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Involvement of the Reparative DNA Polymerase Pol X of African Swine Fever Virus in the Maintenance of Viral Genome Stability *In Vivo*

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The function of the African swine fever virus (ASFV) reparative DNA polymerase, Pol X, was investigated in the context of virus infection. Pol X is a late structural protein that localizes at cytoplasmic viral factories during DNA replication. Using an ASFV deletion mutant lacking the Pol X gene, we have shown that Pol X is not required for virus growth in Vero cells or swine macrophages under one-step growth conditions. However, at a low multiplicity of infection, when multiple rounds of replication occur, the growth of the mutant virus is impaired in swine macrophages but not in Vero cells, suggesting that Pol X is needed to repair the accumulated DNA damage. The replication of the mutant virus in Vero cells presents sensitivity to oxidative damage, and mutational analysis of viral DNA shows that deletion of Pol X results in an increase in the mutation frequency in macrophages. Therefore, our data reveal a biological role for ASFV Pol X in the context of the infected cell in the preservation of viral genetic information.

The existence of mechanisms to repair lesions in the DNA caused by endogenous and exogenous agents is essential to maintain the integrity not only of cellular DNA but also of virus genomes in the infected cell (1–4). Among these mechanisms, the base excision repair (BER) pathway is crucial to eliminate many types of base damage, including alkylated and oxidized bases, and to repair abasic sites as well as single-strand breaks with 3′ blocking ends generated by reactive oxygen species (ROS) (5). This repair system may be of particular importance in the case of African swine fever virus (ASFV) (6, 7), a large and highly complex DNA virus, member of the *N*ucleo*c*ytoplasmic *l*arge *D*NA *v*iruses monophyletic group (NCLDV) (8), which causes a fatal disease in domestic pigs and replicates mainly in swine macrophages, where the virus genome can be exposed to a damaging and mutagenic environment (9–11).

Although an early stage of viral DNA replication in the nucleus has been described, the bulk of virus replication occurs at specific sites in the cytoplasm of the infected cell, designated viral factories (12, 13). The viral genome, a double-stranded DNA molecule of 170 to 190 kbp, encodes more than 150 polypeptides, including several enzymes involved in DNA repair (14). Thus, the ASFV gene O174L encodes a reparative DNA polymerase that belongs to family X of DNA polymerases, like cellular DNA polymerase β, and has been designated ASFV Pol X (15). The gene NP419L encodes a type I DNA ligase that may form part of a viral BER system during ASFV infection. Finally, the gene E296R encodes an AP endonuclease that belongs to the *Escherichia coli* endonuclease IV family of class II AP endonucleases.

ASFV AP endonuclease is a multifunctional enzyme that possesses AP endonucleolytic, 3′-5′ exonuclease, 3′-diesterase, and nucleotide incision repair (NIR) activities (3, 16, 17). The 3′-5′ exonuclease can act as a proofreading activity and can also eliminate 3′ mismatched or damaged nucleotides present at single-strand breaks. These properties make this enzyme most suitable to participate in a BER pathway and in the alternative DNA glycosylase-independent NIR process (18). In support of this, it has been shown that the viral enzyme can protect *E. coli* cells deficient in the two bacterial AP endonucleases, exonuclease III (Exo III) and endonuclease IV (Endo IV), against treatment with oxidative and alkylating drugs, the protection conferred being similar to that provided by the *E. coli* Endo IV enzyme (16, 17). The biological significance of ASFV AP endonuclease for viral infection was established by studying a virus mutant lacking this specific enzyme. Deletion of the AP endonuclease gene leads to impairment of virus replication in its natural host cell, the swine macrophage, and to a higher sensitivity to oxidative and alkylating DNA damage compounds in Vero cells (17), emphasizing the importance of the AP endonuclease *in vivo*.

ASFV Pol X is a highly distributive β-type polymerase able to efficiently repair single-nucleotide gapped DNA, which is consistent with its participation in a BER process during ASFV infection (15, 19, 20). The viral enzyme lacks the 5′-deoxyribose phosphate (dRP) lyase activity characteristic of cellular Pol β that eliminates the 5′-dRP blocking group generated during mammalian BER by the action of the AP endonuclease on the abasic site in the DNA (19, 21). However, Pol X exhibits lyase activity on unincised AP sites, an activity, named AP lyase, which has also been described for human Pol β (19, 22, 23). Taking this into account, the incision of the AP site in the viral BER pathway could be performed by either the AP endonuclease or the Pol X AP lyase activity, as in the case of the bifunctional DNA glycosylases/AP lyases (24). In the latter case, the 3′-phosphodiesterase and 3′-phosphatase activities of ASFV AP endonuclease would excise the 3′-terminal unsatu-

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rated, ring-opened aldehyde produced after AP lyase cleavage, allowing Pol X to fill in the gap.

The three-dimensional structure of ASFV Pol X (25, 26) has shown that unlike other DNA polymerases, Pol X is formed only by a palm with the catalytic site and a C-terminal subdomain involved in deoxynucleoside triphosphate (dNTP) selection, lacking the N-terminal 8-kDa domain of Pol β where the dRP lyase active site resides. 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 protein. This bond is present in the structure reported by Showalter et al. (25) but absent from that described by Maciejewski et al. (26).

