

ROLE OF THE VIRAL mRNA CAPPING ENZYME  
DURING VACCINIA VIRUS INFECTION

By

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To my mother who was not able to see me complete my journey,  
but who I know is with me in spirit.

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Eukaryotic cells and viruses cap mRNA transcripts to increase the stability and translatability of the transcripts. Vaccinia virus, the prototypic member for the poxviridae family, encodes a multifunctional, heterodimeric mRNA capping enzyme. The large subunit is encoded by D1R (97kDa) and the small subunit is encoded by D12L (33kDa). In addition to its role in mRNA capping, the viral capping enzyme has also been implicated by previous biochemical analysis to be an early transcription termination factor and an intermediate transcription initiation factor. A temperature sensitive mutant, Dts36, which has a mutation in the D1 protein, has been analyzed *in vivo* and *in vitro*.

In the phenotypic analysis of Dts36, some early mRNA transcripts and proteins are present in reduced amounts. This is consistent with a defect in one of the enzymatic activities in the formation of the cap structure, most likely the methyltransferase activity. Some early viral transcripts were also longer than expected which is consistent with a defect in the termination function of the capping enzyme. A defect in intermediate gene transcription was also observed during a temperature shift-up experiment. These results represent the first time that the capping enzyme has been shown to be involved in early termination and intermediate initiation *in vivo*. The results of the phenotypic characterization were confirmed by biochemical analysis.

Biochemical assays designed to examine the enzymatic activities of the mRNA capping enzyme were performed and the mutant D1 protein was found to be defective in the methyltransferase activity as predicted by the phenotypic analysis. The results from both analyses when taken together lead to the hypothesis that the mutant enzyme subunits may not be able to properly associate which could explain the capping defect as well as the defects observed in early transcription termination and intermediate transcription.

## CHAPTER 1 INTRODUCTION

### 1.1 Poxviruses

Viruses are extremely small, self-replicating, infectious agents which are parasitic in nature due to their dependence on cellular machinery for viral metabolism. Due to the simplistic nature of viruses, as compared to the complex nature of eukaryotic cells, viruses have been useful tools in the elucidation of basic aspects of molecular and cellular biology. Viruses have been successfully used to study eukaryotic gene regulation and much of what is known about the mechanism of eukaryotic transcription machinery, mRNA processing and DNA replication is due to studies performed with viruses. One example of a virus that has been used to elucidate these processes is vaccinia virus, the prototypical member of the poxviridae family.

The poxviridae is a family of large, double-stranded DNA viruses that are capable of infecting either vertebrate cells (the Chordopoxviruses) or invertebrate cells (the Entomopoxviruses). Vaccinia virus is a member of the Chordopoxvirinae subfamily and the genus orthopoxvirus, and like all poxviruses, encodes all of the factors required for viral transcription and viral DNA replication. As a result of encoding their own transcription and replication machinery, poxviruses do not require entry into the host cell nucleus for these processes and therefore, they replicate in the cytoplasm of the infected cells. This characteristic of vaccinia virus and poxviruses in general, make them extremely useful tools in understanding the cellular processes in eukaryotic cells.

Vaccinia virus possesses a linear, double-stranded DNA genome that is approximately 200 kilobases (kb) in length and which possesses covalently closed ends composed of inverted terminal repeats critical for viral DNA replication. The viral genome encodes approximately 200 genes and is encapsidated in the core of a complex brick-shaped virion surrounded by a single

lipid bilayer. The viral genes are arranged in such a fashion that the highly conserved genes as well as genes critical for the function of the virus are located in the center of the viral genome. The less conserved genes as well as genes involved with host interactions are located at the ends of the viral genome. The genes of orthopoxviruses are named based on the HindIII restriction endonuclease map of the viral genome which gives each HindIII fragment a letter designation (16 HindIII fragments named A-P, see Fig. 3-1 for schematic). Each HindIII fragment is further divided into open reading frames (ORFs) which are numbered from left to right and also labeled with a letter (R or L) to designate the transcriptional orientation of the gene.

## **1.2 Viral Life Cycle**

The vaccinia virus life cycle consists of a number of processes which are temporally regulated. These processes include viral entry, viral gene transcription which is divided into early, intermediate, and late viral transcription, viral DNA replication, and virion morphogenesis and release (Figs. 1-1 and 1-2). These processes are complex in nature and are understood in varying degrees with viral transcription and DNA replication being well-understood whereas the processes involved in viral entry and morphogenesis are continuing to be worked out.

### **1.2.1 Viral Entry**

There are two primary forms of vaccinia virus that are recognized (reviewed in (Moss, 2006); mature virions (MV, formerly called intracellular mature virions (IMV)) are the most abundant form, and extracellular virions (EV, formerly extracellular enveloped virions (EEV)) are simply MVs which possess one additional membrane wrapper. Due to the difference in the number of membranes the two forms of virions possess, the protein composition of the outer membranes is different for each infectious form of the virus (Condit et al., 2006). Although the infectious forms of vaccinia virus have been determined, the exact mechanism of viral entry is still a debatable topic in the field. The current school of thought is that the virus enters through

two distinct mechanisms; fusion of the virion with the plasma membrane or endosomal uptake of the virion followed by fusion of the virion with the endosomal membranes, each of which will be discussed further below.

Independent of the manner of viral entry, the ability of the virion membrane to fuse with a membrane that is cellular in origin is critical for successful entry of the infectious particle into the cell. Recently, a number of viral proteins have been shown to be involved in the entry of virions into the cell. These proteins are A16 (Ojeda et al., 2006b), A21 (Townsend et al., 2005b), A28 (Senkevich et al., 2004), F9 (Brown et al., 2006), G3 (Izmailyan et al., 2006), G9 (Ojeda et al., 2006a), H2 (Senkevich and Moss, 2005), I2 (Nichols et al., 2008), J5 (Senkevich et al., 2005), and L5 (Townsend et al., 2005a). At least nine of these proteins have been shown to interact and form an entry/fusion complex (Brown et al., 2006; Senkevich et al., 2005) and inducible mutants in most of the proteins have shown that these proteins are required for successful entry of the virus into the cell by fusion, be it fusion of the virion with the plasma membrane or fusion of the virion with the endosomal membrane.

Mature virions are thought to enter the cell either through direct fusion with the plasma membrane or through fusion with an endosomal membrane following endosomal uptake of the virion into the cell. Fusion of the virion with the plasma membrane will be addressed here followed by discussion about virion fusion with the endosomal membrane. The simplest manner in which a virion can enter the cell would be for the outer virion membrane to bind and fuse to the plasma membrane of the cell allowing for the virus core to be released into the cytoplasm of the cell. Electron microscopic evidence has shown that MVs are capable of this direct fusion with the plasma membrane and subsequent entry into the cell (Carter et al., 2005). Analysis of EVs by electron microscopy has also provided additional evidence of infectious particles

entering cells through direct fusion with the plasma membrane. After interaction with the cell, the EVs were found to lose their additional outer membrane by a ligand-induced non-fusogenic dissolution process after which the resulting virus particle, now a MV, is able to fuse with the plasma membrane and the viral core is able to enter the cell (Law et al., 2006). This mechanism of virion entry makes it possible for the double-membraned EVs as well as the single-membraned MVs to successfully enter and infect cells.

The other mechanism of virion entry is through endosomal uptake and subsequent fusion of the virion membrane with the endosomal membrane. Recent studies have shed more light on the exact mechanism that vaccinia virions use to enter the cell through endosomes. MVs have been shown to be endocytosed into the cell by macropinocytosis (Mercer and Helenius, 2008) and fluid phase endocytosis (Huang et al., 2008), two processes that consist of non-selective bulk fluid-phase uptake of extracellular material into the endosomes. However, there is controversy on how exactly the virion induces the endocytic process; one school of thought is that the virions mimic apoptotic bodies which are similar in size to MVs (Mercer and Helenius, 2008), whereas, the second school of thought is that the virus exploits a novel cellular protein, vaccinia virus penetration factor (Huang et al., 2008). Successful endocytosis of vaccinia virions is dependent on two distinct low pH processes; the first step promotes the activation of entry/fusion complex and the second low pH step induces the fusion of the virion membrane with the endosomal membrane (Townesley et al., 2006; Townesley and Moss, 2007). Much like fusion of the virion with the plasma membrane, fusion of the virion with the endosomal membrane allows the viral core to enter the cytoplasm of the cell.

### **1.2.2 Viral Transcription**

Vaccinia virus transcription is temporally regulated with each class of genes (early, intermediate, and late) having a distinct promoter and trans-acting factors. The genes are

expressed in a cascade such that each class of genes encodes for factors that are necessary for the transcription of the subsequent class. Early genes also encode factors required for DNA replication and late genes, in addition to encoding transcription factors for early transcription during the next round of viral infection, also encode factors required for virion morphogenesis. Viral DNA replication (discussed further in section 1.2.4) separates the transcription cascade into early transcription and post-replicative transcription which includes both the intermediate and late classes of viral transcripts. This division of the transcription cascade necessitates that the virus possess two forms of the RNA polymerase. However, independent of the form of the RNA polymerase utilized in transcription, all viral transcripts are capped at their 5' end and polyadenylated at their 3' ends.

#### **1.2.2.1 Viral RNA polymerase**

Vaccinia virus possesses two multi-subunit forms of the viral RNA polymerase; one is specific for early transcription and the second is specific for post-replicative transcription. Both forms of the viral polymerase have eight subunits in common including three subunits, rpo147, rpo132, and rpo7, which share homology to eukaryotic and prokaryotic polymerase subunits (Amegadzie et al., 1992; Broyles and Moss, 1986; Patel and Pickup, 1989). However, the early polymerase has an additional polypeptide associated with it, the RNA polymerase-associated protein (RAP94) encoded by the viral gene H4L (Ahn and Moss, 1992). RAP94 is responsible for the recognition of early viral promoters (Ahn et al., 1994; Deng and Shuman, 1994) and plays a role in early transcription termination (Mohamed and Niles, 2001) and at late times during viral infection, RAP94 is responsible for the targeting of the virion components into the forming virions (Zhang et al., 1994). The ability of RAP94 to only recognize early promoters, which are distinct from both intermediate and late promoters, through its association with the vaccinia early transcription factor (VETF) confers specificity to the early form of the viral RNA polymerase.

### 1.2.2.2 Early transcription

There are several differences between early and post-replicative transcription; mainly the cellular location of the transcription process, promoter structure, and transcription termination. Early viral transcription takes place in the viral core upon entry into the cytoplasm of the cell. As such, the infectious virus particle possesses all the factors that are necessary for early transcription including but not limited to the early RNA polymerase, the vaccinia early transcription factor (VETF), the mRNA capping enzyme, and the RNA poly(A) polymerase. The promoter structure of early transcription consist of a 16 base pair (bp) critical region separated from a 7-bp region of initiation by a 11-bp thymidine-rich, less critical region (Davison and Moss, 1989a). The VETF, a heterodimer comprised of the products of the viral genes, A7L and D6R (Gershon and Moss, 1990), recognizes and binds to the early promoter and confers the ATPase activity required for transcription (Broyles and Fesler, 1990).

Transcription termination is another distinction of early viral transcription. The factors that are required for early transcription termination have been determined. The vaccinia termination factor (VTF) is the mRNA capping enzyme, a multifunctional viral enzyme which will be discussed in further detail in section 1.4 (Luo et al., 1991; Shuman et al., 1987). The transcription termination occurs approximately 50 bps downstream of the termination sequence, TTTTNT, in the non-template strand (Yuen and Moss, 1987). The recognition of the complementary sequence, UUUUNU in the nascent mRNA transcript, by VTF signals for transcription termination (Shuman and Moss, 1988). In addition to VTF, several other viral factors are required for early transcription termination. The RAP94 subunit of the RNA polymerase as well as the nucleoside triphosphate phosphohydrolase I (NPH I), encoded by viral gene D11L, are essential for early transcription termination (Christen et al., 1998; Mohamed and Niles, 2001). The currently proposed mechanism for early transcription termination is a two step

model: in the first step VTF and RAP94 recognize the UUUUUNU sequence in the nascent mRNA and in the subsequent step the nascent transcript is released in the presence of NPH I and ATP (Piacente et al., 2008).

### **1.2.2.3 Intermediate and late transcription**

Intermediate and late transcription differs more from early transcription than simply in the form of RNA polymerase that is utilized; post-replicative transcripts possess poly(A) heads at their 5' ends which are due to slippage of the RNA polymerase during initiation (Schwer et al., 1987; Schwer and Stunnenberg, 1988). This characteristic contributes to the heterogeneous length of post-replicative transcripts. Intermediate and late transcription differ from one another in the structure of their promoters as well as the factors that are required for transcription initiation. Intermediate promoters are thirty nucleotides in length and contain a 14 bp critical region found 10-11 nucleotides upstream of the TAAA initiator element (Baldick, Jr. et al., 1992). Late promoters also are approximately thirty nucleotides in length and have a 20 bp thymidine and adenosine rich region separated from the highly conserved TAAAT initiator element by a 6 bp spacer (Davison and Moss, 1989b). The factors that are required for transcription initiation are also unique to each class of post-replicative transcripts. There are four vaccinia intermediate transcription factors (VITF-1, 2, 3, and A) which include both virally encoded and cellular factors. VITF-1 is rpo30, one of the subunits of the viral RNA polymerase (Rosales et al., 1994) and VITF-3 is a heterodimer of viral proteins A8 and A23 (Sanz and Moss, 1999). VITF-2 is a cellular heterodimer consisting of p137 and G3BP proteins and is thought to be involved in the regulation of the switch from early transcription to intermediate transcription (Katsafanas and Moss, 2004). The last intermediate transcription factor, VITF-A, is the viral mRNA capping enzyme which plays a structural role in the formation of the transcription initiation complex (Vos et al., 1991b). There are five vaccinia late transcription factors (VLTF-

1, 2, 3, 4, and X) which like the intermediate factors are both virally (1-4) and host (X) encoded. VLTF-1 is encoded by G8R (Zhang et al., 1992), VLTF-2 is encoded by A1L (Keck et al., 1993), VLTF-3 is encoded by A2L (Passarelli et al., 1996), and VLTF-4 is encoded by H5R (Kovacs and Moss, 1996). The final late transcription factor is host-encoded and is comprised of the ribonucleoproteins A2/B1 and RBM3 (Wright et al., 2001). Another difference between early and post-replicative transcription is, whereas, there is a known early termination signal, there is no known post-replicative termination sequence and this lack of a consensus termination sequence leads to post-replicative transcripts being heterogeneous at the 3' ends. It is important to note that the early transcription termination signal is not recognized as a termination signal in post-replicative transcription.

Although there is no known consensus termination sequence for post-replicative transcription, it is known that several virally encoded factors are required for the 3' end formation of post-replicative transcripts. These factors are A18, G2, J3, and H5, encoded by A18R, G2R, J3R, and H5R, respectively. A18 has been shown to possess DNA helicase (Simpson and Condit, 1995) and DNA-dependent ATPase (Bayliss and Condit, 1995) activities. The A18 protein has also been shown to be required for the release of nascent RNA from transcription elongation complexes *in vitro* (Lackner and Condit, 2000) and mutants in A18 have been shown to synthesize longer than normal post-replicative transcripts (Xiang et al., 1998). These results are consistent with A18 being the post-replicative transcription termination factor. The G2 protein was shown to be a positive post-replicative transcription elongation factor because temperature sensitive mutants in the G2 protein synthesized post-replicative transcripts that were shorter than normal (Black and Condit, 1996). The roles of the A18 and G2 proteins in transcription elongation were further confirmed when it was shown that a mutation in the G2R

gene is able to compensate for a mutation in the A18R gene (Condit et al., 1996b). An additional viral protein, J3, was identified through two independent genetic selections to also be a positive post-replicative transcription elongation factor (Latner et al., 2000). Further analysis with mutants in the J3 protein, which synthesized shorter than normal post-replicative transcripts, confirmed the role of the J3 protein in transcription elongation. The final viral protein with a role in post-replicative transcription is H5. H5 has been identified as a late gene transcription factor (Kovacs and Moss, 1996) and has been shown to interact with G2 through yeast two-hybrid analysis and *in vivo* immunoprecipitation (Black et al., 1998). In the same analysis, the H5 and A18 proteins were also shown to interact during *in vivo* immunoprecipitation. More recently, H5 has been identified as a positive post-replicative elongation factor when an isatin  $\beta$ -thiosemicarbazone (IBT)-resistant mutant was identified (Cresawn and Condit, 2007). IBT is an anti-poxviral drug that is known to promote read-through of post-replicative transcripts. The H5 protein has also been implicated as the endoribonuclease involved in the formation of the 3' end of post-replicative transcripts by a cleavage event (D'Costa et al., 2008).

### **1.2.3 Viral DNA Replication**

Viral DNA replication occurs after early transcription but before intermediate and late transcription. Presumably a second uncoating event occurs after early transcription which allows the release of the viral DNA molecule from the viral core into the cytoplasm of the cell (Joklik, 1964a; Joklik, 1964b; Mallardo et al., 2002). Replication of the viral genome by the viral DNA polymerase, encoded by the gene E9L, (Traktman et al., 1984) and other associated factors results in the formation of an area of electron-dense material that is free of cellular organelles known as viroplasm, where DNA replication and virion morphogenesis occur. The additional viral factors required for viral DNA replication are the processivity factor, A20 (Punjabi et al.,

2001); the uracil DNA glycosylase, D4 (Stanitsa et al., 2006); and a viral ATPase/DNA primase capable of oligomerization, D5 (Boyle et al., 2007; De Silva et al., 2007; Evans et al., 1995).

#### **1.2.4 Virion Morphogenesis and Trafficking**

Virion morphogenesis is the process of virion assembly and maturation. The steps of virion morphogenesis have been determined by electron microscopic examination of vaccinia virus infections (reviewed in (Condit et al., 2006)). The location of virion morphogenesis is the viroplasm where viral DNA replication is occurring as discussed in section 1.2.3. The first discernable structure in morphogenesis is the appearance of crescents which are comprised of at least one lipid bilayer (Fig. 1-2). The crescents continue to grow until they become closed circles forming an immature virion (IV) which is filled with viroplasm. At nearly the same time, immature virions with nucleiod (IVN) appear. The nucleiod is a round region of electron dense material which contains DNA. It has been hypothesized that IVs and IVNs are the same structures that have been sectioned through different planes during sample processing. The proteolytic processing of several virion protein precursors is required for the morphogenesis from IVN to MV and extrusion from the viroplasm into the cytoplasm of the infected cell.

After the MV exits the viroplasm, it is trafficked through the cell to the plasma membrane in order to exit the cell (reviewed in (Smith and Law, 2004)). The MV is transported through the Golgi apparatus on microtubules which results in the formation of wrapped virions (WV) which are MVs that possess two Golgi-derived membrane wrappers (Fig 1-2). The microtubules continue the transport of the WVs to the plasma membrane where the outer membrane wrapper of the WV fuses with the plasma membrane resulting in an EV “sitting” on the cell surface. The EVs can mediate cell-to-cell spread by protrusion of the EV to adjacent cells as actin tail formation drives the EV away from the cell or the EV can be released from the cell surface and mediate long distance dissemination of the virus.

### 1.3 Capping of mRNA

Eukaryotic and viral messenger ribonucleic acid (mRNA) transcripts possess a cap structure at their 5'-ends that is the result of the first post-translation modification of the nascent transcript. The structure and function of the mRNA cap as well as the enzymatic reactions required for the formation of the cap structure have been elucidated and will be discussed in further detail in the following sections.

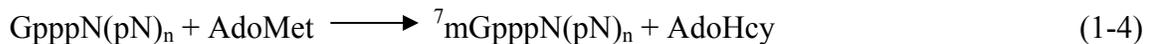
#### 1.3.1 Structure and Function of the mRNA Cap

The mRNA cap is a 7-methylguanosine ( $m^7G$ ) that is linked through an inverted triphosphate bridge (5'-5' linkage) to the initiating nucleoside of the nascent mRNA transcript. There are several different forms of the cap structure depending on the methylation status of the cap (Fig. 1-3); these structures are designated cap 0, cap 1, and cap 2 with the cap 1 structure being the most prevalent structure found in eukaryotic cells. The methylation status of each structure is as follows: the cap 0 structure is methylated at the N7 position on the guanosine residue the cap; the cap 1 structure in addition to the methylated guanosine residue is also methylated at the  $O^2$  position of the initiating nucleoside; and the cap 2 structure in addition to having the same groups methylated as the cap 1 structure is also methylated at the  $O^2$  position of the second nucleoside of the nascent transcript. The function of the mRNA cap structure in eukaryotic cells is to confer stability to the mRNA transcript as well as aid in the translatability of the transcript. The guanosine residue of the cap structure contributes to the transcript stability; it has been previously shown that non-guanylated reovirus RNA is less stable in wheat germ extracts, L cell protein synthesizing extracts as well as in *X. laevis* oocytes (Furuichi et al., 1977). Additionally, it has been demonstrated through the use of a guanylyltransferase mutant in yeast that the inactivation of the mRNA capping activity caused a decrease in the levels of mRNA and protein synthesis. The methylation status of the guanosine residue of the cap

structure contributes to the translatability of the transcript. Methylation of the guanosine is required for efficient translation of the mRNA transcript as was observed in experiments with reovirus and vesicular stomatitis virus (VSV) RNA. Methylated RNA from either virus stimulated protein synthesis in wheat germ extracts better than unmethylated viral RNA (Both et al., 1975). An additional study with RNA from reovirus and VSV removed the methylated guanosine residue from the cap structure by  $\beta$ -elimination and observed that the RNA transcripts were no longer translated (Muthukrishnan et al., 1975). Further studies with vaccinia virus RNA examined the interaction of the methylated guanosine residue of the cap with the ribosome as well as its role in translation. After  $\beta$ -elimination, viral RNA was no longer able to bind to ribosomes or be translated; however, after adding back the  $m^7G$  (but not an unmethylated guanosine residue) to the RNA, ribosome binding and translation activities were restored (Muthukrishnan et al., 1978). The formation of the mRNA cap 1 structure will be discussed in the next section.

### 1.3.2 Formation of the mRNA Cap

The mRNA cap is formed in a series of five enzymatic reactions (Equations 1-1 through 1-5) that are catalyzed by four different enzymes.



The first step in the formation of the cap structure (Equation 1-1) is the removal of a gamma phosphate from the 5' end of the initiating nucleoside of the nascent transcript by RNA

triphosphatase. The product of this reaction is an mRNA transcript with a diphosphate 5'-terminus. The second step in the formation of the cap structure is the transfer of a monophosphate guanosine residue to the di-phosphorylated 5'-terminus of the nascent mRNA transcript. This step, which is catalyzed by mRNA guanylyltransferase, has been shown to be reversible and proceeds in a series of two reactions (Equations 1-2 and 1-3) (Roth and Hurwitz, 1984; Shuman and Hurwitz, 1981). The first guanylyltransferase reaction involves the formation of a covalent capping enzyme-GMP complex intermediate and the release of  $PP_i$  in the presence of a divalent cation. The second guanylyltransferase reaction is the attack and transfer of the guanosine residue to the beta phosphate of the nascent mRNA transcript to yield an mRNA transcript with a guanosine attached to it through a 5'-5' triphosphate bridge (Roth and Hurwitz, 1984; Shuman and Hurwitz, 1981). The third reaction involved in the formation of the mRNA cap structure is the transfer of a methyl group from S-adenosylmethionine (AdoMet) to the N7 position on the guanylylate residue of the nascent mRNA transcript (Equation 1-4). This reaction is irreversible and is catalyzed by mRNA (guanine-N7) methyltransferase (Martin and Moss, 1975; Martin and Moss, 1976). The fourth and final reaction in the formation of the mRNA cap structure is the transfer of a methyl group from AdoMet to  $O^{2'}$  position on the initiating nucleoside of the mRNA transcript (Equation 1-5). This reaction is catalyzed by mRNA (nucleoside- $O^{2'}$ ) methyltransferase and the product of the reaction is an mRNA transcript that possesses a cap 1 structure.

