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Repair of Viral Genomes by Base Excision Pathways: African Swine Fever Virus as a Paradigm

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1. Introduction

African swine fever virus (ASFV) is an enveloped deoxyvirus that infects suids and causes a fatal disease in domestic pigs. ASFV also propagates in ticks of the genus *Ornithodoros*, being the only known DNA arbovirus. Because of its unique features, ASFV is the sole member of the *Asfaviridae* family (Salas 1999; Dixon and Chapman 2008), although comparative genome analyses suggest that ASFV shares a common origin with the members of the proposed nucleocytoplasmic large DNA viruses (NCLDVs), along with poxviruses, iridoviruses and mimivirus, among others (Iyer et al. 2001; Iyer et al. 2006).

The disease, African swine fever (ASF), was reported for the first time in Kenya in the 1920s, as an acute hemorrhagic syndrome of domestic pigs (Montgomery 1921). The infection spread outside Africa to the Iberian Peninsula, initially to Portugal in 1957 and 1960, and subsequently to Spain and several other countries in Europe and Latin America. The virus has been eradicated from all of these regions, apart from sub-Saharan Africa countries and the Mediterranean island Sardinia, where the disease remains enzootic (Gómez-Tejedor Ortíz 1993). In 2007, a new transcontinental spread of ASF occurred with the introduction of ASF to Georgia in the Caucasus region (Beltrán-Alcrudo et al. 2008; Chapman et al. 2008; Rowlands et al. 2008), followed by widespread distribution to neighboring countries, including Armenia, Azerbaijan and several territories in Russia. Currently there is no vaccine available for ASF and the disease is controlled only by animal quarantine and slaughter. Therefore, ASF has potentially devastating effects on the commercial and subsistence pig production sectors, particularly in developing countries (Costard et al. 2009).

The virus particle has an overall icosahedral shape and an average diameter of 200 nm. The ASFV genome is a double-stranded DNA molecule of 170 to 190 kbp, structured in a central constant region of about 125 kbp and two variable regions at the ends (Blasco et al. 1989). The two strands are covalently closed, at both ends, by a 37 nucleotide-long hairpin loops, followed by a perfect terminal inverted repeat (TIR). A comparison of restriction maps of different ASFV isolates has shown that the two variable regions show deletion or additions

up to 8.6 kbp and contain five multigene families that comprise different number of members in different isolates (Blasco et al. 1989). Several complete sequences of the ASFV genome have been published, showing that it encodes more than 150 polypeptides, including a variety of enzymes involved in gene transcription and DNA replication and also in DNA repair (Yáñez et al. 1995; Chapman et al. 2008; de Villiers et al. 2010) (Table 1). The replication cycle occurs mainly in the cytoplasm of the infected cell, but an initial phase of viral DNA replication in the nucleus has been described (García-Beato et al. 1992). Analysis of ASFV replicating DNA molecules has shown the synthesis of DNA fragments of small size in the nucleus and the existence of head-to-head linked molecules that may be replicative intermediates and full length genome molecules in the cytoplasm (Rojo et al. 1999), confined to a specific area termed as viral factory. These factories contain also high amounts of viral structural proteins and ER-derived membranous material needed for particle assembly (Rouiller et al. 1998). A reducing environment in the virus factory is critical for the particle assembly (Cobbold et al. 2007). However, the virus codes for a sulfhydryl oxidase, which might be involved in the formation of the disulfide bonds found in viral proteins (Rodríguez et al. 2006).

The maintenance of genomic integrity is essential not only for the survival of cellular organisms but also viruses. Endogenous aerobic metabolism and a variety of exogenous factors generate reactive oxygen species (ROS), which can damage macromolecules including lipids, proteins and nucleic acids. Macrophages and other immune cells, including monocytes and neutrophils, where ASFV replication mainly occurs (Fernández et al. 1992), have been reported to produce ROS in response to viral infection (Klebanoff and Coombs 1992; Suzuki et al. 1997). Therefore, viral genomes may undergo a highly oxidative stress during its replication, which could generate lesions such as oxidized bases and single-strand breaks bearing 3′-blocking termini in the viral DNA.

Base Excision Repair systems (BER, see Figure 2) are the main pathways that surgically locate and remove damaged bases from DNA and are ubiquitous in Archaea, Bacteria and Eukarya. In the classical BER pathway, a DNA glycosylase recognizes and excises the damaged base. A uracil DNA glycosylase (UNG) or other monofunctional DNA glycosylase liberate the damaged base (typically uracil) and leaves an apurinic/apyrimidinic (AP) site in the DNA. Subsequently, AP endonuclease cleaves the sugar-phosphate backbone at the 5′-side of the AP site resulting in 3′-OH and 5′- deoxyribose phosphate (dRP) groups at the margins of a single nucleotide gap in DNA (Hegde et al. 2008). DNA polymerase β (pol β) inserts a nucleotide into the gap and removes the 5′-dRP group through its associated lyase activity, resulting in nicked DNA that will be sealed by a DNA ligase (Robertson et al. 2009). This sub-pathway is designated as short-patch or single-nucleotide base excision repair (SN-BER). However, if the 5′-sugar group is oxidized or reduced it is not recognized by the pol β dRP lyase and the DNA ligase cannot seal the nick. In this case, the repair occurs through an alternate long patch base excision repair (LP-BER) sub-pathway, involving removal of several nucleotides by a 5′→3′ exonuclease or a flap endonuclease activity prior to their replacement by a DNA polymerase (Sung and Demple 2006; Robertson et al. 2009).

