Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

HHS Public Access

Author manuscript

Virus Res. Author manuscript; available in PMC 2018 April 15.

Published in final edited form as:

Virus Res. 2017 April 15; 234: 193–206. doi:10.1016/j.virusres.2017.01.027.

The Vaccinia Virus DNA Polymerase and its Processivity Factor

Maciej W. Czarnecki[1] and **Paula Traktman**[2,#]

[1]Department of Microbiology and Immunology, Medical University of South Carolina, Charleston SC 29425; Department of Microbiology and Molecular Genetics, Medical College of Wisconsin, Milwaukee WI 53226

[2]Department of Biochemistry & Molecular Biology, Microbiology and Immunology, Hollings Cancer Center, Medical University of South Carolina, Charleston SC 29425

Abstract

Vaccinia virus is the prototypic poxvirus. The 192 kilobase double-stranded DNA viral genome encodes most if not all of the viral replication machinery. The vaccinia virus DNA polymerase is encoded by the E9L gene. Sequence analysis indicates that E9 is a member of the B family of replicative polymerases. The enzyme has both polymerase and 3′–5′ exonuclease activities, both of which are essential to support viral replication. Genetic analysis of E9 has identified residues and motifs whose alteration can confer temperature-sensitivity, drug resistance (phosphonoacetic acid, aphidicolin, cytosine arabinsode, cidofovir) or altered fidelity. The polymerase is involved both in DNA replication and in recombination. Although inherently distributive, E9 gains processivity by interacting in a 1:1 stoichiometry with a heterodimer of the A20 and D4 proteins. A20 binds to both E9 and D4 and serves as a bridge within the holoenzyme. The A20/D4 heterodimer has been purified and can confer processivity on purified E9. The interaction of A20 with D4 is mediated by the N′-terminus of A20. The D4 protein is an enzymatically active uracil DNA glycosylase. The DNA-scanning activity of D4 is proposed to keep the holoenzyme tethered to the DNA template but allow polymerase translocation. The crystal structure of D4, alone and in complex with $A20_{1–50}$ and/or DNA has been solved. Screens for low molecular weight compounds that interrupt the $A20_{1–50}$/D4 interface have yielded hits that disrupt processive DNA synthesis in vitro and/or inhibit plaque formation. The observation that an active DNA repair enzyme is an integral part of the holoenzyme suggests that DNA replication and repair may be coupled.

Keywords

poxvirus; vaccinia; DNA polymerase; processivity factor uracil DNA glycosylase; DNA replication

[#]**Corresponding Author:** Paula Traktman, PhD, Dean of the College of Graduate Studies and Hirschmann Endowed Professor, traktman@musc.edu, 843-876-2405 or 843-876-2414.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

1. Introduction

Poxviruses are one of the defining members of the NCLDV, “nucleocytoplasmic large DNA viruses”, which exhibit some common genetic and functional features (Colson et al., 2013) and have been proposed as a new order, *Megavirales.* Poxviruses (Moss, 2013) are large, complex enveloped viruses with a dsDNA genome. One of their distinguishing characteristics is the fact that they replicate exclusively in the cytoplasm of infected cells. The two subfamilies of the Poxviridae, *Chordopoxvirinae* and *Entomopoxvirinae*, are distinguished by their vertebrate and insect host range, respectively *Chordopoxvirinae* are in turn assigned to 10 genera; the remainder of this chapter will focus on the prototypic member of the *Orthopoxvirus* genus, vaccinia virus (Moss, 2013). Vaccinia virus is closely related to variola virus, the etiological agent of smallpox, and was in fact the virus used as the vaccine in the successful campaign that led to the eradication of variola as a natural pathogen in 1977. Vaccinia enters cells either by micropinocytosis or by direct fusion at the plasma membrane. The internal viral core remains intact during the early phase of infection and serves as the site for the transcription, capping and polyadenylation of early mRNAs, all of which are accomplished by encapsidated viral proteins. The core then uncoats, and early proteins mediate the replication of the viral genome; two subsequent phases of intermediate and late gene expression ensue. A complex process of morphogenesis occurs in the cytoplasm of infected cells; the vast majority of mature virions remain within the infected cell and are thought to mediate host:host spread; a minority of virions acquire an additional envelope and are released by exocytosis, where they mediate cell:cell and distal spread within the host.

2. Genome structure

Members of the *Poxvirdae* family are characterized by large, double-stranded DNA genomes that range in size from 130 to 300 kb and encode more than 200 proteins (Moss, 2007). The 195 kb, AT-rich vaccinia genome is a single contiguous polynucleotide chain which self-anneals into a linear duplex with covalently closed hairpin termini (Figure 1A). The 104 nucleotide telomeric hairpins have an AT-content of approximately 92% and contain 12 unpaired, extrahelical bases (10 on one strand, 2 on the other) (Baroudy et al., 1982; Baroudy et al., 1983; Goebel et al., 1990). They are found in two inverted, complementary isoforms known as flip and flop (Figure 1B) (Du and Traktman, 1996). As will be described below, these hairpins are re-generated during each round of genome replication by cruciform extrusion and Holliday junction resolution of the concatemeric replication intermediate (DeLange et al., 1986; Merchlinsky, 1990). Sequences needed for the process of concatemer recognition and resolution are found proximal to the 104 nucleotide hairpins, and analysis of viral minichromosomes has shown that the terminal 200 bp of the viral genome are necessary and sufficient for replication and resolution (Du and Traktman, 1996). The unique structure of the hairpin termini is recognized by the virally encoded telomere-binding protein (I6), and ultimately facilitates the specific encapsidation of the genome into progeny virions (DeMasi et al., 2001; Grubisha and Traktman, 2003).

Positioned inward from the terminal hairpins and concatemer resolution sequences are two sets of 70 bp, tandem repeats which exhibit high sequence conservation and are

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

hypothesized to play a role in intergenomic recombination (Baroudy et al., 1982; Wittek and Moss, 1980). There is some variability in the number of repeats among various strains of vaccinia virus, with the Western Reserve (WR) isolate exhibiting 13 and 18 repeats, respectively (Baroudy and Moss, 1982; Baroudy et al., 1982). A small number of protein-coding genes are encoded in a diploid manner, with duplicate copies found adjacent to the tandem repeats at each end of the genome (Moss, 2013). The vast majority of the genes are encoded as single copies, however. Interestingly, genes that may be dispensable for replication in tissue culture but encode proteins that modulate the host environment (eg., immunomodulators) are found near the termini. These genes, which are often species-specific, are thought to have been acquired more recently, suggesting that genome evolution via recombinational insertion may be focused within distal regions of the genome. The bulk of the essential genes encoding the machinery required for the viral life cycle are found in the central two-thirds of the genome. Here, tightly packed genes of both polarity are found, with no obvious clustering by temporal class or life cycle function. In contrast, the genes nearest the termini are largely oriented with transcription proceeding toward the hairpin termini. Vaccinia virus genes contain no introns and do not undergo any splicing; moreover, the 5′ UTRs are quite short. The commonly accepted gene nomenclature is based on a scheme originally proposed for the first sequenced vaccinia virus, the Copenhagen strain (Goebel et al., 1990). The method is based on a restriction fragment map generated by HindIII digestion of the mature viral genome. Genes were annotated using an alphanumeric code based on the restriction fragment (ranging in descending size from A to P) in which they initiate, their sequential position within that fragment, and the orientation of the open reading frame with respect to the fully assembled genome. In the case of the gene coding for the viral DNA polymerase, E9L, the gene is the 9th open reading frame within the fifth largest HindIII restriction fragment (E), and it is transcribed in a leftward orientation with respect to the genome. More recently, as complete genomes of other strains are sequenced, a simpler nomenclature in which the ORFs are numbered sequentially from left to right has been used; however, common practice is to then refer to the Copenhagen ortholog for all subsequent discussion.

3. Discovery tools for identification of the DNA polymerase and its processivity factor

The isolation and characterization of collections of temperature-sensitive (*ts*) mutants has been essential to functional studies of poxvirus replication (Condit and Motyczka, 1981; Condit et al., 1983; Dales et al., 1978; Drillien and Spehner, 1983; Ensinger, 1982). The majority of the mutants were isolated by the Dales (D) and Condit (C) laboratories, and these initials are placed before the name/number of the mutant to clarify their origin. Because homologous recombination is robust within infected cells, mapping of these mutations by marker rescue, coupled with complementation analyses, has led to the determination of the genes affected in most of these mutants. Mutants with altered drug sensitivity or altered replication fidelity have also been instrumental in the study of viral replication, and in particular contributed to the structure/function analysis of the DNA polymerase itself. The heterodimeric processivity factor (A20 and D4 [UDG], see below) was identified biochemically, with genetic, biochemical and structural approaches

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

elucidating its structure and function. The other members of the virally encoded replication machinery I3 [ssDNA binding protein], D5 [ATPase, primase/helicase], G5 [FEN-family endonuclease]. A50 [DNA ligase], H5 [abundant hub protein] and B1 [protein kinase], which are largely beyond the scope of this review, were also identified through a combination of genetic and biochemical means.

4. The E9 DNA Polymerase: Identification and Biochemical Properties

The first biochemical characterization of the vaccinia-encoded DNA polymerase was performed in 1979 and the gene encoding the enzyme was described in 1984 and 1985 (Challberg and Englund, 1979a; Challberg and Englund, 1979b; Earl et al., 1986; Traktman et al., 1984). Purification of the virally-induced polymerase enzymatic activity was accomplished by sequential chromatography, which suggested that the polymerase activity originated from a single polypeptide sized between 110 kDa to 115 kDa (Challberg and Englund, 1979b). Subsequent investigations have confirmed that the vaccinia virus E9L gene encodes a 1,006 amino acid, 116 kDa polypepide which has both 5′-to-3′ DNA polymerase activity as well as 3′-to-5′ exonuclease activity (Challberg and Englund, 1979b; Jones and Moss, 1985; McDonald and Traktman, 1994a). Sequence analysis of the vaccinia polymerase reveals evident homology to the replicative B-family of PALM polymerases (Pol α and Pol δ) encoded by mammalian cells and a variety of DNA viruses such as Herpes simplex virus (HSV) (Blanco et al., 1991; Coen, 1996; Earl et al., 1986; Wong et al., 1988; Zhang et al., 1991). Because of a common interest in generating antiviral therapeutics that target the HSV or VV DNA polymerase, comparisons of these two viral polymerases have been common (Gibbs et al., 1988). As described below, we do not yet have a crystal structure of the vaccinia DNA polymerase, however, homology to HSV polymerase has allowed for significant in silico structural modelling to be performed (Sele et al., 2013). In broad terms, as shown in Figure 2, the domain associated with 3′–5′ exonuclease activity (motifs exo I, II and III) lies in the N′ terminal half of the protein. However, the N′ terminal half is not likely to be an autonomous domain, because region IV, a motif associated with DNA polymerase activity is interspersed within the exonuclease-associated motifs. The bulk of the conserved motifs associated with polymerase activity lie within the C′-terminal half of the protein.

4.1 Expression profile

The temporal pattern of E9L expression is consistent with a role early in infection. Transcriptional analysis and immunoprecipitations carried out by McDonald and Traktman indicated that expression of the E9L gene and E9 protein begin within the first hour of infection, peak between 2 and 3.5 hours post infection, and are shortly followed by a decline in nascent synthesis to undetectable levels by 6.5 hours post infection (McDonald et al., 1992). Treatment of infections with cycloheximide, which blocks uncoating and the release of the genome from the intracellular viral core, results in prolonged transcription of E9L mRNAs, confirming that the usually transient profile of transcription is coupled to the process of uncoating (McDonald et al., 1992). Moreover, analysis of infections that fail to progress to intermediate and late gene expression, either through the use of *ts* mutants or via inhibition of DNA synthesis with AraC treatment, revealed no gross alteration in the

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

expression dynamics of E9L. Hence, the transcription of the DNA polymerase mRNA is normally limited to the early phase of the life cycle, and is independent of intermediate and late phases of gene expression as well as the process of DNA replication itself (McDonald et al., 1992).

4.2 dNTP preference and template requirements

Kinetic analysis of purified polymerase revealed K_m values of 0.9, 2.9, 4.0, and 2.7 μM for dGTP, dATP, TTP, and dCTP, respectively (McDonald and Traktman, 1994a). These data indicate that the high AT content of the vaccinia virus genome (~68%) was not driven by a preference of the polymerase for dATP and TTP. The polymerase has also been shown to be able to use dUTP in place of TTP with comparable efficiency (Boyle et al., 2011). As will be described below, the processivity factor of the DNA polymerase has intrinsic uracil DNA glycosylase (UDG) activity, and can remove uracil from dUMP residues present within the DNA. UDG action leaves an abasic site; when the polymerase encounters an abasic site in the template strand, it cannot proceed with synthesis (Boyle et al., 2011). By this definition, the polymerase cannot perform “translesion” synthesis.