#### **1.4 Vaccinia Virus mRNA Capping Enzyme**

Vaccinia virus possesses a multifunctional, virally encoded mRNA capping enzyme. The viral capping enzyme is a heterodimer comprised of a large and small subunit (Guo and Moss, 1990; Martin and Moss, 1975) which are encoded by the D1R gene (97 kilodalton (kDa)) and the D12L gene (33 kDa) respectively (Morgan et al., 1984; Niles et al., 1989). The vaccinia

virus mRNA capping enzyme is responsible for the first three enzymatic steps in the formation of the cap structure; the fourth reaction is catalyzed by a different virally encoded protein, the product of the gene J3R. In addition to its role in mRNA capping, biochemical analysis has demonstrated that the viral mRNA capping enzyme is also a factor required for early gene transcription termination (Luo et al., 1991; Shuman et al., 1987; Shuman and Moss, 1988) as well as a factor required for intermediate gene transcription initiation (Harris et al., 1993; Vos et al., 1991b). These functions of the viral mRNA capping enzyme will be further discussed in the following sections.

#### **1.4.1 Enzymatic Activities of mRNA Capping**

The active sites for the three enzymatic activities catalyzed by the vaccinia virus mRNA capping enzyme are found on the large subunit of the capping enzyme. The active sites for the RNA triphosphatase and the mRNA guanylyltransferase activities are both located on the amino-terminus (amino acids 1-545) of the large subunit (Guo and Moss, 1990; Higman et al., 1992; Shuman, 1990; Shuman and Morham, 1990); however it has been shown by isolating mutants that are defective in one enzymatic activity but not the other, that the active sites for these two enzymatic activities are distinct from one another (Myette and Niles, 1996; Yu and Shuman, 1996). The active site of the RNA triphosphatase activity is comprised of nine charged amino acids: Glu37, Glu39, Arg77, Lys107, Glu126, Asp159, Lys161, Glu192, and Glu194, which are all required for activity (Gong and Shuman, 2003; Yu et al., 1997; Yu and Shuman, 1996). The active site of the mRNA guanylyltransferase is Lys260 which is the residue to which the GMP covalently attaches to during catalysis (Niles and Christen, 1993); however, other amino residues, namely Glu375, Lys392, and Asp400, also play a role in the guanylyltransferase catalysis (Cong and Shuman, 1995). The mRNA (guanine-N7) methyltransferase active site is located in the carboxyl-terminus (amino acids 540-844) of the D1 protein (Higman et al., 1994;

Mao and Shuman, 1994); however, the small subunit of the capping enzyme must be present and interacting with the large subunit to stimulate the intrinsic methyltransferase activity of the large subunit (Higman et al., 1992; Mao and Shuman, 1994). The methyltransferase active site contains amino acid residues Gly600 and His682 and the aromatic ring of Tyr683 has been shown to be critical for the methyltransferase activity (Mao and Shuman, 1994; Mao and Shuman, 1996). It is critical to note that although the subunit association of the D1 and D12 proteins is required for the methyltransferase activity of the viral mRNA capping enzyme, this subunit association is not required for the RNA triphosphatase and mRNA guanylyltransferase activities (Shuman, 1989; Shuman and Morham, 1990).

#### **1.4.2 Early Viral Transcription Termination**

The role of the vaccinia virus mRNA capping in early viral transcription termination is based on biochemical analysis of the virus. The early viral transcription termination factor activity were first shown to co-purify with the mRNA capping activity (Shuman et al., 1987) and further analysis showed that the capping enzyme and the terminator factor were one and the same (Luo et al., 1991). It is hypothesized that the capping enzyme recognizes the early transcription termination signal U<sub>5</sub>NU in the nascent mRNA transcript and signals for termination (Shuman and Moss, 1988). The early transcription termination function of the viral mRNA capping is independent of the capping functions of the enzyme; however, both subunits of the viral mRNA capping enzyme are required for the early transcription termination function (Condit et al., 1996a; Luo et al., 1995; Yu et al., 1997).

#### **1.4.3 Intermediate Viral Transcription Initiation**

Much like the early viral transcription termination function, the role of the vaccinia virus mRNA capping enzyme in intermediate viral transcription is based on biochemical analysis of the virus. Intermediate-specific cytoplasmic extracts from vaccinia infected cells were

fractionated into three components: VITF-A, VITF-B, and the viral RNA polymerase (Vos et al., 1991a). The most purified fractions of each of the three components were used to reconstitute intermediate transcription in an *in vitro* transcription assay. VITF-B was shown to confer promoter specificity and further studies on VITF-A have shown that this component of the intermediate transcription initiation complex is the vaccinia virus mRNA capping enzyme (Vos et al., 1991b). The intermediate transcription initiation function of the capping enzyme also requires both subunits of the capping enzyme (Condit et al., 1996a; Vos et al., 1991b) and much like the early termination activity is independent of the mRNA guanylyltransferase activity of the viral mRNA capping enzyme (Harris et al., 1993).

### **1.5 Previous Mutants in Capping Enzyme Subunits**

Two temperature sensitive mutants in both subunits of the vaccinia virus mRNA capping enzyme have been identified and characterized previously. The mutant in the large subunit of the capping enzyme was characterized both *in vivo* and *in vitro* (Hassett et al., 1997). The *in vivo* analysis showed that the mutant virus synthesized normal amount of proteins and had normal expression of RNA; however, the mutant was defective in telomere resolution, an integral part of DNA replication, as well as in virion morphogenesis. In the biochemical analysis carried out *in vitro*, the mutant D1 viral protein was found to be defective in two of the enzymatic activities of the capping enzyme, guanylyltransferase and methyltransferase. The second capping enzyme mutant possessed a mutation in the small subunit of the capping enzymes and was characterized *in vivo* (Carpenter and DeLange, 1991) and *in vitro* (Condit et al., 1996a). Similar to what was observed with the mutant in the large subunit of the capping enzyme, the mutant in the small subunit of the capping enzyme synthesized normal amounts of viral proteins and had normal expression of viral RNA but was defective in telomere resolution. The biochemical analysis of the mutant D12 protein examined the role of the protein in early

transcription termination and intermediate transcription initiation; both of which were found to be absent in the mutant protein.

## 1.6 Study Objectives

As explained in the previous section of this introduction, in addition to its role in the capping of viral transcripts, the vaccinia mRNA capping enzyme has also been implicated by biochemical analysis to be involved in early viral transcription termination and intermediate viral transcription initiation. Two previous temperature sensitive (ts) mutants in both subunits of the capping enzyme have been characterized by *in vivo* and *in vitro* analyses as described in section 1.5 but these analyses have been unable to confirm the role of the capping enzyme in these additional activities (Carpenter and DeLange, 1991; Condit et al., 1996a; Hassett et al., 1997). During a complementation study that integrated the Dales collection of ts mutants with the Condit collection of ts mutants, a new mutant, Dts36, possessing a mutation in the large subunit of the viral capping enzyme was identified (Lackner et al., 2003). A complete phenotypic characterization of Dts36 was undertaken to further examine the role of the viral capping enzyme in the vaccinia virus life cycle in an attempt to confirm the role of the viral capping enzyme in early transcription termination and intermediate transcription initiation *in vivo*. The results from the phenotypic characterization will be presented in Chapter 3. A biochemical characterization of Dts36 was also undertaken to examine the mRNA capping enzyme activities of the mutant D1 protein from Dts36 as well the early termination and intermediate initiation activities of the mutant protein by *in vitro* analysis. The results from the biochemical characterization will be discussed in Chapter 4. A unified mechanism that explains the results seen in both the *in vivo* and *in vitro* analyses, a defect in the association of the capping enzyme subunits, will be discussed in Chapter 5.

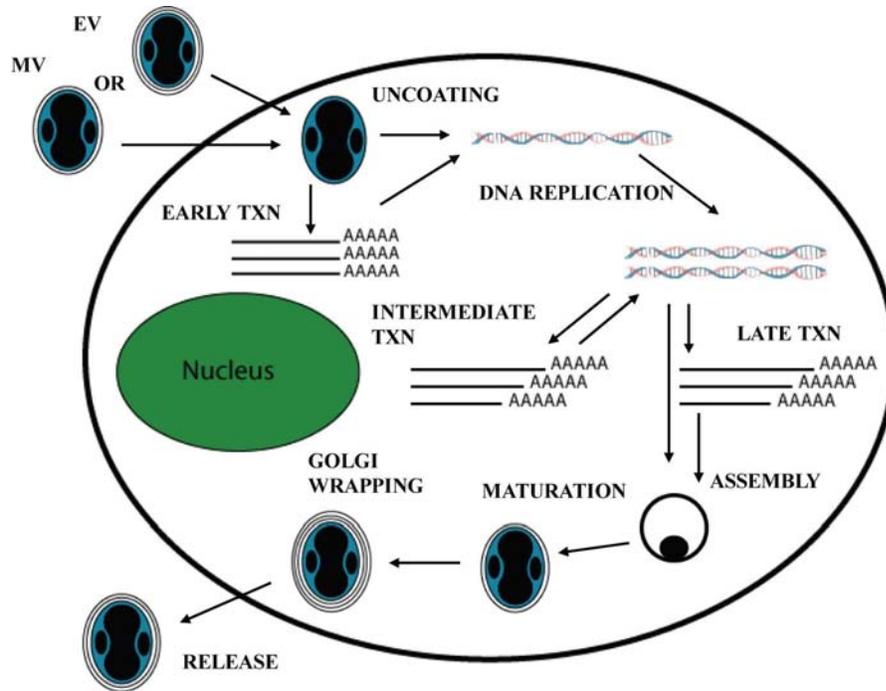


Figure 1-1. Vaccinia virus life cycle. Infectious virus particles in the form of MV or EV enter the cell through fusion; either fusion with the plasma membrane or fusion with an endosomal membrane. This fusion event results in partial uncoating of the virus to produce a viral core in which early transcription (early txn) occurs. Early transcripts encode factors that are required for DNA replication and intermediate transcription. Following early transcription, a secondary uncoating occurs and viral DNA is extruded into the cytoplasm where it can be replicated by the viral DNA polymerase. Following DNA replication, intermediate then late transcription occurs. Intermediate transcripts encode factors that are required for late transcription and late transcripts encode factors that are packaged in the virion for the subsequent infection as well as factors required for assembly and maturation of the infectious particle.

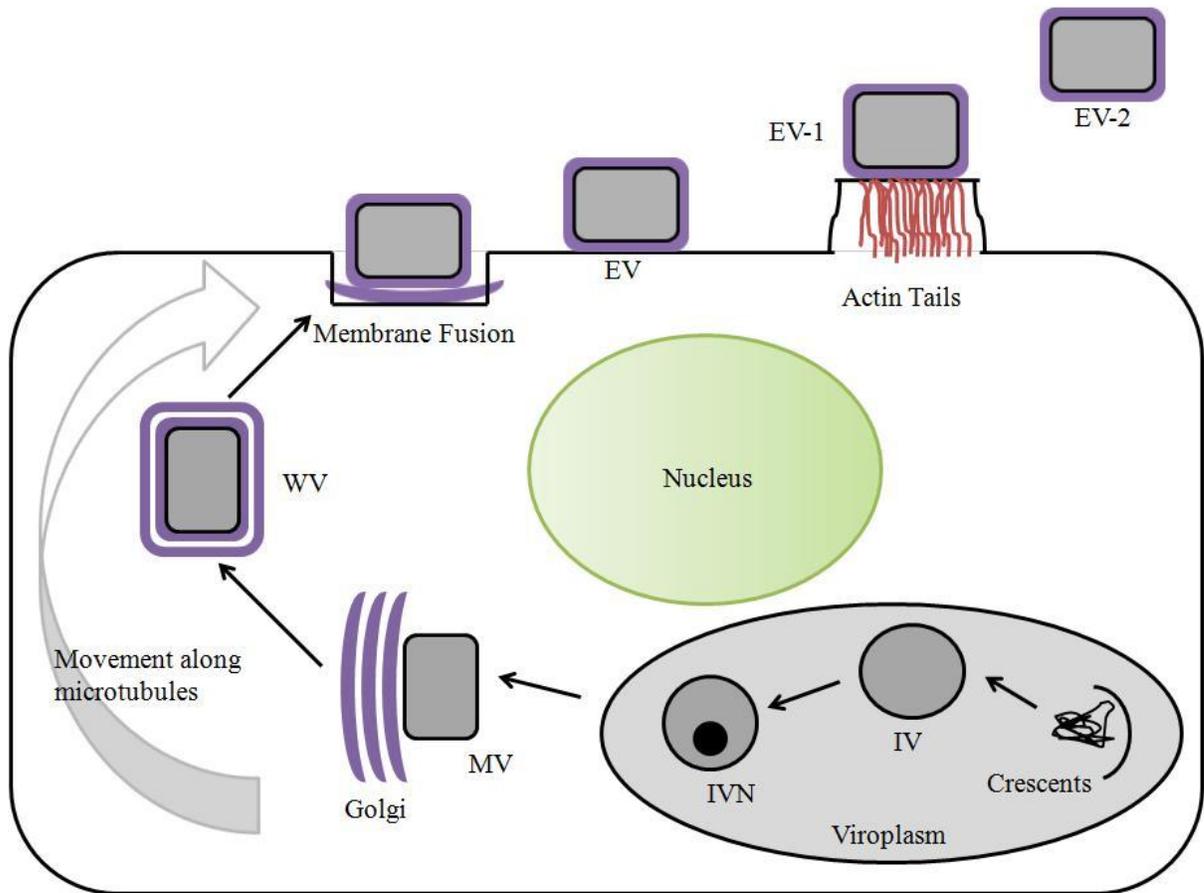


Figure 1-2. Overview of vaccinia virus morphogenesis. Crescents form in the viroplasm of the infected cell and continue to grow until they form closed circles which are IVs containing viroplasm. IVNs appear at nearly the same time that IVs appear; proteolytic processing of several viral proteins occurs and leads to the maturation of the virus particle to MV. MVs are extruded from the viroplasm into the cytoplasm of the infected cell and are transported by microtubules through the Golgi apparatus and to the plasma membrane. The outer membrane wrapper of the MV will fuse with the plasma membrane resulting in EV “sitting” on the surface of the cell. EV-1 is the form of EV that aids in cell-to-cell spread of the virus as actin tail formation drives the EV-1 towards the adjacent cell and EV-2 is the form of EV that is used for long distance dissemination of the virus.

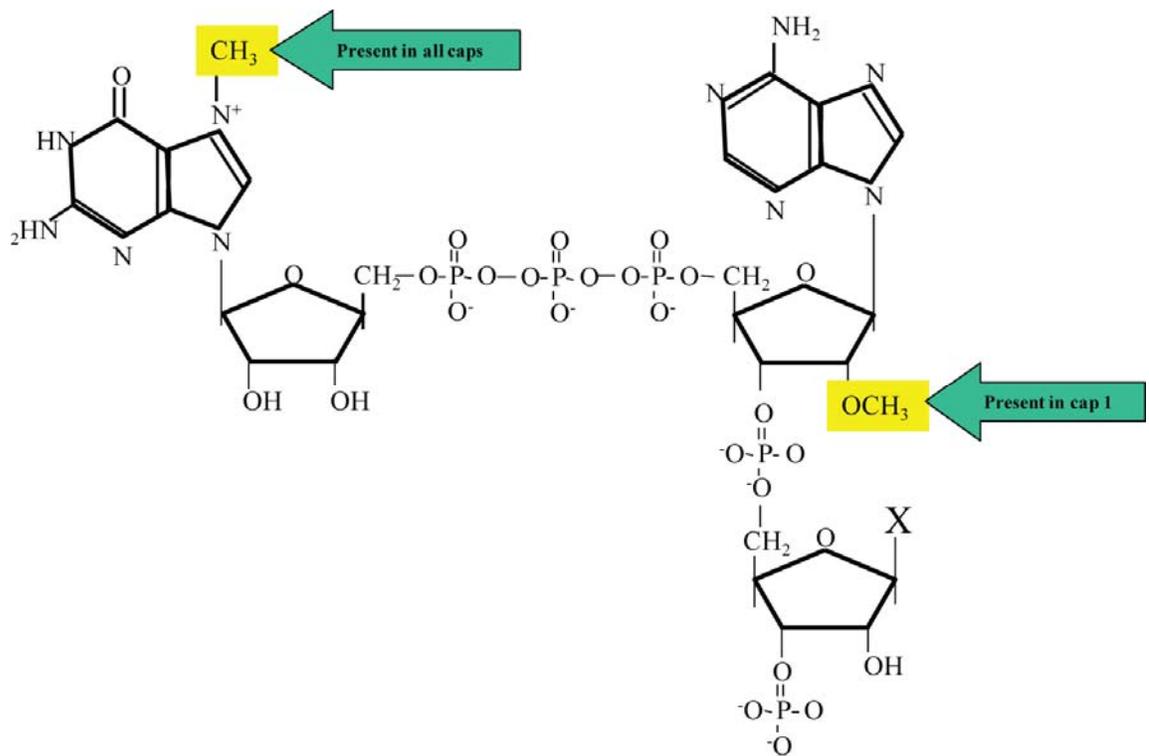


Figure 1-3. The mRNA cap 1 structure. The initiating nucleoside of the nascent mRNA transcript is shown on the right side and is linked to the guanylate residue of the cap structure by a 5'-5' triphosphate bridge. The guanylate residue is methylated at the N7 position as indicated by the arrow labeled, present in all caps. The second site of methylation, the O<sup>2'</sup> position of the initiating nucleoside, is indicated by the arrow labeled, present in cap 1.

## CHAPTER 2 MATERIALS AND METHODS

### 2.1 Methods for Phenotypic Characterization

#### 2.1.1 Cells and Viruses

A continuous line of green monkey kidney cells, BSC40 cells, were grown as previously described (Condit et al., 1983; Condit and Motyczka, 1981). Growth and propagation of the wild type vaccinia virus strain, IHDW, and the temperature sensitive mutant, Dts36, was performed as previously described (Lackner et al., 2003). One-step growth and plaque titration protocols were performed as described previously (Condit et al., 1983; Condit and Motyczka, 1981). A multiplicity of infection (m.o.i) of 10 was used for all experiments unless otherwise noted. The permissive temperature for viral infections was 31°C and the non-permissive temperature was 39.7°C. The non-permissive temperature is the temperature at which the wild type virus grows as expected by the ts viruses do not. Experiments were performed in force draft Hotpack incubators (SP Industries, Warminster, PA) with quick temperature recovery and the temperature inside of the incubator was closely monitored throughout the experimental time course.

#### 2.1.2 Viral DNA Isolation and DNA Sequencing

Isolation of viral DNA from BSC40-infected cells incubated at 31°C was performed as previously described (Lackner et al., 2003) using a Qiagen DNeasy tissue kit (Qiagen, Valencia, CA). The D1R gene was amplified by PCR using primers outside of the open reading frame (ORF) of the gene to yield a 2750 bp-product. An additional eight internal primers were used for the sequencing reactions on the D1R product. The D12L gene was amplified by PCR using primers outside of the ORF of the gene to yield a 1000 bp-product. An additional two internal primers were used in the sequencing reactions on the D12L product. The sequencing reactions were performed by the University of Florida ICBR DNA Sequencing Core Laboratory. The

resulting DNA sequences were assembled and analyzed using the Vector NTI software program (Invitrogen, Carlsbad, CA).

### **2.1.3 Marker Rescue**

One-step marker rescue mapping of Dts36 was performed as previously described (Kato et al., 2004b). BSC40 cells on 60-mm dishes were infected at 31°C with an appropriate m.o.i. as determined by terminal dilution. The infected cells were then transfected with 1.5 µg of DNA PCR-amplified from wild type viral DNA using Lipofectin reagent (Invitrogen). The infected and transfected cells were incubated at 39.7 °C for four days at which time they were stained with crystal violet and the number of wt plaques were counted.

### **2.1.4 Virion Thermostability**

The thermostability of the wild type and mutant virions was assayed as described previously (Fathi and Condit, 1991). Wild type and mutant virions at a concentration of  $1 \times 10^4$  pfu/mL were incubated at 45°C. At various times, aliquots were removed and frozen at -80°C until all samples were collected. In order to determine if the virions were thermolabile, the samples were analyzed by plaque titration at 31°C.

### **2.1.5 Metabolic Labeling of Proteins and Gel Electrophoresis**

Monolayers of infected BSC40 cells on 35-mm dishes were metabolically labeled and samples were analyzed as described previously with slight modifications (Condit and Motyczka, 1981). The infected cells were labeled with 0.5 mL of 100µCi/mL of Redivue Promix<sub>L</sub>-[<sup>35</sup>S] *in vitro* cell labeling mix (1000 Ci/mmol) (GE Healthcare, Piscataway, NJ) for 30 minutes. The labeled cells were lysed with Laemmli sample buffer and samples were analyzed by 11% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were stained with Coomassie blue, destained, dried, and exposed to film.

### **2.1.6 Isolation of Viral RNA**

Isolation of viral RNA from infected cells was performed as described previously (Cresawn et al., 2007). Monolayers of BSC40 cells were infected with an m.o.i. of 10 and incubated at either 31°C or 39.7°C until various times post-infection when RNA was isolated from the infected cells. Total cellular RNA was isolated using the RNeasy RNA isolation kit (Qiagen) following the directions of the manufacturer. The RNA was eluted from the RNeasy column by with 50 µL of RNase-free water. The concentration of the RNA was determined by measuring the optical density (OD) at 260 nm.

### **2.1.7 Synthesis of Riboprobes**

Riboprobes used in Northern blot analysis were synthesized in several different manners. Antisense riboprobes specific for C11R, G8R, and F17R mRNA transcripts were prepared as described previously (Hassett et al., 1997). The template for the F11R riboprobe was amplified by PCR from a pGEM plasmid that contained the open reading frame of the F11R gene linked to the bacteriophage SP6 promoter. The M13 forward and reverse primers were used for the amplification. Riboprobes specific for approximately 500 bp of A20R, D5R, and E9L mRNA transcripts were transcribed from PCR products as previously described with the following modifications (Cresawn et al., 2007). Transcription templates were PCR amplified using a forward primer that was complementary to the 5' end of the ORF and which was tagged with the sequence “*CGTAATACGACTCACTATAGGGAGA*” containing the bacteriophage T7 promoter and a reverse primer complementary for a region 500 bp downstream of the 5' end of the ORF and tagged with the sequence “*CGATTTAGGTGACACTATAGAAGCG*” containing the bacteriophage SP6 promoter. (The essential promoter regions are shown in italics). The riboprobes used in the F11 primer walk experiment were synthesized in the same manner except the regions of complementary were not restricted to the 5' end of the ORF. All riboprobes were

transcribed using the MAXIscript *in vitro* transcription system (Ambion, Austin, TX) and purified using NucAway Spin Columns (Ambion) according to the manufacturer's protocol.

### **2.1.8 Northern Blot Analysis**

Northern blot analysis was performed as described previously with the following modifications (Cresawn et al., 2007). Denaturing formaldehyde gels were run at 80 volts for 2 hours. Following transfer of the RNA to a GeneScreen neutral charge membranes (Perkin Elmer, Waltham, MA), the membranes were prehybridized for at least 6 hours. Following overnight hybridization, the membranes were washed one time at room temperature with 0.1 X SSC/0.1% SDS followed by three washes at 65°C with 0.1 X SSC/1.0% SDS. After all washes were completed, the membranes were exposed to film and to phosphor screen (GE Healthcare) for quantification of viral RNA transcripts. The quantification data was analyzed using a Storm phosphorimager (GE Healthcare) and the ImageQuant software program (GE Healthcare).