A second group of DNA glycosylases, the bifunctional glycosylases also incise the AP site after the base removal, generating a single-stranded DNA break with 3′-sugar phosphate groups that must be removed prior to the gap-filling synthesis step. This cleansing step can be performed either by an AP endonuclease (for 3′-phosphate or 3′-phosphoaldehyde moieties) or by a polynucleotide kinase (only for 3′-phosphate group) (Hegde et al. 2008). The majority of oxidized DNA bases are removed in the BER pathway initiated by

redundant bifunctional DNA glycosylases (Fromme et al. 2004; Zharkov 2008). However, certain types of oxidative DNA damage such as the alpha-anomeric 2′-deoxynucleosides (αdA, αdT and αdC) cannot be repaired by DNA glycosylases but rather by the AP endonucleases in the alternative nucleotide incision repair (NIR) pathway (Ischenko and Saparbaev 2002; Ishchenko et al. 2006). NIR is a DNA glycosylase-independent conserved BER mechanism that is initiated by an AP endonuclease that makes an incision 5′ next to a damaged base, providing a proper 3′-OH group for DNA polymerization and a 5′-dangling damaged nucleotide. Oxidatively damaged pyrimidines including 5,6-dihydrothymine (DHT), 5,6-dihydrouracil (DHU), 5-hydroxyuracil (5OHU) and 5-hydroxycytosine (5OHC) are substrates for both BER and NIR pathways suggesting that the latter pathway can serve as a back-up system to counteract oxidative stress (Couvé-Privat et al. 2007).

Protein function	ORF(s) name(s)	Ref.
DNA polymerase α	G1207R	(Rodríguez et al. 1993)
Thymidine kinase	K196R	(Blasco et al. 1990)
Thymidylate kinase	A240L	(Yáñez et al. 1993)
Ribonucleotide reductase	F778R and F334L	(Boursnell et al. 1991)
DNA primase	C962R	(Yáñez et al. 1995)
DNA helicase	D1133L, Q706L, A859L, B962L, F1055L, QP509L	(Yáñez et al. 1993; Yáñez et al. 1995)
DNA ligase	NP419L	(Yáñez and Viñuela 1993; Lamarche et al. 2005)
dUTPase	E165L	(Oliveros et al. 1999)
DNA polymerase X	O174L	(Yáñez et al. 1995; Oliveros et al. 1997)
AP endonuclease	E296R	(Lamarche and Tsai 2006; Redrejo-Rodríguez et al. 2006)
5′→3′ exonuclease	D345L	(Iyer et al. 2001; de Villiers et al. 2010)
ERCC4-type endonuclease	EP364R	(Yáñez et al. 1995)
PCNA-like	E301R	(Yáñez et al. 1995)

Table 1. ASFV genes involved in DNA replication and repair.

Many DNA viruses, like herpesvirus, poxvirus or mimivirus encode one or more DNA glycosylases that may initiate a putative viral BER pathway (Caradonna et al. 1987; Upton et al. 1993; Raoult et al. 2004). However, only mimivirus, entomopoxvirus (the poxvirus subgroup that infects insects) and the recently described *Cafeteria roenbergensis* virus (CroV), contain ORFs that may code for reparative pol β-like DNA polymerase or AP endonuclease proteins (Afonso et al. 1999; Raoult et al. 2004; Fischer et al. 2010). On the contrary, the ASFV BER system includes a pol X family DNA polymerase, a class II AP endonuclease, a DNA ligase and other factors (Table 1), but lacks a DNA glycosylase homolog. This chapter aims to review the properties of ASFV BER pathway elements, and to provide new data to further characterize the viral BER mechanism(s). The major host cell preference of ASFV for macrophages and other immune cells constitute an important hallmark in ASFV replication cycle environment and might result in a specific variety of oxidative DNA damage, which

may explain the differences with other viruses. The role of DNA repair mechanisms in the viral replication, pathogenesis and evolution of ASFV and other viruses is also discussed.

2. African swine fever virus DNA repair

2.1 DNA damage prevention: dUTPase avoids uridine misincorporation into the viral DNA

dUTPases are enzymes that catalyze the conversion of dUTP to dUMP and PPi. This activity is critical to cell survival because excess dUTP is incorporated into DNA, leading to futile excision repair cycles, DNA breakage, and death. Therefore, dUTPases function is not a DNA repair mechanism itself, but a prophylactic strategy. It is highly conserved in biological kingdoms and has been shown to be essential for DNA replication and consequently for survival (revised in McClure 2001).

Recombinant purified ASFV dUTPase (pE165L, Table 1) is a trimeric enzyme, highly specific for dUTP and with an elevated affinity for its substrate (K_m= 1 μM). The protein is expressed at early and late times of infection and is localized in the cytoplasm of the infected cells, which is consistent with a role in maintaining a high dTTP/dUTP ratio to minimize the introduction of uracil into the viral DNA during the whole replication process (Oliveros et al. 1999). A recombinant virus with a deletion of the dUTPase gene was generated in the Vero cell adapted BA71V ASFV strain (vΔE165R). This mutant virus was successfully purified from cultured Vero cells and further analysis demonstrated that it replicates with the same kinetics and to the same extent than the parental virus. However, the growth of vΔE165R virus was strongly impaired in cultured porcine macrophages, the main target in natural ASFV infections (Oliveros et al. 1999). The differences in virus replication observed between these two cell types could be due to the levels of cellular dUTPase. The differentiated macrophages are quiescent cells, thus they may have very low levels of host cell dUTPase activity, revealing the required biological role of the viral protein, as found for other viruses (Baldo and McClure 1999). Moreover, as already mentioned, sequence analyses have not identified any protein with clear similarity to UNG that might repair incorporated or cytosine deamination-generated uracil bases. Therefore, a proficient dUTPase activity might be especially important to prevent the introduction of deoxyuridine during the replication of the large ASFV genome.

