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Poxvirus DNA Replication

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THE POXVIRAL LIFE CYCLE

Poxviruses, of which vaccinia is the prototype, are complex DNA viruses that display an unusual degree of physical and genetic autonomy from the host cell. The vaccinia genome encodes most, if not all, of the functions required for three temporally regulated phases of transcription and for DNA replication (Moss 1990a,b, 1994; Traktman 1990a,b, 1991). In addition, the 192-kb genome encodes numerous proteins that interact with and modulate the inflammatory and immune responses of the host (Smith 1993). The virus also encodes two protein kinases and a protein phosphatase (Traktman et al. 1989a; Guan et al. 1991; Banham and Smith 1992; Lin et al. 1992; Rempel and Traktman 1992; Lin and Broyles 1994), suggesting that its life cycle, like that of eukaryotic cells, is regulated by dynamic networks of protein phosphorylation.

Infection is initiated by the attachment of virions to an unknown receptor on the cell surface. After direct fusion of cellular and viral membranes, the inner viral core is delivered into the cytoplasmic compartment, where it remains intact for several hours. Early gene expression initiates immediately and is directed by the encapsidated transcriptional machinery. This virally encoded apparatus includes a heterodimeric transcription factor (vETF), a seven-subunit RNA polymerase, the RAP94 polymerase accessory protein, the heterodimeric capping enzyme/termination protein, and the heterodimeric poly(A) polymerase, one of whose subunits also functions as a methyltransferase during mRNA cap maturation (Moss 1990a). An encapsidated RNA helicase is also essential for transcription (Shuman 1992), and two other encapsidated NTPases are reported to affect transcription in vivo and/or in vitro (Kunzi and Traktman 1989; Kahn and Esteban 1990; Diaz-Guerra and Esteban 1993; Simpson and Condit 1994; Bayliss and Condit 1995). Early mRNAs are extruded from the core and translated on host polysomes. A secondary uncoating event then ensues, which releases the viral genome into the cytoplasm where it is accessible to the newly synthe-

DNA Replication in Eukaryotic Cells

sized replication proteins. DNA replication, in addition to providing progeny genomes, is also an obligate step for the switch to intermediate gene expression. Some feature of the replicated template is required in *cis* for the activation of intermediate promoters (Vos and Stunnenberg 1988; Keck et al. 1990) by intermediate transcription factors. Among the products of intermediate gene expression are the transcription factors required for the expression of late genes. Late gene products include encapsidated enzymes and structural proteins.

Virion morphogenesis is a complex process that leads to the production of two major classes of mature virions. Intracellular mature virions (IMV) remain within the cytoplasm of the infected cell and, in the wild, are presumed to play a role in horizontal spread of poxviruses among individuals. They are delimited by a single membrane derived from the intermediate compartment between the endoplasmic reticulum and the Golgi apparatus (Sodeik et al. 1993). A subset of virions acquire an additional wrapping from the *trans*-Golgi network (Schmelz et al. 1994), fuse with the plasma membrane, and are released by exocytosis. Some of these enveloped virions remain tethered to the cell surface (cell-associated virions, CEV) and mediate spread to proximal cells (Blasco and Moss 1992). CEV are responsible for the ability of vaccinia virus to form discrete and localized plaques under liquid overlay in tissue culture. The remainder of the enveloped virions are fully released from the infected cell (extracellular enveloped virions, EEV) and are responsible for distal spread within the host.

DISCUSSION OF POXVIRAL DNA REPLICATION

Spatial and Temporal Localization

The localization of poxvirus replication to the cytoplasm of the infected cell is unique among DNA viruses. DNA replication occurs in viral factories or virosomes, localized regions within the cytoplasm which are devoid of cellular organelles and serve as foci of DNA synthesis and virion morphogenesis. Release of the viral genome into the cytoplasm (Sarov and Joklik 1972) occurs during secondary uncoating and brings the DNA template and the newly synthesized replication proteins into contact. At high multiplicities of infection, the onset of DNA replication is detectable at approximately 2 hpi, as measured by the incorporation of [^{3}H]thymidine into macromolecular, cytoplasmic species. Although thymidine incorporation declines by 4–5 hpi, other assays of viral DNA accumulation reveal that DNA synthesis continues until 10–12 hpi (Rempel et al. 1990; Traktman 1990a, 1991). Changes in nucleotide

pools and feedback inhibition of thymidine kinase (TK) have been invoked to explain this discrepancy. It is now generally accepted that viral DNA accumulation can be most accurately quantitated by dot-blot filter hybridization. Genetic analyses reveal that late gene expression is not required for DNA replication. However, when an inhibitor of protein synthesis is added to infected cultures at 3–5 hpi, the rate of subsequent DNA accumulation is compromised. It is possible that optimal replication requires the continuous production of viral and/or cellular proteins which are also synthesized at intermediate and/or late times after infection. Replication can be categorized as having two functional components, one involving the synthesis of nascent genomes which are present as concatemeric intermediates (see Figs. 1, 2), and the other involving the resolution of these intermediates to mature, monomeric genomes (see Fig. 3). These two processes are separable both temporally and genetically. Replication does not appear to be coupled to genome encapsidation within assembling virions, since it proceeds normally when the latter process is inhibited. Moreover, significantly more DNA is synthesized than is encapsidated into progeny virions.

The Genomic Template: Sequence and Nucleoprotein Complex

Telomeric Sequence Organization

The vaccinia genome is a linear duplex of 192 kbp that has covalently closed hairpin termini (Baroudy et al. 1982; Goebel et al. 1990). The DNA is extremely AT-rich (67%) and is not methylated. The hairpin termini (104 nucleotides) contain almost exclusively A and T residues, and each contains 12 extrahelical residues (10 on one strand and 2 on the other) in addition to 44 fully paired bases and a 4-nucleotide terminal loop. This general organization is retained in all poxviruses, although the number and position of the extrahelical bases vary. Two isoforms of the hairpin are found in any population of genomes; they represent inverted complements of one another and are termed *flip* and *flop*. The maintenance of the extrahelical and mismatched bases within these hairpin termini, and their presence in both isoforms, must be addressed in any replication model proposed.

Internal to the hairpins is an additional 87 bp of sequence which is found at both genomic telomeres. This sequence contains elements required both for replication of exogenous templates and for resolution of concatemeric intermediates (discussed in detail below). Beyond this region are found clusters of tandemly repeated sequences. The vaccinia genome (strain WR) contains 13 copies of a 70-bp element, a 325-bp

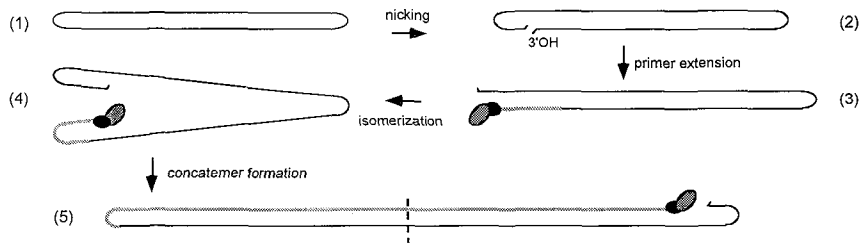

Figure 1 Schematic representation of a working model for poxviral DNA replication. The parental genome, a linear duplex with covalently closed termini, is shown at the top left (*1*). Replication begins with the introduction of a nick near one of the termini; nicking leaves a free 3′-OH group, as shown (*2*). The polymerase/processivity factor complex (shaded and filled ovals, respectively) then performs strand-displacement primer extension (*3*). The nascent strand is shown as a stippled line. This nascent DNA and its template are both self-complementary, and therefore the intermediate can isomerize to the form shown in *4*. The polymerase complex is then poised to continue synthesis along the length of the genome, around the hairpin, and then along the complementary strand. The intermediate generated by this process is a tail/tail dimer, as shown in *5*. The axis of symmetry in this dimeric intermediate is shown by a vertical dashed line.

spacer, an additional 18 copies of the 70-bp element, 2 copies of a 125-bp element, and 8 copies of a 54-bp element. Again, the general scheme is conserved among poxviruses, although the precise number and organization of the repeats do differ. These repeats are thought to participate in frequent inter- and intragenomic recombinational events.

