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An African swine fever virus gene with homology to DNA ligases

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ABSTRACT

Sequence analysis of the *SalI* g region of the genome of a virulent isolate of ASFV (Malawi Lil 20/1) has revealed an open reading frame with the potential to encode a 48 kilodalton (kD) polypeptide which has significant homology with eukaryotic and prokaryotic DNA ligases. This ASFV encoded gene also contains the putative active site region of DNA ligases including the lysine residue which is necessary for enzyme-adenylate adduct formation, but lacks the C-terminal basic region conserved in other eukaryotic DNA ligases. A novel [³²P]-labelled potential DNA ligase-adenylate adduct of M_r 45 kD was observed upon incubation of ASFV infected cell cytoplasmic extracts with α -[³²P]-ATP and subsequent analysis of products by SDS/PAGE. These data together suggest that ASFV encodes its own DNA ligase.

INTRODUCTION

African swine fever virus (ASFV) is a large icosahedral deoxyribonucleic acid containing virus which replicates in the cytoplasm of infected cells [for reviews see (1–3)] and is classified as the only member of a separate virus family (4). The pattern of ASFV gene expression is temporally regulated and early, early/late and late classes of induced proteins are synthesised in infected cells (5,6). In common with the poxviruses, a cytoplasmic site of viral replication necessitates that ASFV encode many of the enzymes required for mRNA transcription and DNA replication. Some of these enzymes are packaged into ASFV particles, such as a DNA topoisomerase (7) and those required for early gene expression such as a DNA-dependent RNA polymerase (8) and mRNA capping, methylation and polyadenylating enzymes (9). Other enzyme activities are induced in ASFV infected cells (3). The genes encoding thymidine kinase (10), both the large and small subunits of ribonucleotide reductase (11) and of a ubiquitin conjugating enzyme (12), have so far been identified on the ASFV genome.

The sequence of vaccinia virus DNA ligase has been reported (13) and is the only mammalian virus-encoded DNA ligase identified until now. It was predicted that ASFV should also encode its own DNA ligase because of its cytoplasmic site of replication, and similar genome structure and mode of replication to poxviruses. DNA ligases join single strand breaks in double stranded DNA by catalyzing the formation of phosphodiester bonds and require a divalent metal ion and either ATP or NAD⁺ as co-factor. They play a major role in joining Okazaki fragments produced during lagging strand DNA synthesis, sealing nicks in repaired DNA and completing recombination events by joining 'crossed over' DNA strands (14,15).

This report describes the identification and primary structure of an African swine fever virus gene encoding a 419 amino acid polypeptide of predicted molecular weight 48 kD with amino acid sequence homology to DNA ligases. In addition, the gene is shown to encode a M_r 45 kD protein which forms a [³²P]-labelled adduct, an intermediate in the DNA ligase reaction mechanism, after incubation with α -[³²P]-ATP. A similar protein is present in cytoplasmic extracts of ASFV infected cells.

MATERIALS AND METHODS

Nucleotide sequencing

A 4.2 kb DNA fragment from within the *SalI* g fragment of the Malawi Lil 20/1 strain of ASFV was isolated from *SalI/BamHI* restricted bacteriophage lambda clone LMw16 (16) (fig 1). This fragment was inserted into the bluescript SK⁺ plasmid (Stratagene) and digested with exonuclease III and SI nuclease to obtain a nested set of deletions for nucleotide sequence analysis (17). Double stranded DNA was sequenced using the M13 reverse primer (18). The second strand was sequenced using 15-mer oligonucleotide primers determined from the first strand sequence, spaced approximately 200 nucleotides apart. The nucleotide sequence data were assembled into contiguous sequences using the programmes DBAUTO and DBUTIL (19).

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The deduced amino acid sequence of the encoded open reading frame (ORF) was compared with the SWISSPROT protein database and multiple protein sequences aligned using the programme Gap (20).