2.2 An early step in base excision repair catalyzed by AP Endonuclease

As previously indicated, the enzymatic activity that cleaves the sugar-phosphate bond in the BER pathways is named AP endonuclease and it generates 3′-OH and 5′-dRP ends. In human cells, AP sites are processed by APE1, whereas in yeast the primary AP endonuclease is termed APN1. These enzymes are the major constitutively expressed AP endonucleases in these organisms and are homologous to the *Escherichia coli* enzymes Exonuclease III (Xth) and Endonuclease IV (Nfo) respectively, which represent the two conserved archetypes of AP endonuclease enzymes.

ASFV protein pE296R is an Nfo-like AP endonuclease, named after the viral gene E296R. It is expressed since early times during the infection and progressively accumulates at later times. The early enzyme is localized in the nucleus and the cytoplasm, while the late protein is detected only in the cytoplasm, supporting a role in BER of viral genomes. The blockage of viral DNA replication results in the accumulation of pE296R in the cell nucleus,

suggesting a function during the nuclear stage of DNA replication, more likely in DNA repair (Redrejo-Rodríguez et al. 2006).

Purified recombinant pE296R protein contains AP endonuclease and 3′→5′ exonuclease activities (Lamarche and Tsai 2006; Redrejo-Rodríguez et al. 2006), as well as 3′-phosphodiesterase, 3′-phosphatase and weak NIR activities against 5ohC and dihydropyrimidines (Lamarche and Tsai 2006; Redrejo-Rodríguez et al. 2009). The 3′→5′ exonuclease activity of pE296R is more efficient against 3′-mismatched substrates (Redrejo-Rodríguez et al. 2006), 3′-damaged nucleotides and pyrimidines over purines (Redrejo-Rodríguez et al. 2009). Strikingly, all DNA repair functions of pE296R protein (AP endonucleolytic, 3′→5′ exonuclease, 3′-diesterase and nucleotide incision repair (NIR) activities) as well as its DNA binding capacity are reversibly inhibited by reducing agents. Furthermore, cysteine residues alkylation experiments showed the presence of bound cysteines in the recombinant protein (Redrejo-Rodríguez et al. 2009). The results suggest that the native protein has one disulfide bond and that the break-up of this cysteine-cysteine bridge by reducing agents may lead to the loss of DNA binding and enzymatic activities of pE296R. Although the in vivo significance of these observations is not well known at present, we propose that the presence of a disulfide bond in the viral AP endonuclease may provide a mechanism for regulation of the enzyme activity in the infected cells by inducing or breaking this bond. In relation to this possibility, it is interesting to mention again that ASFV codes for a sulfhydryl oxidase (Rodríguez et al. 2006), which may be involved in the formation of the disulfide bond.

The biological role of protein pE296R has been studied using two different strategies. A first approach was based in complementation assays of an AP endonuclease deficient *E. coli* xth nfo strain exposed to various genotoxic agents by the expression of the pE296R protein. This is a well-characterized model for the study of the genetic requirements to counteract specific DNA damages that can be repaired by BER mechanisms (Cunningham et al. 1986; Ishchenko et al. 2006). Methylmethanesulfonate (MMS) is an agent that induces alkylation of DNA bases, which can be removed by means of BER, requiring an AP endonuclease activity (Weinberger and Sperling 1986). Among the oxidizing agents, H_2O_2 is produced by the host immune cells infected with certain viruses and therefore might generate oxidative lesions in the ASFV DNA (Israel and Gougerot-Pocidalo 1997; Suzuki et al. 1997). Expression of pE296R protein in the mutant bacteria strain conferred resistance against MMS and H_2O_2 (Lamarche and Tsai 2006; Redrejo-Rodríguez et al. 2009), which strongly suggests that the viral AP endonuclease can repair 3′-blocking groups, 3′-oxidized bases and AP sites *in vivo*. Importantly, the protection against H_2O_2 and MMS provided by both pE296R and Nfo endonucleases is very similar (Redrejo-Rodríguez et al. 2009), suggesting highly efficient properties of the viral AP endonuclease to neutralize DNA damage. Nfo protein NIR activity has been shown to be involved in the repair of oxidizing damage that is produced in the presence of *tert*-butylhydroperoxyde (*t*-BuO_2H) (Ishchenko et al. 2006). Expression of pE296R is also able to complement AP endonucleases in *E. coli* against *t*-BuO_2H-induced DNA damage (Redrejo-Rodríguez et al. 2009). Thereby, although *in vitro* the ASFV AP endonuclease-catalyzed NIR activity is much weaker compared to other DNA repair functions, a role of pE296R in the repair of oxidative DNA base lesions via the DNA glycosylase-independent NIR pathway *in vivo* can be suggested.

A virus mutant lacking the E296R gene allowed additional characterization of the biological role of protein pE296R in the context of the infected macrophage. The viral endonuclease is required for virus growth in swine cultured macrophages but not in Vero cells, supporting

the existence of a viral reparative system to maintain virus viability in macrophages, the ASFV major host cell. Furthermore, the presence of H_2O_2, *t*-BuO_2H and MMS during the infection in Vero cells decreased viral production in a dose-dependent manner (Redrejo-Rodríguez et al. 2009). This corroborates the role of ASFV AP endonuclease in the repair of AP sites and DNA strand breaks in the viral genome, and suggests the involvement of a viral NIR pathway in the maintenance of genome integrity *in vivo*.