The Nucleoprotein Complex

The encapsidated template is thought to be wrapped by at least two DNA-binding proteins of 25 kD and 11 kD. The 25-kD protein is encoded by the L4 gene and is proteolytically processed during virion morphogenesis by removal of 32 amino-terminal amino acids from the 28-kD primary translation product (Yang and Bauer 1988a,b). This abundant protein (7000 molecules per virion) possesses DNA-binding activity and, under physiological salt conditions, exhibits a preference for single-stranded DNA. In the absence of the L4 protein, normal numbers of virions are assembled, but they are severely compromised in infectivity (Wilcock and Smith 1994). It has been hypothesized that the L4 protein stabilizes a subset of the genomic sequences in a single-stranded conformation. The 11-kD protein is encoded by the F18 gene; it is not subject to proteolytic maturation but is phosphorylated on two serine

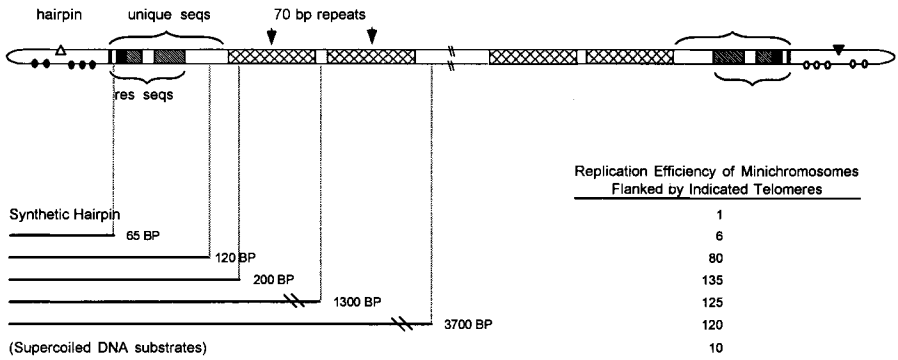

Figure 2 Organization of vaccinia virus genomic termini and analysis of the sequences involved in template activity. The genome is shown at the top, with features of the terminal organization shown in detail. The ovals and triangle shown within the 104-nt hairpin represent the extrahelical bases. The elements comprising the resolution sequence (res seqs) are shown as filled (motifs IA and I) and shaded (motifs II and III) boxes. The extent of the unique sequence block (unique seqs) beyond these resolution motifs is also indicated. The two blocks of 70-bp repeats (13 and 18 copies, respectively) are shown as cross-hatched boxes separated by an intervening spacer sequence. The lower part of the figure represents an analysis of the replication efficiency of exogenous substrates introduced into infected cells. Minichromosome substrates contained a central core of plasmid sequence and telomeres derived from the vaccinia genome. A minichromosome containing synthetic, nonviral hairpin termini was used as a control and assigned a replication level of 1. The substrates tested contained approximately 65, 120, 200, 1300, or 3700 bp of viral sequences at each terminus, as shown; the relative efficiency to which these substrates replicated is shown in the column to the right. The relative length of the telomeric viral sequences contained in each substrate is shown with a horizontal black line; the stippled gray lines extending upward toward the drawing of the genome delimit the terminal elements contained in each substrate. The final group of substrates tested were supercoiled plasmid preparations. In each case, the values shown represent the average of three independent experiments; the differences observed in replication efficiency were statistically significant with the exception of 135, 125, and 120, which were not statistically different from one another. (Data and figure adapted from S. Du and P. Traktman, in prep.)

residues (Kao et al. 1981; Nowakowski et al. 1978; Kao and Bauer 1987; Zhang and Moss 1991). It shows a preference for binding to superhelical DNA substrates in vitro and is thought be present at 27,000 copies per virion. In the absence of the F18 protein, virion maturation is blocked at a stage that appears to coincide with genome encapsidation. The DNA-binding properties of F18, as well as its abundance and its tendency to

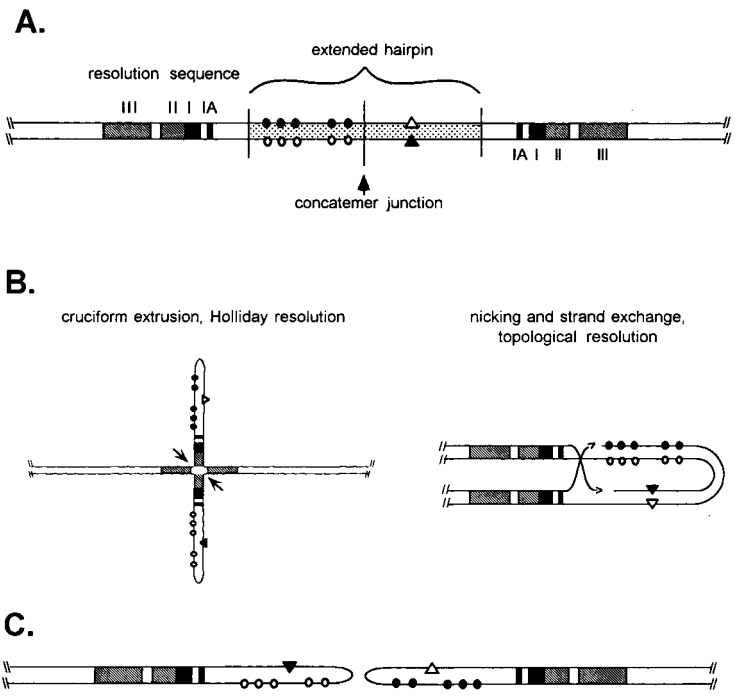

Figure 3 Resolution of concatemeric intermediates to progeny genomes. The central region of the dimeric replication intermediate is shown in panel *A*. The organization of the resolution sequence motifs (IA, I, II, III) on either side of the concatemer junction is illustrated. The extrahelical bases present in the hairpin of the parental genome are shown as ovals or triangles. The hairpin sequences are present in a fully duplexed conformation in the dimeric intermediate; note, however, that there is asymmetry between the hairpin sequences found on the left and right sides of the concatemer junction. Panel *B* shows two possible mechanisms whereby the two copies of the resolution sequences are brought together and resolved. The drawing on the left illustrates extrusion of the hairpins into a cruciform conformation. Cruciform extrusion is shown as extending through box II of the resolution sequence; only extrusion through box I is mandated by the available data. The base of the cruciform would be recognized as a Holliday junction structure, and resolution would be accomplished by asymmetric cleavage (*arrows*) and religation. The drawing on the right illustrates an alternative model involving nicking, strand exchange, and topological resolution. For simplicity, strand exchange is shown as initiating between the hairpin and box IA; however, we know that exchange extends into the genome at least as far as box I of the resolution motif. Panel *C* illustrates the telomeric structure of the mature progeny genomes formed by resolution of the intermediate shown in *A* via either of the mechanisms shown in *B*. Restoration of the hairpin termini with extrahelical bases is indicated; the two forms of the hairpin shown, which are inverted complements of one another, are referred to as "flip" and "flop."

multimerize, have led to the hypothesis that it serves as the major genomic condensing protein. There is some evidence in the literature that these two components of the nucleoprotein complex may be linked by inter-protein disulfide bridges (Ichihashi et al. 1984). Although both of these proteins are bound to the encapsidated genome, it has not been proven rigorously that they remain on the genome after secondary uncoating, and therefore it can only be hypothesized that either or both are present on the DNA replication template.