Assay for the formation of DNA ligase-AMP adduct in ASFV-infected cells

Porcine kidney RSII cells were infected with a tissue-culture adapted Ugandan isolate of ASFV at a multiplicity of infection of 10. Cells were harvested at various times post infection and extracts labelled with α -[32 P]-ATP (21,22). DNA ligase-AMP adduct formation was visualised by 12.5% SDS polyacrylamide gel electrophoresis (SDS/PAGE) of products followed by autoradiography of the dried gels.

Cloning of the ASFV gene in an expression vector

The coding region of the putative ASFV DNA ligase gene was amplified by the polymerase chain reaction (PCR) and inserted into KS⁺ bluescript (Stratagene) to enable expression of the gene under the control of the T7 RNA polymerase promoter. Oligonucleotide primers used in the PCR were 5' TTTCTG-CAGGGATCCTCATGCTAAGTCAATTTCCT 3' and 5' TTTAAAGCTTGAATTCTTAAATAATTCTAAAACGT 3' and included a *Pst*I restriction site upstream of the ATG start site and a *Hind*III site downstream of the stop codon (sites are underlined). The template DNA used in PCR reactions was the plasmid subclone used for sequencing and reactions were carried out for 25 cycles of 2 minutes at 92°C, 2 minutes at 45°C and 3 minutes at 72°C. After amplification the PCR product was digested with *Pst*I and *Hind*III and inserted into *Pst*I/*Hind*III restricted KS⁺. The resultant plasmid is referred to as KS⁺lig.

Expression of the putative ASFV DNA ligase gene in *E. coli*

E. coli BL21 pLysS (23) were transformed with KS⁺lig and expression of the putative ASFV DNA ligase gene induced by the addition of 2mM isopropylthio-B-galactosidase (IPTG). Bacterial extracts were prepared in ligase extraction buffer (21) by sonication and assayed for the formation of a DNA ligase-AMP adduct as above.

RESULTS

Sequence analysis of the ASFV encoded DNA ligase

A 4.2 kb *Sal*I/*Bam*HI fragment was excised from bacteriophage lambda clone LMw16 (16), which contains sequences from the *Sal*I g fragment of ASFV isolate Malawi Lil 20/1 (fig 1a and 1b), and inserted into bluescript SK⁺ for sequence analysis. A contiguous DNA sequence of 4,138 nucleotides was obtained. Translation of this sequence revealed one complete ORF flanked by two incomplete ORF's (fig 1c). The complete ORF covers 1257 nucleotides from position 1778 to 3034 on the *Sal*I/*Bam*HI fragment and encodes a potential polypeptide of 419 amino acids with a predicted molecular weight of 48 kD (SalIg 48 kD ORF). Computer database searches against the SWISSPROT protein library indicated that the 419 amino acid polypeptide shared significant homology with vaccinia virus DNA ligase. Using the programme FASTA (24) with a ktup value of one, an optimised score of 146 was obtained with 20.9% identity in a 292 amino acid overlap.

Table 1 shows a comparison of the predicted molecular weight and percentage identity of the putative ASFV DNA ligase with other DNA ligases which utilise ATP as co-factor. There is great