Controversial results regarding the fidelity of ASFV Pol X have been obtained. We have described that Pol X misinserts nucleotides with frequencies from 10^{-4} to 10^{-5}, these values resembling those described for Pol β (19, 27, 28). In contrast, the fidelity values obtained for Pol X in other studies were from 40 to 700 times lower (20, 29). The low fidelity values reported by Tsai and coworkers prompted these authors to propose a mutagenic role for Pol X.

In this study, we have addressed the biological role of Pol X and found that the growth properties of the wild-type and Pol X-deficient mutant virus, as well as their different sensitivities to oxidative agents, support the role of Pol X in a reparative BER pathway. Furthermore, we analyzed mutations introduced in the ASFV genome during infection in the presence or absence of Pol X. Our results show that Pol X acts as a reparative rather than "mutator" polymerase in ASFV-infected macrophages.

MATERIALS AND METHODS

Cells and viruses. Vero and Vero C1008 cells were obtained from the American Type Culture Collection and grown in Dulbecco modified Eagle medium (DMEM) containing 5% fetal calf serum (FCS). Swine alveolar macrophages, obtained as described previously (30), were grown in DMEM containing 10% swine serum. Cell infections with the ASFV BA71V strain and titrations were carried out as previously described (31). Highly purified ASFV was prepared as described before (32).

The Pol X deletion mutant virus was obtained by insertion of the *gusA* gene of *E. coli* coding for β-glucuronidase into the O174L open reading frame (ORF) of the ASFV strain BA71V. For this, left and right flanking regions of the gene O174L, of 701 and 517 bp, respectively, were amplified by PCR using the following oligonucleotides: ΔO174L_left_f (5′-AAAGGTACCTTTCTAATAGCGCGGTTAAAAAC-3′) and ΔO174L_left_r (5′-GAGAGCTCAAAAAAGACGTATCAACTTGATCTTTT-3′), containing KpnI and SacI restriction sites (underlined), respectively; ΔO174L_right_f (5′-TACTGCAGTCCTAGTCATTAAGCATTTTCTCTTC-3′) and ΔO174L_right_r (5′-CACTCGAGTGGATGAAAAATATATTACGGAAAAT-3′), with PstI and XhoI restriction sites (underlined), respectively. The left flanking region was digested with KpnI and SacI and cloned in plasmid pL29.10T.Gus10T carrying the *gusA* gene under the control of the ASFV late promoter p72.4 (33) to obtain the plasmid pΔpolX.left. The right flanking region was digested with PstI and XhoI and cloned into plasmid pΔpolX.left digested with these restriction enzymes to generate the plasmid pΔpolX. The recombinant virus was obtained by homologous recombination in Vero cells infected with BA71V and transfected with the plasmid pΔpolX as described previously (34). The recombinant virus was purified by sequential rounds of plaque purification in the presence of 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) as indicated before (34). One single clone of vΔpolX was grown and employed in all experiments. The vΔpolX genotype was confirmed by PCR analysis using the oligonucleotides 5′-GCGCGCGGATCCATGTTAACGCTTATTCAAGGAAAAA-3′ and 5′-GCGCGCCCCGGGTTATAAACGTTTCTTAGGTATGCGATA-3′, hybridizing to the 5′ and 3′ ends of the O174L gene, respectively, and oligonucleotides 5′-GATGTGGAGTATTGCCAACGAACC-3′ and 5′-TCATTGTTTGCCTCCCTGCTGCGGTTTTTCACGC-3′, corresponding to the *gusA* gene, and by Western blotting of vΔpol X-infected Vero cells.

The mutant virus vΔE296R, lacking the E296R gene coding for the virus AP endonuclease, has been described before (3).

Antibodies. Antibodies against Pol X were raised in rabbits using recombinant His-tagged Pol X, purified as described previously (15). The rabbit antibody against the protein pE248R and the monoclonal antibody 17LD3 against the protein p72 have been described before (35, 36). The anti-β-actin monoclonal antibody AC-15 was purchased from Sigma.

Analysis of presence of Pol X in ASFV particles and expression in infected cells. Vero cells in DMEM containing 2% FCS were either mock infected or infected with ASFV BA71V at a multiplicity of infection (MOI) of 10 PFU per cell, and at different times postinfection, the cells were lysed in electrophoresis sample buffer at a density of about 3×10^6 cells per ml. Equivalent amounts of the cell lysates were electrophoresed in 12% SDS-polyacrylamide gels and subsequently transferred to nitrocellulose membranes for Western blot analysis. The membranes were incubated with the antibody against Pol X (1:2,000 dilution) and then with a 1:5,000 dilution of peroxidase-labeled anti-rabbit serum (GE Healthcare). The proteins were detected with the ECL system (GE Healthcare) according to the manufacturer's recommendations.

For further analysis of the Pol X presence in ASFV particles, clarified culture supernatants of infections with BA71V were semipurified by using a sucrose cushion and purified by equilibrium sedimentation in a Percoll gradient as described before (37). Aliquots of the gradient fractions were analyzed by Western immunoblotting.