2.3 A minimalist but proficient DNA polymerase X

ASFV gene O174L codes for a highly distributive X-family DNA polymerase, named pol X, that is the smallest naturally occurring DNA-dependent DNA polymerase. Sequence alignment shows that this small protein (20 kDa) contains most of the conserved critical residues involved in DNA binding, nucleotide binding, and catalysis of the polymerization reaction, but lacks the N-terminal 8-kDa domain of pol β that contains the dRP lyase active site. Therefore, ASFV pol X most likely represents the minimal functional version of an evolutionarily conserved pol β-type DNA polymerase core, constituted by only the "palm" and "thumb" subdomains (Oliveros et al. 1997; Showalter et al. 2001).

Pol X is able to efficiently repair single-nucleotide gapped DNA substrates, which is consistent with its participation in a BER process during ASFV infection (Oliveros et al. 1997; Showalter and Tsai 2001; García-Escudero et al. 2003). In agreement with sequence analysis predictions, the recombinant purified enzyme lacks the 5′-deoxyribose phosphate (dRP) lyase activity characteristic of cellular pol β that eliminates the 5′-dRP blocking group generated during the BER process by the action of the AP endonuclease on the abasic site in the DNA. However, pol X, as well as pol β, exhibits lyase activity on unincised AP sites. Taking this into account, the existence of an alternative viral short patch BER pathway has been proposed (García-Escudero et al. 2003) in which the AP lyase activity of pol X would act on AP sites in the viral DNA. Following this, the 3′-phosphodiesterase and 3′-phosphatase activities of the pE296R (Lamarche and Tsai 2006; Redrejo-Rodríguez et al. 2009) protein would excise the 3′-terminal unsaturated aldehyde, allowing the pol X to fill the gap. Nevertheless, a long patch pathway or the existence of a dRP lyase activity in another viral protein that would excise the dRP moiety should be considered.

Pol X binds to single and double stranded DNA (ssDNA and dsDNA). The total site-size of the pol X-ssDNA complex is 16 ± 2 nucleotides, surprisingly large for such a small protein (Jezewska et al. 2007). Regarding BER intermediates, the enzyme forms two different complexes with gapped DNAs, with dramatically different affinities. The high affinity complex is formed preferably with 1-2 nucleotide gaps and engages the total DNA binding site, while in the low affinity complex the enzyme binds to the dsDNA parts of the gapped DNA, using only one of the DNA-binding subdomains (Jezewska et al. 2007). Pol X binds gapped DNAs with cooperative interactions, which increase with the decreasing gap size. Surprisingly, the specific structure necessary to recognize the short gaps is induced by the binding of magnesium to the protein.

The three-dimensional structure of ASFV pol X determined by multidimensional NMR spectroscopy (Maciejewski et al. 2001; Showalter et al. 2001) has confirmed that pol X is formed by only a palm domain (105 amino acids) with the catalytic site and a C-terminal subdomain (69 residues) involved in dNTP selection. The two independently determined pol X structures differ in the presence of a disulfide bond between Cys-81 and Cys-86, the only cysteines present in the protein, located in the catalytic subdomain of the structure

obtained by Showalter et al. (Showalter et al. 2001) and its absence in that described by Maciejewski et al. (Maciejewski et al. 2001).

Controversial results have been obtained regarding the fidelity of ASFV pol X. Table 2 summarizes the fidelity parameters of pol X in a single nucleotide gap BER intermediate with the 5′ end phosphorylated, found by different authors in different experimental conditions. The misinsertion frequency found by Tsai and coworkers (Showalter and Tsai 2001; Lamarche et al. 2006) was much higher than the values reported in our laboratory (García-Escudero et al. 2003). We found values that resemble those described for pol β (Chagovetz et al. 1997; García-Escudero et al. 2003), except for the G:G misinsertion that was higher (7.1×10^{-4} in pol X vs. 2×10^{-6} for pol β), but much less frequent than the reported by Tsai's laboratory (Table 2). Differences in experimental conditions between those reports might somewhat account for the contradictory results. The variances include different salt and pH conditions, and, more importantly, different kinetic analysis and a great difference in enzyme concentration for incorrect nucleotide insertion assays (steady-state and 2 μM pol X in García-Escudero et al. (2003) vs. presteady-state and 50 or 450 nM pol X in Showalter et al. (2001) and Lamarche et al. (2006)). This latter difference may also modify the dNTP insertion fidelity, since, as mentioned above, protein concentration is critical for pol X binding to the gapped DNA substrate (Jezewska et al. 2007). The extremely low fidelity rates reported by Tsai and coworkers prompted these authors to propose a mutagenic role of the pol X, suggesting that an error prone BER system may increase the variability in viral genomes. Furthermore, they speculate that a higher genomic divergence would raise the adaptability of the viral populations. However, genomic variability in ASFV isolates is concentrated in the terminal variable end regions, whereas most of the ORFs in the central segment of the genome are highly conserved (Yáñez et al. 1995; Chapman et al. 2008). Besides, the alterations found in the multigenic families of the variable regions are deletions, duplications and translocations of large fragments of DNA that can be more likely explained by recombination mechanisms rather than due to a mutagenic BER pathway (Blasco et al. 1989; Agüero et al. 1990).

