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An African Swine Fever Virus Gene with Similarity to the Proto-Oncogene *bcl-2* and the Epstein-Barr Virus Gene *BHRF1*

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An open reading frame, *LMW5-HL*, in the African swine fever virus genome displays a high degree of similarity to the proto-oncogene *bcl-2* and, to a lesser degree, the Epstein-Barr virus gene *BHRF1*. A highly conserved central region is found in all three proteins. *LMW5-HL* encodes a protein of 18 kDa that is present in infected porcine macrophages at both early and late times postinfection. The similarity of *LMW5-HL* to *bcl-2* and *BHRF1* suggests a role for it in cell maintenance during productive or persistent viral infection.

African swine fever virus (ASFV) is a large icosahedral DNA virus that shares many structural and biochemical properties with poxviruses. Its genome, a linear double-stranded DNA of 170 to 190 kbp, contains terminal inverted repeats and hairpin loops. Viral replication occurs in the cytoplasm of infected cells, and mature virus particles are thought to contain all the necessary enzymatic machinery for synthesis and processing of early mRNA (for reviews, see references 8 and 45). ASFV is the sole member of an unnamed family of animal viruses.

ASFV infects soft ticks of the *Ornithodoros* genus and members of the Suidae family, including domestic pigs, warthogs, and bush pigs (8, 12, 32, 33, 45). It is the causative agent of African swine fever, a highly significant disease of domestic swine. African swine fever occurs in several disease forms ranging from highly lethal to subclinical infections, depending on contributing viral and host factors. ASFV infects cells of the mononuclear phagocytic system, including fixed tissue macrophages and specific lineages of reticular cells in spleen, lymph node, lung, and liver tissues. The abilities of ASFV to replicate and induce marked cytopathology in these cell types in vivo appear to be critical factors in ASFV virulence (7, 19, 20, 26, 27).

Long-term persistent infection with ASFV occurs in a high percentage of pigs surviving acute infection (4a, 10, 11). Persistently infected pigs remain clinically normal, with rare episodes of transient low-level viremia (11).

ASFV DNA, but not infectious virus, has been detected in peripheral blood monocyte/macrophage cell populations from infected animals at more than 500 days postinfection (p.i.) (4a). The nature of the virus-cell interactions during persistent ASFV infection is unknown.

Here we describe an ASFV gene, *LMW5-HL*, that possesses significant similarity to the proto-oncogene *bcl-2* and the Epstein-Barr virus (EBV) gene *BHRF1*. The similarity of these three genes suggests a role for the ASFV gene in suppression of apoptosis in ASFV-infected cells, thus aiding the survival of the host cell during productive and/or persistent infection.

An 11.2-kbp DNA fragment, located between 22.4 and 29.1 map units, beginning and totally contained within *Sal*I fragment c of the highly virulent ASFV strain Malawi Lil-20/1, was isolated from the recombinant lambda clone LMW5 (13). The DNA fragment was randomly subcloned (3) and sequenced by the dideoxy-chain termination method (34) with an ABI 370A automated DNA sequencer. Sequences were assembled and potential open reading frames (ORFs) were located by using the computer programs of Staden (38).

LMW5-HL is an ORF of 537 bp that begins at position 7440 and ends at position 6914 on the negative strand of the 11.2-kbp DNA fragment (Fig. 1). The codon usage bias of the *LMW5-HL* ORF coincides with the codon frequency over the central region ($P = 0.99$) and at the extremities ($P = 0.75$) for known ASFV genes (4, 16, 23, 24), indicating that *LMW5-HL* may encode a protein (39). The AT-rich nucleotide sequence immediately upstream of the proposed ATG start site falls within the allowed upstream context for both ASFV and vaccinia virus early promoters (2, 9).

The predicted protein encoded by *LMW5-HL* is 179 amino acids in length and has an isoelectric point of 6.8 and a predicted molecular mass of 20 kDa. A potential signal peptide cleavage site was found at the Gly residue at position 18 (46). Comparative analysis of the amino acid sequence with the Prosite data base (release 9) revealed two potential casein kinase phosphorylation sites (47) at residues 28 and 44 and one protein kinase C phosphorylation site (31) at residue 94. A region of LMW5-HL encompassing residues 8 to 52, inclusive, was found to be similar (31% identity in a 45-amino-acid overlap) to a highly conserved sequence present in the catalytic subunit of phosphoprotein phosphatases. This sequence lies adjacent to, but does not include, the highly conserved signature sequence for these phosphatases (6).