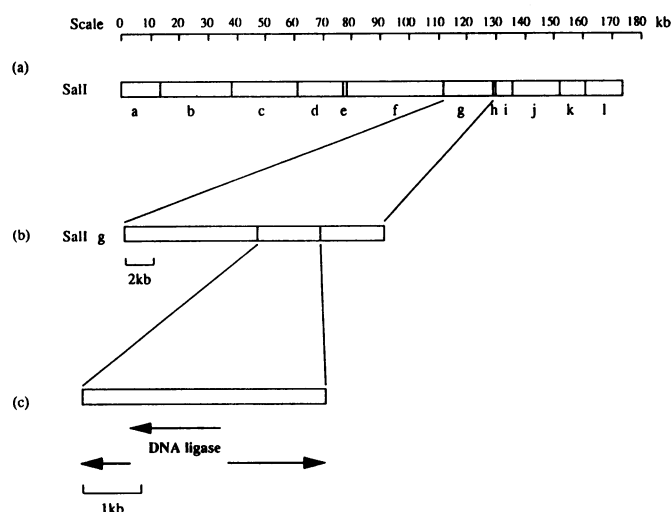


Figure 1. Genome location of ASFV encoded DNA ligase. (a) The *Sal*I map of the complete genome of the ASFV Malawi Lil 20/1 isolate. (b) The *Sal*I g fragment is expanded and the position of the 4.2 kb fragment excised from clone LMw16 (16) is indicated. (c) This 4.2 kb fragment is expanded and the position and direction of ORF's encoded on this fragment are indicated by arrows.

variation in size between the DNA ligases attributed to the highly variable length of the amino terminal region upstream of the active site (25). In contrast the carboxy termini appear well conserved in length and sequence. The bacteriophage T3 enzyme comprises 346 amino acids and has a predicted molecular weight of 39 kD (26) whereas the human DNA ligase I is a 102 kD polypeptide comprising 919 amino acids (27). Due to the highly variable nature of the N-terminal regions of the DNA ligases, comparisons were carried out using both full length sequences and sequences extending towards the C-terminus from the predicted active site lysine. Comparisons over the complete amino acid sequence indicated that the SalIg 48 kD ORF had between 20% (bacteriophage T7) and 25% (bacteriophages T3, 4 and 6) (26,28–30) amino acid identity with all DNA ligases examined (data not shown). The putative ASFV DNA ligase was closest in size and had highest homology with bacteriophage T4 and T6 DNA ligases. A comparison of the known DNA ligase active site (25) and the probable corresponding lysine residue of other DNA ligases, demonstrated that the ASFV polypeptide contains a conserved potential active site region (table 2). The lysine residue predicted to be involved in enzyme-adenylate adduct formation is at position 151. If this lysine is the reactive residue, then its distance from the carboxy terminus, 268 amino acids (table 1), is 44 amino acids shorter than the previously reported conserved terminal region of 332 (+/- 20) residues for all other eukaryotic DNA ligases (25). Including the lysine at position 151, 3 of the next 6 amino acids are identical to those in all eukaryotic DNA ligases at their active sites, with a conservative change (aspartic acid for an asparagine) at a fourth residue 153. Alignment of sequences from the presumed active sites to the carboxy termini showed that this was the most conserved portion of the enzyme and in this region bacteriophage T4 DNA ligase has the highest percentage amino acid identity (32%) when compared with the ASFV DNA ligase (table 1). Figure 2 shows the complete amino acid sequence of the putative ASFV DNA ligase and its alignment from the predicted active site lysine with

Table 1. Comparison of amino acid sequences of DNA ligases.

(a) DNA LIGASE	(b) No. of amino acids	(c) M.WT kD	(d) Position of predicted active site lysine from N-terminus	(e) Distance from lysine to C-terminus	(f) % similarity from lysine to C-terminus	(g) % identity from lysine to C-terminus
ASFV	419	48	151	268		
PT3	346	39.3	34	312	47	26
PT7	359	41.1	34	325	47	21
PT6	487	55.3	159	328	50	25
PT4	487	55.3	159	328	58	32
VV	552	63	231	321	48	22
S.CER	755	84.8	419	336	52	25
HUMAN	919	101.7	568	351	47	21

In panel (a) the source of the DNA ligase gene is indicated: ASFV, bacteriophage T3, T4, T6 and T7 (PT3 to PT7 respectively), vaccinia virus (VV), *Saccharomyces cerevisiae* (S. cer) and human DNA ligase I (Human) (13,26–31). The number of amino acids in each sequence (b), and the predicted molecular weight of encoded proteins (c) are shown. The predicted position of the active site lysine (d) and the number of amino acids between this lysine and the carboxy terminus (e) are indicated. The percentage of similar (f) and identical (g) amino acids are shown after alignment of each DNA ligase sequence with the ASFV DNA ligase from the active site lysine to the C-terminus.

bacteriophage T4 and vaccinia virus DNA ligases. The vaccinia virus enzyme has 22% identical amino acids compared with the putative ASFV DNA ligase. Identical residues are present along the complete length of this region but are fewer at the extreme carboxy termini. In addition, the conserved 16 amino acid motif shared by vaccinia virus, yeast, and human DNA ligase I enzymes adjacent to their carboxy termini is not present in ASFV DNA ligase (13,27,31,32).