A recent study on the relevance of the disulfide bridge for the modulation of the catalytic activity and fidelity of ASFV pol X provides further explanations to the discrepancies reported (Voehler et al. 2009). These authors showed that the oxidized form of pol X containing a disulfide bond between Cys-81 and Cys-86 has about 10-fold lower fidelity than the reduced pol X, when assayed with gapped DNA substrates during dNTP insertion opposite a template G (Table 2). The disulfide linkage is located between two β-strands in the palm domain, nearby the dNTP binding site. Furthermore, even the presence of a reducing agent will not prevent oxidation over time, which may be the reason why Showalter et al. (Showalter et al. 2001) found the disulfide bond in the presence of 1 mM DTT and Maciejewski et al. (Maciejewski et al. 2001) found the reducing form in the presence of 10 mM DTT.

Structural alignment of pol X with a pol β ternary structure suggests that the disulfide bond formation and breakage might modulate fidelity by altering the ability of the palm domain to properly place and stabilize the primer terminus and catalytic metal ion for phosphoryl transfer. Therefore, the structural changes that occur in the palm domain provide molecular basis for the distinct fidelities observed with the oxidized and reduced forms of pol X. Hence, the DNA polymerase fidelity can be modulated by the redox state of the enzyme and its associated conformational changes (Voehler et al. 2009).

Base Pair	Redox conditions (stored protein)	Redox conditions (reaction)	Misinsertion frequency	Reference
G:G	7 mM β-ME	1 mM DTT	7.1×10^{-4}	(García-Escudero et al. 2003)
G:A	7 mM β-ME	1 mM DTT	6.1×10^{-5}	(García-Escudero et al. 2003)
G:T	7 mM β-ME	1 mM DTT	2.4×10^{-4}	(García-Escudero et al. 2003)
G:A	0.5 mM DTT	1 mM DTT	7.1×10^{-3}	(Showalter and Tsai 2001)
G:T	0.5 mM DTT	1 mM DTT	6.2×10^{-2}	(Showalter and Tsai 2001)
G:G	0.5 mM DTT	1 mM DTT	0.52	(Showalter and Tsai 2001)
G:G	0.5 mM DTT	1 mM DTT	5.3×10^{-2}	(Lamarche et al. 2006)
G:G	100 mM DTT	10 mM DTT	9.3×10^{-3}	(Voehler et al. 2009)
G:A	100 mM DTT	10 mM DTT	4.5×10^{-4}	(Voehler et al. 2009)
G:T	100 mM DTT	10 mM DTT	1×10^{-4}	(Voehler et al. 2009)
G:G			7.6×10^{-2}	(Voehler et al. 2009)
G:A			7.6×10^{-2}	(Voehler et al. 2009)
G:T			6.8×10^{-4}	(Voehler et al. 2009)

Table 2. Comparison of pol X fidelity rates reported in a BER intermediate single nucleotide gap. Data from references (García-Escudero et al. 2003; Lamarche et al. 2006; Voehler et al. 2009) were determined by steady state experiments and in ref. (Showalter and Tsai 2001) kinetic parameters were analyzed under pre-steady state conditions. Frequency of misincorporation is calculated as $[k_{cat}(\text{incorrect})/K_m(\text{incorrect})]/[k_{cat}(\text{correct})/K_m(\text{correct})]$.

The redox states of the ASFV AP endonuclease and pol X proteins during viral infection are not known and the biological significance of these findings are currently under investigation in our laboratory. The fact that AP endonuclease is inhibited under reducing agents may provide a fine-tune regulation system in which variations of reducing conditions may increase or reduce nuclease activities and modulate pol X fidelity. It is also tempting to consider a scenario in which low rate but accurate DNA repair activity works in a reducing environment but removal of damaged nucleotide and DNA integrity restoration prevail under oxidized conditions, with lower DNA sequence fidelity.

2.4 DNA ligase and other factors that may play multiple functions in DNA replication, recombination and repair

DNA ligases are found in all free-living organisms and are essential for the maintenance of cellular genome integrity. They are responsible for joining Okazaki fragments on the DNA replication fork and restoring the continuity of the DNA backbone subsequent to nucleotide excision and base excision repair (Timson et al. 2000). DNA viruses, as extrachromosomal replicons, also rely on ligases to accomplish DNA replication and to guard their genomes against breaks introduced during recombination or DNA damage.

ASFV protein pNP419L is an ATP-dependent DNA ligase (Hammond et al. 1992; Yáñez and Viñuela 1993). ATP-dependent ligases are common within NCLDV, they are present in the genomes of many chordopoxviruses and phycodnaviruses, whereas NAD-dependent ligases appear to be more scattered, and are found only in entomopoxvirus and mimivirus genomes. Interestingly, many viruses belonging to NCLDVs lack any ligase gene (Yutin and Koonin 2009). It is likely that ligase loss is counteracted by the host protein, as has been demonstrated for vaccinia virus and cellular DNA ligase I (Paran et al. 2009).

Recombinant purified protein pNP419L has been shown to be a proficient ligase for DNA nicks (Lamarche et al. 2005). It can be detected at early and late times post infection (Yáñez and Viñuela 1993) and seems to be essential for viral replication, since no deletion mutant can be constructed (García-Escudero, R., Salas ML. and Salas J., unpublished results). Altogether, the available data strongly suggest that ASFV ligase function in Okazaki fragment sealing is essential for viral DNA replication process. Under the experimental conditions of Lamarche et al. (2005), the purified protein was found to ligate efficiently 3′ mismatched substrates, which was proposed to be a viral strategy where genome integrity prevails over the sequence fidelity in DNA repair mechanisms (Showalter and Tsai 2001; Lamarche et al. 2006).