FASTA (30) searching of GenPep (release 73.2), Swissprot (release 23.1), and PIR (release 34) data bases revealed that *LMW5-HL* exhibited a remarkable degree of similarity to the alpha and beta forms of the proto-oncogene *bcl-2* (14, 28, 43). LMW5-HL is shorter than either form of Bcl-2, and they all share a central core region which is most highly conserved (Fig. 2). In the protein products, this central core of 93 amino acids, beginning at residue 46 in LMW5-HL and at residue 112 in Bcl-2, contains 31 exact matches and 21 conservative substitutions. Comparison of the entire protein sequence of LMW5-HL with that of Bcl-2 reveals a 26% identity and 47% conservation of amino acids (on the basis of

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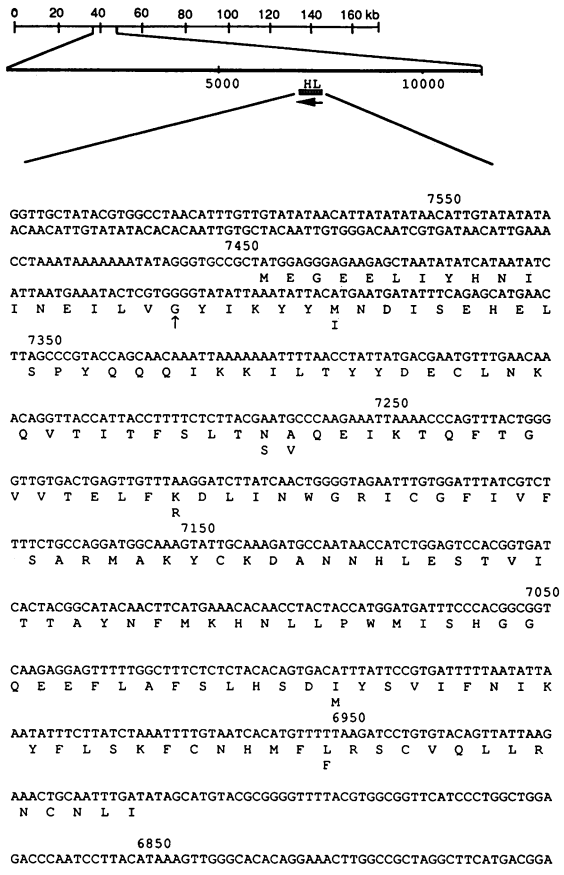

FIG. 1. The *LMW5-HL* locus, depicted by the rectangle labelled HL, on the ASFV genome. The horizontal arrow shows the orientation of the coding strand. Numbering is consistent with the location of the gene within *Sal*I fragment c of *LMW5* (13). The nucleotide sequence of the ASFV ORF *LMW5-HL* is shown, and the deduced amino acid sequences of both the Malawi Lil-20/1 and the MS44 isolates are shown in one-letter code beneath the nucleotide sequence. The substituted amino acid residues in the MS44 isolate are shown below the Lil-20/1 amino acid sequence; all other residues are conserved. A putative signal cleavage site at Gly-18 is indicated by a vertical arrow.

the Dayhoff Pam-250 symbol comparison table [36] with a 0.5 cutoff). Analysis of the observed homology yielded an optimized FASTA score of 152 (a z score of 16.6).

LMW5-HL, like *bcl-2*, has significant homology to the EBV early gene *BHRF1* (5). When the three sequences are aligned (Fig. 2), the central regions of the proteins exhibit the highest level of conservation, with a 20% identity and 40% conservation of amino acid sequence. LMW5-HL and BHRF1 share a hydrophobic region at the amino terminus which contains a potential signal sequence cleavage site. A similar hydrophobic region is not evident in Bcl-2. A putative hydrophobic transmembrane domain, present at the carboxy terminus of BHRF1 and Bcl-2, is absent from LMW5-HL.

The *LMW5-HL* gene from MS44, an avirulent derivative of ASFV strain E70 (42), was amplified by polymerase chain reaction, cloned into KS^+ Bluescript, and then sequenced. The Malawi Lil-20/1 and MS44 genes were found to be highly conserved (96.5% at the DNA sequence level, resulting in a 96.5% amino acid identity). All nonidentical amino acids were found to be conservative substitutions (Fig. 1).

Monospecific antibodies to the *LMW5-HL* protein product were prepared. The entire *LMW5-HL* ORF from the Malawi strain was amplified by polymerase chain reaction and cloned into the commercially available (Novagen) T7 expression vector pET 21b (41). Protein extracts of bacterial cell lysates from a clone expressing the *LMW5-HL* ORF were prepared and used to immunize rabbits (33). Radioimmunoprecipitation experiments using this immune serum were undertaken to analyze LMW5-HL protein expression in ASFV-infected macrophages. LMW5-HL-immune serum specifically recognized a single protein of approximately 18 kDa in ASFV-infected macrophage cell extracts (Fig. 3). This protein was detected in infected macrophages at 3 to 5 h p.i. with increasing levels present at later time points in the infection cycle, and it was synthesized in the presence of cytosine arabinoside, a drug which inhibits DNA replication and late protein synthesis (15) (data not shown). Like the EBV *BHRF1* gene, *LMW5-HL* is expressed early in virus-infected cells. Notably, the immunoprecipitated LMW5-HL protein has an observed size of approximately 18 kDa, while the predicted molecular mass is 20 kDa. A similar size discrepancy exists with BHRF1, which has a predicted