A Working Model for Poxvirus Replication

DNA replication is thought to begin with the introduction of a nick in sequences proximal to a genomic terminus (see Fig. 1). Formation of the nick has been inferred from the increased topological freedom and altered sedimentation properties that are associated with intracellular viral genomes after infection is under way (Pogo 1977). Strand cleavage leaves a free 3′-OH group to serve as a primer terminus for the viral DNA polymerase. Strand displacement synthesis proceeds toward the hairpin telomere, yielding extended template and product strands that are both self-complementary. This duplex terminus can isomerize to form two intrastrand duplexes. The 3′ terminus of the nascent strand is then poised to permit strand-displacement synthesis through the entire genome and around the distal hairpin terminus. The primary product would be a dimeric tail-to-tail concatemer; if the newly synthesized strand again folded back on itself, a tetramer could be formed, and so on. These concatemeric intermediates have been detected by electrophoretic analysis of replicating DNA (Moyer and Graves 1981; Merchlinsky et al. 1988; DeLange 1989; DeLange and McFadden 1990).

The simplest model for genome replication invokes only leading-strand synthesis. Reports of significant levels of single-stranded genomic DNA during virus replication are consistent with this model of strand-displacement synthesis (Esteban and Holowczak 1977; Pogo et al. 1981). However, both leading- and lagging-strand synthesis might occur, with the two meeting at the hairpin loop. Although no recent studies have provided evidence for such a model, early reports intimated that short nascent strands could be chased into larger forms, which was construed as evidence for Okazaki fragment synthesis (Esteban and Holowczak 1977). It also remains unknown whether reinitiation occurs on incompletely synthesized and/or resolved progeny genomes, leading to the formation of rather complex and branched genomic structures. Some evidence for such structures, which fail to enter gels under most conditions of elec-

trophoresis, has been obtained (DeLange 1989; Merchlinsky and Moss 1989b).

Sequences Required for Template Replication

A definition of which *cis*-acting sequences are responsible for vaccinia DNA replication, and/or where DNA synthesis initiates, has remained elusive for many years. When cells infected with *ts* DNA$^-$ mutants were held at the nonpermissive temperature to synchronize infection and then shifted to the permissive temperature, [^{3}H]thymidine was first incorporated within 150 nucleotides of the termini (Pogo et al. 1984). Because both telomeres were labeled, it was supposed that initiation could occur near the left and/or right telomere; the observation that concatemers arise as replication intermediates suggests that, within a given template, one site of initiation is likely.

Attempts to define an origin that could serve as an autonomous replication sequence were not successful and changed the general view of initiation within the field. Any supercoiled, but not linear, plasmid introduced into vaccinia- or Shope fibroma virus-infected cells was seen to replicate to some extent (as judged by the recovery of *Dpn*I-resistant material) (DeLange and McFadden 1986; Merchlinsky and Moss 1988). Replication was not stimulated by the inclusion of any viral sequences within these plasmids. Together, these data suggested that replication might initiate from random nicks present in transfected plasmids and that there might not be any viral sequences that recruited the replication machinery to the viral genome. This unusual feature was in part attributed to the cytoplasmic localization of viral infection, with the rationale that specificity was less important when a virus was not competing with an excess of nuclear DNA.

Clarity regarding the *cis*-acting sequences required for replication has emerged very recently with the analysis of another class of exogenous templates (S. Du and P. Traktman, in prep.). Minichromosomes containing a central region of plasmid DNA and telomeres derived from the vaccinia genome were constructed and introduced into infected cells (see Fig. 2). At 24 hpi, cytoplasmic DNA was harvested and the replication of the minichromosomes was analyzed. The level of replication was normalized to that seen with a minichromosome containing synthetic, nonviral, hairpin termini (assigned a replication level of 1). A sixfold increase in replicated product was seen when the telomeric 65 nucleotides of the viral genome were used; the level of replication increased to 80 when 120 bp of viral sequences were used. A maximum replication level

of 120–135 was seen when 200 bp, 1300 bp, or 3700 bp of telomeric sequences were included. In contrast, several supercoiled substrates replicated to a value of 10. These data suggest that a linear template with hairpin termini containing 200 bp of viral telomeric sequences replicates to a maximum level within vaccinia-infected cells. Within these 200 bp are the hairpin loop and an additional 87 bp of unique sequence which contains boxes IA, I, II, and III of the resolution sequence (58 bp) as well as an additional 29 bp. Comparative analysis of the replication efficiency of the various substrates tested implies that sequence and/or structural elements extending throughout the 87 bp between the hairpin and the 70-bp repeats are necessary and sufficient for efficient replication of an exogenous minichromosome by the vaccinia replication machinery.

Resolution of Concatemeric Intermediates

It is now accepted that concatemeric intermediates arise during poxvirus replication. Because it appears that the viral protein(s) that directs the resolution of these intermediates is a late gene product (see below), the intermediates are more easily detected during the early stages of DNA replication. When late gene expression is impaired genetically or pharmacologically, these concatemers accumulate at the expense of mature genomes (Merchlinsky et al. 1988; DeLange 1989; Merchlinsky and Moss 1989b). The sequence obtained for the genomic junction within these concatemers is consistent with a tail-tail or head-head structure, as predicted previously (Moyer and Graves 1981; Merchlinsky et al. 1988). When resolution is blocked and the half-life of intermediates is therefore extended, head-tail isoforms are generated by homologous intergenomic recombination.

Analysis of the sequences required for resolution (see Fig. 3) was significantly aided by the demonstration that plasmids containing a cloned concatemeric junction fragment were resolved into linear minichromosomes with authentic hairpin termini following transfection into infected cells (DeLange et al. 1986; Merchlinsky and Moss 1986, 1989a; DeLange and McFadden 1987; Merchlinsky et al. 1988; Merchlinsky 1990a,b). Targeted mutagenesis of the terminal sequences permitted a thorough understanding of the sequences required for resolution. Several key regions have been defined: domains IA and I that are absolutely required for resolution, and domains II and III that augment the efficiency of resolution. This entire sequence is reiterated in an inverted repeat conformation on the other side of the resolution axis (see Fig. 3A). The sequence between the two resolution motifs (i.e., the extended duplex copy

of the hairpin loop) must be a palindrome of less than 200 bp. Most of the natural palindrome can be deleted, however, and replaced with a synthetic palindrome. Therefore, neither the precise hairpin sequence nor the characteristic extrahelical bases are important for resolution. Experimental evidence suggests that the two copies of the resolution sequence are brought together during the recognition and resolution event. Sequence parity and spacing between the two elements are crucial. Within each of the two copies of the sequence, the two strands are fully complementary, one having served as the template for the synthesis of the other. On the other hand, the two copies are not identical, since they were independently derived from the two parental strands, which were not fully complementary due to the presence of extrahelical base pairs. Analysis of the resolution of a synthetic concatemeric junction in which one element contains an *Xba*I site in box I and the other element does not (due to a single base substitution) has revealed that the resolved telomeres are each heteroduplexes. Each contains one strand with the *Xba*I recognition sequence and one with the mutated sequence. These studies suggest that either the resolving concatemeric junction fragment extrudes in a cruciform configuration such that resolution at its base via a Holliday resolvase yields the two incompletely base-paired telomeres (Fig. 3B, left), or that recombination and branch migration accomplishes the same event (Fig. 3B, right). Either of these models would explain the need for a central palindromic sequence between the two copies of the resolution sequence. Resolution restores both the flip and the flop forms of the hairpin. Moreover, the recovery of the *Xba*I site in a heteroduplex form marks this site as the most telomeric position at which the resolving nick can occur (within box I, 16 nucleotides interior to the border of the central palindrome).

Site-directed mutagenesis has been employed to arrive at the consensus resolution sequence of boxes I and IA. The sequence $5'T_{6\text{-}7}N_{7\text{-}9}{}^{T}/_{C}AAA^{T}/_{A}\ 3'$, when present in inverted repeat conformation on either side of a double-stranded copy of the hairpin loop, is sufficient to direct telomere resolution. Examination of this sequence reveals that it contains the transcriptional start site associated with late viral genes (TAAAT). Indeed, the authentic Shope fibroma virus resolution sequence has been tested and found to function as a strong late promoter (Stuart et al. 1991), and transcription through the telomeric region has been detected in vivo (Parsons and Pickup 1990). Current models propose that late transcription through the concatemer junction could play a role in opening up the duplex region to facilitate entry of resolution proteins or topological rearrangement. Previously, the observation

that resolution did not proceed when late viral genes were not expressed was interpreted as implicating a late protein in the resolution reaction. The overlap between the resolution sequence and late transcriptional start sites suggests that late transcription per se may be important for resolution. This finding may explain the unexpected observation that a *ts* mutant with a lesion in the capping enzyme appears to be deficient in telomere resolution at the nonpermissive temperature (Carpenter and DeLange 1991).