Immunofluorescence. Vero cells, grown in coverslips, were infected with BA71V or vΔpolX at an MOI of 10 PFU per cell and fixed at different times postinfection with 4% paraformaldehyde in 1× phosphate-buffered saline (PBS) for 15 min at room temperature, followed by 5 min with methanol at −20°C. Fixed cells were incubated for 5 min with 0.1% Triton X-100 in 1× PBS and then incubated for 10 min with 100 mM glycine in 1× PBS. Samples were then blocked for 30 min with blocking buffer (1% cold fish skin gelatin, 0.1% Triton X-100, 10% normal goat serum, and 1× PBS). The cells were then sequentially incubated for 1 h with the primary antibody anti-Pol X or anti-p72 diluted with blocking buffer, washed three times for 5 min with 0.1% Triton X-100 in 1× PBS, incubated with the corresponding secondary antibodies diluted with blocking buffer for 1 h, and finally washed three times for 5 min with 0.1% Triton X-100 in 1× PBS. The primary antibodies were used at the following dilutions: anti-p72, 1:200; anti-Pol X, 1:200; and as a negative control, preimmune serum, 1:200. The secondary antibodies used were goat anti-mouse IgG, goat anti-rabbit IgG coupled to Alexa 488, and Alexa 555, respectively (1:500 dilution) (Molecular Probes). After incubation with the antibodies, the samples were sequentially washed with H_2O and ethanol and dried for 10 min at room temperature. Finally, the coverslips were mounted with Mowiol/Dabco on glass slides. Preparations were examined with a Zeiss LSM 520 confocal laser-scanning microscope. Images were processed using the ImageJ software program (38).

Determination of [^{3}H]thymidine incorporation in infected macrophages. Swine macrophages were mock infected or infected with parental BA71V, mutant vΔpolX, or vΔE296R virus at an MOI of 5 PFU/cell. The cultures were labeled with 5 mCi/ml of [^{3}H]thymidine (2 Ci/mmol), and at different times postinfection, samples were collected and the acid-insoluble radioactivity was measured.

Viral sensitivity to DNA damage agents. Vero cells were infected with parental BA71V or vΔpolX as reported above. Cells were plated at approximately 10^5 cells per cm^2 and infected at an MOI of 5 PFU/cell. After 1 h for viral adsorption, the medium was withdrawn and the cells were washed with DMEM. Fresh medium containing (or not) 0.25 mM methyl methanesulfonate (MMS), 0.3 mM *tert*-butylhydroperoxide (*t*-BuO_2H), or 1.4 mM H_2O_2 was added and maintained for 22 h. Viral replication is

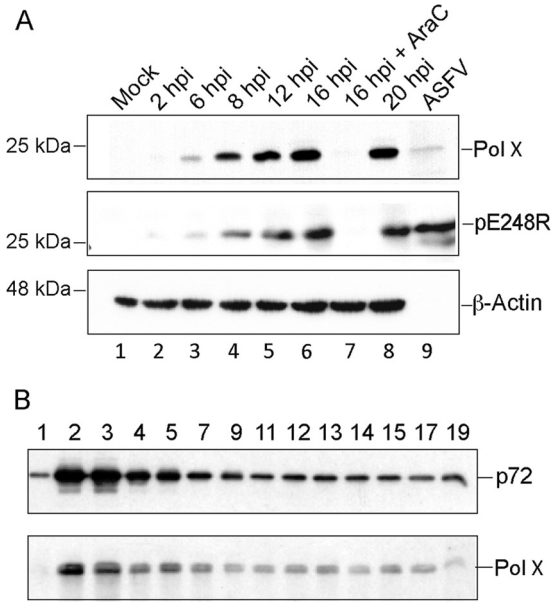

FIG 1 (A) Western blot analysis of cell extracts from ASFV-infected Vero cells with the anti-Pol X antibody. Immunoblotting was carried out for mock-infected cells (Mock) and ASFV-infected cells harvested at the indicated times postinfection, and cells infected for 16 h in the presence of 40 μg/ml of AraC are also shown. A sample with 2 μg of highly purified virus (ASFV) is also included. As a reference, equivalent samples were analyzed using an antibody against the late structural protein pE248R. The levels of β-actin are included as a loading control. (B) Analysis of Pol X presence in ASFV particles by density gradient centrifugation. Aliquots of the indicated gradient fractions were analyzed by Western immunoblotting with antibodies against the protein p72 and Pol X.

estimated by total viral production calculated by plaque assay titrations (31).