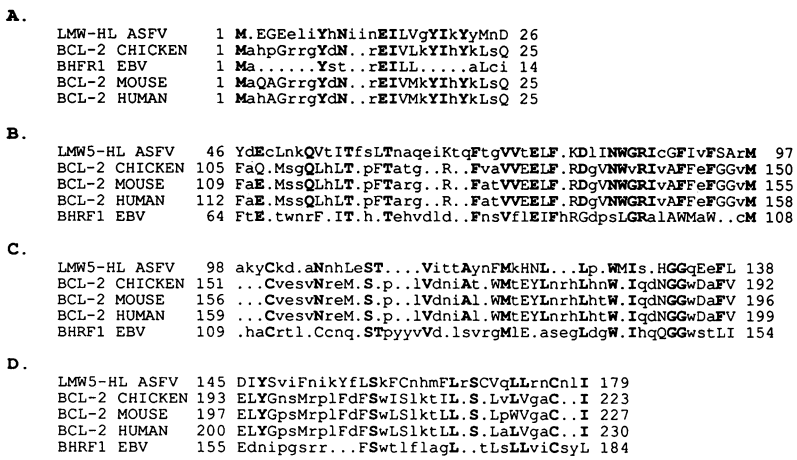

FIG. 2. Conserved regions of LMW5-HL, chicken Bcl-2 (14), mouse Bcl-2 (28), human Bcl-2 (43), and the EBV BHRF1 (29) proteins are shown. Residues identical to those in LMW5-HL are shown in uppercase boldface letters, while conservative substitutions are shown in uppercase lightface letters. The numbering of LMW5-HL is as outlined in Fig. 1. The other proteins are numbered as in the references in which they are described. (A) Amino terminus; (B and C) central core region; (D) carboxy terminus.

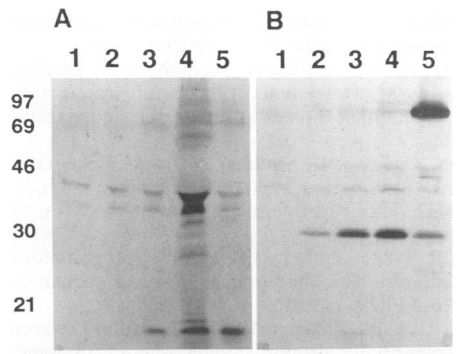

FIG. 3. Expression of *LMW5-HL* in ASFV-infected swine macrophages. Immunoprecipitations were performed at various times p.i. as previously described (1) by using [^{35}S]methionine-labelled primary swine macrophage cultures infected with the virulent ASFV strain Malawi Lil-20/1 (multiplicity of infection, 20). (A) Immunoprecipitations of infected cells with rabbit serum raised against the recombinant LMW5-HL fusion protein at various times p.i. Note the 18- to 19-kDa product precipitated specifically by the antiserum raised against the *LMW5-HL* gene product. (B) Immunoprecipitations with a mixture of two rabbit sera raised against the early ASFV protein p30 and the late ASFV protein p72 at various times p.i. Lane 1, mock-infected cells; lanes 2 through 5, infected cells at 1 to 3, 3 to 5, 5 to 7, and 7 to 9 h p.i., respectively. Molecular size markers (in kilodaltons) are indicated on the left.

molecular mass of 22 kDa and an observed size of 17 kDa (29). Thus, both of these proteins may be processed in some fashion in virus-infected cells.

The *bcl-2* gene has been implicated in the development of follicular lymphoma in humans (5, 43). Expression of *bcl-2* enhances cellular survival in the absence of cytokines by suppressing apoptotic cell death (18, 21, 44), and it is responsible for maintenance of resting B cells and mature T cells (35, 37, 40). The mode of action of *bcl-2* in the suppression of apoptosis is unknown.

The BHRF1 protein is abundantly expressed early in the lytic replication cycle and is also transiently expressed in some latently infected lymphoid cell lines (25). In vitro analysis with *BHRF1* deletion mutants of EBV has failed to elucidate the role of *BHRF1* in EBV infection (25). However, it has been demonstrated that activation of EBV latent genes protects human B cells from apoptotic cell death by inducing the expression of the cellular *bcl-2* gene (17). It has been proposed that *BHRF1* may substitute for *bcl-2* in cells undergoing productive infection, when virus-induced shutdown of host cellular genes may result in *bcl-2* deficiency (22).

In common with the BHRF1 protein, the LMW5-HL protein is expressed in myeloid cells at early times following viral infection (Fig. 3). Although the function of this gene in ASFV infection is unknown, it is tempting to speculate that its role may involve preventing early apoptotic cell death in virus-infected cells, thus allowing productive viral replication to occur. Additionally, this gene may have a role in persistent ASFV infection; it may allow long-term survival of persistently infected monocytic or lymphoid cells.

Nucleotide sequence accession number. The GenBank accession number for *LMW5-HL* (Fig. 1) is L09548.

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