4.3 Intrinsic distributive mode of action

In purified form, the polymerase acts distributively. In the presence of either 10 mM $MgCl_2$ or 40 mM NaCl, DNA synthesis using a primed M13 template occurred at a maximal rate of 8 nucleotides sec^{-1}, with fewer than 10 nucleotides being added per binding event (McDonald and Traktman, 1994b). Furthermore, these reactions required the addition of *E. coli* single strand binding protein, evidence of the enzyme’s lack of inherent strand displacement ability. This hypothesis is reinforced by the observations that the purified polymerase added only 1–2 nucleotides to the 3′ of a nicked template and was unable to replicate through a primed, φX174-derived template due to secondary loop structures at three distinct sequences of the φX174 DNA (Challberg and Englund, 1979a; Challberg and Englund, 1979b; McDonald and Traktman, 1994b). While the adjustment of in vitro conditions was conducive to driving processive polymerization, yielding a reported rate of up to 30 nucleotides sec^{-1} and a 200-fold increase in the number of nucleotides added per binding event, the parameters required were a significant departure from physiological conditions (1 mM $MgCl_2$ in the absence of NaCl) (McDonald and Traktman, 1994b). Cumulatively, these findings reinforced the conclusion that additional cofactors were needed for efficient polymerization in vivo (McDonald and Traktman, 1994b). These analyses suggested that the stability of the template-enzyme interaction played a significant role in the ability of the polymerase to proceed with processive synthesis – as demonstrated by the addition of 40 mM NaCl forcing a reversion to distributive synthesis (McDonald and Traktman, 1994b). Further evidence for this hypothesis stemmed from template switching experiments in which competition of primed templates preincubated with VACV cellular extracts, followed by release into active polymerization conditions via the addition of radiolabeled α-^{32}P-dTTP did not reveal any evidence of polymerase switching to a competitor template (McDonald et al., 1997). The addition of extracts prepared from infected cells that are deficient in functional polymerase, and blocked for intermediate and late gene expression (via hydroxyurea treatment), was also able to reconstitute processive DNA synthesis purified E9 polymerase. These data suggested that the VACV polymerase

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

depends on a virally encoded, early protein to mediate stable interaction with templates and facilitate the shift from distributive to processive DNA synthesis (McDonald et al., 1997). The processivity factor will be discussed in depth below. The aforementioned requirement for the addition of the *E. coli* ssDNA binding protein is also representative of a need for a helix destabilizing protein, and consistent with Challberg's report of an inability of the purified polymerase to move through a template with secondary structure (Challberg and Englund, 1979a).

4.4 Proofreading exonuclease activity

A number of studies from the Evans laboratory have confirmed the 3′-to-5′ exonuclease activity originally ascribed to the VACV DNA polymerase by Challberg in 1979 (Challberg and Englund, 1979a; Challberg and Englund, 1979b; Gammon and Evans, 2009; Hamilton and Evans, 2005). Careful biochemical analysis has revealed that the exonuclease activity has a 3′-to-5′ polarity exclusively, is active on single stranded oligonucleotides or on a 3′ overhanging strand of duplexed DNA, but appears to be significantly less active on nicked, or blunted double stranded DNA (Hamilton and Evans, 2005). In vitro polymerase and nuclease assays reinforce the expectation that at physiological conditions, specifically with respect to dNTP concentration, both functions of the DNA polymerase appear to be active: 5′-to-3′ polymerization and 3′-to-5′ exonuclease (Hamilton and Evans, 2005).

5: The DNA Polymerase: Genetic Dissection

A variety of groups have undertaken forward genetic studies that have further elucidated the domain structure and biochemical activity of the DNA polymerase. These studies have led to the identification of E9L alleles that confer temperature sensitivity, inhibitor resistance and mutator and anti-mutator phenotypes (summarized in Figure 2B). Work in the early 1980's identified two temperature sensitive mutants, C*ts*42 and NG26, both exhibiting defective DNA synthesis at non-permissive temperature (Condit et al., 1983; Sridhar and Condit, 1983). The alleles were later shown to carry G→A transitions that led to a Gly →Asp substitution, at position 392, in NG26 and a Glu→Lys substitution, at position 611, in C*ts*42 (Traktman et al., 1989; Traktman et al., 1984). In vitro studies confirmed that the polymerase activity in NG26-infected cells was temperature sensitive, and that the C*ts*42-encoded enzyme was labile and exhibited no enzymatic activity in vitro (McDonald et al., 1997; Sridhar and Condit, 1983). Two additional *ts* mutants (D*ts*83 and D*ts*20) that led to a DNA-negative phenotype at the non-permissive temperature were shown to contain H185Y and S686N substitutions within the E9 open reading frame, respectively (Kato et al., 2008). These mutations are summarized in Fig 2B (orange text, above the schematic of the DNA polymerase ORF).

5.1 Phosphonoacetate (PAA)-resistant mutants

The NG26 allele (see above) contains a second mutation which results in an additional Gly→Asp substitution at residue 372; this substitution is unrelated to the temperature-sensitive phenotype but is responsible for conferring resistance to phosphonoacetic acid (PAA). PAA is an inhibitor of pyrophosphate exchange, which occurs each time that a dNTP is incorporated into the growing DNA chain. The same amino acid change was identified in

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

a separate, 1986 study, in which Earl and Moss sequenced a 5kb segment of vaccinia genome capable of conferring PAA resistance through marker rescue (Earl et al., 1986). In a 1993 study, Cys356→Tyr and Gly380→Ser substitutions were also shown to confer PAA resistance (Taddie and Traktman, 1993). Together these data indicate the tight cluster of residues 356, 372, and 380 have an intimate relationship with pyrophosphate exchange. These residues lie between the exo II and exo III motifs within a region that is unique to the poxvirus polymerase and has been denoted Pox Insert 2 (Sele et al., 2013).

5.2 Aphidicolin (aphid)-resistant mutants

Aphidicolin, a tetracyclic diterpenoid isolated from the fungus *Cephalosporum aphidicola* has been shown to inhibit the vaccinia DNA polymerase, and other members of the Family B replicative polymerases, by competing with dCTP for binding and displacing the template guanine from the proper position (Baranovskiy et al., 2014; Pedrali-Noy and Spadari, 1980; Taddie and Traktman, 1991). After the first demonstration that vaccinia virus replication was acutely sensitive to aphidicolin, a forward mutagenesis screen was undertaken and resulted in the isolation of an aphid^r VACV population. (DeFilippes, 1984). Aphid^r virus exhibited < 10% decrease in viral yield in the presence of drug, compared to the >99% inhibition seen with WT infection; moreover, DNA synthesis in this isolate was reported to be >4-fold resistant at the typically inhibitory concentrations of aphidicolin (80 μM). In vitro assays of the purified aphid^r virus showed >2-fold increase in the IC_{50} of the inhibitor. Subsequent nucleotide sequence analysis reported the lesion responsible for this resistance to be a Leu670→Met substitution within the E9 polymerase – an amino acid localized within the polymerase III domain of the polypeptide (DeFilippes, 1989). Additional mutations conferring aphid^r (Fig 2B, purple text, below the schematic of the DNA polymerase ORF) were identified after serial passage of a nitrosoguanidine mutagenized stock of vaccinia virus in the presence of 85 μM aphidicolin. From this procedure ten independent isolates of aphidicolin resistant vaccinia virus, each of which exhibited a substitution at Ala498 of the E9 polymerase were isolated (Taddie and Traktman, 1991). The recurrent isolation of mutants at this position strongly suggests that alanine 498 plays a significant role in aphidicolin binding, and likely to be a key residue located in or near the binding pocket for dNTPs. Sequencing of these ten isolates revealed mutations conferring a change to threonine or valine at this position, and both mutations conferred roughly equivalent levels of aphid^r, with viral yield in congenic strains engineered to express the mutations being ~3.4-log greater than WT in the presence of 85 μM aphidicolin (Taddie and Traktman, 1991). Furthermore, the A498V and A498T mutations exhibited, respectively, moderate and acute hypersensitivity to PAA or 9-β-D-arabinofuranosyladenine (AraA), and moderate hypersensitivity to 1-β-D-arabinofuranosylcytosine (AraC). While it is interesting that resistance to a dCTP competitor, aphidicolin, conferred increased sensitivity to, and thus likely altered affinity for, the nucleotide analogues AraA and AraC, previous studies in Herpes simplex virus and other systems have reported similar trends (Gibbs et al., 1988; Hall et al., 1989; Matsumoto et al., 1990; Nishiyama et al., 1985). Similarly, this inverse relationship between aphidicolin and PAA / nucleoside analogue resistance and sensitivity appears to hold true for the three aforementioned mutations conferring PAA resistance: C356T, G372D, G380S. Congenic PAA^r viruses carrying these alleles were tested for sensitivity to aphidicolin treatment, and the reduction of viral yield by 20 μM aphidicolin

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

was 20–1000 fold more severe than what was observed with WT virus (Taddie and Traktman, 1993). In sum, PAA^r appears to confer aphidicolin hypersensitivity, and vice versa, although the mechanism behind this mutual effect remains unclear.

5.3 Cytosine arabinoside (AraC)-resistant mutants

This convoluted relationship between aphid^r and nucleoside / pyrophosphate analogue sensitivity was complicated further by the 1993 Taddie study which concluded that AraC resistance caused by mutation of Phe171→Ser was dependent on the presence of the C356T, G372D, or G380S PAA^r mutations. Detailed analysis revealed that in the absence of any drugs, both DNA replication and viral yield were unperturbed in the dual (F171S plus C356T, G372D, or G380S) mutants. These mutants were PAA^r, and while AraC treatment did reduce viral yield nearly 10-fold, this was still representative of a multi-log increase in resistance when compared to WT virus (Taddie and Traktman, 1993). In contrast to the identification of various residues hypothesized to play a role in dNTP binding, this AraC resistant mutation maps within or adjacent to the exo I motif of the 3′-to-5′ exonuclease domain of the E9 polymerase (Figure 2B, maroon text, below the schematic of the DNA polymerase ORF). AraC, also referred to as cytosine arabinoside, is a deoxycytidine analogue that competitively binds to the viral polymerase and is incorporated into a growing DNA chain. The inhibitory effects of AraC treatment are hypothesized to be mediated by the combination of an inability of susceptible polymerases to extend a DNA chain with a terminal arabinose moiety, and the resistance of incorporated AraC molecules to excision by the 3′-to-5′ proofreading activity of susceptible polymerases (Derse and Cheng, 1981; Kufe et al., 1984; Mikita and Beardsley, 1988; Ohno et al., 1988). Given the mapping of the AraC^r mutation to E9′s 3′-to-5′ exonuclease domain, it stands to reason that F171S confers increased proofreading affinity for incorporated arabinoside bases, although this hypothesis remains to be tested experimentally.

5.4 Cidofivir (CDV)-resistant mutants

Vaccinia virus, and the DNA polymerase itself, is sensitive to inhibition by the acyclic nucleoside phosphonate (ANP) cidofovir (CDV, HPMPC), (S)-1-[3-hydroxy-2-(phosphonomethoxypropyl) cytosine], a well-known and FDA approved ANP for the treatment of HCMV induced retinitis (Andrei and Snoeck, 2010). Work from the Evans laboratory among others has extensively characterized the susceptibility of the vaccinia polymerase to treatment with CDV (Andrei et al., 2006; Gammon and Evans, 2009; Gammon et al., 2008; Julien et al., 2011; Kornbluth et al., 2006; Magee et al., 2005). CDV can likely act as a competitive inhibitor of dCTP binding, while diphosphorylated-CDV can also function as an alternative substrate and become incorporated into a growing polynucleotide chain. CDV does not serve as a direct chain terminator like other nucleoside analogues (ie. cytosine arabinoside). However, in a variety of in vitro assays carried out by Magee et al, it was found that while purified VACV E9 polymerase is capable of incorporating CDVpp into a primed-template, the polymerization reaction was slowed at the N+1 position (where N is the location of CDV incorporation) (Magee et al., 2005). More detailed analysis estimated at least a 3-fold (though likely a gross underestimate) delay in extension of a primer incorporating CDV, a defect that was further exacerbated by the incorporation of two neighboring CDV molecules (Magee et al., 2005). Many of these

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

results were replicated with HPMPA, the acyclic nucleoside phosphonate analogue of adenine; however, E9 did prove to be slightly more amenable to HPMPA incorporation and primer extension. DNA molecules primed with terminal HPMPC and HPMPA were found to be comparable substrates for the 3′ to 5′ exonuclease activity of VACV polymerase when compared with substrates terminating in dCTP and dATP, respectively. However, positioning the nucleoside analogue in the penultimate 3′ position abrogated the 3′ to 5′ exonuclease activity (Magee et al., 2005). While these ANPs were shown to be competent for incorporation into a nascent DNA strand, primer extension assays also clearly demonstrated that polymerization directed by a template incorporating either HPMPC or HPMPA was strongly inhibited, indicating that VACV was unable to perform translesion synthesis in the presence of template ANPs (Magee et al., 2008). These studies culminated in the estimation of the kinetic parameters for VACV polymerase interaction with (S)-HPMPCpp and (S)-HPMPApp: reporting the K_m and V_{max} for (S)-HPMPCpp to be 23 ± 6 μM and 3.0 ± 0.4 pmol min^{-1} versus 3.8 ± 0.7 μM and 2.4 ± 0.2 pmol min^{-1} for dCTP, and K_m and V_{max} for (S)-HPMPApp to be 3.8 ± 0.8 μM and 2.1 ± 0.1 pmol min^{-1} versus 4.6 ± 0.5 μM and 2.0 ± 0.07 pmol min^{-1} for dATP (Magee et al., 2008; Magee et al., 2005).