Determination of mutation frequency during ASFV replication. To perform this analysis, a clonal preparation of parental wild-type BA71V was obtained after 4 successive plaque purification steps in Vero C1008 cells. Vero cells and swine macrophages were infected with clonal preparations of BA71V or mutant vΔpolX at an MOI of 5 PFU/cell. After the adsorption period of 1 h and 22 h of infection, the infected cells were collected by centrifugation, washed with 10 mM Tris-HCl, pH 8, and 1 mM EDTA, centrifuged again, and resuspended in 10 mM Tris-HCl, pH 8. Samples were boiled (15 min at 95°C) and used for PCR amplification using Kappa HiFi DNA polymerase (error rate, 2.8×10^{-7}) (Kappa Biosciences) and oligonucleotides specific for amplification of the ASFV p72 gene, 5′-ATGGCATCAGGAGGAGCTTTTTGTCTTATT and 5′-TTAG GTACTGTAACGCAGCACAGCTGAACCG. The PCR conditions were as follows: one cycle at 94°C for 6 min, three cycles at 94°C for 1 min, annealing at 47°C for 1 min, and extension at 72°C for 5 min. This was followed by 20 cycles of denaturation at 95°C for 1 min, annealing at 54°C for 30 s, and extension at 72°C for 4 min and finally one cycle at 72°C for 15 min. The PCR products were cloned using the CloneJet cloning kit of Fermentas and transformed in XL1 BlueScript. DNA was prepared from 13 to 15 clones from Vero-infected samples and 28 to 29 from infected macrophages, and the full sequence of the p72 gene was determined.

RESULTS AND DISCUSSION

Expression and localization of ASFV Pol X in infected cells and viral particles. We first examined the expression of the reparative DNA polymerase of ASFV, Pol X, during infection of Vero cells with the BA71V strain. As shown in Fig. 1A, Western blot analysis of extracts from infected cells using an antibody prepared against the purified recombinant Pol X revealed the presence of a specific band migrating at the expected position for this enzyme (20 kDa). This band was first observed at 6 h postinfection (hpi) and accumulated for up to 20 hpi. Furthermore, the protein was not detected in the presence of cytosine arabinoside (AraC), an inhibitor of virus DNA replication and late transcription, clearly indicating that it is a late protein. As a reference, the expression pattern and inhibition by AraC of the late structural protein pE248R (35) is also shown.

Interestingly, the presence of Pol X was detected in preparations of highly purified extracellular virus (Fig. 1A, lane ASFV), indicating that Pol X is a component of the virus particle. This structural nature of Pol X was further supported by the finding that protein Pol X cosediments with the virus capsid protein p72 after centrifugation of extracellular virus in Percoll gradients (Fig. 1B). The presence of Pol X-like proteins in the infecting particles has been described for other large DNA viruses (39) and suggests a role for these activities very early during the infection together with the early expressed AP endonuclease (3).

We next determined the subcellular localization of Pol X by confocal immunofluorescence analysis with the anti-Pol X antibody. As a marker of viral factories, the cells were also stained with an antibody against the viral capsid protein p72. To detect viral factories and cell nucleus, 4′,6-diamidino-2-phenylindole (DAPI) staining was also used. At 8 to 9 hpi, when viral DNA synthesis is actively occurring in the cytoplasmic factory (13), Pol X was found to colocalize with p72 and DAPI staining at the viral factory (Fig. 2Aa and Ba). Control experiments with preimmune sera on cells infected with parental BA71V and with the anti-Pol X antibody in cells infected with the Pol X deletion mutant (see below) indicate that the signal observed is due exclusively to the viral protein (Fig. 2Ad and e and Bd). Surprisingly, at later times postinfection (14 to 18 hpi), when virus DNA replication has been completed and the formation of virus particles and genome encapsidation is well under way (13, 40), the fluorescent signal, instead of accumulating in the viral factories, is detected either dispersed throughout the cytoplasm and somewhat accumulated in the nucleus (Fig. 2Ab and Bb) or accumulated only within the cell nucleus (Fig. 2Ac and Bc), suggesting that Pol X is probably released from no longer functional and disrupted repair complexes and progressively accumulated in the nucleus. At these times postinfection, protein synthesis is still active, since Pol X accumulation increases up to 16 hpi (Fig. 1A), which indicates not only that Pol X is being delocalized from the factory but also that the new protein synthesized at very late times postinfection was not found retained in the viral factories in Vero cells. Taking into account the small size of the Pol X protein (20 kDa) and the absence of putative nuclear localization signals in its sequence (analyzed using the NLStradamus [41] and Psort [42] servers), the observed accumulation of Pol X in the nucleus, near the end of the virus replication cycle, is likely to occur by passive diffusion through the nuclear pores, being probably retained in the nucleus because of its affinity for DNA. It is to be noted that other small viral proteins, like the DNA binding protein p10 (43) and the histone-like protein pA104R (J. M. Rodríguez, unpublished results), are also found in the nucleus at very late times postinfection. The significance, if any, of the nuclear import of Pol X during the latest stage of the viral infection cycle is presently unknown.

