Chapter 12
Poxviruses

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Introduction

The Poxviridae family comprises large, complex DNA viruses that infect a wide variety of vertebrate and invertebrate hosts (chordopoxviruses and entomopoxviruses, respectively). Within the chordopoxvirus subfamily, the orthopoxvirus genus is best known for containing variola (VARV), the etiological agent of smallpox, and vaccinia (VACV), the virus used as the vaccine in the successful campaign to eradicate smallpox. Vaccinia has also served as the prototype for experimental investigation of poxvirus biology, and the following review will focus on vaccinia replication. Despite possessing DNA genomes, these viruses replicate exclusively in the cytoplasm of the infected host cell. This unusual physical autonomy from the host nucleus is accompanied by genetic autonomy; the ∼200 viral gene products encode a repertoire of proteins that mediate three temporally regulated phases of gene expression, genome replication, and virion morphogenesis. Poxviruses also encode a plethora of proteins that intersect with, and modulate, many cellular signaling cascades and components of the innate immune response.

Life Cycle

The poxvirus life cycle is shown schematically in Fig. 12.1. Poxvirus virions (∼360 nm × 270 nm × ∼250 nm) are quite complex, containing ∼75 distinct proteins (Condit et al., 2006). They are surrounded by a membrane (protein-rich lipid bilayer) and contain an internal core which houses the viral genome and a complete transcriptional apparatus. The processes of virion binding and entry are still being elucidated, but it appears that poxviruses can enter cells either by direct fusion of the virion membrane with the plasma membrane or by endocytic uptake. In either case, the internal virion core is then released into the cytoplasm and traffics

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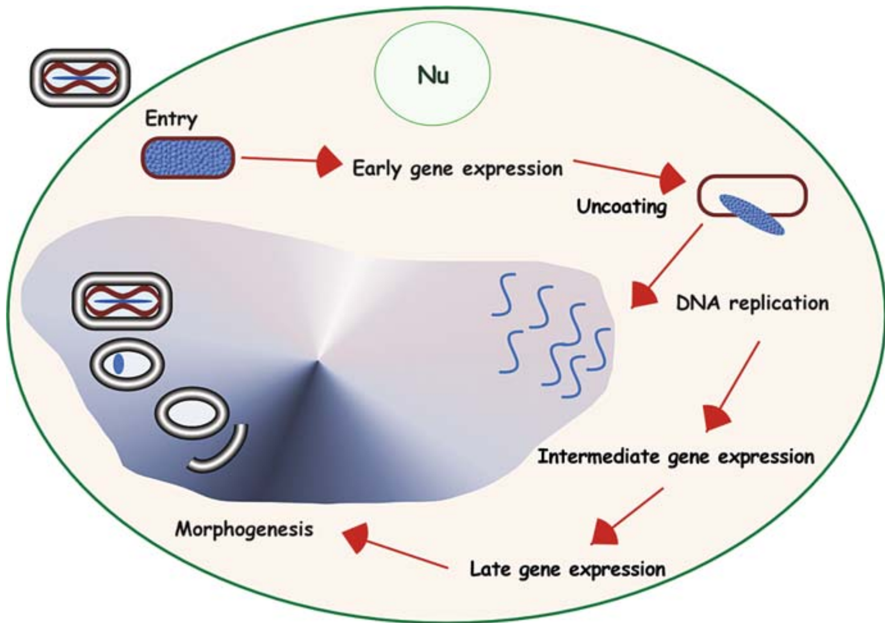

Fig 12.1 *Poxvirus life cycle*. Virion entry results in the deposition of the viral core into the cytoplasm. The remainder of the life cycle is restricted to the cytoplasm and is localized within viral factories. Early gene expression occurs within the core, resulting in the production of proteins involved in DNA replication and intermediate gene transcription. An uncoating event releases the viral genome into the cytoplasm, and viral DNA replication ensues. Intermediate and late gene expression commence, and the complex and highly regulated process of morphogenesis leads to the production of infectious progeny.

on microtubules to a peri-nuclear site (Carter et al., 2003; Mallardo et al., 2001). Within minutes, the encapsidated early transcription machinery is activated and early gene expression initiates within the viral core. Capped and polyadenylated mRNAs, which represent approximately one half of the genome, are extruded into the cytoplasm and translated on host polysomes. Among the early proteins are those needed for genome replication and for the transcription of intermediate mRNAs. Early gene transcription peaks at 1–2 h post-infection, and ceases when "uncoating" occurs, compromising the integrity of the core and releasing the genome into the cytoplasm where it then undergoes replication. Replication continues from ∼2 h post-infection until ∼12 h post-infection, providing a large pool of progeny genomes (∼10,000 per cell, one half of which are encapsidated into nascent virions) (Joklik and Becker, 1964; Salzman, 1960). DNA replication serves as a switch that enables the onset of intermediate, and then late, gene expression. Each phase of gene expression utilizes a unique set of *cis*- and *trans*-acting factors. The synthesis of viral mRNAs, DNAs, and proteins occurs in cytoplasmic domains known as "factories" or "virosomes". Once the late phase of gene expression is underway, the complex process of virion morphogenesis initiates. One of the earliest hallmarks

of virion assembly is the appearance of rigid, membranous crescents that enlarge until they become spherical, enclosing proteins destined to form the internal virion core. These spherical particles are known as immature virions; prior to their closure, the genome is encapsidated and an electron-dense nucleoid appears. Maturation of the immature virions into mature, infectious, virions is accompanied by a complex series of proteolytic cleavages and ultrastructural rearrangements. Recent reviews of virion morphogenesis, and the process whereby a subset of virions acquire additional membrane wrappings and undergo release from the cell to mediate intercellular and distal spread, are available (Condit et al., 2006; Smith et al., 2002).