Consistent with their large genome size and relative replication autonomy, most NCLDVs possess also multiple recombination enzymes. RuvC-like Holliday junction resolvases (HJR) encoded by poxviruses, iridoviruses, phycodnaviruses and the mimivirus could participate in resolution of concatemer replication intermediates and recombination. However, in ASFV, this resolvase is replaced by a ERCC4/Mus81-like nuclease (EP364R, Table 1), which is related to the principal Holliday junction resolvase of the eukaryotes, Mus81 (Iyer et al. 2006). A predicted Fen-1/FLAP-like endonuclease has been reported in poxviruses (G5R), iridoviruses and the mimivirus, and might participate in DNA replication and repair (Da Silva et al. 2006; Iyer et al. 2006). A lambda-like exonuclease, which might be involved in processing DNA ends for strand exchange or single-strand annealing during recombination, has been predicted in ASFV (pD345L, Table 1), phycodnaviruses, CroV and other viruses (Iyer et al. 2006; Fischer et al. 2010; Moreau et al. 2010; Weynberg et al. 2011). Unfortunately, none of those annotated protein sequences have been characterized functionally or biochemically.

Lambda-like viral recombinase paradigm is SPP1 Chu exonuclease (Vellani and Myers 2003). Chu protein, as well as lambda exonuclease, forms an oligomer and functions as a highly processive alkaline exonuclease that digests linear double-stranded DNA in a Mg^{2+}-dependent reaction, showing a preference for 5'-phosphorylated DNA ends. In SPP1 and other phages it forms part of the synaptase/exonuclease two-component viral recombinase functional unit. The other component is a single strand binding protein, named synaptase, that protects the single stranded DNA and favors the strand invasion required for the recombination process. However, this element has not been identified in any ASFV genome.

A structural model of ASFV pD345L protein, based upon the lambda exonuclease protein (Kovall and Matthews 1997) is shown in Figure 1. The model corresponds to two thirds of the N-terminus of the pD345L protein sequence and displays a strong correlation in the position of the catalytic center residues Asp119, Lys131 and Glu129 (Kovall and Matthews 1997), supporting the existence of common features and roles in DNA recombination and repair processes.

Failed attempts to purify a virus deletion mutant by plaque isolation, led us to conclude that ASFV mutants lacking the D345L gene cannot be isolated, even though the protein should be present for the first recombination process. This indicates that the virus mutants are not

viable or enough competitive when compared with the parental virus, suggesting that the protein may be essential for successful viral genome replication.

A biochemical characterization of protein pD345L using a recombinant purified histidine-tagged protein shows that it has a 5′→3′ exonuclease activity on a single stranded substrate and a much weaker activity on double stranded or BER intermediate with a flap structure. The exonucleolytic activity is strongly stimulated by 5′-phosphate ends. Moreover, mismatched nick and gaps are also substrates for protein pD345L-catalyzed 5′→3′ exonuclease activity (Redrejo-Rodríguez, M., Rodríguez J.M., Salas, J. and Salas M.L., unpublished results). The preference for single stranded substrates suggests also a possible function in the degradation of the flap structure in LP-BER and NIR, that may be removed by a FEN specific endonucleolytic activity or by sequential 5′→3′ exonuclease steps.

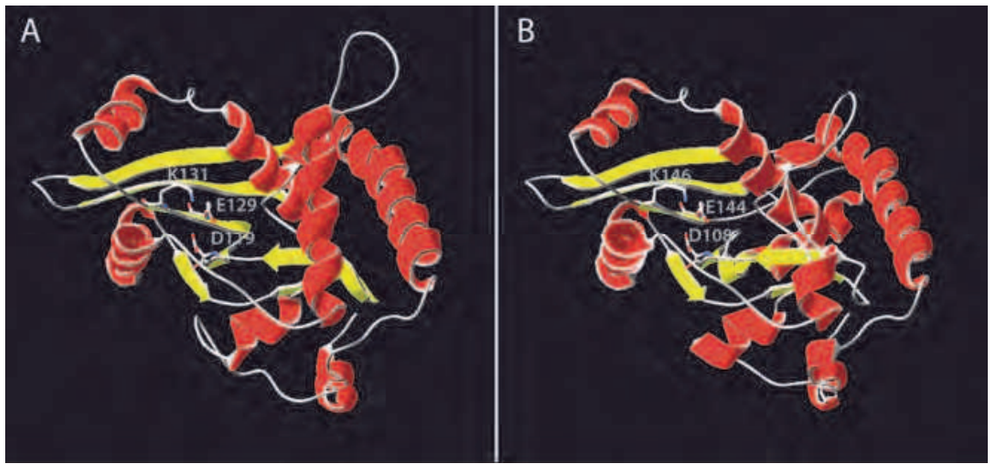


Fig. 1. Three-dimensional structure prediction for ASFV pD345L protein (Schwede et al. 2003). ASFV pD345L structure model (B) was inferred from the structure of lambda phage exonuclease (A, accession no. 1AVQ in the RCSB Protein Data Bank). The model was obtained with the SwissModel server (*Schwede et al. 2003*) and rendered with Swiss-PDB Viewer sotfware. Catalytic center residue disposition in the 1AVQ template (A) and the model obtained for the ASFV pD345L protein is also represented (B).

The ASFV genome also contains a gene, named E301R (Table 1), that has sequence similarity to the proliferating cell nuclear antigen (PCNA) and thus could be a processivity factor of the viral replicative DNA polymerase holoenzyme (Yáñez et al. 1995). Although most of DNA polymerases holoenzymes of NCLDVs contain a processivity factor, they are very divergent, which may be related with the additional functions performed by some of those proteins, like the role of poxvirus G8R in transcription (Iyer et al. 2001; Da Silva and Upton 2009).