Altogether, the expression and localization results are consistent with the involvement of Pol X in a DNA damage repair pathway during the different stages of the ASFV life cycle, mainly dur-

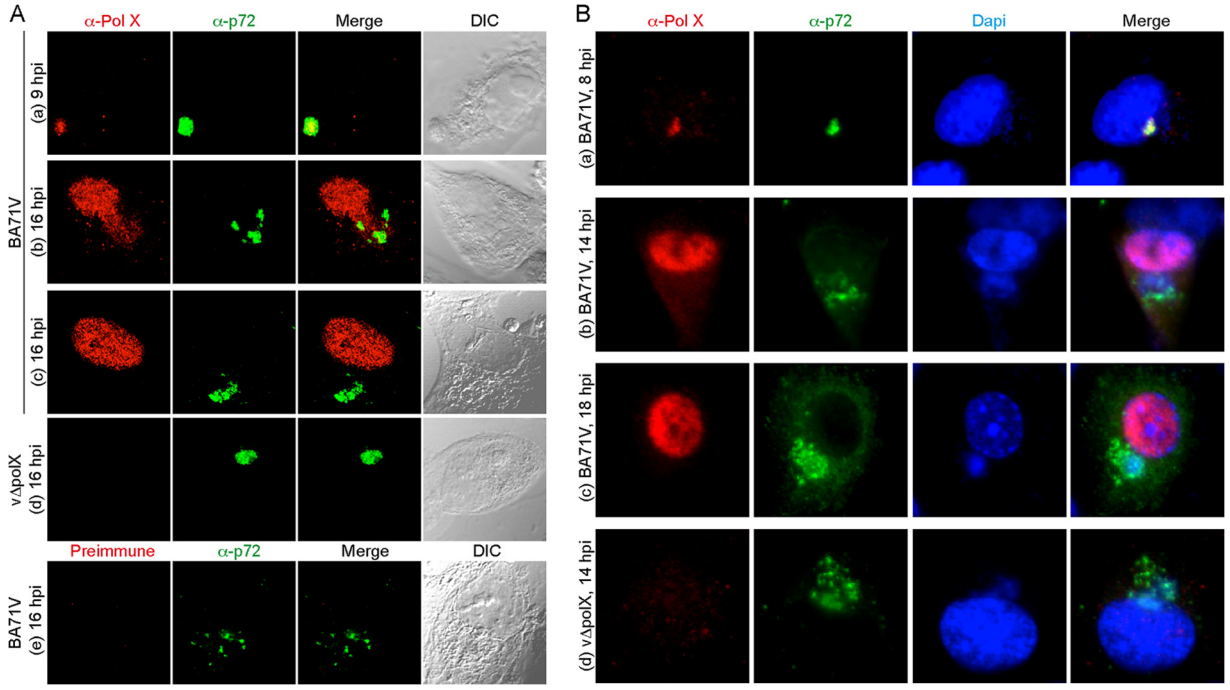

FIG 2 Subcellular localization of Pol X in ASFV-infected cells. (A) Confocal immunofluorescence microscopy images. ASFV-infected Vero cells were fixed at 9 and 16 hpi with parental BA71V or vΔpolX virus and labeled with anti-p72 and anti-Pol X antibodies. An image of infected Vero cells stained with preimmune sera is also shown. Antigens were visualized with secondary antibodies coupled to Alexa 488 (p72; green channel) and 555 (Pol X; red channel). Differential interference contrast (DIC) microscopy of the samples is also shown. (B) Immunofluorescence microscopy analysis of ASFV-infected Vero cells fixed at 8, 14, and 18 hpi. Cells were labeled with anti-p72 and anti-Pol X antibodies and subsequently with secondary antibodies coupled to Alexa 488 (p72; green channel) and Alexa 555 (Pol X; red channel). Cells were also stained with DAPI to visualize cellular and viral DNA (DAPI; blue channel).

ing the cytoplasmic DNA replication process, along with the viral AP endonuclease, which is also found in the cytoplasm at these postinfection times (3). Since Pol X is present in the infective particle and the AP endonuclease is encoded by an early gene, both enzymes may participate in an early viral BER during the nuclear phase of viral DNA replication. It is also tempting to speculate on the existence of a prereplicative repair mechanism, in which both enzymes might cleanse incoming damaged genomes, along with other enzymes that may also be present in the viral particle, like the viral DNA ligase, or even with host factors.

Construction and analysis of replication of the deletion mutant vΔpolX in Vero cells and swine macrophages. To further examine the role of the protein Pol X during viral infection, we constructed a deletion mutant of the BA71V strain, vΔpolX, in which the gene encoding the protein Pol X (gene O174L) was deleted as described in Materials and Methods. The fact that we were able to generate and isolate vΔpolX virus in cultured Vero cells indicates that Pol X is not essential for virus replication in this cell type. Thus, when comparing the growth curves in Vero cells infected at a high or low multiplicity of infection of the parental BA71V with those of the deletion mutants for the Pol X and AP endonuclease (vΔE296R virus), we found that the three viruses replicated with the same kinetics and essentially to the same extent (Fig. 3A and C). We next examined the replication of the three viruses in swine macrophages, the natural target cell of the virus, infected at high multiplicity (Fig. 3B). As we have previously reported (3), the deletion of the AP endonuclease gene considerably affects the replication of this mutant virus ($P < 0.01$). In contrast and rather unexpectedly, when we examined the growth curve of the mutant vΔpolX virus in porcine macrophages, we found that it was only somewhat delayed compared with that of parental BA71V ($P > 0.05$), although the final titers reached, obtained at 36 hpi, were very similar in the two viruses (Fig. 3B). Since these experiments were done at a high MOI, the results indicate that Pol X is not essential in a single replication cycle. However, when the infection was performed in swine macrophages at low multiplicity, the replication of the mutant vΔpolX virus was clearly affected ($P < 0.05$), and the amount of total virus produced was about 1 log unit lower than that obtained in cells infected with the parental virus (Fig. 3D). Under these conditions, an even greater reduction in virus production (about 2 log units) was seen in cells infected with the recombinant virus in which the AP endonuclease gene has been deleted ($P < 0.01$). The specific requirement of Pol X in swine macrophages but not in Vero cells for optimal virus production under low-MOI conditions could indicate a role for Pol X in the mechanisms that prevent the accumulation of damage in the DNA that may occur after multiple rounds of replication in the genotoxic environment of the macrophage. If DNA damage is occurring, it could be detected by its negative effects on the rate of viral DNA replication. Thus, to investigate whether virus DNA synthesis was affected by the absence of Pol X, we compared the incorporation of [^{3}H]thymidine into acid-insoluble material in macrophages infected with a high MOI of parental BA71V and mutant vΔpolX virus. Figure 4 shows that in cells infected with the mutant virus, the amount of viral DNA synthesized at 12 to 14 hpi was about 50% of that obtained with the parental virus. This decrease in viral DNA synthesis, similar to that observed with vΔE296R virus previously reported (3) and used as a control in a