Genome Structure

Poxviral genomes vary significantly in their AT:GC content, although the orthopoxviruses have a very high AT content; the DNA is not methylated. The large genomes (for VACV, 192 kb) possess a number of unique and distinguishing structural characteristics. A schematic representation of the chromosomal structure is depicted in Fig. 12.2. The telomeres of the double-stranded linear DNA genome are composed of highly AT-rich hairpins which contain extrahelical bases (EHB);

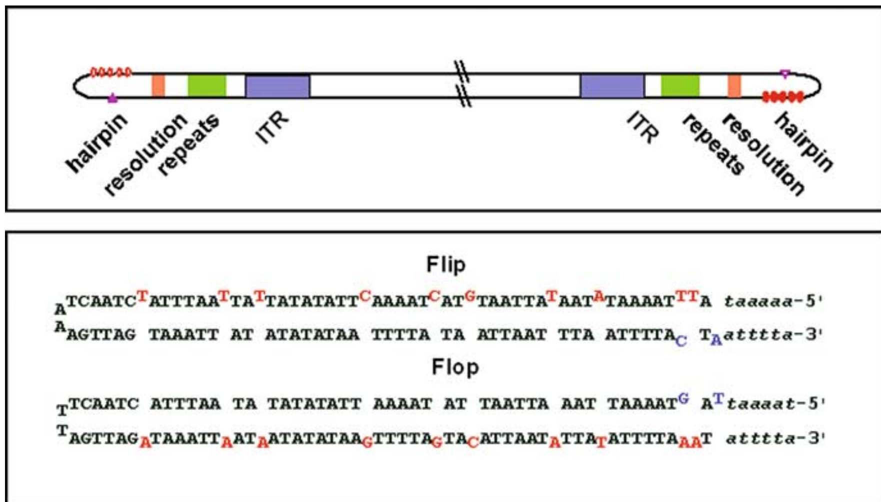

Fig. 12.2 *Poxvirus genome structure*. Top panel: The 192 kb vaccinia genome is a linear DNA duplex flanked by highly AT-rich hairpins which contains 12 extrahelical bases (represented as five *circles* and a *triangle*). Approximately 87 bp regions adjacent to the hairpin contain the motifs required for concatemer resolution. Adjacent to this region are sets of tandem repeats. These motifs, as well as a few genes, are present at both termini of the genome and are referred to as the ITR (inverted terminal repeats). The remainder of the genome encodes ∼200 proteins. Bottom panel: The hairpin sequences exist in two isoforms (flip and flop) that are inverted and complementary to each other; the extrahelical bases are shown in color.

in vaccinia virus, the hairpin is 104 nt in length and has 12 extrahelical bases (10 on one strand, 2 on the other). The presence of EHB is conserved in all poxvirus genomes, although the precise number and position of the bases vary. The hairpin sequences are found in two isoforms, known as flip and flop, that are inverted and complementary with respect to one other. Adjacent to the hairpin loop are 87 bp that are essential for both replication (Du and Traktman, 1996) and concatemer resolution (resolution repeats) (DeLange and McFadden, 1990). Beyond these motifs lie several sets of tandem repeats whose function is not known, although a role in mediating intergenomic recombination has been proposed. This entire region is found at both ends of the genome; in some viruses, the repeated region (inverted terminal repeat [ITR]) also contains protein-encoding genes, which are hence diploid in their inheritance.

The remainder of the poxviral genome is tightly packed with genes; in keeping with the cytoplasmic localization of the life cycle, the genes contain no introns and the transcripts do not undergo splicing. The 5′ and 3′ untranslated regions are minimal, as are the intergenic spaces. In the central portion of the genome, the polarity of the genes is somewhat random, but toward the ends of the genome, transcription is almost always oriented toward the telomere. There is no clustering of the genes by temporal class or protein function.

The complete sequences for 27 individual poxviruses (not counting different strain variants) have been determined (see http://poxvirus.org). The first poxvirus genome to be solved was the Copenhagen strain of vaccinia virus (Goebel et al., 1990), upon which the current nomenclature is based. The open reading frames are named according to their location within a given *Hin*dIII restriction fragment (A–P) and their transcriptional orientation (R or L) relative to the standard genomic map. For example, the DNA polymerase gene (E9L) is the ninth open reading frame in the *Hin*dIII E restriction fragment and it is transcribed in a leftward orientation. The protein name uses the same notation without the transcriptional polarity (E9 protein). For complete genomes that have been sequenced more recently, the open reading frames are often numbered sequentially, but most investigators in the field refer to orthologous genes using the Copenhagen nomenclature.

Analysis of Viral Replication Within Infected Cells

Pioneering electron microscopy provided the first evidence that poxviruses establish a distinct region in the cytoplasm of cells, referred to as the “virosome” or “DNA factory”, where viral DNA replication occurs (Cairns, 1960). These viral DNA factories have since been visualized by electron microscopy, staining with fluorescent dyes such as DAPI or Hoechst, or indirect immunofluorescent detection using anti-sera directed against BrdU or the viral single-stranded DNA-binding protein I3 (Domi and Beaud, 2000; Rochester and Traktman, 1998; Welsch et al., 2003). Cytoplasmic sites of replication have also been visualized by using DNA templates that bear multiple copies of the *Escherichia coli* lac operator and co-expressing a

GFP-lac repressor (De Silva and Moss, 2005). Recent data suggest that the perinuclear sites of replication are surrounded by membranes derived from the endoplasmic reticulum (Tolonen et al., 2001); clearly, much remains to be learned about how poxviruses establish sub-cellular compartments that enable efficient transcription, replication, and assembly.