### **2.1.9 Western Blot Analysis**

Whole cell, infected cell lysates were electrophoresed on 11% SDS-PAGE and transferred to nitrocellulose membranes as described previously (Kato et al., 2004b). After transfer, the membranes were blocked overnight in TBS-T/NFDM (0.15 M NaCl, 0.05 M Tris-HCl, pH 7.5, 0.1% Tween-20, and 5% nonfat dry milk). The membranes were incubated with an appropriate dilution of primary antibody in TBS-T/NFDM for at least 1.5 hours after which the membranes were rinsed with TBS-T. Following the rinse step, the membranes were incubated for 1.5 hours with anti-rabbit Ig-conjugated to horseradish peroxidase (GE Healthcare) at a dilution of 1:5000 in TBS-T/NFDM. The membranes were then rinsed six times with TBS-T and developed with the Enhanced chemiluminescence detection kit (GE Healthcare) according to the manufacturer's protocol. The primary antisera used were as follows: anti-E9 (1:500), anti-D5 (1:1000), and anti-A20 (1:1000) rabbit sera supplied by Dr. Paula Traktman (Medical College of Wisconsin). Anti-

D1 (1:500) and anti-D12 (1:500 or 1:6000 depending on the experiment) rabbit antisera were supplied by Dr. Ed Niles (SUNY-Buffalo). The anti-F11 (1:2000) rabbit serum was previously described (Kato et al., 2004a). Western blot autoradiograms were quantified using Image J (Abramoff et al., 2004).

#### **2.1.10 Isolation of Viral DNA Replication Samples**

Viral DNA was isolated and viral DNA replication was analyzed as previously described (Traktman and Boyle, 2004). The DNA was isolated from virally infected cells by scraping the infected cell monolayer into the culture media and recovering the cells by centrifugation. The cells were washed once with phosphate buffered saline (PBS) and after washing, the cells were resuspended in a solution of 10 X SSC (1.5 M NaCl, 0.15 M sodium citrate) and 1 M ammonium acetate. Samples were subject to three cycles of freeze/thawing to disrupt the cells and were subsequently stored at -20°C.

#### **2.1.11 Slot Blot Hybridization for Viral DNA Replication**

Isolated DNA replication samples were analyzed as previously described (Traktman and Boyle, 2004). The samples were applied to a hydrated and equilibrated Nytran Supercharge nylon transfer membrane (GE Healthcare) on a Minifold II Slot-Blotter apparatus (GE Healthcare). While the membrane was still on the slot blot apparatus, the DNA was denatured with a solution containing 0.5 M NaOH and 1.5 M NaCl and then neutralized by two washes of 10 X SSC. After removal of the membrane from the slot blot apparatus, the membrane was prehybridized at 42°C in a hybridization oven (Labnet International, Inc., Edison, NJ) for at least 2 hours in a solution of 6 X SSC, 50% formamide, 0.5% SDS, 5 X Denhardt's solution (0.1% BSA, 0.1% polyvinylpyrrolidone, 0.1% Ficoll), and 100 µg/mL denatured salmon sperm DNA. After the prehybridization,  $2.25 \times 10^6$  cpm of the randomly <sup>32</sup>P-labeled (DECAprime II kit (Ambion)) HindIII E fragment was added to fresh hybridization solution and incubated with the

membrane overnight at 42°C. After the overnight incubation, the membrane was washed three times with 2 X SSC at room temperature followed by two washes at 55°C with 0.2 X SSC/0.1%SDS. Following the washes, the membrane was exposed to film and was quantified using a phosphor screen (GE Healthcare) and analyzed using a Storm phosphorimager (GE Healthcare) and the ImageQuant software program (GE Healthcare).

#### **2.1.12 AraC Experiment**

Confluent monolayers of BSC40 cells were infected with an m.o.i. of 10 in the presence of 40 µg/mL of cytosine β-D-arbinofuranoside (araC). At various times post-infection, metabolic labeling of proteins, RNA isolation, and DNA isolation were performed as described above.

#### **2.1.13 Shift-Up Experiment**

Confluent monolayers of BSC40 cells were infected with an m.o.i. of 10 and incubated at 31°C for eight hours. At 8 hours post-infection (hpi), the dishes of infected cells were moved to 39.7°C. At various times post-infection, metabolic labeling of proteins, RNA isolation, and DNA isolation were performed as described above.

### **2.2 Methods for Biochemical Characterization**

#### **2.2.1 Virus Purification**

Wild type and mutant viruses were purified as previously described (Kato et al., 2004b). Briefly, confluent monolayers of BSC40 cells in 150 mm dishes were infected with an m.o.i. of 0.01 and were incubated at 31°C until a complete CPE was observed. Cell-associated virus was purified by differential centrifugation through a 36% sucrose cushion followed by banding of the virus on a 24-40% sucrose gradient. The purified virions were quantified by OD at 260 nm (1 OD<sub>260</sub> = 68 µg protein) and by the Bradford assay (Bio-Rad protein assay). Infectivity of the purified virus was measured by plaque titration of the virus on BSC40 cell monolayers at 31°C.

### **2.2.2 Preparation of Virion Extracts**

Virion extracts were prepared essentially as previously described but with the following modifications (Gross and Shuman, 1996). Purified virus, 5 OD units, was incubated for 10 minutes at room temperature in 0.8 mL of a solution containing 50 mM Tris-HCl, pH 8, 10 mM dithiothreitol (DTT), and 0.05% nonidet P40 (NP-40). Virus cores were recovered by centrifugation and the resulting pellet was resuspended in 150  $\mu$ L of buffer N (300 mM Tris-HCl, pH 8, 250 mM KCl, and 50 mM DTT). The samples were adjusted to 0.15% sodium deoxycholate and incubated on ice for 30 minutes with occasional mixing. After the 30 minute incubation, the samples were adjusted to 10% glycerol and insoluble material was removed by centrifugation. The supernatants were applied to a 150  $\mu$ L DEAE column equilibrated with buffer M (50 mM Tris-HCl, pH 8, 250 mM KCl, 1 mM EDTA, 0.1% Triton X-100, 3 mM DTT, and 10% glycerol) and eluted with buffer M. The protein concentrations of the eluted fractions were measured by Bradford assay and the peak fractions were combined and stored in aliquots at -80°C.

### **2.2.3 *In vitro* Transcription by Permeabilized Virions**

Permeabilized virion transcription reactions were essentially performed as previously described (Kato et al., 2004b; Kato et al., 2007). In order to determine the amount of incorporation into RNA, reaction mixtures containing 60 mM Tris-HCl, pH 8, 0.05% NP-40, 10 mM DTT, 10 mM MgCl<sub>2</sub>, 5 mM ATP, 1 mM GTP, 1 mM UTP, 0.2 mM CTP, 40 nM  $\alpha$ -<sup>32</sup>P-CTP (3000  $\mu$ Ci/nmole), and 2 OD/mL of purified virus were incubated at 31 or 37°C. At various times, 50  $\mu$ L aliquots were removed, precipitated with 5% trichloroacetic acid (TCA), filtered on glass microfibre filters (934-AH, GE Healthcare), and TCA-precipitable radioactivity was determined by liquid scintillation counting. In order to determine virion core-associated RNA versus released RNA, reactions were performed as above except that the 50  $\mu$ L aliquots were

added to 150  $\mu$ L of a solution containing 50 mM Tris-HCl, pH 8, 10 mM EDTA, 10 mM DTT, and 0.05% NP-40 and centrifuged at full speed for 3 minutes. The resulting supernatants were removed and were precipitated with 5% TCA. The pellets were resuspended in 200  $\mu$ L of a solution of 50 mM Tris-HCl, pH 8 and 0.1% SDS and precipitated with 5% TCA.

#### **2.2.4 Enzyme-GMP Complex Formation in Permeabilized Virions and Virion Extracts**

The formation of the covalent intermediate between the D1R subunit and the GMP molecule was analyzed as described previously (Hassett et al., 1997; Shuman and Moss, 1990). Various amounts of either purified virus or soluble virion extracts were incubated for 10 minutes at 31°C in a solution containing 50 mM Tris-HCl, pH 8.2, 0.05% NP-40, 5 mM MgCl<sub>2</sub>, 10 mM DTT, and 21 nM  $\alpha$ -<sup>32</sup>P-GTP (3000  $\mu$ Ci/nmole). The reaction was stopped by the addition of 5 X Laemmli sample buffer and the samples were analyzed by 10% SDS-PAGE. The gels were fixed, stained and destained, dried, and exposed to film and the amount of labeled D1 protein was quantified by exposing the dried gel to a phosphor screen (GE Healthcare). The data was quantified using a Storm phosphorimager (GE Healthcare) and the ImageQuant software program (GE Healthcare).

#### **2.2.5 Coupled Transcription and Methyltransferase Assay in Permeabilized Virions**

The methyltransferase activity of the capping enzyme was analyzed by measuring the transfer of a radiolabeled methyl group from S-adenosylmethionine (SAM) to newly synthesized and guanylated virion transcripts as described previously with the following modifications (Gershowitz and Moss, 1979; Hassett et al., 1997). Equal amounts of either a 2 X transcription mixture (10 mM ATP, 2 mM GTP, 2 mM UTP, 0.4 mM CTP, and 80 nM  $\alpha$ -<sup>32</sup>P-CTP (3000  $\mu$ Ci/nmole)) or a 2 X methyltransferase mixture (10 mM ATP, 2 mM CTP, 2 mM GTP, 2 mM UTP, and 4  $\mu$ M <sup>3</sup>H-SAM (10  $\mu$ Ci/nmole)) were combined with 0.5 OD/mL of purified virus in a solution of 60 mM Tris-HCl, pH 8, 0.05% NP-40, 10 mM DTT, 10 mM MgCl<sub>2</sub>. The samples

were incubated at either 31 or 37°C and at various times, 20 µL aliquots were removed. The transcription samples were processed as described above in the *in vitro* transcription section. The methyltransferase samples were spotted onto DE81 filters (GE Healthcare) and unincorporated radioactivity was removed by washing two times with 0.5 M sodium phosphate, once with water, once with ethanol, and air dried. All filters were analyzed by liquid scintillation counting.

### **2.2.6 Uncoupled Methyltransferase Assay in Permeabilized Virion and Virion Extracts**

The transfer of a radiolabeled methyl group from <sup>3</sup>H-SAM to GTP was measured in permeabilized virions and soluble virion extracts essentially as previously described (Hassett et al., 1997; Shuman and Moss, 1990). Purified virions at a concentration of 5 OD/mL were incubated at 31 or 37°C in a solution containing 60 mM Tris-HCl, pH 8, 0.05% NP-40, 10 mM DTT, 10 mM MgCl<sub>2</sub>, 10 mM GTP, and 4 µM <sup>3</sup>H-SAM (10 µCi/nmole). At various times, 20 µL aliquots were removed and spotted on DE81 filters. Various amounts of soluble virion extracts were incubated at 31 or 37°C for 30 minutes in 25 µL reactions containing 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 10 mM GTP, and 4 µM <sup>3</sup>H-SAM (10 µCi/nmole). At various times, 20 µL aliquots were removed and spotted on DE81 filters. Filters were washed four times with 25 mM ammonium formate, once with water, twice with ethanol, dried, and analyzed by liquid scintillation counting.

### **2.2.7 Non-Specific RNA Polymerase Activity**

The non-specific RNA polymerase activity in soluble virion extracts was assayed as previously described with the following modifications (Hassett et al., 1997; Kato et al., 2007). Various amounts of soluble virion extracts were incubated for 30 minutes at 31 or 37°C in 100 µL reactions containing 60 mM Tris-HCl, pH 8, 10 mM DTT, 3 mM MnCl<sub>2</sub>, 1 mM ATP, 1 mM GTP, 1 mM UTP, 0.1 mM CTP, 10 nM α-<sup>32</sup>P-CTP (3000 µCi/nmole), and 1µg single-stranded

M13 DNA. At the end of the incubation period, the samples were precipitated with 5% TCA, filtered on glass microfibre filters (934-AH, GE Healthcare), and TCA-precipitable radioactivity was determined by liquid scintillation counting.

CHAPTER 3  
PHENOTYPIC ANALYSIS OF A TEMPERATURE SENSITIVE MUTANT IN THE LARGE  
SUBUNIT OF THE VACCINIA VIRUS MRNA CAPPING ENZYME

**3.1 Introduction**

The vaccinia virus mRNA capping enzyme is a multifunctional enzyme involved in several aspects of viral transcription in addition to the capping of newly transcribed mRNA transcripts. These additional functions of the capping enzyme, as an early transcription termination factor and an intermediate transcription initiation factor, have been hypothesized as functions of the capping enzyme based on previous biochemical analyses as discussed in the introduction (Chapter 1). As mentioned in the introduction (Section 1.5), although there has been a ts mutant in each of the capping enzyme subunits that has been previously characterized, neither of these mutants have shown defects in early termination or intermediate initiation functions *in vivo* (Carpenter and DeLange, 1991; Hassett et al., 1997). In this chapter, the role of the viral mRNA capping enzyme in early transcription termination and intermediate transcription initiation *in vivo* will be explored through the phenotypic characterization of two temperature sensitive vaccinia virus mutants which both have a mutation in the large subunit of the vaccinia virus capping enzyme. Characterization of one of the two viruses, Dts36, shows that this virus possesses an early phenotype. Specifically, in Dts36 at the non-permissive temperature, early mRNA metabolism is defective, early protein expression is irregular, and both DNA replication and post-replicative gene expression are absent. Furthermore, temperature shift up experiments reveal a defect in intermediate mRNA metabolism in Dts36 infected cells. The results presented in this study indicate that at the non-permissive temperature, the mRNA capping, the early transcription termination, and the intermediate transcription initiation activities of the vaccinia virus capping enzyme are affected in Dts36.

## 3.2 Results

### 3.2.1 Marker Rescue and DNA Sequencing

Dts36 and Dts50 temperature sensitive vaccinia virus mutants comprise a newly discovered complementation group (Lackner et al., 2003). In order to determine the site of the mutation in these viruses, three rounds of a one-step marker rescue were utilized (Kato et al., 2008). Briefly, confluent monolayers of BSC40 cells were infected with either the Dts36 or the Dts50 virus and transfected with DNA fragments that were PCR-amplified from wild type vaccinia virus DNA. The infected and transfected cells were incubated at 39.7°C for four days and subsequently stained with crystal violet. A successful rescue of the ts virus is evident from the appearance of plaques on the dishes that were infected and transfected. The first round of marker rescue used PCR products comprising overlapping 20 kilobase (kB) fragments spanning the vaccinia virus genome (Yao and Evans, 2003). The second round of marker rescue used PCR products that used overlapping 5kB PCR fragments spanning the vaccinia virus genome (Luttge and Moyer, 2005) and the third round used PCR products for individual open reading frames of vaccinia virus genes. Marker rescue of Dts36 (Fig. 3-1) and Dts50 (data not shown) shows that each mutant was rescued by a PCR product amplified from the wild type D1R gene which indicates that both viruses possess a mutation in D1R, the large subunit of the viral mRNA capping enzyme.

The D1R gene from Dts36, Dts50, and two parental strains of vaccinia virus, IHDW and WR, was sequenced as described in materials and methods. IHDW was sequenced because it is the parental strain from which the ts mutants in the study were made and WR was sequenced to determine if there are any differences in the D1R gene between the two parental strains of vaccinia virus. The sequence of D1R in the WR strain was found to be identical to the previously published sequence. By contrast, there are 24 polymorphisms in D1R comparing the

WR and IHDW parental strains. Of these 24 polymorphisms, only three result in changes in the protein sequence of D1 (V5I, T202K, and R812K). Both Dts36 and Dts50 were found to have an identical single point mutation at nucleotide residue 2114 which causes a glycine to aspartic acid coding change at amino acid residue 705 in the protein sequence (G705D) (Fig. 3-2). Thus, Dts36 and Dts50 are sibling viruses and therefore, for the remainder of this study, only Dts36 was used.

The D12L gene, which encodes the small subunit of the viral mRNA capping enzyme, of Dts36, IHDW, and WR was also sequenced as described above. The D12L gene from all viruses sequenced were found to have identical sequences to each other as well as to the previously published sequence.

### **3.2.2 Growth Properties and Virion Thermostability of Dts36**

A one-step growth experiment was used to determine the growth properties of Dts36 and IHDW. Confluent monolayers of BSC40 cells were infected with a high m.o.i. and incubated at 31 or 39.7°C and infected cells and media were harvested and used to measure viral yield in plaque assays at 31°C (Fig. 3-3). At 24 hours post-infection, IHDW grown at either the permissive or the non-permissive temperature produced between 16 and 29 pfu per cell. Dts36 grown at 31°C was virtually indistinguishable from IHDW grown at either temperature. However, Dts36 grown at 39.7°C produced no detectable infectious progeny when analyzed by plaque assays at 31°C.

The viral mRNA capping enzyme is a virion protein so it was critical to determine if the Dts36 virions were thermolabile; a characteristic that could impact further experiments. A virion thermostability assay was used to determine the stability of the Dts36 and the IHDW viruses at 45°C. A known amount of virus,  $1 \times 10^4$  pfu/mL, was incubated for up to 4 hours and the stability of the viruses was measured by plaque titration (Fig. 3-4). Throughout the entire time

course, both the wild type virus, IHDW, and the mutant virus, Dts36, are equally stable with only a slight decrease in thermostability seen as soon as two hours into the incubation with either virus.

### **3.2.3 Basic Phenotypic Characterization of Dts36**

The basic phenotypic characterization of Dts36 included examining viral protein synthesis, viral DNA replication, and viral mRNA synthesis at the permissive and non-permissive temperatures.

#### **3.2.3.1 Viral protein synthesis**

Viral protein synthesis was used as a measure of overall viral gene expression in virus infected cells. Cells infected with either Dts36 or IHDW at 31 or 39.7°C were metabolically labeled with <sup>35</sup>S methionine, samples were analyzed by SDS-PAGE, and the radiolabeled proteins were detected by autoradiography (Fig. 3-5A). The normal temporal expression of viral genes is observed in the wild type infection. Early viral proteins are visible at two hpi and their synthesis peaks at 4 hpi when infected cells are incubated at 31°C and at 2 hpi when infected cells are incubated at 39.7°C. The shut-off of host cell protein synthesis is also evident in wild type infections as early as 2 hpi and extends throughout the remainder of the infection. Post-replicative protein synthesis is marked by the appearance two viral proteins, p4a and p4b, beginning at 6 hpi in infected cells incubated at 31°C and at 4 hpi in infected cells incubated at 39.7°C and continuing throughout the remainder of the experiment. The pattern of protein expression in Dts36 infections at 31°C is indistinguishable from IHDW at either temperature. By contrast, non-permissive Dts36 infections showed extended early viral protein synthesis, decreased shut-off of host protein synthesis, and no post-replicative viral protein synthesis. The synthesis of the D1 protein was verified by Western blot analysis and quantified using Image J (Fig. 3-5B, C). Dts36 synthesized 50-60% less D1 protein compared to IHDW at both

temperatures; however, at both the permissive and the non-permissive temperatures, Dts36 synthesized similar amounts of the D1 protein, as did IHDW (Fig. 3-5C).

### **3.2.3.2 Viral DNA synthesis**

Viral DNA synthesis in Dts36 and IHDW infected cells at both the permissive and the non-permissive temperatures was also examined. DNA was isolated from infected cells, applied to a nylon membrane, probed with a radiolabeled probe specific for a region of the vaccinia virus genome, and quantified using a phosphorimager (Fig. 3-5D). The kinetics of DNA replication in IHDW infected cells at both 31 and 39.7°C and in Dts36 infected cells at 31°C were virtually indistinguishable. In non-permissive infections, no viral DNA replication was observed in Dts36 infected cells.

### **3.2.3.3 Viral RNA synthesis**

The synthesis of viral mRNA in Dts36 infections at the non-permissive temperature was analyzed by northern blot. RNA was isolated from infected cells incubated at either 31 or 39.7°C, separated by gel electrophoresis, transferred to a neutral membrane and probed for viral gene expression. The riboprobes used were specific for a gene from each of the three classes of viral genes. Autoradiograms of northern blots for the early gene (C11R, the vaccinia growth factor), the intermediate gene (G8R, a vaccinia late transcription factor) and the late gene (F17R, a DNA binding phosphoprotein) are shown in Fig. 3-6A. In infections with the WR strain of vaccinia virus, early gene expression peaks and then declines for the remainder of the infection (Baldick, Jr. and Moss, 1993). The current study is the first time that gene expression has been examined in IHDW infections and much to our surprise, in wild type IHDW infections, early gene expression peaks, decreases and then resumes at times late in infection. Early gene expression as analyzed by northern analysis for IHDW infected cells at 31 and 39.7°C and for Dts36 infected cells at 31°C are virtually indistinguishable from one another. The peak of synthesis of the

C11R transcript occurs at 6 hpi at 31°C for both viruses and at 3 hpi at 39.7°C for IHDW. The peak of synthesis of C11R transcript in Dts36 at 39.7°C occurs at 3 hpi as in IHDW at this temperature; however, the mutant produces only 35% the amount of C11R transcript compared to wild type virus infections at this time. The C11R transcript in the Dts36 infection incubated at 39.7°C also does not reappear at times late in infection. It should also be noted that the C11R transcript in both viruses at both temperatures is of the correct size, approximately 0.5kb (Fig. 3-6B). Much like the analysis of C11R gene expression, the intermediate and late gene expression for IHDW infections at 31 and 39.7°C and for Dts36 infections at 31°C are virtually indistinguishable from one another; however, the analysis of post-replicative gene expression in mutant infections at 39.7°C showed no intermediate or late viral transcripts, consistent with the absence of viral DNA replication and post-replicative proteins discussed earlier. These results also suggest that the resumption of early gene expression observed in permissive infections requires late gene expression.

#### **3.2.3.4 Viral protein and RNA synthesis in the presence of araC**

Dts36 appeared to synthesize the same amount of early viral proteins as IHDW at the non-permissive temperature when measured by protein pulse labeling (Fig. 3-5A). However, the lack of host cell protein synthesis shut-off in Dts36 infections incubated at 39.7°C and the earlier switch to post-replicative protein synthesis in IHDW infections incubated at 39.7°C made it difficult to accurately compare protein synthesis levels in the two viruses. Therefore, an additional pulse labeling and RNA isolation was performed in the presence of cytosine  $\beta$ -D-arabinofuranoside (araC), an inhibitor of DNA synthesis that would allow accumulation of early viral message and protein (Fig. 3-7). The pattern of early viral protein synthesis in infections with Dts36 and IHDW at both temperatures was indistinguishable with both viruses apparently synthesizing the same proteins in the same amounts (Fig. 3-7A). In the analysis of early viral

mRNA synthesis, cells infected with IHDW at both temperatures and cells infected with Dts36 at 31°C were indistinguishable; however, the amount of C11R message at 3 hpi in the Dts36 infections was 15% of that measured in the IHDW infections (Fig. 3-7B).

### **3.2.4 Early gene transcription termination is defective in Dts36-infected cells**

Analysis of other early viral genes by northern and western blots was performed to investigate whether the observed decrease in steady state early viral RNA is a global phenomenon or if it is specific for the C11R gene. The early viral genes examined were F11L (a gene involved in cell signaling (Valderrama et al., 2006)), E9L (the DNA polymerase), A20R (a DNA polymerase processivity factor (Klemperer et al., 2001)), and D5R (an ATPase/DNA primase (Boyle et al., 2007; De Silva et al., 2007)).