Assay for DNA ligase-AMP adduct formation

DNA ligases catalyze phosphodiester bond formation at single strand breaks in double stranded DNA. The initial stage of the joining reaction is the formation of a covalently linked enzyme-adenylate intermediate with the simultaneous release of pyrophosphate. The nucleotide derivative required for this reaction has been shown to be either ATP for eukaryotic and bacteriophage enzymes or NAD⁺ for bacterial enzymes (14). Formation of this enzyme-adenylate intermediate can be monitored by addition of α -[³²P]-ATP to reactions followed by analysis of protein products by SDS/PAGE and detection by autoradiography. ASFV infected cell extracts were assayed for proteins forming covalent interactions with [³²P]-AMP. A unique radio-labelled polypeptide of M_r 45 kD was observed after SDS/PAGE of ASFV-infected cell extracts prepared late during infection were incubated with α -[³²P]-ATP (fig 3a). No such product was observed in mock-infected cell extracts. The predicted molecular weight from the DNA ligase gene sequence (48 kD) is similar to the size (M_r 45 kD) by SDS/PAGE of the novel α -[³²P]-ATP radio-labelled polypeptide. Formation of a covalent protein-AMP adduct is not a common reaction (22) and observation of such a radio-labelled polypeptide is indicative of the presence of a DNA ligase enzyme. Further evidence that this α -[³²P]-ATP radio-labelled polypeptide is a DNA ligase-AMP adduct was provided by addition of nicked DNA to the reaction mixture. This resulted in loss of radioactivity from the polypeptide as expected, due to the formation of a DNA-AMP intermediate (data not shown). Our results also indicate that ATP rather than NAD⁺ is used as co-factor, as might be predicted for the ASFV polypeptide from the conserved active site region described above. Direct evidence for the formation of an AMP-adduct with the product of the ASFV DNA ligase gene was obtained by transformation of *E. coli* BL21 pLysS with a plasmid containing

Table 2. Amino acid sequence comparison at the active sites of DNA ligases.

ASFV	V	H	G	K	R	N	G	V	R	A	V	A	C
HUMAN	C	E	Y	K	Y	D	G	Q	R	A	Q	I	H
S.POMBE	C	E	Y	K	Y	D	G	E	R	A	Q	V	H
S.CEREVISIAE	S	E	Y	K	Y	D	G	E	R	A	Q	V	H
PT3	A	D	C	K	Y	D	G	V	R	G	N	I	V
PT4	A	Q	L	K	A	D	G	A	R	C	F	A	E
PT6	A	Q	L	K	A	D	G	A	R	C	F	A	E
PT7	A	E	I	K	Y	D	G	V	R	G	N	I	C
VV	A	E	V	K	Y	D	G	E	R	V	Q	V	H

The amino acid sequences surrounding the active site lysine of DNA ligases from ASFV, human DNA ligase I (Human), *S. pombe*, *S. cerevisiae*, bacteriophage T3, T4, T6 and T7 (PT3 to PT7) and vaccinia virus (VV) are shown (13,26–32). Amino acids that are identical in all eukaryotic DNA ligases are shown in bold.

the putative ASFV DNA ligase gene (KS⁺lig) and incubation of bacterial extracts with α -[³²P]-ATP (fig 3b). A unique radio-labelled polypeptide of M_r 45 kD was detected in bacterial extracts following transformation with KS⁺lig. No such product was detected in extracts prepared from bacteria transformed with plasmid KS⁺ lacking the ASFV DNA insert. This M_r 45 kD polypeptide migrated with similar mobility to the α -[³²P]-ATP labelled DNA ligase-AMP product observed in ASFV-infected cell extracts.