Replication of viral DNA in vivo can be quantitated using one of several techniques. Metabolic labeling of nascent DNA with ^{3}H-thymidine can be used to assess the rate of viral DNA synthesis and is helpful in monitoring the immediate impact of adding pharmacological inhibitors or shifting the incubation temperature during infections with temperature-sensitive mutants. However, due to changes in the nucleotide pool that occur as infection progresses, and because of the feedback inhibition of the thymidine kinase (TK, see below), this technique cannot provide an accurate picture of the full extent of viral DNA accumulation. Furthermore, this method of analysis is limited to TK+ viruses, and many recombinants used for experimental analysis are TK–. To assess the steady-state levels of viral DNA synthesis, dot-blot Southern hybridization can be used. Accurate data can be obtained from ∼5 h to 24 h post-infection. Most recently, real-time PCR has been used to detect viral DNA sequences; this technique is likely to have a larger dynamic range than those described above.

In addition to monitoring the replication of viral genomes introduced by infection, investigators have also introduced exogenous templates in order to dissect the *cis*- and *trans*-acting components required for optimal replication. One approach that has been taken involves the generation of minichromosomes that mimic the topology of the viral genome and its unique telomeric features (Du and Traktman, 1996). In this assay, a linear plasmid stuffer sequence was flanked by telomeric variants ranging in size from 65 bp to 3000 bp. 150–200 bp were shown to be necessary and sufficient for optimal replication, as assessed by the accumulation of *Dpn*I-resistant DNA. Within this region are the terminal hairpin with its extrahelical bases (∼50 bp) and the motifs known to be essential for concatemer resolution (∼70 nt). Minichromosomes bearing these telomeres were shown to replicate 10-fold better than supercoiled plasmids. However, other investigators have reported that supercoiled plasmids lacking any viral sequences can be replicated as efficiently as minichromosomes (De Silva and Moss, 2005). Thus, further investigation is required in order to definitively assess the contributions, if any, that *cis*-acting sequences make to the efficient replication of the viral genome. Interestingly, analyses of plasmid replication within infected cells have revealed that both processes rely on the same repertoire of viral replication proteins, which will be described below in greater detail.

Working Model for Poxvirus DNA Replication

Poxviral DNA replication is thought to initiate with the introduction of a nick near one or both genomic termini (Fig. 12.3, step 1). Introduction of this nick is inferred from the observed increase in topological freedom as well as the change in the

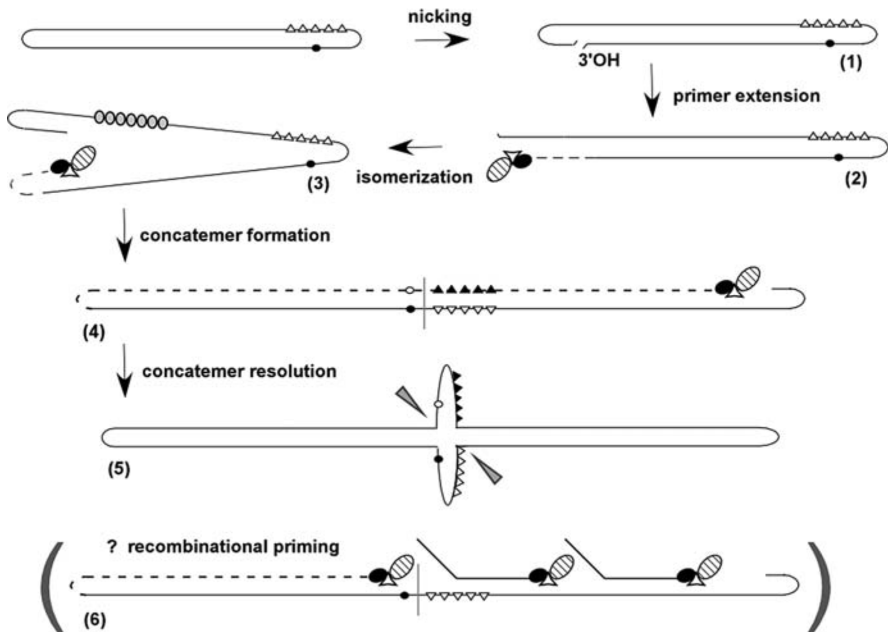

Fig 12.3 *Working model of poxvirus DNA replication*. The linear genome with covalently closed hairpin termini is shown; for convenience, the extrahelical bases (*triangles* and *circle*) are only shown for the right telomere. Replication is thought to begin with the introduction of a nick which exposes a free 3′ OH group that serves as a primer for the trimeric DNA polymerase holoenzyme. The nascent (*dashed lines*) and displaced strands (coated with viral SSB, represented by the *light gray circles*) can each form self-complementary hairpins, which allows leading strand synthesis to replicate the entire molecule. This process generates a tail:tail dimer; a fully duplexed, imperfect palindrome is found at the concatemer junction. The extended duplex form of the palindrome can undergo cruciform extrusion, which creates hairpins that contain extrahelical bases and resemble the viral telomeres. The virally encoded resolvase then cleaves this Holliday junction-like structure, generating monomeric genomes. During later phases of replication, it is proposed that recombination priming events may occur.