To date, the proteins responsible for recognizing the resolution sequence and/or executing resolution have not been identified, nor has the resolution reaction been reconstituted in vitro. It is presumed that one or more late proteins may be involved in this facet of DNA metabolism. An enzyme capable of concerted DNA nicking and cross-linking has been proposed as a possible participant in resolution (Traktman 1990a, 1991), but no clear data have emerged to confirm such an involvement.

Proteins Involved in Poxviral DNA Replication

A variety of genetic, biochemical, and molecular biological techniques have been applied to the identification of proteins involved in poxviral DNA replication. Confidence that most if not all of these proteins would be virally encoded was strengthened by the cytoplasmic localization of poxvirus replication and the observation that replication proceeded normally upon infection of enucleated cells. Three complementation groups of *ts* mutants have been shown to have primary and severe defects in DNA synthesis at the nonpermissive temperature; the E9, D5, and B1 gene products were identified by analysis of these mutants (Traktman et al. 1984; Evans and Traktman 1987; Roseman and Hruby 1987; Rempel et al. 1990). One additional mutant (D4 gene) appears to exhibit diminished DNA synthesis when infections are initiated and maintained at the nonpermissive temperature (Millns et al. 1994). Other genes have been identified by reverse genetics or by recognition of conserved motifs in the predicted amino acid sequence of genomic regions. A description of these proteins is provided below; a summary of the proteins is shown in Table 1.

DNA Polymerase

The 116-kD catalytic DNA polymerase is encoded by the E9 gene. Conserved domains I–VII common to the α family of polymerases are found within the enzyme, as are sequences diagnostic of the δ polymerases

Table 1 Vaccinia virus proteins involved in DNA replication

Protein	Gene	Molecular mass (kD)	Expression	Genetic analysis
DNA polymerase	E9	116	early	*ts* mutants exhibit fast-stop DNA$^-$ phenotype
Processivity factor	A20	49	early	
D5 NTPase	D5	90	early	*ts* mutants exhibit fast-stop DNA$^-$ phenotype, recombination defect
B1 protein kinase	B1	34	early	*ts* mutants exhibit DNA$^-$ phenotype; severity varies with host cell
DNA ligase	A50	63	early	deletion confers resistance to etoposide and sensitivity to UV and bleomycin in tissue culture and attenuation in vivo
Thymidine kinase	J2	19	early	nonessential in tissue culture; deletion confers BrdU resistance in tissue culture and attentuation in vivo
Thymidylate kinase	A48	23	early	nonessential in tissue culture
Ribonucleotide reductase	F4, 14	34, 87	early	nonessential in tissue culture; HU-resistance conferred by amplification of small subunit; deletion causes attenuation in vivo
Topoisomerase I	H6	36	late, encapsid	essential
Uracil DNA glycosylase	D4	25	early	*ts* mutant exhibits reduced DNA synthesis only if replication is initiated at the nonpermissive temperature
dUTPase	F2	16	early	virus with large deletion encompassing F2 is viable, but precise deletion of F2 has not been possible

(Wang et al. 1989; Traktman 1990a; Taddie and Traktman 1993). The presence of domains Exo I, Exo II, and Exo III is consistent with the observation that the enzyme has an intrinsic 3′→5′ exonuclease activity (Challberg and Englund 1979; McDonald and Traktman 1994a). The en-

zyme has been overexpressed within the context of a vaccinia virus infection, and biochemical analysis indicates that it is a highly distributive enzyme under most assay conditions (McDonald and Traktman 1994a,b). It is not capable of strand displacement in vitro. Two *ts* mutants have been characterized, and aphidicolin^r, phosphonoacetic^r, and cytosine arabinoside^r mutants have also been generated (Defilippes 1984, 1989; Traktman et al. 1989b; Taddie and Traktman 1991, 1993). Among the drug^r mutants are those that exhibit mutator and antimutator phenotypes in vivo.

Processivity Factor

Unlike the purified E9 polymerase, the activity present within cytoplasmic extracts of vaccinia-infected cells is highly processive (W.F. McDonald and Traktman, unpubl.). This processive polymerase activity can be reconstituted by mixing recombinant E9 protein and extracts from cells infected with a *ts* mutant carrying a defective E9 protein. Activity could not be reconstituted using uninfected cell extracts, strongly suggesting that the processivity factor was virally encoded. The processivity activity has recently been purified from infected cell extracts and appears to have a native molecular mass of 45 kD. Amino acid analysis of the purified material has led to the assignment of the A20 open reading frame (ORF) as the processivity factor (N. Klemperer and P. Traktman, unpubl.). The structure of the gene is consistent with its being expressed early during infection, and the predicted translation product of the ORF has a molecular mass of 49 kD.

D5 NTPase

Four *ts* mutants with lesions in the D5 gene (*ts*17, 24, 69, and 6389) have a DNA$^-$ phenotype at the nonpermissive temperature (Evans and Traktman 1987, 1992; Roseman and Hruby 1987). When cultures infected with *ts*17 are shifted to the nonpermissive temperature in the midst of DNA replication, further synthesis of DNA ceases within 5 minutes. Together, these data suggest that D5 plays an essential and intimate role in the process of DNA elongation. Three of the *ts* mutants with lesions in the amino-terminal portion of the 90-kD protein also show defects in homologous recombination. In addition, the feedback inhibition of the viral TK that normally develops during vaccinia infection was not seen during nonpermissive infections with *ts*17 or *ts*24. This phenotype was not common to all infections in which DNA synthesis was blocked, suggesting that D5 might play a role in nucleotide biosynthesis. Recently,

the D5 protein has been overexpressed within the context of vaccinia virus infection and purified to homogeneity. The D5 protein possesses intrinsic nucleic-acid-independent NTPase activity (Evans et al. 1995). All of the common ribonucleotides and deoxyribonucleotides are hydrolyzed, and no stimulation with any RNA or DNA cofactor has been observed. In light of the possible correlation of D5 inactivation with altered dNTP metabolism, purified D5 was tested for nucleoside kinase activity, but none was found. Further insight into the functional contribution of D5 to DNA synthesis has proven elusive, suggesting perhaps that interactions with other, as yet unidentified, proteins may be important.

B1 Protein Kinase

Members of the third complementation group of *ts* mutants with a DNA^- phenotype (*ts*2, *ts*25) contain lesions within the B1 gene (Rempel et al. 1990), which encodes a 34-kD serine/threonine protein kinase expressed only at early times after infection (Traktman et al. 1989a; Banham and Smith 1992; Lin et al. 1992; Rempel and Traktman 1992). Recombinant B1 protein produced in bacteria is enzymatically active in the absence of any other viral proteins. The only viral protein identified as a B1 substrate to date is the H5 protein (Beaud et al. 1995). This amphipathic protein shows affinity for both membranes and DNA, but its functional role in vivo is unknown. The phenotype of the B1 mutants is complex and confusing (Rempel and Traktman 1992). In vitro, recombinant *ts*2 protein has no detectable kinase activity, whereas *ts*25 protein has 3% of the specific kinase activity of the *wt* protein. Neither protein is temperature-sensitive. In mouse L cells, both *ts*2 and *ts*25 are strictly DNA^- at the nonpermissive temperature; this restriction is much leakier in primate BSC40 cells. However, whereas the *wt* protein is stable throughout infection, the mutant proteins are extremely labile in both cell types at both permissive and nonpermissive temperatures. Because B1 synthesis ceases by 3 hpi, the B1 protein is effectively absent by the midstages of infection. Nevertheless, when cultures were shifted to the nonpermissive temperature at 7 hpi in the midst of DNA replication, further DNA synthesis ceased after a lag of approximately 30 minutes. These data have elicited a model which proposes that one or more components of the replication complex behave in a temperature-sensitive fashion in the absence of appropriate phosphorylation by B1. There is ample precedent to support the hypothesis that phosphorylation might be required to stabilize protein-protein interactions at 39.5°C to a far greater extent than at 32°C.

