Thus, deletion of *5EL* from the Malawi Lil-20/1 genome did not affect disease course nor viral virulence in domestic pigs.

These data demonstrate that the ASFV IκB homolog *5EL* is highly conserved among pathogenic ASFV isolates and that its protein product p28 is expressed at early times in virus-infected macrophages. Early expression of p28 is consistent with an early host range function for the protein in the infected macrophage and, its expression is coincident with the previously described early suppression of NFκB-activated proinflammatory cytokines in ASFV-infected macrophages (Powell *et al.,* 1996).

Efficient replication of ASFV in cells of the mononuclear-phagocytic system, including fixed tissue macrophages and reticular cells appears to be a critical factor in ASFV virulence in domestic swine (Colgrove *et al.,* 1969; DeKock *et al.,* 1994; DeTray, 1957; Sanchez-Botija,

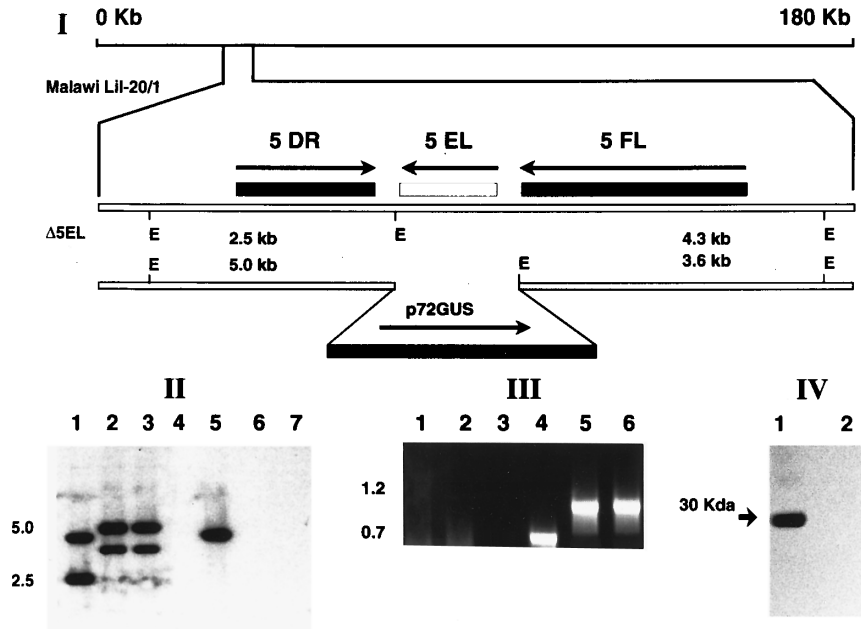

FIG. 3. Characterization of an ASFV *5EL* gene deletion mutant Δ5EL. (I) Diagram of 5EL gene region in the parental Malawi Lil-20/1 isolate and the deletion mutant virus, Δ5EL. (II) Southern blot analysis of parental virus (lanes 1 and 5) and two Δ5EL viruses (lanes 2, 3, 6, and 7). Purified viral DNA digested with *Eco*RI was electrophoresed, blotted, and hybridized with a *5EL* gene probe (lanes 5 to 7) or a probe including both *5EL* gene sequences and flanking genomic regions on either side (lanes 1 to 3). Molecular weight size markers are in kilobase pairs on the left. (III) PCR analysis of Malawi Lil-20/1 (lane 4) and two Δ5EL viruses (lanes 1 and 2) for *5EL* gene sequences. Positive control PCR reaction for the Δ5EL viral DNAs (lanes 5 and 6) using primers for a region of the genome immediately flanking the *5EL* gene. Lane 3 represents a nontemplate control. Molecular weight size markers are in kilobase pairs on the left. (IV) Immunoprecipitation analysis of lysates from macrophage cell cultures infected with Malawi Lil-20/1 (lane 1) and the null mutant, Δ5EL (lane 2), using monospecific anti-5EL serum. Molecular mass size markers are given on the left and p28 is highlighted by the arrow.

1963; Thomson *et al.,* 1981). Although highly conserved, *5EL* is clearly nonessential for virus replication in macrophage cell cultures *in vitro.* And, our pig virulence data indicate that it is nonessential for replication in these critical target cells types *in vivo.* Assuming there is no redundant viral function, these observations indicate that ASFV replication in macrophages does not require suppression of NFκB-activated gene expression.