sedimentation properties of viral DNA at the onset of replication (Pogo, 1977). Furthermore, the initial site of ^{3}H-thymidine incorporation was mapped to a region within 150 bp from the telomeres (Pogo et al., 1984). The enzyme involved in this nicking event remains elusive, although it is proposed that the nick must leave a free 3′ hydroxyl group to serve as a primer terminus for the viral DNA polymerase. Strand displacement synthesis proceeds toward the hairpin terminus, yielding an intermediate in which the termini of both the nascent and template strands are self-complementary (step 2). This intermediate could assume a conformation that would generate a self-priming hairpin structure (step 3), facilitating the replication of the remainder of both the top and bottom strands of the genome. The initial product would be a tail–tail dimer (step 4). Larger tetrameric molecules may be formed if this process was repeated. Recombinational priming of replicating molecules has

also been posited (step 6). Indeed, electrophoretic analysis of replicating DNA has detected the accumulation of large concatemeric and branched intermediates (DeLange, 1989; DeLange and McFadden, 1990; Merchlinsky et al., 1988; Moyer and Graves, 1981).

This current model for poxvirus replication proposes that only leading strand synthesis is employed. This is consistent with the significant levels of single-stranded DNA that have been observed during infection (Esteban and Holowczak, 1977; Pogo et al., 1981). However, early reports suggested that both leading and lagging strand synthesis were involved since it was observed that short nascent DNA strands could be chased into larger forms suggestive of Okazaki fragment formation (Esteban and Holowczak, 1977). Resolution of this issue is an area for further study.

As stated above, the initial products of replication are concatemeric intermediates. The concatemer junction is an imperfect palindrome that can isomerize from a lineform structure to a cruciform structure (step 5). Conversion of the concatemer to monomers that preserve the unique telomeric structure involves a virally encoded Holliday-junction resolvase; this process will be described below in greater detail.

Core Replication Machinery

The repertoire of proteins that mediate vaccinia virus DNA replication has been identified by a blend of genetic, genomic, and biochemical analyses (Table 12.1). Temperature-sensitive (*ts*) viruses, generated by either chemical mutagenesis or targeted clustered charge-to-alanine mutagenesis, reveal that five genes are essential for DNA replication in tissue culture. These encode the DNA polymerase (E9), the two components of the processivity factor (A20 and D4), a nucleic acid-independent NTPase (D5), and a serine/threonine protein kinase (B1).

Table 12.1 Vaccinia-encoded proteins with known or predicted roles in DNA replication are shown

Protein	Function
E9	DNA polymerase
A20	Processivity factor
D4	Uracil DNA glycosylase; processivity factor
D5	DNA independent NTPase; primase; superfamily III helicase homology
B1	Ser/thr protein kinase
I3	Single-strand DNA-binding protein
A22	Holliday-junction resolvase
I6	Telomere-binding protein
A32	Putative ATPase
H6	Topoisomerase I
A50	DNA ligase
F2	dUTPase
F4, I4	Ribonucleotide reductase
J2	Thymidine kinase
A48	Thymidylate kinase

E9, The Catalytic DNA Polymerase

The E9L gene encodes the catalytic DNA polymerase, the core of the trimeric polymerase complex. The 116 kDa E9 protein is expressed early during infection and contains conserved motifs found within the α family of replicative DNA polymerases (Earl et al., 1986; Taddie and Traktman, 1993). E9 has both 5′–3′ polymerization and 3′–5′ exonuclease activities, but lacks intrinsic strand displacement activity (Challberg and Englund, 1979a, b). Structure–function analysis of the protein has been facilitated by phenotypic characterization of two *ts* mutants and drug-resistant mutants that confer resistance to aphidicolin, phosphonoacetic acid, and cytosine arabinoside (DeFilippes, 1984, 1989; Taddie and Traktman, 1991, 1993; Traktman et al., 1989). Aphidicolin-resistant mutants presented a mutator phenotype (Taddie and Traktman, 1991). Lastly, mutations within the E9L gene conferred resistance to the broad spectrum antiviral agent, cidofovir (CDV), a dCMP analog (Kornbluth et al., 2006). It has also been shown that purified E9 can incorporate CDV into the nascent strand adjacent from a G residue, promoting chain termination (Magee et al., 2005). Exploiting E9 as an enzymatic target is an active area of antipoxviral research.

Purified E9 is an inherently distributive enzyme under physiological conditions (McDonald and Traktman, 1994), synthesizing only 10 nt per primer/template-binding event. Since this behavior would not be conductive to efficient and faithful duplication of an ~200 kb genome in vivo, it was not surprising that cytoplasm extracts of infected cells contained a highly processive form of the polymerase that is able to catalyze the synthesis of >7000 nt per primer/template-binding event (McDonald et al., 1997). As described below, the processive polymerase contains the A20 and D4 proteins as well as E9.

Genetic analyses have shown that there is a clear overlap between the proteins involved in viral replication and homologous recombination. Consistent with these findings, the viral DNA polymerase has been shown to participate in both single-strand annealing and duplex-strand joining reactions (Hamilton and Evans, 2005; Willer et al., 1999; Willer et al., 2000).

A20, A Component of the Polymerase Processivity Factor

Chromatographic purification of the processive form of the viral DNA polymerase revealed that an ~48 kDa protein was an intrinsic component of this complex (Klemperer et al., 2001). This protein was identified as the product of the A20 gene. A20 and E9 were shown to interact by co-immunoprecipitation experiments, and overexpression of both A20 and E9 during infection led to increased levels of processive polymerase activity. Site-directed, clustered charge-to-alanine mutagenesis of the A20 gene resulted in the generation of *ts* mutants that were defective in DNA replication and defective in the formation of processive polymerase activity (Ishii and Moss, 2001; Punjabi et al., 2001). However, purification of recombinant

A20 protein proved elusive, and it seemed plausible that A20 might interact with, and require, additional proteins to fulfill its role as a processivity factor. Indeed, A20 was shown to interact with the D5 protein (see below), the H5 protein (an abundant phosphoprotein implicated in transcription and morphogenesis), and the D4 protein (see below) in the yeast two-hybrid assay (Ishii and Moss, 2002; McCraith et al., 2000).