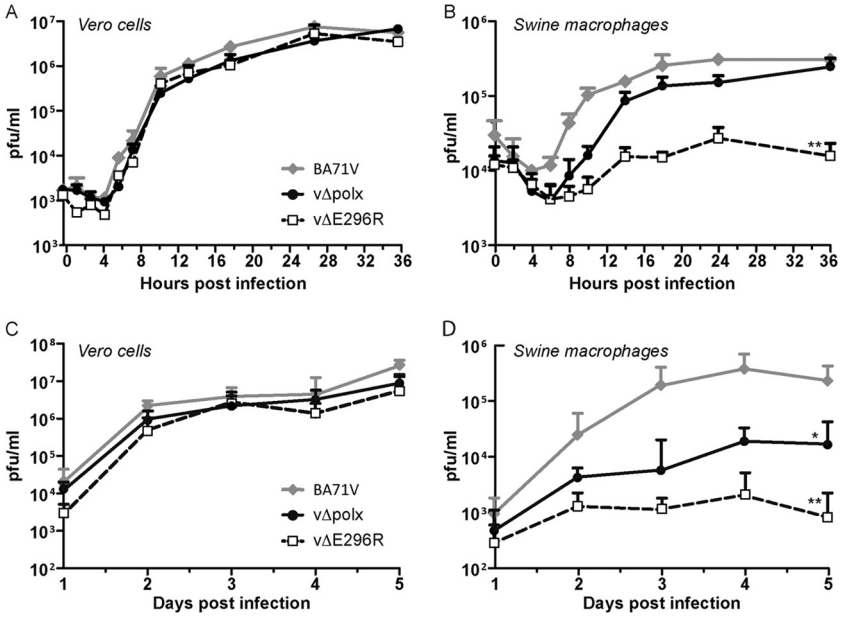

FIG 3 Growth curves of parental BA71V or mutant vΔpolX and vΔE296R virus in Vero cells and swine macrophages. Vero cells or swine macrophages were infected with BA71V, vΔpolX, or vΔE296R at an MOI of 2 PFU per cell (A and B) or at an MOI of 0.01 PFU per cell (C and D). At different times postinfection, samples were collected and titrated by plaque assay on fresh Vero cells as described previously (31). Means and standard errors for at least two experiments are shown. Titers were analyzed by an unpaired *t* test in order to determine the statistical significance of the difference between results for the mutant viruses and those for parental BA71V, with $P < 0.1$ (*) or $P < 0.05$ (**).

parallel experiment, could be due to an accumulation of replication-blocking lesions in the DNA in the absence of Pol X, although this effect is not directly reflected in viral growth under single-replication-step conditions.

Effect of genotoxic drugs on replication of the deletion mutant virus vΔpolX in Vero cells. We previously showed that the oxidizing agents H_2O_2 and *t*-BuO_2H, as well as the alkylating drug MMS, impaired the growth of the mutant virus vΔE296R, lacking the AP endonuclease gene, in Vero cells (17). Therefore, to further investigate the biological role of Pol X in ASFV infection, we exposed Vero cells and macrophages infected with parental BA71V and mutant vΔpolX virus to those DNA-damaging agents. As shown in Fig. 5, the total number for infective virus produced in Vero cells infected by wild-type BA71V or vΔpolX virus was not significantly affected by the presence of the alkylating agent MMS, whereas treated macrophages showed reduced viral production in both cases, suggesting a cumulative or cooperative genotoxic effect of the macrophage-generated ROS and the MMS. These results indicate that the level of viral DNA damage that could be specifically induced by MMS under these conditions may be low, at least under a single replication cycle.