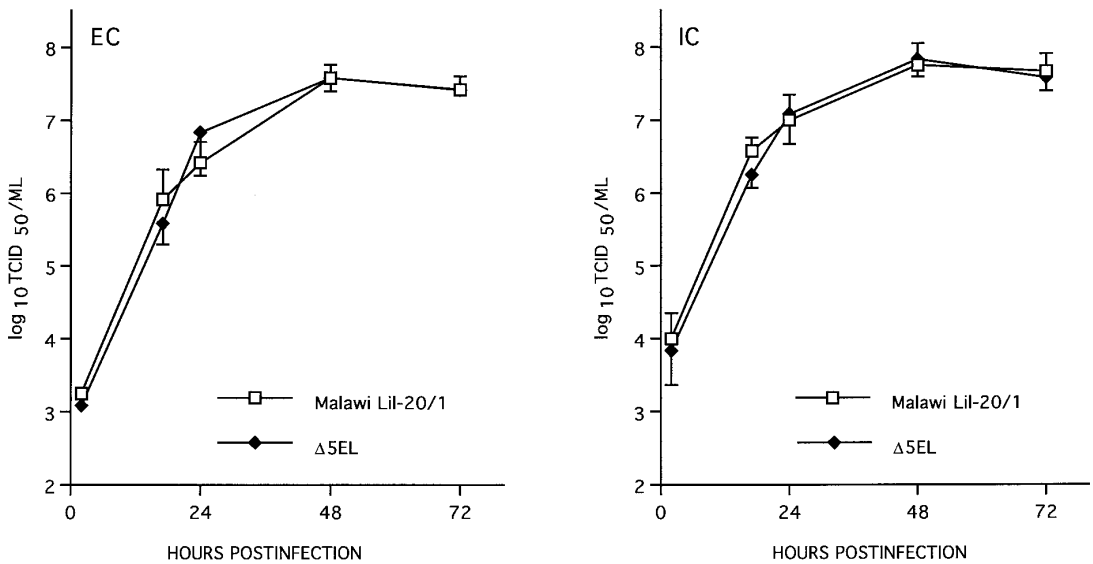

FIG. 4. Growth characteristics of ASFV Malawi Lil-20/1 and Δ5EL in swine macrophage cell cultures. Primary swine macrophages were infected with Malawi Lil-20/1 and Δ5EL (m.o.i. = 1). At indicated times, duplicate samples were collected and titrated for extracellular (EC) and intracellular (IC) virus yield. Titers are expressed as $\log_{10}TCID_{50}$/ml. Data represent means and standard errors of two independent experiments.

TABLE 1

Swine Survival, Viremia, and Fever Response Following Infection with Malawi Lil-20/1 and Δ5EL

Virus	Number surviving	Days to death	Fever	Viremia		
			Days to onset	Days to onset	Mean titer $log_{10}/TCID_{50}$/ml	Max. titer $log_{10}/TCID_{50}$/ml
Malawi Lil-20/1 ($N = 4$)	0/4	8 ± 0	4.3 ± 0.3	4	8.0 ± 0.1	8.5 ± 0.2
Δ 5EL ($N = 4$)	0/4	7.8 ± 0.3	4.0 ± 0	4	8.0 ± 0.1	8.8 ± 0.1

Acute ASF in domestic pigs is a rapid and highly lethal hemorrhagic disease with mortality rates approaching 100% (Mebus, 1988; Plowright *et al.,* 1994; Thomson *et al.,* 1981). Given the central role of NFκB in coordinately regulating the rapid expression of many genes encoding immunomodulatory proteins in monocytes and macrophages following activating stimuli (Baeuerle and Henkel, 1994) it was surprising that deletion of *5EL* from the Malawi Lil-20/1 genome had no affect on disease onset, disease course, or viral virulence. If significant downregulation of NFκB-activated gene expression, including those encoding proinflammatory cytokines, occurs in ASFV-infected animals, as it is proposed to occur in phorbol myristic acid-activated virus-infected macrophages *in vitro* (Powell *et al.,* 1996) it is inconsequential to acute disease and viral virulence.

5EL may, however, be of significance where virus and host are more adapted to one another, as occurs in the natural cycle of the virus. Here, the perpetuation of ASFV involves the cycling of virus between two highly adapted hosts, *Ornithodoros* ticks and warthogs/bushpigs, in sub-Saharan Africa (Plowright *et al.,* 1994; Thomson *et al.,* 1981). In the warthog host, acute ASFV infection is subclinical and characterized by low titered viremias (Plowright *et al.,* 1994; Thomson *et al.,* 1980). This high degree of virus–host adaptation may necessitate a viral immune evasion strategy that will ensure that sufficient levels of viral replication occur in the warthog and that resulting viremia levels will be high enough to infect new populations of feeding ticks. Given that *5EL* gene sequences have been selected for under these natural conditions and that the gene is highly conserved in field isolates, it is possible that the protein performs a host range function involving immune evasion in the warthog host. Additionally, although poorly understood, persistent ASFV infections in warthogs have been reported (Heuschele and Coggins, 1969; Plowright *et al.,* 1994). Conceivably, *5EL* could have a role either in regulating cellular or viral gene expression in the persistently infected monocyte/macrophage or by functioning in immune evasion.

Interestingly, an NFκB-IκB-like system for regulating gene expression has been described in arthropods (for review, see reference Baeuerle and Henkel, 1994 and Georgel *et al.,* 1986). Many insect immune response genes contain in their promoter regions sequences similar to the binding site for mammalian NFκB and these genes are thought to be transcriptionally regulated by proteins similar to NFκB-IκB (Engstrom *et al.,* 1993; Georgel *et al.,* 1986; Kapper *et al.,* 1993). In nature, ASFV persistently infects *Ornithodoros* ticks (Plowright *et al.,* 1969; Sanchez-Botija, 1963). Mechanisms regulating this long-term persistent infection in the tick are unknown. It is tempting to speculate that the ASFV IκB homolog *5EL* may play a role in the tick host, involved in either regulating viral replication in persistently infected tick tissues or perhaps in interfering with possible immune-like host responses to virus infection.

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