#### **3.2.4.1 Analysis of F11L transcript in Dts36 non-permissive infections**

Analysis of the F11L transcript showed that similar patterns of expression were evident in infections with IHDW at both temperatures and Dts36 at the permissive temperature (Fig. 3-8A). In the wild type infections, the major F11L transcript migrates at 1 kb. A second, slightly slower migrating band is seen at 1.35 kb (band 4 in Fig. 3-8A) and most likely represents termination at the next downstream termination signal past the F11L termination signal. Both bands peak at 3 hpi, decrease, and reappear at late times during the wild type infection at both the permissive and the non-permissive temperatures. At late times during the wild type infection, read through from an upstream late gene, most likely F13L, is evident as a smear from 1 kb to greater than 4 kb. The Dts36 infections at 31°C are similar to wild type infections; however, there are two differences. First, by visual inspection, the ratio of the 1.35 kb band to the 1 kb band (band 4 to the F11L band in Fig. 3-8A) appears to be greater in the Dts36 31°C infections than in the wild type infections. Second, there is an additional band at 2.4 kb in the mutant infections at the permissive temperature indicating that the mutant is slightly leaky at the permissive temperature.

The pattern of F11L transcript expression was different in Dts36 infected cells incubated at 39.7°C. The F11L transcript was present but was no longer the predominant transcript. There were four additional transcripts: band 1 (3.6 kb), band 2 (2.4 kb), band 3 (1.65 kb) and band 4 (1.35 kb) (Fig 3-8A). The F11L transcript and the additional transcripts all peaked at 3 hpi, decreased slowly, and did not reappear at times late during infection. The read through from the upstream late genes was also not apparent in mutant infections at the non-permissive temperature. The size of these additional bands corresponds to the predicted length of transcripts that initiate at the F11L promoter and terminate at termination sequences downstream suggesting that these bands result from read-through of termination sequences (Fig. 3-8B). In order to confirm that these bands were indeed longer-than-expected F11L transcripts, further northern analyses were performed using riboprobes designed to detect the longest two additional transcripts seen in the initial F11L northern analysis (Fig. 3-9). One riboprobe, designated F10, was capable of detecting the two longest of the additional transcripts (bands 1 and 2) and a second riboprobe, designated F8/F7, was capable of detecting only the longest additional transcript (band 1). The results showed that when the F10 riboprobe was utilized only the two largest transcripts were seen and when the F8/F7 riboprobe was utilized only the largest transcript was seen in Dts36 at 39.7°C. The leakiness seen in permissive infections with Dts36 in Fig. 3-9 is also apparent in this experiment. In addition to detecting band 1 in non-permissive Dts36 infections, the F8/F7 riboprobe was also able to detect the F7L transcript. This transcript behaved much like the C11R transcript in cells infected with Dts36 and incubated at 39.7°C: it was present in decreased amounts compared to the wild type infections at either temperature and the mutant at the permissive temperature.

#### **3.2.4.2 Analysis of the E9L, A20R, and D5R transcripts in Dts36 non-permissive infections**

The analysis of the E9L transcript yielded results similar to those seen in the analysis of the F11L transcript (Fig. 3-10A). The E9L transcript is visible at 3 hpi in wild type infections at both the permissive and the non-permissive temperatures; however, it is difficult to determine if the transcript reappears late during infections because of the large amount of read through from an upstream late gene (most likely E11L). In infections with Dts36 at 31°C, the E9L transcript is visible as is an additional, slower migrating band at 4.7 kb (band 2 in Fig. 3-10A). Both of these transcripts peak at 3 hpi, decrease, and reappear at times late during infection. Much like the wild type infections, there is read through of a late gene that is located upstream of E9L in the Dts36 infections incubated at 31°C. In mutant infections incubated at 39.7°C, the amount of the E9L transcript was decreased at 3 hpi, expression of E9L did not resume late during infection, and longer-than-expected E9L transcripts appeared. As with F11L, the size of the additional transcripts corresponded to termination sequences downstream of the E9L termination sequence (Fig. 3-10B). Northern analyses were also used to examine the viral transcripts encoded by A20R and D5R (data not shown). In Dts36 infected cells at 39.7°C, the A20R transcript was terminated correctly but was present in decreased amounts and did not reappear late during infections. The D5R transcript in mutant infections at the non-permissive temperature was present in decreased amounts, did not reappear late during viral infection, and did not terminate correctly.

#### **3.2.4.3 Analysis of the F11, E9, A20, and D5 protein synthesis in Dts36 non-permissive infections**

It was of interest to determine the effect that the longer-than-expected transcripts and the decreased amount of transcripts had on viral protein synthesis. Therefore, the synthesis of proteins encoded by these transcripts was analyzed by western blot (Fig. 3-11). For all proteins

examined, the protein of interest was expressed in similar amounts in the wild type infections at both temperatures and the mutant infections at 31°C. When F11 was examined in Dts36 infected cells at 39.7°C, the mutant expressed at least the same amount if not more of the protein than IHDW at the same temperature. On the other hand, the E9, A20, and D5 proteins were expressed in decreased amounts in Dts36 infected cells at 39.7°C compared to IHDW at 39.7°C.

### **3.2.5 Intermediate Gene Transcription is Compromised in Dts36-infected Cells**

The multifunctional vaccinia virus capping enzyme has been implicated in intermediate gene transcription initiation; therefore, it was of interest to determine whether intermediate gene transcription was defective in Dts36 infections at the non-permissive temperature. Because Dts36-infected cells are defective in DNA replication and post-replicative gene expression, a typical time course of infection does not provide any insight into the impact of the mutation on intermediate gene expression. However, we were able to circumvent this problem by performing a shift-up experiment in which the infection was initiated and incubated at 31°C for 8 hours and then shifted to 39.7°C for the remainder of the experiment (Fig. 3-12A). Viral protein synthesis was analyzed by pulse labeling (Fig. 3-12B) and viral DNA replication was assayed by slot blot analysis (Fig. 3-12C). Before and after the shift to 39.7°C, similar amounts of post-replicative viral proteins and of DNA replication were seen for both IHDW and Dts36 infections. It is important to note that Dts36 continues both post-replicative protein synthesis and DNA replication.

Viral RNA synthesis was also examined in the shift-up experiment by northern analysis in which autoradiograms were used to qualitatively assess RNA synthesis (Fig. 3-13A) and phosphorimager analysis was used to quantitatively assess RNA synthesis (Fig. 3-13B-D). Early and late transcripts were synthesized in similar amounts by both viruses after the shift to 39.7°C. When an intermediate transcript was analyzed, the IHDW infections showed a constant amount

of intermediate synthesis whereas the Dts36 infections showed a striking decrease in intermediate synthesis after the shift to the non-permissive temperature.

### **3.3 Summary**

In this chapter, the phenotypic characterization of a temperature sensitive mutant with a mutation in the large subunit of the vaccinia virus mRNA capping enzyme is discussed. The detailed analysis of Dts36 showed that this mutant possesses an early phenotype. At the non-permissive temperature, Dts36 infections show aberrant early gene expression, are DNA negative, and show no post-replicative gene expression. Analysis of various early mRNA transcripts in Dts36 infections incubated at the non-permissive temperature reveals that some but not all early viral mRNAs are synthesized in reduced amounts. This phenomenon extends to early viral protein synthesis as well. It is important to note that we have not directly measured the cap status of viral mRNAs in mutant infected cells. However, given the role of the D1R gene in mRNA capping, the reduction in amounts of both early mRNA and proteins in Dts36 infected cells is consistent with a loss of stability of the nascent mRNA transcripts resulting from a defect in mRNA capping. Additional analysis of early viral mRNA transcripts from non-permissive Dts36 infections also reveals that some early mRNA transcripts are longer than expected. This phenomenon indicates that at the non-permissive temperature the mutant capping enzyme encoded by Dts36 is defective in early viral transcription termination. Finally, analysis of post-replicative viral transcripts produced during an infection that was initiated at the permissive temperature and then shifted to the non-permissive temperature reveals that the intermediate transcript examined in this study decreases in amount after the temperature shift while the late viral transcript examined is not affected. This observation is consistent with a defect in the intermediate transcription initiation function of the mutant capping enzyme encoded by Dts36.

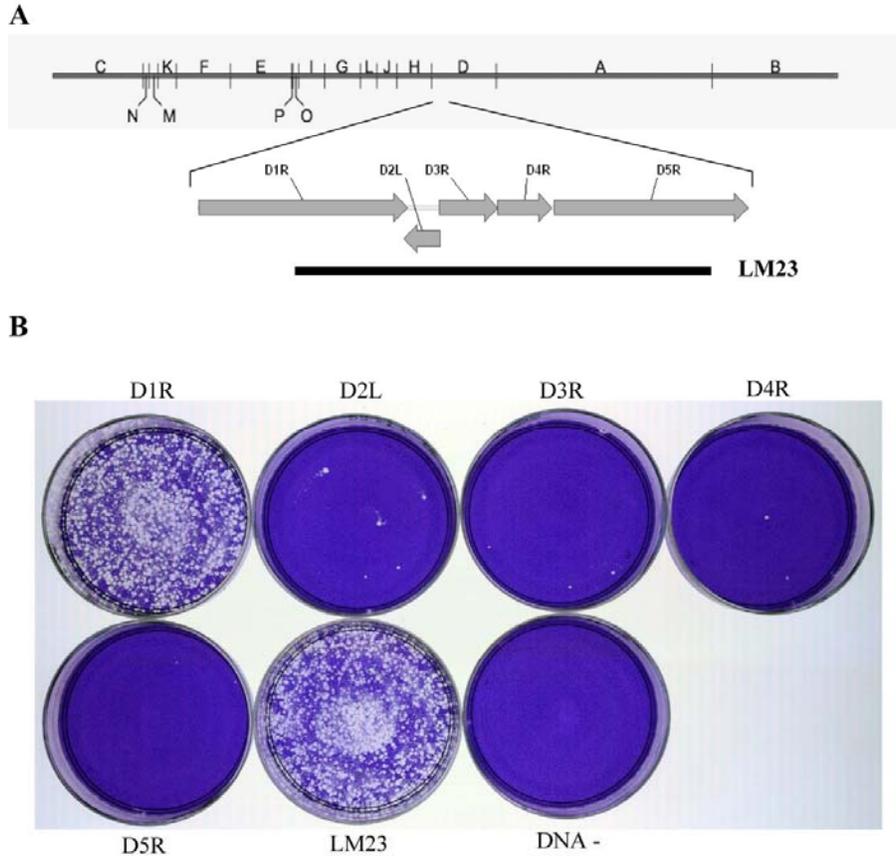


Figure 3-1. Marker rescue of Dts36. A) Hind III restriction map of the vaccinia virus genome. The area of the genome that comprises LM23, the 5 kb PCR product that successfully rescued the mutant infection at the non-permissive temperature, is shown. B) Dishes stained with crystal violet are shown labeled with the gene-specific PCR product used in the transfection.

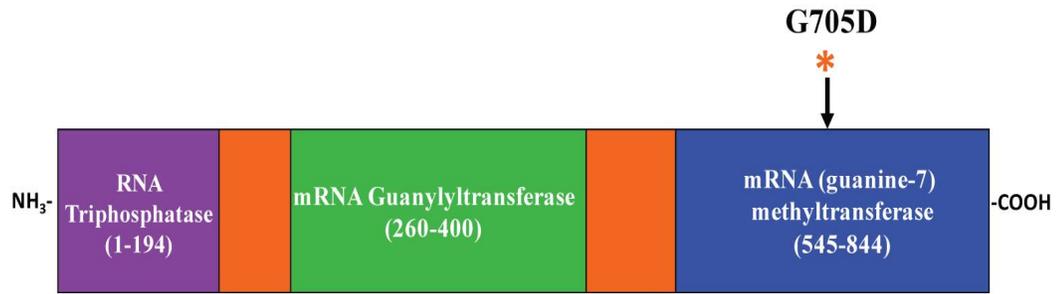


Figure 3-2. The D1 protein. The enzymatic domains of the vaccinia virus mRNA capping enzyme are shown. The location of the mutation in the D1 protein from Dts36 is also indicated by the arrow and \*.

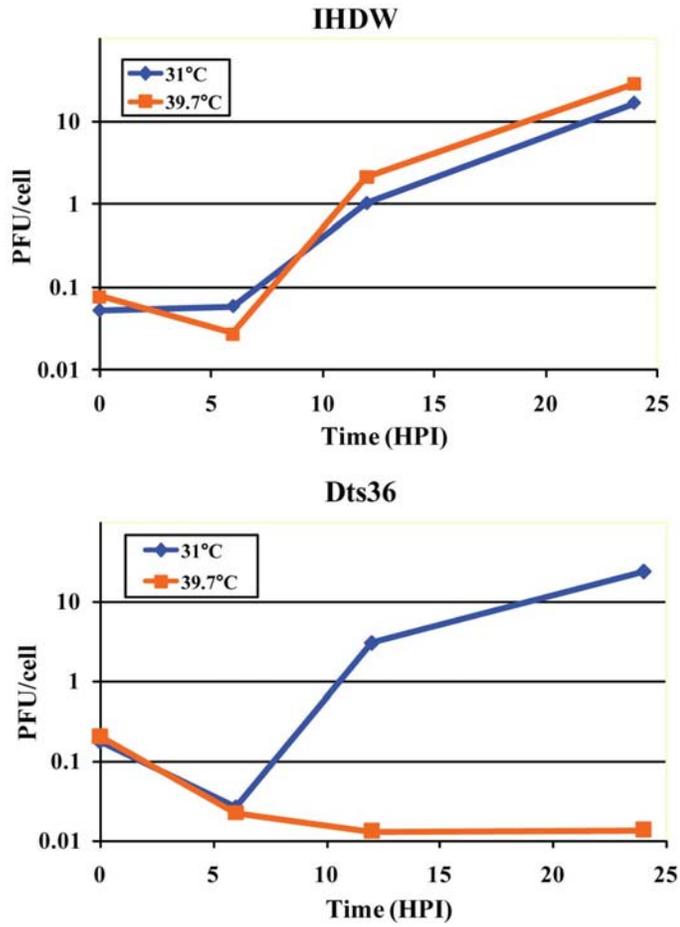


Figure 3-3. One-step growth of IHDW and Dts36 viruses. Cells were infected with an m.o.i. of 10 and incubated at either the permissive (31°C) or non-permissive (39.7°C) temperature. Samples were collected at various times post-infection (x-axis) and viral yields were determined by plaque titration at 31°C (y-axis).

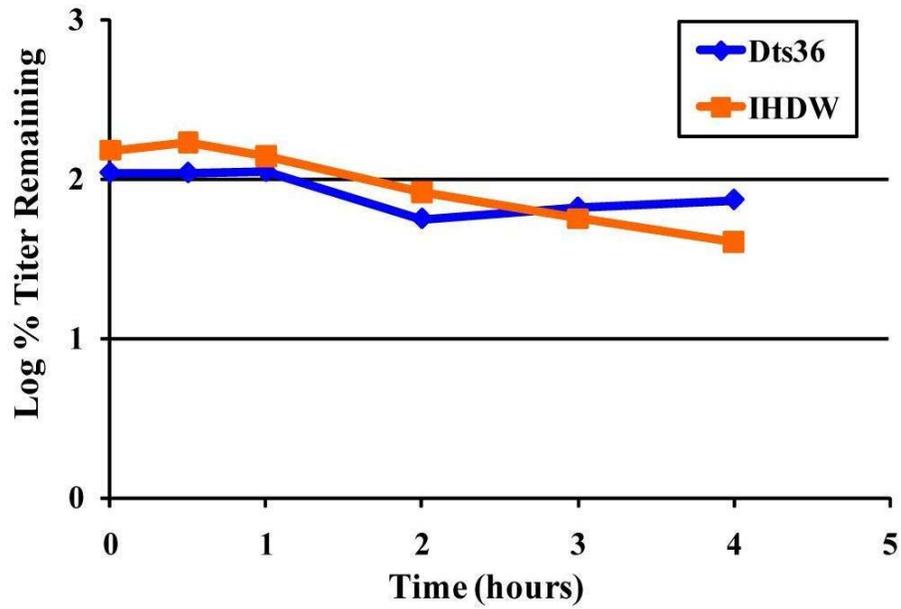


Figure 3-4. Virion thermostability of Dts36 and IHDW at 45°C. Wild type or mutant virus was incubated at 45°C for various amounts of time (x-axis), aliquots were removed, and the amount of virus titer remaining (y-axis) was determined by plaque titration at 31°C.

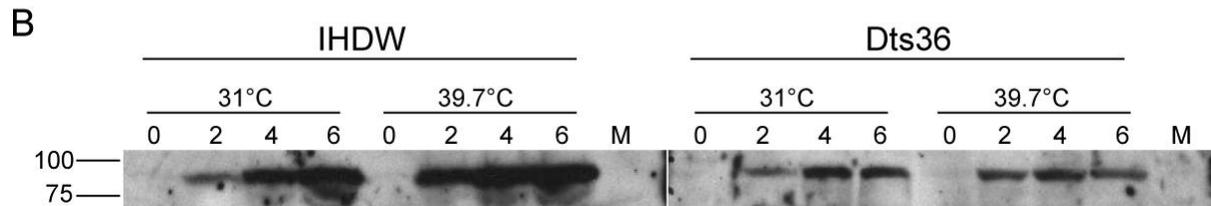
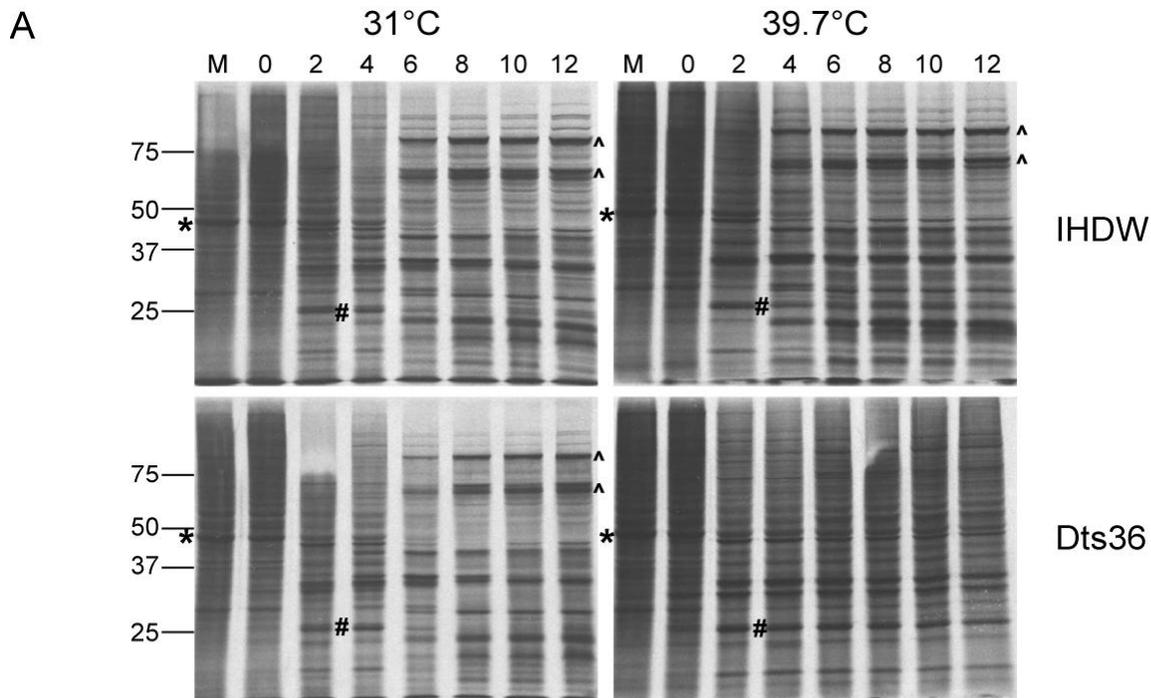
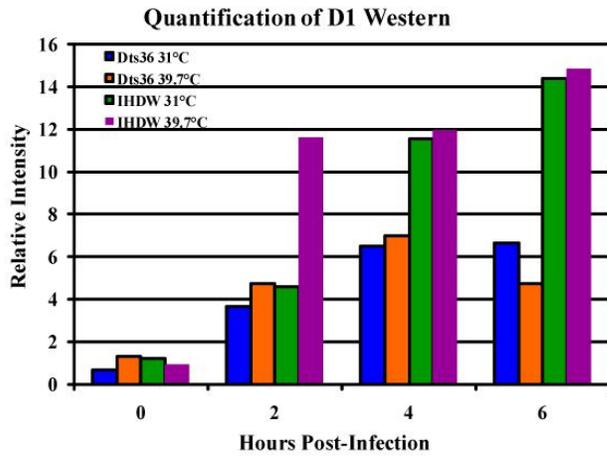


Figure 3-5. Basic phenotypic characterization of Dts36: protein synthesis, D1 synthesis, and DNA replication. A) Autoradiograms of viral protein synthesis in Dts36 and IHDW-infected cells. The virus with which the cells were infected is listed to the right of the autoradiograms and the temperature at which the infected cells were incubated is listed above the autoradiograms. The molecular weights in kD are listed to the left of the autoradiograms. Examples of the different classes of viral proteins are labeled as follows: \* = host protein, # = early viral protein, ^ = post-replicative viral protein. B) Western blot analysis of the amount of D1 protein present in Dts36 and IHDW-infected cells. Listed above each autoradiogram is the virus used to infect the cells, the temperature at which the infected cells were incubated, and the hour post-infection at which the sample was isolated. The molecular weights in kDa are listed to the left of the autoradiogram. C) Quantification of the autoradiograms of the D1 western blots. The hour post-infection when the sample was isolated is on the x-axis and the y-axis shows the relative intensity. D) Synthesis of viral DNA in cells infected with Dts36 or IHDW. Samples were analyzed in triplicate and the resulting plot of the quantified data represents an average of these values. Error bars represent the standard deviations based on the data.