D4, Uracil DNA Glycosylase and a Component of the Polymerase Processivity Factor

The product of the D4 gene is an enzymatically active uracil DNA glycosylase (UDG) (Scaramozzino et al., 2003; Stuart et al., 1993; Upton et al., 1993). Conservation of motifs associated with enzymatic activity in other UDG molecules has enabled the generation of viral recombinants that express stable, soluble, but catalytically inactive variants of UDG. These mutants are viable in tissue culture, but attenuated in vivo, suggesting that the repair function of UDG is important during infection of key cell types in vivo (De Silva and Moss, 2003; Stanitsa et al., 2006). In contrast, deletion of the UDG gene is not compatible with viability in tissue culture, underscoring the fact that the UDG protein plays an essential role in viral replication that is independent of its catalytic activity (De Silva and Moss, 2003; Stanitsa et al., 2006).

Phenotypic analysis of two *ts* mutants with lesions in the D4 gene revealed that, like A20, impairment of UDG led to a defect in DNA replication and a defect in the assembly of processive DNA polymerase activity (Stanitsa et al., 2006; Stuart et al., 1993). As mentioned, D4 and A20 interact tightly in the absence of other viral proteins, as assessed by yeast two-hybrid and co-immunoprecipitation assays (Ishii and Moss, 2002; McCraith et al., 2000). Overexpression of 3XFLAG-UDG, A20, and DNA Pol in the context of infected cells has enabled the purification of dimeric UDG/A20 and trimeric UDG/A20/Pol complexes (Stanitsa et al. 2006). These complexes are quite stable, remaining intact in the presence of 750 mM NaCl. The trimeric complex has processive polymerase activity, and the addition of purified 3XFLAG-UDG/A20 to a purified preparation of E9 leads to the reconstitution of processive polymerase activity. In sum, these data support the conclusion that the vaccinia DNA polymerase holoenzyme is comprised of E9, A20, and D4 (Stanitsa et al., 2006). While analysis of the UDG crystal structure has revealed the propensity of UDG to form dimers (Schormann et al., 2007), the higher order structure of the UDG/A20 and UDG/A20/Pol complexes remains to be determined, as does the dissection of the protein:protein interfaces. It is known that the D4:A20 interaction involves the N' terminal 25 amino acids of the A20 protein (Ishii and Moss, 2002). The participation of a UDG as an essential component of a processivity factor is unique to processes and raises the intriguing possibility that replication and repair may be coupled.

I3, Single-Stranded DNA-Binding Protein

A variety of data suggest that the I3 protein is the replicative single-stranded DNA-binding protein (SSB), although this has not yet been proven definitively. I3 is a highly abundant 34 kDa phosphoprotein that is expressed at both early and intermediate times post-infection (Rochester and Traktman, 1998). Purified I3, both endogenous and recombinant, binds to single-stranded DNA with high affinity and specificity. Gel shift assays have derived a binding site size of approximately 10 nt/I3 molecule, and formation of beaded protein/DNA structures can be seen by electron microscopy (Rochester and Traktman, 1998; Tseng et al., 1999). Depletion of I3 during infection using short-interfering RNA (siRNA) technology leads to a reduction in DNA accumulation as assessed by both immunofluorescence and dot-blot hybridization analysis (PT, unpublished). Definitive proof that I3 is the replicative SSB awaits genetic data. Interestingly, the I3 protein has also been shown to bind to the viral ribonucleotide reductase (see below); this interaction could enhance the efficiency of replication by enabling nucleotide precursors to be synthesized at the site of replication (Davis and Mathews, 1993).

D5, Nucleoside Triphosphatase

The 90 kDa D5 protein possesses intrinsic nucleoside triphosphatase activity (NTPase) that is neither dependent on nor stimulated by nucleic acid cofactors (Evans et al., 1995). Genetic analysis of *ts* mutants with lesions in D5 has revealed an essential role for D5 during DNA replication (Boyle et al., 2007; Evans and Traktman, 1992). Temperature shift experiments demonstrate that DNA synthesis arrests immediately (~5 min) after the shift of *ts*D5-infected cultures to the non-permissive temperature, which is indicative of a role of D5 at the replication fork (Evans and Traktman, 1992; McFadden and Dales, 1980). Evidence that D5 can interact with A20 provides additional indirect evidence that D5 might function at the replication fork (McCraith et al., 2000). Although *ts*D5 mutants are defective in DNA replication, they are not defective in the assembly of a processive DNA polymerase complex.