DNA Ligase

Vaccinia virus encodes a DNA ligase that is not essential for virus replication in tissue culture (Kerr and Smith 1989, 1991; Colinas et al. 1990). The 63-kD enzyme is expressed at early times after infection and utilizes an ATP-dependent mechanism of catalysis. The ability of ligase-deficient viruses to replicate normally in culture suggests either that the mode of DNA synthesis is independent of ligase action, arguing against the existence of lagging-strand synthesis, or that one of the cellular ligases can substitute for the viral enzyme. Support for the latter possibility comes from the observation that ligase-deficient isolates are attenuated in vivo (Kerr et al. 1991). Viruses lacking the DNA ligase appear to be sensitive to DNA-damaging agents in tissue culture, suggesting that the ligase may play an important role in DNA repair. This observation is interesting in light of the recent observation that the human ligase II, a repair enzyme, shows significant homology with the viral gene product (Wang et al. 1994). Another mystery involving the viral DNA ligase is the recent observation that viral mutants resistant to etoposide, a drug most frequently identified as an inhibitor of type II topoisomerases and also shown to inhibit the viral topoisomerase in vitro (J. Countryman and P. Traktman, unpubl.), contain lesions within the DNA ligase gene (DeLange et al. 1995). Presence of a *wt* ligase gene is correlated with etoposide^S, and null alleles of the ligase confer resistance. Particular amino-terminal point mutations confer an even higher level of resistance. No mechanistic explanation for this unusual set of observations has been put forward, but it is clear that the role of the viral ligase remains to be elucidated.

Thymidine Kinase and Thymidylate Kinase

Vaccinia encodes two enzymes involved in thymidine biosynthesis, both of which are dispensable in tissue culture. The TK (Hruby and Ball 1982; Weir et al. 1982; Hruby et al. 1983; Weir and Moss 1983) is synthesized early after infection and functions as a homotetramer. The enzymatic activity of the enzyme declines as infection proceeds because of a sensitivity to feedback inhibition by accumulating dTDP and dTTP (Hruby 1985). This sensitivity to feedback inhibition is diagnostic of type II TK enzymes (as distinct from the type I enzymes encoded by herpesviruses); mutational studies indicate that a single amino acid replacement in conserved domain IV can eliminate feedback inhibition without impairing enzyme activity (Black and Hruby 1992). The substrate specificity of the viral TK is limited to thymidine and BrdU. The ability

to phosphorylate BrdU has been exploited for the construction of vaccinia virus recombinants; insertion of foreign DNA into the TK locus inactivates TK and leads to a BrdU-resistant phenotype. Viruses deficient in TK activity are attenuated in vivo (Buller et al. 1985). The second step of dTTP biosynthesis is accomplished by the viral thymidylate kinase enzyme (Hughes et al. 1991). The vaccinia genome also contains sequences bearing homology with known guanylate kinases, but no intact reading frame remains (Smith et al. 1991).

Ribonucleotide Reductase

Vaccinia encodes a heterodimeric ribonucleotide reductase (RR) that directs the conversion of rNDPs to dNDPs (Slabaugh et al. 1988; Tengelsen et al. 1988). The structure of the viral enzyme is highly homologous to that of its mammalian and bacterial counterparts. The large regulatory subunit is responsible for the allosteric modulation of the enzyme by nucleotides. The small, catalytic subunit contains the active-site *tyrosine* and is sensitive to the action of hydroxyurea (HU). Amplification of the gene encoding this subunit confers HU^r. Phenotypic resistance to HU can also be accomplished by inclusion of deoxyadenosine in the culture medium, consistent with the observation that dATP pools are decreased most dramatically by HU treatment (Slabaugh et al. 1991). Viral mutants lacking an intact RR are severely attenuated in vivo (Child et al. 1990); in tissue culture, the cellular enzyme is apparently sufficient to support the normal replication of these mutants.

Topoisomerase I

Vaccinia virus encodes and encapsidates a type I topoisomerase (Bauer et al. 1977; Shaffer and Traktman 1987; Shuman and Moss 1987). The 36-kD product of the H6 gene directs the relaxation of supercoiled DNA in a manner analogous to the yeast and mammalian enzymes. Transient cleavage of a single DNA strand occurs at a highly conserved consensus site, $^{C}/_{T}CCTT\downarrow$ (Shuman and Prescott 1990; Shuman 1991). The gene is apparently essential for virus viability in tissue culture (Shuman et al. 1989a), but the absence of conditionally lethal mutants with H6 lesions has hampered definitive analyses of the biological roles played by the enzyme. By analogy with other systems, a role in relieving topological strain during transcription and DNA replication is posited. Participation in recombination has also been proposed. In contrast to the paucity of data regarding function in vivo, the small size of the vaccinia enzyme and the ability to produce active recombinant protein have facilitated the

accumulation of a significant body of data regarding structure/function considerations. The active-site *tyrosine* has been identified (Shuman et al. 1989b), and numerous mutations that impair DNA cleavage and relaxation have been characterized (Morham and Shuman 1990, 1992; Klemperer and Traktman 1993; Wittschieben and Shuman 1994).

Presumed Functions

At a minimum, it is also to be expected that ongoing viral DNA synthesis will involve a single-strand DNA-binding protein (SSB) and a DNA helicase. A helicase is invoked to permit the processive DNA polymerase complex to travel through a duplex substrate. However, no data regarding the nature of the enzyme involved have so far been obtained. Regarding an SSB, the best candidate to date is the 34-kD phosphoprotein encoded by the I3 gene. This protein shows a strong preference for single- versus double-stranded DNA, and gel-shift assays have been used to derive a binding site size of approximately 10 nt/I3 molecule (S.C. Rochester and P. Traktman, unpubl.). The protein is essential (S.C. Rochester and P. Traktman, unpubl.) and is synthesized at high levels both before and during DNA synthesis. A potential interaction between the I3 protein and the ribonucleotide reductase has also been observed (Davis and Mathews 1993). Additional proteins are expected to be involved in the initiation of replication and the resolution of concatemeric intermediates; no compelling candidates have been identified for either function.

A Potential Link between DNA Replication and DNA Repair

Comparative analysis of distinct vaccinia strains, as well as isolates maintained in different laboratories, has indicated that the genomes of poxviruses have a high degree of sequence stability. The DNA polymerase has an active proofreading exonuclease and exhibits a high level of fidelity. The ability of some polymerase lesions to confer mutator or antimutator phenotypes suggests that the fidelity of the polymerase plays a significant role in determining the net mutation rate of the virus (Taddie and Traktman 1991, 1993). Efforts to detect virus-specific mismatch repair enzymes have not been successful. However, the virus is known to encode two enzymes that are classically associated with DNA repair. A DNA replication defect is seen in a *ts* mutant with a lesion in one of these repair enzymes, suggesting that replication and repair might be physically and/or functionally linked.

dUTPase

The vaccinia virus F2 gene is expressed early after infection and encodes a functional enzyme that has homology with diverse dUTPases and shows a strict preference for dUTP as substrate (Broyles 1993). By analogy with other systems, the dUTPase is presumed to lower the intracellular concentration of dUTP and so prevent its incorporation into nascent viral genomes. The observation that a viral isolate containing an extensive terminal deletion encompassing the F2 gene (as well as many others) is viable (Perkus et al. 1991) led to the conclusion that the F2 gene is not essential for virus replication in tissue culture. However, efforts to isolate a virus whose sole alteration is a null mutation in the F2 gene have not been successful (N. Roseman, pers. comm.), suggesting that this conclusion might have been erroneous. It is possible that the elimination of other genes in the original, extensive deletion somehow obviated the need for the viral dUTPase.