C



D

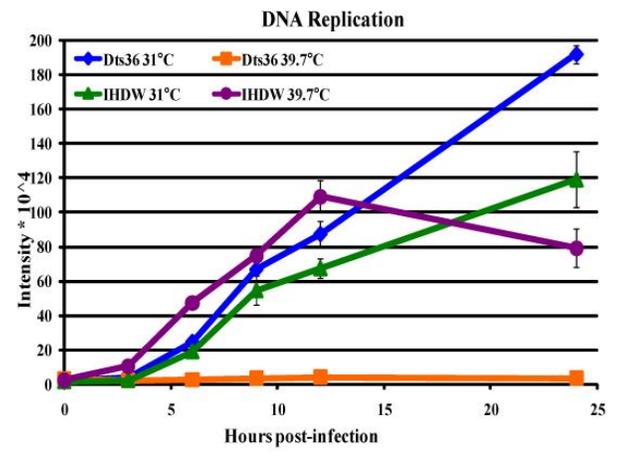


Figure 3-5. Continued

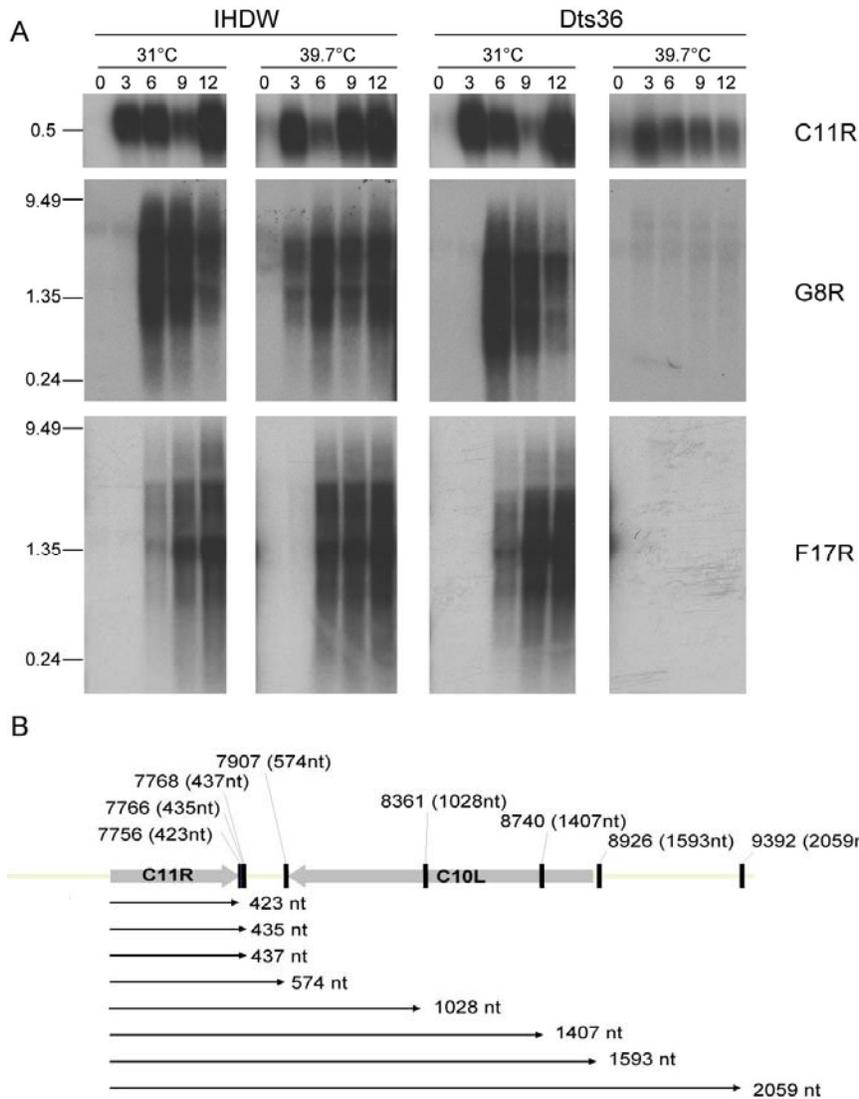


Figure 3-6. Analysis of early, intermediate and late RNA synthesis in cells infected with Dts36 or IHDW. A) Autoradiograms of northern blot analysis of total RNA isolated from cells infected with Dts36 or IHDW. Riboprobes used in this analysis were specific for early (C11R), intermediate (G8R), and late (F17R) viral transcripts. The virus with which the cells were infected, the temperature at which the infected cells were incubated, and the hour post-infection at which the RNA was isolated are listed above the autoradiograms. The riboprobe which was used for each northern is listed to the right and the approximate molecular weights in kb is listed to the left of the autoradiograms. B) A map of the region of the vaccinia virus genome which contains the C11R gene. The C11R gene and the downstream C10L gene are shown as arrows which point in the direction in which each gene is transcribed. The early transcription termination signals are shown as black boxes. Below the genome map are arrows which represent viral transcripts that initiate at the C11R initiation site and terminate at each of the shown termination signals. The length of each of these transcripts is listed to the right of each transcript.

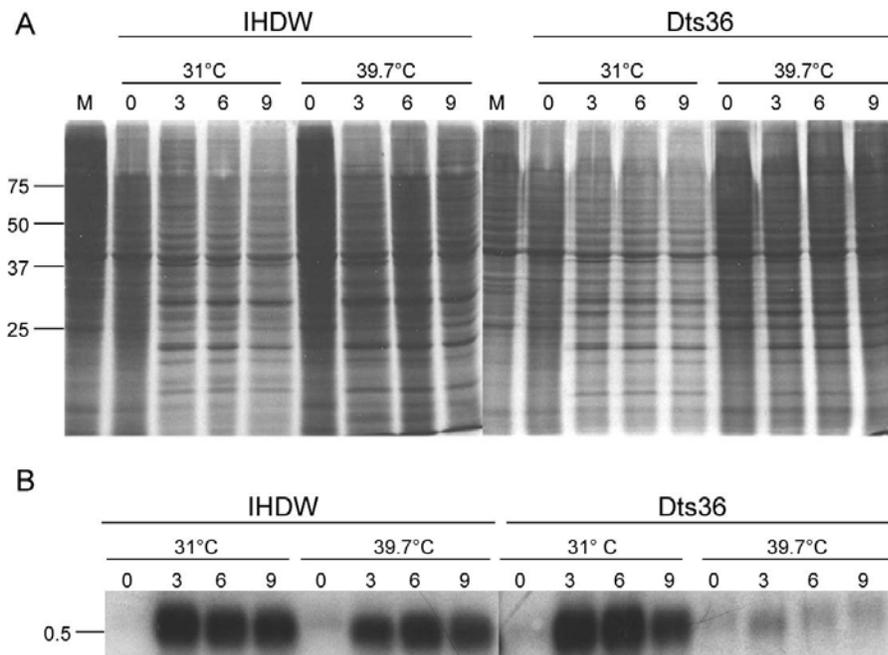


Figure 3-7. Viral protein and mRNA synthesis in cells infected Dts36 or IHDW in the presence of araC. A) Autoradiograms of protein synthesis in cells infected with Dts36 or IHDW in the presence of araC. Labeled above the autoradiograms are the virus with which the cells were infected, the temperature at which the infected cells were incubated, and the hour post-infection at which the samples were isolated. The molecular weights in kDa are listed to the left of the autoradiograms. B) Synthesis of the early viral transcript, C11R, in cells infected with Dts36 or IHDW as determined by Northern blot analysis. The riboprobe used in this analysis was specific for C11R. Labeled above the autoradiograms are the virus with which the cells were infected, the temperature at which the infected cells were incubated, and the hour post-infection at which the RNA was isolated. The molecular weights in kb are listed to the left of the autoradiograms.

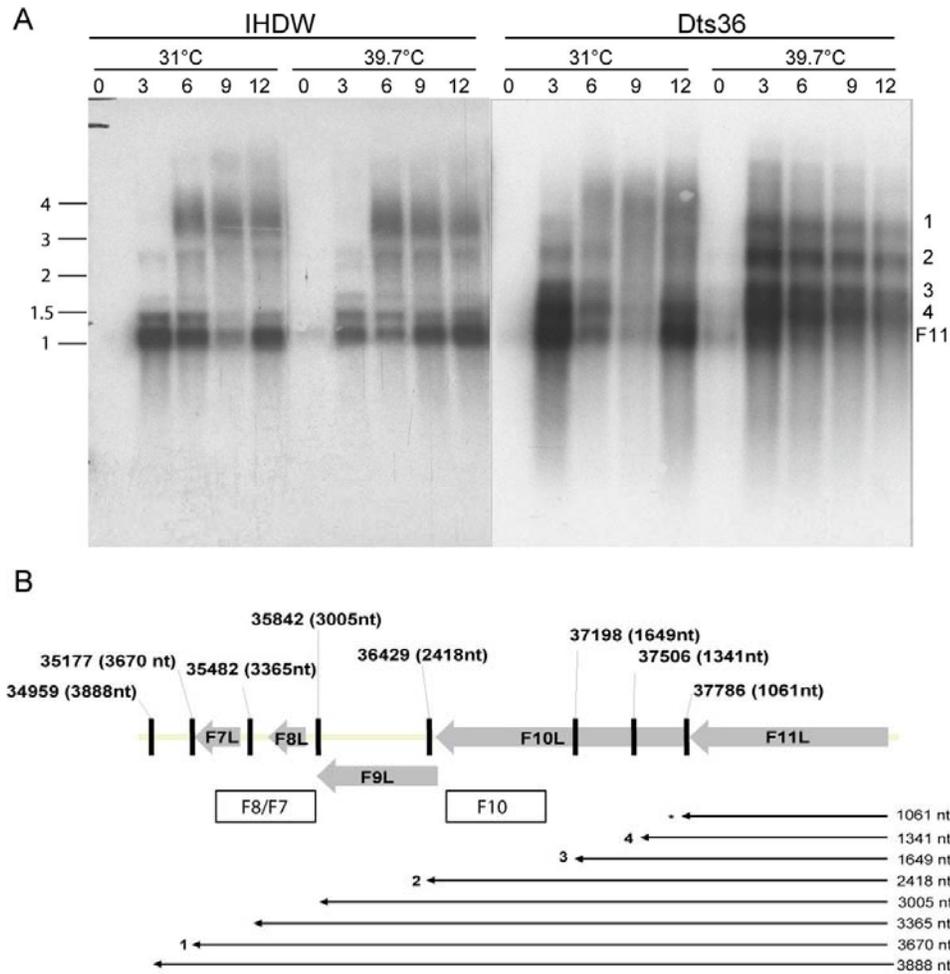


Figure 3-8. Analysis of the early viral transcript, F11L, in cells infected with Dts36 or IHDW. A) Autoradiograms of northern blot analysis using a riboprobe specific for F11L. Labeled above the autoradiogram are the virus with which the cells were infected, the temperature at which the infected cells were incubated, and the hour post-infection at which the viral RNA was isolated. The approximate molecular weight in kb is found to the left of the autoradiogram. The F11L transcript, as well as the longer than expected F11L transcripts, are labeled to the right of the autoradiogram. B) A map of the region of the vaccinia virus genome which contains the F11L gene. The F11L gene and the downstream genes are shown as arrows which point in the direction in which each gene is transcribed. The early transcription termination signals are shown as black boxes. Below the genome map are arrows which represent viral transcripts that initiate at the F11L initiation site and terminate at each of the shown termination signals. The length of each of these transcripts is listed to the right of each transcript and those transcripts which correspond to bands 1-4 in part A are labeled at the left end of the transcript. The regions of the genome where the F10 and F8/F7 riboprobes hybridize are shown in boxes above the transcripts.

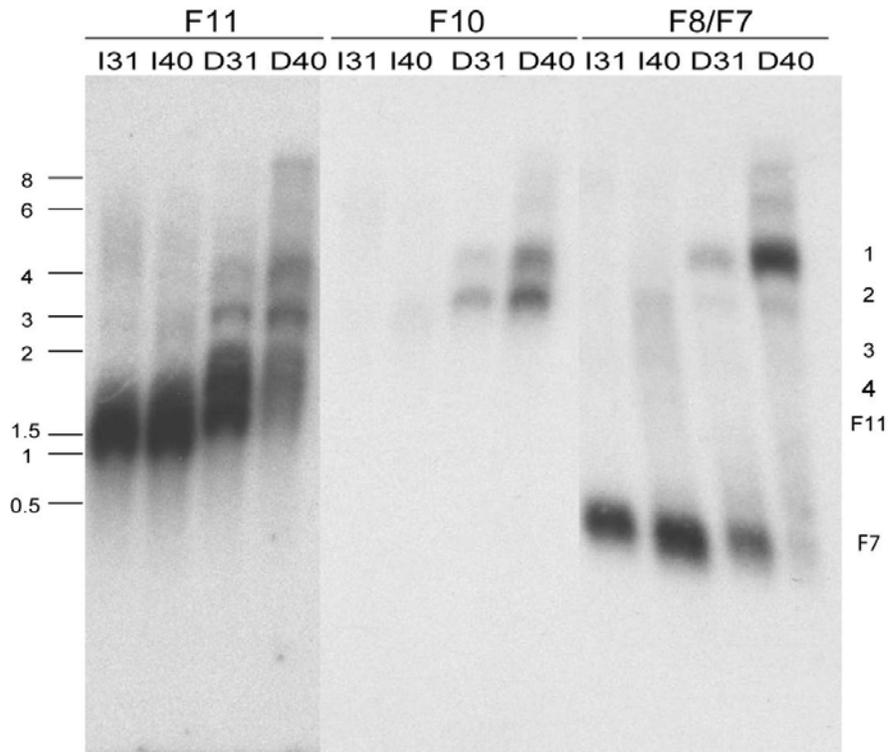


Figure 3-9. Primer walk experiment with various riboprobes for the analysis of the longer than expected F11L transcripts. The RNA analyzed in the northern blot was isolated at 3 hpi. The riboprobe used in the analysis and the virus with which the cells were infected is listed above the autoradiograms. I31 = IHDW, 31°C; I40 = IHDW, 39.7°C; D31 = Dts36, 31°C; D40 = Dts36, 39.7°C. The molecular weights in kb are listed to the right of autoradiograms and bands 1-4, the F11L transcript, and the F7L transcript are labeled to the right of the autoradiogram.

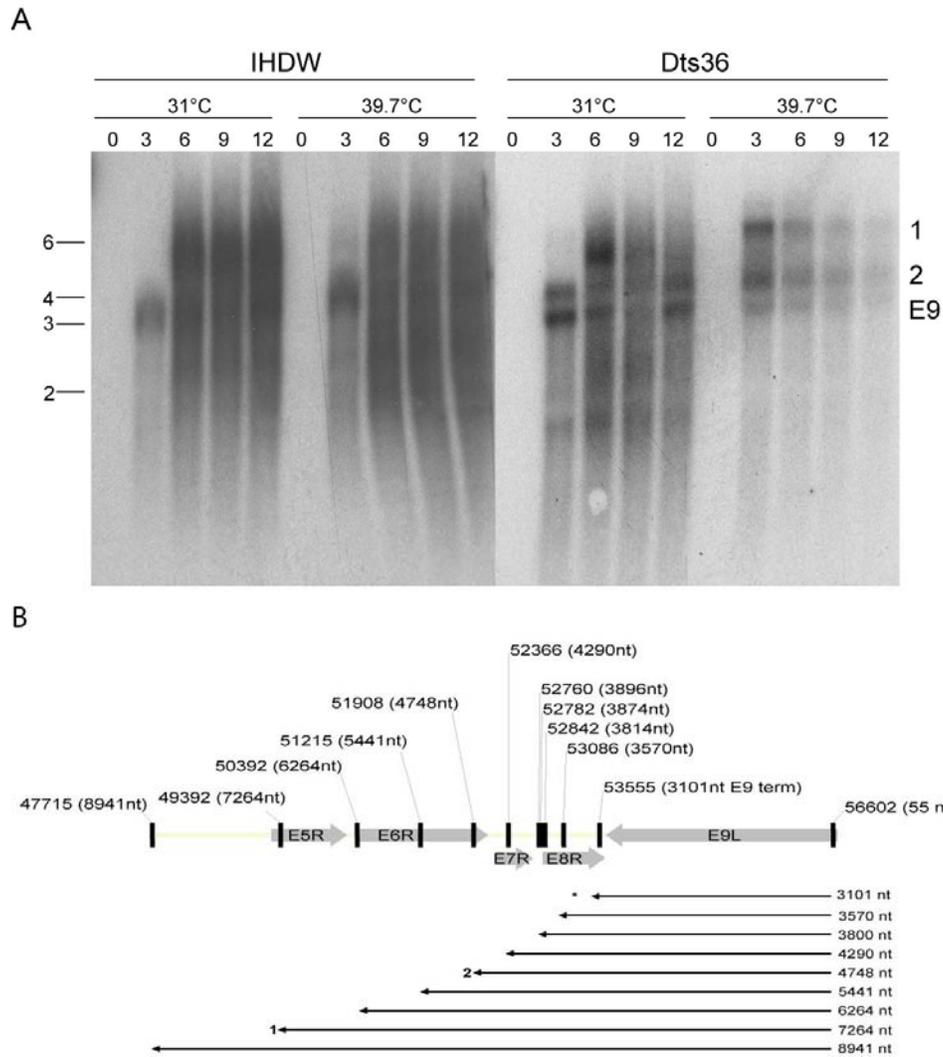


Figure 3-10. Analysis of the early viral transcript, E9L, in cells infected with Dts36 or IHDW. A) Autoradiograms of northern blot analysis using a riboprobe specific for E9L. Labeled above the autoradiogram are the virus with which the cells were infected, the temperature at which the infected cells were incubated, and the hour post-infection at which the viral RNA was isolated. The approximate molecular weight in kb is found to the left of the autoradiogram. The E9L transcript, as well as the longer than expected E9L transcripts, are labeled to the right of the autoradiogram. B) A map of the region of the vaccinia virus genome which contains the E9L gene. The E9L gene and the downstream genes are shown as arrows which point in the direction in which each gene is transcribed. The early transcription termination signals are shown as black boxes. Below the genome map are arrows which represent viral transcripts that initiate at the E9L initiation site and terminate at each of the shown termination signals. The length of each of these transcripts is listed to the right of each transcript and those transcripts which correspond to bands 1 and 2 in part A are labeled at the left end of the transcript.

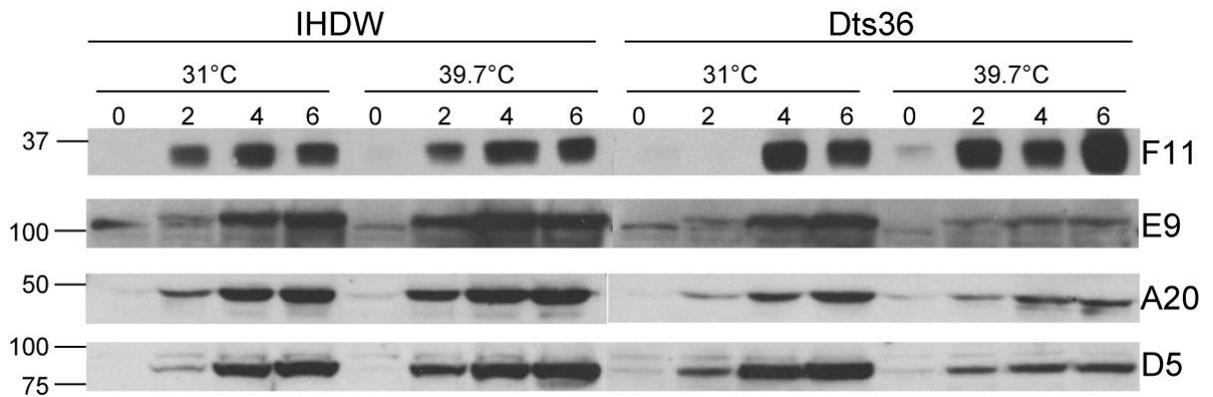


Figure 3-11. Synthesis of the F11, E9, A20, and D5 viral proteins during Dts36 and IHDW infections. Samples were analyzed and blots were prepared and probed. The virus with which the cells were infected, the temperature at which the infected cells were incubated, and the hour post-infection at which the samples were isolated are listed above the autoradiograms. The primary antibody used to probe each membrane is listed to the right of the autoradiograms and the molecular weights in kDa are listed to the left.

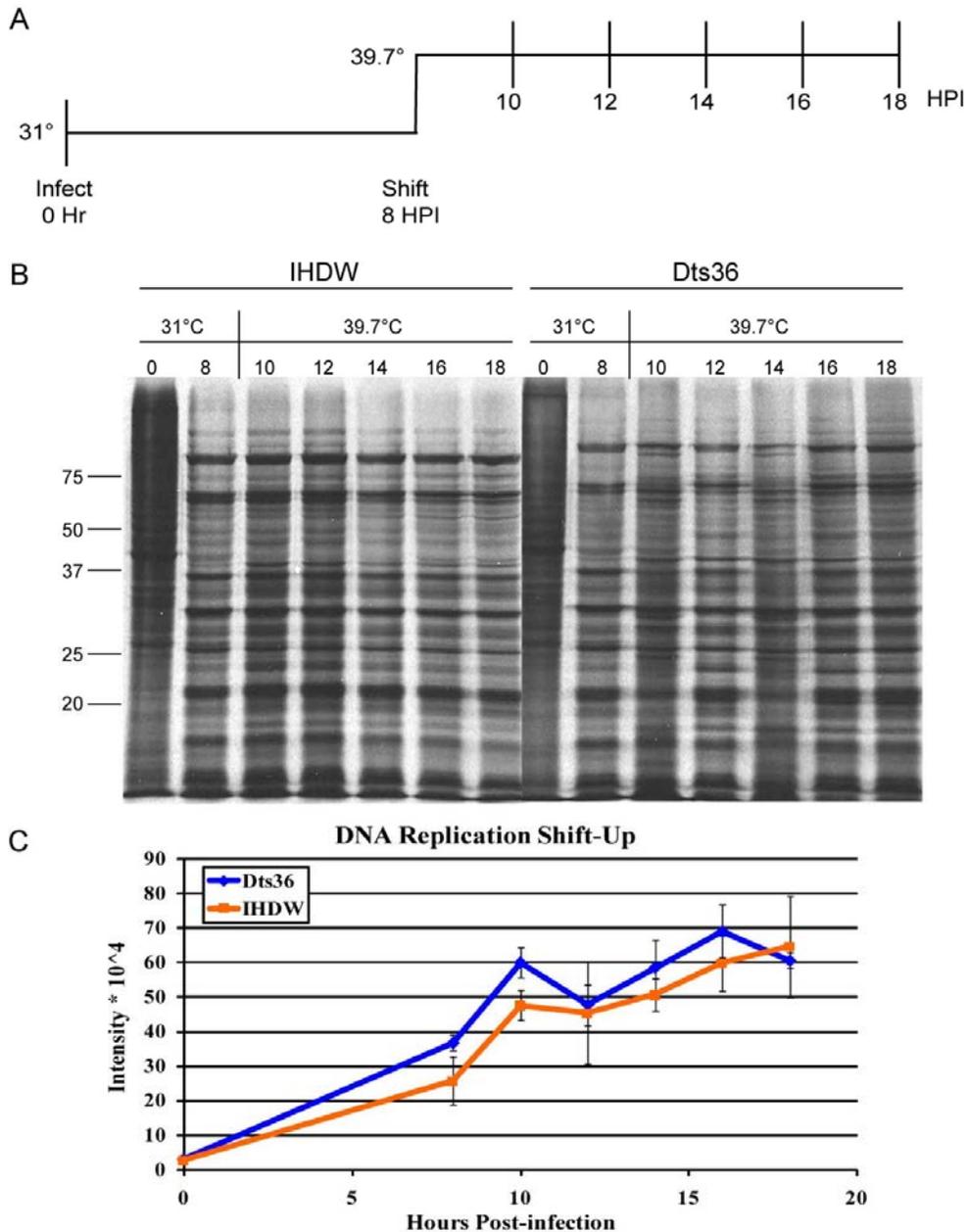


Figure 3-12. Analysis of protein synthesis and DNA replication in Dts36 and IHDW-infected cells during a shift-up experiment. A) Schematic of the shift-up experiment in which cells were infected and incubated at 31°C for 8 hours and then shifted to 39.7°C. B) Autoradiogram of protein synthesis in which the virus with which the cells were infected, the temperature at which the infected cells were incubated, and the hour post-infection at which the samples were isolated are listed above. The molecular weights in kDa are labeled to the left of the autoradiogram. B) Synthesis of viral DNA in cells infected with Dts36 or IHDW. Samples were analyzed in triplicate and the resulting plot of the quantified data represents an average of these values. Error bars represent the standard deviations based on the data.