DISCUSSION

We have sequenced and identified an ASFV gene encoding a polypeptide of 419 amino acids with a predicted molecular weight of 48 kD which has homology with DNA ligases.

A potential active site region containing a lysine residue predicted to be involved in enzyme-adenylate adduct formation is present in the ASFV polypeptide. Amino acid comparisons across this region show that 3 out of 6 residues are identical and that there is a conservative change of a fourth residue in the ASFV DNA ligase compared with other DNA ligases (table 2). The 419 amino acid polypeptide encoded by ASFV shares approximately 20% (bacteriophage T7) to 25% identity (bacteriophages T3, 4 and 6) (26,28–30) over the complete predicted amino acid sequence compared with other DNA ligases. The amino terminal regions of all DNA ligases are diverse and vary greatly in length and sequence. Identity increases to a

ASF	MLSQFPGQCSNNVFCFPIESETKNGKASWIIICVQMQHNTIL
ASF	PITDEMFTDVKDAVAEIFTKFFVEGAVRISKTRVTEGKNLGGKKNATTVVH
ASF	QAFKDALSRYNRHARQKRGHTNRGMIPMLVKYFNIIPKTFEEETDPIVHG
ASF	KRNGVRAVA CQQGD..GSI LLYSRTEKEF LGLD.NIKKE LKQLY....L
Pt4	KadGaRcfA evrGDeiddv rLlSRagnEy LGLDl.lKeE Likmtaearq
Vac	KyGGeRvqv hknenn...ef affSRnmKpv LshkvdyLKEYipkaf
ASF	FIDVRVYLDG ELYLE...RKPLQWI AGQANAKA..
Pt4	ihpegVliDG ELVYHeqvKk epegldflfd AypeNsKake faevaesrta
Vac	kkatsivLds EivLvdehn.vPLpfg slgihhK...
ASFDSSE LHFYVFDC..FWSQDL QM...PSNKR
Pt4	sngiankslk gtisekeaqc mkFqVwDyvp lveiyLpaf rlykdvrfek
VackeyknSn mclfvFDC..lyfDgf dMtdiPlyeR
ASF	QQLLTNIFKQ KEDLTFIHQV ENFSVKNED EALRLKTFIK EGYEGAIVRN
Pt4	legmTsgy...dkviii... ENqvVnNlDE AkviykkyId qGLEGIllkN
Vac	rsfLkdvme ipnrivfseL tNis..NESq ltdvldalt rklEGlrvld
ASF	ANGPYEPG...YNNYHSPHL AKLKPLLDAAE FILVD..YTQ GKKGKDLGAI
Pt4	idqlwE....WarSkNl yKfKevIdvd lkiVg.iYph rKdptkaGqf
Vac	iNgvYEPGkr rwlkikrdyL negsmadaAd lrvlgayYgk GaKGgimavf
ASF	LWVC..ELPN KKRfV.....VTP KHLTYADRYA LFQKLTPALF
Pt4	ilesecgkik vnagsglkdk agvksheldr trimengnY. yigKilecec
Vac	LmgCyddesg KwktVtkcsg hddntlrVlq dqlTmvlkink dphKkipewLv
ASF	KKHLYGKELT VEYA.....ELSPKGTGIPL ...QARAVGF R.....
Pt4	ngwLksdgrT .dYv.....kLflpiaIrL redtkkAntF
Vac	vnkiYipdfv VEdpkqsqiW EISgaefteS kshTAngisi Rfrprfrire
ASFEPINV LEII
Pt4edvfgd fhvvtgl
Vac	dktwkEsthl ndlvnltsa