Uracil DNA Glycosylase

The vaccinia virus D4 gene is also expressed at early times after infection and encodes a functional uracil DNA glycosylase (Stuart et al. 1993; Upton et al. 1993). This enzyme hydrolyzes incorporated UMP residues at the N-glycosylic bond; removal of the uracil moiety targets the site for cleavage by an apurinic/apyrimidinic (AP) nuclease and repair by a DNA polymerase. The viral enzyme is apparently essential, as shown both by the inability to isolate a viral recombinant containing an insertionally inactivated D4 allele and by the existence of a *ts* mutant containing a D4 lesion (Stuart et al. 1993; Millns et al. 1994). Interestingly, this mutant (*ts*4149) shows a partial DNA^- phenotype. When infections are initiated and maintained at the nonpermissive temperature, 1–2% of permissive levels of DNA are synthesized, and these DNA products do not mature into monomer genomes. When infections are initiated at the permissive temperature, a shift to nonpermissive temperature in the midst of DNA replication does not lead to a cessation of DNA synthesis. These data suggest that either (1) the enzyme plays a role only prior to, or during the initiation of, DNA replication or, more likely, (2) the enzyme is assembled into a multiprotein complex, and the thermolability of the mutant protein is masked once complex formation is complete. The latter model suggests that a bifunctional replication/repair complex might track along the DNA during viral DNA synthesis. Further elucidation of the potential role of this enzyme in replication and/or repair awaits the analysis of additional *ts* alleles. No data are available regarding AP

endonucleases that might act subsequent to the action of the uracil DNA glycosylase during DNA repair.

ACKNOWLEDGMENTS

I thank the members of the poxvirus field who generously communicated their unpublished results to me; I also apologize for the inevitable lapses in providing sufficient acknowledgment of published work. The scientific contributions and support provided by the members of my own laboratory have been invaluable. Some of the work cited in this chapter was supported by a grant to P.T. from the National Institutes of Health (AI-21758); the generous support of Dr. David Cofrin and a special group of donors from the Dorothy Rodbell Cohen foundation is also acknowledged.

REFERENCES

Banham, A.H. and G.L. Smith. 1992. Vaccinia virus gene B1R encodes a 34-kDa serine/threonine protein kinase that localizes in cytoplasmic factories and is packaged into virions. *Virology* **191:** 803–812.

Baroudy, B.M., S. Vankatesan, and B. Moss. 1982. Incompletely base-paired flip-flop terminal loops link the two DNA strands of the vaccinia virus genome into one uninterrupted polynucleotide chain. *Cell* **28:** 315–324.

Bauer, W.R., E.C. Ressner, J. Kates, and J.V. Patzke. 1977. A DNA nicking-closing enzyme encapsidated in vaccinia virus: Partial purification and properties. *Proc. Natl. Acad. Sci.* **74:** 1841–1845.

Bayliss, C.D. and R.C. Condit. 1995. The vaccinia virus A18R gene product is a DNA-dependent ATPase. *J. Biol. Chem.* **270:** 1550–1556.

Beaud, G., R. Beaud, and D.P. Leader. 1995. Vaccinia virus gene H5R encodes a protein that is phosphorylated by the multisubstrate vaccinia virus B1R protein kinase. *J. Virol.* **69:** 1819–1826.

Black, M.E. and D.E. Hruby. 1992. A single amino acid substitution abolishes feedback inhibition of vaccinia virus thymidine kianse. *J. Biol. Chem.* **267:** 9743–9748.

Blasco, R. and B. Moss. 1992. Role of cell-associated enveloped vaccinia virus in cell-to-cell spread. *J. Virol.* **66:** 4170–4179.

Broyles, S.S. 1993. Vaccinia virus encodes a functional dUTPase. *Virology* **195:** 863–865.

Buller, R.M., G.L. Smith, K. Cremer, A.L. Notkins, and B. Moss. 1985. Decreased virulence of recombinant vaccinia virus expression vectors is associated with a thymidine kinase-negative phenotype. *Nature* **317:** 813–815.

Carpenter, M.S. and A.M. DeLange. 1991. A temperature-sensitive lesion in the small subunit of the vaccinia virus-encoded mRNA capping enzyme causes a defect in viral telomere resolution. *J. Virol.* **65:** 4042–4050.

Challberg, M.D. and P.T. Englund. 1979. Purification and properties of the deoxyribonucleic acid polymerase induced by vaccinia virus. *J. Biol. Chem.* **254:**

7812–7819.

Child, S.J., G.J. Palumbo, R.M. Buller, and D.E. Hruby. 1990. Insertional activation of the large subunit of ribonucleotide reducase is associated with reduced virulence in vivo. *Virology* **174:** 625–629.

Colinas, R.J., S.J. Goebel, S.W. Davis, G.P. Jonson, E.K. Norton, and E. Paoletti. 1990. A DNA ligase gene in the Copenhagen strain of vaccinia virus is nonessential for viral replication and recombination. *Virology* **179:** 267–275.

Davis, R.E. and C.K. Mathews. 1993. Acidic C terminus of vaccinia virus DNA binding protein interacts with ribonucleotide reductase. *Proc. Natl. Acad. Sci.* **90:** 745–749.

Defilippes, F.M. 1984. Effect of aphidicolin vaccinia virus: Isolation of an aphidicolin-resistant mutant. *J. Virol.* **52:** 474–482.

———. 1989. Site of the base change in the vaccinia virus DNA polymerase gene which confers aphidicolin resistance. *J. Virol.* **63:** 4060–4063.

DeLange, A.M. 1989. Identification of temperature-sensitive mutants of vaccinia virus that are defective in conversion of concatemeric replicative intermediates to the mature linear DNA genome. *J. Virol.* **63:** 2437–2444.

DeLange, A.M. and G. McFadden. 1986. Sequence-nonspecific replication of transfected plasmid DNA in poxvirus-infected cells. *Proc. Natl. Acad. Sci.* **83:** 614–618.

———. 1987. Efficient resolution of replicated poxvirus telomeres to native hairpin structures requires two inverted symmetrical copies of a core target DNA sequence. *J. Virol.* **61:** 1957–1963.

———. 1990. The role of telomeres in poxvirus DNA replication. *Curr. Top. Microbiol. Immunol.* **163:** 71–92.

DeLange, A.M., M.S. Carpenter, J. Choy, and V.E. Newsway. 1995. An etoposide-induced block in vaccinia virus telomere resolution is dependent on the virus-encoded DNA ligase. *J. Virol.* **69:** 2082–2091.

DeLange, A.M., M. Reddy, D. Scraba, C. Upton, and G. McFadden. 1986. Replication and resolution of cloned poxvirus telomeres in vivo generates linear minichromosomes with intact viral hairpin termini. *J. Virol.* **59:** 249–259.

Diaz-Guerra, M. and M. Esteban. 1993. Vaccinia virus nucleoside triphosphate phosphohydrolase I controls early and late gene expression by regulating the rate of transcription. *J. Virol.* **67:** 7561–7562.

Esteban, M. and J.A. Holowczak. 1977. Replication of vaccinia DNA in mouse L cells. III. Intracellular forms of viral DNA. *Virology* **82:** 308–322.

Evans, E. and P. Traktman. 1987. Molecular genetic analysis of a vaccinia virus with an essential role in DNA replication. *J. Virol.* **61:** 3152–3162.

———. 1992. Characterization of vaccinia virus DNA replication mutants with lesions in the D5 gene. *Chromosoma* **102:** S72–S82.

Evans, E., N. Klemperer, R. Ghosh, and P. Traktman. 1995. The essential replication protein encoded by the vaccinia virus D5 gene is a nucleic acid independent NTPase. *J. Virol.* **69:** 5353–5361.

Goebel, S.J., G.P. Johnson, M.E. Perkus, S.W. David, J.P. Winslow, and E. Paoletti. 1990. The complete DNA sequence of vaccinia virus. *Virology* **179:** 247–266.