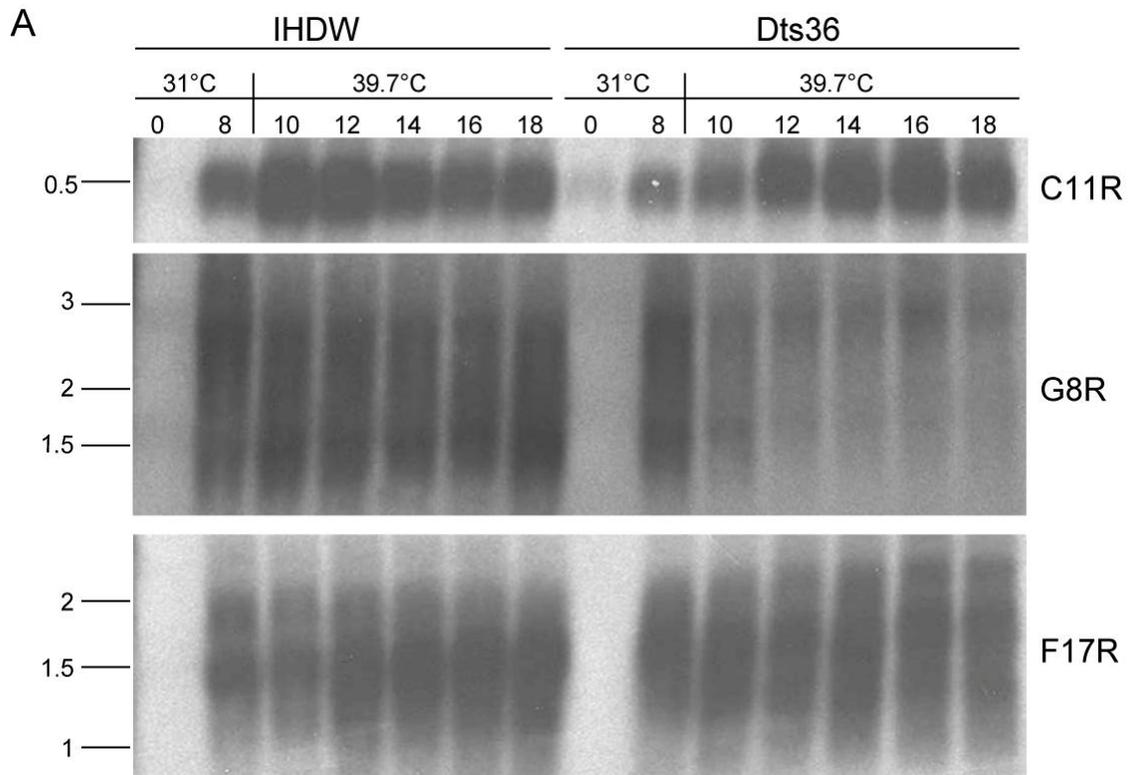
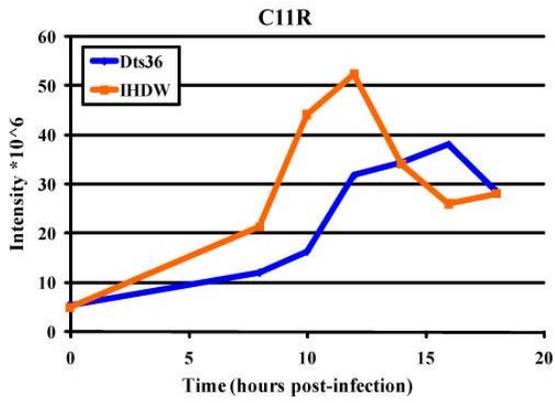
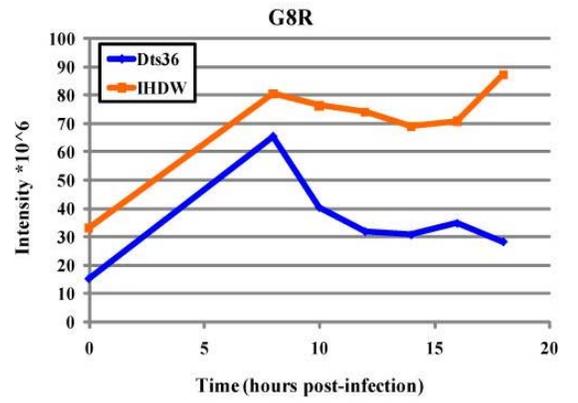


Figure 3-13. Analysis of viral RNA synthesis in Dts36 and IHDW-infected cells during a shift-up experiment. A) Autoradiograms of northern blot analysis of total RNA isolated from cells infected with Dts36 or IHDW. Riboprobes used in this analysis were specific for C11R, G8R, and F17R viral transcripts. The virus with which the cells were infected, the temperature at which the infected cells were incubated, and the hour post-infection at which the RNA was isolated are listed above the autoradiograms. The riboprobe which was used for each northern is listed to the right and the approximate molecular weights in kb is listed to the left of the autoradiograms. B-D) Quantification of the viral mRNA synthesis by phosphorimager analysis. B) C11R, C) G8R, and D) F17R.

B



C



D

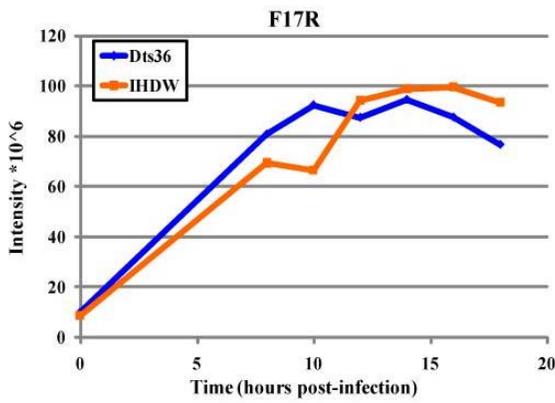


Figure 3-13. Continued

CHAPTER 4  
BIOCHEMICAL ANALYSIS OF A TEMPERATURE SENSITIVE MUTANT IN THE  
LARGE SUBUNIT OF THE VACCINIA VIRUS MRNA CAPPING ENZYME

**4.1 Introduction**

As discussed in Chapter 3, the temperature sensitive virus Dts36 has an early phenotype and is defective in both early transcription termination and intermediate transcription. Based on the phenotypic analysis, it was possible that Dts36 had a defect in one of the three enzymatic activities that are required for the formation of a cap structure on the nascent mRNA transcript. The decreased message stability observed in the phenotypic characterization (Figs. 3-5 – 3-9) is consistent with a defect in the guanylyltransferase activity and the decreased translation efficiency of some viral proteins (Fig. 3-10) is consistent with a defect in the methyltransferase activity. In this chapter, the mRNA guanylyltransferase and the mRNA (guanine-N7)-methyltransferase activities of Dts36 will be explored through biochemical assays *in vitro*. Characterization of Dts36 shows that the mutant capping enzyme has a slight defect in guanylyltransferase activity as measured by the formation of the enzyme-GMP complex and that the mutant capping enzyme is completely defective in methyltransferase as measured by two separate assays. The results presented in this chapter show that the Dts36 mRNA capping enzyme is not able to properly cap newly synthesized viral transcripts.

**4.2 Results**

**4.2.1 Analysis of D1 Protein in Virions and Virion Extracts**

After purification of the IHDW and Dts36 viruses, it was of great interest to determine the protein composition of the purified virions as well as the amount of D1 and D12 proteins present in the purified virions (Fig. 4-1). Analysis of a coomassie-stained SDS-polyacrylamide gel determined that the protein composition of the purified virions as well as the amount of protein present in the purified virions were indistinguishable between IHDW and Dts36 virions (Fig. 4-

1A). The relative amount of D1 and D12 proteins, as compared to the amount of F17, present in the purified virions was determined by western blot analysis (Fig. 4-1B) and quantified using Image J (Fig. 4-1C). Purified virions from both viruses were shown to synthesize similar amounts of both viral capping enzyme subunits and the core protein, F17. The amount of D1 and D12 proteins present in the extracts of IHDW or Dts36 virions was also determined by western blot analysis (Fig 4-2) and it was revealed that equivalent amounts of extracts contained equivalent amounts of both subunits of the viral capping enzyme.

#### **4.2.2 Analysis of mRNA Capping Enzyme Activities in Permeabilized Virions**

The purified vaccinia virus virions can be treated with NP-40, a non-ionic detergent, and DTT, a reducing agent, which will permeabilize the virus by removing the outer membrane but the virus core is left intact. The resulting permeabilized virions are functionally active and when incubated with nucleotide triphosphates, the permeabilized virions are capable of all stages of early viral transcription including modification of the nascent transcripts. In this study, permeabilized virions were used to assess two of the enzymatic functions of the D1 protein, guanylyltransferase and methyltransferase activities, as well as a preliminary assessment on the termination function of the vaccinia virus mRNA capping enzyme.

##### **4.2.2.1 *In vitro* transcription in permeabilized virions**

In this study, preliminary *in vitro* transcription experiments were performed to determine the thermal-inactivation profile of the wild type and mutant virions. From these preliminary experiments, it was determined that the wild type and mutant virions were producing similar amounts of transcripts at all temperatures analyzed (31°, 37°, and 42°C); however, the amount of transcripts synthesized at 42°C by either wild type or mutant virions plateaued very quickly and therefore, this temperature was not used in further experiments. The initial *in vitro* transcription reactions were used as controls for the coupled methyltransferase assay that will be discussed in

further detail subsequently (in section 4.2.2.3). Additional *in vitro* transcription experiments were performed to determine if the RNA that was being transcribed in the permeabilized virions was being released or if it was being retained in the viral core due to a lack of termination (Shuman and Moss, 1989). *In vitro* transcription reactions were incubated at 31° or 37°C for various amounts of time, core and released fractions were separated by centrifugation, and incorporation of radiolabeled CMP was measured by TCA precipitation (Fig.4-3). Both the wild type and the mutant virions showed the same pattern of transcription at all temperatures assayed; the synthesized viral transcripts were released from the core as expected if termination is occurring.

#### **4.2.2.2 Enzyme-GMP complex formation**

In permeabilized virions, guanylyltransferase activity can be assessed through the use of an enzyme-GMP complex formation protocol. The guanylyltransferase reaction in the mRNA cap formation proceeds in two steps; the first of which involves the formation of a covalent capping enzyme-GMP intermediate. It is the formation of the covalent capping enzyme-GMP intermediate which is used as a measure of guanylyltransferase activity in permeabilized virions. The successful formation of the D1-GMP complex implies that the second step of the guanylyltransferase reaction, the transfer of the guanylylate residue to the beta phosphate of the 5' end of the nascent RNA transcript, has occurred allowing the formation of a suitable substrate for the subsequent methyltransferase assay that will be discussed in section 4.2.2.3. Varying amounts of IHDW and Dts36 permeabilized virions were incubated with [ $\alpha$ -<sup>32</sup>P]-GTP at 31°C and the radiolabeled D1-GMP complexes were analyzed by SDS-PAGE, autoradiography and phosphorimager analysis (Fig. 4-4). The autoradiographic analysis, as well as the phosphorimager analysis in which the amount of enzyme-GMP complex formation was compared to the amount of D1 protein present in the purified virions as determined by western

blot (Fig. 4-1), showed that there was a slight difference in the formation of the D1-GMP complex between the wild type and mutant virions, with the Dts36 D1 protein only expressing approximately 80% of the activity of the wild type protein.

#### **4.2.2.3 Analysis of (guanine-N7)-methyltransferase activity**

The (guanine-N7)-methyltransferase activity of the vaccinia virus mRNA capping enzyme can be measured in one of two ways: by a coupled transcription, methyltransferase assay or by an uncoupled methyltransferase assay. The coupled methyltransferase assay uses newly synthesized and guanylated transcripts from the virion cores as the substrate for the (guanine-N7)-methyltransferase reaction (Gershowitz and Moss, 1979). The wild type or Dts36 purified virions were permeabilized with NP-40 and DTT and either analyzed for methyltransferase activity or *in vitro* transcription (Fig. 4-5). The methyltransferase activity was assayed by following the incorporation of a radiolabeled methyl group from SAM into acid insoluble material and the transcription activity was assayed by following the incorporation of radiolabeled CMP into nascent transcripts. The transcription activities for both the wild type and mutant virions at both temperatures examined, 31° and 37°C, were virtually indistinguishable from one another. This results shows that virions from both viruses were able to synthesize substrates that were suitable for the methyltransferase reactions that were being examined in parallel reactions. In stark contrast to the transcription results, the mutant virions have no methyltransferase activity at either temperature tested whereas the wild type virions have comparable amounts of methyltransferase activity at both temperatures.

The second assay that is used to examine the (guanine-N7)-methyltransferase activity of the viral mRNA capping enzyme is an uncoupled assay. In this assay, instead of using a newly synthesized transcript as the substrate for the methylation reaction, a GTP residue is used as the methyl acceptor which allows for the direct measurement of the (guanine-N7)-methyltransferase

activity (Fig. 4-6). Much like the coupled assay, the methyltransferase activity was determined by following the incorporation of radiolabeled methyl group from SAM to a GTP molecule (Fig. 4-7). In agreement with the results from the coupled assay, mutant virions had no appreciable (guanine-N7)-methyltransferase activity compared to the wild type virions. The results from the two methyltransferase assays clearly show that the capping enzyme found in the Dts36 virions is defective in the (guanine-N7)-methyltransferase activity which is also consistent with data from the phenotypic characterization of Dts36.

### **4.2.3 Analysis of mRNA Capping Enzyme Activities in Virion Extracts**

Permeabilized virions can be further solubilized upon treatment with deoxycholate, an ionic detergent. The resulting soluble extracts are less stable than permeabilized virions but still contain the functional viral proteins involved in the transcription of early genes including both subunits of the mRNA capping enzyme (reviewed in (Condit and Niles, 2002)). In the study presented in this chapter, soluble virion extracts were used to further assess the mRNA guanylyltransferase and the mRNA (guanine-N7)-methyltransferase activities of the mutant capping enzyme.

#### **4.2.3.1 Non-specific RNA polymerase activity**

The solubilization of the enzymes from the wild type and mutant virions was determined to be equivalent by testing the RNA polymerase activity of the extracts in a non-specific RNA polymerase assay. Equivalent amounts of soluble extracts were tested for incorporation of [ $\alpha$ - $^{32}$ P]-CTP into RNA transcripts programmed with single stranded DNA in the presence of manganese (Fig. 4-8). The non-specific RNA polymerase activity was found to be equivalent in the wild type and mutant extracts over a range on extract concentrations as well as at different temperature. These data, as well as the western blots analysis of the capping enzyme proteins (Fig. 4-2), confirm that the protein composition of the wild type and mutant extracts was similar,

and that viral proteins not affected by the mutation in Dts36 appear to function normally in the soluble extracts.

#### **4.2.3.2 Enzyme-GMP complex formation**

The formation of D1-GMP complexes were analyzed in soluble virion extracts using the same assay as described for the permeabilized virions. Autoradiographs and phosphorimager analysis showed that when equivalent amounts of IHDW or Dts36 soluble extracts were tested for formation of D1-GMP complexes, the D1 protein from Dts36 possesses approximately 60% of the guanylyltransferase activity of the D1 protein from IHDW (Fig. 4-9). This is a moderate decrease in the formation of the D1-GMP complex in the mutant and is a more pronounced defect than what was seen in the permeabilized virions.

#### **4.2.3.3 Analysis of methyltransferase activity**

The IHDW and Dts36 soluble extracts were tested for methyltransferase activity using the uncoupled assay which measures the transfer of a radiolabeled methyl group to a GTP acceptor (Fig. 4-6). Both wild type and mutant extracts were examined for methyltransferase activity over a range of extract concentrations at 31°C and 37°C (Fig. 4-10). The Dts36 extracts had no measurable methyltransferase activity above the negative controls. These results were consistent with the results seen when permeabilized virions were used in the assay.

### **4.3 Summary**

In this chapter, the biochemical characterization of a temperature sensitive mutant in the large subunit of the vaccinia virus mRNA capping enzyme is presented. The detailed analysis of the D1 protein from Dts36 showed that this protein is defective in several of the enzymatic activities involved in the formation of the mRNA cap structure. The mutant D1 protein has reduced guanylyltransferase activity in both permeabilized virions and virion extracts as measured by the formation of the covalent enzyme-GMP intermediate as well as no measurable

methyltransferase activity in either permeablized virions or virion extracts. As was the case in the phenotypic study, the cap structure *in vitro* has not yet been directly determined. However, based on the results presented in this chapter, the mutant D1 protein is defective in the (guanine-N7)-methyltransferase activity which would result in the formation of a guanylated but unmethylated cap structure. These results confirm the results from the phenotypic analysis of Dts36 (chapter 3) which pointed to a defect in the mRNA capping of viral transcripts as well as the possibility that the capping defect may be a result of inefficient subunit association in the vaccinia virus mRNA capping enzyme which will be discussed in more detail in chapter 5.

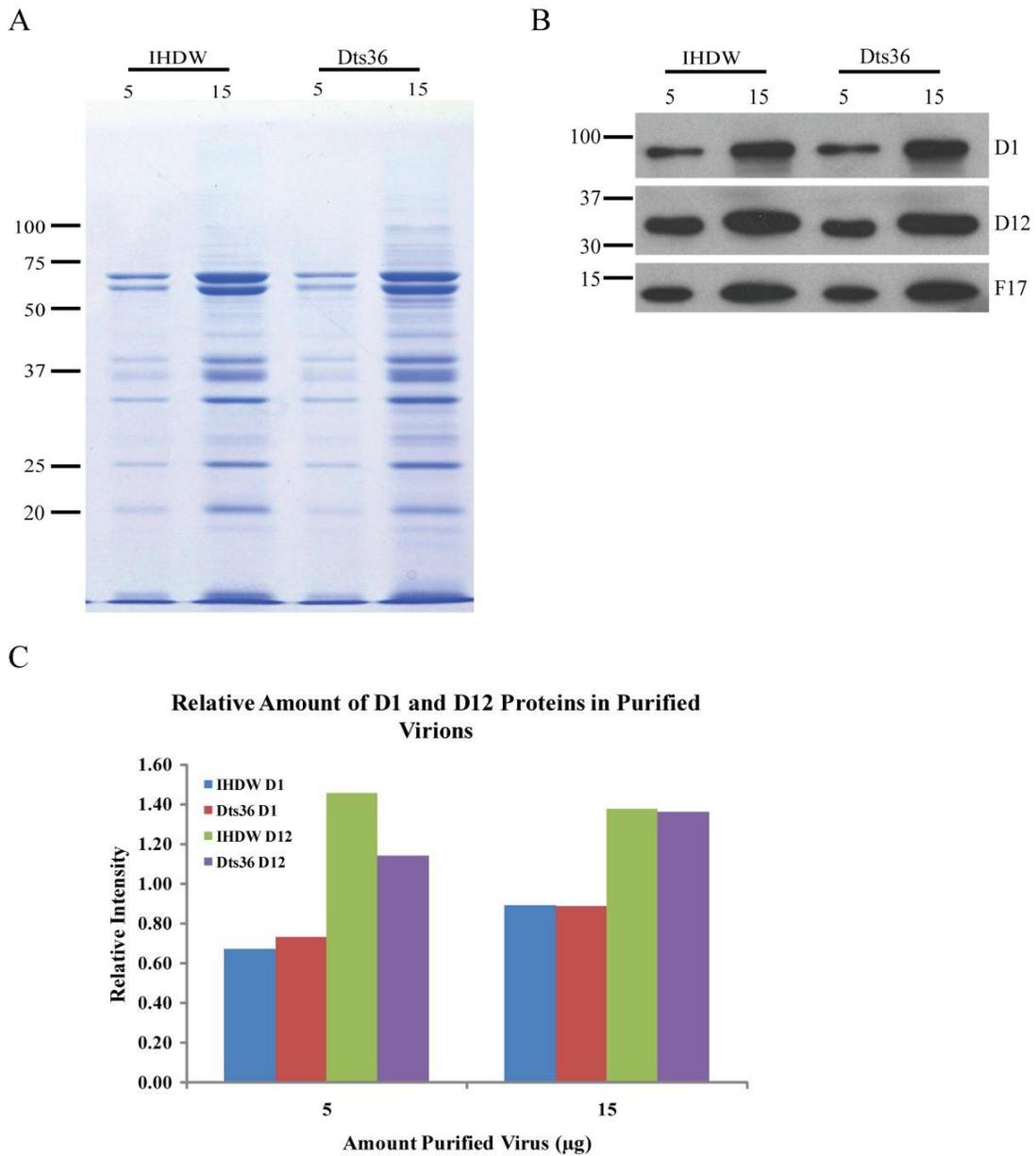


Figure 4-1. SDS-PAGE and western blot analysis of purified IHDW and Dts36 virions. A) The protein composition of purified virions was analyzed by electrophoresis through an 11% gel which was subsequently stained with coomassie blue. The amount of protein in µg that was loaded is labeled above each lane as is the identity of the purified virion. B) Purified virions were analyzed and blots were prepared and probed. The source of the purified virions and the amount of protein in µg are listed above the autoradiograms. The primary antibody used to probe each membrane is listed to the right of the autoradiograms and the molecular weights in kDa are listed to the left. C) Quantification of the western blots by Image J analysis comparing the amount of either capping enzyme subunit to amount of F17 protein in purified virus.

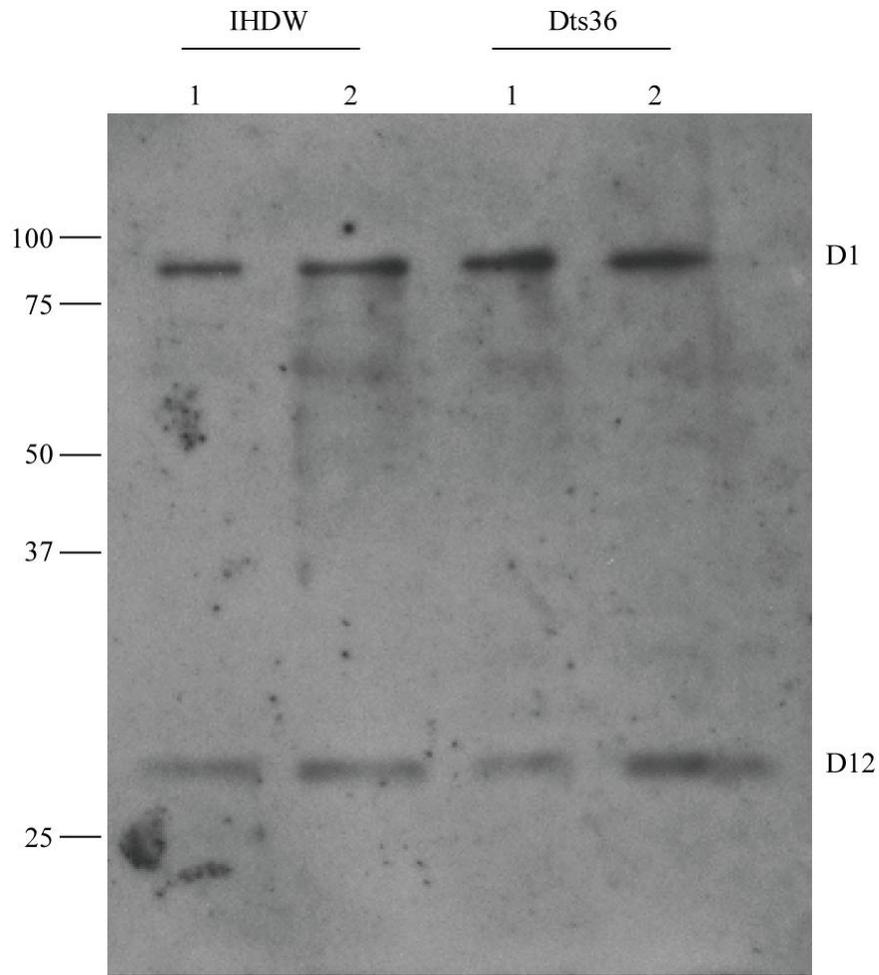


Figure 4-2. Western blot analysis of IHDW and Dts36 virion extracts. Virion extracts were electrophoresed and blots were prepared and probed. The identity of the virion extracts and the amount of protein analyzed in  $\mu\text{g}$  are listed above the autoradiograms. The membrane was probed first with the D1 antibody and then probed with the D12 antibody. The identity of the D1 and D12 proteins are shown to the right of the autoradiogram and the molecular weight in kDa are listed to the left of the autoradiograms.