As with PAA, aphidicolin and AraC, a variety of CDV resistant (CDR^r) mutants have been isolated by several different groups. These include substitutions in both the 3′–5′ exonuclease domain (H296Y, A314T, A314V, H319W, S338F) as well as the 5′–3′ polymerization domain of E9 (R604S, M671I, A684V) (Andrei et al., 2006; Becker et al., 2008; Kornbluth et al., 2006) (Figure 2B, blue text below the schematic of the DNA polymerase ORF). The best characterized of these mutations are the A314T, and A684V substitutions. Individually, A684V and A314T conferred an intermediate (EC_{50} 140 ± 20 μM) and strong (EC_{50} 240 ± 20 μM) resistance to CDV, respectively, as well as cross-resistance to some “second” and “third” generation ANPs (Andrei et al., 2006). The resistance profiles for each of these mutations, as well as the S851Y and T831I mutations which confer preferential resistance to deoxyadenosine analogs, have been analyzed in detail (Duraffour et al., 2012; Gammon et al., 2008). This complex pattern of cross-resistance indicates that many of the “second” and “third” generation ANPs may function in unique ways. During the characterization of these mutants Andrei et al. also reported a strong synergy between these two residues, with a double mutant exhibiting exceptional levels of resistance (EC_{50} 790 ± 40 μM) and the further addition of the Y232H substitution pushing resistance even higher (EC_{50} 1,340 ± 50 μM) (Andrei et al., 2006). Interestingly, when tested for $aphid^r$ the A314T mutation conferred resistance, whereas the A684V mutation conferred 2-fold hypersensitivity (compared to WT); transfer of both mutations into an otherwise WT background appeared to bring the effects of each mutation alone into equilibrium, resulting in a near WT sensitivity to aphidicolin (Andrei et al., 2006). Alanine 684 maps to the polymerization domain of E9, and based on structural modeling of VACV E9 to other B-family DNA polymerases, is hypothesized by Andrei et al. to be positioned proximally to Tyr668, a residue important for correct base pairing to the template strand (Andrei et al., 2006). Furthermore, as Andrei points out, studies of the RB69 polymerase indicate that perturbation of amino acids neighboring Thr688 in 3D space significantly altered the equilibrium constant of the polymerase for dNTPs (Andrei et al., 2006) In contrast, the Ala314 mutation maps to the 3′-to-5′ exonuclease domain of VACV polymerase. While the

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

details of how this mutation confers resistance to HPMPC remain unknown, the clear implication is that the substitution of a larger uncharged amino acid (valine or threonine) in this position augments the ability of VACV polymerase to excise nucleoside analogues in in the penultimate 3′ primer position.

5.5 Polymerase fidelity: mutator and antimutator phenotypes

Detailed analysis and discussion of how each of the mutations discussed above confer resistance to anti-poxviral drugs awaits the publication of an E9 structure. However, based on structural prediction and sequence alignment, many of these mutations can be localized to the polymerase or exonuclease domains of the vaccinia virus DNA polymerase. The conjecture follows that the substitutions confer a reduced ability to incorporate nucleoside analogues, alter the rate of translesion synthesis, or affect the innate 3′–5′ exonuclease proofreading ability. These mechanisms might be expected to influence the overall fidelity of the polymerase enzyme, and in fact some of the mutations reviewed above have been documented to bestow mutator or anti-mutator phenotypes (Figure 2B, * or #, below the schematic of the DNA polymerase ORF). Three alleles, those encoding A498V, A684V, and S851Y substitutions, were found to confer an increased mutation frequency in forward-mutagenesis screens for resistance to unrelated inhibitors of the viral life cycle (Andrei et al., 2006; Taddie and Traktman, 1991). The A498V mutant, which is an aphid^{r} and PAA^{hs} isolate, exhibited a 20- to 40- fold increase in the development of spontaneous resistance to isatin-β-thiosemicarbazone (IBT) or rifampicin, inhibitors of viral transcription and virion assembly, respectively. Interestingly, while the A498T mutation conferred a comparable aphid^{r} phenotype, it did not display a comparable increase in mutation rate – suggesting that substitution of valine for alanine 498 has a unique and specific effect on the misincorporation of erroneous nucleotides during viral DNA replication. Similarly, as part of their isolation of CDV resistant mutants, Andrei et al. screened the A684V and A314T isolates for increased mutation rate and found a roughly 4-fold increase in spontaneous resistance to IBT treatment in the A684V isolate (Andrei et al., 2006). The A314T mutation, which also confers CDV^{r}, did not confer an increased frequency of IBT resistant mutants. As described above, Ala684 is hypothesized to lie close to the catalytic core of the enzyme, and thus mutation of residues in this pocket may reasonably be expected to generate mutator / anti-mutator phenotypes. In a subsequent study assessing the cross resistance to adenosine analogues of CDV (HPMPA), a Y851S substitution was shown to exhibit preferential resistance to dAMP analogs (Gammon et al., 2008). Because this mutation was found to synergize with the previously identified A684V substitution to generate HPMPA resistance, the group assessed the forward mutation rate of both the S851Y mutant and the A684V+S851Y double mutant. Interestingly, while the S851Y mutant displayed a roughly 10-fold increase in rate of acquisition of IBT resistance, the addition of the hypermutator A684V substitution appeared to attenuate the overall rate of mutation – though that frequency remained significantly higher than that of WT (Gammon et al., 2008).

During the course of investigating the C356Y, G372D, and G380S PAA^{r} mutations and their relationship to the AraC^{r} F171S mutation, congenic viruses were also investigated for the frequency of spontaneous acquisition of resistance to rifampicin and IBT (Taddie and Traktman, 1993). The three mutations responsible for PAA^{r} in this context conferred 10- to

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

40-fold decreases (antimutator) in the frequency of spontaneous rifamipicin resistance, while the addition of the F171S mutation, conferring AraC^r, had no detectable impact on mutation frequency. Furthermore, in Taddie's testing, the hypomutator phenotype was puzzlingly confined to rifampicin resistance; no difference in the frequency of IBT^r was identified. The apparent lack of impact from the F171S substitution on the overall rate of mutation strongly suggest that the hypomutator phenotype of these congenic viruses is unrelated to any changes in exonuclease proofreading capacity caused by the F171S mutation. As Taddie and Traktman go on to hypothesize, since the mutations conferring PAA^r are sufficient to reduce the rate of mutation, it is tempting to speculate that an altered interaction with pyrophosphate may dampen the overall rate of polymerization, thereby increasing overall fidelity by providing a longer period of time to achieve stable enzyme-dNTP binding, or proofreading.

6. Pol in replication, but also in recombination

One feature of poxvirus replication is an inherent link between nascent DNA synthesis and homologous recombination. In fact, this process of homologous recombination is at least in part facilitated by the viral DNA polymerase, specifically requiring the 3′-to-5′ proofreading exonuclease functionality of the polymerase at multiple points throughout the recombination reaction (Gammon and Evans, 2009). First, it was hypothesized that the 3′-to-5′ exonuclease activity would be required to prepare substrates for strand invasion / transfer. A series of studies from the Evans laboratory have confirmed that DNA polymerase is sufficient to mediate a strand-transfer reaction between two recombination substrates in vitro (Willer et al., 1999; Willer et al., 2000). Initially, coincubation of DNA polymerase together with fully duplexed DNA oligonucleotide, as well as circular-single-stranded DNA, resulted in the formation of a distinct, joint molecule. Electrophoretic and EM analysis of these complexes revealed this product to be the result of a strand exchange reaction; in effect the product molecule is representative of base-pairing between the circularized, single stranded DNA and a number of bases from the previously blunted duplex (Willer et al., 1999). In depth, biochemical analysis of these reactions suggested that the synapsis step required stoichiometric amounts of the E9 polymerase, but was also dependent on the use of catalytically active DNA polymerase (Willer et al., 1999). Subsequent studies of end-labeled DNA duplexes showed that this process was mediated by 3′ end resection of the invading DNA, required at least 12 bp of sequence homology between substrates, was both stimulated and stabilized by the viral single strand binding protein, I3 (Willer et al., 2000). These studies, as well as previous work suggesting that the majority of VACV recombination events depend on 5′ strand invasion for single strand annealing, suggest that the 3′-to-5′ exonuclease activity of the viral DNA polymerase mediates the initial steps in synapsis formation.

Second, coincubation of imperfectly duplexed junctions with the E9 polymerase was shown to result in processing of branched, 3′ DNA overhangs into nicked, fully duplexed substrates competent for ligation by T4 DNA ligase. These data suggested that the DNA polymerase might play a role in resolving 3′ overhangs generated during the course of viral recombination (Hamilton and Evans, 2005). This finding is in congruence with the possibility that vaccinia DNA polymerase may metabolize the products of in vivo single-

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

strand annealing reactions into ligatable substrates, in effect facilitating the post-synaptic steps of recombination.

Lastly, a catalytically active 3′-to-5′ exonuclease domain within the VACV DNA polymerase was conclusively shown to be indispensable for the process of recombination in vivo (Gammon and Evans, 2009). Selectively inhibiting the proofreading activity of the DNA polymerase while leaving intact an active polymerization domain significantly reduced recombinatorial frequency in vitro and in vivo. By carefully exploiting the polymerase's inability to excise a terminally incorporated cidofovir molecule, Gammon et al. were able to assay the recombination frequency on substrates derived from linearized plasmids containing two fragments of the luciferase gene. This system clearly demonstrated robust homologous recombination in VACV infected cells: transfection of both fragments of the luciferase gene resulted in reconstitution of the full-length luciferase gene, as assessed by demonstrable luciferase activity. In contrast, when the exonuclease activity of polymerase was inhibited using the terminal incorporation of CDV moieties, a significant reduction in luciferase activity, ergo homologous recombination, was observed. This same assay showed superior (compared to WT) levels of recombination with CDV-incorporated substrates in cells infected with the A314T CDV-resistant virus, in which the DNA polymerase exhibits an increased ability to excise CDV molecules. In sum, the viral DNA polymerase serves both an integral role in DNA synthesis and recombination during viral DNA replication. The observation of tight linkage between replication and recombination suggests that recombination-based priming may be an inherent feature of poxvirus DNA replication. Indeed, the inability to isolate viruses encoding exonuclease-deficient alleles of E9 strongly suggests that exo activity plays an essential role during infection (Gammon and Evans, 2009).

7. Assembly of a Processive Holoenzyme: the A20 and D4 (UDG) proteins

As described above, the vaccinia virus DNA polymerase is inherently distributive, adding <10 nt per primer/template binding event (McDonald and Traktman, 1994b). However, extracts from virally infected cells contain a highly processive form of the polymerase (McDonald et al., 1997), and indeed all replicative polymerases associate with a processivity factor. Two main types of processivity factors predominate (Hedglin et al., 2013; Weller and Coen, 2006; Weller and Coen, 2012). The primary prokaryotic and eukaryotic replicative polymerases associate with a protein that multimerizes to form a toroidal ring that encircles the DNA; the best studied protein of this type is PCNA. An "opened" form of the processivity factor is loaded onto the DNA by a clamp loader in an ATP-dependent fashion. The closed toroidal ring lacks DNA binding activity, but is topologically tethered to the DNA and, through its polymerase-binding activity, keeps the polymerase from disassociating from the template. In contrast, many prokaryotic and eukaryotic viruses (eg., Herpes simplex virus) associate with a processivity factor that has intrinsic DNA binding activity; by associating with the polymerase these processivity factors keep the polymerase loosely tethered to the DNA primer/template to facilitate rapid long-chain synthesis.

We now know that the processivity factor for the vaccinia E9 polymerase is a heterodimer of two virally encoded proteins, A20 and D4. As initially characterized by a number of studies

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

from the Traktman laboratory, A20 was shown to co-purify with the processive form of the vaccinia E9 polymerase, and the overexpression of A20 led to an increase in processive polymerase activity (Klemperer et al., 2001; McDonald et al., 1997). In addition, A20 and E9 were shown to interact in vivo as well as in vitro. Moreover, A20 was found to interact with a number of viral replication proteins in yeast two-hybrid analyses (McCraith et al., 2000). Temperature-sensitive mutants were isolated by both the Moss and Traktman laboratories after targeted mutagenesis of A20 (Ishii and Moss, 2001; Punjabi et al., 2001) and were shown to be grossly defective for DNA replication at the non-permissive temperature; in some cases, the absence of processive polymerase activity at the non-permissive temperature was also verified. However, expression and purification of A20 on its own was not feasible, suggesting that there might be another component of the holoenzyme. Two groups (Ishii and Moss, 2002; McCraith et al., 2000) identified a strong interaction between the A20 protein and the D4 protein in two-hybrid, co-immunoprecipitation and MBP-pull down assays. These studies revealed that residues 1–25 of A20 (in both yeast-two hybrid and MBP-A20 pull down assays) were sufficient to interact with D4, although more efficient binding was seen when residues 1–50 were used as the bait (Figure 3A, maroon box). The D4 protein had already been characterized as a uracil DNA glycosylase, the initial enzyme in the base excision repair pathway (Stuart et al., 1993; Upton et al., 1993). The A20/D4 interaction data suggested that the VACV UDG might play an integral role in DNA synthesis as well as DNA repair. Indeed, De Silva and Moss reported that a catalytically null form of D4 could sustain wild-type replication of vaccinia virus in tissue culture (but not in mice), whereas viral DNA replication was abrogated during infections with a D4-null virus (De Silva and Moss, 2003). Insight into the role played by D4 emerged from a key 2006 study (Stanitsa et al., 2006) which clarified that the previously observed insolubility of recombinant A20 protein could be circumvented through the co-purification of A20 in complex with 3xFlag-tagged UDG. This work presented the in vitro reconstitution of a highly processive DNA polymerase complex, made up of E9 / A20 / UDG in apparent 1:1:1 stoichiometry, and demonstrated that the A20 / UDG heterodimer was sufficient to confer processivity on the vaccinia E9 DNA polymerase. Further detailed discussion of each of these factors can be found below. Briefly, A20 is thought to bridge Pol and D4, and D4 is thought to maintain holoenzyme/DNA association in a manner that facilitates rapid and processive DNA synthesis.