D5 homologs can be identified in every poxvirus sequenced to date, but the protein bears no strong similarity to any cellular protein. Indeed, the D5 protein appears to be a defining member of the D5-like helicase family, which can be found in all poxviruses, certain bacteriophages, and the 1.2 megabase genome of mimivirus (Iyer et al., 2001; Raoult et al., 2004). The sequence of the D5 protein contains conserved motifs that have led to its classification as a peripheral member of the AAA+ family of proteins, within the subfamily of superfamily III DNA helicases (Iyer et al., 2001). This latter group contains several viral proteins that function as replicative helicases. Phenotypic characterization of D5 variants containing amino acid substitutions at key positions within these family-defining motifs has revealed that these motifs are essential, for both NTPase activity and biological function

(Boyle et al., 2007). Structure–function analyses have also revealed that multimerization of the D5 protein is a prerequisite for enzymatic activity and that catalytic activity is necessary but not sufficient for biological activity (Boyle et al., 2007). The catalytic domain comprises the C' terminal half of the protein, while the function of the N' terminal half of the protein, which contains most of the residues affected in the four available *ts*D5 mutants, has not been defined. Given D5's essential role in DNA replication, its NTPase activity, and its similarity to the superfamily III group of helicases, it is tempting to postulate that D5 functions as a helicase in vivo. There is no experimental data to support this hypothesis. However, the sequence of D5 also has motifs associated with DNA primases (Iyer et al., 2005). Indeed, purified D5 has recently been shown to have primase activity in vitro, and the motifs associated with primase activity are important for the biological function of D5 in vivo (De Silva et al., 2007). Finally, it is quite possible that D5 may also participate in homologous recombination, since marker rescue experiments using small (<2 kb) intragenic fragments were impaired during *ts*D5 infections (Evans and Traktman, 1992).

B1, Serine/Threonine Protein Kinase

The importance of dynamic protein phosphorylation in the regulation of the viral life cycle is illustrated by the fact that vaccinia encodes two protein kinases and one protein phosphatase. The B1R gene encodes a 34 kDa serine/threonine protein kinase that is expressed early in infection and encapsidated at low levels in virions. Analysis of two *ts*-B1 mutants revealed a temperature-dependent defect in DNA synthesis whose severity varied with host cell (Boyle and Traktman, 2004; Rempel et al., 1990). The proteins encoded by both mutants had greatly diminished kinase activity and appeared to be labile in vivo at all temperatures. A defect in intermediate gene expression upon impairment of B1 was also reported (Kovacs et al., 2001). Clarification of the role of B1 in the viral life cycle proved elusive for many years; although B1 was shown to phosphorylate the viral H5 protein and two ribosomal proteins, none of the components of the replication machinery appeared to be B1 substrates. Recently, bioinformatic analysis indicated that B1 shows significant similarity to a group of cellular kinases classified as peripheral branch of the casein kinase family. These kinases are now known as vaccinia-related kinases (VRKs) due to the high degree of identity within the catalytic domains of the viral and cellular proteins (Nezu et al., 1997; Nichols and Traktman, 2004; Zelko et al., 1998). This sequence identity implied that the proteins might have overlapping substrate specificities, and indeed, the incorporation of an hVRK1 cDNA into the genome of *ts*B1 fully rescued the DNA replication defect (Boyle and Traktman, 2004). Complementation was not obtained when a catalytically inert variant of hVRK1 was used. The endogenous VRK1 is found only in the nucleus and therefore is not available for complementation.

We now know that the cellular BAF protein is the key substrate whose phosphorylation by B1 regulation is imperative for viral DNA replication to ensue (Wiebe and Traktman, 2007). BAF is an abundant protein, found in both the nucleus and

the cytoplasm, that binds to double-stranded DNA avidly and without sequence specificity. Binding of BAF dimers to DNA leads to cross-bridging and condensation. However, when BAF is phosphorylated on key residues within its N' terminus, its ability to bind DNA is abrogated (Nichols et al., 2006). Since vaccinia DNA replication occurs in the cytoplasm, it is accessible to BAF, which can associate with viral DNA within the factories and blocks its replication. BAF appears to be a heretofore unknown host defense against foreign DNA; BAF's repressive action can be reversed by B1-mediated phosphorylation. Recruitment of BAF to the factories does not occur during wild-type infections, but is readily observed during *ts*B1 infections. If BAF is depleted using lentivirus-mediated RNAi, the temperature-sensitive phenotype of the *ts*B1 viruses is largely reversed. Thus the primary, if not only, role of B1 in tissue culture cells is to combat the repressive effect of BAF.

Viral Proteins Involved in Nucleotide Biosynthesis and Precursor Metabolism

Thymidine Kinase and Thymidylate Kinase

Thymidine biosynthesis is directed by two viral enzymes, the thymidine kinase (J2) and thymidylate kinase (A48), neither of which is essential for propagation in tissue culture (Buller et al., 1985; Hughes et al., 1991). The 19 kDa viral thymidine kinase (TK) is a homotetramer whose activity is subject to feedback inhibition with high levels of TTP or TDP (Hruby, 1985; Wilson et al., 1989). This feedback inhibition loop can be bypassed, without compromising enzymatic activity, by altering the key glutamine residue in conserved domain IV (Black and Hruby, 1992). The ability of the viral TK to also utilize BrdU as a substrate has been exploited in the generation of recombinant vaccinia viruses. Insertion of foreign DNA into the TK locus inactivates TK, leading to a BrdU-resistant phenotype which can be selected for by propagation on human 143 TK– cells. TK– viruses are attenuated in vivo (Buller et al., 1985), underscoring the importance of the precursor biosynthetic machinery in key cell types in vivo.

The 23 kDa viral thymidylate kinase functions as a dimer to phosphorylate dTMP. The viral dTMP kinase shares ~40% identity to the human enzyme, retaining several conserved catalytic motifs and the overall conserved core structure (Topalis et al., 2005). The high degree of homology between the viral and cellular enzymes is well illustrated by the finding that A48 can complement the dTMP-deficient *Saccharomyces cerevisiae* cdc8 mutant (Hughes et al., 1991).