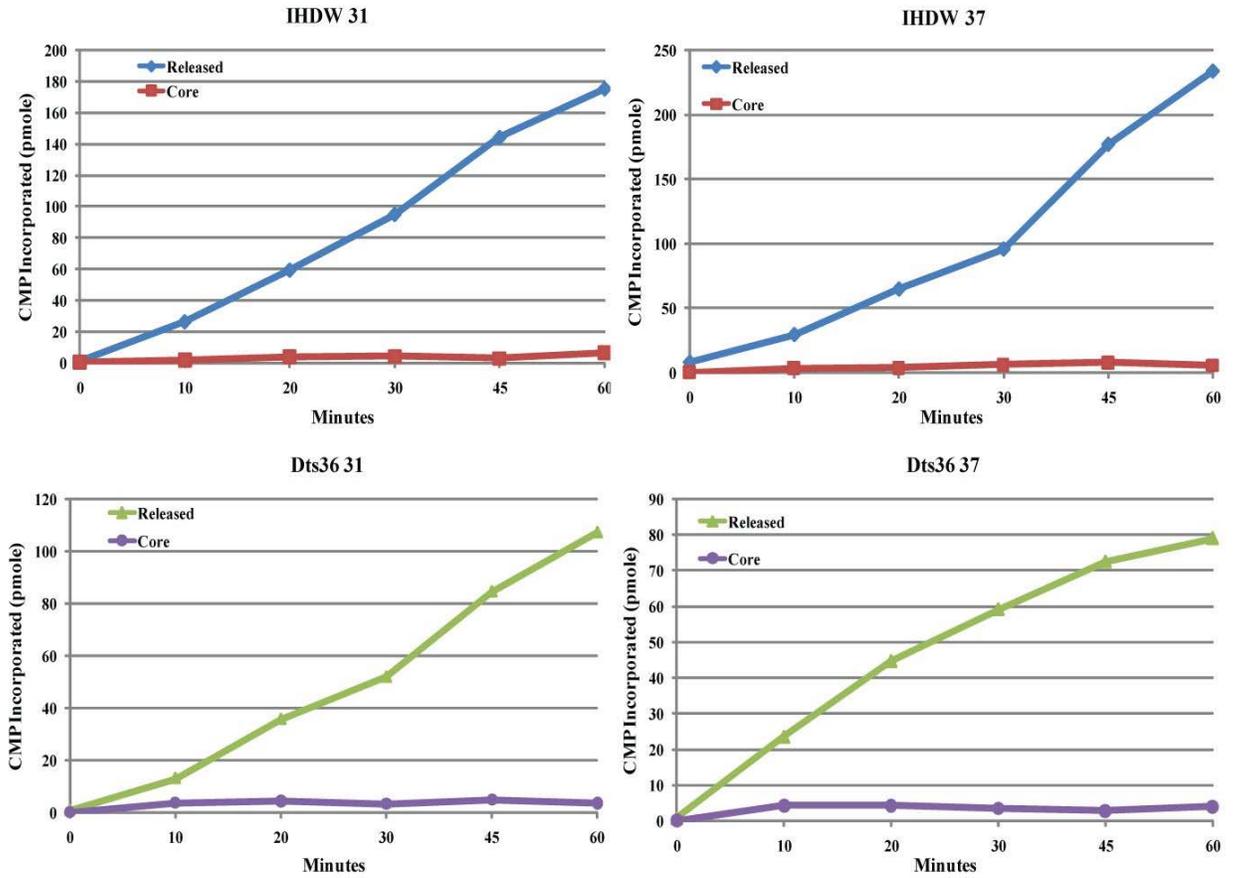


Figure 4-3. *In vitro* transcription in permeabilized IHDW and Dts36 virions. The incorporation of  $[\alpha^{32}\text{P}]\text{-CMP}$  into viral transcripts was measured for 60 minutes at either 31°C or 37°C. At the times indicated, the transcription reactions were separated into core and released fractions and the acid precipitable radioactivity was measured.

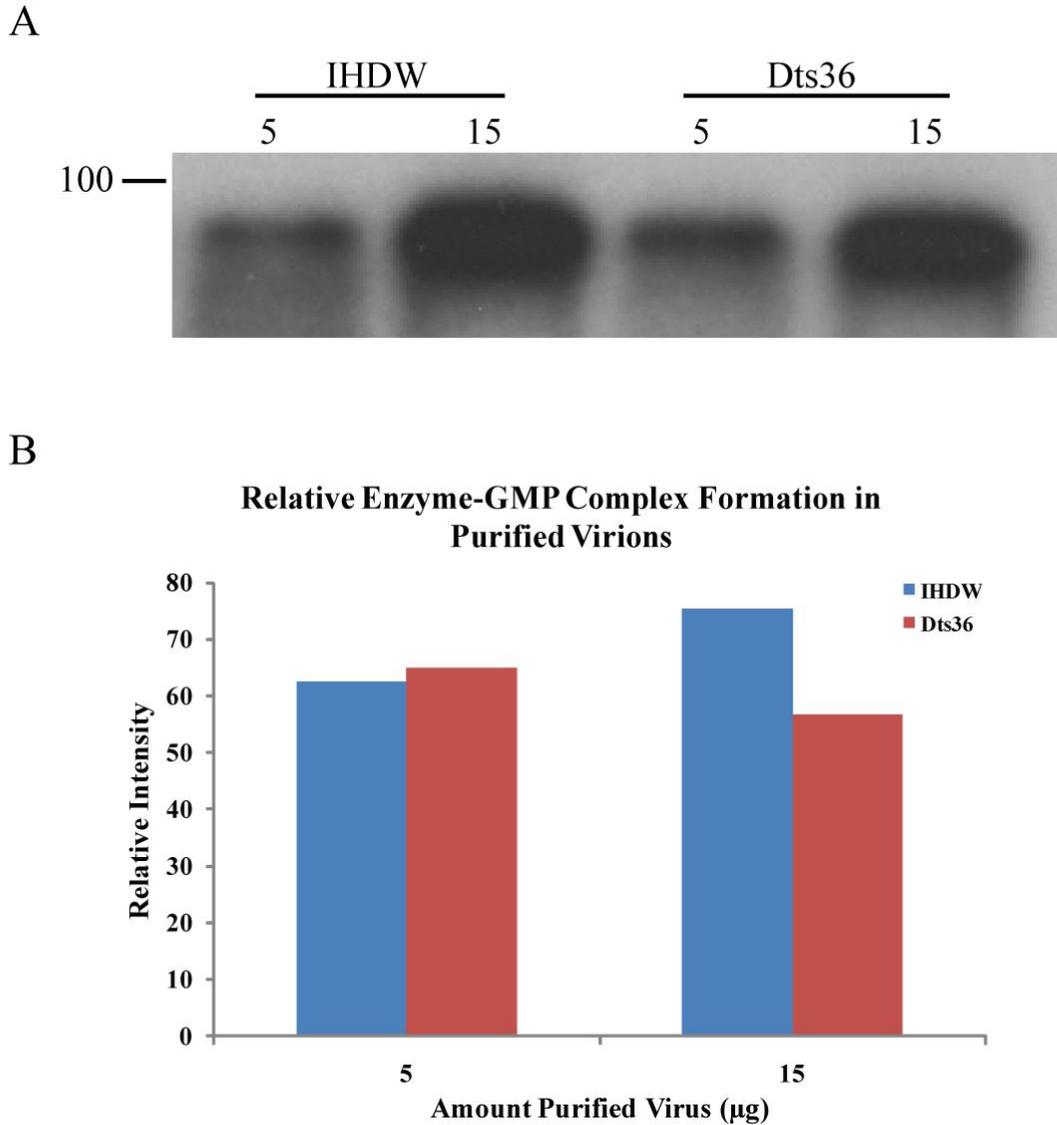
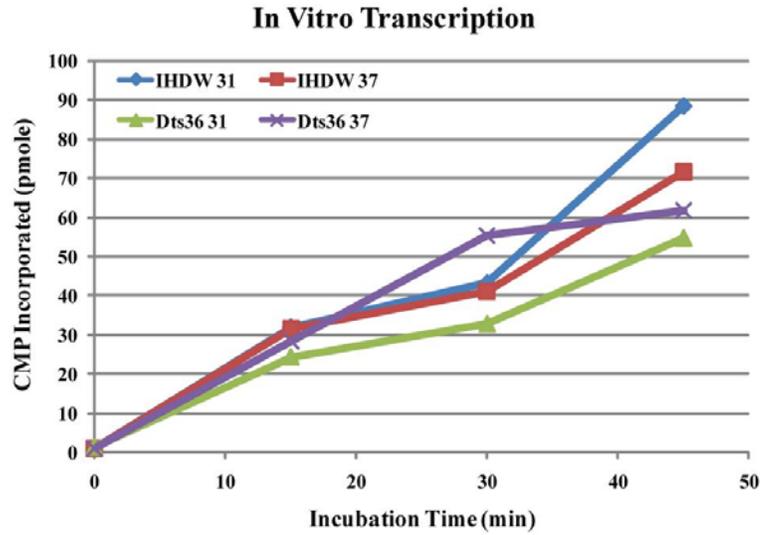


Figure 4-4. Enzyme guanylylate complex formation in purified virions. A) Varying amounts of purified virions were incubated with [ $\alpha^{32}\text{P}$ ]-GTP and analyzed by SDS-PAGE. The autoradiogram shows the radiolabeled D1 protein. The source of purified virions and the amount of protein in  $\mu\text{g}$  analyzed are both listed above the autoradiogram. The molecular weights in kDa are listed to the left of the autoradiogram. B) The relative amount of enzyme-GMP complex formation in purified virions as determined by comparing the amount of complex formation as determined by phosphorimager analysis to the amount of D1 protein in the purified virions as determined by western blot analysis.

A



B

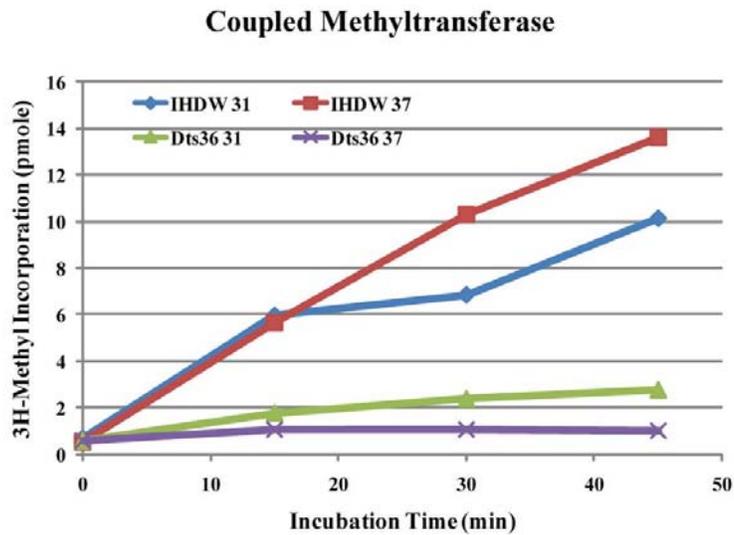


Figure 4-5. Coupled transcription and methyltransferase assay in permeabilized IHDW and Dts36 virions. Purified virions were permeabilized by treatment with NP-40 and DTT and split in two; one sample was assayed for *in vitro* transcription (A) and the other was assayed for methyltransferase activity (B). A) Incorporation of [ $\alpha^{32}\text{P}$ ]-CMP into viral transcripts was measured by TCA precipitation. B) The transfer of a radiolabeled methyl group to *in vitro* synthesized and guanylated transcripts was measured.

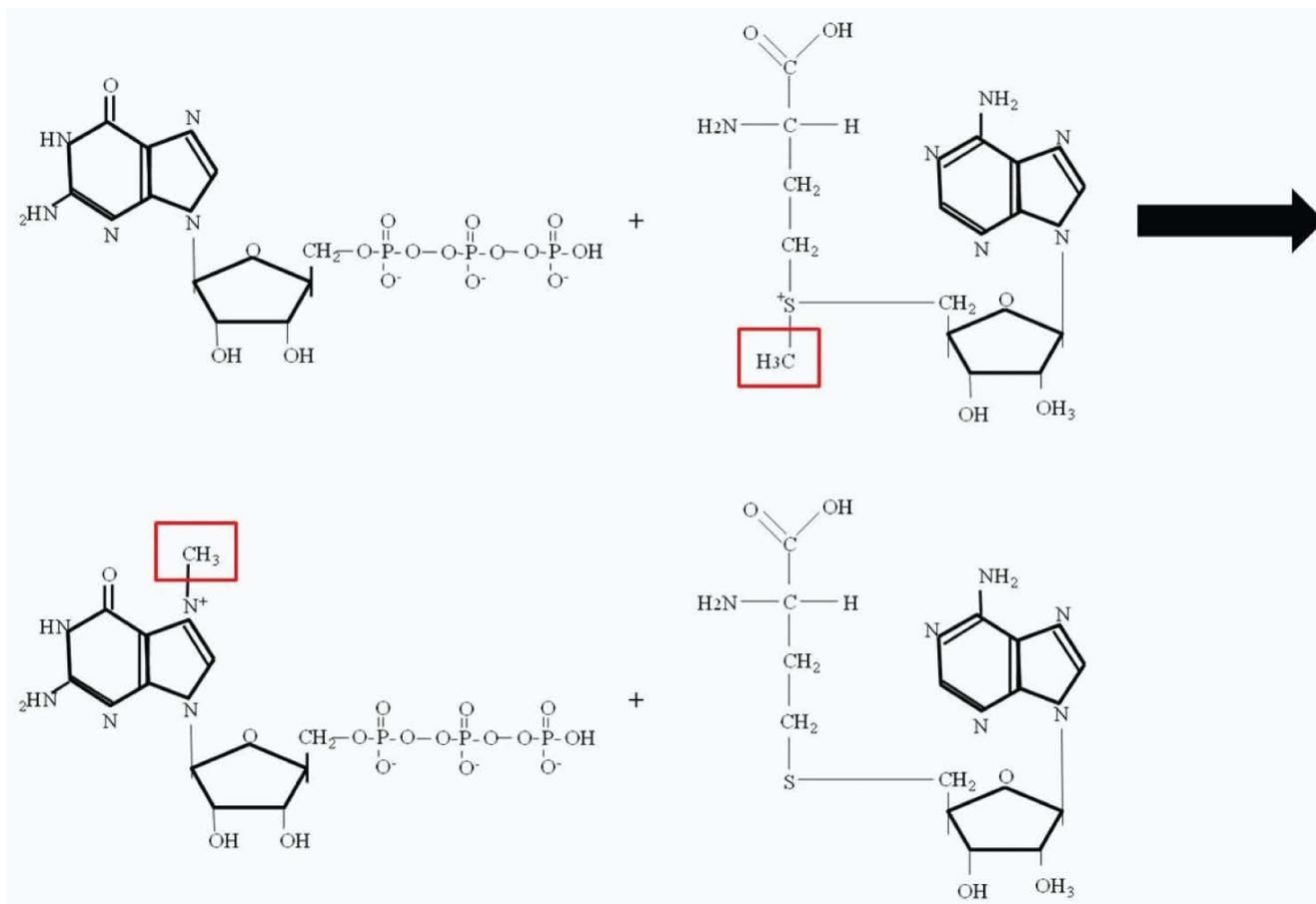


Figure 4-6. Uncoupled methyltransferase assay schematic. In the presence of an enzyme that possesses methyltransferase activity, a guanylylate residue (top left) and [<sup>3</sup>H]-S-adenosylmethionine (top right) interact and the radiolabeled methyl group from the SAM (shown in red box) is transferred to the guanylylate residue. The products of this reaction are a guanylylate residue possessing a tritium labeled methyl group at the N7 position (bottom left) and S-adenosylhomocysteine (bottom right).

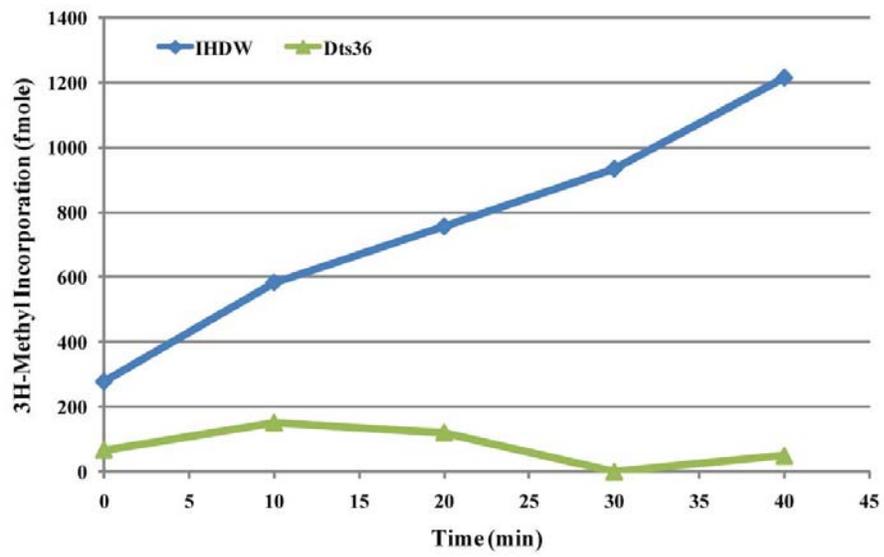


Figure 4-7. Uncoupled methyltransferase assay in IHDW and Dts36 permeabilized virions. The transfer of a radiolabeled methyl group from SAM to a GTP molecule was measured in permeabilized virions.

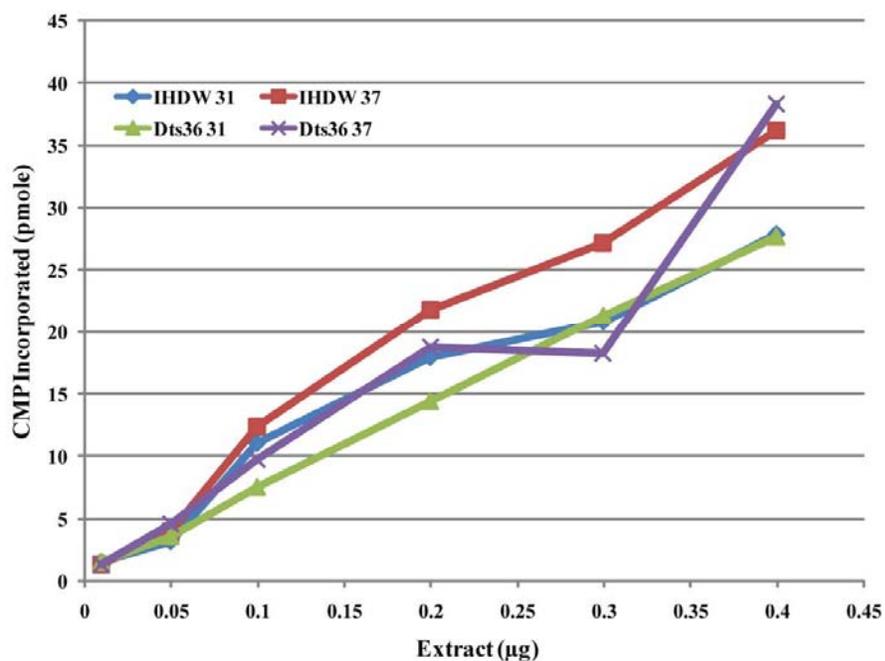


Figure 4-8. Non-specific RNA polymerase activity in IHDW and Dts36 virion extracts. Varying amounts of wild type and mutant virion extracts were assayed for the incorporation of [ $\alpha^{32}\text{P}$ ]-CMP into acid-insoluble material in the presence of  $\text{Mn}^{2+}$  and using single-stranded DNA as a template.

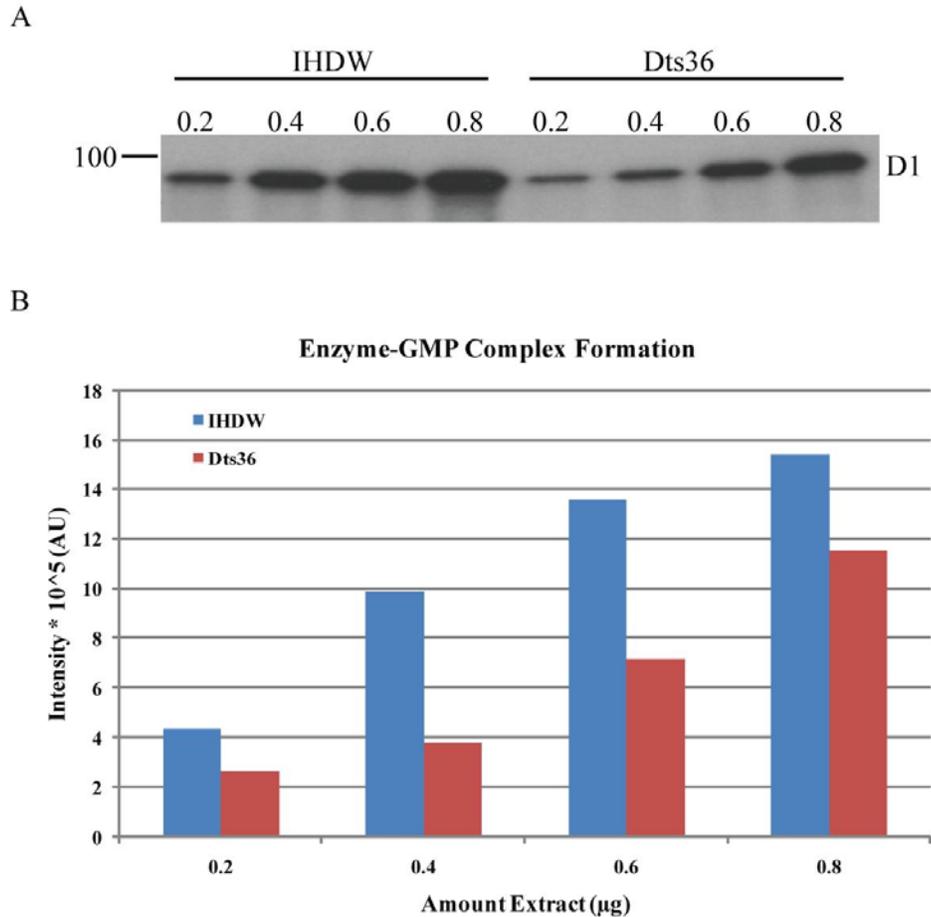


Figure 4-9. Enzyme-guanylate complex formation in IHDW and Dts36 virion extracts. A) Varying amounts of virion extracts were incubated with [ $\alpha^{32}\text{P}$ ]-GTP and analyzed by SDS-PAGE. The autoradiogram shows the radiolabeled D1 protein. The source of virion extracts and the amount of protein in  $\mu\text{g}$  analyzed in the reaction are both listed above the autoradiogram. The molecular weights in kDa are listed to the left of the autoradiogram and the location of the D1 protein is labeled to the right of the autoradiogram. B) Quantification of enzyme-GMP complex formation in virion extracts by phosphorimager analysis.