7.1 Structure/function analyses of A20

In the pursuit of identifying the processivity factor for the viral DNA polymerase, Klemperer et al. subjected an ~45 kDa protein that co-purified with processive DNA polymerase to mass spectrometry and identified it as the product of the A20R open reading frame (Klemperer et al., 2001). Consistent with a role in viral DNA replication, A20R is expressed early in infection; synthesis of the 49 kDa protein is maximal between 1.5 and 4.5 hours post infection and is unperturbed by the addition of 20 μM araC, which inhibits DNA replication and therefore blocks intermediate and late gene expression. The protein does not appear to undergo phosphorylation in vivo, nor does it appear to become encapsidated in mature virions. Bioinformatic analysis reveals that the genomes of all members of the chordopoxvirus subfamily encode A20 homologs. However, homologs in some distant family members such as Yaba poxvirus and Molluscum contagiosum virus, exhibit an

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

overall sequence identity of only 44 and 26% respectively, with the N′ and C′ termini of the protein being the most conserved (Klemperer et al., 2001). Interestingly, no A20 homolog is present in the entomopoxvirus genomes. The A20 protein does not exhibit sequence similarity to any other non-poxviral proteins in the available public databases.

Two parallel studies (Ishii and Moss, 2001; Punjabi et al., 2001) reported the isolation of *ts* mutants with lesions in the A20R gene after performing clustered-charge-to-alanine mutagenesis. Overlapping mutants were produced in the two studies, with *ts*A20-ER5 and *ts*A20-6 (Punjabi et al., 2001) (mutants 185 and 265 respectively in (Ishii and Moss, 2001)) exhibiting the most stringent temperature sensitivity (Fig 3A, orange text above the schematic of the A20 ORF). Alteration of E^{181}RSFDKK to AASFAAA (*ts*A20-ER5) or K^{265}VKKK to AVAAA (*ts*A20-6) resulted in marked attenuation of plaque formation and multi-log decreases in viral yield at non-permissive temperature. Detailed analysis of DNA accumulation in these infections confirmed the hypothesis that attenuated viral DNA accumulation was responsible for the decreased viral yield. Specifically, *ts*A20-6 and tsA20-ER5 only achieved about 17% and 7% of the levels of DNA generated in control WT infections by 24 hours post infection. Radioactive thymidine incorporation assays confirmed that a shift to non-permissive temperature at 6 hours post infection resulted in a fast-stop cessation in DNA replication, both in *ts*A20-6 and *ts*A20-ER5 infections (Punjabi et al., 2001). Lastly, and perhaps most significantly with respect to viral DNA polymerase activity, the same study confirmed that extracts prepared from *tsA20* infections lacked processive DNA polymerase activity.

7.2 Structure/function analyses of D4 (UDG)

Although the contribution of A20 to E9 processivity seemed certain following these studies, the inability to purify recombinant A20 for in vitro reconstitution assays implied a requirement for additional viral factors to stabilize A20. Furthermore, the aforementioned yeast two-hybrid and MBP pull-down screens had confirmed that A20 interacted with the viral D4 protein, a uracil DNA glycosylase. Like A20, the enzymatically active D4 protein shows an early pattern of expression, with accumulation peaking by 4 hpi (Stuart et al., 1993). A detailed review of the structure function relationship of the D4 uracil DNA glycosylase is available (Schormann et al., 2016). In brief, sequence analysis has revealed that while the 218 amino acid VACV UDG is a member of the Family I type uracil DNA glycosylases, and is highly conserved within the poxvirus family, homology to non-poxviral Family I UDGs appears to be significantly more limited. In fact, D4 shares only ~ 20% sequence identity with human and E. coli uracil DNA glycosylases (Schormann et al., 2013; Schormann et al., 2007) and is resistant to the UGI protein, a well-studied inhibitor of many prokaryotic and eukaryotic UDGs (Ellison et al., 1996). D4 has been the subject of numerous crystallography studies (see below), the results of which have revealed that despite the limited primary amino acid sequence homology to other Family I UDGs, the structure of the VACV protein adopts the characteristic alpha / beta fold of DNA glycosylases (Burmeister et al., 2015; Contesto-Richefeu et al., 2014; Schormann et al., 2013; Schormann et al., 2007).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

D4's uracil glycosylase activity has been shown to be active in the context of the polymerase holoenzyme, as evidenced by the generation of abasic sites in a uracil-containing oligonucleotide after incubation with purified polymerase holoenzyme in vitro (Boyle et al., 2011). Moreover, analysis of an in vitro primer extension assay using purified DNA polymerase holoenzyme indicated that misincorporation of dUTP also resulted in the generation of piperidine-sensitive abasic sites, suggesting that viral DNA replication and repair could be coupled, at least in vitro (Boyle et al., 2011). Although studies initially suggested that UDG activity was indispensable for viral replication, those studies were complicated by the thermolability of the D4 mutants being studied or the inability to isolate D4 deletion strains through transient dominant selection (Ellison et al., 1996; Stuart et al., 1993). In contrast, more recent mutational analyses have revealed the glycosylase activity of D4 to be dispensable in tissue culture, but not in vivo, suggesting that the excision of uracil moieties may be necessary for productive infection in certain key cell types, although not in all (Boyle et al., 2011; De Silva and Moss, 2003). In vitro transcription-translation reactions of A20 and flag-tagged alleles of D4 indicate that the interaction between A20 and UDG is preserved in vitro, and furthermore, mutational inactivation of UDG catalytic activity (D68N and H191L) (Figure 3B, purple text, above the schematic of the D4 ORF) had no effect on this interaction (Stanitsa et al., 2006). Evidence of this interaction, as well as the apparent dispensability of UDG catalytic activity for replication, led to the hypothesis that the D4 protein might be required as a structural, stoichiometric component of the vaccinia processivity factor. This hypothesis stimulated further characterization of the contribution of UDG to viral DNA replication. In 2006, Stanitsa et al. confirmed that two previously identified mutants, D*ts*30 and D*ts*27, did in fact contain lesions in the D4 gene (G179R and L110F, respectively) (Figure 3B, orange text, above the schematic of the D4 ORF). These lesions resulted in the attenuation of viral yield by over 1000- and 100-fold respectively (Stanitsa et al., 2006). Using dot-blot DNA accumulation assays it was determined that at the non-permissive temperature both mutants were profoundly deficient for DNA accumulation. Furthermore, cytoplasmic extracts prepared from cells infected with D*ts*27 and D*ts*30 at non-permissive temperature (and also permissive temperature in the case of D*ts*30) were unable to sustain processive replication of primed M13 templates in vitro. While the absence of useful anti-UDG antibodies has hampered the determination of whether these viruses encode a D4 protein deficient in stability or just in function, temperature shift and in vitro transcription translation assays were applied to address some of these concerns. Interestingly, in contrast to the phenotype seen in *ts*A20 mutants, a shift to non-permissive conditions in the midst of DNA replication did not appear to significantly disrupt further viral DNA synthesis. This finding suggests that the temperature sensitivity of these two mutants may arise from an inability of the nascent UDG protein to fold properly or to form the A20/D4 heterodimer at the non-permissive temperature, and that once in the proper conformation the protein remains stable and active. Together, these data provided evidence consistent with the hypothesis that VACV UDG plays a role establishing a processive DNA polymerase complex. This hypothesis was further reinforced by the finding that the temperature sensitive alleles of UDG (D*ts*30, specifically) and A20 (*ts*5ER, specifically) exhibited significantly reduced rates of co-immunoprecipitation (in vitro) when compared to WT alleles.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Further characterization of this interaction in the context of VACV infected cells overexpressing E9 DNA polymerase, A20, and 3xFLAG-tagged UDG (fUDG) revealed that binding to an anti-Flag affinity resin resulted in the purification of all three proteins, even after a stringent 750 mM NaCl wash, supporting the conclusion of an in vivo trimeric complex (Stanitsa et al., 2006). When fUDG and A20 were overexpressed, the A20/fUDG dimer was retrieved, as well as excess free fUDG. However, when only E9 DNA polymerase and fUDG were overexpressed, only 3X-FLAG-UDG alone was purified: no detectable E9 was retrieved in complex with fUDG (Stanitsa et al., 2006). These studies strongly suggest that E9, A20, and D4 participate in an “ordered” trimeric complex, in which A20 is present in a constitutive A20/D4 dimer, and that within this dimer it functions to bridge the interaction with E9, facilitating the assembly of an E9/A20/D4 holoenzyme. Only the trimeric holoenzyme exhibits processive and rapid DNA synthesis in vitro.

Collaborative studies between the Ricciardi and Chattopadhyay groups has focused on probing the A20 – D4 interaction further. Specifically, a 2010 study by Shudofsky et al. reported the screening of 21 point mutants with substitutions in D4 that were designed to neutralize positive residues hypothesized to facilitate DNA synthesis. Three D4 mutants were identified that were defective in supporting E9 processivity while retaining the ability to excise uracil from ssDNA oligonucleotides: the lesions identified were K126V, K160V, and R187V (Druck Shudofsky et al., 2010) (Figure 3B, green text below the schematic of the D4 ORF). This defect was confirmed in two in vitro assays: the M13 extension assay, and a custom ELISA in which reconstituted holoenzyme was challenged to replicate a plate-bound template. The possibility that the reduced processivity observed might be due to a defect in forming the A20/D4 dimer was ruled out by confirming that the three mutant forms of D4 retained the ability to bind A20. The sustained interaction was shown not only by co-immunoprecipitation but also through a poisoning experiment, in which coincubation of WT DNA polymerase holoenzyme with an excess of mutant D4 (K126V, K160V, R187V but not K126R) resulted in decreased processive polymerase activity, a finding consistent with competition by the mutant D4 for binding to the E9/A20 heterodimer. The mutant D4 proteins were also shown to retain uracil excision activity and to exhibit a similar kinetic response to competition by undamaged, competitor DNA as was seen with wild-type D4 protein. In sum this was the first report of D4 mutants that appeared to be specifically defective in conferring E9 processivity.

In a series of productive studies the Ricciardi and Chattopadhyay groups identified a range of small molecule inhibitors of both the VACV polymerase activity and processivity, some of which displayed comparable efficacy and selectivity to the antiviral compound cidofovir (Ciustea et al., 2008; Nuth et al., 2011; Schormann et al., 2011; Silverman et al., 2008). Via two high-throughput screens the groups focused on identifying and validating non-nucleoside inhibitors of DNA synthesis and/or polymerase processivity, assessing previously FDA-approved compounds, natural extracts and synthetic compounds. In total, Ciustea et al. identified 16 synthetic compounds which inhibited E9 mediated DNA synthesis in highly selective manner. Silverman et al. identified NSC 55636 (Fentichlor) as a potent inhibitor of VACV DNA polymerase, as well as NSC 123526, an inhibitor of processivity, both of which conferred protection in a VACV plaque forming assay (Ciustea et al., 2008; Silverman et al., 2008). The second approach (Nuth et al., 2011; Schormann et al., 2011), which was based

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

on Alpha Screen technology, was targeted at identifying small molecules which would disrupt the A20/D4 heterodimer, thereby reducing E9 processivity. Using this screening methodology the group identified 23 compounds representing 11 chemical scaffolds, of which several compounds inhibited vaccinia and cowpox infection at single digit μM concentrations. A subsequent lead optimization study, combining in silico modeling and chemical synthesis, yielded a compound that exhibited an IC50 of 42 nM in a plaque reduction assay (Nuth et al., 2013). It is worth noting that in this endeavor, an in silico model of D4/A20$_{1–100}$ was utilized rather than the empirically determined structure of D4/A20$_{1–50}$ suggesting that the N′-terminus of A20 on its own may yield an incomplete picture of the A20/D4 interface. This optimized compound prevented late gene expression in infected cells and appeared to block the formation of BrdU+ foci of nascent viral DNA. Future studies of this and other potent compounds for their mechanism of action will be highly useful and important.