Ribonucleotide Reductase

The vaccinia ribonucleotide reductase (RNR) is composed of small (F4) and large (I4) subunits; it catalyzes the reduction of rNDPs to the corresponding dNDPs (Slabaugh et al., 1988; Tengelsen et al., 1988). The allosteric behavior and

regulatory mechanism of the viral enzyme are comparable to those of mammalian RNRs (Slabaugh and Mathews, 1984). The large subunit (I4) contains binding sites for nucleotide substrates and allosteric effectors (Slabaugh et al., 1984; Slabaugh and Mathews, 1984), while the small subunit (F4) carries the tyrosyl radical required for catalysis (Howell et al., 1992). It is this tyrosyl radical that is affected by the DNA synthesis inhibitor hydroxyurea (HU) (Ehrenberg and Reichard, 1972). While it would be reasonable to assume that there is an equivalent depletion in the rates of formation of all four dNDPs, experimental evidence has shown that this is not the case. The most significant decrease is seen for dATP levels, and HU-mediated inhibition can be largely overcome by the addition of exogenous deoxyadenosine in the presence of an adenosine deaminase inhibitor (Slabaugh et al., 1991).

RNR-deficient vaccinia mutants replicate normally in tissue culture (Child et al., 1990), suggesting that the host enzyme can provide sufficient precursors for viral replication to proceed; these mutants are somewhat attenuated in vivo. In tissue culture, HU inhibits vaccinia replication; HU-resistant mutants can be isolated, and these display amplification (2–15 copies) of the F4L gene (Slabaugh et al., 1988).

dUTPase

As mentioned above, the D4 protein of vaccinia virus is an active uracil DNA glycosylase, which removes uracil moieties in DNA that can arise either from cytosine deamination or from the incorporation of dUTP by the viral DNA polymerase. The importance of controlling the presence of dUMP within DNA is underscored by the fact that vaccinia also encodes a dUTPase. The F2 protein of vaccinia acts as a trimer to catalyze the hydrolysis of dUTP to dUMP (Broyles, 1993; Roseman et al., 1996). In addition to minimizing the concentration of dUTP within infected cells, dUTPase activity provides increased levels of dUMP, which serves as a precursor in the synthesis of TTP. The F2 sequence shows a high degree of similarity to the human enzyme, and comparisons between the cellular and viral enzymes are facilitated by the recent solution of the crystal structure of the viral dUTPase (Samal et al., 2007).

DNA Replication Accessory Proteins

Topoisomerase

The vaccinia virus H6 protein is type 1B topoisomerase that is expressed late during infection and is encapsidated into the virion core (Bauer et al., 1977; Shaffer and Traktman, 1987; Shuman and Moss, 1987). The poxvirus topoisomerase has a number of unique properties that distinguish it from other enzymes of this class: resistance to the drug camptothecin, sensitivity to the DNA gyrase inhibitors novobiocin and coumermycin, and remarkable specificity for a pentapyrimidine target sequence

for DNA cleavage (Hwang et al., 1998; Klemperer et al., 1995; Sekiguchi et al., 1996; Shuman et al., 1988; Shuman and Moss, 1987; Shuman and Prescott, 1990). An extraordinary amount of insight has been gained into the biochemical mechanism used by this enzyme; in contrast, discerning the biological role of the protein proved difficult. Roles in transcription, replication, recombination, and concatemer resolution were posited. Initially, the inability to isolate an H6 deletion mutant led to the conclusion that H6 was essential in tissue culture (Shuman et al., 1989); more recently, however, such a virus has been isolated using a different selection protocol. The topoisomerase-null virus exhibits reduced infectivity due to a diminution in early transcription; neither DNA replication nor genome maturation appears to be affected (Da Fonseca and Moss, 2003). This data suggest that the primary biological role for the vaccinia topoisomerase is to facilitate early gene transcription.

DNA Ligase

The vaccinia virus A50 protein is a 61 kDa ATP-dependent DNA ligase (Kerr and Smith, 1989) with significant similarity to the mammalian type II and III DNA ligases (Chen et al., 1995; Husain et al., 1995; Wang et al., 1994; Wei et al., 1995). The functional redundancy between the viral and cellular ligases was confirmed by the demonstration that the vaccinia DNA ligase can compensate for the loss of the *S. cerevisiae* DNA ligase (Kerr et al., 1991). A significant amount of biochemical and structure/function analyses has been performed on the viral ligase. An A50 deletion mutant has been constructed: the enzyme is not essential for propagation in tissue culture but the deletion mutant is attenuated in vivo (Colinas et al., 1990; Kerr and Smith, 1991). In some contexts, however, deletion of the ligase does compromise viral replication (Parks et al., 1998). Deletion of A50 leads to UV- and bleomycin sensitivity but to etoposide resistance; increasing the copy number of the ligase gene confers etoposide sensitivity (DeLange et al., 1995; Kerr et al., 1991). Clearly, we do not fully appreciate the role(s) that the viral DNA ligase might play during infection.