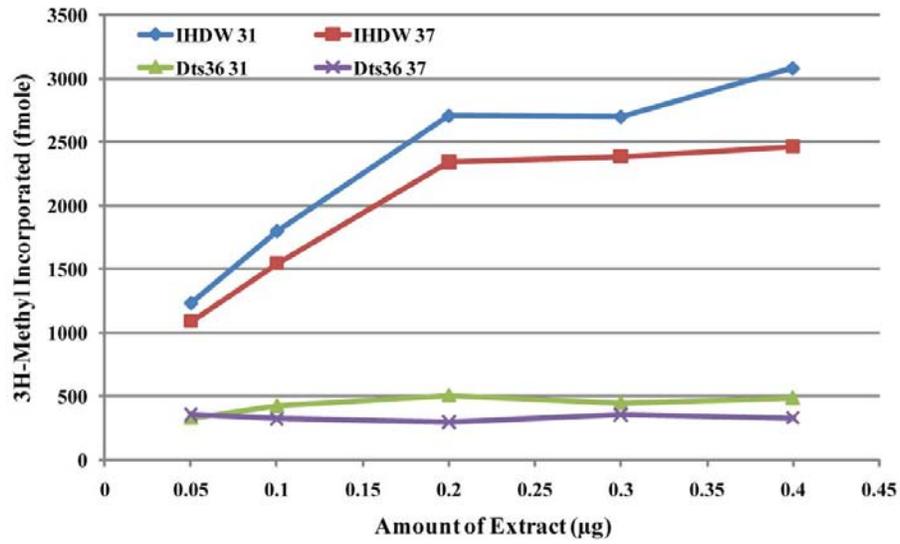


Figure 4-10. Uncoupled methyltransferase assay in IHDW and Dts36 virion extracts. The transfer of a radiolabeled methyl group from SAM to a GTP molecule was measured in wild type and mutant virion extracts.

## CHAPTER 5 DISCUSSION

### 5.1 Introduction

The role of the vaccinia virus mRNA capping enzyme during viral infection has been analyzed both phenotypically and biochemically using a temperature sensitive mutant that encodes a mutated D1 protein (chapters 3 and 4). The *in vivo* analysis showed that in Dts36 infections early gene transcription termination is compromised, early genes are expressed at late times during infection, DNA replication is absent, and intermediate gene transcription is compromised. These findings will be discussed further in Sections 5.3-5.6. The *in vivo* analyses also showed that *in vivo* the mutant possessed defects that were consistent with a defect in mRNA capping; the observed instability of the viral mRNA transcripts could signify a defect in the guanylyltransferase activity of the capping enzyme and the decreased translation efficiency of some viral proteins could signify a defect in the methyltransferase activity of the capping enzyme. The biochemical analysis of the D1 protein encoded by Dts36 showed that the mutant protein was defective in the methyltransferase activity of the viral capping *in vitro*. Together these results point to a specific defect in mRNA methyltransferase activity of the mutant protein; however, it is not known if this defect is due to a change in the enzyme active site or if the mutant D1 protein is unable to efficiently interact with the small subunit of the viral capping enzyme. Both of these possibilities will be discussed further in Sections 5.7 and 5.8.

### 5.2 Temperature Sensitivity

A temperature sensitive virus is a virus that can grow at a permissive temperature but is not able to grow at a non-permissive temperature. In the study presented in Chapter 3, Dts36 was determined to be temperature sensitive *in vivo* by a one-step growth experiment (Fig. 3-3) and these results were confirmed with the remainder of the phenotypic characterization which

showed that Dts36 possessed transcription defects at the non-permissive temperature. An intriguing development in the biochemical characterization of the mutant virus (Chapter 4) showed that the mutant virus is not temperature sensitive *in vitro* but instead shows defects in the enzymatic activities at all temperatures examined in the course of this study. This is not an unprecedented phenomenon; in a study examining a another temperature sensitive mutant in the large subunit of the vaccinia virus mRNA capping enzyme, the virus was shown to be ts *in vivo* but possessed defects in the enzymatic activities at low temperatures as well as higher temperatures *in vitro* (Hassett et al., 1997). The differences in the temperature sensitivities can most likely be explained by a decrease in the stability of the samples. The virus particle that is analyzed in the phenotypic studies is the most stable form of the virus. This is most likely due to the stabilizing environment of the infected cell. As the virus becomes more and more soluble, such as in permeablized virions followed by the soluble virus extracts, the stabilization that was present in the environment of the infected cell is lost which may lead to the phenomenon of the defects being observed at lower temperatures.

### **5.3 Early Viral Gene Transcription Termination**

Early gene transcription termination is compromised in Dts36 mutant infections. This represents the first *in vivo* evidence supporting a role for the mRNA capping enzyme in early gene transcription termination, a phenomenon well established *in vitro* (Condit and Niles, 2002). The data presented in Chapter 3 reveals that termination occurs with varying efficiency at some but not all predicted downstream termination signals in Dts36 mutant infections. These observations are consistent with *in vitro* experiments which demonstrate that early viral transcription termination signals are only 75-80% efficient (Earl et al., 1990; Yuen and Moss, 1987). Interestingly the C11R transcript appeared to be terminated properly in Dts36 mutant infections. Analysis of the termination signals of this transcript showed that there are two

additional termination signals present within twelve nucleotides of the first termination signal (Fig. 3-6B). This is consistent with previous *in vitro* studies which predicted that multiple termination signals within a small region enhance termination (Rohrmann et al., 1986; Yuen and Moss, 1986). There has been no direct *in vitro* analysis of the early termination function encoded by the mutant D1 protein. However, based on the phenotypic characterization of Dts36, in which termination occurs but seems to be less efficient at certain termination sites, it is believed that the *in vitro* transcription experiments described in Chapter 4 are showing that termination is occurring but these experiments are not able to show if the synthesized transcripts are properly terminated. Further experimental evidence is required to determine if the *in vitro* synthesized viral transcripts are longer than expected which would show that the mutant D1 protein is also less efficient at early transcription termination *in vitro*.

#### **5.4 The Reactivation of Early Viral Messages Late during Viral Infection**

The reappearance of early transcripts late during viral infections with IHDW and Dts36 could be due to genetic differences between the commonly used wild type strain of vaccinia virus, WR, and the wild type used in this study, IHDW. In vaccinia virus, early transcription factors are encoded by late genes (Gershon and Moss, 1990) and are packaged into newly formed virions late during infection. Although the factors required for early transcription are present late in infection (Wright and Moss, 1989), vaccinia apparently possesses a mechanism that inhibits transcription from early promoters late during infection. It has been hypothesized that this repression of transcription results either from formation of DNA superhelical structures in newly formed virions or association of the viral genome with DNA-binding proteins (Masternak and Wittek, 1996). However, previous studies have shown that a small subset of early promoters, including the vaccinia virus 7.5k promoter, are reactivated at times late during infection with the WR strain of virus (Garces et al., 1993). In the study presented here, both IHDW and permissive

Dts36 infections were shown to express early mRNA transcripts at times late in infection for all transcripts analyzed. Numerous IHDW genes have been sequenced and no significant differences have been found in the promoter regions relative to the WR strain. Therefore, one possibility is that the repression mechanism that is present in infections with the WR strain of vaccinia is not present in the IHDW strain of vaccinia virus.

Another possible cause of the reactivation of early transcripts late during infections with IHDW and Dts36 is superinfection. Superinfection occurs when virions are able to enter previously infected cells. There are several examples of viruses encoding factors that prevent superinfection; these include the neuraminidase of the influenza virus (Palese et al., 1974), the G protein of VSV (Whitaker-Dowling et al., 1983), and glycoprotein D of herpes simplex virus (Johnson and Spear, 1989). Prevention of superinfection is also present in vaccinia virus based on experiments using the WR wild type vaccinia virus strain that showed that although a superinfecting virus could enter a previously infected cell, the superinfecting virus was incapable of early gene transcription (Christen et al., 1990). This is indicative of a block somewhere between virus adsorption and early transcription and recent advances in experimental technology has allowed the determination of the process that is blocked during superinfection as discussed further below. Although this initial study did not address the mechanism of prevention of superinfection in vaccinia virus, other studies with vaccinia virus have implicated the non-essential, virally-encoded hemagglutinin (HA) in the inhibition of fusion and superinfection (Ichihashi and Dales, 1971; Seki et al., 1990). Recent work using cowpox virus has shown that SPI-3, a viral serine proteinase inhibitor, and HA colocalize on the surface of infected cells and prevent superinfection (Brum et al., 2003; Turner and Moyer, 2006). A recently published study has shown that HA and SPI-3 also interact on the surface of vaccinia virus infected cells and that

the resulting complex reduces superinfection by slowing the entry-fusion process (Turner and Moyer, 2008). The wild type vaccinia virus strain used in this study, IHDW, has been shown previously to be HA-negative and to show extensive cellular fusion as early as 12 hpi (Ichihashi and Dales, 1971). Therefore, it is more conceivable that the early transcripts seen late during infections with IHDW and Dts36 are the result of superinfection as opposed to the reactivation of early genes late during infection.

### **5.5 DNA Negativity of Dts36**

We believe that the defect in DNA replication observed during Dts36 mutant infections results from the observed generalized decrease in early viral protein synthesis and does not reflect a direct role for the mRNA capping enzyme in DNA replication. As discussed above, western analysis of several early viral proteins involved in DNA replication, A20, D5 and E9, showed that these proteins were present in decreased amounts in Dts36 infected cells incubated at the non-permissive temperature. A previous study has shown that D5 interacts with A20 by yeast 2-hybrid analysis (McCraith et al., 2000). Additional studies of D5 have shown that D5 is capable of oligomerization and more importantly, that this oligomerization is required for ATPase activity (Boyle et al., 2007). The A20 and E9 proteins have also been shown to interact with each other by co-immunoprecipitation (Klemperer et al., 2001). Additional analysis of the A20/E9 interactions have shown that A20 interacts with D4, the uracil DNA glycosylase, and that this dimeric complex interacts with E9 to form a complex which possesses processive DNA polymerase activity (Stanitsa et al., 2006). The reduced amounts of the A20, D5 and E9 proteins in non-permissive Dts36 infections could prohibit the formation of viable DNA replication complexes which would therefore explain the lack of DNA replication in these infections. Alternatively, it may be that one or another of these or another unidentified early viral protein

required for DNA replication are normally present in critically limiting amounts, such that a moderate decrease in protein synthesis causes a profound defect in DNA replication.

An intriguing alternative explanation for the DNA negative phenotype of Dts36 relates to the early stages of virion uncoating. Previous studies have shown that vaccinia virus uncoating proceeds in two steps; an initial rapid uncoating after which viral DNA is DNase-resistant and a secondary uncoating after which the viral DNA is no longer DNase-resistant. The second stage of uncoating requires early viral protein synthesis and presumably releases the viral DNA from the core, making it available as a template for DNA replication (Joklik, 1964a; Joklik, 1964b; Mallardo et al., 2002). The proteins required for secondary uncoating have not been identified. The observed decrease in early viral protein synthesis could theoretically affect the secondary uncoating of the virus particle in non-permissive infections with Dts36. A defect in secondary uncoating would prevent release of viral DNA from the core and a halt of the virus life cycle at the DNA replication stage.

Although it is not possible to determine which of the two possibilities hypothesized here is the correct explanation for the DNA negative phenotype of Dts36, it has been shown that the capping enzyme is not involved in the process. If the defect in the capping enzyme encoded by Dts36 did cause the defect in DNA replication observed in the non-permissive infections, when the shift-up experiment (Fig. 3-12) was performed, DNA replication in the mutant infections should have terminated. This was not the case; DNA replication in the Dts36 infections continued after the shift to the non-permissive temperature. These results confirm that viral mRNA capping enzyme is not involved in the process of viral DNA replication.

## **5.6 Intermediate Viral Gene Transcription in Dts36**

Biochemical experiments have shown that the vaccinia mRNA capping enzyme is an intermediate gene transcription initiation factor (Vos et al., 1991b). In the shift-up experiment

presented in this study, the steady state levels of the G8R intermediate transcript decreased drastically in Dts36-infected cells after a shift to the non-permissive temperature, whereas the steady state levels of the early and late viral transcripts examined did not show a similar decrease. The decrease in steady state levels of mRNA could theoretically result either from a decrease in mRNA synthesis or an increase in mRNA degradation. While the cap status of postreplicative mRNAs in mutant infections is not known, and while a defect in capping of postreplicative mRNAs could obviously destabilize the RNAs, it is expected that such a destabilization would affect all postreplicative RNAs rather than a specific subclass. Since the decrease observed is specific to the intermediate transcript it is believed that the intermediate gene transcription initiation function of the capping enzyme is defective in Dts36. This represents the first *in vivo* evidence in support of a role for the vaccinia capping enzyme in intermediate gene transcription initiation.

## 5.7 mRNA Capping

In the phenotypic study presented in chapter 2, Dts36 infected cells were found to synthesize decreased amounts of several early viral proteins at the non-permissive temperature as analyzed by western blot. By contrast, no differences were observed between early viral protein synthesis in wild type and mutant infections as assayed by metabolic labeling. This discrepancy is most likely due to the specificity of each experiment: metabolic labeling focuses predominantly on the most abundantly synthesized viral proteins while western analysis examines steady state levels of specific proteins, regardless of abundance. It may be that for the most actively transcribed genes, mRNA is synthesized in amounts in excess of the translation capacity of the infected cell, so that decreases in the steady state levels of mRNA due to defects in the capping of the mRNA transcripts would not affect the levels of synthesis of the cognate proteins, as assayed by pulse labeling. Several of the proteins that we have analyzed by western

blot, notably E9, D5 and A20, may be from genes expressed at relatively lower levels in infected cells. The mRNA for these genes may be more limiting during infection so that decreases in steady state levels of active mRNA could have a larger impact on accumulation of the cognate proteins.

In addition to a decrease in amount of some early viral proteins, some early viral transcripts were also reduced in amount in non-permissive Dts36 infections. As noted above, we have not measured directly the cap status of viral mRNAs in mutant infected cells. However, both the decrease in the amount of early viral RNA and protein are consistent with a defect in the ability of the capping enzyme to cap newly synthesized viral transcripts. The defect in the capping activity could represent a specific defect in either the guanylyltransferase or the methyltransferase activity of the mutant enzyme. A defect in the guanylyltransferase activity of the capping enzyme would result in nascent mRNA transcripts that are not guanylylated and therefore, not capped. It has been shown previously that unblocked, i.e. non-guanylylated, reovirus mRNA is less stable in *X. laevis* oocytes, wheat germ extracts and L cell protein synthesizing extracts (Furuichi et al., 1977). Furthermore it has been demonstrated that a decrease in mRNA levels and protein synthesis is observed when mRNA capping activity is inactivated in a yeast guanylyltransferase mutant (Schwer et al., 1998). A defect in methyltransferase activity could affect mRNA function or stability either directly by affecting translation efficiency or indirectly by affecting cap stability. Previous studies in vaccinia virus, reovirus, and vesicular stomatitis virus have shown that methylation of the guanylylate residue of the cap structure of viral transcripts is critical for translation (Both et al., 1975; Muthukrishnan et al., 1975; Muthukrishnan et al., 1978). Thus a defect in the methyltransferase function of the Dts36 enzyme could result in synthesis of viral mRNA with decreased translation efficiency. A

defect in methyltransferase activity could indirectly affect the guanylated state of the nascent transcript. The guanylyltransferase reaction is reversible in the absence of methylation (Martin and Moss, 1975). Thus a defect in the methyltransferase activity in Dts36 could destabilize the mRNA cap which in turn could lead to degradation of the viral mRNA transcripts and a decrease in viral protein synthesis. During the phenotypic analysis, it had not been possible to distinguish which, if any, of the two enzymatic activities is defective *in vivo* in the mutant capping enzyme encoded by Dts36. However, the favored hypothesis was that the mutant capping enzyme encoded by Dts36 is defective in the methyltransferase activity. This hypothesis was confirmed upon biochemical analysis of Dts36 when the mutant D1 protein was found to be defective in methyltransferase activity. The methyltransferase defect and the role that the mRNA capping enzyme subunit association may play in this defect are discussed further in the following section.

### **5.8 mRNA Capping Enzyme Subunit Association**

In Dts36, the large subunit of the mRNA capping enzyme contains a glycine to aspartic acid substitution at residue 705. G705 is conserved in all poxviruses and cellular cap methyltransferases (Schwer and Shuman, 2006) as well as those from Shope fibroma virus and African swine fever virus (Mao and Shuman, 1994). Recent X-ray crystallographic data has shown that G705 is located in strand B5 of a seven stranded beta sheet that comprises the conserved core of the enzyme (Fig. 5-1) (De la et al., 2007). Thus G705 is not directly involved in the D1-D12 subunit interaction (Fig. 5-2), as was previously suggested based on biochemical data (Schwer and Shuman, 2006). Nevertheless, a triple mutant of the large subunit of the vaccinia virus capping enzyme (G704, G705A, V707A) was shown to have no interaction with the small subunit and no methyltransferase activity when both proteins were synthesized in reticulocyte lysates (Mao and Shuman, 1994). A yeast complementation assay which assesses vaccinia virus methyltransferase activity (Saha et al., 2003) has shown that the G705D mutation

is temperature sensitive in yeast (S. Shuman, personal communication). Based on these studies, it is possible that the G705D mutation found in Dts36 affects the structure of the D1 protein in such a way that the two subunits of the capping enzyme are no longer able to associate efficiently at the non-permissive temperature.

The lack of efficient subunit association in the vaccinia virus capping enzyme could affect the methyltransferase activity as well as the early transcription termination and intermediate transcription initiation functions of the enzyme. Inefficient subunit association would directly affect the methyltransferase activity of the enzyme since the small subunit is required to stimulate the intrinsic methyltransferase activity of the large subunit by causing a predicted conformation change in the methyltransferase active site of the large subunit (Higman et al., 1992; Niles et al., 1994). Both subunits of the capping enzyme have been shown previously to be required for early gene transcription termination (Luo et al., 1995) and intermediate gene transcription initiation (Condit et al., 1996a). However, it is not known if these activities simply require the presence of both subunits or if subunit association is also required. If in fact subunit association is required for both early gene transcription termination and intermediate gene transcription initiation, then a mechanistic explanation that could account for all of the phenotypic features of non-permissive Dts36 infections, as well as the lack of methyltransferase activity *in vitro*, is a defect in association of the capping enzyme subunits.

## **5.9 Future Studies**

The phenotypic and biochemical analysis of the mutant D1 protein from Dts36 has provided additional insights into the function of vaccinia virus mRNA capping enzyme. The mutant protein was thought to be defective in the methyltransferase activity based on *in vivo* analysis and this was confirmed by *in vitro* analysis. However, the other results from the *in vivo* analysis have yet to be substantiated by *in vitro* analysis. Future work with Dts36 will attempt to

determine if the mutant D1 protein possesses guanylyltransferase activity in soluble enzyme extracts. This experiment utilizes a synthetic RNA molecule as the substrate of the guanylyltransferase reaction and can be used to examine the enzymatic activity of the protein and with a slight modification of the assay, the cap status of *in vitro* synthesized transcripts can be determined. Work will also be performed in order to confirm that the mutant D1 protein is defective in the early transcription termination and intermediate transcription initiation functions of the capping enzyme. The early transcription termination function will be analyzed by two distinct methods; the visualization of *in vitro* transcription products on an agarose-formaldehyde gel or through use of cell-free extracts in a well-described termination assay. The intermediate transcription initiation function will be analyzed through use of cell-free extracts in a well-described initiation assay. Finally, the association of the vaccinia virus capping enzyme subunits in the mutant virus will be determined by co-precipitations which will allow it to be determined if the subunit interaction of the D1 and D12 proteins from Dts36 is less efficient, leading to the defects that have been observed throughout the course of this study.

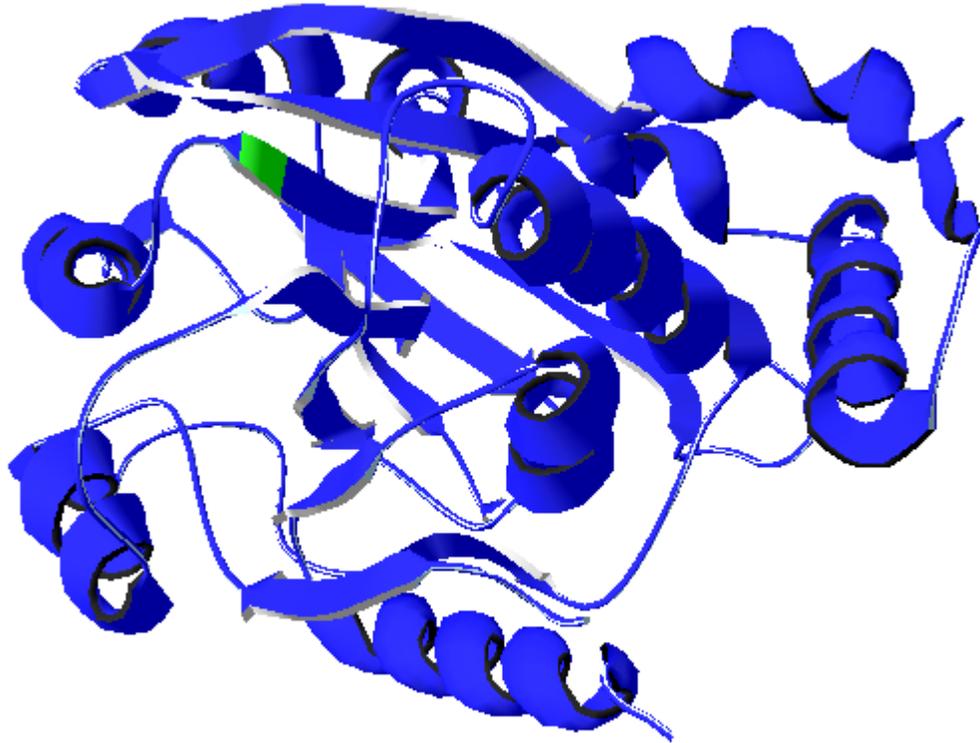


Figure 5-1. Structure of the carboxyl-terminus (aa 545-844) of the large subunit of the vaccinia virus mRNA capping enzyme. The Swiss-Pdb Viewer, version 4.0.1, was used to view the protein structure (Protein Data Bank code 2vdw) and export the figure. The location of amino acid residue 705 is highlighted in green.

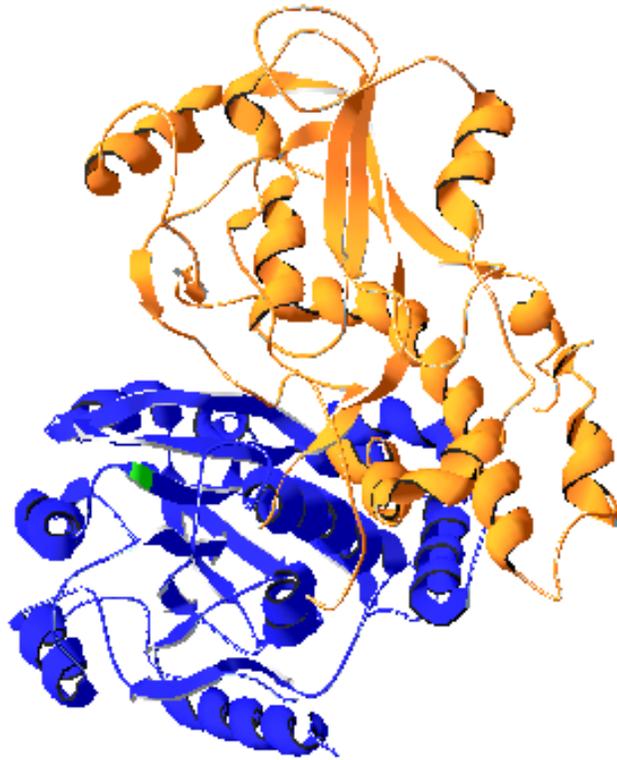


Figure 5-2. Structure of the interaction of the carboxyl-terminus of the large subunit with the entire small subunit of the vaccinia virus mRNA capping enzyme. The Swiss-Pdb Viewer, version 4.0.1, was used to view the protein structure (Protein Data Bank code 2vdw) and export the figure. The D1 subunit is colored blue and the D12 subunit is colored orange. The location of amino acid residue 705 is highlighted in green.

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## BIOGRAPHICAL SKETCH

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