Guan, K., S.S. Broyles, and J.E. Dixon. 1991. A tyr/ser phosphatase encoded by vaccinia virus. *Nature* **350:** 359–362.

Hruby, D.E. 1985. Inhibition of vaccinia virus thymidine kinase by the distal products of its own metabolic pathway. *Virus Res.* **2:** 151–156.

Hruby, D.E. and L.A. Ball. 1982. Mapping and identification of the vaccinia virus

thymidine kinase gene. *J. Virol.* **43:** 403–409.

Hruby, D.E., R.A. Maki, D.B. Miller, and L.A. Ball. 1983. Fine structure analysis and nucleotide sequence of the vaccinia virus thymidine kinase gene. *Proc. Natl. Acad. Sci.* **80:** 3411–3415.

Hughes, S.J., L.H. Johnston, A. deCarlos, and G.L. Smith. 1991. Vaccinia virus encodes an active thymidylate kinase that complements a cdc8 mutant of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **266:** 20103–20109.

Ichihashi, Y., M. Oie, and T. Tsuruhara. 1984. Location of DNA-binding proteins and disulphide-linked proteins in vaccinia virus structural elements. *J. Virol.* **50:** 929–938.

Kahn, J.S. and M. Esteban. 1990. Identification of the point mutations in two vaccinia virus nucleoside triphosphate phosphohydrolase I temperature-sensitive mutants and role of this DNA-dependent ATPase enzyme in virus gene expression. *Virology* **174:** 459–471.

Kao, S.Y. and W.R. Bauer. 1987. Biosynthesis and phosphorylation of vaccinia virus structural protein VP11. *Virology* **159:** 399–407.

Kao, S., E. Ressner, J. Kates, and W.R. Bauer. 1981. Purification and characterization of a superhelix binding protein from vaccinia virus. *Virology* **111:** 500–508.

Keck, J.G., C.J. Baldick, Jr., and B. Moss. 1990. Role of DNA replication in vaccinia virus gene expression: A naked template is required for transcription of three late trans-activator genes. *Cell* **61:** 801–809.

Kerr, S.M. and G.S. Smith. 1989. Vaccinia virus encodes a polypeptide with DNA ligase activity. *Nucleic Acids Res.* **17:** 9039–9050.

———. 1991. Vaccinia virus DNA ligase is nonessential for virus replication: Recovery of plasmids from virus-infected cells. *Virology* **180:** 625–632.

Kerr, S.M., L.H. Johnston, M. O'Dell, S.A. Duncan, K.M. Law, and G.L. Smith. 1991. Vaccinia DNA ligase complements *Saccharomyces cerevisiae* cdc9, localizes in cytoplasmic factories and affects virulence and virus sensitivity to DNA damaging agents. *EMBO J.* **10:** 4343–4350.

Klemperer, N. and P. Traktman. 1993. Biochemical analysis of mutant alleles of the vaccinia virus topoisomerase I carrying targeted substitutions in a highly conserved domain. *J. Biol. Chem.* **268:** 15887–15899.

Kunzi, M.S. and P. Traktman. 1989. Genetic evidence for involvement of vaccinia virus DNA-dependent ATPase I in intermediate and late gene expression. *J. Virol.* **63:** 3999–4010.

Lin, S. and S.S. Broyles. 1994. Vaccinia protein kinase 2: A second essential serine-threonine protein kinase encoded by vaccinia virus. *Proc. Natl. Acad. Sci.* **91:** 7653–7657.

Lin, S., W. Chen, and S.S. Broyles. 1992. The vaccinia virus B1R gene product is a serine/threonine protein kinase. *J. Virol.* **66:** 2717–2723.

McDonald, W.F. and P. Traktman. 1994a. Overexpression and purification of the vaccinia virus DNA polymerase. *Protein Express. Purif.* **5:** 409–421.

———. 1994b. Vaccinia virus DNA polymerase: In vitro analysis of parameters affecting processivity. *J. Biol. Chem.* **269:** 31190–31197.

Merchlinsky, M. 1990a. Mutational analysis of the resolution sequence of vaccinia virus DNA: Essential sequence consists of two separate AT-rich regions highly conserved. *J. Virol.* **64:** 5029–5035.

———. 1990b. Resolution of poxvirus telomeres: Processing of vaccinia virus concatemer junctions by conservative strand exchange. *J. Virol.* **64:** 3437–3446.

Merchlinsky, M. and B. Moss. 1986. Resolution of linear minichromosomes with hairpin ends from circular plasmids containing vaccinia virus concatemer junctions. *Cell* **45:** 879–884.

———. 1988. Sequence-independent replication and sequence-specific resolution of plasmids containing the vaccinia virus concatemer junction: Requirements for early and late *trans*-acting factors. *Cancer Cells* **6:** 87–93.

———. 1989a. Nucleotide sequence required for resolution of the concatemer junction of vaccinia virus DNA. *J. Virol.* **63:** 4354–4361.

———. 1989b. Resolution of vaccinia virus DNA concatemer junctions requires late gene expression. *J. Virol.* **63:** 1595–1603.

Merchlinsky, M., C.F. Garon, and B. Moss. 1988. Molecular cloning and sequence of the concatemer junction from vaccinia virus replicative DNA. *J. Mol. Biol.* **199:** 399–413.

Millns, A.K., M.S. Carpenter, and A.M. DeLange. 1994. The vaccinia virus-encoded uracil DNA glycosylase has an essential role in viral DNA replication. *Virology* **198:** 504–513.

Morham, S.G. and S. Shuman. 1990. Phenotypic selection and characterization of mutant alleles of a eukaryotic DNA topoisomerase I. *Genes Dev.* **4:** 515–524.

———. 1992. Covalent and noncovalent DNA binding by mutants of vaccinia DNA topoisomerase I. *J. Biol. Chem.* **267:** 15984–15992.

Moss, B. 1990a. Regulation of vaccinia virus transcription. *Annu. Rev. Biochem.* **59:** 661–688.

———. 1990b. Poxviridae and their replication. In *Virology*, 2nd edition. (ed. B.N. Fields and D.M. Knipe), pp. 2079–2111. Raven Press, New York.

———. 1994. Vaccinia virus transcription. In *Transcription: Mechanisms and regulation* (ed. R.C. Conaway and J.W. Conaway), pp. 185–206. Raven Press, New York.

Moyer, R.W. and R.L. Graves. 1981. The mechanism of cytoplasmic orthopoxvirus DNA replication. *Cell* **27:** 391–401.

Nowakowski, M., W. Bauer, and J. Kates. 1978. Characterization of a DNA-binding phosphoprotein from vaccinia virus replication complex. *Virology* **86:** 217–225.

Parsons, B.L. and D.J. Pickup. 1990. Transcription of orthopoxvirus telomeres at late times during infection. *Virology* **175:** 69–80.

Perkus, M.E., S.J. Goebel, S.W. Davis, G.P. Johnson, I.K. Norton, and E. Paoletti. 1991. Deletion of 55 open reading frames from the termini of vaccinia virus. *Virology* **180:** 406–410.

Pogo, B.G. 1977. Elimination of naturally occurring crosslinks in vaccinia virus DNA after viral penetration into cells. *Proc. Natl. Acad. Sci.* **74:** 1739–1742.

Pogo, B.G.T., E.M. Berkowitz, and S. Dales. 1984. Investigation of vaccinia virus DNA replication employing a conditional lethal mutant defective in DNA. *Virology* **132:** 436–444.

Pogo, B.G.T, M. O'Shea, and P. Freimuth. 1981. Initiation and termination of vaccinia virus DNA replication. *Virology* **108:** 241–248.

Rempel, R. and P. Traktman. 1992. Vaccinia virus B1 kinase: Phenotypic analysis of temperature-sensitive mutants and enzymatic characterization of recombinant proteins. *J. Virol.* **66:** 4413–4426.

Rempel, R.E., M.K. Anderson, E. Evans, and P. Traktman. 1990. Temperature-sensitive vaccinia virus mutants identify a gene with an essential role in viral replication. *J. Virol.* **64:** 574–583.