Figure 2. Amino acid comparison of ASFV DNA ligase with bacteriophage T4 and vaccinia virus DNA ligases aligned downstream from their predicted active site lysine residues. The sequences downstream from the active site of the ASF, T4 and VV DNA ligases were aligned using the programme GAP (20). The complete amino acid sequence of ASFV DNA ligase is shown in upper case. Amino acid residues identical between the ASFV sequence and one or both of the other DNA ligase sequences are highlighted in bold upper case (13,29). The conserved active site region leave the alignment is underlined. The 16 amino acid peptide conserved at the carboxy termini of vaccinia virus, yeast and human DNA ligase I (13,27,31,32) is also underlined.

maximum of 32% with bacteriophage T4 DNA ligase when just the carboxy terminal amino acids downstream from the putative active site lysine are compared (table 1). The conservation of both the sequence at the carboxy termini of eukaryotic DNA ligases and the distance between this conserved carboxy terminal region and the active site lysine suggest that these features play an important role in the function of the enzyme (25). Although the level of identity between the ASFV and other known DNA ligases is higher in the carboxy terminal region downstream from the active site lysine residue, the carboxy terminus of the putative ASFV DNA ligase is at least 44 amino acids shorter than the other DNA ligases in this region (table 1) and does not contain the highly conserved 16 amino acid sequence present in vaccinia virus, yeast and human DNA ligase I enzymes (figure 2) (13,27,31,32). This region is immunologically cross-reactive between vaccinia and mammalian cell DNA ligases (33). The shorter length of the putative ASFV DNA ligase carboxy terminal region compared with other DNA ligases may indicate differences in enzyme function, specificity in DNA binding or in targeting of the enzyme within cells. In particular, cellular DNA ligases function in the nucleus, whereas the ASFV DNA ligase probably functions in the cytoplasm and therefore may not contain a nuclear targeting signal. However, the C-terminal conserved basic region is not sufficient to dictate nuclear localization in vaccinia virus-infected cells since the vaccinia DNA ligase which contains this region is located in cytoplasmic virus factories. Perhaps when removed from the context of a virus infection the vaccinia enzyme becomes partially nuclear and this might explain the ability of vaccinia virus DNA ligase to complement a yeast mutant (33).

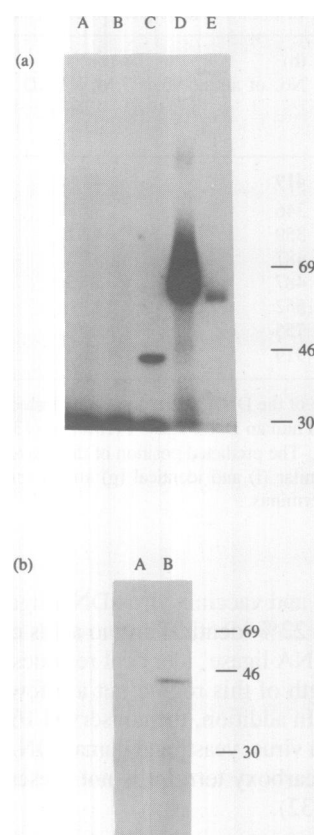


Figure 3. (a) ASFV DNA ligase-AMP adduct formation in the cytoplasm of ASFV infected cells. Cytoplasmic extracts were prepared from ASFV-infected cells at various times post infection (21). Extracts were incubated with α -[32 P]-ATP (22) and labelled products detected by SDS/PAGE and autoradiography of dried gels. Lane (a) uninfected cell extract, lane (b) ASFV infected cell extract 4 hours post infection, lane (c) ASFV infected cell extract 16 hours post infection. Lane (d) T4 DNA ligase and lane (e) vaccinia virus infected cell extract. The positions of molecular weight markers are shown in kD. (b) Expression of ASFV DNA ligase in *E. coli* BL21 pLysS. *E. coli* BL21 pLysS (23) were transformed with bluescript KS⁺ or KS⁺lig DNA containing the ASFV DNA ligase gene under the control of the T7 RNA polymerase promoter. Expression of the ASFV gene was induced by the addition of 2mM IPTG to cultures. Bacterial extracts were prepared by sonication, incubated with α -[32 P]-ATP (22) and labelled products detected by SDS/PAGE and autoradiography of dried gels. Lane (a) *E. coli* BL21 pLysS transformed with KS⁺ and lane (b) *E. coli* BL21 pLysS transformed with KS⁺lig DNA. The positions of molecular weight markers are shown in kD.