8: Structural Analysis of the D4, D4/DNA, D4/A20, D4/A20/DNA and E9 Polymerase

8.1 E9 Polymerase

To date, the crystal structure of the E9 polymerase has not been solved. The overall conservation of the domains of the B-family of replicative polymerases within the E9 sequence suggests that the structure of E9 will show significant similarity with that of the HSV DNA polymerase and cellular replicative polymerases. In fact, when the E9 sequence was analyzed by the iTASSER structural prediction protocol (Yang et al., 2015), DNA polymerase δ, DNA polymerase α and the HSV DNA polymerase were the most closely related structural analogs. In 2013, Selé et al.were successful in generating a low resolution model of recombinant E9 protein (expressed using the baculovirus system) using small-angle X-ray scattering and electron microscopy (Sele et al., 2013). The E9 envelope obtained using SAXS indicates that the monomeric polymerase assumes an overall “half-avocado” shape with a central depression. A model of the E9 sequence (modeled on the HSV Pol) fit well within this envelope. Selé et al. also purified the A20/D4 heterodimer, and could reconstitute the holoenzyme, which showed a 1:1:1 stoichiometry of each of the three components. Surface plasmon resonance analysis indicated this was a stable complex, and the K_d for the binding of A20/D4 to E9 was reported to be 3 nM. SAXS / EM analysis of the holoenzyme was consistent with the structure being an elongated handle (D4/A20) with a bulky head (E9). Assuming that A20 occupies a central position and bridges E9 and D4, the distance between the catalytic sites of Pol and D4 is estimated at 150 Å, corresponding to 50–60 base pairs (Figure 4B). This model has implications for the ability of D4 to excise any dUMP moieties that might be present in the nascent strand as a result of misincorporation of dUTP by E9 (Boyle et al., 2011).

8.2 D4

Several groups have undertaken a detailed investigation of the protein structure of D4 alone, in complex with the N′-terminus of A20 and/or in complex with DNA oligonucleotides (Burmeister et al., 2015; Contesto-Richefeu et al., 2014; Contesto-Richefeu et al., 2016;

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Sartmatova et al., 2013; Schormann et al., 2013; Schormann et al., 2015; Schormann et al., 2007). In 2007, the first crystal structure of the D4 uracil DNA glycosylase was published (Schormann et al., 2007). This report as well as subsequent studies, revealed that highly concentrated preparations of recombinant UDG adopt a homodimeric structure. As will be described below, a consensus has emerged that UDG is not dimeric in vivo, and indeed the interface involved in forming the homodimer seen by Schormann et al. is the same interface through which D4 interacts with the A20 protein, D4's physiological partner in the processivity complex (Contesto-Richefeu et al., 2014).

Although D4 exhibits poor primary amino acid sequence homology to Family I UDGs from outside the poxvirus family, it clearly adopts the common α/β fold of Family I UDGs. Specifically, the protein consists of a core β-sheet, made up of two anti-parallel β-strands, surrounded by two α-helices, one on the N′- and one on the C′-terminus of the central β-sheet (Schormann et al., 2007). Comparison of this crystal structure, as well as those of UDG in complex with uracil and dsDNA, to those available for human and E coli uracil DNA glycosylases, reveals that the catalytic pocket of UDG is nearly identical to other Family I members, including the conservation of two key catalytic residues, Asp68 and His181 (Schormann et al., 2013; Schormann et al., 2015; Schormann et al., 2007; Schormann et al., 2011; Schormann et al., 2016). In 2015, Schormann et al. defined the D4 residues responsible for mediating protein-DNA interactions, with the interface being made up of Ile67, Pro71, Gly128, Glu129, Thr130, Lys131, Gly159, Lys160, Thr161, Asp162, Tyr180, His181, and Ala183. These residues are ascribed to three regions which overlap well with other Family I UDGs, including the extended Pro-rich DNA binding loop (D4 aa's 126–132), Gly-Ser loop (D4 aa's 159–162) and Leu-intercalation loop (D4 aa's 180–187) (Figure 3B, maroon, blue and pink shaded boxes). These three motifs have been shown to mediate the Family I "pinch-push-pull" mechanism of uracil excision. In D4, the substitution of an Arg residue for the canonical Leu in the "Leu intercalation loop" is responsible for the striking resistance of D4 to the pan-UDG inhibitor, UGI (Burmeister et al., 2015). Aside from this core UDG-like fold, D4 also retains two additional β-sheets which flank both boundaries of the core region. It is hypothesized that these additional areas of secondary structure may facilitate other protein:protein interactions, and at least one of these regions has been shown to contribute to D4 stability. Deletion or mutation of the C′-terminal residues 213–217, which are highly conserved among poxvirus homologs, resulted in decreased solubility of recombinant D4 and a loss of processive DNA polymerase activity in cell free DNA synthesis assays (Nuth et al., 2016).

8.3 D4/A20 interaction

In studies performed by Contesto-Richefeu et al., the D4 homodimer that had been observed in purified preparations of D4 was shown to be readily disrupted in the presence of a peptide representing the N-terminal 50 amino acids of A20, as demonstrated by SEC-MALLS and crystallography studies (Contesto-Richefeu et al., 2014). Under these conditions, D4 was present nearly exclusively in a heterodimeric complex with the interaction motif of A20. This shift is apparently driven by the greater specificity and extent of the heterodimeric interaction. Analysis of the D4/A20$_{1–50}$ crystal structure supports this assertion. While the D4/D4 homodimer and D4/A20$_{1–50}$ heterodimer were found to share the same hydrophobic-

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

contact-driven interaction interface on D4, the D4/A20 was found to participate in a number of intramolecular hydrogen bonds and as well as a base stacking interaction coined a “tongue and groove connection” between Trp43 of A20 and Pro173 and Arg167 of D4. The crystal structures of D4 in complex with A20 also reveal that two C-terminal regions of D4 are responsible for interaction with A20; the interaction motifs are made up of amino acids 167 – 180 and 191 – 206 (Burmeister et al., 2015; Contesto-Richefeu et al., 2014) (Figure 3B, cyan shaded box). These data clearly indicate that when expressed in the presence of A20, as would be the case in vivo, the biophysics of the D4 interaction surface strongly favor a heterodimeric interaction with A20 rather than a D4/D4 homodimer (as reported when D4 is overexpressed and isolated alone).

To further dissect which residues in the N′-terminal region of A20 are responsible for mediating the interaction with D4, Boyle et al. performed targeted mutagenesis of several nonpolar and charged residues within this region. They found that mutation of two clusters of leucine residues (L710A and L13,14,16A) reduced the A20/D4 interaction (Figure 3A, blue text below the schematic of the A20 ORF). The crystal structure of the D4/A20$_{1–50}$ complex (Contesto-Richefeu et al., 2014) confirmed the importance of these residues in the A20/D4 interface. This structural analysis also revealed that residues 40–47 of A20 make significant contact with D4; with the exception of Trp43, which protrudes, the extensive contact surface is strikingly flat.

A more recent study (Contesto-Richefeu et al., 2016) investigated the contribution of the so called “tongue and groove” interaction on the structure and heterodimer formation in greater detail. In short, it was found that while mutation of Trp43 (A20), Pro173 (D4) and Arg167 (D4) all reduced the stability of the complex, as demonstrated by a reduction of up to 5 °C in melting temperature in thermal shift assays, it was clear that Trp43 and Pro173 were the critical residues directing complex formation. Mutation of Trp43 to alanine, or Pro173 to glycine was found to cause a detrimental effect on the rate of A20/D4 complex formation (Figure 3A, blue text below the schematic of the A20 ORF, and Figure 3B, blue text below the schematic of the D4 ORF), a decrease which was not observed upon co-expression of WT D4 with an R167A mutant. None of the three mutations caused any appreciable perturbation of global protein structure, thus reemphasizing the major contribution of this “tongue and groove” connection to the strength of the overall D4/A20$_{1–50}$ interaction. As described previously, the G179R lesion in the D4 mutant D*ts*30 (see above) reduced the A20/D4 interaction, as would be predicted for the insertion of a bulky residue in the midst of the protein:protein interaction surface.

Despite the evidence supporting the importance of this “tongue and groove” interaction, Schormann et al. observed that when in complex with DNA, both Arg167 and Pro173 appear to move in an orientation that would not be favorable for the formation of this “groove and pocket” interaction (Schormann et al., 2015). This finding may imply that formation of a heterotrimeric complex requires the formation of the D4:A20 heterodimer prior to binding to DNA. In sum, however, the refinement of our understanding of the interaction of A20 and D4, which is essential for DNA replication in vivo, underscores the importance and promise of identification and optimization of inhibitors of this interaction as effective anti-poxvirus therapeutics.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

8.4 D4/DNA interaction

The structure of D4 bound to a 12-mer undamaged DNA duplex, as well as D4 bound to a 10-mer DNA duplex containing an abasic site, were both reported in 2015 (Burmeister et al., 2015; Schormann et al., 2015). In the Schormann study, three regions of D4 were implicated in DNA binding: extended DNA binding loop (residues 126–132), Gly-Ser loop (residues 159–172), and the Leu-intercalation loop (residues 180–187), which as mentioned above has an Arg in place of the canonical Leu residue. This structure could represent the initial interaction of a UDG as it contacts undamaged DNA and prior to forming a more stable interaction with uracil-containing DNA. Since D4 plays an unusual role in conferring processivity to the E9 replicative polymerase, this structure may be of particular import. The Burmeister study investigated the binding of D4/$A20_{1–50}$ to a variety of DNA oligonucleotides and presented the structure of D4/$A20_{1–50}$ to an oligonucleotide containing an abasic site. Surface plasmon resonance studies indicated that, at physiological salt concentrations, the K_D for a dsDNA oligomer was >50 μM, whereas the K_D for a dsDNA oligomer with an abasic site was ~1 μM.

9: Summary, Experimental Highlights and Unanswered Questions

In sum, this review presents the current state-of-the-art understanding of the vaccinia virus E9 DNA polymerase and its processivity factor, a heterodimer of the A20 and D4 (UDG) proteins. Since its initial purification (Challberg and Englund, 1979b) and genetic mapping (Earl et al., 1986; Traktman et al., 1984), E9 has been subjected to a variety of biochemical and genetic analyses focused on its inherently distributive mode of synthesis (see section 4), it's fidelity, and its sensitivity to a variety of inhibitors (summarized in Fig 2 and section 5). DNA polymerase retains much of the organization of type B replicative polymerases, and is sensitive to inhibition by aphidicolin, cytosine arabinoside, phosphonoacetic acid, and cidofovir. The isolation of drug-resistant mutants has elucidated some of the regions within the protein that are responsible for nucleotide binding and exonuclease activity. Both the polymerase and exonuclease function are essential for viral replication in vivo, and the polymerase is involved in not only DNA synthesis but also in DNA recombination.

The polymerase is inherently distributive, and relies on its association with the A20/D4 heterodimer for rapid and processive synthesis. Identification of the processivity factor components (Ishii and Moss, 2001; Ishii and Moss, 2002; Klemperer et al., 2001; McDonald et al., 1997; Stanitsa et al., 2006) enabled a new chapter in the study of vaccinia replication, both because A20 bears no homology to any other known proteins and because D4 is an active DNA repair enzyme. We have no knowledge of what region(s) of E9 bind to A20, although binding in vitro and in vivo has been demonstrated (Boyle et al., 2011; Stanitsa et al., 2006). The HSV DNA polymerase binds to its processivity factor via C′-terminal sequences, but there is no evidence for a similar organization in E9. In contrast, it has been suggested that "poxvirus-specific" insertions (Sele et al., 2013) in the DNA polymerase sequence could mediate the interaction with A20, and when modeled on the HSV polymerase, these two insertions appear to be adjacent to each other and exposed on the surface of the protein (Sele et al., 2013). Determining how E9 and A20 interact is clearly an important area for future study. For this and other areas of study, it would be extremely

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

beneficial to have a crystal structure of the E9 enzyme. The A20 protein cannot be expressed and purified in isolation: it requires its association with D4 for proper folding and stability. The knowledge that the N′-terminal 50 amino acids of A20 mediate much of the interaction with D4 has been extremely valuable (Ishii and Moss, 2002), and the crystal structures of UDG alone, in complex with $A20_{1–50}$, and/or with a DNA oligonucleotide have been a major advance within the field (Burmeister et al., 2015; Schormann et al., 2015; Schormann et al., 2007); the structure D4/$A20_{1–50}$/DNA is shown in Figure 4A. The next crucial step is to obtain the structure of the intact A20/D4 heterodimer, so that we can visualize the full A20/D4 interface but also gain insight into the structure of A20 itself. The work of Ricciardi et al. has provided genetic insight into key residues in the D4 protein that affect processive synthesis and/or the interaction with A20, and has highlighted the possibility of using inhibitors of this interface as antiviral agents (Druck Shudofsky et al., 2010; Nuth et al., 2013; Nuth et al., 2011; Schormann et al., 2011; Silverman et al., 2008).

Within the processivity factor, A20 appears to serve as a bridge or a linker, binding to both E9 and D4, which do not appear to interact with each other directly (Figure 4B). Moreover, no DNA-binding activity has been found for A20. Hence, D4 is presumed to be responsible for enabling the polymerase holoenzyme to translocate along the DNA template in a processive manner. D4 is an enzymatically active uracil DNA glycosylase, although this enzymatic activity is dispensable for D4's role in conferring processivity. Although the mechanics of the recognition and excision of uracil by UDGs by a pinch-push-pull mode of action is well understood, the scanning of DNA by UDGs that precedes uracil recognition is poorly understood. Do UDGs scan long stretches of DNA processively, or do they undergo cycles of binding and release? At least in the case of poxviruses, it seems clear that D4 must be able to remain bound to the DNA template in a manner that is stable but doesn't hinder polymerase movement. This question, too, is of primary importance for future studies, especially in light of the finding that the affinity of D4 for undamaged DNA may be insufficient (Burmeister et al., 2015) to retain the holoenzyme on the DNA template. Consideration of whether A20 or E9 augment DNA binding is a question for future study.