Roseman, N.A. and D.E. Hruby. 1987. Nucleotide sequence and transcript organization

of a region of the vaccinia virus genome which encodes a constitutively expressed gene required for DNA replication. *J. Virol.* **61:** 1398–1406.

Sarov, I. and W.K. Joklik. 1972. Characterization of intermediates in the uncoating of vaccinia virus DNA. *Virology* **50:** 593–602.

Schmelz, M., B. Sodeik, M. Ericsson, E.J. Wolffe, H. Shida, G. Hiller, and G. Griffiths. 1994. Assembly of vaccinia virus: The second wrapping is derived from the trans Golgi network. *J. Virol.* **68:** 130–147.

Shaffer, R.S. and P. Traktman. 1987. Vaccinia encapsidates a novel topoisomerase with the properties of a eukaryotic type I enzyme. *J. Biol. Chem.* **262:** 9309–9315.

Shuman, S. 1991. Site-specific DNA cleavage by vaccinia virus DNA topoisomerase I. *J. Biol. Chem.* **266:** 1796–1803.

———. 1992. Vaccinia virus RNA helicase: An essential enzyme related to the DE-H family of RNA-dependent NTPases. *Proc. Natl. Acad. Sci.* **89:** 10935–10939.

Shuman, S. and B. Moss. 1987. Identification of a vaccinia virus gene encoding a type I DNA topoisomerase. *Proc. Natl. Acad. Sci.* **84:** 7478–7482.

Shuman, S. and J. Prescott. 1990. Specific DNA cleavage and binding by vaccinia virus DNA topoisomerase I. *J. Biol. Chem.* **265:** 17826–17836.

Shuman, S., M. Golder, and B. Moss. 1989a. Insertional mutagenesis of the vaccinia virus gene encoding a type I topoisomerase: Evidence that the gene is essential for virus growth. *Virology* **170:** 302–306.

Shuman, S., E.M. Kane, and S.G. Morham. 1989b. Mapping the active-site tyrosine of vaccinia virus DNA topoisomerase I. *Proc. Natl. Acad. Sci.* **86:** 9793–9797.

Simpson, D.A. and R.C. Condit. 1994. The vaccinia virus A18R protein plays a role in viral transcription during both the early and late phases of infection. *J. Virol.* **68:** 3642–3649.

Slabaugh, M.B., N. Roseman, and C. Mathews. 1988. Vaccinia virus encoded ribonucleotide reductase: Sequence conservation of the gene for the small subunit and its amplification in hydroxyurea resistant mutants. *J. Virol.* **62:** 519–527.

Slabaugh, M.B., M.L. Howell, Y. Wang, and C.K. Mathews. 1991. Deoxyadenosine reverses hydroxyurea inhibition of vaccinia virus growth. *J. Virol.* **65:** 2290–2298.

Smith, G.L. 1993. Vaccinia virus glycoproteins and immune evasion. *J. Gen. Virol.* **74:** 1725–1740.

Smith, G.L., S.Y. Chan, and S.T. Howard. 1991. Nucleotide sequence of 42 kbp of vaccinia virus strain WR from near the left inverted terminal repeat. *J. Gen. Virol.* **72:** 1349–1376.

Sodeik, B., R.W. Doms, M. Ericsson, G. Hiller, C.E. Machamer, W. van't Hof, G. van Meer, B. Moss, and G. Griffiths. 1993. Assembly of vaccinia virus: Role of the intermediate compartment between the endoplasmic reticulum and the Golgi stacks. *J. Cell Biol.* **121:** 521–541.

Stuart, D., K. Graham, M. Schreiber, C. Macaulay, and G. McFadden. 1991. The target DNA sequence for resolution of poxvirus replicative intermediates is an active late promoter. *J. Virol.* **65:** 61–70.

Stuart, D.T., C. Upton, M.A. Higman, E.G. Niles, and G. McFadden. 1993. A poxvirus-encoded uracil DNA glycosylase is essential for virus viability. *J. Virol.* **67:** 2503–2512.

Taddie, J.A. and P. Traktman. 1991. Genetic characterization of the vaccinia virus DNA polymerase: Identification of point mutations conferring altered drug sensitivities and reduced fidelity. *J. Virol.* **65:** 869–879.

———. 1993. 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.* **67:** 4323–4336.

Tengelsen, L.A., M.B. Slabaugh, J.K. Bibler, and D.E. Hruby. 1988. Nucleotide sequence and molecular genetic analysis of the large subunit of ribonucleotide reductase encoded by vaccinia virus. *Virology* **164:** 121–131.

Traktman, P. 1990a. The enzymology of poxvirus DNA replication. *Curr. Top. Microbiol. Immunol.* **163:** 931–123.

———. 1990b. Poxviruses: An emerging portrait of biological strategy. *Cell* **62:** 621–626.

———. 1991. Molecular genetic and biochemical analysis of poxvirus DNA replication. *Semin. Virol.* **2:** 291–304.

Traktman, P., M.K. Anderson, and R.E. Rempel. 1989a. Vaccinia virus encodes an essential gene with strong homology to protein kinases. *J. Biol. Chem.* **264:** 21458–21461.

Traktman, P., M. Kelvin, and S. Pacheco. 1989b. Molecular genetic analysis of vaccinia virus DNA polymerase mutants. *J. Virol.* **63:** 841–846.

Traktman, P., P. Sridhar, R.C. Condit, and B.E. Roberts. 1984. Transcriptional mapping of the DNA polymerase gene of vaccinia virus. *J. Virol.* **49:** 125–131.

Upton, C., D.T. Stuart, and G. McFadden. 1993. Identification of a poxvirus gene encoding a uracil DNA glycosylase. *Proc. Natl. Acad. Sci.* **90:** 4518–4522.

Vos, J.C. and H.G. Stunnenberg. 1988. Derepression of a novel class of vaccinia virus genes upon DNA replication. *EMBO J.* **7:** 3487–3492.

Wang, T.S.-F., S.W. Wong, and D. Korn. 1989. Human DNA polymerase α: Predicted functional domains and relationships with viral DNA polymerases. *FASEB J.* **3:** 14–21.

Wang, Y.C., W.A. Burkhart, Z.B. Mackey, M.B. Moyer, W. Ramos, I. Husain, J. Chen, J.M. Besterman, and A.E. Tomkinson. 1994. Mammalian DNA ligase II is highly homologous with vaccinia DNA ligase. Identification of the DNA ligase II active site for enzyme-adenylate formation. *J. Biol. Chem.* **169:** 31923–31928.

Weir, J. and B. Moss. 1983. Nucleotide sequence of the vaccinia virus thymidine kinase gene and the nature of spontaneous frameshift mutations. *J. Virol.* **46:** 530–537.

Weir, J.P., G. Bajszar, and B. Moss. 1982. Mapping of the vaccinia virus thymidine kinase gene by marker rescue and by cell free translation of selected mRNA. *Proc. Natl. Acad. Sci.* **79:** 1210–1214.

Wilcock, D. and G.L. Smith. 1994. Vaccinia virus core protein VP8 is required for virus infectivity, but not for core protein processing or for INV and EEV formation. *Virology* **202:** 294–304.

Wittschieben, J. and S. Shuman. 1994. Mutational analysis of vaccinia DNA topoisomerase defines amino acid residues essential for covalent catalysis. *J. Biol. Chem.* **269:** 29978–29983.

Yang, W.-P. and W.R. Bauer. 1988a. Biosynthesis and post-translational cleavage of vaccinia virus structural protein VP8. *Virology* **167:** 585–590.

———. 1988b. Purification and characterization of vaccinia virus structural protein VP8. *Virology* **167:** 578–584.

Zhang, Y. and B. Moss. 1991. Vaccinia virus morphogenesis is interrupted when expression of a gene encoding an 11-kilodalton phosphorylated protein is prevented by the *Escherichia coli* lac repressor. *J. Virol.* **65:** 6101–6110.