Protein pE301R is a non-structural late protein (Redrejo-Rodríguez 2009); therefore it cannot be involved in the early nuclear DNA replication stage. It should be noted that at early times post infection only small DNA fragments are detected, whereas genome size DNA fragments are synthesized during the cytoplasmatic late DNA replication stage, thus DNA polymerase processivity may be more important in the late stage. E301R gene deletion-mutants are reluctant to purification (García-Escudero, R., Salas ML. and Salas J., unpublished results), suggesting an essential requirement for successful genome replication. At late times post-infection pE301R protein signal is detected in the cytoplasm and

accumulates in the viral factories, in agreement with a role in the stimulation of the replicative DNA polymerase processivity. Besides, it may also act as coordination factor for polymerase switching at repair processes.

3. Conclusion and perspectives

BER pathways in mammalian cells and the ASFV-encoded proteins that can be responsible of each step in a viral pathway(s) are summarized in Figure 2. Early steps of a viral BER mechanism might require the pE296R protein to remove AP sites and a number of oxidatively modified bases, through BER and NIR pathways. To date, the required 5′-dRP lyase or hydrolase activity necessary for SN-BER has not been identified in ASFV. Though the 5′- dRP group can be lost spontaneously via β-elimination, the half-life of this reaction under physiological conditions is rather long (on the order of 30 h), suggesting that LP-BER and NIR pathways are more likely to happen after an endonucleolytic cleavage on 5′-side of the lesion. ASFV Pol X AP lyase activity could initiate an alternative pathway for the reparation of abasic sites (García-Escudero et al. 2003) and besides, since there is no viral DNA glycosylase, the participation of host monofunctional or bifunctional glycosylase(s) cannot be ruled out. The 3′-phosphatase and 3′-phosphodiesterase activities of pE296R protein are able to cleanse 3′-moieties derived from pol X or bifunctional glycosylases AP-lyase activities, providing the proper 3′-OH ends that pol X needs to fill the gap. The viral 5′→3′ exonuclease pD345L and the putative processivity factor pE301R might participate in LP-BER or NIR pathways and the ATP-dependent ligase pNP419L would seal the nick. Moreover, the 3′→5′ exonuclease activity of the pE296R protein might act as editing activity that would increase the repair fidelity, as proposed for *E. coli* and human AP endonucleases (Chou and Cheng 2002; Kerins et al. 2003).

ROS can induce also single and double strand breaks. BER pathway is involved in single strand breaks repair but double strand breaks must be repaired by homologous recombination (HR) or non-homologous end joining (NHEJ) pathways. Oxidatively induced DNA breaks usually contain damaged bases and/or 3′-phosphate ends that can be removed by the 3′-activities of pE296R protein. The 5′→3′ exonuclease activity of pD345L could generate the single stranded homologous end for the strand invasion in HR. On the other hand, a viral NHEJ mechanism might require also the pNP419L ligase and pol X. An oxidative environment that might induce double strand breaks may also be compatible with the pol X Cys-81 - Cys-86 disulfide bond. Therefore, it is tempting to speculate that a putative viral NHEJ pathway may favor genome structural stability over fidelity.

The existence of a viral BER pathway involved in ASFV genome maintenance was proposed as a result of the analysis of the first complete genome sequence (Yáñez et al. 1995), based upon two main reasons. First, the presence of ORFs with homology to several DNA repair genes, particularly a class II AP endonuclease and a pol β-like DNA polymerase; second, the fact that ASFV mainly infects macrophages and other immune cells suggests that the viral enzymes may be required to cope with a potentially highly oxidative environment of the infected cells. Subsequent biochemical and genetic evidences further support that model. Still, the specific DNA repair mechanisms that constitute the viral BER pathway(s) must be confirmed. Current and future work on ASFV genome repair mechanisms should pursue a double objective. First, a deeper knowledge of the biochemical and genetic mechanisms of BER pathways, and second, a study on the fidelity and biological role of the pol X in the context of the potentially genotoxic environment of the infected macrophage.