Figure 5 also shows that the total number of infective viruses produced by infection with vΔpolX of cells exposed to H_2O_2 decreased more than that of those produced by cells infected under the same conditions with parental BA71V. More importantly, mutant vΔpolX growth showed a significantly higher sensitivity to *t*-BuO_2H than the wild-type virus in both Vero cells ($P < 0.05$) and swine macrophages ($P < 0.1$), which strongly supports a role for Pol X in counteracting oxidative DNA damage produced by these agents. However, similar to the results obtained in swine macrophages, the viral titers obtained in cells infected with vΔpolX in the presence of oxidizing agents are reduced to a smaller extent than those obtained from cells infected with the virus lacking the AP endonuclease (17). These results support a role for both enzymes in the same viral repair mechanisms, although the lack of the AP endonuclease leads to more severe consequences for viral growth, probably because it is a multifunctional enzyme that might participate in more than one repair pathway. In line with this, it is worth mentioning that in the case of cellular enzymes, deletion of AP endonucleases gives rise to stronger impaired phenotypes than deletion of reparative polymerases (44–48), indicating a redundant role of reparative DNA polymerases. Thus, the possibility that a cellular DNA polymerase or even the viral replicative DNA polymerase may also participate in some viral repair mechanisms cannot be ruled out.

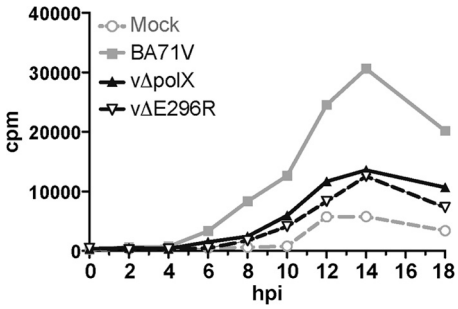

FIG 4 Impaired DNA replication in the absence of viral Pol X. DNA synthesis in macrophages infected with parental BA71V and mutant vΔpolX or vΔE296R virus was analyzed. Swine macrophages infected with parental or mutant virus at an MOI of 5 PFU/cell, as well as mock-infected cultures, were labeled with 5 mCi/ml of [^{3}H]thymidine as described in Materials and Methods. At the indicated times postinfection, samples were collected and the acid-insoluble radioactivity was determined.

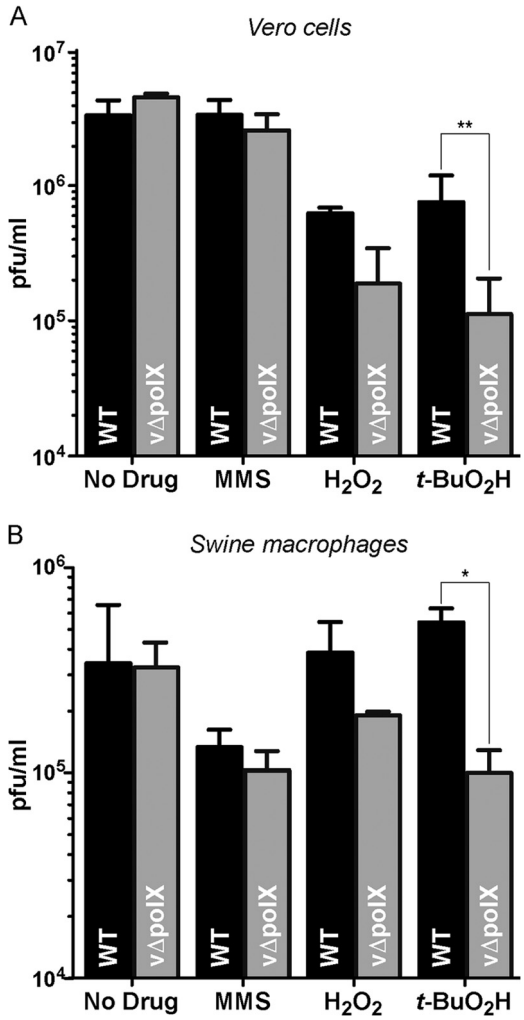

FIG 5 Sensitivity of ASFV Pol X deletion mutant to genotoxic agents. Vero cells (A) or swine macrophages (B) were infected with BA71V or vΔpolX at an MOI of 5 PFU/cell and treated or not with 0.25 mM MMS, 0.3 mM *t*-BuO_2H, or 1.4 mM H_2O_2, as indicated in Materials and Methods. Samples were collected at 22 hpi and titrated by plaque assay in Vero cells without drugs. Titers were analyzed by a Mann-Whitney U test in order to determine the statistical significance of the difference between the data, with $P < 0.1$ (*) or 0.05 (**).

Mutation analysis of DNA synthesized in cells infected with parental BA71V and mutant vΔpolX virus. Lesions caused in the DNA by endogenous or exogenous damaging agents can exert a number of deleterious effects, including the induction of mutations. However, since viral titrations detect a loss of viral viability due to lethal mutations, analysis of viral growth in cell culture may not be sensitive enough to detect the accumulation of mutations in viral DNA. To investigate the importance of Pol X in maintaining the informational integrity of the viral genome, we carried out a mutation analysis of the DNA synthesized in infected cells. For this, clonal preparations of BA71V and vΔpolX were used to infect Vero cells and two different preparations of swine macrophages. The presence of mutations in the gene coding for the capsid protein p72 was then analyzed by PCR amplification (using total DNA from infected cells as described in Materials and Methods), cloning, and sequencing of 13 to 15 clones for Vero cell infections (25,000 to 30,000 nucleotides [nt]) and 28 to 29 clones for macrophages (54,000 to 56,000 nt). We selected the p72 gene for this analysis taking into account that it is a well-characterized gene used for genotyping of ASFV isolates.