Lastly, the role of D4 within the holoenzyme as both a processivity factor component and a repair enzyme remains to be clarified. In the cellular replication machinery, hUNG2 associates with PCNA and RPA at the replication fork, allowing it to scan for dUMP residues in nascent DNA (Otterlei et al., 1999). Vaccinia virus (and other poxviruses) have taken this association to another level by incorporating the UDG in the DNA polymerase holoenzyme. The Traktman laboratory has shown that D4 retains its UDG activity within the holoenzyme (Boyle et al., 2011). Moreover, when dUTP is present during processive DNA synthesis assays in vitro, abasic sites are present within the nascent DNA strands. These data imply that the UDG is serving a repair function at the replication fork. If the A20/D4 complex is positioned on the ssDNA template behind the moving polymerase and there are ~50–60 bp between the active site of the polymerase and the UDG active site [as suggested in (Sele et al., 2013)], then the UDG would be in a position to recognize and excise the uracil moiety as it translocates behind the polymerase. (Experimental confirmation that the holoenzyme is oriented in this manner has not yet been obtained, and other orientations are possible.) Because UDG is the first enzyme in the base excision repair (BER) pathway, it remains to be determined how subsequent excision of the abasic site and repair is

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

accomplished. The vaccinia virus genome does not encode any proteins with homology to apurine/apyrimidinic endonucleases (APEs), implying that either a cytoplasmic pool of the cellular APE is able to perform this next step of BER, or that repair is accomplished via a non-canonical mechanism.

Over the past few decades, genetic screens, inhibitor studies, biochemical analyses and structural biology have greatly enhanced our understanding of the vaccinia virus DNA polymerase and its heterodimeric processivity factor. Future insights into this novel holoenzyme will provide further understanding of poxvirus replication and inform the development of antiviral therapeutics.

Acknowledgments

Some of the work described above (from the Traktman laboratory) was supported by NIH grant AI 21758 (awarded to PT). We thank members of the Traktman laboratory for helpful conversations and the many investigators whose work we have referenced here for the contributions.

ABBREVIATIONS

aa	amino acid
ANP	acyclic nucleoside phosphonate
APE	apurinic/apyrimidinic endonuclease
aphid	aphidicolin
AraA	9-β-D-arabinofuranosyladenine
AraC	cytosine arabinsodie/1-β-D- arabinofuranosylcytosine BER, base excision repair
bp	base pair
BrdU	bromodeoxyuridine
CDV	cidofovir/(S)-1-[3-hydroxy-2-(phosphonomethoxypropyl) cytosine]
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dNTP	deoxynucleoside triphosphate
dsDNA	double stranded deoxyribonucleic acid
dUMP	deoxyuridine monophosphate
dUTP	deoxyuridine triphosphate
EM	electron microscopy

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

FDA	Food and Drug Administration
HCMV	human cytomegalovirus
hpi	hours post infection
HPMPA	(S)-1-[3-hydroxy-2-(phosphonomethoxypropyl) adenosine]
HPMPC	(S)-1-[3-hydroxy-2-(phosphonomethoxypropyl) cytosine]
HSV	herpes simplex virus
hUNG	human uracil-DNA glycosylase
IBT	isatin-β-thiosemicarbazone
kb	kilobase
kDa	kilodalton
MBP	maltose-binding protein
NCLDV	nucleocytoplasmic large DNA virus
nt	nucleotide
PAA	phosphonoacetic acid
PCNA	proliferating cell nuclear antigen
RPA	replication protein A
SAXS	small-angle X-ray scattering
ssDNA	single stranded deoxyribonucleic acid
ts	temperature sensitive
TTP	thymidine triphosphate
UDG	uracil-DNA glycosylase
UTR	untranslated region
VACV	vaccinia virus
VV	vaccinia virus
WR	Western Reserve
WT	wild type

References

Andrei G, Gammon DB, Fiten P, De CE, Opdenakker G, Snoeck R, Evans DH. Cidofovir resistance in vaccinia virus is linked to diminished virulence in mice. J Virol. 2006; 80(19):9391–9401. [PubMed: 16973545]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Andrei G, Snoeck R. Cidofovir Activity against Poxvirus Infections. Viruses. 2010; 2(12):2803–2830. [PubMed: 21994641]

Baranovskiy AG, Babayeva ND, Suwa Y, Gu J, Pavlov YI, Tahirov TH. Structural basis for inhibition of DNA replication by aphidicolin. Nucleic Acids Res. 2014; 42(22):14013–14021. [PubMed: 25429975]

Baroudy BM, Moss B. Sequence homologies of diverse length tandem repetitions near ends of vaccinia virus genome suggest unequal crossing over. Nucleic Acids Res. 1982; 10(18):5673–5679. [PubMed: 6292846]

Baroudy BM, Venkatesan S, Moss B. Incompletely base-paired flip-flop terminal loops link the two DNA strands of the vaccinia virus genome into one uninterrupted polynucleotide chain. Cell. 1982; 28(2):315–324. [PubMed: 7060133]

Baroudy BM, Venkatesan S, Moss B. Structure and replication of vaccinia virus telomeres. Cold Spring Harb Symp Quant Biol. 1983; 47(Pt 2):723–729. [PubMed: 6574869]

Becker MN, Obraztsova M, Kern ER, Quenelle DC, Keith KA, Prichard MN, Luo M, Moyer RW. Isolation and characterization of cidofovir resistant vaccinia viruses. Virol J. 2008; 5:58. [PubMed: 18479513]

Blanco L, Bernad A, Blasco MA, Salas M. A general structure for DNA-dependent DNA polymerases. Gene. 1991; 100:27–38. [PubMed: 2055476]

Boyle KA, Stanitsa ES, Greseth MD, Lindgren JK, Traktman P. Evaluation of the role of the vaccinia virus uracil DNA glycosylase and A20 proteins as intrinsic components of the DNA polymerase holoenzyme. J Biol Chem. 2011; 286(28):24702–24713. [PubMed: 21572084]

Burmeister WP, Tarbouriech N, Fender P, Contesto-Richefeu C, Peyrefitte CN, Iseni F. Crystal Structure of the Vaccinia Virus Uracil-DNA Glycosylase in Complex with DNA. J Biol Chem. 2015; 290(29):17923–17934. [PubMed: 26045555]

Challberg MD, Englund PT. The effect of template secondary structure on vaccinia DNA polymerase. J Biol Chem. 1979a; 254(16):7820–7826. [PubMed: 381293]

Challberg MD, Englund PT. Purification and properties of the deoxyribonucleic acid polymerase induced by vaccinia virus. J Biol Chem. 1979b; 254(16):7812–7819. [PubMed: 468791]

Ciustea M, Silverman JE, Druck Shudofsky AM, Ricciardi RP. Identification of nonnucleoside DNA synthesis inhibitors of vaccinia virus by high-throughput screening. J Med Chem. 2008; 51(20): 6563–6570. [PubMed: 18808105]

Coen, DM. Viral DNA Polymerases. In: DePamphilis, editor. DNA Replication in Eukaryotic Cells. Cold Spring Harbor Laboratory Press; New York: 1996. p. 495-523.

Colson P, De Lamballerie X, Yutin N, Asgari S, Bigot Y, Bideshi DK, Cheng XW, Federici BA, Van Etten JL, Koonin EV, La Scola B, Raoult D. “Megavirales”, a proposed new order for eukaryotic nucleocytoplasmic large DNA viruses. Arch Virol. 2013; 158(12):2517–2521. [PubMed: 23812617]

Condit RC, Motyczka A. Isolation and preliminary characterization of temperature-sensitive mutants of vaccinia virus. Virology. 1981; 113(1):224–241. [PubMed: 7269240]

Condit RC, Motyczka A, Spizz G. Isolation, characterization, and physical mapping of temperature-sensitive mutants of vaccinia virus. Virology. 1983; 128(2):429–443. [PubMed: 6577746]

Contesto-Richefeu C, Tarbouriech N, Brazzolotto X, Betzi S, Morelli X, Burmeister WP, Iseni F. Crystal structure of the vaccinia virus DNA polymerase holoenzyme subunit d4 in complex with the a20 N-terminal domain. PLoS Pathog. 2014; 10(3):e1003978. [PubMed: 24603707]

Contesto-Richefeu C, Tarbouriech N, Brazzolotto X, Burmeister WP, Peyrefitte CN, Iseni F. Structural analysis of point mutations at the Vaccinia virus A20/D4 interface. Acta Crystallogr F Struct Biol Commun. 2016; 72(Pt 9):687–691. [PubMed: 27599859]

Dales S, Milovanovitch V, Pogo BG, Weintraub SB, Huima T, Wilton S, McFadden G. Biogenesis of vaccinia: isolation of conditional lethal mutants and electron microscopic characterization of their phenotypically expressed defects. Virology. 1978; 84(2):403–428. [PubMed: 622807]

De Silva FS, Moss B. Vaccinia virus uracil DNA glycosylase has an essential role in DNA synthesis that is independent of its glycosylase activity: catalytic site mutations reduce virulence but not virus replication in cultured cells. J Virol. 2003; 77(1):159–166. [PubMed: 12477821]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

DeFilippes FM. Effect of aphidicolin on vaccinia virus: isolation of an aphidicolin-resistant mutant. J Virol. 1984; 52(2):474–482. [PubMed: 6436508]

DeFilippes FM. Site of the base change in the vaccinia virus DNA polymerase gene which confers aphidicolin resistance. J Virol. 1989; 63(9):4060–4063. [PubMed: 2503622]

DeLange AM, Reddy M, Scraba D, Upton C, McFadden G. Replication and resolution of cloned poxvirus telomeres in vivo generates linear minichromosomes with intact viral hairpin termini. J Virol. 1986; 59(2):249–259. [PubMed: 3016294]

DeMasi J, Du S, Lennon D, Traktman P. Vaccinia virus telomeres: interaction with the viral I1, I6, and K4 proteins. J Virol. 2001; 75(21):10090–10105. [PubMed: 11581377]

Derse D, Cheng YC. Herpes simplex virus type I DNA polymerase. Kinetic properties of the associated 3′–5′ exonuclease activity and its role in araAMP incorporation. J Biol Chem. 1981; 256(16):8525–8530. [PubMed: 6167579]

Drillien R, Spehner D. Physical mapping of vaccinia virus temperature-sensitive mutations. Virology. 1983; 131(2):385–393. [PubMed: 6318438]

Druck Shudofsky AM, Silverman JE, Chattopadhyay D, Ricciardi RP. Vaccinia virus D4 mutants defective in processive DNA synthesis retain binding to A20 and DNA. J Virol. 2010; 84(23): 12325–12335. [PubMed: 20861259]

Du S, Traktman P. Vaccinia virus DNA replication: two hundred base pairs of telomeric sequence confer optimal replication efficiency on minichromosome templates. Proc Natl Acad Sci U S A. 1996; 93(18):9693–9698. [PubMed: 8790393]

Duraffour S, Andrei G, Topalis D, Krecmerova M, Crance JM, Garin D, Snoeck R. Mutations conferring resistance to viral DNA polymerase inhibitors in camelpox virus give different drug-susceptibility profiles in vaccinia virus. J Virol. 2012; 86(13):7310–7325. [PubMed: 22532673]

Earl PL, Jones EV, Moss B. Homology between DNA polymerases of poxviruses, herpesviruses, and adenoviruses: nucleotide sequence of the vaccinia virus DNA polymerase gene. Proc Natl Acad Sci U S A. 1986; 83(11):3659–3663. [PubMed: 3012524]

Ellison KS, Peng W, McFadden G. Mutations in active-site residues of the uracil-DNA glycosylase encoded by vaccinia virus are incompatible with virus viability. J Virol. 1996; 70(11):7965–7973. [PubMed: 8892920]

Ensinger MJ. Isolation and genetic characterization of temperature-sensitive mutants of vaccinia virus WR. J Virol. 1982; 43(3):778–790. [PubMed: 7143565]

Gammon DB, Evans DH. The 3′-to-5′ exonuclease activity of vaccinia virus DNA polymerase is essential and plays a role in promoting virus genetic recombination. J Virol. 2009; 83(9):4236–4250. [PubMed: 19224992]

Gammon DB, Snoeck R, Fiten P, Krecmerova M, Holy A, De Clercq E, Opdenakker G, Evans DH, Andrei G. Mechanism of antiviral drug resistance of vaccinia virus: identification of residues in the viral DNA polymerase conferring differential resistance to antipoxvirus drugs. J Virol. 2008; 82(24):12520–12534. [PubMed: 18842735]

Gibbs JS, Chiou HC, Bastow KF, Cheng YC, Coen DM. Identification of amino acids in herpes simplex virus DNA polymerase involved in substrate and drug recognition. Proc Natl Acad Sci U S A. 1988; 85(18):6672–6676. [PubMed: 2842788]

Goebel SJ, Johnson GP, Perkus ME, Davis SW, Winslow JP, Paoletti E. The complete DNA sequence of vaccinia virus. Virology. 1990; 179(1):247–263. [PubMed: 2219722]

Grubisha O, Traktman P. Genetic analysis of the vaccinia virus I6 telomere-binding protein uncovers a key role in genome encapsidation. J Virol. 2003; 77(20):10929–10942. [PubMed: 14512543]