The ASFV DNA ligase is the smallest eukaryotic DNA ligase yet identified and it remains possible that it may be more highly homologous to the as yet uncloned mammalian DNA ligases II and III (34).

ASFV DNA ligase [32 P]-AMP-adduct formation is detectable late in infection, although it is not yet known when transcription of this ASFV gene occurs. The vaccinia DNA ligase enzyme initiation codon forms part of a late transcriptional initiation sequence (13), however, the gene is transcribed early and the enzyme appears to accumulate at late times, implying stability of the protein. The nucleotide sequence upstream of the ASFV DNA ligase gene start codon does not contain obvious homology with the well documented vaccinia virus early or late promoter regions (35,36) although it has been suggested that some ASFV promoters may be similar to those of poxviruses (37). However, two regions containing a high proportion of A-residues are present

upstream of the ASFV DNA ligase gene at positions -3 to -7 and -30 to -36 with respect to the ATG. It is possible that one or both of these regions may be analogous to a critical vaccinia virus early promoter region thought to be required for the binding of a transcription factor (35). No homology with either the eukaryotic polyadenylation signal (AATAAA) or the vaccinia virus early termination signal (TTTTTNT) (38,39) is apparent within the nucleotide sequence adjacent to the stop codon of the ASFV ligase gene.

The conservation of the active site lysine and surrounding sequences and the identification of a Mr 45 kD protein with AMP-binding activity in cytoplasmic extracts of ASFV infected cells is consistent with the DNA ligase being functional. The presence of an ASFV encoded DNA ligase was predicted (13) since ASFV replicates in the cytoplasm of infected cells. This enzyme may be involved in the replication, repair or recombination of viral DNA. Vaccinia virus DNA ligase is not essential for viral replication or recombination *in vitro* (40,41), although it does play an important role *in vivo* (33), and DNA ligase negative virus has an increased sensitivity to DNA damaging agents *in vitro*.

Very little is known about the mechanism of ASFV DNA replication, which occurs in the cytoplasm approximately mid-way through the infection cycle. The genome structure of ASFV and poxviruses is similar and consists of linear double stranded DNA closed by terminal hairpin loops which are present in two forms that are inverted and complementary with respect to each other. Head to head concatameric forms of DNA are also found in both ASFV and vaccinia virus infected cells (42,43), and thus, it is probable that the mechanism of ASFV DNA replication is similar to that utilised by the poxviruses. Two basic models have been proposed for poxviral DNA replication (reviewed in 44). The simplest model comprises unidirectional synthesis after a nick is introduced at one or both ends of the genome in the terminal hairpin(s) followed by self-priming at the free 3' ends. This mechanism does not require a DNA ligase, as a nicking-joining enzyme (45) is capable of creating and sealing the necessary breaks in the DNA. A second model suggests leading and lagging strand synthesis. After the introduction of nick(s) and self-priming at the hairpin termini, DNA synthesis would proceed along both DNA strands. Okazaki-like fragments formed on the lagging strand would then require joining by a DNA ligase. Since vaccinia virus DNA ligase is not essential for virus replication in tissue culture cells, the first of these mechanisms is more likely, unless the vaccinia enzyme is complemented by cellular DNA ligase I (33), or by the immunologically unrelated DNA ligase II or III (34).

Genetic recombination between the genomes of orthopoxviruses is well documented (46-49) and genetic recombination between capripoxviruses during natural transmission has been suggested (50). Recombination between ASF viruses has also been demonstrated *in vitro* (51), and therefore, it is possible that poxvirus and ASFV DNA ligases augment the generation of recombinant viruses in the field. A function for these DNA ligases in either DNA recombination or repair could denote an instrumental role in viral evolution.

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