The pattern of distribution of mutations in the p72 gene appears to be random since there is no visible accumulation of mutations in certain regions of the gene (not shown). As shown in Table 1, the frequencies of mutations in viral DNA from infected Vero cells were very low and were similar in the genomes of the two viruses. Previous analysis of ASFV genome sequence stability in Vero cells showed a very low mutation frequency in a dispensable region after repetitive sequencing, since no mutations were detected in a total of 54,026 nt screened (49). The frequencies we found, on an essential gene, are consistent with those, even though we performed amplification and cloning steps.

On the other hand, for both viruses, viral DNA from infected macrophages showed an increased mutation frequency, in agreement with the previously hypothesized production of genotoxic ROS in the ASFV-infected macrophage (3). Thus, wild-type genomes had an increased number of transitions, which led to a mutation frequency 1.8 times higher in macrophages than in Vero cells. Importantly, vΔpolX DNA showed not only more transitions but also some transversions in macrophages, leading to an increase in the mutation frequency of 4.6 times with respect to that found in Vero cells. Moreover, in macrophages, the frequency of mutations was significantly higher (2.2 times) in the vΔpolX genome than in BA71V wild-type DNA. Altogether, these results underline the requirement of Pol X for genomic stability.

DNA base lesions induced by ROS are mediated mainly by cytosine deamination or guanosine oxidation to 7,8-dihydro-8-oxoguanine (8-oxo-G) (50–52), leading to CG → TA and TA → CG transitions, which are more abundant in viral DNAs from infected macrophages. TA → GC transversions, which can arise from the wrong incorporation of dATP opposite 8-oxo-G (51), from oxidation of thymine to thymine glycol or methylation to

TABLE 1 Viral genome mutation analysis in BA71V and vΔpolX[a]

Virus	Cells	$f_{transitions}$, $\times 10^{-5}$		$f_{transversions}$, $\times 10^{-5}$				No. of nt screened	f, $\times 10^{-5}$
		CG → TA	TA → CG	TA → AT	TA → GC	CG → GC	CG → AT		
BA71V	Vero		3.97 (1)					25,194	3.97
	Macrophages[b]	5.53 (3)	1.84 (1)					54,264	7.37
vΔpolX	Vero		3.44 (1)					29,070	3.44
	Macrophages[b]	7.12 (4)	5.34 (3)		3.56 (2)			56,202	16.01

[a] Analyses of mutation frequencies (f) were performed for Vero cells and swine macrophages infected with BA71V or vΔpolX virus as described in Materials and Methods. The number of mutations found is indicated in parentheses.

[b] The Mann-Whitney U test was used to compare the mutation frequencies of samples from each virus, with the result that the samples from infected macrophages for BA71V and for vΔpolX were significantly different ($P = 0.04$).

O^4MeT and N^3MeT, or from the formation of adenine oxidation and deamination products (52–54), are also found in viral sequences from vΔpolX-infected macrophages. Altogether, these results support a role for Pol X in the repair of oxidative or alkylating lesions in the DNA in infected macrophages. It is important to notice that no GC → CG mutations were found under any of the conditions tested, indicating that no incorporation of G opposite template G occurs. This is discrepant with the results obtained by Tsai and coworkers in *in vitro* studies (20, 29), with purified recombinant Pol X indicating that the enzyme can catalyze misincorporation of G opposite template G with an efficiency comparable to that of the correct incorporation of C. Rather, the result is in conformity with our previous fidelity studies showing that ASFV Pol X misinserts G opposite template G with an efficiency about 1,000 times lower than that for the correct insertion of C opposite G (19).

Function of ASFV DNA repair mechanism. As mentioned in the introduction, *in vitro* analysis of Pol X fidelity by biochemistry approaches has led to a great controversy in the literature (19, 20, 29). Moreover, two different structures of Pol X were reported, with the only difference being the presence or not of the disulfide bond between Cys-81 and Cys-86. This cysteine-cysteine bridge has been shown to reduce about 10-fold the fidelity of Pol X in *in vitro* assays compared with results with the reduced form (55). These results might explain the divergence in the biochemical results previously published by Tsai and coworkers (20, 29) and our group. However, *in vivo*, even in the potentially oxidant environment of the infected cultured macrophages, the natural host cell of ASFV, the presence of Pol X prevents the accumulation of mutations (Table 1). Thus, our results strongly suggest that Pol X can protect the virus genome in the macrophage against oxidative-damage lesions, likely through a reparative viral BER pathway with the participation of the virus AP endonuclease, which can provide an increased fidelity through the proofreading activity of its 3′–5′ exonuclease in this system.

Taken together, our data reveal a biological role of ASFV Pol X in the context of the infected macrophage, rather than as a mutagenic repair system that would introduce variability in the virus genome, in the preservation of ASFV genomic information during the infection following exposure to oxidative stress.

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