Hall JD, Wang YS, Pierpont J, Berlin MS, Rundlett SE, Woodward S. Aphidicolin resistance in herpes simplex virus type I reveals features of the DNA polymerase dNTP binding site. Nucleic Acids Res. 1989; 17(22):9231–9244. [PubMed: 2555788]

Hamilton MD, Evans DH. Enzymatic processing of replication and recombination intermediates by the vaccinia virus DNA polymerase. Nucleic Acids Res. 2005; 33(7):2259–2268. [PubMed: 15843688]

Hedglin M, Kumar R, Benkovic SJ. Replication clamps and clamp loaders. Cold Spring Harb Perspect Biol. 2013; 5(4):a010165. [PubMed: 23545418]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Ishii K, Moss B. Role of Vaccinia Virus A20R Protein in DNA Replication: Construction and Characterization of Temperature-Sensitive Mutants. J Virol. 2001; 75(4):1656–1663. [PubMed: 11160663]

Ishii K, Moss B. Mapping interaction sites of the A20R protein component of the vaccinia virus DNA replication complex. Virology. 2002; 303(2):232–239. [PubMed: 12490386]

Jones EV, Moss B. Transcriptional mapping of the vaccinia virus DNA polymerase gene. J Virol. 1985; 53(1):312–315. [PubMed: 2981354]

Julien O, Beadle JR, Magee WC, Chatterjee S, Hostetler KY, Evans DH, Sykes BD. Solution structure of a DNA duplex containing the potent anti-poxvirus agent cidofovir. J Am Chem Soc. 2011; 133(7):2264–2274. [PubMed: 21280608]

Kato SE, Moussatche N, D'Costa SM, Bainbridge TW, Prins C, Strahl AL, Shatzer AN, Brinker AJ, Kay NE, Condit RC. Marker rescue mapping of the combined Condit/Dales collection of temperature-sensitive vaccinia virus mutants. Virology. 2008; 375(1):213–222. [PubMed: 18314155]

Klemperer N, McDonald W, Boyle K, Unger B, Traktman P. The A20R protein is a stoichiometric component of the processive form of vaccinia virus DNA polymerase. J Virol. 2001; 75(24): 12298–12307. [PubMed: 11711620]

Kornbluth RS, Smee DF, Sidwell RW, Snarsky V, Evans DH, Hostetler KY. Mutations in the E9L polymerase gene of cidofovir-resistant vaccinia virus strain WR are associated with the drug resistance phenotype. Antimicrob Agents Chemother. 2006; 50(12):4038–4043. [PubMed: 16982794]

Kufe DW, Munroe D, Herrick D, Egan E, Spriggs D. Effects of 1-beta-D- arabinofuranosylcytosine incorporation on eukaryotic DNA template function. Mol Pharmacol. 1984; 26(1):128–134. [PubMed: 6431261]

Magee WC, Aldern KA, Hostetler KY, Evans DH. Cidofovir and (S)-9-[3-hydroxy-(2-phosphonomethoxy)propyl]adenine are highly effective inhibitors of vaccinia virus DNA polymerase when incorporated into the template strand. Antimicrob Agents Chemother. 2008; 52(2):586–597. [PubMed: 18056278]

Magee WC, Hostetler KY, Evans DH. Mechanism of inhibition of vaccinia virus DNA polymerase by cidofovir diphosphate. Antimicrob Agents Chemother. 2005; 49(8):3153–3162. [PubMed: 16048917]

Matsumoto K, Kim CI, Kobayashi H, Kanehiro H, Hirokawa H. Aphidicolin-resistant DNA polymerase of bacteriophage phi 29 APHr71 mutant is hypersensitive to phosphonoacetic acid and butylphenyldeoxyguanosine 5′-triphosphate. Virology. 1990; 178(1):337–339. [PubMed: 2117830]

McCraith S, Holtzman T, Moss B, Fields S. Genome-wide analysis of vaccinia virus proteinprotein interactions. Proc Natl Acad Sci U S A. 2000; 97(9):4879–4884. [PubMed: 10781095]

McDonald WF, Crozel-Goudot V, Traktman P. Transient expression of the vaccinia virus DNA polymerase is an intrinsic feature of the early phase of infection and is unlinked to DNA replication and late gene expression. J Virol. 1992; 66(1):534–547. [PubMed: 1727498]

McDonald WF, Klemperer N, Traktman P. Characterization of a processive form of the vaccinia virus DNA polymerase. Virology. 1997; 234(1):168–175. [PubMed: 9234958]

McDonald WF, Traktman P. Overexpression and purification of the vaccinia virus DNA polymerase. Protein Expr Purif. 1994a; 5(4):409–421. [PubMed: 7950389]

McDonald WF, Traktman P. Vaccinia virus DNA polymerase. In vitro analysis of parameters affecting processivity. J Biol Chem. 1994b; 269(49):31190–31197. [PubMed: 7983061]

Merchlinsky M. Resolution of poxvirus telomeres: processing of vaccinia virus concatemer junctions by conservative strand exchange. J Virol. 1990; 64(7):3437–3446. [PubMed: 2352329]

Mikita T, Beardsley GP. Functional consequences of the arabinosylcytosine structural lesion in DNA. Biochemistry. 1988; 27(13):4698–4705. [PubMed: 2458756]

Moss, B. Poxviridae: The Viruses and Their Replication. In: Knipe, DM., Howley, PM., editors. Fields Virology. 5th. Lippincott Williams & Wilkins; Phila: 2007. p. 2905-2946.

Moss, B. Poxviridae. In: Knipe, DM., Howley, PM., editors. Fields Virology. 6th. Vol. 2. Lippincott Williams & Wilkins; 2013. p. 2129-2159.2 vols

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Nishiyama Y, Yoshida S, Tsurumi T, Yamamoto N, Maeno K. Drug-resistant mutants of herpes simplex virus type 2 with a mutator or antimutator phenotype. Microbiol Immunol. 1985; 29(4): 377–381. [PubMed: 2991710]

Nuth M, Guan H, Ricciardi RP. A Conserved Tripeptide Sequence at the C-terminus of the Poxvirus DNA Processivity Factor D4 is Essential for Protein Integrity and Function. J Biol Chem. 2016

Nuth M, Guan H, Zhukovskaya N, Saw YL, Ricciardi RP. Design of potent poxvirus inhibitors of the heterodimeric processivity factor required for viral replication. J Med Chem. 2013; 56(8):3235–3246. [PubMed: 23527789]

Nuth M, Huang L, Saw YL, Schormann N, Chattopadhyay D, Ricciardi RP. Identification of inhibitors that block vaccinia virus infection by targeting the DNA synthesis processivity factor D4. J Med Chem. 2011; 54(9):3260–3267. [PubMed: 21438571]

Ohno Y, Spriggs D, Matsukage A, Ohno T, Kufe D. Effects of 1-beta-D- arabinofuranosylcytosine incorporation on elongation of specific DNA sequences by DNA polymerase beta. Cancer Res. 1988; 48(6):1494–1498. [PubMed: 3345522]

Otterlei M, Warbrick E, Nagelhus TA, Haug T, Slupphaug G, Akbari M, Aas PA, Steinsbekk K, Bakke O, Krokan HE. Post-replicative base excision repair in replication foci. EMBO J. 1999; 18(13): 3834–3844. [PubMed: 10393198]

Pedrali-Noy G, Spadari S. Mechanism of inhibition of herpes simplex virus and vaccinia virus DNA polymerases by aphidicolin, a highly specific inhibitor of DNA replication in eucaryotes. J Virol. 1980; 36(2):457–464. [PubMed: 6253671]

Punjabi A, Boyle K, DeMasi J, Grubisha O, Unger B, Khanna M, Traktman P. Clustered charge-to-alanine mutagenesis of the vaccinia virus A20 gene: temperature-sensitive mutants have a DNA-minus phenotype and are defective in the production of processive DNA polymerase activity. J Virol. 2001; 75(24):12308–12318. [PubMed: 11711621]

Sartmatova D, Nash T, Schormann N, Nuth M, Ricciardi R, Banerjee S, Chattopadhyay D. Crystallization and preliminary X-ray diffraction analysis of three recombinant mutants of Vaccinia virus uracil DNA glycosylase. Acta Crystallogr Sect F Struct Biol Cryst Commun. 2013; 69(Pt 3):295–301.

Schormann N, Banerjee S, Ricciardi R, Chattopadhyay D. Structure of the uracil complex of Vaccinia virus uracil DNA glycosylase. Acta Crystallogr Sect F Struct Biol Cryst Commun. 2013; 69(Pt 12):1328–1334.

Schormann N, Banerjee S, Ricciardi R, Chattopadhyay D. Binding of undamaged double stranded DNA to vaccinia virus uracil-DNA Glycosylase. BMC Struct Biol. 2015; 15:10. [PubMed: 26031450]

Schormann N, Grigorian A, Samal A, Krishnan R, DeLucas L, Chattopadhyay D. Crystal structure of vaccinia virus uracil-DNA glycosylase reveals dimeric assembly. BMC Struct Biol. 2007; 7:45. [PubMed: 17605817]

Schormann N, Sommers CI, Prichard MN, Keith KA, Noah JW, Nuth M, Ricciardi RP, Chattopadhyay D. Identification of protein-protein interaction inhibitors targeting vaccinia virus processivity factor for development of antiviral agents. Antimicrob Agents Chemother. 2011; 55(11):5054–5062. [PubMed: 21844323]

Schormann N, Zhukovskaya N, Bedwell G, Nuth M, Gillilan R, Prevelige PE, Ricciardi RP, Banerjee S, Chattopadhyay D. Poxvirus uracil-DNA glycosylase-An unusual member of the family I uracil-DNA glycosylases. Protein Sci. 2016; 25(12):2113–2131. [PubMed: 27684934]

Sele C, Gabel F, Gutsche I, Ivanov I, Burmeister WP, Iseni F, Tarbouriech N. Low-resolution structure of vaccinia virus DNA replication machinery. J Virol. 2013; 87(3):1679–1689. [PubMed: 23175373]

Silverman JE, Ciustea M, Shudofsky AM, Bender F, Shoemaker RH, Ricciardi RP. Identification of polymerase and processivity inhibitors of vaccinia DNA synthesis using a stepwise screening approach. Antiviral Res. 2008; 80(2):114–123. [PubMed: 18621425]

Sridhar P, Condit RC. Selection for temperature-sensitive mutations in specific vaccinia virus genes: isolation and characterization of a virus mutant which encodes a phosphonoacetic acid-resistant, temperature-sensitive DNA polymerase. Virology. 1983; 128(2):444–457. [PubMed: 6612992]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Stanitsa ES, Arps L, Traktman P. Vaccinia virus uracil DNA glycosylase interacts with the A20 protein to form a heterodimeric processivity factor for the viral DNA polymerase. J Biol Chem. 2006; 281(6):3439–3451. [PubMed: 16326701]

Stuart DT, Upton C, Higman MA, Niles EG, McFadden G. A poxvirus-encoded uracil DNA glycosylase is essential for virus viability. J Virol. 1993; 67(5):2503–2512. [PubMed: 8474156]

Taddie JA, Traktman P. Genetic characterization of the vaccinia virus DNA polymerase: identification of point mutations conferring altered drug sensitivities and reduced fidelity. J Virol. 1991; 65(2): 869–879. [PubMed: 1898973]

Taddie JA, Traktman P. Genetic characterization of the vaccinia virus DNA polymerase: cytosine arabinoside resistance requires a variable lesion conferring phosphonoacetate resistance in conjunction with an invariant mutation localized to the 3′–5′ exonuclease domain. J Virol. 1993; 67(7):4323–4336. [PubMed: 8389930]

Traktman P, Kelvin M, Pacheco S. Molecular genetic analysis of vaccinia virus DNA polymerase mutants. J Virol. 1989; 63(2):841–846. [PubMed: 2911123]

Traktman P, Sridhar P, Condit RC, Roberts BE. Transcriptional mapping of the DNA polymerase gene of vaccinia virus. J Virol. 1984; 49(1):125–131. [PubMed: 6317886]

Upton C, Stuart DT, McFadden G. Identification of a poxvirus gene encoding a uracil DNA glycosylase. Proc Natl Acad Sci U S A. 1993; 90(10):4518–4522. [PubMed: 8389453]

Weller, SK., Coen, DM. Herpes simplex virus. In: DePamphilis, editor. DNA replication and human disease. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY: 2006. p. 663-686.