Genome Maturation

Cis-*Acting Sequences*

As described above, the initial product of genomic replication is the generation of head-to-head or tail-to-tail concatemers (DeLange, 1989; DeLange and McFadden, 1990; Merchlinsky et al., 1988; Moyer and Graves, 1981). These concatemers contain an imperfect palindromic junction that can be extruded into a cruciform structure that mimics a DNA Holliday junction (Dickie et al., 1987, 1988; Merchlinsky et al., 1988). Indeed, within infected cells, plasmids containing these inverted repeats are processed into linear minichromosomes with hairpin termini (DeLange and McFadden, 1987; DeLange et al., 1986; Merchlinsky, 1990a, b;

Merchlinsky et al., 1988; Merchlinsky and Moss, 1986, 1989a). At the concatemer junction, the extended duplex version of the hairpin sequence is flanked on each side by sequences required for resolution. Within these sequences, four *cis*-acting motifs have been defined that are either essential (domains 1A and 1) or stimulatory (domains II and III) in this resolution process. The canonical resolution sequence within domains 1A and 1 has been identified, and site-directed mutagenesis has revealed that resolution is exquisitely dependent on its sequence (DeLange and McFadden, 1987; Merchlinsky, 1990a; Merchlinsky and Moss, 1989a). The resolution sequences are strongly conserved among poxviruses (Merchlinsky, 1990a). Interestingly, the resolution sequence can also function as a late promoter, suggesting that transcription through the region might stimulate resolution (Parsons and Pickup, 1990; Stuart et al., 1991).

Resolvase

The observation that concatemer resolution fails to occur when late protein synthesis is blocked provided the initial hint that a late viral protein might mediate concatemer resolution (DeLange, 1989; Merchlinsky and Moss, 1989b). The A22 protein was subsequently identified as a likely candidate because of its homology to a known Holliday-junction resolvase, the *E. coli* Ruv C protein (Garcia et al., 2000). A22 is a 23 kDa protein that is expressed at late times during infection (Garcia and Moss, 2001), and purified recombinant A22 can bind to and cleave a synthetic Holliday junction, yielding nicked duplex molecules (Garcia et al., 2000). The protein forms a dimer, which most likely mediates the symmetrical cleavage of the concatemeric junction (Garcia et al., 2006). In vitro, the viral enzyme exhibits only weak sequence specificity, which does not explain the strict sequence specificity that characterizes resolution in vivo (Culyba et al., 2006; Garcia et al., 2006). Nevertheless, repression of A22 expression leads to a significant block to concatemer resolution: the majority of newly synthesized viral DNA remains concatemeric and virion morphogenesis is not completed (Garcia and Moss, 2001). Clearly, A22 is necessary for concatemer resolution; whether other viral proteins impart specificity to the reaction remains to be resolved.

Genome Encapsidation

To complete the infectious cycle, progeny DNA genomes must be encapsidated into nascent virions. Genome encapsidation is associated with the appearance of an electron-dense nucleoid within immature virions. Although much remains to be learned about this process, we have gained some insights into some of the proteins that participate in this process (Condit et al., 2006). Repression of the 8 kDa A13 protein, which is a component of the virion membrane, leads to a morphogenesis arrest characterized by the accumulation of immature virions lacking nucleoids

and the accumulation of DNA crystalloids in the cytoplasm (Unger and Traktman, 2004). Two components of the virion core are more directly associated with genome encapsidation. The A32L gene encodes a 34 kDa protein with some sequence similarity to proteins involved in adenovirus and bacteriophage DNA encapsidation (Koonin et al., 1993). Based on conserved Walker A and Walker B motifs, the protein has been predicted to have ATPase activity; although no empirical evidence of this activity has been reported. When expression of the A32 protein is repressed, the biochemical events of the viral life cycle progress normally. The late stages of morphogenesis are aberrant; however, resulting in the production of abnormal spherical particles which are devoid of viral DNA (Cassetti et al., 1998). The 44 kDa I6 protein is also directly implicated in genome encapsidation; this protein binds to the telomeric hairpins of the genome with great specificity and stability (DeMasi et al., 2001). During nonpermissive infections with a temperature-sensitive I6 mutant, the viral life cycle again progresses normally until the later stages of morphogenesis. Again, aberrant spherical particles are formed which lack viral DNA, and DNA crystalloids accumulate in the cytoplasm (Grubisha and Traktman, 2003). The I6 protein encoded by this mutant appears to be defective in its telomere-binding capacity (Traktman, unpublished). The A32 protein is encapsidated at wild-type levels in the DNA-deficient particles that assemble during *ts*I6 infections, whereas I6 is not encapsidated in the A32-deficient particles (Traktman, unpublished). It seems plausible to propose that the binding of I6 to the telomeres of the viral genome imparts specificity to the encapsidation process and that entry of the genome maybe facilitated by I6-A32 interactions or by A32-mediated ATP hydrolysis.

Questions for Future Study

Poxvirus DNA replication is unique in that it occurs within the cytoplasm of infected cells and relies almost exclusively on viral proteins. Much has been learned about this fascinating process (Moss and De Silva, 2006; Traktman, 1996), but many unsolved puzzles remain. The development and organization of the cytoplasmic factories in which replication occurs are poorly understood. Replication is thought to initiate with the introduction of a nick, but neither the *cis*- nor *trans*-acting factors that contribute to this initiation process have been identified. Confirmation that replication relies solely on leading strand synthesis is also pending, and disparities between the specificity of concatemer resolution in vivo and the lack of specificity of the resolvase in vitro need to be understood. Processivity is conferred upon the catalytic subunit of the polymerase (E9) by a heterodimeric protein complex (A20 + UDG), one component of which is an active repair enzyme. This is a unique model for a processive holoenzyme, and both the mechanism by which processivity is engendered and the possible coupling of replication and repair remain to be elucidated. The D5 NTPase is likely to be the replicative helicase, and the I3 protein is likely to be the replicative SSB, but further studies are required to support these predictions. The viral B1 kinase plays a unique role in replication, serving to combat

the antiviral action of the cellular BAF protein, which binds to the viral genome and prevents replication unless it is disarmed by phosphorylation. Encapsidation appears to rely upon some components of the virion membrane, a telomere-binding protein, and a putative ATPase, and further study of this important process is also needed.

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