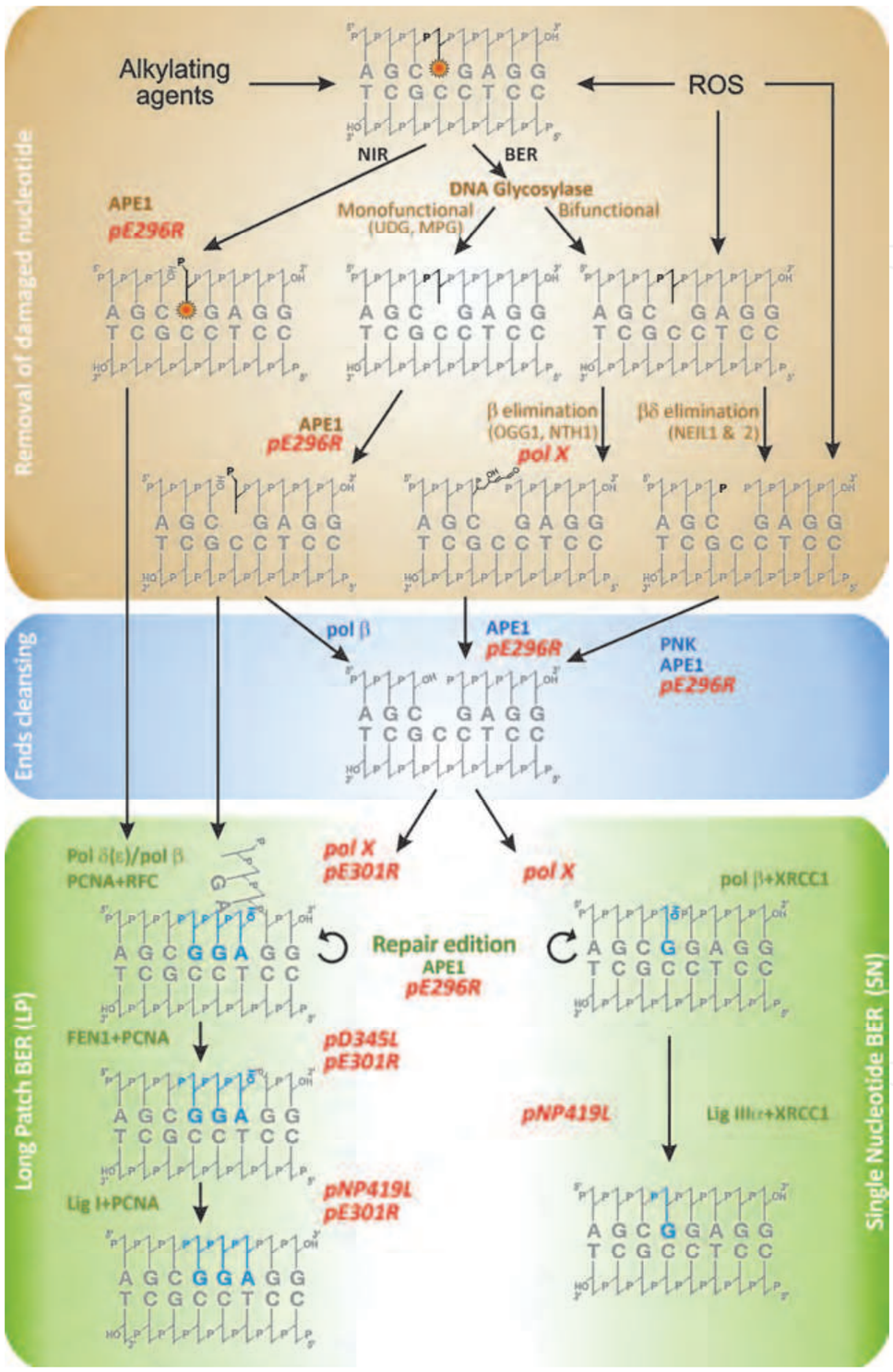


Fig. 2. African swine fever virus possible repair pathways in the framework of mammalian BER mechanism. Damaged nucleotide stands out in black. Different repair pathways or even some DNA damaging agents −like ROS− can induce breaks in the DNA backbone (brown upper zone). These processes converge to a key step in which a 3′-OH end should be

generated (blue zone) and subsequently extended by a reparative DNA polymerase that inserts the correct nucleotide (blue). The 3′→5′ exonucleolytic activity of the AP endonuclease might remove misincorporated nucleotides (circular arrow), thus increasing the repair fidelity. SN-BER consists in a surgical removal of a single damaged nucleotide (right green zone at the bottom), whereas NIR and LP-BER require additional factors that collaborate to replace a few nucleotides (left green zone). The viral proteins that might play a role in each step are indicated in red. As reference, some of the major human proteins involved in each stage are also indicated.

Different viruses present alternative strategies that might aim to control the stability of genetic information. Some retroviruses, including HIV, incorporate a host DNA glycosylase (Willetts et al. 1999) to avoid uridine misincorporation in the retrotranscribed DNA (Priet et al. 2005). On the other hand, flexivirus, a RNA virus, encodes a AlkB-like glycosylase that removes methylated bases from genomic RNA (van den Born et al. 2008).

Alternative or complementary hypothesis may be argued to justify the constant presence of DNA repair systems in different viruses. Several host nucleic acid modification proteins can destabilize viral genomes through deamination or direct degradation. This strategy has been described as a host "intrinsic immunity" to impair replication of some retroviruses (Bieniasz 2006; Lloyd et al. 2006). It would be extremely interesting to evaluate its role in a wider range of viral infections and whether it can be counteracted by viral DNA repair mechanisms. This strategy reminds the bacterial restriction enzymes and it may have played a striking role during evolution, as recently reported for SUKH protein superfamily that includes a number of nucleases and nucleic acid deaminases, that have evolved to different functions in various eukaryotic and DNA viral systems (Zhang et al. 2011).

Finally, we wish to share some hypothesis about the practical lessons that can be learned from a detailed understanding of mechanisms to maintain viral genome stability. First, an engineered virus with highly stable genome would increase the biosecurity of viruses for multiple applications (vaccines, gene therapy or other biotechnology purposes). On the other hand, a controlled or predictable deterioration of genetic information may be useful in vaccine development, since it would allow generating virus mutants able to accomplish only one or a few rounds of replication and therefore producing abortive infections that may be enough to immunize the organism but not enough to trigger the disease.

4. Acknowledgements

This work has been supported by the Spanish Ministerio de Ciencia e Innovación (grant nº AGL2010-22229-C03-02) and by an institutional grant from Fundación Ramón Areces.

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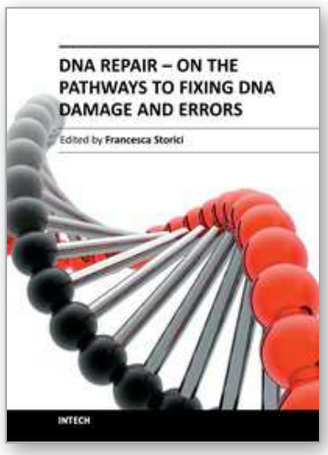
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Edited by Dr. Francesca Storici

ISBN 978-953-307-649-2
Hard cover, 380 pages
Publisher InTech
Published online 09, September, 2011
Published in print edition September, 2011

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