Weller SK, Coen DM. Herpes simplex viruses: mechanisms of DNA replication. Cold Spring Harb Perspect Biol. 2012; 4(9):a013011. [PubMed: 22952399]

Willer DO, Mann MJ, Zhang W, Evans DH. Vaccinia virus DNA polymerase promotes DNA pairing and strand-transfer reactions. Virology. 1999; 257(2):511–523. [PubMed: 10329561]

Willer DO, Yao XD, Mann MJ, Evans DH. In vitro concatemer formation catalyzed by vaccinia virus DNA polymerase. Virology. 2000; 278(2):562–569. [PubMed: 11118378]

Wittek R, Moss B. Tandem repeats within the inverted terminal repetition of vaccinia virus DNA. Cell. 1980; 21(1):277–284. [PubMed: 6250716]

Wong SW, Wahl AF, Yuan PM, Arai N, Pearson BE, Arai K, Korn D, Hunkapiller MW, Wang TS. Human DNA polymerase alpha gene expression is cell proliferation dependent and its primary structure is similar to both prokaryotic and eukaryotic replicative DNA polymerases. EMBO J. 1988; 7(1):37–47. [PubMed: 3359994]

Yang J, Yan R, Roy A, Xu D, Poisson J, Zhang Y. The I-TASSER Suite: protein structure and function prediction. Nat Methods. 2015; 12(1):7–8. [PubMed: 25549265]

Zhang J, Chung DW, Tan CK, Downey KM, Davie EW, So AG. Primary structure of the catalytic subunit of calf thymus DNA polymerase delta: sequence similarities with other DNA polymerases. Biochemistry. 1991; 30(51):11742–11750. [PubMed: 1721537]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Highlights

- Vaccinia DNA polymerase (E9) is a member of the B-family of replicative polymerases
- E9 polymerase has both DNA polymerase and 3′–5′ exonuclease activities
- E9 mutants with ts, drug resistant or altered fidelity phenotypes have been studied
- Polymerase processivity is conferred by interaction with the A20/D4 heterodimer
- A20 bridges E9 and D4; D4 confers processivity and is an active UDG repair enzyme

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

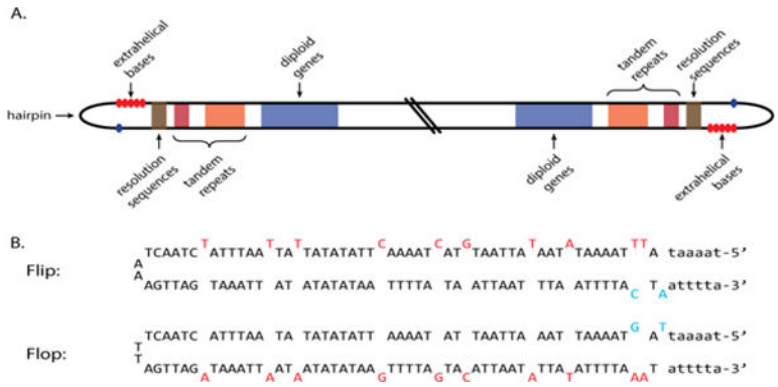

Figure 1. Structure of the poxvirus genome

A.) Schematic representation of the linear, 195 kb vaccinia genome with covalently closed hairpin ends. Several features of the ~10 kb inverted terminal repeats are illustrated. The 104 nt telomeric regions are represented in greater detail, with 12 extrahelical bases symbolized by the five red and one blue circles (10 and 2 bases on each strand respectively). Proceeding inward, key sequences involved in progeny genome resolution, two sets of 70bp tandem repeats, and several genes that are present at each terminus of the genome (therefore diploid) are marked. B.) Sequence diagram of the two, complementary isoforms of terminal hairpin sequences (flip and flop) each containing 12 extrahelical bases.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

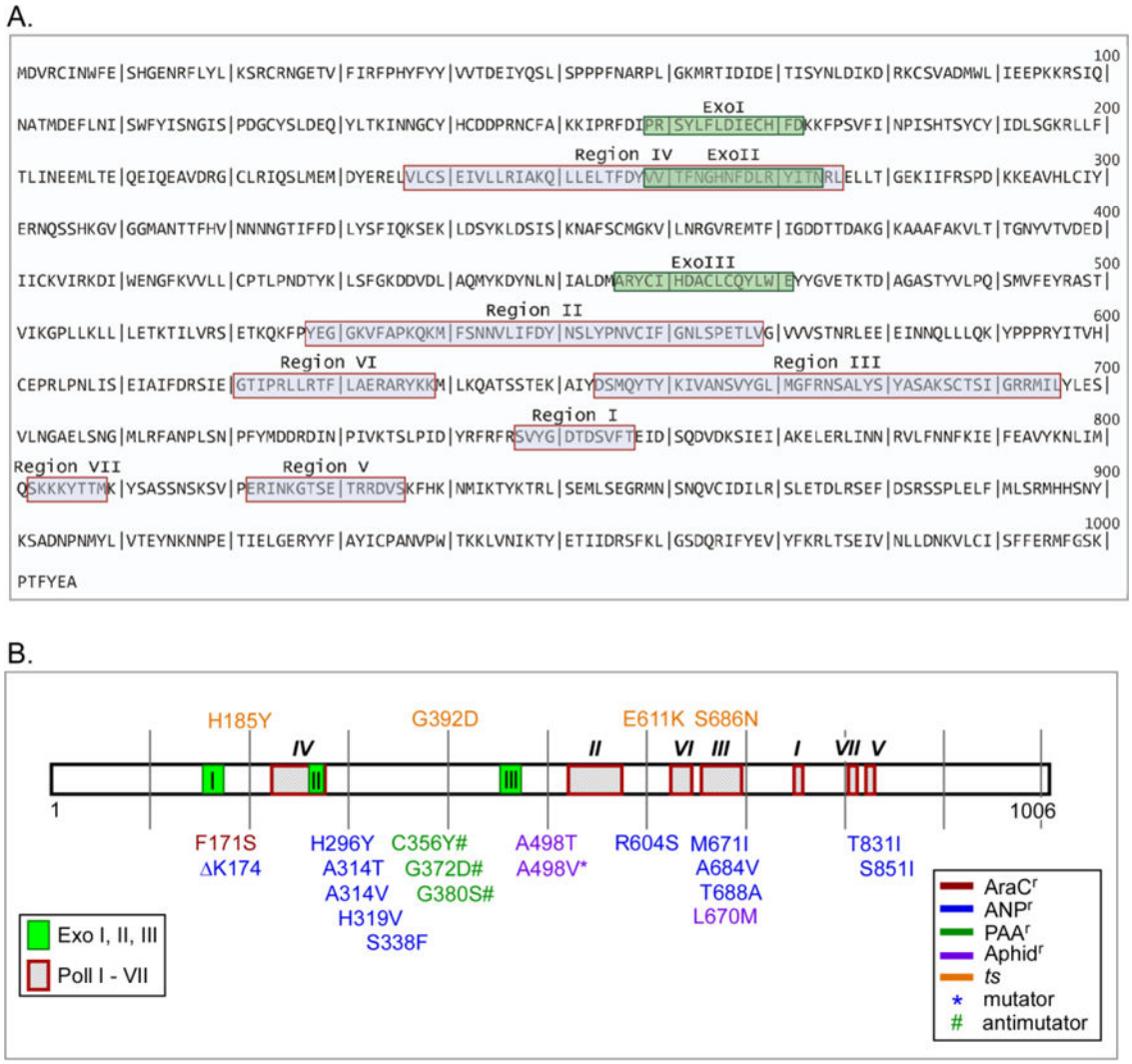

Figure 2. Vaccinia virus E9 protein

A.) The full, 1006 amino acid sequence of the E9 DNA polymerase is shown, with conserved motifs highlighted. The amino acids comprising the three motifs (exo I, II and III) that contribute to the exonuclease domain of E9 are delineated in green shaded boxes. Motifs associated with DNA polymerase activity (Regions I – VII) are marked in blue shaded boxes with red borders. B.) Schematic of the E9 protein with exonuclease motifs, polymerase motifs, and amino acid substitutions/deletions conferring drug resistance, temperature sensitivity, mutator or antimutator phenotypes marked. Characteristics of the motifs or mutations are shown in the left and right boxes, respectively.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

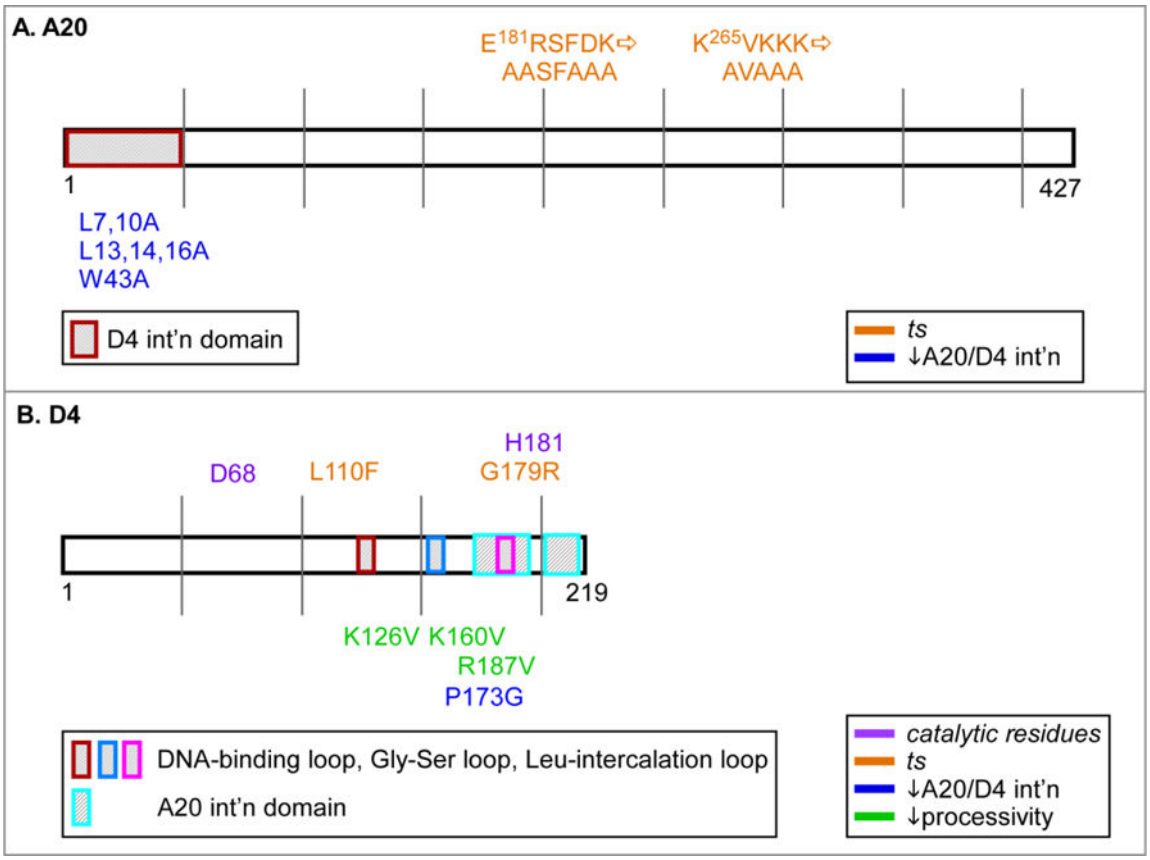

Figure 3. Vaccinia virus A20 and D4 proteins

A.) Schematic of the A20 protein with D4 interaction motif framed in red, as well as amino acid substitutions conferring a temperature-sensitive phenotype or decreased interaction with D4. Characteristics of the motifs or mutations are shown in the left and right boxes, respectively; int'n is an abbreviation for interaction. B.) Schematic of the viral D4 protein (UDG), with motifs associated with DNA binding or interaction with A20, and mutations associated with a temperature-sensitive phenotype, reduced interaction with A20, or reduced processivity marked. Characteristics of the motifs or mutations are shown in the left and right boxes, respectively; int'n is an abbreviation for interaction.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

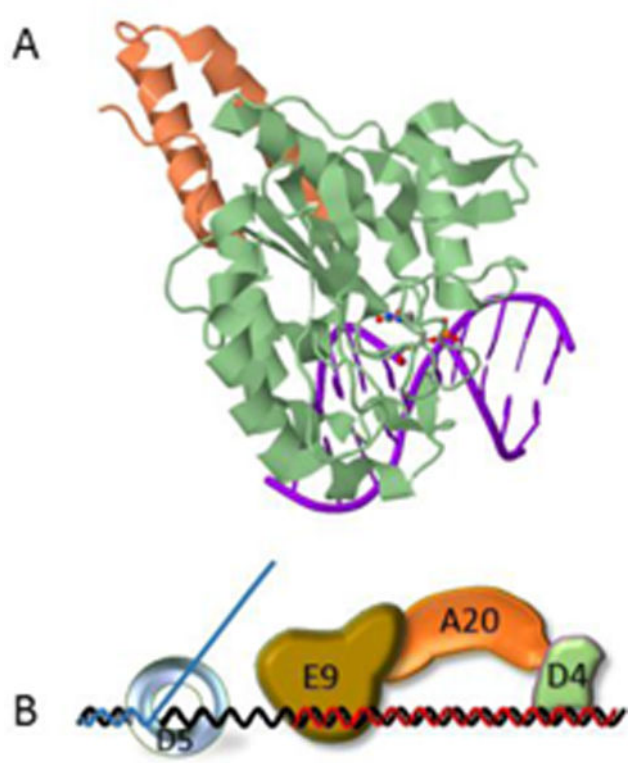

Figure 4. Vaccinia virus DNA polymerase holoenzyme

A.) Cartoon depiction of the crystal structure of D4 (green) in complex with the 50 N-terminal amino acids of A20 (orange) and dsDNA (purple) (PDB entry: 4YIG). B.) A model depicting the VACV DNA polymerase holoenzyme. In this model A20 serves as a bridge between the E9 polymerase and the D4 uracil DNA glycosylase. As a consequence of this orientation, D4 would be in a position to proof-read the newly polymerized genome for misincorporation of uracil moieties. In light of E9's inability to mediate strand displacement, D5, the hypothesized viral primase / helicase is positioned upstream of the polymerase holoenzyme to serve a replicative helicase. The DNA template strand is shown in black and the nascent daughter strand in red.